PLANT RESPONSES TO PHYTOPHAGOUS MITES/THRIPS AND SEARCH FOR RESISTANCE

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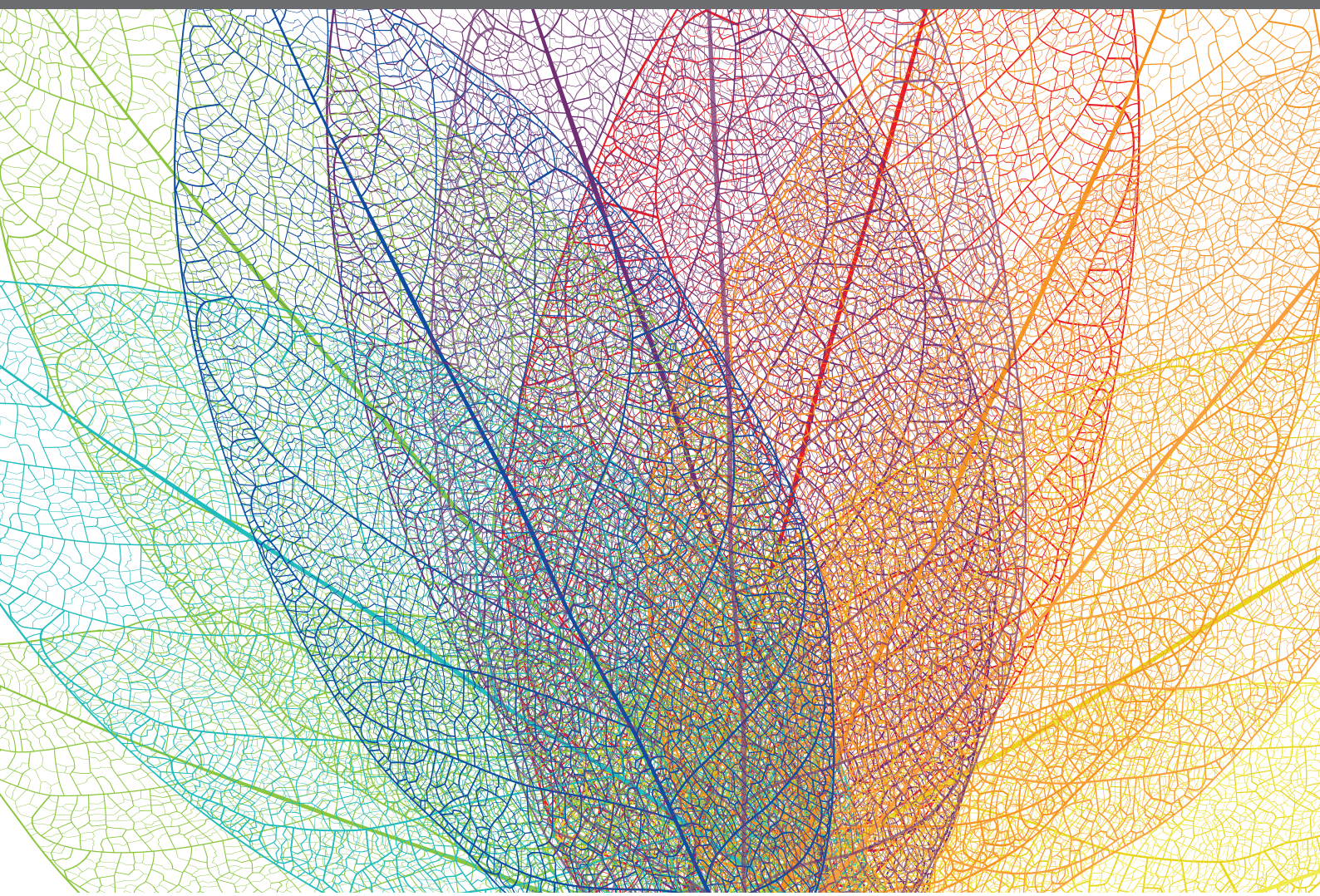

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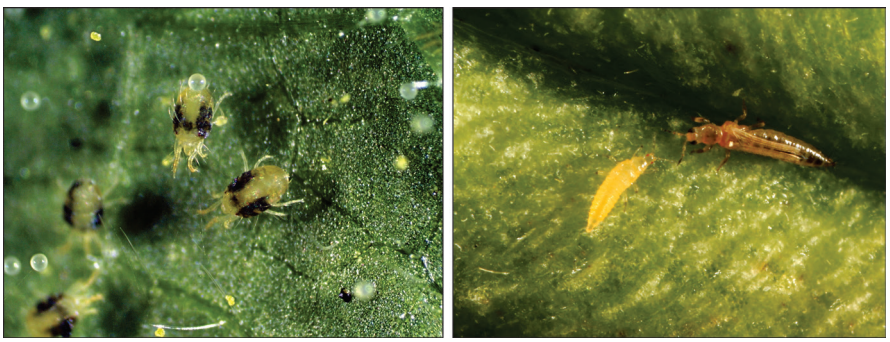

Two-spotted spider mite (TSSM), *Tetranychus urticae* Koch (Acari: Tetranychidae) and Western flower thrips (WFT), *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae).
Images: Vladimir Zhurov, The University of Western Ontario, Canada; Yulin Gao, Chinese Academy of Agricultural Sciences, China

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EDITORIAL
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Editorial: Plant Responses to Phytophagous Mites/Thrips and Search for Resistance

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Editorial on the Research Topic

Plant Responses to Phytophagous Mites/Thrips and Search for Resistance

Phytophagous mites and thrips are global pests affecting a wide range of agricultural crops (Mouden et al., 2017; Agut et al., 2018). Among the arthropods, they are phylogenetically distant, but both classes harbor species ranging from highly specialized to extremely polyphagous (Rioja et al., 2017; Wu et al., 2018). Through convergent evolution, mites and thrips evolved stylets to facilitate feeding from mesophyll or epidermal cells (Bensoussan et al., 2016; Rioja et al., 2017; Wu et al., 2018). Despite large crops losses (Agut et al., 2018; Steenbergen et al., 2018) that are expected to become more severe with global warming (Ximenez-Embún et al., 2017; Urbaneja-Bernat et al., 2019), the interactions between mites/thrips and their host plants have been understudied. Hence, understanding how plants defend themselves against these pests is essential for developing crop protection strategies. This Research Topic provides an update on recent advances in the plant molecular and physiological mechanisms associated with phytophagous mite/thrips-plant interactions, and provides an overview of different approaches for improving crop resistance sustainably, either through repellence, feeding disruption or prevention of feeding damage. Here, we highlight some of the major points arising from these reports.

MITE-RELATED

Plant Resistance

The ability of zoophytophagous predators such as the mirid *Macrolophys pygmaeus* to induce plant defenses and indirectly affect spider mites is well-known (Pappas et al., 2015; Zhang et al., 2018). Recently, Cruz-Miralles et al. (2019) showed that *Euseius stipulatus*, a zoophytophagous phytoseiid common in citrus, can trigger plant-genotype specific defensive responses and affect their prey beyond predation through plant-mediated effects. In this topic, Pérez-Hedo et al. report reduced infestation of tomato plants exposed to the mirid predator *Nesidiocoris tenuis* by the spider mite *Tetranychus urticae*. This effect correlated with the upregulated expression of a jasmonic acid-responsive gene (*PIN2*) and two protease inhibitors markers (*PI-II1* and *PI-II2*) in tomato plants, indicating that crop protective benefits of zoophytophagous predators include both herbivore predation and the induction of plant innate defenses. Furthermore, the negative correlation of

the zingiberene content of tomato trichomes and spider mite performance was described by de Oliveira et al. demonstrating a multifaceted role of this terpene in defense response. Glandular trichomes and zingiberene contribute greatly to the resistance of wild tomatoes against herbivores (Glas et al., 2012). The ability to accumulate zingiberene in glandular trichomes is being transferred to commercial varieties to increase resistance to mites and whiteflies (Bleeker et al., 2012).

The red spider mite, *Tetranychus neocaledonicus*, can be an important pest on lima bean (*Phaseolus lunatus*) (Gomes Neto et al., 2017). de França et al. assessed antibiosis and antixenosis effects of nine lima bean genotypes against the red spider mite and identified two distinct groups. The authors propose that one set of these genotypes can be used in trap cropping strategies, and the second can be used as a source of resistance against *T. neocaledonicus*.

T. urticae genome contains a large number of cysteine- and serine-proteases, indicating their importance in spider mite physiology (Santamaria et al., 2015). However, cystatins and serine-protease inhibitors are plant defense proteins that target these mite proteins (Santamaria et al., 2012; Martel et al., 2015). Arnaiz et al. investigated the role of Arabidopsis Kunitz Trypsin Inhibitors (KTI) on plant defense against spider mite and showed that KTI confer resistance to *T. urticae*. Moreover, transient overexpression of *KTI4* and *KTI5* in tobacco demonstrated their bifunctional ability to inhibit cysteine- and serine-proteases. It is expected that KTI impact mite gut cysteine-proteases involved in the hydrolysis of dietary proteins and can thus be used as a potential tool to engineer plant resistance/tolerance to *T. urticae*.

Monocots, including grasses, are attacked by generalist and specialist herbivores. Several spider mite species are key pests on cereals, including the generalist *T. urticae* (Grbić et al., 2011) and the Poaceae specialist *Oligonychus pratensis* (Bynum et al., 2015). Bui et al. provided evidence that maize and barley defenses induced by *T. urticae* and *O. pratensis* herbivory are similar. However, a functional benzoxazinoid pathway negatively affected only the performance of the generalist *T. urticae* and not the specialist *O. pratensis*, suggesting that *O. pratensis* adapted to the maize as a plant-host by evading benzoxazinoid defenses. Similarly, Arena et al. used RNAseq to assess the global response of Arabidopsis upon the infestation of the false spider mite *Brevipalpus yothersi*. *Brevipalpus* feeding induced jasmonic acid (JA)- and salicylic acid (SA)-regulated Arabidopsis responses, very similar to those resulting from *T. urticae* feeding (Zhurov et al., 2014). However, Arena et al. demonstrated that Arabidopsis defenses affect *Brevipalpus* differently than *T. urticae*, since *Brevipalpus* is insensitive to JA-regulated defenses, and requires the SA pathway for maximal performance. The comparative analysis of mechanisms underlying differential responses of *T. urticae*, *O. pratensis,* and *Brevipalpus* to plant defenses will provide new insights into mechanisms of mite adaptation to different host plants.

The phytohormones gibberellic acid (GA) and JA regulate plant growth/development and defense, respectively (Hou et al., 2013). Several studies have addressed the plant dilemma between "to grow" and "to defend" in response to various stimuli, indicating that plants prioritize GA- or JA-induced responses (Heinrich et al., 2013; Qin et al., 2013; De Bruyne et al., 2014; Wang et al., 2015; Campos et al., 2016; Guo et al., 2018). Sperotto et al. observed that wild rice species are highly susceptible to the mite *Schizotetranychus oryzae*. This was possibly due to a high GA:JA ratio, since the tested wild species are relatively tall (1.5–5 m). Therefore, authors suggest the use of short and stocky *Oryza* species as primary sources of mite resistance.

While resistance is aimed at maximizing plant fitness by targeting herbivores, other traits may maximize fitness through compensatory physiological responses (Koch et al., 2016; Erb, 2018), while tolerating the herbivore. Very little is known about the genetic mechanisms of tolerance (Peterson et al., 2017). In an Opinion article, Sperotto et al. reason that crops are tolerant when they produce acceptable yields and maintain fitness when infested. By evaluating rice morphology and productivity, Buffon et al. subsequently identified a rice cultivar tolerant to *S. oryzae*. Together they argue that tolerance, as a mechanism for maximizing yield and productivity, should be more at the forefront of crop protection research, since this is what really matters to farmers.

Responses to Mixed Stimuli

Plants are exposed to numerous biotic and abiotic stresses. To survive these challenges they have to undergo distinct physiological and structural transformations that can be costly (Wang et al., 2003). Plant responses to abiotic stress may also affect the performance of herbivores (Scheirs et al., 2006). Some plants evolved the ability to accumulate heavy metals from the soil in their shoots. It was suggested that this can provide protection against herbivores (Boyd, 2007; Hörger et al., 2013). Godinho et al. showed that tomato plants can accumulate cadmium (Cd) to levels that negatively affect spider mite performance, while not affecting the plant. They observed detrimental Cd effects on mites, regardless of their ability to induce or suppress plant defenses. This suggests that Cd-accumulation could provide a plastic plant resistance trait that can also counteract defense-suppression by herbivores. Furthermore, the study of *Tetranychus evansi* performance on drought-adapted tomatoes showed a positive link between the induction of soluble carbohydrates and amino acids used by the plant for osmotic adjustment during drought and mite performance (Ximénez-Embún et al.). *Tetranychus evansi* downregulated the accumulation of defense-related phytohormones in control and drought-adapted plants, indicating that drought promotes *T. evansi* tomato infestation.

Beneficial microorganisms are known to promote plant growth and confer resistance to biotic and abiotic stressors (Pieterse et al., 2014; Finkel et al., 2017). Pappas et al. studied the effect of a beneficial endophytic fungus (*Fusarium solani*, FsK) on tomato defenses against *T. urticae*, and showed that both direct and indirect defenses can be enhanced on endophyte-colonized plants. Defense-related genes were differentially expressed and *T. urticae* performance was negatively affected by FsK-colonization. Furthermore, FsK-colonized plants emitted different volatiles in response to *T. urticae* compared to control plants, and were more attractive to *Macrolophus pygmaeus*, a natural enemy of spider mites. Therefore, three-way interactions (such as tomato-FsK-*T.*

urticae) may offer the opportunity for the development of novel tools for spider mite control.

Wheat curl mite (WCM), *Aceria tosichella*, is a major pest of wheat that vectors damaging plant viruses (Navia et al., 2013). Skoracka et al. reviewed the current knowledge on WCM-wheat-virus interactions and identified gaps in underlying mechanisms of mite infestation, viral epidemiology, and plant responses. They emphasize the application of molecular techniques in mite-wheat-virus studies and discuss the possibilities for breeding cereal cultivars carrying resistance genes against WCM and viruses.

Modulation of Plant Defense Response

Some herbivores evolved the ability to counteract plant defenses by producing effectors that disrupt plant signaling and induce effector-triggered susceptibility (Hogenhout and Bos, 2011; Ferrari et al., 2013; Pel and Pieterse, 2013). Accordingly, several species of spider mites were shown to suppress plant defenses (Kant et al., 2008) via effectors (Villarroel et al., 2016). While under laboratory conditions this can promote mite performance, it can also encourage competition and predation (Ataide et al., 2016). Blaazer et al. asked why the suppression trait is common among mites and argue that buffering traits may shield it from natural selection. Thus, mites in nature may have to work hard to limit the ecological costs associated with suppression and to keep a monopoly on their feeding site.

Others (Digestive System of *Tetranychus urticae* and Eriophyoid Mites)

Plants evolved several strategies to deter herbivory. These traits, in turn, have selected for counter-adaptations in herbviores to cope with plant defenses (Heidel-Fischer and Vogel, 2015). The wide host range of *T. urticae* suggests that it may have evolved general traits that allow digestion and detoxification of a wide range of different plant compounds (Rioja et al., 2017). Bensoussan et al. described the organization and properties of *T. urticae* alimentary system, as well as the functional properties of digestive compartments relative to their ability to parcel out molecules of different weights. Together with genomic and reverse genetics tools, this will enable a functional dissection of the *T. urticae* gut to identify the specific features that enabled the evolution of *T. urticae* extreme generalist feeding strategy.

Eriophyoid mites are extremely small phytophagous arthropods with unusual morphological, biological and behavioral specialization compared to other Acari (Skoracka et al., 2010). Many of them are major plant pests, and some increase their impact by transmitting plant viruses (Stenger et al., 2016; Skoracka et al.). De Lillo et al. reviewed current knowledge on agriculturally relevant eriophyoids with emphasis on sources for host plant resistance. This review aims to guide future efforts for achieving basic, specific, and applied goals in plant protection against these mites.

THRIPS-RELATED

Plant Resistance

Constitutive resistance to thrips across all plant life stages is essential for more sustainable and successful cultivation of *Capsicum* (Ssemwogerere et al., 2013). Visschers et al. screened 40 *Capsicum* accessions for resistance to *Frankienella occidentalis* and *Thrips tabaci* over the plant's ontogenetic development by measuring leaf damage. Results show that resistance in *Capsicum* is species-specific and its levels determined by the plant's developmental stage, suggesting that breeding for resistance should not rely on screening in only one ontogenetic stage.

Thrips are also major pests of peanut worldwide and serve as vectors of devastating orthotospoviruses (Riley et al., 2011). Srinivasan et al. describe field resistance to different thrips species in peanut based on morphological or chemical traits. They also discuss screening methods, marker-assisted selection and genetic modifications that can be integrated to manage thrips and associated viruses, and layout future directions in peanut thrips management.

Some thrips species can induce gall formation, thereby altering the development of host tissues (Hori, 1992). Galls are obtained via manipulation of the host's cellular communication system and often include suppression of defenses (Oates et al., 2016). Jorge et al. studied structural and chemical changes in *Myrcia splendens* plants associated with galls induced by *Nexothrips* sp. Major structural changes during gall formation included alteration of the number and size of oil glands, which could affect leaf volatile production. Comparing the headspace volatiles, over 80 different compounds were differentially detected. It was concluded that presence of methyl salicylate in non-galled samples may be a bioindicator for host resistance.

Responses to Mixed Stimuli (Induced Resistance Against Thrips)

Fungal endophytes may prime plant defenses resulting in a stronger and/or faster response to attack by herbivores (Brotman et al., 2010). Muvea et al. demonstrated that colonization with the endophyte *Hypocrea lixii* plays an important role in mediating induced resistance to *Thrips tabaci* in onion. Plants colonized with the endophyte showed substantially decreased thrips feeding activity, as well as reduced replication of Iris yellow spot virus (IYSV), resulting in decreased disease incidence.

Inducible plant defense against western flower thrips (WFT) have recently been proposed as a promising tool for thrips control (Steenbergen et al., 2018). Chen et al. showed that *Pseudomonas syringae* pv. tomato DC3000 (Pst) activates the JA signaling pathway through the production of phytotoxin coronatine (COR). Infection of tomato plants with non-pathogenic concentrations of Pst or spray treatments with COR led to significant reduction of WFT feeding damage.

New Technologies for Recording and Analysis of Insect Behavior

The search for genetic resistance against thrips has been ongoing for a long time (Douglas, 2018). However, the toolbox for studying genetic resistance to thrips is limited. Abd-El-Haliem et al. present a method for the identification of genes relevant to thrips performance. They utilized an *Agrobacterium tumefaciens*-mediated expression of candidate thrips genes within leaf discs

of the target host on which subsequently thrips performance was assessed. The methodology allows for testing of multiple candidate genes without the need for production of stable transformed plants.

Host-plant resistance to insects, like thrips, is a complex trait difficult to phenotype quickly and reliably. A crucial element is the accurate estimation of resistance level, which requires robust phenotyping systems that can accurately screen many different plant lines in a high-throughput manner (Kloth et al., 2012; Goggin et al., 2015). Jongsma et al. introduce novel hardware and software to facilitate insect choice-assays and to automate the acquisition and analysis of movement tracks. The analysis resulted in much larger contrasts in behavior traits than previously reported. Compared to leaf damage assays on whole plants or detached leaves, this method is faster and more reliable than previous methods.

MITE/THRIPS-RELATED

There are indications that plant responses against herbivores may be influenced by bacteria associated with herbviores (Chung et al., 2013; Su et al., 2015). Schausberger discussed the abundance and diversity of bacteria associated with spider mites and thrips, and argued that these can have a profound impact on herbivore-induced plant defense responses. Gut/saliva-associated and endosymbiotic bacteria introduced into plants during feeding can induce plant defenses, such as the activation of SA pathway, which decreases the expression of JA pathway. Schausberger stressed there is need for further research to pinpoint the effects that different bacterial groups—and their elicitors—have on plant defense against mites and thrips.

FINAL COMMENT

In summary, the work presented here documents recent advances in the interface between plants and mites or thrips. Now, the challenge is to move forward, to integrate this information and to develop knowledge-driven sustainable control strategies against a diverse range of mite and thrips pests.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Induced Tomato Plant Resistance Against *Tetranychus urticae* Triggered by the Phytophagy of *Nesidiocoris tenuis*

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The zoophytophagous predator *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) is capable of inducing plant defenses in tomato due to its phytophagous behavior. These induced defenses, which include the release of herbivore-induced plant volatiles (HIPVs), have been proven to affect the oviposition behavior and reduce the subsequent performance of some tomato pests. However, the effect of induction of plant defenses by *N. tenuis* on the preference, development, and reproduction of the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) remains unknown. In this research, *T. urticae* did not show preference for the odor source emitted by intact tomato plants when compared with *N. tenuis*-punctured plants and jasmonic acid (JA) deficient mutant tomato plants. Furthermore, the number of eggs laid by *T. urticae* on intact tomato plants or on *N. tenuis*-punctured plants was similar. However, in a greenhouse experiment conducted to evaluate whether the defense induction mediated by *N. tenuis* had an effect on *T. urticae* the infestation of *T. urticae* was significantly reduced by 35% on those plants previously activated by *N. tenuis* when compared to the control. The expression of a JA-responsive gene that was upregulated and the transcription of the plant protein inhibitor II was higher on activated plants relative to the control. These results can serve as a basis for the development of new management strategies for *T. urticae* based on plant defense mechanisms induced from the phytophagous behavior of *N. tenuis*.

Keywords: two-spotted spider mite, oviposition, jasmonic acid, protein inhibitors, biological control

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INTRODUCTION

In recent years the use of omnivorous natural enemies in horticultural crops, and in particular, the zoophytophagous predators that can feed on both plant and prey, has given rise to some of the most resounding successes in biological control in Southern Europe (Jacas and Urbaneja, 2009). In sweet pepper, for example, the release and conservation of the predatory mite *Amblyseius swirskii* (Athias-Henriot) (Acari: Phytoseiidae) together with the anthocorid *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae) allows successful management of populations of the key pests of this crop: the whitefly *Bemisia tabaci* Gennadius and the thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) (van der Blom et al., 1997, 2009; Sanchez et al., 2000; Calvo et al., 2009, 2015). Similarly, in tomato the cosmopolitan predatory mirid *Nesidiocoris tenuis* (Reuter)

(Hemiptera: Miridae) enables effective control of *B. tabaci* and the tomato borer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Calvo et al., 2012; Urbaneja et al., 2012; Pérez-Hedo and Urbaneja, 2015, 2016; Pérez-Hedo et al., 2017), an important invasive tomato pest detected for the first time in Spain in 2007 (Desneux et al., 2010).

It is widely known that plants respond to herbivory through several signal transduction pathways that are mediated by phytohormones. The accumulation in the plant of the main phytohormones related to plant defenses, the jasmonic acid (JA), the salicylic acid (SA), the abscisic acid (ABA), and the ethylene (ET), activates signaling cascades that regulate transcriptional responses. These defenses can cause the production of secondary metabolites and proteins that have toxic, repellent and/or anti-nutritive effects on herbivores (direct defenses) (Kant et al., 2015). Furthermore, when the production and release of plant volatiles (Herbivore Induced Plant Volatiles; HIPVs) are triggered they can modify the behavior of both phytophagous pests and their natural enemies (indirect defenses) (Paré and Tumlinson, 1999; Kessler and Baldwin, 2001; Dicke, 2009). Recently, some of these zoophytophagous predators have been found to activate the same defense mechanisms as those triggered by herbivorous arthropods (Halitschke et al., 2011; Pappas et al., 2015, 2016; Pérez-Hedo et al., 2015a,b; Bouagga et al., 2018a,b; Zhang et al., 2018).

The mirid *N. tenuis* is capable of inducing plant defenses in tomato due to its phytophagous behavior. In a previous study, we verified how the phytophagy of the predator *N. tenuis* activated the metabolic pathway of ABA and JA in tomato plants, which made them less attractive to the whitefly *B. tabaci* and more attractive to the whitefly parasitoid *Encarsia formosa* (Gahan) (Hymenoptera: Aphelinidae) (Pérez-Hedo et al., 2015b). In addition, we observed how the volatiles emitted by the *N. tenuis* punctured plants induced defenses in neighboring untouched plants by activating the JA pathway. This induction also resulted in the attraction of parasitoids by these intact plants that had not been exposed to *N. tenuis* (Pérez-Hedo et al., 2015b). Later, we were able to confirm that all stages of development of *N. tenuis* (from young nymphs to adults) are able to trigger these defensive responses (Naselli et al., 2016). However, we show not all zoophytophagous predators have the same ability to induce such responses in tomato plants. Tomato plants may have different degrees of attraction for pests and natural enemies depending on whether phytophagous behavior occurs, for example, by *N. tenuis*, *Macrolophus pygmaeus* (Rambur) or *Dicyphus maroccanus* Wagner (Hemiptera: Miridae) (Pérez-Hedo et al., 2015a). Thus, while plants punctured by *N. tenuis* are rejected by *B. tabaci* and *T. absoluta*, the phytophagy of *M. pygmaeus* and *D. maroccanus* has no effect on repellence in *B. tabaci* and in fact attracts *T. absoluta*. In contrast, the feeding activity of these three mirids results in the attraction of *E. formosa*. This fact could be elucidated by identifying the volatiles (HIPVs) involved in the defensive responses of tomato plants induced by *N. tenuis* and *M. pygmaeus*; in general, plants exposed to *N. tenuis* emitted more volatiles than plants exposed to *M. pygmaeus*, and the latter emitted more volatiles than intact plants. Furthermore, six green leaf volatiles (GLVs) together with the methyl salicylate were found to be repellent to *B. tabaci* and attractive to *E. formosa*, whereas no effect on *T. absoluta* was observed. Octyl acetate, which was only significantly present in plants exposed to *M. pygmaeus*, was significantly attractive for *T. absoluta*, repellent for *E. formosa* and indifferent to *B. tabaci* (Pérez-Hedo et al., 2018). Similarly, in sweet pepper the phytophagy of the anthocorid *O. laevigatus* and the mirids *N. tenuis* and *M. pygmaeus* also trigger defense responses in this crop (Bouagga et al., 2018a,b).

Pappas et al. (2015, 2016) demonstrated a reduction in oviposition and the subsequent performance of the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) by *M. pygmaeus*. These authors attributed the reduction in *T. urticae* performance to a consequence of direct defense induction mediated by *M. pygmaeus*. *M. pygmaeus*-punctured tomato plants were observed to increase locally and systematically the accumulation of transcripts and the activity of protease inhibitors that are known to be involved in plant responses, resulting in detrimental effects on the life history traits of *T. urticae*.

However, the effect of these *N. tenuis* mediated plant defenses on plant selection, development, and reproduction of *T. urticae* remains unknown. In this research, we evaluated the olfactory response of *T. urticae* females exposed to *N. tenuis*-punctured tomato plants, JA-deficient mutant tomato plants and intact tomato plants, for comparison, in a Y-tube olfactometer. Secondly, the oviposition of *T. urticae* was evaluated on *N. tenuis*-punctured tomato plants and on intact tomato plants. Thirdly, a greenhouse experiment was conducted to evaluate whether the defense induction mediated by *N. tenuis* had an effect on *T. urticae*. Finally, we used gene expression analysis to assess whether *N. tenuis* activated JA signaling pathways and increased accumulation of transcripts of two proteinase inhibitor II markers which are known to be involved in plant defense.

MATERIALS AND METHODS

Plants and Insects

Tomato plants *Solanum lycopersicum* cv. Moneymaker, JA-deficient tomato mutants (def-1) and their respective near-isogenic wild type (cv. Castlemart) parental lines were used to determine the responses of *T. urticae* and *N. tenuis* to the distinct experimental treatments described below. Seeds were sown in soil. Two weeks after germination seedlings were individually transplanted into pots (8 cm × 8 cm × 8 cm). Plants were maintained undisturbed at 25 ± 2°C, with constant relative humidity of 65% ± 5% and a photoperiod of 14:10 h (light: dark). Pesticide-free tomato plants were used for the experiments at 4 weeks of age (approximately 20 cm high). *N. tenuis* was provided directly by Koppert Biological Systems, S.L. (Murcia, Spain) and *T. urticae* adults were obtained from a culture established at IVIA in 2011 originally collected from the region of La Plana (Castelló, Spain). Mites were maintained on tomato plants kept in a climatic chamber at 25 ± 2°C, and 65% ± 5% RH and 14:10 h (light: dark).

Nesidiocoris tenuis-punctured plants were obtained by exposing tomato plants to 20 fourth instar nymphs for 24 h

in a 30 cm × 30 cm × 30 cm plastic cage (BugDorm-1 insect tents; MegaView Science Co., Ltd., Taichung, Taiwan). Naselli et al. (2016) demonstrated that *N. tenuis* nymphs had the same potential to induce plant defenses in tomatoes as adults. Therefore, to avoid, on one hand, induction of defenses by adult oviposition and on the other hand accumulation and hatching of eggs along with interference in performance experiments, nymphs were used to induce defenses instead of adults. All motile individuals were removed from plants before the beginning of each trial.

Y-Tube Bioassays

A Y-tube olfactometer experiment was conducted to test the olfactory responses of *T. urticae* and *N. tenuis* females to tomato plants that were previously punctured by *N. tenuis* relative to intact plants; to JA-deficient tomato mutant *def-1* and its near-isogenic wild type (*wt*) parental line. The Y-tube olfactometer (Analytical Research Systems, Gainesville, FL, United States) consisted of a 2.4-cm-diameter Y-shaped glass tube with a 13.5-cm long base and two arms each 5.75 cm long (Pérez-Hedo and Urbaneja, 2015). Both side arms were connected via high-density polyethylene (HDPE) tubes to two identical glass jars (5 L volume) each of which were connected to an air pump that produced a unidirectional humidified airflow at 150 ml/min (Pérez-Hedo and Urbaneja, 2015).

A single individual female was introduced into the tube (entry array) and observed until she had walked at least 3 cm up one of the arms or until 15 min had elapsed. A total of 30–40 valid replicates for each species were recorded for each pair of odor sources. Each individual was tested only once. Females that did not choose a side arm within 15 min were recorded as "no-choice" and were excluded from data analysis. After recording five responses, the Y-tube was rinsed with soapy water then acetone and left to dry for 5 min. The odor sources were subsequently switched between the left and right side arms to minimize any spatial effect on choice. Three types of plants (intact, mutant, and punctured) were used only once to test the response of 10 females and then were replaced with new plants. The Y-tube experiment was conducted under the following environmental conditions: 23 ± 2°C and 60 ± 10% RH.

Tetranychus urticae Oviposition Mediated by the Exposure of the Plants to *N. tenuis*

The oviposition of *T. urticae* was evaluated on 10 *N. tenuis*-punctured tomato plants and on 10 intact tomato plants (cv. Moneymaker). Each of the plants were isolated inside a plastic cage (60 cm × 60 cm × 60 cm) (BugDorm-2 insect tents) maintained in a climate chamber at 25 ± 2°C and 60–80% RH with a 14:10 h (light: dark) photoperiod. For each of the plant types, two fully expanded leaflets were selected on which approximately one clip-cage was gently placed (3 cm on diameter). Inside each clip-cage, 10 presumably mated females of *T. urticae* were released and left undisturbed for 48 h. After this time, the clip-cages were removed and the number of *T. urticae* eggs was counted.

Tetranychus urticae Survival and Reproductive Performance Mediated by the Exposure of the Plants to *N. tenuis*

The experiment was conducted in a 40 m × 10 m greenhouse equipped with drip irrigation system located at IVIA in Moncada (Valencia, Spain). The greenhouse was accessed through a double door and was divided into 12 experimental cages, six for *N. tenuis*-punctured plants and six for intact plants. Each cage represented one replicate. Cages were screened with "anti-thrips" polyethylene mesh with 220 μm × 331 μm interstices and the floor was covered with a 2 mm thick woven white polyethylene ground cloth. Each experimental cage was 2.5 m × 2 m × 2.5 m (L × W × H) and was accessed by a separate door secured with a zipper. One Datalogger (model TESTO 175-H2, Amidata S.A., Madrid, Spain) was placed in a central cage to record temperature and relative humidity. The average temperature during the experiment ranged between 23.5°C on the 31st of May, 2017 and 25.7°C on the 14th of June, 2017 with a minimum and maximum temperature of 20.4 and 34.8°C, respectively.

Eight tomato plants (cv. Moneymaker) were introduced into each cage. To avoid spider mite movement from plant to plant, plants were individually isolated, without touching either each other or the cage walls. Additionally, plants were placed on top of a brick inside a plastic tray full of water, and all pots and drip lines were painted with a band of glue. Plants were artificially infested with *T. urticae* from the previously mentioned laboratory population. Twenty *T. urticae* females were released per plant, distributed equally throughout the leaves with the aid of a fine brush (24th May, 2017). Seven and 14 days after *T. urticae* release samplings were conducted. Samplings involved counting the total number of *T. urticae* females on each plant. This was done with the naked eye, *in situ*, without removing leaves from the plant.

Plant Gene Expression

The transcriptional response of the PIN2 wound-induced proteinase inhibitor II precursor (PIN2), a marker gene for JA, and two plant Proteinase Inhibitor II (PI-II1 and PI-II2) markers were studied on six *N. tenuis*-punctured tomato plants and on six intact tomato plants (Lopez-Raez et al., 2010; Pappas et al., 2015). The apical part of the tomato plant samples were immediately ground in liquid nitrogen. Portions of the ground samples were used for RNA extraction. Total RNA (1.5 μg) was extracted using a Plant RNA Kit (Omega BioTek Inc., Doraville, GA, United States) and was treated with RNase-free DNase (Promega Corporation, Madison, WI, United States) to eliminate genomic DNA contamination. The RT reaction and the PCR SYBR reaction were performed as described by Pérez-Hedo et al. (2015b). Quantitative PCR was carried out using the LightCycler® 480 System (Roche Molecular Systems, Inc., Basel, Switzerland) sequence detector with standard PCR conditions. Expression of EF1 was used for normalization as a standard control gene. The nucleotide sequences of the gene specific primers are described in **Table 1**.

TABLE 1 | Forward and reverse sequences of *PIN2* (Wound-induced proteinase inhibitor II precursor) marker gene for JA, PI-II1, and PI-II2 markers of plant proteinase inhibitor II, and the constitutive gene EF1.

Primers	Forward	Reverse
PIN2	5′-GAAAATCGTTAATTTAT CCCAC-3′	5′-ACATACAAACTTTCCAT CTTTA-3′
PI-II1	5′-CATCATGGCTGTTCA CAAGG-3′	5′-ATCCCGAACCCAAGAT TACC-3′
PI-II2	5′-GGCCAAATGCTTGCAC CTTT-3′	5′-CAACACGTGGTACATC CGGT-3′
EF1	5′-GATTGGTGGTATTGG AACTGTC-3′	5′-AGCTTCGTGGTGC ATCTC-3′

Data Analysis

Chi-square (χ^2) goodness of fit tests based on a null model were used to analyze data collected from the olfactory responses where the odor sources were selected with equal frequency. Individuals that did not make a choice were excluded from the statistical analysis. Two-tailed Student's *t*-test ($P < 0.05$) was performed to compare oviposition between the two treatments and to compare the quantified expression of defense genes between intact plants and *N. tenuis*-punctured plants. Two measurements on two sample dates (7 and 14 days after *T. urticae* infestation) were analyzed using a generalized linear mixed model (GLMM) with repeated measures. Treatment was considered as a fixed factor and replicates nested within treatment was used as random factor to correct for pseudoreplication. The GLMM used a Poisson distribution with the logarithm as the link function. Results are expressed as the mean ± standard error.

RESULTS

N. tenuis-Punctured Plants Do Not Alter *T. urticae* Plant Selection

The two-spotted spider mite, *T. urticae*, showed no preference for the odor source emitted by intact tomato plants when compared with *N. tenuis*-punctured plants. ($\chi^2 = 0.842$; $P = 0.1794$) (**Figure 1A**). Similarly, *T. urticae* had no preference when given a choice between the JA-mutant tomato plants and their near-isogenic *wt* that had and had not been exposed to mirids

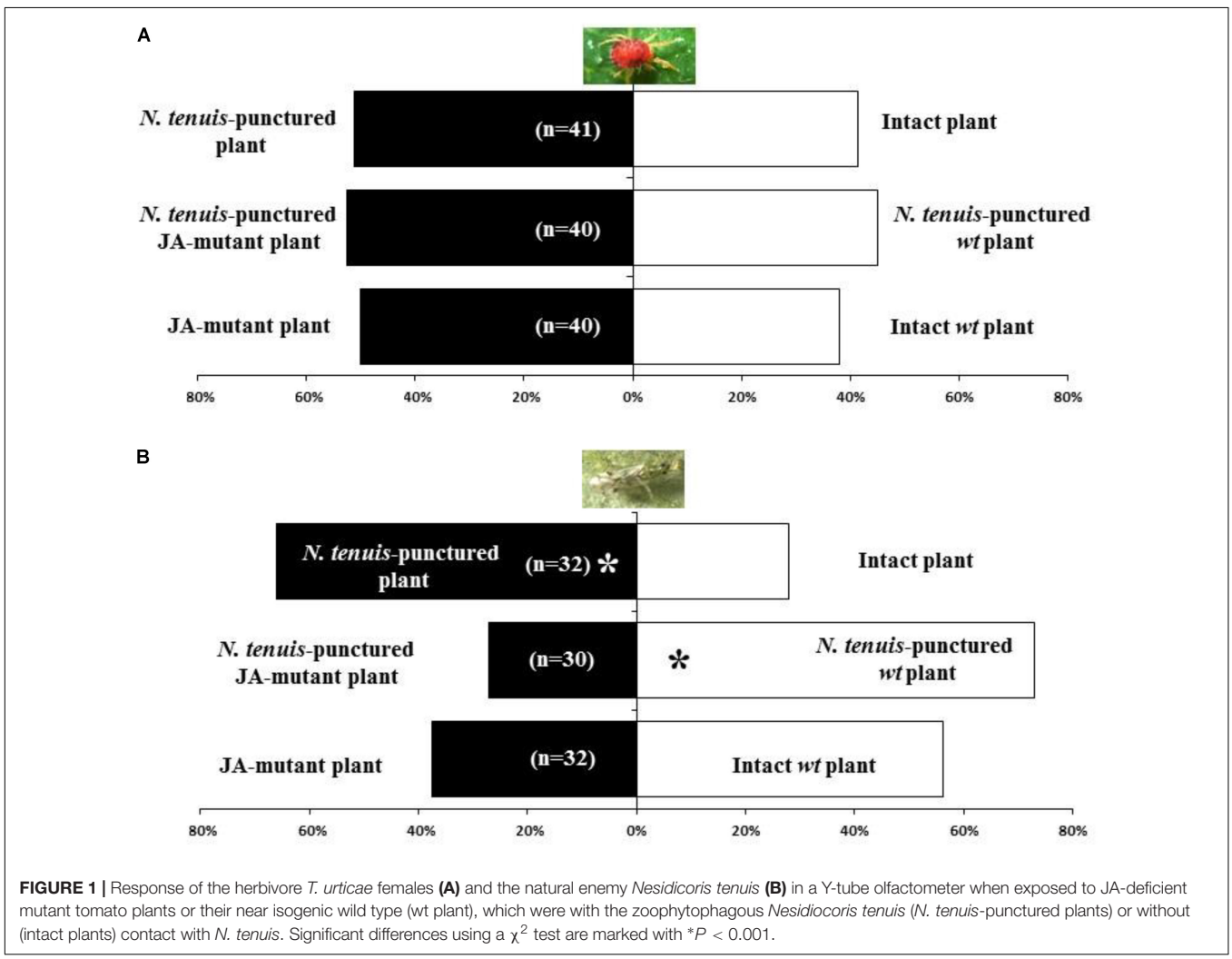

FIGURE 1 | Response of the herbivore *T. urticae* females **(A)** and the natural enemy *Nesidicoris tenuis* **(B)** in a Y-tube olfactometer when exposed to JA-deficient mutant tomato plants or their near isogenic wild type (wt plant), which were with the zoophytophagous *Nesidiocoris tenuis* (*N. tenuis*-punctured plants) or without (intact plants) contact with *N. tenuis*. Significant differences using a χ^2 test are marked with $^*P < 0.001$.

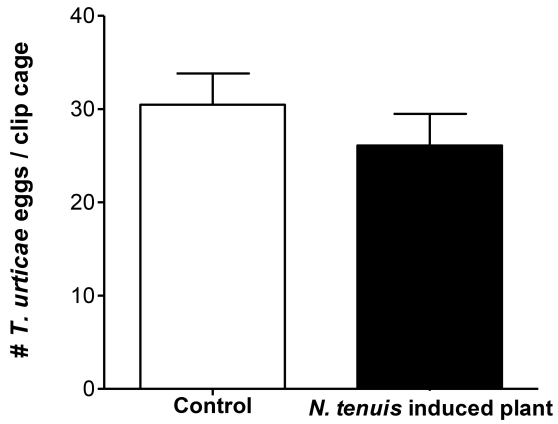

FIGURE 2 | Number of eggs (mean ± SE) laid by 10 *T. urticae* females in a clip cage in 48 h on *N. tenuis*-punctured tomato plants and on intact tomato plants at 25 ± 2°C and 14:10 h L:D.

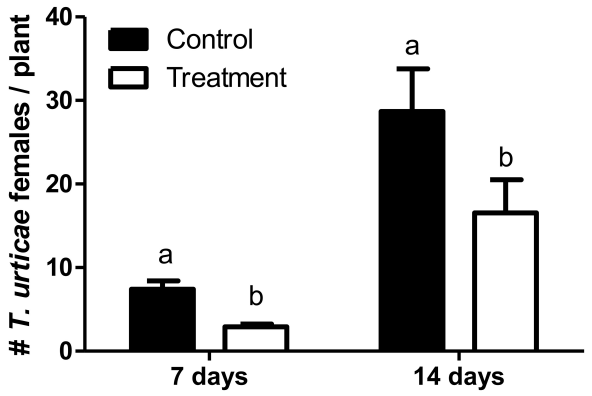

FIGURE 3 | Number (mean ± SE) of *Tetranychus urticae* females per tomato plant in a glasshouse experiment comparing the mite development on *N. tenuis*-punctured tomato plants in comparison to intact tomato plants (Control). Bars with different letters are significantly different (GLMM, repeated measures; $P < 0.05$).

(χ^2 = 0.461; P = 0.248; χ^2 = 1.429; P = 0.116, respectively) (**Figure 1A**).

The mirid *N. tenuis* clearly chose *N. tenuis*-punctured plants when given a choice between intact plants and *N. tenuis*-punctured plants (χ^2 = 9.600; P = 0.001; **Figure 1B**). The *N. tenuis*-punctured *wt* plants were also preferred to JA-mutant tomato plants previously punctured by *N. tenuis* (χ^2 = 13.07; P = 0.0002). The mirid did not show a significant preference (χ^2 = 2.400; P = 0.0607) when given a choice between JA-mutant plants or intact *wt* tomato plants (**Figure 1B**).

N. tenuis-Punctured Plants Do Not Affect *T. urticae* Oviposition

The number of eggs laid by *T. urticae* within each clip cage during 48 h was not significantly different when the females laid the eggs on intact tomato plants or on *N. tenuis*-punctured plants (t = 0.9165; df = 1, 36; P = 0.3655) (**Figure 2**).

N. tenuis-Punctured Plants Reduce *T. urticae* Performance

The number of *T. urticae* per plant was significantly lower in those tomato plants that were pre-exposed to *N. tenuis* (F = 16.612; df = 1, 166; $P < 0.0001$) (**Figure 3**). At day 14 the number of *T. urticae* per plant was significantly reduced by 35% on those plants previously activated by the feeding punctures of *N. tenuis* when compared to the control. The JA was significantly up-regulated and the concentration of protein inhibitors was higher on activated plants relative to the control (**Figure 4**). The analysis of the relative expression genes involved in indirect defense showed transcriptional differences between *N. tenuis*-punctured plants and intact tomato plants. The PIN2 gene (a marker for JA) was significantly up-regulated (t = 2.344; df = 10; P = 0.043) and the concentration of two plant protein inhibitors (PI-II1 and PI-II2) was higher on activated plants relative to the control (t = 2.260; df = 10; P = 0.047 and t = 5.924; df = 10; $P < 0.0001$, respectively) (**Figure 4**).

DISCUSSION

We have verified how *N. tenuis* is the activator of direct defense mechanisms responsible for reducing the performance of a key tomato pest such as the spider mite *T. urticae*. However, a clear effect on the *T. urticae* female choice mediated by HIPV's by an *N. tenuis*-induced or by intact plant was not illustrated. It is known that the two-spotted spider mite uses odors to locate or avoid plants. Pallini et al. (1997) demonstrated that spider mites were weakly but significantly attracted to cucumber plants infested with conspecific herbivores, whereas strongly repelled by cucumber plants with heterospecific herbivores (i.e., the thrips *F. occidentalis*). Contrarily, Dicke (1986) observed that *T. urticae* dispersed when exposed to the odors of bean plants infested with spider mites. However, in our work *T. urticae* showed no repellence or attraction to the volatiles emitted by the defensive induction of *N. tenuis* which are mediated by the activation of the JA pathway. This conclusion was further confirmed with the use of JA-mutant plants, with and without previous punctures by *N. tenuis*, on which no response of *T. urticae* was obtained either. Therefore, in view of our results it seems that *T. urticae* does not respond to the volatiles induced by the phytophagy of *N. tenuis* through the JA pathway. These divergent results could be explained by the different composition of the volatile blends of each particular experimental situation aforementioned. Bouagga et al. (2018a) and Pérez-Hedo et al. (2018) showed that both of the mirid predators, *M. pygmaeus* and *N. tenuis* activated the JA pathway due to their phytophagous behavior in both of the crops, tomato, and sweet pepper. However, the composition of the volatile blend was specific at the species and plant level. In this work, through the use of JA-mutant plants, we demonstrate that the signaling pathway of JA is responsible for the attraction of the predator *N. tenuis*. Lins et al. (2014) previously illustrated that plants previously exposed to *N. tenuis* resulted attractive to *N. tenuis*. Pappas et al. (2015) observed that *T. urticae* deposited a lower number of eggs on plants previously

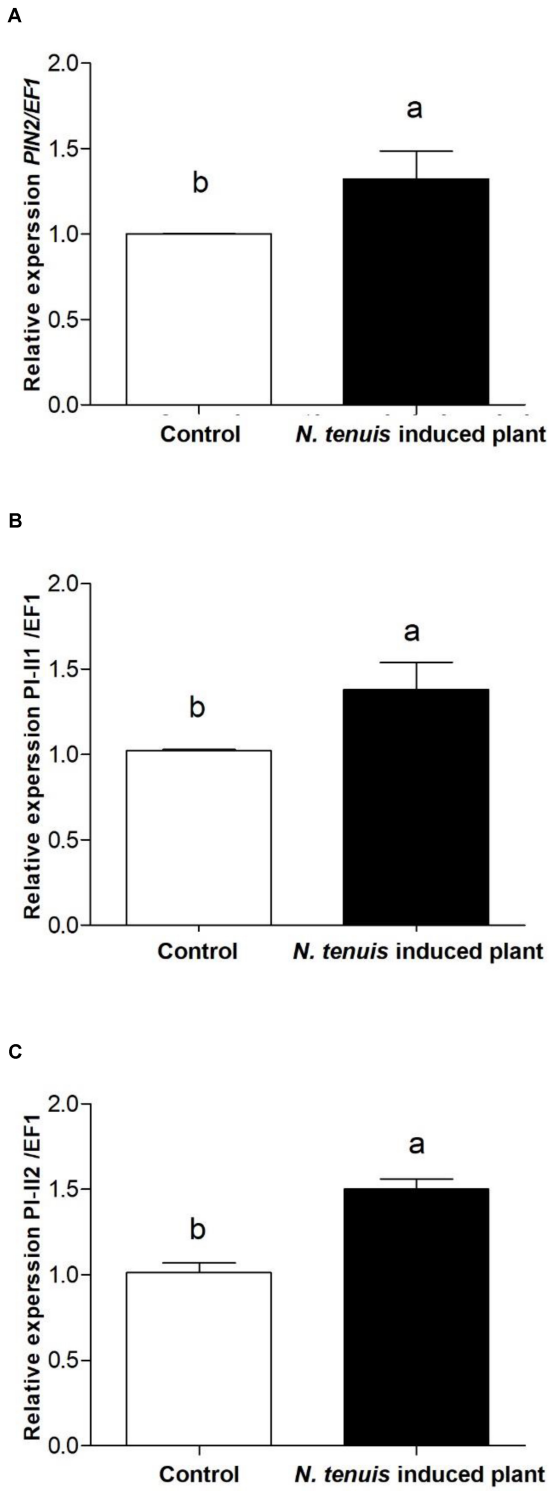

FIGURE 4 | Transcriptional response of the defensive gene PIN2, a JA-responsive gene **(A)**, and two plant Proteinase Inhibitor (PI-II1 and PI-II2) markers **(B,C)**, in control tomato plants with comparison to plants pre-exposed to *N. tenuis* for 24 h. Data is presented as the mean of eight independent analyses of transcript expression relative to the constitutive EF1 gene ± SE ($n = 8$). Bars with different letters are significantly different (t-test; $P < 0.05$).

exposed to the zoophytophagous predator, *M. pygmaeus*. Indeed, these authors found that this fecundity reduction was dependent on the predator density. However, in our study the oviposition of *T. urticae* was not affected when the mite was left undisturbed to lay eggs on either of the plants activated by *N. tenuis* or on intact plants. The methodology employed in our study and the one by Pappas et al. (2015) was quite different such as different mirid species, different time of mirid exposition and different number of mirids used to induce plants so the differences obtained between the studies could be due to the distinct way the mirid species, *N. tenuis* and *M. pygmaeus* activated the plants. Similar to our results, Ament et al. (2004) found that *T. urticae* laid as many eggs on JA-mutant plants as on wild-type plants.

Several previous studies demonstrated and explained the relationship between the activation of the JA pathway and the reduction in *T. urticae* performance (Arimura et al., 2000; Gols et al., 2003; Kant et al., 2004; Pappas et al., 2015) and even come to show that *T. urticae* infests and reproduces much better in JA-mutant plants than in wild plants (Li et al., 2002; Ament et al., 2004). Ament et al. (2004) suggested that JA-dependent direct defenses enhanced egg mortality or increase the time needed for embryonic development. In our research, *T. urticae* infestation was significantly lower in those plants that had been previously activated by *N. tenuis*. The activation of *N. tenuis* resulted in an up-regulation of the defensive gene PIN2, a JA-responsive gene, and two plant Proteinase Inhibitor (PI-II1 and PI-II2) markers. Pappas et al. (2015) already suggested that the decreased performance of *T. urticae* could be attributed to the higher concentration of PI in the induced plants by *M. pygmaeus* as occurred in our case with *N. tenuis*. Despite this and the effect of these PIs on other agricultural pests such as *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae) (Abdeen et al., 2005) or *Heliothis obsoleta* (Fabricius) (Lepidoptera: Noctuidae) (Abdeen et al., 2005) and bacterial diseases such as *Pseudomonas syringae* pv Tomato (Zhang et al., 2012), the exact role they play in the digestive physiology of phytophagous mites has yet to be clarified (Rehman et al., 2017).

The widespread use of *N. tenuis* in tomato greenhouses in southeastern Spain has ensured less pest pressure as well as fewer diseases in those crops where the mirid is well established (Calvo et al., 2012; Urbaneja et al., 2012; Pérez-Hedo and Urbaneja, 2016). The results of this study could partly explain how the incidence of *T. urticae* in crops where *N. tenuis* is being used is lower. Analogously, the direct induction triggered by the feeding punctures of *N. tenuis* could be affecting other key pests in this crop such as the whitefly *B. tabaci* and the lepidopteran *T. absoluta*. Preliminary results of our group suggest that plants activated by *N. tenuis* would also reduce the performance of both pests. Even more interesting would be to relate the activation of defenses and specifically the activation of the jasmonic pathway with the lower incidence of viruses. Since the use of zoophytophagous predators in horticultural crops has been promoted, lower incidence of some phytopathogenic viruses has been observed (Téllez et al., 2017). Recently, Escobar-Bravo et al. (2016) have shown that tomato plants with high expression of methyl jasmonate are less infected with the tomato yellow leaf curl virus (TYLCV). This led us to hypothesize that the defenses

induced by *N. tenuis* in tomato could be altering the acquisition and multiplication of phytopathogenic viruses. However, further research is required to confirm this novel hypothesis.

It has been more than two decades since the activation of the jasmonic route has been shown to reduce the incidence of agricultural pests. Field studies have shown the application of exogenous JA to plants leads to a reduction in herbivore abundance and performance (Thaler, 1999) and increases plant fitness (Baldwin, 1998). Gols et al. (2003) also obtained a repellent effect for *T. urticae* when treating Lima bean plants directly with JA. The application of exogenous JA to cotton plants reduced spider mite oviposition rates by more than 75% (Omer et al., 2001). However, as far as our knowledge is concerned, this defense activation has not been put into practice nor adopted by growers for the improvement of pest management, except for the activation by zoophytophagous predators. With the widespread use of omic techniques and the increasingly vertiginous breakdown of the gene editing technique (CRISPR-Cas9), we think that the activation of defenses in plants will become a key tool for sustainable control of agricultural pests and diseases. To this end, our results can serve as a basis for the new management development of strategies for *T. urticae*, based on resistance mechanisms induced from the phytophagous behavior of *N. tenuis*.

AUTHOR CONTRIBUTIONS

MP-H and AU conceived and designed the research. All authors participated in data collection and analyses, wrote the manuscript, and read and approved the manuscript.

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Trichomes and Allelochemicals in Tomato Genotypes Have Antagonistic Effects Upon Behavior and Biology of *Tetranychus urticae*

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Tomato genotypes selected for their high foliar zingiberene (ZGB) contents in a segregating F_2 population were assessed to determine their effect on behavior and biology of *Tetranychus urticae* Koch, the putative resistance mechanisms involved and the role of trichomes on that resistance. Genotypes with contrasting ZGB content (RVTZ-09 = low ZGB, RVTZ-79 = high ZGB, RVTZ-142 = high ZGB, and RVTZ-331 = high ZGB) were selected from an interspecific cross between wild *S. habrochaites* var. *hirsutum* accession PI-127826 (high ZGB content and resistant to mites) and *S. lycopersicum* cv. Redenção (low ZGB content and susceptible to mites). To determine the effect of these genotypes on mite behavior and biology, free- and no-choice tests, as well as biological studies were performed. Types and densities of trichomes on the foliar surface and their correlation with ZGB contents was determined. Genotypes rich in ZGB (RVTZ-79, RVTZ-142, and RVTZ-331) presented a high number of types IV and VI glandular trichomes, and both type IV and VI densities were positively correlated with ZGB content. In the free-choice test, *T. urticae* showed a high preference toward *S. lycopersicum* cv. Redenção and the genotype RVTZ-09 (low ZGB content), whereas, genotypes with high ZBG content were less preferred. Moreover, on high ZGB genotypes, increase in the egg incubation period and in total mortality of nymphs, and decrease of fecundity rate were observed, indicating deleterious effects in mite biology. Results indicated that high ZGB/high glandular trichome densities genotypes present both non-preference and antibiosis mechanisms of resistance to the mite.

Keywords: ***Solanum lycopersicum*, *Solanum habrochaites* var. *hirsutum*, zingiberene, glandular trichomes, biology, plant-resistance**

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INTRODUCTION

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is a phytophagous and polyphagous mite species (Clotuche et al., 2011). In tomato, *Solanum lycopersicum* L. (formerly *Lycopersicon esculentum* Mill.), this arthropod causes severe damage on leaves and on fruits, especially when they are grown in greenhouses, where the mites find favorable environmental

conditions (Meck et al., 2013). Considering the need for reduction of chemical applications (i.e., synthetic acaricides) for mite control, tomato breeding programs aimed to develop resistant cultivars should be considered an important contribution for the integrated management of this pest.

Studies have shown the potential use of some accessions of *S. lycopersicum* as resistance sources against arthropod pests. For instance, *S. lycopersicum* var. *cerasiforme* (Dunal), known as cherry tomato, has been studied for this purpose (Sánchez-Peña et al., 2006; Lucini et al., 2016). However, wild *Solanum* (section *Lycopersicon*) species are the most exploited as sources of resistance genes that may be deployed in tomato cultivars (Resende et al., 2009; Silva et al., 2009; Maluf et al., 2010; Oliveira et al., 2012; Dias et al., 2013; Lima et al., 2015).

Wild tomato accessions have been shown to affect behavior and biology of lepidopterans (Gurr and McGrath, 2001; Dias et al., 2013; Lima et al., 2015), coleopterans (Carter et al., 1989), hemipterans (Simmons et al., 2003; Resende et al., 2009) and mites *Tetranychus* spp. (Carter and Snyder, 1985; Gonçalves et al., 2006; Resende et al., 2008; Lucini et al., 2015). The main factor associated with resistance to pests in wild tomatoes, is reportedly the presence of glandular trichomes (especially, types IV and VI), which are responsible by storing and releasing allelochemical compounds (Maluf et al., 2001; Freitas et al., 2002; Simmons and Gurr, 2005). The zingiberene (ZGB) (a sesquiterpene) is an allelochemical stored and exuded by type IV and VI glandular trichomes present on plant surface of *Solanum habrochaites* Knapp and Spooner var. *hirsutum* Dunal (Maluf et al., 2001; Freitas et al., 2002; Gonçalves et al., 2006). The repellent effect of this wild tomato species against mites, imparted by presence of glandular trichomes containing ZGB, has been reported (Weston et al., 1989; Gonçalves et al., 2006).

Behavioral and biological bioassays are required to determine the mechanism(s) involved in the resistance against pests, in order to provide information on the extent of genotype resistance to herbivory. In this study, we selected tomato genotypes which contrasting ZGB contents in the F_2 generation of the interspecific cross *S. lycopersicum* cv. Redenção × *S. habrochaites* var. *hirsutum* accession PI-127826, and evaluated their effects on behavior and biology of the spider mite *T. urticae*, the putative resistance mechanisms involved (i.e., antixenosis and/or antibiosis), and the role of trichomes in the resistance.

MATERIALS AND METHODS

Spider Mite Population Maintenance

Adults of *T. urticae* were field-collected at the experimental area of the Universidade Estadual do Centro Oeste do Paraná, Guarapuava, Brazil (25°23′S; 51°29W). Mites were taken to the Laboratory of Entomology where a colony was established under controlled conditions (25 ± 2°C, photoperiod of 12L:12D hours). Plants of *Canavalia ensiformis* (L.) (Fabaceae), grown in plastic pots (5L), were used as mite food source, and replaced when necessary. For use in the laboratory bioassays, a mite colony was established in a BOD incubator chamber at 25 ± 2°C; 70 ± 10% relative humidity, and photoperiod of 12L:12D hours.

The tomato genotypes used in bioassays were the acessions *S. lycopersicum* cv. Redenção (low ZGB content and susceptible to mites), *S. habrochaites* var. *hirsutum* accession PI-127826 (wild accession rich in ZGB and resistant to mites), and additional genotypes selected from the interspecific cross between PI-127826 × cv. Redenção.

These additional genotypes comprised one F_1 plant (Redenção × PI 127826), and four plants selected from the F_2 (Redenção × PI 127826) population, three of which (RVTZ-79, RVTZ-142, RVTZ-331) selected for high ZGB contents and one (RVTZ-09) selected for low ZGB.

The selected genotypes were cloned through rooting of axillary shoots in a tray filled with commercial substrate that was kept moist. Seedlings were transplanted into 10L plastic pots containing a mixture of commercial substrate: soil (1:1) and fertilized with NPK (04:14:08). The plants were kept in a greenhouse with daily irrigation. Expanded leaflets of 40/50-day-old plants were sampled and used in the laboratory bioassays.

Zingiberene Contents and Trichomes Densities

In order to select for ZGB contents, 553 plants were analyzed [433 plants of the F_2 generation (PI-127826 × cv. Redenção), 40 plants of the F_1 generation, 40 plants of cv. Redenção and 40 plants of PI-127826]. The methodology proposed by Freitas et al. (2000) was used to quantify ZGB: six leaf disks (diameter 1 cm) were sampled from each plant, placed in tubes containing 2 ml of hexane, and then vortexed for 30 s. After that, the leaf disks were removed and the absorbance of the solution was measured using a spectrophotometer (Cary series – UV-Vis Spectrophotometer) at 270 nm of wavelength. The selection of genotypes was carried based on the absorbance values, which were above 0.700 for the three high ZGB genotypes, and below 0.250 for the low ZGB genotype.

Identification and quantification of trichomes on each genotype was based on images obtained using a scanning electron microscope (SEM) (Hitachi High-Tech TM3000 with tungsten filament, low vacuum and 15 kV). For this purpose, leaf disks (diameter 1 cm) taken from leaflets were inserted into the microscope. Luckwill's (1943) and Toscano et al.'s (2001) classifications were followed to identify the trichome types.

Types IV and VI glandular trichomes, and type VIII non-glandular trichomes were counted separately. Counting was performed on both foliar surfaces (abaxial and adaxial) of four equal quadrants of each leaf disk, and numbers of trichomes per mm^2 were recorded.

Free- and No-Choice Tests

For free- and no-choice test, arenas (petri dishes with 6 cm diameter) were coated inside with a layer of household sponge topped with a layer of cotton-wool, both moistened in distilled water. In the free-choice test, leaf disks (diameter 3 cm) of the different genotypes evaluated were placed in pairs (one pair per arena) onto the cotton wool layer with abaxial side up. Leaf disks in each pair were connected each other using a transparent plastic coverslip (18 × 18 mm). After that, six 10-day-old adult

T. urticae females collected from the colony were transferred into the center of the coverslip under a stereomicroscope (Nikon SMZ745T), allowing for free choice and mite access to both leaf disks. Because there were seven treatments, a full replication comprised 21 pairwise combination of genotypes (i.e., 21 arenas). Five full replications, totaling 105 petri dishes, were used, in a completely randomized design. After 24 h, the number of mites on each leaf disk were counted, and used to calculate the mite preference (%) for the respective genotype.

In the no-choice test, leaf disks (diameter 3 cm) of each genotype were placed separately onto the cotton wool layer, with abaxial side up, into the arenas. Six 10-day-old adult *T. urticae* female mites collected from the colony were transferred to each leaf disk under a stereomicroscope, and kept for 24 h. After that period, the number of eggs laid on each leaf disk was recorded. This test was conducted in a completely randomized design with 10 replications per genotype, comprising a total of 210 arenas. For both tests, the arenas were kept in a walk-in chamber at 25 ± 2°C; 70 ± 10% relative humidity, and photoperiod of 12L:12D hours.

In addition, another no-choice experiment was performed in order to evaluate the total distance traveled by the mite on leaflets using Weston and Snyder's (1990) methodology. Leaflets (replications) from each genotype were sampled and fixed with abaxial side up on polystyrene sheets, using a metallic thumbtack (diameter 10 mm). Seven tomato genotypes (treatments) with 20 replications each were tested in a completely randomized design. Ten 6-day-old females were transferred onto each thumbtack, using a fine paint brush. After 10, 20, 40, and 60 min, the distances traveled by the females on the leaflet surface were recorded. The mean distances traveled by the mites after 60 min were determined for each genotype. This test is based on the assumption that lower distances covered by the mites indicate higher levels of mite repellence (e.g., negative effects on movement behavior).

Biological Parameters of *Tetranychus urticae* on Tomato Genotypes

This study comprised two experiments. In the first, the incubation period and viability of the eggs, the duration and viability of young stages (larvae, protonymph, and deutonymph) and the longevity of adults were measured for the seven genotypes tested. Six leaf disks (diameter 2 cm) of each genotype, with abaxial side up, were equidistantly distributed into arenas (petri dishes, diameter 10 cm) coated inside with a layer of household sponge topped with a layer of cotton-wool, both moistened in distilled water. A 10-day-old *T. urticae* female + male pair was transferred and kept for 24 h onto each leaf disk; after that, the mites and eggs (except one), were carefully removed with a fine artist's brush under a stereomicroscope, leaving one single egg per disk. Eggs, young stages and adults were daily observed to determine the parameters previously cited. Each set of six leaf disks within an arena was considered one replication. Altogether, 10 replications per treatment were used, in a randomized complete design.

In a second experiment, mite fecundity rate was evaluated. Ten leaf disks (diameter 2 cm) of a same tomato genotype, with abaxial side up, were equidistantly placed into plastic boxes (11 × 11 × 3.5 cm) coated inside with a layer of household sponge topped with a layer of cotton-wool, both moistened in distilled water. A 10-day-old *T. urticae* female was transferred onto each leaf disk, and was observed daily for 10 days to record the total number of eggs laid. During this period, the leaf disks were replaced by new disks of the same genotype every 2 days keeping the same female on the new disk.

Each plastic box with ten leaf disks of the same genotype, was considered a replication. Ten replications distributed in a completely randomized design were used for each of the seven genotypes tested.

Altogether, 60 and 100 observations (leaf disks) were taken per genotype respectively in the first and second experiments.

Both bioassays were carried out in a BOD incubator chamber at 25 ± 2°C; 70 ± 10% relative humidity, and photoperiod of 12L:12D.

Statistical Analyses

Data were previously tested using Bartlett's test to check for homogeneity of variance ($p < 0.05$), and then transformed to $(x + 0.5)^{1/2}$ when necessary to fulfill the pre-requisites of analysis of variance (ANOVA). The mean number of trichomes and data related to behavioral and biological parameters of the mites were submitted to ANOVA, and treatment means were compared by Tukey test ($p < 0.05$). Data of *T. urticae* preferences (%) from the free-choice test were compared using Pearson's Chi-Square test (χ^2). The types and densities of trichomes and ZGB contents were submitted to Pearson's correlation analysis and compared by the Student's *t*-test using Microsoft Excel® program. The biological and behavioral parameters evaluated were submitted to similarity grouping using the cluster analysis according to Linkage's method. Statistical analyses were performed using the Sisvar® program (Ferreira, 2011).

RESULTS

Trichomes and Correlations With Zingiberene Content

Densities of type VIII non-glandular trichomes and types IV/VI glandular trichomes varied among the genotypes in both abaxial and adaxial leaflet surfaces (**Table 1**). The highest non-glandular trichome density was observed in *S. lycopersicum* cv. Redenção, followed by the genotype RVTZ-09 (low ZGB content); in contrast, no non-glandular trichomes were found on wild accession (*S. habrochaites* var. *hirsutum* PI-127826) (**Table 1** and **Figures 1A,B**), and very low densities in the high ZGB genotypes.

On the other hand, the wild accession PI-127826 and genotypes selected for high ZBG content (RVTZ-79, RVTZ-142, and RVTZ-331) presented high densities of types IV and VI glandular trichomes, on both surfaces, when compared to *S. lycopersicum* cv. Redenção (in which no glandular trichomes were observed) and the low ZGB genotype RVTZ-09 (**Table 1**). Densities of both types IV and VI glandular trichomes were significantly and positively correlated with ZGB content ($r = 0.81$, $p < 0.01$; and $r = 0.80$, $p < 0.05$, respectively) (**Table 1**).

TABLE 1 | Zingiberene content (ZGB) and mean number (± SE) of glandular (types IV and VI) and non-glandular (type VIII) trichomes per mm^2 present on abaxial (Ab) and on adaxial (Ad) surfaces of leaflets obtained from different tomato genotypes.

Genotype	ZGB content[1]	Glandular trichome type IV[2]			Glandular trichome type VI[2]			Non-glandular trichome type VIII[2]	Glandular types IV and VI (Ab + Ad)[2]
		Ab	Ad	Total	Ab	Ad	Total	Total	
S. lycopersicum (cv. Redenção)	0.084	0.0 ± 0.0d	0.0 ± 0.0d	0.0 ± 0.0d	0.0 ± 0.0d	0.0 ± 0.0d	0.0 ± 0.0d	84.5 ± 1.0a	0.0
S. habrochaites var. *hirsutum* (PI-127826)	1.099	20.7 ± 1.1bc	39 ± 2.7a	59.7 ± 1.4ab	23.2 ± 2.7a	4.0 ± 1.2ab	27.2 ± 3.0a	0.0	87.0 ± 4.0a
F1 plant (Redenção × PI-127826)	0.328	24.0 ± 1.3b	12.2 ± 0.9bc	36.2 ± 1.1c	6.0 ± 0.9b	4.5 ± 0.6a	10.5 ± 1.2bc	0.2 ± 0.2cd	46.7 ± 1.4b
RVTZ-09 (= Low)	0.247	8.0 ± 2.7c	4.7 ± 0.0c	12.7 ± 2.5d	0.0 ± 0.0d	1.5 ± 1.0ab	1.5 ± 0.9cd	13.5 ± 2.1b	14.2 ± 2.0c
RVTZ-79 (= High)	0.715	35.7 ± 6.2ab	8.2 ± 1.1b	44.0 ± 7.1bc	7.7 ± 1.1b	0.7 ± 0.5ab	8.5 ± 1.0bcd	5.7 ± 0.2c	52.5 ± 7.9b
RVTZ-142 (= High)	0.813	33.5 ± 6.5ab	16.5 ± 1.3b	50.0 ± 7.3bc	7.5 ± 1.3b	4.5 ± 2.9ab	12.0 ± 4.1b	3.5 ± 0.6cd	62.0 ± 4.7b
RVTZ-331 (= High)	0.746	51 ± 7.4a	35.2 ± 0.9a	86.2 ± 7.8a	1.5 ± 0.9c	2.7 ± 1.0ab	4.2 ± 1.6bcd	0.2 ± 0.2cd	90.5 ± 7.4a
Correlation coefficient with ZGB (r)		–	–	0.81**	–	–	0.80*	–0.66^{ns}	0.90**
CV (%)		17.5	16.9	11.8	16.6	35.5	20.6	9.1	9.4

Means followed by the same lowercase letter within a column do not differ significantly by the Tukey test ($p < 0.05$).
*^{ns}Non-significant,*and **Significant different by Student's t-test ($p < 0.05$ and $p < 0.01$, respectively).*
[1]Zingiberene content determined at 270 nm (see section Materials and Methods).
[2]Original data presented [for analysis, data were transformed in $(x + 0.5)^{1/2}$].

Free- and No-Choice Tests

In the free-choice test, behavior of *T. urticae* females differed among the genotypes tested (**Figure 2**). Mites had significantly higher preference toward *S. lycopersicum* cv. Redenção, when paired with all other genotypes evaluated: over 60% of preference, reaching 100% when paired to the wild accession PI-177826. PI-177826 and F_1 plant were significantly the least preferred ($p < 0.01$) by the mites at all genotype pairs assessed, even when compared to genotypes selected for high ZGB content. In addition, no statistical difference was observed between wild accession and F_1 plant, indicating that F_1 plant mite resistance level is similar to that of the resistant check treatment (**Figure 2**).

Genotypes selected for high ZGB content (RVTZ-79, RVTZ-142, and RVTZ-331) remained in an intermediary position between the least preferred genotypes PI-127826/F1 and the most preferred *S. lycopersicum* cv. Redenção/RVTZ-09 (low ZGB content). This latter genotype, showed similar responses to *S. lycopersicum* cv. Redenção when paired with genotypes rich in ZGB; therefore, it was also considered susceptible to the mite (**Figure 2**).

The no-choice test showed that the fecundity (eggs laid during 24 h) was reduced on genotypes with high ZGB content: (PI-127826, RVTZ-79, RVTZ-142, RVTZ-331, and F1) when compared to low-ZGB genotypes *S. lycopersicum* cv. Redenção and RVTZ-09. Fecundity in the high ZGB genotypes was ca. ≥ 4 times lower than in low ZGB genotypes (**Table 2**).

Significant effects were observed on the movement behavior of the *T. urticae* released on the different tomato genotypes. Females released on leaflets of *S. lycopersicum* cv. Redenção were able to travel a significant higher distance (35 mm) after 60 min of evaluation than all other genotypes (<18 mm), except the low ZGB genotype RVTZ-09, which did not differ significantly from cv. Redenção (**Table 2**). The lower distances covered by the mite on genotypes with high ZGB content, was associated the presence of glandular trichomes (IV and VI), which were absent on *S. lycopersicum* cv. Redenção, and present at low density on RVTZ-09 (**Table 1**).

Biological Parameters of *Tetranychus urticae* on Tomato Genotypes

The duration of egg incubation on genotypes with high ZGB content, RVTZ-142 (4.8 days), wild accession PI-127826 (4.5 days) and RVTZ-79 (4.3 days) tended to be significantly longer compared to genotypes with low ZGB content, (cv. Redenção and RVTZ-09). However, the viability of eggs in all genotypes evaluated was high (≥98%) (**Table 3**).

The nymph stage was only completed in mites placed either on *S. lycopersicum* cv. Redenção or RVTZ-09; on both, all nymphs evaluated reach adulthood in a similar time (11.9 and 11.8 days, respectively), and adult longevities were also similar. In genotypes with high ZGB content (PI-127826, RVTZ-79, RVTZ-142, and RVTZ-331) and in F_1 plants mites did not

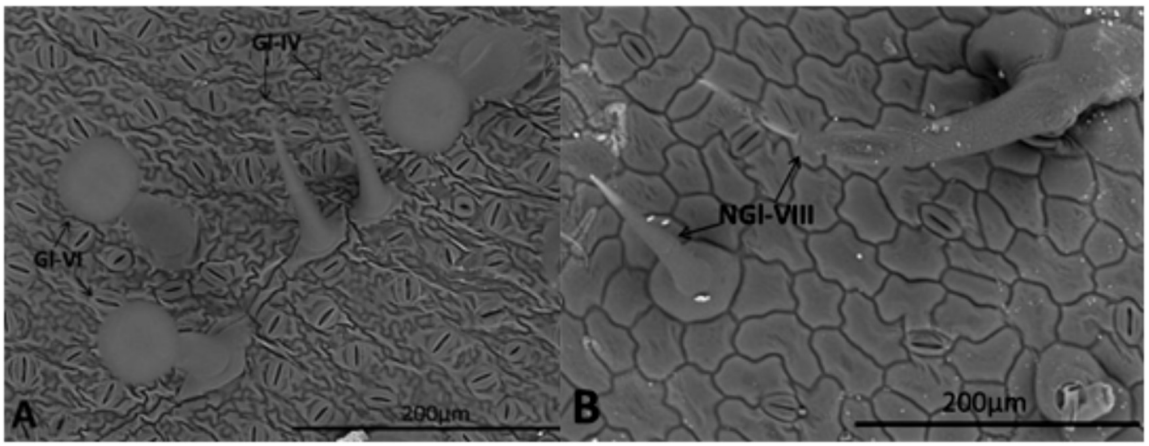

FIGURE 1 | Leaflet surface of *S. habrochaites* var. *hirsutum* **(A)** and *S. lycopersicum* cv. Redenção **(B)** obtained in a scanning electron microscope (SEM) showing different trichome types. Type IV (GI-IV) and VI (GI-VI) glandular trichomes **(A)**, and type VIII (NGI-VIII) non-glandular trichome **(B)** according to Luckwill (1943) and Toscano et al. (2001) classification.

TABLE 2 | Mean number (± SE) of eggs laid on leaf disks from different tomato genotypes during first 24 h of the no-choice test, and total distance traveled (mm) by the mites on the abaxial leaflet surface of the genotypes after 60 min.

Genotypes	Mean (± SE) of eggs laid[1]	Total distance traveled (mm)
S. lycopersicum (cv. Redenção)	19.1 ± 2.2a	35.0 ± 4.2a
S. habrochaites var. *hirsutum*	2.2 ± 1b	10.0 ± 2.1b
F_1 (Redenção × PI-127826)	2.9 ± 0.6b	9.9 ± 2.3b
RVTZ-09 (= Low)	17.5 ± 1.7a	21.0 ± 5.3ab
RVTZ-79 (= High)	3.7 ± 0.9b	16.2 ± 2.5b
RVTZ-142 (= High)	3.4 ± 1.0b	18.2 ± 3.2b
RVTZ-331 (= High)	4.4 ± 1.3b	13.7 ± 1.9b
CV (%)	29.2	38.0
F	23.9**	7.9**

Means followed by the same lowercase letter within a column do not differ significantly by the Tukey test ($p < 0.05$).
***Significant different by F-test ($p < 0.01$).*
[1]Original data presented [for analysis, data were transformed in $(x + 0.5)^{1/2}$].

reach adulthood, because the mortality of the nymphs was total in the third instar. On those genotypes, nymphal stage had a short time span until death, ranging from 5.4 to 6.1 days – a span corresponding to the second nymphal stage (**Table 3**).

The fecundity rate was also adversely affected by the tomato genotypes with high ZGB content (fecundity rate from 0.2 to 0.8 eggs/female/day), whereas on *S. lycopersicum* cv. Redenção and RVTZ-09 the mites laid significantly more eggs (3.8 and 3.7 eggs/female/day, respectively) (**Table 3**).

Cluster Analysis

Cluster analysis of the tomato genotypes showed a clear contrasting relationship between the genotypes evaluated (**Figure 3**), and two clear groups were formed: one group comprised by *S. lycopersicum* cv. Redenção and RVTZ-09 (low ZGB content), and a second group formed by the genotypes with high ZGB content (RVTZ-79, RVTZ-142, RVTZ-331, and PI-127826) and the F1. Within-group differences between Redenção and RVTZ-09 could be at least partly explained by the slightly higher ZGB concentration in the latter (**Table 1**). However, ZGB content in RVTZ-09 is only slightly lower than in the F_1 (**Table 1**), indicating that factors other than ZGB content in RVTZ-09 may account for its dissimilarity with Redenção.

All genotypes selected for high ZGB content might be considered close to the resistant check (wild accession PI-127826), as shown in the cluster analysis (**Figure 3**). The genotypes selected for high ZGB content formed two distinctive pairs strongly related each other __ first pair composed by RVTZ-331 and PI-127826, and the second one by RVTZ-79 + RVTZ-142.

The presence of glandular trichomes on genotypes selected for high ZGB content (**Table 1**) was strongly and negatively associated with the behavioral and biological parameters of the mite, except with egg incubation period (**Table 3**), which was the parameter less affected by the genotypes rich in ZGB. Thus, the results indicate that the presence of glandular trichomes lead to negative effects on mite behavior and biology.

TABLE 3 | Biological parameters [duration (days ± SE) of egg incubation and nymphal development time, egg and nymph viability (%), longevity of adults, and fecundity rate] of *Tetranychus urticae* kept on leaf disks of different tomato genotypes.

Genotype	Egg		Nymph		Adult	
	Incubation (days)[1]	Viability (%)	Duration (days)[1]	Viability (%)	Longevity (days)	No. eggs/female/day[1]
S. lycopersicum (cv. Redenção)	3.4 ± 0.1c	100	11.9 ± 0.2a	100	12.7 ± 0.5a	3.8 ± 0.2a
S. habrochaites var. *Hirsutum* (PI-127826)	4.5 ± 0.3ab	100	5.8 ± 0.3[2]b	0.0[3]	–	0.2 ± 0.1c
F_1 plant (Redenção × PI-127826)	3.8 ± 0.2bc	100	6.1 ± 0.2[2]b	0.0[3]	–	0.2 ± 0.1c
RVTZ-09 (= Low)	3.4 ± 0.1c	100	11.8 ± 0.1a	100	12.0 ± 0.5a	3.7 ± 0.2a
RVTZ-79 (= High)	4.3 ± 0.2ab	100	5.5 ± 0.2[2]b	0.0[3]	–	0.6 ± 0.1b
RVTZ-142 (= High)	4.8 ± 0.2a	100	5.4 ± 0.3[2]b	0.0[3]	–	0.8 ± 0.2b
RVTZ-331 (= High)	3.8 ± 0.2bc	98.3	5.8 ± 0.2[2]b	0.0[3]	–	0.4 ± 0.1bc
CV (%)	32.6		17.6		31.1	14.9
F	9.9**		298.7**		–	99.0**

Means followed by the same lowercase letter within a column do not differ significantly by the Tukey test ($p < 0.05$), except for longevity of adults, in which was applied Student's t-test ($p < 0.05$) to compare between S. lycopersicum cv. Redenção and RVTZ-09.
***Significant different by F-test ($p < 0.01$).*
[1]Original data presented [for analysis, data were transformed in $(x + 0.5)^{1/2}$].
[2]Nymph development time up to 2nd stadium.
[3]Total mortality of nymphs in the 3rd stadium.

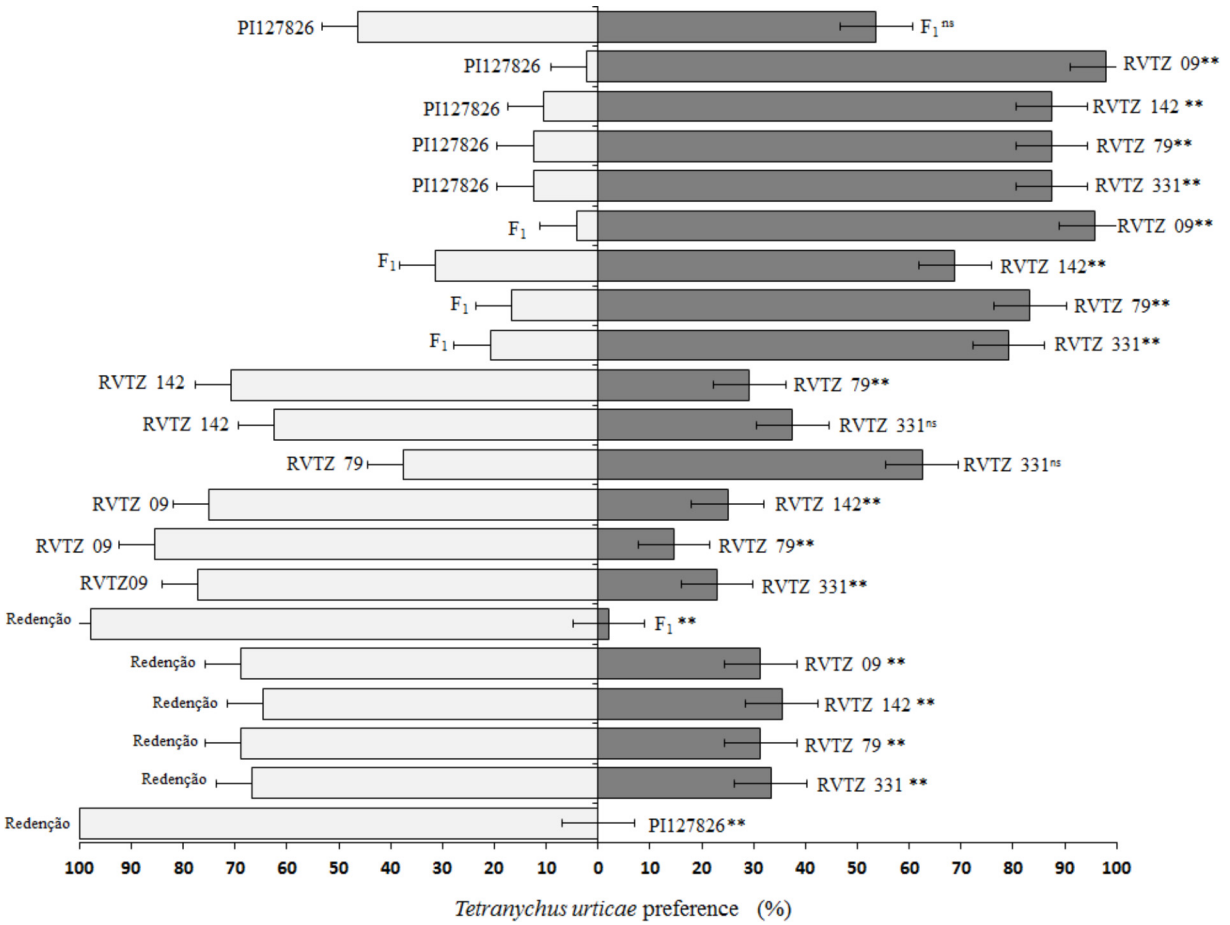

FIGURE 2 | Preference ratios (%) of *Tetranychus urticae* on the 21 different pairwise combinations of tomato genotypes during the free-choice test. Redenção = *S. lycopersicum* cv. Redenção, PI127826 = *S. habrochaites* var. *hirsutum* accession PI-127826, F_1 = F_1 plant (PI-127826 × cv. Redenção), RVTZ-09 = RVTZ 2011-09 (low ZGB content), and RVTZ-79, RVTZ-142, and RVTZ-331 = RVTZ2011-79, RVTZ2011-142, and RVTZ2011-331, respectively (genotypes with high ZGB content). [ns] non-significant different, and ** significant different ($p < 0.01$) using Pearson's Chi-Square test (χ^2).

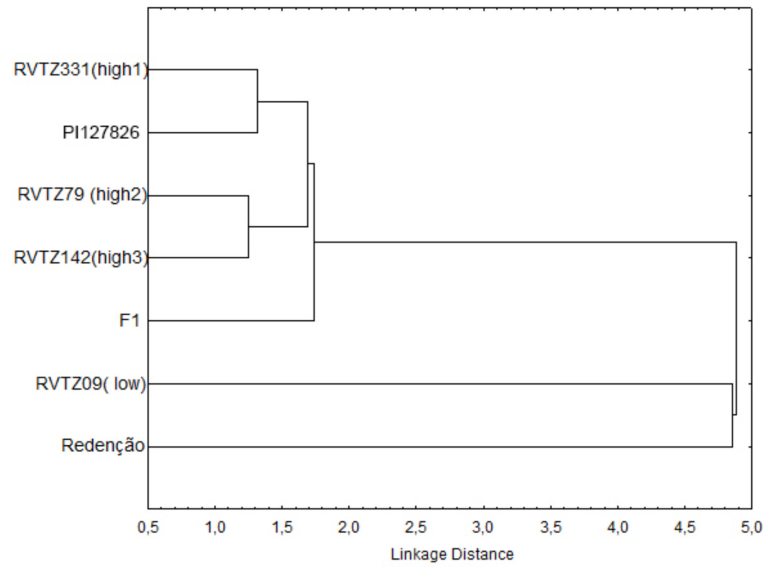

FIGURE 3 | Relationships (dendrogram) between seven tomato genotypes with different contents of zingiberene using hierarchical cluster analysis (Linkage's method). The cladogram has been based on number and type of trichomes, zingiberene content, and behavioral and biological parameters of *Tetranychus urticae*.

DISCUSSION

Plants that produce and release chemical compounds (allelochemicals) affecting arthropod behavior and biology, may express resistance through both antixenosis (non-preference) and/or antibiosis (War et al., 2012). In tomato, resistance has been extensively reported in the literature as mediated by different allelochemicals against arthropod pests such as mites (Gonçalves et al., 2006; Resende et al., 2008; Lucini et al., 2015; Lima et al., 2016), whiteflies (Freitas et al., 2002; Silva et al., 2009; Lima et al., 2016), and lepidopterans (Maluf et al., 2010; Dias et al., 2013; Lima et al., 2015).

In the behavioral test (free-choice test), our results indicated lower preference of *T. urticae* toward the wild ZGB-rich genotype (PI-127826), followed by the F_1 plant and genotypes selected for high ZGB content (RVTZ-79, RVTZ-142, and RVTZ-331). In contrast, *S. lycopersicum* cv. Redenção and RVTZ-09 (low ZGB content) were the most preferred genotypes by *T. urticae*. Lima et al. (2016) evaluating these same ZGB-rich genotypes observed a strong repellency effect against *T. urticae* and whitefly *Bemisia tabaci* (Genn.). The results indicated that non-preference is a mechanism by which ZGB-rich genotypes express resistance to the two-spotted spider mite.

Nonetheless, the genotypes rich in ZGB also presented the antibiosis type of resistance, indicated by their deleterious effects on biological parameters of *T. urticae*. In general, with ZGB-rich genotypes there was an increase of the egg incubation period, total mortality of nymphs, and a strong decrease in the fecundity rate. The adverse effect of tomato genotypes with high allelochemical contents on arthropod biology parameters is also documented in the literature for several species (Eigenbrode and Trumble, 1993; Azevedo et al., 2003; Fancelli et al., 2005; Moreira et al., 2009; Silva et al., 2013). Evaluating tomato genotypes with high content of the allelochemical acylsugar, Lucini et al. (2015) observed that they also presented both mechanisms non-preference and antibiosis against *T. urticae*, a situation analogous to our current findings for ZGB-rich genotypes. No similar reports on the deleterious effect of high-ZGB tomato genotypes on *T. urticae* mite survival, fecundity and longevity could be found in the literature.

The high density of glandular trichomes and its associated high ZGB content were therefore responsible for adverse effects upon both the behavior and the biology of *T. urticae* mites, indicating that both resistance mechanisms, non-preference and antibiosis, are present. In addition, *S. lycopersicum* cv. Redenção presented a high number of non-glandular trichomes, which did not cause negative effects on mite behavior and biology.

Other studies have demonstrated the effect of glandular trichomes present on *S. habrochaites* var. *hirsutum* on *Tetranychus* spp. (Carter and Snyder, 1985; Maluf et al., 2001; Freitas et al., 2002). Several other studies have also reported negative effects on behavior and biology of arthropod pests associated with glandular trichomes present in wild tomato accessions and in genotypes selected for high allelochemical contents (Freitas et al., 2002; Maluf et al., 2007; Alba et al., 2009; Lucini et al., 2015).

In this study, we demonstrated that ZGB content was positively correlated with both types of glandular trichomes (IV and VI), although the density of type IV was much higher than that type VI trichomes. These results agree with reports in the literature (Freitas et al., 2002; Gonçalves et al., 2006). Allelochemicals other than ZGB have been associated with glandular trichomes; e.g., acylsugar stored in type IV glandular

trichomes of *Solanum pennellii* Correll accession LA-716 (Lucini et al., 2015) and *Solanum pimpinellifolium* accession TO-937 (Alba et al., 2009).

The results support that genotypes selected for high ZGB content (RVTZ-79, RVTZ-142, and RVTZ-331) are potential sources of resistant genes against *T. urticae* mites and possibly against other pests, in tomato breeding programs.

AUTHOR CONTRIBUTIONS

JdO designed and performed the experiments, carried out the statistical analyses, and wrote the project and the manuscript with support of TL and CN. JdR and WM devised the project, the main conceptual ideas and provided the plants and laboratory apparatus. TL and CN contributed to the design and implementation of the research, to the analysis of the results, and to the writing of the manuscript. CN were involved in planning and supervised the work. RdLF and IdL performed the plants and acari experiments with support of JdO and contributed in discussion of the manuscript in consultation with JdO. All authors provided critical feedback, helped in discussion, and contributed to the final manuscript.

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Resistance of Lima Bean (*Phaseolus lunatus* L.) to the Red Spider Mite *Tetranychus neocaledonicus* (Acari: Tetranychidae)

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The red spider mite, *Tetranychus neocaledonicus* (Acari: Tetranychidae) can be an important pest on lima bean (*Phaseolus lunatus* L.). Thus, the objective of this work was to assess the antibiosis and antixenosis effects of lima bean genotypes on *T. neocaledonicus*, through the evaluation of performance parameters as well as the host preference for food and oviposition. Nine lima bean genotypes from the Active Bank of Germplasm of the Federal University of Piauí – BGP / UFPI were screened. To assess antibiosis parameters, eggs of *T. neocaledonicus* were individually placed on leaf disks of each genotype. The period and survival of the different stages of development (larvae, protonymph, deutonymph and adult), pre-oviposition, oviposition and post-oviposition period, longevity and fecundity of females were evaluated, and fertility life table parameters were calculated. In choice tests, adult females of *T. neocaledonicus* were used. The numbers of mites and eggs were counted for each genotype. The protonymph, egg-adult, longevity and oviposition period, fertility life table parameters, as well as the food and oviposition preference were affected by lima bean genotypes. We found that some genotypes reduced adult female longevity, increased the larval and egg-adult period, decreased oviposition period, negatively affected the fertility life table parameters, reducing the net reproductive rate (R_o), the intrinsic rate of increase (r_m) and the finite rate of increase (λ), while increasing the population doubling time (DT), exhibiting a reliable antibiosis effect upon *T. neocaledonicus*. Nevertheless, these same genotypes were the most preferred for food and oviposition. By contrast, some other genotypes reduced the adult female longevity and oviposition period, elongated

the larval period and affected fertility life table parameters, demonstrating an antibiosis effect upon *T. neocaledonicus.* Moreover, these other genotypes were among the less preferred for food and oviposition, exhibiting an additional antixenosis effect. Thus, our results demonstrate that the genotypes of lima bean may present distinct levels of resistance to *T. neocaledonicus*, and this resistance may be an important tool for Integrated Pest Management. This is one of the first studies aiming to describe mite resistance sources in lima bean.

Keywords: antibiosis, antixenosis, population growth, host choice, *Phaseolus lunatus*, Tetranychidae

INTRODUCTION

The use of host plant resistance is widely known as an efficient, economical, ecological and socially advantageous control method within Integrated Pest Management (IPM) programs (Stenberg, 2017). Plant resistance against herbivores is composed of two parts: antibiosis, affecting the pest's fitness, resulting in reduced population growth, longevity and higher mortality; and antixenosis, the non-preference behavior of the pest for feeding, oviposition or shelter (Dehghan et al., 2009; Silva et al., 2011; Stenberg and Muola, 2017).

Some studies aiming to select host plant resistance to pest mites have been carried out in recent decades. Sources of resistance to *Tetranychus evansi* Baker & Pritchard (Acari: Tetranychidae) were investigated in tomato varieties (Silva et al., 1992); strawberry and vines sources were tested for *T. urticae* Koch (Acari: Tetranychidae) resistance (Lourenção et al., 2000; Valadão et al., 2012; Breda et al., 2016) evaluated sweet pepper genotypes' resistance to the broad mite, *Polyphagotarsonemus latus* (Banks) (Acari: Tarsonemidae). Silva et al. (2011) evaluated Rubber tree clones' resistance to Eriophyiidae and Tenuipalpidae mites, among other studies. Here, for the first time, we study the resistance of lima bean (Fabaceae) genotypes to pest mites.

Lima bean has a significant economic and social importance, with features of tolerance to drought and heat, which justify its economic exploitation, primarily in family farming, as one of the main sources of income and livelihood, contributing to food security (Baudoin, 1988; Vieira, 1992; Santos et al., 2002). Therefore, several studies aiming to increase the genetic knowledge about lima bean have been carried out, through the collection of traditional and wild genotypes, molecular characterization and maintenance of gene banks (EMBRAPA, 2016).

In addition, several species of Tetranychidae mites are reported in association with lima bean, including *T. urticae*, *Eutetranychus banksi*, and *T. neocaledonicus* (Mendonça et al., 2011; Silva and Gondim, 2016; Gomes Neto et al., 2017). Throughout the world, *T. neocaledonicus* can be found on more than 400 different host plants, presenting itself as a species of considerable economic importance for several crops, with a wide distribution in the intertropical zone (Gutierrez and Zon, 1973; Bonato and Gutierrez, 1999).

The present study aimed to evaluate host plant resistance aspects of nine genotypes of lima bean from the Active Bank of Germplasm of the Federal University of Piauí – BGP / UFPI to the red mite, *T. neocaledonicus*.

MATERIALS AND METHODS

Phaseolus lunatus L. (Fabaceae) Genotypes

Nine lima bean genotypes from the Active Bank of Germplasm of the Federal University of Piaui – BAGF / UFPI were used. The genotypes and their features are included in **Table 1**. The genotypes were chosen based on widespread use by farmers and prior studies of agronomic and molecular features, as well as disease resistance developed in the region.

Tetranychus neocaledonicus André (Acari: Tetranychidae) Rearing

The mites were reared in the Phytotechnical Department of the Federal University of Piaui (UFPI), under greenhouse and laboratory conditions, on lima bean. Plants of the genotype UFPI-971-PI were grown in plastic containers of 3.8 liters in the greenhouse. Thirty days after emergence, the plants were infested with *T. neocaledonicus* females. Weekly, during the whole period of bioassays, new infestations were made through direct contact between plants infested with mites and uninfested plants. Under laboratory conditions, leaf disks of lima bean UFPI-971-PI were infested on filter paper moistened by a water-saturated sponge. The leaf disks were surrounded by hydrophilic cotton wool to prevent mite escape. The bioassays and *T. neocaledonicus* rearing were carried out with temperature and relative humidity monitored daily by a thermohygrograph, and 12 h photoperiod.

TABLE 1 | Genotypes of lima bean from the Active Bank of Germplasm of the Universidade Federal do Piauí- BGP / UFPI used in the bioassays, and their features.

Genotype	Origin	Common name	Weight* (g)
UFPI-822	Puxinanã – PB	Coquinho bean	38.81
UFPI-883	Esperantina – PI	White bean	48.49
UFPI-887	Novo Oriente – CE	Mestiça bean	69
UFPI-888	Tianguá – CE	White bean	56.64
UFPI-891	Uruçuí – PI	White bean	56.64
UFPI-908	Pedra Branca – CE	Butter bean	49.62
UFPI-909	Crato – CE	White bean	57.84
UFPI-915	Miguel Alves – PI	White bean	57.91
UFPI-971	Barras – PI	Boca de Moça bean	64.57

**Weight of 100 g of seeds.*

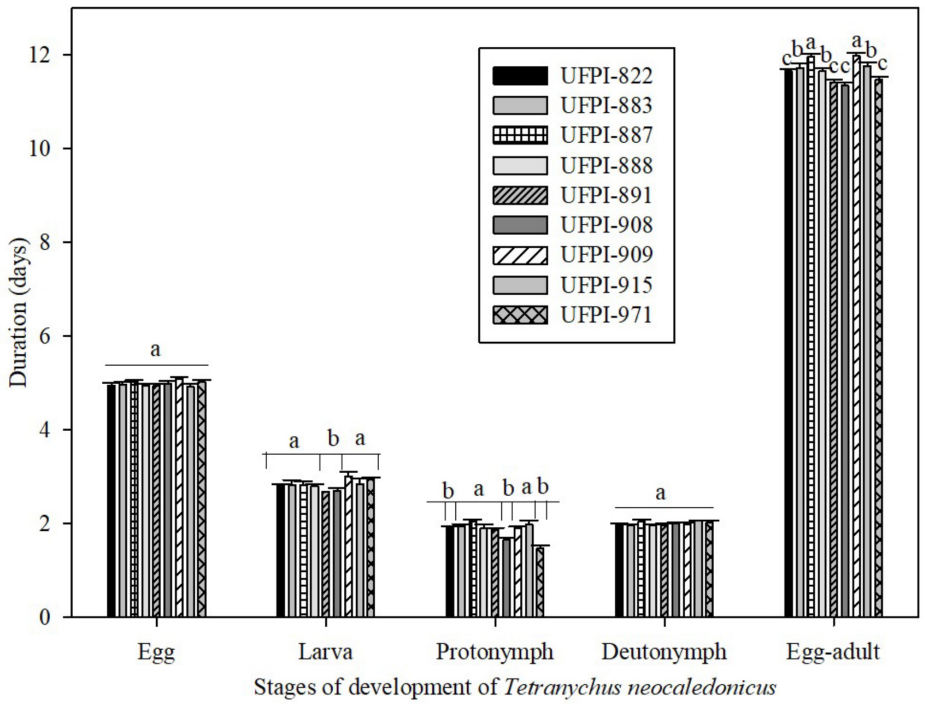

FIGURE 1 | Effects lima bean genotypes on the development period (days; Means ± SE) of *Tetranychus neocaledonicus* females. 25 ± 1°C, RH 70 ± 10% and 12 h photophase. Means followed by the same letters in stage of development do not differ statistically by the Scott-Knott at 5% probability.

Antibiosis of Lima Bean Genotypes

To assess the antibiosis effects of the nine genotypes of lima bean on *T. neocaledonicus*, the performance aspects of the red spider mite were evaluated. Leaf disks of 3 cm diameter, from 30-day-old lima bean plants, medium leaves, of each genotype, were kept on filter paper over a moistened sponge in Petri dish arenas. Three *T. neocaledonicus* adult females were left for an oviposition period of 16h in each disk. After this period, the females were removed, and one egg was kept per arena.

The following parameters were evaluated: incubation period (days) (period between egg oviposition and larval hatching), egg viability (%), period of development stage (larva, protonymph, deutonymph and adult) and egg-adult period. Two scorings were performed per day, every 12 h (7 a.m. and 7 p.m.) during larval stage. At the adult stage, the following parameters were daily evaluated: pre-oviposition (period prior to the first oviposition), oviposition and post-oviposition periods (days), female fecundity, fertility and longevity.

TABLE 2 | Effects of lima bean genotypes on the survival rate (%) and adult female longevity (days) of *Tetranychus neocaledonicus*. 25 ± 1°C, RH 70 ± 10% UR and 12 h photophase.

Genotypes	Survival rate (%)				Longevity
	Egg-larvae	Protonymph	Deutonymph	Egg-adult	
UFPI-822	95	94.74	88.89	80	39.47 ± 1.50 b
UFPI-883	100	95	89.47	85	41.30 ± 0.49 b
UFPI-887	100	95	89.47	85	43.25 ± 0.78 b
UFPI-888	100	95	94.44	85	47.90 ± 0.21 a
UFPI-891	96	95	94.74	90	41.02 ± 0.45 b
UFPI-908	100	100	100.00	95	47.55 ± 0.88 a
UFPI-909	100	95	89.47	85	43.55 ± 1.66 b
UFPI-915	100	95	94.74	90	47.25 ± 0.72 a
UFPI-971	100	95	94.74	90	44.32 ± 2.38b

**Means followed by the same letters in the columns do not differ statistically by Scott–Knott at 5% probability.*

TABLE 3 | Effects of lima bean genotypes on the preovipostion, oviposition and post-oviposition periods (days; means ± SE) of *T. neocaledonicus*. 25 ± 1°C, RH 70 ± 10% and 12 h photophase.

Genotypes	N[1]	Pre-oviposition ± SE	Oviposition ± SE	Post-oviposition ± SE[2]
UFPI-822	10	1.21 ± 0.12a	37.02 ± 0.60c	1.30 ± 0.42*a*
UFPI-883	11	1.10 ± 0.14a	39.02 ± 0.40b	1.20 ± 0.23*a*
UFPI-887	11	1.41 ± 0.10a	40.12 ± 0.30b	1.31 ± 0.33*a*
UFPI-888	12	1.51 ± 0.18a	45.19 ± 0.33a	1.29 ± 042*a*
UFPI-891	15	1.11 ± 0.04a	39.02 ± 0.44b	1.23 ± 0.19*a*
UFPI-908	13	1.23 ± 0.09a	45.02 ± 0.60a	1.22 ± 0.20*a*
UFPI-909	11	1.39 ± 0.14a	41.02 ± 0.60b	1.27 ± 0.42*a*
UFPI-915	14	1.36 ± 0.13a	45.22 ± 0.49a	127 ± 0.19*a*
UFPI-971	12	1.81 ± 0.18a	42.02 ± 0.47a	1.28 ± 0.42*a*

[1]*Number of observations;* [2]*Means followed by the same letters in the columns do not differ statistically by Scott–Knott at 5% probability.*

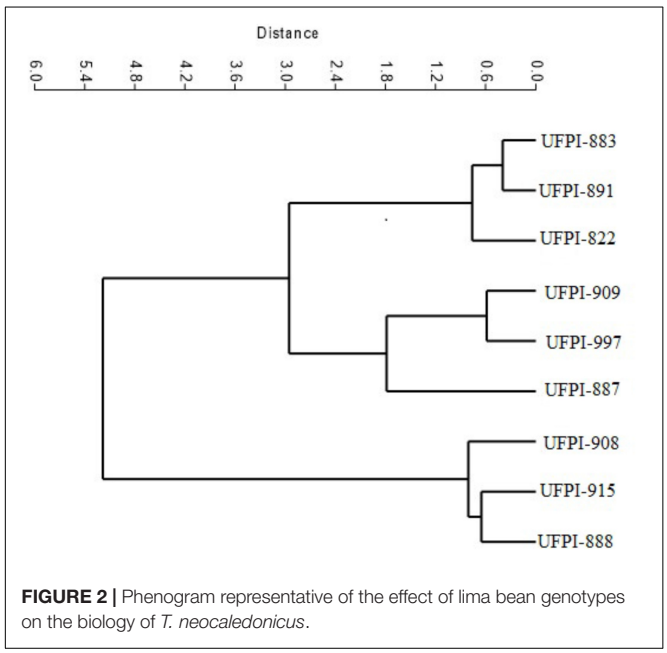

FIGURE 2 | Phenogram representative of the effect of lima bean genotypes on the biology of *T. neocaledonicus*.

Every 7 days the leaf disks were replaced, and the mites transferred with the aid of a fine brush. The experiment was kept in a BOD incubator (Bio-Oxygen Demand) with a temperature of 25 ± 1° C, relative humidity of 70 ± 10% and photophase of 12 h. A completely randomized design was used with nine treatments (lima bean genotypes) and 20 replicates. The data were $(x + 1)^{0.5}$ transformed to satisfy the ANOVA assumptions. The Scott and Knott grouping test was performed, using the SISVAR statistical program (Ferreira, 1998).

The cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), which groups individuals (lima bean genotypes) according to similarity (antibiosis and antixenosis effects on *T. neocaledonicus*), considering the parameters of incubation period, period of development stages (larva, protonymph, deutonymph), egg-adult period and longevity. The analysis was performed by the PAST software (Hammer et al., 2001).

For fertility life table parameters, the survival rate (l_x), specific fertility (m_x), net reproductive rate (R_o), intrinsic rate of increase (r_m), mean generation time (T), finite rate of increase (λ) and population doubling time (DT) were calculated to provide accurate data for the determination of host quality, thus helping the identification of possible resistance sources (Gomes Neto et al., 2017). The data were submitted to Analysis of variance with the Duncan test at 5% probability, by the statistical program SAS (SAS Institute, 2001).

Antixenosis of Lima Bean Genotypes

To assess the antixenosis effects of the nine genotypes of lima bean on *T. neocaledonicus*, food and oviposition choice tests were performed. Petri dishes of 15 cm diameter with filter paper over a moistened sponge were used as arenas. In the center of each arena, a plastic disk of 8 cm diameter was placed and surrounded by leaf disks of 3 cm diameter of each genotype. The leaf disks were placed equidistant from the center of the arena in contact with the plastic disk. Twenty adult females of *T. neocaledonicus* were released in the central plastic disk. After 1, 3, 6, 12, 24, and 48 h, the number of mites and eggs were evaluated. The bioassay was developed at 25 ± 1°C, 70 ± 10% RH and 12 h of photophase. The analysis was performed by the Scott and Knott (1974) grouping test at 5%, using the statistical program ASSISTAT.

RESULTS

Antibiosis of Lima Bean Genotypes

Regarding the antibiosis effect of lima bean on the development period of *T. neocaledonicus*, the nine tested genotypes did not affect the incubation (eggs) and the deutonymph period of the mite, with mean values of 4.98 and 1.81 days, respectively. However, the lima bean genotypes significantly affected the larval, protonymph and egg-adult periods (**Figure 1**).

Genotypes UFPI-891 and UFPI-908 significantly reduced the larval period of *T. neocaledonicus*, with means of 2.68 and 2.70 days. The highest value for larval period was observed on the UFPI-909 genotype (3.0 days), not

TABLE 4 | Fertility life table parameters of *T. neocaledonicus* (means ± SE) on lima bean genotypes. 25 ± 1 °C, RH 70 ± 10% and 12h photophase.

P[1]	UFPI-822	UFPI-883	UFPI-887	UFPI-888	UFPI-891	UFPI-908	UFPI-909	UFPI-915	UFPI-971
r_m	0.27 ± 0.03*a*	0.19 ± 0.013*b*	0.14 ± 0.01*c*	0.18 ± 0.02*b*	0.26 ± 0.03*a*	0.25 ± 0.03*a*	0.13 ± 0.06*c*	0.20 ± 0.032*b*	0.25 ± 0.02*a*
T	19.24 ± 0.17*a*	17.12 ± 0.009*b*	18.32 ± 0.17*b*	15.44 ± 0.11*c*	20.21 ± 0.21*a*	21.09 ± 0.09*a*	18.29 ± 0.33*b*	20.18 ± 0.19*a*	21.23 ± 0.31*a*
R_0	29.13 ± 0.67*a*	27.25 ± 0.53*a*	21.08 ± 0.13*c*	25.19 ± 0.29*b*	27.13 ± 0.37*a*	27.27 ± 0.49*a*	20.18 ± 0.36*c*	25.33 ± 0.27*b*	26.17 ± 0.67*a*
λ	1.30 ± 0.01*a*	1.20 ± 0.01*b*	1.12 ± 0.02*c*	1.19 ± 0.01*b*	1.22 ± 0.32*b*	1.28 ± 0.03*a*	1.14 ± 0.02*c*	1.21 ± 0.037*b*	1.29 ± 0.018*a*
DT	4.58 ± 0.06*b*	4.59 ± 0.18*b*	5.33 ± 0.10*a*	3.43 ± 0.012*c*	4.14 ± 0.017*b*	3.42 ± 0.014*c*	5.32 ± 0.12*a*	4.65 ± 0.13*b*	4.69 ± 0.06*b*

[1] *P: Fertility life table parameters; r_m: Intrinsic rate of increase; T: Mean generation time; R_0: Net reproductive rate; λ: Finite rate of increase and DT: Population doubling time. Means followed by the same letters in the rows do not differ statistically by the Scott–Knott test, at 5% probability*

differing from the other genotypes. Genotypes UFPI-908, UFPI-822 and UFPI-971 significantly reduced the protonymph period (1.65, 1.57 and 1.47 days). The longest egg-adult periods were observed on genotypes UFPI-909 and UFPI-887 (11.98 and 11.96 days), significantly differing from the other genotypes, while the shortest egg-adult period occurred for UFPI-908 (11.35 days), although not differing from UFPI-882, UFPI-891, UFPI-908, and UFPI-971.

None of the lima bean genotypes affected egg-larval, protonymph, deutonymph or egg-adult survival rates. However, for genotypes UFPI-888, UFPI-908, and UFPI-915 a higher female longevity was observed, with mean values of 47.90, 47.55, and 47.25 days, respectively. However, UFPI-822 caused the lowest adult female longevity (39.47 days), although not differing from UFPI-883, UFPI-887, UFPI-891, UFPI-909, and UFPI-971 (**Table 2**).

Regarding the effect of lima bean upon *T. neocaledonicus* oviposition periods, genotypes UFPI-915, UFPI-888, UFPI-908, and UFPI-971 significantly lengthened the oviposition period, with values of 45.22, 45.19, 45.02 and 42.02 days, respectively. Among the genotypes, UFPI-822 significantly promoted the lowest oviposition period (37.02 days). Nevertheless, the lima bean genotypes did not affect pre and post-oviposition periods (**Table 3**).

The cluster analysis allowed the formation of three distinct groups of lima bean genotypes: the first group with genotypes UFPI-908, UFPI-915, and UFPI-888 (Group 1), the second group with genotypes UFPI-909, UFPI-887, and UFPI-971 (Group 2) and the third with genotypes UFPI-883, UFPI-891, and UFPI-822 (Group 3) (**Figure 2**). Among the tested parameters for *T. neocaledonicus*, the greatest influence on cluster analysis was adult female longevity. In the first group are the lima bean genotypes which provided the highest female longevity. In the

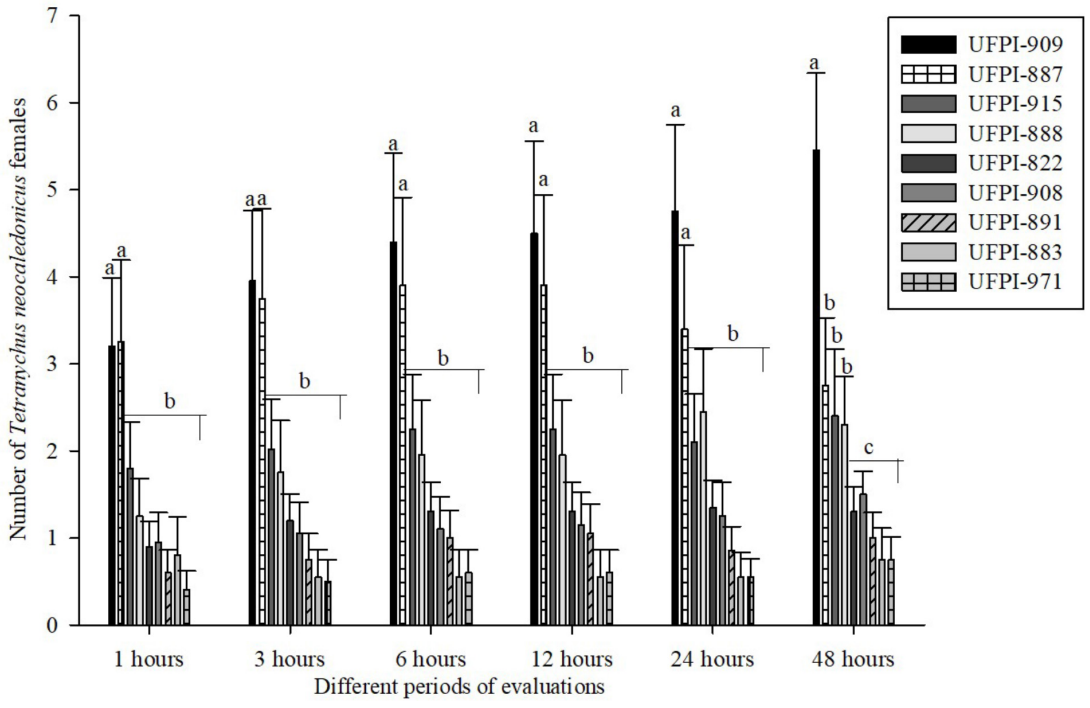

FIGURE 3 | Food preference of *T. neocaledonicus* adult female (Means ± SE) on lima bean genotypes after 1, 3, 6, 12, 24, and 48 h. Means followed by the same letters in stage in each time interval do not differ statistically by the Scott-Knott at 5% probability.

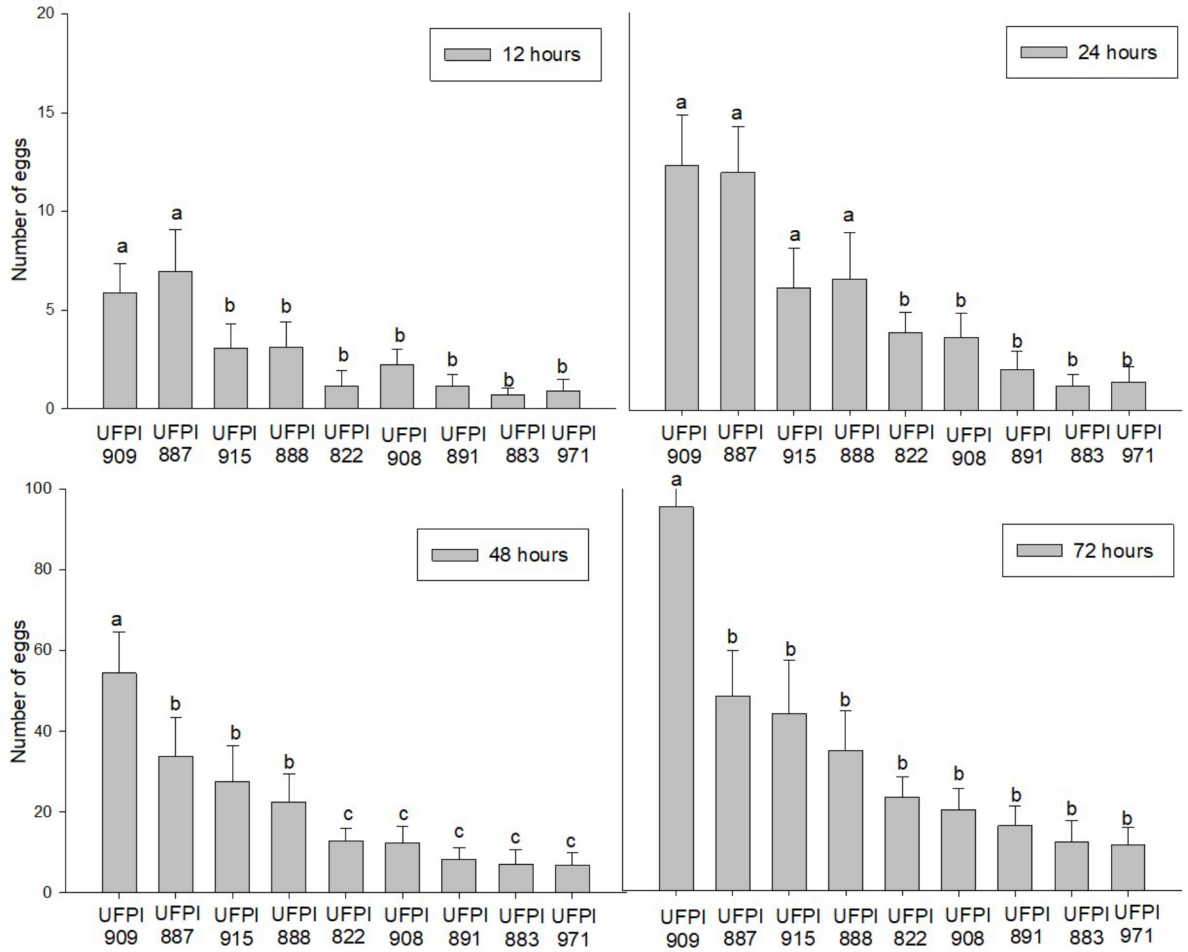

FIGURE 4 | Number of eggs (Means ± SE) of *T. neocaledonicus* on lima bean genotypes after 12, 24, 48, and 72 h. Columns with the same letter at each evaluation interval do not present statistically differences by the Scott-Knott at 5% probability.

second group, there are genotypes with intermediate values for female longevity and, in the third group, the genotypes with the lowest female longevity values.

Lima bean genotypes significantly affected the fertility life table parameters of *T. neocaledonicus* (**Table 4**). For the net reproductive rate (R_o) genotypes UFPI-887 and UFPI-909 presented the lowest values (21.08 and 20.18), significantly differing from the other genotypes. The mean generation time (T) ranged from 15.44 to 21.23 days for UFPI 888 and UFPI-971, with genotypes UFPI-882, UFPI-891, UFPI-908, UFPI-915, and UFPI-971 presenting significantly higher values. The intrinsic rate of increase (r_m) was significantly reduced by genotypes UFPI-909 and UFPI-887, with values of 0.13 and 0.14 individuals/female/day, and the population doubling time (DT) was significantly increased by genotypes UFPI-887 and UFPI-909, with values of 5.33 and 5.32 days, respectively.

Antixenosis of Lima Bean Genotypes

Regarding the antixenosis effect of lima bean genotypes on *T. neocaledonicus*, the choice tests for food preference demonstrated that host selection started 1 h after exposure to the lima bean genotypes. Genotypes UFPI-909 and UFPI-887 were the ones most chosen among the nine tested genotypes after 24 h of bioassay. After 48 h, UFPI-822, UFPI-908, UFPI-891, UFPI-883, and UFPI-971 were the least chosen among the nine lima bean genotypes (**Figure 3**).

The number of eggs was directly proportional to the time of exposure for the nine tested lima bean genotypes. However, UFPI-909 and UFPI 887 presented the highest number of eggs after 24 h, among the tested genotypes. On the other hand, UFPI-822, UFPI-908, UFPI-891, UFPI-883, and UFPI-971 presented the lowest number of eggs during the whole evaluation period, indicating a possible antixenosis effect for oviposition (**Figure 4**).

DISCUSSION

Overall, genotypes UFPI-887 and UFPI-909, group 2 in the cluster analysis, reduced adult female longevity, increased the larval and egg-adult period, decreased oviposition period and

negatively affected the fertility life table parameters, reducing the net reproductive rate (R_o), the intrinsic rate of increase (r_m) and the finite rate of increase (λ), while increasing the population doubling time (DT). Fertility life table parameters have been used successfully to determine host-plant quality and to identify sources of antibiosis resistance (Razmjou et al., 2009; Lin, 2013). For that, it is possible to determine that genotypes UFPI-887 and UFPI-909 exhibited a reliable antibiosis effect upon *T. neocaledonicus*. Nevertheless, these same genotypes were the most preferred for food and oviposition. According to Valladares and Lawton (1991), a poor link between host plant choice by the adult insect/mite and offspring performance has been widely observed and may be explained by several hypotheses, as host preference is often based on many factors, such as competition, microclimate, host density, size, age and chemical features, among others.

Based on the findings of the present study, genotypes UFPI-887 and UFPI-909 could be suggested as promising plants for trap cropping development strategies in IPM programs for *T. neocaledonicus*. Trap cropping is based on distinct herbivore preference among plant species, genotypes or crop stages (Hokkanen, 1991). The strategy's development may consist of offering the preferred genotype in space and time of the main crop, manipulating the pest mite population. Crop protection could be achieved by preventing the mite population from colonizing the main crop or by trapping them on a genotype with strong antibiosis, which could be colonized without affecting the main crop. Javaid and Joshi (1995) also reported that trap cropping might involve early planting of border strips of a genotype to attract the pest mites to a place where they may well be exposed to chemical control. For that, additional studies should be performed to confirm the genotype preference and antibiosis effect upon *T. neocaledonicus* at field scales.

By contrast, genotypes UFPI-882, UFPI-891, and UFPI-883, group 3 in the cluster analysis, reduced the adult female longevity and oviposition period, elongated the larval period and affected fertility life table parameters, demonstrating an antibiosis effect upon *T. neocaledonicus*. Moreover, these genotypes were among the less preferred for food and oviposition, exhibiting an additional antixenosis effect.

Combining strong antibiosis and antixenosis, the three genotypes UFPI-882, UFPI-891, and UFPI-883 could be used as sources of resistance to *T. neocaledonicus* in future programs. According to Sharma and Ortiz (2002), one of the major attractive features of plant resistance to herbivorous predators is that it does not require farmers to have any specific skill for the employment of the technique. Furthermore, the financial investment by farmers is very low. For the lima bean crop context of family farming in underdeveloped regions, the advance of resistant genotypes to a potential pest threat presents itself as a management practice that ensures food sovereignty.

Altogether, the nine lima bean genotypes increased the egg-adult development period of *T. neocaledonicus,* ranging from 11.35 to 11.98 days at 25°C, when compared to values found in the literature for other Tetranychidae mites on *Phaseolus* hosts. According to Rivero and Vásquez (2009), *T. desertorum* Banks on lima bean presented an egg-adult period of 6.8 days at 28°C. Morros and Aponte (1995) reported that *T. ludeni* Zacher also presented an egg-adult period of 6.8 days on *Phaseolus vulgaris* L. at 26°C.

According to Ballhorn et al. (2005), lima bean is already well described as emitting a toxic compound, cyanide, as well as volatile organic compounds (VOCs) for plant defense against herbivorous predators. Such defenses are assumed to protect the plant directly, reducing oviposition and/or feeding; and indirectly affecting the herbivore's development. Furthermore, the effects of VOCs are varied and may even include repellence in herbivores (Moraes et al., 2001; Heil, 2004).

Although some evidence may infer that piercing-sucking herbivores do not promote enough tissue disruption to activate plant defense process (Schreiner et al., 1984), laboratory studies recognize that the release of VOCs may be induced by minor injuries from small piercing-sucking herbivores, for instance, spider mites (Dicke, 1999; Ballhorn et al., 2008).

Besides that, in the present study, the nine lima bean genotypes significantly affected the performance and population growth parameters as well as the food and oviposition preference of *T. neocaledonicus* at different rates. Ballhorn et al. (2008) quantified the defense mechanism of lima bean to herbivores and found a substantial variation among 16 lima bean genotypes for cyanide and VOC emissions as defense mechanisms.

Thus, our findings support the hypothesis that lima bean genotypes may affect the performance, populational parameters and the host selection of *T. neocaledonicus*, presenting itself as the first study to describe sources of resistance to pest mites in lima bean in Brazil. Therefore, the results of the present study may be characterized as a basis for further studies aiming to develop a strategy of host plant resistance for IPM programs.

AUTHOR CONTRIBUTIONS

SdF and AG-N conceived and designed the research. RG provided the lima bean genotypes. JdSM, PS, MB, SdF, RG, and AG-N wrote the manuscript and analyzed all data. All authors read and approved the manuscript.

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Arabidopsis Kunitz Trypsin Inhibitors in Defense Against Spider Mites

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Tetranychus urticae (two-spotted spider mite) is a striking example of polyphagy among herbivores with an extreme record of pesticide resistance and one of the most significant pests in agriculture. The *T. urticae* genome contains a large number of cysteine- and serine-proteases indicating their importance in the spider mite physiology. This work is focused on the potential role of the Kunitz trypsin inhibitor (KTI) family on plant defense responses against spider mites. The molecular characterization of two of these genes, At*KTI4* and At*KTI5*, combined with feeding bioassays using T-DNA insertion lines for both genes was carried out. Spider mite performance assays showed that independent *KTI* silencing Arabidopsis lines conferred higher susceptibility to *T. urticae* than WT plants. Additionally, transient overexpression of these inhibitors in *Nicotiana benthamiana* demonstrated their ability to inhibit not only serine- but also cysteine-proteases, indicating the bifunctional inhibitory role against both types of enzymes. These inhibitory properties could be involved in the modulation of the proteases that participate in the hydrolysis of dietary proteins in the spider mite gut, as well as in other proteolytic processes.

Keywords: plant-herbivore interphase, *Tetranychus urticae*, *Arabidopsis thaliana*, serine protease inhibitors, cysteine protease inhibitors, spider mite digestion

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INTRODUCTION

The fact that higher plants are sessile organisms has favored the acquisition of sophisticated resources to prevent or hamper pest feeding (Walling, 2000; Wu and Baldwin, 2010). Such defenses can be constitutive and/or induced upon attack by herbivore pests. Induced defenses include morphological and metabolic changes with a negative impact on phytophagous arthropod behavior (Walling, 2000; Howe and Jander, 2008; Alba et al., 2011) or the attraction of natural enemies of the herbivore (Sabelis et al., 1999; Dicke and Baldwin, 2010). Plant receptors recognize Herbivore-Associated Molecular Patterns (HAMPs), Microbe-Associated Molecular Patterns (MAMPs) and Damage-Associated Molecular Patterns (DAMPs) and trigger the induction of defenses (Mithöfer et al., 2005; Frost et al., 2007; Staudacher et al., 2017; Santamaria et al., 2018a). Plant responses are specific to the phytophagous pest species (Stout et al., 1998; de Vos et al., 2005; Rodriguez-Saona et al., 2010) and dependent on the duration of the infestation (Kant et al., 2004). However, the perception of herbivory is not well understood and few plant receptors have been identified (Bonaventure, 2012; Santamaria et al., 2018a).

Among plant defenses, protease inhibitors (PIs) exert direct effects on herbivores by interfering with their physiology (Diaz and Santamaria, 2012; Martinez et al., 2016). When an arthropod ingests PIs in its diet, the inhibition of proteolytic activities takes place in its gut avoiding the degradation of proteins. In this context, PI classification may be based on the type of protease they inhibit. There are three main subclasses of proteases involved in arthropod digestion, serine-, cysteine-, and aspartic-proteases, grouped according to the reactive amino acid of their active site group (Terra and Ferreira, 2012). The proteolytic activity is dependent on the pH of the gut (Ortego, 2012; Martinez et al., 2016). Most lepidopteran, orthopteran and hymenopteran and some coleopteran possess alkaline midguts and their digestive systems are largely based on serine-proteases and exopeptidases (Wolfson and Murdock, 1990; Ortego et al., 1996; Johnson and Rabosky, 2000). The majority of coleopteran, hemipteran and some phytophagous acari have slightly acidic midguts providing cysteine- and aspartic-proteases and exopeptidases their major proteolytic activity (Murdock et al., 1987; Cristofoletti et al., 2003; Carrillo et al., 2011). Since Green and Ryan (1972) reported that wound-inducible PIs inhibited digestive herbivore gut proteases, numerous plant PIs have been characterized for their potential to control herbivorous insects (Hilder et al., 1987; Alfonso-Rubi et al., 2003; Tamhane et al., 2005; Telang et al., 2009; Chen et al., 2014). According to MEROPS database, there are currently 85 families of PIs (Rawlings et al., 2018), being the Kazal, Kunitz, Bowman-Birk, Potato I and II, Cystatin, Cereal trypsin/α-amylase, and Serpin families the most represented in plants (Santamaria et al., 2014). Most of them specifically inhibit a mechanistic class of proteases but some may act as multifunctional inhibitors (Grosse-Holz and van der Hoorn, 2016). The first successful PI gene used to improve resistance against larvae of *Heliothis virescens* when expressed in transgenic tobacco was the cowpea trypsin inhibitor gene (CPTI) from the Bowman–Birk family (Hilder et al., 1987). Then, the CPTI gene was inserted in the genome of other plants like cotton, rice, cabbage, strawberry, sweet potato, potato or pigeon pea enhancing the resistance to different lepidopteran species (reviewed in Diaz and Santamaria, 2012). The I3 Kunitz Trypsin Inhibitor (KTI) gene family is a complex family composed by versatile protease inhibitors. Most of them inhibit serine proteases (families S1 and S8), but some of them are able to inhibit cysteine proteases (families C1 and C13) as well as other hydrolases (Renko et al., 2012). This family has been studied in different plants and contexts but most works have been focused on their potential role in defense against insect attack since their gene expression is up-regulated in response to wounding, jasmonates and insect feeding (Major and Constabel, 2008; Philippe et al., 2009; Botelho-Junior et al., 2014). *In vitro* assays with KTIs from poplar and soybean expressed as recombinant proteins differentially inhibited midgut proteases from *Mamestra configurata* and *Malacosoma disstria*, lepidopteran pests from *Populus* and crucifers, respectively (Major and Constabel, 2008). KTIs from the passion fruit displayed activity against midgut serine and cysteine proteases from the sugarcane borer *Diatraea saccharalis* and the coleopteran *Callosobruchus maculatus* on artificial diets (Botelho-Junior et al., 2014). In addition, the heterologous expression of a good number of KTIs in poplar, sweet corn, potato, rice, tobacco, and tomato conferred resistance to lepidopteran (Confalonieri et al., 1998; Cipriani et al., 1999; Gatehouse et al., 1999; Lee et al., 1999; McManus et al., 1999; Nandi et al., 1999; Marchetti et al., 2000; Rufino et al., 2013; Guimarães et al., 2015), coleopteran (Major and Constabel, 2008) and acari (Castagnoli et al., 2003). Likewise, serine-PIs from other different families overexpressed in several plant species have conferred resistance to lepidopteran, coleopteran, homopteran (reviewed in Diaz and Santamaria, 2012) and acari (Santamaria et al., 2012).

Tetranychus urticae is an extreme polyphagous pest with more than 1,100 documented host plants and an extraordinary ability to develop pesticide resistance (Van Leeuwen and Dermauw, 2016). These features, along with the predicted expansion of spider mites under climate change conditions, make *T. urticae* one of the most significant pests in the agriculture (Luedeling et al., 2011). Phytophagous mites pierce parenchymatic plant cells using stylets to suck their nutrients, and cause severe chlorosis leading to a reduction in crop yield (Park and Lee, 2002; Farouk and Osman, 2011; Bensoussan et al., 2016). *T. urticae* is a model within chelicerate herbivores with its genome sequenced and a broad range of tools and protocols developed (Grbic et al., 2011; Cazaux et al., 2014; Suzuki et al., 2017). Besides, mite ability to feed on *Arabidopsis thaliana* and the wide available toolkits for this plant species have provided an outstanding opportunity for functional studies of plant-mite interaction (Santamaria et al., 2012, 2015a, 2017; Zhurov et al., 2014). Among plant PIs, cystatins and serine-protease inhibitors have been reported to be involved in Arabidopsis defense against spider mite. According to Santamaria et al. (2012), the over-expression of barley cystatin (*Icy6* gene) and/or trypsin inhibitor (*Itr1* gene) conferred Arabidopsis resistance by producing an increase in mite mortality. In addition, members from I3 and I13 Potato Inhibitor I families are induced upon *T. urticae* infestation in tomato and Arabidopsis (Martel et al., 2015). In the case of plant cystatins, it is well known that their targets in mites are digestive cysteine-proteases (Carrillo et al., 2011; Santamaria et al., 2012, 2015b, 2018b). In contrast, mite targets for serine-protease inhibitors remain unknown. The fact that an Arabidopsis KTI is able to inhibit papain-like cysteine proteases and participates in the defense against herbivorous arthropods (Boex-Fontvieille et al., 2016; Rustgi et al., 2017) prompted us to examine the role of the Arabidopsis KTI protease inhibitor family in plant defense against mites. Among the seven KTIs identified in Arabidopsis, AtKTI4 and AtKTI5 were selected because of the induction of their corresponding genes after spider mite feeding, and the differences in their amino acid sequences suggesting tridimensional structure dissimilarities. Knock down lines for these two Arabidopsis *KTIs* genes were used to analyze plant phenotypes after spider mite infestation. Behavior of mites fed on knock down lines was also evaluated to verify KTI effect on mite performance. Furthermore, transient overexpression of these inhibitors in *Nicotiana benthamiana* was performed to test their ability to inhibit both serine- and cysteine-proteases.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana Columbia (Col-0), Kondara (Kon) and Bla-2 (Bla-2) accessions (Nottingham Arabidopsis Seed Collection) were used as wild-types (WT). *A. thaliana* T-DNA mutants (SALK_131716C, SALK_067224, SALK_115805C and SALK_009101C, referred as *kti4.1*, *kti4.2, kti5.1,* and *kti5.2*, respectively) were obtained from the Arabidopsis Biological Resource Centre, through the European Arabidopsis Stock Centre. For soil growth, a mixture of peat moss and vermiculite (2:1 v/v) was used. Sterilized seeds were stratified in the dark at 4°C for 5 days. Plants were then grown in growth chambers (Sanyo MLR-350-H) under control conditions (23°C ± 1°C, >70% relative humidity and a 16 h/8 h day/night photoperiod).

Spider Mite Maintenance

A colony of *T. urticae*, London strain (Acari: Tetranychidae), provided by Dr. Miodrag Grbic (UWO, Canada), was reared on beans (*Phaseolus vulgaris*) and maintained on growth chambers (Sanyo MLR-350-H, Sanyo, Japan) at 25°C ± 1°C, >70% relative humidity and a 16 h/8 h day/night photoperiod.

Sequence Analysis and Molecular Modeling

Multiple protein alignment was performed by MUSCLE program (Edgar, 2004). Arabidopsis Kunitz proteins were named following Santamaria et al. (2014) changing the Kun family term for the more commonly used KTI term. The KTI of *Delonix regia* (PDB ID 1R8N) was included in the alignment to infer secondary structure locations. Displayed multiple sequence alignment was made by the ESPript 3.0 web server (Robert and Gouet, 2014). 3D modeling was performed using SWISS-MODEL online protein structure prediction tool (Biasini et al., 2014). The known structures of two KTIs (PDB IDs: 3I2A and 3IIR) were used to construct the models for AtKTI4 (At1g73260) and AtKTI5 (At1g17860), respectively. Predictions on papain–AtKTI4 and papain–AtKTI5 interactions were made by using the obtained 3D models and the 3D structure of papain (PDB ID: 1PPN) in the ClusPro 2.0 server (Kozakov et al., 2017). Molecular models were visualized and analyzed by Chimera 1.12 program (Pettersen et al., 2004).

Nucleic Acid Analysis

Genomic DNA was isolated from Arabidopsis T-DNA insertion and control lines essentially as described by Sambrook and Russell (2001). The presence and homozygous status of the T-DNA insertion lines were validated by conventional PCR (Bio-Rad) (Supplementary Figure S1). Specific primers were designed through the Salk Institute website[1]. Primer sequences are indicated in Supplementary Table S1.

For quantitative real time PCR (RT-qPCR) studies, Arabidopsis rosettes from T-DNA insertion and control lines were collected, frozen into liquid N_2 and stored at −80°C until used for RNA isolation. Total RNA was extracted by the phenol/chloroform method, followed by precipitation with 8 M LiCl (Oñate-Sánchez and Vicente-Carbajosa, 2008). Regarding *N. benthamiana* assays, total RNA was extracted from plants agroinfiltrated with 35S::GFP, 35S::KTI4-GFP and 35S::KTI5-GFP by the TRIZOL reagent following manufacturer instructions (Ambion, Austin, TX, United States). Complementary DNAs (cDNAs) were synthesized from 2 μg of RNA using the Revert Aid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas) following manufacturer's instructions. The RT-qPCR conditions used were 40 cycles with 15 s at 95°C, 1 min at 60°C and 5 s at 65°C using FastStart Universal SYBR Green Master (Rox) (Roche). RT-qPCR was performed for three samples coming from three independent experiments as previously described (Santamaria et al., 2017) using a SYBR Green Detection System (Roche) and the CFX Manager Software 2.0 (Bio-Rad). mRNA quantification was expressed as relative expression levels (2^{-dCt}) or fold change (2^{-ddct}) normalized to ubiquitin or actin for *Arabidopsis* and *Nicotiana* samples, respectively (Livak and Schmittgen, 2001). Specific primers were designed through PRIMER3[2]. Primer sequences are indicated in Supplementary Table S1.

Enzymatic Assays

Total protein extracts from the T-DNA insertion lines and control Arabidopsis rosettes were resuspended in 50 mM sodium phosphate pH 6.0, 0.15 M NaCl 2 mM EDTA, for 1 h at 4°C and treated as described in Santamaria et al. (2012). Total protein content was determined according to the method of Bradford (1976). Cathepsin B- and L-like activities were assayed using *N*-carbobenzoxy-Arg-Arg-7-amido-4-methylcoumarin (Z-RR-AMC) and *N*-carbobenzoxy-Phe-Arg-AMC (Z-FR-AMC) commercial substrates, respectively. Trypsin- and chymotrypsin-like activities were analyzed using Z-L-Arg-AMC (ZLA-AMC) and Suc-Ala-Ala-Pro-Phe-AMC (Suc-A-A-P-F-AMC) commercial substrates, respectively.

Inhibitory activity of plant protein extracts was tested *in vitro* against commercial trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), papain (EC 3.4.22.2), and bovine cathepsin B (EC 3.4.22.1) from Sigma. Basically, 20 μg of protein extracts were preincubated for 10 min with 100 ng of cathepsin L- and B-like in a buffer containing 100 mM sodium phosphate pH 6.0, L-cysteine, 10 mM EDTA, and 0.01% (v/v) Brij35, or with 100 ng of trypsin/chymotrypsin in the buffer 0.1 M Tris-HCl pH 7.5. Subsequently, substrates were added at a final concentration of 25 μM and incubated for 1 h at 28°C or 37°C for cysteine and serine proteases, respectively. Fluorescence was measured using an excitation filter of 365 nm and an emission filter of 465 nm (Tecan GeniusPro). The system was calibrated with known amounts of 7-amido-4-methylcoumarin (AMC) hydrolysis product in a standard reaction mixture. Specific enzymatic activity was represented as nmoles of substrate hydrolyzed/min/mg of protease. Inhibitory activity was expressed as percentage of protease activity relative to that in the absence of

[1] http://signal.salk.edu/tdnaprimers.2.html

[2] http://bioinfo.ut.ee/primer3-0.4.0/

the inhibitor. All assays were carried out in triplicates and blanks were used to account for spontaneous breakdown of substrates.

Subcellular Location

To create the translational fusions of At*KTI4* and At*KTI5* genes to the Green Fluorescent Protein (*GFP*) reporter gene, the corresponding cDNAs were amplified by conventional PCR using specific primers (Supplementary Table S1). The amplicons were independently cloned in-frame with the *GFP* gene into the Gateway binary vector pGWB5 (Invitrogen), under the CaMV35S promoter. 35S-Red Fluorescent Protein (RFP)-HDEL plasmid was used as a control of ER location (Shockey et al., 2006). Transient transformation of onion (*Allium cepa*) epidermal cells was performed by particle bombardment with a biolistic helium gun device (DuPont PDS-1000; Bio-Rad) as described by Diaz et al. (2005). Fluorescent images were acquired after 24 h of incubation at 22°C in the dark, using a Leica TCS-SP8 confocal microscope. GFP and RFP signals were acquired sequentially using the following settings: GFP, excitation 488 nm and emission 492–552 nm; RFP, excitation 561 nm, emission 581–665 nm.

For *N. benthamiana* agroinfiltration, the *Agrobacterium tumefaciens* strain C58CI Rif^R (GV3101) carrying the constructs 35S::*KTI4*-GFP (pGWB5), 35S::*KTI5*-GFP (pGWB5) or 35S::GFP (pEAQ-HT-GFP) was co-incubated with the construct 35S::P19 (pBIN61) that carries the helper P19 (Voinnet et al., 2015) to a final optical density of 0.3 nm. Bacterial suspensions were infiltrated into the abaxial side of the third-youngest fully expanded *N. benthamiana* leaf using a syringe. Fluorescent images were acquired 3 days post-infiltration (dpi), using a Leica TCS-SP8 confocal microscope. GFP and chlorophyll autofluorescence signals were acquired sequentially using the following settings: GFP, excitation 488 nm and emission 500–600 nm; chlorophyll, excitation 633 nm, emission 639–727 nm.

Plant Damage Determination

Damage quantification analyses were done on *A. thaliana* plants from T-DNA insertion lines and WT control. Three week-old plants were infested with 20 *T. urticae* adults per plant. After 4 days of infestation, leaf damage was assessed by scanning the entire rosette using a hp scanjet (HP Scanjet 5590 Digital Flatbed Scanner series), according to Cazaux et al. (2014). Leaf damage was calculated in mm^2 using Adobe Photoshop CS software. Six replicates were used for each genotype. Plant damage was also evaluated by analyzing accumulation of hydrogen peroxide (H_2O_2) and the cell death in response to spider mite attack. Leaf disks (9 mm diameter) from 3 week-old plants from the five studied genotypes, were infested with 10 mites during 24 h. The H_2O_2 accumulation was analyzed using 3,3-diaminobenzidine tetrachloride hydrate (DAB) substrate which produces a brown precipitate after oxidation in the presence of H_2O_2 (Martinez de Ilarduya et al., 2003). The staining procedure was performed according to Rodríguez-Herva et al. (2012), and observed under a Zeiss Axiophot microscope. Damage was quantified using Image J software.

Cell death was quantified by trypan blue staining as described by Sanchez-Vallet et al. (2010). Leaves were boiled in trypan blue solution [10 ml lactic acid (Sigma)], 10 ml phenol (Sigma), 10 ml glycerol (Duchefa), 10 ml water and 10 mg trypan blue (Sigma) diluted with 96% (1:2 v/v) ethanol in a 15 ml tube for 1 min. Tissues were incubated in the staining solution overnight at room temperature. Subsequently, leaves were cleared four times with 2.5 g/ml of chloral hydrate solution (PRS). Cleared leaves were prepared for imaging in 50% (v/v) glycerol, 0.1 M sodium phosphate buffer at pH 7.0, and observed under a Zeiss Axiophot microscope. Damage was quantified using Adobe Photoshop CS6 and values relativized to control plants. Six replicates were used for each genotype.

Spider Mite Performance

Mite bioassays were conducted on entire detached Arabidopsis leaves from the T-DNA insertion and control plants. Entire leaves were fit into a closed system with 100 eggs. Samples were maintained under controlled conditions at 25 ± 1°C, >70% relative humidity and a 16 h/8 h day/night photoperiod. To study mortality, the percentage of neonate larvae (<24 h of hatching) that became adults was recorded after 10 days of feeding. Eight replicates were used for each genotype. For fecundity assays detached entire leaves were infested with 12 synchronized females each, and the number of eggs laid was counted after 36 h. Female mite synchronization was conducted on bean leaves. Entire detached leaves were placed onto wet cotton, surrounded by wet filter paper to avoid mite escape in confined special dishes (11.5 cm diameter with ventilation). 50 female mites were placed on each leaf and removed after 36 h. After 11 days, same age females were used to infest Arabidopsis leaves for the fecundity assay.

Inhibitory Ability of *KTIs* Overexpressed in *Nicotiana*

To test the ability of AtKTI4 and AtKTI5 to inhibit cysteine/serine proteases, we used extracts from *N. benthamiana* plants co-agroinfiltrated with 35S::KTI4-GFP (pGWB5), 35S::KTI5-GFP (pGWB5) or 35S::GFP (pGWB5), and 35S::P19 (pBIN61). Entire agroinfiltrated leaves were collected at 3 dpi (days post infiltration). Fluorescent images were acquired using a Leica Fluorescence Stereoscope MZ10F. Leaves were homogenized in a buffer containing 50 mM Tris-HCl pH 7.25, 150 mM NaCl, 2 mM EDTA and 0.1% (v/v) Triton X-100, centrifuged at 16,630 *g* at 4°C for 10 min and the supernatants were pooled to obtain soluble protein extracts for inhibition assays. The ability to inhibit commercial papain, cathepsin-B, trypsin and chymotrypsin activities was *in vitro* tested as indicated above but using 15 μg of plant protein extracts for the assays. In addition, E-64 [*trans*-Epoxysuccinyl-L-leucylamido (4-guanidino)butane], TLCK (N_α-Tosyl-L-lysine chloromethyl ketone hydrochloride) and chymostatin [*N*-(N_α-Carbonyl-Cpd-X-Phe-al)-Phe] inhibitors (Sigma) were added as positive inhibitory controls for papain and cathepsin-B, trypsin and chymotrypsin inhibition, at a final concentrations of 0.02, 100, and 0.1 μM, respectively, and incubated for 10 min before the addition of substrates.

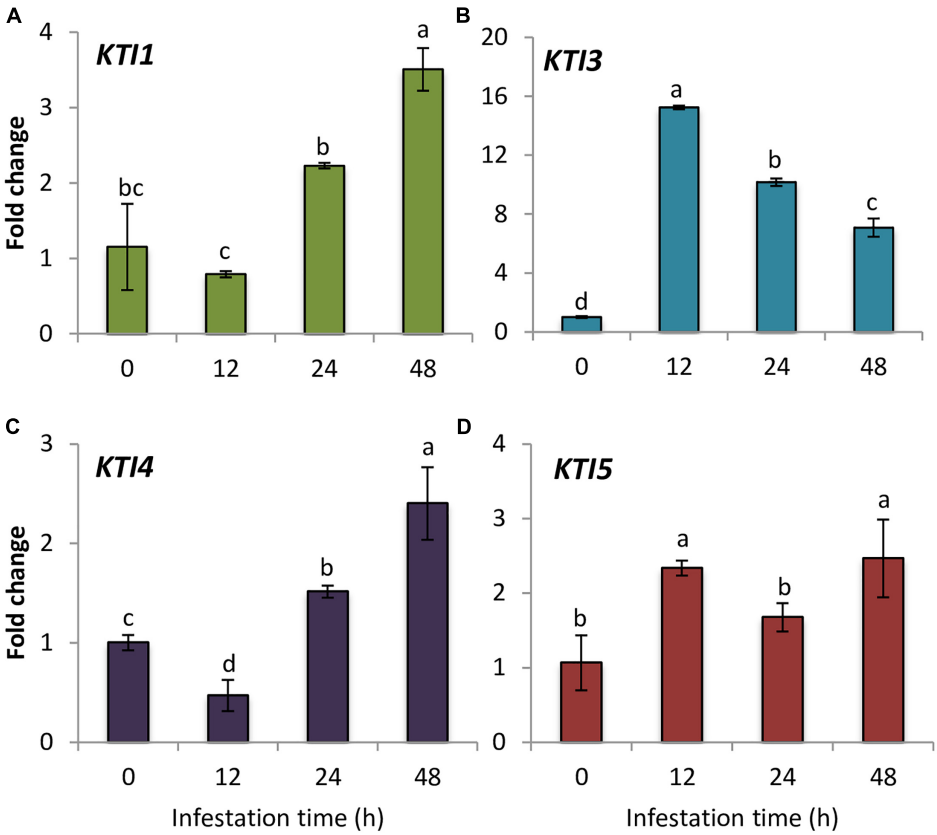

FIGURE 1 | Gene expression of Arabidopsis Kunitz-type inhibitors upon spider mite infestation. Fold change in Col-0 plants at 0, 12, 24, and 48 hpi using time 0 as a calibrator sample. **(A)** At*KTI1* **(B)** At*KTI3* **(C)** At*KTI4,* and **(D)** At*KTI5*. Data are means of three replicates ± SE. Different letters indicate significant differences ($P < 0.05$, One-way ANOVA followed by Student–Newman–Keuls test).

Statistical Analysis

Differences in gene expression, leaf damage, mortality and fecundity assays were compared by One-Way ANOVA, followed by Student Newman–Keuls multiple comparison test ($p < 0.05$). Compensation effects and enzymatic assays were compared by One-Way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$).

RESULTS

Gene Expression of Arabidopsis I3 Kunitz Inhibitors in Response to *T. urticae* Infestation

As a first estimation of the importance of the I3 Kunitz inhibitors in Arabidopsis defense against spider mite, the gene expression of the whole Kunitz family members in infested and non-infested Col-0 plants was studied. This family included seven genes previously identified (Ma et al., 2011; Santamaria et al., 2014). One of these genes, At*KTI4* was previously reported as a differential expressed gene between the resistant Bla-2 and the susceptible Kondara Arabidopsis accessions upon spider mite feeding (Zhurov et al., 2014). RT-qPCR results showed that At*KTI5* and At*KTI3* genes were induced at 12 hpi, whereas At*KTI4* and At*KTI1* genes were up-regulated at 24 hpi, and presented their highest gene expression peak at 48 hpi (**Figure 1**). In contrast, At*KTI2,* At*KTI6,* and At*KTI7* gene expression was not detected in Arabidopsis leaves neither in non-infested plants nor upon mite infestation.

Sequence and Structural Features of Arabidopsis KTIs

To obtain some clues on the inhibitory capacities of the seven Arabidopsis KTIs, their amino acid sequences were aligned. The seven inhibitors presented a signal peptide in the N-terminal region and their sequences were more similar in the regions aligned with the amino acids involved in secondary structures of a Kunitz inhibitor from *D. regia* (**Figure 2A**). The AtKTI2 did not present the positively charged residue (Lys or Arg) in the loop between strands β4 and β5, essential to inhibit trypsin. This residue was at the right position in AtKTI3, 4, 5, and 6. The amino acid pair Trp-Pro, located in the loop between strands β5 and β6, and putatively involved in cysteine-protease inhibitory capability of AtKTI2 was only partially conserved in AtKTI1

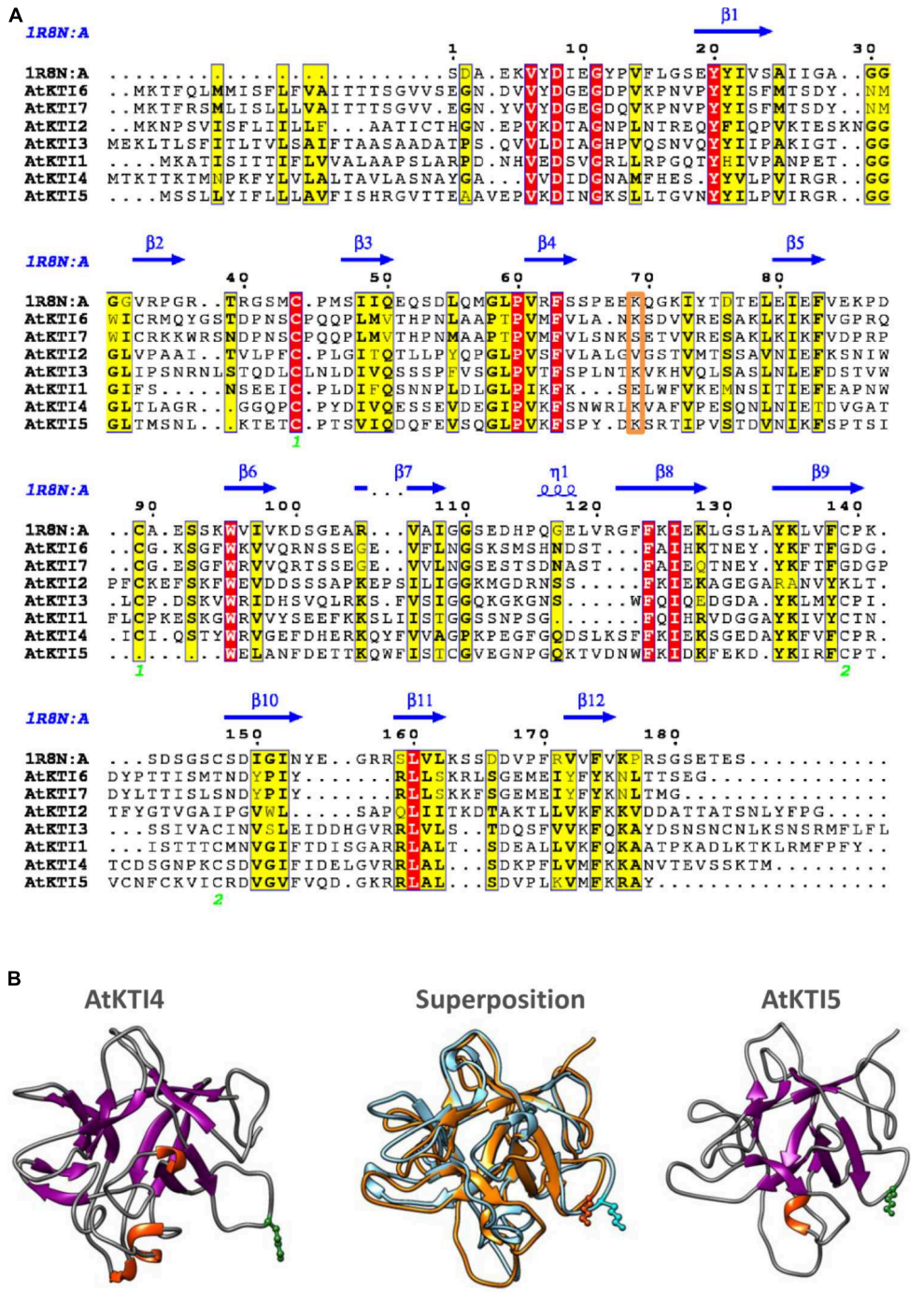

FIGURE 2 | Sequence-structure analysis of Kunitz-type inhibitors. **(A)** Multiple sequence alignment of Arabidopsis KTIs. The Kunitz type inhibitor of *Delonix regia* (PDB ID 1R8N) was included in the alignment to infer secondary structure locations. Conserved residues are in red boxes. Similar residues are indicated in black bold characters and boxed in yellow. Green numbers at the bottom indicate disulphide bridge topology. Putative reactive site is boxed in orange. The figure was made with the ESPript 3.0 web server. **(B)** Ribbon diagrams showing structural models for AtKTI4 and AtKTI5, and their superposition (blue, AtKTI4; orange, AtKTI5). Conserved reactive Lys residue is colored in green. Modelization and visualization were made by SWISS-Model and Chimera tools.

and AtKTI3. Whereas AtKTI2, AtKTI6, and AtKTI7 lacked the two cysteines involved in the first disulphide bridge, AtKTI5 is deficient in one of them but had two additional cysteines between strands β9 and β10 that could form a novel disulphide bridge. To determine the possible effect of this variation on the protein structure, tridimensional structures for AtKTI4 and 5 were made by homology modeling. Predictions showed structural differences. This predicted structural dissimilarity was

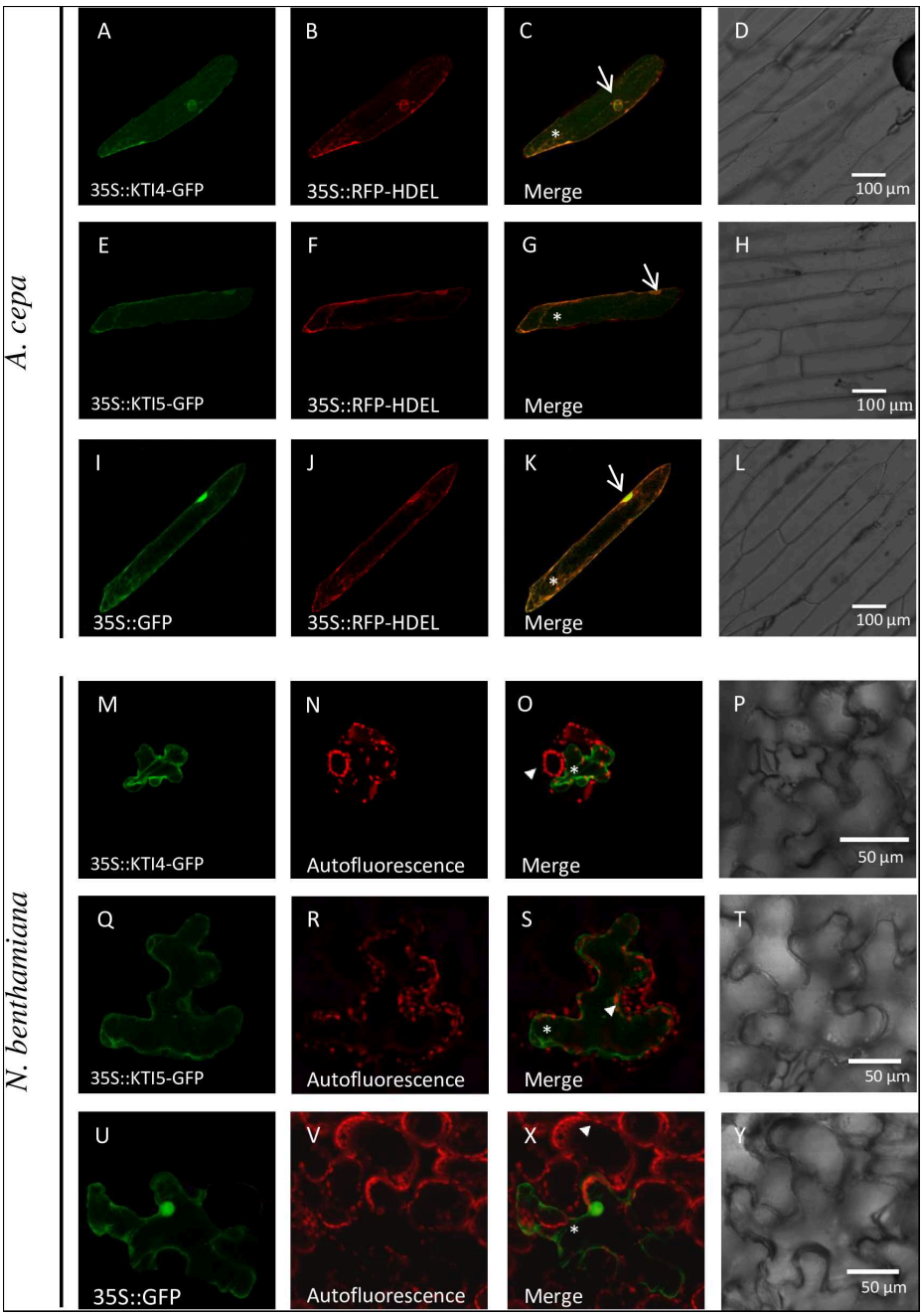

FIGURE 3 | AtKTI4 and AtKTI5 subcellular localization. Confocal stacks spanning epidermal onion cells co-transformed with 35S::KTI4-GFP, 35S::KTI5-GFP and 35S::GFP and 35S::RFP-HDELcontrols. Confocal images and projections of the KTI4 **(A)** and KTI5 **(E)** are shown. Projections from GFP **(A,E,I)**, RFP **(B,F,J)**, merged **(C,G,K)** and the corresponding Nomarski snapshots **(D,H,L)**. Confocal stacks spanning *N. benthamiana* cells agroinfiltrated with 35S::KTI4-GFP **(M)**, 35S::KTI5-GFP **(Q)** and 35S::GFP control **(U)**. Projections from GFP **(M,Q,U)**, chlorophyll auto fluorescence **(N,R,V)**, merged **(O,S,X)** and the corresponding Nomarski snapshots **(P,T,Y)**. Bars are indicated in images. Arrows indicate nuclei, asterisks signal ER and arrowheads the chlorophyll autofluorescence.

mainly observed in the loops connecting secondary structures and leads to a distinct spatial orientation of the Lys reactive residue in the β4–β5 loop (**Figure 2B**). The predicted sequence-structure plasticity of these inhibitors could lead to different inhibitory properties. Thus, AtKTI4 and 5 were selected to further characterization.

AtKTI4 and AtKTI5 Protein Subcellular Location

Signal peptides found in every AtKTI indicate a targeted transport to the endoplasmatic reticulum. To determine the final AtKTI4 and AtKTI5 subcellular localization, transient expression assays were performed in onion epidermal layers by microparticle

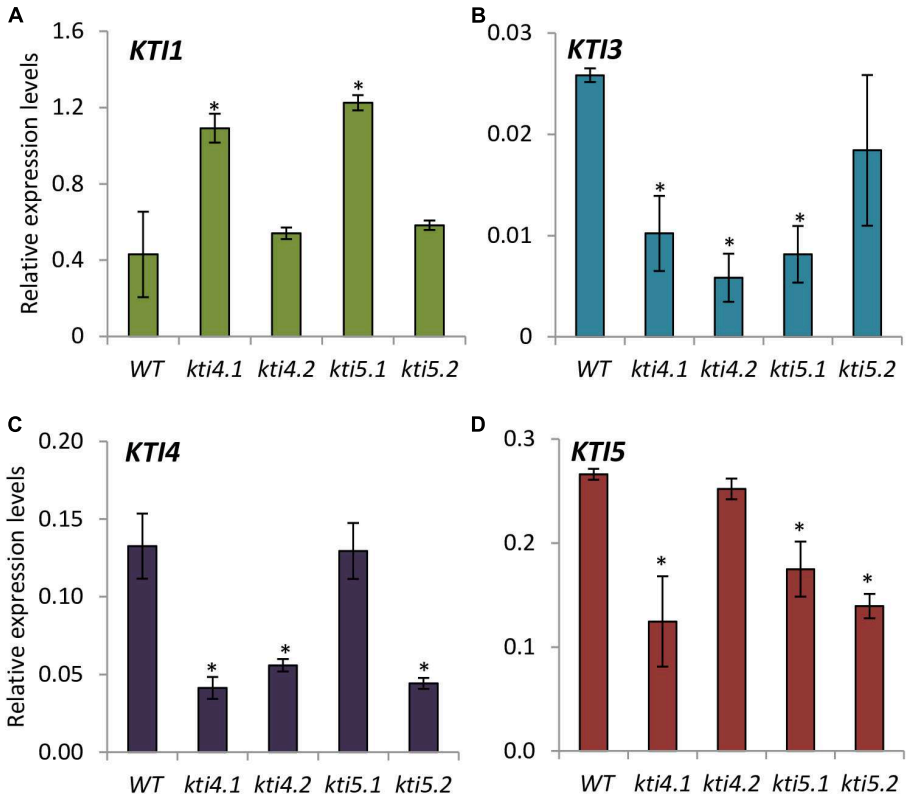

FIGURE 4 | Gene expression of Kunitz-type inhibitors in Arabidopsis T-DNA insertion lines and WT plants. Relative gene expression levels of: **(A)** At*KTI1* **(B)** At*KTI3* **(C)** At*KTI4* and **(D)** At*KTI5*. Data are means ± SE of three replicates. Asterisk indicates significant differences with the WT ($P < 0.05$, One-way ANOVA followed by Dunnett's test).

bombardment. AtKTI4 and AtKTI5 were fused to GFP. To determine the subcellular localization of the GFP signal, the cells were co-transfected with the 35S::RFP-HDEL plasmid to reveal the ER. Both green and red signals were found in the nuclear area, tracking along threads of cytoskeleton elements and the cell periphery. These locations are consistent with the subcellular distribution of the ER (**Figures 3A–C,E–G**). The 35S::GFP control showed an intense fluorescence in the cell nucleus, entering due to its small size, whereas the ER marker is seen in the nuclear periphery (**Figures 3I,J**). Agroinfiltration of *N. benthamiana* plants with the same constructs confirmed the location of both AtKTI4 and AtKTI5 inhibitors in the endomembrane system. In this case, the red signal corresponds to the autofluorescence of chlorophyll (**Figures 3M–V,X,Y**).

Effects of Knock-Down AtKTI4 and AtKTI5 Lines on KTI Expression and Protease Inhibitory Properties

To investigate the role of AtKTI4 and AtKTI5 proteins in plant defense, silenced lines for these genes (*kti4.1, kti4.2*, *kti5.1, kti5.2*) were ordered. The characterization of the homozygous mutant lines revealed the insertion of the T-DNA at the promotor region for *kti4.1* and *kti5.2* lines, at the coding region for *kti5.1* line and at the 3′UTR for the *kti4.2* line (Supplementary Figure S1A).

The expression of At*KTI1,* At*KTI3,* At*KTI4,* and At*KTI5* genes was analyzed in the *kti4* and *kti5* mutants and in the WT plants. As expected, the expression of At*KTI4* and At*KTI5* genes was reduced in their cognate T-DNA insertion lines (**Figures 4C,D**). Moreover, statistical differences in mRNA quantification revealed variations in the expression of other At*KTI* genes in these lines. Regarding the mutant lines for At*KTI4* gene, *kti4.1* plants were knocked down for At*KTI3,* At*KTI4,* and At*KTI5* genes and up regulated for At*KTI1* gene (**Figures 4A–D**) while *kti4.2* plants were down regulated for At*KTI3 and* At*KTI4* genes (**Figure 4C**). In the case of the T-DNA insertion lines for At*KTI5* gene, *kti5.1* plants were knocked down for At*KTI3* and At*KTI5* genes while the expression of At*KTI1* gene was induced (**Figures 4A,B,D**), and *kti5.2* mutant plants were down regulated for At*KTI4* and At*KTI5* genes (**Figures 4B–D**).

Compensatory effects described above were also studied by analyzing the effect of T-DNA insertions on the protease activities of these plants. Results showed higher trypsin activity in all mutants than in the WT plants (**Figure 5A**). For chymotrypsin activity, *kti4.1* and *kti4.2* plants presented higher proteolytic levels in comparison to WT plants while *kti5.1* and *kti5.2* did not (**Figure 5A**). In contrast, no significant differences on both, cathepsin L- and B-like activities were detected between mutant and WT plants. Only the *kti4.1* line showed slightly higher levels of cathepsin B-like activity than the WT plants (**Figure 5B**). The

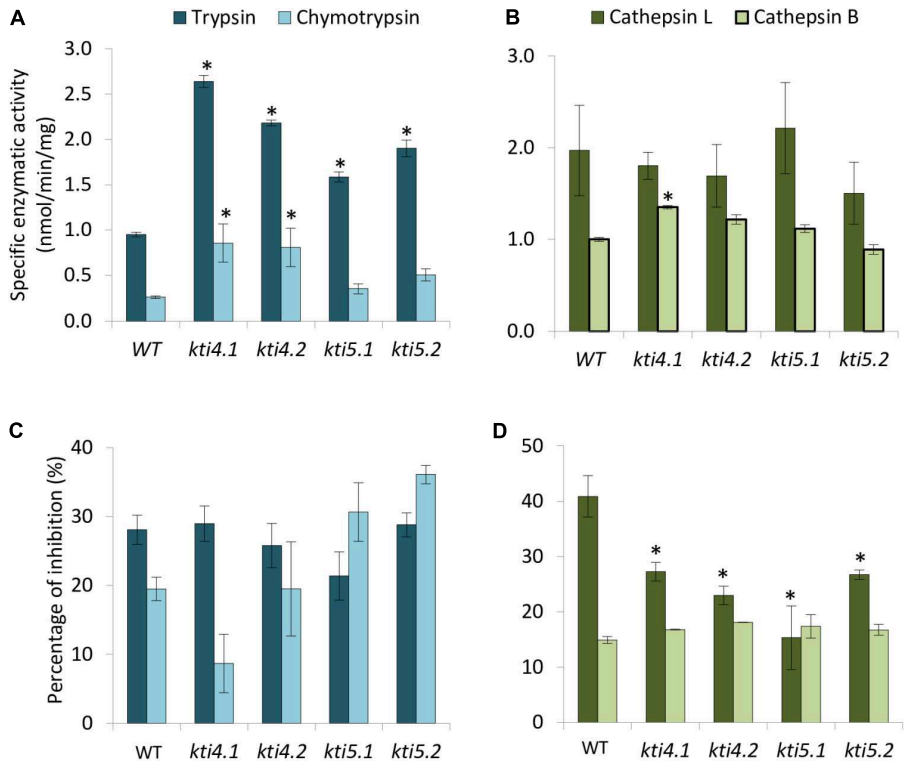

FIGURE 5 | Proteolytic patterns of T-DNA insertion lines. Specific proteolytic activities of protein extracts from Arabidopsis T-DNA insertion lines and control WT using specific substrates. **(A)** Trypsin- and chymotrypsin-like specific activities. **(B)** Cathepsin L- and B-like specific activities. Data are expressed as nmoles/min/mg. Inhibitory activity of protein extracts from T-DNA insertion lines and control plants against commercial proteases. **(C)** Inhibitory activity against trypsin and chymotrypsin. **(D)** Inhibitory activity against commercial papain (cathepsin L-like) and bovine cathepsin B. Data are expressed as a percentage of inhibition. Data are means ± SE of three replicates. Asterisk indicates significant differences with the WT ($P < 0.05$, One-way ANOVA followed by Dunnett's test).

capability of the different Arabidopsis genotypes to inhibit serine- and cysteine-proteases was also tested by using commercial proteases. Significant differences were not found for trypsin, chymotrypsin and bovine cathepsin B assays (**Figures 5C,D**). Interestingly, the commercial papain (cathepsin L-like) was less inhibited by protein extracts from mutant lines than from the WT plants (**Figure 5D**).

AtKTI4 and AtKTI5 Are Able to Inhibit Both Serine and Cysteine Proteases

To further explore the serine- and cysteine-protease inhibitory capacity of AtKTI4 and AtKTI5 inhibitors, *Agrobacterium*-mediated transient over-expression for At*KTI4* and At*KTI5* genes fused to GFP was performed in *N. benthamiana* plants. GFP detection demonstrated the expression of the GFP gene and the location of the GFP protein in all agroinfiltrated plants (**Figures 6A,B**). Protein extracts from plants expressing the AtKTI4 and AtKTI5 proteins fused to GFP presented higher capability to inhibit serine- (trypsin and chymotrypsin) and cysteine- proteases (papain and bovine cathepsin B) compared with the extracts from the plants expressing only the GFP protein (**Figure 6C**). These results support the consideration of AtKTI4 and AtKTI5 as bifunctional inhibitors of both serine and cysteine proteases.

Docking analyses using ClusPro program were carried out to figure out the putative interaction between papain and the inhibitors AtKTI4 and AtKTI5. Models suggest an intrusion of different residues into the reactive site of papain stabilized by hydrogen bonds (Supplementary Figure S2). In the AtKTI4-papain interaction, the residues Glu122 and the Lys175 at the β6–β7 and β9–β10 loops, respectively, would form hydrogen bonds with the Cys and His amino acids of the papain reactive site. A similar interaction by the residues Val164 and Lys165 at the β9–β10 loop of AtKTI5 was predicted. Additional hydrogen bonds between residues of protease and inhibitor are predicted, which probably are involved in the preservation of the interaction (data not shown).

Effects of At*KTI4* and At*KTI5* Genes on Plant Resistance and Mite Performance

To further explore into the role of AtKTI4 and AtKTI5 in plant defense against *T. urticae*, homozygous T-DNA insertion lines and WT plants were infested with spider mites and the plant damage (chlorotic area) was visualized and quantified 4 days upon mite infestation. All knock down lines showed more damage than the WT plants. The injury was between 1.2 and 1.5 times higher in the knock down lines than in the WT plants (**Figures 7A,D**). Cell death caused by spider mite feeding was

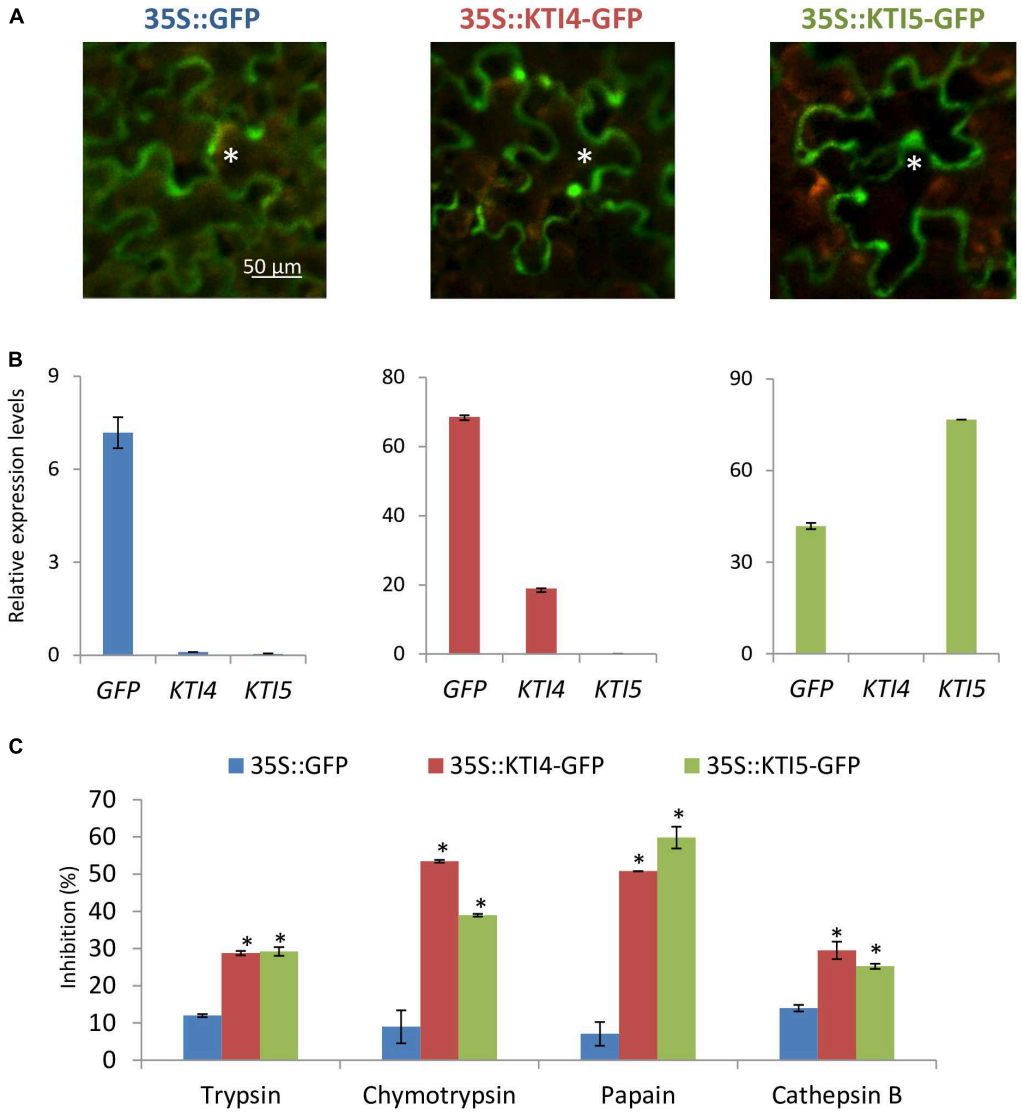

FIGURE 6 | Characterization of the expression, location and inhibitory properties of At*KTI4* and At*KTI5* genes by using transient expression assays in *N. benthamiana* plants. **(A)** GFP signal after 3 days of agroinfiltration. Asterisks indicate GFP signal of the epidermal cells. **(B)** Relative expression levels of *GFP*, *KTI4,* and *KTI5* genes in the *N. benthamiana* plants after 3 days of infiltration. **(C)** Inhibitory ability of *N. benthamiana* protein extracts expressing *KTI4* and *KTI5* genes against commercial trypsin, chymotrypsin, papain and bovine cathepsin B 3 days post-agroinfiltration. Data are mean ± SE of triplicate measurements of each sample. Asterisk indicates significant differences with the 35S::GFP plants ($P < 0.05$, One-way ANOVA followed by Dunnett's test).

evaluated by trypan blue staining of leaves from all Arabidopsis genotypes after 24 h of infestation. All knock down lines showed higher levels of staining than WT plants with *kti5.1* and the *kti5.2* lines being the most prominent (**Figures 7B,D**). As the production of H_2O_2 is used as a plant damage indicator, H_2O_2 concentrations were determined in the five Col-0 genotypes. The quantification of H_2O_2 in infested plants, expressed as DAB relative units, demonstrated that *kti5.1* and *kti5.2* lines accumulated more H_2O_2 than *kti4.1, kti4.2* lines and WT plants (**Figures 7C,D**).

To ensure that the chlorotic area correlated with mite feeding, mite performance was analyzed after feeding on mutants for At*KTI4* and At*KTI5* lines. Fecundity assays carried out on leaves from different Col-0 genotypes showed that synchronized mites fed on insertion lines had higher fecundity rates than the ones fed on WT plants (**Figure 8A**). To evaluate the KTI toxicity for mites, the mortality was recorded after feeding. Mites fed on mutant lines exhibited lower mortality rates than those on the WT plants (**Figure 8B**).

DISCUSSION

PIs from plants are proteins of particular interest because of their putative involvement in the natural defense system to phytophagous pests and pathogens. Particularly, the defense

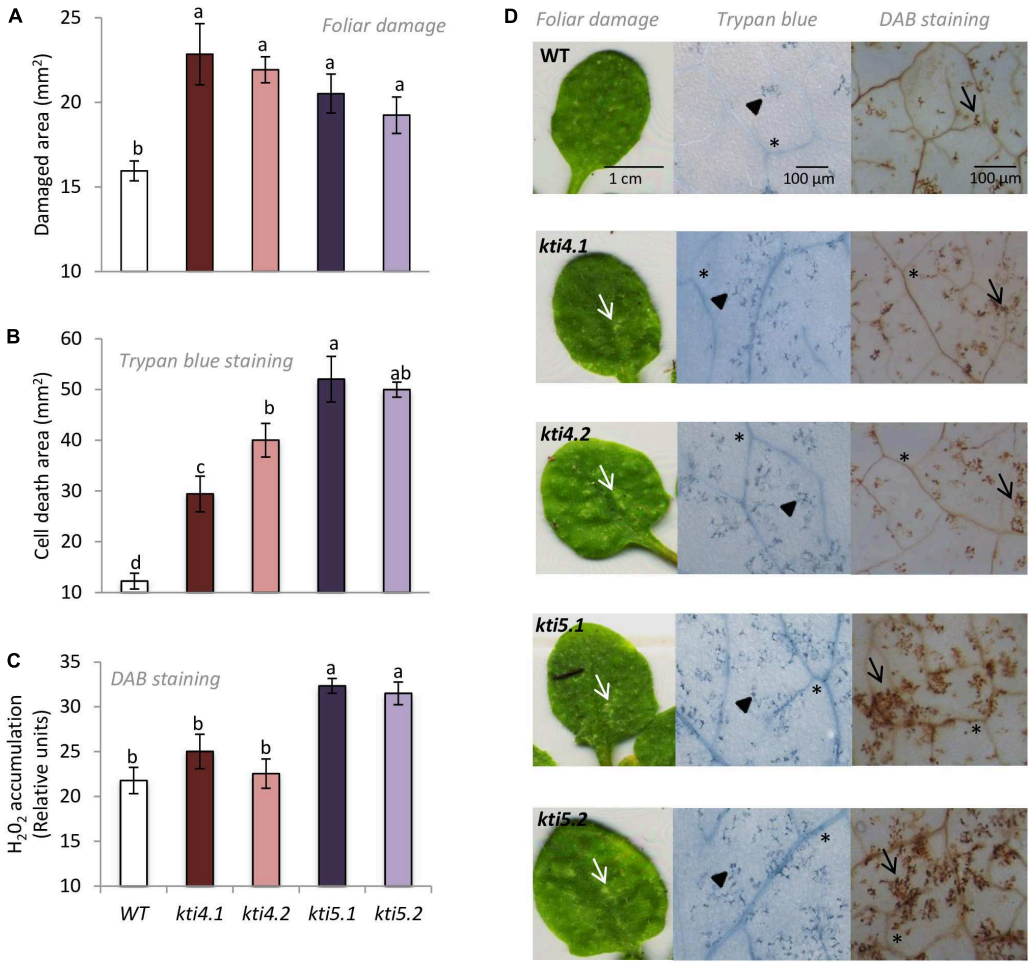

FIGURE 7 | Plant damage and hydrogen peroxide production of Arabidopsis genotypes infested with *T. urticae*. **(A)** Foliar area damaged on Arabidopsis T-DNA insertion lines (*kti4.1, kti4.2, kti5.1, kti5.2*) and WT plants after 4 days of spider mite infestation. **(B)** Trypan blue stained area on leaf disks from Arabidopsis T-DNA insertion lines and WT plants after 1 day of spider mite infestation. **(C)** Hydrogen peroxide production on leaf disks from Arabidopsis T-DNA insertion lines and WT plants after 1 day of spider mite infestation. **(D)** Example of leaf phenotypes of Col-0 genotypes after spider mite infestation. Data are means ± SE of six replicates. Different letters indicate significant differences ($P < 0.05$, One-way ANOVA followed by Student–Newman–Keuls test). Bars are as indicated in images. White arrows indicate plant damage, arrowheads cell death, black arrows H_2O_2 accumulation and asterisks signal indicate vascular tissues.

role of the plant KTI family against insects has been proven, although most studies involved individual Kunitz-type members. Determining the functional diversity of all members of the At*KTI* gene family against an important pest such as the polyphagous mite *T. urticae* may provide alternatives to spider mite control. From the 7 At*KTI* genes identified in Arabidopsis, four of them, At*KTI1,* At*KTI3*, At*KTI4,* and At*KTI 5* responded to spider mite feeding (**Figure 1**). At*KTI2,* At*KTI6,* and At*KTI7* genes were not expressed in Arabidopsis rosettes either under control or infested conditions. In the case of the At*KTI2*, this result was in agreement with a recent report showing a restricted expression to flowers and etiolated seedlings (Boex-Fontvieille et al., 2016).

Generally, KTIs are small proteins of about 20 kDa folded in β-trefoil manner with two disulphide bonds, but small differences in sequence or structure may make them different in their role as inhibitors of serine- and/or cysteine-proteases (Bendre et al., 2018). The low similarity observed among the amino acids residues of the 7 AtKTI members when their sequences were aligned may justify specific inhibitory roles against mite proteases (**Figure 2**). Trypsin inhibition is expected as the S1-binding site is negatively charged and accepts the conserved Lys at the P1 position. Likewise, chymotrypsin inhibition could be due to the presence of Tyr, Leu, and Phe residues in the β4–β5 reactive loop. The ability to inhibit papain could not be *a priori* predicted from the analysis of the amino acid sequences due to the multiple ways that Kunitz and Kunitz-structurally related inhibitors follow to inhibit C1 cysteine-proteases (Renko et al., 2012). These inhibitors may use different loops to occlude the reactive site of the cysteine protease. For instance, residues Trp88 and Pro89 of the AtKTI2 β5–β6 loop have been proposed to intrude into the catalytic triad of the Arabidopsis cysteine-protease RD21, blocking its protease activity (Boex-Fontvieille et al., 2015). This interaction is stabilized by hydrogen bonds between RD21 amino acids and

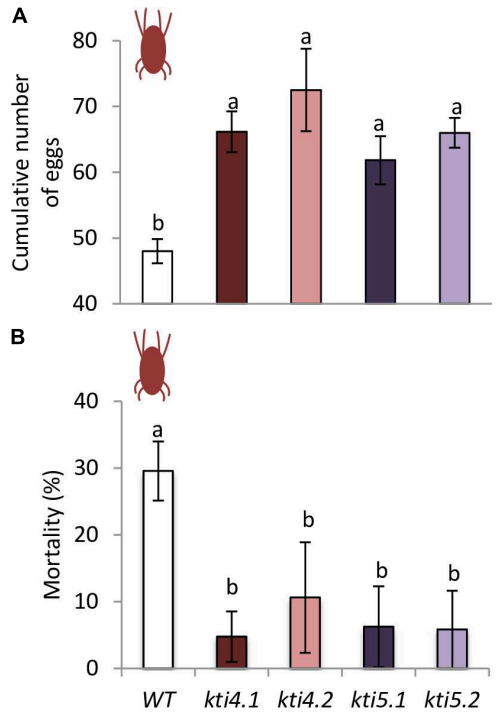

FIGURE 8 | Analysis of spider mite performance. **(A)** Effects of T-DNA insertion lines and control plants on *T. urticae* fecundity, 36 h after infestation with synchronized females. **(B)** Effects of T-DNA insertion lines and control plants on *T. urticae* mortality, 10 days after infestation with neonate larvae. Data are means ± SE of eight replicates. Different letters indicate significant differences ($P < 0.05$, One-way ANOVA followed by Student–Newman–Keuls test).

residues in the AtKTI2 β2–β3 and β5–β6 loops. The comparative alignment of amino acid sequences of the AtKTI members revealed that AtKTI2, AtKTI6, and AtKTI7 were the most different among the whole family. These inhibitors presented the cysteine residues essential to form only one disulphide bridge instead of the two bridges predicted in the rest of AtKTIs. Besides, the AtKTI2 lacked the positive residue in the loop between strands β4 and β5 essential for trypsin inhibition, which correlated with its inability to inhibit serine-proteases. In contrast, although several dissimilarities were found in the tridimensional structures of AtKTI4 and AtKTI5, both proteins conserved the Lys residue in the β4–β5 loop and are putatively able to interact with the catalytic site of papain. This plasticity of the loops coming out of the stable β-trefoil scaffold maybe the reason of the versatility of these inhibitors, which display several different mechanisms of inhibition involving different positions of the loops and their combinations (Renko et al., 2012). Thus, the wide sequence-structure variability of KTIs supports the bifunctional action shown for AtKTI4 and AtKTI5 against trypsin and chymotrypsin serine-proteases as well as against cathepsin B- and L-like cysteine-proteases. Many protease inhibitors have to be targeted to the endomembrane system to reach their functional location (Martinez et al., 2009). As expected for proteins with signal peptide that would end up as vesicle-secreted proteins, AtKTI4 and AtKTI5 were subcellular located in the endomembrane system (**Figure 3**). Previous reports have detected AtKTI2 in the cell wall, apoplast spaces and tonoplast (Boex-Fontvieille et al., 2015).

To elucidate the response of At*KTI4* and At*KTI5* genes after mite feeding, we studied their effect on the spider mite performance using T-DNA insertion lines for both genes. First, we confirmed that the reduction of At*KTI4* and At*KTI5* transcripts detected in the mutant lines was associated to an increase of commercial trypsin activity, detected by *in vitro* assays using plant extracts, a promising result to perform bioassays (**Figure 5**). Besides, we found compensation effects in T-DNA insertion lines through the alteration of the expression of other At*KTI* genes, suggesting that AtKTI1, AtKTI3, AtKTI4, and AtKTI5 proteins might participate in the defense process against spider mites in a concerted manner to modulate protease target activities (**Figure 4**).

Feeding assays conducted with the spider mite resulted in a significant increase of leaf damage either quantified as total chlorotic area or detected by trypan blue staining in comparison to control plants (**Figure 7**). However, only the knock down lines for the At*KTI5* gene accumulated higher levels of H_2O_2 than the WT plants. These findings indicate that these mutant lines either produced more H_2O_2 when infested, or alternatively, were not able to detoxify H_2O_2 efficiently, and triggered the cell death. Santamaria et al. (2017) demonstrated that an increase in H_2O_2 during *T. urticae* feeding was associated to sharp reduction in the accumulation of thiol groups and a parallel promotion of cell death. It is generally accepted that moderate levels of reactive oxygen and/or nitrogen species may differentially sense defense signaling while an excess of oxidative stress results to programmed cell death (Foyer and Noctor, 2005; Baxter et al., 2014). Therefore, these changes in the redox status have a potential impact on mite behavior. Accordingly, mite performance improved when fed on knock down lines (**Figure 8**). Our results confirm a significant reduction in mite mortality and higher fecundity rates upon feeding on mutant lines compared to control plants. Previous literature indicated that the role of At*KTI4* and At*KTI5* in Arabidopsis defense was not specifically associated to mites. Both genes were induced by *Botrytis cinerea* treatment (Coolen et al., 2016). At*KTI4* was also triggered by pathogen-derived elicitors and antagonized pathogen-associated cell death in Arabidopsis (Li et al., 2008), which may explain the higher cell death observed in our T-DNA insertion lines after spider mite infestations. Additionally, the expression of At*KTI4* and At*KTI5* was modulated by *Pieris rapae* infestation (Coolen et al., 2016). Interestingly, the expression pattern of At*KTI4* gene showed a highly localized response to *P. brassicae* eggs (Little et al., 2007) and was also induced upon nematode infestations in Arabidopsis (Jammes et al., 2005).

A second, but probably the most important mechanism of defense mediated by the AtKTIs is based on their capability to inhibit mite protease activities. The sequence and annotation of *T. urticae* genome revealed a large proliferation of serine- and cysteine-protease gene families in comparison to other sequenced arthropod species (Grbic et al., 2011). The 70 gene members identified in the serine-protease family pointed out an

essential role in the spider mite physiology (Grbic et al., 2011). In many phytophagous insects, particularly in lepidopteran, the participation of serine proteases in the gut digestion has been demonstrated (Lara et al., 2000; Patankar et al., 2001; Fan and Wu, 2005; Srinivasan et al., 2006; Chougule et al., 2008; Tabatabaei et al., 2011). However, the lack of detection of trypsin- and chymotrypsin activities in mite extracts (Carrillo et al., 2011) together with the fact that serine-protease genes did not show a clear developmental pattern of expression correlated with feeding stages (Santamaria et al., 2012, 2015b), and the absence of this activity in mite feces suggested the association of this protease type with physiological processes other than the hydrolysis of dietary proteins (Santamaria et al., 2015b). Jonckheere et al. (2016) found some genes that presumably code for serine-proteases expressed in the salivary glands of *T. urticae* suggesting a pre-digestive function in the saliva. Based on these data, an alternative target for KTIs could be the serine-proteases present in the mite saliva. However, other putative roles involved in the regulation of mite growth and development have been suggested for these enzymes (Santamaria et al., 2012). In any case, plant inhibitors might get access to the endogenous proteases through the mite gut, as has been described for some insects (Down et al., 1999; Azzouz et al., 2005). The induction of members of the PIN-I and PIN-II serine PI families after spider mite attack (Li et al., 2002; Kant et al., 2004, 2008; Martel et al., 2015) and the enhanced resistance to spider mites showed by Arabidopsis when overexpress a barley trypsin inhibitor (Carrillo et al., 2011; Santamaria et al., 2012) strongly support the potential defense role of KTIs. Interestingly, the transient expression of At*KTI4* and At*KTI5* genes in *Nicotiana* plants showed their bifunctional features to inhibit cysteine- and serine-protease activities (**Figure 6**), which it should substantiate the impact of AtKTIs on cysteine-proteases from the mite gut involved in the hydrolysis of dietary proteins.

CONCLUSION

Our results confirm a wide role of the Arabidopsis KTI proteins in defense against spider mite based on: (i) four out of the seven *KTIs* identified in *A. thaliana* are induced upon spider mite infestation; (ii) transcriptional *KTI* compensation effects take place among the silencing At*KTI4* and At*KTI5* lines; (iii) *T. urticae* inflicts more leaf damage in *kti4* and *kti5* mutant lines than in WT plants; (iv) mites feeding on At*KTI* silencing lines improve their performance; (v) AtKTI4 and AtKTI5 show a bifunctional inhibitory activity against both serine and cysteine proteases. In consequence, the inhibition of proteolytic process mediated by AtKTIs may decrease the mite access to essential amino acids and consequently to impair protein functions and to disrupt crucial physiological events needed by *T. urticae* performance. These effects finally increase mite mortality and reduce mite reproduction. Further research is needed to elucidate if the ability to inhibit serine protease activity contributes to the defense role of these inhibitors against spider mite.

AUTHOR CONTRIBUTIONS

ID and MM conceived the research. AA, LT-M, and MS performed most of the experimental research. ID, MM, PG-M, and MS participated in the design, the acquisition, analysis, or interpretation of data for the work. MS, MM, and ID wrote the manuscript. All authors contributed to final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00986/full#supplementary-material

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frontiers in Plant Science

ORIGINAL RESEARCH
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Generalist and Specialist Mite Herbivores Induce Similar Defense Responses in Maize and Barley but Differ in Susceptibility to Benzoxazinoids

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While substantial progress has been made in understanding defense responses of cereals to insect herbivores, comparatively little is known about responses to feeding by spider mites. Nevertheless, several spider mite species, including the generalist *Tetranychus urticae* and the grass specialist *Oligonychus pratensis*, cause damage on cereals such as maize and wheat, especially during drought stress. To understand defense responses of cereals to spider mites, we characterized the transcriptomic responses of maize and barley to herbivory by both mite species, and included a wounding control against which modulation of defenses could be tested. *T. urticae* and *O. pratensis* induced highly correlated changes in gene expression on both maize and barley. Within 2 h, hundreds of genes were upregulated, and thousands of genes were up- or downregulated after 24 h. In general, expression changes were similar to those induced by wounding, including for genes associated with jasmonic acid biosynthesis and signaling. Many genes encoding proteins involved in direct defenses, or those required for herbivore-induced plant volatiles, were strongly upregulated in response to mite herbivory. Further, biosynthesis genes for benzoxazinoids, which are specialized compounds of Poaceae with known roles in deterring insect herbivores, were induced in maize. Compared to chewing insects, spider mites are cell content feeders and cause grossly different patterns of tissue damage. Nonetheless, the gene expression responses of maize to both mite herbivores, including for phytohormone signaling pathways and for the synthesis of the benzoxazinoid 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside, a known defensive metabolite against caterpillars, resembled those reported for a generalist chewing insect, *Spodoptera exigua*. On maize plants harboring mutations in several benzoxazinoid biosynthesis genes, *T. urticae* performance dramatically increased compared to wild-type plants. In contrast, no difference in performance was observed between mutant and wild-type plants for the specialist *O. pratensis*. Collectively, our data provide little evidence that maize and barley

defense responses differentiate herbivory between *T. urticae* and *O. pratensis*. Further, our work suggests that the likely route to specialization for *O. pratensis* involved the evolution of a robust mechanism to cope with the benzoxazinoid defenses of its cereal hosts.

Keywords: Maize (*Zea mays* L.), *Hordeum vulgare*, *Tetranychus urticae*, *Oligonychus pratensis*, benzoxazinoid, spider mite, herbivore, HDMBOA

INTRODUCTION

Cereal crops of the grass family (Poaceae) account for the majority of human calories, and reductions in their yield dramatically impact human welfare. Abiotic factors, such as drought, are a major source of unrealized yield (Boyer, 1982), while another well-characterized source of loss is from herbivory by insects (Oerke, 2006). Spider mites (Acari: Tetranychidae) belong to the Chelicerata, an arthropod lineage that diverged more than 450 million years ago (Dunlop, 2010), and hence evolved herbivory independently from insects. Crops including maize (*Zea mays*) and wheat (*Triticum* sp.) are susceptible not only to insects but also to spider mites, especially during drought conditions (Al-Kaisi et al., 2013), where yield losses as high as 47.2% for maize have been reported (Bacon et al., 1962). Nevertheless, relatively little is known about the molecular nature of the defenses plants use to deter spider mites, especially for grasses.

As shown by molecular studies of plant–herbivore interactions, largely with insects and dicots such as *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*), many plants complement constitutive defenses (like trichomes) with rapid, inducible ones that negatively impact herbivores (Howe and Jander, 2008). For instance, herbivore-associated triggers like physical damage, oral secretions, or frass, alone or in combination, lead to changes in the production of specialized metabolites or defensive proteins that deter herbivores (Howe and Jander, 2008; Ray et al., 2015). In dicots, molecular responses to insect herbivores are mediated largely by phytohormones, especially jasmonates (jasmonic acid, or JA, and its derivatives or conjugates), which induce transcriptomic reprogramming within hours (Howe and Jander, 2008). Some defenses act directly, such as toxic compounds or protease inhibitors that retard digestion in an herbivore's gut. Others act indirectly, like plant volatiles, which can serve as olfactory cues for predators to locate herbivores at feeding sites (Turlings and Erb, 2018).

The type and magnitude of inducible defenses is influenced by several factors. One of these is feeding guild. Chewing insects like caterpillars, for instance, cause extensive tissue damage and elicit different defense responses compared to phloem-feeding insects like aphids, which cause minimal loss of plant tissue (Howe and Jander, 2008). Additionally, plant responses to generalist herbivores, to which ∼10% of plant-feeding insects belong (Ali and Agrawal, 2012), can differ from those induced by specialists. Generalist herbivores feed on hosts in many plant families. They are typically thought to rely on broad detoxification capabilities to overcome the challenges they encounter on phylogenetically (and chemically) divergent plant hosts (Dermauw et al., 2013), or to potentially suppress plant defense responses that are broadly conserved (Ali and Agrawal, 2012). Alternatively, some specialists have evolved the ability to suppress or otherwise circumvent plant defenses, potentially ameliorating the role of detoxification, or instead have evolved highly specialized detoxification abilities to cope with the toxins they encounter in their preferred plant hosts (Dobler et al., 2012; Glas et al., 2014; Maag et al., 2014; Wouters et al., 2014).

Like dicots, monocots, including grasses, are attacked by generalist and specialist herbivores of diverse feeding guilds, including leaf-chewing (e.g., caterpillars) and piercing-sucking (e.g., aphids and whiteflies). As for dicots, JA signaling and the production of specialized compounds feature prominently in monocot responses to insect herbivory (Meihls et al., 2012; Tzin et al., 2015a, 2017). Of the downstream specialized compounds in grasses, the best studied are benzoxazinoids, which are 1,4-benzoxazin-3-one derivatives produced by cereals including maize, wheat, and rye (Zúñiga et al., 1983; Niemeyer, 2009). In maize, levels of benzoxazinoids are highest in seedlings (Cambier et al., 2000), but can be locally induced at feeding sites in the leaves of older plants (Köhler et al., 2015; Maag et al., 2016). The most studied benzoxazinoid, 4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), is stored in vacuoles as an inactive glucoside (Glc) conjugate. Upon tissue damage by herbivores, DIMBOA-Glc, as well as derivatives such as 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc), are exposed to glucosidases in plastids (Meihls et al., 2012). This leads to the release of the aglucones, which are toxic to herbivores, potentially by several modes of action (Wouters et al., 2016).

Several spider mite species are significant field pests on cereals. These include *Tetranychus urticae* (the two-spotted spider mite) on maize, and *Oligonychus pratensis* (the Banks grass mite) on both maize and distant relatives including wheat (**Figure 1A**; Brandenburg and Kennedy, 1982; Mansour and Bar-Zur, 1992; Archer and Bynum, 1993; Tadmor et al., 1999; Blasi et al., 2015). *T. urticae* is an extreme generalist that has been documented on more than 100 plant families (Grbić et al., 2011). In contrast, *O. pratensis* is a specialist on plants in the Poaceae, though it has been reported on a few non-grass hosts including date palm (which is also a monocot) (Ward et al., 1972; Foster et al., 1977; Chandler et al., 1979; Holtzer et al., 1984; Archer and Bynum, 1993; Bynum et al., 2015; Negm et al., 2015). As cell-content feeders, spider mites belong to a different feeding guild than the best studied insect herbivores (Bensoussan et al., 2016). Currently, knowledge of plant responses to spider mites comes mainly from *A. thaliana*, tomato, and grapevine (*Vitis vinifera*), where *T. urticae* feeding was shown to induce robust JA responses

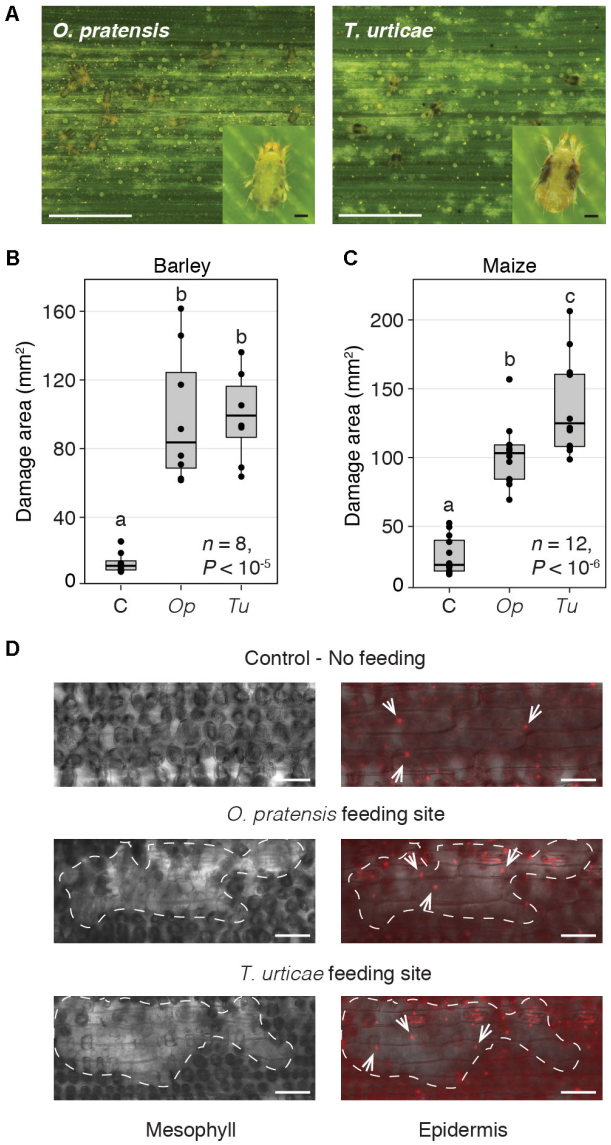

FIGURE 1 | *O. pratensis* and *T. urticae* morphology, tissue damage, and feeding strategy. **(A)** *O. pratensis* and *T. urticae* feeding causes light colored (chlorotic) spotting on barley leaves, as well as on maize (see also **Supplementary Figure 2**) (scale bars: 2.5 mm). Insets show adult *O. pratensis* and *T. urticae* females (scale bars: 100 μm). **(B,C)** Total area of damage on barley and maize leaf enclosures, respectively, for uninfested leaves (C, control), and those subjected to *O. pratensis* (*Op*) and *T. urticae* (*Tu*) feeding at 24 h. *P*-values are from an ANOVA, and letters indicate significant differences between comparisons ($P < 0.05$, Tukey's HSD test; *n*: number of biological replicates). In the boxplots, circles represent individual data points. **(D)** Leaf cell damage caused by *O. pratensis* and *T. urticae* feeding on barley. Clusters of empty mesophyll cells at mite feeding sites (left) are denoted by dashed lines; a focal plane through the epidermis is shown at right with nuclei visualized by propidium iodide staining (white arrows). Scale bar: 100 μm.

(Zhurov et al., 2014; Martel et al., 2015; Díaz-Riquelme et al., 2016). This suggests that *T. urticae* relies in large part on a broad detoxification capacity to enable its exceptionally wide host range. Nonetheless, the sister species *T. evansi*, a specialist of plants in the Solanaceae, has been documented to suppress plant defenses in tomato (Alba et al., 2015). Further, another specialist mite herbivore of tomato, *Aculops lycopersici*, is able to potently suppress plant defenses (Glas et al., 2014). While *O. pratensis* is a pest on diverse cereal crops like maize and wheat, little is known about its ancestral host range within Poaceae. Whether the grass specialist *O. pratensis* relies on detoxification, suppression of plant defenses, or both, to colonize its Poaceae hosts is unknown.

In this study, we have characterized defense responses to herbivory by *O. pratensis* and *T. urticae* in grasses. As a plant host, we chose maize, for which defense responses to insects have been comparatively well studied (Meihls et al., 2012; Tzin et al., 2015a, 2017). We also included barley (*Hordeum vulgare*) as it is a close relative of wheat with a more tractable genome (Mayer et al., 2012). By including both maize and barley, we were able to compare variation in plant defense responses to both mite species in each of two major cereal crops in phylogenetically distant lineages within Poaceae (subfamilies Panicoideae and Pooideae, respectively).

MATERIALS AND METHODS

Biological Materials and Maintenance of Stocks

Seeds for barley (cultivar Morex) were kindly provided by A. Fischer (Montana State University, Bozeman, MT, United States), while those for maize inbred B73 were kindly provided by G. Drews (University of Utah, Salt Lake City, UT, United States). Seed stocks for maize inbred W22, as well as homozygous lines for previously characterized *Ds* insertions in *BX1*, *BX2*, and *BX6* on the W22 background (Tzin et al., 2015a), were kindly provided by G. Jander (Boyce Thompson Institute, Ithaca, NY, United States). For the study, a *T. urticae* colony (strain W-GR) was established from *T. urticae* collected from a community garden and from a greenhouse site in Salt Lake City, UT, United States. Prior to this study, the W-GR strain was maintained on whole kidney bean plants (*Phaseolus vulgaris*) for more than 30 generations using established rearing methods (Van Leeuwen et al., 2012). Before being used as a source of mites for plant infestation experiments (see below), mites of W-GR were acclimated on barley (Morex) or maize (B73) for at least two generations to remove possible maternal effects on host use that might impact plant responses. For propagation, 8–10 week old barley and maize plants were used to maintain bulk populations of at least several thousand mites. For *O. pratensis*, a field-collected strain was acquired from maize (Logan, UT, United States); propagation of the *O. pratensis* strain, and acclimation prior to the respective experiments on barley and maize, was as for *T. urticae* except that *O. pratensis* was never maintained on kidney beans, which are not a host.

For maintaining mite colonies, barley and maize plants were germinated and grown in Metro-Mix® 900 growing mix (Sun Gro®, Agawam, MA, United States) and watered from below as needed (pots were placed in trays to which water was

added). Barley plants were germinated and grown in a walk-in growth chamber with a 16-h light/8-h dark photoperiod with 170–200 μmol m^{-2} s^{-1} light at 20°C and 60% humidity. Maize plants were germinated and propagated in a greenhouse with a 16-h light/8-h dark photoperiod at an approximate temperature of 25°C. Maize and barley plants were fertilized weekly with 200 ppm NutriCulture Cal-Mag Special 16N-3P-16K (for maize) and NutriCulture Mag-Iron Special 18N-6P-18K (for barley) (Plant Marvel Laboratories, Chicago Heights, IL, United States). At the respective leaf stage, plants were moved to an isolated room maintained at room temperature (∼22°C) for mite propagation, with new plants placed next to and touching previously infested plants to allow mites to move to fresh hosts. Mites from these colonies were used for all experiments unless otherwise noted.

Quantification of Feeding Damage

To assess the extent of foliar damage caused by mite feeding, mites of each species were placed on either the fifth leaves of 30-day-old barley plants or on the fourth leaves of 22-day-old maize plants. The studies for barley and maize were performed in a walk-in growth chamber or a greenhouse, respectively, under the conditions described above. Briefly, barley and maize seeds were sown 1–2 cm deep in 5 × 5 cm plastic pots in Metro-Mix® 900 growing mix. Ten days after sowing, seedlings were transplanted into 20 cm diameter pots. As the maize plants were grown in a greenhouse bay, plants were maintained inside insect-free enclosures until the day before the experiment to exclude potential greenhouse pests (insect enclosures were constructed with PVC piping and No-See-Um nylon netting, BioQuip Products, Compton, CA, United States, catalog number 7250NSB). To minimize the impact of environmental variation, a randomized block design was employed.

While mites cannot fly, they can disperse quickly by crawling on leaves. Therefore, for both control plants and those used for mite infestations, we established leaf enclosures using non-phytotoxic wax barriers that mites do not cross, an approach previously employed in studies with both mite species (Feese and Wilde, 1977; Mansour and Bar-Zur, 1992). For our study, we used Tree Tanglefoot wax (The Scotts Miracle-Gro Company, Marysville, OH, United States), with two barriers established perpendicular to the length of the leaf blades (Tanglefoot was placed in stripes across the adaxial and abaxial surfaces of leaves and joined at the edges; **Supplementary Figure 1**). Mites added between the two barriers were thus free to move along and between the leaf surfaces internal to the Tanglefoot boundaries. Maize leaves are well separated, and hence enclosures were easily established on free-standing plants. However, the plant architecture of barley presented an additional challenge, necessitating immobilization of leaf-harboring enclosures as shown in **Supplementary Figure 1**.

Twenty-four hours after establishing 12.5 cm Tanglefoot enclosures, 250 and 350 female mites were used to infest barley and maize leaves, respectively; as maize leaves are wider, more mites were added to keep mite density roughly constant (no mites were added to enclosures on the control plants). Adult mites of both species, which are only ∼0.6 mm in length (**Figure 1A**, insets), were collected under dissection microscopes from the bulk populations maintained on the respective hosts. To do this, defined numbers of adult females were harvested into 200 μL barrier pipet tips by suction (the large ends of pipet tips were attached to vacuum lines). At the time of collection, tips with mites were immediately placed onto ice. On ice, mites are less physically active and less likely to spin webs, which can otherwise lead to complications for subsequent release. To release mites onto leaf enclosures, pipet tips with mites were removed from ice, the tops of the tips were cut off with scissors (to increase the bore size for release), and mites were gently poured onto the leaf surface along the length of the enclosures.

At 24 h post-infestation, leaf enclosures were harvested by cutting across the leaf blade immediately internal to the Tanglefoot barriers. Both sides of each leaf segment were then scanned at 1200 dpi using an Epson Perfection V550 Photo scanner (Epson, Suwa, Japan). To ensure the mite damage was quantified from leaf segments of exactly the same length, the leaf segments were immobilized to a frame to reveal the middle 7.5 and 10 cm for barley and maize enclosures, respectively, before scanning. Barley leaf segments were sufficiently flat to be scanned without additional manipulation. In contrast, maize leaf segments could not be scanned directly due to pronounced midribs; therefore, leaf segments harvested from the enclosures were cut along the midrib, and the midrib and two halves of the leaf blade were pressed flat for scanning (**Supplementary Figure 2**). The resulting images were processed using the thresholding function of the FIJI software (Schindelin et al., 2012), which allowed selection of light colored spots caused by mite feeding. The total area of damage on the adaxial and abaxial leaf surfaces was then quantified. Statistical analyses of leaf feeding damage were performed with analysis of variance (ANOVA) in R version 3.3.2 (R Core Team, 2016), with pairwise comparisons among treatments subsequently performed with Tukey's HSD test.

Cell Imaging at Mite Feeding Sites

Barley leaves were infested with *T. urticae* and *O. pratensis* for 24 h as described for quantification of mite feeding damage. Uninfested leaves were used as controls. Barley leaf segments with and without mite feeding were dehydrated through an ethanol series (15, 50, 75, and 95%) for 30 min each under vacuum, then decolorized in 200 proof ethanol overnight at 4°C. Decolorized leaf segments were rinsed twice in water, then incubated in 1 μg/mL propidium iodide for an hour. Stained leaf segments were then rinsed and mounted in water for imaging, and Z-series images were collected at 2 μm intervals through the epidermis and mesophyll layers of the leaf specimens using a Zeiss Axio Observer Z1 microscope (Zeiss, Baden-Württemberg, Germany). Fluorescence signal from propidium iodide was detected using an excitation filter of 559–585 nm and an emission filter of 600–690 nm. Images were processed using FIJI (Schindelin et al., 2012), Adobe Photoshop CC 2017, and Adobe Illustrator CC 2017 (Adobe, San Jose, CA, United States).

Collection of Plant Tissue for Transcriptomic Studies

Plant leaf tissue was collected from barley (Morex) and maize (B73) plants with the following treatments: (1) control (uninfested leaves), (2) wounding, (3) *T. urticae* infestation, and (4) *O. pratensis* infestation. As a time course, we selected 2- and 24-h time points because dynamic expression changes in defense genes in response to *T. urticae* were observed in the dicots *A. thaliana*, tomato, and grapevine within 24 h (Zhurov et al., 2014; Martel et al., 2015; Díaz-Riquelme et al., 2016). Further, in maize, time points for analogous studies with insects from several feeding guilds were similar, and included 24-h time points (Tzin et al., 2015a, 2017). The experimental design employed for assessing transcriptomic responses was similar to that for assessing quantification of feeding damage with several modifications. For barley, 30-day-old plants were used with enclosures of length 10 cm on the fifth leaves, and for maize, 22-day-old plants were used with enclosures of length 9 cm on the fourth leaves (mite density was therefore held roughly constant). A randomized block design was used to minimize environmental variation. For the wounding treatments, plant tissue in enclosures was gently pressed between 2 pieces of 60 grit sandpaper (particle size ∼260 μm; the blades on each side of the midrib were wounded along the lengths of the enclosed leaf segments). For mite infestation, 400 and 200 adult female mites were used per leaf enclosure for the 2- and 24-h time points, respectively. Briefly, because it takes tens of minutes to several hours for released mites to fully untangle themselves, disperse, and start feeding, the density of applied mites was doubled for the 2-h time point to ensure feeding by 2 h. For RNA preparation, we collected tissue from within the enclosures as a major component of induced plant defense responses is local (Maag et al., 2016; Tzin et al., 2017). Following best practices in the field (Tzin et al., 2015a, 2017), mite infestation and wounding treatments were staggered prior to a single collection time (**Supplementary Figure 3**). In all cases, a biological replicate consisted of the entire leaf enclosure from a single leaf. To minimize the effects of the circadian cycle, for both the barley and maize experiments all samples were collected within a 15-min time window 3 h into the 16-h light period.

RNA Isolation, RNA-Seq Library Construction, and Sequencing

Total RNA was prepared from leaf material from Tanglefoot enclosures with the DirectZol RNA extraction kit (Zymo Research, Irvine, CA, United States). Barcoded RNA-seq libraries were constructed at the High-Throughput Genomics Core Facility (University of Utah) using the Illumina (Illumina, San Diego, CA, United States) TruSeq Stranded mRNA Library Preparation Kit with poly(A) selection, and 125 bp paired-end reads were generated on an Illumina HiSeq 2500 sequencer with HiSeq SBS Kit v4 sequencing reagents. Briefly, four lanes were run in total for each of the barley and maize experiments, each consisting of 28 samples (four replicates each for the control, wounding at 2 and 24 h, *T. urticae* infestation at 2 and 24 h, and *O. pratensis* infestation at 2 and 24 h). Biological replicates were evenly distributed across the four sequencing lanes to reduce the possibility of confounding due to lane-level effects.

Detection of Differentially Expressed Genes

The barley genome version ASM3268v1 (Release 33) (Mayer et al., 2012) and the maize genome version AGPv3 (Release 31) (Schnable et al., 2009) were downloaded from Ensembl (Yates et al., 2016). To examine expression of benzoxazinoid biosynthesis genes in maize, the gene model for *BX7* (*GRMZM2G441753*) was taken from an earlier annotation (Ensembl Release 20), as it was considered a low-confidence model and not included in the newer AGPv3 release. RNA-seq reads were aligned to their respective genomes using the two-pass alignment mode of STAR 2.5.2b (Dobin et al., 2013) with a maximum intron size of 20 kb. The number of reads uniquely aligned to each locus was counted using HTSeq 0.6.0 (Anders et al., 2015) with "--strand reverse" and "--feature transcript". Differentially expressed genes (DEGs) were detected using the DESeq2 package (version 1.14.0) (Love et al., 2014) with a false discovery rate (FDR) adjusted *P*-value of 0.01 and an absolute value $\log_2$ fold change cutoff of 1. Fragments per kilobase of transcript per million mapped reads (FPKM) (Mortazavi et al., 2008; Trapnell et al., 2010) values were calculated with Python scripts using the BCBio[1] GFF parser.

Cluster Analyses

Independently for each of the maize and barley time course RNA-seq data sets, gene-level *k*-means clustering was performed on the set of genes that was differentially expressed in at least one treatment. Briefly, the $\log_2$ fold change estimates of each gene across treatments compared to the control were used as the input to the *kmeans* function of the base "stat" package in R with the default Hartigan and Wong algorithm. Different *k* numbers were tested with $k = 6$ chosen as it resulted in clusters with distinct expression profiles across the treatments. The *kmeans* function was run with 100 iterations (nstart = 100) of the algorithm to optimize the clustering output. To compare the expression profiles across host species, the centers of the barley and maize gene *k*-means clusters were subjected to hierarchical clustering using the *hclust* function in R (distance: squared Euclidean; linkage: complete). While the *k*-means clusters were numbered arbitrarily by the *kmeans* function, barley and maize gene clusters with similar expression patterns identified by hierarchical clustering were manually renumbered for simplicity of comparison.

Gene Ontology Annotations and Gene Set Enrichment Analysis

Gene ontology (GO) annotations of the barley genome version ASM3268v1 (Release 33) and maize genome version AGPv3 (Release 31) were obtained by parsing the respective EMBL flat files provided by Ensembl (Biopython package SeqIO, version 1.69; Cock et al., 2009). The GO annotations were used to

[1]https://Github.com/chapmanb/bcbio-nextgen

classify gene sets using the biological process (BP) and molecular function (MF) ontologies. The MF ontology was used to identify genes encoding defensive proteins and enzymes (i.e., protease inhibitors, chitinases, and terpene synthases).

Gene set enrichment analysis was performed using the BP ontology. To enable a comparison of gene set enrichments between barley and maize gene sets, we modified the BP ontology annotation of each species so that one-to-one orthologs between barley and maize were associated with the same GO terms. The one-to-one orthologs were determined by reciprocal BLASTP (BLAST+ version 2.5.0+; Camacho et al., 2009) searches with an *E*-value cut-off $<10^{-5}$. The search was limited to the longest isoform of each protein. For each gene with a best reciprocal BLASTP hit, the list of BP ontology terms was updated to contain the union of annotated BP ontology terms from the barley and maize orthologs. Gene set enrichment analyses were performed using the "weight01" algorithm with Fisher's test statistic as implemented in the topGO 2.32.0 package (Alexa et al., 2006).

Hormonometer Analyses

We adapted the Hormonometer tool (Volodarsky et al., 2009) to assess plant hormone signaling in barley and maize following the approach used by Tzin et al. (2015a, 2017) in studies of maize responses to herbivory by insects. This tool searches for the similarity of gene expression changes to signatures of transcriptomic responses diagnostic for specific plant hormone signaling pathways. Briefly, experimentally assessed reference expression data sets are available from *A. thaliana*, and were generated by exogenous application of plant hormones (or hormone precursors) that induce JA, salicylic acid (SA), ethylene, abscisic acid (ABA), brassinosteroid, cytokinin, auxin, and gibberellic acid (GA) signaling (Volodarsky et al., 2009). As the Hormonometer tool was developed with *A. thaliana* expression data, to use the tool with barley and maize we performed reciprocal BLASTP searches with *A. thaliana* proteins to identify orthologs in both grass species. As a result, we identified 8236 barley genes and 8904 maize genes expressed in our study that had corresponding *Arabidopsis* Probeset IDs (**Supplementary Data Sheets 1**, **2**, respectively), and we used these as input for the Hormonometer tool to assess the similarity of gene sets induced by spider mite feeding and wounding to those induced by specific phytohormones.

Peroxidase Activity Assays

Plants were grown and infested with mites as described for damage quantification. The leaf samples were collected 24 h after infestation and ground to a fine powder in liquid nitrogen. Peroxidase activity in leaf samples was quantified with a microplate reader (Biotek EPOCH, Winooski, VT, United States) as described previously (Ramirez and Spears, 2014). Briefly, frozen leaf powder was thawed and suspended in 1 mL of sodium phosphate buffer (0.05 M, pH 7.0). Leaf homogenates were centrifuged for 12 min at 12,000 rpm to extract soluble leaf proteins in the supernatant. Peroxidase activity was detected in soluble protein at 470 nm following the oxidation of guaiacol for 1 min (Moran and Cipollini, 1999), and expressed as the change in absorbance per mg of total protein. The impacts of the treatments on peroxidase activity were assessed by ANOVA factoring in the block design; where significant effects were observed, Tukey's HSD tests were performed.

Mite Productivity on *bx* Mutant Plants

To assess the impact of mutations in genes in the maize benzoxazinoid pathway on mite infestation, seeds of W22, *bx1::Ds*, *bx2::Ds*, and *bx6::Ds* (Tzin et al., 2015a) were germinated directly in 15 cm diameter pots. At 2 weeks (approximately the three-leaf stage), plants of the four genotypes were arranged in a randomized block design with 16 replicates of each genotype (eight plants per block). Three 1–2-day-old adult females from synchronized mite populations were then added to each plant. To do this, three mites were sucked into barrier pipet tips as described previously, and the mites were tapped to the bottom of the tips against the barriers. The tops of the tips were then cut off to allow mites to escape, and single tips were immediately taped to the bottom of individual plants with the top of the cut tip pointing up and touching the second leaf. Synchronized mite populations were obtained by placing fertilized female mites on detached B73 maize leaves, allowing the females to lay eggs for 2 days, and then removing them (the resulting population of female mites was thus approximately synchronized). To prevent detached leaves from drying out, they were placed on wet cotton and their edges were sealed with Tanglefoot wax. Eighteen days after adding tips with mites to plants, entire plants were collected and kept at 4°C (which arrests mite reproduction and development). The number of eggs and viable mites (larvae, nymphs, and adults) found on each plant was then subsequently counted under a dissecting microscope. The impact of maize genotype on mite productivity by species per plant was assessed with ANOVA factoring in the block design. Subsequent pairwise tests were performed with Tukey's HSD method.

RESULTS

O. pratensis and *T. urticae* Cause Similar Patterns of Tissue Damage

To assess responses of barley (cultivar Morex) and maize (inbred B73) to specialist and generalist spider mite herbivores, we examined transcriptomic responses to *O. pratensis* and *T. urticae* feeding at 2 and 24 h. As the magnitude of wounding can impact interpretation of transcriptomic responses, we first assessed the extent of plant tissue damage from both mite species. For *O. pratensis* and *T. urticae*, and for barley and maize, macroscopic damage from mite feeding at 24 h consisted of fine white stippling (**Figure 1A** and **Supplementary Figure 2**). For both plant hosts and for both mite herbivores, significant plant damage was observed compared to uninfested leaves following damage quantification from leaf scans ($P < 0.05$, ANOVA with Tukey's HSD method to correct for multiple tests; **Figures 1B,C**). At 24 h, areas of stippling caused by *O. pratensis* and *T. urticae* on barley were not significantly different (**Figure 1B**). In contrast, a significant albeit modest increase in damage was observed for maize leaves infested with *T. urticae* relative to those exposed to *O. pratensis* (**Figure 1C**). It should be noted, however, that

the sample size, and hence power to detect an effect, was larger for maize; this was a function of performing maize studies in a large greenhouse as opposed to a growth chamber as was used for barley (see the section "Materials and Methods").

We also examined the microscopic pattern of damage caused by *O. pratensis* and *T. urticae* on barley leaves, for which the even epidermis is straightforward to image with differential interference contrast and fluorescence microscopy in cleared tissue. For both mite species, clusters of mesophyll cells were empty at feeding sites (or minimally, were devoid of chloroplasts that are dark in appearance; **Figure 1D**). The overlying epidermis, in which cells lack chloroplasts, nonetheless appeared to be intact as assessed by the presence of propidium iodide stained nuclei in pavement cells.

Spider Mite Herbivores Induce Similar Transcriptomic Responses on Barley and Maize

To examine transcriptomic responses to herbivory by *O. pratensis* and *T. urticae*, we collected plant tissue from within leaf enclosures at 2 and 24 h after mite infestation. Quantification of gene expression from alignments of the resultant RNA-seq reads from four biological replicates for controls and treatments revealed that 21,472 of 26,066 (82.4%) high-quality barley genes were expressed (i.e., had non-zero read counts), as were 30,279 of 39,625 (76.4%) high-quality genes in maize (see the section "Materials and Methods").

As assessed with a principal component analysis (PCA) using controls and all treatments, biological replicates were tightly clustered (**Figures 2A,B**). For the 2-h time point, replicates for both *O. pratensis* and *T. urticae* clustered nearby but were separate from controls in both barley and maize. A similar but more distinct pattern was observed at 24 h. Although dramatic differences in plant responses to the two mite species were not readily apparent for most contrasts, *O. pratensis* replicates at 24 h clustered farther away from control samples in barley than did those for *T. urticae*, albeit along the same PCA axis.

We used DESeq2 (Love et al., 2014) to detect DEGs – as assessed with a FDR-adjusted *P*-value of 0.01 and an absolute value $\log_2$ fold change cutoff of 1 – between treatments and controls for barley and maize (**Figures 2C,D**, respectively; results of DEG analyses for all comparisons are given in **Supplementary Data Sheet 3** for barley and **Supplementary Data Sheet 4** for maize). In response to feeding by both *O. pratensis* and *T. urticae*, hundreds and thousands of DEGs were detected in both barley and maize at 2 and 24 h, respectively. In contrast to the 2-h time point, for which nearly all expression changes involved upregulation, both up- and downregulation were observed at 24 h.

The partial overlap of DEGs between mite treatments (**Figures 2C,D**) raised the possibility that components of plant defense responses to the generalist and specialist mites differed. To investigate this further, we generated scatter plots of $\log_2$ fold changes for genes responding to mite herbivory at the 2- and 24-h time points for combinations of plant and mite species (**Figure 3**). Consistent with the PCAs (**Figures 2A,B**), transcriptomic responses to feeding by *O. pratensis* and *T. urticae* were highly correlated at both time points in each species (R^2-values between 0.8 and 0.9, all *P*-values $< 10^{-16}$; **Figure 3**). Our findings, therefore, revealed no compelling evidence for large-scale, qualitative differences in plant gene expression responses between the specialist and generalist mites.

Nonetheless, at the quantitative level, *O. pratensis* induced modestly stronger transcriptomic responses than did *T. urticae*, as assessed by the magnitude of fold changes, in barley at 24 h (**Figure 3B**). Likewise, in maize at the 2-h time point, a similar pattern was observed for the grass specialist (**Figure 3C**). These trends were also apparent in measurements of the activity of peroxidase, which has been reported to increase in response to *T. urticae* herbivory in several dicots (Hildebrand et al., 1986; Liang et al., 2017). In both barley and maize at 24 h, herbivory by both *O. pratensis* and *T. urticae* elevated peroxidase activity relative to control leaves ($P < 0.05$, ANOVA and Tukey's HSD test; **Figure 4**). Paralleling the difference in the relative magnitude observed for gene expression responses in barley (**Figure 3B**), including for genes encoding peroxidase enzymes (**Supplementary Figure 4**), *O. pratensis* induced significantly greater peroxidase activity compared to *T. urticae* at 24 h (**Figure 4A**).

Comparison of Plant Responses Induced by Mites to Those Induced by Mechanical Wounding

While mechanical wounding cannot fully replicate the intricacies of physical damage caused by herbivores (Howe and Jander, 2008), our wounding treatment, which consisted of gently pressing leaf blades with sandpaper, mimicked the dispersed nature of spider mite damage to plant leaves (**Figure 1A** and **Supplementary Figure 2**). As assessed by PCAs, at 2 h wounding treatments in both barley and maize clustered separately and further from controls compared to the mite treatments, and more genes were differentially regulated (**Figure 2**). In contrast, at 24 h, biological replicates for wounding treatments clustered nearer control samples in both barley and maize, and fewer genes were detected as differentially expressed compared to wounding at 2 h, or to *O. pratensis* and *T. urticae* feeding at 24 h (**Figure 2**). However, despite differences in the number of DEGs between the wounding and mite feeding treatments, the direction of changes in gene expression and their fold-induction were correlated in both barley and maize between wounding and mite feeding at 2 as well as 24 h (R^2-values between 0.12 and 0.52, with all *P*-values $< 10^{-15}$; **Supplementary Figure 5**).

Defense Pathways That Respond to Mite Herbivory in Barley and Maize

To further characterize gene expression changes across treatments, time points, and plant hosts, we performed *k*-means clustering in barley and maize using expression levels for all genes significantly differentially expressed in at least one treatment. For $k = 6$, and as assessed subsequently with hierarchical clustering (**Figure 5A**), analogous clusters with similar patterns of RNA abundances were readily observed

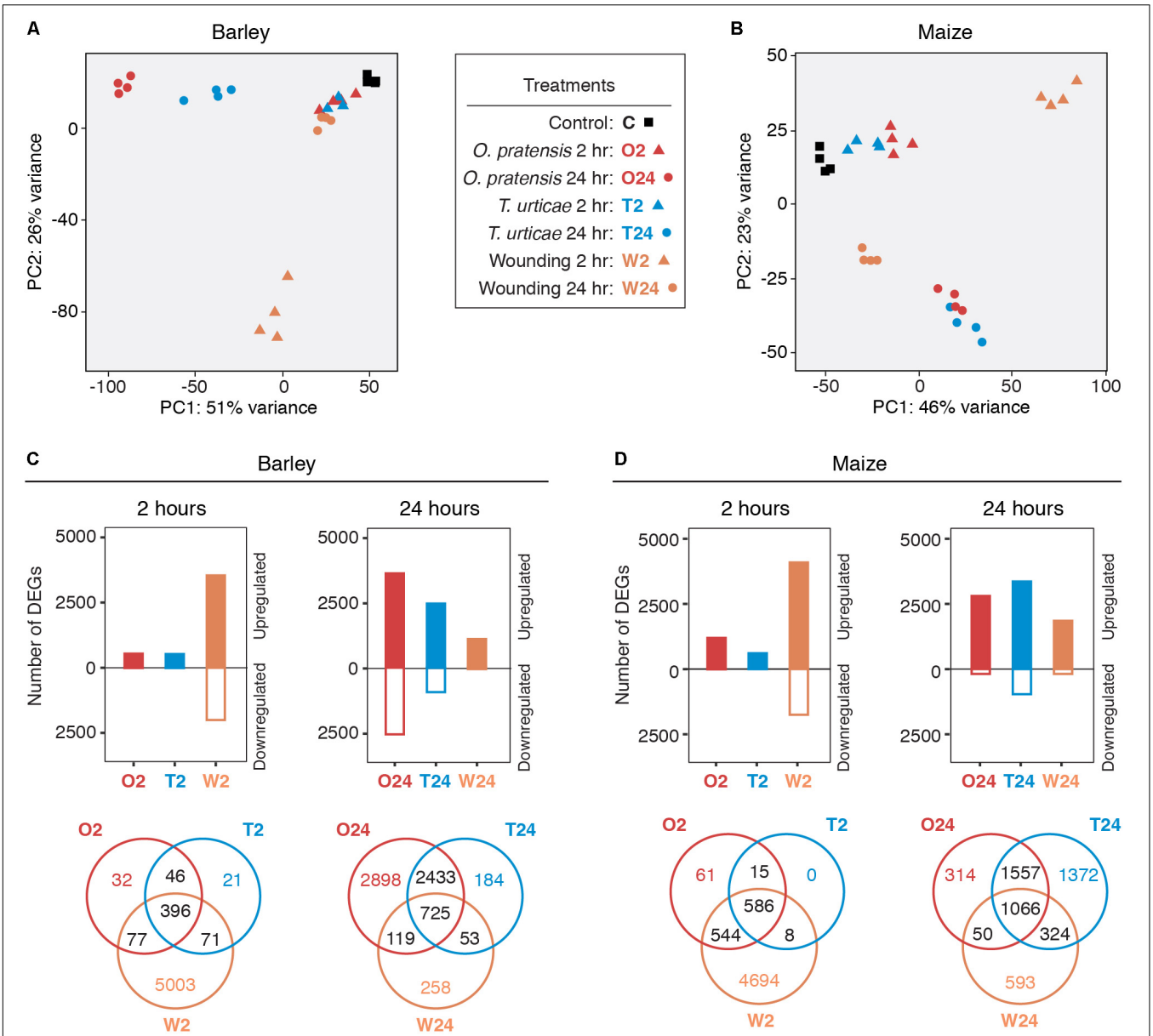

FIGURE 2 | Dynamic gene expression changes in barley and maize in response to mite herbivory and wounding. **(A,B)** PCAs of expression data for control, mite infested, and wounded leaves in barley and maize, respectively. Treatments and time points are as indicated in the legend (center; hour: hr). **(C,D)** Number of significantly up- and downregulated genes detected in each treatment for barley and maize, respectively (FDR-adjusted *P*-value of 0.01, absolute value $\log_2$ fold change cutoff of 1). Expression differences are broken out by time points (2 and 24 h, left and right for each plant host), and Venn diagrams show the overlap of DEGs among treatments (bottom).

between barley and maize (**Figure 5B**). Clusters 1–5 contain genes that were mostly upregulated in response to either herbivory or wounding, whereas genes in cluster 6 were mostly downregulated (**Figures 5A,B**; clusters 4 and 5, which contain genes with relatively modest fold changes, are shown in **Supplementary Figure 6**).

To relate genes in clusters to biological functions, we performed a GO enrichment analysis for BPs as implemented in the topGO package (Alexa et al., 2006). Significantly enriched GO terms for all clusters for both barley and maize are given in **Supplementary Tables 1**, **2**, respectively. This analysis revealed that herbivory by *O. pratensis* and *T. urticae*, as well as wounding, led to a general switch in transcriptomic programs from processes such as development to those associated with response to the environment. For example, cluster 6, representing mostly downregulated genes in the treatments, was enriched for GO terms related to photosynthesis and development. In contrast, clusters with genes upregulated in response to mite herbivory or wounding, such as clusters 1 and 2, were enriched for GO terms associated with abiotic or biotic defense responses in

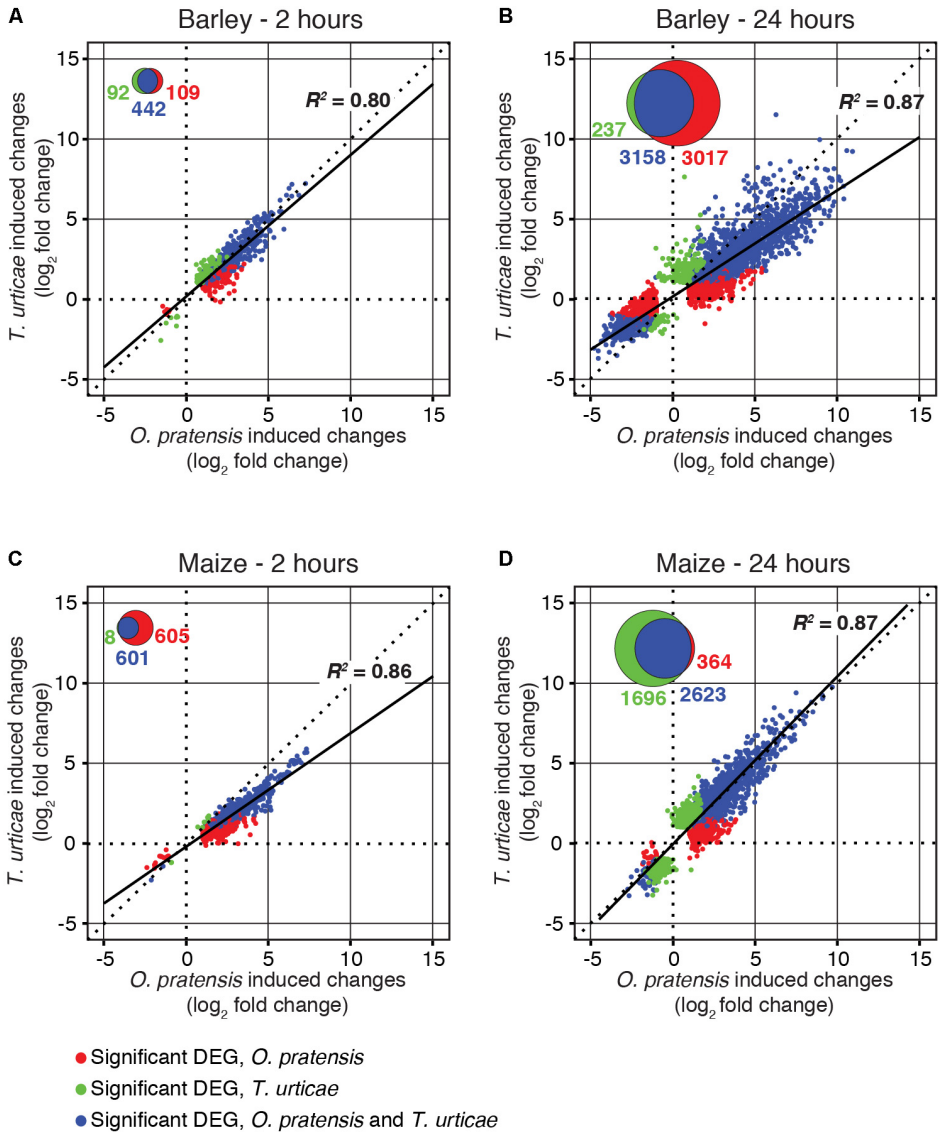

FIGURE 3 | *O. pratensis* and *T. urticae* induce similar changes in gene expression in both barley and maize. Scatter plots of $\log_2$ fold changes for DEGs (FDR-adjusted *P*-value of 0.01, absolute value $\log_2$ fold change cutoff of 1) in barley **(A,B)** and maize **(C,D)** in response to *T. urticae* and *O. pratensis* herbivory at two time points (2 h: **A**,**C**; 24 h: **B**,**D**). For inclusion in a given analysis, a gene had to be detected as differentially expressed in response to at least one herbivore (see Venn diagram insets, and legend, bottom left).

barley, maize, or both. These included ontology terms such as "JA biosynthetic process," "response to JA," "response to SA," "response to fungus," "response to wounding," and "hyperosmotic salinity response." Of the genes in the six clusters, fold changes for those in cluster 1 were most dramatic with respect to mite feeding across the time course (strong upregulation at 2 h, and even greater upregulation at 24 h). Genes in this cluster were also highly upregulated in response to wounding, especially at 2 h. A reduced correspondence between the magnitude of gene expression changes to mite feeding and wounding was observed in other clusters (e.g., clusters 2 and 3).

We also examined whether the composition of analogous clusters between barley and maize was enriched in orthologous genes, which would suggest common response pathways. To do this, we identified 14,087 genes as orthologs between barley and maize as assessed with a reciprocal best BLASTP hit analysis. For each of clusters 1–6, orthologous gene pairs were enriched between barley and maize (all *P*-values $< 10^{-12}$ as determined with hypergeometric tests; **Supplementary Table 3**).

Genes for JA Biosynthesis and Signaling Respond Rapidly to Mite Herbivores and Wounding

The GO enrichment analysis suggested a prominent role for phytohormone signaling in responses to both mite herbivores

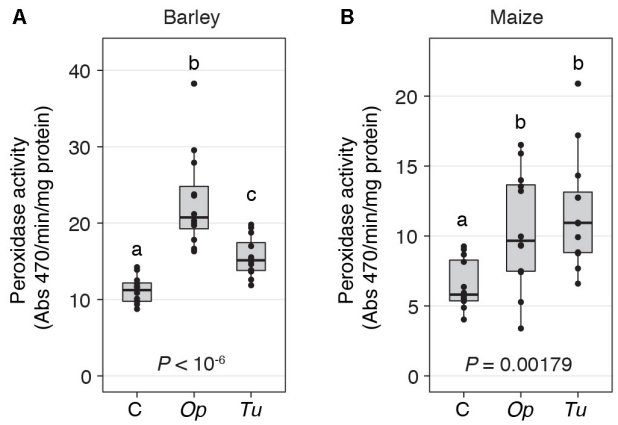

FIGURE 4 | Peroxidase activity increases in response to spider mite herbivory. Peroxidase activity in response to *O. pratensis* (*Op*) and *T. urticae* (*Tu*) herbivory at 24 h in barley **(A)** and maize **(B)**. *P*-values are from an ANOVA, and different letters reflect significant differences among controls (C) and the treatments ($P < 0.05$, Tukey's HSD test). In the boxplots, circles represent individual data points.

and wounding. Therefore, we adapted the Hormonometer tool (Volodarsky et al., 2009) to relate mite- and wounding-induced changes in gene expression to those induced by diverse phytohormones. As displayed in dendrograms for both plant hosts (**Figures 6A,B**), changes in most phytohormone response pathways were readily apparent by 2 h in response to herbivory by *O. pratensis* and *T. urticae*, as well as wounding. In general, patterns of up- or downregulation for plant hormone responsive genes were similar between barley and maize, and at 24 h responses were generally attenuated. Response genes for JA, SA, ABA, and auxin were most dramatically upregulated, while response genes associated with ethylene, GA, cytokinin, and brassinosteroid signaling were either downregulated, variable, or largely unaffected.

Because of the strong JA responses, and functional-genetic studies demonstrating a role for JA signaling in deterring *T. urticae* feeding on *A. thaliana* and tomato (Zhurov et al., 2014; Martel et al., 2015), we characterized transcriptomic responses of this pathway further. While the JA pathway is well characterized in dicots, less about it is known in monocots. However, candidate genes for JA biosynthesis, signal transduction, and mediation of transcriptional changes have been identified in maize (Borrego and Kolomiets, 2016), and genetic studies provide functional evidence for the involvement of several of these genes in JA biosynthesis and signaling (Yan et al., 2012; Tzin et al., 2017). Initially, we focused our analysis on the JA pathway in maize (**Figure 6C**), describing general patterns of responses to both mite species as induced gene expression is highly correlated between them (**Figure 3**).

Lipoxygenases (LOXs) catalyze the production of oxylipins, which are involved in multiple aspects of plant defense (Borrego and Kolomiets, 2016). The first step in the JA biosynthesis pathway is the production of the oxylipin 13(*S*)-hydroperoxylinolenate (13-HPOT) from α-linolenic acid. Putative LOXs for catalyzing this reaction belong to the 13-LOX group, of which maize has five (*LOX7*, *8*, *9*, *10*, *11*, *13*) (Borrego and Kolomiets, 2016). Of these, all but *LOX11* were induced at one or more time points, albeit with modest fold changes. As assessed by normalized expression values (FPKMs), basal levels of *LOX10* were ∼90-fold higher than for other 13-LOX genes, consistent with its role in the production of green leaf volatiles (Christensen et al., 2013), which are abundant in grasses. The next two steps in JA synthesis involve the conversion of 13-HPOT to 12,13(*S*)-epoxylinolenic acid (12,13-EOT), followed by cyclization to yield 12-oxo-phytodienoic acid (12-OPDA) (Borrego and Kolomiets, 2016). Differential expression was also observed for putative genes mediating these steps, including the upregulation of four *ALLENE OXIDE SYNTHASE* (*AOS*) genes and two *ALLENE OXIDE CYCLASE* (*AOC*) genes, respectively. As compared to *13-LOX* and *AOC* genes, the *AOS* genes were more highly upregulated at both 2- and 24-h time points. This included genes in the *AOS1* clade, but also genes in clade 2 for which a role in JA biosynthesis is uncertain (Borrego and Kolomiets, 2016). The next step in JA synthesis involves the conversion of 12-OPDA to 3-oxo-2-(*cis*-2′-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0), which is mediated by oxophytodienoate reductase (OPR). In maize, this step requires the products of *OPR7* and *OPR8*, for which JA levels are dramatically reduced in tissues of the double mutant, including in leaves (Yan et al., 2012). One of these genes, *OPR7*, was modestly upregulated at 24 h in response to mite herbivory. The conjugation of JA to isoleucine, which is carried out by the JASMONATE RESISTANT1 (JAR1) protein in *A. thaliana* (Staswick, 2002), produces JA-Ile, the most biologically active form of JA in plants (Koo and Howe, 2012). One maize gene encoding a JAR protein, *JAR2a*, was weakly downregulated at 24 h after *T. urticae* feeding, mirroring its downregulation in response to *S. exigua* herbivory (Tzin et al., 2017).

To assess JA signaling, we examined expression changes for genes downstream of JA-Ile, including ones that were identified as not being inducible (or being less induced) in response to wounding in *OPR7/8* double mutant maize plants (Yan et al., 2012; Borrego and Kolomiets, 2016). Many of these genes increased in expression in response to mite feeding, albeit moderately (i.e., the transcriptional regulators *MYC7*, *WRKY14*, and *WRKY46*). Nevertheless, genes in several families showed dramatic upregulation. For instance, *JAZ3*, *4*, *5*, *6*, *7*, *8*, and *12* were strongly upregulated at both the 2- and 24-h time points. Further, members of the 9-LOX clade, including *LOX3*, *4*, and *5*, were also upregulated at both time points, with *LOX3* and *5* exhibiting strong upregulation at 24 h. *LOX1*, which encodes a dual activity lipoxygenase (13-LOX and 9-LOX) of unknown function (Borrego and Kolomiets, 2016), was also upregulated at both time points.

Collectively, gene expression changes for JA biosynthesis and downstream responses were induced similarly between *O. pratensis* and *T. urticae*, with patterns at 2 and 24 h essentially identical where strong differential gene expression was observed (e.g., for most *LOX*, *AOS*, and *JAZ* genes). Further, responses to wounding at 2 h closely mirrored those of responses to mite herbivory (**Figure 6C**). While we focused on the JA pathway

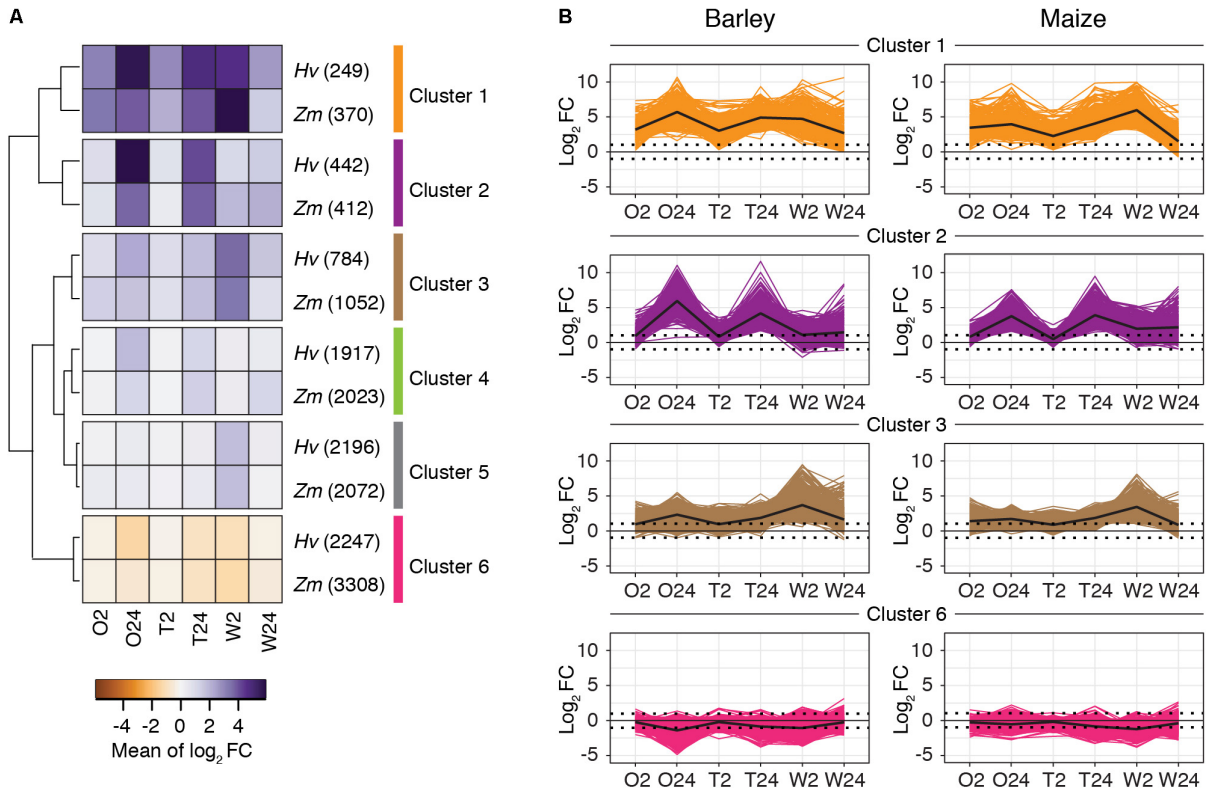

FIGURE 5 | Global patterns of gene expression in response to spider mite herbivory and wounding are similar between barley and maize. **(A)** By plant host, *k*-means clusters ($k = 6$) for DEGs (FDR-adjusted *P*-value of 0.01, absolute value $\log_2$ fold change cutoff of 1) between treatments and controls (gene numbers in clusters are given in parentheses). For inclusion in the analysis for either barley or maize, a gene had to be significantly differentially expressed in at least one treatment relative to the respective control. Shown is a hierarchical clustering of the barley and maize clusters based on the mean expression values of each cluster. **(B)** Gene expression profiles for barley and maize genes in clusters 1, 2, 3, and 6 as indicated (for clusters 4 and 5, see **Supplementary Figure 6**). Lines reflect expression changes ($\log_2$ fold change relative to controls) for individual genes across treatments. The mean changes of expression by cluster are shown with black lines. *Hv*, *Hordeum vulgare* (barley); *Zm*, *Zea mays* (maize); O2 and O24, *O. pratensis* herbivory at 2 and 24 h; T2 and T24, *T. urticae* herbivory at 2 and 24 h; and W2 and W24, wounding at 2 and 24 h.

in maize, for which more is known, an analysis of putative orthologs in barley revealed similar responses (**Supplementary Figure 7**). Finally, mirroring our analysis of genes involved in JA signaling, we performed a similar one for SA, another key plant hormone mediating biotic interactions. Many candidate genes for SA biosynthesis (Martel et al., 2015; Tzin et al., 2015a) were upregulated in response to mite herbivory and wounding at both the 2- and 24-h time points. Examples from maize included genes encoding putative prephenate dehydratases (*GRMZM2G437912* and *GRMZM2G125923)*, Phe ammonia-lyase *(GRMZM2G063917)*, and *trans*-cinnamate 4-monooxygenases (*GRMZM2G147245* and *GRMZM2G010468)* (**Supplementary Figure 8**).

Upregulation of Defensive Proteins

Expression of protease inhibitors and chitinases has been shown to retard the growth of insects, spider mites, or both (Lawrence and Novak, 2006; Carrillo et al., 2011; Santamaría et al., 2012), and about half of cysteine protease inhibitors (cystatins) and serine protease inhibitors were expressed in barley or maize leaves in controls or treatments (**Supplementary Data Sheets 3**, **4**, respectively). In barley, no cystatin genes changed in expression in response to herbivory by *O. pratensis* or *T. urticae*, although two genes were moderately upregulated in response to wounding at 2 h (**Supplementary Figure 9A**). In contrast, eight maize cystatin genes were modestly upregulated, primarily at 24 h, in response to *O. pratensis* or *T. urticae* herbivory (**Supplementary Figure 9A**). Most of these were also upregulated at either 2 or 24 h in response to wounding. As opposed to the pattern observed for cystatins, higher-fold upregulation of serine protease inhibitors was observed in leaves of both plant hosts in response to feeding by *O. pratensis* and *T. urticae*. Among the upregulated genes were several previously reported to be responsive to wounding or JA signaling, including *WOUND INDUCED PROTEIN1* (*WPI*) and *MAIZE PROTEASE INHIBITOR* (*MPI*) (Eckelkamp et al., 1993; Rohrmeier and Lehle, 1993; Tamayo et al., 2000) (**Supplementary Figure 9B**). A pattern shared with the cystatins in maize was that serine protease inhibitor genes were upregulated at the 24-h time point (or if they were significantly induced at 2 h, induction was higher at 24 h). Most serine protease inhibitors induced by mite herbivory were upregulated by wounding at 2 or 24 h. Twenty-one chitinase genes changed in expression in response

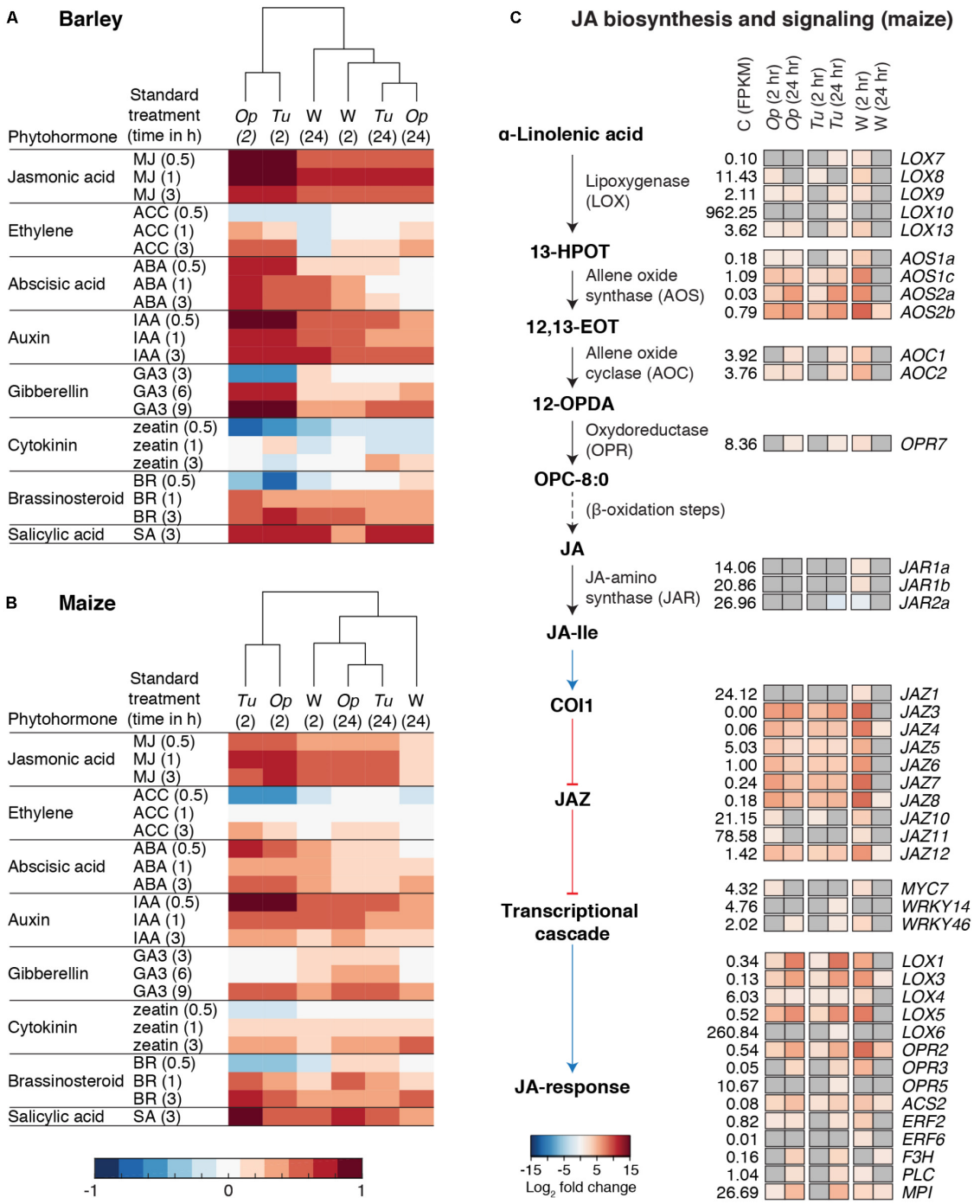

FIGURE 6 | Phytohormone responses to spider mite herbivory and wounding. **(A,B)** Hormonometer analyses for plant hormone signatures based on transcriptomic responses of barley **(A)** and maize **(B)** to *O. pratensis* (*Op*) and *T. urticae* (*Tu*) herbivory as well as wounding (W) at 2 and 24 hours (hr) as indicated in parentheses. The colors indicate similarity between the herbivory/wounding treatments and a particular hormone response (blue and red for negative and positive correlations, respectively, see bottom). MJ: methyl jasmonate; ACC: 1-aminocyclopropane-1-carboxylic acid (a precursor of ethylene); ABA: abscisic acid; IAA: indole-3-acetic acid; GA3: gibberellin A3; BR: brassinosteroid; and SA: salicylic acid. **(C)** Schematic of the JA biosynthesis and signaling pathway after Borrego and Kolomiets (2016) (left) with heat maps of $\log_2$ fold changes for DEGs, right (FDR adjusted *P*-value of 0.01, absolute value $\log_2$ fold change cutoff of 1). Upregulation or downregulation of marker genes for JA signaling is given in red and blue, respectively, see bottom, for treatments as compared to the control (only genes differentially regulated in at least one contrast are shown). The mean expression level of each gene among control replicates, as assessed by FPKM values, is as indicated (C, control). Where no differential expression was observed for a given gene and treatment, cells are shaded gray. Black arrows indicate chemical transformations (solid: one reaction; dashed: multiple reactions) and blue and red arrows indicate signal transduction (activation and inhibition, respectively).

to herbivory by *O. pratensis* or *T. urticae*, or wounding, in maize (**Supplementary Figure 10A**). The majority of genes were upregulated, many at 24 h only, although *GRMZM2G129189*, *GRMZM2G145461*, and *GRMZM2G162359* were induced at both time points. The upregulated genes belonged to several glycoside hydrolase families that hydrolyze chitin (GH-18 and GH-19) (Hawkins et al., 2015). A similar pattern of dynamic responses of chitinase genes to mite herbivory and wounding was also apparent for barley (**Supplementary Figure 10B**).

A Functional Benzoxazinoid Pathway Deters the Generalist but Not the Specialist Mite Herbivore

Domesticated varieties of barley, including the cultivar Morex used in our study, lack benzoxazinoids (Glawischnig et al., 1999; Niemeyer, 2009). In maize, however, at least 14 genes are involved in the benzoxazinoid biosynthesis pathway, and several participate at multiple steps, as shown in **Figure 7A** (the schematic of the pathway is after Tzin et al., 2017). In B73, the *BX12* gene is disrupted, although HDMBOA-Glc can still be produced (Meihls et al., 2013).

In all cases, genes for the synthesis of DIMBOA-Glc were expressed in control B73 tissue, with *BX1* and *BX2* having the lowest expression levels (**Figure 7B**). In response to herbivory by either *O. pratensis* or *T. urticae*, six of the genes needed for synthesis of DIMBOA-Glc were induced weakly at 24 h (**Figure 7B**; *BX6* was also differentially expressed at 2 h in response to *O. pratensis*, albeit with a small fold change). Seven of these genes were also induced weakly by wounding at 2 h, but none remained upregulated at 24 h post-wounding. In contrast to genes needed for DIMBOA-Glc synthesis, those for the modification of DIMBOA-Glc to produce HDMBOA-Glc and HDM2BOA-Glc had very low basal expression levels in control tissue (**Figure 7B**). However, two genes involved in the production of HDMBOA-Glc, *BX10* and *BX11*, were dramatically upregulated in response to *O. pratensis* and *T. urticae* herbivory as well as wounding at 2 h, and remained strongly induced at 24 h. Two other genes, *BX13* and *BX14* that are needed for the production of HDM2BOA-Glc, were also induced by mite herbivory, but with upregulation only observed at 24 h; in response to wounding, *BX13* was also upregulated at 24 h, albeit only moderately. We also compared the relative upregulation of benzoxazinoid biosynthesis genes in response to spider mite herbivory at 24 h to those reported for *S. exigua* feeding at the same time point (Tzin et al., 2017). Although different stage plants were used (see the section "Discussion"), genes for the synthesis of DIMBOA-Glc (e.g., *BX1* and *BX2*) were less strongly induced by spider mites, while those for the conversion of DIMBOA-Glu to HDMBOA-Glc and HDM2BOA-Glc (especially *BX10* and *BX11*) were induced strongly by both mites as well as the caterpillar herbivore (**Supplementary Table 4**).

In the W22 maize inbred, *Ds* transposon insertions have been recovered in three genes responsible for DIMBOA-Glc synthesis – *BX1*, *BX2*, and *BX6* (Tzin et al., 2015a). For *O. pratensis*, reproductive performance, as assessed by the number of progeny per female, did not differ significantly following infestation of wild-type and *bx* mutant plants (ANOVA, $P = 0.313$; **Figure 7C**). In contrast, for *T. urticae* stark differences were observed (ANOVA, $P < 10^{-7}$; **Figure 7D**). While the number of *T. urticae* progeny did not differ between wild-type (W22) and *bx6::Ds* plants, significantly more progeny were observed on both *bx1::Ds* and *bx2::Ds* plants compared to wild-type, and significantly more progeny were observed on *bx2::Ds* compared to *bx1::Ds* plants ($P < 0.05$ after correction for multiple comparisons with Tukey's HSD method).

Genes Required for the Production of Volatile Plant Compounds Are Induced by Mite Herbivory

In addition to green leaf volatiles and methyl salicylate (MeSA) – volatile organic compounds whose biosynthesis genes were either constitutively expressed or induced by mite herbivory (**Figure 6** and **Supplementary Figure 8**) – terpenes are well-characterized herbivore-induced plant volatiles (HIPVs) that mediate plant responses to herbivory, including indirect defenses (Kessler and Baldwin, 2002; Singh and Sharma, 2015). In maize, the *TERPENE SYNTHASE* (*TPS*) genes *TPS10*, *TPS2*, and *TPS3* were highly induced at 2 h in response to *O. pratensis* and *T. urticae*, as well as by wounding (each gene remained induced at 24 h; **Supplementary Figure 11**). Additionally, 14 other putative maize *TPS* genes were induced with lesser fold changes, primarily at 24 h, in response to mite herbivory, or at 2 h in response to wounding. Patterns were similar for barley *TPS* genes including *MLOC_56812* and *MLOC_76989*, which were strongly induced by *O. pratensis* and *T. urticae* herbivory at both time points, and *MLOC_13618* at 24 h. In maize, TPS2 and TPS10 synthesize multiple products. For TPS2, two products are (E)-nerolidol and (E,E)-geranyllinalool, which are subsequently converted to the homoterpenes (E)-3,8-dimethyl-1,4,7-nonatriene (DMNT) and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), respectively. Recently, Richter et al. (2016) implicated the cytochrome P450 genes *CYP92C5* and *CYP92C6* in the final step for the production of DMNT and TMTT. Both genes were induced in response to mite herbivory at 2 or 24 h, and *CYP92C5* was upregulated by wounding at 2 h (**Supplementary Figure 11**).

In maize, *IGL* is responsible for volatile indole, which is inducible by an insect trigger, and was recently shown to prime defenses within and between maize plants (Erb et al., 2015). In response to herbivory by *O. pratensis* and *T. urticae* in maize, *IGL* was modestly upregulated at 24 h ($\log_2$ fold changes $\sim$2) but not at 2 h; in the wounding treatment, *IGL* was upregulated by a similar fold change at 2 h, but was not significantly changed in expression at 24 h (**Supplementary Data Sheet 4**).

DISCUSSION

Whether specialist and generalist herbivores induce different plant responses, and if so, to what degree the herbivore or the plant benefits, has attracted long-standing interest (Ali and Agrawal, 2012). Experimental approaches to tackle these questions have often been confounded by factors

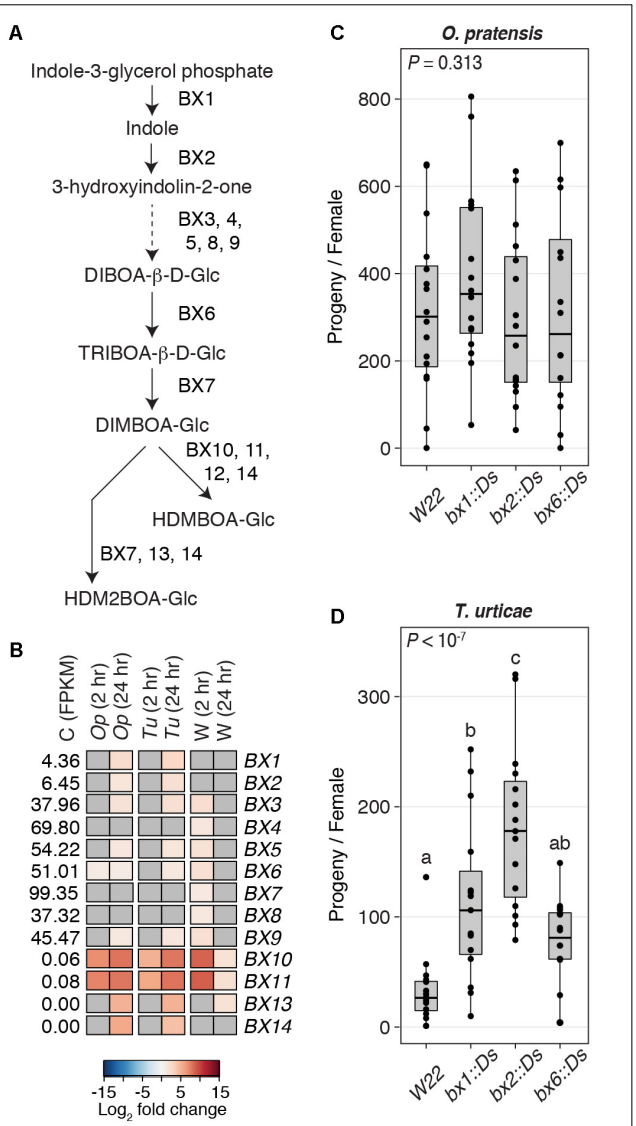

FIGURE 7 | A role for benzoxazinoids in deterring the generalist spider mite, *T. urticae*. **(A)** Biosynthesis pathway for benzoxazinoids after Tzin et al. (2017). Arrows indicate chemical transformations (solid lines, one reaction; dashed line, multiple reactions). **(B)** Heat maps for DEGs (FDR-adjusted *P*-value of 0.01, absolute value $\log_2$ fold change cutoff of 1) in the biosynthesis pathway for benzoxazinoids in response to herbivory by *O. pratensis* (*Op*) and *T. urticae* (*Tu*) or to wounding (W) at 2 or 24 hours (hr) as indicated. For the control samples (C), the mean expression level among replicates of each gene, as assessed by FPKM values, is given. The colors correspond to $\log_2$ fold changes of individual genes in each treatment (blue, downregulation; red, upregulation). Where genes were not significantly differentially expressed, cells are gray. **(C,D)** Boxplots showing the number of progeny produced by *O. pratensis* and *T. urticae*, respectively, on W22 (wild-type) and homozygous Ds transposon insertion mutants in *BX1*, *BX2*, and *BX6* on the W22 background. Circles represent individual data points. *P*-values were determined by ANOVA, with letters indicating significant differences among contrasts ($P < 0.05$, Tukey's HSD test).

including differences in feeding guild, comparisons between phylogenetically divergent herbivores, inclusion of single plant hosts, variation in the extent of tissue damage, and the lack of an expectation of plant defense responses in the absence of (potential) manipulation by herbivores (Ali and Agrawal, 2012). Our study addresses several of these confounding factors. While *O. pratensis* and *T. urticae* are in different genera, they are closely related within the family Tetranychidae (Matsuda et al., 2014), differ little in size and morphology, and cause similar levels of tissue damage on barley and maize leaves. At the cellular level, we found that both species feed on mesophyll cells. This pattern of damage agrees with reports for *Tetranychus* mites in dicots, where empty mesophyll cells were reported at feeding sites (Bensoussan et al., 2016, and references therein). Further, Bensoussan et al. (2016) showed that adult *T. urticae* females feed on mesophyll cells in *A. thaliana* and bean by inserting their stylets either through stomata or between epidermal pavement cells. Our observation of a seemingly intact epidermis overlying empty mesophyll cells in barley suggests that spider mites use a similar feeding mechanism in grasses.

Although spider mites cause less dramatic tissue damage than insects like caterpillars, both *O. pratensis* and *T. urticae* induced pronounced changes in gene expression and peroxidase activity in barley and maize. Strikingly, the DEG sets induced by the two mite species in both plant hosts were similar in composition as well as in the direction (up- or downregulation) and magnitude of fold changes. For the latter, several modest differences in magnitude were apparent. For instance, *O. pratensis* induced a slightly stronger transcriptomic response than *T. urticae* at 24 h in barley, and at 2 h in maize. Whether these differences reflect modulation of plant responses by either herbivore, or alternatively arise from behavioral differences or other factors, is not clear. Nevertheless, neither mite species appears to differentially manipulate barley or maize defenses to a great extent (or alternatively, the two plants do not distinguish between the two mite species to mount different responses). A caveat is that our study does not rule out the possibility that manipulation could occur post-transcriptionally. It should further be noted that our study used only one strain of *T. urticae*, which we maintained on bean until several generations before collecting barley and maize transcriptomic data. Several experimental studies have documented that the generalist *T. urticae* can adapt to its hosts when continuously maintained over many generations (Gould, 1979; Fry, 1989; Agrawal, 2000; Magalhães et al., 2007). If *T. urticae* (and possibly *O. pratensis*) populations adapt to specific grass hosts, potentially by gaining the ability to modulate plant defenses, is not known. Therefore, whether our findings generalize to all *T. urticae* and *O. pratensis* populations is an outstanding question. However, our experimental design mimicked the agriculturally relevant setting for cereal crops in which spider mites invade fields from weeds or other crops, persist during the growing season for a modest number of generations, and then move to other hosts for overwintering (Margolies and Kennedy, 1985).

Our findings of similar barley and maize responses to *O. pratensis* and *T. urticae* herbivory do not rule out the possibility that both suppress plant responses similarly. To test this, as well as to understand how grasses perceive mite herbivores, we also included a wounding treatment against which potential suppression of defenses could be assessed (Howe and

Jander, 2008; Ali and Agrawal, 2012). The utility of a wounding treatment depends largely on how well the treatment mimics patterns of herbivore tissue damage (Mithofer et al., 2005). In the case of mites, mimicking mechanical damage to individual mesophyll cells is not possible. Further, our wounding treatments did not replicate the continuous nature of mite feeding, and consisted instead of marked and instantaneous tissue damage at the beginning of treatments. Despite these caveats, in both barley and maize, genes that responded most strongly to herbivory at both 2 and 24 h also responded strongly to wounding alone. This suggests that grasses readily perceive physical tissue damage by spider mites and mount strong defense responses. Additionally, for many known defensive genes, it was striking that expression changes induced by wounding (up- or downregulation, as well as magnitude of fold changes) were similar to those induced by mite feeding. Therefore, within the limits of our experimental design, we found no obvious signs that *O. pratensis* or *T. urticae* suppress plant defenses associated with tissue disruption.

In both barley and maize, reprogramming of the transcriptome in response to mite feeding was dynamic over a 24-h time period. At 2 h, most DEGs were upregulated, including genes associated with JA and other phytohormone signaling, the production of some specialized metabolites, and the synthesis of HIPVs. More dramatic changes in gene expression were observed at 24 h, including a mix of up- and downregulated genes. These dynamics resemble those reported previously in studies with *T. urticae* in dicots including *A. thaliana*, tomato, and grapevine (Zhurov et al., 2014; Martel et al., 2015; Díaz-Riquelme et al., 2016). Recently, Santamaría et al. (2018) examined transcriptomic responses to *T. urticae* in barley in a design that examined both biotic and abiotic stresses. Although their experimental design differed markedly from ours, with transcriptomic responses assessed at long time points (RNA-seq data were collected after 7 days of herbivore exposure), they also observed upregulation of genes associated with JA biosynthesis and signaling. In maize, our findings are also consistent with those of Szczepaniec et al. (2013), who found by reverse transcription quantitative polymerase chain reaction that several marker genes for JA and SA signaling were upregulated after 3 days of herbivory by *T. urticae*. More generally, at the whole transcriptome level for maize, the transcriptomic changes we observed following herbivory by spider mites are also similar to those reported for herbivory by the caterpillar *S. exigua* (Tzin et al., 2017). These similarities encompassed rapid induction of diverse LOX genes, including but not limited to those involved in JA biosynthesis and signaling. This suggests that chewing insects and mesophyll-feeding mites induce globally similar transcriptomic responses even though patterns of tissue damage differ radically. The downregulation of genes involved in photosynthesis that we observed in response to mite herbivory has been reported to be a general response to biotic stress (Bilgin et al., 2010).

We found that genes in several families encoding defensive proteins, including protease inhibitors and chitinases, were upregulated in our study. Transgenic expression of plant protease inhibitors has been shown to reduce *T. urticae*'s performance on several plant hosts (Carrillo et al., 2011; Santamaría et al., 2012). The same was also observed for transgenic expression of a chitinase, albeit from an insect source (McCafferty et al., 2006), although it should be noted that plant-produced chitinases in the frass of *S. frugiperda* were found to suppress plant defenses and favor the herbivore (Ray et al., 2016). These transgenic studies relied on overexpression, however, and the extent to which endogenous production of protease inhibitors or chitinases in barley and maize leaves impacts spider mites is not known. In insects, protease overexpression or expression of alternative proteases is one route to overcome ingested, plant-produced inhibitors (e.g., Kuwar et al., 2015). This mechanism is likely relevant for *T. urticae*, as sequencing of the *T. urticae* genome revealed expansions of protease families, some of which were found to be highly induced upon plant host shifts (Grbić et al., 2011). Whether protease families are expanded in *O. pratensis*, or whether this specialist has evolved specialized digestive proteases to overcome the inhibitors produced by its hosts in Poaceae, is an outstanding question.

Beyond defensive proteins, HIPVs released from feeding sites on grass leaves may play important roles in indirect defenses against mites. In both barley and maize, genes for the synthesis of SA (the precursor to the volatile MeSA), as well as for the synthesis of terpenes, were upregulated in response to mite herbivory. These can serve as cues to predators of spider mites, which include predatory mites as well as winged ladybird beetles (family Coccinellidae). Within Coccinellidae, minute species of the tribe Stethorini feed primarily on mites in the Tetranychidae family (Biddinger et al., 2009). For instance, *Parastethorus nigripes* is an introduced species that has established on *O. pratensis* on maize in the southern United States (Pollock and Michels, 2002). In Y-tube olfactometer experiments, predatory mites were attracted by MeSA or terpenes (De Boer et al., 2004; Kappers et al., 2005), and synthetic MeSA was shown to attract a Stethorini species (James and Price, 2004). *IGL* was also modestly upregulated in maize in response to herbivory, suggesting that volatile indole is released at mite feeding sites. While indole is a priming agent in maize (Erb et al., 2015), whether it also serves as an attractant for predators of spider mites is unknown.

Apart from defensive proteins and HIPVs, several specialized compounds are likely to play roles in defense against mite herbivores. Mirroring findings reported for herbivory by *S. exigua* (Tzin et al., 2017), genes encoding 9-LOX proteins were rapidly induced by mite feeding. Unlike 13-LOX proteins involved in JA synthesis or the production of green leaf volatiles, LOX3, 4, and 5 belong to the 9-LOX clade, which likely has diverse functions including the production of "death acid" compounds; 10-OPEA, one such compound, was shown to reduce the performance of fungal pathogens and the lepidopteran herbivore *Helicoverpa zea* (Christensen et al., 2015). More recently, it was shown that *S. exigua* growth increased on maize plants with transposon insertions in *LOX4* as compared to wild-type plants (Woldemariam et al., 2018). These findings, coupled with the upregulation of *LOX3*, *4*, and *5* that we observed in our study, suggest that members of the 9-LOX clade should also be assessed for roles in deterring spider mites.

Additionally, we observed modest upregulation of genes involved in the synthesis of DIMBOA-Glc. However, we found dramatic and rapid upregulation of *BX10* and *BX11* that modify DIMBOA-Glc to produce HDMBOA-Glc, which has been associated with resistance to multiple lepidopteran species that feed on maize (Glauser et al., 2011; Tzin et al., 2015b). In contrast, we did not observe as rapid or as strong an induction for genes needed for the synthesis of HDM2BOA-Glc. This may suggest different transcriptional regulation of the biosynthesis genes for major classes of benzoxazinoids derived from DIMBOA. Further, despite globally similar transcriptomic responses to mite herbivory in our study compared to those reported for *S. exigua* (Tzin et al., 2017), some *BX* genes, especially those required for DIMBOA-Glc and HDM2BOA-Glc synthesis (e.g., *BX1* and *BX14*, respectively), were more strongly induced by *S. exigua*. In Tzin et al.'s (2017) study, younger plants were used for the transcriptomic analysis, and the inducibility of benzoxazinoids has been shown to decrease as maize plants age (Köhler et al., 2015). Whether differences in plant stage, the scope of tissue damage, or other factors explain differences in relative induction for some benzoxazinoid synthesis genes between our study and that of Tzin et al. (2017) warrants additional investigation.

Mutations in the benzoxazinoid pathway in maize allowed us to test if benzoxazinoids deter *O. pratensis* or *T. urticae*, both, or neither. For *T. urticae*, performance was markedly reduced on wild-type plants compared to homozygous *bx1::Ds* and *bx2::Ds* plants. Our finding that *T. urticae* performed better on *bx2::Ds* plants than on *bx1::Ds* plants was unexpected, as both are reported to reduce DIMBOA to the same low level in W22 (Tzin et al., 2017). One possibility is that indole produced by BX1, which would be anticipated to accumulate in *bx2::Ds* mutant plants, negatively affects *T. urticae*. Previously, Tzin et al. (2015a) found that DIMBOA-Glc levels were only modestly reduced (∼70%) in *bx6::Ds* plants, suggesting substantial functional redundancy at the respective step in the benzoxazinoid pathway. This likely explains our finding that mite performance was not significantly different on *bx6::Ds* compared to wild-type plants.

In line with our finding for *T. urticae* and benzoxazinoids in maize, *A. thaliana* plants unable to make indole glucosinolates – a class of specialized compounds in the Brassicaceae – are less resistant to *T. urticae* (Zhurov et al., 2014). Combined with results from our study, this observation is at odds with the supposition, for which there is mixed experimental support in insects, that generalists should be good at suppressing phylogenetically conserved plant defense pathways (like canonical phytohormone signaling upstream of plant family-specific defensive compounds) (Ali and Agrawal, 2012). It is consistent, however, with an important role for detoxification in underlying *T. urticae*'s extreme host range, as supported by the finding of expansions of diverse detoxification genes in this species' genome (Grbić et al., 2011), and its known ability to detoxify compounds from diverse chemical classes (Van Leeuwen and Dermauw, 2016). However, specialized compounds that typify plant families, like benzoxazinoids in Poaceae, nonetheless appear to be costly for *T. urticae*. It should also be noted that our performance assessments for *T. urticae* on *bx* mutants were performed on young plants, in which levels of benzoxazinoids are expected to be higher than in older plants (Cambier et al., 2000; Köhler et al., 2015). Although benzoxazinoid synthesis can be induced in maize (Köhler et al., 2015; Maag et al., 2016), screens of maize lines for resistance to *T. urticae* have shown that while many lines are resistant when young, resistance is ameliorated or essentially lost in older plants for some, although not all, inbred lines (Tadmor et al., 1999). Therefore, determining the extent to which benzoxazinoids deter herbivory by *T. urticae* in field settings – where infestation and significant plant damage typically occur later in the growing season – requires further study.

In contrast, our characterization of *O. pratensis* performance on wild-type and *bx* mutant plants suggests that *O. pratensis* is not affected by benzoxazinoids. The simplest interpretation of this finding is that the route to specialization for *O. pratensis*, at least with respect to this class of toxic compounds, lies in a specialized mechanism of detoxification or inactivation. Precedence for this comes from insects, where several caterpillars have been shown to enzymatically render ingested benzoxazinoids non-toxic (or less toxic) by glucosylation (Glauser et al., 2011; Maag et al., 2014). Our findings with *O. pratensis* contrast with those of other specialist mite herbivores like *T. evansi* and *A. lycopersici*, for which suppression of host plant defenses has been documented (Glas et al., 2014; Alba et al., 2015). Thus, multiple paths to specialization appear to have been taken by different herbivorous mites.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Our findings revealed dynamic yet highly correlated transcriptomic responses of two major cereal crops to two spider mite herbivores. Further, the plant responses resembled those observed for wounding, a physical component of herbivory. Taken together, these results suggest that neither mite species manipulates defense responses of these grasses, nor that the grass hosts distinguish between the generalist and specialist mites to initiate different (and potentially adaptive) defensive programs. Nevertheless, we found that the generalist *T. urticae* is negatively impacted by the benzoxazinoid defenses of maize. Our study included the widely used maize inbreds B73 and W22, both of which are readily fed upon by *T. urticae*. These lines are representative of most maize inbred lines in that they are comparatively sensitive to *T. urticae*; nevertheless, a small number of lines have been reported to be resistant throughout their ontogeny (Tadmor et al., 1999). Our findings suggest that variation in benzoxazinoid levels or types should be investigated as a potential factor in explaining resistance in maize to *T. urticae*, and intraspecific variation in benzoxazinoid biosynthesis or accumulation in maize has been documented (Meihls et al., 2012, 2013). Further, in agriculture, economic damage to maize from spider mites is typically observed under drought conditions. As we did not include drought stress as a factor in our experimental design, a future challenge will be to test whether this key abiotic stress influences the relative defense responses of cereals to generalist and specialist spider

mites. Given the importance of resistant germplasm for breeding programs, these studies should be extended as well to examine maize varieties previously reported to be mite resistant.

DATA AVAILABILITY

The RNA-seq data sets for barley and maize generated and analyzed for this study are available through Gene Expression Omnibus (GEO) under accession numbers GSE83676 and GSE100121, respectively.

AUTHOR CONTRIBUTIONS

HB, RR, and RC conceived the research. HB, RG, RC, SL, AR, GG, and RR performed the experiments. Analysis of the data was performed primarily by HB and RG. HB, RG, and RC assumed primary responsibility for writing the manuscript, with input from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01222/full#supplementary-material

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Making a Better Home: Modulation of Plant Defensive Response by *Brevipalpus* Mites

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False-spider mites of the genus *Brevipalpus* are highly polyphagous pests that attack hundreds of plant species of distinct families worldwide. Besides causing direct damage, these mites may also act as vectors of many plant viruses that threaten high-value ornamental plants like orchids and economically important crops such as citrus and coffee. To better understand the molecular mechanisms behind plant-mite interaction we used an RNA-Seq approach to assess the global response of *Arabidopsis thaliana* (Arabidopsis) plants along the course of the infestation with *Brevipalpus yothersi*, the main vector species within the genus. Mite infestation triggered a drastic transcriptome reprogramming soon at the beginning of the interaction and throughout the time course, deregulating 1755, 3069 and 2680 genes at 6 hours after infestation (hai), 2 days after infestation (dai), and 6 dai, respectively. Gene set enrichment analysis revealed a clear modulation of processes related to the plant immune system. Co-expressed genes correlated with specific classes of transcription factors regulating defense pathways and developmental processes. Up-regulation of defensive responses correlated with the down-regulation of growth-related processes, suggesting the triggering of the growth-defense crosstalk to optimize plant fitness. Biological processes (BPs) enriched at all time points were markedly related to defense against herbivores and other biotic stresses involving the defense hormones salicylic acid (SA) and jasmonic acid (JA). Levels of both hormones were higher in plants challenged with mites than in the non-infested ones, supporting the simultaneous induction of genes from both pathways. To further clarify the functional relevance of the plant hormonal pathways on the interaction, we evaluated the mite performance on Arabidopsis mutants impaired in SA- or JA-mediated response. Mite oviposition was lower on mutants defective in SA biosynthesis (*sid2*) and signaling (*npr1*), showing a function for SA pathway in improving the mite reproduction, an unusual mechanism compared to closely-related spider mites. Here we provide the first report on the global and dynamic plant transcriptome triggered by

Brevipalpus feeding, extending our knowledge on plant-mite interaction. Furthermore, our results suggest that *Brevipalpus* mites manipulate the plant defensive response to render the plant more susceptible to their colonization by inducing the SA-mediated pathway.

Keywords: plant–herbivore interaction, plant hormones, defense pathways, salicylic acid, jasmonic acid, cross-talk, *Tetranychus*, RNA-Seq

INTRODUCTION

Plants are frequently threatened by arthropods herbivores from different feeding guilds causing variable tissue injuries. Chewers consume a significant amount of plant tissue thus promoting extensive damage, while sap-suckers and cell-content-feeders pierce to ingest plant fluids, inflicting minimal physical damage. To further enhance self-protection against attackers, plants display receptors that recognize conserved molecules associated with herbivores (herbivore-associated molecular patterns – HAMPs) or even self-molecules released after cell damage inflicted by the attack (damage-associated molecular patterns – DAMPS) and mount appropriate defense response. Some adapted herbivores have evolved the ability to counteract plant defenses by producing effectors that disrupt plant signaling and induce effector-triggered susceptibility (Hogenhout and Bos, 2011; Ferrari et al., 2013; Pel and Pieterse, 2013). The plant counterattack involves resistance proteins (R proteins) which directly bind the effectors, or the plant proteins they modify, and elicit a second layer of the immune response. The outcome of induced defenses includes the production of toxins that interfere with herbivore feeding, growth, reproduction or fecundity and/or volatile compounds that attract natural enemies of the attacker (Pieterse et al., 2012).

Upon recognition a cascade of phytohormone-dependent signals, modulated by the nature of the damage, orchestrates specific plant defense responses. Generally, arthropods such as chewing insects that greatly damage the plant tissue integrity trigger the jasmonic acid (JA) pathway, whilst herbivores causing minimal tissue disruption, i.e., piercing-sucking arthropods induce salicylic acid (SA) mediated response (Arimura et al., 2011). The SA pathway is typically associated with resistance against biotrophic pathogens and can often antagonize JA-mediated defenses. Ethylene (ET) and abscisic acid (ABA) also control plant responses to herbivore through the modulation of JA signaling branches. ABA regulates the MYC transcription factor branch (MYC-branch) acting in the defenses against herbivores, whereas ET regulates the ethylene responsive factor branch (ERF-branch) to defend against necrotrophic invaders. The ET- and ABA-regulated branches antagonize each other to fine tune JA pathway against the specific invader (Pieterse et al., 2012).

Herbivores can take advantage of the natural cross-talk between hormonal pathways to circumvent plant defenses. *Bemisia tabaci* activates SA responses to suppress effective JA defenses and improve whitefly performance (Zarate et al., 2007; Zhang et al., 2013). Likewise, some insect eggs induce high levels of SA that leads to reduced protein levels of MYC2, subsequent suppression of JA defenses, and the enhancement of larvae performance (Bruessow et al., 2010; Schmiesing et al., 2016). The ERF-MYC branch antagonism is also occasionally exploited by herbivores. Oral secretions of *Pieris rapae* activates the ERF-branch to rewire JA signaling toward the insect preferred branch (Verhage et al., 2011). Beyond through cross-talk, other herbivores are capable of directly suppressing several defense pathways. The mite *Tetranychus evansi* repress both JA and SA signaling in tomato, dramatically reducing the levels of defense compounds (Sarmento et al., 2011; Alba et al., 2015).

Manipulation of plant defenses by herbivores has been shown to frequently occur through saliva-contained effectors. Salivary proteins able to modulate defenses and improve herbivore performance have been identified in insects (Hogenhout and Bos, 2011) and mites (Villarroel et al., 2016). Moreover, proteins from arthropod-associated microorganisms such as endosymbiont bacteria (Casteel et al., 2012; Chung et al., 2013) and viruses (Casteel et al., 2014; Li et al., 2014) may also be present in the saliva and modulate plant defenses to promote herbivore performance.

Current understanding of the mechanisms involved in plant response to herbivores comes mainly from studies of plant–insect interactions. Relatively little is known about molecular responses to other arthropods as mites, most of them focusing on the two-spotted spider mite *T. urticae* (Rioja et al., 2017). False spider mites of the genus *Brevipalpus* (Acari: Tenuipalpidae) are economically important phytophagous mites that attack hundreds of plant species of very distinct families, including large-scale plantations of high-value crops and several ornamental plants (Childers et al., 2003; Kitajima et al., 2010). Besides causing direct damage to some plant species, the negative impacts of infestation are often exacerbated by their ability to vector numerous plant-infecting viruses, the so-called *Brevipalpus*-transmitted viruses (BTVs) (Kitajima and Alberti, 2014). *Brevipalpus yothersi* vectors both cileviruses and tentative dichorhaviruses (Ramos-González et al., 2016; Chabi-Jesus et al., 2018) being the main vector of citrus leprosis virus C (CiLV-C), the prevalent virus causing citrus leprosis disease. Chemical control of *B. yothersi* mites costs millions of dollars each year in Brazil, the world leading producer of sweet orange juice, frozen concentrated orange juice (FCOJ) and not-from-concentrate orange juice (NFC) (Bastianel et al., 2010). The cosmopolitan distribution of *Brevipalpus* spp. poses a major threat to the worldwide citrus industry and to other crops such as coffee (Rodrigues and Childers, 2013; Beard et al., 2015). In addition to their agricultural relevance, *Brevipalpus* mites are also prominent because of their unusual biology. Several species of the genus

are haploid during their entire life cycle, an exclusive feature amongst higher organisms, and are essentially female due to the presence of the endosymbiont bacterium *Cardinium* sp. (Weeks et al., 2001).

Despite the economic and biological significance, many aspects of the *Brevipalpus* mite-plant interaction remain largely unknown. A previous study showed that plants respond to the presence of *B. yothersi* non-viruliferous mites with a ROS burst and induction of specific genes from SA and JA-dependent pathways (Arena et al., 2016). Upon infestation with CiLV-C viruliferous mites, both SA- and JA-responsive genes are reduced, and mite behavior is affected (Arena et al., 2016). To achieve a wider understanding of the molecular mechanisms behind plant-mite interaction, we used an RNA-Seq approach assessing the global response of *Arabidopsis thaliana* plants along the course of the infestation with *B. yothersi*. Transcriptome analysis was complemented with the measuring of the SA and JA hormone levels in plants upon mite feeding. Finally, to further clarify the functional relevance of hormone-triggered plant defense on the interaction, we evaluated the *B. yothersi* oviposition on Arabidopsis mutants impaired in SA or JA-mediated response. Current work provides a comprehensive picture of the plant response to *Brevipalpus* mite feeding.

RESULTS

Brevipalpus Mites Elicit a Significant Transcriptome Reprogramming on Infested Plants

A time-course RNA-Seq experiment was set up to assess the global response of *A. thaliana* plants along the course of infestation with non-viruliferous *B. yothersi* mites. The transcriptome of infested plants was compared with that from non-infested ones (control) at 6 h after infestation (hai), 2 and 6 days after infestation (dai). Overall, 995 million paired-end reads were obtained by Illumina sequencing, with an average of 41.5 million per library and higher average number of reads in samples from the infested treatment (**Supplementary Table S1** and **Figure 1A**). Roughly 94% of the reads mapped against the *A. thaliana* reference genome, with a 91% average of uniquely mapped reads (**Supplementary Table S1** and **Figure 1B**).

Biological variability between samples was verified by principal component analysis (PCA) using the normalized count data (**Figure 1C**). Infested and control samples grouped separately, suggesting a globally distinct expression profile, as expected. Even though a classification of the first principal components as treatment or time of infestation was not clear, the first component (PC1), which accounts for 52% of the variance, apparently separated the samples by the intensity of stimuli. Except for two out of four control samples at 6 dai, all control samples grouped together with those of mite-infested treatments from 6 hai, whose plants were stimulated by a short period of mite feeding. Samples from plants challenged by longer mite feeding period (2 and 6 dai) grouped separately. Hierarchical clustering of samples within each time point confirmed a clear separation between mite-infested and non-infested treatments over the course of the experiment (**Figure 1D**).

By using the negative binomial-based DESeq2 package and FDR correction of *p*-values for multiple comparisons, 5005 differentially expressed genes (DEGs, $\alpha \leq 0.05$) were detected (**Supplementary Table S2**). Mite infestation deregulated 1755, 3069 and 2680 genes at 6 hai, 2 dai, and 6 dai, respectively (**Figure 1E**). At the earliest stage of the interaction (6 hai), the majority of the DEGs was up-regulated. The number of down-regulated genes progressively increased during the interaction reaching its highest rating at 6 dai (**Figures 1E,F**). Analysis performed here show an intense modulation of the plant transcriptome in response to *Brevipalpus* mite infestation.

To validate the RNA-Seq data, 10 genes were selected for Real Time RT-qPCR analysis. Expression of these genes was assessed in a new experiment with mite-infested and non-infested Arabidopsis plants at 6 hai, 2 dai, and 6 dai (**Supplementary Figure S1**). Altogether, expression profiles of selected genes obtained by RT-qPCR were consistent with those obtained by the RNA-Seq, supporting the results described in this work. Additionally, some of these genes had an expression profile similar to that revealed by a qPCR-driven analysis during a comparable experiment previously described (Arena et al., 2016).

The Plant Immune System Is Modulated by *Brevipalpus* Mite Infestation

Gene ontology (GO) enrichment analysis was performed with all 5005 DEGs to identify the most relevant biological processes (BPs), molecular functions (MFs) and cellular components (CC) disturbed during *Brevipalpus* mite-plant interaction. This study identified 264 BPs, 83 MFs and 78 CCs that were over-represented (hypergeometric test, $\alpha \leq 0.001$) in the list of DEGs (**Supplementary Table S3**). Enriched BPs were further visualized as a network using the app BinGO from Cytoscape, where color and size of the nodes identify *p*-values and number of DEGs from each category, respectively (**Supplementary Figure S2**).

The GO network revealed a striking deregulation of plant defensive responses. BP categories were clustered in two major groups comprising metabolism and response to stimuli. BP-metabolism was sub-clustered into two branches separately harboring the primary and secondary metabolisms. Secondary metabolism group was represented by processes related to the biosynthesis and metabolism of "flavonoids," "glucosinolates," "toxins," and "camalexins," which are known to exert anti-herbivory roles and be induced by SA or JA. Primary metabolism included categories associated to the metabolism of: (i) "aminoacids" and "proteins," connected to processes involved in the control of gene expression (such as "protein modification," "phosphorylation," and "transcription"); (ii) "organic acid," whose sub-categories included the "SA metabolism" and "JA biosynthesis and metabolism"; and (iii) "carbohydrates," edged to several photosynthesis-associated categories and processes related to "cell wall modification" such as "callose deposition," a well-known defense against herbivores (Jander, 2014).

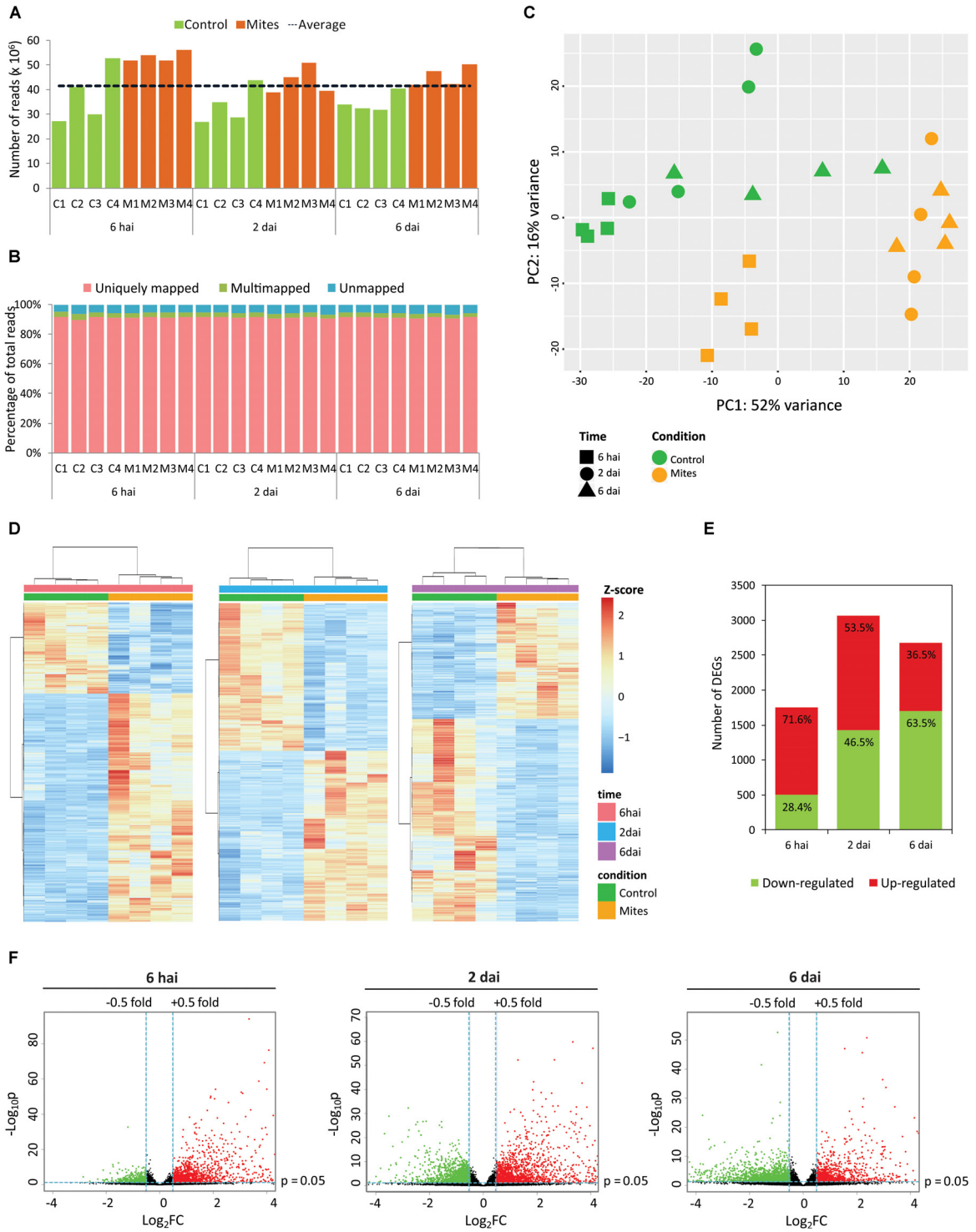


FIGURE 1 | Overview of *Arabidopsis thaliana* transcriptome upon infestation by *Brevipalpus* mites. **(A)** Number of *paired-end* reads generated for each library by Illumina HiSeq sequencing. C, control (non-infested plants); M, mite-infested-plants. Dashed line represents the average of *paired-end* reads from all 24 libraries. **(B)** Proportion of uniquely mapped, multi-mapped and unmapped reads obtained for each library. Reads were mapped in the *A. thaliana* (TAIR 10) genome using *TopHat2*. C, control; M, mite-infected plants. **(C)** Principal component analysis of normalized count data from all samples. **(D)** Hierarchical clustering analysis of normalized count data *z*-scores exhibited by differentially expressed genes (DEGs) of each sample within each time point. **(E)** Numbers of up- and down-regulated DEGs in mite-infested plants in comparison to non-infested control at each time point. DEGs were identified using *DESeq2* and defined by $\log_2$ fold-change ≥ 0.5 and false discovery rate (FDR)-corrected *p*-value ≤ 0.05. **(F)** Volcano-plots of $-\log_{10}$p and $\log_2$FC exhibited by each gene in mite-infested plants compared to non-infested control at each time point. Up- and down-regulated genes are presented in red and green, respectively. FC, fold-change; p, FDR-corrected *p*-value, hai, hours after infestation; dai, days after infestation.

Biological processes cluster centralized in "response to stimulus" was fully represented by processes associated with defense responses. Nodes from the cluster included response to "stress," "abiotic," and "biotic" stimulus (linked to "defense response" and to the subcategories of response to "wounding," "insects," and other pathogens). A large "response to hormone" branch displayed all main hormone-mediated pathways, including SA and JA, but also abscisic acid (ABA), ethylene (ET), auxins (IAA), cytokinins (CK) and gibberellins (GA). Other nodes included in the "response to stimuli" group were "response to chitin," commonly triggered in plants colonized by chitin-rich organisms such as arthropods and fungi (Libault et al., 2007), and "oxidative stress," typically induced in plant-biotic interactions (Arena et al., 2016; Camejo et al., 2016). Several processes related to defense response were also present in "biological regulation" nodes, such as "regulation of hormone levels," "defense response," "immune response," and, "JA pathway."

Specific and Common Transcriptomic Changes Occur at Different Time Points After *Brevipalpus* Mite Infestation of Plants

A comparison of the DEGs deregulated across the experiment revealed both common and specific changes at each time points (**Figure 2A**). Few DEGs were common to all time points, whereas the highest percentage of them were found to be exclusively modulated at 2 or 6 dai suggesting intense reprogramming steps of plant transcriptome throughout the course of the *Brevipalpus* mite infestation (**Figure 2A**).

Most of the BPs (84 terms) over-represented during mite infestation overlap at all time points (**Figure 2B** and **Supplementary Table S4**). These processes included most of the general terms of plant response to stresses and hormones, indicating a continuous and lasting reprogramming of the plant immune system since the beginning of the interaction until at least 6 dai. Several categories were common between 6 hai and 2 dai (75 terms), and 2 dai and 6 dai (20 terms), but no biological process was shared between the first and the last evaluated time points (**Figure 2B**).

Even though processes related to plant defense responses were markedly enriched over the time course of the experiment, time point-specific ontologies were also identified. From all the over-represented BP categories, 49, 47, and 24 were uniquely identified at 6 hai, 2 dai, and 6 dai, respectively (**Figure 2B** and **Supplementary Table S4**). Hormone biosynthesis ("salicylic acid biosynthetic process," "oxylipin biosynthetic process"), early signaling ("activation of MAPK activity"), and structural defenses ("callose deposition," "cell wall thickening," and "lignin biosynthetic process") were processes exclusively enriched at 6 hai (**Figure 2C**). At 2 dai, unique categories were related to the metabolism of defense-related secondary metabolites ("indole glucosinolate metabolic process," "pigment," and "flavonoid biosynthetic and metabolic process"), photosynthesis and oxidative stress ("photosynthesis," "carbon fixation," "photosynthetic electron transport," "response to light intensity," "response to oxidative stress") (**Figure 2C**). Exclusive ontologies that came up with the late infestation state (6 dai) were detoxification processes ("cellular detoxification," "cellular response to toxic substance"), and other associated to cell wall components and structure ("plant-type cell wall organization," "cell wall loosening," and "pectin," "galacturonan," "glucan," "carbohydrate," and "polysaccharide" metabolic process) (**Figure 2C**).

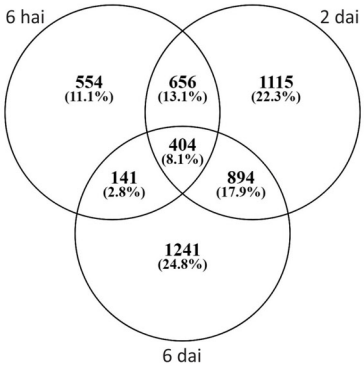
Brevipalpus Mite Infestation Induces Plant Defensive Responses and Represses the Plant Growth-Related Processes

The vast majority of DEGs detected in more than one time point were strictly kept up- or down-regulated. Among the 5005 DEGs identified throughout the analysis, only 201 of them (4%) shift their expression patterns across the experiment (**Supplementary Table S2**). In agreement with this, results of the hierarchical clustering analysis revealed two major clusters of DEGs, which mainly encompassed 2762 and 2243 up-regulated and down-regulated genes, respectively (**Figure 3A**).

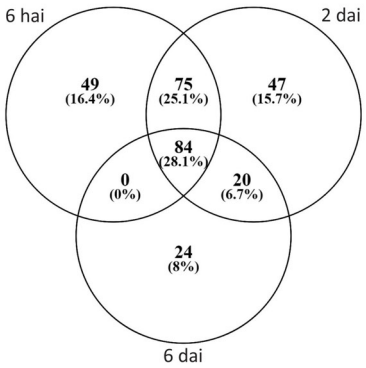
Gene ontology enrichment analysis separately performed with DEGs within each of the predefined clusters identified only 31 common categories between the up- and down-regulated groups (**Figure 3B** and **Supplementary Table S5**). These categories represented higher GO levels and included general BPs such as "regulation of biological quality," "response to stimulus," "metabolic process," "signal transduction," among others. BPs such as "response to hormones" and "hormone-mediated signaling pathway" were also shared between the up- and down-regulated clusters but GO-terms identifying a particular hormonal pathway were always detected in just one of the two groups.

The up-regulated cluster was enriched in 264 exclusive BPs (**Figure 3C** and **Supplementary Table S5**). Network topology was similar to that obtained using all the DEGs (**Supplementary Figure S2**), with two major clusters centralized in metabolic processes and response to stimuli. GO terms within metabolic process cluster involved several BPs related to secondary metabolism, whilst response to stimuli cluster presented terms associated to stress and defense and hormonal pathways. Over-represented categories were massively typified by defensive responses. Besides broad immune system-related terms (e.g., "immune response"), other common categories displayed by the general network (**Supplementary Figure S2**) included responses to hormones, oxidative stress and the production of secondary metabolites, e.g., glucosinolates, flavonoids, and camalexin. Only SA, JA, ET, and ABA-mediated hormonal pathways were represented in the up-regulated cluster network. Induced GO network also included other over-represented processes that were unidentified in the general network (**Supplementary Figure S2**). Among these are included: "response to herbivore," "response to virus," "multi-organism process," "modification of morphology/physiology of other organism," "lignin biosynthetic and metabolic process," "defense response by callose deposition," and "phytoalexin biosynthetic and metabolic process."

A



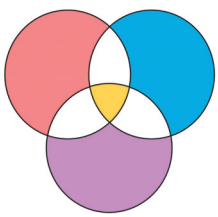
B



C

6 hai	p-value
cellular response to salicylic acid stimulus	3,93E-05
regulation of multi-organism process	9,29E-05
response to endoplasmic reticulum stress	1,25E-04
response to heat	1,26E-04
cell death	1,41E-04
cellular response to salicylic acid stimulus	2,24E-04
salicylic acid biosynthetic process	2,86E-04
defense response by cell wall thickening	2,86E-04
defense response by callose deposition in cell wall	3,01E-04
lignin biosynthetic process	4,25E-04
auxin metabolic process	5,02E-04
oxylipin biosynthetic process	5,16E-04
salicylic acid mediated signaling pathway	6,64E-04
defense response by callose deposition	6,90E-04
oxylipin metabolic process	7,35E-04
activation of MAPK activity	7,61E-04
negative regulation of RNA biosynthetic process	7,61E-04
response to molecule of bacterial origin	9,04E-04
modulation by symbiont of host innate immune response	9,68E-04
activation of protein kinase activity	9,84E-04

2 dai	p-value
cell surface receptor signaling pathway	2,31E-11
fatty acid metabolic process	2,27E-07
flavonoid metabolic process	4,51E-07
flavonoid biosynthetic process	1,32E-06
pigment metabolic process	1,50E-06
reductive pentose-phosphate cycle	2,50E-06
photosynthesis, dark reaction	5,68E-06
carbon fixation	8,55E-06
photosynthetic electron transport in photosystem I	1,38E-05
response to hydrogen peroxide	2,17E-05
transmembrane receptor protein kinase signaling pathway	3,03E-05
pigment biosynthetic process	3,79E-05
response to light intensity	6,39E-05
photosynthetic electron transport chain	7,75E-05
hormone metabolic process	1,46E-04
response to high light intensity	2,85E-04
regulation of stomatal movement	3,92E-04
fatty acid derivative metabolic process	6,67E-04
hormone transport	7,00E-04
indole glucosinolate metabolic process	8,20E-04



6 dai	p-value
polysaccharide metabolic process	5,49E-08
plant-type cell wall organization	2,06E-06
carbohydrate catabolic process	1,57E-05
cellular oxidant detoxification	3,90E-05
cellular detoxification	4,37E-05
cellular response to toxic substance	4,89E-05
oligopeptide transport	8,13E-05
plant-type cell wall loosening	2,10E-04
steroid metabolic process	5,14E-04
response to gibberellin	5,48E-04
lipid biosynthetic process	5,67E-04
response to low light intensity stimulus	5,97E-04
plant-type cell wall organization or biogenesis	6,33E-04
pectin metabolic process	6,35E-04
syncytium formation	6,38E-04
sterol biosynthetic process	6,38E-04
galacturonan metabolic process	6,97E-04
carbohydrate biosynthetic process	7,48E-04
cellular glucan metabolic process	9,93E-04
glucan metabolic process	9,93E-04

6 hai x 2 dai x 6 dai	p-values at respective times		
response to stimulus	2,49E-75	1,69E-98	6,89E-50
response to chemical	4,46E-58	9,46E-70	1,81E-38
response to stress	2,84E-57	1,85E-52	4,57E-19
defense response	1,07E-42	3,09E-40	9,84E-12
response to wounding	3,02E-42	1,18E-33	3,91E-06
response to external biotic stimulus	7,21E-37	6,48E-39	7,52E-10
response to biotic stimulus	1,59E-36	6,48E-39	1,60E-09
response to jasmonic acid	1,66E-33	2,76E-33	1,22E-10
response to hormone	5,57E-33	1,39E-50	1,82E-29
response to bacterium	1,08E-32	1,12E-33	4,78E-06
response to chitin	9,52E-24	1,64E-27	1,72E-09
cell communication	1,09E-23	1,22E-32	1,35E-18
signaling	9,18E-22	1,12E-33	3,81E-18
hormone-mediated signaling pathway	1,71E-16	4,00E-21	4,66E-13
biological regulation	2,68E-14	1,59E-16	4,66E-13
response to abiotic stimulus	1,54E-13	3,28E-32	6,55E-20
secondary metabolic process	8,94E-13	9,29E-14	3,81E-04
response to salicylic acid	1,35E-11	3,60E-11	2,23E-07
response to ethylene	1,45E-08	1,88E-10	2,48E-08
response to abscisic acid	6,36E-04	4,67E-09	1,36E-04

FIGURE 2 | Transcriptomic changes at different time points after *Brevipalpus* mite infestation of *A. thaliana* plants. **(A)** Venn diagram of DEGs in mite-infested plants compared to non-infested control at each time point. DEGs were identified using *DESeq2* and defined by $\log_2$ fold-change ≥ 0.5 and FDR-corrected *p*-value ≤ 0.05. **(B)** Venn diagram of overrepresented BPs of DEGs at each time point. Overrepresented BPs were identified for each time point based on a hypergeometric test with FDR-adjusted *p*-values ≤ 0.001. **(C)** Lists of overrepresented BPs exclusive to each experimental time point (6 hai, 2 dai or 6 dai) and those commons between them (6 hai × 2 dai × 6 dai). BPs corresponding *p*-values obtained in the Gene ontology (GO) enrichment analysis are included in the right column of the tables. Twenty BPs of each list are presented in each table. Complete lists of exclusive and common BPs are available in **Supplementary Table S4**.

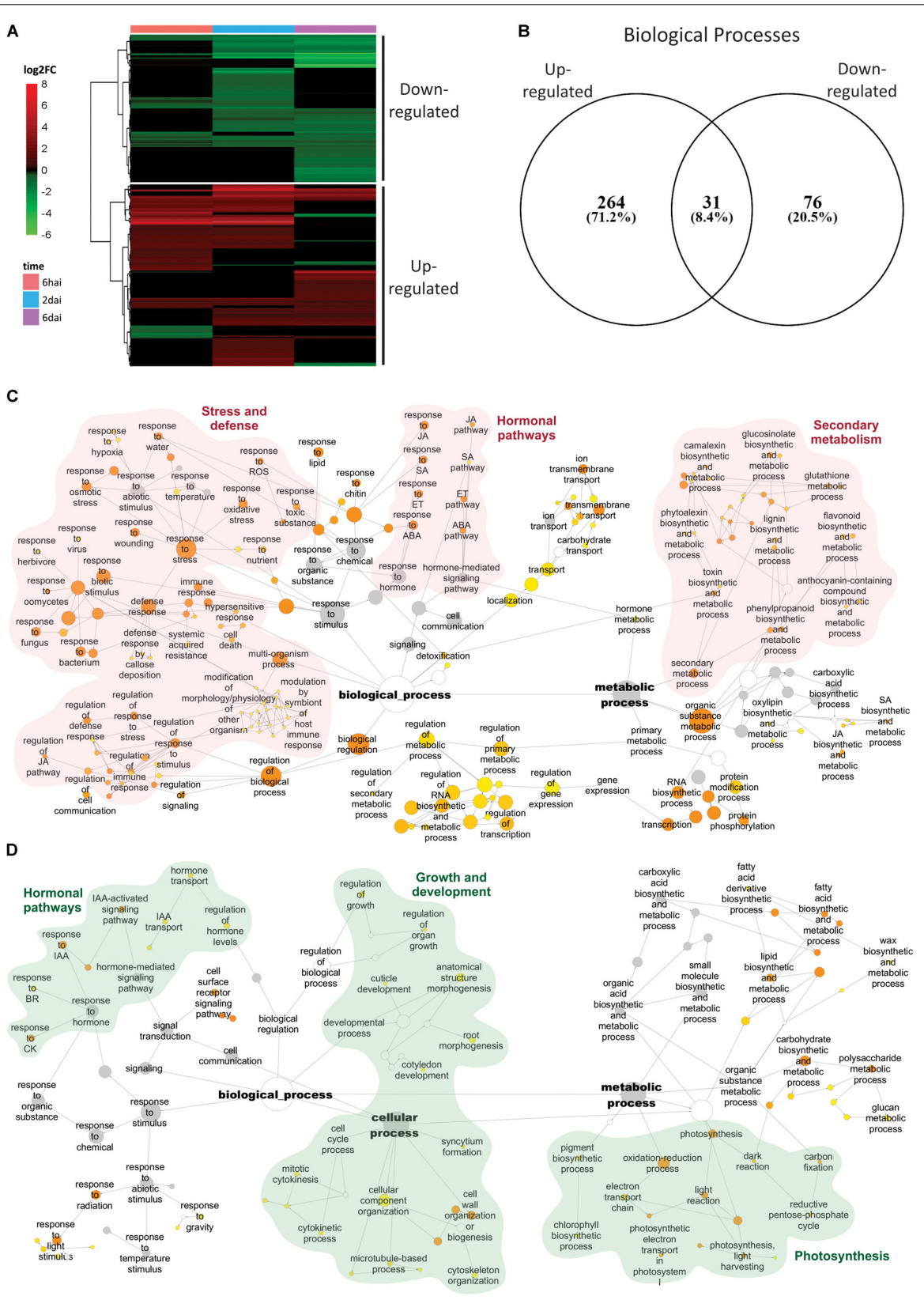


FIGURE 3 | Continued

FIGURE 3 | Induced and repressed responses on *A. thaliana* infested by *Brevipalpus* mites. **(A)** Hierarchical clustering analysis of $\log_2$ FC exhibited by DEGs on mite-infested plants compared to non-infested control. DEGs were identified using *DESeq2* and defined by $\log_2$ fold-change ≥ 0.5 and FDR-corrected p-value ≤ 0.05. hai, hours after infestation; dai, days after infestation. **(B)** Venn diagram of overrepresented BPs of DEGs at each cluster composed by up- and down-regulated genes. Overrepresented BPs were identified for each cluster based on a hypergeometric test with FDR-adjusted p-values ≤ 0.001. **(C,D)** Networks of enriched BPs from clusters of up-regulated **(C)** and down-regulated **(D)** DEGs, generated using the app BinGO in Cytoscape. Size of the nodes correlates with the number of DEGs. Color of the nodes reveals p-values of enriched categories. Nodes in gray represent categories that were shared between clusters of up- and down-regulated genes. Names of some BPs were simplified for clarity; full names are displayed in **Supplementary Table S5**. ROS, reactive oxygen species; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; ABA, abscisic acid; IAA, auxin; CK, cytokinin; BR, brassinosteroid.

The down-regulated cluster was enriched in 76 exclusive BPs, which were predominantly associated with the plant growth and development (**Figure 3D** and **Supplementary Table S5**). Over-represented terms included broad categories, for instance "developmental process" and "regulation of growth," and also those directly related to plant growth such as "cytokinetic process," "cell cycle process," "mitotic cytokinesis," or indirectly related to growth such as "cell wall organization or biogenesis" and "cytoskeleton organization". Among the enriched BPs there were also processes associated to morphogenesis and development of specific plant components such as "root morphogenesis," "cuticle development," and "cotyledon development." The other major class of over-represented BPs uniquely detected in the down-regulated cluster comprised photosynthesis-related processes such as "photosynthesis," "electron transport chain," "carbon fixation," "photosynthesis, dark and light reaction," "light harvesting," "photosynthetic electron transport," and "chlorophyll biosynthetic process." Finally, the only hormones represented in the down-regulated cluster network were the major growth regulators IAA, CK and brassinosteroids (BR).

Co-expression of Genes Correlates With Classes of Transcription Factors (TFs) Involved in SA, JA and Developmental Processes

Since transcriptional reprogramming is mainly controlled by TFs, the regulation of the expression dynamics of DEGs by specific classes of TFs was tested by two different approaches.

First, over-represented TF families were searched based on up- and down-regulated DEGs that encode TFs (**Figure 4A** and **Supplementary Table S6**). Within the cluster of up-regulated DEGs, 254 (9.2%) TFs from 30 different families were identified. From those, 16 over-represented families were detected (hypergeometric test, $\alpha \leq 0.001$). The largest and most significant of them were the WRKY (33 genes, p-value = 2.47E-33) and the AP2/ERF (40 genes, p-value = 8.46E-34), known to act as regulators of SA pathway and ERF-branch of the JA pathway, respectively. From the analysis using the cluster of down-regulated DEGs, 141 (6.3%) TFs belonging to 30 families were detected. Twenty-three of these families were also found in the cluster of up-regulated DEGs. TFs were evenly distributed among 18 over-represented families (hypergeometric test, $\alpha \leq 0.001$), with lower significance (higher p-values). The largest and most significantly over-represented families were bHLH (22 genes, p-value = 3.21E-25), which comprises the regulators of the MYC-branch of the JA pathway, and C3H family (17 genes, p-value = 1.46E-17).

Second, TF families that potentially regulate the expression of the DEGs were searched based on over-represented target genes within the DEGs (**Figure 4B** and **Supplementary Table S7**). Enriched target genes and their corresponding TFs were identified by using a TF enrichment tool that takes advantage of previously identified *cis*-regulatory elements and regulatory interactions from literature mining (Jin et al., 2017). As a result, WRKY was the largest identified family with potential targets within the up-regulated DEGs. Twenty-one out of its 42 TF members were also induced during *Brevipalpus* mite-plant interactions. Targets from WRKY TFs were exclusively enriched in the up-regulated cluster. The next largest families with targets within the induced DEGs were MYB, bZIP, and bHLH, with 29, 26, and 24 TFs, respectively. Targets for MYB, bZIP, and bHLH, however, were not exclusively enriched in the analysis of the cluster of up-regulated DEGs. These families represented by 34, 24, and 14 TFs, respectively, were also among the largest with potential targets within down-regulated DEGs. MYC2, the marker TF from the MYC-branch of the JA pathway, was one of the bHLH TF with targets exclusively enriched in the down-regulated cluster. Notably, the analysis of the down-regulated cluster also revealed the TCP family, which is typically involved in the control of plant development. This family involved 15 and 4 TFs that potentially regulate targets within the assortment of repressed and induced DEGs, respectively.

Overall, the analysis of co-expressed genes with its corresponding TFs showed a correlation of up- and down-regulated genes with TFs that regulates SA, JA and developmental processes. The SA-related WRKY family was the largest one with target genes exclusively enriched in the up-regulated cluster and most of its TF members were also up-regulated. The analysis of enriched TFs settles the involvement of plant hormonal pathways and developmental processes in the plant response to *Brevipalpus* mites, with highlight on the participation of the SA pathway solely on the up-regulated responses.

Focus on Defense Pathways: SA- and JA-Mediated Responses Are Induced in *Brevipalpus* Mite-Infested Plants

Over-represented genes from GO-terms associated with SA and JA-dependent responses were thoroughly reviewed to confirm their induced status. Data from genes included in the categories "response to SA" and "SA metabolic process,"

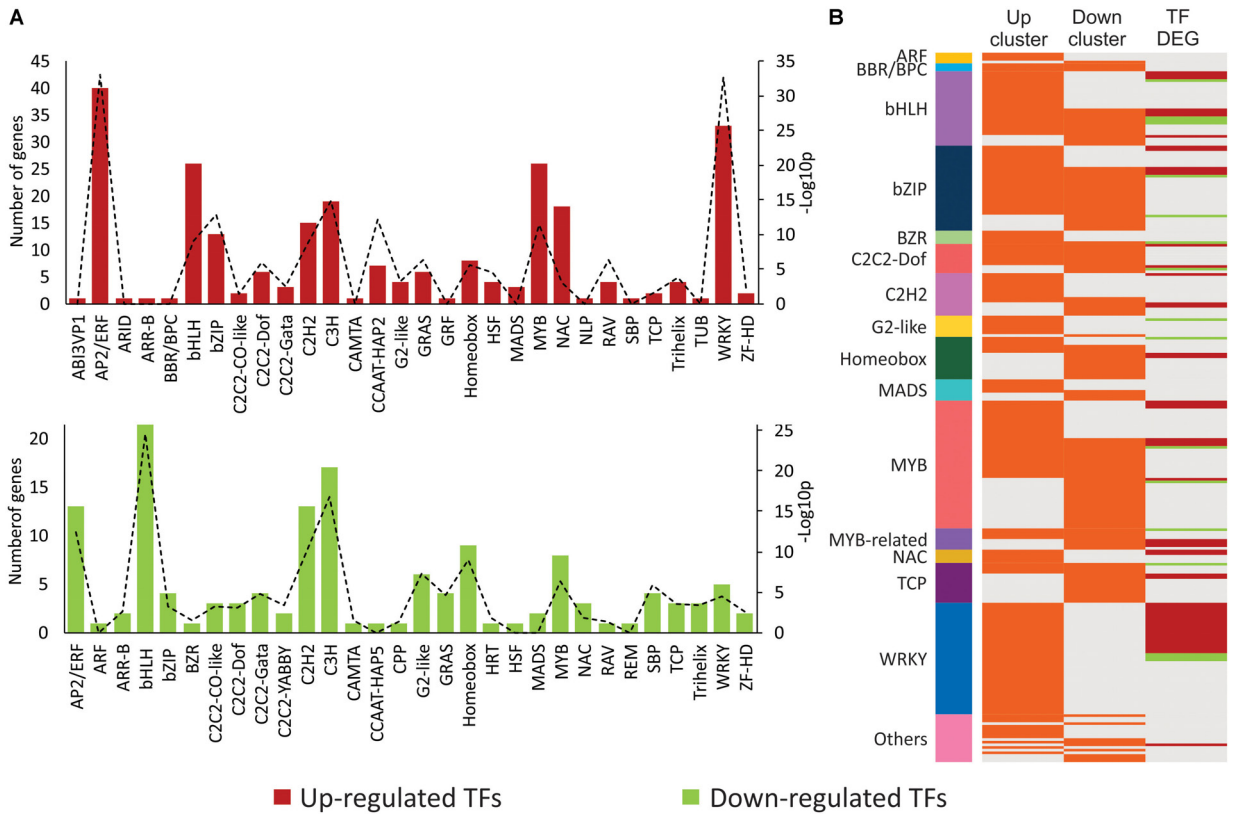


FIGURE 4 | Enriched transcription factors (TFs) and TF targets in clusters of mite-responsive co-expressed genes. **(A)** Number of DEGs coding for TFs within each TF family identified in the clusters of up- and down-regulated DEGs. Up- and down-regulated DEGs are presented in red and green, respectively. Levels of enrichment ($-\text{Log}_{10}\ p$, with p: p-value) of each family (hypergeometric test, $\alpha \leq 0.001$) are presented by a dashed line with its corresponding values in the secondary axis. **(B)** TFs with enriched targets within each cluster of up- and down-regulated DEGs, identified by TF enrichment tool. TFs are grouped according to their families. Each line identifies one TF. In the first and second row ("Up" and "Down" clusters, respectively), orange lines correspond to TFs with enriched targets within each cluster. In the third row ("TF DEGs"), red and green lines represent up- and down-regulated differentially expressed genes, respectively, encoding TFs. Gray lines indicate absence of enriched targets for a given TF- and/or TF not differentially expressed. Families encompassing two or less TFs were grouped in "Others."

or "response to JA," "regulation of JA-mediated pathway," and "JA metabolic process" were processed by a Hierarchical cluster analysis.

The SA-dependent pathway was represented by 103 DEGs (**Supplementary Table S8**). Eighty-one of these DEGs were up-regulated in at least one of the experimental time points. Some of these genes were induced at either early or late stages of the response, but they were not down-regulated in any of the other analyzed time points (**Figures 5A,B**). Examples of these expression patterns are the genes coding for the signaling protein for SA activation *EDS1* (*enhanced disease susceptibility 5*) and the SA-biosynthetic enzyme *ICS1* (*isochorismate synthase 1*) that were up-regulated at the beginning of the interaction, whilst the SA-responsive proteins *PR1* (*pathogenesis-related protein 1*) and *GLIP1* were induced at later time points. Moreover, the expression profile analysis of some DEGs revealed the quick regulation of some SA-responsive genes since the initial steps of the plant-mite interaction. For instances, *PR2/BGL2* (*pathogenesis-related protein 2/beta-1,3-glucanase 2*) was up-regulated as soon as 6 hai and remained activated at least till 2 dai. Among the induced genes associated to the SA pathway there were also several signaling kinases such as the receptor-like kinase (RLK) *CRK9* (*cysteine-rich RLK 9*), the *wall-associated kinases WAK1* and *WAKL10*, and the *L-type lectin receptor kinase LCRK-S.2, LCRK-IV.1* and *LCRK-IX.2*. Other up-regulated DEGs from this cluster were genes encoding the SA biosynthetic enzymes *ICS2* and *PAL1* (*Phenyl ammonia lyase 1*), the transporter of SA from chloroplast to cytoplasm *EDS5* (*enhanced disease susceptibility 5*), the regulator of SA responses *GRX480* (*glutaredoxin 480*), the methyl-salicylate (MeSA) esterase proteins *MES7* and *MES9* (*methyl esterase 7* and *9*), the defense protein *PR5*, and several TFS from WRKY and MYB families.

Another small cluster of SA-related genes comprised a group of 22 DEGs that were mainly down-regulated, particularly at the two latest time points of the experiment (**Figure 5A**). This cluster was largely formed by TFs that are also responsive to JA. Repressed TFs included members of the ERF/AP2, MYB and GRAS/DELLA families. Other repressed genes beyond TFs were *UGT1* (*UDP-glucose transferase 1*), involved in the callose formation, and the

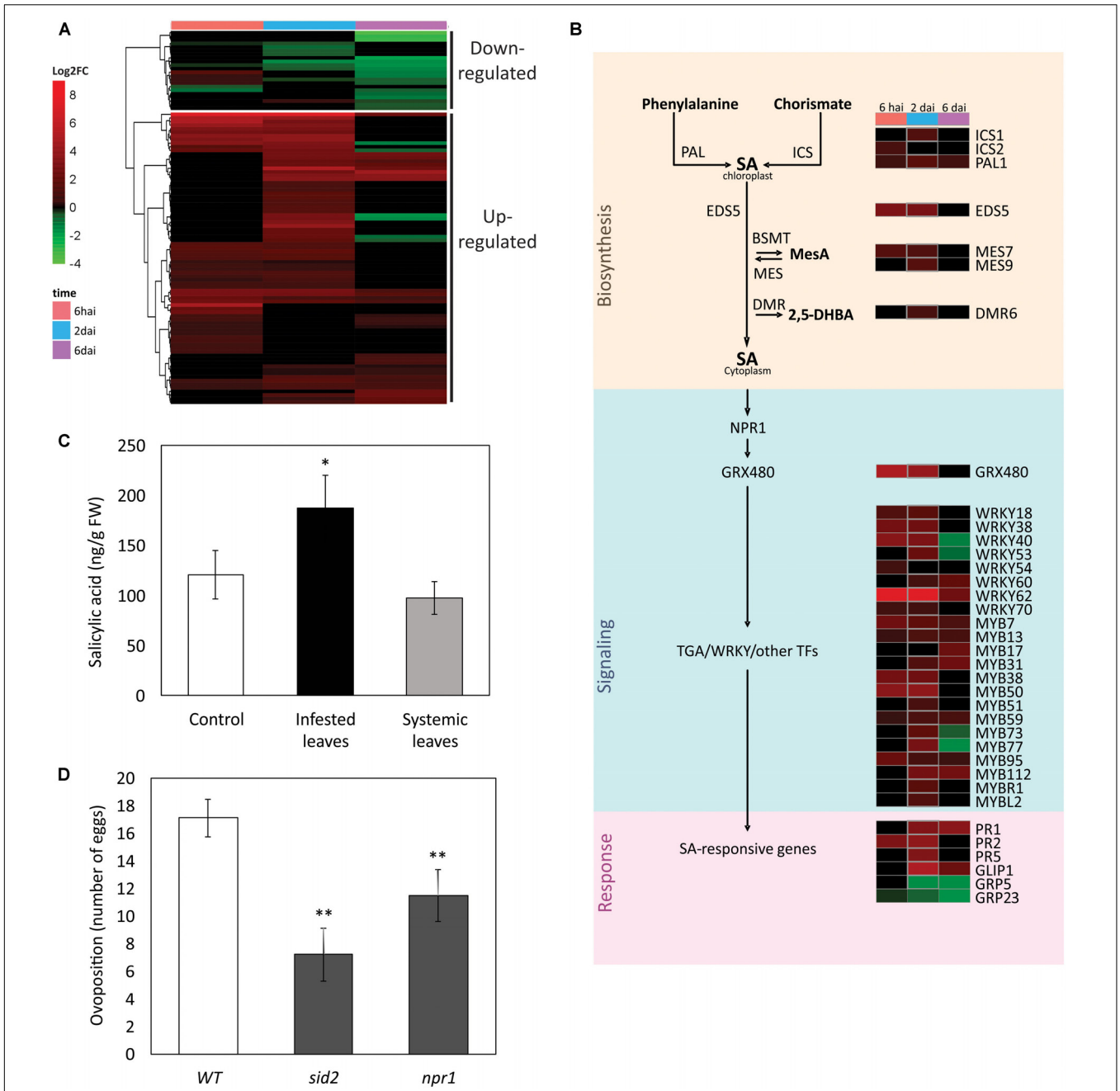


FIGURE 5 | Salicylic acid (SA) pathway in the *A. thaliana* response to *Brevipalpus* mites. **(A)** Hierarchical clustering analysis of $\log_2$ FC exhibited by DEGs involved in the SA pathway. hai, hours after infestation; dai, days after infestation. **(B)** Schematic representation of SA pathway, where a sub-set of DEGs is presented. Colors identifies $\log_2$FC of DEGs at each experimental time point, according to the scale presented in **(A)**. **(C)** SA levels in infested and systemic leaves of mite-infested plants, and in non-infested control plants. Hormone levels were quantified by LC-MS/MS at 6 dai. Error bars represent standard errors. Statistically significant difference at p-value ≤ 0.05 (*) is indicated. FW, fresh weight. **(D)** Mite performance in Arabidopsis mutants compromised in the SA pathway. Data represent the average number of eggs deposited after 4 days of infestation with five *Brevipalpus yothersi* mites/plant. Error bars represent standard errors. Statistically significant differences at p-values ≤ 0.01 (**) are indicated. WT, wild type.

genes encoding the *GRP23* and *GRP5* proteins *(glycine-rich proteins 23 and 5*), which are components of the plant cell wall.

The JA-mediated pathway was composed by 137 DEGs that, similarly to what was observed in the SA-pathway analysis, were mainly up-regulated (**Supplementary Table S8**) (**Figures 6A,B**). DEGs were subdivided in three clusters: two larger groups formed by 60 and 54 highly and mildly up-regulated genes, respectively, and a small one composed by 23 genes that were mostly down-regulated (**Figure 6A**).

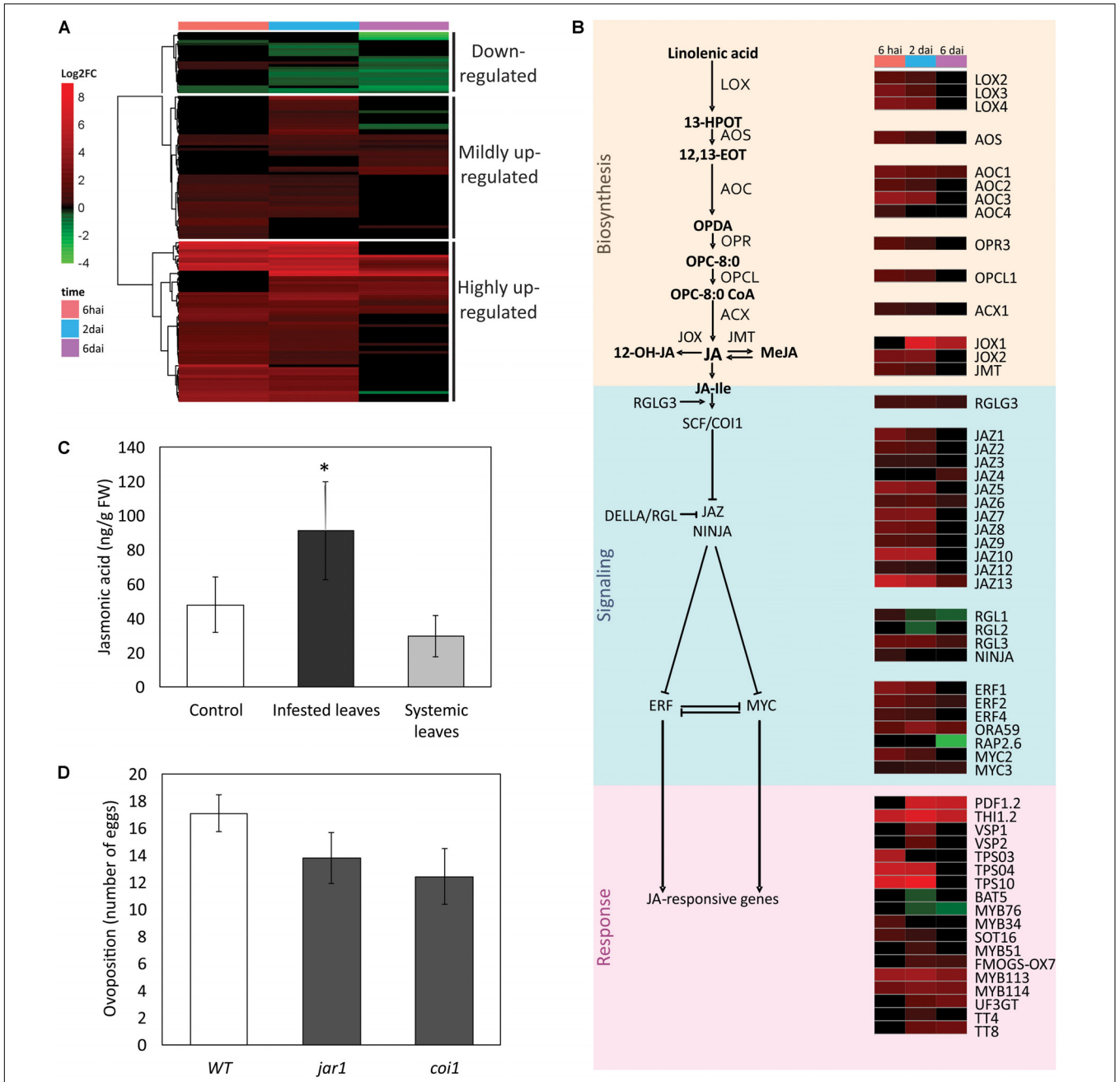


FIGURE 6 | Jasmonic acid (JA) pathway in the *A. thaliana* response to *Brevipalpus* mites. **(A)** Hierarchical clustering analysis of $\log_2$ FC exhibited by DEGs involved in the JA pathway. hai, hours after infestation; dai, days after infestation. **(B)** Schematic representation of JA pathway showing a sub-set of DEGs. Colors identifies $\log_2$FC of DEGs at each time point, according to the scale presented in **(A)**. **(C)** JA levels in infested and systemic leaves of mite-infested plants, and in non-infested control plants. Hormone levels were quantified by LC-MS/MS at 6 dai. Error bars represent standard errors. Statistically significant differences at p-value ≤ 0.05 (*) is indicated. FW, fresh weight. **(D)** Mite performance in Arabidopsis mutants compromised in the JA pathway. Data represent the average number of eggs deposited after 4 days of infestation with five *Brevipalpus yothersi* mites/plant. Error bars represent standard errors. WT, wild type.

Highly induced JA-related genes (**Supplementary Table S8**) at the beginning of the infestation code for proteins acting upstream on the JA pathway such as the DAMP receptor *PEPR2* (*PEP1 receptor 2*) and the JA-biosynthetic and modifying enzymes *AOS* (*allene oxide synthase*), *AOC2* and *AOC3* (*allene oxide cyclase 2* and *3*), *LOX2, LOX3,* and *LOX4* (*lipoxygenase 2*, *3,* and *4*), *OPCL1* (*OPC-8:0 CoA ligase 1*) and *JMT* (*JA carboxyl methyltransferase*) (**Supplementary Table S8**). DEGs induced at the two first experimental time points also included terpene synthases (*TPS03*, *TPS04,* and *TPS10*), several JAZ (*jasmonate-zim-domain*) proteins (*JAZ1, JAZ2, JAZ5, JAZ7, JAZ8, JAZ9/TIFY7,* and *JAZ10*) and the TFs *MYC2* and *ERF1*.

Genes with high expression at later time points encode proteins directly involved in defense such as the marker responsive protein of the ERF-branch *PDF1.2* (*plant defensin 1.2*), and proteins involved in the anthocyanin biosynthesis such as *UF3GT* (*UDP-glucose:flavonoid 3-o-glucosyltransferase*). Other highly up-regulated DEGs included MYB TFs, the DELLA protein *RGL3* that contributes to JA/ET-mediated defenses, the ERF-branch TFs *ERF2* and *ORA59* (*octadecanoid-responsive Arabidopsis AP2/ERF59*), the responsive gene *THI2.1* (*thionin 2.1*), and the JA oxidases *JOX1* and *JOX2* that down-regulate plant immunity by hydroxylation and inactivation of JA.

Genes from the JA pathway that were mildly induced (**Supplementary Table S8**) at the first time points included those coding for the JA-biosynthetic enzymes *ACX1* (*acyl-CoA oxidase 1*) and *AOC4*, the TF *WRKY70* that acts on the SA-JA antagonism, the negative regulators of JA-responsive genes *NINJA* (*novel interactor of JAZ*) and *JAZ3*, and the proteins *MYB34* and *SOT16* (*sulfotransferase 16*) involved in the synthesis of glucosinolates. Later in the infestation, the group of mildly induced genes included those coding for the proteins *MYB75/PAP1* and *TT4* that act in the biosynthetic pathway of anthocyanin, the *MYB51* and *FMOGS-OX7* proteins involved in the synthesis of glucosinolates, and the responsive marker genes of the MYC-branch *VSP1* and *VSP2* (*vegetative storage proteins 1* and *2*), which directly act during the anti-herbivory defense. Two essential modulator genes of the JA signaling, i.e., *RGLG3* (ring domain ligase 3) and the *MYC3* TF were induced at all the assessed time points although at low expression levels. *RGLG3* encodes for a RING-ubiquitin ligase acting upstream of JA-Ile recognition and MYC3 operates together with MYC2 coordinating the expression of responsive genes from the MYC-branch.

The down-regulated JA cluster comprised DEGs that were mainly repressed at 2 and 6 dai (**Supplementary Table S8**). Some of them encode TF commonly acting in the SA-pathway such as members of the families: ERF (*RAP2.6* and *DREB26*), MYB (*MYBS1*, *MYB28*, *MYB29,* and *MYB16*), and GRAS/DELLA (*RGL1* and *RGL2*). Other repressed genes were those encoding the *BAT5* (*bile acid transporter 5*) protein and the *MYB76* TF, both required for the biosynthesis of glucosinolates, the DAMP receptor *PEPR1*, and the JA-repressed protein *AGP31* (arabinogalactan protein 31).

SA and JA Levels Increase in Mite-Infested Plants

Both SA and JA biosynthetic and responsive genes were induced in *Brevipalpus* mite-infested plants. To confirm the consistency of the observed molecular data, the profiles of the SA and JA hormones were determined in Arabidopsis plants challenged with *Brevipalpus* mites. SA (**Figure 5C**) and JA (**Figure 6C**) levels were 1.5- and 2.8-fold higher, respectively, on infested leaves when compared to the control ones (Student's *t*-test, $\alpha \leq 0.05$).

Salicylic acid and JA levels were also verified in systemic leaves of mite-infested plants. No difference was observed between the levels of both hormones in systemic and non-infested control leaves, suggesting a local rather than a systemic response to *Brevipalpus* mite infestation.

Brevipalpus Mites Have a Decreased Performance on Plants Impaired in SA Responses

Salicylic acid- and JA-mediated pathways were clearly induced upon *Brevipalpus* mite infestation. To further explain the functional relevance of the plant hormonal pathways on the interaction, the performance of *B. yothersi* mites was evaluated on Arabidopsis plants impaired in SA or JA-mediated response. Mite oviposition was assessed on mutants defective in SA biosynthesis (*salicylic acid induction deficient2, sid2*) and signaling (*non-expressor of pathogenesis-related protein1, npr1*), and JA signaling (*jasmonate resistant1, jar1* and *coronatine-insensitive1, coi1*). Plants were infested with adult female mites and the number of laid eggs was counted after 6 days.

The number of eggs per plant was 2.4- and 1.5-fold lower on SA-mutants *sid2* and *npr1*, respectively, when compared to the mite's performance in the infested wild-type *A. thaliana* Col-0 plants used as control (Student's *t*-test, $\alpha \leq 0.05$) (**Figure 5D**). No difference was observed between the number of eggs on the mutants affected in the JA pathway (*jar1* and *coi1*) and the wild-type control (Student's *t*-test, $\alpha \leq 0.05$) (**Figure 6D**). These results point to a role of SA-mediated response promoting the *Brevipalpus* mite colonization.

DISCUSSION

False-spider mites of the genus *Brevipalpus* are serious and cosmopolite phytophagous pests with a unique biology (Weeks et al., 2001). They directly provoke injuries in some plant species, but the major consequence of their feeding behavior ensues from the transmission of several cile- and dichorha- viruses that infect economically important crops (Kitajima and Alberti, 2014). Almost 10 species of *Brevipalpus* mites are known to act as virus vector, but, among them, mites of the species *B. yothersi* stands out due to their involvement in transmission of viruses causing citrus leprosis, a severe disease that threatens the citrus industry in the Americas (Beard et al., 2015; Ramos-González et al., 2016). To disentangle the *Brevipalpus*-mite interaction, in the current paper we provide data that extensively describe the response of Arabidopsis plants during their colonization by *Brevipalpus* mites. Changes in the plant transcriptome profile are complemented with the analysis of the accumulation of defense hormones and the results are discussed emphasizing the role of particular plant defense genes during the *Brevipalpus* infestation process.

Our results showed that mite infestation clearly triggers the plant immune system. Processes related to the response to herbivory and other biotic stresses dominate a large number of the over-represented GO categories among all DEGs. Most of the BPs were common between all the time points, although specific changes were also identified. Plant response during the initial 6 h included the induction of genes involved in the hormone

biosynthesis and signaling, consistent with an early recognition of the mite feeding. Transcriptome changes were followed by the up-regulation of a wide range of genes involved in defense and synthesis of secondary metabolites at 2 and 6 dai. The major dissimilarity was between the first and last time points, which do not share any BPs except the ones that were present throughout the infestation. GO enrichment analysis revealed that defense responses were up-regulated and mainly involved the SA- and JA-mediated pathways. Deeper analysis on SA/JA-related DEGs and quantification of hormone contents confirmed that these pathways were distinctly induced upon the infestation by *Brevipalpus* mites. On mite-infested plants, genes involved in the biosynthesis, signaling, and response of the SA and JA pathways were mostly up-regulated, and SA and JA hormone levels were increased. In this regard, simultaneous induction of SA and JA plant response to *B. yothersi* infestation follows a similar pattern to those observed during plant colonization by several spider mites (Kant et al., 2004; Zhurov et al., 2014; Alba et al., 2015; Rioja et al., 2017). Induced defense-related processes also included a clear transcriptional response to oxidative stress. Previous histochemical analysis of infested tissues revealed the production of ROS upon mite feeding (Arena et al., 2016). Induction of ROS production and related transcripts also resembled plant response to spider mite feeding and, in both cases, the role of ROS signaling remains to be determined (Agut et al., 2018).

Gene ontology enrichment analysis revealed an extensive genetic expression adjustment throughout the JA pathway including the hormonal biosynthesis and metabolism, and downstream regulation and response. Mite presence induced the DAMP-receptor PEPR2, suggesting the perception capacity of damaged tissues by Arabidopsis plants. Although minimal, mite feeding causes tissue disruption on infested leaves (Arena et al., 2016). Individual or very few dead cells are observed after mite feeding activity, probably as consequence of punctures by the mite stylets. Upon recognition, JA-biosynthetic enzymes such as AOC, AOSs, and LOXs were up-regulated. Higher JA content in mite-infested leaves confirmed activity of this biosynthetic pathway. Downstream of JA production, several signaling proteins and regulators were induced, including many TFs from MYB, AP2/ERF, and bHLH families. Downstream responses were represented by an array of up-regulated transcripts involved in the production of terpenes, anthocyanin, and glucosinolates. Since induced JA responses to *Brevipalpus* mites are similar to the ones that mediate Arabidopsis response to spider mites (Zhurov et al., 2014), our results indicate a conservation of mite-induced JA regulatory mechanisms. Moreover, several negative regulators of JA response were induced on plants infested by *B. yothersi*, including genes encoding NINJA and numerous JAZ proteins, which interact to repress the TFs that regulates the expression of JA-responsive genes (Wager and Browse, 2012), and JA oxidases, which down-regulates downstream responses by hydroxylation and inactivation of JA (Caarls et al., 2017). In this context, the induced JA pathway might be attenuated, and consequently, the observed data reflect a somewhat mitigated rather than a fully-induced JA-mediated response.

Even though the JA pathway was largely induced upon mite infestation, distinct activation profiles of JA branches were observed. First, TFs from the ERF- and MYC-branches were differentially regulated. AP2/ERF family with TFs that control the ERF-branch was the largest and most enriched family within up-regulated DEGs, whilst bHLH family that includes the TFs that regulates the MYC-branch was the largest and most enriched one within down-regulated DEGs. Particularly, MYC2 that is the major regulator of the MYC-branch responsive genes, was induced, although its target genes were enriched within the cluster of down-regulated genes. Second, the expression levels of defensive genes from the ERF-branch were much higher than that from genes of the MYC-branch. The gene encoding the ERF-responsive anti-microbial protein PDF1.2 figures among the most highly up-regulated DEGs (e.g., FC = 94-fold at 2 dai), whilst those coding for the MYC-responsive anti-herbivory proteins VSP2 and VSP1 were only mildly or not induced at the same experimental time points (e.g., FC = 4- and 10-fold, respectively, at 2 dai). The preferential activation of the ERF-branch over the MYC-branch was described as an herbivore strategy to induce a harmless response in expense of a harmful defense (Verhage et al., 2011; Pieterse et al., 2012). The strongest activation of the ERF-branch reported here corroborates a previous study proposing that *Brevipalpus* mites might mitigate effective defenses by manipulating the plant resistance mechanisms toward herbivore preferred JA responses (Arena et al., 2016). However, further analysis of ERF and MYC mutants are required to clarify the actual role of each one the JA branches in plant response to *Brevipalpus* mites.

Within the induced hormonal pathways in response to *Brevipalpus* infestation, the SA-mediated pathway plays a conspicuous role. On these plants SA levels were elevated, the vast majority of SA-related genes were up-regulated, and the SA-related WRKY TFs as well as their target genes were exclusively over-represented in the cluster of up-regulated genes. Induction of SA response has been associated with stealthy arthropods such as piercing-sucking insects (Nguyen et al., 2016; Patton et al., 2017). Likewise, *Brevipalpus* mite feeding behavior causes minimal tissue disruption. During feeding, mites pierce epidermal cells using interlocked stylets, sometimes through leaf stomata, and suck out overflowed cell content (Kitajima and Alberti, 2014). Activation of the SA pathway by *Brevipalpus* mites agrees with the common pattern observed for herbivores causing little overt tissue damage (Arimura et al., 2011).

Interestingly, an increasing number of evidence indicate that activation of SA pathway favors herbivore performance rather than acts as an effective defense against herbivory. For instance, *Bemisia tabaci* nymphs performs better in the *cpr6* mutants pre-activated for SA-mediated defenses (Zhang et al., 2013), and SA exogenous application render Arabidopsis plants more attractive to thrips (Abe et al., 2012). Using Arabidopsis mutants, we found that the performance of *Brevipalpus* mites is compromised in plants with lower SA content (*sid2*, mutant for ICS1) and defective SA signaling (*npr1*). In comparison with wild-type plants, the number of laid eggs was 2.4- and 1.5-fold lower on *sid2* and *npr1* mutant plants, respectively. Whilst SA levels during

plant-biotic stresses is mainly produced through ICS1-mediated isochorismate pathway (Wildermuth et al., 2001), responses downstream SA accumulation are branched in NPR1-dependent and -independent genes (Uquillas et al., 2004; Shearer et al., 2012). The milder phenotype from *npr1* in comparison with *sid2* might be related to intact NPR1-independent responses. Influence of SA response in *Brevipalpus* mites seems contrary to its role against spider mites. Even though a few reports showed no influence of SA against *Tetranychus* species (Zhurov et al., 2014), a recent study showed that *T. urticae* mites have an increased performance on SA-deficient NahG tomato plants (Villarroel et al., 2016). Lower oviposition of *B. yothersi* mites in either the SA -synthesis or -signaling impaired plants suggests the manipulation by *Brevipalpus* mites of the SA pathway aiming the promotion of host colonization. The positive influence of the SA pathway over *B. yothersi* also has implications for the role of the mite as a vector. We previously reported that infestation with CiLV-C-carrying *B. yothersi* induces even stronger SA response than that reached during non-viruliferous mites feeding (Arena et al., 2016). Higher up-regulation of SA pathway in response to viral infection might further enhance mite colonization and, probably, contribute to the viral transmission.

Salicylic acid-mediated improvement of herbivore performance is usually associated with the antagonistic interactions between the SA and JA signaling pathways (Bruessow et al., 2010; Thaler et al., 2012; Zhang et al., 2013; Caarls et al., 2015). Some arthropods induce SA as a strategy to repress JA effective defenses exploiting the natural cross-talk between signaling pathways. JA defenses, and specifically the production of indole glucosinolates, are central to Arabidopsis defense against *Tetranychus* species (Rioja et al., 2017). Higher reproduction rate of *Brevipalpus* mite due to the induction of the SA pathway could be associated with the reduction on the JA pathway, as previously suggested (Arena et al., 2016). However, our current results show that *Brevipalpus* mite oviposition was not increased in Arabidopsis mutants impaired in JA-responses (*jar1* and *coi1*), therefore, the role of JA pathway against *Brevipalpus* mite colonization is not as obvious as against spider mites, or it does not directly affect oviposition. Molecularly, our results might suggest that the induction of SA antagonizes a set of JA responses which are independent of JAR1 and COI1, or that the SA pathway might improve mite performance by mechanisms alternative to the SA–JA crosstalk. It is noteworthy that upon mite infestation both *JAR1* and *COI1* genes were not induced (**Supplementary Table S2**), consequently, at least at transcriptional level, evidence of involvement of these two gene in response against *Brevipalpus* mite colonization was not revealed. Furthermore, it is possible that the JA pathway influences other aspects rather than mite oviposition such as host preference or mite development. The deeper analysis of other JA mutants and features of mite behavior will help to disentangle the role of the JA on mite infestation.

Some arthropod herbivores are capable of manipulating host responses to circumvent defenses (Stahl et al., 2018). Even though most of the known examples of defense suppression by herbivores involves plant–insect interactions, some cases of suppressive mites have been described (Agut et al., 2018). Defense counteraction has been shown to occur by secreted proteins, called effectors, injected into host cells through herbivores' saliva to interfere with plant responses (Hogenhout and Bos, 2011). Effectors from *Tetranychus* saliva that suppress harmful defenses and increase spider mite performance were recently described (Villarroel et al., 2016). *Brevipalpus* mites likely inject saliva inside host cells through a tube formed between its interlocked stylets (Kitajima and Alberti, 2014). The ability of *B. yothersi* mites to manage the plant response favoring their own performance suggests that, similarly as spider mites do, *Brevipalpus* mites might also deliver saliva-borne effector proteins into plant cells. It is noteworthy, however, that mites from *Brevipalpus* genus employ such a distinct strategy of modulation of plant responses compared to closely-related spider mites. Even though feeding by both *Brevipalpus* and *Tetranychus* mites induce SA and JA pathways simultaneously, the effectiveness of such responses diverges between the two systems. Whilst JA pathway defend plants against *Tetranychus* mites (Zhurov et al., 2014), SA pathway has been described as neutral or detrimental to spider mite species (Villarroel et al., 2016). On the contrary, adverse effect of JA responses to counteract *Brevipalpus* mite infestation was not revealed in the present study, but SA pathway has a positive effect over the colonization of these false-spider mites, pointing to a unique response within described plant-mite interactions.

Polyphagous arthropods likely posses a larger collection of salivary proteins due to their exposure to a wide range of host plants with distinct morphology and physiology (Vandermoten et al., 2014). Large groups of proteins families identified in *T. urticae* saliva were proposed to facilitate the expansion of the host range of these highly polyphagous mites (Jonckheere et al., 2016). Like *T. urticae*, *Brevipalpus* mites infest a wide range of hosts that includes almost a thousand of plant species in more than a hundred of different families (Childers et al., 2003). Collectively, our results suggest that *Brevipalpus* mites manipulate the plant defensive response to render the plant more susceptible to the colonization by inducing the SA-mediated pathway, a mechanism unusual to spider mite species. Mite's ability to modulate the plant physiology in their favor might support the high polyphagous nature of false-spider mites.

Independent GO enrichment analysis from up- and down-regulated DEGs revealed not only the up-regulation of defensive responses, but also the repression of plant growth-related processes. Defensive responses have been long considered to impose a cost that results in reduced plant growth and reproduction (Züst and Agrawal, 2017). Growth-defense trade-off comes from a reallocation of resources to optimize fitness when plants are exposed to environmental changes. Upon herbivory, the plant metabolism is frequently reconfigured. While the secondary metabolism is enhanced to produce defenses, the primary metabolism is suppressed. For instance, induction of JA pathway by *Manduca sexta* results in down-regulation of photosynthesis in *Nicotiana attenuata* (Halitschke et al., 2011). Plant growth genes repressed during the elicitation

of defenses comprise, among others, those associated with cell wall (e.g., expansins), cell division (e.g., cyclins), and DNA replication and photosynthesis (such as components of the light-harvesting complex, photosystem subunits, electron transport chain, chlorophyll biosynthesis, etc.) (Attaran et al., 2014). On *Brevipalpus* mite-infested plants, several of those growth-related genes were down-regulated. Over-represented GO terms within repressed DEGs included processes associated to the cell wall, morphogenesis of cell components, cell division, and photosynthesis.

The plant growth-defense trade-off is modulated through the interplay between defense hormonal pathways mediated by SA and JA and the hormones that act as the major plant growth regulators, i.e., IAA, BR, and GA. Some molecular players that regulate the trade-off have been identified (Huot et al., 2014; Lozano-Durán and Zipfel, 2015; Campos et al., 2016; Züst and Agrawal, 2017). DELLA proteins are key negative regulators of GA signaling that inactivates growth-promoting phytochrome-interacting factors (PIFs). Upon GA elicitation, DELLA proteins are degraded, releasing PIFs and allowing them to activate expression of growth-promoting genes (Huot et al., 2014; Züst and Agrawal, 2017). DELLA and JAZ proteins interact, inhibiting each other actions over the repression of growth and defense-related genes (Yang et al., 2012). The degradation of JAZ proteins triggered by JA accumulation releases DELLA from JAZ binding, thereby strengthens the suppression of PIFs and plant growth (Yang et al., 2012; Züst and Agrawal, 2017). Likewise, overexpression of the DELLA protein RGL3 reduces GA-mediated growth while increases MYC2-dependent expression of JA-responsive genes (Wild et al., 2012). Our results indicate that markers from the molecular mechanism behind the trade-off, such as RGL3, were induced. The SA- and JA-dependent defense responses were up-regulated and IAA-, BR-, and GA-mediated growth processes were downregulated, suggesting that the growth-defense trade-off occurs during Arabidopsis-*Brevipalpus* interaction.

Results obtained here extend our previously proposed model on the Arabidopsis response to non-viruliferous *Brevipalpus* mites (Arena et al., 2016). Beyond the responses focused here, the large-scale transcriptome we obtained will provide a valuable resource to further explore unknown molecular components involved in plant interaction with false-spider mites.

MATERIALS AND METHODS

Plant Material

Wild-type A. thaliana ecotype Columbia (Col-0) was obtained from the Arabidopsis Biological Resource Center[1]. Arabidopsis mutants in the Col-0 background (sid2-1, npr1, and jar1) were obtained from Georg Jander. The Arabidopsis mutant coi1-16 was obtained from Kirk Overmyer. Plants were grown in controlled growth chambers (Conviron, Winnipeg, Canada) at 23 ± 2°C and a 12 h light/dark photoperiod. Four-week-old plants were used in the experiments.

[1]http://www.arabidopsis.org

Mite Rearing

Non-viruliferous mites were initially obtained from citrus orchards and further confirmed as *B. yothersi* using phase contrast microscopy as reported elsewhere (Beard et al., 2015). Mites were reared onto fruits of 'Tahiti' acid lime (*Citrus latifolia* Tanaka), a genotype immune to citrus leprosis virus C, as previously described (Arena et al., 2016). Mites were reared for several generations and were confirmed as non-viruliferous by RT-PCR using primers for CiLV-C (Locali et al., 2003) before their use in the experiments.

RNA-Seq Experiment

A time course experiment was conducted on plants infested with non-viruliferous mites and on non-infested control plants at 6 h after infestation (hai), 2 and 6 dai. For each time point, Arabidopsis Col-0 plants were grouped in sets of 16 individuals assigned to each treatment (infested and control). Plants from both treatments were kept at the same growth chamber. Plants from the infested treatment were challenged with 15 mites (5 mites per each of 3 rosette leaves), transferred with a small brush under a stereoscopic microscope. Mites were not caged. Infested or control leaves were collected at each time-point. From mite-infested plants, only the leaves where mites were originally deposited were collected. Leaves from two plants were pooled, totaling eight biological replicates per treatment per time point, flash-frozen in liquid N_2 and stored at −80°C until RNA extraction. Plant RNA was purified using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) and treated RNAse-free DNAse (Qiagen, Venlo, Netherlands) for removal of residual plant DNA. RNA quality was assessed in Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, United States). All samples had an RNA integrity number (RIN) above eight and were considered suitable for RNA-Seq. RNA extracts from two samples (100 ng/μL each) were pooled in a single sample, totaling four replicates per treatment per time point for library construction and independent sequencing. cDNA libraries were prepared using Illumina TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, United States). Sequencing was performed with HiSeq SBS v4 High Output Kit (Illumina, San Diego, CA, United States) in an Illumina HiSeq 2500 system (Illumina, San Diego, CA, United States) and generated 2 × 125 bp paired-end reads.

Bioinformatics Analysis of RNA-Seq Data

RNA-Seq data were analyzed using R and Bioconductor according to Anders et al. (2013) with some modifications. Quality of the sequences was confirmed using ShortRead (Morgan et al., 2009) and FASTQC. Reads were mapped to the *A. thaliana* TAIR10 genome using TopHat2 (Kim et al., 2013). The number of reads per gene was counted with HTSeq (Anders et al., 2015) and normalized by size factors obtained from the negative binomial-based DESeq2 package (Love et al., 2014). After normalization, clusterization profiles of the samples were assessed by hierarchical clustering (with Euclidean distance metric and Ward's clustering method) and principal component analysis (PCA). Differentially expressed

genes (DEGs) between infested and control treatments were identified at each time point using likelihood ratio tests after negative binominal fittings using the package DESeq2 (Love et al., 2014). Genes with False Discovery Rate (FDR)-corrected p-values ≤ 0.05 and fold-change ($\log_2$) threshold of 0.5 were classified as differentially expressed. To identify mechanisms potentially involved in the plant response to mite feeding, GO Enrichment Analysis was performed. A gene set was defined as all DEGs (unless otherwise noted) and the universe comprised all genes of the *A. thaliana* TAIR10 genome expressed in at least one of the observed conditions. Overrepresented BPs, MFs, and CCs were identified based on a hypergeometric test with FDR-adjusted p-values ≤ 0.001. GO networks were generated using the app BinGO in Cytoscape (Maere et al., 2005).

Identification of Enriched Transcription Factors

A hierarchical clustering was performed with all DEGs to identify up- and down- regulated clusters, using Euclidean distance metric and Ward's clustering method. Two approaches were used to identify enriched TFs on each cluster. First, we searched for genes coding for TFs within DEGs using PlantTFDB database (Jin et al., 2017). Over-represented TFs on each cluster were identified using a hypergeometric test ($\alpha \leq 0.001$). Second, we searched for enriched TF targets using the TF enrichment tool, based on previously identified *cis*-regulatory elements and regulatory interactions from literature mining (Jin et al., 2017).

Validation of Gene Expression Data by RT-qPCR

Another time course experiment was set with plant infested with non-viruliferous mites and non-infested control plants at 6 hai, 2 and 6 dai. For each time point, Arabidopsis Col-0 plants were grouped in sets of 16 individuals assigned to each treatment (infested and control). Plants from the infested treatment were challenged with 15 mites (5 mites per each of 3 rosette leaves). Infested or control leaves were collected at each time-point. Leaves from two plants were pooled, totaling eight biological replicates per treatment per time point, and flash-frozen in liquid N_2. Plant RNA was purified using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) and treated with RNAse-free DNAse (Qiagen, Venlo, Netherlands). RNA concentration was assessed using NanoDrop ND-8000 microspectrophotometer (Thermo Scientific, Waltham, MA, United States) and RNA quality was verified in 1.2% agarose gels. cDNA were generated for each RNA sample (500 ng) using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, United States) as described by the manufacturer. RT-qPCR were prepared with 6.5 μL of GoTaq qPCR Master Mix (Promega, Madison, WI, United States), 120 nM of each gene-specific primer pair and 3 ng of cDNA. Primer sequences are available on **Supplementary Table S9**. Reactions were performed in a 7500 Fast Real-Time PCR System (Thermo Scientific, Waltham, MA, United States) device, using the standard settings. Each sample was analyzed in triplicate and melting curves were included to confirm the absence of genomic DNA and unspecific reactions. Quantification cycle (*C*q) values and primer pairs efficiencies were determined for each individual reaction using Real-time PCR Miner (Zhao and Fernald, 2005). Gene expression analyses were performed according the ΔCq model using multiple reference genes (Hellemans et al., 2007) as previously described (Arena et al., 2016). Statistical significances between infested and control samples within each time point were assessed using Student's t-test ($\alpha \leq 0.05$).

Quantification of Hormone Levels

Four-week-old Arabidopsis Col-0 plants were infested with 10 mites (two leaves with five mites each) or kept without mites. Infested leaves were collected after 6 days. Leaves from two plants were pooled together in one sample, totaling six replicates per treatment. Harvested leaves were weighted, flash frozen in liquid nitrogen and ground in a paint shaker. The SA and JA contents at local and systemic leaves of mite-infested plants were compared with those from the non-infested control as previously described (Casteel et al., 2015). For analysis, 5 μL of each extract were analyzed on a triple-quadrupole liquid chromatography-tandem mass spectrometry system (Agilent 6420A triple-quadrupole with Infinity II HPLC). Extracts were separated on a Zorbax Extend-C18 HPLC column (Agilent, 3.5 μm, 150 mm × 3.00 mm) using 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Statistical significance was assessed using Student's t-test ($\alpha \leq 0.05$).

Mite Performance in Arabidopsis

The mite performance was evaluated on Arabidopsis mutants impaired in SA- (*sid2* and *npr1*) or JA- (*jar1* and *coi1*) mediated response. Plants were infested with five female adult *B. yothersi* mites in a single leaf, caged to prevent escape, and a completely randomized design was set. After 4 days of infestation, plant leaves were carefully detached, and the number of mite eggs was counted. Data from each mutant genotype was compared to the wild-type plants using Student's t-test ($\alpha < 0.05$).

RNA-Seq Raw Data

The RNA-Seq raw data are available at sequence read archive (SRA) with the ID SRP144249.

AUTHOR CONTRIBUTIONS

GA, PR-G, CC, JF-A, and MM conceived and designed the experiments. GA, PR-G, and LR performed the experiments. GA, PR-G, and MR-A analyzed the data. MR-A, CC, MM, and JF-A contributed with reagents, materials, and analysis tools. GA, PR-G, and JF-A wrote the paper. All authors discussed the results and reviewed the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01147/full#supplementary-material

FIGURE S1 | RT-qPCR validation of the RNA-Seq data. Expression profile of selected *Arabidopsis thaliana* genes was analyzed in *Brevipalpus yothersi* mite-infested plants by RNA-Seq and RT-qPCR. Data are presented as $\log_2$ fold-change (FC) values in comparison with non-infested plants (with $\log_2$FC set to zero). Statistically significant differences of mite-infested versus non-infested control at p-values ≤ 0.01 (**) and ≤ 0.05 (*) are indicated. hai, hours after infestation; dai, days after infestation. Note that the gene coding for the SA-responsive protein *PR1* was up-regulated at 2 and 6 dai, following the same profile obtained by RNA-Seq. The SA-related genes *GRX480*, *WRKY70,* and *PR2* were also identified as induced by RT-qPCR at the beginning of the interaction, but statistical significance was not confirmed in the later time points. Expression of the JA-responsive TFs *MYC2* and *ORA59* was also validated by RT-qPCR. Even though *MYC2* transcripts were significantly higher at 6 dai by RT-qPCR but not RNA-Seq, up-regulation of this TF at 6 hai and 2 dai was identified using both techniques. Likewise, *ORA59* gene was up-regulated at 6 hai and 6 dai by both RNA-Seq and RT-qPCR. RT-qPCR results also confirmed the mild induction of the MYC-branch responsive gene *VSP2* at 2 dai, and the high induction of the ERF-branch marker gene *PDF1.2* at 2 and 6 dai. The growth-related gene *EXP3* was down-regulated at 6 hai and 6 dai, while the negative regulator *RGL3* was induced at the same time points, according the RNA-Seq data.

FIGURE S2 | Main biological processes (BPs) affected by *Brevipalpus* mites in *Arabidopsis thaliana* plants. Overrepresented BPs were identified based on a hypergeometric test with false discovery rate (FDR)-adjusted p-values ≤ 0.001. Gene ontology (GO) networks were generated using the app BinGO in Cytoscape. Color and size of the nodes identify the number and p-values of differentially expressed genes (DEGs) from each category. Names of some BPs were simplified for clarity; full names are shown on **Supplementary Table S3**. ROS, reactive oxygen species; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; ABA, abscisic acid; IAA, auxin; CK, cytokinin; GA, gibberellic acid.

TABLE S1 | Sequencing and alignment statistics for all *Arabidopsis thaliana* samples. hai, hours after infestation; dai, days after infestation.

TABLE S2 | Total differentially expressed genes (DEGs) in *A. thaliana* plants infested with *Brevipalpus yothersi* mites at 6 hours after infestation (hai), 2 days after infestation (dai) and 6 dai. FC, fold-change; FDR, False Discovery Rate-corrected p-values. Gene symbols and descriptions were retrieved from ThaleMine (https://apps.araport.org/thalemine).

TABLE S3 | Enriched Biological processes (BPs), molecular functions (MFs), and cellular components (CCs) in the set of DEGs of *A. thaliana* plants infested with *B. yothersi* mites. Overrepresented BPs, MFs, and CCs were identified based on a hypergeometric test with False Discovery Rate (FDR)-adjusted p-values ≤ 0.001. GO, Gene ontology; FDR, FDR-corrected p-values.

TABLE S4 | Enriched BPs at each time point assessed after the infestation of *A. thaliana* plants with *B. yothersi* mites. Overrepresented BPs were identified based on a hypergeometric test with FDR-adjusted p-values ≤ 0.001. GO, Gene ontology; FDR, FDR-corrected p-values; hai, hours after the infestation; dai, days after the infestation.

TABLE S5 | Enriched BPs at each cluster formed by DEGs that were mainly up- or down-regulated during the *A. thaliana* interaction with *B. yothersi* mites. Clusters were defined after a hierarchical clustering analysis of all DEGs. Overrepresented BPs were identified based on a hypergeometric test with FDR-adjusted p-values ≤ 0.001. GO, Gene ontology; FDR, FDR-corrected p-values.

TABLE S6 | Differentially expressed genes (DEGs) coding for TF at each cluster formed by DEGs that were mainly up- or down-regulated during the *A. thaliana* interaction with *B. yothersi* mites. Clusters were defined after a hierarchical clustering analysis of all DEGs.

TABLE S7 | Transcription factors (TFs) with enriched targets within each cluster formed by DEGs that were mainly up- or down-regulated during the *A. thaliana* interaction with *B. yothersi* mites. TFs with enriched targets were identified by TF enrichment tool (Jin et al., 2017). Up- and down-regulated clusters were defined after a hierarchical clustering analysis of all DEGs.

TABLE S8 | Differentially expressed genes (DEGs) involved in salicylic acid (SA) and jasmonic acid (JA) pathways, identified in *A. thaliana* plants infested with *B. yothersi* mites at 6 hai, 2 dai and 6 dai. FC, fold-change; FDR, False Discovery Rate-corrected p-values. Gene symbols and descriptions were retrieved from ThaleMine (https://apps.araport.org/thalemine).

TABLE S9 | Selected genes and corresponding primer pairs of *A. thaliana* used for gene expression analyses by RT-qPCR.

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Checkmite!? Is the Resistance to Phytophagous Mites on Short and Stocky Wild *Oryza* Species?

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Plants must effectively defend against environmental stresses to survive in nature. However, immunity to disease is costly and often comes with a significant growth inhibition and yield penalty (Yang et al., 2012; Huot et al., 2014; Huang et al., 2017; Ning et al., 2017). Hormones play important roles in regulating plant growth and stress responses (Heinrich et al., 2013). Gibberellins (GAs) and jasmonates (JAs) are two types of essential phytohormones that control many aspects of plant growth and development in response to environmental and endogenous signals. GA regulates many essential plant developmental processes (including stem and leaf elongation), while JA plays a dominant role in mediating plant defense, especially to herbivores attack (Hou et al., 2013). Even though GA and JA antagonize each other in regulating plant growth and defense response via interaction between JAZs and DELLAs proteins (De Bruyne et al., 2014; Song et al., 2014; Chaiwanon et al., 2016), the exactly way how plants coordinate the fluctuating growth-defense dynamics is not well understood. Especially, the role of GA in growth-defense conflicts during herbivory is yet to be characterized. Several works have been addressing the plant dilemma between "to grow" and "to defend" in response to various stimuli, clearly indicating that plants need to prioritize GA- or JA-induced responses. Heinrich et al. (2013) showed that high levels of JA antagonize the biosynthesis of GA and inhibit the growth of *Nicotiana attenuata* stems. In rice, GA application was found to decrease resistance to the hemibiotrophic rice pathogens *Magnaporthe oryzae* (Mo) and *Xanthomonas oryzae* pv. *oryzae* (Xoo) (Yang et al., 2008; Qin et al., 2013). The GA biosynthetic pathway and signaling cascade were shown to be regulated by JA during Mo and Xoo interactions with rice plants. It was also shown that the only DELLA protein in rice, SLR1, is crucial to integrate GA and JA (as well as salicylic acid) crosstalk (De Vleesschauwer et al., 2016). In agreement with that, rice plants overexpressing a GA deactivating enzyme accumulated low levels of GA and displayed enhanced resistance to Mo and Xoo, whereas plants harboring loss-of-function mutations in the same gene were more vulnerable to these pathogens (Yang et al., 2008; De Bruyne et al., 2014).

Upon attack by the chewing herbivore *Chilo suppressalis*, rice plants activate the expression of OsWRKY70, a transcription factor that physically interacts with W-box motifs and prioritizes defense over growth by positively regulating JA and negatively regulating GA biosynthesis (Li et al., 2015). Two groups investigated the growth/defense response of rice plants infested by brown planthopper (BPH) insect: (1) Wang et al. (2015) detected a shift from growth to defense in response to BPH infestation, evidenced by down-regulation of GA-related genes, decreased GA levels, increased JA levels, and reduced plant growth; (2) Qi et al. (2016) showed that plants over-expressing *OsJMT1* (JA carboxyl methyltransferase, which is up-regulated by BPH infestation and is a key enzyme in methyl-JA biosynthesis pathway) exhibited increased MeJA levels and reduced height. In line with these findings, we previously detected lower expression of three GA biosynthetic

pathway-related genes (*OsGA2ox1*, *OsGA2ox3*, and *OsGA20ox1*) in rice leaves infested with the phytophagous mite *Schizotetranychus oryzae*, when compared with control leaves. The expression of *OsAOS* (allene oxide synthase), which catalyzes the committed step in JA biosynthesis, was only detected in infested leaves (Buffon et al, 2016). Altogether, these results suggest that JA-related responses antagonize the biosynthesis of GA and GA-related responses during herbivory.

Wild plant species have been widely recognized as valuable source of resistance genes for developing herbivore-resistant cultivars. For example, *Oryza brachyantha* is resistant to the rice leaf folder *Cnaphalocrocis medinalis* (Ramachandran and Khan, 1991; Ricachenevsky et al., 2018). To date, 10 QTLs and one causative gene have been identified from six wild rice species (*O. officinalis*, *O. eichingeri*, *O. minuta*, *O. latifolia*, *O. rufipogon*, and *O. australiensis*—Huang et al., 2013; Zhang et al., 2014; Hu et al., 2015; Ji et al., 2016). With this in mind, we asked ourselves whether wild rice cultivars could also present some degree of resistance to *Schizotetranychus oryzae* mite infestation. Surprisingly, the wild rice genotypes tested (*O. glaberrima* and *O. barthii*) were characterized as highly sensitive to *S. oryzae* infestation, being even more sensitive than cultivated *O. sativa* genotypes (**Figures 1A,B**). Similar results were reported by Veasey et al. (2008), which tested the infestation of *S. oryzae* in four wild rice species (*O. glumaepatula*, *O. latifolia*, *O. alta*, and *O. grandiglumis*), and Chandrasena et al. (2016), which tested the infestation of panicle rice mite *Steneotarsonemus spinki* in five wild rice species (*O. nivara*, *O. eichingeri*, *O. rufipogon*, *O. granulata*, and *O. rhizomatis*), with no signs of mite resistance.

Taking into account the antagonism between JA and GA, and that breeding of cultivated rice during the Green Revolution has selected for low GA/GA-insensitive genotypes, as shown by the semi-dwarf phenotype of modern rice cultivars (Spielmeyer et al., 2002), we believe the mite-sensitivity presented by the wild rice species could be explained, at least partially, by a presumable high GA:JA ratio in these plants. Tested wild species are tall plants, varying from 1.5 to 5 m, and probably have high levels of GA synthesis. It is important to highlight that a plant's height is not only dependent on the GA level, and resistance to herbivores is not only dependent on the JA level. However, considering the high variability found on the genomes of wild rice species and the high number of already identified insect-resistance genes, we hypothesize that a short (or a semi-dwarf) wild rice species could present a significant level of mite-resistance. In line with our assumption, the semi-dwarf IR36 rice cultivar (one of many of the Green Revolution which replaced many local strains and genetic diversity previously found in rice paddies, resulted from a cross-breeding of IR8 with 13 parent varieties from six nations and a wild species of rice, *O. nivara*) is resistant to many pests and diseases, including green leafhopper (*Nephotettix virescens*), BPH, stem borer (*Chilo* sp.), blast blight (*Pyricularia oryzae*), bacterial blight (*Xanthomonas campestris* pv *oryzae*), tungro, and grassy stunt viruses (Innes, 1992).

Therefore, we would like to suggest short *Oryza* species and genotypes as primary sources of herbivory tolerance, including mites. We should expect low GA levels/sensitivity, and therefore high SLR1 (the sole DELLA protein in rice) levels in these plants. Accumulated SLR1 will amplify the JA-response, driving plant resources toward defense instead of growth (Chaiwanon et al., 2016; De Vleesschauwer et al., 2016). Obviously, we do not expect the GA-JA switch to be the sole determinant of resistance. However, plants with high GA stimulus are more likely to have lower levels of SLR1, freeing JAZ proteins to sequester JA-response activation transcription factors and in turn attenuate JA-mediated resistance (**Figure 1C**). Thus, we should focus our efforts in searching for useful genes in genotypes that are already primed to JA defense responses (i.e., plants with low GA level/sensitivity and therefore high SLR1 levels, which would amplify JA-responses and drive plant resources toward defense instead of growth).

We suggest *O. minuta*, *O. meyeriana*, *O. neocaledonica*, and *O. schlechteri* (http://www.knowledgebank.irri.org/images/docs/wild-rice-taxonomy.pdf) as possible sources of mite-resistance genes. *O. minuta* ($2n = 48$, BBCC genome, 1 m tall, perennial) exhibits significant potential to resist to several pests/diseases (http://archive.gramene.org/species/oryza_species/o_minuta.html), including blast blight, bacterial blight (BB), white-backed planthopper (WBPH), and brown planthopper (BPH) (Amante-Bordeos et al., 1992; You et al., 2007; Rahman et al., 2009; Asaf et al., 2016, 2017), which are damaging to the growth and yield of cultivated rice. Few studies have been conducted to identify and transfer the resistance genes from *O. minuta* to cultivated rice (Amante-Bordeos et al., 1992; Rahman et al., 2009). However, no hybrid with commercial rice cultivar with elevated resistance to herbivores have been developed so far. *O. meyeriana* ($2n = 24$, GG genome, about 50 cm tall, perennial) is adapted to survive in harsh environments and possesses many useful traits absent in cultivated rice, including high resistance to rice blast and bacterial blight, which has been transferred to cultivated rice (*O. sativa*) (Yan et al., 2004; Han et al., 2014; He et al., 2015; Chen et al., 2016; Cheng et al., 2016). *O. neocaledonica* ($2n = 24$, GG genome, 60–80 cm tall, perennial), is the latest species described in the genus *Oryza* (Nayar, 2014). First considered a subspecies/variety/population of *O. meyeriana* (Vaughan, 2003), it was later recognized as a valid *Oryza* species (Clayton et al., 2010 - https://www.kew.org/data/grasses-db.html). Surprisingly, no studies are known to have been done on *O. neocaledonica* (Nayar, 2014), evidencing an unexplored gene diversity. *O. schlechteri* ($2n = 48$, HHKK genome, 30–90 cm tall, annual), found in undisturbed forests, is the least studied species in the genus (Brar and Singh, 2011). Based on bioclimatic analysis, Atwell et al. (2014) pointed *O. schlechteri* as a candidate species for flooding tolerance. Regarding biotic stress response, no studies have been done with this species, and for this reason is an irreplaceable material for improving the cultivated varieties.

Even though herbivore resistance is commonly a genetically determined trait that shows heritable genetic variation (Muola et al., 2010), it is important to highlight that before including a plant material as a primary source in a breeding program, is essential to know the heritability of the trait and how stable the trait would be when transmitted to the offspring. Therefore, it would be interesting to examine whether and how environmental factors regulate GA:JA ratio/crosstalk and mite resistance in

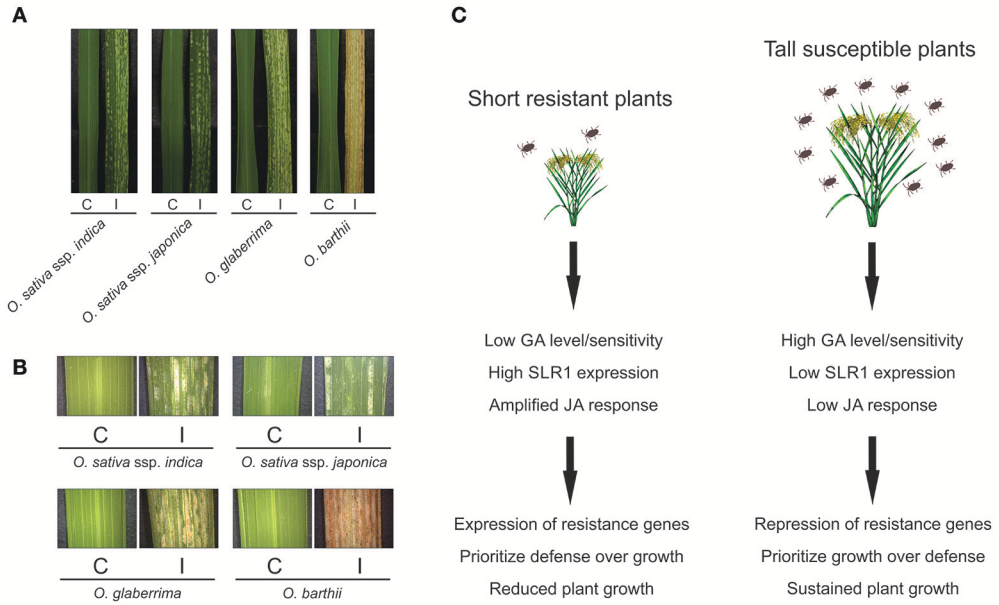

FIGURE 1 | Visual characteristics of leaves from *O. sativa* ssp. *indica*, *O. sativa* ssp. *japonica*, *O. glaberrima*, and *O. barthii* plants, under control (C) and infested (I) conditions **(A)**. Detailed view of these leaves under stereomicroscope **(B)**. Schematic model of the different responses probably employed by short resistant and tall susceptible plants **(C)**.

these short and stocky wild rice species. Also, we are aware that crossing often fails to generate fertile hybrids between cultivated rice and wild species because of reproductive barriers (Han et al., 2014). However, the use of asymmetric somatic hybridization has proved to be effective (Jena, 2010). Therefore, searching for mite resistance genes taking GA-JA crosstalk into account, together with comprehensive screens in the wild rice diversity, should be fruitful to develop mite resistance in susceptible cultivated rice lines.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Crops Responses to Mite Infestation: It's Time to Look at Plant Tolerance to Meet the Farmers' Needs

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It is common to see crop farmers struggling to solve herbivore infestations. Normally, worry starts when herbivore population increases and leaf damage is apparent. Whatever the treatment chosen by the farmer (chemical or biological) to control it, yield and productivity are the final and most important characteristics they would like to see unaffected by herbivory. Certainly, farmers would be less interested in percentage of leaf damage or mite population increase, which are useful but limited approximations of tolerance, than maintenance of crop production even under infested conditions. If a crop can stand infestation and/or larger mite populations while still producing the same seed set, the crop is, from a farmer standpoint, tolerant. On the other hand, academic studies on the field of plant-herbivore interaction are still based on resistance mechanisms in vegetative tissues and herbivore performance, rather than the plant reproductive success. We should be aware that genetic breeding for tolerance should focus on comparing seed production when dealing with most crops, not indirect measures such as leaf damage or mite population dynamics.

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RESISTANCE/TOLERANCE MECHANISMS

The interactions between herbivores and plant hosts result from an elaborate evolutionary interplay: plants have developed strategies to arrest attackers and reduce pest fitness as a defense against herbivory, while herbivores have evolved mechanisms to overcome that (Rioja et al., 2017). Such defense strategies include several modifications that reduce the negative impact of herbivores on a plant's reproductive success (i.e., the production of fertile offspring) and increase the plant's fitness (i.e., its contribution to the gene pool of the next generation) as a function of herbivory (Erb, 2018). Plants that efficiently and effectively use these defense strategies are called "pest-resistant" (which can be broadly classified into two different mechanisms, antibiosis and antixenosis - Stenberg and Muola, 2017), or "pest-tolerant" (Mitchell et al., 2016). Antibiosis mechanisms affect pest biology in a deleterious manner (Peterson et al., 2017), decreasing herbivore fitness or performance (e.g., fertility rate or larval development time - Stenberg and Muola, 2017). Antixenosis mechanisms direct a pest away from the plant (Peterson et al., 2017), decreasing the herbivore presence (number of eggs, larvae, or adults) and, consequently, the herbivore damage (e.g., percentage of leaf area removed - Stenberg and Muola, 2017). On the contrary, tolerance mechanisms allow the plants to withstand pest injury and produce acceptable yields, maintaining the fitness under stressful conditions, without affecting pest biology or behavior, which creates

little selective pressure on pest populations and therefore does not generate resistant variants to the tolerant plants (Peterson et al., 2017).

TOLERANCE: MUCH LESS STUDIED THAN ANTIBIOSIS OR ANTIXENOSIS

Recently, a tricky and artful question was made by Peterson et al. (2017): is tolerance the forgotten child of plant resistance? This questioning came from the fact that it has received the least attention of the three types of plant defenses (or the three types of plant resistance, according to some authors that consider tolerance as the third type of plant resistance). According to Erb (2018), most plant defenses are still characterized by proximate variables such as herbivore performance or plant damage rather than actual fitness, which means that antibiosis and antixenosis (resistance subtypes) are more commonly used than tolerance to describe plant behavior against herbivorous pests. This is evidenced by the data presented in **Table 1**. Since 2009, we found 25 articles describing phytophagous mite interaction with crop species, and only four analyzed plant yield under infested condition (Karmakar, 2009; Vichitbandha and Chandrapatya, 2011; Nyoike and Liburd, 2013; Warabieda, 2015). The most common group of measures to assess defenses are herbivore performance traits, including mite population, survival, development and oviposition rates, along with leaf damage (**Table 1**). Peterson et al., 2017) list five reasons why tolerance has not been developed as successfully as antibiosis and antixenosis: (1) tolerance is difficult to identify, and the mechanisms conferring it are poorly understood; (2) the genetics of tolerance is mostly unknown; (3) high-throughput phenotyping methods for large-scale screening of tolerance are still missing; (4) most of the entomologists are interested in mechanisms which affect pest biology, not plant biology (highlighting the need for interdisciplinary research between plant scientists and entomologists); and (5) plant resistance efforts are still directed at controlling pest populations rather than managing plant stress.

WHY RESISTANCE ANALYSIS CAN BE PROBLEMATIC?

Even though the importance of data on plant reproductive success and yield in plant defense studies has been previously emphasized (Clavijo McCormick et al., 2012; Poelman, 2015), the plant-herbivore community still have difficulties to use the appropriate fitness analysis in combination with recent methodological advances to increase our understanding of plant defense traits (Erb, 2018). Therefore, herbivore performance traits and leaf damage—proximate variables, according to Erb (2018)—still are the first options. However, proximate variables has several strong limitations. For example, herbivore population can be a poor predictor of feeding damage (Lu et al., 2015), while herbivore weight gain can be inversely related to herbivore survival (Veyrat et al., 2016). Host plant preference have been widely used to assess plant resistance against herbivores, through the analysis of feeding/oviposition reduction (antixenosis - Stenberg and Muola, 2017). Unfortunately, this is a reliable analysis only for large herbivores, which can directly determine the formation of reproductive structures and plant survival (Huber et al., 2016; Machado et al., 2016; Erb, 2018). For small herbivores such as arthropods, antixenosis as a proximate variable for the study of plant defenses can have limitations, mostly related to the different preferences of adults and juveniles (Clark et al., 2011), difficult to mimic the field situation in lab conditions (Schuman et al., 2015) or the complexity of interaction between the plant and several other herbivores (Kessler and Baldwin, 2004). Leaf damage assessments are often used because they accurately mirror the severity of attack (Erb, 2018). Even though in some cases there is a clear relation between plant damage and yield penalty (Vichitbandha and Chandrapatya, 2011), the relationship between the two variables is not always linear, and many plants can sustain herbivore damage without suffering significant yield penalties through tolerance (Scholes and Paige, 2014; Lehndal and Ågren, 2015; Erb, 2018). We have seen such behaviour in our lab with a rice cultivar infested by *Schizotetranychus oryzae*, in which mite population and leaf damage increased consistently throughout the vegetative and reproductive stages, resulting in no grain yield reduction, while other cultivars showed reductions of more than 60% (unpublished data). Furthermore, recording the exact extent of plant damage caused by most of the herbivores remains challenging and somewhat subjective, due to the need of a visual damage scale, unless quantified in standardized units of leaf area consumed relative to herbivore size (Fragoso et al., 2014) or using image softwares.

IT'S TIME TO LOOK AT PLANT TOLERANCE

Another point that favors the more widely exploitation of tolerance mechanisms in crop protection strategies is the fact that antibiosis and antixenosis typically deter herbivore feeding, likely imposing a strong selection pressure on the herbivore to overcome plant resistance (similar to what happen with pesticides). On the other hand, plant tolerance have no effect on herbivore biology or behavior, and therefore is unlikely to impose selection on the herbivore (Mitchell et al., 2016). Thus, plant tolerance is considered a more stable management strategy for pests (Weis and Franks, 2006; Peterson et al., 2017), with greater chance of providing long-lasting pest control (Mitchell et al., 2016). Therefore, based on all these points, we suggest the analysis of plant tolerance mechanisms and yield/productivity in every crop-mite interaction (which could probably be extended to most of the herbivorous pests). In fact, this is what really matters to crop farmers, and academic studies should be aligned with these needs.

It is important to highlight that we are not suggesting the replacement of crop resistance studies by crop tolerance ones.

TABLE 1 | Studies on crop-mite interactions since 2009.

Crop	Mite species	Analyzed trait	Yield analysis	References
Rice	*Steneotarsonemus spinki*	Mite population, leaf sheath and grain damage, and yield loss	X	Karmakar, 2009
Maize	*Tetranychus urticae* and *Brevipalpus chilensis*	Mite population	–	Carrillo et al., 2011
Tomato	*Tetranychus evansi*	Mite survival, development and oviposition rates	–	Onyambus et al., 2011
Chili	*Polyphagotarsonemus latus*	Shoot damage and yield loss	X	Vichitbandha and Chandrapatya, 2011
Tomato	*Tetranychus urticae* and *Tetranychus evansi*	Mite survival, development and oviposition rates	–	Bleeker et al., 2012
Strawberry	*Tetranychus urticae*	Mite population and yield loss	X	Nyoike and Liburd, 2013
Tomato	*Tetranychus urticae*	Leaf damage and mite population	–	Salinas et al., 2013
Maize and tomato	*Tetranychus urticae*	Mite population	–	Szczepaniec et al., 2013
Grapevine	*Colomerus vitis*	Leaf damage and mite population	–	Khederi et al., 2014
Wheat	*Aceria tosichella*	Leaf damage and mite population	–	Richardson et al., 2014
Common bean	*Tetranychus urticae*	Leaf damage, mite population and oviposition rate	–	Tahmasebi et al., 2014
Citrus	*Tetranychus urticae*	Mite oviposition rate	–	Agut et al., 2015
Tomato	*Tetranychus urticae*	Traveled distance on the leaf surface	–	Baier et al., 2015
Tomato	*Tetranychus urticae*	Mite population and oviposition rate	–	Pappas et al., 2015
Apple	*Tetranychus urticae*	Mite population and yield loss	X	Warabieda, 2015
Citrus	*Tetranychus urticae*	Mite oviposition rate	–	Agut et al., 2016
Cherry tomato	*Tetranychus urticae*	Traveled distance on the leaf surface	–	Lucini et al., 2016
Wheat	*Aceria toschiella*	Leaf damage and mite population	–	Aguirre-Rojas et al., 2017
Wheat	*Aceria toschiella*	Leaf damage and mite population	–	Chuang et al., 2017
Rice	*Schizotetranychus oryzae*	Mite survival, development and oviposition rates	–	Gonçalves et al., 2017
Cassava	*Tetranychus urticae*	Leaf damage	–	Liang et al., 2017
Cassava	*Tetranychus cinnabarinus*	Mite survival, development and oviposition rates	–	Lu et al., 2017
Mini tomato	*Tetranychus urticae*	Traveled distance on the leaf surface	–	Maciel et al., 2017
Common bean	*Tetranychus kanzawai*	Leaf damage and mite oviposition rate	–	Ozawa et al., 2017
Maize	*Tetranychus urticae*	Mite survival and development rates	–	Paulo et al., 2017

We believe that both defense strategies should be extensively analyzed. We agree with the statement of Peterson et al. (2017): "Before substantial work on tolerance development can occur, we must conduct basic research on the physiological and biochemical mechanisms of tolerance. This must involve interdisciplinary research between plant scientists and entomologists." On the other hand, in a couple of years we would like to say: no, plant tolerance is not "the forgotten child of plant resistance." We believe that basic understanding of how plants cope with herbivory and the identification of tolerant genotypes from important crops will have a major impact in pest control and grain yield.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Unraveling Rice Tolerance Mechanisms Against *Schizotetranychus oryzae* Mite Infestation

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Rice is the staple food for over half of the world's population. Infestation of *Schizotetranychus oryzae* (Acari: Tetranychidae) causes great losses in rice productivity. To search for rice genotypes that could better tolerate *S. oryzae* infestation, we evaluated morphological and production parameters in Brazilian cultivars, and identified two cultivars with contrasting responses. Leaf damage during infestation was similar for all cultivars. However, infestation in Puitá INTA-CL resulted in reduction in the number of seeds per plant, percentage of full seeds, weight of 1,000 seeds, and seed length, whereas infestation in IRGA 423 increased weight of 1,000 seeds and seed length. Reduction in seed weight per plant caused by infestation was clearly higher in Puitá INTA-CL (62%) compared to IRGA 423 (no reduction detected), thus Puitá INTA-CL was established as susceptible, and IRGA 423 as tolerant to *S. oryzae* infestation. Photosynthetic parameters were less affected by infestation in IRGA 423 than in Puitá INTA-CL, evidencing higher efficiency of energy absorption and use. *S. oryzae* infestation also caused accumulation of H_2O_2, decreased cell membrane integrity (indicative of cell death), and accelerated senescence in leaves of Puitá INTA-CL, while leaves of IRGA 423 presented higher levels of total phenolics compounds. We performed proteomics analysis of Puitá INTA-CL and IRGA 423 leaves after 7 days of infestation, and identified 60 differentially abundant proteins (28 more abundant in leaves of Puitá INTA-CL and 32 in IRGA 423). Proteins related to plant defense, such as jasmonate synthesis, and related to other mechanisms of tolerance such as oxidative stress, photosynthesis, and DNA structure maintenance, together with energy production and general metabolic processes, were more abundant in IRGA 423. We also detected higher levels of silicon (as amorphous silica cells) in leaves of infested IRGA 423 plants compared to Puitá INTA-CL, an element previously linked to plant defense, indicating that it could be involved in

tolerance mechanisms. Taken together, our data show that IRGA 423 presents tolerance to *S. oryzae* infestation, and that multiple mechanisms might be employed by this cultivar. These findings could be used in biotechnological approaches aiming to increase rice tolerance to mite infestation.

Keywords: phytophagous mite, rice infestation, *Schizotetranychus oryzae*, proteomics, silicon, tolerance

INTRODUCTION

Rice is one of the most important sources for global food security and socioeconomic stability (FAO, 2017). Research directed to this crop are important for the development of technologies that increase productivity and assist farmers who depend on it for subsistence, as is the case in several developing countries such as Brazil (Zeigler and Barclay, 2008), which is the ninth largest rice producer and the main producer outside Asia (FAO, 2017). In the last years, Brazil produced around 10 million tons of rice, with Rio Grande do Sul (RS) state accounting for approximately 70% of this amount. However, monoculture and intensive use of fertilizers benefit the appearance of pest arthropods, which are the main competitors of humans for the resources generated by agriculture (Oerke and Dehne, 2004).

Interactions between plants and herbivores are important determinants of plant productivity in managed and natural vegetation. In response to attack, plants have evolved a range of defenses to reduce the threat of injury and seed set. Crop losses from damage caused by arthropod pests can exceed 15% annually (Mitchell et al., 2016). In order to quantify the pest resistance of the cultivars, the best tool does not seem to be the increase of the arthropod population, but the measurement of the damages caused to the plants, since the reduction of the leaf damage is normally followed by an increase in yield and quality of the grain, and these are the ultimate objectives of most crop breeding programs (Smith, 2005; Erb, 2018). Thus, the plant resistance/tolerance to arthropods is the sum of genetically inherited traits that result in an adapted species that suffers less damage compared to susceptible ones (Stenberg and Muola, 2017). These resistance/tolerance qualities should be measured on a relative scale by comparing levels of damage and productivity with susceptible plants that are severely damaged when exposed to similar experimental conditions (Smith, 2005; Sperotto et al., 2018b). Plant tolerance to arthropods has been indicated as a category of resistance. However, very little is known about the genetic mechanisms of tolerance to arthropods (Peterson et al., 2017).

Tolerance is distinctive in terms of the plant's ability to withstand or recover from herbivore injury through growth and compensatory physiological processes (Koch et al., 2016; Erb, 2018). Since plant tolerance involves compensatory behavior, the plant is able to bear a large number of herbivores without interfering with the pest's physiology or behavior (Mitchell et al., 2016; Peterson et al., 2017; Sperotto et al., 2018b). Some studies observed that tolerant plants can compensate photosynthetically by avoiding feedback inhibition and impaired electron flow through PSII that occurs as a result of arthropod feeding. Similarly, the up-regulation of peroxidases and other oxidative enzymes during pest feeding, together with elevated levels of phytohormones, can play an important role in plant tolerance to phytophagous pests (Koch et al., 2016). Tolerance is also currently believed to be caused by other general physiological mechanisms such as pre-existing high levels of carbon storage in roots and increased resource allocation from root to shoot after damage (Peterson et al., 2017).

Phytophagous mites (Acari) comprise a diverse group of arthropods with several species that are pests in crop plants (Blasi et al., 2015). Within this group, the spider mites of the Tetranychidae family are of special interest since they cover a broad host–plant range (Rioja et al., 2017; Blaazer et al., 2018) and can develop into devastating outbreaks (Blasi et al., 2015; Van Leeuwen et al., 2015). Adult spider mites feed from leaves by piercing mesophyll cells with their cheliceral stylets, and sucking the cell content (Villarroel et al., 2016). During feeding, stylets transverse the leaf epidermis either in between epidermal pavement cells or through stomatal openings (Rioja et al., 2017). Such feeding behavior can severely damage leaf tissues (Bensoussan et al., 2016; Rioja et al., 2017). To control such damage there is an indiscriminate use of acaricides. However, mites of the Tetranychidae family have been reported for developing resistance to various acaricides (Osakabe et al., 2016). Physical barriers, such as thick cuticle or wax depositions on the leaf surface (and also around stomatal openings) of some plant hosts impede mites' ability to penetrate their stylets and feed (Beard et al., 2012; Rioja et al., 2017). Although physical defense plays an important role in preventing mite attack, chemical defenses are recognized as crucial to the plant defense against phytophagous mites (Blasi et al., 2015). Their first chemical defense is to synthesize toxic metabolites (e.g., cyanogenic glycosides, glucosinolates, alkaloids, terpenoids, latex, proteinase inhibitors) with antinutritional, deterrent, repellent, and toxic properties that can reduce plant digestibility for a wide range of potential consumers, and interfere with the metabolism, development, and fecundity of phytophagous mites (Strauss and Zangerl, 2002; Wu and Baldwin, 2010; Mithöfer and Boland, 2012; War et al., 2012; Santamaria et al., 2013, 2018; Rioja et al., 2017; Blaazer et al., 2018). Following this first chemical defense, several proteins are expressed as part of the plant defenses. These molecules are described as anti-insect proteins which negatively affect development or population growth (Blasi et al., 2015). Additionally, several volatiles are produced to attract predators of the phytophagous mites (Blasi et al., 2015; Santamaria et al., 2018). Defense genes involved in the pathways of jasmonic acid (JA), salicylic acid (SA), and ethylene are responsible for the

Abbreviations: EI, early infestation; II, intermediate infestation; JA, jasmonate; LI, late infestation; PSII, photosystem II; SEM, scanning electron microscopy.

production of defense proteins (glucanases, chitinases, proteases, polyphenol oxidases, protease inhibitors) that can limit the damage of the attacked plant (Blasi et al., 2015; Rioja et al., 2017; Santamaria et al., 2018). Signaling components of the JA and SA pathways can interact with each other, but can also interact with signaling components of growth-regulating hormonal pathways (Pieterse et al., 2012; Blaazer et al., 2018; Sperotto et al., 2018a).

Among the mites from the Tetranychidae family found in rice crops that cause economic damage is *Schizotetranychus oryzae* Rossi de Simons, which has been reported in several South American countries, and generates damages in irrigated rice fields (Ferla et al., 2013). To date, there is little information about the damage and economic loss caused by *S. oryzae* infestation in rice crops, and the available information is usually related to visual effects of the plant. Recently, our group described differentially abundant proteins in rice leaves early infested (EI) (Buffon et al., 2016) and late-infested (LI) (Blasi et al., 2017) by *S. oryzae*, along with the physiological changes induced by such different mite populations. However, the response variability of distinct rice genotypes to *S. oryzae* infestation is unknown, and the molecular and physiological changes caused by infestation in resistant/tolerant and susceptible rice cultivars have not yet been elucidated. Even though *S. oryzae* being the phytophagous mite most commonly found in rice cultivation in the RS state (Ferla et al., 2013), field observations show that some rice cultivars present different levels of infestation, suggesting a possible resistance mechanism. Therefore, we evaluated different rice cultivars commonly cultivated in different regions of RS state aiming to identify different rice responses to *S. oryzae* infestation, in order to understand the molecular and physiological mechanisms behind resistance/tolerance and susceptibility to this mite. Our results may be useful for future breeding programs aiming at resistance/tolerance to phytophagous mite *S. oryzae* infestation.

MATERIALS AND METHODS

Plant Growth Conditions and Mite Infestation

Seeds of rice (*Oryza sativa* L. ssp. *indica*) from IRGA 426, BRS Atalanta, Puitá INTA-CL, IRGA 424, BRS 7 Taim, IRGA 410, and IRGA 423 cultivars were surface sterilized and germinated for 4 days in an incubator (28°C) on paper soaked with distilled water. After germination, plantlets were transferred to vermiculite/soil mixture (1:3) for additional 14 days in greenhouse conditions, and then transferred to plastic buckets containing soil and water. Plastic buckets containing rice plants highly infested by *S. oryzae* were kindly provided by Instituto Rio-Grandense do Arroz (IRGA, Cachoeirinha, RS, Brazil), and were used to infest rice plants in our experiment. Fifty plants (V7-9 stage, according to Counce et al., 2000) of each cultivar (five plants per bucket) were infested by proximity with the bucket containing the highly infested plants placed in the center of the other buckets. For greater homogeneity of infestation and contact, buckets of each cultivar were rotated at a 90° angle counterclockwise every 2 days. Fifty plants of each cultivar were cultivated without infestation (control condition).

The level of damage caused by *S. oryzae* was analyzed from V7-9 stage until the plants reach its final stage of reproductive development (panicle maturity, R9 stage; Counce et al., 2000). Evaluation of damage in the abaxial and adaxial faces of leaves was based on a classification of four levels of infestation: control condition, without any sign of infestation; early infested (EI) leaves, 10–20% of damaged leaf area, average of 168 h of exposure to the mite; intermediate infested (II) leaves, 40–50% of damaged leaf area, average of 360 h; and late infested (LI) leaves, >80% of damaged leaf area, average of 720 h, according to **Supplementary Figure S1**.

Plant Height and Tiller Number

Plant height and tiller number were evaluated on the seven previously mentioned cultivars during the vegetative stage (V7-9, before being infested) and during the last reproductive stage (R9, control and infested plants). Puitá INTA-CL, BRS 7 Taim, and IRGA 423 cultivars were selected for further analysis based on their different morphological responses to *S. oryzae* infestation.

Chlorophyll a Fluorescence Transients

The chlorophyll a fluorescence transient was measured on the third upper leaves of control and infested plants of the cultivars Puitá INTA-CL, BRS 7 Taim, and IRGA 423 in three different exposure times: EI, II, and LI, using a portable fluorometer (OS30p, Optisciences, United Kingdom). Before the measurements, plants were dark adapted for 20 min and the fluorescence intensity was measured by applying a saturating pulse of 3,000 μmol photons m^{-2} s^{-1} and the resulting fluorescence of the chlorophyll a measured from 0 to 1 s. The chlorophyll fluorescence intensity rises from a minimum level (the O-level), to a maximum level (the P-level) via two intermediate steps labeled J and I (Stirbet and Govindjee, 2011), also known as OJIP curve (Strasser et al., 2000). These data were used to calculate parameters of the JIP Test (Strasser et al., 2000; Tsimilli-Michael and Strasser, 2008), which are highly studied for *in vivo* investigation of intact photosynthetic apparatus (Jafarinia and Shariati, 2012). Puitá INTA-CL and IRGA 423 cultivars were selected for further analysis based on their different chlorophyll fluorescence responses to *S. oryzae* infestation.

Seed Analysis

Seeds from Puitá INTA-CL and IRGA 423 cultivars were collected in R9 stage and the following agronomical parameters were evaluated: number of seeds (empty + full) per plant, percentage of full seeds, weight of 1,000 full seeds, and seed length. Yield reduction caused by *S. oryzae* infestation was calculated using the following equation for each cultivar and each condition (control and infested): number of seeds (empty + full) per plant × percentage of full seeds × weight of one seed = seed weight per plant. The seed weight per plant of the infested condition was divided by the seed weight per plant of the control condition, showing an estimative of yield loss percentage in each cultivar caused by *S. oryzae* infestation.

In situ Histochemical Localization of H_2O_2 and Loss of Plasma Membrane Integrity

In situ accumulation of H_2O_2 in control and LI leaves of Puitá INTA-CL and IRGA 423 cultivars was detected by histochemical staining with diaminobenzidine (DAB), according to Shi et al. (2010), with minor modifications. For H_2O_2 localization, leaves were immersed in DAB solution (1 mg ml^{-1}, pH 3.8) in 10 mM phosphate buffer (pH 7.8), and incubated at room temperature for 8 h in the light until brown spots were visible, which are derived from the reaction of DAB with H_2O_2. Leaves were bleached in boiling concentrated ethanol to visualize the brown spots, and kept in 70% ethanol for photo documentation with a digital camera coupled to a stereomicroscope. To determine changes in cell viability (indicative of cell death), another set of control and LI leaves were immersed for 5 h in a 0.25% (w/v) aqueous solution of Evans Blue (Romero-Puertas et al., 2004). Leaves were discolored in boiling concentrated ethanol to develop the blue precipitates, which were photo documented with a digital camera coupled to a stereomicroscope.

Phenolic Compounds

Phenolic compounds were quantified according to Fett-Neto et al. (1992), with minor modifications. Approximately 50 mg of control and EI leaves from Puitá INTA-CL and IRGA 423 cultivars were pulverized in liquid nitrogen, extracted in 2 ml 0.1 N HCl, and submitted to sonication in a water bath for 30 min. The extracts were centrifuged at 9,000 rpm for 10 min at 4°C. The supernatant was collected and the pellet was re-extracted. The supernatants were pooled and the final volume was completed to 1.5 ml with 0.1 N HCl. For quantification, 300 µl of 20% (w/v) Na_2CO_3 and 150 µl of Folin–Ciocalteu reagent were added, mixed, and then incubated at 100°C for 1 min. Absorbance was read at 750 nm. The standard curve was established with gallic acid in 0.1 N HCl.

Microscope Observation of Amorphous Silica Cells and Silicon Quantification

Control and LI leaves from Puitá INTA-CL and IRGA423 cultivars were used in observation of amorphous silica cells and SiO_2 quantification. Morphology of silica cells on the leaf surfaces (abaxial and adaxial faces) was observed using SEM. A fresh specimen (0.3–0.5 cm in length) part of the reciprocal fourth leaf was sampled and wiped with tissue paper to remove moisture. The leaf segment was fixed and coated with metal and then loaded onto the SEM. Pictures (at 700× magnification) were obtained to illustrate the differences in amorphous silica cells of rice leaves.

Plant Protein Extraction and Quantification

Three biological samples (250 mg of fresh matter) of control and EI leaves from Puitá INTA-CL and IRGA 423 cultivars, each containing three leaves from three different plants, were subjected to protein extraction using Plant Total Protein Extraction Kit (Sigma-Aldrich). The protein concentration was measured using 2-D Quant Kit (GE Healthcare, Piscataway, NJ, United States).

Protein Digestion

For protein digestion, three biological replicates of 100 µg of proteins from Puitá INTA-CL and IRGA 423 leaves were used. Before the trypsin digestion step, protein samples were precipitated using the methanol/chloroform methodology to remove any detergent from samples (Nanjo et al., 2012). Then, samples were resuspended in Urea 7 M and Thiourea 2 M buffer, and desalted on Amicon Ultra-0.5 3 kDa centrifugal filters (Merck Millipore, Germany). Filters were filled to maximum capacity with buffers and centrifuged at 15,000 × *g* for 10 min at 20°C. The washes were performed twice with Urea 8 M and then twice with 50 mM ammonium bicarbonate (Sigma-Aldrich) pH 8.5, remaining approximately 50 µl per sample after the last wash. The methodology used for protein digestion was as previously described (Calderan-Rodrigues et al., 2014). For each sample, 25 µl of 0.2% (v/v) RapiGest® (Waters, Milford, CT, United States) was added, and samples were briefly vortexed and incubated in an Eppendorf Thermomixer® at 80°C for 15 min. Then, 2.5 µl of 100 mM DTT (GE Healthcare, Piscataway, NJ, United States) was added, and the tubes were vortexed and incubated at 60°C for 30 min under agitation. Next, 2.5 µl of 300 mM iodoacetamide (GE Healthcare, Piscataway, NJ, United States) was added, and the samples were vortexed and then incubated in the dark for 30 min at room temperature. The digestion was performed by adding 20 µl of trypsin solution (50 ng/µl; V5111, Promega, Madison, WI, United States) prepared in 50 mM ammonium bicarbonate, and samples were incubated at 37°C during 15 h. For RapiGest® precipitation and trypsin activity inhibition, 10 µl of 5% (v/v) trifluoroacetic acid (TFA, Sigma-Aldrich) was added and incubated at 37°C for 30 min, followed by a centrifugation step of 20 min at 16,000 × *g*. Samples were transferred to Total Recovery Vials (Waters, Milford, CT, United States).

Mass Spectrometry Analysis

A nanoAcquity UPLC connected to a Synapt G2-Si HDMS mass spectrometer (Waters, Manchester, United Kingdom) was used for ESI–LC–MS/MS analysis. First was performed a chromatography step by injecting 1 µl of digested samples (500 ng/µl) for normalization to relative quantification of proteins. To ensure standardized molar values for all conditions, normalization among samples was based on stoichiometric measurements of total ion counts of MS^E scouting runs prior to analyses using the ProteinLynx Global SERVER v. 3.0 program (PLGS; Waters, Milford, CT, United States). Runs consisted of three biological replicates. During separation, samples were loaded onto the nanoAcquity UPLC 5 µm C18 trap column (180 µm × 20 mm) at 5 µl/min during 3 min and then onto the nanoAcquity HSS T3 1.8 µm analytical reversed phase column (75 µm × 150 mm) at 400 nl/min, with a column temperature of 45°C. For peptide elution, a binary gradient was used, with mobile phase A consisting of water (Tedia, Fairfield, OH, United States) and 0.1% formic acid (Sigma-Aldrich), and mobile phase B consisting of acetonitrile (Sigma-Aldrich) and 0.1% formic acid. Gradient elution started at 7% B, then ramped from 7% B to 40% B up to 91.12 min, and from 40% B to 99.9% B

until 92.72 min, being maintained at 99.9% until 106.00 min, then decreasing to 7% B until 106.1 min and kept 7% B until the end of experiment at 120.00 min. Mass spectrometry was performed in positive and resolution mode (V mode), 35,000 FWHM, with ion mobility, and in data-independent acquisition (DIA) mode; ion mobility separation ($HDMS^E$) using IMS wave velocity of 600 m/s, and helium and IMS gas flow of 180 and 90 ml/min, respectively; the transfer collision energy ramped from 19 to 55 V in high-energy mode; cone and capillary voltages of 30 and 2750 V, respectively; and a source temperature of 70°C. In TOF parameters, the scan time was set to 0.5 s in continuum mode with a mass range of 50–2,000 Da. The human [Glu1]-fibrinopeptide B (Sigma-Aldrich) at 100 fmol/μl was used as an external calibrant and lock mass acquisition was performed every 30 s. Mass spectra acquisition was performed by MassLynx v4.0 software.

Bioinformatics Analysis

Spectra processing and database searching conditions were performed by Progenesis QI for Proteomics Software V.2.0 (Nonlinear Dynamics, Newcastle, United Kingdom). The analysis used the following parameters: Apex3D of 150 counts for low energy threshold, 50 counts for elevated energy threshold, and 750 counts for intensity threshold; one missed cleavage, minimum fragment ion per peptide equal to two, minimum fragment ion per protein equal to five, minimum peptide per protein equal to two, fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M) and phosphoryl (STY), and a default false discovery rate (FDR) value at a 1% maximum, peptide score greater than four, and maximum mass errors of 10 ppm. The analysis used the *O. sativa* protein databank from Phytozome[1]. Label-free relative quantitative analyses were performed based on the ratio of protein ion counts among contrasting samples. After data processing and to ensure the quality of results, only proteins present or absent (for unique proteins) in three out of three runs were accepted and submitted to differentially abundance analysis. Proteins were considered to be up-regulated if the fold change (FC) was greater than 1.5 and down-regulated if the FC was less than 0.6667, and both with significantly *P*-value ANOVA ($P < 0.05$). The Blast2GO tool[2] was used to identify proteins with known Gene Ontology (GO) annotations (Conesa et al., 2005). Detected proteins were also analyzed using the B2G Kegg maps (Ashburner et al., 2000).

RNA Extraction and cDNA Synthesis

Total RNA was extracted from control and EI rice leaves of Puitá INTA-CL and IRGA 423 cultivars using NucleoSpin RNA Plant (Macherey-Nagel). First-strand cDNA synthesis was performed using the SMART PCR cDNA Synthesis Kit (Clontech Laboratories, Mountain View, CA, United States) with reverse transcriptase (M-MLV, Invitrogen, Carlsbad, CA, United States) and 2 μg of RNA quantified using Qubit RNA Assay Kit (Invitrogen, Carlsbad, CA, United States) and Qubit 2.0 Fluorometer.

[1]https://phytozome.jgi.doe.gov/

[2]https://www.blast2go.com

Quantitative RT-PCR and Data Analysis

RT-qPCRs were carried out in a StepOne Real-Time Cycler (Applied Biosystems). All primers (listed in **Supplementary Table S1**) were designed to amplify 100–150 bp of the 3′-UTR of the genes (*2,3-bisphosphoglycerate-independent phosphoglycerate mutase*, *hexokinase*, and *glutathione reductase*) and to have similar Tm values (60 ± 2°C). Reaction settings were composed of an initial denaturation step of 5 min at 94°C, followed by 40 cycles of 10 s at 94°C, 15 s at 60°C, 15 s at 72°C, and 35 s at 60°C (fluorescence data collection); samples were held for 2 min at 40°C for annealing of the amplified products and then heated from 55 to 99°C with a ramp of 0.1°C/s to produce the denaturing curve of the amplified products. RT-qPCRs were carried out in 20 μl final volume composed of 10 μl of each reverse transcription sample diluted 100 times, 2 μl of 10× PCR buffer, 1.2 μl of 50 mM $MgCl_2$, 0.1 μl of 5 mM dNTPs, 0.4 μl of 10 μM primer pairs, 4.25 μl of water, 2.0 μl of SYBRGreen (1:10,000, Molecular Probe), and 0.05 μl of Platinum Taq DNA Polymerase (5 U/μl, Invitrogen, Carlsbad, CA, United States). Gene expression was evaluated using a modified $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008), which takes into account the PCR efficiencies of each primer pair (Relative expression TESTED GENE/CONTROL GENE = $(PCR_{eff}\ CG)^{CtCG}/(PCR_{eff}\ TG)^{CtTG}$). *OsUBQ5* gene expression was used as an internal control to normalize the relative expression of tested genes (Jain et al., 2006). Each data point corresponds to three biological and four technical replicate samples. The expression of a senescence marker gene (*Staygreen* gene, *OsSGR*, a chloroplast protein which regulates chlorophyll degradation by inducing LHCII disassembly through direct interaction; Park et al., 2007) was also analyzed in control and early/intermediate infested leaves.

Statistical Analysis

Data were analyzed using the Student's *t*-test ($P \leq 0.05$, 0.01, and 0.001) or One-Way ANOVA followed by Tukey's test, using SPSS Base 21.0 for Windows (SPSS Inc., United States).

RESULTS

Different Physiological Responses of Rice Cultivars to *S. oryzae* Infestation

The first screening of rice responses to *S. oryzae* infestation showed that all tested cultivars present similar pattern of infestation kinetics (**Supplementary Figure S2**). After 5 weeks, high infestation levels were detected in all cultivars. Therefore, none of the tested cultivars seems to be resistant to *S. oryzae* infestation. On the other hand, plant height and tiller number were differentially affected by mite infestation (**Figure 1**). *S. oryzae* affected the plant growth of IRGA 426, Puitá INTA-CL, and IRGA 410 cultivars (**Figures 1A,C,F**), and also the tillering in BRS Atalanta, Puitá INTA-CL, and IRGA 423 cultivars (**Figures 1B,C,G**). Even though we were not able to find any sign of resistance in these cultivars, we decided to further characterize the response to *S. oryzae* of three cultivars that showed different

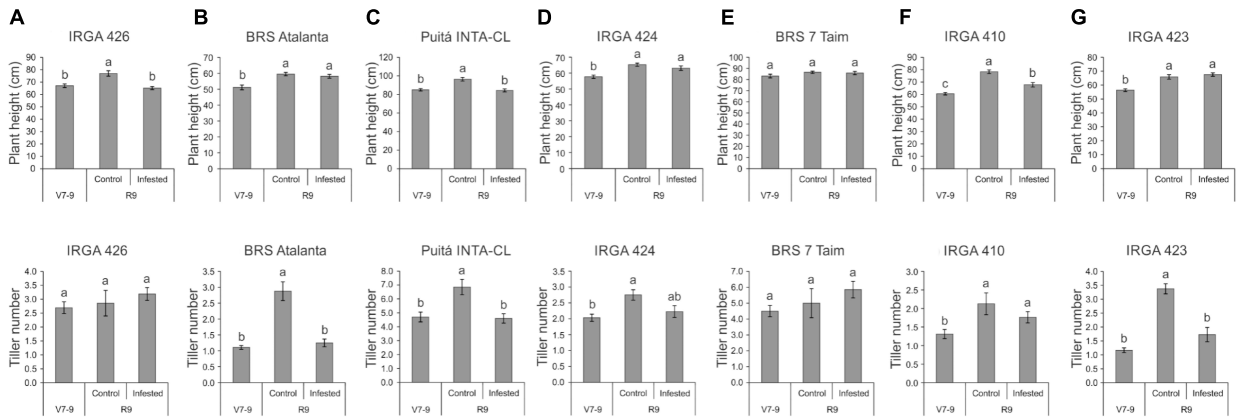

FIGURE 1 | Plant height (cm) and tiller number of the seven tested cultivars, **(A)** IRGA 426, **(B)** BRS Atalanta, **(C)** Puitá INTA-CL, **(D)** IRGA 424, **(E)** BRS 7 Taim, **(F)** IRGA 410, and **(G)** IRGA 423, at the vegetative stage (no infestation, V7-9) and full maturity stage (control or infested conditions, R9). Represented values are the averages of 50 samples ± SE. Different letters indicate that the means are different by the Tukey's HSD test ($P \leq 0.05$).

responses to mite infestation. Therefore, we selected Puitá INTA-CL, BRS7 Taim, and IRGA 423 cultivars to further analysis. Even though BRS Atalanta cultivar presented similar response of IRGA 423 (plant height not affected and tiller number deeply affected by infestation), we selected IRGA 423 for being commonly considered as more productive in southern Brazilian properties.

Chlorophyll a fluorescence analysis showed that EI and II conditions were not enough to change any parameter on the three tested cultivars (data not shown). On the other hand, several parameters were affected by LI condition. Puitá INTA-CL decreased the energy flow in PSII throughout the four OJIP curve-times when comparing control and LI plants, while both Puitá INTA-CL and BRS 7 Taim did not show any decrease in the same parameter (**Figures 2A–D**). On the other hand, IRGA 423 LI plants increased the net rate of reaction centers closure (M_0), reducing the energy needed to close all the reaction centers of the thylakoidal membrane (S_m), thus showing greater efficiency in energy use, while Puitá INTA-CL and BRS 7 Taim did not present any difference compared to control plants (**Figures 2E,F**). Also, Puitá INTA-CL LI plants reduced the fluorescence intensity at F300 (0.30 ms intensity, at the maximum point of fluorescence emission), where the plastoquinone is reduced and the reaction centers are closed, showing that this cultivar will have less energy to be used in the next phases of photosynthesis, whereas the other two cultivars present no changes in the same parameter (**Figure 2G**).

The energy absorption per reaction center (ABS/RC) is significantly increased only in infested leaves of IRGA 423 cultivar (**Figure 2H**), evidencing that these plants try to obtain more energy to tolerate the herbivory stress. The light energy capture per reaction center (TR_0/RC), which is converted in chemical energy during photosynthesis, is also increased only in infested leaves of IRGA 423 cultivar (**Figure 2I**). Puitá INTA-CL was the only cultivar that decreased light energy capture per active leaf area (TR_0/CS_0 – **Figure 2J**). The electron transport per active leaf area (ET_0/CS_0) decreased in infested leaves of IRGA 423 cultivar (**Figure 2K**), probably decreasing ATP production as a mean of favoring defense over energy production in this cultivar. Puitá INTA-CL and BRS 7 Taim presented increased energy dissipation per reaction center (DI_0/RC – **Figure 2L**), evidencing that these two cultivars are more affected by *S. oryzae* infestation than IRGA 423, due to less efficient energy use. Puitá INTA-CL also presented a decrease in energy dissipation per active leaf area (DI_0/CS_0 – **Figure 2M**). Based on these data, we concluded that Puitá INTA-CL and IRGA 423 show contrasting responses to *S. oryzae* infestation. Therefore, we selected these cultivars for further analysis.

The impaired chlorophyll fluorescence of Puitá INTA-CL suggests that *S. oryzae* infestation can promote an earlier senescence process on the leaves of this cultivar when compared to IRGA 423. Such hypothesis was confirmed by the higher expression of *OsSGR* gene (a senescence marker) in II leaves of Puitá INTA-CL (**Figure 3**).

Seeds from both cultivars were evaluated in order to verify whether *S. oryzae* infestation can decrease rice yield. As seen in **Figure 4**, seeds from Puitá INTA-CL cultivar were more affected by *S. oryzae* infestation than seeds from IRGA 423, showing a decrease in the number of seeds per plant (**Figures 4A,B**), percentage of full seeds (**Figure 4C**), weight of 1,000 full seeds (**Figure 4D**), and seed length (**Figures 4E,F**), resulting in approximately 62% reduction in seed weight per plant, which is an estimate of yield loss (**Figure 5**). On the other hand, seeds from IRGA 423 presented an increase in the weight of 1,000 full seeds (**Figure 4D**), explained by an increase in seed length (**Figures 4E,F**), resulting in no yield loss (**Figure 5**). Based on these data, we suggest that Puitá INTA-CL is susceptible to *S. oryzae* infestation, while IRGA 423 can be considered tolerant. From now on, we will call Puitá INTA-CL and IRGA 423 as "susceptible" and "tolerant" cultivars, respectively.

As seen in **Figure 6A**, leaves of the tolerant cultivar accumulate lower levels of H_2O_2 and less evidence of cell death (higher level of plasma membrane integrity) than the susceptible cultivar. Therefore, *S. oryzae* infestation differentially affect the generation of oxidative stress and the

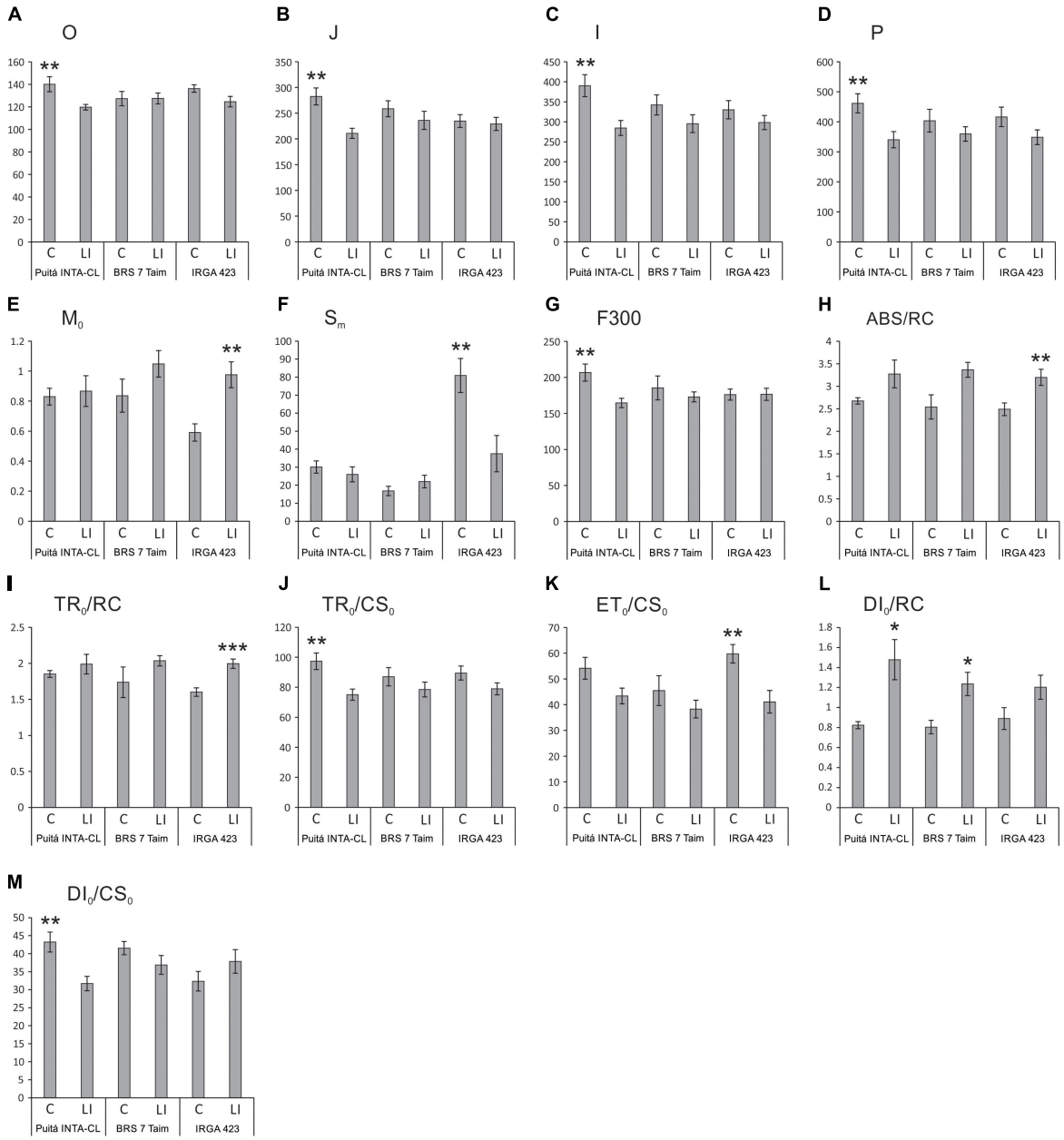

FIGURE 2 | OJIP-test parameters calculated from the chlorophyll a fluorescence transient in control (C) and late infested (LI) leaves of the three tested cultivars, (Puitá INTA-CL, BRS 7 Taim, and IRGA 423). **(A)** O, **(B)** J, **(C)** I, **(D)** P, **(E)** M_0, **(F)** S_m, **(G)** F300, **(H)** ABS/RC, **(I)** TR_0/RC, **(J)** TR_0/CS_0, **(K)** ET_0/CS_0, **(L)** DI_0/RC, and **(M)** DI_0/CS_0. Represented values are the averages of 10 samples ± SE. Mean values (from each cultivar) with one, two, or three asterisks are significantly different as determined by the Student's *t*-test ($P \leq 0.05$, 0.01, and 0.001, respectively).

cell death level on the LI leaves of susceptible and tolerant cultivars. Such low levels of oxidative stress on the leaves of tolerant cultivar could be explained, at least partially, by the higher level of phenolic compounds on the infested leaves of tolerant cultivar when compared to susceptible one (**Figure 6B**).

We used SEM to visualize the leaf surfaces of susceptible and tolerant plants during *S. oryzae* infestation. Under control condition, both cultivars presented similar levels of amorphous silica cells on the abaxial face and diminute amounts on the adaxial face (data not shown). However, under infested condition, adaxial face of tolerant IRGA 423 cultivar presents higher levels of amorphous silica cells than the susceptible Puitá INTA-CL (**Figure 7A**), while similar amounts were found on the abaxial face (data not shown). Also, adaxial surface of the tolerant cultivar accumulates more SiO_2 (major component of the amorphous silica cells) than the susceptible one (**Figure 7B**).

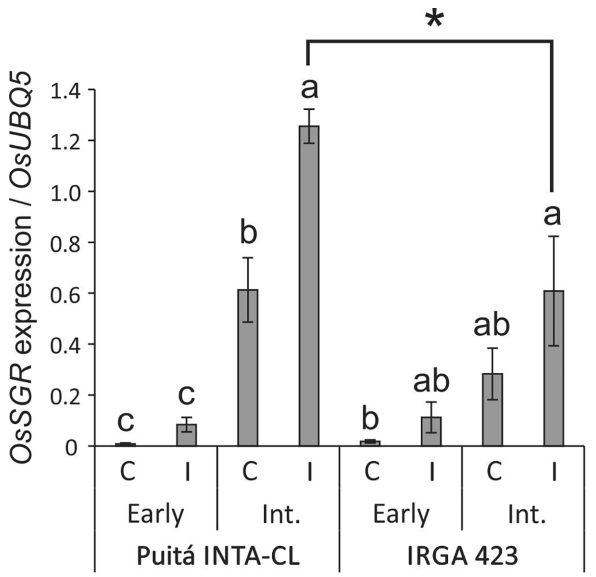

FIGURE 3 | *OsSGR* expression in control and early and intermediate infested leaves of the two tested cultivars (Puitá INTA-CL and IRGA 423). Represented values are the averages of three samples ± SE. Different letters indicate that the means (from each cultivar) are different by the Tukey's HSD test ($P \leq 0.05$). Mean values with one asterisk are significantly different as determined by the Student's *t*-test ($P \leq 0.05$).

Overview of Proteomic Analysis

A crucial step in plant defense is the early perception of stress in order to respond quickly and efficiently (Rejeb et al., 2014). A total of 728 proteins were identified comparing control and infested conditions in both cultivars, with 332 (45.6%) unique to or differentially abundant between cultivars. As seen in **Supplementary Figure S3**, comparing control and infestation leaves of susceptible cultivar, we detected 118 proteins, being 63 more abundant (and one unique) in control condition and 54 more abundant in infested condition. We identified 217 proteins in control and infestion conditions of tolerant cultivar, being 84 more abundant (and two uniques) in control condition and 82 more abundant (and one unique) in infested condition. When we compared both cultivars in control condition, we identified 137 proteins, with 97 more abundant (and one unique) in susceptible cultivar and 96 more abundant in tolerant one. Comparison of both cultivars in infested condition generated 60 proteins, with 28 more abundant in susceptible cultivar and 32 more abundant in tolerant one.

The corresponding sequence of each identified protein was compared to NCBI using BLASTp to identify specific domains, molecular functions, and protein annotations. Afterward, proteins were categorized in functional categories, according to its putative molecular function. The lists of all unique or differentially abundant proteins identified in this work are presented in **Supplementary Tables S2–S5**.

Several metabolic processes seem to be inhibited by *S. oryzae* infestation on the susceptible Puitá INTA-CL cultivar, including translation, carbohydrate metabolism and energy production (especifically glycolysis), photosynthesis, and response to stress. On the other hand, the higher abundance of oxidative stress- and ATP synthesis-related proteins in infected leaves suggest an attempt to respond to *S. oryzae* infestation (**Supplementary Table S2**). On the tolerant IRGA 423, *S. oryzae* infestation seems to be less damaging and to generate a more complex defense response. Several proteins involved with protein modification and degradation, general metabolic processes, carbohydrate metabolism and energy production (especially galactose and polysaccharide metabolism), oxidative stress, response to stress, photosynthesis, amino acid metabolism, and DNA structure maintenance were identified as more abundant in infested than in control condition. Even though, some categories are still inhibited by infestation, as translation, transport, and lipid metabolism (**Supplementary Table S3**). Surprisingly, when we compare both cultivars in control condition, the susceptible Puitá INTA-CL seems to present all the metabolic processes more active than the tolerant IRGA 423 (**Supplementary Table S4**). However, when both cultivars are compared in infested conditions, the tolerant IRGA 423 presents higher expression of proteins related to carbohydrate metabolism/energy production and general metabolic processes than the susceptible Puitá INTA-CL, which in turn, shows increased expression of proteins related to translation and transport. Also, under infested condition, the susceptible cultivar seems to prioritize growth over defense, due to the higher expression of a gibberelin (GA) receptor, while the tolerant one seems to prioritize defense over growth, due to the higher expression of jasmonate *O*-methyltransferase (**Supplementary Table S5**), a key enzyme for jasmonate-regulated plant responses.

GO Enrichment and KEGG Pathways

Gene Ontology analysis provided an overview of rice molecular response to *S. oryzae* infestation in susceptible and tolerant plants. The GO annotations of all 332 differentially abundant and unique proteins identified are shown in **Supplementary Figures S4**, **S5**. As expected, several biological processes are regulated when control and infested conditions are compared in both cultivars (control condition: Puitá INTA-CL × IRGA 423; infested condition: Puitá INTA-CL × IRGA 423), with a higher number of regulated biological processes during infestation. Two biological processes (cellular component organization and regulation of cellular processes) are more regulated on the tolerant cultivar (IRGA 423: control × infested) when compared to the susceptible one, and could be related to a more efficient plant defense (**Supplementary Figure S4**). The molecular function of structural constituent of ribosome is only regulated on the susceptible cultivar (Puitá INTA-CL: control × infested), and the protein binding is only regulated on the tolerant one (IRGA 423: control × infested) (**Supplementary Figure S5**).

To identify specific pathways affected by *S. oryzae* infestation in susceptible and tolerant rice plants, we also analyzed KEGG pathways. The following KEGG pathways (involving five or more proteins) were identified as associated with proteins differentially abundant only on the tolerant IRGA 423 cultivar (control × infested conditions): pyruvate metabolism (11), glyoxylate and dicarboxylate metabolism (8), amino sugar and

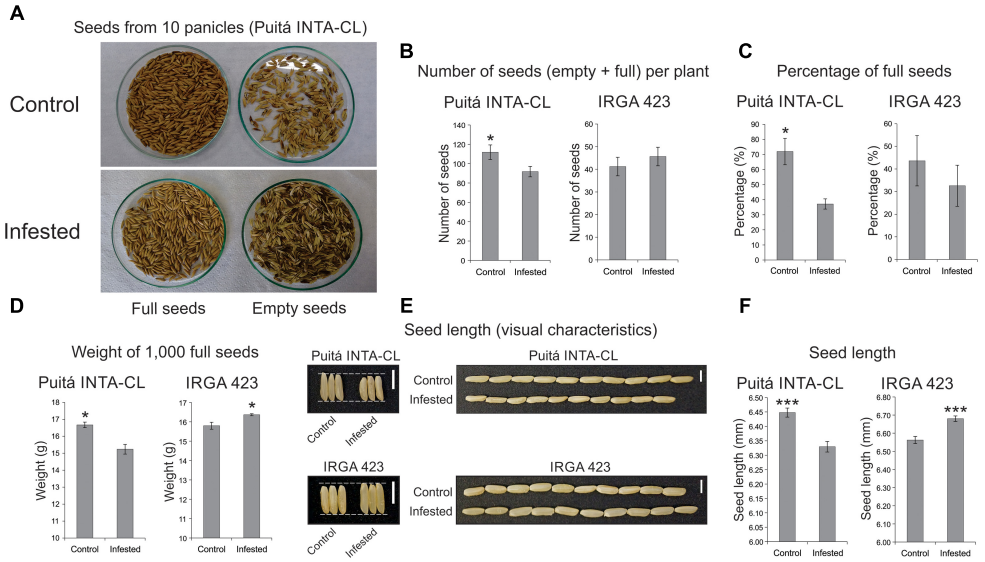

FIGURE 4 | Seeds analysis of the two tested cultivars (Puitá INTA-CL and IRGA 423). **(A,B)** Number of seeds (empty + full) per plant. **(C)** Percentage of full seeds. **(D)** Weight of 1,000 full seeds. **(E)** Seed length (visual characteristics). **(F)** Seed length (mm). Represented values are the averages of 50 samples ± SE. Mean values with one or three asterisks are significantly different as determined by the Student's *t*-test ($P \leq 0.05$ and 0.001). Bars in panel **(E)** indicate 0.5 cm.

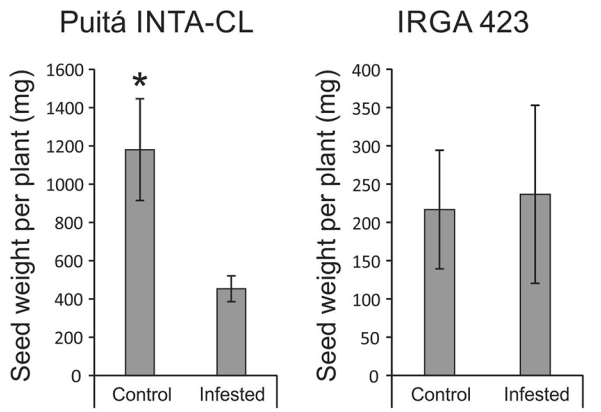

FIGURE 5 | Seed weight per plant (estimative of yield) of the two tested cultivars (Puitá INTA-CL and IRGA 423). Represented values are the averages of 10 samples ± SE. Mean values with one asterisk are significantly different as determined by the Student's *t*-test ($P \leq 0.05$).

nucleotide sugar metabolism (8), and methane metabolism (5). On the other hand, the following KEGG pathways (involving five or more proteins) were identified as associated with proteins differentially abundant in control condition (susceptible Puitá INTA-CL × tolerant IRGA 423): Glyoxylate and dicarboxylate metabolism (8), citrate cycle (TCA cycle) (5), starch and sucrose metabolism (5), carbon fixation in photosynthetic organisms (5), amino sugar and nucleotide sugar metabolism (5), and frutose and mannose metabolism (5). The only pathway identified as associated with proteins differentially abundant in infested condition (susceptible Puitá INTA-CL × tolerant IRGA 423) is gycolysis/gluconegenesis (5), suggesting a complete different pattern of energy use employed by the cultivars in both tested conditions.

Validation of Proteomic Data

The mRNA expression of three randomly selected genes (*2,3-bisphosphoglycerate-independent phosphoglycerate mutase*, *hexokinase*, and *glutathione reductase*) was evaluated in control and EI leaves (**Supplementary Figure S6**). The proteomic profiles were confirmed for the three tested genes, even though the ratio between conditions detected at the mRNA and protein levels was different, probably due to regulation at the post-transcriptional level.

DISCUSSION

In our first screening of rice responses to *S. oryzae* infestation using seven different cultivars, it was clearly shown that none of these cultivars present a classical resistance response, due to the rapid and somewhat similar infestation kinetics throughout the analyzed period (**Supplementary Figure S2**). Even though, physiological analysis and agronomical parameters showed that two cultivars (Puitá INTA-CL and IRGA 423) present different responses to *S. oryzae* infestation (**Figures 1–7**), being considered susceptible and tolerant, respectively. In fact, there is no consensus about the requirement for a trait be considered as a plant defense mechanism (Karban, 2011; Poelman, 2015), and most plant defenses are still characterized by proximate variables such as herbivore performance or plant damage (Wetzel et al., 2016; Erb, 2018). However, plant defenses can be surely defined as traits that reduce the negative impact of herbivores on plant reproductive success or that increase plant fitness (Erb, 2018). Tolerance mechanisms allow the plants to withstand pest injury

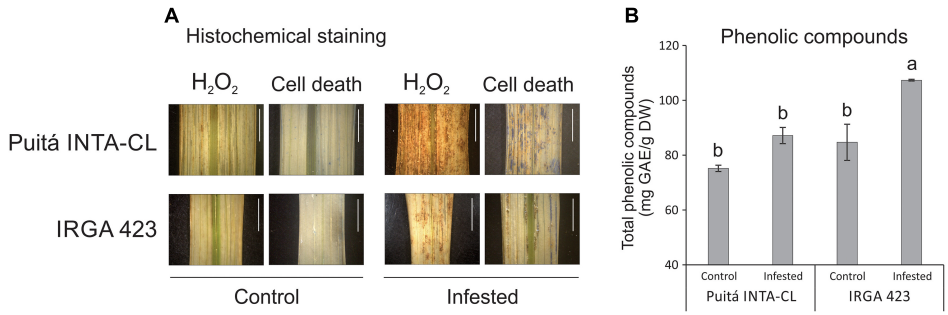

FIGURE 6 | Histochemical staining assay **(A)** of H_2O_2 and loss of plasma membrane integrity (indicative of cell death), by DAB and Evans Blue, respectively, in control and LI leaves of the two tested cultivars (Puitá INTA-CL and IRGA 423). The positive staining (detected in higher levels on infested leaves) in the photomicrographs shows as bright images (brown-color for DAB and blue-color for Evans Blue). Bars in figures indicate 0.5 cm. Total phenolic compounds **(B)** of control and EI leaves of the two tested cultivars (Puitá INTA-CL and IRGA 423). Represented values are the averages of three samples ± SE. Different letters indicate that the means are different by the Tukey's HSD test ($P \leq 0.05$). GAE, gallic acid equivalents; DW, dry weight.

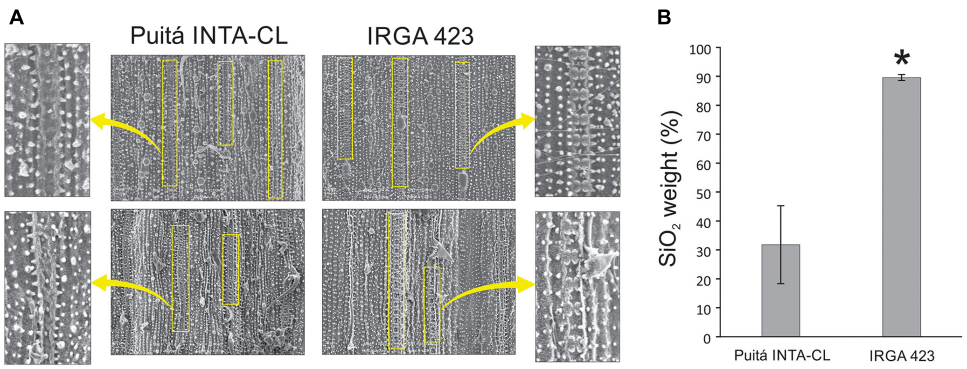

FIGURE 7 | Microscope observation of amorphous silica cells and SiO_2 quantification. **(A)** SEM of the infested leaf surfaces (adaxial face) from susceptible Puitá INTA-CL and tolerant IRGA 423 cultivars, highlighting the amorphous silica cells. **(B)** Quantification of SiO_2 using SEM. Figures in **(A)** are representatives of 10 analyzed leaf surfaces from each cultivar. Represented values in **(B)** are the averages of 10 samples ± SE. Mean values with one asterisk are significantly different as determined by the Student's *t*-test ($P \leq 0.05$). DW, dry weight.

and produce acceptable yields, maintaining the fitness under stressful conditions (Peterson et al., 2017; Sperotto et al., 2018b). For this reason, the fact that IRGA 423 did not decrease the yield under infested condition (compared to 62% in Puitá INTA-CL) was the main characteristic that encouraged us to define IRGA 423 as tolerant to *S. oryzae* infestation. It is important to highlight that under control condition (without infestation), the tolerant IRGA 423 cultivar presented a much lower yield than the susceptible Puitá INTA-CL (**Figure 5**). Although genetic immunity provides an economical method for the control of crop diseases, high levels of resistance/tolerance usually carry yield penalties (Brown, 2002). Reported in several crops, our understanding of the "cost of resistance/tolerance" on yield has improved in recent years, and novel breeding strategies to rapidly and efficiently select highly resistant/tolerant cultivars without yield penalties are being developed (Ning et al., 2017).

Chlorophyll fluorescence is a non-invasive tool commonly used for determining the behavior of the photosynthetic apparatus of control and abiotic stressed plants (Gururani et al., 2015; Rapacz et al., 2015). Our group previously detected a reduction in Pi_{ABS}, S_m, and N parameters (related to the donor and acceptor sides of PSII) in rice leaves EI by *S. oryzae* in IRGA 424 cultivar (Buffon et al., 2016). However, to the best of our knowledge, this is the first work that uses this type of photosynthetic analysis to differentiate plant susceptibility and tolerance to a biotic stress. Several parameters related to chlorophyll a fluorescence were affected in the susceptible Puitá INTA-CL cultivar during infestation, showing a worse photosynthetic performance than IRGA 423 (**Figure 2**). Puitá INTA-CL increased its energy dissipation per reaction center (DI_0/RC – **Figure 2I**) and decreased energy flow in PSII at the four OJIP-curve/times (**Figures 2A–D**), fluorescence intensity at F300 (**Figure 2G**), energy dissipation per active leaf area (DI_0/CS_0 – **Figure 2M**), and light energy capture per active leaf area (TR_0/CS_0 – **Figure 2J**), the last one probably linked to enhanced cell death in their leaves (**Figure 6A**). Zhang et al. (2013) demonstrated that excess Ca^{2+} increased the toxicity of Hg^{2+} to PSII of cyanobacterium *Synechocystis* sp. through the increase of energy flux dissipation per reaction center (DI_0/RC), leading to dysfunction of PSII. Rapacz et al. (2015) showed that DI_0/RC parameter increases with increasing levels of PSII damage in wheat under low temperature. The I and P

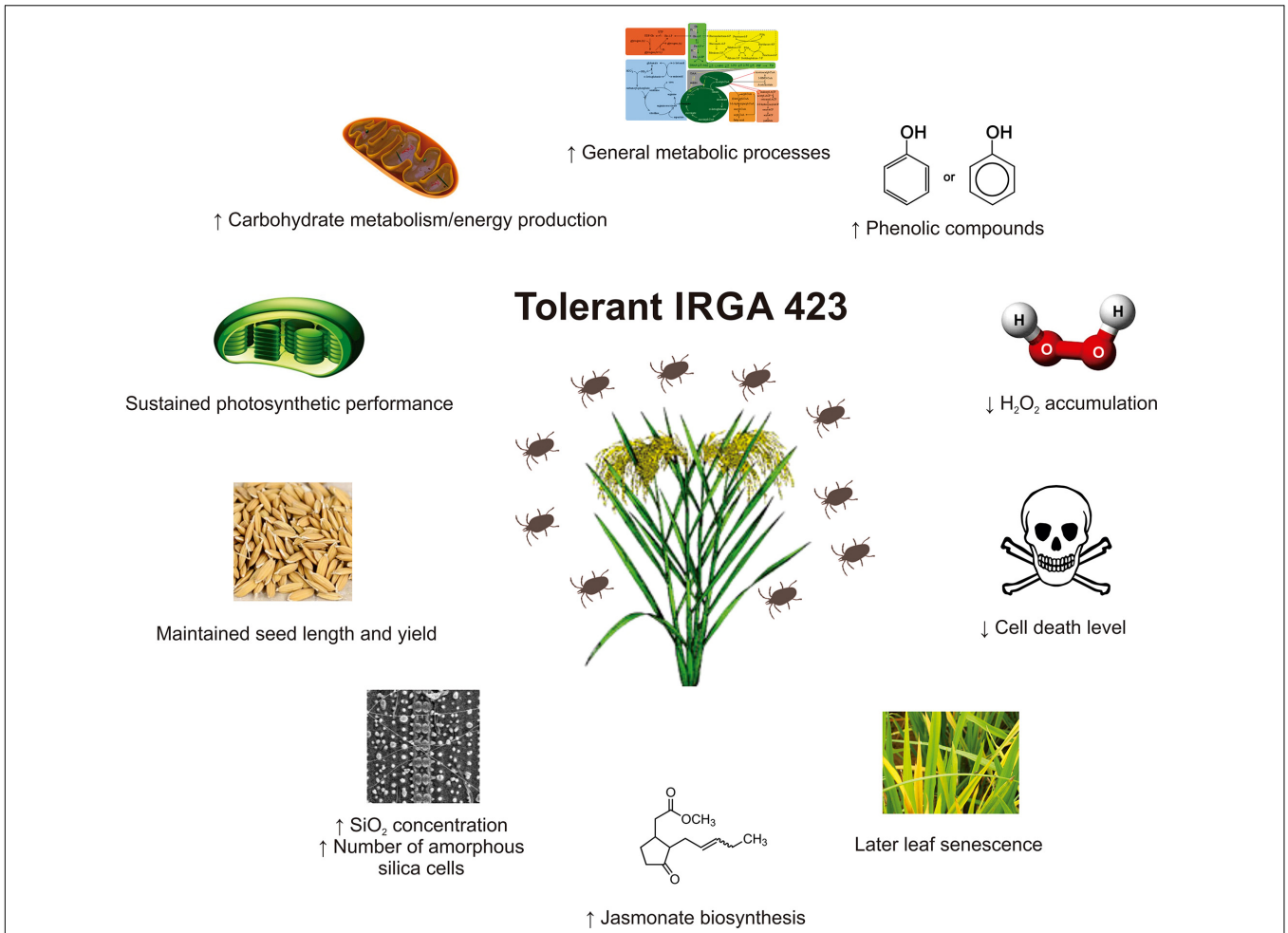

FIGURE 8 | Rice mechanisms employed by IRGA 423 cultivar to tolerate *Schizotetranychus oryzae* infestation.

fluorescence intensities on the OJIP induction curve and the F300 parameter also decrease in wheat plants exposed to Pb stress (Kalaji and Loboda, 2007). Interestingly, tall fescue *Festuca arundinacea* leaves show a great decrease at all steps of OJIP and F300 parameters in response to high-temperature stress, but pre-acclimation treatment inhibit such declines (Hu et al., 2015). Intriguingly, Rapacz et al. (2015) suggest that DI_0/CS_0 values increase with increasing levels of PSII damage, which is the opposite to what we found (**Figure 2M**). More studies are needed to clarify the impact of DI_0/CS_0 in rice photosynthetic performance. In common wheat, TR_0/CS_0 values correlate well with plant survival after freezing, being an excellent indicator for prediction of winter field survival or estimation of freezing tolerance (Rapacz et al., 2015). According to Gururani et al. (2015) and Rapacz et al. (2015), the OJIP test is a reliable indicator of cold tolerance in the turfgrass *Zoysia japonica* and freezing tolerance in wheat, respectively. Therefore, we suggest for the first time that rice tolerance to *S. oryzae* (and probably to other herbivores) can also be estimated by OJIP test. According to Peterson et al. (2017), increased net photosynthetic rate after herbivory is one of the general physiological mechanisms involved in plant tolerance. The differences in photosynthetic performance presented by the susceptible Puitá INTA-CL and tolerant IRGA 423 cultivars are supported by decreased and increased numbers of photosynthesis-related proteins detected in response to *S. oryzae* infestation, respectively (**Supplementary Tables S2**, **S3**).

As a result of impaired chlorophyll a fluorescence in infested leaves of Puitá INTA-CL, an earlier senescence process was established in their leaves upon *S. oryzae* infestation (**Figure 3**). Leaf senescence is a natural and important developmental process, responsible for great part of the nitrogen mobilized to the seeds. Late senescence, which means a prolonged and maximum period of photosynthetic activity, should lead to higher yields (Jagadish et al., 2015; Diaz-Mendoza et al., 2016). However, senescence processes are also closely linked to stress conditions, which commonly anticipate this process (Wojciechowska et al., 2017). Therefore, manipulation of senescence events can be a rationale way to obtain higher yield and quality of grains (Egli, 2011; Jagadish et al., 2015). We believe that the late senescence process detected on the leaves of tolerant IRGA 423 cultivar is at least partially responsible for the better seed characteristics

presented by this cultivar under *S. oryzae* infestation (**Figure 4**), including the absence of yield loss. Yet, infested leaves of susceptible Puitá INTA-CL cultivar express atATG18b protein (**Supplementary Table S5**), which is required for the formation of autophagosomes during nutrient stress and senescence in Arabidopsis (Xiong et al., 2005).

Even though we detected a lower level of H_2O_2 accumulation in infested leaves of the tolerant IRGA 423 cultivar (**Figure 6A**), we were not able to find a clear difference in oxidative stress-related proteins identified on the both cultivars under infested condition (**Supplementary Tables S2**, **S3**, **S5**), except a peroxidase protein 2.6-fold more abundant in tolerant IRGA 423 infested leaves (**Supplmentary Table 5**). However, infested leaves of the tolerant cultivar accumulate higher levels of phenolic compounds than the susceptible one (**Figure 6B**). In plants, it is well established that phenolics can act as antioxidants by donating electrons to guaiacol-type peroxidases for the detoxification of H_2O_2 produced under different stress conditions, including biotic ones (Sakihama et al., 2002; Shalaby and Horwitz, 2015; Hung, 2016). Also, many structurally different phenolics rapidly accumulate to higher levels as components of an induced defense arsenal against herbivore attack (Gaquerel et al., 2014; Karabourniotis et al., 2014). For example, the larval development of the pea aphid is longer, the reproduction period is shorter, the fecundity is decreased, and the aphid population is reduced on alfalfa lines containing high levels of phenolics (Goławska and Łukasik, 2009). Therefore, we believe that part of the tolerance mechanism to *S. oryzae* infestation by IRGA 423 cultivar is due to phenolics accumulation in their leaves. Interestingly, the accumulation of phenolic compounds along with enhancement of phenylpropanoid metabolism has been observed under different environmental stress conditions (Michalak, 2006). Phenylpropanoid metabolic pathway synthesizes flavonoids, which have many diverse functions, including plant responses to stress conditions (as cold and drought – Schulz et al., 2016; Shojaie et al., 2016) and defense (Buer et al., 2010), functioning as powerful antioxidants. Under infested condition, we detected flavonol-3-*O*-glycoside-7-*O*-glucosyltransferase 1 protein, involved with flavonol biosynthesis (Sun et al., 2016), 3.6-fold more abundant on the tolerant IRGA 423 cultivar than the susceptible one (**Supplementary Table S5**), suggesting that flavonoids can also contribute to tolerate *S. oryzae* infestation.

The beneficial effects of silicon (Si) on plant resistance against biotic stresses, including insect herbivory, have been well documented in rice plants, showing positive correlations between increased Si content and enhanced insect resistance (Sidhu et al., 2013; Ye et al., 2013; Han et al., 2016). We detected higher number of amorphous silica cells and higher accumulation of SiO_2 in infested leaves of the tolerant IRGA 423 cultivar when compared to the susceptible one (**Figure 7**). Based on these data, we strongly suggest that enhanced Si levels can also contribute to the more effective defense of IRGA 423 cultivar against *S. oryzae* mite infestation. According to Ye et al. (2013), there is a strong interaction between Si and JA in rice defense against insect herbivores involving priming of JA-mediated defense responses by Si and the promotion of Si accumulation by JA. This is reinforced by the higher expression of jasmonate *O*-methyltransferase protein on the infested leaves of tolerant IRGA 423 cultivar (**Supplementary Table S5**). Such enzyme catalyzes the methylation of JA into Me-JA, which controls plant defenses against herbivore attack (Qi et al., 2016; Huang et al., 2017). On the other hand, we detected higher expression of a GA receptor protein on the infested leaves of susceptible Puitá INTA-CL cultivar (**Supplementary Table S5**). GA regulates many essential plant developmental processes, including growth (Hou et al., 2013). As GA and JA antagonize each other in regulating plant growth and defense (Chaiwanon et al., 2016; Ning et al., 2017), we suggest that under infested condition, the susceptible Puitá INTA-CL cultivar prioritize growth over defense (showing a significant yield penalty), while the tolerant IRGA 423 prioritize defense over growth (showing no yield penalty), and this difference might contribute to the *S. oryzae* susceptiblity or tolerance, as hypothetically occurs with wild *Oryza* species of different heights (Sperotto et al., 2018a).

Physiological analyses showed that the tolerant IRGA 423 cultivar under infested condition presents better photosynthetic performance, later leaf senescence period, less affected seeds and lower yield loss, lower levels of H_2O_2 accumulation and cell death, higher levels of phenolic compounds (and probably flavonoids), higher level of SiO_2 concentration in leaves, and higher number of amorphous silica cells on the leaf surface than the susceptible Puitá INTA-CL cultivar. Proteomic analysis showed that the tolerant IRGA 423 cultivar under infested condition presents a more complex and efficient response to *S. oryzae* infestation, with carbohydrate metabolism/energy production, general metabolic processes, and JA biosynthesis more active than the susceptible Puitá INTA-CL cultivar. The model in **Figure 8** summarizes the rice tolerance mechanisms employed by IRGA 423 cultivar.

CONCLUSION

This is the first report evaluating the defense responses of two contrasting rice cultivars to *S. oryzae* mite infestation. Even though we were not able to find obvious signs of plant resistance in any of the tested cultivars, infested condition did not affect the number of seeds, percentage of full seeds, weight of 1,000 full seeds, seed length, and ultimatelly seed weight per plant (estimate of yield) of IRGA 423 cultivar. Therefore, this cultivar can be characterized as tolerant to *S. oryzae* infestation. Altogether, our findings are helpful to reveal the different mechanisms involved in the rice response to *S. oryzae* infestation, and could be used in future breeding programs or genetic engineering attempts aiming to increase mite tolerance in rice plants.

AUTHOR CONTRIBUTIONS

JS, FR, and RS conceived and designed the research. GB, EB, AR, TL, RG, JA, and AH conducted the experiments. VS and ML contributed with analytical tools. GB, EB, JA, AH, VS, and RS analyzed the data. GB and RS wrote the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01341/full#supplementary-material

FIGURE S1 | Classification of infestation levels according to visual characteristics of leaves.

FIGURE S2 | Pattern of infestation kinetics based on leaf damage after 5 weeks in the seven tested cultivars.

FIGURE S3 | Venn diagram showing the overlap of rice proteins identified in control and early infested (EI) leaves of susceptible Puitá INTA-CL and tolerant IRGA 423 cultivars. **(a)** Puitá INTA-CL (control × infested); **(b)** IRGA 423 (control × infested); **(c)** control condition (Puitá INTA-CL × IRGA 423); **(d)** infested condition (Puitá INTA-CL × IRGA 423). In **(a)** and **(b)** dark green circles, control leaves; yellow circles, infested leaves. In **(c)** and **(d)** dark green circles, Puitá INTA-CL; yellow circles, IRGA 423. Light green means overlap in **(a)**–**(d)**.

FIGURE S4 | Gene ontology annotation. Biological processes of differentially abundant and unique proteins obtained in control and EI leaves from susceptible Puitá INTA-CL and tolerant IRGA 423 cultivars.

FIGURE S5 | Gene ontology annotation. Molecular functions of differentially abundant and unique proteins obtained in control and EI leaves from susceptible Puitá INTA-CL and tolerant IRGA 423 cultivars.

FIGURE S6 | Relative expression levels (RT-qPCR, relative to *OsUBQ5* expression) of three randomly selected genes **(a)** *2,3-bisphosphoglycerate-independent phosphoglycerate mutase*, **(b)** *hexokinase*, **(c)** *glutathione reductase*, for which the encoded proteins were identified by proteomics as differentially abundant between the control and EI leaves from Puitá INTA-CL and IRGA 423 cultivars. Represented values are the averages of three samples ± SE. Different letters indicate that the means are different by the Tukey's HSD test ($P \leq 0.05$).

TABLE S1 | Gene-specific PCR primers used for RT-qPCR analyses.

TABLE S2 | Differentially abundant proteins in susceptible Puitá INTA-CL cultivar (control × infested condition).

TABLE S3 | Differentially abundant proteins in tolerant IRGA 423 cultivar (control × infested condition).

TABLE S4 | Differentially abundant proteins in control condition (susceptible Puitá INTA-CL × tolerant IRGA 423).

TABLE S5 | Differentially abundant proteins in infested condition (susceptible Puitá INTA-CL × tolerant IRGA 423).

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Effect of Cadmium Accumulation on the Performance of Plants and of Herbivores That Cope Differently With Organic Defenses

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Some plants are able to accumulate in their shoots metals at levels that are toxic to most other organisms. This ability may serve as a defence against herbivores. Therefore, both metal-based and organic defences may affect herbivores. However, how metal accumulation affects the interaction between herbivores and organic plant defences remains overlooked. To fill this gap, we studied the interactions between tomato (*Solanum lycopersicum*), a model plant that accumulates cadmium, and two spider-mite species, *Tetranychus urticae* and *Tetranychus evansi* that, respectively, induce and suppress organic plant defences, measurable via the activity of trypsin inhibitors. We exposed plants to different concentrations of cadmium and measured its effects on mites and plants. In the plant, despite clear evidence for cadmium accumulation, we did not detect any cadmium effects on traits that reflect the general response of the plant, such as biomass, water content, and carbon/nitrogen ratio. Still, we found effects of cadmium upon the quantity of soluble sugars and on leaf reflectance, where it may indicate structural modifications in the cells. These changes in plant traits affected the performance of spider mites feeding on those plants. Indeed, the oviposition of both spider mite species was higher on plants exposed to low concentrations of cadmium than on control plants, but decreased at concentrations above 0.5 mM. Therefore, herbivores with contrasting responses to organic defences showed a similar hormetic response to metal accumulation by the plants. Additionally, we show that the induction and suppression of plant defences by these spider-mite species was not affected by the amount of cadmium supplied to the plants. Furthermore, the effect of cadmium on the performance of spider mites was not altered by infestation with *T. urticae* or *T. evansi*. Together, our results suggest no interaction between cadmium-based and organic plant defences, in our system. This may be useful for plants living in heterogeneous environments, as they may use one or the other defence mechanism, depending on their relative performance in each environment.

Keywords: metal accumulating plants, plant defence, tomato, spider mites, elemental defence hypothesis

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INTRODUCTION

Plants are exposed to an array of abiotic and biotic stresses. The mechanisms that allow them to survive these adversities imply physiological and structural transformations that can be costly to the plants, affecting negatively their growth and fitness (Boyer, 1982; Wang et al., 2003). One such stress is high bioavailable metal concentrations in soil, either naturally (geochemical anomalies) or due to anthropogenic activities. Although these high concentrations are toxic to most organisms, some plant species or populations, termed metallophytes, thrive in such environments. They achieve this either by limiting the metal uptake or the translocation to the shoot (excluders), or by storing metals in their shots (accumulators; Baker, 1987). However, these strategies entail costs that may be reflected in the plant performance, namely in plant biomass, in the water content of the shoots, and/or in the root to shoot ratio (Kastori et al., 1992; Das et al., 1997; Larbi et al., 2002; Chaffei et al., 2004; Devi et al., 2007). In addition, the stress caused by metal toxicity may lead to disturbances in the carbon and nitrogen metabolism, affecting the nutritional status of various plant parts (Larbi et al., 2002; Chaffei et al., 2004; Wahid et al., 2007), and potentially changing the accumulation of soluble sugars, either leading to increased (Devi et al., 2007; Rosa et al., 2009; Mishra et al., 2014) or decreased (Scheirs et al., 2006; Shackira and Puthur, 2017) sugar concentrations in the shoots. These physiological changes in the plant may also affect the performance of the herbivores feeding on those plants (White, 1984; Scheirs et al., 2006).

Besides being costly for the plant, accumulation of some metals is highly toxic to herbivores as well. Indeed, due to their elemental nature, they cannot be degraded by chemical counter-defenses of the herbivores (Boyd, 2004). Therefore, metal accumulation by the plants may be detrimental to herbivores (Martens and Boyd, 1994; Boyd and Moar, 1999; Behmer et al., 2005; Kazemi-Dinan et al., 2014) and this accumulation has thus been suggested to serve as a defense against herbivory (Boyd, 2004; Poschenrieder et al., 2006; Hörger et al., 2013). If metal accumulation does not compromise the production of organic defenses, the combination of both defense strategies may give accumulating plants an advantage over non-accumulating competitors (Boyd, 2007). It has been shown that metal exposure may directly increase the activity of some organic plant defenses, such as proteases (Pena et al., 2006; Lin et al., 2010), having possible indirect effects on herbivores. However, because both types of defenses may be costly to the plant, the production of effective metal-based defenses may lead to fewer organic plant defenses being produced. Indeed, some studies show that metal-accumulating plants produce fewer organic defenses upon pathogen attack when they are supplied with metals (Farinati et al., 2011; Fones et al., 2013). This suggests a trade-off between metal-based and organic defenses, although more evidence is needed to establish causality and determine its prevalence.

Most herbivores induce the production of organic plant defenses (Karban and Myers, 1989; Walling, 2000; Awmack and Leather, 2002). However some are able to suppress them (Musser et al., 2002; Abramovitch et al., 2006; Sarmento et al., 2011). Likewise, metal defenses vary in their effects upon herbivores. For example, the effectiveness of metal accumulation as an anti-herbivore defense varies with herbivore feeding guilds (Jhee et al., 2005; Vesk and Reichman, 2009; Konopka et al., 2013), as well as between specialist and generalist herbivores (Kazemi-Dinan et al., 2014). However, it is yet unknown whether metal-based defenses affect differently herbivores that induce or suppress organic defenses, and this may shed new light into the study of potential interactions between metal-based and organic defenses.

The model system composed of tomato plants (*Solanum lycopersicum,* L.) and herbivorous mites is ideal to test the abovementioned issues. When growing on soils with cadmium (Cd), tomato plants show higher tolerance than other species (Bingham et al., 1974; Khan and Khan, 1983; Kuboi et al., 1986) and inclusively are able to accumulate this metal in their shoots, sometimes over the Cd-hyperaccumulation threshold (100 $mg.kg^{-1}$; Gratão et al., 2008; López-Millán et al., 2009). Among spider mites, *Tetranychus urticae* is negatively affected by the accumulation of different metals by some host plants (Jhee et al., 2005; Quinn et al., 2010), but information concerning the effects of metals on other spider-mite species is as yet lacking. Additionally, different species within the Tetranychidae show contrasting effects on the induction of organic defenses of tomato plants. Indeed, *T. urticae* induces the production of jasmonate defenses, such as proteinase inhibitors, leading to lower performance of herbivores infesting those plants (Li et al., 2002; Ament et al., 2004; Kant et al., 2004). In contrast, *Tetranychus evansi* suppresses the production of such defenses (Sarmento et al., 2011; Alba et al., 2015), leading to higher performances of herbivores on subsequent infestations (Sarmento et al., 2011; Godinho et al., 2016). These differences allow testing the possible effect of metal accumulation on the inducibility of organic plant defenses. To this aim, we assessed the effects of Cd accumulation on the performance of tomato plants and on the spider mites that infest those plants. Additionally, we evaluated the effect of herbivory on jasmonate defenses and subsequent infestations by spider mites, on plants exposed to different Cd concentrations.

MATERIALS AND METHODS

Biological Materials and Rearing Conditions

Plants

Tomato plants (*Solanum lycopersicum*, var. Moneymaker) were sowed in a climate chamber (25°C, photoperiod 16/8 h light/darkness), in a soil (pH 5.0–6.0; Siro, professional substrates, Portugal)/vermiculite mixture (4:1) and watered 3 times per week for the first 2 weeks. In the third and fourth weeks, plants were watered once a week with tap water and twice a week with 60 mL of a Cd chloride solution with two ranges of concentrations: a wide range: 0, 0.01, 0.1, 0.5, 1, 2, or 10 mM; and a narrow range: 0, 0.1, 0.25, 0.5, 0.75, 1, or 1.5 mM. Using the wide range, we tested the effects of high Cd concentrations in the plant and on spider mites. Using the narrow range allowed us to

measure plant and spider-mite traits with higher resolution. At the end of the fourth week, plants were used in the experiments.

Spider Mites

Tetranychus urticae was collected from tomato plants in Portugal in 2010, and reared on bean plants (*Phaseolus vulgaris*, L.) since then (Clemente et al., 2016). In January 2016, a sub-set of the population (>300 mated females) was transferred to tomato plants and maintained on this host for six generations, before being used in the subsequent experiments. *T. evansi* was collected from *Datura stramonium*, L. in 2013, in Portugal, and reared on tomato plants ever since (Zélé et al., 2018). The two species were maintained, separately, in plastic boxes containing two entire tomato plants, in a climate chamber with conditions identical to those of the plant growing compartment (25°C, photoperiod 16/8 h light/darkness). Once a week, one plant was removed, and its leaves were cut and placed on top of the leaves of a new plant, allowing spider mites to migrate to new intact plants. To ensure that females used in the experiments were approximately of the same age, adult females where isolated on separate leaves and allowed to lay eggs for 48 h. Twelve days later, the adult females resulting from these cohorts were used in the experiments.

General Methodology

The performance of plants and spider mites was assessed using plants exposed to both ranges of Cd supply. For every assay the plants were between 4 and 5 weeks old, but to control for the effect of leaf age, we also always used the third leaf from below (third older) for the several measurements.

Plant Performance

Because the plant material collected was not enough to use in every assay, measurements were performed with different plants: Plants exposed to the wide range of concentrations (0–10 mM, $N = 6$ per Cd concentration) were used to determine Cd accumulation on the leaf, as well as the amount of calcium (Ca) and magnesium (Mg). As Cd^{2+} uses the same transporters as these ions, their assimilation by the plant may be hampered by Cd, which is not the case in hyperaccumulating plants (Gomes et al., 2013). From the narrow range (0–1.5 mM), half the plants ($N = 6$ per Cd concentration) were used to obtain the biomass parameters (root/shoot; specific leaf area and water content), however, due to technical problems, the plants supplied with 1.0 mM of Cd could not be used in this assay. The remaining plants ($N = 6$ per Cd concentration) were used to measure the amount of soluble sugars and to determine the carbon (C) to nitrogen (N) ratio. Nevertheless, for each plant, and before any destructive assay, we determined the spectral reflectance of the leaf, a non-invasive method that provides a general assessment of plant stress (Carter, 1993; Carter and Knapp, 2001).

Spectral analysis

The spectral reflectance was measured on one leaf from each plant, five measurements per leaf, using a UniSpec spectroradiometer (PP Systems, Haverhill, MA, United States). The spectral data generated by these measurements was analyzed by calculating spectral reflectance factors (R) for each wavelength (between 300.4 and 1148.1 nm with intervals of 3.4 nm). These factors were obtained by normalizing the reflected radiation from the leaves by a reflectance white standard. Several vegetative indices can be determined using reflectance data and used as a proxy of plant stress, being the most commonly used the Normalized Difference Vegetation Index (NDVI) as it reflects the efficiency of the photosynthetic system (Sridhar et al., 2007). Therefore, we here measured NDVI ((R810–R680)/(R810+R680)). In addition, we measured the SC index, which is representative of structural changes (SC) in leaf cells caused by accumulation of Cd (R1110/R810; Sridhar et al., 2007). Moreover, as it has been proposed that plants respond similarly to UV-B light exposure and herbivory, such as producing phenolic compounds (Roberts and Paul, 2006; Izaguirre et al., 2007), we also analyzed the spectral data under those wavelengths. For that we averaged, for each plant, the spectral reflectance factors of all UV-B wavelengths (R300.4–R313.9), referred afterward simply as UV-B reflectance.

Cadmium, calcium, and magnesium quantification

One leaf from each plant was dried for 72 h at 60°C until constant mass and uniformly ground. The elements were then quantified using Inductively Coupled Plasma – Atomic Emission Spectrometry (ICP – AES, Agilent 7500ce – Eurofins, Spain), after nitric acid digestion, with a detection limit of 0.1 μg/L.

Root to shoot ratio, specific leaf area and plant water content

All leaves and roots of each plant were collected, then the area of each leaf was measured with a laboratory leaf meter (LI-COR Biosciences). Next, the fresh weight of leaves and roots was obtained. Each leaf and the roots were then separately dried for 72 h at 60°C until constant mass and again weighed. The ratio between the dry weight of the roots and the dry weight of the leaves (root/shoot) was determined as well as the specific leaf area (SLA, total leaf area/total leaf dry weight) and plant water content (fresh weight–dry weight/fresh weight).

Carbon to nitrogen ratio

One leaf from each plant was dried at 60°C until constant mass and again weighed. The total carbon (C) and nitrogen (N) contents (grams of C or N per 100g of leaf dry weight) of each leaf was determined by dry combustion using an elemental analyser (EuroVector, Italy; Rodrigues et al., 2009).

Soluble sugar contents

One leaf disk (Ø 12 mm) was stored at −80°C and subsequently used to quantify the amount of soluble sugars. These were extracted from the leaf disk using 2 mL of 80% ethanol at 80°C and then quantified through changes in absorbance, at 405 nm for sucrose, using the resorcinol (1,3-dihidroxybenzene) method (de Carvalho et al., 2015), and 490 nm for glucose and fructose, using DNS (de-nitrosalicilic acid) as an oxidizing agent (Santos et al., 2017).

Spider-Mite Performance

Six leaf disk arenas (Ø 12 mm) were cut from one leaf (third from below) of each plant ($N = 6$ per Cd concentration for the wide range, $N = 12$ per Cd concentration for the narrow range) and placed on a petri dish on top of wet cotton wool.

One female spider mite of one of the two species was placed on each arena (three arenas per species per plant) and allowed to feed and oviposit for 4 days. Daily survival and fecundity of each female were recorded. The daily fecundity of spider mites was obtained by dividing the number of eggs laid by the number of days the female lived. In a previous study, it has been shown that this measurement is highly correlated with total lifetime fecundity (Clemente et al., 2018). Therefore, this measure can also be considered as an indication of the overall performance of spider mites.

Interaction Between Cd Accumulation and Inducibility of Jasmonate Organic Defenses

To test whether the effect of Cd and jasmonate organic defenses on herbivores are independent, tomato plants were exposed to three different Cd concentrations (0, 0.5, and 1.5 mM) as described before. Next, plants from the three treatments were infested for 48 h with either 100 *T. evansi* or *T. urticae* females on the third leaf (from below), or they were left un-infested (N = 12 plants per treatment; 9 treatments: 3 Cd concentrations vs. 3 infestation status – un-infested plants, plants infested with *T. urticae* and plants infested with *T. evansi*). Afterward, the plants were cleaned by removing all the mites, web, and eggs with a brush.

The performance of spider mites was determined as above.

Activity of trypsin inhibitors (TIs)

Plant material from the leaf used to determine the performance of spider mites was stored at −80°C and used, later, to quantify the activity of TIs, as a proxy for inducibility of the jasmonic acid pathway by spider mites (Sarmento et al., 2011; Godinho et al., 2016; Paulo et al., 2018). Approximately 300 mg of the leaf material stored at −80°C was weighed, ground, and homogenized with 600 μL of extraction buffer (0.1 M Tris-HCl, pH 8.2; 20 mM $CaCl_2$; 1:3). Each sample was centrifuged at 4°C, 16.0 × g for 25 min, and the supernatant was separated from the pellet and used in the spectrophotometer assay. This assay, adapted from Paulo et al. (2018) consisted in measuring the changes in absorbance at 405 nm caused by the activity of trypsin upon its substrate N-α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BApNA).

Statistical Analyses

All statistical analyses were performed with the software package R 3.0.2. The normality of the residuals of each model was tested using a Shapiro–Wilk normality test and, when needed, a Box-Cox transformation to the data was performed. Models were simplified by sequentially removing non-significant interactions and factors. Due to logistic constraints, each experiment was repeated in blocks of three plants per treatment. Block was thus included in the models as a random factor.

The effects of Cd exposure on NDVI, SC index (R1110/R810) and reflectance under the UV-B spectrum were determined using general linear mixed models (lmm) with, respectively NDVI, SC index or UV-B reflectance as response variables, Cd supplied as a fixed factor and block as a random factor.

The relation between the Cd contained in the solution administrated to the soil and the Cd contained in the leaves was determined with a Spearman correlation, due to the non-normality of the data. Furthermore, the relation between Cd contents and the amount of calcium (Ca) and magnesium (Mg) present on the leaves was assessed with a Pearson correlation.

The effects of Cd on specific leaf area, water and soluble sugar contents were tested using general linear mixed models (lmm) with Cd supplied as a fixed factor and block as a random factor, whereas differences in root/shoot and in C/N were determined using a generalized linear mixed model (glmm) with a binomial distribution, and the same factors as above.

The effects of Cd on daily fecundity of spider mites were determined for each range, using general linear mixed models (lmm) with species tested and Cd supplied as fixed factors and block as a random factor. Additionally, because the soluble sugar contents and the spectral SC index (R1110/R810) were affected by Cd, we tested whether changes in those traits influenced the daily fecundity of spider mites using a multivariate analysis of variance with distance matrices (adonis function, vegan package; Oksanen et al., 2013). The fecundity of *T. evansi* and *T. urticae* were used as response variables, the amount of sucrose and glucose plus fructose or the spectral SC index (R1110/R810), were used as fixed factors.

The statistical analysis of the interactions between Cd accumulation and jasmonate organic defenses were performed using general linear mixed models (lmm) with daily fecundity of *T. evansi* or the amount of trypsin inhibited as response variables, Cd supplied (0 mM; 0.5 mM; 1.5 mM) and infestation status (un-infested plants; plants previously infested with *T. urticae*; plants previously infested with *T. evansi*) used as fixed factors and block as a random factor.

RESULTS

Effect of Cd on the Performance of Tomato Plants

Cadmium exposure had no effect on NDVI (**Table 1**). However, significant differences were detected for the SC index (R1100/R810), (**Table 1**) on plants exposed to 2 mM or 10 mM of Cd (**Table 2**), suggesting structural changes in the leaf cells. The same pattern was detected when analyzing the narrow range of Cd concentrations (**Table 1**) but only for plants exposed to 1 mM and not for plants exposed to 1.5 mM (**Table 2**). Additionally, the UV-B reflectance of plants was significantly affected by Cd exposure (**Table 1**), for concentrations higher than 1 mM in the wide range (**Table 2**) and higher than 0.75 mM for the narrow range (**Table 2**).

The concentration of Cd accumulated in tomato leaves correlated positively with the Cd concentrations that plants were exposed to, in a linear way ($y = 52.299x + 21.165$, rho = 0.945, $P < 0.001$; **Figure 1**). The amount of Ca and Mg in the leaves did not change significantly with Cd accumulation ($R^2 = 0.25$, $P = 0.11$ for Ca and $R^2 = 0.23$, $P = 0.14$ for Mg).

Cadmium supplied to plants did not significantly affect water content of the leaves, SLA and root/shoot ratio (**Tables 3**, **4** and

TABLE 1 | Statistical analyses of the effect of cadmium on leaf reflectance.

Variable of interest	Data subset	Exploratory variable	*Df*	*F*	*P*-value
NDVI	Wide range	Cd supplied	6	0.21	0.97
	Narrow range		5	0.83	0.53
SC index	Wide range	Cd supplied	6	3.19	**0.015**
	Narrow range		5	4.49	**0.001**
UV-B reflectance	Wide range	Cd supplied	6	11.44	**<0.001**
	Narrow range		5	10.15	**<0.001**

p-values < 0.05 are indicated in bold.

TABLE 2 | *A posteriori* contrasts on the effect of cadmium on leaf reflectance.

Variable of interest	Data subset	Contrast	*Z*-value	*P*-value
SC index	Wide range	0 mM vs. 0.01 mM	1.23	0.88
		0 mM vs. 0.1 mM	1.80	0.54
		0 mM vs. 0.5 mM	2.87	0.06
		0 mM vs. 1 mM	−1.89	0.49
		0 mM vs. 2 mM	−0.18	**0.001**
		0 mM vs. 10 mM	−0.13	**0.008**
	Narrow range	0 mM vs. 0.1 mM	−0.79	0.97
		0 mM vs. 0.25 mM	−0.20	0.99
		0 mM vs. 0.5 mM	0.84	0.96
		0 mM vs. 0.75 mM	−3.38	**0.009**
		0 mM vs. 1.5 mM	−1.43	0.71
UV-B reflectance	Wide range	0 mM vs. 0.01 mM	1.67	0.64
		0 mM vs. 0.1 mM	2.12	0.34
		0 mM vs. 0.5 mM	3.91	**0.002**
		0 mM vs. 1 mM	−0.11	**<0.001**
		0 mM vs. 2 mM	−0.13	**<0.001**
		0 mM vs. 10 mM	−0.17	**<0.001**
	Narrow range	0 mM vs. 0.1 mM	1.50	0.66
		0 mM vs. 0.25 mM	2.35	0.17
		0 mM vs. 0.5 mM	4.07	**<0.001**
		0 mM vs. 0.75 mM	−5.28	**<0.001**
		0 mM vs. 1.5 mM	−5.74	**<0.001**

p-values < 0.05 are indicated in bold.

Figures 2A,B). No effect of Cd exposure was observed in the C/N content of the tomato leaves up to 1.5 mM (**Table 3** and **Figure 2C**). However, the amount of soluble sugars in the leaves was affected by the concentration of Cd to which plants were exposed (**Table 3** and **Figure 2D**). The amount of both sucrose and glucose plus fructose decreased in plants exposed to low concentrations of Cd, having the lower values at 0.5 mM (**Table 5** and **Figure 2D**). In plants exposed to 0.75 mM of Cd, the levels of sugars peaked to values higher than in control un-exposed plants but then decreased again for higher concentrations to values lower than on control plants (**Table 5** and **Figure 2D**).

Effect of Cd Accumulation on the Performance of Spider Mites

The oviposition of spider mites on leaf disks was significantly affected by the Cd supplied to the plants used to make those disks (**Table 6** and **Figure 3**). Additionally, both spider-mite species were similarly affected by the Cd concentration that plants were exposed to (**Table 6**). Both species increased their oviposition with low amounts of Cd until a threshold concentration, 0.5 mM (**Table 6** and **Figure 3**). From this concentration onward, Cd had a negative effect on the oviposition rate of spider mites, reaching, values lower than in control plants at 2 mM for the wide range and at 1.5 mM for the narrow range, respectively (**Table 6** and **Figure 3**).

The amount of sucrose in the leaves did not affect the fecundity of spider mites ($F_1 = 0.008$, $P = 0.71$). In contrast, the amount of glucose plus fructose affected this trait ($F_1 = 0.19$, $P = 0.003$). Additionally, the reflectance SC index (R1110/R810) affected the fecundity of spider mites ($F_1 = 0.07$, $P = 0.005$).

Interaction Between Cd Accumulation and Inducibility of Jasmonate Organic Defenses

The oviposition rate of *T. evansi* was affected by both Cd concentration and previous infestation with conspecifics or

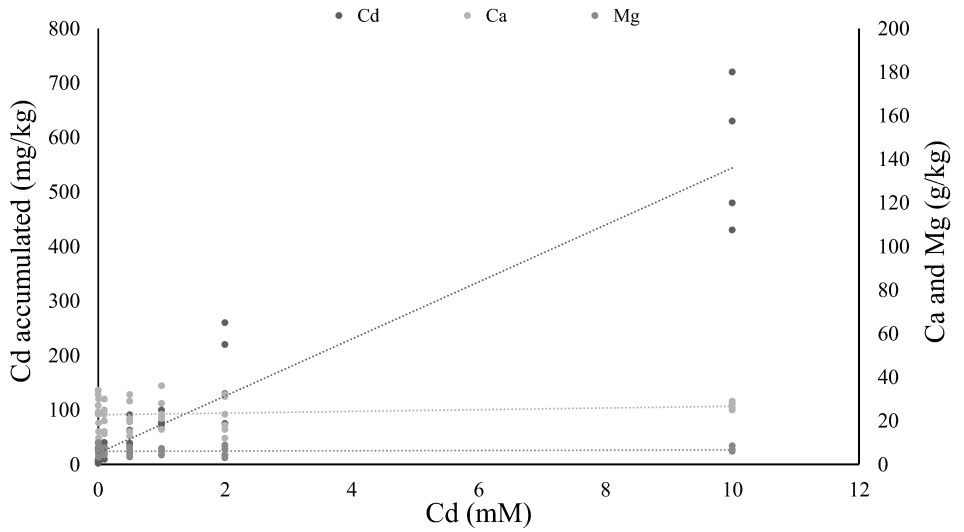

FIGURE 1 | Relation between cadmium supplied in soil solution in relation to cadmium, calcium, and magnesium concentration on tomato leaves. Lines represent linear regressions between the concentration of cadmium, calcium or magnesium solutions supplied to the plant (0, 0.01, 0.1, 0.5, 1, 2, and 10 mM; six plants per concentration) and the cadmium accumulated on the leaves, or the calcium and magnesium present on those leaves.

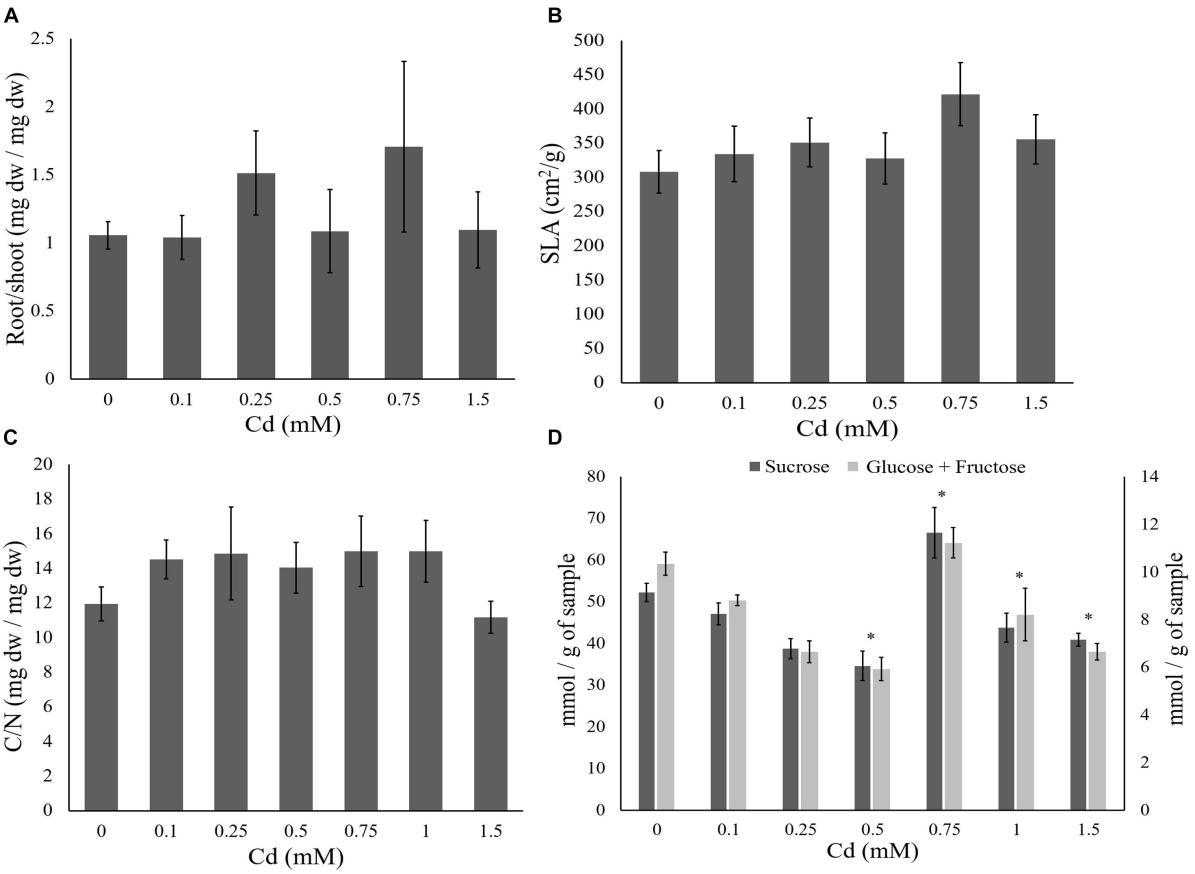

FIGURE 2 | Effect of cadmium on the performance of tomato plants. Tomato plants were supplied with different cadmium concentrations (0, 0.1, 0.25, 0.5, 0.75, 1, or 1.5 mM). The plant traits (±standard error – vertical bars; six plants per concentration) were: **(A)** average root to shoot ratio; **(B)** average specific leaf area (SLA, cm^2/g); **(C)** average carbon to nitrogen ratio of the leaves; **(D)** average glucose and fructose (light gray bars) and sucrose (dark gray bars) concentration (mmol per gram of leaf fresh weight). *Represents significant differences from the control plants.

TABLE 3 | Statistical analyses of the effects of cadmium on plant performance traits.

Variable of interest	Explanatory variable	*df*	χ^2/F	*P*-value
Water content	Cd supplied	5	34.03	0.31
SLA (specific leaf area)	Cd supplied	5	1.70	0.16
Root/shoot	Cd supplied	5	34.01	0.81
C/N	Cd supplied	6	4.42	0.65
Sucrose	Cd supplied	6	9.77	**<0.001**
Glucose + fructose	Cd supplied	6	13.21	**<0.001**

p-values < 0.05 are indicated in bold.

TABLE 4 | Effect of cadmium on plant biomass.

Biomass	Contrast	Fresh weight (mg ± SE)	Dry weight (mg ± SE)
Shoots	0 mM	48.44 ± 0.35	5.34 ± 0.06
	0.1 mM	52.23 ± 0.61	5.23 ± 0.09
	0.25 mM	41.52 ± 0.68	4.48 ± 0.09
	0.5 mM	47.00 ± 0.48	5.23 ± 0.08
	0.75 mM	37.04 ± 0.76	3.65 ± 0.09
	1.5 mM	44.83 ± 0.54	4.41 ± 0.08
Roots	0 mM	91.34 ± 0.94	5.56 ± 0.07
	0.1 mM	79.23 ± 0.90	5.00 ± 0.08
	0.25 mM	88.87 ± 0.89	5.79 ± 0.08
	0.5 mM	100.41 ± 1.04	5.00 ± 0.09
	0.75 mM	78.48 ± 0.62	4.73 ± 0.13
	1.5 mM	72.98 ± 0.80	4.36 ± 0.08

Average biomass of plants exposed to the narrow range of cadmium (N = 6).

TABLE 5 | *A posteriori* contrasts for the effects of cadmium on soluble sugar contents.

Variable of interest	Contrast	*t*-Value	*P*-value
Sucrose	0 mM vs. 0.1 mM	−1.07	0.29
	0 mM vs. 0.25 mM	−2.81	**0.008**
	0 mM vs. 0.5 mM	−3.67	**<0.001**
	0 mM vs. 0.75 mM	2.98	**0.005**
	0 mM vs. 1.5 mM	−2.36	**0.024**
Glucose + fructose	0 mM vs. 0.1 mM	1.61	0.12
	0 mM vs. 0.25 mM	4.72	**<0.001**
	0 mM vs. 0.5 mM	−6.06	**<0.001**
	0 mM vs. 0.75 mM	−0.79	**0.043**
	0 mM vs. 1.5 mM	−4.59	**<0.001**

p-values < 0.05 are indicated in bold.

heterospecifics (**Table 7** and **Figure 4**). However, the interaction between these factors was not significant (**Table 7**). The oviposition rate of *T. evansi* increased with previous infestation by conspecifics and decreased with previous infestation by *T. urticae* (**Table 7** and **Figure 4**), independently of the concentration of Cd to which plants were exposed before. Moreover, the oviposition rate of *T. evansi* increased on plants exposed to 0.5 mM of Cd and decreased on plants exposed to 1.5 mM of Cd (**Table 7** and **Figure 4**), compared to control plants, as observed in the previous results (**Figure 3**).

Additionally, the activity of trypsin inhibitors was modified by infestation by spider mites, independently of the concentration of Cd supplied to the plants (**Table 7** and **Figure 5**). Cadmium accumulation did not significantly affect the activity of trypsin inhibitors (**Table 7** and **Figure 5**).

DISCUSSION

Our results show that within the tested ranges, Cd exposure did not affect tomato growth (specific leaf area, root/shoot, water content, NDVI). However, variables related to leaf structure (SC index) or sugar content were affected, suggesting structural and biochemical changes in leaf cells. Spider mites infesting those plants were affected by Cd concentrations, albeit in a non-linear way. Indeed, both spider mite species had increased performance on plants mildly exposed to Cd, as compared to un-exposed plants, but lower performance after a given threshold, revealing a hormetic effect, which is a dose response phenomenon with stimulatory effects of mild concentrations and inhibitory effects at higher concentrations (Calabrese and Blain, 2009). Finally, the interaction of both spider mites with jasmonate defenses was not affected by the level of Cd that the plants were exposed to. Together, these results suggest that metal accumulation and the production of the studied plant organic defenses against herbivores do not interact with each other.

Studies regarding Cd accumulation by tomato plants reveal high variability in this trait (Hartke et al., 2013), with some plants accumulating amounts below the hyperaccumulation threshold (<100 mg/kg, Pollard, 2000), even at high concentrations of Cd supply (Ammar et al., 2007, 2008) and others accumulating above this threshold (Dong et al., 2006; Gratão et al., 2008; López-Millán et al., 2009). Here we observe that Cd accumulated linearly in the leaves of tomato plants, up to values above the hyperaccumulation threshold, suggesting that *Moneymaker*, the variety of tomato used in our study, is as a facultative hyperaccumulator. This is further confirmed by the values of Ca and Mg in the leaves which remain stable with increasing Cd in the leaves, as seen for other hyperaccumulator plants (Gomes et al., 2013; Pereira et al., 2017).

Here we report the absence of an immediate negative impact on plant growth, although there was an effective uptake of Cd into the leaves. Moreover, we also observe no differences in the carbon to nitrogen ratio in leaves of plants exposed to different Cd concentrations, indicating no shifts in the growth/defense balance (Herms and Mattson, 1992). This contrasts with previous studies showing a negative impact of Cd on tomato plant growth, for Cd accumulation values within the ranges used here (Dong et al., 2006; Ammar et al., 2007; Gratão et al., 2008; López-Millán et al., 2009). Possibly, the variety of tomato we used in this experiment is more tolerant to Cd than most other varieties. Indeed, the few studies using this variety observe no signs of toxicity (Petit and Van de Geijn, 1978; Petit et al., 1978). Another possibility is that the growing substrate affected these results. Indeed, most studies on this topic used continuously aerated hydroponics, creating an artificial situation for the plants such as

TABLE 6 | Statistical analyses on the effect of cadmium on the performance of spider mites.

Variable of interest	Data subset	Explanatory variable	*df*	*F*	*P*-value
Daily fecundity	Wide range	Cd supplied × tested species	6	47.04	0.14
		Cd supplied	6	64.13	**<0.001**
Daily fecundity	Narrow range	Cd supplied × tested species	6	0.12	0.99
		Cd supplied	6	10.62	**<0.001**
Variable of interest	**Data subset**	**Contrast**		***Z*-value**	***P*-value**
Daily fecundity	Wide range	0 mM vs. 0.01 mM	–	0.32	0.99
		0 mM vs. 0.1 mM		−0.94	0.96
		0 mM vs. 0.5 mM	–	−3.14	**0.028**
		0 mM vs. 1 mM		−1.34	0.83
		0 mM vs. 2 mM	–	−3.99	**0.012**
		0 mM vs. 10 mM	–	−4.82	**<0.001**
Daily fecundity	Narrow range	0 mM vs. 0.1 mM	–	−0.58	0.99
		0 mM vs. 0.25 mM	–	−1.45	0.77
		0 mM vs. 0.5 mM	–	−3.26	**0.018**
		0 mM vs. 0.75 mM	–	0.50	0.99
		0 mM vs. 1 mM	–	−1.43	0.78
		0 mM vs. 1.5 mM	–	3.93	**0.001**

p-values < 0.05 are indicated in bold.

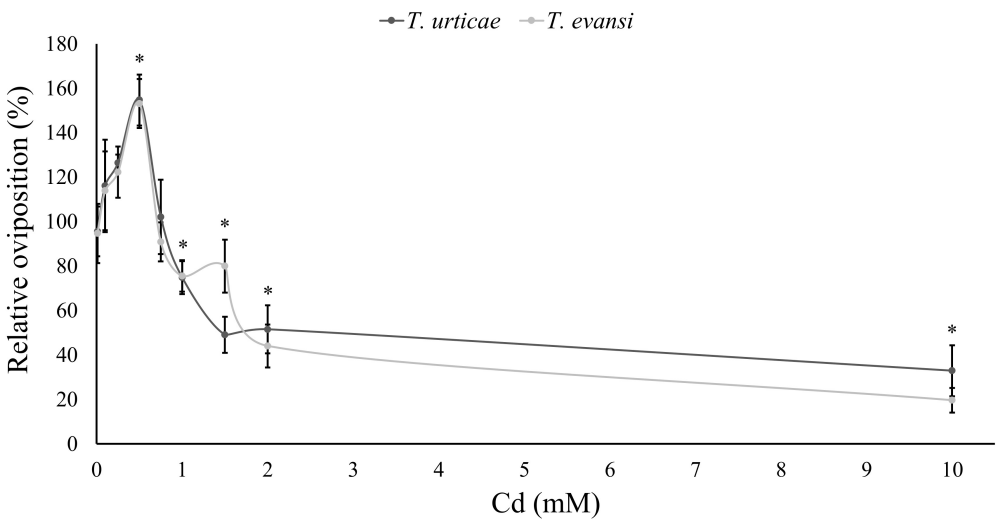

FIGURE 3 | Performance of spider mites on leaves of tomato plants exposed to cadmium. Average relative oviposition rate of *T. evansi* (light gray) and *T. urticae* (dark gray) females on tomato plants (±standard error – vertical bars; 6/12 plants, 3 disks per species per plant). For each range of cadmium solutions, a (0, 0.01, 0.1, 0.5, 1, 2, or 10 mM, *N* = 6) and b (0, 0.1, 0.25, 0.5, 0.75, 1, or 1.5 mM, *N* = 12) the oviposition of spider mites was normalized to the control (no cadmium) and merged in the same panel. *Represent significant differences to the control.

the absence of microbiota around the root, and here we used soil as a substrate as in natural conditions. Growing in soils may be advantageous to plants, given that soil microbiota may regulate the process of metal accumulation in the shoots (de Souza et al., 1999; Farinati et al., 2009), reducing the costs involved in this process for the plant (Farinati et al., 2009).

In contrast to most plant traits that did not respond to Cd, we found changes in soluble sugar contents and leaf reflectance. Changes in the amount of soluble sugars in the leaves with Cd were non-linear. Soluble sugars are generally associated with an initial response to plant stress, with changes in their accumulation, either increasing or decreasing, affecting the REDOX reactions originated by environmental stress (Couée et al., 2006). Cadmium supply may lead to either an increase (Mishra et al., 2014) or a decrease (Scheirs et al., 2006; Shackira and Puthur, 2017) in the amount of soluble sugars in the shoots of exposed plants. The fluctuations we observe in the soluble sugars content may indicate that these are being affected by different processes in the plant, and this may help to reconcile the contrasting observations in the literature. Additionally, plants

TABLE 7 | Statistical analyses of the effect of cadmium and spider mite infestation on daily fecundity and concentration of trypsin inhibitors.

Variable of interest	Explanatory variable	*df*	*F*	*P*-value
Daily fecundity	Cd supplied x infestation status	4	0.47	0.76
	Infestation status	2	27.56	**<0.001**
	Cd supplied	2	32.18	**<0.001**
Trypsin inhibitors	Cd supplied × infestation status	4	0.03	0.97
	Infestation status	2	4.49	**0.014**
	Cd supplied	2	0.77	0.38
Variable of interest	**Contrast**		***Z*-value**	***P*-value**
Daily fecundity	Un-infested vs. *T. evansi*	–	–3.22	**0.021**
	Un-infested vs. *T. urticae*	–	3.49	**0.009**
	0 mM vs. 0.5 mM	–	4.19	**<0.001**
	0 mM vs. 1.5 mM	–	–3.88	**<0.001**
Trypsin inhibitors	Un-infested vs. *T. evansi*	–	–3.26	**0.018**
	Un-infested vs. *T. urticae*	–	3.93	**0.021**

p-values < 0.05 are indicated in bold.

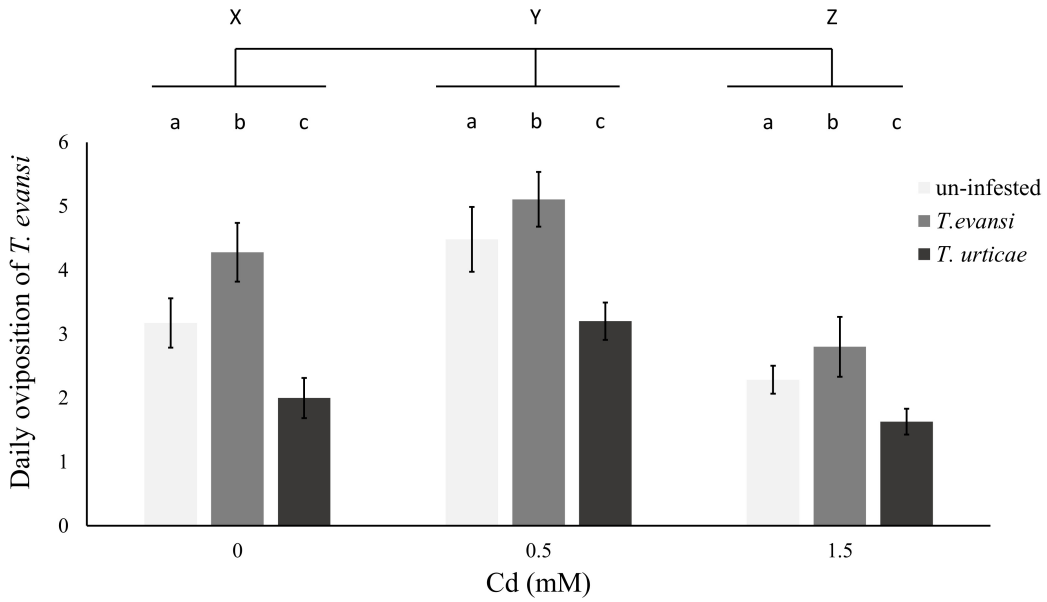

FIGURE 4 | Effect of cadmium exposure and herbivory on the performance of subsequent infestations. Average number of eggs laid per day by *T. evansi* females on un-infested plants (light gray), plants infested with 100 *T. evansi* females (gray) or with 100 *T. urticae* females (dark gray) for 48 h. Plants (±standard error – vertical bars; 12 plants, 3 disks per species per plant) were exposed to a range of cadmium concentrations (0, 0.5, or 1.5 mM). Small case letters (a,b,c) represent significant differences between infestation treatments and upper case letters (X,Y,Z) between cadmium treatments, there were no significant interactions between the two factors.

may also have a hormetic response to abiotic stressors, increasing their performance with small amounts of Cd until a threshold where the negative effects caused by this metal exceed the positive ones (Siddhu et al., 2008; Poschenrieder et al., 2013). If this is the case, the response of the plant to this stress may be different below and above this threshold, and this in turn could be reflected in the soluble sugar content. Corroborating this hypothesis requires more controlled experiments and a systematic measurement of Cd concentrations in the leaves. Moreover, higher concentrations of Cd exposure caused changes in leaf reflectance (SC index), which have been linked to structural changes on their leaf cells (Sridhar et al., 2007). Additionally, plants exposed to the higher concentrations of Cd showed a significant increase in the reflectance of UV-B light, which is possibly related to the production of phenolic compounds known to protect plants against abiotic stresses (Roberts and Paul, 2006; Izaguirre et al., 2007). Our results thus highlight the need to collect different measures of plant performance in response to a single abiotic stress.

The performance of spider mites was also affected by Cd accumulation in tomato leaves. Both species had a non-linear, hormetic response to this metal. Most herbivores are negatively

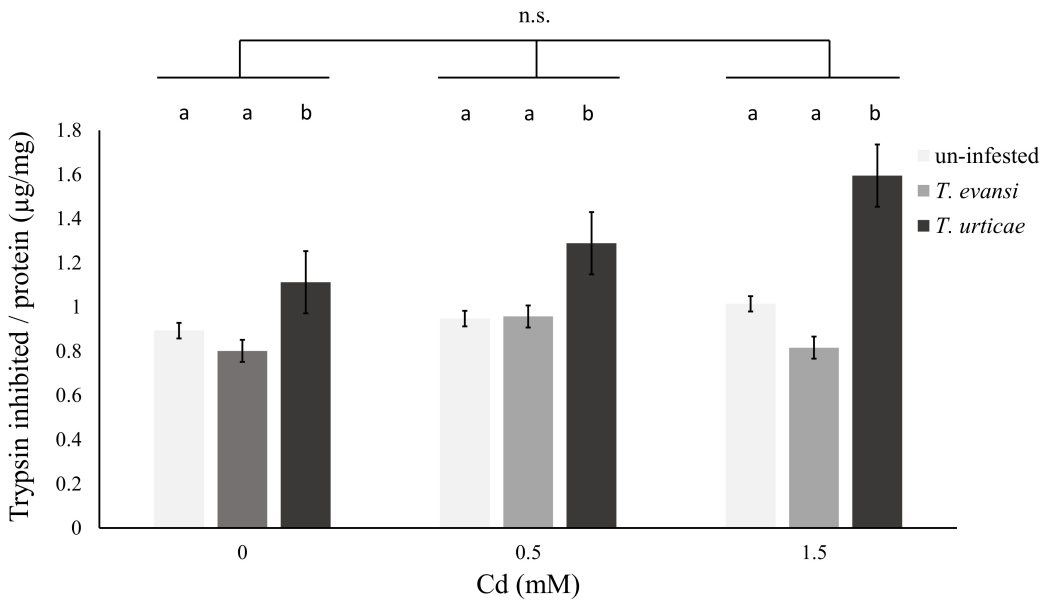

FIGURE 5 | Effect of cadmium exposure and herbivory on organic plant defenses. Amount (µg) of trypsin inhibited per mg of protein in leaf samples of un-infested plants (light gray), plants infested by 100 *T. evansi* females (gray) for 48 h or 100 *T. urticae* females (dark gray) for 48 h. Plants (±standard error – vertical bars; 12 plants, 3 disks per species per plant) were exposed to a range of Cd concentrations (0, 0.5, or 1.5 mM). Lower case letters (a,b) represent significant differences between infestation treatments, Cd supplied had no significant effect, neither was the interaction between the two factors significant.

affected by metal accumulation in the leaves of their host plants (Hanson et al., 2003; Freeman et al., 2007; Quinn et al., 2010; Stolpe et al., 2017). Still, there are some examples of higher abundances of herbivores on sites with intermediate concentrations of toxic metals (Zvereva et al., 1995; Kozlov, 2003), under natural conditions. Nevertheless, this is, to our knowledge, the first report of a hormetic effect of metals on herbivore performance. This phenomenon may significantly affect the evolution of metal accumulation, as selection will favor plants that accumulate amounts of metal above the threshold for herbivore inhibition. Such pattern may complement the "defense enhancement hypothesis" (Boyd, 2007). Indeed, given the hormetic effect of metals upon herbivores, plants are expected to reach the protective threshold of metal accumulation only when the costs of accumulation (i.e., a positive effect of metals upon herbivores) are surpassed by the benefits of metals reducing herbivory (Boyd, 2012; **Figure 6**). This hormetic pattern may be due to direct effects of the metal on the spider mites, or indirectly, through changes in plant quality. We observed no effect of Cd on plant biomass and C/N ratio, however, the amount of soluble sugars in the leaf significantly affected the performance of spider mites. The performance of spider mites has been reported as positively (Ximénez-Embún et al., 2016, 2017) or negatively (Wermelinger et al., 1985; Joutei et al., 2000; Scheirs et al., 2006) correlated to sugar content, indicating that this may depend on the host plant species or on other physiological responses that were not assessed. Although this correlation does not imply causation, it does suggest that Cd may indirectly affect mite performance via an effect on sugars or other physiological changes associated with them, a hypothesis that requires further tests. Additionally, we observed that the daily fecundity of both species correlated with the leaf spectral SC index, indicating a possible effect of structural changes in leaf cells caused by Cd. Still, our experiments do not exclude a possible direct effect of the metal on the performance of spider mites. Whether these correlations imply causality is a relevant question that calls for future studies.

The similarity in the hormetic pattern of the two spider mite species suggests they both may prefer to establish on plants with intermediate Cd concentrations rather than on un-contaminated plants. Moreover, their higher oviposition probably indicates that their growth rate is higher on those plants (Clemente et al., 2018). This may entail a faster saturation of that environment, relative to others. If this hormetic effect is extended to other herbivore and pathogenic species, then plants are expected to pay a high cost of mild Cd accumulation. Thus, given enough time, plants may be selected to "avoid" the level of Cd accumulation that results in better performance for the herbivores, being selected to accumulate higher amounts of metal, becoming hyperaccumulators, or to not accumulate metal at all, becoming excluders.

Because the two spider-mite species have dissimilar interactions with jasmonate organic defenses, the fact that they perform best on plants with the same Cd supply also suggests no interaction between metal accumulation and the inducibility of jasmonate defenses. Furthermore, the contrasting effects of these two spider-mite species on the activity of trypsin inhibitors and its effect on subsequent infestations were consistent across Cd environments, revealing no interference of Cd on protease activity, in contrast to what was seen in other

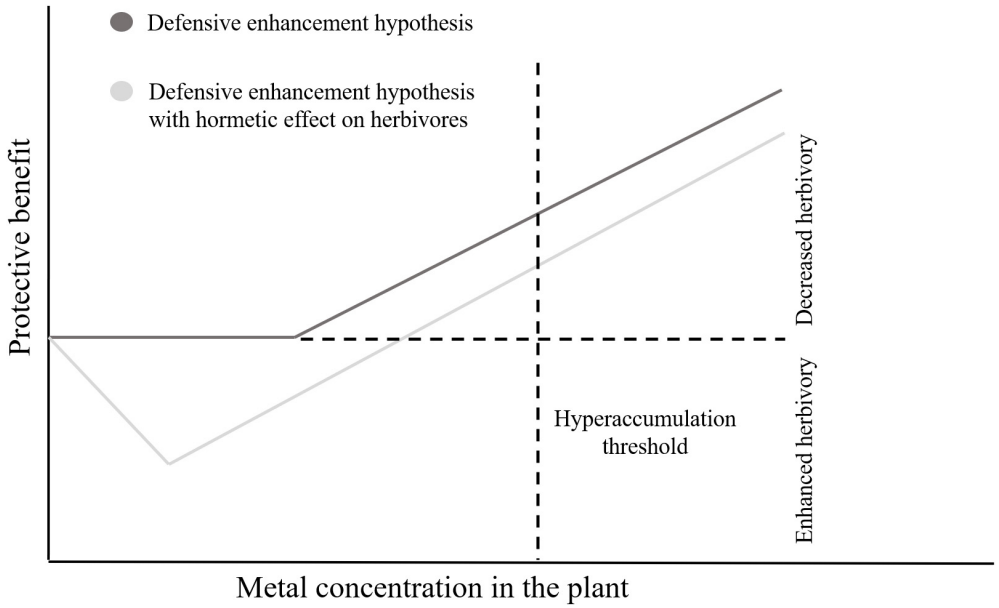

FIGURE 6 | The impact of hormesis on the protective effect of metals. Schematic comparison of the protective benefit of metal accumulation to the plant between the defensive enhancement scenario and a scenario with a hormetic effect of the metal on herbivores.

plant species (Pena et al., 2006; Lin et al., 2010). The plants used in these experiments showed little evidence of Cd toxicity. Possibly, the effect of Cd was not strong enough to induce the protective protease activity reported for other plants (Pena et al., 2006; Lin et al., 2010). Additionally, the effect of metal supply on spider mite performance was not affected by previous infestation. Together, these results suggest that metal based and organic plant defenses do not interfere with each other, serving the same purpose. Although some studies reveal that the expression of organic defenses is lower with high metal supply (Davis et al., 2001; Tolra et al., 2001; Farinati et al., 2009, 2011; Sun et al., 2009; Fones et al., 2013; Stolpe et al., 2017; Tewes et al., 2018), how herbivores are affected by the interaction between metal accumulation and organic defenses remains poorly studied (but see Stolpe et al., 2017). Still, if metal accumulation provides the same function as organic defenses, and if the production of organic defenses is costly, this may select for a reduction in organic defenses in plants under high metal supply. The opportunity for such selection to be effective is much higher on obligate metal accumulators (Poschenrieder et al., 2006), which is not the case of tomato. Alternatively, plants may be suffering from metal accumulation, hence they may lack the necessary resources to trigger organic defenses (Farinati et al., 2009, 2011; Fones et al., 2013). As we did not observe negative effects of Cd on plant growth, it may be that cost on tomato plants due to metals was not sufficient to lead to a trade-off between these two types of defenses. Possibly, long-term exposure to this contaminant, or exposure to higher concentrations, would cause significant costs to the plant affecting its growth rate or posing constrains in fruit production. Still, a recent field work found little evidence for trade-offs between organic and inorganic defenses (Kazemi-Dinan et al., 2015).

Another possible explanation for the absence of a trade-off in our study is that the effect of metal accumulation on herbivores was non-linear. Thus, if plants would produce fewer organic plant defenses, as metal accumulation increased, herbivores would have an extra-advantage at intermediate metal concentrations, benefiting from both a high performance in response to metals, and a low exposure to organic defenses. This, in turn, would pose a strong selective pressure upon plants not to shut down organic defenses. In the absence of an interaction between metal-based and organic defenses, plants occurring in heterogeneous environments may fine tune these strategies depending on their relative performance in each environment. Possibly, plants accumulate more metal when exposed to herbivores that suppress their organic defenses, overcoming the positive effects that low concentrations may have on these herbivores. This hypothesis awaits to be tested.

In sum, our results show that spider mites with different effects on the organic defenses of tomato plants have a similar hormetic response to Cd accumulation. This suggests that the community of spider mites on tomato plants will be similar in contaminated and un-contaminated soils. Our results highlight the importance of studying the interactive effects of metal based and organic plant defenses on herbivores, using metal concentrations below the hyperaccumulating threshold, which allows using more facultative accumulator species, including some of agricultural importance, such as tomato plants.

DATA AVAILABILITY

All data used in this work is archived in Dryad at doi: 10.5061/dryad.f274gs3.

AUTHOR CONTRIBUTIONS

DG, SM, and CB conceived the study. DG, SM, and CB designed the experiments with help from HS and ADS. DG collected the data with assistance from HS and ADS. DG analyzed the data with help from all authors. DG and SM led the writing of the manuscript, with significant help from CB and contributions by all authors. All authors gave final approval for publication.

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Plant-Mediated Effects of Water Deficit on the Performance of *Tetranychus evansi* on Tomato Drought-Adapted Accessions

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Climate change is expected to increase drought periods and the performance and dispersal of some invasive species such as *Tetranychus evansi*, which has been reported to take advantage of the nutritional changes induced by water-shortage on the tomato cultivar Moneymaker (MM). We have examined the implications for mite's biology of four accessions of the drought-adapted tomatoes, "Tomàtiga de Ramellet" (TR), under moderate drought stress. Mite performance was enhanced by drought in two accessions (TR61 and TR154), but not in the other two accessions (TR58 and TR126). We selected one accession of each outcome (i.e., TR154 and TR126) to further analyze plant nutritional parameters. We found that free sugars and most essential amino acids for mites were induced by drought and/or mite infestation on MM and TR154 plants, whereas sugars were not altered and a reduced number of essential amino acids were induced by drought in TR126. Remarkably, mite performance was enhanced by leaf infiltration of free sugars, essential amino acids mixture, and L-proline on well-watered MM and by free sugars on drought-stressed TR126 plants. These results indicate a positive link between the induction of soluble carbohydrates and amino acids used by the plant for osmotic adjustment and mite performance. The effects of drought and/or mite infestation on the defense response of plants was analyzed at three levels: phytohormone accumulation, the transcript levels of marker genes linked to jasmonates (JAs), salicylic acid (SA), and abscisic acid (ABA) pathways, and the activity of defense proteins. The ability of *T. evansi* to downregulate the accumulation of defense-related phytohormones was noted on MM and the two TR accessions analyzed (TR126 and TR154), though differences in the induction of protein defense genes and activities by drought and/or mite infestation were observed among them. These results emphasize the importance of studying plant biotic and abiotic stress factors in combination and provides an experimental framework for screening drought-tolerant tomato accessions that will be also resistant to herbivore mites.

Keywords: plant-herbivore interaction, abiotic stress, drought stress, spider mites, Tomàtiga de Ramellet

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a major vegetable crop grown all over the world in outdoor fields and greenhouses. During cultivation, tomato plants are exposed to a combination of biotic and abiotic stresses, soil water deficiency, and arthropod pests being among the most critical. Its cultivation is mainly concentrated in semiarid zones, like the Mediterranean, where it needs to be cultivated under irrigation (Rivelli et al., 2013), and where drought events associated with climate change are expected to be more frequent (Nankishore and Farrell, 2016). Thus, water shortage caused by drought periods can have important consequences for tomato production, as it might produce yield reduction of up to a 50% in the case of an equivalent reduction in irrigation (Cantore et al., 2016). Tomato plants are attacked by a number of insect and mite pests which significantly reduce fruit yield and quality. Worldwide, losses due to these pests are estimated to be about 34.4% of attainable tomato yield under current production practices (Zalom, 2003). Moreover, the sustainability of tomato production is threatened by an increasing number of invasive arthropod pests (Navajas et al., 2013; Tonnang et al., 2015).

The high sensitivity of tomato to water deficit and the need for irrigation in the Mediterranean basin has prompted different approaches for breeding drought-resistant crops, including the search for drought tolerant/adapted varieties (Hu and Xiong, 2014). The term "drought-adapted" refers to higher yield in crop plants under water shortage conditions (Verslues and Juenger, 2011). In this regard, finding tomato drought-adapted varieties might become an important approach to improve water use efficiency under future climate change scenarios. Hereof, the "Tomàtiga de Ramellet" tomatoes, which represent a population of landraces from the Balearic Islands (Spain), have been traditionally cultivated outdoors under low water availability conditions during the Mediterranean summer, and thus they represent tomatoes adapted to cultivation under water deficit (Galmes et al., 2011, 2013). Several studies have compared groups of tomato varieties, landraces or wild species, observing a differential expression on some drought-associated traits between varieties, but without having a consistent response in the literature. For instance, the osmolite L-proline is usually induced by drought, but the level of induction is a variety-dependent trait, whereas leaf water content is generally less reduced on tolerant plants (Sanchez-Rodriguez et al., 2010; Tapia et al., 2016). The accumulation of osmolites, mainly soluble free sugars and amino acids, which facilitate water uptake and retention, have been reported in tomato leaves during restricted watering (English-Loeb et al., 1997; Tapia et al., 2016). Other traits such as stomatal conductance and the maximum quantum yield of photosystem II photochemistry (Fv/Fm) are parameters commonly used as drought response indicators. In general, a fast decrease in stomatal conductance and a long maintenance of the Fv/Fm value, are indicative of tolerance (Thompson et al., 2007; Mishra et al., 2012; Nankishore and Farrell, 2016; Tapia et al., 2016). To our knowledge, molecular mechanisms driving drought adaptation on the aforementioned drought-adapted landraces are unknown.

The red tomato spider mite, *Tetranychus evansi* Baker and Pritchard, first recorded in Brazil, has emerged as a serious invasive pest in some areas of Africa and Europe (Navajas et al., 2013), because it is highly tolerant to hot and dry conditions. Thus, it is expected to spread northward across Europe (Meynard et al., 2013) under a climate change scenario. Furthermore, it is able to suppress tomato plant defenses (Kant et al., 2015) by downregulating the accumulation of defense-related phytohormones and the expression of genes involved in the regulation of secondary metabolites and defense proteins (Sarmento et al., 2011; Alba et al., 2015; Ataide et al., 2016; Schimmel et al., 2017). In addition, we have found that both drought and *T. evansi* infestation induced significant changes in the nutritional quality of tomato plants of the commercial cultivar, Moneymaker (MM) (Ximénez-Embún et al., 2016). Specifically, we found that more essential amino acids and free sugars were available in leaf tissues under drought conditions, and that L-proline, the amino acid highest induced by drought in tomato, had a phagostimulant effect on *T. evansi*. These physiological changes trigger a bottom-up effect on key biological traits of *T. evansi* causing a highly significant increase in leaf damage and mite performance, thus it represents an increasing threat to tomato crop production in the face of climate change (Ximénez-Embún et al., 2016).

A complex phytohormonal network drives plant responses to biotic and abiotic stresses and it is responsible of fine-tuning plant physiology response to a specific stress (Spoel et al., 2007; Bostock et al., 2014). Plant responses to drought stress are mainly controlled by abscisic acid (ABA) (Cutler et al., 2010), whereas plant responses to biotic stresses are mediated by salicylic acid (SA), jasmonic acid, and derivates named as jamonates (JAs) and ethylene (ET). Plant response to a combination of stresses is a complex trait that cannot be directly deduced from that to each of the different stress applied individually (Atkinson and Urwin, 2012; Suzuki et al., 2014), but both stresses contribute to the final responses (Coolen et al., 2016). However, despite the wealth of sources of variation for drought tolerance in accessions of tomato and wild-related species, the mechanisms that govern their responses to water stress are not well characterized (Egea et al., 2018). Moreover, the consequences that these mechanisms may impose on their interaction with biotic stresses are largely unknown. We report here a holistic approach that considers both drought and spider mites. Accordingly, we investigate the performance of *T. evansi* on drought-adapted "Tomàtiga de Ramellet" tomatoes subjected to a moderate water deficit. The mite performance response when feeding on some of the selected accessions was linked to the observed changes in plant nutritional composition and plant defense responses as compared to the commercial cultivar MM that we have used as model in previous works (Ximénez-Embún et al., 2016). Furthermore, by imposing these two stresses in combination, the possible directions of the multiple physiological interactions could be examined.

MATERIALS AND METHODS

Plant Material and Mite Rearing

Four tomato accessions (provided by Dr. Granell, IBMCP, Valencia, Spain) were used in this study: TR58, TR61, TR126, and TR154 (from a population of local landraces "Tomàtiga de Ramellet" (TR) from Mallorca, Balearic Islands, Spain) traditionally grown under non-irrigated conditions during the Mediterranean summer and therefore adapted to water deficit conditions; as well as the commercial cultivar MM. Tomato plants were grown from seeds in 40-well trays. Plants with three expanded leaves were transferred to 2.5 l pots (diameter: 16 cm, height: 15 cm) (Maceflor©, Valencia, Spain) filled with 600 g of Universal growing medium "Compo sana®" (Compo GmbH, Münster, Germany) and watered to saturation.

A colony of *T. evansi* derived from the Nice strain collected in Beausoleil (South of France) was provided by Dr. Maria Navajas (CBGP, France). Mites were maintained on detached MM tomato leaves placed on ventilated plastic cages (22 cm × 30 cm × 15 cm) for about 50 generations. Inside the cages, leaves were placed on a plastic plate that was on top of a soaked sponge. The petioles of the leaves were in contact with a thin layer of water in the bottom of the cages to maintain the leaf turgor and to contain the mites.

Plants and mite cages were maintained in climate rooms at 25 ± 1°C, 50 ± 5% relative humidity and a 16 h light/8 h dark photoperiod.

Drought Stress Regime

Drought stress was attained by water deficiency as described by Ximénez-Embún et al. (2016). In brief, tomato plants were well-watered until they developed four to five fully expanded leaves, then we imposed two irrigation regimes, defined as control and moderate drought stress. Control plants were watered regularly to maintain the soil volumetric water content (θ) up to 74%. For moderate drought stress, watering was stopped for 7 days and thereafter plants were watered to maintain θ between 21 and 30%. Steady stress conditions were reached at about 7–9 days after ceasing irrigation. Water-stressed plants from the four accessions were over the wilting point associated with severe drought stress, established at $\theta \leq 16\%$ for MM in our experimental conditions (Ximénez-Embún et al., 2016). θ was determined gravimetrically by recording single plant pot weight (balance BSH 6000, PCE Iberica, Tobarra, Spain).

The severity of drought stress was assessed by measuring the following parameters on the sub-terminal leaflet of the fourth leaf: (a) stomatal conductance (gs) using a leaf porometer (SC-1 Decagon-T, Pullman, WA, United States); and (b) variations in maximum quantum yield of photosystem II photochemistry (Fv/Fm), using a FluorPen FP 100 (PSI, Drasov, Czech Republic). Plant growth was estimated by measuring the stem length (distance between the soil and the terminal bud).

Bioassays

Three different experiments were carried out: (1) to measure the effect of moderate drought on mite performance and leaf damage; (2) to determine the effects of moderate drought and mite infestation on tomato nutritional composition and plant defenses; and (3) to test the stimulatory effect of free sugars and amino acids in mite performance. All experiments were carried out in a climate room under the same environmental conditions, as those described above for mite rearing.

Experiment 1

Effect of drought on mite performance and leaf damage. Tomato plants from each of the four TR accessions were randomly assigned to control or moderate drought treatments. At about 7–9 days, after stopping irrigation, the drought stress conditions had stabilized, corresponding with the phenological stage of six to seven expanded tomato leaves. Then, plants were infested with *T. evansi* females of random age collected from the laboratory colony by using a vacuum pump D-95 (Dinko S.A., Barcelona, Spain) with a sucking power of 10–50 mmHg connected to a modified polypropylene microtube. They were placed on the two sub-terminal leaflets of tomato leaves three, four, and five (eight mites per leaflet, 48 mites per plant). All plants (infested and non-infested) were confined with a ventilated metacrylate cylinder, fitting the pot diameter, to avoid mites escaping from the infested plants and to simulate similar environmental conditions in the non-infested ones. They were set up in a climate room following a complete randomized block design. A total of nine replicates per treatment were simultaneously performed. Mite performance was assessed at 4 days post infestation (dpi) to avoid overlapping generations, as eggs need at least 5 days to hatch in our experimental conditions. All leaves were detached from the plants, and the number of eggs and mobile mite stages (larvae, nymphs, and adults) were counted under a stereomicroscope M125 (Leica Mycrosystem, Wetzlar, Germany). The leaf damaged area (mm^2 of chlorotic lesions) was determined by scanning the damaged leaflets using hp scanjet (HP Scanjet 5590 Digital Flatbed Scanner series, United States) and analyzing the scanned leaflets with the program GIMP 2.8[1], as described in Ximénez-Embún et al. (2016).

Experiment 2

Effect of drought and *T. evansi* on plant nutritional composition and plant defenses. Plants of the accessions TR126 and TR154 and of the cultivar MM were assigned to four different groups combining two treatments: uninfested well-watered plants (Control); uninfested drought-stressed plants (Drought); infested well-watered plants (*T. evansi*); and uninfested drought-stressed plants (Drought + *T. evansi*). When drought stress conditions had stabilized, plants were infested as described above. Four days after infestation, plant material was collected. The left leaflets from leaves three, four, and five were pooled, ground in liquid nitrogen to a fine powder, and stored at −80°C for the analysis of total protein, free amino acids, and plant defense responses (phytohormone accumulation, transcript levels of stress marker genes, and enzymatic activity of defense proteins). The right leaflets from the same leaves were pooled and immediately

[1] www.gimp.org

weighed, oven-dried at 70°C for 3 days and weighed again to assess the percentage of water, ground using a mortar and pestle to obtain a fine powder, and stored at room temperature for free sugar analysis. Six replicates per treatment were used.

Experiment 3

Stimulatory effect of free sugars and amino acids in mite performance. Tomato leaves of the cultivar MM and the accession TR126 were infiltrated with solutions of sugars, essential amino acids, and L-proline. The accession TR126 was chosen as mite performance on it differs to the observed on MM, and we wanted to test whether this difference remains when infiltrating nutrients. Concentrations were chosen to simulate their induction by both drought and *T evansi* infestation on tomato plants of the cultivar MM at 4 dpi (see Section "Results" "Changes in Plant Nutritional Composition Induced by Drought and *T. evansi*"), corresponding to 38 mg of free sugars, 9.66 mg of essential amino acids, and 2.42 mg of L-proline/g of leaf dry weight, respectively. Accordingly, a solution of essential amino acids containing L-valine (0.39 g/l), L-isoleucine (0.29 g/l), L-leucine (0.27 g/l), L-tyrosine (0.27 g/l), L-phenylalanine (0.21 g/l), L-histidine (0.19 g/l), and L-arginine (0.83 g/l) was prepared, based on their relative proportions in the tomato leaves (see **Table 1**) and an estimated fresh leaflet weight of 0.5 g and a ratio of 0.06 dry/fresh leaflet weight. In the case of L-proline, its concentration in the solution was 0.61 g/l. For the free sugars, a solution including sucrose (4.62 g/l), glucose (2.78 g/l), and fructose (1.85 g/l) was prepared, based on the total amount of free sugars (see **Figure 2**) and the relative amount of these sugars in tomato leaves as reported by Khodakovskaya et al. (2010). Both, control and moderate drought-stressed plants were infiltrated, with five replicates per treatment. The infiltration protocol was as follows: the sub terminal leaflet of the third leaf was infiltrated with approximately 0.2 ml of an aqueous solution using a 1 ml needleless syringe. It was allowed to dry for 1 h and then 100 females of *T. evansi* were placed on the infiltrated leaflet. A barrier of Lanolin (Manuel Riesgo, Madrid, Spain) was placed on the petiolule of the leaflet to avoid the mites to escape. After 24 h, the number of eggs per leaflet was determined. To measure the incorporated free amino acids and sugars into the tomato leaf by infiltration, MM leaves were infiltrated but not infested with mites and leaflets were collected at 1 h post infiltration (hpi), dried at 70°C for 3 days, ground using a mortar and pestle to obtain a fine powder for the analysis of free sugars and L-proline.

Chemical and Biochemical Analysis

Chemicals and Equipment

Unless specified otherwise, all chemical compounds were obtained from Sigma-Aldrich (St Louis, MO, United States). Fluorimetric measurements were made using a Varioskan Flash reader (Thermo Fisher Scientific, Wilmington, DE, United States), and spectrophotometric measurements with a VERSAmax microplate reader (Molecular Devices Corp., Sunnyvale, CA, United States).

Free Sugars

Samples of 40 mg of leaf powder were homogenized in 650 µl of 95% (v/v) aqueous ethanol and heated at 80°C for 20 min. Samples were then centrifuged at 10,000 rpm for 10 min, and the supernatant collected. The process was repeated two more times and the three supernatants were pooled. A volume of 750 µl of the mixture was dried on a SpeedVac Concentrator Savant SVC-100H (Thermo Fisher Scientific) and redissolved in 500 µl of water. Soluble carbohydrate concentration was estimated by the anthrone method (Maness, 2010) using glucose as standard. In brief, 1 ml of anthrone reagent (0.2% v/v anthrone on 95% sulfuric acid) was added to the extract, heated at 90°C for 15 min, and the absorbance measured at 630 nm.

Free Amino Acids

The extraction of free amino acids was done as described by Hacham et al. (2002). Samples of 50 mg of frozen leaf powder were homogenized with 600 µl of water:chloroform:methanol (3:5:12 v/v/v). After centrifugation at 12,000 rpm for 2 min, the supernatant was collected and the residue was re-extracted with 600 µl of the same mixture, pooling the two supernatants. A mixture of 300 µl of chloroform and 450 µl of water were added to the supernatants, and after centrifugation the upper water:methanol phase was collected and dried in the SpeedVac. The samples were dissolved on 100 µl of sodium citrate loading buffer pH 2.2 (Biochrom, United States) and 10 µl were injected on a Biochrom 30 Amino Acid Analyser (Biochrom, United States) at the Protein Chemistry Service at CIB (CSIC, Madrid, Spain).

Soluble Protein

Samples of 100 mg of leaf frozen powder were homogenized in 500 µl of 0.15 M NaCl and ground with fine sand. The homogenate was centrifuged at 12,000 rpm for 5 min at 4°C, and the soluble protein quantified by absorbance at 280 nm on a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States).

Plant Defense Proteins

Samples of 100 mg of leaf frozen powder were homogenized with 500 µl of extraction buffer (0.15 M NaCl for protease inhibitors, and 0.1 M phosphate buffer, pH 7.0; 5% w:v polyvinylpolypyrrolidine for oxidative enzymes) and soluble protein quantified as explained above.

The inhibitory activity of plant protein extracts was tested against commercial enzymes: papain (EC 3.4.22.2), cathepsin B from bovine spleen (EC 3.4.22.1), trypsin from bovine pancreas (EC 3.4.21.4), α-chymotrypsin from bovine pancreas (EC 3.4.21.1), cathepsin D from bovine spleen (EC 3.4.23.5), and leucine aminopeptidase from porcine pancreas (EC 3.4.11.1), as described by Ximénez-Embún et al. (2016). Reaction conditions are summarized in **Supplementary Table S1**. Results were expressed as a percentage of protease activity inhibited.

Polyphenol oxidase (PPO) activity was analyzed by incubating 20 µl of enzyme extract with cathecol (40 mM final concentration) in 160 µl of Tris-HCl pH 8.5 buffer at 30°C for 1 h. Absorbance was read at 420 nm. Peroxidase (POD)

activity was determined incubating 20 μl of a 1:10 dilution of the enzyme extract with guaiacol (5 mM final concentration) and H_2O_2 (2.5 mM final concentration) in 150 μl of potassium phosphate pH 6 buffer at 30°C for 10 min. Absorbance was read at 470 nm. PPO and POD activities were expressed as nmol substrate metabolized relative to time and total protein content.

L-Proline Analysis

The L-proline content of infiltrated leaves was analyzed adapting the protocol described by Bates et al. (1972). Samples of 4 mg of dry leaf powder were homogenized in 1 mL of a solution of sulfosalicylic acid 3% (w/v). After centrifugation at 10,000 rpm for 10 min, the supernatant was collected. A mix of 250 μl of supernatant, 250 μl of glacial acetic acid (AcH) and 250 μl of acid ninhydrin reagent [acid ninhydrin 2.5% (w/v), AcH 60% (v/v), H_3PO_4 2.5 M] was incubated for 1 h at 100°C. The reaction was stopped in an ice bath for 10 min. The products of the reactions were extracted by adding 500 μl of toluene and mixing vigorously for 15-20 s. The absorbance of the aqueous supernatant was measure at 520 nm. The L-proline concentration was determined from a commercial L-proline standard curve.

Quantification of Phytohormones

Freeze-dried plant material (c.a. 10 mg) was spiked with 25 μl of an internal standard mixture (containing ABA-d_6, DHJA, and $^{13}C_6$-SA) to correct for analyte losses and extracted in 1 mL ultrapure water for 10 min in a ball mill at room temperature. After extraction, homogenates were centrifuged at 10,000 rpm for 10 min at 4°C to remove debris. Supernatants were recovered, pH adjusted to 3.0 with 30% acetic acid, and partitioned twice against an equal volume of di-ethyl ether. The combined organic layers were evaporated under vacuum in a centrifuge concentrator (Jouan, Saint Germain Cedex, France). The dry residues were subsequently reconstituted in 0.5 mL of 10% aqueous methanol and the resulting solutions filtered through 0.20 μm syringe membrane filters. Filtered extracts were analyzed by tandem LC/MS in an Acquity SDS UPLC system (Waters Corp., United States) coupled to a TQS triple quadrupole mass spectrometer (Micromass Ltd., United Kingdom) through an electrospray ionization source. Separations were carried out on a C18 column (Gravity C18, 50 mm × 2.1 mm, 1.8 μm particle size, Macherey-Nagel, Germany) using a linear gradient of ultrapure methanol and water, both supplemented with acetic acid to a 0.1% concentration, at a constant flow rate of 0.3 $mL.min^{-1}$. During analyses, column was maintained at 40°C and samples at 10°C to slow down degradation. Plant hormones were detected in negative electrospray mode by their specific precursor-to-product ion transitions (ABA, 263 > 153; JA, 209 > 59; SA, 137 > 93; 12-oxo-phytodienoic acid OPDA, 291 > 165; and SA glucosyl ester SAGE 299 > 137) and quantitated using an external calibration curve with standards of known amount.

Quantification of Gene Expression *via* qRT-PCR

The expression levels of drought response *RAB18* gene, SA-dependent *PR1a* gene, and JA-dependent *MYC2*, *CDI*, *PPO-F*, and *PI-Ia* genes were measured by qRT-PCR. Samples of 100 mg of frozen leaf powder were taken for total RNA extraction using the TRIZOL Reagent (Molecular Research Center, Cincinnati, OH, United States) according to the manufacturer's instructions. RNA quantification was done using NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, United States). Two micrograms of total RNA previously treated with RQ1 DNase (Promega, Madison, WI, United States) during 35 min at 37°C followed by 10 min at 75°C was used for single strand cDNA synthesis. Reverse transcription was carried out using the Revert Aid H Minus First Strand cDNA Synthesis Kit (k1632, Thermo Fisher Scientific) according to the manufacturer's instructions and with some minor modifications. qPCR was carried out in a Corbett Rotor Gene 6000 real-time cycler (Qiagen) using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, United States) as previously described (Vidal-Quist et al., 2015). Raw gene expression data were efficiency-corrected and gene expression was transformed to normalized relative quantities (NQR) by using the reference genes (Hellemans et al., 2007). The primers used are listed in **Supplementary Table S2**.

Statistical Analysis

All plant and mite data were checked for the assumptions of normality and heteroscedasticity, and transformed if necessary. Stem length, stomatal conductance, *T. evansi* eggs and mobile forms, leaf damaged area, and phytohormone data were $\log_{10}(x)$ transformed, Fv/Fm was $\log_{10}(x + 1)$ transformed; gene-expression data (NRQ values) were $\ln(x + 1)$ transformed; and the percentage of water, free sugars, protein, total free amino acids, and protease inhibition activities were arcsine square root transformed. Different types of statistical analysis were performed depending on the design and purpose of each experiment. Student's *t*-test were performed to determine whether moderate drought has an effect on plant functional traits (stomatal conductance, Fv/Fm, and stem length) and mite performance (*T. evansi* eggs and mobile forms, and leaf damaged area) for each plant cultivar/accession tested (Experiment 1). Two-way ANOVA were performed to test the effect of drought condition (D), mite infestation (M), and their combination on plant nutritional parameters (percentage of water, free sugars, protein and free amino acids, and amount of specific amino acids) and plant defenses (phytohormone levels, gene-expression, protease inhibition, and oxidative enzyme activities) for a given cultivar/accession (Experiment 2). When the interaction D × M was significant, Newman-Keuls *post hoc* test were performed to compare treatments. The infestation by *T. evansi* did not affect plant functional traits (stomatal conductance, Fv/Fm, and stem length) in Experiment 2, and thus, data from infested and non-infested plants were pooled and re-analyzed also by Student's *t*-test for each plant cultivar/accession tested. One-way ANOVA followed by Newman-Keuls *post hoc test* was performed to compare the effect of the agroinfiltration of leaflets with different nutrients on mite performance (number of eggs) and the content of free sugars and L-proline recovered from infiltrated leaflets at 1 and 24 h post-infiltration (Experiment 3). The correlation between the levels of plant nutrients (free

sugars and L-proline) and the number of mite's eggs in infiltration experiments was analyzed by using the Pearson's correlation coefficient. For the statistical analysis, the IBM SPSS Statistics 24.0 software (Chicago, IL, United States) was used.

RESULTS

Effect of Drought on Stomatal Conductance, Photosynthetic Efficiency, and Tomato Plant Growth

The effect of drought stress on stomatal conductance, photosynthetic efficiency, and stem length observed in Experiment 1 (**Supplementary Figure S1** and **Supplementary Table S3**) and Experiment 2 (**Supplementary Figure S2** and **Supplementary Table S4**) indicates that the severity of drought stress attained under our experimental conditions can be considered as moderate. In all plant cultivar/accessions, drought induced a significant reduction of stomatal conductance that was, on average, about 3.5 and 4.5 times lower at mite infestation time and at 4 dpi, respectively (**Supplementary Figures S1A**, **S2A**). The maximum efficiency of PSII (Fv/Fm) was not affected by drought in any cultivar/accession, except for a significant increase at mite infestation for TR126 in Experiment 2 (**Supplementary Figures S1B**, **S2B**). Moreover, the Fv/Fm values were never below 0.7, corroborating that severe drought stress conditions were never reached (Ritchie, 2006; Mishra et al., 2012). Moderate drought stress reduced plant growth, as stem length was smaller on drought-stressed plants at the time of infestation for TR58 and TR61 in Experiment 1 and for MM and TR154 in Experiment 2, and in all cultivar/accessions at 4 dpi in both experiments (**Supplementary Figures S1C S2C**).

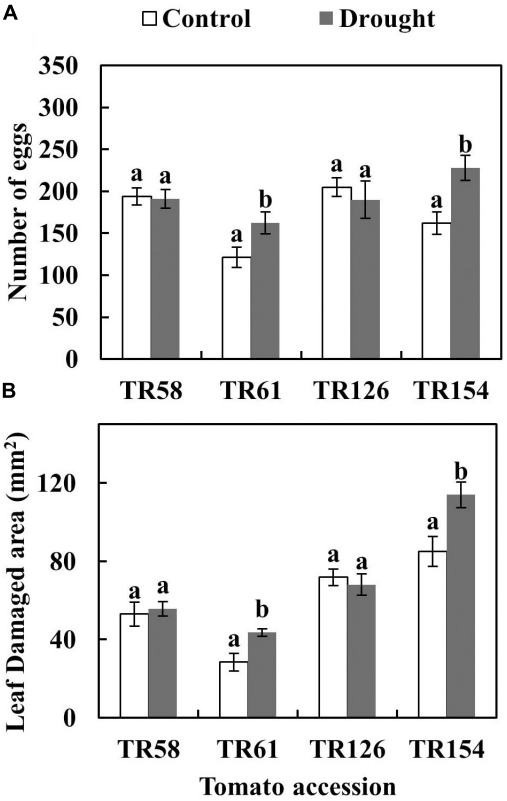

FIGURE 1 | Performance of *T. evansi* in the tomato accessions TR58, TR61, TR126, and TR154. The number of eggs **(A)** and leaf damaged area **(B)** on well-watered (Control) and moderate drought-stressed (Drought) tomato plants at 4 days post infestation were measured. Data are mean ± SE of nine replicates/treatment. Different lowercase letters indicate significant differences within each cultivar/accession (Student's *t*-test, $p < 0.05$). The detailed results (*t* and *p* values and degrees of freedom) are shown in **Supplementary Table S5**.

Effect of Drought on *T. evansi* Performance

A differential effect of moderate drought was observed on *T. evansi* performance depending on the tomato TR accessions on which they were feeding (**Figure 1** and **Supplementary Table S5**). The number of eggs laid by females on drought-stressed plants was 1.3- and 1.4-fold higher than on control plants when feeding on TR61 and TR154, respectively (**Figure 1A**). Likewise, 1.5- and 1.3-fold more damaged area was measured on drought-stressed than on control plants when feeding on TR61 and TR154, respectively (**Figure 1B**). By contrast, similar numbers of eggs and damaged area values were obtained in control and drought-stressed plants when *T. evansi* fed on TR58 and TR126. The number of mobile forms recovered at 4 dpi (surviving females, since eggs need at least 5 days to hatch in our experimental conditions) was not significantly different (**Supplementary Table S5**) between water-stressed and control plants for all TR accessions (data not shown).

The accessions TR154 and TR126 were selected for further chemical, biochemical, and molecular analyses. The rationale for this selection is that they represent the two types of plant-mediated effects of water deficit on mite performance: enhanced (TR154) and no effect (TR126); and that in both cases, the biological parameters obtained on control plants are closer to the values obtained on MM under identical experimental conditions (Ximénez-Embún et al., 2016 observed a 180/341 eggs and 107/133 mm^2 of damaged area on control/moderate drought-stressed plants, respectively).

Changes in Plant Nutritional Composition Induced by Drought and *T. evansi*

The nutritional composition of tomato leaves was determined by analyzing water, free sugars, protein, and total free amino acid content (**Figure 2** and **Supplementary Tables S6–S8**). Drought stress was the most significant factor for MM, inducing the amount of total free sugars and amino acids, whereas *T. evansi* infestation had no significant effect (**Figures 2B,D**). TR154 was affected by *T. evansi* infestation that induced an increase on free sugars, though not when combined with drought stress, and all treatments had lower levels of protein than the

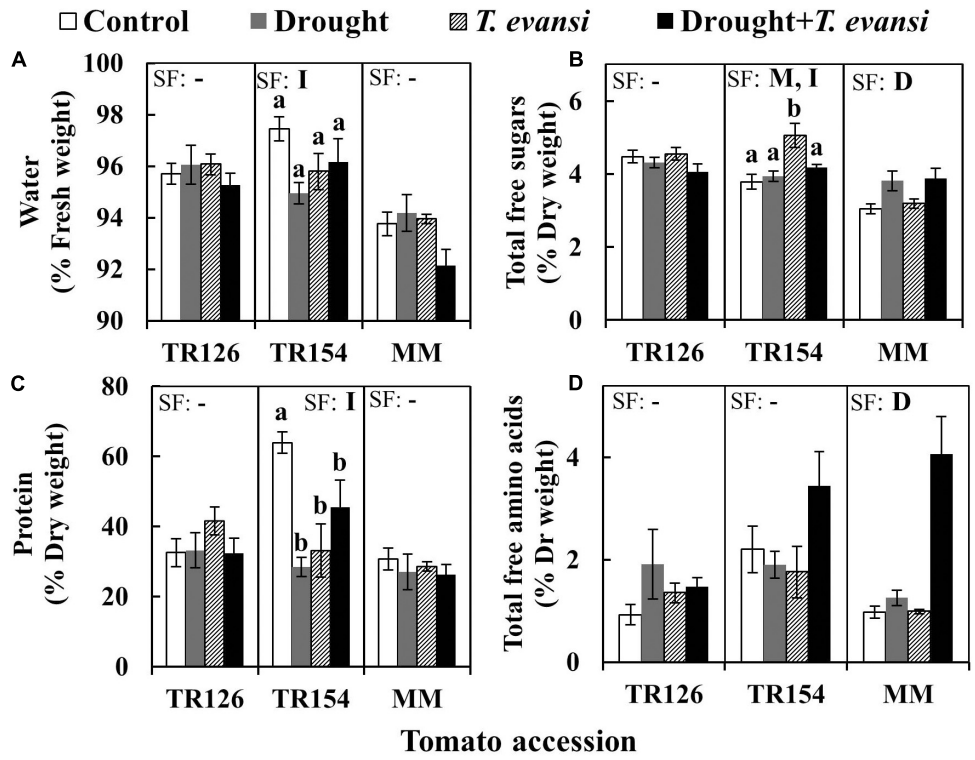

FIGURE 2 | Effect of moderate drought, *T. evansi* infestation and their combination on nutritional composition of tomato accessions TR126 and TR154 and the cultivar Moneymaker (MM) at 4 days post infestation: **(A)** water, **(B)** total free sugars, **(C)** protein, and **(D)** total free amino acids. Data are mean ± SE of six replicates/treatment: uninfested well-watered plants (Control); uninfested drought-stressed plants (Drought); infested well-watered plants (*T. evansi*); and uninfested drought-stressed plants (Drought + *T. evansi*). Significant factors (SF) indicates whether either of the two independent factors D (drought condition) and M (mite infestation) and/or their interaction I (D × M) are statistically significant for a given cultivar/accession (two-way ANOVA, $p < 0.05$). When the interaction D × M was significant, Newman-Keuls *post hoc* test were performed (different lowercase letters indicate significant differences among treatments). The detailed results of the two-way ANOVA (*F* and *p* values and degrees of freedom) are shown in **Supplementary Tables S6–S8**.

control (**Figures 2B,C**). By contrast, drought and/or *T. evansi* infestation did not cause any significant change in the nutritional composition of TR126.

The levels of specific free amino acids, classified as essential or non-essential for the related species *Tetranychus urticae* according to Rodriguez and Hampton (1966), were analyzed (**Table 1** and **Supplementary Tables S6–S8**). L-Proline, an indicator of drought stress, was induced in the three cultivar/accessions by drought. With respect to the rest of amino acids, similar results were obtained with TR154 and MM, which responded to drought stress with an increase of most of the essential amino acids (valine, isoleucine, leucine, tyrosine, histidine, lysine, and arginine) and serine. An increase of threonine, cysteine, and the essential amino acid phenylalanine was also induced by drought in MM, but not in TR154. A comparatively reduced number of essential amino acids (valine, isoleucine, leucine, and phenylalanine) and threonine were induced in TR126 by drought. Mite infestation was only a significant factor for glutamic acid in the case of MM. In addition, a significant interaction between the two factors occurred for glutamic acid in the three varieties/accessions. Thus, the levels of glutamic acid increased under drought conditions but not when combined with mite infestation in TR126, only when both stresses were combined in MM, and no differences among treatments were found in the case of TR154.

Effect of Drought and *T. evansi* on Tomato Hormones and Stress Response Genes and Defense Proteins

Hormone profiling in leaf tissues of MM and TR accessions under moderate drought, *T. evansi* infestation, and their combination were determined (**Figure 3** and **Supplementary Tables S6–S8**). As expected, ABA levels in MM and TR154 tomato plants were induced by drought stress, though in the case of TR154 these levels were significantly higher in uninfested drought-stressed plants than in infested drought-stressed plants (**Figure 3A**). On the contrary, TR126 did not show any significant increase in ABA levels, indicating that their molecular response to drought stress is different from that of MM or TR154. The levels of JA were not significantly affected by any stress condition on MM or TR154, whereas a significant reduction of JA levels occurred on uninfested drought-stressed TR126 plants (**Figure 3B**). OPDA levels were reduced in MM in response to all stress conditions and in TR126 in response to drought stress, whereas no significant changes on TR154 could be observed (**Figure 3C**). Interestingly,

TABLE 1 | Effect of moderate drought stress, *T. evansi* infestation, and their combination (Dr + Te) on the amino acid composition of the tomato accessions TR126, TR154, and the cultivar Moneymaker at 4 days post infestation.

	TR126					TR154					Moneymaker				
	Control	**Drought**	***T. evansi***	**Dr + Te**	**SF**	**Control**	**Drought**	***T. evansi***	**Dr + Te**	**SF**	**Control**	**Drought**	***T. evansi***	**Dr + Te**	**SF**
Non-essential amino acids															
Asp	2.10 ± 0.50	2.79 ± 0.91	2.97 ± 0.52	1.91 ± 0.18	–	4.00 ± 0.89	2.02 ± 0.17	2.84 ± 1.12	3.80 ± 0.83	–	1.47 ± 0.11	2.57 ± 1.28	1.32 ± 0.08	3.04 ± 0.80	–
Thr	0.40 ± 0.10	0.98 ± 0.36	0.58 ± 0.09	0.71 ± 0.08	D	1.10 ± 0.28	0.89 ± 0.11	0.78 ± 0.27	1.48 ± 0.27	–	0.54 ± 0.07	1.70 ± 0.94	0.57 ± 0.03	1.83 ± 0.37	D
Ser	2.00 ± 0.70	2.68 ± 1.02	2.77 ± 0.92	2.72 ± 0.51	–	2.49 ± 0.45	4.42 ± 0.97	2.44 ± 0.62	7.36 ± 1.53	D	1.87 ± 0.43	8.42 ± 5.52	1.80 ± 0.26	11.8 ± 3.41	D
Glu	3.20 ± 0.40a	10.00 ± 3.60b	4.79 ± 0.25ab	5.13 ± 0.90ab	D,I	12.00 ± 2.60a	7.33 ± 0.52a	8.82 ± 2.63a	14.09 ± 3.00a	I	4.21 ± 0.50a	4.34 ± 1.00a	4.34 ± 0.30a	8.38 ± 1.15b	D,M,I
Gly	0.03 ± 0.01	0.05 ± 0.02	0.04 ± 0.01	0.07 ± 0.03	–	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	–	0.02 ± 0.01	0.13 ± 0.11	0.02 ± 0.01	0.05 ± 0.01	–
Ala	0.35 ± 0.09	0.43 ± 0.16	0.43 ± 0.08	0.49 ± 0.21	–	0.41 ± 0.12	0.26 ± 0.04	0.28 ± 0.09	0.47 ± 0.10	–	0.22 ± 0.06	0.92 ± 0.73	0.25 ± 0.06	0.56 ± 0.13	–
Cys	0.03 ± 0.01	0.05 ± 0.02	0.04 ± 0.01	0.05 ± 0.01	–	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.10 ± 0.02	–	0.02 ± 0.01	0.12 ± 0.1	0.02 ± 0.01	0.09 ± 0.02	D
Pro	0.08 ± 0.02	0.79 ± 0.27	0.13 ± 0.02	0.84 ± 0.32	D	0.28 ± 0.06	0.75 ± 0.09	0.2 ± 0.05	1.39 ± 0.44	D	0.11 ± 0.01	1.20 ± 0.79	0.11 ± 0.01	2.42 ± 0.75	D
Essential amino acids															
Val	0.16 ± 0.04	0.36 ± 0.13	0.22 ± 0.04	0.51 ± 0.16	D	0.36 ± 0.07	0.65 ± 0.17	0.41 ± 0.1	1.10 ± 0.22	D	0.26 ± 0.08	1.55 ± 1.16	0.29 ± 0.04	1.55 ± 0.41	D
Met	0.02 ± 0.01	0.03 ± 0.02	0.03 ± 0.01	0.08 ± 0.05	–	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	–	0.02 ± 0.01	0.21 ± 0.18	0.02 ± 0.01	0.07 ± 0.02	–
Ile	0.10 ± 0.02	0.20 ± 0.07	0.15 ± 0.04	0.33 ± 0.10	D	0.21 ± 0.04	0.47 ± 0.14	0.28 ± 0.08	0.80 ± 0.18	D	0.18 ± 0.05	1.01 ± 0.75	0.21 ± 0.04	1.38 ± 0.24	D
Leu	0.08 ± 0.02	0.14 ± 0.05	0.11 ± 0.03	0.46 ± 0.25	D	0.20 ± 0.04	0.38 ± 0.13	0.25 ± 0.06	0.65 ± 0.14	D	0.15 ± 0.05	1.45 ± 1.23	0.18 ± 0.03	1.32 ± 0.24	D
Tyr	0.07 ± 0.02	0.1 ± 0.03	0.11 ± 0.03	0.27 ± 0.12	–	0.13 ± 0.02	0.36 ± 0.13	0.22 ± 0.07	0.55 ± 0.12	D	0.13 ± 0.05	0.86 ± 0.66	0.16 ± 0.03	1.46 ± 0.22	D
Phe	0.13 ± 0.02	0.26 ± 0.09	0.19 ± 0.02	0.42 ± 0.17	D	0.34 ± 0.07	0.35 ± 0.09	0.34 ± 0.10	0.62 ± 0.12	–	0.19 ± 0.03	1.04 ± 0.82	0.2 ± 0.02	0.84 ± 0.20	D
His	0.06 ± 0.02	0.09 ± 0.03	0.10 ± 0.04	0.15 ± 0.04	–	0.11 ± 0.02	0.33 ± 0.08	0.18 ± 0.04	0.68 ± 0.19	D	0.12 ± 0.04	0.45 ± 0.28	0.13 ± 0.03	0.88 ± 0.16	D
Lys	0.09 ± 0.03	0.12 ± 0.05	0.15 ± 0.06	0.37 ± 0.20	–	0.17 ± 0.03	0.36 ± 0.09	0.19 ± 0.05	0.63 ± 0.12	D	0.11 ± 0.03	1.17 ± 0.97	0.13 ± 0.02	1.09 ± 0.24	D
Arg	0.30 ± 0.13	0.07 ± 0.04	0.73 ± 0.55	0.24 ± 0.12	–	0.16 ± 0.03	0.40 ± 0.15	0.27 ± 0.10	0.63 ± 0.15	D	0.15 ± 0.05	2.10 ± 1.64	0.18 ± 0.04	3.93 ± 1.33	D

Data are the mean amount of amino acid (mg) per gram of dry weight ± SE of six replicates/treatment. The division between essential and non-essential amino acids is based on a study with the closely related species Tetranychus urticae (Rodríguez and Hampton, 1966). Significant factors (SF) indicates whether either of the two independent factors D (drought condition) and M (mite infestation) and/or their interaction I (D × M) are statistically significant for a given cultivar/accession (two-way ANOVA, $p < 0.05$). When the interaction D × M was significant, Newman-Keuls post hoc test were performed (different lowercase letters indicate significant differences among treatments). The detailed results of the two-way ANOVA (F and p values and degrees of freedom) are shown in ***Supplementary Tables S6–S8****.*

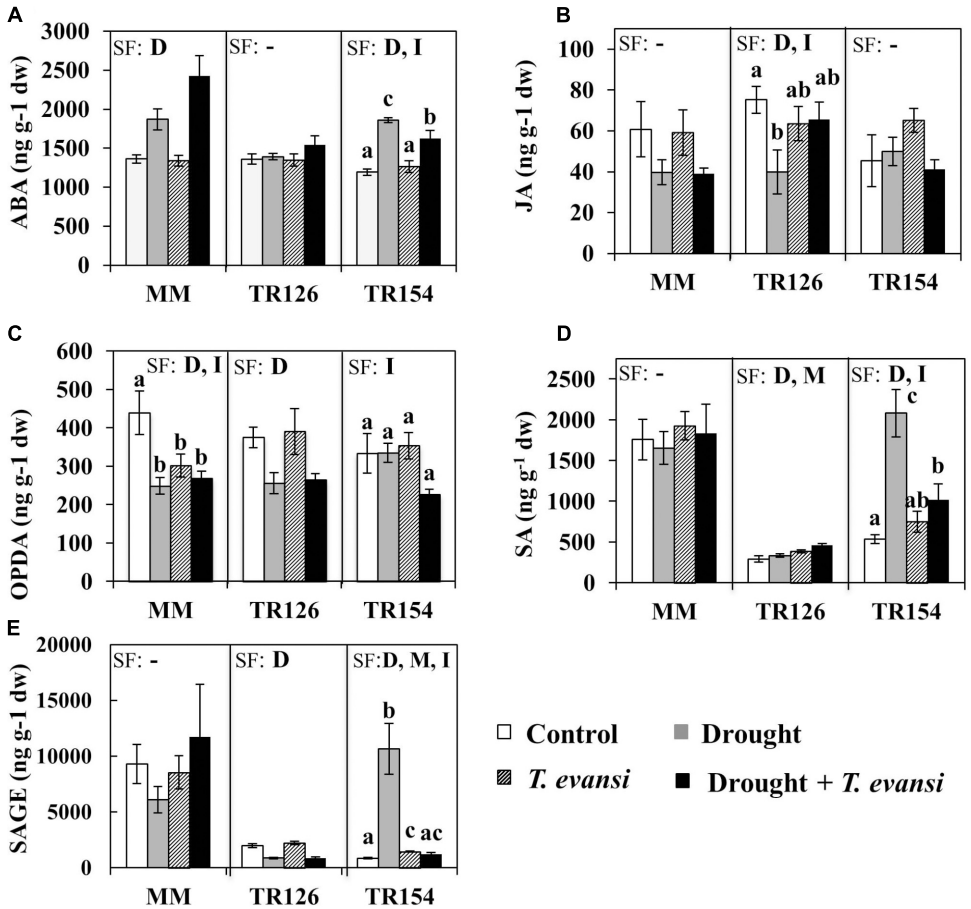

FIGURE 3 | Hormone profiling of tomato accessions TR126 and TR154 and the cultivar Moneymaker (MM) under moderate drought, *T. evansi* infestation, and their combination. Levels of phytohormones **(A)** ABA, **(B)** JA, **(C)** OPDA, **(D)** SA, **(E)** and SAGE were determined at 4 days post infestation. Data are mean ± SE of six replicates/treatment: uninfested well-watered plants (Control); uninfested drought-stressed plants (Drought); infested well-watered plants (*T. evansi*); and uninfested drought-stressed plants (Drought + *T. evansi*). Significant factors (SF) indicates whether either of the two independent factors D (drought condition) and M (mite infestation) and/or their interaction I (D × M) are statistically significant for a given cultivar/accession (two-way ANOVA, $p < 0.05$). When the interaction D × M was significant, Newman-Keuls *post hoc* test were performed (different lowercase letters indicate significant differences among treatments). The detailed results of the two-way ANOVA (*F* and *p* values and degrees of freedom) are shown in **Supplementary Tables S6–S8**.

levels of SA and SAGE in TR126 and TR154 were threefold to fivefold lower than in MM, when plants were maintained under control conditions (**Figures 3D,E**). However, their levels were not significantly affected by any stress condition on MM, whereas (i) both drought stress and mite infestation induced accumulation of SA and drought stress reduced SAGE levels in TR126 and (ii) SA was induced by drought in TR154, but to a lower extent in infested drought-stressed plants, and SAGE was induced by drought stress and mite infestation, but not when both stresses were combined.

Concerning the expression of stress marker genes, the analysis shows different hormone-dependent stress responses in TR accessions and MM (**Figure 4** and **Supplementary Tables S6–S8**). The drought responsive gene *RAB18* was overexpressed under drought and/or in combination of drought and *T. evansi*. This later interaction was especially relevant in the case of MM. However, under *T. evansi* infestation alone, MM and TR accessions showed an opposite pattern, indicating a putative differential ABA-dependent response (**Figure 4A**). The SA-dependent *PR1a* gene expression was significantly induced by drought in TR126 and by mite infestation in TR154, but not in MM (**Figure 4B**). We found also differences in the expression of *CDI* and *PI-Ia* genes, which encode enzymatic activities putatively involved in the response to mite infestation that are under the control of JAs. Thus, *CDI* was overexpressed in response to both drought stress and mite infestation in all varieties/accessions (**Figure 4D**). On the contrary, *PI-Ia* was induced by both stresses in MM and TR154, but only by mite infestation in TR126 (**Figure 4F**). However, the JA-dependent *MYC-2* and PPO-F genes were not significantly differentially expressed in MM or TR accessions (**Figures 4C,E**).

Tomato plant defense proteins were affected by drought stress and mite infestation, but different responses were obtained depending on the cultivar/accessions (**Table 2** and **Supplementary Tables S6–S8**). In the case of MM, drought stress induced an increase in POD activity, but not when

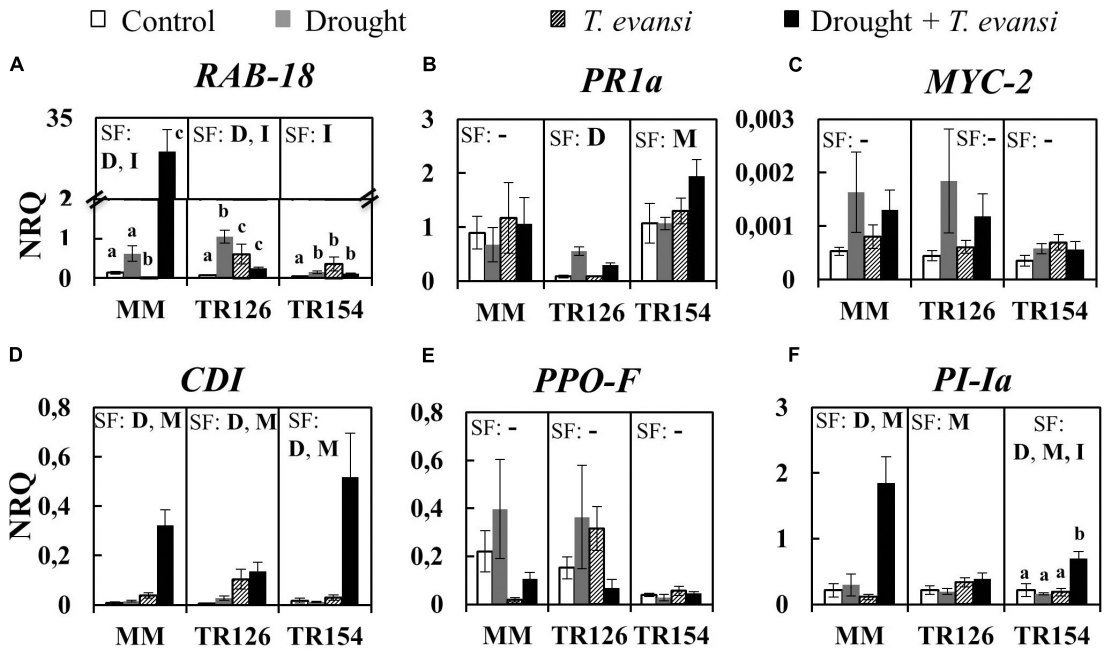

FIGURE 4 | Stress response gene expression analysis of tomato accessions TR126 and TR154 and the cultivar Moneymaker (MM) under moderate drought, *T. evansi* infestation, and their combination. The expression of drought response **(A)** *RAB18* gene, SA-dependent **(B)** *PR1a* gene, and JA-dependent **(C)** *MYC2*, **(D)** *CDI*, **(E)** *PPO-F*, **(F)** and *PI-Ia* genes was analyzed at 4 days post infestation. Data are mean ± SE of six replicates/treatment: uninfested well-watered plants (Control); uninfested drought-stressed plants (Drought); infested well-watered plants (*T. evansi*); and uninfested drought-stressed plants (Drought + *T. evansi*). Significant factors (SF) indicates whether either of the two independent factors D (drought condition) and M (mite infestation) and/or their interaction I (D × M) are statistically significant for a given cultivar/accession (two-way ANOVA, $p < 0.05$). When the interaction D × M was significant, Newman-Keuls *post hoc* test were performed (different lowercase letters indicate significant differences among treatments). The detailed results of the two-way ANOVA (*F* and *p* values and degrees of freedom) are shown in **Supplementary Tables S6**–**S8**.

combined with mite infestation, and *T. evansi* infestation induced the inhibition activity against papain, cathepsin D, and chymotrypsin. TR154 showed a similar response: drought induced a decrease on the cathepsin B inhibitory activity; and *T. evansi* induced the inhibition of cathepsins B and D and papain. The accession TR126 showed the highest changes in the levels of defense proteins in response to stress: drought induced the inhibitory activity against cathepsin D, trypsin, and aminopeptidase; and *T. evansi* increased the inhibition against cathepsin B and chymotrypsin and reduced the inhibition against aminopeptidase.

Effect of Sugars, Amino Acids, and L-Proline Infiltration on *T. evansi* Performance

In order to test if the accumulation of nutrients in plants by drought stress is responsible for the enhanced mite performance, plant leaflets were infiltrated with free sugars and amino acids and used to fed mites. In the case of MM, the number of eggs laid by *T. evansi* on leaflets from well-watered (control) plants and infiltrated with free sugars, essential amino acids and L-proline was significantly higher than on leaflets from the same plants infiltrated with water, and similar to those laid on leaflets from drought-stressed plants and infiltrated with water (**Figure 5A**). We checked the amount of free sugars and L-proline in leaflets infiltrated with each of these nutrients, and their levels were significantly higher than on leaflets from control and drought-stressed plants infiltrated with water for at least 24 h, when the number of eggs was recorded (**Supplementary Figures S3A,B**). Interestingly, a significant correlation was found between the number of eggs laid and the levels of sugars (Pearson's correlation coefficient (*r*): 0.678; *p*: 0.005) and L-proline (*r*: 0.608; *p*: 0.016) in the leaflets at 24 h post infiltration (**Supplementary Figures S3C,D**). When the accession TR126 was tested, no significant differences were observed between control plants infiltrated with free sugars, essential amino acids or L-proline, and the water-infiltrated controls (**Figure 5B**). Thus, the experiment was repeated using drought-stressed plants, and in this case, a significant increase in the number of eggs was obtained only with sugar-infiltrated plants (**Figure 5C**).

DISCUSSION

Our data reveal that drought stress has a differential effect on the performance of *T. evansi* in drought-adapted "Tomàtiga de Ramellet" tomatoes. Thus, the changes induced by drought stress on two of the TR accessions (TR61 and TR154) triggered a bottom up effect on *T. evansi*, increasing mite performance and the damage to the plant, following a similar pattern to that reported for the tomato cultivar MM

TABLE 2 | Effect of moderate drought, *T. evansi* infestation, and their combination (Dr + Te) on plant defense proteins of the tomato accessions TR126 and TR154 and the cultivar Moneymaker at 4 days post infestation.

	TR126				TR154				Moneymaker			
	Control	Drought	*T. evansi*	Dr + Te	SF	Control	Drought	*T. evansi*	Dr + Te	SF	Control	Drought	*T. evansi*	Dr + Te	SF
Protease inhibitors (% inhibition)															
Cathepsin B	47 ± 3	41 ± 2	55 ± 3	53 ± 3	M	46 ± 4	36 ± 2	58 ± 2	53 ± 3	D,M	42 ± 4	38 ± 5	54 ± 4	45 ± 5	–
Papain	66 ± 5	46 ± 7	71 ± 5	61 ± 11	–	49 ± 5	40 ± 6	70 ± 7	61 ± 11	M	50 ± 8	45 ± 9	72 ± 7	64 ± 5	M
Cathepsin D	36 ± 3	55 ± 4	47 ± 3	59 ± 5	D	47 ± 3	47 ± 5	65 ± 4	59 ± 5	M	45 ± 4	55 ± 5	67 ± 6	61 ± 5	M
Trypsin	24 ± 1	32 ± 2	27 ± 1	33 ± 2	D	36 ± 5	31 ± 1	36 ± 2	33 ± 2	–	32 ± 5	29 ± 3	36 ± 4	29 ± 4	–
Chymotrypsin	33 ± 5	35 ± 3	52 ± 5	49 ± 5	M	44 ± 3	38 ± 3	51 ± 5	49 ± 5	–	40 ± 2	29 ± 5	49 ± 5	43 ± 3	M
Aminopeptidase	36 ± 2	42 ± 1	34 ± 2	36 ± 1	D,M	31 ± 2	39 ± 6	41 ± 1	36 ± 1	–	37 ± 3	36 ± 3	43 ± 5	41 ± 3	–
Oxidative enzymes (specific activity)															
Polyphenol oxidases[1]	5.8 ± 0.7	7.4 ± 1.0	5.2 ± 0.7	5.1 ± 0.7	–	4.8 ± 0.6	5.5 ± 0.7	6.2 ± 1.0	5.1 ± 0.6	–	4.8 ± 0.5	6.2 ± 0.4	5.4 ± 0.7	5.0 ± 0.5	–
Peroxidases[2]	3.5 ± 0.4	3.2 ± 0.5	3.3 ± 0.4	3.6 ± 0.5	–	2.9 ± 0.3	3.8 ± 0.3	4.0 ± 0.6	4.0 ± 0.2	–	2.4 ± 0.3*a*	3.6 ± 0.4*b*	2.9 ± 0.3*ab*	2.8 ± 0.3*ab*	I

Data are mean ± SE of 6 replicates/treatment. Significant factors (SF) indicates whether either of the two independent factors D (drought condition) and M (mite infestation) and/or their interaction I (D × M) are statistically significant for a given cultivar/accession (two-way ANOVA, $p < 0.05$). When the interaction D × M was significant, Newman-Keuls post hoc test were performed (different lowercase letters indicate significant differences among treatments). The detailed results of the two-way ANOVA (F and p values and degrees of freedom) are shown in ***Supplementary Tables S6–S8****.* [1]*PPO: nmol cathecol metabolized/mg protein*min.* [2]*POD: nmol guaiacol metabolized/mg protein* min.*

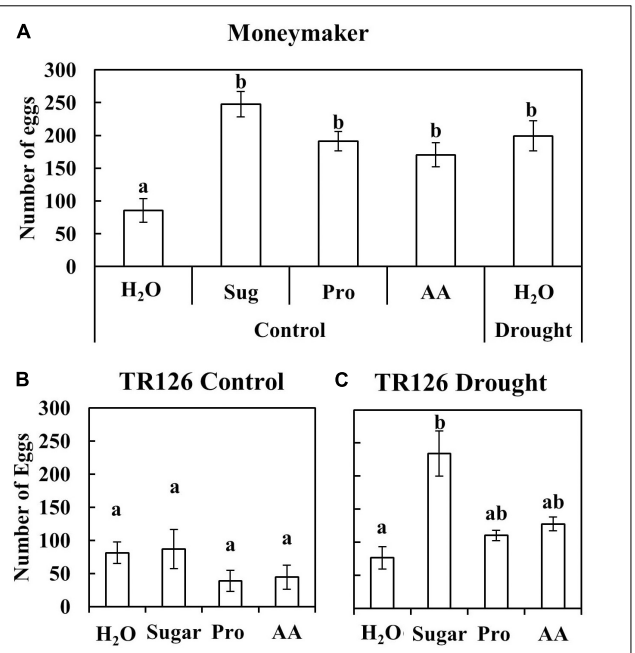

FIGURE 5 | Number of eggs laid by *T. evansi* on leaflets from well-watered (Control) and drought-stressed (Drought) Moneymaker **(A)**, TR126 (Control) **(B)**, and TR126 (Drought) **(C)** tomato plants infiltrated with water (H_2O), free sugars (Sug), amino acids (AA), and L-proline (Pro). Data are mean ± SE of five replicates/treatment at 24 h post infiltration. Different lowercase letters indicate significant differences among treatments (one-way ANOVA, Dunnett *post hoc* test, $p < 0.05$. **(A)** Moneymaker: $F_{4,20}$: 9.52, $p < 0.001$; **(B)** control TR126: $F_{3,16}$: 1.22, p: 0.335; **(C)** drought-stressed TR126: $F_{3,15}$: 5.94, p: 0.007).

(Ximénez-Embún et al., 2016). By contrast, no increase in mite performance was observed in TR58 and TR126, since no differences in the number of eggs laid and leaf damage were found between drought-stressed and control tomato plants. Drought stress has been reported to be positive, negative, or to have no effect on mite performance, depending on the mite species, the host plant, and the stress level (see Ximénez-Embún, 2017 for a review). However, in the case of tomato, the effect of drought conditions has been reported to be positive for three different mite species: *T. evansi* (Ximénez-Embún et al., 2016), *T. urticae* (Ximénez-Embún et al., 2017a), and *Aculops lycopersici* (Gispert et al., 1989; Ximénez-Embún et al., 2017b). This could have significant implications for mite outbreaks under future climate change scenarios, when longer periods of drought and less water availability are expected for irrigated crops such as tomato in semiarid environments. The finding that some accessions (such as TR126) were not more susceptible under stress conditions offers alternatives for pest management and provides an experimental framework for screening for drought-tolerant tomato accessions that are also resistant to herbivore mites.

A link could be observed between the nutritional composition of MM and the two accessions analyzed (TR154 and TR126) and *T. evansi* performance on these plants. Tomato plants have been reported to metabolize protein and starch into amino acids and free sugars, respectively, in response to drought stress

(Bauer et al., 1997; Khodakovskaya et al., 2010; Ximénez-Embún et al., 2016). We have recorded an increase in the levels of free sugars and most essential amino acids for mites in MM (eight of a total of nine) and of most essential amino acids in TR154 (seven of nine) in response to drought stress, associated with a better performance of *T. evansi* on these plants. However, sugars were not altered and a comparatively reduced number of essential amino acids (four of nine) were induced by drought in TR126, an accession in which mite proliferation was not enhanced by drought stress. The positive effect of essential amino acids concentration on mite performance has been previously observed in *T. evansi* (Ximénez-Embún et al., 2016) and *T. urticae* (Tulisalo, 1971; Dabrowski and Bielak, 1978; Ximénez-Embún et al., 2017a). A special mention should be made to L-proline, traditionally used as a good indicator of plant response to drought (Claussen, 2005). We have found that this non-essential amino acid was induced in both TR126 and TR154, as well as in MM, indicating that the induction of L-proline alone is not sufficient for explaining the observed increase in the performance of *T. evansi*. Remarkably, we have shown that free sugars, L-proline, and essential amino acids had a stimulatory effect on *T. evansi* performance, when supplemented to control tomato MM leaflets, similar to that obtained in plants subjected to drought, supporting their role in enhancing mite performance as already reported for L-proline (Ximénez-Embún et al., 2016). However, only free sugars improved mite performance in TR126 when infiltrated into drought-stressed plants, whereas no stimulatory effect was observed when infiltrated in non-stressed plants. These results suggest that free sugars have a stronger stimulatory effect than L-proline and essential amino acids or that the response of the mites to these different nutrients depends on the tomato genetic background. Taken together, our results indicate that the accumulation of plants nutrients appears to play a key role in the higher suitability of drought-stressed tomato plants for *T. evansi*, though the contribution of L-proline, essential amino acids, and free sugars may vary among tomato genotypes. However, the fact that the infiltration of nutrients did not increase *T. evansi* performance under well-watered conditions in TR126 suggest that other factors can be involved. Thus, additional studies will be necessary to explain the mechanisms behind this association.

Plant defense is an important issue to be considered when assessing the plant response to biotic and abiotic stresses. We have investigated plant defense at three levels: at the phytohormone level, at the marker gene expression level and at the defense-protein activity level. The levels of defense-related phytohormones (OPDA, JA, SA, and SAGE) and the expression of SA-dependent (*PR1a*) and JA-dependent (*MYC2* and *PPO-F*) genes were not induced in MM after *T. evansi* infestation. This is in line with the reported suppression of plant defenses by *T. evansi* in tomato (Kant et al., 2015), including the downregulation of phytohormones and most of the defense genes analyzed (Sarmento et al., 2011; Alba et al., 2015; Ataide et al., 2016; Schimmel et al., 2017). However, we have found that mite infestation induced the expression of *CDI* and *PI-Ia* genes, which encodes for protease inhibitors, and increased cathepsin D, chymotrypsin, and papain inhibitory activities in MM. Thus, our results with MM corroborate the downregulation of defense-related phytohormones by *T. evansi*, but show that some defense genes can still be induced and plat defenses are not fully suppressed. Indeed, the induction of protease inhibitory activities in MM by *T. evansi* infestation has been previously reported (Ximénez-Embún et al., 2016). The ability of *T. evansi* to suppress the induction of defense-related phytohormones was also observed in the two TR accessions, with the exception of an increase in SA levels in TR126, though SAGE levels were not altered. The expression of the *CDI* and *PI-Ia* genes was also induced by *T. evansi* in both TR126 and TR154, as well as *PR1a* in TR154 and some of the protease inhibitory activities (cathepsin B and chymotrypsin in TR126 and cathepsin B, papain, and cathepsin D in TR154). This may have implications for tomato-mite interactions, since PIs are recognized as key components of the defensive response of tomato to mite infestation (Kant et al., 2004; Santamaria et al., 2012, 2015; Alba et al., 2015). *T. evansi* relies mostly on cysteine (cathepsin B, and L- and legumain-like) and aspartyl (cathepsin D-like) proteases and aminopeptidases for proteolytic digestion (Ximénez-Embún et al., 2016). Thus, the ingestion of PIs targeting some of these proteases may be harmful, as already demonstrated for *T. urticae* (Carrillo et al., 2011; Santamaria et al., 2012). Serine proteases do not appear to be directly involved in the hydrolysis of dietary proteins in this species, but tomato serine PIs may target other physiological processes, as has been indicated for *T. urticae* which has a similar digestive proteolytic profile (Santamaria et al., 2012, 2015). Since differences in the induction of protein defense genes and activities were found among the different plant genotypes tested, it will be of interest for the control of this invasive mite species to broaden the range of tested commercial and wild tomato cultivars, including other drought-adapted tomato accessions and varieties.

Our results suggest an association between ABA induction and the changes triggered by drought stress in MM and TR154 tomato plants, causing an increase in mite performance. Thus, ABA levels were significantly increased in MM and TR154 tomato plants by drought stress, but not in TR126. It is known that ABA lead to the accumulation of free amino acids, including L-proline, and to the conversion of starch to maltose, though subsequent free sugars accumulation appears to be regulated in an ABA-independent manner (Krasensky and Jonak, 2012). In addition, free sugars such as glucose modulate vital processes that are controlled by plant stress hormones and have been involved in the control of ABA biosynthesis and signaling, exemplifying the complex interplay of sugar and hormone signaling (Cheng et al., 2002; León and Sheen, 2003). However, the observed differences may also result from differential time responses to the ABA accumulation and signaling pathway, since the ABA-responsive *RAB18* gene was induced in all cultivar/accessions in response to drought stress. Moreover, ABA may modulate plant responses to mite infestation since it has been involved in the response to several biotic stresses with a positive or negative effect on the plant responses depending of the biotic stress (de Torres-Zabala et al., 2007; Ton et al., 2009). Further studies will be required to confirm the contribution of ABA in enhancing mite performance under drought conditions.

Salicylic acid and jasmonate, the two main regulators of biotic stress responses, have been also reported to be involved in the plant response to drought, interacting at different levels with the ABA signaling pathway on tomato plants (Muñoz-Espinoza et al., 2015; De Ollas and Dodd, 2016). However, our results suggest that the changes operated in MM and TR154 tomato plants by drought stress, causing an increase in mite performance, are independent of SA and JA signaling. The SA signaling pathway regulates the expression of a large set of defense-related genes, including pathogen-related proteins (PRs), and has been involved in tomato response defense to mites (Glas et al., 2014; Alba et al., 2015), though the mechanism of action is not clearly understood. We have found that the levels of SA and SAGE on TR126 and TR154 plants were threefold to fivefold lower than on MM under control conditions. However, SA levels were strongly induced by drought in both TR accessions, whereas drought induced SAGE levels in TR154 and reduced them in TR126. On the contrary, the levels of these two hormones were not altered by drought stress in MM, as previously reported (Ximénez-Embún et al., 2017b). In addition, the expression of the SA-dependent *PR1a* gene was induced by drought in TR126 and by mite infestation in TR154, but not in MM. The observed differences between the two TR accessions and MM may be related to their distinct antioxidant status during control and/or drought stress conditions, since SA has been reported to mediate the response to drought stress providing protection against oxidative damage in tomato plants (Hayat et al., 2008). The JA pathway has been reported to play a key role in tomato plant resistance to mites, mediated by the induction of plant defense marker genes and proteins (Glas et al., 2014; Alba et al., 2015). However, our results suggest a complex regulation of the JA pathway in the two TR accessions and MM in response to drought stress. We found that drought stress reduced the levels of the JA precursor OPDA in MM and TR126 and that significantly lower JA levels occurred on uninfested drought-stressed TR126 plants. However, the *CDI* gene was induced by drought in all tomato cultivar/accessions tested, and the *PI-Ia* gene by drought in MM and when both stresses were combined in TR154. Moreover, trypsin, cathepsin D, and aminopeptidase inhibitory activities were induced in TR126 by drought stress, cathepsin B inhibitory activity was reduced by drought stress in TR154, and POD activity was induced in uninfested drought-stressed MM plants. The potential implications of the ingestion of the induced PIs in mite performance have been discussed above. With regard to antioxidant enzymes, POD activity has been reported to be induced in tomato by drought stress (English-Loeb et al., 1997; Inbar et al., 2001). The production of PODs is an adaptive mechanism for the scavenging and detoxification of reactive oxygen species in drought-stressed plants (Rai et al., 2013), but its induction might vary depending on the tomato plant genotype (Sanchez-Rodriguez et al., 2010). This might explain the absence of induction in the case of TR126 and TR154 plants.

Altogether, our data revealed differential plant-mediated effects of water deficit on the performance of *T. evansi* on drought-adapted "Tomàtiga de Ramellet" tomatoes. These findings have important implications for decision making in the selection of tomato cultivars to be planted in a forthcoming climate change scenario, as their differential response to water deficit might speed up or slow down the expansion of this important invasive mite species in extensive tomato production. In addition, the plant-mediated effects reported here may have ecological implications, since it is known that the suppression of tomato plant defenses by *T. evansi* have effects on its competition with other spider mites (Oliveira et al., 2016).

AUTHOR CONTRIBUTIONS

PC, FO, MX-E, and MG-G conceived and designed the experiments. MX-E and MG-G performed the experiments. VA and AG-C performed the phytohormone experiments. PC, FO, MX-E MG-G, VA, and AG-C analyzed the data. PC, FO, MG-G, VA, and AG-C contributed reagents, materials, and analysis tools. PC, FO, MX-E, and MG-G wrote the paper. All authors revised the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01490/full#supplementary-material

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The Beneficial Endophytic Fungus *Fusarium solani* Strain K Alters Tomato Responses Against Spider Mites to the Benefit of the Plant

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Beneficial microorganisms are known to promote plant growth and confer resistance to biotic and abiotic stressors. Soil-borne beneficial microbes in particular have shown potential in protecting plants against pathogens and herbivores via the elicitation of plant responses. In this study, we evaluated the role of *Fusarium solani* strain K (FsK) in altering plant responses to the two spotted spider mite *Tetranychus urticae* in tomato. We found evidence that FsK, a beneficial endophytic fungal strain isolated from the roots of tomato plants grown on suppressive compost, affects both direct and indirect tomato defenses against spider mites. Defense-related genes were differentially expressed on FsK-colonized plants after spider mite infestation compared to clean or spider mite-infested un-colonized plants. In accordance, spider mite performance was negatively affected on FsK-colonized plants and feeding damage was lower on these compared to control plants. Notably, FsK-colonization led to increased plant biomass to both spider mite-infested and un-infested plants. FsK was shown to enhance indirect tomato defense as FsK-colonized plants attracted more predators than un-colonized plants. In accordance, headspace volatile analysis revealed significant differences between the volatiles emitted by FsK-colonized plants in response to attack by spider mites. Our results highlight the role of endophytic fungi in shaping plant–mite interactions and may offer the opportunity for the development of a novel tool for spider mite control.

Keywords: endophyte, *Fusarium*, gene expression, performance, spider mites, tomato, volatiles

INTRODUCTION

Plants have evolved sophisticated mechanisms to defend themselves against biotic stressors such as pathogenic microorganisms and herbivorous arthropods. In particular, the ways plants respond to herbivory involve the expression of direct defenses such as toxins and anti-digestive proteins that target the herbivore but also indirect defenses to attract the natural enemies of the attacker to the

plant via, for example, the emission of herbivore-induced plant volatiles itself (Karban and Baldwin, 1997; Schaller, 2008; Dicke and Baldwin, 2010). Direct and indirect defenses can be constitutively produced and/or specifically induced after attack (Karban and Baldwin, 1997; Erb et al., 2012). For example, many defense mechanisms are initiated upon recognition of the attacker after which downstream defense signaling is activated leading to, for example, the production of defensive compounds that negatively affect the attacker (Wu and Baldwin, 2010). The phytohormones jasmonic acid (JA) and salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) are key regulators in plant defense against herbivores, modulating afterwards the expression of defense-related genes and the production of defensive compounds (Erb et al., 2012; Pieterse et al., 2014). Importantly, cross-talk among the phytohormonal pathways (e.g., antagonistic relationships between the JA and SA pathways) allows plants to fine-tune their defensive responses depending on the organisms encountered in a multi-species environment (Pieterse et al., 2012).

Plant defense production is generally assumed to be a costly process that requires the allocation of valuable resources to resistance at the expense of growth and reproduction (Cipollini et al., 2003; Walters and Heil, 2007; Pappas et al., 2017). In addition to physiological costs, i.e., those related to energy investment, ecological costs, such as the disturbance of plant interactions with other organisms (Agrawal et al., 1999; Thaler et al., 1999; Ballhorn et al., 2014; Ohm and Miller, 2014), both, may ultimately result in reduced plant performance (Herms and Mattson, 1992). To minimize plant defense related costs, the majority of defenses are activated after herbivore attack only. Besides energy savings, defense induction may also protect plants from auto-toxicity and, importantly, allows tailoring of plant responses against specific attackers (Baldwin and Callahan, 1993; Pappas et al., 2017). In addition, defense priming, a physiological state of readiness that takes place after initial exposure to a stressor that prepares plants for a subsequent stress, is an additional strategy that plants have evolved against herbivory (Heil and Kost, 2006; Frost et al., 2008). Eventually, primed plants are able to respond faster, stronger and thus more effectively to certain attackers compared to non-primed plants, often at a lower cost to the plant (Martinez-Medina et al., 2016).

Priming of defenses can occur after initial exposure of plants to harmful herbivores or pathogens but also, when plants are exposed to beneficial non-pathogenic organisms. Selected root-colonizing microbes (e.g., bacteria and fungi) have long been recognized for their ability to antagonize soil-borne pathogens, facilitate nutrient uptake, improve plant growth, and also prime the plant immune system against aboveground future attackers in return for carbohydrates secreted by the plant (Smith and Smith, 2011; Pineda et al., 2013; Pieterse et al., 2014; Finkel et al., 2017). For example, defense priming by plant-growth promoting rhizobacteria (PGPR), generally referred to as induced systemic resistance (ISR), is characterized by increased acceleration of defense-related genes upon herbivore and pathogen attack and generally known to be JA-regulated, not shown to trade-off with plant fitness (Rosenblueth and Martínez-Romero, 2006; Van Wees et al., 2008). In addition, other microbes such as plant-growth promoting fungi (PGPF) and arbuscular mycorrhizal fungi (AMF) have been shown to variously impact herbivorous arthropods on aboveground plant parts. As such, soil-borne beneficial microbes are of particular interest as 'vaccination' agents, capable of enhancing plant resistance to biotic stressors most possibly without compromising crop production.

Mechanisms involved in plant defense induction by beneficial soil microbes mediate both direct and indirect responses against aboveground herbivores (Pineda et al., 2010; Rasmann et al., 2017; Shikano et al., 2017). Microbe-ISR can be directly effective against insects and mites because it involves an increased sensitivity to JA (Rosenblueth and Martínez-Romero, 2006; Van Wees et al., 2008). Thus, chewing herbivores but also phloem feeders (e.g., aphids, whiteflies), that normally counteract JA-defenses via crosstalk, can be negatively impacted by JA-mediated plant responses induced by beneficial microbes (Pineda et al., 2010). In addition, such plant-mediated effects have been shown to not only depend on the microbe group (e.g., PGPR or AMF) but also on the feeding specialization of the herbivore. For example, AMF are believed to show negative effects against generalists and mesophyll feeders and positive or neutral effects on specialist chewers and phloem feeders (Hartley and Gange, 2009; Pineda et al., 2010; Shikano et al., 2017). On the other hand, JA is also involved in indirect defense responses against herbivores and thus it is reasonable to expect that microbe-ISR is capable of altering the composition or the emission rate of the volatile blend emitted by microbe-colonized plants in response to herbivory (Pineda et al., 2010; Rasmann et al., 2017). Indeed, selected soil-borne microbes have been shown to modify the volatile blends thereby increasing the attractiveness of the infested plants to the natural enemies of the attacker (e.g., Fontana et al., 2009; Schausberger et al., 2012; Pineda et al., 2013). Whether both direct and indirect defenses of a plant against a particular herbivorous species can be affected by a single microbe species via ISR remains largely unknown.

Despite the vast diversity of soil-borne beneficial microbes that are associated with plants, much of our current knowledge about microbe-ISR effects on herbivores derives from studies on two microbial groups mainly, PGPR and AMF. Nevertheless, a number of diverse endophytic fungi are known to also inhabit roots, forming variable associations with the plants, ranging from parasitic to mutualistic, without, however, causing apparent disease symptoms in plants (Wilson, 1995; Schulz and Boyle, 2005; Hartley and Gange, 2009; Rodriguez et al., 2009). In contrast to AMF, the ecological roles of the most common endophytic fungi, especially those that are horizontally transmitted via spores (e.g., Ascomycetes), currently remain elusive although generally believed to also play an important role in plant protection against herbivores (Jaber and Vidal, 2009; Rodriguez et al., 2009; Gan et al., 2017). Indeed, certain root endophytic fungi have been shown to increase the expression of defense-related genes and the production of secondary metabolites that may be relevant to plant defense (Pieterse et al., 2014). In addition, a few studies involving endophytic fungi have reported negative effects on above ground herbivores thus enhancing their potent role in plant resistance to biotic stressors

(Jallow et al., 2004; Jaber and Vidal, 2009, 2010; Muvea et al., 2014; Coppola et al., 2017; Contreras-Cornejo et al., 2018). Nevertheless, our understanding of endophytic fungi – plant – herbivore interactions is still at its infancy thus calling for more empirical studies on the significance of horizontally transmitted endophytes in plant–herbivore interactions (Gan et al., 2017).

In this study, we hypothesized that tomato responses to spider mites can be enhanced by soil-borne beneficial microbes, particularly endophytic fungi. Spider mites are mesophyll cell-content feeders and many species are major pests in agriculture. Specifically, the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) is a cosmopolitan species that infests a high number of crops belonging to different plant families. In tomato, *T. urticae* induces JA and SA defenses simultaneously and has been shown to be highly sensitive to JA-mediated defenses (Kant et al., 2008; Alba et al., 2015; Ataide et al., 2016). Besides direct defense responses, tomato also activates volatile production in response to *T. urticae* feeding. This results in spider mite-infested plants being highly attractive to its natural enemies, such as the predatory mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) (Kant et al., 2004). To the best of our knowledge, plant-mediated effects of soil-borne microbes on spider mites have been scarcely addressed so far, mainly for AMF (Hoffmann et al., 2009, 2011; Schausberger et al., 2012; Khaitov et al., 2015), and no study has ever dealt with beneficial root endophytic fungi in tomato.

We thus, assessed the impact of the endophytic fungus *Fusarium solani* strain K (FsK) on the performance of *T. urticae* on tomato and recorded the changes in defense-related gene expression on FsK-colonized compared to control plants. Furthermore, we analyzed the volatile blends emitted from FsK-colonized and control plants and recorded the responses of the zoophytophagous predator *Macrolophus pygmaeus*, a natural enemy of spider mites, toward these plants. *Fusarium solani* strain K is an horizontally transmitted endophytic fungal isolate that colonizes tomato roots, including vascular tissues to the crown area, without fungal growth progressing to aboveground tissues (Kavroulakis et al., 2007). In tomato, FsK is known to confer ethylene-dependent resistance against fungal root and foliar pathogens (Kavroulakis et al., 2007). In addition, FsK-colonized plants are more resistant to plant damage caused by the zoophytophagous predator *Nesidiocoris tenuis*, possibly via the JA and/or ethylene signaling pathways (Garantonakis et al., 2018). We thus, hypothesized that FsK may be effective against herbivores too and included spider mites in our experiments to first, assess FsK potential on impacting spider mite performance but also, to explore putative mechanisms involved in FsK-tomato-spider mite interactions.

MATERIALS AND METHODS

Plants and Growing Conditions

Tomato [*Solanum lycopersicum* L., cv. Ace 55 (Vf)] plants were used in all experiments as well as in herbivore and predator rearing. Experimental plants were grown from seeds that were surface-sterilized in 2.5% NaOCl and sown directly in pots (Ø 12 cm), each containing approximately 300 cm^3 of a sand mixture with vermiculite (2:1) and a N-P-K fertilizer (20–20–20) to a total concentration of 0.8 $gl^{-}1$ of potting mix. Plants used for rearing arthropods were grown from seeds in pots (Ø 12 cm) with soil (Klasmann-TS2). All plants were maintained in separate climate chambers (25 ± 2°C, 16:8 LD, 60–70% RH) and watered every other day and once a week fertilized. Experimental plants were fertilized with a balanced nutrient solution (Hoagland 100%) and a N-P-K fertilizer (20–20–20) was used to fertilize plants grown to rear arthropods. Plants used in the experiments were 4–5 weeks old.

Fungal Strain, Plant Inoculation and Quantification of Fungal Colonization

Experimental tomato plants were inoculated with the endophytic non-pathogenic *F. solani* strain FsK (Kavroulakis et al., 2007) routinely cultured on potato dextrose broth (PDB) at 25°C for 5 days in the dark. Following removal of mycelium fragments by sieving, conidia were recovered by centrifugation at 4000 *g*, counted using a haemocytometer and suspended in an appropriate volume of 0.85% NaCl in order to achieve the desired inoculum concentration. Application of the inoculum of strain FsK with 10^2 conidia cm^{-3} of potting mix was performed as water drench 1 week after seed sowing. FsK colonization was verified with destructive sampling of 10 plants per batch and treatment (FsK-colonized and control plants) for all experiments by PCR 2 weeks after seed sowing and colonization levels were estimated 4 days after spider mite infestation by means of qPCR.

Samples were used for whole genomic DNA extraction using the "NucleoSpin® Plant II genomic DNA extraction" kit (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany). FsK colonization of root tissues after infestation and spider mite feeding was assessed via qPCR by using primers pair for a ca 170 bp fragment of the *Nectria haematococca* translation elongation factor 1a (*Tef-1a*) gene (**Supplementary Table S1**). An external standard curve was generated in order to quantify the copy number of *Tef-1a* gene in total DNA extracted from root tissues of FsK-colonized plants. The standard curve was generated as follows: *Tef-1a* gene was amplified using FsK genomic DNA as template, the PCR product was purified and ligated into pGEM-T Easy vector (Promega, Madison, United States) and transformed to competent *Escherichia coli* DH5a cells. The recombinant plasmid was extracted again (NucleoSpin Plasmid, Macherey Nagel) and its concentration was determined via Qubit 3.0 Fluorometer. The copy numbers of the targeted gene were calculated from the concentration of the extracted plasmid DNA. Amplification occurred in a 10 μl reaction mixture containing Kapa SYBR FAST qPCR Master Mix (1x) Universal, 200 nM of each primer, and 1 μl of DNA, using the following thermocycling protocol: 3 min at 95°C; 45 cycles of 15 s at 95°C, 20 s at 58°C followed by a melting curve to check the specificity of the products. PCR products were further analyzed on a 1.5% agarose gel in order to check for potential non-targeted amplifications. Data were analyzed using the Student's two-tailed homoscedastic *t*-test to compare the colonization of FsK with the +/− spider mite-infested group.

Herbivore and Predator Rearing

Spider mites (*T. urticae*) were reared on detached tomato leaves on wet cotton wool inside plastic trays that were kept in a climate room at 25 ± 2°C, 16:8 LD, 60–70% RH. Fresh tomato leaves were provided every 3 days and the trays were filled with water to maintain leaf vigor. In this study, we used the 'KOP' spider mite line kindly provided by Dr. Merijn Kant (University of Amsterdam). This is a tomato-adapted line previously shown to resist JA defenses in tomato (Ament et al., 2004; Kant et al., 2008). For all experiments, adult female mites (2–4 days old) were used. These were obtained by infesting tomato plants with a high number (approximately 200) of female spider mites that were allowed to lay eggs for 48 h at 25 ± 2°C, 16:8 LD. The next day, the adult mites were carefully removed and the plants were maintained at the same conditions till adult spider mites emerged (after approximately 16 days).

Macrolophus pygmaeus, a zoophytophagous predator that feeds both on prey and plant was reared on young tomato plants (2-weeks old) in plastic cages (47.5 cm × 47.5 cm × 47.5 cm, BugDorm MegaView Science Co., Ltd.) maintained at 25 ± 2°C, 16:8 LD, 60–70% RH, as described by Pappas et al. (2015). The rearing was established with adults of the commercially available product MIRICAL (Koppert B.V. Berkel en Rodenrijs, Netherlands). Bee pollen and eggs of *Ephestia kuehniella* were provided *ad libitum* as supplementary food for the predators. For the olfactometer experiments, we used young female predators (7–10 days old) that were obtained by allowing 5 predator females to lay eggs on young tomato plants for 48 h. Emerging nymphs were fed with *E. kuehniella* eggs sprinkled on tomato plants until adulthood.

Herbivore Performance and Feeding Damage

Spider mite performance on tomato plants that were colonized by the endophyte was assessed by infesting FsK-colonized and control (un-colonized) tomato plants with 45 female spider mites per plant on 3 leaflets [15 females per leaflet, leaflets were selected as described by Alba et al. (2015)] for a period of 4 days. Subsequently, the number of eggs and live females per plant were recorded. Spider mites were prevented from escaping by a lanolin circle applied around the petiolule of each leaflet. Thirteen plants from two independent experiments were used per treatment.

Feeding damage inflicted by spider mites on FsK- colonized and control plants was recorded after 10 days when 10 female spider mites per leaflet (3 infested leaflets, thus 30 females per plant) had been feeding on tomato plants, as described above. Eight plants from two independent experiments were used per treatment. All spider mite-infested leaflets were collected and scanned digitally. Leaf area damage was assessed as described by Cazaux et al. (2014).

Means (number of surviving spider mites, number of eggs, feeding damage) were compared by Student's *t*-test (SPSS, 2011). Shapiro–Wilk test was used to verify the normality of error distribution.

Plant Growth Parameters

To assess the extent to which plant growth parameters (root and shoot biomass) are affected by fungus colonization and/or spider mite infestation, 4–5 weeks old FsK-colonized and control (un-colonized) tomato plants were infested with 30 female spider mites (10 females per leaflet, 3 leaflets per plant) for a period of 10 days. Subsequently, plant shoot tissue was harvested and weighed on a microbalance. In addition, roots were harvested, cleaned in water, dried on tissue paper and weighed. Four treatments in total were included in this experiment: FsK-colonized plants (+F/−T), FsK-colonized and spider mite infested plants (+F/+T), un-colonized and spider mite-infested plants (−F/+T) and clean plants (−F/−T). Eight plants from two independent experiments were used per treatment. Differences in shoot and root weight among treatments were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's HSD *post hoc* tests ($P < 0.05$). Prior to data analysis Shapiro–Wilk and Levene's tests were used to verify the assumptions of parametric analysis, i.e., normality of error distribution and equality of variances, respectively (R Core Team, 2016).

Tomato Defense-Gene Expression

FsK-colonized and control tomato plants (4–5 weeks old) were infested with 45 spider mites, as described above for the performance experiments. Another set of FsK-colonized and control plants received no spider mite treatment. This experiment was conducted in a climate room at 25 ± 2°C, 16:8 LD and 60–70% RH. After 4 days of spider mite feeding, infested leaflets as well as leaflets of the same position on uninfested plants, were harvested, flash frozen on dry ice and stored at −60°C until mRNA extraction ($n = 6$ biological replicates per treatment). The three leaflets harvested from the same plant were pooled to form one biological replicate. The experiment was repeated with the same experimental set-up one month later.

To explore tomato defenses, we analyzed the expression of the following genes: *JIPI-21*, *WIPI-II*, *PI-IIc*, *PPO-D*, *PPO-F*, *LOXD*, *PR-1A*, *PR-P6*, *GGPS1*, *GLU-A*, *GLU*-B, *CHI3,* and *CHI9*. RNA was extracted from plant tissues using a LiCl protocol according to Brusslan and Tobin (1992). RNA samples were treated with DNase I from Thermo Scientific as follows: samples were incubated in 37°C for 30 min, the tubes were transferred on ice and 1 μl EDTA 50 mM was added before the inactivation of the DNase at 65°C for 10 min. In order to ensure no genomic DNA was left, a PCR was performed using primers specifically designed to amplify the tomato housekeeping gene *ubiquitin*. cDNA was made with a 1st-strand cDNA synthesis kit from TAKARA using an oligo-dT primer according to the manufacturer's instructions. Quantitative PCR was conducted with a SYBR-Fast kit from Kappa Biosystems according to the manufacturer's instructions on a Bio-Rad CFX Connect Real Time thermo-cycler. The sequences of gene-specific primers used in RT-PCR analysis are shown in **Supplementary Table S1**. The resulting first-strand cDNA was normalized based on expression of the housekeeping gene *ubiquitin* (*UBQ*). Analysis was carried out as described in Delis et al. (2011) using the geometric mean of *ubiquitin* as reference gene. To calculate the fold-change in transcript

levels, the relative expression of each target gene was calculated for each sample as described, and the ratio of each transcript's relative expression was normalized to its expression in control samples. Differences in gene expression between treatments were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's HSD *post hoc* tests ($P < 0.05$). Data were tested for normality using the Shapiro–Wilk test (SPSS, 2011). Results of the two independent experiments are presented in **Figures 4**, **5**, **Supplementary Table S2** and **Supplementary Figure S2**. For the visualizations, data manipulation was performed in RStudio with the pheatmap package (version 1.0.8) (R Core Team, 2016; Kolde, 2018). Sample/gene grouping was based on hierarchical clustering (complete linkage algorithm) of the Euclidean sample/gene distances of the differentially expressed genes detected by ANOVA (Tukey's *post hoc* tests, $P < 0.05$).

Headspace Collection and Analysis of Tomato Volatiles

The collection of volatile organic compounds (VOCs) was performed at 25 ± 1°C and 60–70% RH between 10:00 am and 17:00 pm for treatments (1)–(5) mentioned below in 'Olfactometer Assays' (5–6 biological replicates in total per treatment) with a push-pull dynamic volatile collection system. The system consisted of 5 L glass chambers each containing one tomato plant. Pots were wrapped in aluminum foil to avoid trapping soil and plastic volatiles. Five independent chambers containing randomly assigned plants were run simultaneously. Charcoal-filtered, humidified air was pumped in the containers at a rate of 1 L/min and pull out at 0.6 L/min passing through stainless steel tubes loaded with 200 mg of Tenax (MARKES, Llantrisant, United Kingdom). The sampling duration was adjusted to 30 min. In addition, we also sampled volatiles from empty glass chambers. Those "air blanks" were used in the further data processing to exclude systemic contamination compounds.

Tomato volatiles were analyzed by a thermal desorption-gas chromatograph-mass spectrometer (TD-GC-MS) consisting of a thermodesorption unit (MARKES, Unity 2, Llantrisant, United Kingdom) equipped with an autosampler (MARKES, Ultra 50/50). Tubes were desorbed with helium as carrier gas and a flow path temperature of 150°C using the following conditions: Dry Purge 5 min at 20 ml/min, Pre Purge 2 min at 20 ml/min, Desorption 8 min at 280°C with 20 ml/min, Pre Trap fire purge 1 min at 30 ml/min, Trap heated to 300°C and hold for 4 min. The VOCs were separated on a gas chromatograph (Bruker, GC-456, Bremen, Germany) connected to a triple-quad mass spectrometer (Bruker, SCION). Separation took place on a DB-5MS column (30 m × 0.25 mm × 0.25 μm. Restek, Germany). The conditions of the GC were as follows: 40°C for 5 min, 5°C/min to 185°C, 30°C/min to 260, and hold for 0.5 min. The mass spectrometer was operated in full scan mode with the following parameters: transfer line temperature 280°C, ion source temperature 260°C, scan time 250 ms, scan range 40–550 m/z, ionization 70 eV.

We selected the most prominent peaks in the chromatograms (signal to noise ratio > 10). Peaks that were also present in air blanks were regarded as systemic contamination and were excluded from further analysis. This procedure resulted in 41 compounds. The peak areas of these compounds were calculated using the Bruker Workstation software (v8.0.1). PCA analysis was performed on autoscaled data in R (v3.3.2) (R Core Team, 2016) using the packages ggplot2 (v2.2.1) (Wickham, 2009) and ggfortify (v0.4.4) (Tang et al., 2016). Differences in the emissions of the selected compounds were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's HSD *post hoc* tests ($P < 0.05$). Prior to data analysis Levene's tests was used to verify the assumption of equality of variances (R Core Team, 2016).

Olfactometer Assays

To assess the extent that the volatile blend emitted by clean or spider mite-infested plants may be changed by FsK-colonization and eventually, the responsiveness of the mirid predator *M. pygmaeus* to these plants, we performed a series of vertical Y-tube olfactometer assays, as described by Lins et al. (2014). The Y-tube olfactometer (4.0 cm diameter, main arm 20 cm long, side arms 23 cm long, 75° angle between the side arms) was connected to a volatile collection system. Each side arm of the olfactometer was connected to a 4 L glass vessel containing one tomato plant. Each pot was wrapped with aluminum foil to restrict the emission of soil/plastic volatiles. Pressurized air was purified by passing through a wash bottle filled with activated charcoal pellets, humidified and entered the odor chambers at a rate regulated by means of a flowmeter of 2 L/min. From the outlet port at the top of the odor chamber the air was led to the arms of the olfactometer. At the base of the Y-tube the air was sucked off by means of a vacuum peristaltic pump, producing an air flow of 0.4 L/min in each side arm and 0.8 L/min in the base of main arm of the Y-tube. Teflon tubing was used for the connections between different parts of the set-up.

One predator female (5–7 days old) was introduced into the main arm of the olfactometer and allowed to make a choice between the two arms, i.e., volatile sources. Each female was considered to have made a choice when covering more than 12 cm inside each chosen arm. The females that did not make a choice within 10 min were excluded from data analysis. Each predator was used only once and had no visual contact with the plants during the bioassay since they were separated with a white panel. Before the bioassays the predators were starved for 24 h. We recorded 67–70 replicates (individuals) depending on the treatment (i.e., odor) combination. Every two replicates, the olfactometer side arms were switched to exclude positional effects. Every 10 female predators the Y-tube and the glass vessels were washed with ethanol (70%) and neutral soap and were allowed to dry before use. Olfactometer assays were performed in a room at 25 ± 1°C and 60–70% RH between 10:00 am and 17:00 pm. Predator responses were assessed for combinations of the following treatments: (1) FsK-colonized plants (+F/−T), (2) spider mite-infested, un-colonized plants (−F/+T), (3) FsK- colonized, spider mite-infested plants (+F/+T), (4) clean plants (−F/−T), (5) clean air (blank, i.e., no plant). For the Y-tube olfactometer bioassays, the null hypothesis that females of *M. pygmaeus* showed no preference for either arm of the olfactometer (i.e., 50:50 response) was tested using χ^2 test (SPSS, 2011).

RESULTS

Spider Mite Performance, Fungal Colonization and Feeding Damage

FsK-colonization affected spider mite performance with the number of eggs recorded on leaves of colonized plants within 4 days being significantly less than those on control (un-colonized) plants [**Figure 1A**; $t_{(24)} = -6.527$; $P < 0.001$]. In contrast, FsK-colonization did not affect the number of mites found alive on these compared to untreated control plants [**Figure 1B**; $t_{(24)} = 1.376$; $P = 0.182$]. Notably, spider mite infestation had no effect on FsK colonization compared to non-infested un-colonized plants [**Supplementary Figure S1**; $t_{(10)} = 0.179$; $P = 0.861$].

Tomato colonization by FsK had a significant effect on the damage inflicted by spider mites over the 10 days of feeding, which was reduced on colonized compared to un-colonized plants [**Figure 2A**; $t_{(14)} = 2.91$; $P < 0.05$]. Total leaflet area was similar between FsK-colonized and control plants [$t_{(14)} = 0.63$; $P = 0.535$] and feeding damage was reduced by approximately 28.7% resulting in a significant decrease in the proportion of damaged to total leaflet area compared to control plants [**Figure 2B**; $t_{(14)} = 2.89$; $P < 0.05$].

Shoot and Root Biomass

We tested whether tomato colonization by the endophyte would affect plant growth parameters of spider mite-infested or control (non-infested) plants. We found a significant endophyte effect ($F_{1,28} = 5.084$, $P = 0.032$), a highly significant herbivore effect ($F_{1,28} = 50.178$, $P < 0.001$) and no significant interaction effect ($F_{1,28} = 1.632$, $P = 0.212$) on shoot fresh weight. Spider mite-infested plants were heavier compared to control (non-infested) plants and FsK-colonization significantly affected shoot fresh weight (**Figure 3A**). In addition, two-way ANOVA revealed a significant endophyte effect ($F_{1,28} = 13.372$, $P = 0.001$), a highly significant herbivore effect ($F_{1,28} = 82.266$, $P < 0.001$) and a significant interaction effect ($F_{1,28} = 8.796$, $P = 0.0061$) on root fresh weight. Roots of spider mite-infested plants were heavier and this effect was significantly enhanced when these plants were also colonized by FsK (**Figure 3B**).

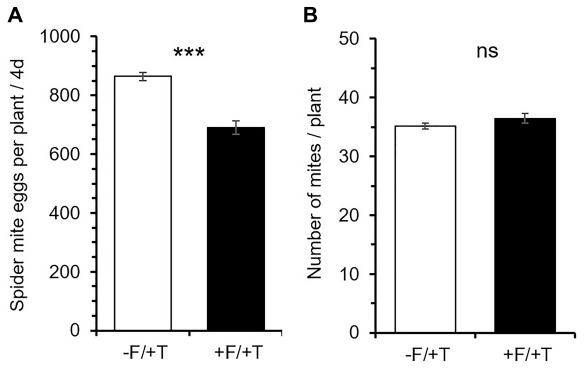

FIGURE 1 | Effects of tomato colonization by the endophyte *Fusarium solani* strain K (FsK) on spider mite (*Tetranychus urticae*) performance. One week after seed sowing plants were either inoculated with the endophyte (black bars, +F) or not (white bars, –F). Subsequently, plants were infested with 45 spider mites (15 mites per leaflet, 3 leaflets per plant, +T) when 4–5 weeks-old for a period of 4 days. Mean ± SE ($n = 13$) of the **(A)** number of spider mite eggs per plant and **(B)** number of live adult females per plant recorded on FsK-colonized and control plants after 4 days. Significant differences between treatments are indicated by asterisks after Student's *t*-test: ***$P < 0.001$, ns, not significant.

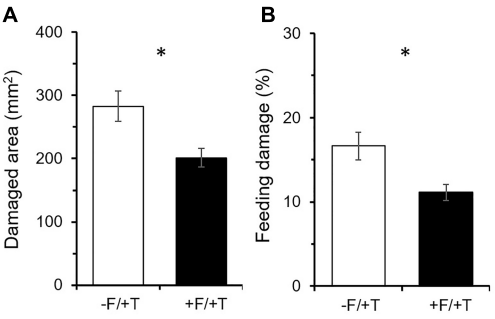

FIGURE 2 | Effects of tomato colonization by the endophyte *Fusarium solani* strain K (FsK) on the feeding damage inflicted by spider mites (*Tetranychus urticae*). One week after seed sowing plants were either inoculated with the endophyte (black bars, +F) or not (white bars, –F). Subsequently, plants were infested with 30 spider mites (10 mites per leaflet, 3 leaflets per plant, +T) when 4–5 weeks-old for a period of 10 days. Mean ± SE ($n = 8$) of the **(A)** damaged area per plant and **(B)** proportion of damaged to total leaflet area inflicted by spider mites on FsK-colonized compared to control plants over a period of 10 days. Significant differences between treatments are indicated by asterisks after Student's *t*-test: *$P < 0.05$.

Tomato Defense-Gene Expression

To assess the effects of FsK-colonization on defense-gene expression in response to spider mite feeding, we used well-established defense marker genes that are known to mark mite-activated JA and SA defenses in tomato (Li et al., 2002; Ament et al., 2004; Kant et al., 2004, 2008; Alba et al., 2015; Martel et al., 2015). In addition, our study included the genes *GLU-A* and *GLU-B*, *CHI3* and *CHI9*, typically induced against fungi or other herbivores [e.g., whiteflies, Puthoff et al. (2010)].

We found that the expression levels of genes *JIP-21*, *WIPI-II*, *PI-IIc,* and *LOXD,* previously shown to be induced by spider-mites (Kant et al., 2008; Alba et al., 2015; Martel et al., 2015), were also up-regulated in response to spider mite feeding in our study (**Figures 4**, **5**, **Supplementary Figure S2** and **Supplementary Table S2**). Nevertheless, other genes previously reported to be activated or induced by spider mites, such as the *PR-1A*, *PR-P6, PPO-D/F,* and *GGPS1* (Kant et al., 2004; Alba et al., 2015) were not consistently altered by the herbivore (**Figures 4**, **5**, **Supplementary Figure S2** and **Supplementary Table S2**).

Tomato colonization of spider mite-infested plants by FsK resulted in a significant further up-regulation in the expression of genes *WIPI-II* and *PPO-D* compared to the un-colonized but spider mite-infested plants (**Figures 4**, **5** and **Supplementary Table S2**). No effect was recorded in the expression of the other mite-defense related genes such as *JIP-21*, *PPO-F, LOXD, PR-1A*,

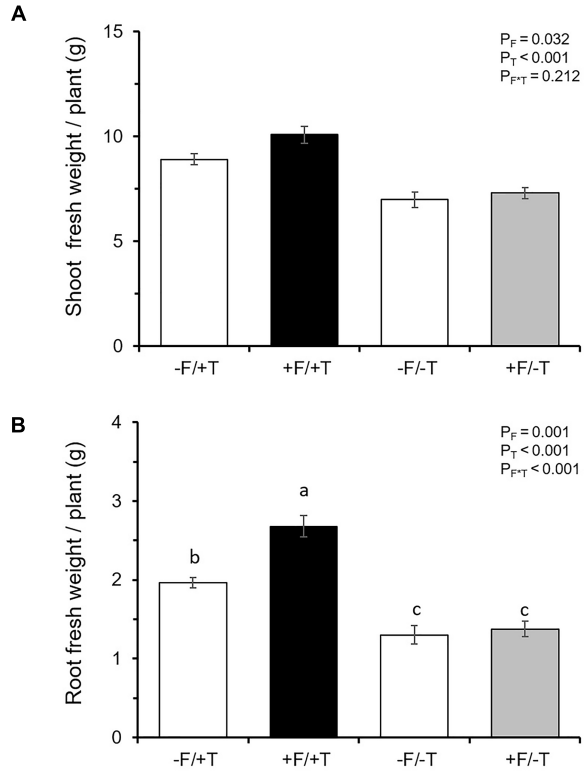

FIGURE 3 | Effects of spider mite infestation and tomato colonization by the endophyte *Fusarium solani* strain K (FsK) on plant growth parameters. One week after seed sowing plants were either inoculated with the endophyte (black and gray bars, +F) or not (white bars, –F). Subsequently, plants were either infested with 30 spider mites (10 mites per leaflet, 3 leaflets per plant, +T) or not (–T) when 4–5 weeks-old for a period of 10 days. Mean ± SE (n = 8) of **(A)** shoot and **(B)** root fresh weight per plant across all treatments. Significant differences between treatments are indicated by different letters by Tukey's *post hoc* tests after ANOVA: $P < 0.001$.

PR-P6, and *GGPS1* compared to the respective levels on spider mite-infested plants, except for *PI-IIc* which was shown to be differentially expressed on FsK-colonized plants in response to spider mite feeding depending on the experiment (**Figures 4**, **5** and **Supplementary Table S2**). Finally, FsK colonization of spider mite-infested plants significantly affected the expression levels of genes *GLU-A* and *GLU-B* and *CHI9* which were up-regulated on these plants, albeit to similar levels as observed to non-infested plants.

Tomato Volatiles

Having demonstrated significant effects of FsK-colonization of tomato plants on induced direct defense against spider mites, we subsequently investigated whether the endophyte can alter the emission of volatile compounds and thus also contribute to tomato's indirect defense. We sampled the volatile profiles of FsK-colonized plants with (+F/+T) and without (+F/−T) spider mites. In addition, we sampled volatiles of plants experiencing only herbivory (−F/+T) and plain control plants (−F/−T). This resulted in 41 compounds that were further analyzed. Volatile emissions were different between the treatments when compared with a principal component analysis (PCA) (**Figure 6**). The first two principal components explained 76.48% combined variance and separated samples between treatments. Control (−F/−T) plants cluster separated from the other treatments and plants experiencing herbivory (−F/+T and +F/+T) grouped together as well. The first principal component can be attributed to between-sample variability, maybe due to sampling effects. The second principal component can be clearly attributed to the effect of the treatments. Compounds with a high loading on the second principal component were highly interesting since they might be the reason for the attraction of the generalist predator *M. pygmaeus* recorded in the olfactometer assays described below.

The analysis of the loadings revealed two distinct groups of compounds. Three (decanal, 5-heptene-2-one-6-methyl and geranyl acetone) had high negative loadings on PC 2 pointing toward the control samples. These compounds (**Figure 7A**) showed a trend to be suppressed by herbivory or the fungal treatments, even though only significant in the case of decanal (**Table 1**). Another set of eight compounds (sesquiterpenes, C6 VOCs and MeSA) had a high positive loading on PC 2 pointing toward the herbivory treatment (**Figure 7B**). Especially, the *cis*-3-hexenyl acetate, C6 VOC B, *cis*-3-hexenyl butyrate and MeSA showed a significant effect of herbivory, whereas the interaction of the endophyte with the herbivore for these compounds was found not significant. On the other hand, a significant interaction effect was recorded for C6 VOC A, the two unknown sesquiterpenes A and B, as well as longifolene (**Table 1**). The two sesquiterpenes could not be matched to a RI and mass spectra and thus their identity remains elusive. Both sesquiterpenes and longifolene were significantly affected by the presence of the endophyte and the herbivore (**Table 1**).

Besides the 11 compounds depicted in the PCA, another 30 compounds were also identified for which two-way ANOVA revealed no significant interaction effect (**Supplementary Table S3**). Of these 30 compounds, 13 were found to be significantly affected by the presence of spider mites only, and none of the endophyte suggesting that, in total, 17 of the sampled volatiles were identified to be herbivore-specific (**Table 1** and **Supplementary Table S3**).

Predator Choice

To test whether the differences in volatile emissions caused by FsK colonization could be functionally significant in indirect defense, we used a Y-tube olfactometer choice test with the generalist predator *M. pygmaeus*. Only a few predators (1.5–5.7% depending on the treatment, $\chi^2 = 3.84$; df = 5; $P = 0.573$) used in the olfactometer assays did not make a choice within the time frame of 12 min and, thus, were excluded from data analysis (**Figure 8**). *M. pygmaeus* females preferred volatiles from plants (either FsK-colonized or not) over clean air (**Figure 8**; −F/−T vs. air: $\chi^2 = 8.73$; $P = 0.003$; +F/−T vs. air: $\chi^2 = 21.88$; $P < 0.001$). In addition, females preferred volatiles from spider mite-infested plants that were either FsK-colonized or not (**Figure 8**; −F/+T vs. −F/−T: $\chi^2 = 10.24$; $P = 0.001$; +F/+T vs. +F/−T: $\chi^2 = 15.51$; $P < 0.001$). Volatiles from FsK-colonized plants were more

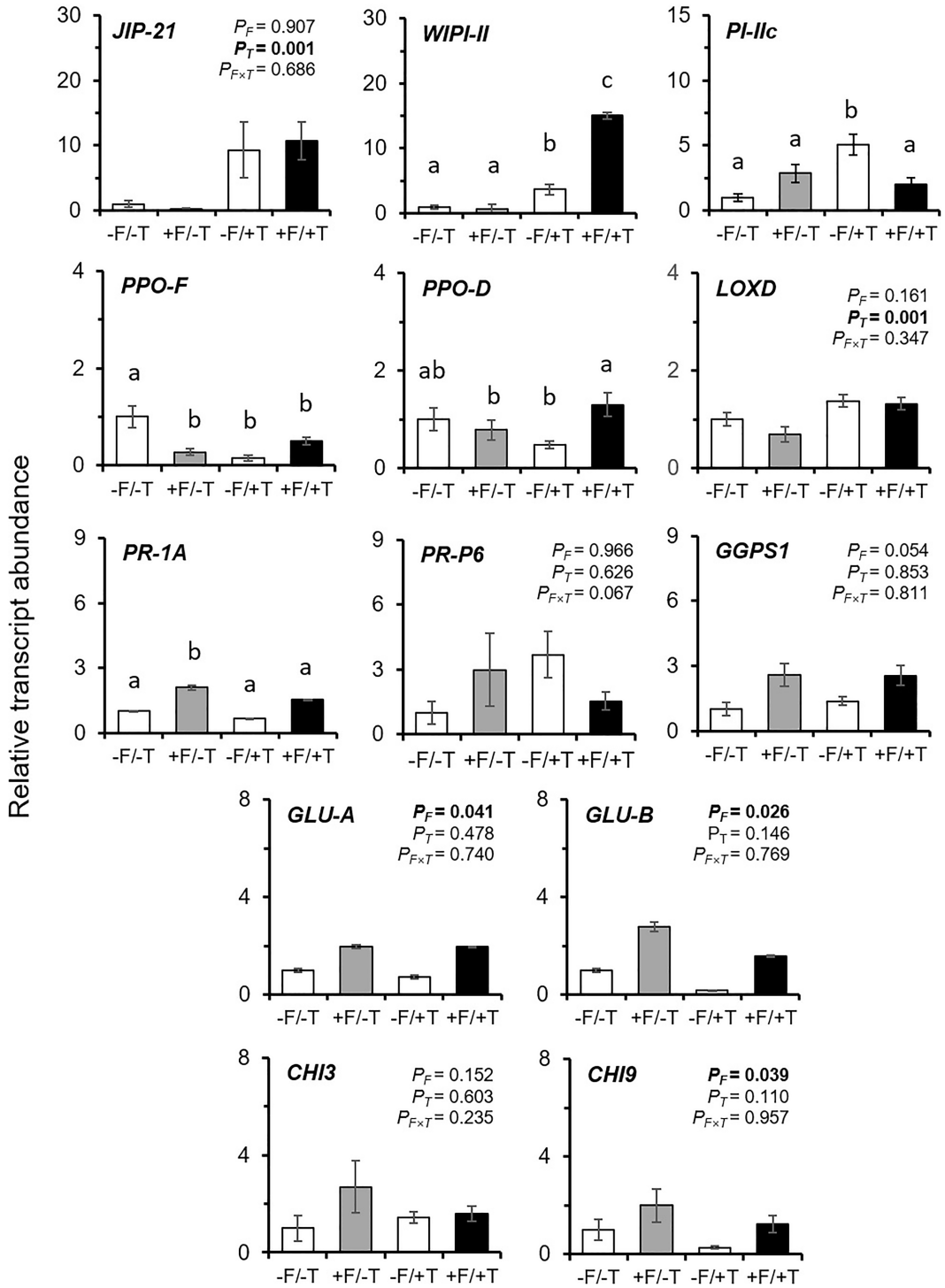

FIGURE 4 | Effects on the transcript levels of defense marker genes in tomato plants colonized by the endophyte *F. solani* strain K (+F) and/or infested with spider mites (*T. urticae*, +T) compared with the untreated control (–F/–T). Values are the average ± SE of two technical replicates for each of six biological replicates of an independent experiment (**Supplementary Table S2**). Expression levels for all target genes were normalized to the geometric mean of ubiquitin and actin expression levels in each sample as a reference. Two-way ANOVA ($\alpha = 0.05$) *P*-values are shown for each gene on each panel in the case of non-significant interaction effects ($P_{F*T} > 0.05$) and significant *P*-values (<0.05) are indicated in bold: P_F, probability value for the endophyte (FsK) effect; P_T, probability value for the herbivore (T) effect; P_{F*T}, probability value for FsK × T interaction. In the case of significant interaction ($P_{F*T} < 0.05$) significant differences between treatments are indicated by different letters by Tukey's *post hoc* tests after two-way ANOVA: $P < 0.05$. *JIP-21*, *WIPI-II*, *PI-IIc*, *PPO-F/D,* and *LOXD* are JA-marker genes; *PR-1A* and *PR-P6* are SA-marker genes; *GLU-A/B* and *CHI3/9* are fungal (or herbivore, e.g., whitefly)-marker genes; *GGPS1* has been reported as responsive to spider mite infestation in tomato (see relevant references in manuscript).

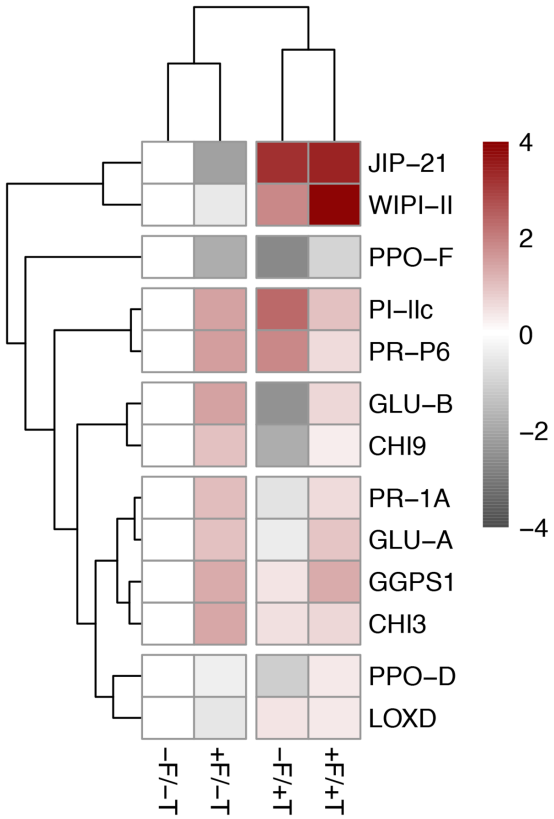

FIGURE 5 | Heatmap of the differentially expressed plant defense genes in tomato plants colonized by the endophyte *F. solani* strain K (+F) and/or infested with spider mites (*T. urticae*, +T) compared with the untreated control (–F/–T). Pink indicates upregulation of gene expression levels and a strong up-regulation is indicated in dark red. Light gray indicates downregulation and dark gray indicated strong downregulation of gene expression levels. White indicates the control group. The sample/gene grouping is based on hierarchical clustering (complete linkage algorithm) of the Euclidean sample/gene distances of the differentially expressed genes detected by ANOVA (Tukey's *post hoc* tests, $P < 0.05$). Values are the mean fold-change compared to relative gene expression in the control plants (–F/–T) of two technical replicates for each of six biological replicates. Expression levels for all target genes were normalized to the geometric mean of ubiquitin and actin expression levels in each sample as a reference. Values and statistically significant differences compared to control samples are presented in **Supplementary Table S2**. *JIP-21*, *WIPI-II*, *PPO-F/D*, *PI-IIc,* and *LOX-D* are JA-marker genes; *PR-P6* and *PR-1A* are SA-marker genes; *GLU-A/B* and *CHI3/9* are fungal (or herbivore, e.g., whitefly)-marker genes (see relevant references in manuscript).

attractive than those from un-colonized plants, either when these were spider mite-infested or not (**Figure 8**; +F/−T vs. −F/−T: $\chi^2 = 6.06$; $P = 0.014$; +F/+T vs. −F/+T: $\chi^2 = 4.90$; $P = 0.027$).

DISCUSSION

In the present study, we tested to which extent the colonization of plants by the endophytic fungus *F. solani* strain K affects tomato responses against spider mites. We found that defense-related genes were differentially expressed on colonized plants. In accordance, spider mites laid a lower number of eggs on FsK-colonized plants. We also observed that feeding damage inflicted by spider mites was lower on FsK-colonized plants compared to control plants. However, plant biomass was increased in the presence of the endophyte and the spider mites. Therefore, we argue that there are indications for the existence of plant growth promotion capabilities in the endophyte but also for the expression of herbivore-induced compensatory growth in tomato. Finally, we also recorded substantial differences in the volatiles emitted by endophyte-colonized plants that were either spider mite-infested or not. As a result, tomato indirect defense was enhanced and the zoophytophagous predator *M. pygmaeus*, a natural enemy of spider mites, was able to identify spider mite-infested but also FsK-colonized plants in all colonization/infestation-type combinations.

Tomato Colonization by the Endophyte Leads to Reduced Spider Mite Performance and Increased Plant Biomass

Plant-mediated effects of beneficial soil microbes on spider mites have been scarcely addressed so far. To the best of our knowledge, AMF are the most, if not the main, group of soil microbes that have been studied in this regard. In contrast to what is generally known about the negative effects of several AMF on generalists and mesophyll feeders (Hartley and Gange, 2009; Pineda et al., 2010; Shikano et al., 2017), the two-spotted spider mite is a generalist parenchyma cell-content feeder but has been previously shown to be positively affected by AMF in symbiosis with different plant species. For example, *T. urticae* performance was shown to be enhanced on common bean plants *Phaseolus vulgaris* by the AMF *Glomus mosseae* and/or the nitrogen-fixing bacteria *Azotobacter chroococcum* (Hoffmann et al., 2009; Hoffmann et al., 2011; Khaitov et al., 2015). In these cases, the benefit for the herbivore was attributed to the improved nutritional value of the plant tissue which correlated to enhanced uptake of P and K by AMF and improved N uptake by *A. chroococcum*. Another study, however, that explored the effects of four different AMF species belonging to different genera, found that spider mite (*T. urticae*) performance in *Lotus japonicus* was differentially impacted depending on the AMF species. In the same study, defense-related compounds were also differentially altered by the various AMF species, suggesting that AMF effects on aboveground herbivorous mites and plant responses are species-specific and variable (Nishida et al., 2010). Nevertheless, symbioses of soil microbes with other plants such as tomato and their interactions with aboveground mites remain unexplored.

Our study is a first report of a beneficial soil endophytic fungus that negatively affects spider mite performance in tomato. *Fusarium solani* strain K has been previously shown to mediate tomato systemic resistance against other foliar organisms, such as the pathogen *Septoria lycopersici* (Kavroulakis et al., 2007) as well as against the feeding damage inflicted by the zoophytophagous predator *N. tenuis* (Garantonakis et al., 2018). Similarly, a

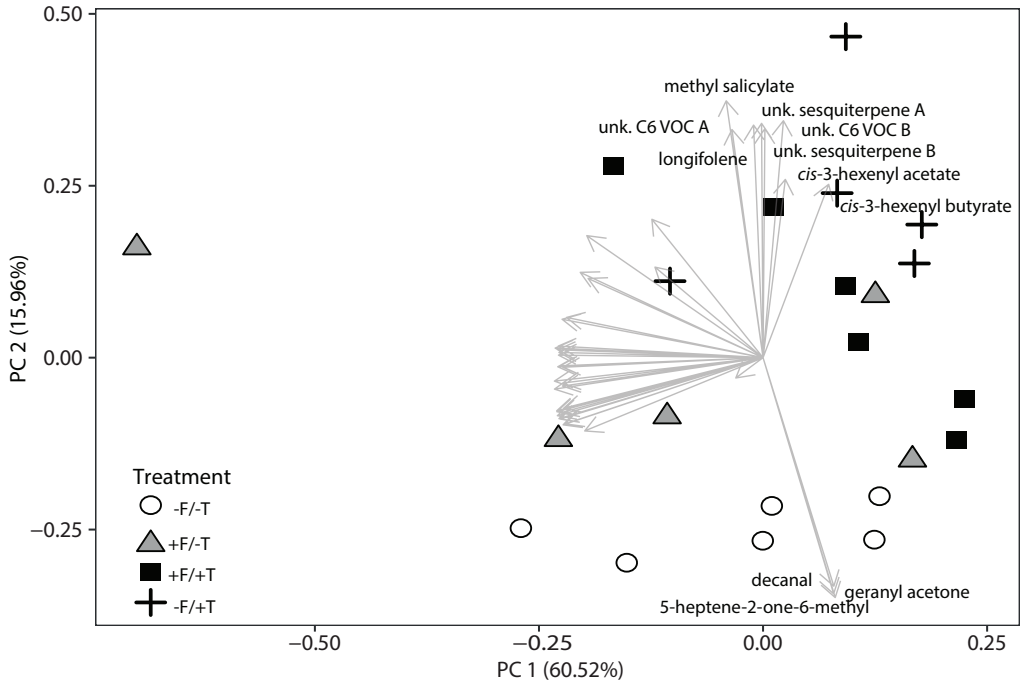

FIGURE 6 | Changes in volatile emissions from tomato plants colonized by the endophyte *F. solani* strain K (+F) and/or infested with spider mites (*T. urticae*, +T). Principal Component Analysis of the autoscaled data of 41 selected volatiles as measured by TD-GC-MS 2 days after spider mite infestation. Analysis was performed on the average peak data. Symbols represent the different biological replicates within each treatment (control plants –F/–T, open circles; FsK-colonized plants +F/–T, gray triangles; FsK-colonized and spider mite infested plants +F/+T, black squares; and only spider mite infested plants –F/+T, black crosses, 5–6 biological replicates per treatment). Arrows represent the loadings of a single volatile compound. Volatiles that show high loading on PC 2, representing the FsK and spider mite treatment (treatment effect), are labeled by their names.

pathogenic *Fusarium* species in tomato (*F. oxysporum* f. sp. *lycopersici* race 1) was shown to induce plant resistance against spider mites that led to reduced oviposition on a *Fusarium*-susceptible tomato line, however, when plants were also water-stressed (Jongebloed et al., 1992). The exact mechanism(s) involved in FsK-mediated resistance to foliar organisms is not known. Yet, there is evidence toward the involvement of the ethylene and JA signaling pathways in FsK-mediated resistance to *N. tenuis* feeding (Garantonakis et al., 2018). Furthermore, an earlier study has shown that tomato resistance to a root pathogen by FsK was only expressed in the presence of intact ethylene signaling pathway, independently of JA (Kavroulakis et al., 2007). In the latter study, root colonization by FsK did not induce elevated ethylene production in the root and aerial parts whereas SA-mediated responses in roots (but not in leaves) were suppressed. This data, although limited, suggest the involvement of all three important signaling pathways, which also mediate plant responses to herbivory, in the FsK-resistance cases reported so far. Nevertheless, a thorough study of the phytohormone accumulation in the aerial parts of FsK-colonized plants is required to cast light on the mechanisms involved in FsK-mediated tomato resistance against spider mites.

When it comes to herbivory the net benefit of a mutualistic relationship among soil microbes and plants depends on the trade-off between microbe-induced plant defenses versus plant nutritional quality or quantity alteration (Pozo and Azcón-Aguilar, 2007; Gehring and Bennett, 2009; Kempel et al., 2010; Pineda et al., 2010; Shikano et al., 2017). In the present study, however, putative mechanisms involved in FsK-mediated resistance against spider mites were shown to be related to both defense elicitation and plant growth promotion by FsK. Endophytes in general have been shown to promote plant growth and to affect resource allocation in ways that impact the host's ability to compensate for herbivory (Vessey, 2003; Hartley and Gange, 2009; Rodriguez et al., 2009; Bever et al., 2013; Dupont et al., 2015). We herein recorded shoot but not root growth promotion by FsK in 5–6 weeks-old tomato plants in the absence of spider mites (**Figure 3**). This effect was not evident for plants that were 10 days younger (data not shown). On the other hand, compensatory plant growth in response to herbivory was also recorded for the endophyte or the spider mites alone whereas root weight was further stimulated in response to their combined effect (**Figure 3**). Herbivore-induced plant growth may be promoted in the plant's attempt to compensate for herbivory and depends on herbivore characteristics and herbivore density (Järemo and Palmqvist, 2001). For example, photosynthetic activity was shown to be stimulated in cucumber and chrysanthemum at low populations of spider mites (Tomczyk et al., 1991). Although additional plant growth and developmental factors would be needed to evaluate the biological significance of

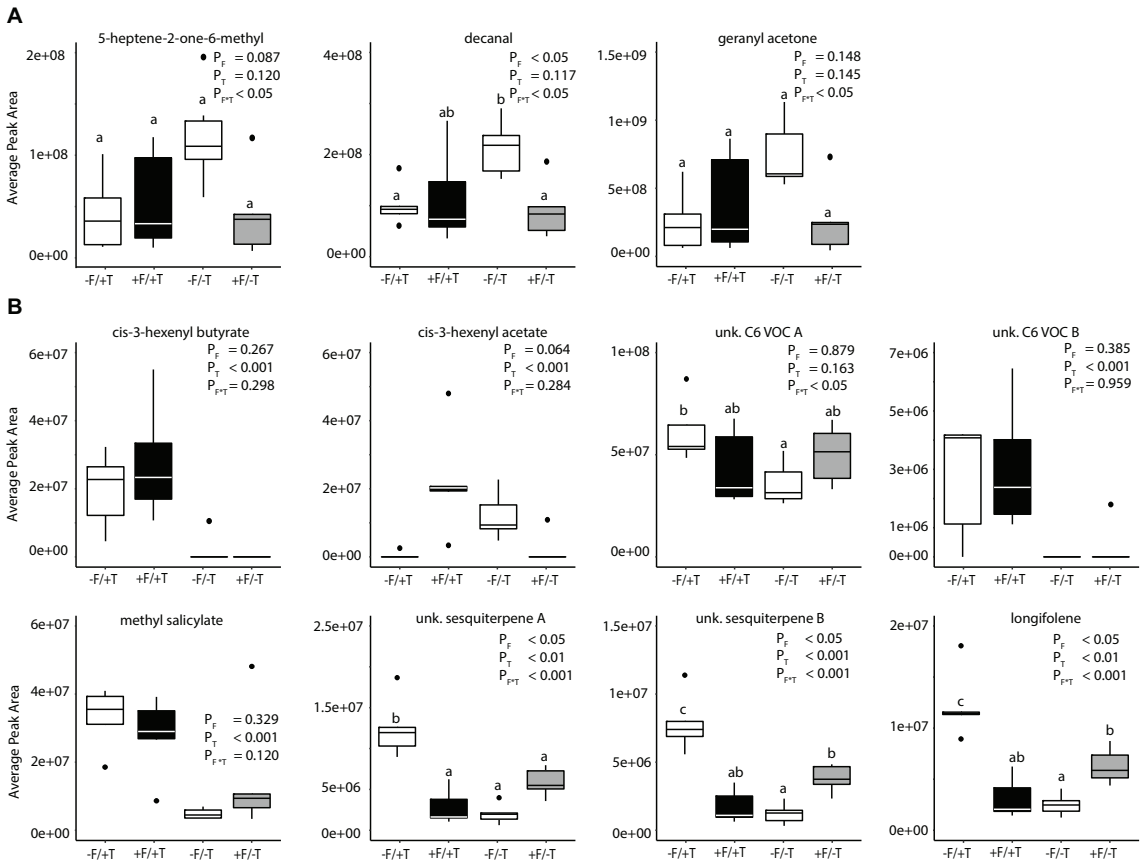

FIGURE 7 | Changes in volatile emissions from tomato plants colonized by the endophyte *F. solani* strain K (+F) and/or infested with spider mites (*T. urticae*, +T). Boxplot of 11 compounds that showed high loading on PC 2. Treatments are: control plants –F/–T; FsK-colonized plants +F/–T; FsK- colonized and spider mite infested plants +F/+T; and only spider mite infested plants –F/+T (5–6 biological replicates per treatment). **(A)** Depicts compounds with negative loadings on PC 2 (treatment effect), while panel **(B)** depicts the compounds with positive loadings. Significant differences between treatments are indicated by different letters by Tukey's *post hoc* tests after two-way ANOVA: $P < 0.05$.

this observation, it is clear that FsK further stimulates plant growth, acting complementary to plant responses to spider mites alone. Spider mites were adversely impacted on FsK-colonized plants (**Figures 1**, **2**), suggesting the absence of nutritional benefits and/or that defense induction outcompetes the putative benefits of improved nutrition to the herbivore. It should be noted, however, that leaflet area was found not to be different among FsK-colonized and un-colonized plants suggesting that spider mites had no access to additional food supply on either plant.

The number of live spider mites on plants was not different among FsK-colonized and control plants (**Figure 1**), suggesting that recorded differences can be attributed to plant responses affecting spider mite reproduction. Spider mites are known to be sensitive mainly to JA- and to a lesser extent to SA-mediated defenses (Li et al., 2002; Kant et al., 2008; Alba et al., 2015; Ataide et al., 2016; Villarroel et al., 2016). In accordance, both JA and SA defense marker genes were shown to be up-regulated in the presence of FsK in spider mite-infested plants in the present study (**Figures 4**, **5** and **Supplementary Table S2**). Hence, we may assume that JA and SA effectual defenses are induced in FsK-colonized plants against spider mites. JA-activation is common in other mutualistic symbioses such as with PGPR and AMF. In these cases, symbiotic plants show a JA-related 'primed' state of defense that results in plant resistance to chewing herbivores and necrotrophic pathogens (Pineda et al., 2010; Zamioudis and Pieterse, 2012; Pieterse et al., 2014; Shikano et al., 2017). Hence, in addition to the clarification of JA and SA involvement, whether priming is also involved in FsK-mediated resistance to spider mites is yet to be determined.

Tomato Defense-Gene Expression Is Altered by the Endophyte

In tomato, JA signaling is the most important regulator of spider-mite induced defenses (Kant et al., 2015; Martel et al., 2015). Similarly to previous reports, we also recorded the up-regulated expression of genes *JIP-21*, *WIPI-II* [or *PI-IIf,* see Alba et al. (2015)], *LOXD* and *PI-IIc* in response to spider mite feeding (**Figures 4**, **5**, **Supplementary Figure S2** and **Supplementary Table S2**) that are known to mark JA defenses in tomato

TABLE 1 | Volatiles emitted by tomato plants according to their calculated Kovats retention index (RI).

Compound	Calculated RI	Two-way ANOVA			Tukey HSD *post hoc*					
		Endophyte (F)	Herbivore (T)	Interaction (F*T)	−F/+T	−F/−T vs. +F/−T	+F/+T	−F/+T vs. +F/−T	−F/+T vs. +F/+T	+F/−T vs. +F/+T
5-hepten-2-one-6-methyl[c]	990	0.087	ns	*	0.061	0.060	ns	ns	ns	ns
cis-3-hexenyl acetate[b]	1011	0.064	***	ns						
unk. C6 VOC A	1101	ns	ns	*	0.050	ns	ns	ns	ns	ns
unk. C6 VOC B	1147	ns	***	ns						
cis-3-hexenyl butyrate[b]	1189	ns	***	ns						
Methyl salicylate[a,b]	1194	ns	***	ns						
Decanal[b]	1209	*	ns	*	*	*	0.062	ns	ns	ns
unk. sesquiterpene A	1394	*	**	***	***	0.056	ns	**	***	ns
unk. sesquiterpene B	1407	*	***	***	***	*	ns	***	***	0.085
Longifolene[d]	1411	*	**	***	***	*	ns	**	***	0.099
geranyl acetone[b]	1451	ns	ns	*	0.062	0.077	ns	ns	ns	ns

[b]Adams (1995); [c]Lucero et al. (2003); [d]Kant et al. (2004). Compounds presented are from the PCA in ***Figure 7*** *and the same as shown in* ***Figure 8*** *and were identified by comparison to an authentic standard[a] or tentatively identified by comparison to RI values in the literature[b,c,d] when possible. Two-way ANOVA (df = 1) with Endophyte (F) and Herbivore (T) as factors was performed on the average peak area. Tukey HSD was carried out as post hoc analysis for compounds showing a significant interaction (F*T) effect. P-values marked with an asterisk represent *P < 0.05, **P < 0.01, ***P < 0.001, ns represent values P > 0.1. For P-values 0.1 > P > 0.05 the values are shown.*

(Kant et al., 2004; Alba et al., 2015; Martel et al., 2015). It should be noted, however, that the KOP spider mite strain used in the present study is a tomato-adapted line previously shown to resist JA defenses in tomato (Ament et al., 2004; Kant et al., 2008). Importantly, KOP mites have been shown to perform equally well on *def-1* and *PS* cultivars, a JA biosynthesis mutant and a transgenic tomato plant *35S::prosystemin*, respectively, and on WT plants (Kant et al., 2008). Hence, despite the ability of KOP spider mites to induce JA-dependent responses, as also verified in our study, we expected these mites to display a resistant phenotype on our tomato plants. Indeed, our results show that KOP mites produce a similar or slightly lower number of eggs compared to previous reports (Ament et al., 2004; Kant et al., 2008) that decreased to approx. 3.5 eggs/female/day on FsK-colonized plants in our study (**Figure 1**). On the other hand, other genes such as *PR-1A*, *PR-P6, PPO-D/F,* and *GGPS1* that have been previously shown to be up-regulated by other spider-mite strains [KMB or *T. urticae* Santpoort-2 in Kant et al. (2008) and Alba et al. (2015), respectively] were not shown to be consistently affected in our study (see for example, *PR-1A* expression in **Figures 4**, **5**, **Supplementary Figure S2** and **Supplementary Table S2**). This could be attributed to differences in our experimental set-up with previous studies (e.g., different tomato cultivar and spider mite line, soil-less experimental plants in our study). It also indicates the limitations of marker genes to deduce conclusions as regards to the molecular mechanisms that govern multi-partite interactions, such the ones studied here. Hence, the dynamics that result in the reduced performance of spider mites we were able to show in independent experimental set-ups require a more detailed analysis. On the other hand, *PPO* genes encode proteins that normally reduce amino acid availability from ingested plant tissue in the gut of herbivores. As such, these compounds are believed to have little or no effect on herbivores with acid guts such as spider mites (Erban and Hubert, 2010; Carrillo et al., 2011; Martel et al., 2015). This may explain why spider-mite infestation may not induce a consistent induction of these genes.

Tomato colonization by the endophyte resulted in differential expression of defense-related genes (**Figures 4**, **5**, **Supplementary Figure S2** and **Supplementary Table S2**). As expected, genes *GLU-A* and *CHI9* that are typically induced against beneficial and pathogenic fungi or other herbivores [e.g., whiteflies, Puthoff et al. (2010)] were shown to be up-regulated in FsK-colonized plants. Up-regulation was also recorded for *PI-IIc* and *PR-1A* whereas *PPO-F* was down-regulated on FsK-colonized compared to un-colonized plants. In the presence of the herbivore, FsK retains the capability to induce these genes as compared to uncolonized plants. In addition, FsK colonization led to a significant further increase of the transcript levels of *WIPI-II*. These results correlate well with the significant decrease in spider mite oviposition recorded on these plants in our study (**Figure 1**) and to the rather conserved mechanism reported for microbe-induced JA-mediated defenses in response to herbivory (Pineda et al., 2010; Shikano et al., 2017). KOP is a tomato-resistant spider mite line that performs equally well on JA-defended and WT plants (Kant et al., 2008) whereas spider mites (*T. urticae*) have been shown to be negatively impacted by JA defenses up to the endogenous JA-IIe levels of around 10 ng.gFW^{-1} (Ataide et al., 2016). Most possibly this plateau was not reached on spider mite-infested plants thus enabling FsK to prime or, further induce, effectual JA defenses such as *WIPI-II* expression against KOP mites. This effect was also consistently observed in *WIPI-II* expression levels in our second independent experimental set-up (**Supplementary Figure S2**).

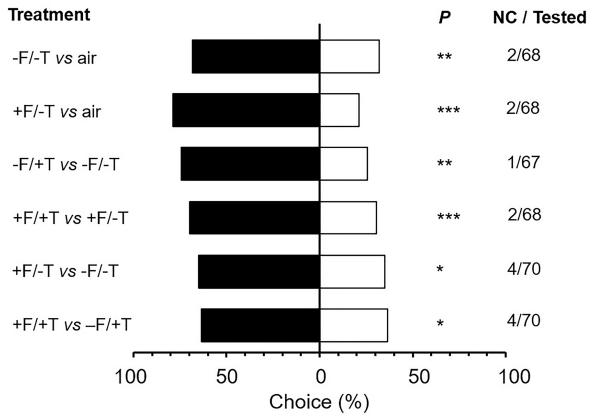

FIGURE 8 | Responses of the generalist predator *Macrolophus pygmaeus* to volatiles emitted by tomato plants colonized by the endophyte *Fusarium solani* strain K (+F) and/or infested with spider mites (*T. urticae*, +T) or clean air (air). One week after seed sowing plants were either inoculated with the endophyte (+F) or not (–F). Subsequently, plants were either infested with 200–300 spider mite females (+T) or not (–T) when 4–5 weeks-old for a period of 2 days. Shown are the percentages of predator females that moved toward the volatile sources in the corresponding choice situations indicated on the left (under 'Treatment') in a vertical Y-tube olfactometer. 'NC/Tested' indicates the number of individuals that did not make a choice over the total number of tested individuals (67–70 individuals depending on the treatment combination). Significant differences are indicated by asterisks after a χ^2 test: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

In tomato, the increasing emission of certain volatiles such as the homoterpene (E,E)-4,8,12-trimethyltridecane-1,3,7,11-tetraene (TMTT) and methyl salicylate (MeSA) in response to spider mite feeding is well-documented (Ament et al., 2004, 2006; Kant et al., 2004; Kant et al., 2008; Nagaraju et al., 2012). TMTT has been shown to play a prominent role in indirect defense since it is attractive to predators of spider mites (Dicke et al., 1990, 1998, 1999; Ament et al., 2004; Sarmento et al., 2011) but we were unable to detect it in our analysis. This may be attributed to the sampling time (volatiles were collected 2 days after spider mite feeding in our study), whereas Kant et al. (2004) reported that a 3-day delay is required for indirect defense mounting. This compound is synthesized by the precursor molecule geranylgeranyl diphosphate by the activity of the enzyme geranylgeranyl diphosphate synthase. Gene *GGPS1* encoding this biosynthetic enzyme is known to be induced by *T. urticae* (Kant et al., 2004; Ament et al., 2006). In the present study, we could not detect consistently this up-regulation of *GGPS1* expression levels at 4 days after spider mite feeding (**Figures 4**, **5**, **Supplementary Figure S2** and **Supplementary Table S2**). The same was reported by Martel et al. (2015) for plants infested with spider mites for only 1 day. It is plausible that a time-delay for mounting an indirect defense in response to spider mite feeding in tomato is required, and may explain the inconsistencies both in the gene expression levels and the lack of TMTT emission in our study (**Figures 4**, **5**, **Table 1** and **Supplementary Table S3**) Nevertheless, other volatiles were found to be emitted by FsK-colonized plants corroborating the hypothesis that FsK may also mediate indirect tomato defense to attract natural enemies.

Indirect Tomato Defense Against Spider Mites Is Enhanced by the Endophyte

Beneficial soil microbes are known to be capable of affecting indirect plant defense by mediating the expression of plant traits that impact natural enemies (Rasmann et al., 2017). Volatile emission is one such trait that can be directly impacted by soil microbes through their impact on plant metabolites. On the other hand, increased volatile emission and eventually indirect defense, could result from microbe-induced promotion of growth rate, plant vigor or size when linked to increased prey density and volatile emission. In contrast to microbe-mediated effects to plant morphology, the effects of beneficial soil microbes on plant volatile production have been better documented so far (Rasmann et al., 2017). As with direct defenses, however, most of our current knowledge derives from studies on AMF-activated effects on volatiles (e.g., Guerrieri et al., 2004; Fontana et al., 2009; Babikova et al., 2014) and the impact of PGPR, rhizobia and endophytic fungi have been scarcely addressed so far. Beneficial soil microbes are generally assumed to affect constitutive and inducible volatile production in response to herbivory but the outcome of these tri-trophic interactions seems to be vastly species-specific and context dependent. Considering spider mites, only one study reporting the enhanced production of the sesquiterpenes β-omicene and β-caryophyllene induced by *T. urticae* on AMF-colonized bean plants that resulted in an enhanced attraction of the predatory mite *P. persimilis* toward these plants has been reported so far (Schausberger et al., 2012). Hence, our knowledge on microbe-mediated effects on indirect plant defense to spider mites is limited, even more on such effects of endophytic fungi on herbivores in general and, spider mites in particular.

Very little is known about the effects of endophytic fungi on plant volatile production [mainly focused on *Trichoderma* species, e.g., Battaglia et al. (2013), Coppola et al. (2017), Contreras-Cornejo et al. (2018)] and to the best of our knowledge, no previous study has ever explored how plant colonization by an endophytic fungus mediates the volatile-regulated attraction of insect predators to spider mite-infested plants. We herein show that the generalist predator *M. pygmaeus* was attracted to FsK-colonized plants irrespectively of the presence of spider mites (**Figure 8**). This predator is known to be attracted by volatiles emitted by tomato plants in response to herbivory (e.g., by *Tuta absoluta* or *Bemisia tabaci*) (Lins et al., 2014; De Backer et al., 2015; De Backer et al., 2017). From the volatile blend emitted by *T. absoluta*-infested tomato plants, (*E*)hex-2-enal, 2-carene, α-pinene, β-phellandrene, hexanal, and linalool were found to evoke positive attraction in *M. pygmaeus* (De Backer et al., 2017). Our volatile analyses clearly show that the volatile blends of tomato plants are affected by the presence of the endophyte and the same holds for the volatile blends emitted by spider mite-infested plants compared to control plants (**Figure 6**, **Table 1** and **Supplementary Table S3**).

The TMTT and MeSA constitute the most abundant volatiles emitted by tomato in response to spider mite feeding (Ament et al., 2004; Ament et al., 2006). Nevertheless, as noted earlier, TMTT was not detected in our volatile sampling 2 days after spider mite infestation, which is surprising since *GGPS1* was also not expressed after another 4 days. On the other hand, JA-dependent MeSA emission by tomato plants was significantly increased in response to spider mite feeding in our study (**Figure 7** and **Table 1**) in accordance with a previous report showing a two-fold increase in MeSA emission in response to KOP mites feeding (Kant et al., 2008). In addition, another 16 volatiles were significantly affected by the presence of spider mites alone in our study (**Table 1** and **Supplementary Table S3**) suggesting that these compounds are spider mite-specific. Of these volatiles, β-myrcene is known to increase upon spider mite infestation in tomato, although not always significantly (Kant et al., 2004). In addition, β-caryophyllene and β-phellandrene were found to be significantly affected by spider mite infestation in our study although previously reported to be produced constitutively by tomato plants, and not induced by spider mites (Kant et al., 2004; Sarmento et al., 2011). In contrast, the induction of *trans*-nerolidol and *trans*-β-ocimene by spider mites, shown to be JA-dependent by Ament et al. (2004), was not significantly affected by spider mites in our study. Finally, α-terpinene, recently suggested for its putative role in the attraction of *M. pygmaeus* to tomato (Cortés et al., 2016) was also found herein to be significantly affected by spider mites.

The fact that the predators were able to identify FsK-colonized from un-colonized plants even when these were infested with spider mites (**Figure 8**) suggests that endophyte effects on volatile blend alteration are functionally important for tritrophic interactions. Identifying the specific volatiles mediating the predator attraction to FsK-colonized plants might be difficult since arthropods are known to respond to volatile blends, rather than specific chemicals (Gols et al., 2011; van Wijk et al., 2011). Nevertheless, we should note that endophyte colonization of tomato plants led to increased emissions of one sesquiterpene (unknown sesquiterpene B, calculated RI: 1407, **Figure 7** and **Table 1**) and longifolene, whereas the emission of decanal was significantly suppressed (**Figure 7** and **Table 1**). On the other hand, spider mite infestation of FsK-colonized plants led to a decreased emission of unknown sesquiterpenes A and B, as well as longifolene (**Figure 7** and **Table 1**). Notably, these plants displayed a stronger attraction of the predators over uninfested, FsK-colonized plants (**Figure 8**). Taken together, it would be interesting to further assess the role of the two sesquiterpenes as well as decanal and longifolene in shaping tomato indirect defense.

Collectively, our data support the hypothesis that the endophytic fungus *F. solani* strain K alters tomato responses to spider mites to the benefit of the plant. Putative mechanisms involved are shown herein to vary between defense induction and/or priming to plant growth promotion and tolerance. In the present study, we observed these mechanisms to be separately displayed. Hence, the temporal dynamics of FsK-related resistance/tolerance mechanisms in tomato should be further explored, following non-targeted transcriptomics approaches as well. Moreover, a detailed study is required to cast light on the dynamics and interactions of phytohormones underlying the endophyte's role in shaping tomato-spider mite interactions. Besides direct defense activation, FsK was also shown to enhance indirect tomato defense. The putative adaptive value of predator attraction to FsK-colonized plants lies in the fact that plants might have to invest less in direct defense activation, provided that volatile production does not entail major energetic costs. On the other hand, the attraction of mirids to the well-defended FsK-colonized plants might impose no harm to the predators when, similarly to AMF-colonized plants (Pineda et al., 2010; Shikano et al., 2017), FsK-colonized plants also display susceptibility to sucking insects (e.g., aphids and whiteflies). Hence, to draw safe conclusions about the protective role of FsK in tomato, it is imperative to assess its effects against other herbivores as well, also in relation to its impact on root-feeding organisms (e.g., arthropods or nematodes). Ultimately, the net benefit of FsK-colonization for the plant and its potential as a novel tool in spider mite control should be confirmed by studying the effects of FsK-mediated resistance on plant fitness and reproductive output. Finally, it must be noted that our experiments were performed with plants grown on a sand mixture with vermiculite. Hence, some of the differences observed between the present study and previous works may be related to the absence of other soil microbes in our study that in different settings (i.e., when plants are grown in soil) could interact with the endophyte and/or impact gene expression and volatile emission in aboveground plant parts (Benítez et al., 2017).

AUTHOR CONTRIBUTIONS

MP, GB, and KP conceived and designed the experiments. ML, MP, DP, and MA performed the experiments. GB, MP, KP, NK, and AW analyzed the data. MP wrote the manuscript with input from KP, GB, and AW. All authors read, edited, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01603/full#supplementary-material

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MINI REVIEW
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The Interface Between Wheat and the Wheat Curl Mite, *Aceria tosichella*, the Primary Vector of Globally Important Viral Diseases

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Wheat production and sustainability are steadily threatened by pests and pathogens in both wealthy and developing countries. This review is focused on the wheat curl mite (WCM), *Aceria tosichella*, and its relationship with wheat. WCM is a major pest of wheat and other cereals and a vector of at least four damaging plant viruses (*Wheat streak mosaic virus*, *High plains wheat mosaic virus*, *Brome streak mosaic virus*, and *Triticum mosaic virus*). The WCM–virus pathosystem causes considerable yield losses worldwide and its severity increases significantly when mixed-virus infections occur. Chemical control strategies are largely ineffective because WCM occupies secluded niches on the plant, e.g., leaf sheaths or curled leaves in the whorl. The challenge of effectively managing this pest–virus complex is exacerbated by the existence of divergent WCM lineages that differ in host-colonization and virus-transmission abilities. We highlight research progress in mite ecology and virus epidemiology that affect management and development of cereal cultivars with WCM- and virus-resistance genes. We also address the challenge of avoiding both agronomically deleterious side effects and selection for field populations of WCM that can overcome these resistance genes. This report integrates the current state of knowledge of WCM–virus-plant interactions and addresses knowledge gaps regarding the mechanisms driving WCM infestation, viral epidemics, and plant responses. We discuss the potential application of molecular methods (e.g., transcriptomics, epigenetics, and whole-genome sequencing) to understand the chemical and cellular interface between the wheat plant and WCM–virus complexes.

Keywords: cereals, eriophyoid mites, pathogen vector, plant viruses, phytophagous mites

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INTRODUCTION

Wheat, *Triticum aestivum* L., is the most abundant source of calories and protein in the human diet (Braun et al., 2010; Arzani and Ashraf, 2017). It is grown annually on 215 million acres, an area larger than for any other crop, and remains the most traded on world markets and the most important crop in the 21st Century (Curtis and Halford, 2014).

However, wheat production is affected by a number of pests, including insects, fungi, nematodes, and mites, that can severely reduce yields and lead to crop failures. One of the most important global pests of wheat, occurring in North and South America, Europe, Asia, and Oceania, is the wheat curl mite (WCM), *Aceria tosichella* Keifer (**Figures 1**, **2A**) which belongs to the superfamily Eriophyoidea (Navia et al., 2013). WCM is minute (about 0.2 mm long) and occupies sheltered niches on the plant, such as leaf sheaths and rolled and curled leaves, making its detection difficult, and limiting its exposure to acaricides (Navia et al., 2013). Moreover, its reproduction by arrhenotokous parthenogenesis (Miller et al., 2012), short developmental time, and high thermal tolerance (Kuczyński et al., 2016) enable great colonization potential.

The greatest economic impact of WCM results from its ability to transmit at least four damaging plant viruses to several different cereal crops. In this review we integrate the current state of knowledge of WCM–virus-plant interactions and address knowledge gaps regarding the mechanisms driving WCM infestation, viral epidemics, and plant responses.

WHAT CURL MITE FEEDING AND VIRUS TRANSMISSION

Almost 90 grass species worldwide have been reported as host plants for WCM including cereals such as wheat, oats, barley, pearl millet, corn, and rye, as well as other cultivated (pasture) and uncultivated grasses (Navia et al., 2013). WCM has very short chelicerae (ca. 0.02 mm) and can feed only on leaf epidermal tissues. On wheat they colonize the plant by feeding within the whorl of a developing leaf on thin-walled epidermal tissue known as bulliform cells. Feeding on these cells by mites prevents leaves from unfurling causing leaf curling (**Figure 2B**) that promotes a humid environment generally preferred by WCM. WCM feeding also reduces photosynthetic capacity (Royalty and Perring, 1996).

The WCM has been shown to be the only transmitter of four distinct viruses to wheat and numerous other grass hosts (Stenger et al., 2016). These viruses occur across two virus families and three virus genera. Slykhuis (1955) first identified WCM as the vector of *Wheat streak mosaic virus* (family *Potyviridae*/genus *Tritimovirus*; acronym WSMV). The mite was also shown to transmit *High plains wheat mosaic virus* (*Fimoviridae*/*Emaravirus*; HPWMoV) (Seifers et al., 1997). Transmission of *Brome streak mosaic virus* (*Potyviridae*/*Tritimovirus*; BrSMV) by WCM was verified by Stephan et al. (2008). Most recently, Seifers et al. (2009) identified the WCM as the vector of *Triticum mosaic virus* (*Potyviridae*/*Poacevirus*; TriMV).

Of these viruses, WSMV is the most widely distributed and studied and it has been identified from every major wheat growing region around the world (Navia et al., 2013). The greatest and most consistent impact from WSMV occurs across the Great Plains of North America with more sporadic impact in other regions. BrSMV has only been found in Europe and no economic impact from the virus has been reported (Stephan et al., 2008).

Wheat streak mosaic virus infection of wheat results in a light and dark green mosaic pattern on the youngest emerged leaves (**Figure 2C**; Wegulo et al., 2008). As the plant adds new leaves, the newest leaves will first show these subtle mosaic symptoms while older leaves will become progressively more yellow. The tight curling at the leaf edge resulting from mite feeding is often apparent. The severity of symptoms and subsequent yield impact from virus infection in wheat depends on the plant stage at first infection (Hunger et al., 1992; Wosula et al., 2018). Plants infected prior to or during tillering will eventually become severely stunted, discolored, and take on a very prostrate growth pattern. These severe symptoms indicate that extreme yield loss will occur.

In the North American Great Plains co-infection of the viruses is common (Burrows et al., 2009; Byamukama et al., 2013, 2016) and may result in more spotted appearance on leaves but

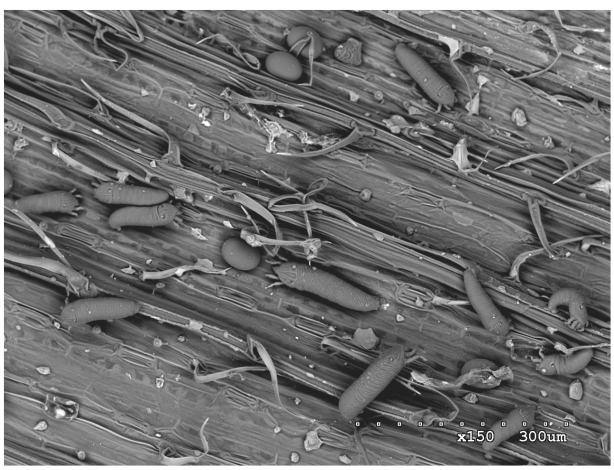

FIGURE 1 | Scanning electron microscopy (SEM) image of wheat curl mite (*Aceria tosichella*) specimens on a wheat leaf.

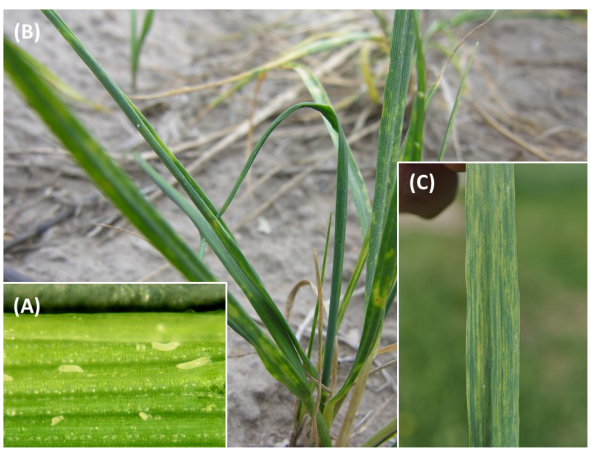

FIGURE 2 | Wheat curl mite (WCM) and WSMV symptoms: **(A)** specimens of WCM on a wheat leaf; **(B)** leaf curls caused by WCM; and **(C)** WSMV symptoms on wheat leaf.

distinguishing symptoms of co-infections is not possible. Co-infection of WSMV and TriMV have been shown to increase the severity of symptoms and yield impacts (Tatineni et al., 2010; Byamukama et al., 2012, 2014). HPWMoV is not manually transmissible and this has limited study of this virus both independently and in combination with other viruses (Tatineni et al., 2014; Stenger et al., 2016).

WCM DIVERSITY AND ITS IMPLICATIONS

Understanding the relationships between WCM, viruses, and their hosts is challenging since WCM is a cryptic species complex. It includes multiple lineages that are distinguishable using mitochondrial (mtDNA COI, 16S) and nuclear (28S rDNA D2, ITS1–ITS2, and ANT) DNA sequences, differing also in host preference (Skoracka et al., 2012, 2013; Miller et al., 2013; Szydło et al., 2015). Some lineages are highly host-specific and locally distributed, whereas others are generalists with wider geographic ranges (Skoracka et al., 2014). Two WCM genotypes associated with wheat are the most polyphagous and widespread, having been found in the Middle East, Europe, Australia, and North and South America (Skoracka et al., 2014; Wosula et al., 2016). They are known as type 1 and type 2 in Australia (Carew et al., 2009) with corresponding genotypes occurring in North America (Hein et al., 2012), as well as in Europe and South America where they are known as MT-8 and MT-1, respectively (Skoracka et al., 2014). Hereafter this latter nomenclature will be used.

In North America these two lineages have been shown to transmit WSMV (Wosula et al., 2016). However, MT-1 had a higher reproductive capacity in the presence of WSMV and vectored it more efficiently than MT-8 (Seifers et al., 2002; Siriwetwiwat, 2006; Oliveira-Hofman et al., 2015). In Australia, among these two lineages only MT-1 has been observed to transmit WSMV (Schiffer et al., 2009). MT-1 is also the most effective vector of HPWMoV and TriMV (Seifers et al., 2002; McMechan et al., 2014; Wosula et al., 2016). Mixed-virus infections further confound virus–mite studies, e.g., transmission by MT-1 was more frequent from WSMV infected source plants than from those co-infected with TriMV (Oliveira-Hofman et al., 2015).

MT-8 and MT-1 have been found coexisting in mixed populations in wheat-producing areas in North America, Australia, and Europe, where plants from a single wheat field contained both MT-1 and MT-8 (Siriwetwiwat, 2006; Schiffer et al., 2009; Hein et al., 2012; Skoracka et al., 2017), further complicating management of viruses vectored by WCM. This sympatry combined with differential virus-transmission accentuates the need for efficient identification methods.

WCM Management

To date, research to manage this mite–virus complex has focused mainly on the development of classical host plant resistance (HPR) to both the mite and viruses by introgressing favorable traits from resistant germplasm into advanced breeding lines (Whelan and Hart, 1988; Chen et al., 1998; Harvey et al., 2003; Malik et al., 2003a; Hakizimana et al., 2004; Carrera et al., 2012; Carver et al., 2016), in addition to cultural practices such as planting date and summer control of volunteer wheat plants (McMechan and Hein, 2016). The search for genes conferring WSMV resistance to wheat began shortly after the virus was identified in the 1950s (McKinney and Sando, 1951). With few sources of resistance available in wheat, the search eventually targeted close relatives culminating with the chromosome translocation of the *Wsm1* gene from *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey to the short arm of chromosome 4D in wheat (Friebe et al., 1991).

Continued efforts resulted in release of the first germplasm: KS96HW10-3 (Seifers et al., 1995) and first commercial cultivar 'Mace' (Graybosch et al., 2009) with the *Wsm1* gene. This gene has demonstrated resistance to both WSMV and TriMV (Friebe et al., 2009), however, its value has been limited due to linkage drag that reduces yields (Sharp et al., 2002). Similar issues have impacted a second gene, *Wsm3*, transferred into wheat from *T. intermedium* but efforts continue to improve its effectiveness and identify genetic markers (Friebe et al., 2009; Danilova et al., 2017).

A germplasm line, CO96093-2, was identified by Haley et al. (2002) as resistant to WSMV, but the gene's origin was uncertain. Lu et al. (2011) found this gene to be a new gene (*Wsm2*) of wheat origin. Four varieties have thus far been released with the *Wsm2* gene: 'RonL' (Seifers et al., 2007), 'Snowmass' (Haley et al., 2011), 'Clara CL' (Martin et al., 2014), and 'Oakley CL' (Zhang et al., 2015). Studies with both *Wsm1* and *Wsm2* have demonstrated that both genes are temperature-sensitive with high levels of resistance below 20°C but breaking down as temperatures approach 25°C (Seifers et al., 1995, 2007).

Additional sources of WSMV resistance in wheat have recently been identified and hold promise for incorporation into commercial wheats (Seifers et al., 2007, 2013), including increased temperature stable resistance (Fahim et al., 2012a; Kumssa et al., 2017). Lu et al. (2011) has hypothesized the presence of a minor gene in wheat that confers partial resistance or tolerance in some commercial cultivars.

Early efforts to identify resistance to the WCM in wheat were not successful (Harvey and Livers, 1975), and this led to efforts to target close wheat relatives for resistance. Thus far, four WCM-resistance genes have been identified. The earliest of these genes (*Cmc3*) was translocated to wheat from rye (*Secale cereale* L.) (Martin et al., 1983; Malik et al., 2003a). It was present in 'TAM 107,' a commercial release that became widely used in the 1980s and 1990s across the Great Plains (Porter et al., 1987). However, the extensive use of TAM 107 led to loss of effectiveness of the gene (Harvey et al., 1995, 1997). A mite-resistance gene (*Cmc1*) translocated from *Aegilops tauschii* (Coss.) Schmal. to wheat (Thomas and Conner, 1986; Whelan and Thomas, 1989) has been used to develop breeding material (Cox et al., 1999) and the recent release of 'Radiant' in Canada (Thomas et al., 2012). A third source of resistance, *Thinopyrum ponticum* (Podp.) Barkworth & D.R. Dewey, contributed with gene *Cmc2* (Whelan and Hart, 1988). A second gene originating from *A. tauschii* (*Cmc4*) was found to be independent of *Cmc1* (Cox et al., 1999;

Malik et al., 2003a) and has been used in the breeding release OK05312 (Carver et al., 2016). Additional resistance genes have been proposed but not yet isolated from common wheat (Harvey and Martin, 1992), rye (Cainong et al., 2010), and *A. tauschii* (Malik et al., 2003b; Dhakal et al., 2017).

The value of mite-resistance lies in the potential for reduced virus transmission and spread through the field, as well as in the reduction of mite buildup in the volunteer wheat that serves as a bridge host to the following wheat crop (Martin et al., 1984; Conner et al., 1991; Harvey et al., 2005). However, mite response to resistance genes has often been variable (Harvey et al., 1999) and the stability of resistance genes is a concern due to the apparent adaptation to *Cmc3* by mite populations (Harvey et al., 1995, 1997, 1999). Greater understanding of the variability in mite genotype responses to resistance genes is needed to evaluate potential stability of resistance genes. Genetic characterization of the mites used in resistance studies has become critical to understanding mite-gene response (Richardson et al., 2014; Aguirre-Rojas et al., 2017; Dhakal et al., 2017). Future efforts to pyramid *Wsm* and *Cmc* genes may enhance the utility and stability of these management options.

Molecular tools, such as *in situ* hybridization and genetic marker maps have improved the efficiency and precision of HPR introgression efforts. In addition, RNAi techniques have been used to produce transgenic wheat lines with resistance to WSMV (Fahim et al., 2010, 2012b; Cruz et al., 2014) and TriMV (Shoup Rupp et al., 2016) although no commercial wheat cultivars with this resistance have been released. With current advances in DNA sequencing technology, the whole genome sequences (WGSs) of wheat, WCM, WSMV, HPWMoV, TriMV, and BrSMV (Gustafson et al., 1987; Seifers et al., 2008; Stewart, 2016; Tatineni et al., 2016; Zimin et al., 2017) are all now available, presenting the opportunity to study these tripartite host–vector–virus relationships at the level of genome sequence and gene expression.

FUTURE DIRECTIONS

Wheat–WCM Interactions

Like many eriophyoid mites that attack grasses, WCM is vagrant, i.e., inhabiting the leaf surface rather than inducing galls, and there is very little published information regarding its direct molecular or physiological interactions with its hosts. Given the availability of its genome sequence and those of several of its hosts, such as wheat (Zimin et al., 2017), maize (Schnable et al., 2009), and barley (Mascher et al., 2017), WCM is a good candidate to be a model for such studies in grass-infesting Eriophyoidea. For example, using available genomic and transcriptomic (Ozsolak and Milos, 2011; Jänes et al., 2015) resources, it will be possible to determine whether the ability of polyphagous genotypes (e.g., MT-1, MT-8) to change from one host to another is genetically or epigenetically (Laird, 2011) controlled. Similarly, the factors that determine which plant species are accepted by a host-specific WCM genotype can be dissected (Gompert et al., 2010; Narum et al., 2013). Moreover, novel genomic technologies and high-throughput phenotyping of wheat varieties can accelerate germplasm improvement (see Mondal et al., 2016 for examples).

Proteomic analyses of rice leaves from control plants and those infested with *Schizotetranychus oryzae* (Acari: Tetranychidae) revealed a wide range of intracellular physiological changes induced by this mite although the specific source(s) of induction (e.g., salivary components) are not known (Buffon et al., 2016). Similar analyses of WCM on one or more of its hosts could take advantage of the mite's and host plants' genomic resources, as well as recent techniques developed to characterize the salivary proteins of a tetranychid mite (Jonckheere et al., 2016), to assess mite–host interactions from both sides. Effects of individual proteins could be assessed through knockout genotypes created by the CRISPR-Cas9 mutagenesis (Ran et al., 2013). Complementary studies of other eriophyoids and mite species from other families that attack cereal crops would identify similarities and differences in these interactions that could shed light on prospective control strategies against multiple mite species, e.g., via RNAi in the host plant to block production of essential mite proteins.

WCM–Virus Interactions

Regarding the ability of mites to transmit WSMV, a genotyping-by-sequencing study (e.g., Narum et al., 2013) incorporating all known WCM genotypes with variable WSMV transmission ability and anchored to an annotated WGS of WCM would identify candidate genomic regions associated with WSMV transmission variability. This could also be used to explore the differential transmission of TriMV and HPWMoV by WCM genotypes. Complementary transcriptomic and epigenetic studies could further identify the candidate gene(s) involved in this variability and tease apart genetic and epigenetic factors.

Different strains of WSMV have also been detected that are differentially transmitted by individual WCM genotypes (Wosula et al., 2016). Mutations to the helper component proteinase (HC-Pro) gene of WSMV have been shown to alter transmission from mite to plant or prevent it altogether (Stenger et al., 2006; Young et al., 2007) although the precise physiological mechanism of transmission is unknown. Given that WSMV is a circulative virus that is transmitted via the salivary glands of WCM (Paliwal, 1980), the development of salivary protein characterization techniques (Jonckheere et al., 2016) may enable association of specific WCM salivary proteins with successful or unsuccessful WSMV transmission. If other WCM-transmitted viruses have a persistent circulative type of relationship with the vector, understanding the mechanisms (receptors) by which these viruses cross the midgut epithelium and salivary gland barriers to reach the stylet channel may yield basic information regarding the traffic of these viruses within the mite body.

WCM Colonization Potential

The spread of WCM and its associated plant viruses to cereal-producing regions worldwide is of increasing scientific and economic importance (Navia et al., 2013). Colonization and

invasive potential of any organism is inevitably associated with its dispersal ability and its degree of ecological specialization (Ehrlich, 1986). WCM disperses passively by air currents (Sabelis and Bruin, 1996) and wheat-associated lineages are characterized by low host-specificity (Skoracka et al., 2013). Generalists with high dispersal ability are typically successful invaders (Wilson et al., 2009). But relationships between WCM dispersal potential, degree of host specialization, and colonization success have never been tested. To do so, it will be necessary to understand the mechanisms of successful WCM wheat colonization, including long-established and recent invasions. Research on the relationship between WCM host specialization and dispersal ability revealed trade-offs in plant performance between different host plant species after mite dispersal (Laska et al., 2017). Also it has been shown that a small number of WCM specimens landing on wheat plants after aerial dispersal (about 2% of an initial source population) were able to establish dense colonies very quickly, indicating great colonization potential (Kiedrowicz et al., 2017). Understanding how interactions between dispersal and local adaptation shape WCM distribution is crucial because predicting spread of potentially invasive organisms, particularly under current anthropogenic environmental changes, is a key to managing pest outbreaks and minimizing ecosystem degradation.

AUTHOR CONTRIBUTIONS

AS, BR, and GH designed the conception, wrote the manuscript, and read and approved the submitted version with equal contribution.

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REVIEW
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Why Do Herbivorous Mites Suppress Plant Defenses?

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Plants have evolved numerous defensive traits that enable them to resist herbivores. In turn, this resistance has selected for herbivores that can cope with defenses by either avoiding, resisting or suppressing them. Several species of herbivorous mites, such as the spider mites *Tetranychus urticae* and *Tetranychus evansi*, were found to maximize their performance by suppressing inducible plant defenses. At first glimpse it seems obvious why such a trait will be favored by natural selection. However, defense suppression appeared to readily backfire since mites that do so also make their host plant more suitable for competitors and their offspring more attractive for natural enemies. This, together with the fact that spider mites are infamous for their ability to resist (plant) toxins directly, justifies the question as to why traits that allow mites to suppress defenses nonetheless seem to be relatively common? We argue that this trait may facilitate generalist herbivores, like *T. urticae*, to colonize new host species. While specific detoxification mechanisms may, on average, be suitable only on a narrow range of similar hosts, defense suppression may be more broadly effective, provided it operates by targeting conserved plant signaling components. If so, resistance and suppression may be under frequency-dependent selection and be maintained as a polymorphism in generalist mite populations. In that case, the defense suppression trait may be under rapid positive selection in subpopulations that have recently colonized a new host but may erode in relatively isolated populations in which host-specific detoxification mechanisms emerge. Although there is empirical evidence to support these scenarios, it contradicts the observation that several of the mite species found to suppress plant defenses actually are relatively specialized. We argue that in these cases buffering traits may enable such mites to mitigate the negative side effects of suppression in natural communities and thus shield this trait from natural selection.

Keywords: defense suppression, host plant manipulation, resistance, *Tetranychus*, effectors, jasmonate, herbivore, buffering trait

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INTRODUCTION

Among the diverse organisms that parasitize plants are numerous species of mites (Arachnida: Acari). With a body size of usually less than a millimeter, these mites are among the smallest herbivores. They feed by piercing an epidermal or mesophyll cell with their stylet-like mouthparts, after which they suck up the cellular contents (Helle and Sabelis, 1985; Lindquist et al., 1996;

Bensoussan et al., 2016). Despite their relatively limited per capita consumption, herbivorous mites are a pest on nearly every agriculturally or horticulturally important plant species, causing massive economic losses worldwide (Helle and Sabelis, 1985; Lindquist et al., 1996; Van Leeuwen et al., 2015). That is because herbivorous mites generally have high fecundity, a short developmental time and a female-biased offspring ratio, which, among others, allows them to build up populations large enough to destroy entire plants within just a few weeks (Helle and Sabelis, 1985; Lindquist et al., 1996).

In order to protect themselves from mites and other phytophagous organisms, plants have evolved a plethora of defensive traits. A subset of these traits is aimed at deterring, inhibiting or killing the parasite via mechanisms that range from physical obstruction to the production of (volatile) metabolites or proteins that either directly harm the attacker, e.g., due to their toxic or antinutritional properties, or that do so indirectly via facilitating the recruitment of the attacker's natural enemies (**Figure 1A**) (Jones and Dangl, 2006; Heil, 2008; Mithöfer and Boland, 2012; Schuman and Baldwin, 2016). A major source of resistance to small arthropod herbivores, including mites, are the glandular trichomes, as these represent physical barriers that also produce, store and/or exude large amounts of (volatile) defensive metabolites and proteins (Glas et al., 2012). Many defensive traits, however, are more specific in the sense that they: (a) are most effective against a relatively narrow range of attackers, and; (b) are confined to a single plant species, -family, -order or -clade (Mithöfer and Boland, 2012; Couto and Zipfel, 2016; Schuman and Baldwin, 2016). Furthermore, in many cases the amounts of defensive metabolites and proteins are low or absent under unstressed conditions but increase considerably upon attack. Probably, inducible defenses have evolved to save resources and/or to limit autotoxic effects (Mithöfer and Boland, 2012; Couto and Zipfel, 2016; Schuman and Baldwin, 2016). Such inducibility requires a rapid and robust signaling machinery to activate the appropriate defenses in a timely manner, which starts with detection of the attacker.

How plants recognize mite-feeding or the extent to which they can tell mites apart from other herbivores, is yet unknown. Plants are thought to recognize their attacker based on the perception of two classes of molecules: The first one being damage-associated molecular patterns (DAMPs), which are plant-derived and modified or dislocated as a consequence of wounding (Heil and Land, 2014; Gust et al., 2017). Spider mites seem to avoid causing unnecessary damage and, hence, the release of DAMPs, by inserting their stylet in between epidermal cells or via open stomata to reach the mesophyll (Bensoussan et al., 2016). The second one being microbe- or herbivore-associated molecular patterns (MAMPs or HAMPs, respectively), which can be attacker-derived, plant-derived (but often modified by the attacker) or conjugates of the two (Boller and Felix, 2009; Acevedo et al., 2015). DAMP-, MAMP-, and presumably also HAMP-recognition is mediated by pattern-recognition receptor (PRR) proteins (Couto and Zipfel, 2016). Such recognition activates an intracellular signaling cascade that, within minutes, results in the induction of defenses (Couto and Zipfel, 2016). This entire process is known as pattern-triggered immunity (PTI). Whereas PTI has been well established for plants attacked by diverse microbial phytopathogens and is expected to function in plant–herbivore interactions as well, experimental evidence for the latter hypothesis is still scarce and often indirect. For instance, while several HAMPs have been characterized at the molecular level, no matching PRR has been identified (Acevedo et al., 2015; Schmelz, 2015). Likewise, some plant PRRs have been implicated in plant resistance to herbivores (Gilardoni et al., 2011; Cheng et al., 2013; Gouhier-Darimont et al., 2013; Liu et al., 2015; Hu et al., 2018) but the matching herbivory-derived ligands remain elusive. Hence, while it is likely that they exist, mite-derived HAMPs and cognate plant PRRs have not been identified yet.

The PTI signaling cascade critically depends on the action of several phytohormones, the most important ones are jasmonic acid (JA) and salicylic acid (SA) (Pieterse et al., 2012; Couto and Zipfel, 2016). Generally, JA is required to mount effective defense responses against herbivores as well as microbial pathogens with a necrotrophic life style (Pieterse et al., 2012). By contrast, resistance against biotrophic microbial pathogens depends on SA (Glazebrook, 2005; Pieterse et al., 2012). Curiously, some mites, including the extremely polyphagous cosmopolitan pest species *Tetranychus urticae*, simultaneously induce JA and SA-regulated defenses (**Figure 1A**) (Kant et al., 2004; Zhurov et al., 2014; Alba et al., 2015; Schimmel et al., 2017a,b), although plant resistance to these mites predominantly depends on JA (Kant et al., 2008; Zhurov et al., 2014; Villarroel et al., 2016). Signaling components of the JA and SA pathways can interact with each other, synergistically or antagonistically, but can also interact with signaling components of growth-regulating hormonal pathways (Pieterse et al., 2012). Hormonal crosstalk is thought to adaptively tailor defense responses to different enemies as well as to minimize wasting resources on unnecessary defenses (Thaler et al., 2012). Finally, many of the defense responses activated during PTI are induced not only in the attacked tissue but also in non-attacked, systemic tissues (Fu and Dong, 2013; Schuman and Baldwin, 2016). Herbivorous mites, too, induce defense responses in systemic leaves (Sarmento et al., 2011a), which may increase the resistance of these tissues when attacked later on (Agut et al., 2016).

Pattern-triggered immunity confers resistance to the majority of all plant parasites, meaning to those that induce defenses and are susceptible to them. Many phytophagous organisms, however, have acquired traits that enable them to overcome plant defenses (Kant et al., 2015). These traits can roughly be divided into three categories: (1) avoidance, (2) metabolic resistance, and (3) suppression, i.e., host plant manipulation. The avoidance of (induced) plant defenses entails a behavioral strategy that has been observed for diverse arthropod herbivores (Dussourd, 2017) at spatial resolutions ranging from between individual plants (Kessler et al., 2004) to within a single leaflet (Shroff et al., 2008). Metabolic resistance to plant defenses can arise from target-site insensitivity or from detoxification mechanisms that may include metabolite modification, degradation and/or secretion (Despres et al., 2007; Heckel, 2014). Defense suppression is achieved via sabotage of the host plant's molecular machinery. To do so, plant-feeding organisms have evolved specialized molecules which they secrete into or onto their host and which interfere in various

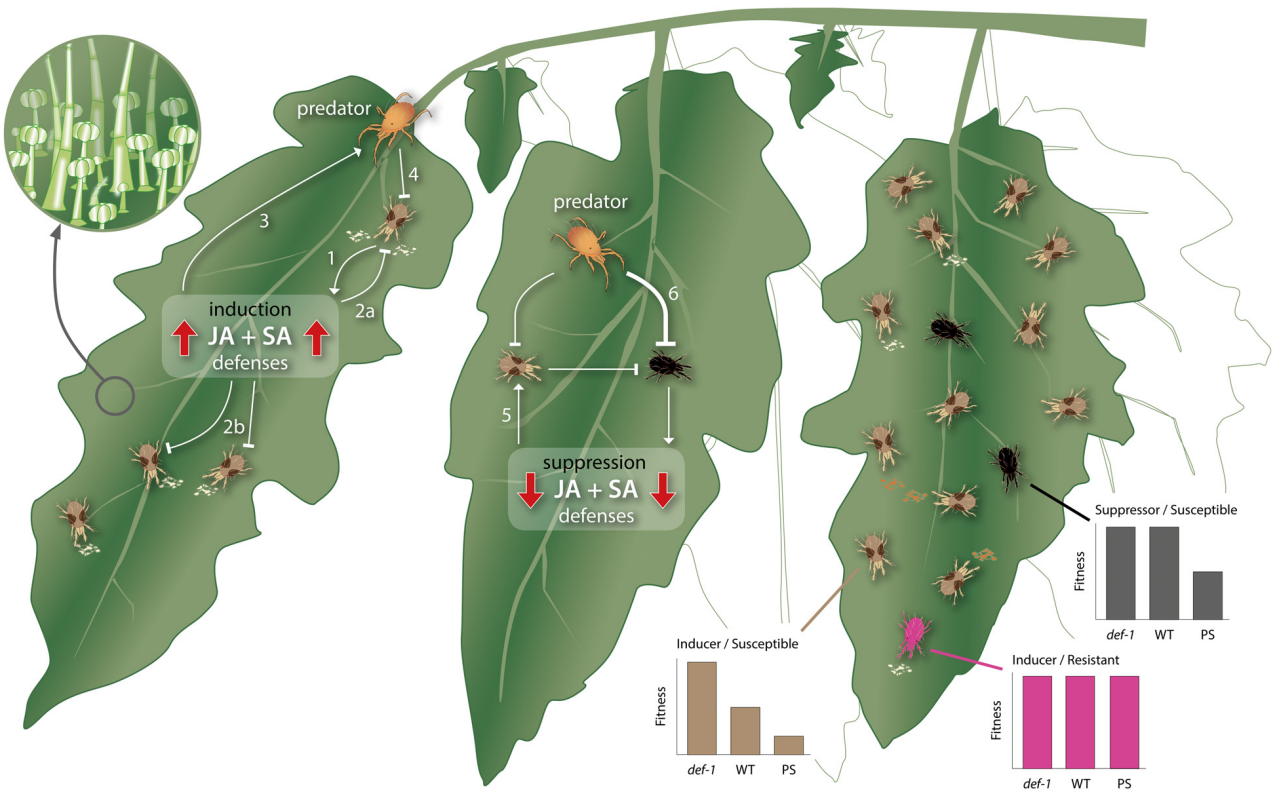

FIGURE 1 | Schematic, simplified overview of the direct and indirect interactions between cultivated tomato plants, herbivorous spider mites, and carnivorous predatory mites. **(A)** Mite herbivory induces direct and indirect plant defenses that hamper mite performance. While feeding, *Tetranychus urticae* triggers the accumulation of the phytohormones jasmonic acid (JA) and salicylic acid (SA), which, in turn, promotes the production of (volatile) metabolites and proteins (arrow 1), aimed at harming or deterring the mite (inhibition line 2a). Such plant-produced metabolites and proteins may similarly affect mites that simultaneously or subsequently feed from the same or from not previously attacked, systemic tissues (inhibition line 2b). Additionally, they may mediate the attraction and arrestment of the attacker's natural enemies (arrow 3), thereby facilitating predation of the herbivore (inhibition line 4). The areal surface of tomato plants contains glandular trichomes (inset), which not only represent a physical barrier for mites, but also produce large amounts of defense-associated (volatile) metabolites and proteins. **(B)** In natural communities, herbivorous mites that suppress plant defenses may suffer from negative side effects associated with this trait. That is, by suppressing defenses, the host becomes a better food source, including for competitors, which may therefore be promoted on a shared leaf (arrow 5). Furthermore, defense-suppressing mites themselves may become a better food source for their natural enemies, i.e., better than defense-inducing mites (inhibition line 6). **(C)** For more polyphagous mite species, intraspecific variation in traits related to mite–plant interactions exists within natural populations. Three *T. urticae* genotypes have, for instance, been described: (i) Mites that induce defenses on wild type (WT) tomato and that are susceptible to these defenses (brown mites). Consequently, these mites have a low fitness on WT plants, a much higher fitness on JA-impaired mutants (*def-1*) and an extremely low performance on transgenic 35S::prosystemin (PS) plants that constitutively display JA defense responses. (ii) Mites that are susceptible to tomato defenses but nevertheless maintain a high fitness because they suppress these defenses (black mites). These mites perform as well on WT as on *def-1*, but have a lower fitness on PS, presumably because they cannot suppress the extraordinarily strong defenses of PS plants. Mites from this genotype appear to be relatively rare as compared to the defense-inducing/susceptible ones. (iii) Mites that induce defenses but that are metabolically resistant to plant-produced defensive metabolites or proteins and, therefore, have an equally high fitness on WT, *def-1* and PS (pink mites). This genotype appears to be rare as well. Note that mites are not drawn to scale and are distinctively colored for illustrative purposes only. In nature, *T. urticae* from the three described genotypes are, by eye, morphologically indistinguishable.

ways with the host's ability to defend itself (Hogenhout and Bos, 2011; Kant et al., 2015; Khan et al., 2018). Such molecules are referred to as "effectors" or "virulence factors," but it is important to point out that effectors that suppress defenses in one host plant may elicit defenses in another (in the latter case they are also referred to as "avirulence factors") (Hogenhout et al., 2009). Here we will use the term "effector" in the context of host-plant defense suppression. Finally, it is of note that symbioses with microorganisms, or alternatively horizontal gene transfer events from microbes, may underlie a herbivore's ability to overcome plant defenses (Douglas, 2015; Wybouw et al., 2016).

Among herbivorous arthropods, suppression of defenses has been observed in several insect species (Stahl et al., 2018), three spider mites species (*T. urticae*: Kant et al., 2008; *T. evansi*: Sarmento et al., 2011a; *T. ludeni*: Godinho et al., 2016) and an eriophyoid mite species (*Aculops lycopersici:* Glas et al., 2014). Research on defense suppression by arthropods has almost exclusively focused on the source of suppression (i.e., effectors)

and the effect of suppression on plant physiology (Stahl et al., 2018). Defense suppression has obvious benefits for herbivores as the down-regulation of defenses coincides with an increase in herbivore fitness (Kant et al., 2008; Sarmento et al., 2011a; Alba et al., 2015; Schimmel et al., 2017a). This observation comes mostly from laboratory experiments lacking the natural ecological context. Yet, for understanding which factors drive the emergence, persistence, and disappearance of this trait this context may be crucial. For example, defense suppression appeared not only to benefit the herbivore doing it but also its competitors residing on the same leaf (Kant et al., 2008; Sarmento et al., 2011b; Alba et al., 2015) and may underlie patterns of mite species-succession observed in the field (Glas et al., 2014). In addition, defense suppression may promote predation (**Figure 1B**), given that the predatory mite *Phytoseiulus longipes* consumed more spider mite eggs that had been produced on defense-suppressed plants than eggs produced on plants with induced defenses (Ataide et al., 2016). This suggests that plant defensive substances, produced in response to herbivory, are transferred to the mite's eggs and may hamper predators, unless defenses are suppressed. In nature, mites commonly live in close proximity to other herbivorous- and predatory mites, i.e., on the same plant or even on the same leaf (De Moraes and Lima, 1983; Rosa et al., 2005; Ferragut et al., 2013; Glas et al., 2014). If so, then how do defense-suppressing mites control the apparent ecological costs of suppression? This question becomes even more puzzling when considering that species like *T. urticae* possess an extraordinary number of genes associated with metabolic resistance (Grbić et al., 2011; Dermauw et al., 2013) and, hence, may not need to suppress defenses in the first place. By means of this review, we propose scenarios that may explain why defense suppression seems to be a relatively common trait among specialist as well as generalist plant-feeding mites.

We will first present the mechanistic background of defense suppression by herbivorous mites, including how to experimentally tell suppression apart from induction or from stealth feeding. Then, we will explore the eco-evolutionary scenario's that may favor this trait. Finally, we will outline which traits may enable herbivorous mites to counteract the negative side effects of defense suppression that can occur when living in natural communities. We will focus on the direct and indirect interactions between cultivated tomato (*Solanum lycopersicum*), the generalist two-spotted spider mite (*T. urticae*), and the specialist tomato red spider mite (*T. evansi*), because these three species have become a model for addressing mechanistic or ecological questions on the costs and benefits of defense induction versus suppression by arthropod herbivores.

MECHANISTIC BACKGROUND OF PLANT DEFENSE SUPPRESSION BY MITES

The ability to suppress plant defenses is a trait that allows a phytophagous organism to lower the magnitude of a defensive process, either constitutive or induced, such that it gains a reproductive advantage. Although this definition could include behavioral sabotage such as vein-cutting (Dussourd, 2017), we will focus here on the suppression of molecular processes. The definition also excludes stealth feeding (Walling, 2008), because this does not affect the defensive process as such. It is important to realize that suppression does not need to be absolute, i.e., down to- or below levels of non-attacked plants, as it can already be effective when defenses are down-regulated to intermediate levels (Glas et al., 2014; Alba et al., 2015). In our experience, such absolute suppression is rare. Defense-suppressors rather reduce the extent to which a subset of defenses are induced (Glas et al., 2014; Alba et al., 2015). For example, when compared to non-infested controls, an infestation with defense-suppressing *T. urticae* or *T. evansi* typically results in the increased accumulation of JA and SA, as well as in the increased expression of defense-associated genes, yet the magnitude of these defense responses is very small when compared to an infestation with non-adapted *T. urticae* (Alba et al., 2015; Schimmel et al., 2017a,b). These properties make it challenging to experimentally tell suppression apart from induction as well as from stealth feeding. However, there are three selection criteria that, together, enable researchers to identify defense-suppressors.

The first of these criteria is that defense-suppressing mites should have a similar fitness on wild-type (WT) plants versus on defense-deficient mutants (**Figure 1C**). That is because suppression renders WT plants phenotypically equivalent to such mutants in terms of their susceptibility to herbivores. Indeed, whereas non-adapted *T. urticae* performed much better on the JA-biosynthesis mutant *defenseless-1* (*def-1*) than on WT tomato (Li et al., 2002), defense-suppressing *T. urticae* and *T. evansi* mites performed just as well on WT as on *def-1* plants (Kant et al., 2008; Alba et al., 2015). Since defense-resistant mites will also have an equally high fitness on WT and defense-deficient mutants, this assay can be expanded with a set of hyper-defended plants, such as transgenic *35S::prosystemin* (PS) plants, to further discriminate the suppressor mites from the defense-resistant ones (Kant et al., 2008). The idea behind this is that suppressors can no longer suppress the extraordinarily strong defenses of PS plants, while resistant mites remain unaffected by them (**Figure 1C**).

The second criterion is that, on a shared host, defense-suppressing mites should be able to facilitate conspecific and/or heterospecific mites, including non-adapted ones. The reasoning behind this is threefold: (1) Plants attacked by suppressor mites are a better food source than plants attacked by defense-inducing mites. This will translate into a higher herbivore fitness on the former. (2) Suppression is most likely not free of costs for mites, i.e., it requires resources/energy, thus also suppressors will benefit when defenses are already suppressed by others. (3) Mites that have adapted to plant defenses by not inducing them (avoidance) or by evolving insensitivity (metabolic resistance) will not pass this test, as they are unable to facilitate other mites. Accordingly, compared with their respective controls, non-adapted *T. urticae* had a higher reproductive performance when their tomato host was either previously or simultaneously infested with defense-suppressing *T. urticae* (Kant et al., 2008; Alba et al., 2015) or *T. evansi* (Sarmento et al., 2011b; Alba et al., 2015). Similar experiments have identified *T. ludeni* (Godinho et al., 2016) and

A. lycopersici (Glas et al., 2014) as defense-suppressors. Despite the reported success of these co-infestation assays, they may also deliver variable results because the outcome strongly depends on the infestation conditions, such as timing of the infestations and the number of mites used (de Oliveira et al., 2016; Schimmel et al., 2017a,b). In addition, these co-infestation assays cannot discriminate between effects due to induced/suppressed defenses on the one hand and, for example, effects on plant resources on the other.

Whereas the first two criteria are bioassay-based and, thus, have mite performance as readout, the third criterion is based on a molecular assay and aimed to assess the impact of an alleged suppressor on an induced defense via an ask-the-plant approach. In practice this means that defense-suppressing mites should be able to suppress defenses that are induced by non-adapted mites or, in principle, by any other type of induction. The magnitude of defenses in plants that were infested with suppressor mites during or after the induction treatment should be lower than in plants that only received the induction treatment. For example, expression levels of defense-associated genes were significantly lower in tomato leaflets simultaneously infested with defense-suppressing *T. evansi* and defense-inducing *T. urticae* than in leaflets solely infested with defense-inducing *T. urticae* (Alba et al., 2015), even though the mite density was two-fold higher on the dual-infested leaves. This assay should be combined with one or both of the other methods as statistically significant down-regulation of defenses is by itself not proof for a biologically relevant effect. Finally, this assay may overlook relatively weak suppressors or suppressors with a primarily local effect.

How suppression of plant defenses by mites, or by herbivorous arthropods in general, works at the molecular level is still poorly understood. Suppression by *T. urticae*, *T. evansi*, and *A. lycopersici* was found to act downstream from phytohormone accumulation and independently of JA–SA crosstalk (Glas et al., 2014; Alba et al., 2015). While feeding, mites secrete saliva, which contains effector proteins that sabotage the host's defenses, resulting in effector-triggered susceptibility (**Figures 2A,B**) (Jonckheere et al., 2016; Villarroel et al., 2016). Combined genomic and transcriptomic analyses have revealed that spider mites are likely capable of producing and secreting several hundreds of salivary proteins (Jonckheere et al., 2016; Villarroel et al., 2016). Further proteomic analyses of salivary secretions collected using an artificial diet system have thus far identified 95 proteins from *T. urticae*'s saliva (Jonckheere et al., 2016). It remains unknown, though, how many of the (putative) salivary proteins actually interfere with the host's defenses. Firstly, because the sequence identity of effectors is usually very species-specific thus hampering *in silico* identification (Arnold et al., 2009; Lo Presti et al., 2015). Secondly, effectors not necessarily target plant defenses to trigger host susceptibility (Van Schie and Takken, 2014; Macho, 2016). Thirdly, salivary proteins may have effector-unrelated functions. For example, several mite salivary proteins were predicted to be carbohydrate or protein catabolic enzymes, suggesting a role in the degradation of plant material, possibly prior to ingestion (Jonckheere et al., 2016). Lastly, salivary proteins may be multifunctional. Salivary proteases of insects, for instance, may serve to (pre)digest proteins as food but may additionally target plant defensive proteins (Zhu-Salzman and Zeng, 2015).

Recent microscopic observations indicate that spider mites probably have much lower consumption rates than was hitherto assumed (Bensoussan et al., 2016). On common bean (*Phaseolus vulgaris*), the average duration of a single *T. urticae* feeding event was found to last nearly 14 min (Bensoussan et al., 2016), i.e., considerably longer than the roughly 3 s reported earlier (Liesering, 1960). If feeding events indeed last several minutes instead of seconds, pre-digestive functions of secreted salivary proteins would be conceivable. Likewise, this amount of time could allow effectors to interfere with host defenses in the pierced cell prior to ingestion. Additionally, it may allow effectors or their secondary signals to translocate to neighboring cells or to the apoplast to suppress defenses in plant tissues beyond the attacked cell (**Figure 2B**) (Bensoussan et al., 2016; Rioja et al., 2017). Indeed, there is empirical evidence for defense suppression to occur systemically within leaflets (Alba et al., 2015) and within compound leaves (Sarmento et al., 2011a). However, suppression appears to be a predominantly local event, i.e., largely restricted to the mite's multicellular feeding patch (Schimmel et al., 2017a,b). Molecular studies at single cell resolution are required to assess the true spatial extent of suppression.

We can only speculate about how mite salivary effector proteins operate inside the host plant. Most likely they interact with plant proteins to modulate their function such that the plant becomes more suitable as food. Numerous of such *in planta* targets have been identified for effectors from diverse microbial phytopathogens and for many of these their mode of action has been characterized as well, providing valuable insights into the molecular mechanisms underlying pathogen virulence (Khan et al., 2018; Xin et al., 2018). The majority of phytopathogen effectors as well as their *in planta* targets appear to be of proteinaceous nature (Khan et al., 2018), but note that this could be due to a methodological bias. Large scale protein-protein interaction assays have revealed that a subset of the effectors deployed by phytopathogens targets and modifies a relatively small but conserved set of plant signaling "hubs," which represent highly connected nodes within the plant protein network, as each of them (potentially) interacts with dozens of other plant proteins (Mukhtar et al., 2011; Wessling et al., 2014). Examples of effector-targeted hub proteins are: TCP transcription factors, which function at the nexus of plant development and defense (Lopez et al., 2015); subunits of the ubiquitin-proteasome system, which are crucial for protein turnover including during phytohormone signaling (Banfield, 2015); JAZ proteins, which are transcriptional repressors of JA responses (Howe et al., 2018), and; papain-like cysteine proteases, which have diverse functions in immune signaling (Misas-Villamil et al., 2016). Consistent with their role in PTI, mutations in effector-targeted hub proteins generally have dramatic consequences for plant resistance to phytopathogens (Mukhtar et al., 2011; Wessling et al., 2014). Other components of the PTI machinery, i.e., that are (relatively) less well-connected, are manipulated by phytopathogen effectors as well. These include conserved detection and signaling components (e.g., PRRs, co-receptors, receptor-like cytoplasmic kinases, MAP kinases, transcriptional

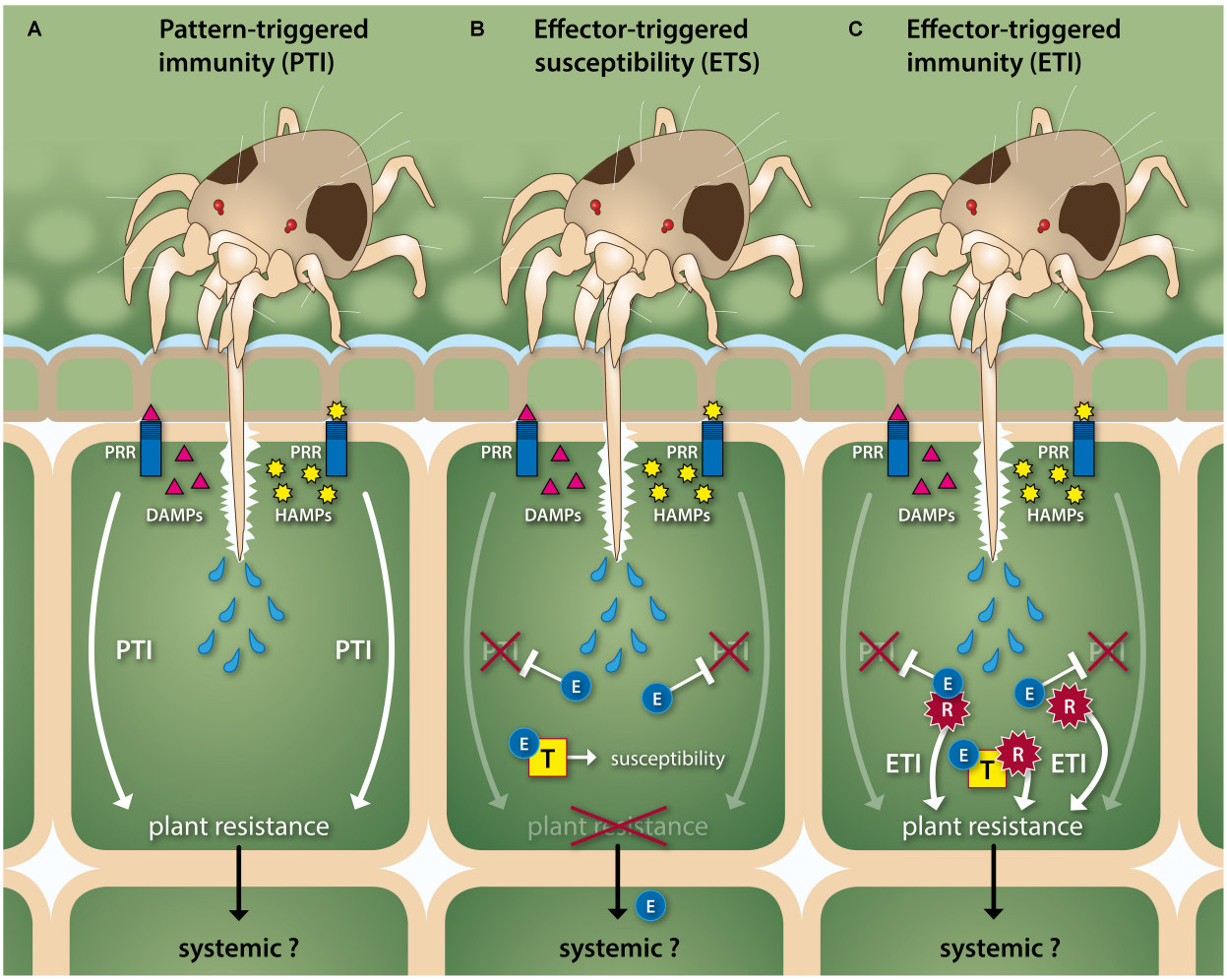

FIGURE 2 | Schematic, simplified overview of the *in planta* molecular interplay between herbivorous spider mites and their host. **(A)** Spider mites use their stylet-shaped mouthparts to retrieve the contents of mesophyll cells, which may trigger the activation of plant defense responses that render the plant resistant. Mites pierce mesophyll cells and inject them with saliva prior to ingestion of their contents. This may cause the release of damage- and/or herbivore-associated molecular patterns (DAMPs and HAMPs, respectively) that are recognized by plant pattern-recognition receptors (PRRs) and which leads to pattern-triggered immunity (PTI). Spider mites seem to minimize the release of DAMPs by inserting their stylet via open stomata (not shown) or in between epidermal cells to reach the mesophyll. **(B)** Mites may interfere with PTI and other host processes by injecting salivary effector molecules (E) that target and interact with various plant proteins (T) to inhibit or to exploit their function and, thereby, render the plant susceptible. This process is termed effector-triggered susceptibility (ETS). **(C)** Plants have evolved receptor proteins (R) that detect effectors directly or indirectly and subsequently restore PTI responses plus induce additional defenses that altogether render the plant resistant again. This process is referred to as effector-triggered immunity (ETI). Herbivory by mites likely induces PTI- or ETI-associated defense responses beyond the attacked cell, i.e., also in non-attacked tissues, but the spatiotemporal dynamics of such systemic responses are not fully understood. Likewise, mites are thought to manipulate their host plant beyond the attacked cell, for instance via the intracellular transport of effectors. Note that mite-HAMP and plant-PRR pairs have not been identified yet. Mites, plant cells and their (secreted) components are not drawn to scale.

regulators; Macho and Zipfel, 2015; Khan et al., 2018) as well as proteins that are produced by the plant to actually fight off the pathogen (e.g., proteases and protease inhibitors; Jashni et al., 2015). Finally, phytopathogen effectors have been found to target proteins with less obvious connections to plant immunity but whose manipulation is nevertheless essential for virulence and pathogen proliferation (Van Schie and Takken, 2014; Macho, 2016). Examples of such so-called susceptibility proteins are: nutrient transporters (Chen et al., 2010), proteins involved in vesicular trafficking (Xin et al., 2016) and cell cycle regulators (Wildermuth et al., 2017). The first reports of proteinaceous plant targets of effectors from herbivorous arthropods indicate that at least some members of this diverse group of plant-feeders may have evolved mechanisms to manipulate their host that are similar to those of microbial phytopathogens. That is, effectors secreted by larvae of the Hessian fly (*Mayetiola destructor*) were shown to interact with the wheat (*Triticum* spp.) Skp subunit of the ubiquitin-proteasome system (Zhao et al., 2015) and effector Mp1 secreted by the aphid *Myzus persicae* was found to interact with Arabidopsis (*Arabidopsis thaliana*) as well as potato (*Solanum tuberosum*) VPS52, which is thought to be involved in vesicular trafficking (Rodriguez et al., 2017). Since mites are

not highly mobile, small, and feed from one cell at a time, we hypothesize that there will be considerable overlap between the effector targets of biotrophic microbial pathogens and those of mites, in particular for generalists like *T. urticae*. Specifically, we predict effectors of generalist mites to target conserved plant targets. If so, this would allow the mite to manipulate different hosts using a relatively small set of effectors as compared to the large number of metabolic resistance-conferring genes that would be needed to overcome the defenses of all its different hosts. Specialized mite species may have evolved effectors more specific for their host and as a consequence they may have lost redundant effector paralogs.

Despite the convincing genomic, transcriptomic and proteomic data on mite salivary proteins, the far majority still awaits functional characterization (as effectors). Four mite proteins have been identified as plant defense-suppressing effectors so far; Tu28 and Tu84 from *T. urticae* and the orthologous Te28 (66% identical) and Te84 (63% identical), respectively, from *T. evansi* (Villarroel et al., 2016; Schimmel et al., 2017a). As indicated by their numbers, these proteins represent members from two putative effector families: in *T. urticae* family 28 has 10 members (paralogs), whereas *T. evansi* has only one; family 84 has two paralogs in both mite species (Villarroel et al., 2016). Proteins from these families have also been recovered from the saliva of *T. urticae* feeding on artificial diet (Jonckheere et al., 2016). When transiently overexpressed in *Nicotiana benthamiana* these effectors suppressed SA-defenses (Villarroel et al., 2016) as well as JA-defenses (Schimmel et al., 2017a) and three of the four homologs promoted the fitness of non-adapted *T. urticae* (Villarroel et al., 2016). Te28 did not enhance the performance of *T. urticae* on *N. benthamiana*, probably due to the severe chlorosis that coincided with *Te28* overexpression (Villarroel et al., 2016). In line with this reasoning, on tomato, *Te28* transcript abundance in *T. evansi* correlated negatively with the magnitude of JA and SA defenses in the plant, and positively with mite performance (Schimmel et al., 2017a). Similar correlations were found for *Te84* (Schimmel et al., 2017a), strongly suggesting that Te28 and Te84 are indeed used by *T. evansi* to suppress tomato defenses. The fact that defense-inducing *T. urticae* possess gene copies that encode the functional effectors Tu28 and Tu84 suggests that these mites, too, can suppress defenses. Expression analysis of the corresponding effector genes, though, revealed stunning quantitative differences between *T. urticae* and *T. evansi*, especially for effectors of family 84. On tomato, across different -but comparable- infestation conditions, the relative expression of *Tu28* versus *Te28* ranged from similar levels in the two species to *Te28* transcripts being up to six times more abundant in *T. evansi* than *Tu28* transcripts in non-adapted *T. urticae* (Schimmel et al., 2017a). Transcripts of effector 84 were much more abundant in *T. evansi* regardless of infestation conditions, i.e., *Te84* was roughly 60 to 140 times higher expressed than *Tu84* (Schimmel et al., 2017a). Thus, in addition to differences in the amino acid sequences between orthologous effectors of non-adapted *T. urticae* and specialist *T. evansi*, there are probably also differences at the effector abundance level. Something similar was observed for the spider mite-specific SHOT gene family, which is thought to encode effector proteins (Jonckheere et al., 2017). The genome of generalist *T. urticae* contains 12 SHOT paralogs while Solanaceae-specialist *T. evansi* possess only one ortholog and the Fabaceae-specialist *Tetranychus lintearius* only two (Jonckheere et al., 2017). The expression of several *T. urticae* SHOT genes appeared strongly host-dependent and remarkably plastic, as they were both rapidly and massively induced upon transfer to Fabaceae hosts but were not expressed on plants from other families (Jonckheere et al., 2016, 2017). Together this underscores that the ability of mites to suppress plant defenses via secreted effectors, and possibly to dodge detection by plants, may be tremendously plastic and cannot simply be inferred from the absence/presence of (putative) effectors in the mite's genome.

As indicated before, there is no information yet on the *in planta* targets of spider mite effectors but based on our knowledge of effectors from microbial phytopathogens (Mukhtar et al., 2011; Wessling et al., 2014; Khan et al., 2018), we speculate that: (a) a subset of the mite effectors will target and manipulate conserved plant proteins that function as signaling hubs in defense and/or development; (b) multiple mite effectors will be able to interact with the same plant protein, while simultaneously; (c) individual mite effectors will be able to interact with multiple plant proteins. Finally, thus far research has been focused on the identification of mite salivary proteins and their characterization as effectors, but effectors are not necessarily of proteinaceous nature. For example, certain bacterial phytopathogens secrete metabolites that function as plant hormones and exploit the conserved hormonal crosstalk mechanism of the host to trigger susceptibility (Zheng et al., 2012; McClerklin et al., 2018). Some eriophyid mites have been suggested to produce and secrete functional plant hormones (De Lillo and Monfreda, 2004). There are no indications that spider mites do so (Grbić et al., 2011). As another example, fungal phytopathogens (Weiberg et al., 2013) and parasitic plants (Shahid et al., 2018) can secrete small RNAs that exploit the host's RNA interference machinery to silence defense-associated genes. Whiteflies have been predicted to do the same, as they also secrete small RNAs into their host (van Kleeff et al., 2016). The involvement of small RNAs in defense suppression by mites cannot be excluded. Taken together, the mite effector repertoire may extend well beyond their salivary proteins.

To counteract effector-triggered susceptibility, plants have evolved sensory molecules (receptors) often referred to as R-genes/proteins that can by-pass the effector's manipulation. R-genes usually encode intracellular nucleotide-binding leucine-rich-repeat (NLR) proteins or cell surface-localized receptor-like proteins/kinases (RLPs/RLKs) that detect effectors or effector-activity and subsequently restore PTI plus induce additional defenses that altogether render the plant resistant again (Cui et al., 2015; Kourelis and van der Hoorn, 2018; Su et al., 2018). This R-gene mediated process is referred to as effector-triggered immunity (ETI) (**Figure 2C**). Plant genomes typically contain hundreds of NLR- and RLP/RLK-encoding genes that are fast-evolving and belong to expanded families (Jacob et al., 2013; Kourelis and van der Hoorn, 2018; Su et al., 2018). Consequently, most of these sensory proteins appear to be highly specific, meaning distinct variants are present in each plant species,

putatively reflecting the effector repertoire of the biotic attackers they are commonly confronted with (Jacob et al., 2013; Kourelis and van der Hoorn, 2018; Su et al., 2018). This implies that the occurrence of effective R-gene mediated resistance can differ greatly across genotypes (varieties) within plant species. As with PTI, ETI has been well established for plants in response to attacks by microbial phytopathogens, while its involvement during interactions with herbivores is still being explored. For instance, only a small fraction of the R-genes that have been implicated in plant resistance to arthropod herbivores has been characterized to date, i.e., *Mi-1.2* in tomato (Milligan et al., 1998; Rossi et al., 1998; Vos et al., 1998), *Vat* in melon (*Cucumis melo*) (Dogimont et al., 2014) and several *Bph* genes in rice (*Oryza sativa*) (Du et al., 2009; Tamura et al., 2014; Wang et al., 2015; Ji H. et al., 2016; Ren et al., 2016; Zhao Y. et al., 2016; Guo et al., 2018). With respect to mites and ETI, spider mite feeding was shown to rapidly affect the expression of large groups of putative RLK-encoding genes in tomato and Arabidopsis (Martel et al., 2015), suggesting these may play an important role in the detection of mite feeding, i.e., as (co-)receptors for DAMPs, HAMPs, or effectors (Couto and Zipfel, 2016; Kourelis and van der Hoorn, 2018). Other than this report, the involvement of ETI in plant–herbivorous mite interactions (**Figure 2C**) remains hypothetical and requires experimental verification.

The strong selective pressures enforced by such plant receptors is reflected in the characteristics of effector-encoding genes of phytophagous organisms: such genes are usually highly abundant in their genomes, are fast-evolving and belong to expanded families (Jiang et al., 2008; Raffaele et al., 2010; Zhao et al., 2015). This appears to be the case for (putative) spider mite effector genes as well (Jonckheere et al., 2016; Villarroel et al., 2016). Under pressure of ETI, plant-parasites have evolved various counter-adaptations to overcome it, including: (a) the acquisition of sequence mutations in 'betraying effectors' that do not interfere with their function yet attenuate recognition by NLRs; (b) the loss of 'betraying effectors' via gene silencing or gene removal; (c) the gain of novel effectors that serve as decoys for- or that mask 'betraying effectors' (Aggarwal et al., 2014; Dong et al., 2014; Huang et al., 2014; Wei et al., 2015; Ji Z. et al., 2016; Zhao C. et al., 2016; Inoue et al., 2017; Ma et al., 2017; Menardo et al., 2017). Not surprisingly, plant-feeding organisms deploy distinct sets of effectors depending on which host species they attack, likely to deal with the specific defenses they encounter (Yoshida et al., 2016; Mathers et al., 2017; Rivera-Vega et al., 2017; Lorrain et al., 2018). The available data for spider mites is consistent with this hypothesis (Jonckheere et al., 2016, 2017).

ECO-EVOLUTIONARY BACKGROUND OF PLANT DEFENSE SUPPRESSION BY MITES

Plants and herbivores are probably regularly engaged in a co-evolutionary arm's race. If so, there should be heritable variation in traits that allow plants to resist herbivores as well as heritable variation in traits that allow herbivores to cope with these defenses, for natural selection to act on (Bolnick et al., 2011; Gloss et al., 2016). For interactions between generalists and multiple host plants such interactions are predicted to be more diffuse than for specialists (Futuyma and Agrawal, 2009). Given the tremendous diversity among the more than 200,000 defensive metabolites/proteins found across the plant kingdom, it is hypothesized that the larger the host range of a herbivore is, the smaller is the chance it will evolve metabolic resistance-conferring traits (Becerra, 1997; Despres et al., 2007; Ali and Agrawal, 2012). There are two main arguments to support this hypothesis: (1) Mechanistically, metabolic adaptations to each individual class of defensive metabolites/proteins encountered on diverse hosts do not seem feasible or seem too costly. (2) By changing host species the selective pressure required to evolve and/or maintain metabolic adaptations to specific plant defensive compounds will decrease or disappear. Hence, metabolic resistance-conferring traits are most often found in specialized herbivores, as these feed from a single or a few closely related plants and, thus, continuously encounter the same defensive compounds. By contrast, generalists are hypothesized to increase their fitness across multiple plant taxa by actively interfering with conserved defense signaling components (Ali and Agrawal, 2012; Kant et al., 2015). Concurrently, plant-produced defensive metabolites/proteins are expected to have a different impact on generalist versus specialist herbivores. Whereas generalists are negatively affected at an intermediate level by any class of defensive compounds, specialists are less affected by metabolites/proteins produced by the plant species they have specialized on, but on average suffer more from those produced by non-host plants (Ali and Agrawal, 2012; Heckel, 2014). Studies on various plant-insect systems have found empirical evidence to support these hypotheses (Ali and Agrawal, 2012; Kant et al., 2015), but the available data on plant-mite interactions does not seem to do this for several reasons.

Firstly, among the mites species that have been found to suppress plant defenses, only *T. urticae* is a true generalist, whereas *T. ludeni*, *T. evansi,* and *A. lycopersici* are all (relatively) specialized herbivores, i.e., on Solanaceae (Helle and Sabelis, 1985; Lindquist et al., 1996). Additionally, within natural populations of *T. urticae* the defense-suppressors do not appear to be the dominant genotype (**Figure 1C**) (Kant et al., 2008; Alba et al., 2015). So far, all sampled populations of *T. evansi*, covering both haplotypes, were found to be potent suppressors of tomato defenses (Sarmento et al., 2011a; Alba et al., 2015), suggesting that the defense suppression trait is fixed in this species. These observations apparently contradict the hypothesis that most defense-suppressing herbivores should be generalists. It is worth pointing out, though, that defense suppression by *T. ludeni*, *T. evansi,* and *A. lycopersici*, respectively, has only been demonstrated on cultivated tomato (Sarmento et al., 2011a; Glas et al., 2014; Alba et al., 2015; Godinho et al., 2016) and that, although these mites predominantly infest Solanaceae, they have been found on plants belonging to other families as well. Specifically, *T. ludeni* has been recorded on plants from as many as 62 other families, *T. evansi* on plants from 35 other families, and *A. lycopersici* on one other family, i.e., the Convolvulaceae (Helle and Sabelis, 1985; Lindquist et al., 1996;

Migeon et al., 2010). The extent to which *T. evansi* and *T. ludeni* feed from- and reproduce on these non-solanaceous plants is not known. The identification of these mites on non-solanaceous hosts might actually be incorrect (i.e., many *Tetranychus* spp. are hard to distinguish by eye) or be an incidental consequence of passive dispersal (i.e., mediated by wind) from nearby overexploited solanaceous plants (Navajas et al., 2013). Nonetheless, it would be exciting to find out if these (relatively) specialized mites are able to also suppress defenses of plants that do not belong to the Solanaceae.

Secondly, *T. urticae* mites collected from diverse hosts have frequently been shown to be able to adapt to novel hosts. Strangely, most often this adaptation does not seem to go at the expense of their fitness on the ancestral or other hosts (Gould, 1979; Agrawal, 2000; Magalhaes et al., 2009; Wybouw et al., 2015), suggesting *T. urticae* to be a jack-of-all-trades. A comparative genome analysis has revealed that *T. urticae*'s genome harbors expansions in multiple gene families that have been implicated in xenobiotic metabolism, while such expansions were less dramatic, or not found at all, in the genomes of the specialized mites *T. evansi*, *T. lintearius,* and *A. lycopersici*, which suggests that metabolic resistance is a prominent trait underlying *T. urticae*'s adaptive abilities and enormous host range (Grbić et al., 2011; Van Leeuwen and Dermauw, 2016). Accordingly, (experimental evolution) studies that have analyzed the adaptation mechanism(s) of *T. urticae* to novel, challenging host plants have demonstrated large transcriptional plasticity in the mite's xenobiotic metabolism machinery (Dermauw et al., 2013; Zhurov et al., 2014; Wybouw et al., 2015). Very similar findings have been reported for generalist versus specialist aphids (Ramsey et al., 2010; Silva et al., 2012; Bansal et al., 2014; Mathers et al., 2017; Wenger et al., 2017). However, *T. urticae*'s adaptation to a novel, challenging host plant was also associated with the partial attenuation of a set of plant defense-associated transcriptomic responses, indicative of defense suppression (Wybouw et al., 2015). Something similar was observed for the generalist Kanzawa mite, *Tetranychus kanzawai*, by Ozawa et al. (2017). This suggests that the plasticity in the mite's effector repertoire (Jonckheere et al., 2016, 2017; Schimmel et al., 2017a) may augment the plasticity in its xenobiotic metabolism to rapidly overcome the resistances of novel hosts. Such a dual mechanism has also been suggested for aphids, whose ability to colonize novel host plants is correlated with transcriptional plasticity of a conserved set of genes, several of which encode (putative) host plant-specific effectors (Elzinga et al., 2014; Thorpe et al., 2016; Eyres et al., 2017; Mathers et al., 2017; Rodriguez et al., 2017). Collectively, the available data suggest that mite traits enabling an improved xenobiotic metabolism are functionally linked, at least partially, with traits related to host defense manipulation.

Tetranychus urticae appears to harbor distinct intraspecific variation for traits that cause these mites to induce defenses as well traits that allow them to suppress or to resist tomato JA defenses (Kant et al., 2008; Alba et al., 2015). Both Kant et al. (2008) and Alba et al. (2015) sampled natural populations of *T. urticae* from various non-solanaceous host plants. Subsequently they created near-isogenic lines from individual mites, which were then submitted to a novel host, i.e., WT tomato plants, *def-1* and PS, as described earlier. These assays revealed the existence of three distinct phenotypes (**Figure 1C**): (1) Mites that induce defense responses to which they are also susceptible (i.e., these lower their fitness). This was the most common phenotype. (2) Mites that induced defense responses to which they are resistant (i.e., absence/presence of defense did not affect their fitness). This was a rare phenotype, not found in all populations. (3) Mites that were susceptible to induced defenses but nevertheless had a high performance because they could suppress these defenses. This phenotype was found at low frequencies in all populations. These results suggest that especially the defense-suppression traits could be maintained as a polymorphism by frequency-dependent selection in populations of *T. urticae* living on a mosaic of plant environments. This supports the scenario that defense suppression is a generalist trait that allows it to behave as a jack-of-all-trades, provided that the traits that allow mites to suppress defenses are effective on unrelated hosts. This would be possible if effectors target proteins or processes conserved across multiple host taxa. Yet, since suppression of defenses may come at high ecological costs (Sarmento et al., 2011b; Glas et al., 2014; Alba et al., 2015; Ataide et al., 2016) it may in time be replaced -via natural selection- by resistance traits, which not only appear to be more 'safe' in an ecological context, but may also promote fitness stronger than suppression does (Kant et al., 2008). In this scenario, defense suppression will allow populations that shift their host plant frequently to act as jack-of-all-trades but master-of-none. Sub-populations confined to a single host may gain resistance to that host at the expense of suppression and become a master-of-some (i.e., specialized).

Although this scenario predicts that suppression will be rare among specialist this does not seem to be the case for mites, as indicated earlier. This justifies the question why the suppression-traits of mites have not been replaced by resistance-traits during the course of specialization? We argue that these species possess buffering traits that can shield suppression-traits from natural selection imposed by facilitated competitors and/or natural enemies.

BUFFERING TRAITS THAT ENABLE MITES TO MITIGATE NEGATIVE SIDE EFFECTS OF HOST DEFENSE SUPPRESSION IN NATURAL COMMUNITIES

Probably the most obvious of such buffering traits of *T. evansi* concerns the production of web. As a family characteristic, spider mites produce silk, which is among others used to construct a web that shields the mites from unfavorable abiotic conditions as well as from competitors and predators (Helle and Sabelis, 1985). Silk production quantitatively differs between spider mite species and *T. evansi* is known to synthesize extraordinarily large amounts of it (Helle and Sabelis, 1985). Shortly after colonization of a new host plant, when the population size is small, only

local feeding patches are covered with web, but as the population grows, entire plants get readily encapsulated (Liu et al., 2017). The particularly dense web of *T. evansi* effectively hinders competing herbivorous mites, such as *T. urticae* (Sarmento et al., 2011b), as well as predatory mites, like *Euseius concordis* (De Moraes and Lima, 1983) (**Figure 3A**), but there is more to it than that. Results from another study suggest that *T. evansi* may actively increase the exclusion of competitors, as *T. evansi* females were found to produce a denser web in response to cues emanating from nearby *T. urticae* feeding sites (Sarmento et al.,

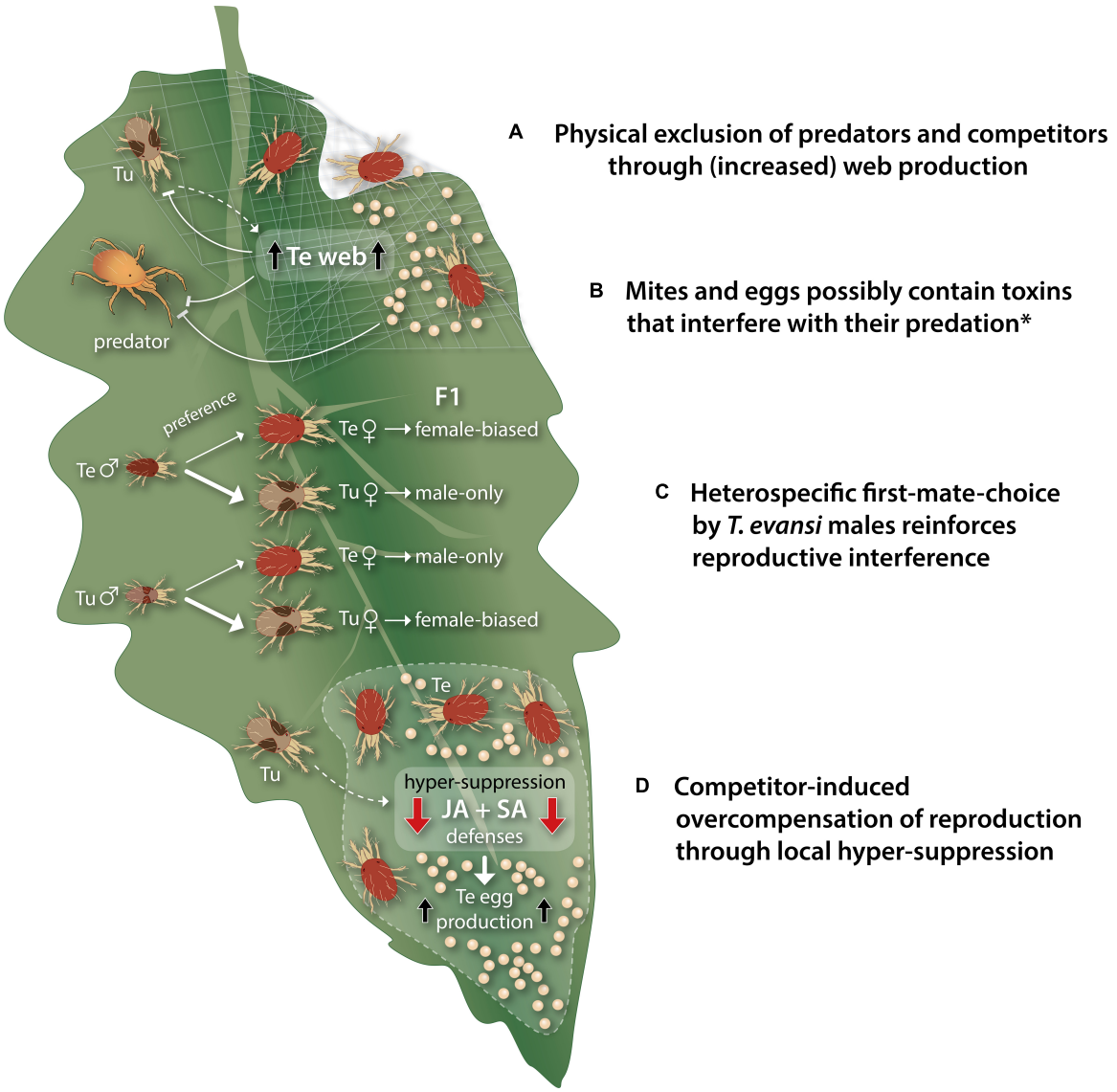

FIGURE 3 | Schematic, simplified overview of the buffering traits that may enable *Tetranychus evansi* mites to mitigate negative side effects of host defense suppression that can occur when living in natural communities. **(A)** *T. evansi* (Te) covers its feeding site with copious amounts of silken web, which shields off competing *Tetranychus urticae* (Tu) as well as predatory mites. Moreover, upon perception of *T. urticae* cues (dashed arrow), *T. evansi* produces a denser web, presumably to increase exclusion of its competitor. **(B)** Multiple species of predatory mites have an extremely poor performance on a *T. evansi* diet, possibly because *T. evansi* mites and their eggs contain one or more host plant-derived toxins that interfere with their predation. **(C)** Contrary to *T. urticae* males, *T. evansi* males prefer to copulate with heterospecific (i.e., *T. urticae*) females, this asymmetric mating preference reinforces reproductive interference by *T. evansi*, which can negatively impact the population growth of competing *T. urticae*. Spider mites are haplodiploid organisms and females show a strong first-male sperm precedence. Heterospecific mating events, therefore, result in (near) male-only offspring (hybrid females are not fertile), whereas conspecific mating events yield strongly female-biased offspring. **(D)** Upon perception of cues from nearby *T. urticae* (dashed arrow), *T. evansi* mites hyper-suppress jasmonate (JA) and salicylate (SA)-regulated plant defenses, albeit only at their feeding site, and this is paralleled by an increased oviposition rate for *T. evansi*, while the invading *T. urticae* does not benefit (yet). This rapid overcompensation response likely boosts *T. evansi*'s competitive population growth. The asterisk denotes that there is currently no empirical evidence for a causal relationship between the sequestration of toxins by *T. evansi* and the increased predation risk that may be associated with defense suppression by these mites.

2011b) (**Figure 3A**). The same happened in response to local *T. urticae* cues (Sarmento et al., 2011b). Vice versa, *T. evansi* does not appear to be hindered by *T. urticae*'s web, nor does *T. urticae* produce a denser web when *T. evansi* feeds close by (Sarmento et al., 2011b). Surprisingly, local cues from the predatory mite *P. longipes* did not trigger an increased production of web by *T. evansi* (Lemos et al., 2010). Different predator-induced behavioral changes were observed instead: not only did *T. evansi* lay fewer eggs, about a third of its eggs were suspended in the web, whereas nearly all eggs were deposited on the leaf surface under predator-free conditions (Lemos et al., 2010). Compared to eggs on the leaf surface, web-suspended eggs were less likely to be eaten by *P. longipes* (Lemos et al., 2010), providing a clear explanation for *T. evansi*'s altered behavior.

Predation of *T. evansi* eggs is actually a relatively rare event in nature, especially outside *T. evansi*'s native area (Navajas et al., 2013). For numerous naturally co-occurring as well as commercially available predatory mites, *T. evansi* is an unsuitable prey, meaning that aside from the adverse effects of the silken web and host-plant trichomes, most predators have an extremely poor performance on a diet of *T. evansi*, likely because it is toxic (De Moraes and Mcmurtry, 1985; Escudero and Ferragut, 2005; Rosa et al., 2005; Ferrero et al., 2014). This toxicity has been attributed to one or more plant-derived metabolites, which are probably modified and/or sequestered by *T. evansi* and passed on to their eggs as well (**Figure 3B**) (De Moraes and McMurtry, 1986; Koller et al., 2007; Ferrero et al., 2014). Selection for this toxin sequestration has possibly been promoted by an increased predation risk due to suppression of defenses as suggested by Ataide et al. (2016). It should be noted that defense suppression by *T. evansi* does not necessarily prevent the attraction of predatory mites, i.e., indirect plant defenses, despite their interference with the herbivory-induced production of volatile organic compounds (Sarmento et al., 2011a; Lemos, 2015). Hence, the toxin sequestration may be a buffering trait.

The third buffering trait of *T. evansi* concerns the direct interference with *T. urticae*'s reproduction due to asymmetric mating preferences. Even though the two species are reproductively incompatible, *T. evansi* males prefer to mate with *T. urticae* females instead of with conspecific females, whereas *T. urticae* males *do* preferentially mate with conspecifics (Sato et al., 2014, 2016). Since spider mites are haplodiploid organisms and females show a strong first-male sperm precedence (Helle and Sabelis, 1985), this asymmetric mating preference can have a strong negative effect on *T. urticae*'s population growth when mites from both species co-occur (Sato et al., 2014), a phenomenon known as reproductive interference (**Figure 3C**). That is because although heterospecific mating events do not affect the total number of eggs laid, females produce predominantly male offspring upon mating with a heterospecific male, as opposed to strongly female-biased offspring when fertilized by a conspecific (Sato et al., 2014; Clemente et al., 2016). The few hybrid females derived from interspecific mating events between *T. urticae* and *T. evansi* are not fertile (Clemente et al., 2016). Reproductive interference has also been observed between *T. urticae* and *T. ludeni* (Clemente et al., 2017), but it is not known which effects this has on the population growth of both species.

The fourth buffering trait of *T. evansi* involves plasticity in its reproductive performance -possibly resulting from plasticity in the magnitude of suppression- in response to the presence of competitors (**Figure 3D**). Analogous to the competitor-induced increased web production, *T. evansi* females on a well-established feeding site were found to suppress plant defenses stronger, albeit only locally, when *T. urticae* was introduced to adjacent leaf tissue (Schimmel et al., 2017a). This local hyper-suppression coincided with the increased expression of effector-encoding genes in *T. evansi* (*Te28* and *Te84*) and, moreover, was paralleled by an increased production of eggs by *T. evansi* -but not by the invading *T. urticae* (Schimmel et al., 2017a). Also Orsucci et al. (2017) found evidence for an increase in *T. evansi*'s reproductive performance when *T. urticae* was present on the same tomato leaf. In the opposite experimental situation, no significant changes were detected in the plant's defense responses, nor did *T. urticae* females produce more eggs upon introduction of *T. evansi* to adjacent leaf tissue (Schimmel et al., 2017a). This competitor-induced, plant-mediated overcompensation response of *T. evansi* therefore likely promotes its competitive population growth on tomato.

The discovery and characterization of *T. evansi*'s buffering traits has raised numerous questions, in particular whether similar traits can be found in other defense-suppressing mites (or insects)? For *A. lycopersici* the answer is a partial no, because this species does not produce web at all. However, this mite is extremely small and resides exclusively within the trichome forest on tomato stems and leaves, which is neither accessible for *T. urticae* nor for predatory mites. This may represent a behavioral trait that buffers facilitating competitors or natural enemies. Interestingly, after a few days of feeding by *A. lycopersici* glandular- and non-glandular trichomes on tomato deteriorate and this exposes the mite to its natural enemies, such as the predatory mite *Amblydromalus limonicus*. On such plants the russet mites were observed to rapidly move toward plant parts with intact trichomes (Van Houten et al., 2013).

Taken together, although natural selection may act against defense suppression under pressure of competition and predation this trait may also escape selection when shielded by buffering traits. These buffering traits may allow defense suppressors to remain suppressors, i.e., to counteract the evolution of resistance, during periods of specialization by enabling them to maintain the monopoly on their feeding site and to exclude natural enemies.

CONCLUSIONS AND PERSPECTIVES

So why do herbivorous mites suppress plant defenses?

(1) Not all herbivorous mites seem to suppress plant defenses but those that do obviously benefit from suppression as it increases their performance under laboratory conditions.
(2) Under natural conditions the benefits of suppression are less obvious since the ecological risks (costs) that come with suppression can be considerable.

(3) Suppression of defenses by herbivores is facilitated by secreted salivary effector proteins that manipulate plant processes to turn their host into a better food source.
(4) In our view the ability to suppress defenses facilitates a generalist life style and for these generalists -that move across environments with variable ecological risks- the advantage of being able to colonize multiple hosts may, on average, outweigh the costs.
(5) Existence of intraspecific variation suggests suppression-traits of generalist herbivores that live on a mosaic of plant environments to be maintained by frequency-dependent selection.
(6) We predict the effectors of generalists to target elements (e.g., proteins) of plant processes (e.g., defense pathways), that are conserved across their multiple hosts and thereby facilitate their multiple-host life style. This in contrast to xenobiotic resistance that will usually only facilitate a herbivore's compatibility with a limited set of (related) plant hosts.
(7) Evidence suggests that upon colonization of a novel host by the generalist *T. urticae* the ability to suppress defenses rapidly emerges possibly due to plasticity and/or selection.
(8) We predict that in generalists confined to a host for extended periods of time the suppression trait will be replaced by resistance traits, because these traits are ecologically more safe and, according to the data available, may promote mite performance more strongly.
(9) We argue that the existence of specialists that suppress defenses rather than resist them may represent 'accidents' facilitated by buffering traits that shield suppression from natural selection. We predict these specialists to possess a smaller set of effectors/effector paralogs than generalists do and these to more often target less conserved (i.e., more host-specific) plant proteins or processes.
(10) We speculate that under the umbrella of the buffering traits, the suppression traits of specialists may still erode because of physiological costs and/or drift, yet at a relatively slow pace.
(11) Finally, we argue that defense suppression traits and their buffering traits can be, but not necessarily are, co-adaptations.

AUTHOR CONTRIBUTIONS

MK and BS conceptualized the manuscript. CB, EV-P, and RC drafted the manuscript. TVL, MK, and BS supervised the writing, critically revised the manuscript, and contributed to its final version. CB, EV-P, RC, and BS designed the figures.

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The Digestive System of the Two-Spotted Spider Mite, *Tetranychus urticae* Koch, in the Context of the Mite-Plant Interaction

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The two-spotted spider mite (TSSM), *Tetranychus urticae* Koch (Acari: Tetranychidae), is one of the most polyphagous herbivores, feeding on more than 1,100 plant species. Its wide host range suggests that TSSM has an extraordinary ability to modulate its digestive and xenobiotic physiology. The analysis of the TSSM genome revealed the expansion of gene families that encode proteins involved in digestion and detoxification, many of which were associated with mite responses to host shifts. The majority of plant defense compounds that directly impact mite fitness are ingested. They interface mite compounds aimed at counteracting their effect in the gut. Despite several detailed ultrastructural studies, our knowledge of the TSSM digestive tract that is needed to support the functional analysis of digestive and detoxification physiology is lacking. Here, using a variety of histological and microscopy techniques, and a diversity of tracer dyes, we describe the organization and properties of the TSSM alimentary system. We define the cellular nature of floating vesicles in the midgut lumen that are proposed to be the site of intracellular digestion of plant macromolecules. In addition, by following the TSSM's ability to intake compounds of defined sizes, we determine a cut off size for the ingestible particles. Moreover, we demonstrate the existence of a distinct filtering function between midgut compartments which enables separation of molecules by size. Furthermore, we broadly define the spatial distribution of the expression domains of genes involved in digestion and detoxification. Finally, we discuss the relative simplicity of the spider mite digestive system in the context of mite's digestive and xenobiotic physiology, and consequences it has on the effectiveness of plant defenses.

Keywords: gut, digestion, detoxification, plant-pest interaction, pest, histology

INTRODUCTION

Plant-pest interactions represent an elaborate interplay between organisms whose complexity reflects a constant evolutionary arms-race between a plant and a guild of its pests. Plants evolved a myriad of strategies to deter herbivory, ranging from the manipulation of multitrophic interactions through the recruitment of pest natural enemies, to the placement of physical or chemical

barriers that repel herbivores and prevent consumption of plant tissues. In addition, as herbivores feed on plants to acquire nutrients, they also ingest a wide range of plant defense compounds that interfere with essential herbivore physiological processes (Howe and Jander, 2008). The exposure to plant-imposed barriers has been an important driver of herbivore evolution for millions of years, resulting in the development of counter mechanisms that enable herbivores to overcome plant defenses (Heidel-Fischer and Vogel, 2015). As the alimentary tract is one of the major sites where plant-herbivore warfare takes place, knowledge of its cellular organization and physiology is essential for the functional understanding of plant-herbivore interactions.

Insect herbivores have been excellent models for understanding the interplay between plant- and insect-derived molecules acting in the gut. Identification of plant toxic metabolites and their detoxification counterparts have been described for several plant-pest interactions. For example, allelic variation, gene duplication, overexpression and the subfunctionalization of cytochrome P450 proteins enabled the parsnip webworm (*Depressaria pastinacella*) and the black swallowtail (*Papilio polyxenes*) to feed on furanocoumarin-containing plants (Mao et al., 2006, 2007; Wen et al., 2006), and the aphid *Myzus persicae* to utilize nicotine-accumulating tobacco as a host (Bass et al., 2013). In addition to compounds that act as xenobiotics, plants synthesize defensive proteins that directly target gut physiology. Lectins, chitin-binding proteins, chitinases and some proteases disrupt the peritrophic matrix (Zhu-Salzman et al., 1998; Vandenborre et al., 2011), a structure that lines luminal surface of the alimentary canal and aids in digestion and protection of the midgut epithelial cells. In addition, plants synthesize enzymes like proteinase inhibitors (PIs) that interfere with the digestion of plant nutrients, and amino acid-degrading enzymes (e.g., arginase (ARG2) and threonine deaminase (TD2) (Chen et al., 2005; Gonzales-Vigil et al., 2011), which reduce the availability of nutrients that herbivores require for development. The knowledge of gut physiology and its cellular organization is of primary importance for understanding the interactions between plant defense molecules and the cellular environment of insect guts. For example, gut pH profoundly affects the effectiveness of ARG2 and TD2. As these enzymes are most active within an alkaline pH, they are effective in restricting herbivory of insects with alkaline guts, such as lepidopteran larvae (Gu et al., 1999; Chen et al., 2004, 2007; Fowler et al., 2009; Chung and Felton, 2011; Gonzales-Vigil et al., 2011), but are ineffective against herbivores with acidic guts, like the Colorado potato beetle (Felton et al., 1992; Gonzales-Vigil et al., 2011). Recent advances in DNA and RNA sequencing technologies further enabled mapping of the expression of genes to different gut domains, thereby correlating the anatomical and genomic features in some insect herbivores (Neira Oviedo et al., 2008; Chung et al., 2009).

The two-spotted spider mite (TSSM), *Tetranychus urticae* (Koch), is a chelicerate herbivore with an exceptionally wide host range (Migeon et al., 2010). The TSSM's extreme polyphagy indicates that TSSM has an outstanding ability to adapt its digestive physiology and to overcome a wide range of defenses imposed by different host plants (Rioja et al., 2017). TSSM's xenobiotic responsiveness, an ability to detoxify many diverse phytochemicals, is also associated with its ability to readily develop resistance to pesticides (Dermauw et al., 2018). Characterization of the TSSM feeding pattern showed that TSSMs consume the content of single mesophyll cell at a time (Bensoussan et al., 2016). As TSSMs ingest plant cellular content, they also take up mesophyll parenchyma-localized defense compounds, suggesting that both nutrient digestion and detoxification of plant defense compounds occur in digestive tract.

The two-spotted spider mite was named for its two distinct dark spots that originate from the internal gut content visible through the semitransparent cuticle (**Figures 1A–C**). TSSMs, like insects, have a complete digestive system that consists of a foregut, midgut and hindgut. However, TSSM digestive physiology deviates from a characteristic insect digestive system. For example, insects digest nutrients extracellularly (Lemaitre and Miguel-Aliaga, 2013), while in TSSMs, the remnants of plant cellular contents have been observed in vesicles of cellular origin, suggesting "intracellular" digestion (Wiesmann, 1968; Orlob and Takahashi, 1971; Mothes and Seitz, 1981). Furthermore, it is not clear if TSSMs have a peritrophic membrane or a hemolymph (Mothes and Seitz, 1981), further questioning mechanisms that underlie the distribution of acquired nutrients to different tissues.

The analysis of the TSSM genome revealed that its herbivorous pest adaptations at least partially associate with the expansion of gene families that encode proteins involved in digestion and detoxification (Grbić et al., 2011). Subsequent studies established that changes in the expression of these genes associate with TSSM host shifts (Dermauw et al., 2013; Zhurov et al., 2014; Wybouw et al., 2015), leading to the proposition that they are one of the main determinants of TSSM's xenobiotic responsiveness. However, a direct connection between gene family expansions and TSSM host adaptation has so far been demonstrated only in the case of cysteine synthase (Tu-CAS), which confers resistance of spider mites to cyanogenic glycosides (Wybouw et al., 2014). The establishment of forward (Van Leeuwen et al., 2012) and reverse (Khila and Grbić, 2007; Kwon et al., 2013, 2016; Suzuki et al., 2017b) genetic approaches should facilitate the understanding of the molecular basis of the TSSM digestive physiology, and mechanisms underlying host-adaptation and xenobiotic responsiveness. Although there have been several detailed ultrastructural studies that described the TSSM digestive tract (Mothes and Seitz, 1981; Andre and Remacle, 1984; Mothes-Wagner, 1985), a more comprehensive understanding of the TSSM digestive system is needed to support the functional analysis of its digestive and detoxification physiology. Here, we describe the organization of the TSSM digestive tract, complementing previously published ultrahistological studies by putting cellular details in a broader tissue-organization context. In addition, we follow a variety of tracer dyes to define the properties of compartments within the alimentary system. This study provides a histological framework for the functional analysis of TSSM digestive and

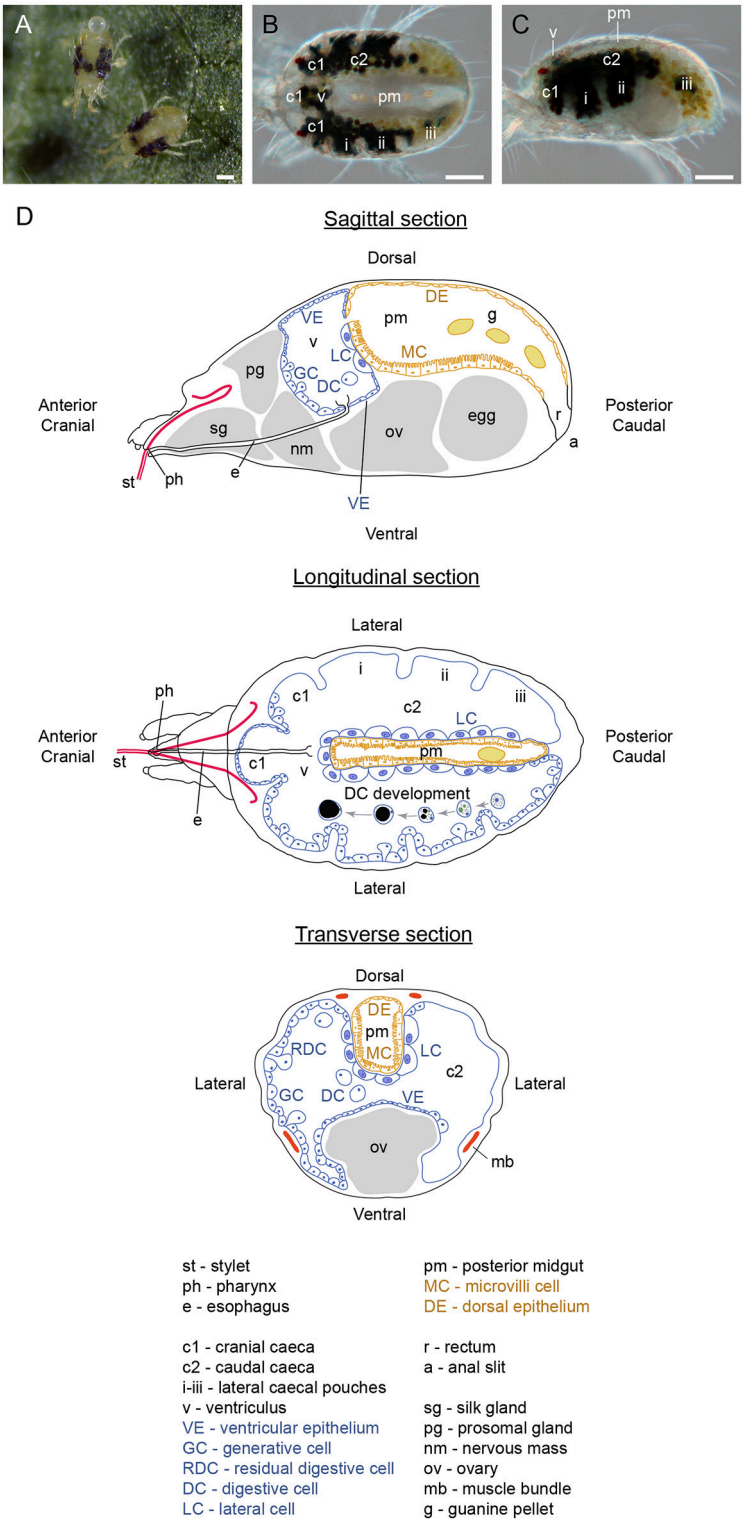

FIGURE 1 | Organization of spider mite body. **(A)** Spider mite females on bean (*Phaseolus vulgaris*) leaf. **(B)** Dorsal view of female spider mite. **(C)** Lateral view of female spider mite. **(D)** Schematics of spider mite internal anatomy. Scale bars **(A–C)**: 100 μm.

detoxification physiology, hallmarks of TSSM extreme polyphagy and xenobiotic responsiveness.

MATERIALS AND METHODS

Mite Rearing

The London mite population was maintained on the bean, *Phaseolus vulgaris*, cultivar California Red Kidney (Stokes, Thorold, ON) for more than 10 years. Bean plants were grown in a peat–vermiculite growing mix (Pro-Mix® BX Mycorrhizae™; Premier Tech, Rivière-du-Loup, QC). The mites and their host plants were maintained at 26°C, under 100–150 μmol m^{-2} s^{-1} cool-white fluorescent light and a 16/8 h (light/dark) photoperiod.

Paraffin Embedding and Sectioning of Mite Tissues

Spider mite adult females were collected and fixed overnight at 4°C in 4% formaldehyde in 10 mM phosphate buffer saline (PBS), pH 7.4 with 1% (v/v) Triton X-100. Mites were washed twice in 10 mM PBS and were subsequently dehydrated in an ethanol series (10%, 30%; 50% and 70%; v/v in H_2O). Further dehydration and paraffin embedding were performed in tissue processor (Leica ASP300TP). Embedded mites were sectioned on a microtome (Leica RM2255 Microtome) at a thickness of 5 μm. Sections were dewaxed in two 10 min changes of 100% xylene and were progressively rehydrated. The following histological dyes were used as general staining: 0.1% Safranin O (C.I. 50240; MilliporeSigma) and 0.05% Fast Green (FCF, C.I. 42053, MilliporeSigma), 1% hematoxylin (C.I. 75290, MilliporeSigma) solution and 1% eosin (C.I. 45400, MilliporeSigma), and Movats' pentachrome solution. Images were obtained using a Zeiss AxioCam Color HRc CCD Camera 412-312 and Zeiss Axioplan II microscope. Finally, 300 nM of DAPI (D1306, Invitrogen) mixed in 10 mM PBS was used to visualize nuclei under epifluorescence Zeiss Axioplan II microscope.

Cryo-Sectioning of Mite Tissues

Adult female spider mites were fixed as described above, then frozen in OCT compound (Tissue-Tek) and stored at −80°C. Sections of approximately 10 μm in thickness were cut using the Leica CM3050 S cryostat (Leica, Austria). Sections were stained with 0.5% Oil Red O (102419, MilliporeSigma).

Semi-thin Sectioning of Mite Tissues

Adult female mites were fixed in solution of 2.5% (v/v) glutaraldehyde in 100 mM sodium phosphate buffer at pH 7. After 24 h, mites were washed in 100 mM sodium phosphate buffer at pH 7 and dehydrated in a graded alcohol series: 25, 50, 75, 95, 100 and 100% (v/v in H_2O), for 15 min in each solution. Mites were embedded in LR White resin (Electron Microscopy Science, USA) and were cured overnight at 55°C. Specimens were cut with a Reichert Ultracut S ultramicrotome (Leica, Austria) into 1 μm serial sections, using a glass knife. Sections were stained with toluidine blue, 0.5% (w/v) in 0.1% (w/v) Na_2CO_3, for 5 min on a slide warmer at 60°C and dried overnight at room temperature.

DAPI Staining of Digestive Cells, Feces and Guanine Pellets

Digestive cells were dissected from adult female mites by disrupting one of the caeca using a fine tungsten needle. All gut content, including digestive cells, was released into a solution of 75% (v/v) glycerol with 10 μM of DAPI (D1306, Invitrogen) in 10 mM PBS. Images were captured using an epifluorescence Zeiss Axioplan II microscope fitted with a DAPI filter cube (350–400 nm excitation, 417–477 nm emission) and an AxioCam Color HRc CCD Camera 412-312. For the collection of feces and guanine pellets, a small square arena of about 1.5 cm^2 was created on a microscope glass slide using a wet Kimwipe. 100 adult female mites from the general rearing population were collected mid-day and were allowed to excrete for 1 h. A solution of 75% glycerol and 10 μM of DAPI (D1306, Invitrogen) in 10 mM PBS, was applied to deposits. Slides were examined under an epifluorescence Zeiss Axioplan II microscope fitted with a DAPI filter cube. Images were taken using AxioCam Color HRc CCD Camera 412-312. Images captured were further refined using the mask layer option from Adobe Photoshop® to highlight nucleus in digestive cells (**Figures 5C–H**; overlay) and "screen" as a blending mode for the nucleus in feces (**Figure 5K**).

Guanine, Chlorophyll and Digestive Products Autofluorescence

Guanine particles localized in digestive cells and the posterior midgut were visualized with confocal microscopy using an argon laser at a 543 nm wavelength excitation and a LP of 650 nm. Chlorophyll and its degradation products were visualized with a He-Ne laser using a 633 nm excitation wavelength and a LP of 650 nm. A red false color was used to show the chlorophyll and its degradation product while a green color was used to highlight guanine crystals. Epifluorescence microscopy was also used to visualize expelled guanine crystals, using UV light with a FITC filter cube (450–490 nm excitation, 515–586 nm emission). Images were taken with a Zeiss Axioplan II microscope using AxioCam Color HRc CCD Camera 412-312.

Microsphere Size Exclusion Assay

To assess the size range of particles that can be ingested by adult female spider mites, a device for delivering water (control) or an aqueous suspension of polystyrene fluorescent microspheres (FMs; 50 nm to 1 μm in diameter; Polysciences) was designed using a 0.2-mL tube cap filled with either 50 μL of water or 2.5% (w/v) aqueous suspension of FMs and covered with stretched Parafilm® (see **Supplementary Figure S1**). Ten newly molted adult female mites were placed per device and incubated for 24 h at 25°C. Fluorescent images were taken with a digital camera (EOS Kiss X7, Canon) installed on a Leica M205FA microscope fitted with a GFP filter (395-455 nm excitation, >480 nm emission) with an exposure time of 5 s (ISO: 200). Bright-field images were taken using the same system without filters with an exposure time of 1 s (ISO: 200).

Size Exclusion Properties of Ventriculus-Posterior Midgut Connection

To test the filtration properties of the ventriculus-posterior midgut connection, polystyrene FMs of 50 nm (Polysciences) and a fluorescent tracer dye (Alexa Fluor 555, Thermo Fisher Scientific) were delivered using the method described above. In addition, fluorescein-labeled dextran of various sizes (500, 40, and 4 kDa, MilliporeSigma) were delivered to adult female mites as mixtures with 6% blue dye (erioglaucine; McCormick, Sparks Glencoe, MD) using the leaf coating method (Suzuki et al., 2017a). To ensure the intactness of fluorescein-labeled dextran, the fluorescein-12-UTP (Roche) was delivered as a control. In both delivery systems, mites were allowed to feed for 24 h and fluorescence was visualized using an epifluorescence Zeiss Axioplan II microscope fitted with a FITC filter cube. Images were taken using an AxioCam Color HRc CCD Camera 412-312.

Phalloidin Staining

Approximately 100 adult spider mite females were incubated in 150 μM phalloidin solution (Alexa Fluor® 546 Phalloidin, ThermoFisher Scientific, USA) in 10 mM PBS, overnight at 4°C (Jiang et al., 2007). Phalloidin-stained actin filaments were visualized using a Zeiss Axioplan confocal microscope (Carl Zeiss AG, Germany) with the following settings: 543 nm excitation, Filter: Ch1, LP 560 nm.

Lumen Movement Within TSSM Gut

To visualize the lumen movement in the TSSM gut, a solution of 50% (v/v) glycerol diluted in 10 mM PBS and 0.1% (v/v) Tween 20 was gently applied to the back of an adult female mite with a fine brush to improve the transparency of the cuticle. Movement within the TSSM gut was recorded using a Canon EOS Rebel T5i camera (Canon, Japan).

Estimation of Gene Expression Level Enrichment in Proterosoma

For estimation of gene expression level enrichment in proterosoma, we have retrieved previously published transcriptome data from the Sequence Read Archive (SRA SRP074404, BioProject PRJNA320686) (Jonckheere et al., 2016). Reads were mapped to the reference *T. urticae* genome using STAR aligner version 2.5.3a (Dobin et al., 2013), allowing only unique mapping, up to five mismatches per read mapped, a minimum intron size of 20 bp, and a maximum intron size of 15,000 bp, in per-sample 2-pass mode. Read counts were generated using HTSeq at the level of gene locus in "union" mode (Anders et al., 2015). Estimation of gene expression fold changes and associated adjusted (BH) *p*-values was conducted using edgeR (Benjamini and Hochberg, 1995; Robinson et al., 2010) for genes that were expressed at the level of at least 1 CPM in at least one sample library. This procedure resulted in 12,814 genes retained for subsequent analysis. Lists of genes implicated in digestion and detoxification were obtained from previous publications (Santamaría et al., 2015a; Jonckheere et al., 2016), and high quality manual annotation of spider mite genome (Grbić et al., 2011). Identification of MFS and lipocalin genes was based on the significantly assigned PFAM domains PF07690, and PF00061, PF08212, respectively (Finn et al., 2014).

RESULTS

Terminology

TSSMs, like insects, have a complete digestive system that consists of a foregut, midgut and hindgut. We have adapted the terminology used by Mothes-Wagner (1985) to describe the TSSM digestive tract, as it takes into account the embryonic origin of gut tissues. Accordingly, the foregut and hindgut contain compartments that are derived from the embryonic ectoderm and are consequently lined with the cuticle, while the midgut that is derived from the embryonic endoderm does not have a cuticular lining. The foregut consists of the stylet, buccal cavity, pharynx, esophagus and esophageal valve, the midgut includes the ventriculus, caeca and posterior midgut, and the hindgut is comprised of rectum and anal slit (**Figure 1D**). For the histology of the TSSM digestive system, we analyzed transverse, longitudinal, and sagittal sections of adult female TSSMs. We prepared serial sections, with examples shown in **Supplementary Figures S2–S4**, that allowed for a reconstruction of the TSSM alimentary tract. As our analysis of the TSSM digestive system complements previous ultrahistological studies (Mothes and Seitz, 1981; Andre and Remacle, 1984; Alberti and Crooker, 1985; Mothes-Wagner, 1985), we integrated observations from previous studies in describing different parts of the TSSM alimentary tract.

Foregut

Determination of the Particle Sizes Ingestible by the Adult Female TSSM

The first step in digestion of plant nutritive fluid within the TSSM alimentary tract is ingestion. Two scenarios for TSSM food ingestion were proposed: (i) TSSMs ingest food via the stylet (Summers et al., 1973; Hislop and Jeppson, 1976; Andre and Remacle, 1984; Bensoussan et al., 2016), (albeit connection of stylet and buccal cavity has not been demonstrated), or (ii) plant liquefied material is brought to the plant surface by capillary action where TSSM "laps" it into its buccal cavity (Alberti and Crooker, 1985; Nuzzaci and de Lillo, 1989, 1991). Regardless of the mode of nutrient uptake, the size of the ingestible particles is expected to be limited by the diameter of the stylet and/or the preoral groove/food canal. To determine the size cut off of ingestible particles, we used an aqueous suspension of polystyrene fluorescent microspheres (FMs) ranging in diameter from 50 nm to 1 μm. We designed a feeding device, shown in **Supplementary Figure S1**, where the liquid with FMs is sealed off by Parafilm®, requiring a TSSM to penetrate through the membrane with its stylet and to ingest the suspension. The mites' ability to ingest the FMs was assessed by detection of fluorescence within the digestive tract. As shown in **Figure 2**, FMs up to 500 nm in diameter accumulate in the midgut, however, no accumulation of fluorescent microspheres of 750 nm and greater was observed, indicating that TSSM can uptake only particles that are smaller than 750 nm. This is consistent with the size limitation imposed by the stylet and the preoral

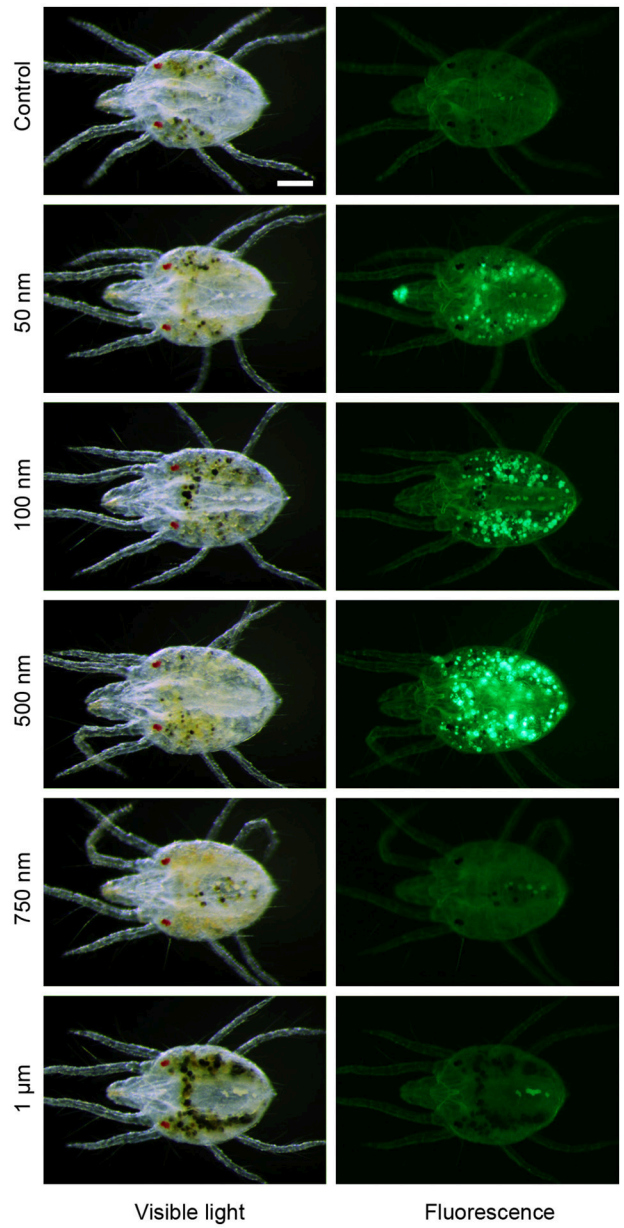

FIGURE 2 | Size limitation of spider mite feeding apparatus. Accumulation of fluorescent microspheres of 50 nm to 1 µm in diameter in midgut of the spider mites after 24 h feeding at 25°C. Scale bar: 100 µm.

groove/food canal (Mothes and Seitz, 1981; Andre and Remacle, 1984; Nuzzaci and de Lillo, 1989, 1991).

Pharynx, Esophagus

On its way to the midgut, the ingested plant cell content passes through the pharynx and the esophagus (**Figure 3**). In longitudinal cross sections, the pharynx (ph) cavity is seen as an opening with dark-stained walls (**Figure 3A**, and Figure 3 in Mothes and Seitz, 1981), while the esophagus (e) is a tube that runs at median along the TSSM anterior-posterior axis (**Figure 3**). The esophagus initially passes ventrally through the silk glands (sg in **Figure 3A**) and then arches upwards as it traverses through the nervous mass (nm in **Figures 3A–D**). The esophagus enters into the ventriculus (v) of the midgut through the ventral layer of gut epithelial cells (**Figures 3C,E–G**) and terminates with the esophageal valve (asterisk in **Figures 3C,E,F**). The esophageal valve is cup-shaped and secures the inflow of the ingested plant content into the ventriculus of the midgut (**Figure 3G**). Ultra-high magnification cross sections through the esophagus, with clearly visible inner chitin lining, and the cup-shaped esophageal valve are also shown in Figures 11 and 12 in Mothes and Seitz (1981).

Midgut

The midgut (caeca, ventriculus, and the posterior midgut) comprises the greatest volume of the alimentary tract, filling almost the entire opisthosoma. Of five caeca, three project cranially (c1 in **Figures 1B–D**) and two are caudal, running laterally along the anterior-posterior axis of the TSSM body (labeled c2 in **Figures 1B–D**). Each caudal caeca forms three lobes (i, ii, iii), visible in **Figures 1B–D, 4A**. The caeca are interconnected through the ventriculus (v, **Figures 1B–D, 4C**). The ventriculus protrudes dorsally, above the caecal plane to form a separate compartment, **Figures 1C, 4E**. In addition, the ventriculus connects to the posterior midgut (pm) (**Figures 1B–D, 4C**), and thus, is a central hub that joins different parts of the midgut. The posterior midgut is the terminal part of the midgut that is nested dorsally between two caudal caeca (**Figures 1B–D, 4 C,I,K,M**). It enlarges and curves downwards at the posterior end (**Figure 4C**), where it transitions into the hindgut (rectum, r in **Figures 1D, 4C**).

The midgut is comprised of a single-layered epithelium that encircles the midgut lumen. The midgut epithelium is composed of five cell types. In the caeca, three types of epithelial cells can be differentiated based on their shape. We refer to them as: (a) generative cells (GC, **Figures 1D, 4A, B**), (b) ventricular epithelial cells (VE in **Figure 1D**, red arrowheads in **Figures 4C,E–H**), and (c) large epithelial cells (LC) that form an inner wall of caudal caeca, adjacent to the posterior midgut (**Figures 1D, 4I–N**). GCs are the most abundant cell type in the midgut epithelium, building the outer midgut wall. They are cuboidal and densely stained in histological preparations. GCs detach from the epithelial wall and form free-floating vesicles in the midgut lumen (see below and **Figure 5**). No obvious histological differences were observed among the GC populations in different caeca. In addition, we did not observe any clear spatial patterning in the formation of the free-floating vesicles. Thus, it appears that GCs may all have generative capabilities. However, this should be tested in future experiments, e.g., by bromodeoxyuridine staining. Even though GCs form the majority of the caecal outer wall, there are few areas in the midgut outer epithelium that are composed of a different cell type—the ventricular epithelium (VE). The VE is located at the cranial caeca (**Figures 4C,D,G**), and in the dorsal (**Figures 4E,G**) and ventral (**Figures 4F,H**) areas of the ventriculus. VE cells form a thin squamous epithelium that does not detach into the lumen. The cranial caeca are in direct contact with head

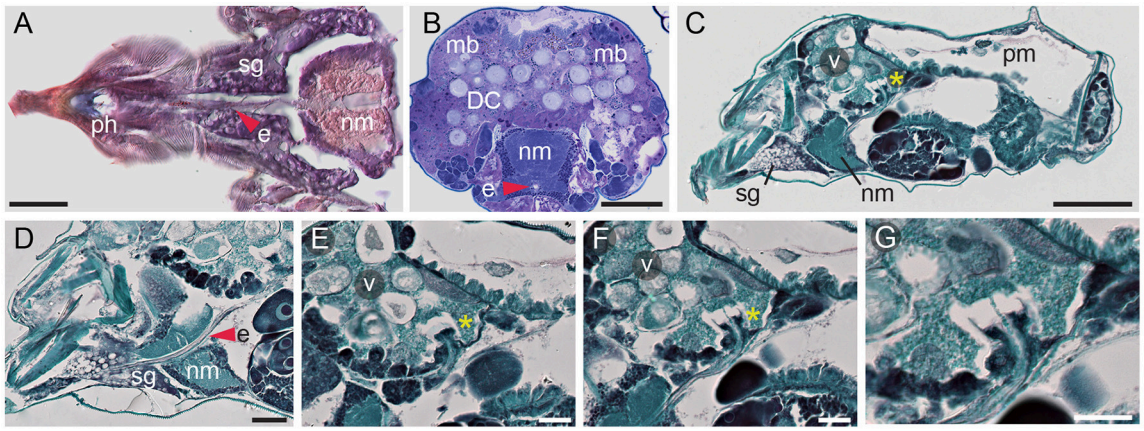

FIGURE 3 | Esophagus and esophageal valve of spider mite. **(A)** Longitudinal cryosection of ventral part of proterosoma showing pharynx (ph) connected to esophagus (e) that passes through the silk gland (sg) and the nervous mass (nm) stained with Oil Red O. **(B)** Transverse section showing esophagus passing through nervous mass. Dorsal muscle bundles (mb) that facilitate midgut movement are clearly visible. LR White plastic embedding and Toluidine Blue staining. DC, digestive cells. **(C)** Sagittal section of female spider mite. Esophageal valve (asterisk) entering ventriculus (v). pm, posterior midgut. **(D)** Sagittal section through proterosoma of spider mite showing esophagus (red arrowhead) passing through silk gland and nervous mass toward ventriculus. **(E,F)** Consecutive sections through the esophageal valve (asterisk) entering the ventriculus. **(G)** Detail of esophageal valve entering ventriculus. Paraffin embedding and Fast Green and Safranin O staining were used in **(C–G)**. Scale bars: **(A)**: 25 μm; **(B)**: 50 μm; **(C)**: 100 μm; **(D)**: 25 μm; **(E–G)**: 20 μm.

glands and head tissues (Mothes and Seitz, 1981), while the ventral VE is adjacent to developing ovaries/eggs (**Figures 4F,H, Supplementary Figures S3, S4**) and silk glands (**Figure 4H**). LCs are the third cell type that characterize the caecal epithelium. These are large, rounded cells that line the caudal caeca in contact with the posterior midgut (**Figures 4I–N**). In paraffin-embedded and sectioned tissue preparations, vesicles within the LCs are not readily visible. However, these vesicles are apparent in plastic-embedded specimens (**Figures 4K, L**, and Figure 22 in Mothes and Seitz, 1981).

The posterior midgut is seen as a V-shaped tube on transverse sections (**Figures 4I,K**). It runs along the dorsal midline, originating above the posterior portion of the ventriculus, and continues posteriorly above ovaries (**Figure 4C**) and between the caudal caeca (**Figures 4M,N**). At its anterior end, the posterior midgut connects to the ventriculus, while posteriorly, it enlarges and connects to the rectum (**Figure 4C**). The lateral walls of the posterior midgut are made of the fourth midgut cell type, squamous epithelial cells, with microvilli projecting into the lumen (MC in **Figures 1D, 4C,I–N**). These cells share the basement membrane (yellow arrowheads in **Figures 4J,N**) with the LCs that delimit the caeca. Posteriorly and dorsally, microvilli-containing cells are replaced with the flat cells that represent the fifth midgut cell type, DE (**Figure 1D** and blue arrowheads in **Figures 4C,N**). At the posterior transition of MC to DE cells, the caecal LCs are also replaced with GCs (**Figures 4C,N**), resulting in a coordinated change in the caeca-posterior midgut cellular superimposition.

The lumens of the caeca and the ventriculus are filled with large vesicles that were referred to as "food balls," "floating cells" or "phagocytes" (Wiesmann, 1968; Mothes and Seitz, 1981; Alberti and Crooker, 1985). The lack of precision in the terminology describing these vesicles reflects the uncertainty of their origin and their fate within the TSSM digestive tract. Previous studies were not able to differentiate whether these vesicles arise from holocrine or apocrine secretions. In addition, while nuclei have been observed in some of these vesicles (for example see Figure 16 in Mothes and Seitz, 1981), they may not be present in others. Nevertheless, these vesicles have been firmly linked to the digestion of plant nutrients, as thylakoid and starch granules were observed within them (Orlob and Takahashi, 1971; Figures 16–20 in Mothes and Seitz, 1981). To determine the origin and fate of these vesicles within the TSSM digestive tract, we used a variety of approaches. In cryo-sectioned specimens, the GCs are strongly stained with pentachrome (**Figure 5A**, top). DAPI (4',6-diamidino-2-phenylindole) fluorescence staining of DNA (**Figure 5A**, bottom) confirms the presence of a single, large nucleus in each GC. GCs, while still attached to the basement membrane, enlarge and protrude into the caecal lumen (**Figure 5B**). They have nuclei and are referred to as Residual Digestive Cells (RDC). Ultimately, the RDCs detach from the epithelium and become free-floating cells, filling the lumen of caeca (**Figures 1B,C, 3B, 4A–C**). Free-floating cells dissected from the gut lumen were examined by brightfield microscopy (top row in **Figures 5C–H**), in DAPI-stained whole mount preparations (two middle rows in **Figures 5C–H**), and in paraffin sections (two bottom rows in **Figures 5C–H**). Our observations using the three visualization strategies suggest five stages in the development of the free-floating cells. In stage 1, upon detachment from the caecal epithelium, the cells are spherical and filled with many transparent vesicles (**Figure 5C**). In stage 2, most cells internalize the gut lumen content, resulting in the presence of two types of vesicles–transparent and pigmented ones (red arrowhead, **Figure 5D**). The internalization probably occurs through pinocytosis and/or phagocytosis as starch and thylakoid membranes had been observed within floating cells (Mothes and

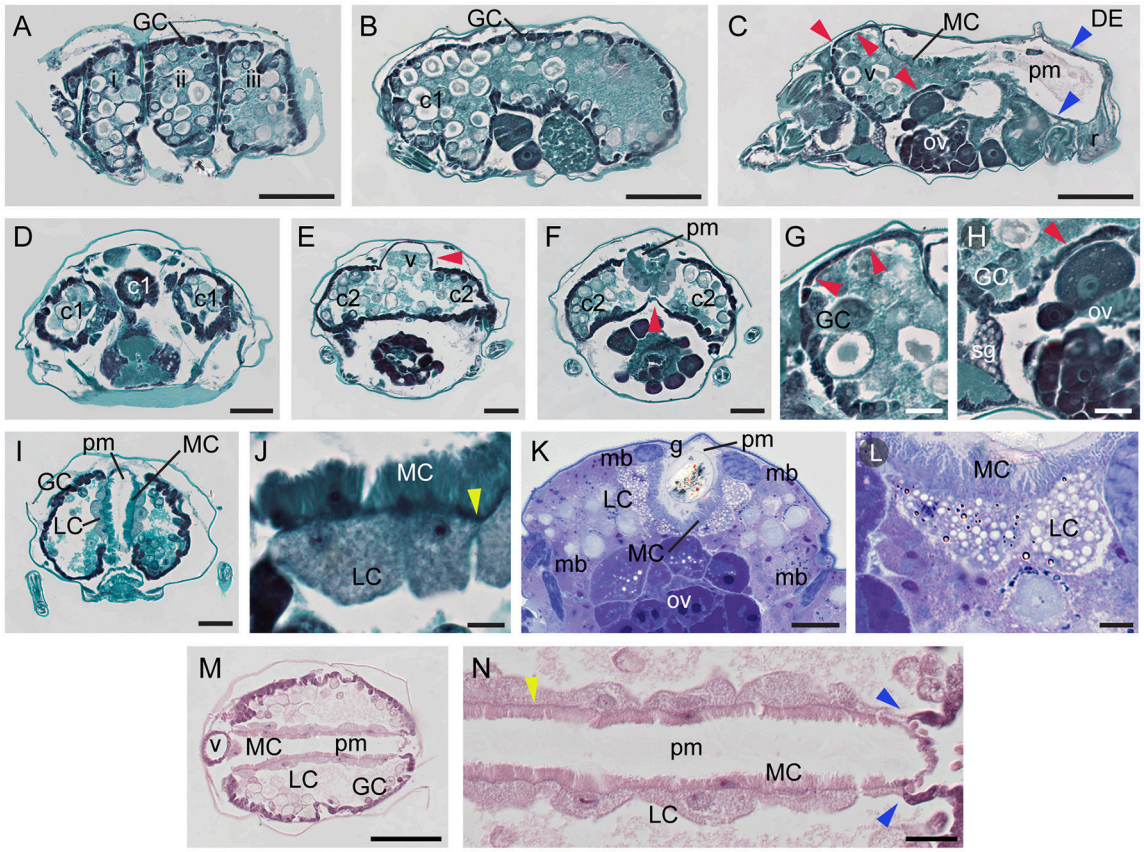

FIGURE 4 | Midgut of spider mite. **(A–C)** Serial sagittal sections of spider mite female progressing from outside part of the body **(A)** showing lateral caecal pouches (i–iii) to the middle **(C)** where posterior midgut (pm) becomes visible. Red arrowheads point to the ventricular epithelium at cranial caeca and ventriculus (v). Blue arrowheads point to the thin single-layered squamous cells that lack microvilli projections at the dorsal and posterior region of the posterior midgut, DE. r, rectum. **(D–F)** Transverse sections through region of cranial caeca (c1), ventriculus (v), and anterior portion of posterior midgut (pm). **(G)** Detail of thin dorsal ventricular epithelium (red arrowheads) which transitions to cuboid generative cell (GC) epithelium anteriorly. **(H)** Detail of thin ventral ventricular epithelium (red arrowhead) adjacent to silk gland (sg) and ovary (ov). **(I)** Representative transverse section of mite digestive system showing generative cells (GC) of midgut epithelium, large cells (LC) of midgut epithelium adjacent to posterior midgut (pm), and microvilli cells (MC) of posterior midgut. **(J)** Large cells (LC) of midgut epithelium opposite of microvilli cells (MC) of posterior midgut epithelium. Yellow arrowhead points to basement membrane. **(K)** Transverse section of LR White plastic embedded and Toluidine Blue stained spider mite. **(L)** Large cells (LC) of midgut epithelium opposite of microvilli cells (MC) of posterior midgut epithelium. **(M)** Longitudinal section through ventriculus (v), midgut and posterior midgut (pm) stained with hematoxylin and eosin. **(N)** Detail of the interface between LCs of midgut and MCs of posterior midgut. Basement membrane (yellow arrowhead) is very well defined in this preparation. Notice the immediate change of cell types in both midgut and posterior midgut epithelia as two become separated in the posterior region (blue arrowheads). Scale bars: **(A–C)**: 100 μm; **(D–F)**: 50 μm; **(G,H)**: 20 μm; **(I)**: 50 μm; **(J)**: 10 μm; **(K)**: 30 μm; **(L)**: 10 μm; **(M)**: 100 μm; **(N)**: 20 μm.

Seitz, 1981). In stage 3, the internal dark vesicles increase in size (**Figures 5E,F**). In stage 4, the cells have a single large vesicle with dense deposits that appear dark brown or black in bright-field optics. These deposits have been proposed to be waste products of digestion (Liesering, 1960; Wiesmann, 1968) (**Figure 5G**). The cytoplasmic ring is still visible in these cells and contains small vesicles. Lastly, in stage 5, the volume of the cell is almost completely occupied by the single dark-colored vesicle that is surrounded by a very thin cytoplasm (**Figure 5H**). Importantly, cells of all stages contain nuclei that are progressively flattened and displaced to the cell periphery as the central internal vesicle enlarges (see DAPI and Overlay rows in **Figures 5C–H**). Thus, we refer to these cells as digestive cells (DC). DCs of mixed stages are excreted as a fecal pellet. Even upon excretion, DCs stain with DAPI (**Figures 5I–K**), indicating that they retain nuclei and may be metabolically active.

Functional Anatomy of TSSM Digestive Tract

The Filtration Properties of the Ventriculus-Posterior Midgut Transition

The caeca and the posterior midgut are two distinct compartments of the midgut that differ with regard to the continuous presence of digestive cells and epithelial cell types. Contraction of the paired dorsal longitudinal muscles positioned along the boundary between the caeca and the posterior midgut (marked as mb in **Figures 3B, 4K, Supplementary Figure S5**)

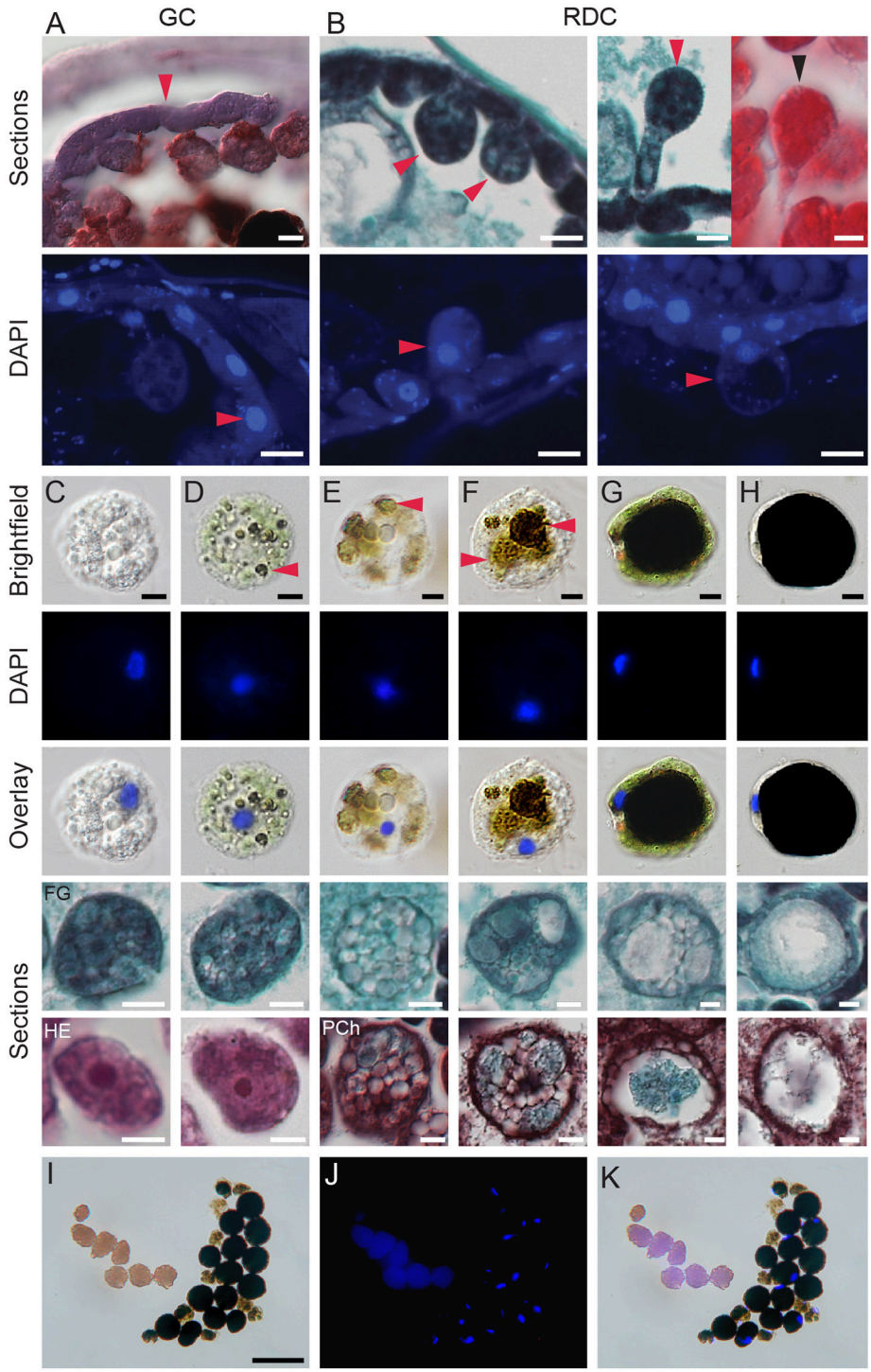

FIGURE 5 | Development of digestive cells in spider mite midgut. **(A)** Brightfield microscopy (cryosection, Oil Red O staining) and fluorescence microscopy (paraffin sections, DAPI staining) of generative cells (GC) forming midgut epithelium. **(B)** Brightfield microscopy (paraffin sections, Fast Green and Safranin O, and Hematoxylin and Eosin staining) and fluorescence microscopy (paraffin sections, DAPI staining) of residual digestive cells (RDC) as they grow into the midgut lumen and detach from the midgut epithelial layer. **(C–H)** Digestive cells (DC) at various stages of development. DCs were dissected from the midgut lumen of female spider mites, stained using DAPI, and observed using brightfield and fluorescence microscopy. Lower panels, paraffin sections of DCs from different staining preparation: FG, Fast Green/Safranin O; HE, Hematoxylin/Eosin and Pch, Pentachrome. **(C)** Stage 1, the early stage of DC development with clear but numerous vesicles and large

(Continued)

FIGURE 5 | nucleus. **(D)** Stage 2, DC starting to uptake midgut lumen and plant pigments. Arrowhead marks colored vesicles within DC. **(E,F)** Stage 3, colored vesicles enlarge. **(G)** Stage 4, a large vesicle with dark content is surrounded by a thin layer of cytoplasm. Numerous small vesicles are still visible in cytoplasm, nucleus is displaced to the periphery. **(H)** Stage 5, the terminal stage of DC development characterized by a dense dark brown/black vesicle, a very thin crescent of cytoplasm and condensed nucleus on side of a DC. **(I–K)** Brightfield and fluorescence microscopy of spider mite feces. Feces are represented by guanine pellets and DCs at various stages of development. There is no apparent sorting of DCs based on developmental stage and amount of waste products accumulated. Guanine pellets readily absorb DAPI and also exhibit autofluorescence. Scale bars: **(A)**: 10 μm; **(B–H)**: 5 μm; **(I–K)**: 50 μm.

creates caecal lumen movement (see **Supplementary Movie**). The ceacal lumen dynamically moves from one caudal caeca to another, readily seen by the movement of digestive cells. However, even though digestive cells pass through the ventriculus, they do not enter into the posterior midgut. Thus, the translocation of the material from the ventriculus into the posterior midgut seems to be regulated. A sphincter muscle (Figure 2 in Mothes-Wagner, 1985) was proposed to tightly control the passage from the ventriculus into the posterior midgut. We were unable to identify these muscles at the junction between the ventriculus and the posterior midgut in serial transverse sections or with confocal microscopy of phalloidin-stained tissues. However, the junction between the ventriculus and the posterior midgut can be seen in anteriorward serial sections shown in **Figures 6A–D**. In these sections, it is apparent that the posterior midgut terminates anteriorly in the complex that includes LCs from the caecal epithelium, the ventral edge of the posterior ventriculus, and microvilli cells of the posterior midgut epithelium (**Figure 6D**). Whether these epithelial cells and/or the sphincter muscle control the entrance to the posterior midgut is presently unknown. However, as the guanine and/or liquid excretion is frequent, it was proposed that water and small ions can transverse the ventriculus-posterior midgut barrier (McEnroe, 1961b). To test the permeability of this barrier we fed TSSMs with solutions containing a mixture of conjugated and free dyes, and followed their distribution within the TSSM digestive tract. **Figures 6E,F** show the distribution of the 50 nm fluorescent microspheres and Alexa Fluor 555 fluorescent tracer dye, and the 500 kDa fluorescein-conjugated dextran and erioglaucine (blue dye), respectively, when provided to TSSMs as mixtures. In both cases, the small molecules [Alexa Fluor 555 fluorescent tracer dye (**Figure 6E**) and erioglaucine (**Figure 6F**)] readily passed into the posterior midgut. On the contrary, the FMs and conjugated fluorescent dyes were retained in the caeca. This demonstrates the existence of differential filtering of the caecal lumen content by size, regulating entrance into the posterior midgut. To more precisely test the cut off size of the molecules that can enter into the posterior midgut, we tested the distribution of the 40 and 4 kDa fluorescein-conjugated dextran molecules that were each provided to TSSMs in a mixture with the erioglaucine. As seen in **Figure 6F**, both 40 and 4 kDa fluorescein-conjugated dextrans remained in the caeca, while erioglaucine passed into the lumen of the posterior midgut. Since fluorescein-12-UTP (**Figure 6F**), Alexa Fluor 555 fluorescent tracer dye and erioglaucine have molecular weights of 1.03, 1.25, and 0.8 kDa, respectively, these results indicate that the filtering cut-off between the ventriculus and the posterior midgut is between 4 and 1 kDa. It should be noted that the mobility of a particular compound does not only depend on its molecular weight, but also depends on its chemical properties. Regardless, these results indicate that small non-complex chemicals and metabolites can readily move from the ventriculus to the posterior midgut while more complex molecules, with higher molecular weights, such as proteins, will be retained in the caeca.

Excretion of Nitrogenous Waste

The Malpighian tubules mark the transition between the midgut and the hindgut in insect digestive tracts (Nation, 2015). They serve as an excretory organ that removes nitrogenous waste from the hemolymph and passes it to the hindgut for elimination, as part of the fecal excretions. TSSMs were reported to lack both the Malpighian tubules and the hemolymph (Blauvelt, 1945), raising the question of where is the nitrogenous waste formed and how is it disposed. TSSMs form guanine birefringent spherules that are disposed of as nitrogenous waste. They can be observed using brightfield (**Figures 4K, 7A,G,H**), confocal (**Figures 7C–F**) or epifluorescence (**Figures 7J,L**) microscopy. Guanine fluorescence is associated with two distinct forms that can be seen in the posterior midgut and caeca. In the posterior midgut, pellets of approximately 20–30 μm are made exclusively of guanine (g, **Figures 1D, 4K** and arrowhead in **Figure 7G**). The guanine pellets are excreted as guanine fecal droplets alone or together with digestive cells (**Figures 7G–L**). In the caeca, guanine particles are smaller (**Figure 7C**). The overlay of chlorophyll (associated with DC phagocytosis of the ingested plant cell content) and guanine fluorescence indicates that nitrogenous waste accumulates within digestive cells (**Figures 7D,E** and close up in **Figure 7F** showing individual guanine particles within DCs). The guanine within the digestive cell is excreted along with the chlorophyll waste products (for example see DCs labeled with arrowheads in **Figures 7K,L**). Note that neither LCs of the caecal epithelium nor microvilli-bearing cells (MC) forming the lateral walls of the posterior midgut show any fluorescence (**Figure 7E**), suggesting that they may not be involved in the digestion of plant cell contents and do not accumulate guanine waste products. It is currently unknown how guanine pellets form in the posterior midgut, yet they are the most abundant form of fecal deposits. Over the course of 1 h, we observed that a population of 100 mites excretes an average of 102 ± 13 guanine-only fecal pellets and an average of 12 ± 3 fecal pellets that contain both DCs and guanine ($n = 4$, mean $\pm$ SD reported).

Expression Pattern of the Digestion/Detoxification Related Genes

Anatomical characterization of the TSSM digestive system provides a cellular framework for the functional characterization of processes associated with the digestion of plant nutrients

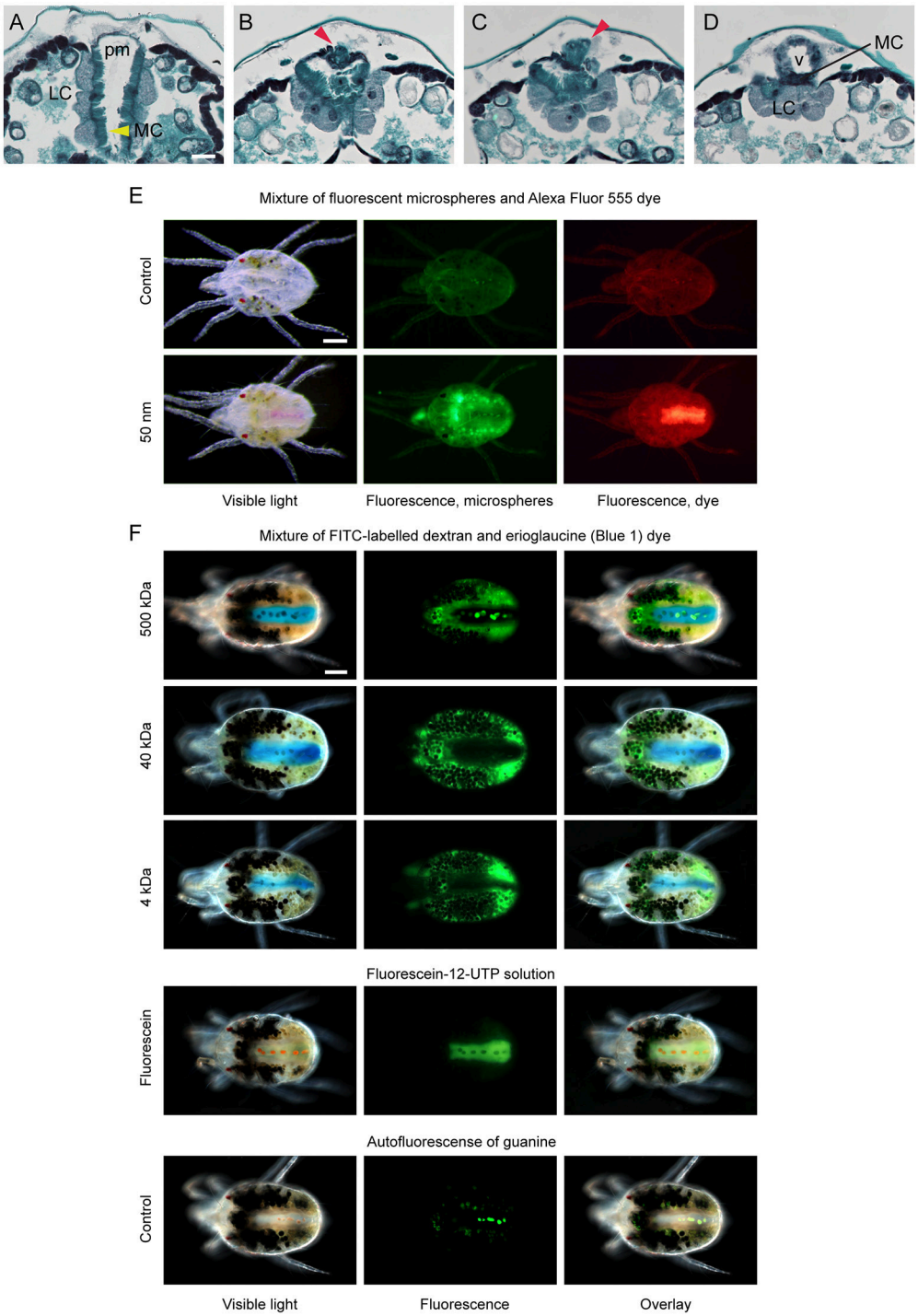

FIGURE 6 | Permeability of ventriculus-posterior midgut barrier in spider mites. **(A–D)** Serial transverse sections of the dorsal region of spider mite body in the area of connection between ventriculus (v) and posterior midgut (pm). **(A)** Typical arrangement of V-shaped posterior midgut positioned between two caudal caeca. LC, large cells of caudal caeca epithelium adjacent to posterior midgut (pm); MC, microvilli cells of posterior midgut (yellow arrowhead). **(B,C)** Anteriorward sections through the posterior midgut that narrows, while the posterior-dorsal portion of the ventriculus appears (red arrowhead). **(D)** Section through the point of ventriculus-posterior midgut connection. **(E)** Distribution of the 50 nm fluorescent microspheres and fluorescent dye Alexa Fluor 555 (1250 Da) in mite midgut upon the ingestion of their mixture. Alexa Fluor 555 readily passes through midgut and accumulates within posterior midgut, while 50 nm microspheres are retained in the caeca lumen. **(F)** Distribution of the FITC-labeled dextrans of different sizes and erioglaucine dye in mite midgut upon the ingestion of their mixture. Erioglaucine (793 Da) and fluorescein-12-UTP (1034 Da) accumulate in posterior midgut while FITC-labeled dextrans (4, 40, and 500 kDa) are retained in the caeca lumen. Guanine autofluorescence can be seen within digestive cells in midgut and in pellets in the posterior midgut. Scale bars: **(A–D)**: 25 μm; **(E,F)**: 100 μm.

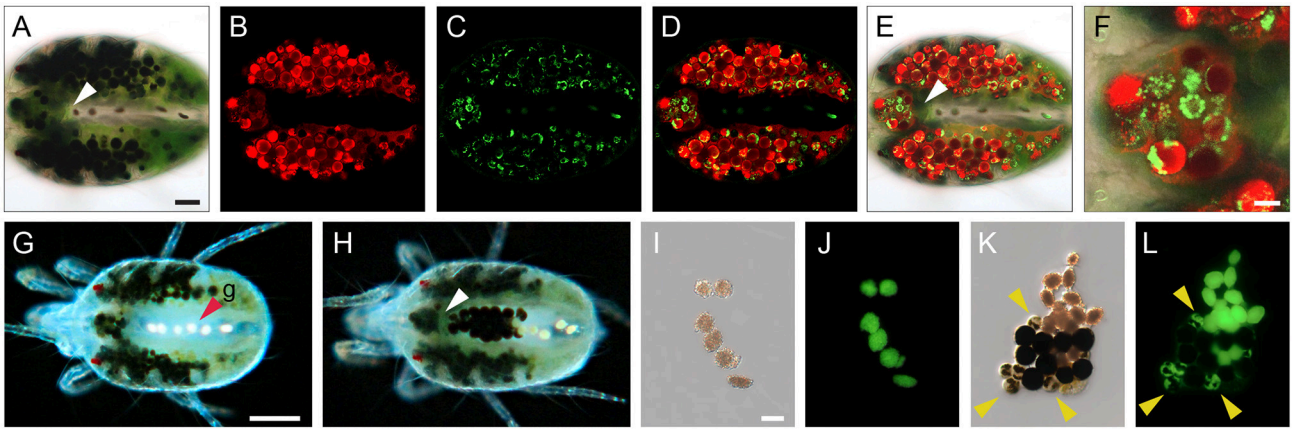

FIGURE 7 | Compartmentalization of digestion and nitrogenous waste in spider mite. **(A)** Brightfield microscopy of a female spider mite showing characteristic spots produced by large numbers of mature DCs, green pigmentation of midgut lumen and colorless posterior midgut with a few guanine pellets within. White arrowheads point at the boundary between the midgut and the posterior midgut in panels **(A, E, H)**. **(B–D)** Confocal images of autofluorescence of **(B)** chlorophyll, red false color, **(C)** guanine, green false color, and **(D)** their overlay within mite midgut lumen, DCs and the posterior midgut. **(E)** Overlay of images in A–C. **(F)** Detail, showing distribution of chlorophyll (red) and guanine (green) in DCs in the ventriculus. **(G)** Guanine pellets (g, red arrowhead) within posterior midgut of female spider mite. **(H)** DCs and guanine pellets within posterior midgut of female spider mite. **(I)** Excreted guanine pellets. **(J)** Autofluorescence of guanine pellets under UV light. **(K)** Mixed excretion containing guanine pellets and DCs at various stages of development. **(L)** Autofluorescence of guanine pellets, and guanine deposits within DCs under UV light. Scale bars: **(A–E)**: 50 μm; **(F)**: 10 μm; **(G,H)**: 100 μm; **(I–L)**: 25 μm.

and detoxification of plant defense compounds. Gene families associated with digestion and detoxification underwent a significant expansion in the TSSM genome (Grbić et al., 2011) and were repeatedly associated with TSSM-plant interactions and pesticide detoxification (Dermauw et al., 2013; Zhurov et al., 2014; Wybouw et al., 2015). To identify genes within these families that have roles in the TSSM gut, we investigated their expression domains through comparisons of transcriptome data from mite proterosoma to whole-body data of bean-reared TSSMs (**Figure 8**). Expression of genes encoding proteins detected in the feces (and thus associated with TSSM gut) (Santamaría et al., 2015a) and salivary glands (Jonckheere et al., 2016), were used as controls to calibrate gene expression depletion and enrichment patterns in the proterosoma, respectively (**Figure 8B**). Peptidases associated with digestive processes in mites such as cathepsins-B and -L, and legumains, were generally strongly depleted in the proterosoma, consistent with their predicted localization in gut cells. Similarly, carboxylesterases, intradiol ring-cleavage dioxygenases, lipocalins, and transporters showed a pattern that is consistent with their greater expression in the TSSM gut. By contrast, the expression of serine proteases, which have not been implicated in mite digestive processes, was found to be enriched in the proterosoma, as were most cytochrome P450 genes. When genes associated with experimental mite adaptation to tomato (Wybouw et al., 2015) or with TSSM responsiveness to *Arabidopsis* indole glucosinolate defense compounds (Zhurov et al., 2014) were highlighted within the classes of analyzed genes, a majority (89 out of 135 genes) corresponded with genes depleted in the proterosoma and potentially associated with the digestive system. However, approximately 1/3 of xenobiotic-responsive genes mapped to the proterosoma in mites reared on beans. Whether these genes undergo tissue-specific changes in their expression, as suggested by this analysis, is at present not known but should be checked by *in situ* localization of their expression in xenobiotically-challenged mites.

DISCUSSION

Using a variety of histological techniques in combination with tracer dye experiments, we analyzed the organization of the TSSM digestive tract as well as the functional properties of digestive compartments relative to their ability to parcel out molecules of different molecular weights. This study lays a histological basis for a better understanding of TSSM-plant interactions at the cellular level. Here, we discuss features of TSSM digestive physiology that are expected to profoundly affect the interaction between the TSSM and its plant hosts.

The Role of Stylet in TSSM Digestive Physiology and in Modulation of Plant-Mite Interactions

The stylet is composed of two cheliceral digits that interlock when protracted, forming a hollow tube-like organ (Andre and Remacle, 1984). Mites protrude the stylet during feeding (Beard et al., 2012; Bensoussan et al., 2016), but withdraw it upon any perturbation. As a consequence, histological studies addressing the involvement of the stylet in plant nutrient ingestion were performed on mites with withdrawn stylets, making the resolution of stylet connectivity with the buccal cavity/pharynx unclear (Summers et al., 1973; Jeppson et al., 1975; Andre and Remacle, 1984; Nuzzaci and de Lillo, 1991; see

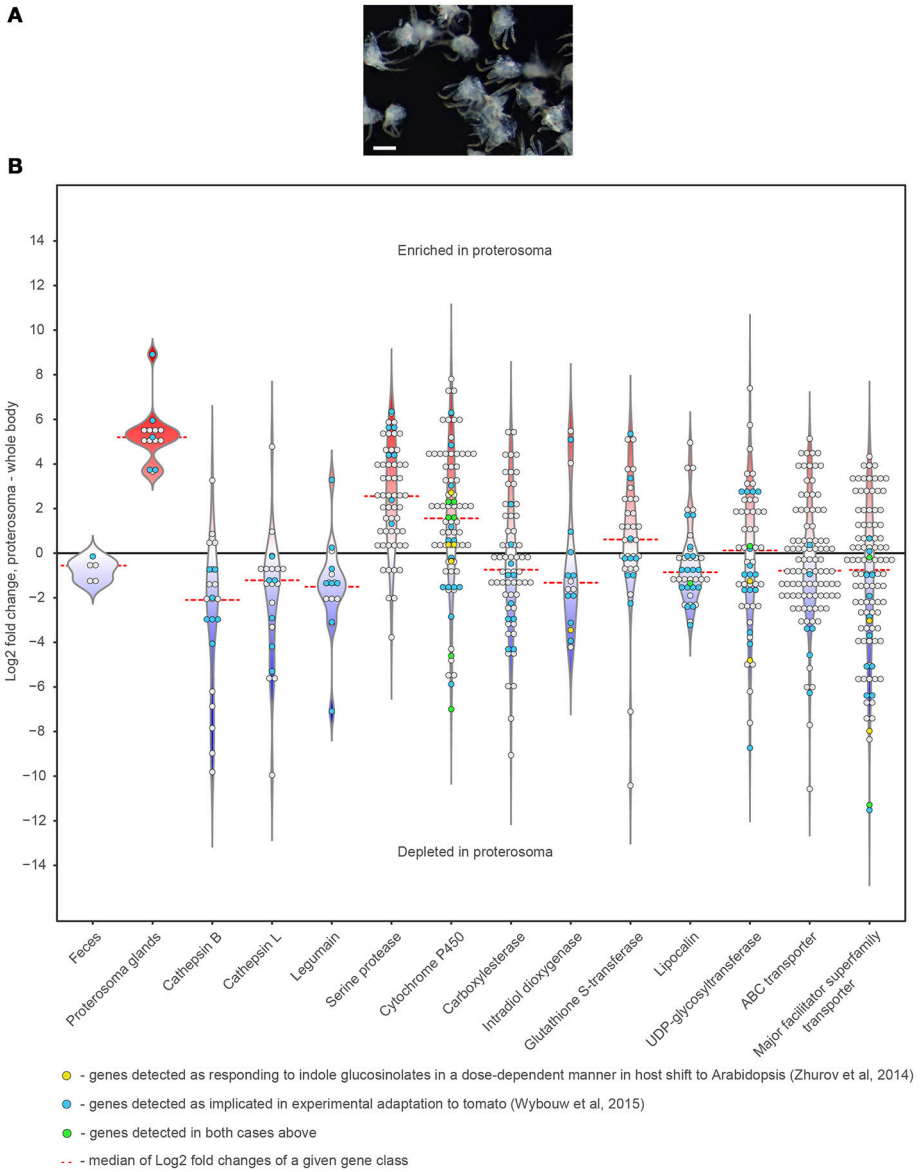

FIGURE 8 | Expression patterns of the digestion/detoxification related genes within spider mite body. **(A)** Dissected spider mite proterosomas used for the RNA extraction. **(B)** Violin and dot plots showing distribution of Log2 Fold Changes of expression of genes implicated in digestion and detoxification in mite proterosoma compared to the whole-body levels of expression. Scale bar: 100 μm.

discussions in Alberti and Kitajima, 2014b; Bensoussan et al., 2016). We have previously captured mites in a feeding position, with their stylets inserted in the plant cells from which they are feeding (Bensoussan et al., 2016). The invariable association of stylets with feeding cells in our preparations is consistent with the possibility that mites use stylets to ingest plant cell content. Here we showed that there is a size limit to particles TSSMs can ingest through their feeding apparatus. Their exclusion size, smaller than 750 nm (**Figure 2**), is consistent with the inner stylet diameter of 1 μm (Andre and Remacle, 1984). Furthermore, the stylet was implicated in the delivery of salivary secretions: (1) stylet is directly connected to the salivary glands (de Lillo et al., 2002), and (2) salivary secretions were detected in a solution that was enclosed in a parafilm bubble accessible only by stylets (Jonckheere et al., 2016), in a similar experimental set up that was used in the feeding experiment shown in **Figure 2**. If TSSMs use stylets to ingest plant nutrients, then the stylet is expected to transport material both inwardly (the plant cell content) and outwardly (the salivary secretions). This is in contrast to other cell-content feeding arthropods that show more specialized stylet structures. For example, Eriophyoid mites have nine stylets that are individually used for piercing, salivary injections, and nutrient intake (Krantz and Lindquist, 1979). In addition, even though phloem-feeding arthropods such as aphids have a single

stylet, their stylets consist of two canals–one that acts as a conduits for salivary secretions and one that allows the ingestion of phloem sap (Tjallingii and Esch, 1993; Garzo et al., 2012). How and whether these anatomical differences affect TSSMs' ability to deliver and potentially take up solutions is not clear. Thus, a further functional understanding of the TSSM feeding apparatus is required to identify TSSM-specific adaptations that support its feeding style.

The identification of the cut off size of ingestible particles is strongly suggestive of liquefaction and partial digestion of plant cellular content prior to its intake by TSSMs (Mothes and Seitz, 1981). Enzymes involved in the preoral digestion of plant cell content may originate from the vacuole of the pierced plant cell, which is expected to burst upon stylet penetration. In addition, digestive enzymes (e.g. cathepsins, serine proteases, glycoside hydrolases, beta-galactosidases, and beta-mannosidases) together with proteins of unknown functions were identified in TSSM salivary secretions (Jonckheere et al., 2016, 2018). Thus, TSSM feeding is associated with partial digestion and decomposition of plant structures, and the presence of TSSM-secreted proteins, generating a rich source of potential Damage and Herbivory Associated Molecular Patterns (DAMPs and HAMPs), some of which can act as elicitors of plant defenses. It has been shown that TSSM salivary secretions also contain effector proteins (Villarroel et al., 2016). Even though functional analysis of TSSM secreted proteins is at its infancy, it is expected that TSSMs have a wide repertoire of effectors that can interfere with plant responses. Thus, the stylet provides an interface between the TSSM and the host plant; as mites feed, they cause cellular damage and contribute elicitors and effectors at the feeding site. On the other hand, plants perceive DAMPs, HAMPs and other stimuli that trigger their responses to mite herbivory. The balance between TSSM- and plant-derived compounds ultimately determines the nature and the intensity of plant defenses.

Digestion of Plant Nutrients

Digestion of plant nutrients in the TSSM gut is another process in which plant and TSSM compounds interact. However, as the TSSM digestive system departs from the canonical insect alimentary tract, it is not clear if, how and where these anti-digestive plant compounds act to reduce TSSMs' ability to retrieve nutrients.

Ingested liquefied plant cell content passes through the esophagus to reach the ventriculus within the TSSM midgut. The esophageal valve enters the ventriculus ventrally and releases the ingested food into the caecal lumen (**Figure 3**). We demonstrated the existence of a regulatory system between the ventriculus and the posterior midgut, capable of separating molecules based on size. Using mixtures of conjugated dextran or fluorescent microspheres, and small tracer dyes, we showed that molecules smaller than approximately 1.25 kDa pass to the posterior midgut, while molecules greater than 4 kDa are retained in the caecal lumen (**Figure 6**). Thus, the ingested mixture of plant cell content can be rapidly separated into a fraction that passes to the posterior midgut (corresponding by size to e.g. ions and plant primary and secondary metabolites), and complex biological molecules that are retained in the caecal lumen (including proteins and remnants of plant cellular structures) (**Figure 6**). Molecules retained in the caecal lumen undergo digestion.

It has been suggested that unlike in insects, digestion in some Acari occurs (at least partially) intracellularly (Sojka et al., 2013). In TSSMs, the midgut lumen is filled with digestive cells that originate from the midgut epithelium. Previously, their ontogeny has been attributed to apocrine or holocrine secretions of the midgut epithelium cells (Mothes and Seitz, 1981). However, we showed that these cells retain their integrity, including the nucleus, throughout their lifespan–from detaching from the midgut epithelium to their final excretion in feces (**Figure 5**). Furthermore, the cytoplasm of these cells is filled with small vesicles that gradually gain the color of the midgut lumen and enlarge, to reach a final state where a thin cytoplasmic layer encircles the large vesicle that appears dark brown/black in bright-field optics. Observation of starch granules and thylakoid membranes in these cells (Mothes and Seitz, 1981), and the identification of dark deposit as a breakdown product of plant pigments (Liesering, 1960; Wiesmann, 1968) strongly suggest that these cells take up the gut lumen content (i.e. ingested plant material) via pinocytosis or phagocytosis and digest it. We referred to them as digestive cells (to avoid confusion of previously used term "phagocytes" that implicates immune function), even though a direct demonstration that they actively carry the digestion process is still missing. Aspartyl and cathepsin L-like proteases have been identified in TSSM feces, implying that they are the most abundant digestive proteases in the TSSM gut (Santamaría et al., 2015a). If lumen-floating cells participate in digestion, then aspartyl and cathepsin L-like digestive proteases should localize in vesicles within these cells, a prediction that should be tested by immunolocalization. In the tick *Ixodes ricinus*, it has been shown that digestive proteases localize in numerous vesicles within digestive cells (Sojka et al., 2013), unequivocally demonstrating that ticks digest hemoglobin intracellularly. In addition, in the herbivorous false spider mite, *Brevipalpus phoenicis,* intracellular digestion is implied, as its midgut is filled with compacted cells and the lumen is reduced to a system of small clefts (Alberti and Kitajima, 2014a). In contrast, in Acari that feed on solid food, like *Acarus siro* and *Archegozetes longisetosus*, ingested food particles form a food bolus (peritrophic membrane/matrix enveloped food particles) whose digestion is at least initially extracellular and restricted to the gut lumen (Alberti et al., 2003; Šobotník et al., 2008).

In response to herbivory, plants synthesize anti-digestive enzymes that are best characterized in the tomato (Gu et al., 1999; Chen et al., 2004, 2007; Fowler et al., 2009; Chung and Felton, 2011; Gonzales-Vigil et al., 2011). These enzymes include protease inhibitors that interfere with the action of digestive proteases, and amino acid-degrading enzymes that eliminate digested nutrients. Plant-host triggered protease inhibition has been demonstrated in the TSSM (Santamaria et al., 2012; Santamaría et al., 2015b; Santamaria et al., 2018). However, even though the efficacy of tomato anti-digestive enzymes in restricting TSSM fitness has not been tested, the predicted pH range of TSSM digestive compartments of 3.5–5.5 (Santamaría et al., 2015a) may not be conducive for their activity.

Distribution of Nutrients and Excretion of Waste Products

During digestion, plant macromolecules are broken down to release nutrients needed to support TSSM metabolism. Egg and silk production are the greatest sinks for released nutrients, as female mites lay 2–10 eggs per day (egg diameter being approximately 1/5 of female body length) (see **Figures 1C,D**) and continuously deposit silk while moving (Saitô, 1977). In insects, the distribution of nutrients occurs through the hemolymph circulating within the hemocoel that is in direct contact with the sink tissues (Nation, 2015). In addition, insects store nutrients in fat bodies in the form of glycogen and lipid droplets (Arrese and Soulages, 2010). However, TSSM tissues are compact (see **Figure 3B**, **Supplemental Figure 5A** for examples) and no cavity that could correspond to the hemocoel could be observed. Likewise, mites do not have fat bodies (Mothes-Wagner, 1982). This raises the question of how nutrients are distributed and stored in TSSMs. The ventricular epithelium (patches of thin cells, **Figures 4E–H**) is in direct contact with sink tissues, indicating that it may be involved in transport of nutrients. However, if digestion is occurring in digestive cells that float in the caecal lumen, then it is unclear how nutrients reach ventricular cells or any other epithelial cells. Alternatively, nutrients may be absorbed in the posterior midgut by the epithelial cells with microvilli, as suggested by Mothes-Wagner (1985), who observed both glycogen depositions and lipid droplets in these cells. If MCs acquire nutrients from the lumen and/or directly from digestive cells as they pass through the posterior midgut, then the transport of nutrients from these cells to sink tissues is likely indirect, as they are not in a direct contact.

Furthermore, remnants of undigested food and byproducts of digestive processes also accumulate in the gut. Guanine, the end product of nitrogen metabolism (McEnroe, 1961a; Wiesmann, 1968) is easily detected due to its refractive (**Figure 4K**) and fluorescent properties (**Figure 7**). Guanine accumulates in digestive cells and large guanine conglomerates form in the posterior midgut (**Figures 5**, **7**, and Occhipinti and Maffei, 2013). Guanine crystals are the most frequent excretory particles, yet the location of their synthesis is still not known. It was proposed that the caecal epithelium adjacent to the posterior midgut (LCs) might synthesize guanine, as these cells have an abundance of vesicles with excretory depositions (Mothes and Seitz, 1981; Mothes-Wagner, 1985). However, these cells do not show guanine fluorescence under fluorescence or confocal microscopy (**Figure 7E**). Alternatively, it was proposed that guanine forms in the caecal lumen and accumulates in the ventriculus from which it is passed to the posterior midgut (Occhipinti and Maffei, 2013). However, we observed no guanine conglomerates in the midgut lumen outside of DCs. In fact, DCs that are excreted in fecal pellets still contained guanine (**Figure 7**). Finally, posterior midgut epithelial cells with microvilli may contribute to the formation of these crystals, as proposed by Mothes and Seitz (1981). However, these cells also lack fluorescence under fluorescence or confocal microscopy (**Figure 7E**). The resolution of the guanine biosynthetic pathway and *in situ* localization of transcripts/proteins of biosynthetic enzymes should help resolve the management of nitrogen metabolism in TSSM.

Detoxification of Xenobiotic Compounds

Adaptation to xenobiotic compounds encompasses many strategies including avoidance, target site modification, metabolism/detoxification, transport/excretion and sequestration (Després et al., 2007). The existence of molecular size regulation in the form of a "shunt" between the ventriculus and the posterior midgut (**Figure 6**) predicts that ingested plant secondary metabolites, the majority of which have a molecular weight <1 kDa, will go directly to the posterior midgut and be excreted. In contrast, insect alimentary canals are linear, forcing all ingested compounds to pass through the whole length of the gut. The ability of TSSMs to expedite small molecules for rapid excretion potentially reduces the accumulation of allelochemicals in the midgut and may contribute to TSSM robustness to xenobiotic exposure. However, some plant toxins, despite being of small size, have chemical properties (e.g. are lipophilic) that allow them to be retained in the TSSM gut (e.g. enter cells through passive diffusion). TSSM resistance to such allelochemicals is likely based on detoxification. The analysis of the TSSM genome identified major proliferations of gene families encoding enzymes involved in oxidation, hydrolysis and/or reduction, and conjugation (Grbić et al., 2011). In addition, studies of TSSM transcriptional changes induced by either a host-shift or the exposure to pesticides established that the expression of detoxification genes changes in response to a xenobiotic challenge (Dermauw et al., 2013; Zhurov et al., 2014; Wybouw et al., 2015). In insects with sufficiently large body sizes, transcriptional analysis of xenobiotic responsiveness was performed directly on dissected gut tissues. For example, host-specific transcriptional reprograming of midgut physiology has been described in *Trichoplusia ni* (Herde and Howe, 2014). In addition, the analysis of dissected gut compartments of *Anopheles gambiae* larvae revealed that even though the expression of detoxification genes occurs throughout the gut, detoxification functions dominate in the anterior midgut and the hindgut/Malpighian tubes (Neira Oviedo et al., 2008). This is consistent with the discrete patterns of expression of cytochrome P450 genes in Drosophila, where they localize in the fat body, Malpighian tubules, and in the midgut (Chung et al., 2009). TSSMs are lacking the fat body and Malpighian tubules. In addition, TSSMs are small and thus not readily amenable to dissections of individual tissues. As a result, tissues/cells that are responsive to xenobiotic challenge have not been determined and are still unknown.

Here, we described how the TSSM digestive physiology deviates from the canonical processes as characterized in insects. This work builds on earlier ultrastructural analyses of the TSSM gut and provides a cell biology context for TSSM-plant interaction studies. Combining this knowledge with genomic and reverse genetics tools will allow a functional dissection of the TSSM gut and the identification of the specific features that enabled the evolution of an extreme

generalist feeding strategy characteristic of two-spotted spider mites.

AUTHOR CONTRIBUTIONS

NB, VZ, TS, MG, and VG conceived and planned the study. NB, CO, and SY performed experimental procedures and collected data. NB, VZ, TS, MG, and VG performed analysis and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01206/full#supplementary-material

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An Intimate Relationship Between Eriophyoid Mites and Their Host Plants – A Review

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Eriophyoid mites (Acari Eriophyoidea) are phytophagous arthropods forming intimate relationships with their host plants. These mites are associated with annual and perennial plants including ferns, and are highly specialized with a dominant monophagy. They can be classified in different ecological classes, i.e., vagrant, gall-making and refuge-seeking species. Many of them are major pests and some of them are vectors of plant pathogens. This paper critically reviews the knowledge on eriophyoids of agricultural importance with emphasis on sources for host plant resistance to these mites. The role of species belonging to the family Eriophyidae as vectors of plant viruses is discussed. Eriophyoid-host plant interactions, the susceptibility within selected crops and main host plant tolerance/resistance mechanisms are discussed. Fundamental concepts, subjects, and problems emerged in this review are pointed out and studies are suggested to clarify some controversial points.

Keywords: plant feeding mites, mite-host plant interactions, plant-virus vectors, economic importance, host-plant resistance mechanisms, cultivar susceptibility

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INTRODUCTION

Eriophyoids are obligatory plant feeders with unusual morphological, biological and behavioral specialization compared to other Acari (Skoracka et al., 2010). Many of them are major pests of agricultural and ornamental crops, wild plants, grasses, and plants of urban and community forestry but they rarely cause their death (Lindquist et al., 1996). Some of them increase their impact by transmitting plant viruses (de Lillo et al., 2016). Other species are efficient in hampering invasive alien plant species (Smith et al., 2010). Mites of the family Diptilomiopidae are vagrants and have trivial interest. Vice versa, about one third of known species in Phytoptidae and Eriophyidae are gall-making and refuge-seeking, considerably affecting the physiology and production of some relevant crops, even though some vagrants in Eriophyidae can injure severely their hosts (Amrine and de Lillo, pers. database). Crop areas and plant distribution are assuming new geographical borders for climatic changes and for new approaches in agricultural and land management (e.g., requests of cultivars resistant to arid and semi-arid conditions). The bio-ecological features of eriophyoids can affect plants in these "new environments."

The current paper would update the most recent reviews on the taxon (Ueckermann, 2010) on selected biological and ecological aspects that may explain the intimate relationships between eriophyoids and their host plants. The information gathered here could be devoted to guide future efforts to explore eriophyoid diversity in order to achieve basic, specific and applied goals in plant protection as well as in understanding more general acarological aspects.

THE ERIOPHYOIDS AS ECONOMICALLY IMPORTANT CROP PESTS

Recent advances on eriophyoids having an economic impact in agriculture and their control deserve to be discussed. Chemical control of eriophyoids has rarely been associated to failures associated with acaricide resistance; this is surprising when compared to worldwide problems encountered in spider mite control (Van Leeuwen and Dermauw, 2016). Nevertheless, a dramatic reduction in pesticide availability has occurred in Europe after the application of the Directive 91/414/EEC and the Regulation (EC) No. 1107/2009 devoted to plant protection products registration (Van Leeuwen et al., 2010) and this tendency is involving also non-EU countries. The reduced number of available acaricides implies potential risks for pesticide resistance and suggests to identify urgently effective non-chemical alternatives. Regarding eriophyoids, most of these studies have been devoted to identify biocontrol agents while research on plant resistance is still lacking or limited to few species.

Aceria tosichella Keifer and *A. guerreronis* Keifer appear the most investigated (119 and 107 documents reported in Web of Science Core Collection, respectively, from 1985 to 2018) among the eriophyoids damaging annual and perennial crops, followed by *Aculus schlechtendali* (Nalepa) (65), *Phyllocoptruta oleivora* (Ashmead) (61), *Cecidophyopsis ribis* (Westwood) (50), *A. tulipae* Keifer (50), *Calepitrimerus vitis* (Nalepa) (48), *Aculops lycopersici* (Tryon) (42), *Abacarus hystrix* (Nalepa) (41), *Colomerus vitis* (Pagenstecher) (36) and *Phytoptus avellanae* Nalepa (26). It should be stressed that the number of documents reported for each species in this database does not reflect strictly their economic importance.

The wheat curl mite, *A. tosichella*, infests a wide range of cultivated and wild Poaceae, and genotype MT-1 was able to colonize onion and garlic in laboratory trials (Skoracka et al., 2014a, 2018). It can inflict direct yield loss to wheat, *Triticum aestivum* L., and other cereal crops inducing a syndrome of curled, looped and trapped leaves (Navia et al., 2010; Petanović and Kielkiewicz, 2010b). The main injuriousness of this mite comes from the transmission of wheat streak mosaic virus (WSMV) and wheat mosaic virus (WMoV) which are severe yield-reducing pathogens (Dumón et al., 2013; Richardson et al., 2014; Skoracka et al., 2018). Brome streak mosaic virus (BrSMV) and triticum mosaic virus (TriMV) can also be transmitted (Navia et al., 2013a). This species can house genetically distinct lineages, including generalist ones (Carew et al., 2009; Skoracka et al., 2012, 2013, 2014a,b, 2018). *Aceria tosichella* and the cereal rust mite, *A. hystrix*, can dominate eriophyoid communities associated to wild and cultivated grasses. In a recent survey the first infested the largest number of grass species while the second had the highest incidence on wheat (Kiedrowicz et al., 2014). Host specialization has been widely reported for both species (Skoracka and Dabert, 2010; Miller et al., 2013; Skoracka et al., 2013). Their involvement in virus transmission has promoted research aimed to finding sources of resistance and developing breeding programs (Dhakal et al., 2018). In contrast, studies on factors affecting the incidence of mite infestation and the severity of virus infection are still limited and appropriate control strategies should be substantially improved (Ranabhat et al., 2018).

The dry bulb mite, *A. tulipae*, is an important pest of bulbous plants (e.g., garlic, onion and tulip). Development is optimal at 25°C but eggs can survive at quite large temperature regimes (6–45°C) (Courtin et al., 2000) with clear implications for crop damage. Host range of *A. tulipae* has been recently explored due to its importance for risk assessment and control (Kiedrowicz et al., 2017). A large variation in mite susceptibility among garlic varieties has been reported and the choice of resistant varieties has dramatically reduced *A. tulipae* infestation (Sapakova et al., 2015). Attempts to identify biocontrol agents have suggested a number of candidates (Duarte et al., 2018) but strategies using them should be delineated more in depth.

The tomato russet mite, *A. lycopersici*, tolerates diversified climatic conditions and completes several generations per year causing alterations in leaves, stems and fruits, often up to plant desiccation (Duso et al., 2010). The identification of non-chemical and effective tools to control mite pests on tomato is urgently needed. The use of predatory mites has been largely investigated but the presence of glandular trichomes hinders their settlement. These trichomes can be degraded in plants infested by *A. lycopersici* allowing predatory mites colonization (van Houten et al., 2013). However, tomato russet mite can find refuges in fresh trichome-dense areas of leaves, escaping to predators. The effectiveness of pathogenic fungi against russet mites has been also tested with promising results (Zanolli et al., 2010). Damage intensity varied among tomato cultivars and mite densities, and was lower on wild *Lycopersicon* spp. (Kitamura and Kawai, 2006). These findings were considered useful for breeding tomato resistant cultivars but a significant progress in this field appears still lacking.

The coconut rust mite, *A. guerreronis*, is highly damaging in the coconut production areas (de Lillo and Skoracka, 2010). Patterns in mite invasion, genetic variability in different countries and features of mite-plant interactions suggest that this species moved from its original host (another palm species?) to coconut after the latter was largely cultivated in the Americas and Africa (Navia et al., 2013b). Severe infestations cause fruit distortion and premature dislodging with reduction in crop yield, coconut fiber length and strength, and husk availability (Navia et al., 2013b). The relationship between damage and mite density at different fruit ages has been investigated in Brazil to improve monitoring techniques (Sousa et al., 2017). Since chemical control is expensive, a number of studies have been devoted to biocontrol strategies with promising results (Navia et al., 2013b).

Aculus schlechtendali feeds on flowers, fruits and leaves of apple inducing fruit russet, cracking on the cheek and color alterations (Easterbrook, 1996). High infestations cause negative effects on the net CO_2 exchange and transpiration rates but the impact of mites on apple yield depends on apple cultivar and environmental conditions (Duso et al., 2010). The identification of economic thresholds for the most popular apple cultivars and the adoption of strategies aimed at preserving predatory mite

populations are crucial to reduce acaricide use in fruit orchards (Simoni et al., 2018).

The big bud mite, *P. avellanae*, includes two cryptic species based on phylogenetic analyses of mitochondrial cytochrome oxidase subunit I (COI) DNA and the nuclear D2 region of 28S rDNA sequences (Cvrković et al., 2016). The first one, *P. avellanae* s.s., lives in hazelnut buds, causes their increase in size (big buds) and drying. The second one is vagrant and should be named after its morphological characterization and more exhaustive study of bio-ecology. *Phytoptus avellanae* s.s. damages 18–20% of buds in North America and over 50% of buds in South Europe and Middle East (Castagnoli and Oldfield, 1996; Özman and Toros, 1996). The impact of the second species on hazelnut production needs to be ascertained. Biocontrol agents of *P. avellanae* have been largely studied but their management can be difficult due to the side-effects of pesticides used to control other pests. Genetic bases of the susceptibility of hazelnut to big bud mite have been explored (Gantner, 2009) but susceptible cultivars are still preferred by hazelnut industry.

Phyllocoptruta oleivora is the most damaging among eriophyoids associated to citrus and frequent acaricide applications are made to reduce its damage (Maoz et al., 2016; Childers et al., 2017). Pesticide contamination and resistance pressed to search for alternatives to acaricides. Most of recent studies deal with biocontrol agents (e.g., Phytoseiidae, Cecidomyiidae, pathogenic fungi) whereas knowledge on cultivar susceptibility/tolerance is quite limited. The pest abundance on sweet oranges can be affected by rootstocks (da Silva et al., 2016). These authors suggested the presence of putative resistance mechanisms to this pest in some genotypes but ad hoc studies are still lacking.

Cecidophyopsis ribis causes abnormal growth of buds (big buds) and transmits blackcurrant reversion virus (BRV) that makes plants unproductive (Jones, 2000). Most of commercial blackcurrant cultivars in Western Europe proved to be susceptible to this mite but the impact of damages and reversion diseases was variable among blackcurrant genotypes (Brennan, 1996; Jones et al., 1998). Resistance detected in *Ribes* spp. to *C. ribis* promoted successful breeding programs (see below).

Vineyards can be infested by gall making and vagrant eriophyoids. Among the former, *Co. vitis* has been considered for long time a minor pest. Recent studies pointed out the negative effects of erineum strain of this mite on plant growth and physiology (Javadi Khederi et al., 2014, 2018a). Evidence that erineum strain of *Co. vitis* is involved in the epidemiology of the Grapevine Pinot gris Virus (GPGV) has been provided (Malagnini et al., 2016). Studies on *Co. vitis* aimed at evaluating the impact on grapevine yield and quality, and mite bio-ecology are needed to assess its real pest status and adopt adequate control measures. A number of generalist phytoseiid mites colonizing vineyards prey upon *Co. vitis* (Duso and de Lillo, 1996). Laboratory studies evaluated the effect of a diet based on *Co. vitis* on the demographic parameters of some predatory mites with a potential impact in controlling this pest in realistic conditions (Lorenzon et al., 2012).

Calepitrimerus vitis is vagrant and can seriously damage grape growth and yield. A relatively low (<10) number of overwintering females (the so-called deutogynes which are morphologically separated by the spring-summer females known as protogynes) per bud have been associated with leaf and shoot distortions, retarded shoot growth and crop losses (Walton et al., 2007, 2010). Knowledge on the biology and ecology of *Ca. vitis* has been substantially improved in the last two decades (Duffner et al., 2001; Walton et al., 2010; Lee et al., 2015, 2018) allowing to delineate the remarkable potential of this species. Chemical control is often requested because grapevine tissues are susceptible in early spring when populations of natural antagonists are not sufficiently dense to contrast the infestation (Duso et al., 2010). Little is known on cultivar susceptibility to this species and possible sources of resistance have been not identified, yet.

This synopsis focuses on eriophyoid species most cited in the literature. It is worth mentioning that a complex of eriophyoid species can be associated with particular crop systems but they are not enough cited/studied even though their relevant importance in agriculture. As an example, *Aceria sheldoni* (Ewing), *Aculops pelekassi* (Keifer) and *Diptilomiopus floridanus* Craemer & Amrine can infest citrus orchards where *P. oleivora* is dominant (Childers et al., 2017). Other eriophyoids are becoming emerging pests in tropical and subtropical crops. *Aceria litchii* (Kiefer) reduces litchi productivity in Brazil where genetic sources for resistance are under investigation (Arantes et al., 2017). An interesting case study shows that *Aceria mangiferae* Sayed increases the severity of infection caused by the fungal pathogen *Fusarium mangiferae* Britz, M. J. Wingf. & Marasas, in mango (Gamliel-Atinsky et al., 2010). Information on other mite pests infesting major crops in these areas (e.g., rice and banana) is also reported.

ERIOPHYOIDS AS VECTORS OF PLANT VIRUSES

Some species of the family Eriophyidae can transmit plant viruses. They belong to seven genera within the subfamilies Eriophyinae, Phyllocoptinae and Cecidophyinae and have vagrant [*A. hystrix*, *Aceria cajani* Channabasavanna, *Aceria ficus* (Cotte), *A. tosichella, Aculus cercidis* (Hall)], refuge-seeking (*A. tulipae*, *Eriophyes insidiosus* Keifer and Wilson, *Phyllocoptes fructiphilus* Keifer, *P. gracilis* (Nalepa) and gall-making behavior (*C. ribis*, *Co. vitis*, *Eriophyes inaequalis* Wilson and Oldfield, *E. pyri* (Pagenstecher)). They are associated to a single host (i.e.: *A. ficus* on fig; *A. cercidis* on Eastern redbud; *E. inaequalis* on wild bitter cherry), to plant species within the same genus (i.e.: *A. cajani* on *Cajanus* spp.; *C. ribis* on *Ribes* spp.; *Co. vitis* on *Vitis* spp.; *P. fructiphilus* on *Rosa* spp.; *P. gracilis* on *Rubus* spp.) and within the same family (i.e., *A. tosichella* and *A. hystrix* on Poaceae; *E. pyri* on Rosaceae). *Aceria tulipae* is an exception; it has been recorded on *Allium* (Amaryllidaceae), *Tulipa* (Liliaceae) and *Xerophyllum* (Melanthiaceae). This wide range of host plants is quite unusual for Eriophyidae but cryptic species are common. They are currently studied for *A. tosichella*, *A. tulipae*,

A. hystrix and are presumed for *E. pyri* (Skoracka, 2009; Skoracka and Dabert, 2010; Skoracka et al., 2014a, 2015, 2017, 2018). Phytoptid and diptilomiopid species have never been suspected to be vectors and there are no published studies suggesting their involvement in the virus transmission even though a new emaravirus, blackberry leaf mottle-associated virus (Hassan et al., 2017), may be related to a new diptilomiopids species (Druciarek, pers. comm. 5 Sept., 2018). The application of new techniques and instruments, like Next Generation Sequencing and Illumina, and a more intense collaboration between acarologists, plant physiologists and virologists could clarify some pathosystems and allow detecting new virus-eriophyoid-plant combinations, confirming or rejecting the exclusive interaction between mites of the family Eriophyidae and plant viruses (Skoracka et al., 2018). About two dozens of serious viral diseases of herbaceous and woody plants are associated to eriophyid mites (de Lillo et al., 2016) and new ones are coming (Hassan et al., 2017). An eriophyid-borne virus is transmitted neither by other mites, nematodes or insects nor by more than one eriophyid species (Oldfield and Proeseler, 1996). Also the previously reported vectors of the BRV, *C. ribis* and *C. spicata* Jones (Lemmetty et al., 2004), were recently suggested to belong to a single species by molecular investigation results (Stalažs and Moročko-Bičevska, 2016). The high degree of specificity between mite vectors and viruses (Oldfield and Proeseler, 1996) might depend on the mode of virus acquisition, transmission and inoculation. Biochemical modifications induced by eriophyid saliva injected into the infected plant cells may influence the virus acquisition. Specific helper proteins might mediate the absorption of virus coats on the mite gut. Membrane proteins might translocate viruses through the gut and salivary gland epithelia. All these issues are fairly conjectural and require a histological and biomolecular approach. Similarly, the effects of mite feeding on the plant physiology and biochemistry are far from being clarified.

The impact of *A. tosichella* on crops can benefit from the susceptible volunteer plants and alternative hosts growing at the field edges, fallow fields, along roadsides and natural environments. These plants can represent green bridge refuges for mites and reservoirs for related viruses during non-growing seasons of the primary or elective hosts (Malik et al., 2003; Skoracka and Kuczyński, 2006). Also seeds of wheat, corn or grasses can be a source of WSMV and WMoV even though at low rates (0.07–1.5%) (Jones et al., 2005; Lanoiselet et al., 2008). Spreading of virus-infected seeds in virus-free areas infested by *A. tosichella* could easily start a new infection by means of the mite. In contrast, PPSMV is transmitted only by *A. cajani* on pigeon pea and not by plant sap, seed or dodder (Latha and Doraiswamy, 2008; Maurya et al., 2017) sustaining the management of the virus disease by means of resistant varieties (Pallavi and Ramappa, 2014). The effectiveness of *A. tosichella* in transmitting viruses to the wheat can be influenced by the mite population composition and origin, and it might be related to the genetic mite lineage (Schiffer et al., 2009; Navia et al., 2013b; McMechan et al., 2014; Skoracka et al., 2018). Mite genotypes differ in vectoring ability also for *A. cajani* in transmitting pigeon pea sterility mosaic virus (PPSMV) and *A. hystrix* in spreading ryegrass mosaic virus (Oldfield and Proeseler, 1996; Kumar et al., 2001; Harvey et al., 2005; McMechan et al., 2014).

The effects of the virus infected plants on the biology of the Eriophyidae are poorly known even though a strict co-evolution of the pathosystem may have produced advantages to mites. *Aceria tosichella* and *A. cajani* increase their fecundity rate and density, respectively, on WSMV and PPSMV infected and susceptible plant genotypes (Reddy and Nene, 1980; Kulkarni et al., 2002; Jones et al., 2004; Siriwetwiwat, 2006; Latha and Doraiswamy, 2008; Murugan et al., 2011; Skoracka et al., 2018). Field populations of *P. fructiphilus* were up to 17 times denser on rose rosette disease-symptomatic multiflora rose than on symptomless ones and the virus transmission was more efficient only when the mite was feeding on rapidly growing plant organs, which are more susceptible to the mite and more receptive for virus infection (Epstein and Hill, 1999). Vice versa, a negative effect was found on the reproduction of *A. tosichella* when feeding on plants infected by TriMV, which may be explained by a shorter co-evolutionary mite-virus path, such as a lower nutritional quality of the host or an increase in the production of secondary plant metabolites induced by the virus that are detrimental to the mite (McMechan, 2012). Recent studies showed also a negative differential off-host survival of *A. tosichella* coming from TriMV-infected plants compared with those tested from non-infected and WSMV-infected plants (Wosula et al., 2015). These data suggest that the TriMV-infected plants decreased *A. tosichella* response to environmental stress factors, like the absence of the elective host plant.

MOLECULAR AND BIOCHEMICAL ERIOPHYOID-PLANT INTERACTIONS

Host plants genotypes, plant age, mite's life style, species and strains are crucial in determining the type of plant changes induced by the eriophyoids (Petanović and Kielkiewicz, 2010a,b; Skoracka et al., 2010; Chakrabarti et al., 2011; Cvrković, 2012).

Morphological, biochemical and physiological responses of plants to eriophyoids are still inadequately studied (Petanović and Kielkiewicz, 2010a,b; Chetverikov et al., 2015). A relevant role has to be played by the salivary secretions injected into the plant tissues, whereas the mechanical action causes only accumulation of chitosan and callose at the feeding site as a wound response of the plant (Petanović and Kielkiewicz, 2010a,b). Saliva of piercing and sucking insects is able to degrade cell walls and middle lamella suggesting cellulolytic and pectinolytic enzyme involvement. Studies on insects suggested a potential involvement of the oligosaccharides produced from pectin components of plant cells as a wound messenger in the induction of plant defense responses (Ryan, 2000; Walling, 2000; Gatehouse, 2002). Polygalacturonase (a pectinase) and cellulase activity was documented in saliva of the gall-making *Aceria caulobia* (Nalepa) (Monfreda and Spagnuolo, 2004; Monfreda and de Lillo, 2010). How the salivary secretions of eriophyoids interact with and disrupt the constitutive plant defenses has to be explained, yet, even though potentiality may come from a secretome investigations such as made in spider mites (Villarroel

et al., 2016). The presence of other enzymes into the eriophyoid saliva is expected. Techniques and protocols for getting saliva from these tiny mites should be improved in order to routinely collect saliva. Preliminary bioassays have been carried out on off-host eriophyoids treated with some neurotransmitters which acted as stimulators of salivary secretions making the collection more easily reproducible (Monfreda and de Lillo, 2010). Collected saliva might be processed by the most advanced instruments (e.g., Spectrophotometry, HeadSpace Gas Chromatography, Gas Chromatography/Mass Spectrometry, etc.) and analytical techniques (e.g., MALDI) which have lower detection limits.

Vagrant mites can be found on all green surfaces of the plants. Their low population densities do not cause damage, but high population densities may be responsible of phytotoxemias and non-distortive alterations. Injured epidermal cells may collapse and die soon after piercing (Petanović and Kielkiewicz, 2010a,b). A repeated attack to close portions of epidermal cells induces typical leaf surface alterations (e.g., bronzing, russeting, silvering, discoloration). In many other cases, cells adjacent to the pierced ones may respond to mite injury primarily with the accumulation of higher amounts of lignin-like compounds and with cell walls thickening that may involve in a spongy parenchyma (Petanović and Kielkiewicz, 2010a). *Aculops lycopersici*, a typical vagrant species, induces hypersensitive reactions in the pierced epidermal cells and accumulation of lignin-like compounds in the walls of the closest intact cells (Royalty and Perring, 1988). Short feeding time of *A. lycopersici* on four-leaf tomato plants induced stronger lipoxygenase (LOX) and peroxidase (POD) responses on leaflets of the same leaf (leaf-systemic spatial response) and a strong increase in the peroxidase (POD) activity on leaflets relatively far from the infested leaves (plant-systemic spatial response) (Stout et al., 1996). On tomato, the effect of *A. lycopersici* infestation on the defense regulated by the jasmonic acid (JA) and the salicylic acid (SA) pathways was studied in detail (Glas et al., 2014). Tomato russet mite suppressed the JA downstream defense responses and this was independent from the production and accumulation of salicylic acid (SA), which is the natural antagonist of JA. They also evaluated the effect of the contemporary presence of *A. lycopersici* and *Tetranychus urticae* Koch, showing that in this condition large colonies of the spider mite developed while population growth of russet mites was inhibited. These phenomena might be related to the altered balance in the plant defense chemicals and accumulation of SA. Furthermore, the authors found that SA was not systemically spread to the whole leaflets and its higher amounts were detected near the mite feeding sites. The accumulation of SA induced by *A. lycopersici* infestation seemed to inhibit the growth of *Pseudomonas syringae* pv. *tomato* DC3000, suggesting a role in limiting the development of secondary infections by pathogens, which could make the substrate unsuitable for *A. lycopersici*. Population densities of *A. lycopersici* can increase faster also on drought-stressed tomato plants (Ximenez-Embun et al., 2017). Several biochemical changes were detected in tomato under mite attack and/or drought stress periods. Levels of total proteins along with some free amino acids and free sugar increased only when mite infestation and drought were combined. It should be emphasized that free sugars act as feeding stimulants for herbivorous mites. *Aculops lycopersici* induced an increase in JA, in its precursor (OPDA) and bioactive form (JA-Ile). When drought was combined with mite infestation, OPDA and SA increased further in contrast with JA accumulation. Mite infestation downregulated the expression of some JA-marker genes, while transcript accumulation for the SA-marker gene increased. Mite infestation alone increased the activity of cysteine protease inhibitors, PPO and POD. Drought contrasted the accumulation of POD and JA induced by *A. lycopersici,* while it synergized the accumulation of SA. These effects have clear implications in the framework of climate change, which are expected to increase the periods of drought. Ximenez-Embun et al. (2017) proved that on drought-stressed tomato plants, *A. lycopersici* can found favorable conditions: high nutritional value and reduced levels of induced defenses (i.e., transcript level of JA-associated genes). Changes in phenolic compound concentrations were observed on olive fruits infested by *Aculus olearius* Castagnoli. The amount of tyrosol and vanillic acid decreased and increased, respectively, and fruits with lower concentrations of tyrosol were more susceptible to mite damage (Çetin et al., 2011). Changes in volatile organic compounds emitted by plants infested by *A. lycopersici* have also been reported and may be related to changes in defense pathways triggered by this vagrant mite (Takayama et al., 2013). Little is known on the molecular mechanisms underlying these interactions.

Refuge seeking mites can exploit preexisting shelters in needles and leaf sheets, in buds and between bulb scales. Gall-making mites induce abnormalities in plant tissues in form of leaf curls, erinea, pouched galls, blisters, witches' brooms, big buds, organ deformations (Chetverikov et al., 2015), which create refuges as well as foraging sites. *Hibiscus vitifolius* L. infested by the gall mite *Acalitus hibisci* Mondal & Chakrabarti showed an increase in leaf hairs density with implications for mite success (Chakrabarti et al., 2001). All these abnormalities are usually induced on young, in-growth and tender tissues of the plant apart from the roots. Eriophyoids are supposed to synthesize and secrete chemicals which cause local changes in the metabolism and balance of plant hormones, as well as can trigger biochemical chain reactions responsible for induction and growth of plant abnormalities (Chetverikov et al., 2015). Saliva of *A. caulobia*, inducing stem galls on shrubby seablight, was proved to have indolacetic acid (IAA) and cytokinin-like activities through wheat-coleoptile and radish-cotyledon growth bioassays (de Lillo and Monfreda, 2004). But little is known on the nature of this activity and if saliva contains plant growth regulators or other chemicals (proteins and peptides) which can alter the synthesis of plant hormones in the infested cells. In any case, pouched galls result from the de-differentiation of the adjacent parenchymal cells in a meristematic tissue, from the proliferation of epidermal and parenchymal cells, and from the protrusion of neoplasic structures from the organ surfaces (Petanović and Kielkiewicz, 2010a; Chetverikov et al., 2015). These histological reactions are in accordance with the changes induced by *Fragariocoptes setiger* (Nalepa) in the expression of transcription factors involved in meristem activity, plant hormone secretion, cell mitosis and

adaxial–abaxial galled leaf polarity during gall morphogenesis on *Fragaria viridis* Weston (Paponova et al., 2018), demonstrating a complex biochemical network which has to locally restrain the mite.

Practically useless fine details are available on mite-plant interactions for gall-making species inducing other plant abnormalities apart pouched galls. Recently, the erineum strain of *Co. vitis* was demonstrated to decrease the leaf area, the plant height, internode length, and chlorophyll content, and to increase the leaf fresh weight as the main effect of hypertrophy and hyperplasia of epidermal and mesophyll cells of the erinea (Javadi Khederi et al., 2014, 2018b,c). The content of chlorophyll decreased also in leaf galls induced by *A. hibisci* on *H. vitifolius*, whereas the amount of carotene was increasing in comparison to the healthy tissues (Chakrabarti et al., 1999). Changes in chemical composition of edge-rolled and erineal leaves were verified on *Tilia* spp. infested by *Phytoptus tetratrichus* (Nalepa) (Kielkiewicz et al., 2011; Soika et al., 2017). High number of starch grains was assessed within the nutritive and hypertrophied parenchyma cells as well as accumulation of antioxidant flavonols, anthocyanins and tannins was detected on densely infested leaves. Starch can suggest a high metabolic activity during abnormality formation. The increase of flavonoid levels, whose protective action from reactive oxygen species (ROS) is well-know, might be the result of an induced defense action triggered by mite saliva injection, even though histochemical analysis evidenced the presence of poliphenolic compounds also inside the mite body (Kielkiewicz et al., 2011). Silencing polyphenol oxidase (PPO) activity, at the early stage of gall formation, might influence the monophenol flavonols stressing the mite-plant interactions and given further evidences of the defense mechanism. But also the regulation of the expression of other factors related to anthocyanins and tannins might help in understanding the gall induction and development mechanisms.

Apart vagrant (e.g., *P. oleivora* and *A. lycopersici*) and gall-making (e.g., *Aceria sheldoni* [Ewing]) species that damage fruits, eriophyoid mites can impact indirectly plant productivity. The physiological processes of transpiration and photosynthesis can be negatively influenced in different plant-mite species combinations involving vagrants (Daud et al., 2012) and gall-makers (Javadi Khederi et al., 2018b). These effects can have also large scale and long term impacts on community forestry where gall inducing mites have proven to be a major driver of age-related declines in tree performance and patterns of net primary productivity (Patankar et al., 2011).

PLANT RESISTANCE MECHANISMS AND SELECTION

Knowledge on the physiological changes in infested plants, their genetic basis and heritability are fundamental for the implementation of resistance/tolerance of the host plants (Mitchell et al., 2016; Stenberg and Muola, 2017). The effects induced by the eriophyoid feeding on their hosts may not be always forecasted. Some of them showed a notable variability depending on mite and plant genotype, mite density, feeding period, plant age and environmental conditions (Royalty and Perring, 1996; Westphal and Manson, 1996; Duso et al., 2008; Petanović and Kielkiewicz, 2010a,b). Resistant plants respond to their feeders mainly by changes in the expression of genes related to defense. For example, the increase of the ratio between the total phenolic compounds (feeding inhibitors) and the total carbohydrates/proteins (feeding stimulators) in leaves of blackberry infested by *Epitrimerus gibbosus* (Nalepa) were suggested to limit mite success (Shi and Tomczyk, 2001). Similarly, *Co. vitis* appeared not thriving on grapevine cultivars with increasead leaf phenol content during mite infestation (Javadi Khederi et al., 2014; **Figure 1**). The levels of epicuticular wax thickness and leaf carbohydrate content were negatively correlated with the mite density and the incidence of erinea (Javadi Khederi et al., 2014; **Figure 1**). *Colomerus vitis*, like other gall-making eriophyoids, redirects the development of pierced cells and their closest ones. Consequently, the plant's physiology, and the shape and size of infested growing organs are modified (Petanović and Kielkiewicz, 2010a,b; Javadi Khederi et al., 2014, 2018b,c,d). Little is known on the expression of defense genes among different genotypes. Six plant marker genes [LOX, stilbene synthase, protease inhibitor, beta-1,3-glucanase, polygalacturonase inhibitor protein and *V. vinifera* proline-rich protein of three Iranian grapevine cultivars displayed differential responses shortly after *Co. vitis* infestation (Javadi Khederi et al., 2018a; **Figure 1**)]. Lower expression of the above mentioned genes was always observed in the susceptible cultivar (Ghalati), whereas the genes for the highly (Atabaki) and the moderately resistant (Muscat Gordo) cultivars were quite differently regulated during that time. The highest upregulated expression of LOX (ethylene-associated gene) in the highly resistant cultivar (Atabaki) is in accordance with the pattern of the same gene in roots of grapevine resistant to the plant-parasitic hemipteran *Daktulosphaira vitifoliae* Fitch (Phylloxeridae) (Blank et al., 2009). The rate of ethylene synthesis in pierced tissues is influenced by the salivary indole-3-acetic-acid (IAA) amount injected or accumulated in them, and ethylene may be involved in incompatible mite-plant interactions (Javadi Khederi et al., 2018a; **Figure 1**).

The involvement of IAA and phenolic compounds in gall making mite-plant interactions was pointed out in *Co. vitis* (Javadi Khederi et al., 2018c), *Aceria cherianii* (Massee), *A. cernuus* (Massee) (Balasubramanian and Purushothaman, 1972a,b; Tandon and Arya, 1980; Tandon, 1985), *F. setiger* (Paponova et al., 2018) and was recently reviewed (Chetverikov et al., 2015). Density of *Co. vitis* on a highly susceptible cultivar (Sezdang) was positively correlated with IAA content of the infested leaves and mites appeared to benefit when leaf IAA increased more than JA and SA, which were negatively correlated with infestation levels (Javadi Khederi et al., 2018c; **Figure 1**). The putative presence of IAA or related compounds was also demonstrated in salivary secretions of *A. caulobia* (de Lillo and Monfreda, 2004). Also, genes encoding cell-wall-proteins (polygalacturonase inhibitor protein and *V. vinifera* proline-rich protein 1) were upregulated in the grapevine cultivar highly resistant to *Co. vitis* (Atabaki) (Javadi Khederi et al., 2018a; **Figure 1**). Both encoded proteins oppose the degradation of cell

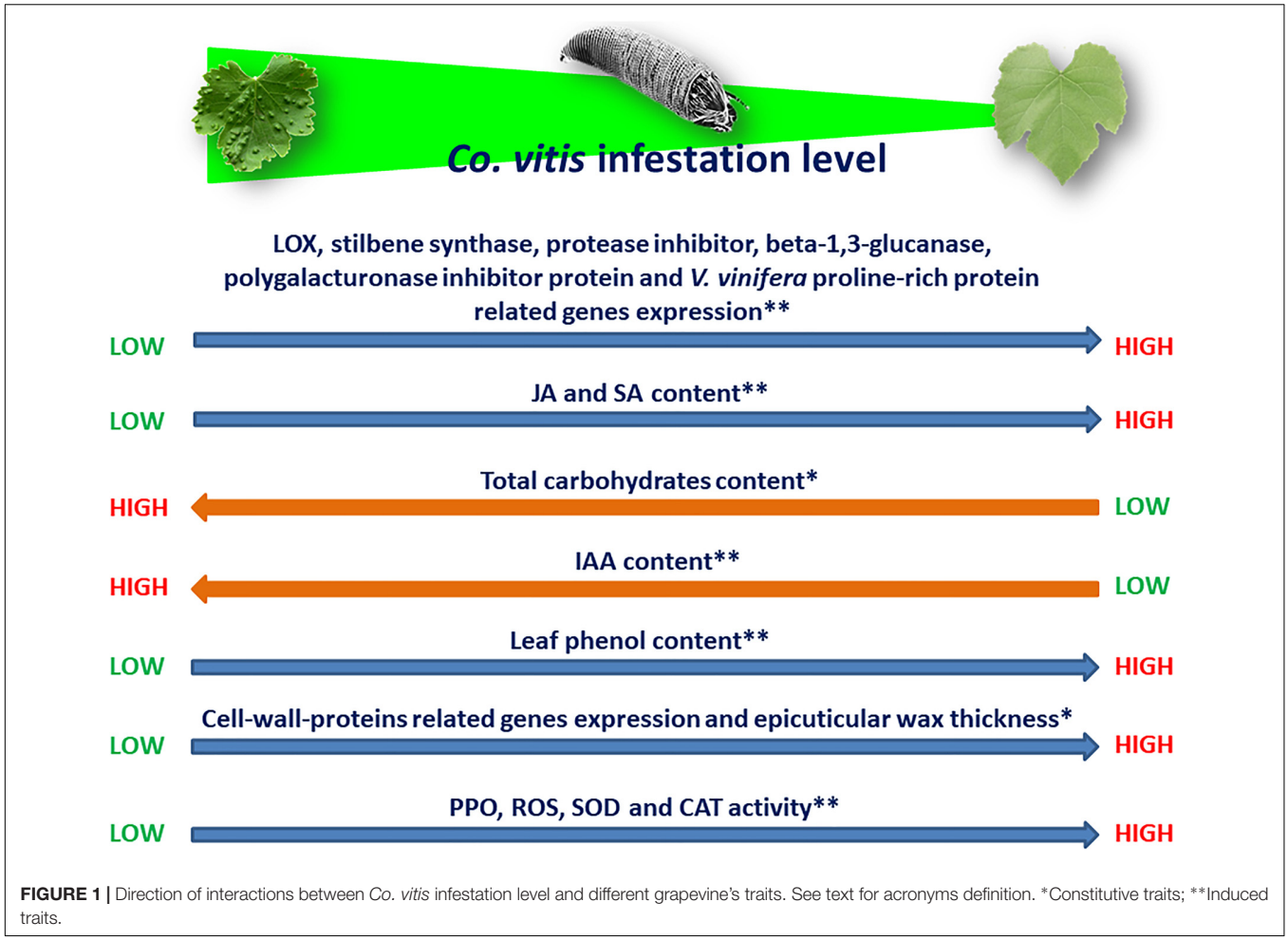

FIGURE 1 | Direction of interactions between *Co. vitis* infestation level and different grapevine's traits. See text for acronyms definition. *Constitutive traits; **Induced traits.

wall architecture caused by the feeder saliva. Hydrogen peroxide and the activity of PPO, superoxide dismutase (SOD) and catalase (CAT) enzymes were found to explain the responses of grape cultivars with a different susceptibility to *Co. vitis* infestation (Javadi Khederi et al., 2018d; **Figure 1**). Hydrogen peroxide is one of the most common ROS induced by environmental stress and was highly and negatively correlated with the mite infestation. SOD and CAT activity was mostly higher in the least susceptible cultivars (**Figure 1**). They are strictly related to each other and both detoxify the overproduction of ROS: SOD transforms the highly toxic free radical superoxide in oxygen and in the less toxic hydrogen peroxide (Petkau et al., 1975); CAT degrades hydrogen peroxide in water (Storey, 1996). Particularly, PPO displayed a highly negative correlation with the infestation. Its high activity on cultivars resistant to *Co. vitis* might be related to the increase of phenols, decrease of the nutritional quality, lignification and hypersensitive responses of the injured tissues (Thipyapong and Steffens, 1997; Mayer, 2006).

The genetic basis of *A. schlechtendali* resistance in apple was investigated in Switzerland, where a number of different genotypes were monitored for mite susceptibility. A Quantitative Trait locus (QTL) analysis was carried out using data available for F1 progeny plants of the cultivars "Fiesta × Discovery." Two QTLs for *A. schlechtendali* resistance on linkage group 7 of "Fiesta" were identified. The identification of a Simple Sequence Repeat (SSR) marker associated to one of the QTLs was considered a first step for the evaluation of resistance to rust mites and the breeding of resistant apple cultivars (Stoeckli et al., 2009). The functional importance of these markers was not fully defined. The "Fiesta" × "Discovery" apple progeny is characterized by adequate fruit firmness, sugar content and acidity but the infestation of various apple pests (excluded *A. schlechtendali*) was positively correlated with apple high quality features. This phenomenon has been explained as a trade-off between resource allocation to defensive secondary metabolites or to fruit (Stoeckli et al., 2011). This study stressed the need to consider pest resistance when breeding for high quality apple cultivars and advantages using genetic markers for fruit quality and pest resistance.

The most important source of constitutive resistance to *P. avellanae* was represented by the content of secondary metabolites, including tannins (Gantner, 2009). Also essential oil components occurring in the buds of differently susceptible cultivars showed allelopathic effects, like in the less affected Mogulus cultivar, in which nerol, α-campholenol, methyl salicylate, spatulenol, β-caryophylene and δ-cadinene were at

higher concentrations compared to the most affected Barra cultivar (Gantner and Najda, 2013). A role of these compounds in deterring mites from feeding was suggested. Differences in varietal susceptibility have also been widely observed for coconut plants against *A. guerreronis* and the physical characteristics of perianth and fruits have been suggested to be involved in plant resistance and reduced plant susceptibility (Navia et al., 2013a). Molecular markers associated with plant resistance have been identified in India (Shalini et al., 2007). However, little is known about the outcome of breeding programs in the selection of mite-resistant coconuts. Experiment design for the evaluation of the assessment of the biological and behavioral responses in mites to different varieties (Stenberg and Muola, 2017) could help in defining future directions in breeding programs.

Currently, breeding program for the selection of resistant plant species have been mainly addressed to viruses and their eriophyid vectors like wheat against *A. tosichella*, pigeon pea and *A. cajani*, black and red currants against *C. ribis* (Jones, 2002; Kumar et al., 2005; Van Leeuwen et al., 2010; Petanović et al., 2017; Chuang et al., 2017; Skoracka et al., 2018) and a few others.

For example, resistance detected in *Ribes* species to *C. ribis* promoted breeding programs focused on Ce and P genes originating from *R. uva-crispa* L. and *R. nigrum* L. var. *sibiricum*, respectively (Anderson, 1971; Knight et al., 1974; Keep et al., 1982; Brennan et al., 2009; Sasnauskas et al., 2009). Eriophyids cannot penetrate the buds of Ce-genotypes, while they cannot survive for long periods of time in buds of P-genotypes. They can transmit BRV to these genotypes but at a low incidence (Jones et al., 1998; Mitchell et al., 2011). Molecular markers for Ce and P genes have been developed (Brennan et al., 2009; Mazeikiene et al., 2012) and recently applied to investigate the origin of resistance to mites in a number of *Ribes* species assessing the inheritance of resistance in genotypes obtained by interspecific hybridization (Mazeikiene et al., 2017). Very recently, Stalažs and Moročko-Bičevska (2016) identified a complex of *Cecidophyopsis* species (based on phylogenetic analyses), besides *C. ribis,* on cultivated and wild blackcurrant in Latvia and some of them could play a significant role in damaging currants. According to these results, future breeding programs for host resistance to Eriophyids mites should also consider other *Cecidophyopsis* species other than *C. ribis*.

The utilization of pest resistant plants is an easy-to-apply strategy, compatible with other control means and ecological friendly for the natural enemies of the target pest. The development of programs for the exploitation of plant defense mechanisms or other finer strategies involving application of non-transgenic methods and genome editing cannot be allowed for the eriophyoids, yet. Most of the research on host resistance to eriophyoid mites has been focused on reducing the potential for pest population development, focusing mostly on antibiosis while scarce attention has been posed on tolerance (Sperotto et al., 2018). Future studies should be aimed at the identification of biological mechanisms underlying the maintenance of crop productivity independently of eriophyoid infestation. It has been suggested that the use of tolerant varieties, having no effect on pest biology are expected to be a more stable and long-lasting strategy (Peterson et al., 2017; Sperotto et al., 2018).

Research on *A. tosichella* may provide an example where identification on tolerant varieties could have interesting perspectives. On this topic, research initially focused on identifying varieties with low trichomes density and length that was thought to reduce landing efficiency of the mites, but breeding program for these traits were never initiated (Navia et al., 2013a). More research effort has been aimed at the identification of genes bearing resistance, mainly antibiosis (Richardson et al., 2014). These were identified in common wheat and related species (Li et al., 2002; Harvey et al., 2003; Navia et al., 2013a; Aguirre-Rojas et al., 2017). Based on these results breeding programs were implemented resulting in commercial wheat cultivars (Martin et al., 1983; Carver et al., 2016). However, different mite population were found to overcome resistance genes in commercial varieties with pest population build-up and this can depend on adaptation of different mite biotypes (Malik et al., 2003; Hein et al., 2012; see Skoracka et al., 2018 for details). In some cases the tolerance has also been evaluated finding varieties which were able to tolerate the wheat curl mite infestation (Carrera et al., 2012). This can provide the basis for breeding program for commercial exploitation of tolerant varieties that could be coupled with plant resistance to viruses for future mite and virus sustainable management options (Carrera et al., 2012).

FUTURE DIRECTIONS

Knowledge on mite-plant relationships with or without virus interactions appears to be scanty and fragmented and it may depend on tiny sizes of mites, difficulties with their manipulation especially for suitable feeding substrate and micro-environmental conditions, low numbers of specialists on eriophyoids, few non-taxonomic studies, etc.

Composition of the plant sap ingested by eriophyoids is unknown and it might be supposed that plant defense compounds may interact with saliva within the cells and may interfere with the physiological processes into the mite gut. Transcriptome and proteome analysis addressed to the salivary compounds appear to give a relevant support in understating abnormality inducing processes in gall-making species as well as for the most intimate interactions with the most noxious vagrant and refuge-seeking species in the attempt to identify potential silencing genes. The recent achievements on dsRNA delivery methods also on mites (Suzuki et al., 2017) may make these studies easier, but eriophyoid transcriptome and genome still need to be processed overcoming technical problems related mainly with their tiny size. The key role of IAA and cytokinins in gall-induction needs to be detailed, as well as sustained by explaining the role of the other phytohormones. Research on stimulating the collection of saliva should be supported in order to achieve a protocol for getting a more purified solution to be analyzed with more sensitive instruments developed in the recent years. The approaches used in other mite-plant models could constitute a framework for future studies. In particular,

transcriptomic and proteomic on *T. urticae*-plant interactions with shaded light on the interplay between host plants and mite can allow the study from the protein constituents of mite saliva and their functions (Jonckheere et al., 2016, 2018; Villarroel et al., 2016; Rioja et al., 2017) to the responses of plants to the mite attack (Santamaria et al., 2013, 2017; Bensoussan et al., 2016; Diaz-Mendoza et al., 2017; Liu et al., 2017). Mite-plant interactions can be influenced by the environment through its effect on the plant status, i.e., drought stress (Ximenez-Embun et al., 2017; Santamaria et al., 2018), and this aspect appear of particular importance for eriophyoids. Potentially, the results could be used to exploit genomic information and new technologies to accelerate breeding program for resistance and tolerance in crops infested by eriophyoids (Salinas et al., 2013; Martel et al., 2015; Díaz-Riquelme et al., 2016; Gascuel et al., 2017; Karkute et al., 2017; di Donato et al., 2018; Haque et al., 2018). One of the main limitation for the implementation of these types of studies on eriophyoids is the absence of efficient mass rearing methods on artificial substrates (Cazaux et al., 2014; Jonckheere et al., 2016) for which cooperation between plant and mite scientists is needed for its development.

This will require additional knowledge on the gut anatomy, cellular organization and physiology, which can explain the interactions with the ingested chemicals as well as the detoxification of plant metabolites, the protective physiology of the gut cells, and the inactivation of enzyme inhibitors. Histological and microscopy techniques combined with the study of the expression pattern of digesting/detoxifying genes may present a valuable research line.

Finally, the microbiome associated to the eriophyoids and its effect on plant-mite interaction is a field completely unexplored. The idea that eriophyoids might transmit gall-inducing viruses or bacteria remains a fascinating and unverified hypothesis till now (Chetverikov et al., 2015), even though the new biotechnologies could assist in looking for entities with very small DNA and RNA. Whether individuals of different generations and morphs (protogynes *versus* deutogynes) have the same efficiency and mechanisms in inducing galls is still an open question.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Thrips Resistance Screening Is Coming of Age: Leaf Position and Ontogeny Are Important Determinants of Leaf-Based Resistance in Pepper

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Capsicum is a genus containing important crop species, many of which severely suffer from thrips infestation. Thrips feeding damages leaves and fruits, and often results in virus infections. Only a few insecticides are still effective against thrips, underlining the importance of finding natural resistance in crops. *Capsicum* is a perennial plant which is usually cultivated for several months, during which time the fruits are harvested. From the young vegetative stage to the mature fruit bearing stage, the plants are at risk to thrips infestation. Constitutive resistance to thrips over the entire ontogenetic development is therefore a key trait for a more sustainable and successful cultivation of the hot and sweet pepper. In addition to ontogeny, leaf position can affect the level of thrips resistance. Pest resistance levels are known to differ between young and old leaves. To our knowledge, no studies have explicitly considered ontogeny and leaf position when screening for constitutive resistance to thrips in *Capsicum*. In this study we analyze whether ontogeny and leaf position affect leaf-based resistance to *Frankliniella occidentalis* and *Thrips tabaci*, in 40 *Capsicum* accessions, comprising five different species. Our results show that resistance to both thrips species in *Capsicum* varies with ontogenetic stage. This variation in resistance among ontogenetic stages was not consistent among the accessions. However, accessions with constitutive resistance in both the flowering and fruit ripening stage could be identified. In addition, we found that thrips resistance is overall similar at different leaf positions within the ontogenetic stage. This implies that resistance mechanisms, such as defense compounds, are constitutively present at sufficient levels on all leaf positions. Finally, we found that resistance to *F. occidentalis* and resistance to *T. tabaci* were not correlated. This indicates that leaf-based resistance in *Capsicum* is thrips species-specific. Because of the variation in resistance over ontogeny, identifying *Capsicum* accessions with

resistance over their entire lifespan is challenging. For resistance screening, accounting for leaf position may be less of a concern. To identify the defense mechanisms responsible for thrips resistance, it is important to further analyze and compare resistant and susceptible accessions.

Keywords: *Frankliniella occidentalis*, *Thrips tabaci*, *Capsicum*, pepper, insect resistance, crop breeding

INTRODUCTION

Thrips are wide-spread piercing-sucking insects which are responsible for severe yield reduction in vegetable crops such as cucumber, strawberry, melon and pepper (Shipp et al., 2000; Park et al., 2007; Peng et al., 2011; Sampson and Kirk, 2013). Crops infected with thrips show stunted growth, leaf deformation and scarring of the fruits, leading to reduced yield and marketing quality (Welter et al., 1990; Tommasini and Maini, 1995; Shipp et al., 1998). In addition, they are an important vector of plant viruses, especially tospovirsuses (Whitfield et al., 2005; Riley et al., 2011; Rotenberg et al., 2015). Their positive thigmotactic nature, fast life cycle and parthenogenetic reproduction makes thrips difficult to combat. Thrips control is mostly achieved by integrated pest management (IPM) (Weintraub, 2007; Mouden et al., 2017), which combines chemical and biological strategies to grow healthy crops (Ehi-Eromosele et al., 2013). Pesticides have lost their effectiveness due to the emergence of resistant thrips populations (Bao et al., 2014; Li et al., 2016; Nazemi et al., 2016). Worldwide there are currently 175 documented cases of *Frankliniella occidentalis* populations and 112 cases of *Thrips tabaci* populations, which are resistant to insecticides (Arthropod Pesticide Resistance Database[1], Michigan State University, East Lansing, MI, United States). In addition, the use of pesticides has been linked to bee colony disorder (Dively et al., 2015; Brandt et al., 2016), decline in insectivorous bird populations (Hallmann et al., 2014), and human health hazards (Kim et al., 2017). The successful application of IPM to minimize pesticide use is dependent on the presence of natural resistance in crop plants.

Capsicum, a genus in the nightshade family that includes hot and sweet pepper, can be severely damaged by thrips. Commonly grown species are *C. annuum* (chili and sweet pepper) and *C. chinense* (aromatic hot pepper). Nowadays, hot and sweet peppers are among the most produced crops, with an estimated production of 34.5 million tons worldwide (FAOSTAT; Data Productions Crops 2016[2]). Due to domestication, commercially grown hot and sweet pepper have lost their resistance to thrips and as a result *Capsicum* is infested by several thrips species (Talekar, 1991; Capinera, 2001; Ssemwogerere et al., 2013). Thrips species commonly found on *Capsicum* include *F. occidentalis* (western flower thrips), *Thrips palmi* (melon thrips), *Scritothrips dorsalis* (chilli thrips) and, to a lesser extent, *T. tabaci* (onion thrips) (Cannon et al., 2007; Weintraub, 2007; Walsh et al., 2012; Ssemwogerere et al., 2013). *F. occidentalis*, also known as Western Flower Thrips, is one of the most wide spread thrips species. It has successfully spread from America to Europe, Africa, Australia and Asia where it has established as a common pest (Tommasini and Maini, 1995; Kirk and Terry, 2003). It causes damage on leaves, flowers and developing fruits by feeding and egg deposition. Moreover, it can transmit at least five types of Tospoviruses (Riley et al., 2011). *T. palmi* and *S. dorsalis* are more problematic in tropical to subtropical regions (Cannon et al., 2007; Weintraub, 2007; Kumar et al., 2013). In Europe, they are observed only occasionally. Consequently, these thrips species are quarantine organisms in the EU (EPPO/CABI, 1998; Vierbergen and van der Gaag, 2009). *T. tabaci* was one of the first recognized vectors of the Tomato Spotted Wilt Virus (Pittman, 1927) and is the most serious pest in onion (Diaz-Montano et al., 2011). It is known to occur on a broad range of hosts, including *Capsicum*, and causes damage on the foliage (Ramakers, 1978; De Klerk and Ramakers, 1986; Walsh et al., 2012). Controlling thrips on *Capsicum* with pesticides is difficult and the identification of resistant accessions is necessary for successful and sustainable production of pepper in the future.

Various studies have explored sources of thrips resistance within the genus *Capsicum* (Fery and Schalk, 1991; Maris et al., 2003a; Maharijaya et al., 2011). Accessions resistant to thrips were characterized based on reduced preference, feeding or reproduction rates, or increased thrips mortality (Fery and Schalk, 1991; Maris et al., 2003b; Maris et al., 2004; Maharijaya et al., 2011). However, the various accessions tested do not always show consistent results throughout these studies. For example, the sweet pepper accession "Yolo Wonder" was reported to be resistant to *F. occidentalis* by Fery and Schalk (1991), but later identified as susceptible to the same thrips species by Maharijaya et al. (2011). These inconsistencies among studies might be explained by the fact that the accessions were screened at different ontogenetic stages. Because plant development is accompanied by physiological changes, resistance levels assessed in one ontogenetic stage cannot simply be extrapolated to other ontogenetic stages (Broekgaarden et al., 2012; Stout et al., 2013; Visschers et al., 2018a). *Capsicum* is a perennial plant, which bears fruits up to at least 160 days after the seedlings are planted (González-Dugo et al., 2007). An individual plant can suffer thrips damage from the early vegetative stage to the mature, fruit-bearing stage. Constitutive leaf-based resistance to thrips over the entire plant's ontogenetic development is therefore a key trait for a more sustainable and successful cultivation of pepper. In addition to ontogeny, leaf position can affect the level of thrips resistance. Resistance to a given pest is known to differ between young and old leaves (van Dam et al., 1995; Visker et al., 2003; Alvarez et al., 2006; Gutbrodt et al., 2012). Moreover, whole plant assays have shown that within plant distribution of thrips is plant species- and cultivar- dependent (Reitz Stuart, 2002; Reay-Jones et al., 2017).

[1]https://www.pesticideresistance.org/

[2]http://www.fao.org/faostat/en/#data/QC

Leaf position and ontogeny have, to our knowledge, not been included when screening for constitutive leaf-based resistance to thrips in *Capsicum*.

In this study we analyze whether ontogeny and leaf position affect leaf-based resistance to two different thrips species, *F. occidentalis* and *T. tabaci*, in *Capsicum*. Feeding damage was used as a parameter to determine leaf-based resistance levels, because this is a widely applied method for identifying resistant accessions (Koschier et al., 2002; Maris et al., 2003b; Leiss et al., 2009; Maharijaya et al., 2011). Moreover, feeding damage is positively correlated with thrips performance (Maharijaya et al., 2012), which makes it a good predictor for overall resistance. Our screening panel consisted of 40 accessions, including previously screened and novel *Capsicum annuum*, *Capsicum baccatum*, *Capsicum chinense*, *Capsicum pubescens* and *Capsicum frutescens* accessions. Using a high throughput leaf-disc based screening approach (Visschers et al., 2018a,b), the accessions were assessed for thrips resistance based on damage inflicted on leaf discs in three ontogenetic stages of the plant: the vegetative, flowering and fruiting stages. In the vegetative stage, only middle leaves were used for resistance screening, while in the reproductive stages apical and basal leaves were also included. Because the same accessions were screened for both *F. occidentalis* and *T. tabaci*, we could also directly correlate the resistance levels to these two species. A positive correlation might be indicative for a general resistance factor effective to multiple thrips species.

TABLE 1 | Overview of the forty *Capsicum* accessions used in this research.

Species	Accession code	Ru-code
C. annuum	CGN22151	1
C. annuum	CGN22830^{I}	2
C. annuum	CGN22120^{S}	3
C. annuum	CGN21543^{R}	4
C. annuum	CGN21534	5
C. annuum	CGN23765^{R}	6
C. annuum	CGN16913	7
C. annuum	CGN21550	8
C. annuum	CGN19226^{I}	9
C. annuum	CGN19240	10
C. annuum	CGN20503^{R}	11
C. annuum	CGN16922^{I}	12
C. annuum	CGN19189^{I}	13
C. annuum	CGN23222^{RS}	14
C. annuum	CGN23098^{IS}	15
C. annuum	CGN19239^{R}	16
C. annuum	CGN23289^{S}	17
C. annuum	CGN16835	18
C. annuum	CGN22151	19
C. annuum	CGN17202	20
C. annuum	CGN17227	21
C. annuum	CGN16975^{R}	22
C. chinense	CGN17220	23
C. chinense	CGN22829^{SR}	24
C. chinense	CGN22862^{I}	25
C. chinense	Hot fatali	26
C. chinense	CGN16994^{I}	27
C. chinense	CGN17004^{S}	28
C. chinense	CGN21557^{S}	29
C. chinense	CGN17222	30
C. chinense	CGN17219^{S}	31
C. chinense	CGN16995^{S}	32
C. chinense	CGN21469^{R}	33
C. frutescens	CGN22839	34
C. pubescens	CGN22108	35
Unknown	VERGASA	36
Unknown	ROXY	37
Unknown	SNOOKER	38
C. chinense	CNG22798	39
C. baccatum	CGN21513	40

Ru-codes are used for presentation in figures and tables. Letters following accession codes indicate previously identified resistance levels (^{R}, resistant, ^{I}, intermediate resistant and ^{S}, susceptible) to thrips according to Fery and Schalk (1991); Maris et al. (2003a), and Maharijaya et al. (2011). Unknown in which ontogenetic stage thrips resistance was determined.

MATERIALS AND METHODS

Plant Material

We used five *Capsicum* species, *C. annuum*, *C. chinense*, *C. baccatum*, *C. pubescens*, *C. frutescens* and a total of 40 accessions (**Table 1**). Several of these accessions have already been tested in Fery and Schalk (1991); Maris et al. (2003a), and Maharijaya et al. (2011) as being resistant or susceptible to thrips (**Table 1**). Seeds were obtained from the Centre for Genetic Resources (CGN), Wageningen University and Research Centre, Netherlands[3]. Accession "Hot fatalli" was obtained from East West Seed (Chiang Mai, Thailand).

Seeds were germinated in closed plastic cups (Ø 7 cm) on sterile glass beads (Ø 1 mm) in a climate cabinet (Snijders Labs, Tilburg, Netherlands) at L16:D8 light: dark regime and temperature set to 30°C/20°C (day/night). When the first two true leaves had developed, the seedlings were transplanted to pots (11 cm × 11 cm × 12 cm) containing potting soil (Potting soil 4, Horticoop, Bleiswijk, Netherlands). The pots were randomly placed on tables in a greenhouse, inside two insect-free net cages (Rovero 0.30 mm gauze, 7.50 m × 3 m × 2.75 m) at 16 h photoperiod and minimum temperatures set to 20°C/17°C (day/night). Natural light was supplemented with Greenpower lights (400 V/1000 W, Phillips, Amsterdam, Netherlands) when below 200 W m^{-2}. Plants were inoculated with biological control agent, *Amblyseius swirskii* (Koppert Biological Systems, Berkel en Rodenrijs, Netherlands) every 4 to 6 weeks to minimize infection with thrips, whitefly and broad mite. Three to four individual plants per accession were maintained throughout the experiments. Sampling of leaves in the vegetative stage started 5 weeks after germination. After plants were sampled in the vegetative stage, they were transferred to larger containers (18 cm × Ø 13 cm) containing potting soil and 4 g L^{-1} Osmocote® Exact Standard 3-4M (Everris, Waardenburg, Netherlands). Plants were watered with additional

[3]http://cgngenis.wur.nl/

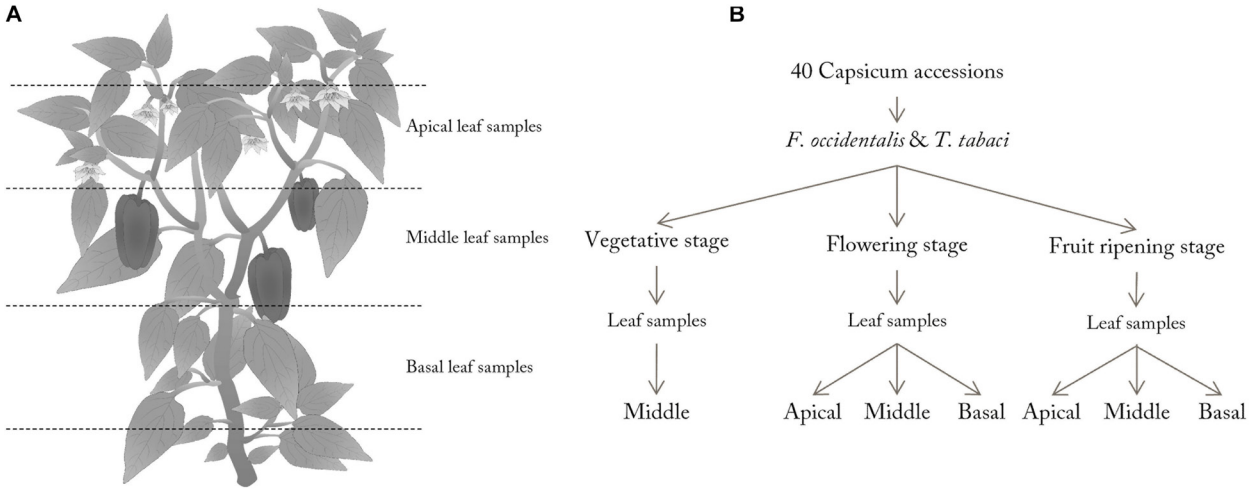

FIGURE 1 | Schematic view of leaf sampling along the vertical axis of the plant **(A)** and overview of the experimental design **(B)**. Three plants per accession and life stage were sampled. Of each sampled plant two leaf discs per leaf position were tested for thrips damage (n = 6–12 leaf discs per accession and leaf position).

nutrient solution as necessary (1.8% Kristalon Label Blue, Yara, Grimsby, United Kingdom). For the flowering stage, leaves were collected after the first fully opened flowers had emerged on the plant. For the fruit ripening stage, leaves were collected when the first fruit started ripening and reached the breaker stage (fruit color changes from green to red). Experiments were conducted in two blocks, February 2015 through July 2015 for screening against *T. tabaci*, August 2015 through January 2016 for screening against *F. occidentalis*. Several accessions suffered from low levels of mite and aphid infestation in the reproductive stages (**Supplementary Table S1**). Plants were not sampled when yellow/white spots caused by spider mite feeding had occurred on leaves or when more than 10 aphids were observed on the plant.

Insect Culture

To establish a colony, *F. occidentalis* and *T. tabaci* stock was obtained from Wageningen University, Netherlands. Cultures were kept in glass jars (11 cm × Ø 7.5 cm) with lids containing fine mesh gauze (45 μM polyester, Ø 6 cm) for aeration. *F. occidentalis* culture was kept on five fresh green beans (*Phaseolus vulgaris* L.) per jar and a 1.5 ml Eppendorf tube with a small amount of pollen grains (De Traay imkerij, Lelystad, Netherlands) to increase oviposition rates (Kirk, 1985; Anjum et al., 2012). *T. tabaci* was reared on two pieces of ±2 cm, Ø 3 cm *Allium ampeloprasum* var. porrum. Three layers of filtration paper were placed on the bottom to absorb excess moisture and to prevent the beans and leek from fouling. Thrips were transferred to clean jars weekly; beans and pieces of leek that were still looking fresh were transferred. The jars with thrips cultures were kept in separate climate cabinets (Economic Delux 432 L with TL lightning, Snijders Labs, Tilburg, Netherlands) at 25°C for *F. occidentalis* and 23°C for *T. tabaci* under a L16:D8 light regime. All experiments were performed using synchronized L1/L2 larvae that were starved for 24 h prior to experiments. Thrips colony rearing conditions and testing conditions were kept constant over time.

No-Choice Leaf Disc Assay

Leaf samples were collected in the greenhouse of the Radboud University as described by Visschers et al. (2018a). In the vegetative stage, leaf samples were selected in the middle of the plant's vertical axis (**Figure 1A**). In the flowering and fruit ripening stage, apical (avoiding young, not fully developed leaves) and basal leaf samples (avoiding old discolored leaves) were taken in addition to middle leaf samples. For each plant, one leaf per position on the plant was collected for the no-choice assay (**Figure 1B**). Using a cork borer, four leaf discs (Ø 1.5 cm) were punched from the leaves, thereby avoiding the mid-vein for optimal image analysis (n = 4 leaf discs per leaf and leaf position on the plant). Each leaf disc was placed in a separate Petri dish on a drop of 1.5% slightly liquid agar (Sigma-Aldrich, United States) with the abaxial side up in the center of the Petri dish. For each sampled leaf, two of the four Petri dishes were inoculated with thrips. Thrips larvae were used for these experiments, since this stage is known to damage leaves the most. For inoculation, five synchronized L1/L2 thrips larvae were placed on the leaf disc using a small painting brush. The Petri dish was sealed with Parafilm and placed in the same climate cabinet as used for insect rearing. Petri dishes without thrips were directly sealed with Parafilm and used for correction during image analysis. After 48 h, the thrips were removed and digital images of treated and untreated leaf discs were acquired as describe by Visschers et al. (2018b). Leaf disc assays were conducted separately for each thrips species.

Determination of Feeding Damage

Feeding damage included all discolorations of the leaf disc caused by thrips feeding, e.g., necrosis, dark green freshly damaged area and the typical silver leaf damage (**Figure 2**). Image

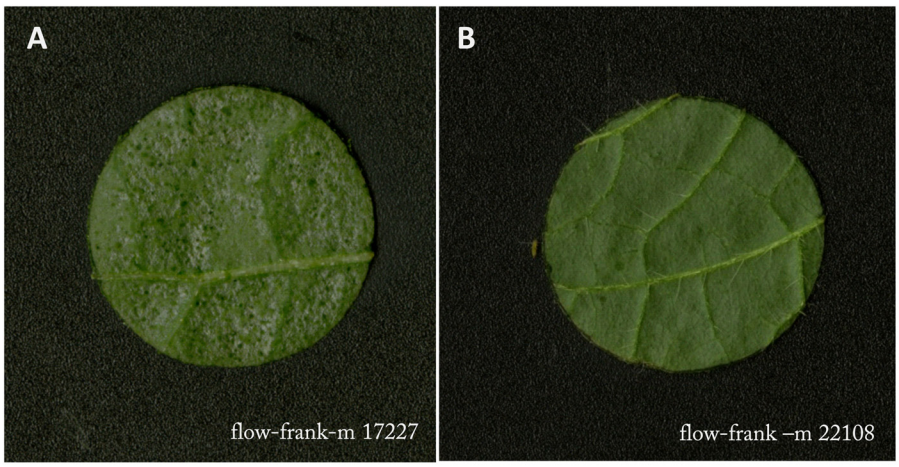

FIGURE 2 | Example of severely damaged **(A)** and very little damaged **(B)** leaf disc by *Frankliniella occidentalis* in the flowering stage. Leaf samples were taken in the middle of the vertical axis of the plant.

processing and quantification of feeding damage in mm^2 was performed using ImageJ Fiji (version 2.0.0 with Java 1.6.0_24) (Schindelin et al., 2012) and Ilastik (version 1.1.3) (Sommer et al., 2011) according to the protocol described by Visschers et al. (2018b). Ilastik was trained using four to six leaf discs per accession and life stage.

Statistical Analysis

All statistical analyses were performed using R Version 1.0.153 (R Core Team, 2016). Statistical analyses were performed using non-parametric tests, since our data did not meet the requirements for parametric tests. Statistical analyses were preformed separately for each thrips species. Biological replicates were treated as independent replicates. Thrips damage (mm^2) was transformed to ranks separately for each thrips species and ontogenetic stage in order to allow comparisons. This was necessary because the accessions differed in their ontogenetic development. Therefore, they were sampled at different time points, which caused random (temporal) variation in the amount of thrips damage. Accessions with equal scores were each assigned the same, average rank. For the *F. occidentalis* experiment, plants were ranked from 1 to 275 for both the vegetative and flowering stage; for the fruiting stage from 1 to 156, because some accessions became infested with greenhouse pests (see above) or did not set fruit. For the *T. tabaci* experiment, plants were ranked from 1 to 274 in the vegetative stage, in the flowering stage from 1 to 258 and in the fruit ripening stage from 1 to 209. Differences in resistance ranks among accessions within each ontogenetic stage were analyzed using the non-parametric Kruskal–Wallis H test. Differences among ontogenetic stage per accession were also analyzed using Kruskal–Wallis H test. Finally, effects of leaf position on thrips damage (in mm^2) were assessed within each ontogenetic stage using Kruskal–Wallis H test. Significant effects were reported with alpha adjusted to 0.015 to correct for multiple comparisons.

Pairwise linear regression correlations of thrips damage between the ontogenetic stages and the leaf positions were performed using the "lm()" function from the Stats package in R. Correlation analyses among ontogenetic stages were based on the accession mean damage rank. Correlation analyses among leaf positions were based on accession-mean of mm^2 damage per leaf position within ontogenetic stage. Correlation analyses among thrips species were based on accession mean of mm^2 damage of middle leaves for each ontogenetic stage separately. Significant effects were reported with alpha set to 0.05.

RESULTS

In this study, 40 *Capsicum* accessions, comprising five species, were screened for resistance to two common thrips species. Using an objective leaf disc test, we analyzed whether ontogeny and leaf position affect resistance. Therefore, plants were screened over three ontogenetic stages and at three different leaf positions within the flowering and fruiting stages.

Effects of Ontogeny on Thrips Damage

Within each ontogenetic stage we analyzed whether thrips damage differed among the forty *Capsicum* accessions. In each ontogenetic stage, accessions significantly differed in thrips damage rank ($p < 0.001$ for each ontogenetic stage, **Figure 3**). This effect was observed for both thrips species (**Figure 3**). As a result, highly resistant and susceptible accessions could be identified in each ontogenetic stage. For example, in the flowering stage, accession CGN22839 (34) was only slightly damaged whereas accession CGN17227 (21) was severely damaged by *F. occidentalis* (mean thrips damage rank, 22.6 and 260, respectively, **Figure 3B**).

Next, we analyzed, within each accession, whether ontogeny significantly influenced thrips damage. For 55% of the accessions, based on the results of both thrips species, damage ranks were not consistent in the vegetative, flowering and fruit ripening stage (**Figure 3** accessions marked by •, **Supplementary Table S2**). In 46% (*F. occidentalis*) and 25% (*T. tabaci*) of the accessions there

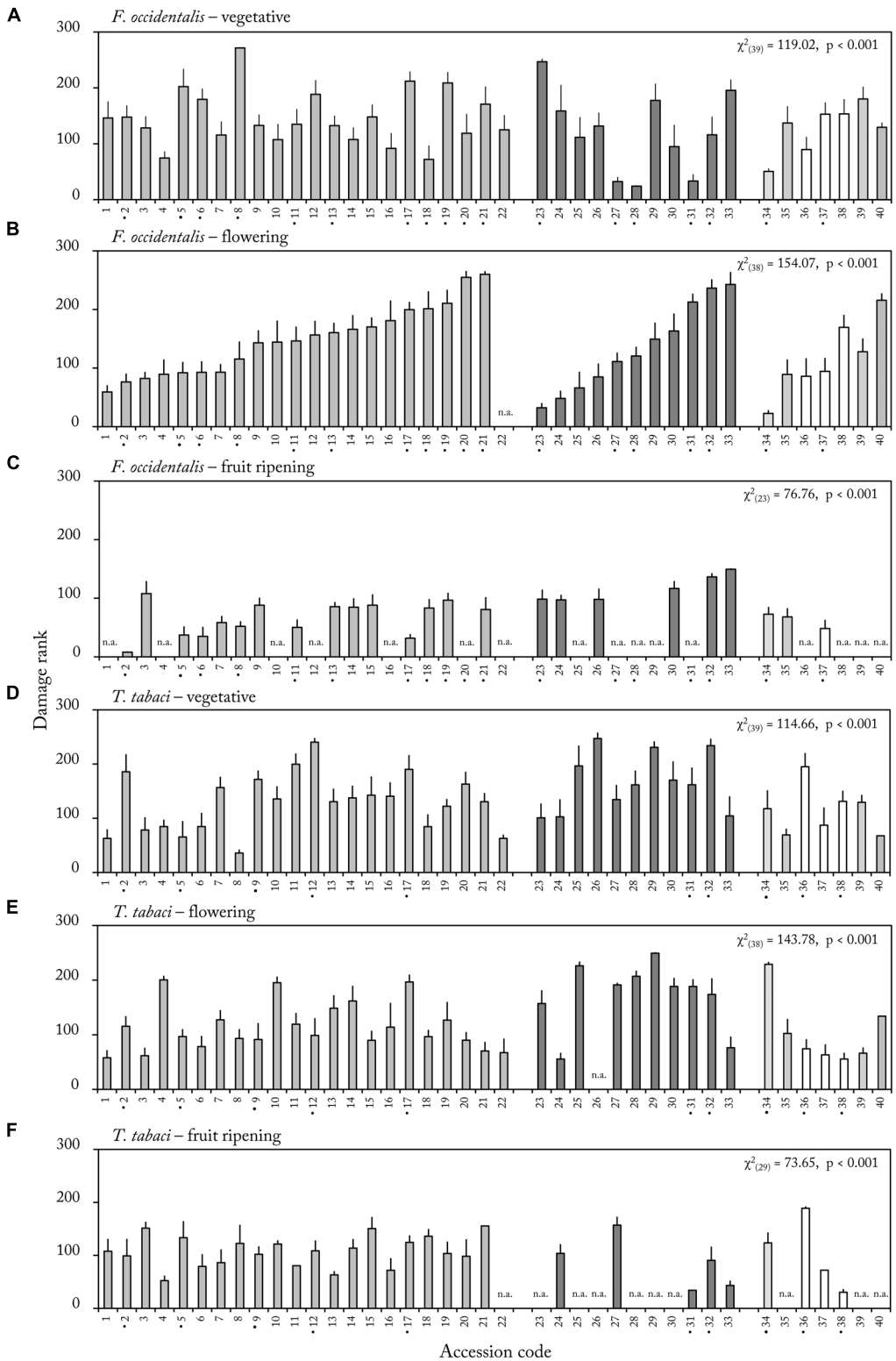

FIGURE 3 | Mean (±SE) thrips damage rank of middle leave samples in the vegetative **(A,D)**, flowering **(B,E)** and fruit ripening stage **(C,F)** of 40 *Capsicum* accessions. Leaf discs were either exposed to *F. occidentalis* **(A–C)** or *T. tabaci* **(D–F)** (n = 4–12). Significant effect of accessions was observed for each life stage and thrips species (p-values of overall Kruskall–Wallis on effect of accession within ontogenetic stage). Accessions labeled with a • on the X-axis showed a significant effect of ontogeny on resistance rank ($p < 0.015$, see **Supplementary Table S2** for details). Accession codes and corresponding CGN numbers can be found in **Table 1**. Different formatting of bars represents different *Capsicum* species. n.a. = not assessed due to mite and aphid infection.

TABLE 2 | Pairwise linear regression correlation of thrips damage rank between the vegetative (veg), flowering (flow) and fruit ripening stage (fruit) for *F. occidentalis* and *T. tabaci*.

	F. occidentalis		*T. tabaci*	
	R^2	*p*-value	R^2	*p*-value
Veg – flow	<0.001	0.953	0.175	**0.008**
Veg – fruit	0.032	0.405	<0.001	0.992
Flow – fruit	0.132	0.087	0.005	0.719

Coefficient of determination (R^2) and the significance level (p) is given. Bold p-values ($p < 0.05$) indicate a significant positive correlation.

was a significant effect of ontogenetic stage on thrips damage rank. For example, in accessions CGN17220 (23) and CGN17227 (21) we found a significant effect of ontogeny ($\chi^2_{(2)} = 13.363$, $p = 0.001$ and $\chi^2_{(2)} = 12.877$, $p = 0.002$, respectively) for resistance to *F. occidentalis*. Accession CGN17227 (21) was highly damaged in the flowering stage, but became less susceptible in the fruit ripening stage (mean thrips damage rank 260 and 80, respectively, **Figures 3B,C**). Accession CGN17220 (23) on the other hand, was one of the most resistant accession in the flowering stage, but among the most susceptible in the vegetative and fruit ripening stages (mean thrips damage rank 246.8, 32.2, and 98.4, respectively, in the vegetative, flowering and fruit ripening stage, **Figures 3A–C**).

To carefully explore this effect of ontogeny on thrips damage, we correlated mean damage ranks between ontogenetic stages. No significant correlation in damage rank was observed between most of the ontogenetic stages (**Table 2**). Only for *T. tabaci* we observed a positive correlation between damage ranks in the vegetative and flowering stage ($p = 0.008$, **Table 2**). Thrips damage, and thus resistance ranking, among the accessions is therefore dependent on ontogenetic stage.

Effects of Leaf Position on Thrips Damage

We quantified thrips damage on leaf discs of apical, middle and basal leaves in the flowering and fruit ripening stage. Overall, middle leaves were damaged the most by both thrips species (mean thrips damage over all accessions for apical, middle and basal leaves were 8.5, 11.8, and 9.8 mm^2, respectively). Significant effects of leaf position were only observed in a few accessions (**Figure 4** accessions marked by •, **Supplementary Table S3**). Of these accessions only accession CGN23098 (15) demonstrated a significant effect of leaf position in both reproductive stages for *F. occidentalis* damage (flowering stage: $\chi^2_{(2)} = 12.21$, $p = 0.002$, and fruit ripening stage: $\chi^2_{(2)} = 9.50$, $p = 0.009$, **Supplementary Table S3**). Since the effect of leaf position was only observed in a few accessions, we tested whether feeding damage at a certain leaf position could be correlated to thrips damage at other leaf positions. Correlation analyses indeed confirmed that feeding damage was highly correlated between leaf positions. With *F. occidentalis*, these correlations were found in both the flowering and fruit ripening stages (**Table 3**). For *T. tabaci* a significant positive correlation was observed between basal and apical leaves and basal and middle leaves only in the fruit ripening stage. Thus, in most of the accessions thrips resistance scores are consistent at different leaf positions within an ontogenetic stage.

Correlation Between Resistance to *F. occidentalis* and *T. tabaci*

Finally, we tested whether thrips damage caused by *F. occidentalis* correlated to damage caused by *T. tabaci* in the vegetative, flowering and fruit ripening stage. We observed no significant correlation in mean damage ranks between these two thrips species. This effect was consistent for all three ontogenetic stages (**Table 4**).

DISCUSSION

In this study we provided evidence that leaf-based resistance to thrips changes over plant ontogeny. Although there was a strong effect of ontogeny, we identified several *C. annuum* and *C. chinense* accessions with consistent constitutive leaf-based resistance in the flowering and fruit ripening stage. We further demonstrate that when the plant is in a certain ontogenetic stage, resistance scores are mostly similar at different leaf positions. In addition, we found no correlation in damage rank for *F. occidentalis* and *T. tabaci*.

Ontogenetic Stage Affects Leaf-Based Resistance to Thrips

Our results provide experimental evidence that leaf-based resistance to thrips in *Capsicum* varies with ontogenetic development. This means that it is important to control for ontogenetic stage when screening for resistance to thrips in *Capsicum*. Previous studies on other plant-insect systems have indicated the importance of ontogenetic stage when screening for resistance (Stout et al., 2013; Hoque and Avila-Sakar, 2015). Among crops, there is variation independent of ontogenetic stage. For example, field studies on cabbage revealed that *T. tabaci* preferred plants with developing cabbage heads over younger plants (Voorrips et al., 2008). Resistance to *F. occidentalis* in cucumber (*Cucumis sativus* L.), on the other hand, was not dependent on plant age (de Kogel et al., 1997a). However, in this study, it was not specified whether these differentially aged plants were also in different ontogenetic stages.

Our results show that the magnitude of the ontogenetic effect depends on the accession that is tested. In other words, we could not identify a general pattern for ontogenetic variation in leaf-based thrips resistance among the 40 *Capsicum* accessions. This is in line with a meta-analysis on the ontogeny of plant defense and herbivory, which also failed to find common patterns (Barton and Koricheva, 2010). This meta-analysis comprised 116 published studies reporting on ontogenetic patterns in defense traits and herbivory. It revealed that ontogenetic patterns were dependent on life form (woody, herbaceous, grass), type of herbivore (insect, mollusk, mammal) and defense trait (secondary chemistry, physical defense, tolerance) (Barton and Koricheva, 2010). Here we show that even within a group of congeneric species no general pattern in leaf-based resistance between ontogenetic stages to insects could be found. This means

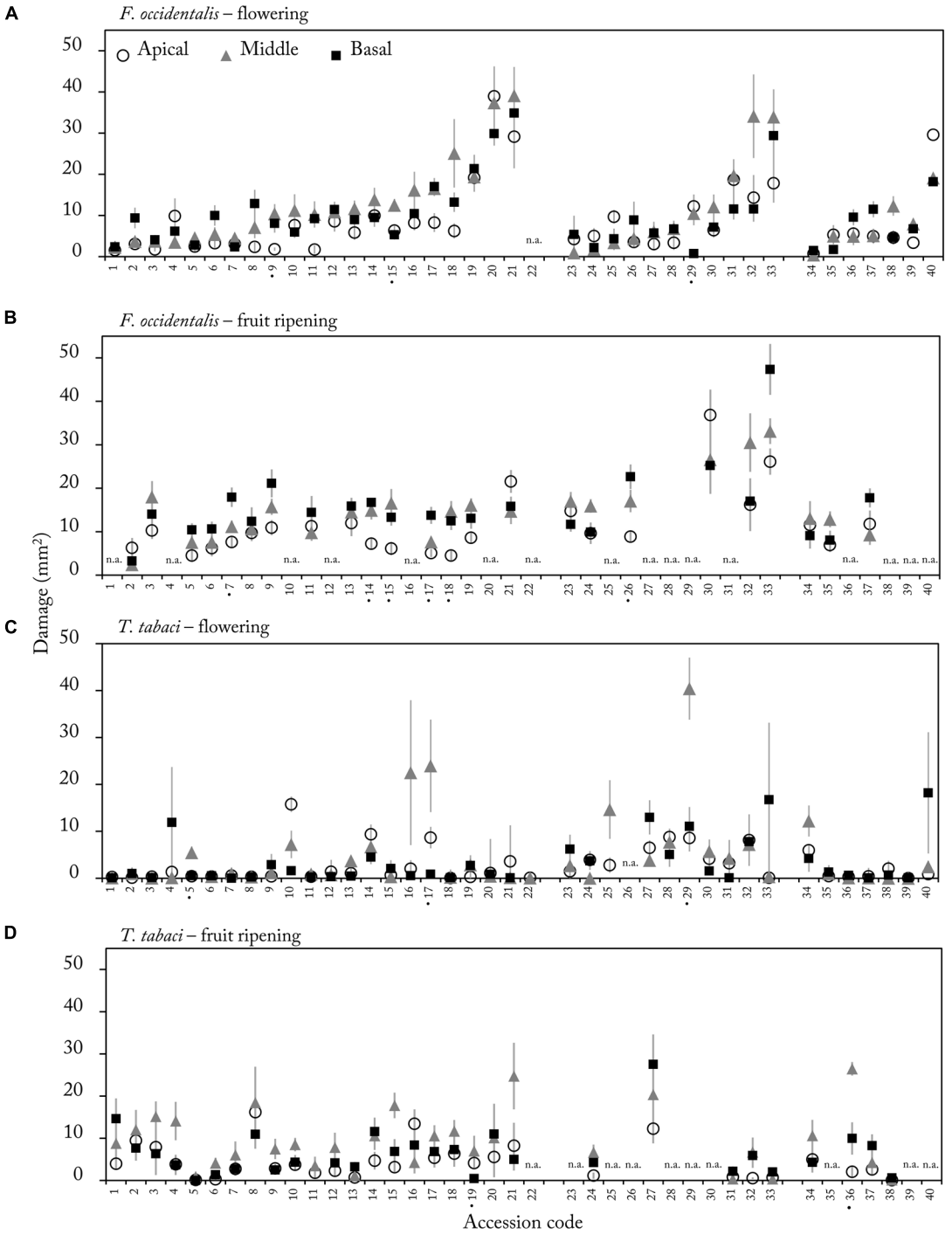

FIGURE 4 | Mean (±SE) thrips damage (mm^2) on apical (white), middle (gray) and basal (black) leaves in the flowering **(A,C)** and fruit ripening stage **(B,D)** on leaf discs (Ø 1.5 cm) of 40 *Capsicum* accession (n = 4–12). Leaf discs were either exposed to *F. occidentalis* **(A,B)** or *T. tabaci* **(C,D)**. Accession codes labeled with a • on the *X*-axis showed a significant effect of leaf position on damage ($p < 0.015$, **Supplementary Table S3**). Accession codes and corresponding CGN numbers can be found in **Table 1**. n.a. = not assessed due to mite and aphid infection.

that identifying *Capsicum* accessions possessing constitutive leaf-based resistance over their entire lifespan is challenging. In *Capsicum* the reproductive stages are the most crucial for fruit set and development and last for several months. It can be argued that these stages are thus the most important for growers. On the other hand, natural enemies of thrips such as *Amblyseius swirskii* and *Orius laevigatus* establish when plants start to flower, due to availability of pollen (Goleva and Zebitz, 2013;

TABLE 3 | Pairwise linear regression correlation of thrips damage between apical, middle and basal leaves in the flowering and fruit ripening stage for *F. occidentalis* and *T. tabaci*.

	Flowering stage				Fruit ripening stage			
	F. occidentalis		*T. tabaci*		*F. occidentalis*		*T. tabaci*	
	R^2	*p*-value	R^2	*p*-value	R^2	*p*-value	R^2	*p*-value
Apical - middle	0.628	**<0.001**	0.280	**<0.001**	0.487	**<0.001**	0.286	**0.002**
Apical - basal	0.608	**<0.001**	0.034	0.273	0.398	**<0.001**	0.349	**<0.001**
Middle - basal	0.695	**<0.001**	0.033	0.278	0.522	**<0.001**	0.285	**0.003**

Coefficient of determination (R^2) and the significance level (p) is given. Bold p-values ($p < 0.05$) indicate a significant positive correlation.

Wong and Frank, 2013). Plants are therefore better protected by natural enemies in the reproductive stages, which makes leaf-based resistance in the vegetative stage more important. Natural leaf-based resistance in the vegetative stage combined with the application of natural enemies in the reproductive stage thus can contribute to successful implementation of IPM in *Capsicum*. As such, accessions such as CGN16994 (27) and CGN17004 (28), provide important leads for resistance breeding programs, because of their strong resistance in the vegetative stage. Our resistance measures are based on leaf disc-assays. Arguably, this may not reflect resistance on the whole plant level, where flowers are available. However, previous studies have shown that thrips damage observed in leaf disc assays and whole plant assays yield comparable results (Maharijaya et al., 2011). Leaf disc assays, especially when combined with computerized data acquisition, thus may be a cost-effective method to quickly identify suitable accessions for breeding thrips resistant crops. Nevertheless, whole plant screening assays including the monitoring of population development can provide additional information on the level of antibiosis in these resistant accessions, especially when plants have entered the generative stage.

Additionally, we have shown that both *C. annuum* and *C. chinense* include accessions with constitutive leaf-based resistance in the flowering and fruit ripening stages such as accession CGN22151 (1), CGN22830 (2) and CGN23765 (6). These accessions provide interesting targets for identifying resistance mechanisms to thrips. Each *Capsicum* species might possess its own unique set of metabolites that confers resistance to thrips. In the two closely related *Solanum* species, *S. galapagense* and *S. cheesmaniaeare*, metabolomics profiles were distinctively different (Vosman et al., 2018). Based on these studies, we expect metabolomics profiles and consequentially metabolites conferring resistance to differ between *C. annuum* and *C. chinense* as well. As both species can be hybridized, heritable (chemical) resistance may be combined to create a multilayered thrips defense which lasts over the plant's reproductive lifetime (Zewdie and Bosland, 2000).

TABLE 4 | Pairwise linear regression correlation of damage rank between *F. occidentalis* and *T. tabaci* in the vegetative, flowering and fruit ripening stage.

	R^2	*p*-value
Vegetative	0.025	0.331
Flowering	0.001	0.820
Fruit ripening	<0.001	0.904

Coefficient of determination (R^2) and the significance level (p) is given. No significant correlations were observed.

Leaf Position Does Not Affect Thrips Resistance

Our study also shows that thrips resistance within ontogenetic stage is mostly constant at different leaf positions. In previous studies on within-plant distribution of insect feeding, leaf position was found to affect resistance to thrips. In cucumber (*C. sativus*), leaf position had a significant effect on reproduction of *F. occidentalis,* and this effect was different among accessions (de Kogel et al., 1997a,b). Similarly, in cotton, thrips abundance within the plant was affected by leaf position and this effect was dependent on the tested cultivar (Reay-Jones et al., 2017). We show that in *Capsicum* leaf position does not affect resistance to *F. occidentalis* or *T. tabaci*. This suggests that resistance mechanisms, such as defense compounds, are constitutively produced in leaves over the whole plant, independent of leaf position. In other studies, presence of defense compounds in apical and basal leaves have shown contrasting patterns. In maize (*Zea mays* L.), the defense compound 1,4-benzoxazin-3-one, an antifeedant and toxic compound to a wide range of herbivores (Niemeyer, 2009), was found to be the lower in young leaves than in old leaves (Kohler et al., 2015). However, in *Cynoglossum officinale* (Houndstongue) opposite patterns were observed. Young leaves contained up to 190 times higher levels of pyrrolizidine alkaloids, which are deterrent to generalists' herbivores (van Dam et al., 1994). In *Capsicum*, capsianoside III-2, a linear diterpene glycoside with a hydroxygeranyllinalool skeleton, has been linked to resistance to thrips in a cross between a resistant *C. annuum* and susceptible *C. chinense* (Maharijaya et al., 2018). The distribution of this compound over different leaf positions is not known, though it has been found in the fruits (Lee et al., 2006). In *Nicotiana attenuata* (coyote tabacco), capsianosides provided an effective direct defense against *Manduca sexta* (tobacco hornworm) (Heiling et al., 2010). Interestingly, the allocation of this defense compound in *N. attenuata* changes with leaf position, with the highest concentrations found in young leaves and reproductive tissues (Heiling et al., 2010). Our results show that resistance to thrips is not affected by leaf position. Therefore, it is likely that thrips-related defense compounds, such as capsianosides, are either

homogeneously allocated or, in resistant accessions, present at sufficient levels at each position to reduce thrips feeding.

Resistance in *Capsicum* Is Thrips Species-Specific

We observed no correlation between damage caused by *F. occidentalis* and *T. tabaci*. This is in contrast with the study of Maharijaya et al. (2011). They determined resistance to *Thrips parvispinus* and *F. occidentalis* in 32 *Capsicum* accessions and found that resistance to these two thrips species was positively correlated. They used this as evidence to argue that there are global resistance mechanisms to thrips (Maharijaya et al., 2011). Our results, which were obtained over a broader panel of accessions and at multiple ontogenetic time points, rather suggest that resistance is thrips species-specific. This makes it less likely that there is a general resistance factor to all thrips species in *Capsicum*. However, this does not preclude that a single accession cannot be resistant to multiple thrips species. A study on resistance to several aphid species in *Medicago truncatula* (barrel clover) showed that one of the tested accessions provided resistance to three aphid species, while failing to provide resistance to two other aphid species (Gao et al., 2007). Their findings indicate some level of aphid species-specificity in the plant's resistance mechanism and a strong influence of the plants genetic background on this specificity. Similar aspects might play a role in resistance mechanisms to thrips in *Capsicum*. Resistance to *T. tabaci* might be driven by a different genetic mechanism than resistance to *F. occidentalis* and *T. parvispinus*. Such aspects might partially explain the dissimilarities between our findings and those of Maharijaya et al. (2011).

In order to identify chemical traits conferring resistance to thrips, accessions with contrasting effects on thrips damage, such as CGN21469 (33), which is susceptible to *F. occidentalis* and moderately resistant to *T. tabaci*, might be interesting models for identifying defense compounds against different thrips species. This can be achieved, for example, by fractionation-driven bioassays (Rimando et al., 2001; Tsao et al., 2005; Wahyuni et al., 2013). This method integrates the process of separation of bio-active compounds by fractionation methods (e.g., liquid or gas chromatography) with results obtained from biological testing, e.g., thrips performance. In *Eurythrina crista-galli* this method was successfully applied to identify botanical insecticides for cotton aphids (*Aphis gossypii*) (Wang et al., 2018). In addition, this method allowed the identification of a novel unsaturated isobutylamide, providing resistance to *F. occidentalis* in *Chrysanthemum* (Tsao et al., 2005).

Altogether, our findings show that identifying accessions with broad scale leaf-based resistance to thrips is a challenging endeavor, even with plants growing under controlled greenhouse conditions. More knowledge on thrips species-specific defense mechanisms might provide important clues for more targeted identification of broad scale thrips resistance.

AUTHOR CONTRIBUTIONS

IV, JP, and NvD contributed conception and design of the study. JP and NvD revised it critically for important intellectual content and provided approval for publication of the content. IV provided substantial contributions to the acquisition, analysis and interpretation of the data. JvdV and RH provided substanial contributions to acquisition and analysis of the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00510/full#supplementary-material

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REVIEW
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Resistance to Thrips in Peanut and Implications for Management of Thrips and Thrips-Transmitted Orthotospoviruses in Peanut

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Thrips are major pests of peanut (*Arachis hypogaea* L.) worldwide, and they serve as vectors of devastating orthotospoviruses such as *Tomato spotted wilt virus* (TSWV) and *Groundnut bud necrosis virus* (GBNV). A tremendous effort has been devoted to developing peanut cultivars with resistance to orthotospoviruses. Consequently, cultivars with moderate field resistance to viruses exist, but not much is known about host resistance to thrips. Integrating host plant resistance to thrips in peanut could suppress thrips feeding damage and reduce virus transmission, will decrease insecticide usage, and enhance sustainability in the production system. This review focuses on details of thrips resistance in peanut and identifies future directions for incorporating thrips resistance in peanut cultivars. Research on thrips–host interactions in peanut is predominantly limited to field evaluations of feeding damage, though, laboratory studies have revealed that peanut cultivars could differentially affect thrips feeding and thrips biology. Many runner type cultivars, field resistant to TSWV, representing diverse pedigrees evaluated against thrips in the greenhouse revealed that thrips preferred some cultivars over others, suggesting that antixenosis "non-preference" could contribute to thrips resistance in peanut. In other crops, morphological traits such as leaf architecture and waxiness and spectral reflectance have been associated with thrips non-preference. It is not clear if foliar morphological traits in peanut are associated with reduced preference or non-preference of thrips and need to be evaluated. Besides thrips non-preference, thrips larval survival to adulthood and median developmental time were negatively affected in some peanut cultivars and in a diploid peanut species *Arachis diogoi* (Hoehne) and its hybrids with a Virginia type cultivar, indicating that antibiosis (negative effects on biology) could also be a factor influencing thrips resistance in peanut. Available field resistance to orthotospoviruses in peanut is not complete, and cultivars can suffer substantial yield loss under high thrips and virus pressure. Integrating thrips

resistance with available virus resistance would be ideal to limit losses. A discussion of modern technologies such as transgenic resistance, marker assisted selection and RNA interference, and future directions that could be undertaken to integrate resistance to thrips and to orthotospoviruses in peanut cultivars is included in this article.

Keywords: *Frankliniella fusca*, peanut, resistance, wild species, vector, *Orthotospovirus*

INTRODUCTION

Thrips Feeding Damage and Virus Transmission in Peanut

Peanut (*Arachis hypogaea* L.) is a major food and oil seed crop that provides high quality human nutrition and is severely affected by thrips and viruses transmitted by them in many parts of the world including the Southern United States, South/Southeastern Asia, and South America (Pappu et al., 2009; Riley et al., 2011; Mandal et al., 2012). Thrips are small (<2 mm in length) and slender insects with fringed wings belonging to the order Thysanoptera. They are hemimetabolous insects with egg, larvae, prepupal (quasi pupal stage), and adult stages. The adults and larvae are the two mobile stages, with adults alone possessing wings (Lewis, 1973, 1997). The two common wing morphs include the brachypterous (short-winged) and macropterous (long-winged) forms. Depending upon seasonal environmental parameters and host availability, thrips alternate wing forms to aid their dispersal. In the United States, two thrips species, Western flower thrips, *Frankliniella occidentalis* (Pergande), tobacco thrips, *Frankliniella fusca* (Hinds), occur in most peanut producing areas (**Figure 1**; Sakimura, 1963; Todd et al., 1995; Riley et al., 2011). In Southeastern United States, where more than half of United States peanuts are grown, *F. fusca* is commonly found on peanut foliage and flowers, and is responsible for almost all the early season feeding injury in peanut (Todd et al., 1995). Western flower thrips is predominantly a flower feeder, and is often found later in the growing season. In other peanut growing areas, such as South and Southeast Asia, thrips species *viz*., common blossom thrips, *Frankliniella schultzei* (Trybom), chili thrips, *Scirtothrips dorsalis* (Hood), melon thrips, *Thrips palmi* (Karny), bean flower thrips, *Megalurothrips usitatus* (Bagnall), and groundnut thrips, *Caliothrips indicus* (Bagnall) are known to infest peanut (Amin et al., 1985; Ekvised et al., 2006a). In South America, besides *Frankliniella* sp. others such as *Enneothrips flavens* (Moulton) are commonly found on peanuts (de Souza et al., 2010; Michelotto et al., 2017).

FIGURE 1 | Two common thrips species in peanuts in Southeastern United States, tobacco thrips, *Frankliniella fusca* (Hinds), on the left, and the western flower thrips, *Frankliniella occidentalis* (Pergande), on the right.

Thrips feeding in peanut is a concern from the time of seedling emergence to a few weeks following emergence. Under severe thrips pressure, thrips feeding injury early in the season can result in yield loss and/or delayed maturity (Todd et al., 1995; de Moraes et al., 2005; Funderburk et al., 2007). Thrips possess asymmetrical mouthparts, due to an atrophied mandible, and generally feed by sucking plant cell contents. In the process, thrips feeding is often characterized by "silvering" of leaves. The silvering appearance is caused by empty epidermal cells following thrips feeding on the cells' contents (**Figure 2A**). Larvae and adults can feed on the peanut foliage. When thrips populations are high early in the growing season, a situation characterized by extensive larval colonization, it is common to find leaf-tip yellowing and necrosis and curling of newly developing leaflets (terminals) at the shoot tip (**Figures 2B,C**). Heavily infested peanut seedlings are often stunted and in severe cases can die (**Figure 2D**). Thrips feeding also results in transmission of viruses. Orthotospoviruses are a major peanut-infecting virus group that is of concern worldwide (Jones, 2005; Pappu et al., 2009; Riley et al., 2011). The main orthotospoviruses (Family *Tospoviridae*; Order *Bunyavirales*) include the *Tomato spotted wilt virus* (TSWV) in Asia and in North America, *Groundnut ring spot virus* (GRSV) and *Groundnut bud necrosis virus* (GBNV) in Asia, and GRSV in South America (Pappu et al., 2009). Increased thrips populations are often correlated with increased virus incidence (Garcia et al., 2000; Culbreath et al., 2003; Sharma et al., 2003).

Thrips Management Options in Peanut and Limitations

Thrips employ haplodiploid sex determination, wherein the fertilized eggs produce diploid females, and the non-fertilized eggs result in haploid males (Moritz, 1997). This mode of

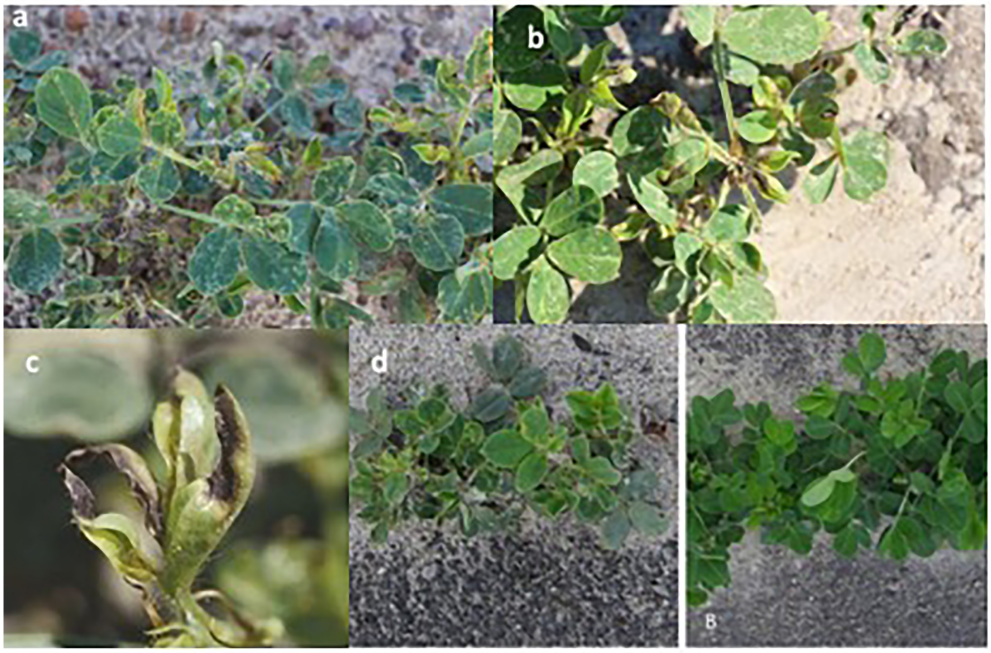

FIGURE 2 | Thrips induced feeding symptoms on peanut. Silvering appearance due to thrips feeding on the epidermal cells' contents **(A)**, leaf-tip yellowing and necrosis and curling of newly developing leaflets (terminals) at the shoot tip **(B)**, a close-up view of tip burning in terminal leaflets **(C)**, and stunting of thrips-infested non-treated peanut plant on the left and treated peanut plant on the right **(D)**.

reproduction and rapid life cycle (dependent on temperature) allows them to build up in large numbers. Their populations are usually characterized by one or two peaks in a typical peanut growing season. Their reproductive capacity, short lifecycles, broad host range, and thigmotactic behavior (seeking refuge in tight spaces such as unfolded peanut terminals), make thrips difficult to manage in peanut. Cultural practices can significantly affect thrips populations in peanut. Manipulating planting date to avoid coincidence of peak thrips dispersal and the susceptible seedling stage results in lower thrips densities and reduces the risk of feeding injury and virus transmission (Brown et al., 1996; McKeown et al., 2001; Culbreath et al., 2010). Likewise, seeding into heavy plant residue in conservation tillage systems reduces thrips abundance on peanut compared with conventional tillage systems with bare soil (Brown et al., 1996; Monfort et al., 2007). Increased plant density and twin row planting have also been shown to reduce thrips infestation and virus incidence, though the mechanism(s) responsible are not well understood (Culbreath et al., 2008; Tubbs et al., 2011). Unfortunately, all the management tactics discussed here have potential negative consequences. Planting dates that minimize risk of thrips infestation are not always optimal for maximizing yield. While conservation tillage offers several recognized agronomic and environmental benefits in addition to thrips management, it may have negative effects on weed management programs compared with conventional tillage systems (Johnson et al., 2001). The additional seed and specialized planting equipment needed to increase plant densities and achieve twin row patterns ultimately increase production costs for farmers.

Chemical management options for thrips in peanut, like many other row crops, are limited to a few insecticide active ingredients (Reddy et al., 1995; Todd et al., 1995, 1996; Herbert et al., 2007; Culbreath et al., 2008; Marasigan et al., 2016). The most commonly used insecticide classes include organophosphates, carbamates, phenylpyrazole, pyrethroids, and neonicotinoids (Todd et al., 1996; Culbreath et al., 2003, 2016; Mandal et al., 2012; Marasigan et al., 2016; Srinivasan et al., 2017). Newer classes of insecticides such as spinosyns and diamides, though effective, are too expensive to justify their use in peanut (Marasigan et al., 2016, 2018). These products are generally reserved for high value crops such as fruits and vegetables. In addition, there is increased concern over environmental and non-target issues associated with older broad-spectrum insecticides such as organophosphates, and carbamates. Even neonicotinoids, long considered to be reduced risk options, are being scrutinized due to their presumed role in pollinator decline (Mullin et al., 2010; Nicodemo et al., 2014). In lieu of the perceived effects, the United States Environmental Agency in 2015 issued a temporary moratorium on new neonicotinoid registrations[1]. This moratorium has not affected any existing registrations. Neonicotinoids applied as liquid in-furrow at peanut planting offer ease of application, are relatively less

[1]https://www.epa.gov/sites/production/files/2015-04/documents/neonicotinoid-new-use.pdf

expensive, and in general provide good efficacy. For these reasons, the neonicotinoid imidacloprid is now used increasingly in Georgia in Southern United States (Marasigan et al., 2016, 2018; Srinivasan et al., 2017). Thrips, in general, have effective pesticide detoxification abilities (Espinosa et al., 2005; Bielza et al., 2007, 2008; Bielza, 2008), and they have developed resistance to several insecticide classes. Increased neonicotinoid usage in cotton has already led to widespread resistance development in the Southeastern United States (Huseth et al., 2016). Preliminary research conducted in Georgia in the United States indicated no evidence of resistance to neonicotinoids in thrips populations collected from peanut (Lai, 2015). The usefulness of these insecticides in the long run remains questionable. Of course, the main concern in peanut production in many parts of the world is thrips-transmitted orthotospoviruses. No insecticide, except phorate, has been found to be effective in suppressing virus transmission significantly in the United States, and the effect of phorate is not consistent (Culbreath et al., 2003, 2008, 2016; Marasigan et al., 2018).

Because of the difficulty associated with managing thrips and the significant economic loss that accompanies virus infection, a tremendous amount of effort has been invested into breeding cultivars with resistance against orthotospoviruses. Much of the information on breeding for virus resistance comes with research conducted with TSWV in peanut in the United States and GBNV in Asia. The cultivars grown at the advent of TSWV in Southern United States, in the 1980s and in early 1990s, such as Florunner and Southern runner, were extremely susceptible to TSWV-induced spotted wilt disease (Culbreath et al., 1992, 1996). Screening and breeding efforts led to incremental increases in resistance, most of which was derived from a single genotype (PI 203363) introduced from Brazil in 1953 (Culbreath et al., 2003). The introduction of this unique genotype had a rapid and profound effect on the United States peanut breeding, as the main runner peanut cultivars have a significant proportion of PI203363 alleles (Clevenger et al., 2018). Current "third generation TSWV-resistant" peanut cultivars are highly field resistant to TSWV, and losses due to the disease have been minimized. Breeding efforts also have led to identification of moderate resistance to GBNV in Asia (Amin et al., 1985; Dwivedi et al., 1995; Reddy et al., 2000; Kesmala et al., 2004; Mandal et al., 2012). In all these instances, resistance to the virus is not complete, and often other management options are integrated. For instance, insecticides are still being employed to reduce thrips feeding injury in early season peanut (Mandal et al., 2012; Marasigan et al., 2016). Identifying and incorporating effective thrips resistance in high yielding peanut cultivars will provide significant economic benefit to producers and result in reduced environmental impact associated with pesticide use.

Factors Contributing to Resistance Against Thrips in Peanut in Relation to Other Crops

Thrips resistance in peanut was more actively pursued in the 1980s and early 1990s in Asia and in the United States until thrips-transmitted viruses became a more pressing issue (Amin and Mohammed, 1980; Campbell and Wynne, 1980; Amin et al., 1985; Mulder and Seuhs, 2002). Most of those early examinations were based on field screening (Young et al., 1972; Stalker and Campbell, 1983; Lynch, 1990). Consequently, thrips resistance contributing factors in peanut are not well understood. Information about thrips resistance stems mostly from work on other crops, where resistance seems to be imparted by morphological as well as biochemical traits. Each trait category is discussed in detail.

Morphological Traits

Early on increased leaf pubescence in crops such as cassava was associated with thrips resistance (Schoonhoven, 1974). Similarly, increased foliar pubescence in diploid cotton such as *Gossypium arboreum* L., *Gossypium thurberi* (Todd), *Gossypium trilobum* (DC.) Skovst resulted in reduced western flower thrips infestation compared with other commonly grown *Gossypium hirsutum* L. cv. Sicot 71 (Miyazaki et al., 2017). Another study found that thrips infestation was less in glandless cotton than in glandular cotton (Zhang et al., 2014). Increased leaf waxiness was associated with resistance against thrips in cabbage (Voorrips et al., 2008a,b). On the contrary, in onion cultivars, glossy (less wax) yellow green foliage provided more protection against thrips than cultivars with non-glossy or waxy blue green foliage (Coudriet et al., 1979; Molenaar, 1984; Diaz-Montano et al., 2010; Damon et al., 2014). Traits such as leaf angle and leaf toughness were also more influential than waxiness on host plant susceptibility to onion thrips (Njau et al., 2017). Onion foliage that was more open and round, allowed more thrips exposure to natural enemies, and thus had fewer thrips compared with onion foliage that was tight and had short angle of deviation. In addition to leaves, floral, and fruiting structures are also vulnerable to thrips. It has been shown in at least two cases that the size of floral structures can be associated with thrips resistance. Smaller flowers in both cowpea and chrysanthemum resulted in reduced incidences of *Megalurothrips sjostedti* (Trybom) and *F. occidentalis*, respectively (de Jager et al., 1995; Abudulai et al., 2006; Omo-Ikerodah et al., 2009).

Field screening of peanut genotypes revealed differences in thrips feeding injury consistently, and it was speculated that foliage color could be influencing thrips host selection patterns (Amin et al., 1985). A relatively recent study found differences in normalized vegetation index among peanut cultivars, which in turn might affect light reflectance off peanut foliage (Navia-Gine, 2012). It is not clear if these differences in light reflectance affect thrips host plant utilization. The hypothesis that reflectance contributes to host plant resistance should be evaluated, and the role of light reflectance in thrips host selection and host utilization in peanut needs to be examined in greater depth. Such differences in foliar reflectance in light could be due to differences in profiles of cuticular waxes. Peanut cultivars' and wild species foliage hues differ substantially from light green to bluish green. Increased cuticular wax in various wild peanut species such as *Arachis batizocoi* Krapov. & W. C. Gregory, *Arachis glandulifera* Stalker, *Arachis ipaensis* Krapov. & W. C. Gregory, *Arachis Chacoense* Krapov., and *Arachis paraguariensis* Chodat & Hassl., is believed to be responsible for suppressing thrips

feeding compared with commonly grown peanut cultivars (Yang et al., 1993; de Souza et al., 2010). Other morphological traits discussed previously such as leaf hairiness, and leaf toughness are also believed to be involved in conferring resistance to insects including thrips in peanut (Campbell and Wynne, 1980; Yang et al., 1993), but their role in suppressing thrips needs to be experimentally demonstrated.

Biochemical Traits

Alkaloids and other secondary metabolites seem to be contributing to thrips resistance either in the presence or absence of morphological traits. In a study with wild tomato species *viz*., *Solanum pennellii* Correll and *Solanum hirsutum* Dunal, acyl sugars were implicated as being involved in conferring resistance to thrips (Mirnezhad et al., 2010; Romero González, 2011). An alkaloid, pyrrolizidine, was associated with thrips resistance in the case of *Jacobaea aquatica* (Hill) G. Gaertn., (Cheng et al., 2011). Similarly, glycoalkaloids provided resistance against thrips in potato, *Solanum tuberosum* L. (Galvez et al., 2005). Evidence for involvement of biochemical compounds besides alkaloids in thrips resistance is found in numerous crops. Isobutylamides of unsaturated fatty acids and chlorogenic acid from chrysanthemum, *Chrysanthemum indicum* L., conferred resistance against western flower thrips (Tsao et al., 2003; Leiss et al., 2009, 2011). A flavonoid luteolin and phenylpropanoid sinapic acid derived from carrot, *Daucus carota* (Hoffm.) Schübl., negatively impacted Western flower thrips development (Leiss et al., 2013). In some instances, biochemicals are thought to be involved in imparting resistance against thrips; however, they have not been identified. For instance, steam distillates from certain resistant cultivars of rice (*Oryza indica* L.) were toxic to *Stenchaetothrips biformis* (Bagnall), but it is not clear which active compound(s) in that distillate was responsible for thrips mortality (Velusamy and Saxena, 1991). Similarly, extensive studies in pepper, *Capsicum* sp., led to identification of resistance believed to be caused by biochemical traits, but the causal agents have not been identified (Maris et al., 2003a,b; Maharijaya, 2013). Also, some odors from rose, *Rosa* sp., that repelled western flower thrips, and are yet to be characterized (Gaum et al., 1994). de Souza et al. (2010) found that certain n-alkanes from wild diploid peanut species could be responsible for *E. flavens* resistance in Brazil. A polyphenolic compound, 2,3-Di-(E)-caffeoyl-(2B,3R)-(+)-tartaric acid, found in peanut terminals was associated with *F. fusca* resistance in the Southeastern United States (Snook et al., 1994). However, this study merely reported a correlation, and did not present a conclusive evidence of the compound's involvement in resistance against thrips. Another study showed a strong negative correlation between phenols and tannins in peanut germplasm with thrips feeding damage (Kandakoor et al., 2014). The correlative roles of these compounds in thrips resistance should further be functionally characterized by isolating these compounds and conducting bioassays with thrips.

Mechanism(s) of Resistance Against Thrips in Peanut

Peanut cultivars have been screened for *F. fusca* and *F. Schultzei* feeding injury in numerous studies in the Southern United States, Asia, and in South America (Young et al., 1972; Kinzer et al., 1973; Amin et al., 1985; Sharma et al., 2003). In the United States, several studies identified runner type and Virginia type peanut germplasm plant introductions with resistance to thrips in Georgia and North Carolina (Lynch, 1990). Results from these studies showed differences in thrips injury rating. However, the peanut plants generally recovered from thrips injury and yield losses only occurred under certain conditions (Funderburk et al., 2007). The losses that were observed were more often associated with Virginia type peanut cultivars than runner type (Amin and Mohammed, 1980; Campbell and Wynne, 1980; Mulder, 1999; Mulder and Seuhs, 2002; Herbert et al., 2005; Whalen et al., 2014). Choice experiments conducted with peanut cultivars and tobacco thrips suggest that cultivars may differently affect thrips density as well as severity of thrips feeding injury (**Figure 3**). Thrips feeding was reduced in some cultivars such as "Tifguard" and "Georganic" when compared with others such as Georgia Green and Georgia 06G (Sundaraj et al., 2014). Thrips feeding was measured by using a thrips feeding damage index, which is a measurement of silvering area on the plant as a proportion of the undamaged area (Maris et al., 2003a). The evaluated peanut cultivars were actually released with increased resistance to TSWV, but none were specifically bred for thrips resistance. These results suggest that there could be non-preference or antixenosis effects present in peanut cultivars that impact the behavior of thrips. The term non-preference was defined by Painter (1951); Kogan and Ortman (1978) later described it as antixenosis, these terms describe the inability of the insect to effectively use a host plant and instead select an alternate host plant (Smith, 2005). It is not clear how these preference patterns would influence thrips in peanut agroecosystems with relatively low genotype diversity such as those in the Southern United States. For instance, in Georgia, more than 80% of the peanut acreage (>600 K acres, NASS, 2017) is often planted with a single cultivar. More research needs to be conducted to evaluate the significance of antixenosis against thrips in commercial peanut production.

Microcosmic "Munger"(45 cm^3 thrips-proof cages) studies indicate that peanut cultivar differences could differentially influence tobacco thrips, *F. fusca* fitness. No-choice tests to monitor thrips development and thrips survival were conducted with commonly grown cultivars in Georgia. Results revealed that cultivar differences significantly influenced tobacco thrips survival (**Figure 4**; Sundaraj et al., 2014). Thrips fitness was consistently reduced in some of the "second-generation TSWV resistant" peanut cultivars compared to ones released earlier such as "Georgia Green." Antibiosis is a resistance mechanism by which a host plant adversely affects the biology of the insect, often resulting in increased mortality or reduced longevity and fecundity (Teetes, 1996). The Munger cage studies revealed that thrips fecundity (adults recovered per adult released) and longevity was reduced in some cultivars such as Georganic and Tifguard. The innate factors in these cultivars that contribute to antibiosis against thrips are yet to be identified. Unique parentage of these cultivars could be influencing resistance to thrips. For instance, "Tifguard," which possesses resistance to nematodes seems to be the most resistant against thrips

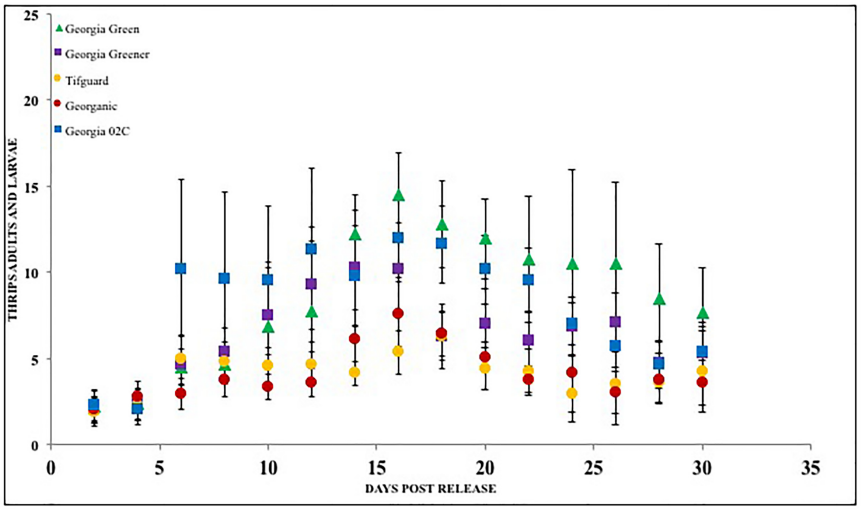

FIGURE 3 | Thrips larvae and adults counted on peanut foliage of various cultivars that are resistant and susceptible to *Tomato spotted wilt virus* under a choice situation. Georgia Green is considered as a TSWV-susceptible cultivar. Thrips counts were taken at two-day intervals since release, and counted for 3 weeks post initial thrips release.

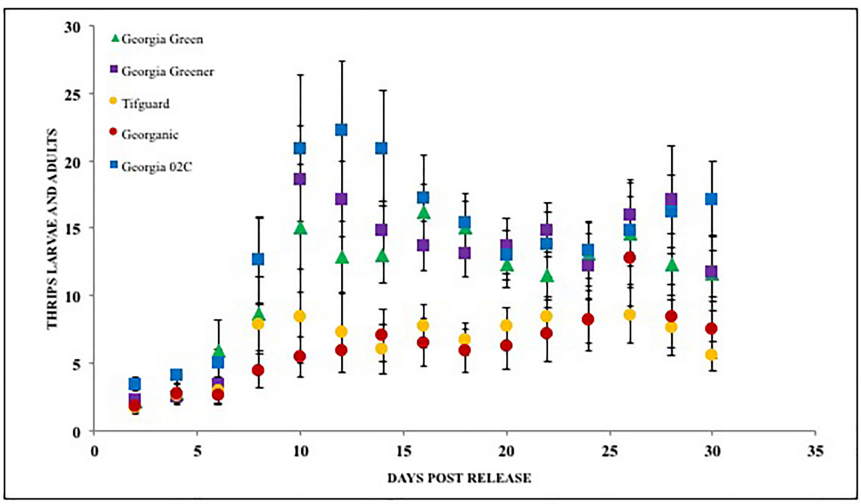

FIGURE 4 | Thrips larvae and adults counted on peanut foliage of various cultivars that are resistant and susceptible to *Tomato spotted wilt virus* under a no-choice situation. Georgia Green is considered as a TSWV-susceptible cultivar. Thrips counts were taken at two-day intervals since release, and counted for 3 weeks post initial thrips release.

(Holbrook et al., 2008; Shrestha et al., 2013). Nematode resistance in Tifguard is derived from a germplasm accession line "COAN"; it is not clear if the nematode resistance and thrips resistance are interlinked (Holbrook et al., 2008). Fitness experiments also revealed that the median thrips developmental time from egg to adult in Tifguard was lower than in any other cultivar evaluated (**Figure 5**; Shrestha et al., 2013). The reduced developmental time (by 2 days) of *F. fusca* on a resistant cultivar could be a strategy used by thrips to overcome unfavorable characteristics in that resistant genotype. This strategy is not unique to *F. fusca* and peanut; variations in thrips developmental time have been associated with *F. occidentalis* on thrips-resistant pepper (Maris et al., 2003b; Maharijaya et al., 2012). This phenomenon has also been observed in other insects (Leather et al., 1998).

Breeding efforts for thrips resistance in peanut has dwindled in the United States and continues on a minor scale in Asia Culbreath et al., 2003; Pappu et al., 2009; Culbreath and Srinivasan, 2011; Mandal et al., 2012; Srinivasan et al., 2017). A study on heritability of thrips resistance in Thailand found that there was a weak correlation between thrips resistance parameters and agronomic traits and predicted that these characters are independently inherited (Ekvised et al., 2006b). With increased adoption of TSWV and other virus-resistant cultivars in most peanut production areas, there is heightened

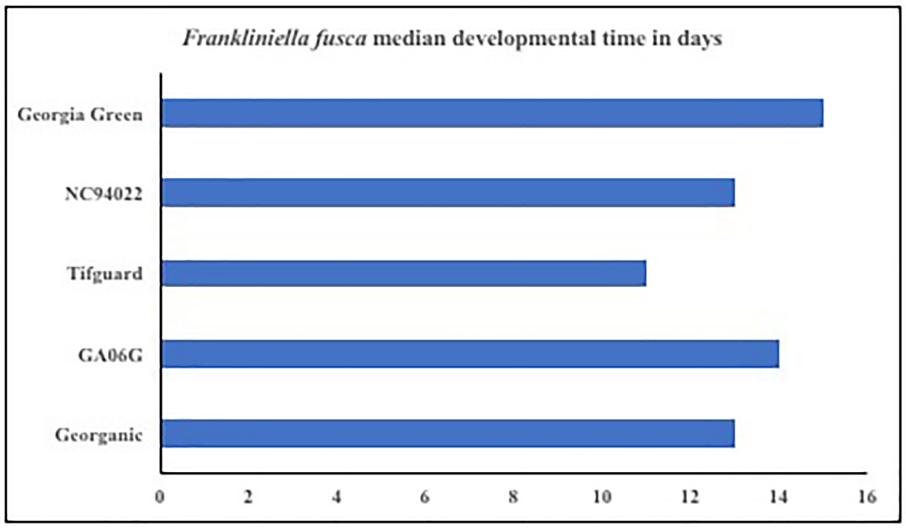

FIGURE 5 | Thrips developmental time on *Tomato spotted wilt virus* resistant and susceptible cultivars. Developmental time refers to the time take from egg to adulthood, and counts were taken once every 2 days. Thrips developmental time was monitored using Plexiglass arenas called Munger cages.

concern about evolution of resistance breaking virus strains (Sundaraj et al., 2014). Prior evidence of TSWV resistance breakdown in solanaceous crop hosts should serve as a warning (Qiu and Moyer, 1999), and all protections should be taken to prevent virus-resistance breakdown in peanut cultivars. Evidence presented above suggests that peanut cultivar-thrips interactions could influence virus transmission by thrips (Sundaraj et al., 2014). Results from Sundaraj et al. (2014) revealed that some of the TSWV-resistant cultivars such as Tifguard and Georganic accumulated less viral copies (up to 1/3rd less) and were infected at a lower percentage (up to 20%) than other TSWV resistant cultivars such as Georgia Green and Georgia Greener. These results suggest that cultivars that negatively affect thrips preference and/or fitness further suppress virus incidence and accumulation in cultivars that are already moderately resistant to TSWV, thereby providing additive effects. Therefore, it is logical to assume that stacking thrips resistance with virus resistance would reduce the amount of selection pressure imparted against the virus itself, delay the development of resistance breaking strains, and prolong the usefulness of these resistant cultivars that are heavily relied upon. In the United States in Georgia and North Carolina, more than 80% of the peanut acreage is planted with TSWV-resistant cultivars. Losing TSWV resistance in these cultivars would be devastating to peanut production. One way to prevent such a resistance breakdown would be to identify and incorporate thrips resistance in conjunction with TSWV resistance.

ROLE OF PEANUT WILD RELATIVES FOR RESISTANCE

Wild species of peanut have been sources of resistance for many pests and diseases in peanut. Resistance to thrips is no exception. The most remarkable being the production of varieties with resistance to root-knot nematode, leaf spot, and to rust, all derived from wild species (Simpson, 1991; Simpson et al., 2003; Khedikar et al., 2010; Chu et al., 2011). Several wild species have been associated with thrips resistance in peanut. Stalker and Campbell (1983) evaluated more than 30 wild species of *Arachis* over 3 years through field screening and identified several species that possess resistance to thrips. The number of thrips damaged leaves per plot on *A. batizocoi*, *Arachis correntina* (Benth.), *Arachis villosa* Benth., *Arachis spegazzini* Greg., *A. chacoense, Arachis cardenasii* Krapov., *A. stenosperma* Krapov. & W. C. Greg., *Arachis duranensis* Krapov, *Arachis rigonii* Krapov., *Arachis paraguariensis* Chodat & Hassl., *Arachis pusilla* Benth., and *Arachis repens* Handro, were two to ∼100 times less than on peanut cultivars. Similar evaluations were conducted in other places in the Southern United States as well as in Asia, and several genotypes were found to possess thrips resistance (Amin and Mohammed, 1980; Lynch, 1990). Diploid wild species such as *Arachis vallsii* Krapov. & W. C. Greg., *Arachis kempff-mercadoi* Krapov., W. C. Greg. & C. E. Simpson, *Arachis williamsii* Krapov. & W. C. Greg., *A. duranensis*, and amphidiploids such as *A. batizocoi* × *A. kempff-mercadoi, A. gregoryi* × *A. stenosperma, A. magna* × *A. cardenasii* also exhibited substantial levels of resistance to *E. flavens* in South America (Michelotto et al., 2017). Most of these evaluations were based on field screening, and the mechanism by which resistance is conferred in some of these wild species is not known. Few studies have examined the resistance contributing factors in these wild species. Epicuticular waxes containing n-alkanes from *A. batizocoi, A. chacoense, A. paraguariensis, A. glandulifera*, and *A. ipaensis* were speculated to confer resistance to thrips and other sucking insects, probably through non-preference/antixenosis (Yang et al., 1993). The same group of compounds, were also identified in *Arachis monticola* Krapov.

& Rigoni and *A. stenosperma*, through gas chromatography (de Souza et al., 2010). In Brazil, another study showed that various wild species exhibited field resistance to *E. flavens* (Michelotto et al., 2017). For a more comprehensive list of wild species with resistance to thrips and TSWV, please refer to review by Stalker (2017).

While there is evidence that wild species exhibit resistance against thrips, their ploidy level, makes it difficult to incorporate their resistance into cultivated tetraploid peanuts. Most wild species are diploids. Resistance introgression from wild species is achieved by crossing and subsequent backcrossing. This process is time consuming, and a major concern has been the reduction of level of resistance while backcrossing. *Arachis diogoi* is a diploid wild species that is known to possess resistance to numerous pests and pathogens including TSWV (Lyerly et al., 2002). Attempts were made to introgress TSWV resistance from *A. diogoi* to the Virginia type cultivar Gregory, using the hexaploid route (Stalker HT et al., 1979; **Figure 6**). Several progenies from those crosses accumulated fewer copies of the virus when compared with Gregory (Lai, 2015). Subsequently evaluations for thrips resistance were done using microcosmic Munger cages (Lai, 2015). Results reiterated that *A. diogoi* was highly resistant to thrips when compared with the cultivar Gregory; however, the genotypes from the intraspecific crosses were not anymore resistant than the recurrent parent Gregory (Lai, 2015). Thrips fitness parameters such as developmental time and fecundity were evaluated, and thrips developmental time was (20%) longer and its fecundity was lower on *A. diogoi* than on tetraploid Gregory. These results revealed direct effects on thrips biology suggesting that there could be antibiosis-based resistance against thrips.

A different route of introgression, called the tetraploid route involves crossing two wild species of complementary genomes and doubling the resultant sterile diploid hybrid with colchicine (Simpson, 1991; Leal-Bertioli et al., 2012, 2015). The resultant synthetic allotetraploid possesses all genes from both wild species and is compatible with peanut, being, therefore, useful for breeding. In Brazil, synthetics developed using this strategy were evaluated for field resistance to thrips, and three of them [(*A. batizocoi* × *A. kempff-mercadoi*)^{4x}, (*A. gregoryi* × *A. stenosperma*)^{4x}, and (*A. magna* × *A. cardenasii*)^{4x}] were found to have superior resistance than the cultivars tested, and are, therefore potentially useful for breeding (Michelotto et al., 2017).

TOOLS AND TECHNOLOGIES TO ENHANCE INTEGRATION OF THRIPS RESISTANCE IN PEANUT

Availability of novel tools and resources could facilitate a renewed interest in breeding for thrips resistant peanut cultivars, a few of them are discussed below.

Thrips Screening

Improvement in traditional and novel breeding approaches will result in hundreds and hundreds of peanut genotypes that need to be screened for thrips resistance. Until recently, most phenotypic evaluations for thrips resistance have focused on field screening for foliar feeding damage. Recently, a few studies have performed laboratory experiments to examine resistance to thrips in peanut (Shrestha et al., 2013; Sundaraj et al., 2014). These assays were used to evaluate both behavior such as preference and end-point parameters such as thrips feeding damage and biological fitness. These tests need to be conducted for weeks if not months, and could be laborious especially if there are many genotypes involved. Until recently, there was no automated high-throughput screening tool available for thrips, but have been available for hemipteran insects such as aphids. Recently, one such automated thrips-resistance screening tool has become available (Thoen et al., 2016). Thoen et al. (2016) used an automated video monitoring parallel choice test platform to screen ∼350 *Arabidopsis* accessions within a week. By using this assay, they could measure parameters that are obtained in traditional choice tests such as leaf damage and number of nymphs produced within a certain time. In addition to these usual parameters, they were also able to get information pertaining to thrips behavior on the host plant using a behavior analyzing software Ethovision® XT 10. The parameters estimated included time spent on the foliage, time not moving on the foliage, time not moving, distance moved, and movement velocity. Assessing these parameters for peanut genotype screening against thrips will provide more insights into thrips-peanut plant interactions facilitate faster and better screening for resistance. Electronic

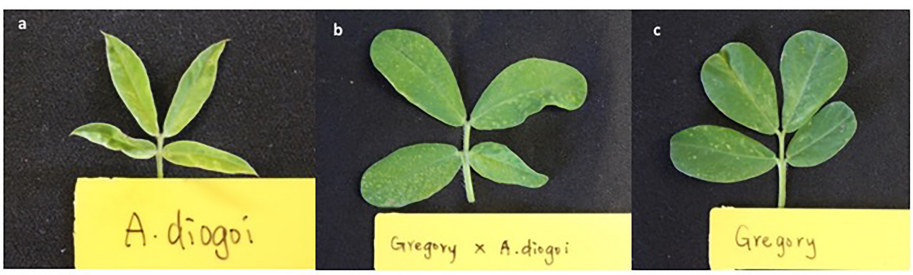

FIGURE 6 | Leaf phenotype of a diploid wild species, *Arachis diogoi* **(A)**, cultivated tetraploid *Arachis hypogaea* cultivar Gregory **(C)**, and a cross between the two **(B)**.

nose is another tool that could be used for quick and efficient screening for thrips resistance in peanut. This technique was found to be useful to discriminate Western flower thrips resistant genotypes from susceptible chrysanthemum genotypes effectively using headspace volatile profiles following simulated thrips feeding and thrips feeding bioassays (McKellar et al., 2005). The usefulness of such a technique needs to be explored in screening for thrips resistance in peanut.

Marker Assisted Selection and Single Nucleotide Polymorphisms

Genomic tools have been used for crop breeding to develop improved varieties. In particular, the application of trait-linked DNA markers to facilitate trait selection (Marker Assisted Selection – MAS) for crop improvement have proved successful for major crops. MAS uses trait-linked markers, instead of trait itself, has the advantage of eliminating plants with undesirable gene combinations, allowing the breeder to concentrate on a lower number of better lines. It has proven successful for cultivar improvement on many major crops. Peanut, however, has lagged behind the major crops due to the intrinsic narrow genetic variability delaying the identification of markers useful for selection. With a concerted effort by the Peanut Genome Initiative, an international group of scientists, genetic and genomic tools became available to the community and made marker assisted selection a reality in for peanut breeding (Stalker and Mozingo, 2001; Wang et al., 2018). Various marker systems have been developed throughout the years, following technology development (for a comprehensive review, see Wang et al., 2018). Recently, the most significant achievement was the sequence of the genome of the two wild progenitors of peanut, *A. duranensis* and *A. ipaensis* (Bertioli et al., 2016) and the development of a publicly available genotyping tool: a chip with nearly 60,000 SNPs (Clevenger et al., 2017; Pandey et al., 2017).

To date, only one study was conducted that showed correlation of a molecular marker with a peanut virus vector, *Aphis craccivora*, which transmits *Groundnut rosette virus* (Genus *Umbravirus*; Family *Tombusviridae*) (Herselman et al., 2004). In other crops (but not peanuts), markers have been associated with resistance to thrips (e.g., cowpea Lucas et al., 2012; pepper Maharijaya et al., 2015; tomato, Escobar-Bravo et al., 2017). For peanut, we still need to understand better the nature of thrips resistance and develop molecular markers.

Research on *Orthotospovirus* resistance, on the other hand, have seen more advances. For instance, TSWV host resistance has been established as a major factor to reduce disease risk, and therefore breeding for resistance has become a major goal in the breeding programs in the United States. Phenotypic selection for TSWV is inaccurate as field resistance expression varies significantly from year to year, depending on the environment (Culbreath et al., 2010; Tseng et al., 2016). *In vitro* transmission is also not considered reliable as it bypasses some possible plant defense responses (Zhao et al., 2018). Therefore, breeding for resistance to orthotospoviruses such as TSWV could greatly be facilitated by implementation of MAS in breeding programs. The first population, major QTLs were found in LG A01, and in the second study, 11 minor QTLs were found in different regions of the genome (Khera et al., 2016; Pandey et al., 2017). Work with another population, Florida EPTM^{13} x Georgia Valencia revealed major QTLs on LG01, and markers tightly linked to TSWV resistance (Tseng et al., 2016; Zhao et al., 2018). Association genetics using these markers on the United States minicore collection, however, did not show association between TSWV resistance and the markers (Li et al., 2018; Zhao et al., 2018). This is probably because the resistance allelic region to which the marker is associated is from a unique source, which is not present in the United States peanut mini core gene pool. This well documented study flags caution on the use of correct and validated molecular markers on genotypes with the same allele variants.

All the studies described above were conducted mainly using Simple Sequence Repeat (SSR) markers, which are costly, time consuming and not very abundant. New genotyping methods using Single Nucleotide Polymorphism (SNPs) are currently available (Bertioli et al., 2014; Clevenger et al., 2017; Pandey et al., 2017) and, together with more precise genotyping, are likely to speed the discovery of tightly linked markers and consequently, the implementation of MAS for in peanut breeding against thrips and viruses. MAS is not the only, or the main one, but it is undoubtedly, a very useful tool in the breeder's tool box.

Transgenic Thrips Resistance and RNAi

Peanut transformation was first reported by Brar et al. (1994) but no resistance tests were performed. Peanut has been transformed with *cry* genes derived from *Bacillus thuringiensis* (Berliner) that confer resistance to several insects, especially of the order Lepidoptera (Krishna et al., 2015). However, to our knowledge, no transgenic peanut has been tested or found to be resistant to thrips. In other crops, transgenesis has been successful to transfer resistance to thrips: resistance to western flower thrips has been engineered in potato plants (Outchkourov et al., 2004). Members of inhibitors of cysteine and aspartic proteases *viz*., stefin, potato cystatin, equistatin, and cystatin were fused and made into a functional unit and expressed in potato plants. The plants containing these multidomain proteins had fewer larvae and adults when compared with non-transgenic control plants (Outchkourov et al., 2004).

Resistance to orthotospoviruses in transgenic peanut has been achieved multiple times. In the United States and in India, both Valencia and runner genotypes were transformed with TSWV/GBNV N-gene or coat protein-based constructs, and pathogen-derived resistance was achieved in these transformants. Transformed peanut seedlings provided substantial field or *in vitro* resistance against TSWV and GBNV (Li et al., 1997; Magbanua et al., 2000; Yang et al., 2004; Rao et al., 2013). Mehta et al. (2013) also obtained transgenic peanut resistant to *Peanut stem necrosis virus*. So, the potential for viral resistance is very significant.

RNA interference has been deployed to provide resistance against a wide range of organisms including viruses and insects (Whyard et al., 2009; Gan et al., 2010; Zhang et al., 2013). In this process, the dsRNA pertaining to an invading organism is

degraded into short interfering (si) RNAs (20–23 nucleotides long) with the help of an enzyme complex, and subsequently the siRNAs block the mRNA translation (Fire et al., 1998; Fire, 2007). RNAi and its usefulness have not been demonstrated in peanuts against viruses and/or thrips. Nevertheless, the usefulness of RNAi has been demonstrated with *F. occidentalis*, wherein double stranded RNA (dsRNA) pertaining to an important enzyme (vacuolar ATP-Synthase or V-ATPase) was silenced, following which a significant reproductive fitness was observed in *F. occidentalis* (Badillo-Vargas et al., 2015). Advancements in thrips genomics and transcriptomics has led to identification of various developmental genes associated with western flower thrips and tobacco thrips in recent years (Schneweis et al., 2017; Shrestha et al., 2017). The usefulness of these genes should first be validated through *in vitro* assays, and further their *in planta* expression and effectiveness against thrips should be attempted to examine the usefulness of RNAi as a management option. Though consumer preference will keep transgenic peanut from coming to the marketplace anytime soon, available transgenic technology does offer some very interesting research possibilities. Technologies such as RNAi could be modified in certain ways to circumvent plant-based transgene delivery. In fact, the versatility of RNAi has been demonstrated in other cropping systems by mere exogenous application. For instance, Gogoi et al. (2017) observed that exogenous application of *Zucchini yellow mosaic* (Genus *Potyvirus*; Family *Potyviridae*) virus specific dsRNA molecules when applied to tomato plants were detected in the plants up to 14 days post application, and they were also detected in phloem feeding insects such as aphids and whiteflies up to 10 days post application (Gogoi et al., 2017). In another study, exogenous application of dsRNA specific to *Tobacco mosaic virus* (TMV) (Genus *Tobamovirus*; Family *Virgaviridae*) limited the systemic movement of TMV and conferred resistance to TMV inoculation (Konakalla et al., 2016). RNAi is very specific to the target organism and should greatly minimize non-target effects, and therefore could be highly useful and acceptable especially when an exogenous application strategy is considered. This RNAi strategy (exogenous application) will help alleviate the regulatory hurdles and consumer concerns associated with peanut or any other crop. However, this technology is just emerging, its suitability and its commercial viability for peanut production need to be developed and explored in greater depth. Nevertheless, it is promising and reassuring that thrips management in peanut may not have to exclusively rely on spraying insecticides in the long run. Stacking thrips resistance with pathogen resistance could have multifaceted benefits to peanut growers worldwide.

CONCLUSION

Even though thrips-transmitted viruses have garnered substantial attention in the last two decades, it is abundantly clear that thrips continue to cause damage by direct feeding in peanut production systems throughout the world. Host-resistance to orthotospoviruses is the main management option adopted in peanut, but it is used in conjunction with cultural practices and insecticide applications targeted at thrips. Unlike other crops such as tomato and pepper where the resistance is governed by a major gene, neither is resistance to orthotospoviruses complete nor is the mechanism of resistance known in peanut (Shrestha et al., 2013; Sundaraj et al., 2014). Studies suggest that virus resistance in peanut may not be an exclusive result of host–virus interactions, but could also be influenced by peanut–thrips interactions (do Nascimento et al., 2006; Sundaraj et al., 2014; Shrestha et al., 2015). *A. hypogaea*, an allotetraploid, has a very narrow genetic base and has little to no inherent resistance to thrips and/or viruses transmitted by them (Ratnaparkhe et al., 2011). Numerous studies have documented that wild species source of resistance for thrips and orthotospoviruses. Therefore, it may be possible to incorporate resistance to both thrips and the virus simultaneously. Differences in ploidy level and the subsequent dilution effect in resistance introgression due to backcrossing represent the biggest hurdle for using wild species in breeding for resistance. This obstacle could be overcome by using novel strategies such as development of synthetic tetraploids (Leal-Bertioli et al., 2015). Molecular markers could also be potentially used to link thrips and virus resistance (Bertioli et al., 2014). Stacking resistance against thrips and orthotospoviruses could reduce selection pressure on the virus itself, delay or prevent evolution of highly virulent strains, prolong the usefulness of resistant cultivars, reduce insecticide usage and allied non-target effects, and promote sustainability in peanut production.

AUTHOR CONTRIBUTIONS

RS developed the idea and contributed to writing. MA co-developed the idea and contributed to writing. P-CL conducted the experiments included in manuscript and contributed to writing. AC contributed to writing. ST was a collaborator on *Arachis diogoi* work and contributed to writing. SL-B contributed to writing.

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Structural and Chemical Profiles of *Myrcia splendens* (Myrtaceae) Leaves Under the Influence of the Galling *Nexothrips* sp. (Thysanoptera)

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Thysanoptera-induced galls commonly culminate in simple folding or rolling leaf gall morphotypes. Most of these galls are induced by members of the suborder Tubulifera, with only a few species of the suborder Terebrantia being reported as gall inducers. The Terebrantia, as most of the gall inducers, manipulates the host plant cellular communication system, and induces anatomical and biochemical changes in its host plant. In an effort to keep its homeostasis, the host plant reacts to the stimuli of the galling insect and triggers chemical signaling processes. In contrast to free-living herbivores, the signaling processes involving galling herbivores and their host plants are practically unknown. Current investigation was performed into two steps: first, we set the structural profile of non-galled and galled leaves, and looked forward to find potential alterations due to gall induction by an undescribed species of *Nexothrips* (suborder Terebrantia) on *Myrcia splendens*. Once oil glands had been altered in size and number, the second step was the investigation of the chemical profile of three tissue samples: (1) non-galled leaves of a control individual, (2) non-galled leaves of galled plants, and (3) galls. This third sample was divided into two groups: (3.1) galls from which the inducing thrips were manually removed and (3.2) galls macerated with the inducing thrips inside. The chemical profile was performed by gas chromatography/ mass spectrometric detector after headspace solid-phase extraction. The galling activity of the *Nexothrips* sp. on *M. splendens* culminates in mesophyll compactness interspersed to diminutive hypersensitive spots, development of air cavities, and the increase in size and number of the secretory glands. Seventy-two compounds were completely identified in the volatile profile of the three samples, from which, sesquiterpenes and aldehydes, pertaining to the "green leaf volatile" (GLVs) class, are the most abundant. The rare event of gall induction by a Terebrantia revealed discrete alterations toward leaf rolling, and indicated

quantitative differences related to the plant bioactivity manipulated by the galling thrips. Also, the content of methyl salicylate has varied and has been considered a potential biomarker of plant resistance stimulated as a long-distance effect on *M. splendens* individuals.

Keywords: methyl salicylate, *Myrcia*-thrips system, plant–insect interactions, rolling galls, volatiles

INTRODUCTION

Among the galling insects, Thysanoptera, commonly known as thrips, are suckers and induce their galls (Meyer, 1987) by means of chemical and/or mechanical stimuli, and alter the development of their host plant tissues (Mani, 1964; Hori, 1992). Galls result from the interaction between a galling organism and its host plant, and demand a high complex and intimate interaction between the associated species (Shorthouse et al., 2005; Raman, 2007). The galling organism manipulates the cellular communication system of the host plant by suppressing its defenses (Oates et al., 2016), and induces anatomical and biochemical changes in the host plant (Raman et al., 2005). Previous studies show that galling insects manipulate plant cells and tissues by the interaction between secondary metabolism and phytohormones (Bedetti et al., 2014). Also, gall induction impairs redox homeostasis, and the accumulation of reactive oxygen species in cell walls is responsible for cell wall loosening and consequent cell redifferentiation and hypertrophy (Isaias et al., 2015). Both of these processes are commonly observed during gall growth and development. Nevertheless, how the insect is able to achieve such an extraordinary level of control over its host plant is perhaps the most intriguing question surrounding plant-galling insect interaction (Oates et al., 2016), and has not been fully described yet.

Along the process of gall induction and the establishment of the galling organism within plant tissues, the plant reacts to the presence of the parasite and chemical signaling mechanisms initiate. Such phenomenon of chemical signaling on plant-herbivore interactions has been widely explored for free-living insects (Rosenthal and Berenbaum, 1992; Dicke et al., 1993), but the signaling mediated by volatile secondary metabolites between galling insects and their host plants is practically unknown (Damasceno et al., 2010).

Gall induction may stimulate the neo-synthesis of secondary metabolites (Oliveira et al., 2006; Guedes et al., 2016) or the standard synthesis of both primary and secondary metabolites may be maintained, but their accumulation is translocated to specific gall tissue compartments (Carneiro et al., 2014; Bragança et al., 2017). Host plants with high potential for the production of volatiles, such as *Myrcia splendens* (Myrtaceae), may come up with novelties regarding the chemical profile of primary metabolites and their involvement in biotic association. The volatile content of the oil glands of *M. splendens* leaves has 95% of sesquiterpenes (Cole et al., 2008; Nakamura et al., 2010), mostly composed of hydrocarbons and oxygenated sesquiterpenes (Cole et al., 2008), this volatile profile can be altered after gall induction.

Our study focuses on a rolling gall morphotype induced by a tiny Thripinae, an undescribed species of *Nexothrips* (suborder Terebrantia) on *M. splendens* (Sw) DC, and it aimed to (1) characterize gall anatomical structure to elucidate how the host plant cell an tissue responses lead to the rolling of leaf lamina; (2) quantify the number and area of the essential oil-producing glands in order to determine whether the gall induction alters the host leaf potential for the production of volatiles; and (3) trace the composition of the volatile compounds emitted by non-galled leaves of a plant totally free of galls (the control individual), by non-galled leaves of galled plants, and by *Nexothrips* sp. galls to detect possible biomarkers of the biotic stress related to gall induction and establishment.

MATERIALS AND METHODS

Non-galled leaves and rolling galls were collected from a population of *M. splendens* (Sw.) DC. in Serra Verde State Park (Parque Estadual Serra Verde, PESV), Belo Horizonte, Minas Gerais, Brazil (19°47′21.8″S 43°57′34.4″W). The characterization of the gall morphotype followed Isaias et al. (2013). Individuals of *M. splendens* ($n = 20$) were tagged, and monitored monthly from February 2015 to March 2016. The gall cycle and the diagnosis of the galled and non-galled condition of the individuals, as well as the occurrence and frequency of the galls were analyzed.

Anatomical Analysis

For anatomical observations, non-galled leaves and mature rolling galls ($n \geq 5$) were fixed in FAA (formalin, acetic acid, 50% ethanol, 1:1:18) (Johansen, 1940), dehydrated in an *n*-buthanolic series and embedded in Paraplast® (Kraus and Arduin, 1997). The material was sectioned (12 μm) in a rotatory microtome (Leica 2035 BIOCUT®), deparaffinized, and stained in astra blue-safranin 9:1 (v/v) (Bukatsch, 1972, modified to 0.5%). The slides were mounted with varnish Acrilex® (Paiva et al., 2006), and the images were obtained with a photomicroscope (Leica ICC50 HP®).

Scanning Electron Microscopy (SEM)

Non-galled leaves and rolling galls fixed in FAA (Johansen, 1940) were dehydrated in an ethanolic series (Johansen, 1940), critical point dried, mounted on stubs, and covered with 15 nm of gold (Balzers SCD 050) (O'Brien and McCully, 1981). The samples were observed in a scanning electron microscope (JEOL JSM - 6360LV) in the Center of Microscopy at the Universidade

Abbreviations: cLRG, clean-leaf rolling galls, where cLRG stands for clean leaf galls; Ctrl, control individual; LRGwT, leaf rolling galls without thrips, where "wT" stands for "with thrips"; NGL, non-galled leaves.

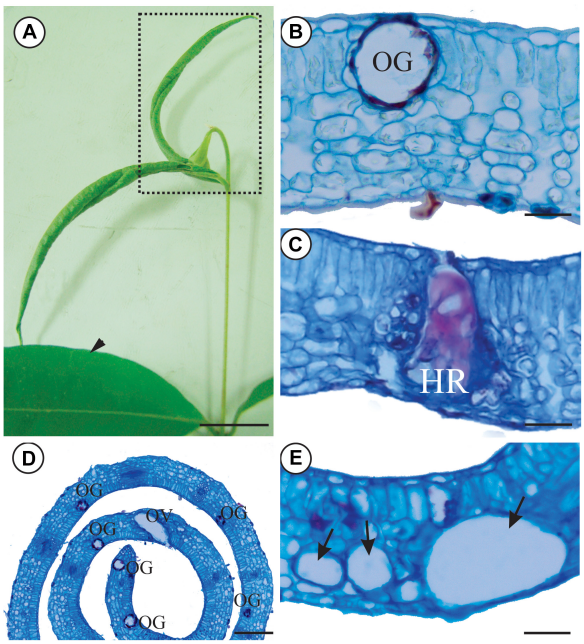

FIGURE 1 | Non-galled leaves and galls induced on leaves of *Myrcia splendens* by an undescribed species of *Nexothrips* (suborder Terebrantia) **(A)** Non-galled leaves and stem branch with leaf rolling galls. **(B)** Anatomy of non-galled leaves, evidencing the mesophyll and oil glands. **(C)** Gall mesophyll, evidencing the hypersensitive reaction in response to the inducer's feeding activity. **(D)** Anatomy of a gall, evidencing the increased number of secretory cavities. **(E)** Gall mesophyll, evidencing the reduction of intercellular spaces and air cavities. Arrowhead: Non-galled leaves, Dashed: Rolling gall, OG: Oil gland, OV: Oviposition, HR: Hypersensitive area, Arrow: Air cavities Scale bars: **A**: 2 cm, **B**,**C**,**E**: 50 μm; **D**: 200 μm.

Federal de Minas Gerais, Belo Horizonte, Minas Gerais state, Brazil[1].

Quantitative Analysis of Glands

For the analysis of oil glands, non-galled leaves (NGL) and mature rolling galls (n = 20 per sample) were clarified in 50% potassium hydroxide until complete bleaching, washed in water (three times), stained with 1% safranin in 95% ethanol, and dehydrated in ethanolic series (Bersier and Bocquet, 1960). The slides were mounted with varnish Acrilex® (Paiva et al., 2006), and the images were obtained with a photomicroscope (Leica ICC50 HP®). The number of glands per area (8.00/mm^2) was counted, and the relative area of the glands on non-galled leaves and rolling galls was measured using the AxioVision 7.4 software (Carl Zeiss® Microscopy GmbH, Jena, Germany). Statistical analyzes were performed using the SigmaStat® software, and t-test was applied considering $p \leq 0.05$.

Profile of Volatile Compounds by HS-SPME-GC/MS

Volatile compounds were analyzed in (1) non-galled leaves (n = 5) from an individual (n = 1), which did not have galls along the year (the control individual), and (2) non-galled leaves (n = 5), and (3) leaf rolling galls (n = 5) from young individuals (n = 14) of *M. splendens* in vegetative phenophase in July 2016. The samples were collected and immediately placed under dry ice, in the field. They were macerated in liquid nitrogen, powdered, and divided into 3 to 5 vials of 20 ml (25 mg/vial), in the laboratory. The vials were immediately stored in ultrafreezer (−80°C) until analysis.

The samples were divided into four composites, as follows: (1) control individual (Ctrl) – composite of all leaves obtained from the non-galled individual; (2) non-galled leaves (NGL) – composite of the leaves of the fourteen individuals; and (3) leaf-rolling galls – composite of the galls of the fourteen individuals, divided into two groups: (3.1) galls from which the inducing thrips were manually removed (cLRG), for the detection of volatiles exclusive to plant tissues, and (3.2) galls macerated with the inducing thrips inside (LRGwT) for detection of volatile compounds exclusive to the inducing thrips.

Volatile profiles were obtained by extracting the compounds from the samples employing HS-SPME with subsequent analyses by gas chromatography coupled to mass spectrometry (Souza Silva et al., 2017). Briefly, 20 mL vial containing 0.025 g of powdered plant material was taken from the ultrafreezer just before extraction. Immediately, 2 μL of internal standard (aqueous solution of 1,4-cineole at 100 ng μL^{-1}) was added to the sample with the use of a 10 μL Hamilton syringe pierced through the vial cap septum, without opening the vial cap. Subsequently, each sample vial was pre-incubated on a home-made heating block at 30 ± 2°C, without agitation, for 30 min prior to exposure of the SPME fiber to the vial HS. After 15 min of extraction, the fiber was desorbed into the GC injector at 240 °C for 15 min. Upon desorption of the volatile compounds, the samples were analyzed by gas chromatography coupled to mass spectrometry (GC/MS) with a Shimadzu 2010Q-Plus GC/MS equipment equipped with a DB-5 column (30 m × 0.25 mm × 0.25 μm).

Peaks in the total ion current chromatograms (TICC) were tentatively identified comparing the experimentally acquired mass spectra and the NIST08 mass spectral library, with minimal mass spectra match threshold of 80%. In addition, retention indices (RI) were determined using data obtained from an n-alkane solution (C8–C28), and compared to RI reported in the literature. For sesquiterpenes, whenever an appropriate match between mass spectra library hit and retention index was not found, only the family group was designated, according to their mass spectra fragmentation. Relative amounts, as percentages of each component, were achieved by peak area normalization measured without any correction factor. The response obtained for the internal standard 1,4-cineole, measured as area counts, was utilized to monitor possible drifts in instrumental response (Adams, 2001, 2007; **Supplementary Material**).

The final data matrix containing the relative percentages of each identified peak was submitted to statistical analysis. All analyses were performed assuming 95% level of confidence (α = 0.05). Since the chromatographic response (area) for each compound had already been normalized, no further data normalization or transformation was performed, and scaling of the data was performed using Pareto scaling (mean-centered and divided by the square root of standard deviation of each

[1]http://www.microscopia.ufmg.br

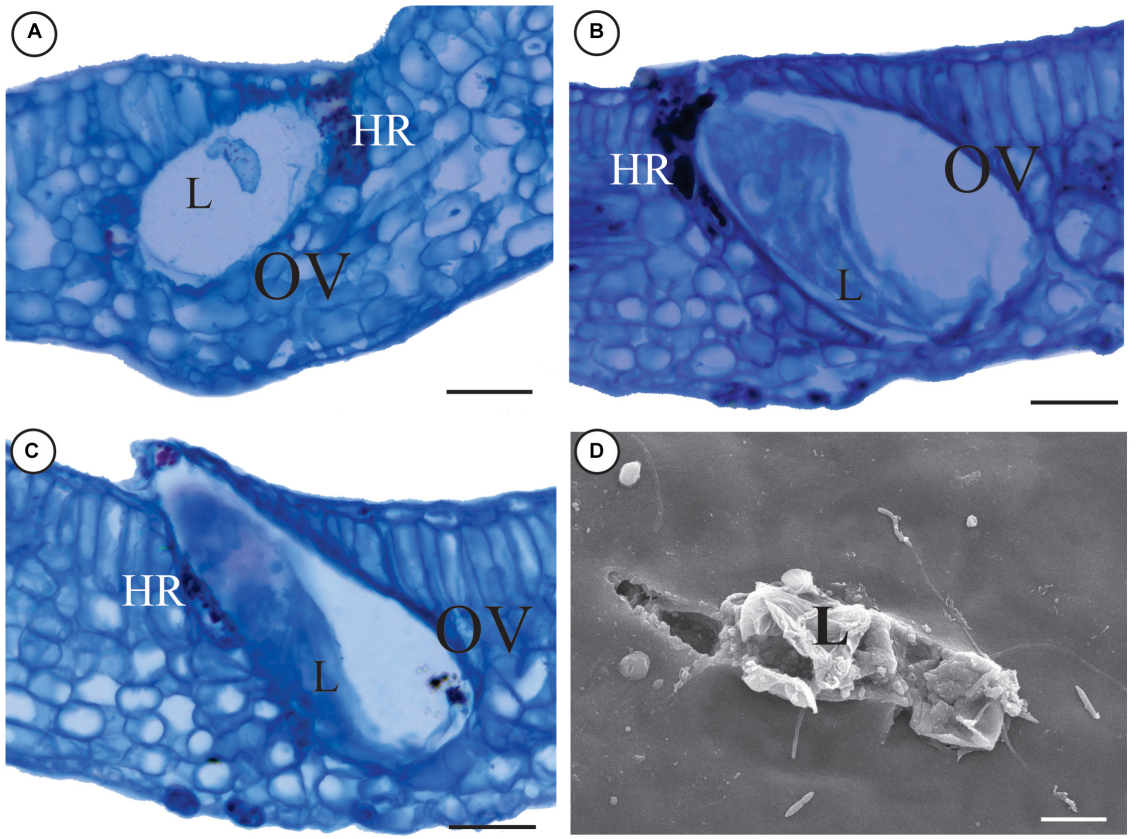

FIGURE 2 | Oviposition inside the tissues of a *M. splendens* rolling gall induced by an undescribed species of *Nexothrips* (suborder Terebrantia). **(A)** Initial development of the larva inside gall tissues. **(B)** Development of the larva. **(C,D)** Larvae hatching out of gall tissues. **(C)** Anatomy of a gall, evidencing hatching out of the leaf tissue. **(D)** MEV slides evidencing the hatching. L: Larva, OV: oviposition, HR: Hypersensitive area. Scale bars: **A–C**: 50 μm; **D**: 10 μm.

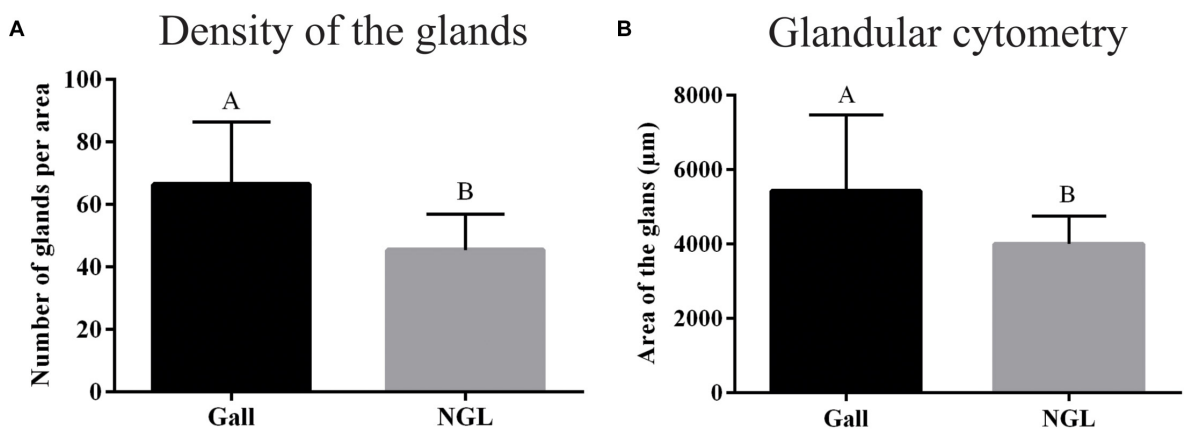

FIGURE 3 | Quantitative analysis of glands. **(A)** Density of the glands per mm^2. **(B)** Glands area. NGL: Non-galled leaves.

variable) in order to give all variables equal weight regardless their absolute value. This procedure is especially useful to generate a sound statistical analysis since the levels of the volatile compounds found may be of very different orders of magnitude. After data pre-processing, Principal Component Analysis (PCA), heat map with hierarchical clustering, and Partial Least Squares Discriminant Analysis (PLS-DA) were performed using web-based metabolomic data processing tool MetaboAnalyst[2]. PCA was used to detect intrinsic clusters and outliers within the data set, while PLS-DA maximized class discrimination.

[2]http://www.metaboanalyst.ca

TABLE 1 | Metabolites tentatively and positively identified in the headspace of galled and non-galled leaves of *Myrcia splendens*.

#	Analyte	Class	RT (min)	RI_{exp}	RI_{Lit}
1	Ethanol	Alcohol	1.864	636	448
2	1-Penten-3-ol	Alcohol (GLV)	3.894	690	684
3	1-Penten-3-one	Ketone (GLV)	3.945	691	686
4	Pentanal	Aldehyde (GLV)	4.203	698	699
5	3-Buten-1-ol, 3-methyl-	Alcohol (GLV)	5.201	724	724
6	1-Butanol, 3-methyl-	Alcohol (GLV)	5.432	730	733
7	3-Penten-2-one	Ketone (GLV)	5.525	733	733
8	2-Pentenal, (E)-	Aldehyde (GLV)	5.666	737	740
9	2-Pentenal, (Z)-	Aldehyde (GLV)	6.017	746	740
10	1-Pentanol	Alcohol (GLV)	6.715	763	763
11	2-Penten-1-ol, (Z)-	Alcohol (GLV)	6.819	767	767
12	3-Hexenal, (Z)-	Aldehyde (GLV)	7.958	797	796
13	Hexanal	Aldehyde (GLV)	8.02	799	800
14	2-Hexenal, (E)-	Aldehyde (GLV)	10.968	855	854
15	3-Hexen-1-ol, (Z)-	Alcohol (GLV)	11.114	858	857
16	2-Hexen-1-ol, (E)-	Alcohol (GLV)	11.597	866	863
17	1-Hexanol	Alcohol (GLV)	11.718	869	871
18	Heptanal	Aldehyde (GLV)	13.317	899	901
19	α-Thujene	Monoterpene	14.786	925	924
20	α-Pinene	Monoterpene	15.106	931	934
21	Benzaldehyde	Aromatic	16.362	952	960
22	4-Hexen-3-one, 5-methyl-	Ketone	16.499	955	961
23	β-Pinene	Monoterpene	17.491	972	974
24	1-Octen-3-one	Ketone	17.657	975	975
25	1-Octen-3-ol	Alcohol	17.827	978	980
26	5-Hepten-2-one, 6-methyl-	Ketone	18.177	984	985
27	2,4-Heptadienal, (E,E)-	Aldehyde	18.613	992	1011
28	D-Limonene	Monoterpene	20.48	1026	1027
29	Benzyl Alcohol	Aromatic	20.696	1030	1032
30	Benzeneacetaldehyde	Aromatic	21.104	1037	1040
31	β-Ocimene	Monoterpene	21.676	1047	1050
32	Linalool oxide	Monoterpene	22.911	1070	1069
33	α-Terpinolene	Monoterpene	23.783	1086	1088
34	Linalool	Monoterpene	24.431	1097	1097
35	Nonanal	Aldehyde	24.648	1101	1101
36	Phenylethyl Alcohol	Aromatic	24.65	1108	1106
37	*cis*-Pinocarveol	Monoterpene	26.384	1135	1136
38	2,4,6-Octatriene, 2,6-dimethyl-, (E,Z)-	Monoterpene	26.663	1128	1131
39	α-Terpinen-4-ol	Monoterpene	28.432	1174	1178
40	Naphthalene	Aromatic	28.551	1176	1169
41	Butanoic acid, 3-hexenyl ester, (Z)-	Ester	28.999	1185	1184
42	Methyl salicylate	Ester	29.234	1190	1193
43	Octanoic acid, ethyl ester	Ester	29.585	1193	1195
44	Decanal	Aldehyde	29.928	1203	1205
45	Dodecane	Hydrocarbon	29.928	1200	1200
46	β-Cyclocitral	Monoterpene	30.594	1217	1217
47	Citronellic acid, methyl ester	Ester	32.691	1260	1261
48	2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester	Ester	35.709	1323	1323
49	**Sesquiterpene 1**	Sesquiterpene	35.975	1329	n/a
50	**Sesquiterpene 2**	Sesquiterpene	36.113	1331	n/a
51	δ-Elemene	Sesquiterpene	36.504	1340	1339

(Continued)

TABLE 1 | Continued

#	Analyte	Class	RT (min)	RI_{exp}	RI_{Lit}
52	α-Cubebene	Sesquiterpene	37.048	1352	1352
53	Cyclosativene	Sesquiterpene	37.761	1367	1368
54	α-Ylangene	Sesquiterpene	38.041	1373	1373
55	α-Copaene	Sesquiterpene	38.353	1380	1380
56	β-Bourbonene	Sesquiterpene	38.689	1387	1388
57	**Sesquiterpene 3**	Sesquiterpene	38.903	1392	n/a
58	β-Elemene	Sesquiterpene	39.035	1395	1394
59	**Sesquiterpene 4**	Sesquiterpene	39.179	1398	n/a
60	α-Gurjenene	Sesquiterpene	39.796	1412	1413
61	β-Caryophyllene	Sesquiterpene	40.339	1424	1424
62	γ-Elemene	Sesquiterpene	40.721	1433	1433
63	α-Caryophyllene	Sesquiterpene	41.076	1441	1443
64	**Sesquiterpene 5**	Sesquiterpene	41.225	1445	n/a
65	**Sesquiterpene 6**	Sesquiterpene	41.884	1460	n/a
66	γ-Muurolene	Sesquiterpene	42.189	1467	1465
67	δ-Muurolene	Sesquiterpene	42.274	1468	1468
68	α-Muurolene	Sesquiterpene	43.432	1499	1498
69	Germacrene D	Sesquiterpene	43.506	1496	1492
70	γ-Cadinene	Sesquiterpene	43.929	1508	1509
71	σ-Cadinene	Sesquiterpene	44.455	1523	1523
72	δ-Cadinene	Sesquiterpene	44.819	1533	1533
73	**Sesquiterpene 7**	Sesquiterpene	45.069	1540	n/a
74	**Sesquiterpene 8**	Sesquiterpene	45.21	1544	n/a
75	3,7(11)-Selinadiene	Sesquiterpene	45.285	1546	1545
76	Germacrene B	Sesquiterpene	45.441	1551	1553
77	**Sesquiterpene 9**	Sesquiterpene	46.098	1569	n/a
78	Caryophyllene oxide	Sesquiterpene	47.063	1597	1599
79	**Sesquiterpene 10**	Sesquiterpene	47.44	1609	n/a
80	**Sesquiterpene 11**	Sesquiterpene	47.668	1617	n/a
81	τ-Cadinol	Sesquiterpene	48.564	1647	1643
82	α-Cadinol	Sesquiterpene	48.949	1660	1657
83	**Sesquiterpene 12**	Sesquiterpene	50.169	1702	n/a
84	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (E,E)	Sesquiterpene	50.712	1723	1722

RESULTS

Anatomical Analysis

The leaves of *M. splendens* in non-galled condition are green (**Figure 1A**), dorsiventral and hypostomatic. The epidermis is uniseriate, the mesophyll has a 1- layered palisade parenchyma and 7–10 layered spongy parenchyma. Secretory cavities occur all over the mesophyll, and in the midrib cortex (**Figure 1B**).

The galls are green with the rolling movement of leaf lamina upward, along both sides of the midrib (**Figure 1A**). Galling thrips in several stages of life occur inside the galls, but parasitoids and predators are rare. Hypersensitive reactive spots form in response to the feeding activity of the thrips in the epidermis and mesophyll (**Figure 1C**). The number of secretory cavities increases and the cells of the epithelium are hypertrophied (**Figure 1D**). The spongy parenchyma is 5–7 layered and compact due to a reduction of intercellular spaces. Large air cavities are evident within mesophyll cells (**Figure 1E**). The oviposition takes place inside leaf tissues, where the larvae develop. Later on, the immature thrips hatch out of the tissues (**Figures 2A–D**) and start feeding.

Quantitative Analysis of Glands

Both the number and the area of the oil glands are statistically different between non-galled leaves and galls. The density of the glands per mm^2 was higher in galls than in non-galled leaves ($p < 0.0001$) (**Figure 3A**), and the area of the glands was larger and more variable ($p = 0.0227$) in galls than in non-galled leaves (**Figure 3B**).

For the quantitative analysis of the oil glands, the ANOVA tests were applied for parametric data, and Kruskal test for non-parametric data.

Volatile Profile Characterization by HS-SPME-GC/MS

A total of 84 compounds were aligned across all samples. Twelve compounds were tentatively identified only as sesquiterpenes due to their characteristic mass spectra fragmentation pattern, inconsistencies between NIST library hit, experimental retention indexes and literature retention indexes. The complete identification of 72 metabolites was possible according to their retention indexes (**Table 1**). The 36 sesquiterpenes

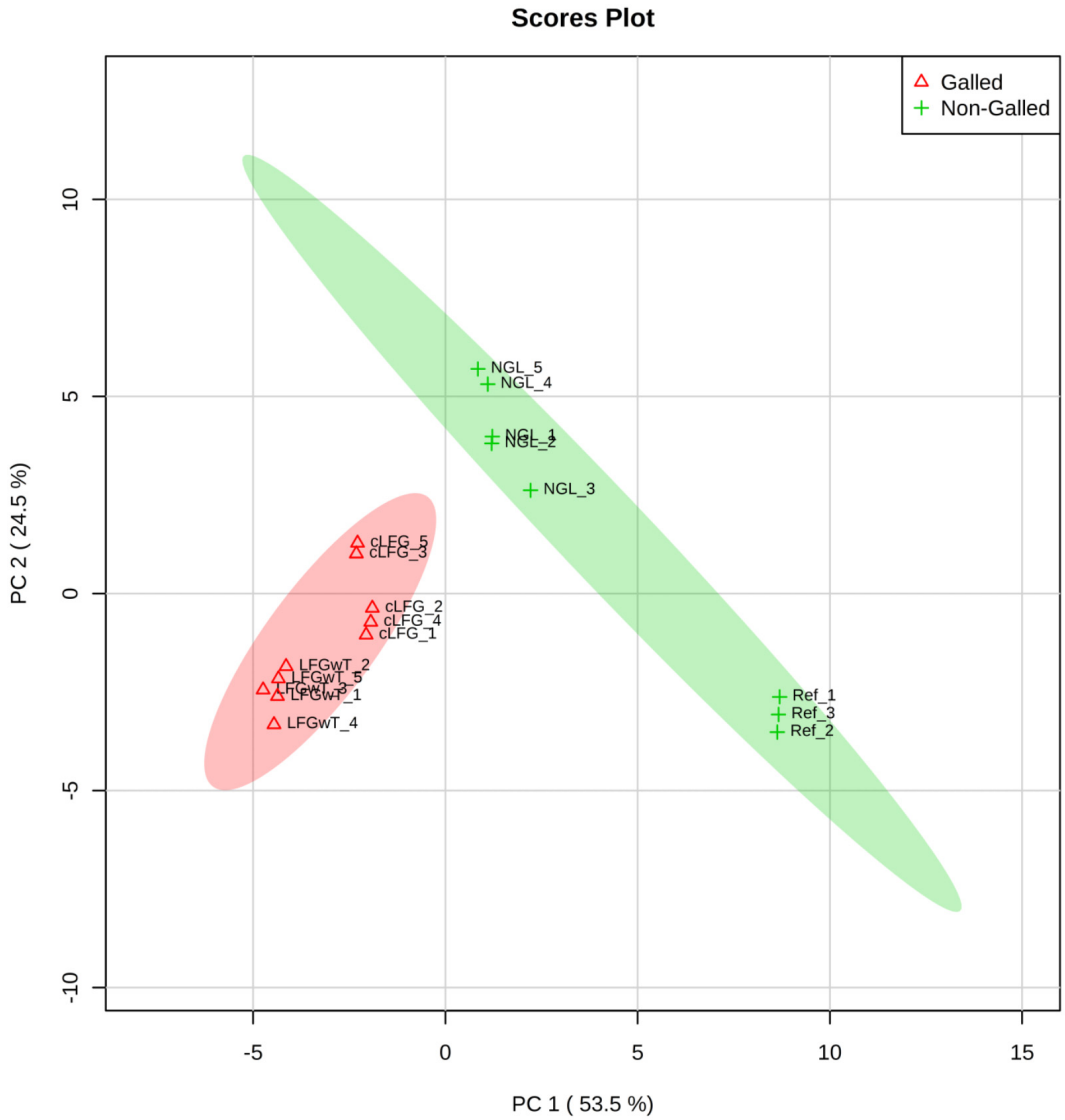

FIGURE 4 | Profile of the distribution of VOCs in non-galled leaves and galls. Plot depicts the distribution of VOC profiles of leaves (non-galled samples, including NGL and Ctrl) and galls (including LRGwT and cLRG samples) over the PCA score plot defined by the first two principal components.

constitute the major class of the identified compounds, followed by monoterpenes (12), alcohols (10), aldehydes (10), esters (5), aromatic compounds (5), ketones (3), and hydrocarbon (1). Green leaf volatiles (GLVs), comprising low molecular weight oxygenated compounds, amounted to 17 compounds.

The profiles of volatile organic compounds (VOC) obtained from the four composites could be separated into two classes by the PCA analysis: (1) leaves of the non-galled individual, comprising the control group (Ctrl) together with the non-galled leaves (NGL), and (2) galls with thrips (LRGwT) and galls without thrips (cLRG) (**Figure 4**).

Even though the four groups could be successfully separated into two classes (PCs 1 and 2 explain 78% of variance in the data), the 25 most discriminating VOCs show that there is a clear distinction between the profile of the groups within a class, i.e., between the Ctrl and the NGL samples, and between the cLRG and the LRGwT (**Figure 5**). The main compounds responsible for the separation of the groups are 2-*E*-hexenal (#14), sesquiterpene 5 (#64), β-caryophyllene (#61), and β-bourborene (#56) that are upregulated in the leaves of non-galled samples, and methyl salicylate (#42), 3-*Z*-hexen-1-ol (#15), 2-*E*-hexen-1-ol (#16) and 1-hexanol (#17), which are upregulated in the samples of galls (**Figure 6**). In fact, there were significant distinguishing features between the control sample (Ctrl) and the non-galled leaves of galled individuals (**Figure 7**). Contrastingly, to the higher levels of sesquiterpenes in the samples of the control individual, there were increased levels of aldehydes in the non-galled samples of galled individuals, mainly of the C6 aldehydes from GLV class.

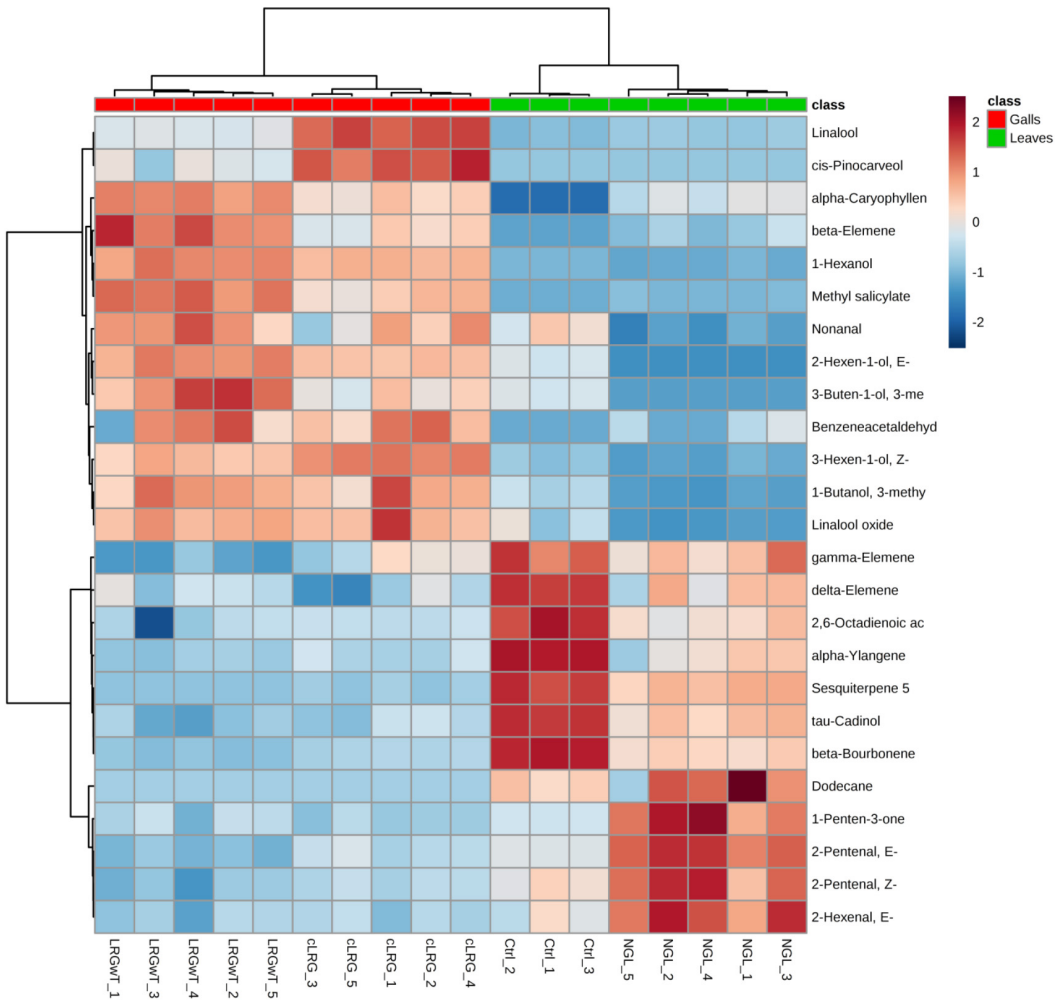

FIGURE 5 | Profile of the distribution of VOCs in non-galled leaves and galls. Plot shows the heat map distribution of the 25 most discriminating VOCs identified by SPME-GC-MS analysis. Color key indicates metabolite expression value, blue: Lowest, red: highest.

In a similar pattern, benzaldehyde (#21) and methyl salicylate (#42) appeared to be upregulated in the LRGwT (galls with inducing thrips inside) composite as compared to the cLRG. Moreover, sesquiterpenes, such as δ-muurolene (#67), β-elemene (#58) and α-caryophyllene (#63), decreased in the composites of galls from which the galling *Nexothrips* were removed (cLRG) (**Figure 8**).

DISCUSSION

The first step in current investigation, i.e., the structural profile of non-galled leaves and galls, revealed that the development of the leaf rolling gall morphotype on *M. splendens* results in discrete alterations in epidermis, and in conspicuous alterations in palisade and spongy parenchymas. These alterations lead to a complete rolling of leaf lamina upward.

The mosaic of tissue alterations of *Nexothrips*-induced galls on *M. splendens* with its peculiar air cavities and compactness of spongy parenchyma is not an exclusive feature, for it has been previously described for galls of *Aneurothrips priesneri* Bhatti on *Cordia obliqua* Willd. This pattern seems to be consequence of cell displacement due to the stretching and folding/rolling of leaf lamina throughout gall development (Ananthakrishnan and Raman, 1989). Even though the origin of the stimuli for gall induction remains unknown (Mound and Kranz, 1997), the insect saliva seems to be involved in gall induction and development of thrips-induced galls (Ananthakrishnan and Raman, 1989). Nevertheless, the role of oviposition has not been considered in gall induction of most galling Thysanoptera, since females of suborder Tubulifera lay their eggs externally to plant tissues. Comparatively, the ovipositor of most female Terebrantia is well-developed, and eggs are inserted in a cavity within mesophyll cells. Although this endophytic process has been shown by almost all phytophagous Terebrantia, only three thrips species are reported as capable of causing plant cell responses and gall induction through oviposition (Ananthakrishnan, 1978a,b; Tree and Mound, 2009).

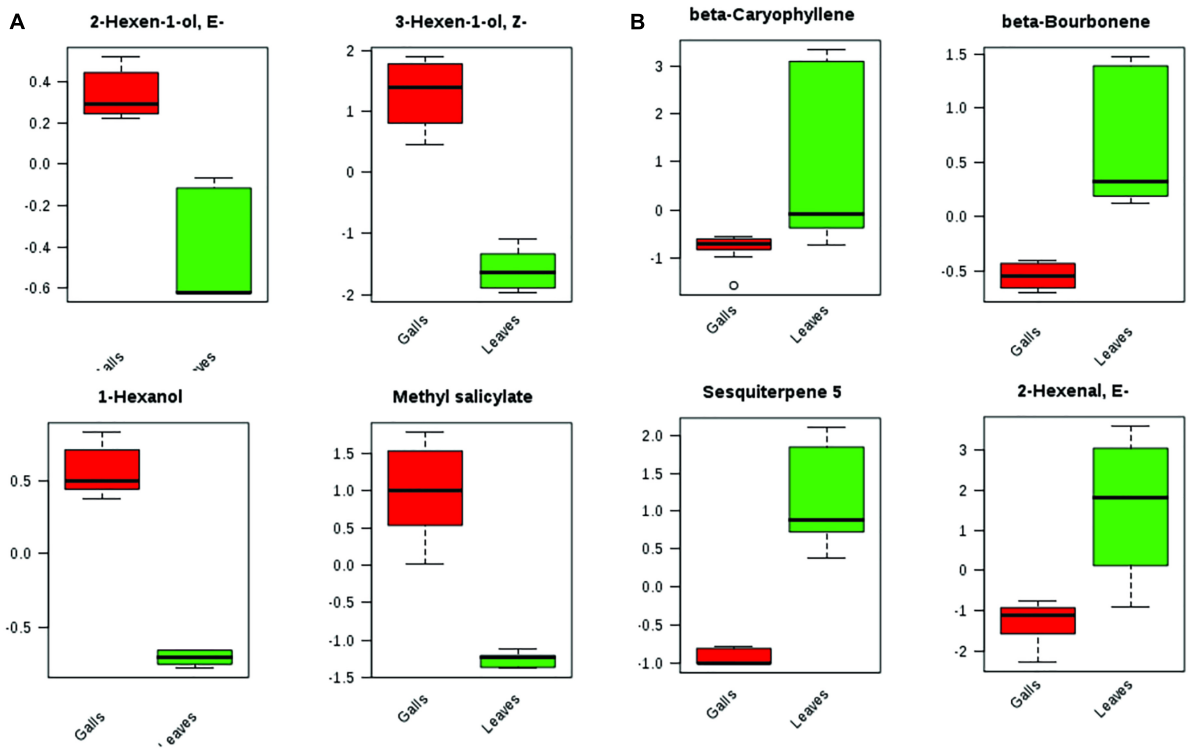

FIGURE 6 | Box plots of top metabolites that significantly differed between leaves and galls. **(A)** VOCs up regulated in gall samples. **(B)** VOCs down regulated in gall samples.

The females of *Nexothrips* oviposit on mature leaves of *M. splendens*, which should be a strategy to avoid the crushing of the egg cavities by the intense cell proliferation common in young developing leaves (Rivnay, 1935). Besides crushing the cavities, the hyperplasia of young leaf tissues should end up pushing eggs out of the leaf, which should not favor the establishment of the galling thrips and gall development. The ability of inducing galls on mature leaves rather than exclusively on young leaves, as is common for most galling insects, guarantees to the individuals of *Nexothrips* sp. a high availability of sites for completing their life cycles. However, the more differentiated is a cell, the less responsive it is. So, inducing galls on mature leaves may impose constraints for the differentiation of high-specialized cells, such as those of true nutritive tissues (Ferreira et al., 2017). Nutritive cells may occur in some Thysanoptera galls (Ananthakrishnan and Raman, 1989), but they are absent in the galls induced by *Nexothrips* studied here. The absence of a true nutritive tissue indicates that the galling *Nexothrips* should feed on epidermal cell contents. Also, the reduced hypersensitive sites next to the oil glands in the galls on *M. splendens* indicate that the *Nexothrips* may access the cells of the oil glands and take advantage of its high energetic content. Taking into consideration the non-occurrence of secretory structures other than the oil glands in leaves of *M. splendens*, we assume their potential for secreting the major portion of the VOCs.

Potential Roles of the Chemical Profile of the Oil Glands

Currently, the second step of investigation revealed that gall induction and establishment caused alterations in the density and area of the oil glands, which are larger in galls than in the non-galled leaves. The increased size of the oil glands indicates an enhancement in the potential for the production of volatiles in galled condition. Such potential can provide a favorable microenvironment to the galling *Nexothrips* sp., which can benefit from the products of the glands, as proposed for other galling insect-host plant systems (Stone and Schonrögge, 2003).

Role of Volatiles as Biomarkers and Chemical Signalers

The volatiles can act by chemical signaling for herbivores, and their biosynthesis can be altered in response to herbivory (Valladares et al., 2002; Banchio et al., 2005), as observed for *M. splendens* regarding the concentration of volatiles. Currently, gall induction alters the size and area of the oil glands, and accordingly the concentration of VOCs in the samples of non-galled leaves and of galls is distinct. The substantial changes in the emissions of volatiles as gall induction consequence is expected (Izzo et al., 2006), and is clearly perceived in the content of sesquiterpenes in

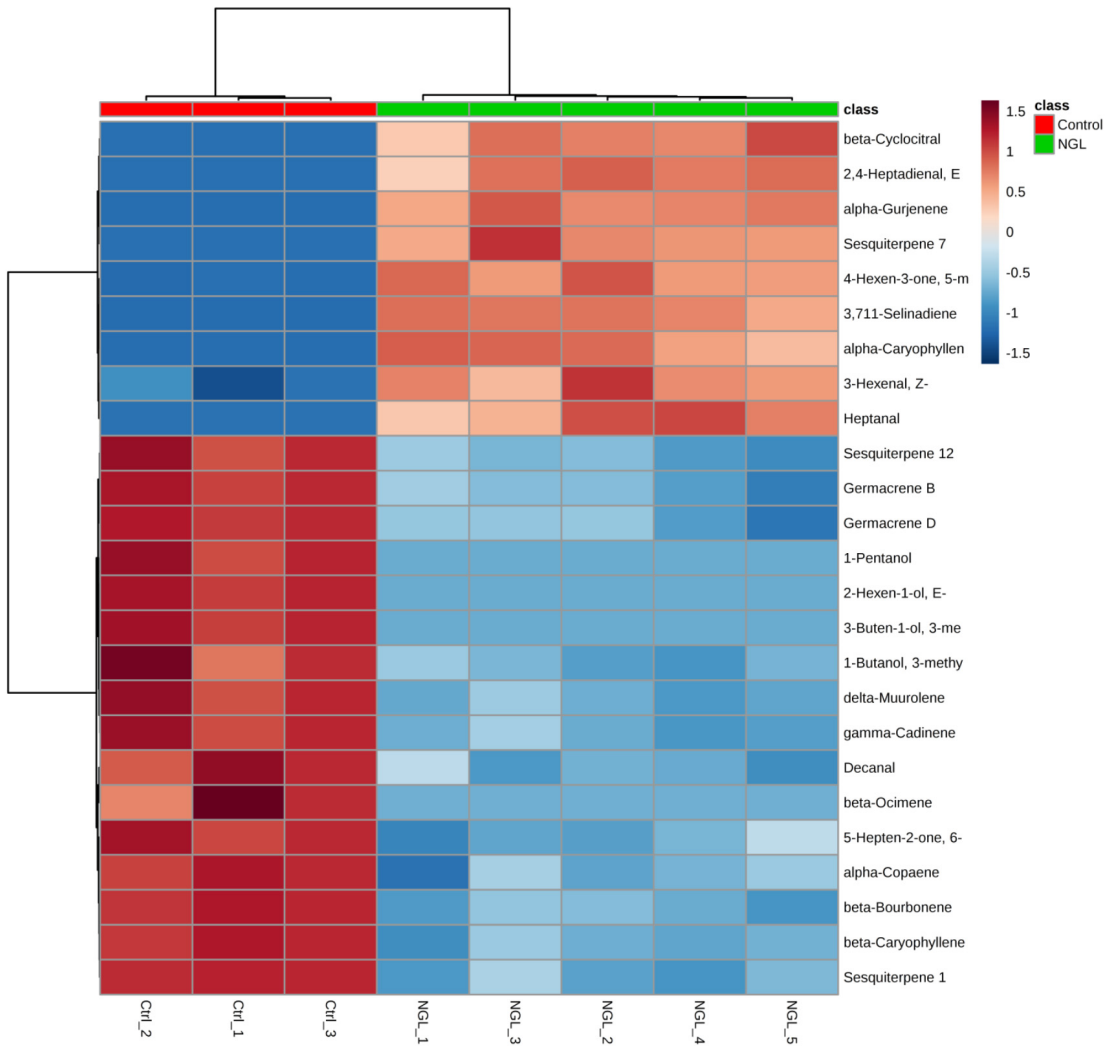

FIGURE 7 | Heat map of non-galled leaves. The heat maps were constructed based on the 25 most discriminating compounds. Compounds identified by HS-SPME-GC/MS. Color key indicates metabolite expression value, blue: Lowest, red: highest.

M. splendens. Such quantitative changes have been reported for other three galling herbivore-host plant systems (*cf.* Tooker et al., 2002; Tooker and Hanks, 2004, 2006). The volatiles, besides attracting reproductive partners (Koschier et al., 2000, 2007) may act in direct or indirect plant defenses against natural enemies (Dudareva et al., 2004; Koschier et al., 2007; Oates et al., 2016). Due to the high frequency of galls in the population of *M. splendens* along the year, we can infer that the repellent properties of the sesquiterpenes were not effective against the associated galling *Nexothrips*. The quantitative differences in the content of sesquiterpenes detected by the SPME analysis in *Nexothrips* sp. galls in comparison to the non-galled condition implied favorable features for the galling Thysanoptera. The volatiles produced in the different samples of *M. splendens* should be related both to insect–plant and to plant–plant interactions.

Chemical signaling, mediated by volatiles, may allow the insects to find and recognize their host plants (Hanula et al., 1985; Tooker and Hanks, 2004), but may also attract natural enemies (James and Chem, 2005). Such inference is based on the ability of the inducers to stimulate host plant responses, which trigger local reactions, and may interfere directly with the insect communication with its host plant (Moura et al., 2009a,b; Oates et al., 2016), but also with other plants in the population. In the population of *M. splendens* at the PESV, there is one plant individual, which has never associated to *Nexothrips* sp. The lowest content observed in the chemical profile of this individual of *M. splendens*, the aforementioned control individual, and the highest content observed in the general composite of the non-galled leaves (NGL) of the other individuals in the population indicates that the galling activity of *Nexothrips* sp. may have caused long-distance effects (Mani, 1964) over the population of *M. splendens*. The secondary effects

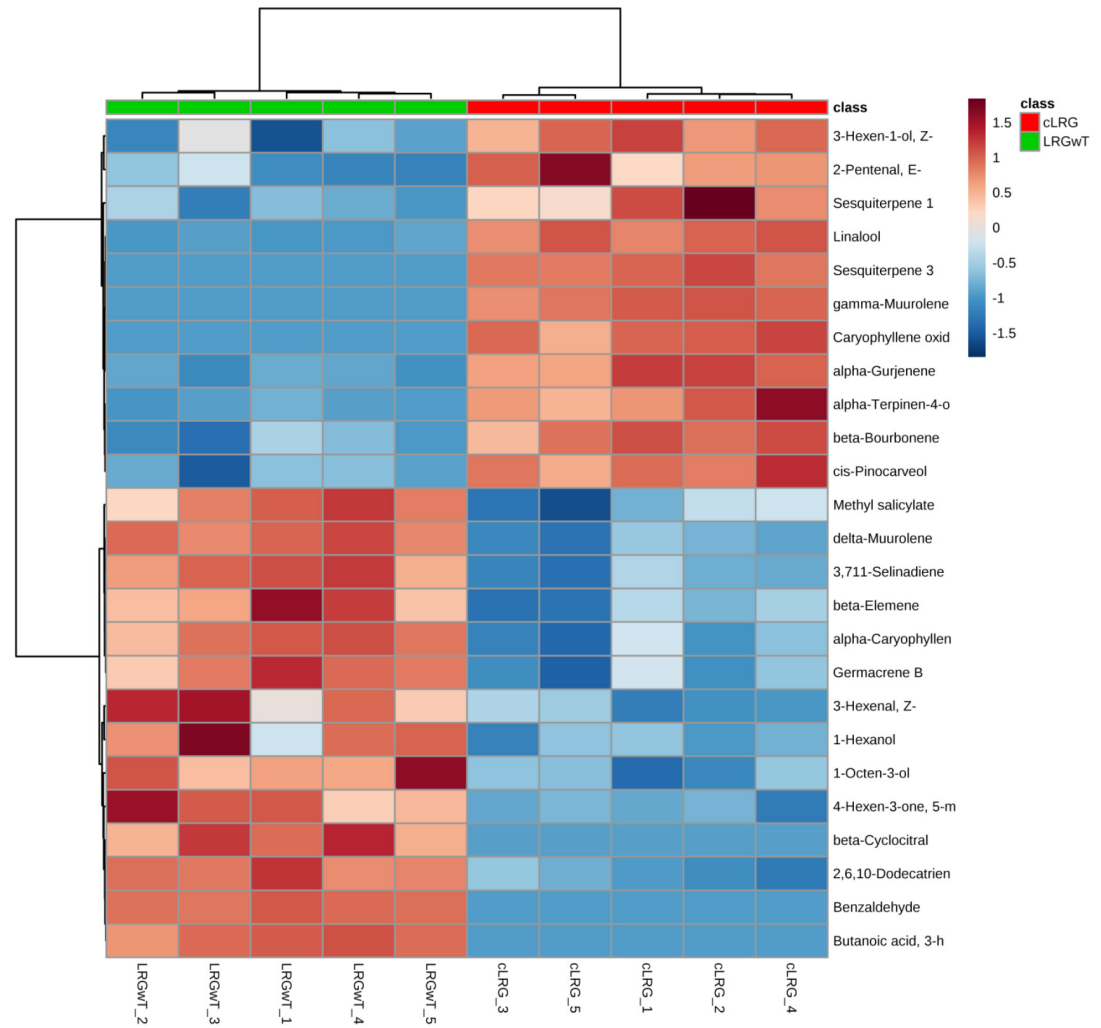

FIGURE 8 | Heat map of galls. The heat maps were constructed based on the 25 most discriminating compounds. Compounds identified by HS-SPME-GC/MS. Color key indicates metabolite expression value, blue: Lowest, red: highest.

or tele-effects were first described for galls induced in roots, but causing changes in flowers of the host plant, *Heterodera marioni* (Mani, 1964), and has recently been reported for *Ditylenchus gallaeformans* galls on *Miconia* spp (Ferreira et al., 2017).

The effect of gall induction on other host plant organs, by the production of secondary metabolites, including volatile compounds, may represent an indirect defense of the plant (Unsicker et al., 2009; Fürstenberg-Hägg et al., 2013; Oates et al., 2016). Despite of their simple molecular structures, the alcohols and aldehydes deriving from the lypoxygenase (LOX) pathway, methyl salicylate, and 3-hexenyl butanoate of low molecular weight pertaining to the GLV class were detected in the samples of *Nexothrips* sp. galls on *M. splendens*, and can act as signaling molecules in plant-herbivore interactions (Yan and Wang, 2006; Damasceno et al., 2010). The chemical signaling between *M. splendens* and its associated galling herbivores may be mediated by some of the terpenes detected in samples of galled leaves. The monoterpenes (geraniol) and the sesquiterpenes may play an attractive role for the adult female of *Nexothrips* sp., as proposed for *Frankliniella occidentalis*, a generalist phytophagous species found worldwide (Koschier et al., 2000, 2007). The geraniol and sesquiterpenes-mediated attraction is yet to be tested for the four galling herbivores reported on *M. splendens* on PESV (Portugal-Santana and Isaias, 2014). The decreasing concentration of δ-muurolene (#67), β-elemene (#58) and α-caryophyllene (#63) in the samples from which the galling thrips were removed (cLRG) could be an indicative that the individuals of *Nexothrips* sp. were manipulating *M. splendens* metabolism and assimilating some of these secondary metabolites.

The detection of methyl salicylate (#42) in the samples of galls had also been related to acquired resistance and indirect plant defense (Oates et al., 2016). Methyl salicylate is a plant semiochemical related to stress signaling (Pickett et al., 2006), and it is generally

described as anti-herbivoric, attractive to beneficial insects that would kill herbivores (Bruinsma et al., 2009), and as a pheromone (James and Price, 2004; Troncoso et al., 2012). In *M. splendens*, the production of methyl salicylate neither affected the life cycle of the inducing thrips nor attracted natural enemies, since individuals in several stages of life occurred inside the galls, and the rate of hyperparasitism was apparently low in comparison to other Neotropical systems (Gonçalves et al., 2009; Carneiro et al., 2013). Such inability of methyl salicylate as an anti-herbivore substance may be effect of its low concentration and consequently its limited potential to stimulate the galling thrips responses, crucial for attractiveness or repellency (Koschier et al., 2000, 2002; Bruhin, 2009).

CONCLUSION

The rare event of gall induction by the Terebrantia studied here revealed mesophyll compactness and formation of air cavities as new features, first described for the Neotropical Thysanoptera-induced galls. The structural profile of *M. splendens* non-galled leaves and galls revealed that the main alteration regards the number and size of the oil glands. As the only secretory structure differentiated in leaves of *M. splendens*, the oil glands should be the main secretory sites responsible for the peculiar chemical profile of the analyzed samples. The main alteration in GLVs concentration in response to *Nexothrips* sp. activity indicates the GLVs as possible stress biomarkers involved in the host plant-galling Thysanoptera signaling. Moreover, the methyl salicylate in the composite of the non-galled individual reveals a potential plant resistance stimulated as a long-distance effect. In addition to the signaling effects of the volatile compounds produced by the non-galled leaves and the galls on *M. splendens*, it can be hypothesized that the individuals of the galling *Nexothrips* sp. may have captured, incorporated, and metabolized some of these VOCs. This hypothesis is based on the increased levels of some sesquiterpenes detected in the composites containing the galling thrips in comparison to the composites without thrips.

AUTHOR CONTRIBUTIONS

NJ and DA did the field sampling. ÉS-S, GS, GLS, and CZ did the chemical analyses. NJ, DA, and RI analyzed the structure. AC did the characterization and ecology of thrips. NJ, ÉS-S, DA, GS, GLS, CZ, AC, and RI analyzed the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01521/full#supplementary-material

DATA SHEET S1 | Area value of each compound identified in *Myrcia splendens* samples by HS-SPME-GC/MS.

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Endophytic Colonization of Onions Induces Resistance Against Viruliferous Thrips and Virus Replication

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In agricultural ecosystems, insect pests, pathogens, weather patterns, and reduced soil fertility pose major challenges to crop productivity and are responsible for significant yield losses worldwide. *Iris yellow spot virus* (IYSV) vectored by *Thrips tabaci* Lindeman, is a major hindrance to onion production in eastern Africa. Control measures often rely on insecticides with deleterious effects. Endophytes are one key alternative as they can play important roles in mediating induced systemic resistance. Hence, we examined the potential effect of endophytic fungus *Hypocrea lixii* (F3ST1) on feeding and replication of IYSV on endophyte-colonized (E+) and endophyte-free (E−) onion plants. For more precise assessment, replication was also tested using leaf disk bioassays and individual thrips. The number of feeding punctures was significantly lower in E+ as compared to E− plants. Disease level was significantly lower in E+ as compared to E− plants for four weeks post-exposure to thrips. IYSV replication was reduced by 2.5-fold in endophytic treatment on both whole plant and leaf disk assays. *Thrips tabaci* showed 2 times higher feeding activities on endophyte-free onion leaf disks as compared to the endophyte-inoculated leaf disks. Our results suggest potential utility of the endophytes to reduce feeding damage and virus infection on onion plants. Further studies should be conducted to elucidate the secondary metabolites involved in such endophyte-thrips-virus mediated interaction and determine whether the interactions extend for this and other onion varieties and viruses under field conditions.

Keywords: *Hypocrea lixii*, *Thrips tabaci*, *Iris yellow spot virus*, onions, systemic, host plant resistance, multi-trophic interactions

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INTRODUCTION

Onion, *Allium cepa* L. (Asparagales: Amaryllidaceae), is an important vegetable crop grown for its benefits in subsistence or commercial farming systems worldwide. In Kenya, onions are grown in all counties by both large- and small-scale farmers (Narla et al., 2011). The major factors limiting onion production are pests and diseases (Pappu et al., 2009; Birithia et al., 2011; Gachu et al., 2012). The onion thrips, *Thrips tabaci* Lindeman, is the most economically important pest of onion in Kenya

and worldwide (Trdan et al., 2005; Waiganjo et al., 2008). They cause direct damage by feeding on leaf tissues resulting in a reduction of photosynthetic ability and consequently reducing onion bulb size and yield (Rueda et al., 2007; Birithia et al., 2014). Bulb onion yield losses of up to 60% have been reported in Kenya due to thrips damage alone (Waiganjo et al., 2008). Thrips feeding lesions also act as a source of secondary infection by pathogenic fungi and bacteria (McKenzie et al., 1993). Tospovirus, *Iris Yellow Spot Virus* (IYSV) (Bunyaviridae: *Tospovirus*) transmitted by onion thrips is also a major threat to economic production of both bulb and seed onion production globally (Gent et al., 2004, 2006; Pappu et al., 2009) and in eastern Africa (Birithia et al., 2014). Farmers mostly rely on synthetic chemical insecticide applications to manage onion thrips vectoring IYSV (Gachu et al., 2012). However, insecticides can lead to serious environmental hazards in addition to causing pesticide resistance in onion thrips populations (Martin et al., 2003). Hence, to remain effective, control programs have to integrate several disease management tactics that explores next-generation agriculture including the use of beneficial micro-organisms such entomopathogenic fungi which have been reported to play multiple roles in nature (Vega et al., 2008).

Iris yellow spot virus is transmitted by *T. tabaci* in a circulative and propagative manner (Whitfield et al., 2005). The virus is acquired by the first or second larval stages and it then multiplies and survives through the later developmental stages (Whitfield et al., 2005; Birithia et al., 2013). Adult thrips emerging from thrips larvae that had acquired IYSV are viruliferous and can transmit the virus. While adults directly feeding on a virus infected plant can acquire the virus, but they cannot transmit it. Strategies that can interrupt this process of acquisition, multiplication and further spread of the virus can lead to development of effective thrips-tospovirus management technologies.

Fungal endophytes are one of such organisms that inhabit and live inside plant tissues without inducing apparent symptoms in their hosts (Rodriguez et al., 2009). In plants primed with endophytes, defense responses are accelerated upon pathogen or insect attack, resulting in enhanced resistance to the attacker (Brotman et al., 2010). Published evidence suggests that endophytic fungi can play symbiotic roles in nature, such as antagonists of plant disease, beneficial rhizosphere colonizers, increased drought tolerance and plant-growth promoters (Vega et al., 2008; Rodriguez et al., 2009; Jaber and Salem, 2014; Jaber and Ownley, 2017). When endophytes colonize plants, they produce enzymes which have the function to suppress plant pathogen activities directly and have the capability of degrading the cell walls of such pathogens (Gao et al., 2010). Emission of secondary metabolites is considered to play an important role during plant defense activities against insects and pathogen attack. Plant colonization by endophytes is also known to influence the population dynamics of insect vectors of diseases. For instance, endophytic isolates of the genus *Neotyphodium* protected meadow ryegrass (*Lolium pretense* = *Festuca pratensis*) from herbivory by bird cherry oat aphid (Lehtonen et al., 2006). A reduction of tunneling in maize by *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) (Bing and Lewis, 1991) and *Sesamia calamistis* Hampson (Lepidoptera: Pyralidae) (Cherry et al., 2004) were attributed to endophytic *Beauveria bassiana* Balsamo (Hypocreales: Clavicipitaceae). Feeding and oviposition were significantly reduced in endophyte–colonized bean plants which in turn affected pupation and adult emergence (Mutune et al., 2016). Similarly, endophytic colonization of banana by *Beauveria bassiana* significantly reduced larval survivorship of banana weevil, *Cosmopolites sordidus* (Coleoptera: Curculionidae), resulting in 42–87% reduction in plant damage (Akello et al., 2008).

Several fungal isolates have been reported to colonize onion plants and confer them protection against thrips through reduced feeding and oviposition resulting in reduced population (Muvea et al., 2014). Further, these authors demonstrated that colonization of onion plants by endophytic fungus, *Hypocrea lixii* F3ST1 triggered antixenotic repellence of *T. tabaci* that impacts their biology (Muvea et al., 2015). On Faba beans Akutse et al. (2013), whilst using the same fungus, reported reduced longevity of progeny, number of pupae and adult longevity of leaf miner flies. Evidence indicates that endophytic fungi can provide plants with protection against plant pathogens (Lehtonen et al., 2006; Ownley et al., 2010; Jaber and Salem, 2014). For instance, *Piriformospora indica* (Hymenomycetes: Basidiomycota), an endophytic root-colonizing fungal species has been shown to repress *Pepino mosaic virus*, which is found widely in tomato greenhouses in many parts of the world, especially at high light intensities (Fakhro et al., 2010).

The mechanism of increased pathogen tolerance in endophyte-inoculated plants is largely speculative, but it is hypothesized that secondary compounds produced by endophytes may play a partial role in this phenomenon (Yue et al., 2000). Mechanisms implicated in plant-endophyte-virus interactions on induced resistance to viral infection may include inhibition of viral multiplication or accumulation (Loebenstein, 1972). The changes in host plant properties can affect viral diseases both directly through host metabolites and indirectly via effects of plant quality on insect vectors transmitting the viruses. Endophytic systemic colonization through intercellular spaces and vascular xylem elements can inhibit or interfere with the systemic movement of plant viruses from cell to cell, which eventually results in delayed multiplication in inoculated plants (Loebenstein, 1972; Martelli, 1980). A case study evaluating the transmission and multiplication of *zucchini yellow mosaic virus* (ZYMV) found lower virus titer levels on endophyte-colonized plants with *Beauveria bassiana* isolates as compared to the endophyte-free plants (Jaber and Salem, 2014). Lehtonen et al. (2006) reported a lower percentage of *barley yellow dwarf virus* (BYDV) infections in endophyte-colonized meadow ryegrass (*Lolium pretense*) compared to endophyte-free plants, indicating that endophyte colonization can protect plants from virus infections and eventual multiplication. Indeed, endophyte-mediated resistance could also impact on the replication of IYSV, in addition to the induced systemic resistance to its vector, *Thrips tabaci* as reported earlier (Muvea et al., 2014, 2015). Extending these findings, in the present study, we hypothesized that endophytic colonization of onion plants by *H. lixii* would induce resistance against viruliferous thrips damage and will

impact on the virus replication on the insects. This is with a view to obtaining a potential environmentally friendly biocontrol agent in the management of IYSV in onion.

MATERIALS AND METHODS

Insect Rearing

Initial cultures of *T. tabaci* were field-collected from onion plants at the International Centre of Insect Physiology and Ecology (*icipe*) organic farm, Duduville, Nairobi, Kenya. Thrips were reared on snow peas pods, *Pisum sativum* L. (Fabales: Fabaceae), for over 35 generations in ventilated plastic jars at the *icipe*'s insectary at 25 ± 1°C, 50–60% relative humidity (RH), 12 h L: 12 h D photoperiod. Adults for the experiment were allowed to lay eggs on snow pea pods for 3 days and were then removed. Neonate first instars (≤ 8 h-old) were used in the subsequent experiments.

Fungal Isolate

Endophytic fungi *Hypocrea lixii* F3ST1, isolated from the aboveground parts of maize, obtained from tropical highland region in Kenya was selected for this study based on its antagonistic effects against thrips (Muvea et al., 2014, 2015). Conidia were obtained from two-week old cultures grown on Potato Dextrose Agra (PDA) plates. The conidia were harvested by scraping the surface of sporulating cultures with a sterile scalpel. The harvested conidia were then placed in a universal bottle with 10 ml sterile distilled water containing 0.05% triton X-100 and vortexed for 5 min to produce homogenous conidial suspension. The conidial concentration was determined using a Neubauer hemocytometer. The conidial concentration was adjusted to 1×10^8 conidia ml^{-1} through dilution prior to inoculation of seeds. To assess the viability of the conidia, 100 µL of conidial suspension was inoculated to the surface of four fresh plates of PDA. Two sterile microscope cover slips were placed on top of the agar in each plate before incubation. The inoculated plates were incubated for 24 h at 20°C. The percentage conidial germination was assessed by counting the number of germinated conidia out of 100 in one randomly selected field.

Seed Inoculation and Endophyte Colonization

Seeds of onions (var. Red Creole, East Africa Seed Co., Ltd., Tanzania) were surface-sterilized in 70% ethanol and then immersed in 2% NaOCl for 2 and 3 min, respectively. The seeds were finally rinsed three times using sterile distilled water to ensure that the seed surface was sterilized free of epiphytes. To confirm the efficacy of the surface sterilization, 100 µl of the last rinse water was spread onto four PDA plates and incubated at 20°C for 14 days. The absence of fungal growth on the medium confirmed the reliability of the sterilization procedure (Schulz and Boyle, 2005). The seeds were then placed on sterile filter paper to dry for 20 min and subdivided into two equal portions one for inoculation and the other to serve as the control. Surface-sterilized seeds were soaked in a conidial suspension of 1×10^8 conidia ml^{-1} for 10 h. In the control, seeds were soaked in sterile distilled water containing 0.05% Triton X 100. The inoculated seeds were air dried on a sterile paper towel for 20 min and then transferred to plastic pots (8 cm diameter × 7.5 cm height) containing planting substrate. The substrate was a mixture of red soil and livestock manure in a 5:1 ratio, sterilized in an autoclave for 2 h at 121°C, and allowed to cool down to ambient temperature before being used. Four seeds per pot were sown 1 cm below the surface of the substrate and maintained at room temperature (∼25°C and 60% RH) in a screen house. Endophytic colonization of the inoculated plants was confirmed using the technique described by Muvea et al. (2014). Plants were randomly selected and carefully removed from the pots 50 days after inoculation and the roots washed with running tap water. Leaves, stems and roots of seedlings were cut into different sections. Five randomly selected leaf, stem and root sections from each plant were surface-sterilized as described above. The different plant parts were then aseptically cut into ∼1cm pieces before placing the pieces 4 cm apart from each other, on PDA plates amended with a 0.05% solution of antibiotic (streptomycin sulfate salt). Plates were incubated at 25 ± 2°C for 10 days, after which the presence of endophyte was determined. Prior to incubation of the different plant parts, the last rinse water was also plated out to assess the effectiveness of the surface sterilization procedure as described above. The colonization of the different plant parts was recorded by counting the number of pieces that showed the inoculated fungal growth. Only the presence of the endophyte used for inoculation was scored. After testing for colonization, the remaining seedlings were thinned to one per pot and watered once per day.

Inoculum Preparation and Virus Transmission Through Mechanical Inoculation

The inoculum was prepared by grinding freshly harvested and virus-infected onion leaves with phosphate buffer at PH 7.4 (1:5 weight/volume). A sterilized mortar and pestle were used for grinding. Forthy four potted whole plant without endophyte and virus were arranged in four groups and placed in separate cages were used for the experiment. Freshly emerging leaves were dusted with carborundum (silicon carbide, 400–600 mesh) to increase infection by providing minute wounds for entry of the virus particles. The inoculum was gently rubbed on the leaves as a source of infection. Simultaneously but in separate cages, the same number of potted plants was used for thrips transmission experiment. However, ten viruliferous thrips were released per plant for this experiment. After 2 weeks, the presence of the virus was tested using IYSV-specific DAS-ELISA (Agdia Biofords) for up to four times.

Acquisition and Transmission of IYSV by *Thrips tabaci*

Virus transmission using *T. tabaci* was adopted for this study because mechanical inoculation attempted as described was not successful and this was also reported previously (Bulajić, 2008; Srinivasan et al., 2010; Naveed and Pappu, 2012). A cohort of 500

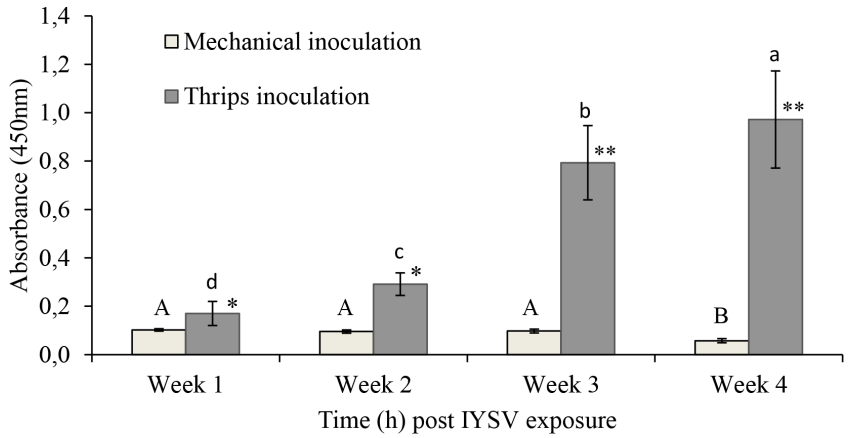

FIGURE 1 | *Iris yellow spot virus* titer levels on onion plants inoculated through mechanical and thrips transmission methods ($n = 11$). Means followed by the same lower or upper case letters indicate no significant differences between different time intervals for IYSV replication through thrips and mechanical transmission, respectively. Asterisks indicate statistically significant differences between treatments at each time point $^{*}P < 0.05$, $^{**}P < 0.01$.

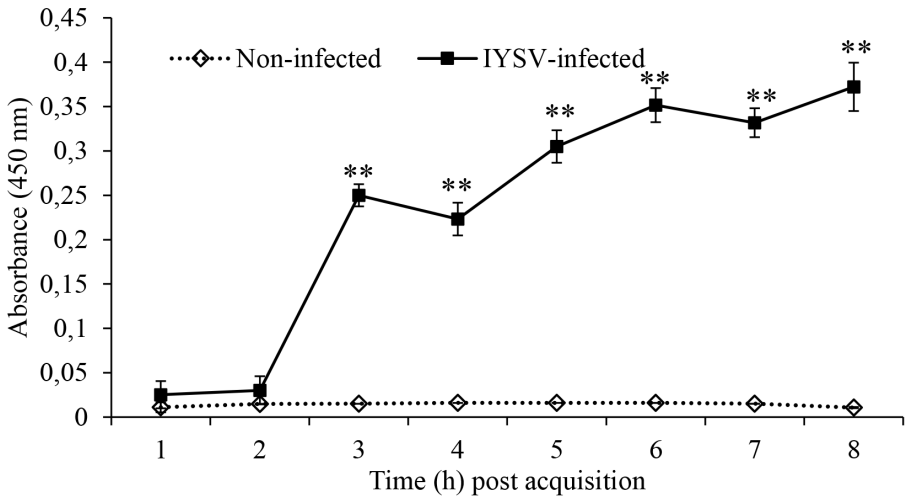

FIGURE 2 | *Iris yellow spot virus* non-structural protein levels in *Thrips tabaci* determined at varying times post-acquisition using specific antibodies. Asterisks indicate a significant difference in NS replication between IYSV-infected and non-infected thrips $^{*}P < 0.05$, $^{**}P < 0.01$.

first instar (≤ 8 h-old) *T. tabaci* obtained from *icipe*'s insectary were allowed to acquire virus by feeding on IYSV-infected *Allium cepa* var. Red creole (plants maintained at *icipe* as virus inoculum source) for an acquisition access period (AAP) of 16 h. The virus infection in the plants leaves (1 g) used for virus acquisition was confirmed using IYSV-specific ELISA Flashkit (Agdia Biofords, Netherlands) (Birithia et al., 2013). Thrips were then transferred and reared on snow pea pods until adults emerged.

Monitoring Replication of IYSV in the Thrips Vector

Virus replication in their vectors has important epidemiological implications as it allows the vector to remain infective throughout their life stages for transmission of viruses (Ullman et al., 1993). In vector thrips of IYSV, *T. tabaci* has shown competence in virus load increase from acquisition as 8 h old larva up to adult stage (Birithia et al., 2013). Moreover, studies on its non-structural (NS) proteins have been reported for their specificity for monitoring IYSV replication (Birithia et al., 2013). The expression of the NSs protein in thrips was monitored by direct antigen-coated (DAC) ELISA (Srinivasan et al., 2012). DAC-ELISA was carried out as described by Wijkamp et al. (1993) with slight modifications. Two cohorts of 200 first instar (≤ 8 h-old) *T. tabaci* were given an AAP of 16 h on IYSV-infected and non-infected *Allium cepa* var. red creole. They were transferred thereafter to non-infected snow pea pods placed in Petri dishes (8 mm diameter). From each cohort five insects were sampled at 0, 4, 12, 24, 48 and 72 h post-acquisition (h.p.a.) and at pre-pupal and 2-day-old adult stages. Thrips were placed in 1.5 mL Eppendorf tubes containing 100 μL coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ in 1 L distilled water, pH 9.6) and

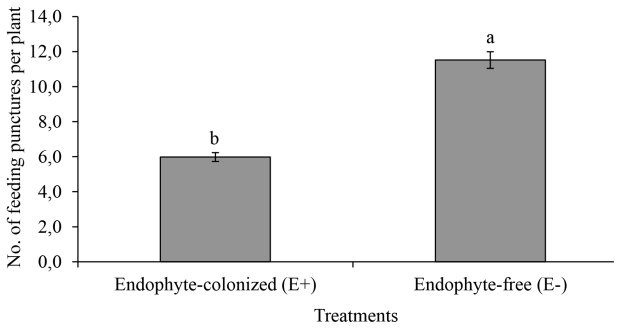

FIGURE 3 | Effect of endophytically colonized onion plants on mean feeding punctures by viruliferous adult *Thrips tabaci*. The figure quantifies mean feeding activity by 20 viruliferous *T. tabaci* exposed for 48 h on endophyte-colonized (E+) and endophyte-free (E–) onion plants. Bars indicate means ± SE at 95% CI (n = 44). Different letters indicate a significant difference between treatments.

stored at −20°C until analysis. Thrips were triturated in the buffer with a sterile blunt-end plastic pestle. The suspension was transferred to flat bottom ELISA plates and incubated for 12 h at 4°C. Plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and blocked with 200 μL 1% bovine serum albumen (BSA) for 2 h at 37°C. After draining the plates and washing once with PBS-T, 200 μL polyclonal anti-NSs antibody diluted (1:4000) in antibody dilution buffer {0.2% BSA, 2% polyvinylpyrrolidone (PVP) mol. wt. 40 000, 0.02% sodium azide, pH 7.4} was added to the wells, and the plates were incubated at 37°C for 2 h. Plates were washed three times with PBS-T and 200 μL goat anti-rabbit IgG-alkaline phosphatase (universal conjugate, Agdia Biofords) in antibody dilution buffer (1:500) was added to each well and the plates were incubated at 37°C for 2 h. Absorbance values were measured at 405 nm 1 h after the addition of substrate (0.5 mg mL^{-1} disodium q-nitrophenyl phosphate in 1 M diethanolamine, 0.5 mM $MgCl_2$, 0.02% sodium azide) on an ELISA plate reader (Biotek-Epoch).

Impact of Endophyte Colonization of Onion Plants on Virus Transmission by Thrips

Four 10-weeks-old plants two each from Endophyte-colonized (E+) and Endophyte-free (E−) plants were placed in an equidistant and alternating pattern in 44 thrips-proof cages (40 cm × 30 cm), cages herein referred to as replicates. Virus transmission was performed by releasing 20 viruliferous adults thrips per cage and allowed to feed for 48 h after which all the thrips were removed from the plants using a Carmel brush. The cages were then randomly divided into four groups (Group 1, 2, 3, and 4) with each group comprising of 11 cages (each cage consists of four plants two each from E+ and E−) whereby for instance, group one represented samples for week one in that order. This was considered important to enable assessment of virus replication over time. To exclude bias in our results on virus transmission on E+ and E− treatments, samples (1 g leaf) for the test were cut from sections of the plant with visible feeding punctures. This was necessary because random selection would imply that E+ will have less titer as feeding punctures are positively correlated with virus transmission (Jiang et al., 2000). Samples were obtained after 2 weeks post thrips exposure. Feeding damage was assessed in both treatments from the 44 replicates by counting the number of punctures from four leaf sections each 3cm long per treatment. The excised leaf sections were then tested for virus using IYSV-specific double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA, Agdia Biofords). Non-colonized plants (without endophyte and virus) were tested simultaneously for baseline titers.

To obtain a more precise assessment, transmission of IYSV was additionally analyzed using leaf disk assays and individual thrips. This was done to confine the thrips to a food source because previous reports suggest that thrips feeding on whole plants can be repelled leading to reduced feeding and eventual virus replication (Muvea et al., 2014, 2015). Twenty two potted onion plants each for E+ and E− raised in separate cages were used for the leaf disk experiment. A single leaf disk was obtained from the second middle leaf of each plant. The assay was done by allowing viruliferous individual adult thrips which were reared and infected as described earlier to feed on 2 cm^2 onion leaf disks placed in Petri dishes (9 cm diameter). The top of the Petri dishes was sealed with Parafilm to prevent escape of thrips. A single adult thrips was allowed to feed on each leaf disk for 48 h period. Feeding damage on the leaf disks was confirmed by counting the number of feeding punctures. The same leaf disks were used to test for the presence of the virus as described earlier. ELISA readings were considered positive when the absorption (OD = 405 nm) of the sample wells was at least two times greater than the mean absorption of negative control samples.

Statistical Analysis

All data were checked for normality and homogeneity of variance before analysis. One-way Analysis of variance (ANOVA) was performed to determine accumulation of NSs protein in thrips and comparisons of means at 95% significance was undertaken with Tukey's Honestly Significant Difference (HSD) test. Feeding was determined by counting the number of punctures on leaf sections and taking the average per treatment (E+ and E−) for all the 44 replicates before analysis by negative binomial regression using package *MASS* (Venables and Ripley, 2002). The negative binomial distribution was deemed appropriate for this kind of study because of its biological appropriateness and ability to handle over distribution in count data and better goodness of fit measurements (deviance and Pearson chi-square closer to 1) compared with Poisson or Gaussian distributions (Candy 2000). Virus titer levels on whole plants over time was analyzed using repeated measure analysis of variance (ANOVA) and a Bonferroni *post hoc* test using package *multcomp* (Hothorn et al., 2008). Treatments were considered as fixed effects and the cages as replicates. Petri dish experiment analysis was performed using a chi-squared test. *P*-values were based on type III chi-square values in all the analyses. All statistical analyses were performed in R 2.15.3 (R Core Team, 2013).

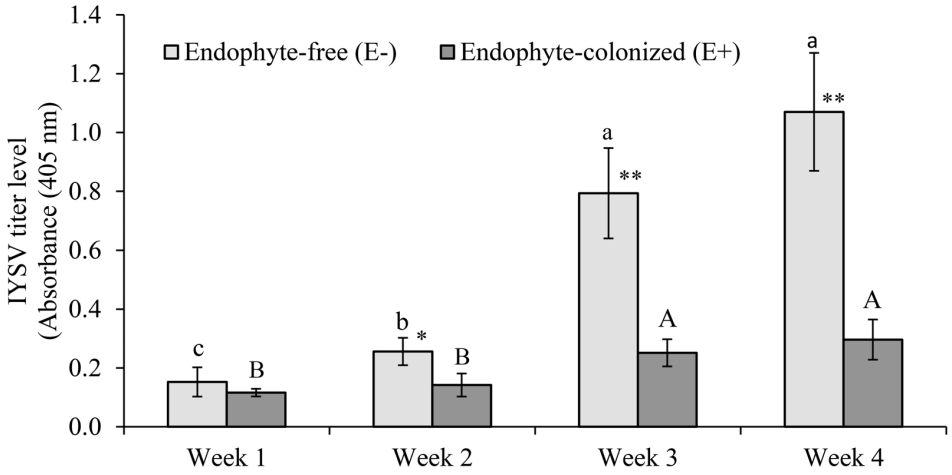

FIGURE 4 | Effect of endophytically colonized onion plants by *Hypocrea lixii* F3ST1 on IYSV replication overtime. Means followed by the same lower or upper case letters indicate no significant differences between different time intervals for IYSV replication on E– and E+, respectively. An evaluation of endophytic fungus for its effect on IYSV transmission by viruliferous thrips after 48 h as measured from samples taken from a whole plant. Means ± (standard error) SE at 95% confidence interval ($n = 11$). Asterisks indicate statistically significant differences between treatments at each time point $^{*}P < 0.05$, $^{**}P < 0.01$.

RESULTS

Evaluating Thrips vs. Mechanical Transmission of IYSV

Biotests on transmission of IYSV using thrips and mechanical modes of transmission showed that the mechanical method of virus transmission is not effective. Hence, the virus transmission method using thrips was selected for this study (**Figure 1**).

Endophytic Colonization

In viability tests, conidial germination of F3ST1 before seed inoculation was 90% which was acceptable. The final rinse of water used for surface sterilization of seeds was effective as no sign of fungal growth was observed on the plating media. The average endophytic colonization of onion plant parts was 81, 53.6, and 48.6% for root, stem and leaf sections, respectively.

Confirmation Test for Acquisition of IYSV by *T. tabaci*

Thrips were tested for positive acquisition of IYSV by quantification of NS proteins from larval to adult stages and our results recorded mean titer level of 0.015 at larval and 0.36 at adult stage. Results of the biotest showed that thrips samples tested positive for the virus and there was a significant increase of NS proteins in thrips fed on IYSV-infected plants ($F_{1,78} = 115.32$, $P < 0.001$; **Figure 2**).

Effect of Endophytic Colonization on Feeding and IYSV Replication

The number of feeding punctures were significantly lower in endophyte-inoculated plants as compared to the control treatment ($\chi 2 = 19.67$, $df = 1$, $P < 0.001$, $n = 44$; **Figure 3**). There was approximately a 2-fold reduction in feeding activities on E+ as compared to E− plants (**Figure 3**). Since feeding was assessed on random sections of the plant, the data was collected and recorded before using the samples for IYSV test. Replication of IYSV was reduced 2.5-fold on endophytically colonized onion plants (**Figure 4**). Endophyte-colonized plants showed lower IYSV titer levels of 0.23 ± 0.07 as compared to 0.58 ± 0.11 from the endophyte-free plants ($F = 5.98$; $df = 1$, 10; $P < 0.001$; **Figure 4**). The effect of time in regard to virus replication was significant for E− plants ($F = 10.98$; $df = 3$, 10; $P < 0.001$). However, there was no significant difference in the level of IYSV replication on E+ plants over time ($F = 1.02$; $df = 3$, 10; $P = 0.39$) (**Figure 4**). The third and the fourth week of data sampling showed a 3.2 and 3.6-fold increase in IYSV replication in E− compared to E+ plants, respectively (**Figure 4**). The average ELISA titer value for non-infected controls for four weeks (without endophyte and virus) was 0.11 ± 0.003 which was 2 and 5-fold lower than the readings for E+ and E− plants, respectively.

Results from the leaf disk assay showed a 2.5 fold reduction of IYSV replication on endophyte-colonized onion plants ($\chi 2 = 4.65$, $df = 1$, $P = 0.03$) (**Figure 5**). *Thrips tabaci* showed 2.5 fold reduction in feeding activities on endophyte-colonized as compared to the endophyte-free onion leaf disks (**Figure 6**).

DISCUSSION

Colonization of the onion plants by fungal endophytes in a previous study by Muvea et al. (2014) was found to significantly reduce feeding, oviposition and survival of onion thrips, *Thrips tabaci*. Among the endophytes tested, *Hypocrea lixii* (F3ST1) was the best performing isolate (Muvea et al., 2014), which was selected for the current study. *H. lixii* F3ST1, isolated from above ground parts of maize and sorghum has been reported to be endophytic on several host plants such as maize, onion

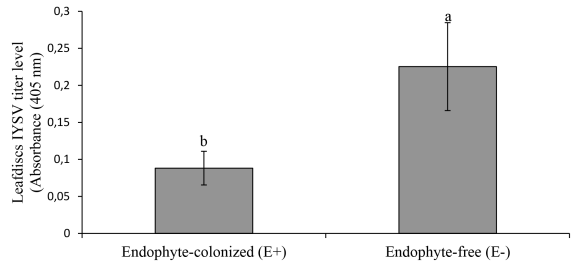

FIGURE 5 | Effect of endophyte-inoculated and endophyte-free onion leaf disks on transmission of IYSV by viruliferous *Thrips tabaci*. Bars indicate average titer levels (±SE) after a 48h feeding period (*n = 22*). Different letters indicate a significant difference between treatments.

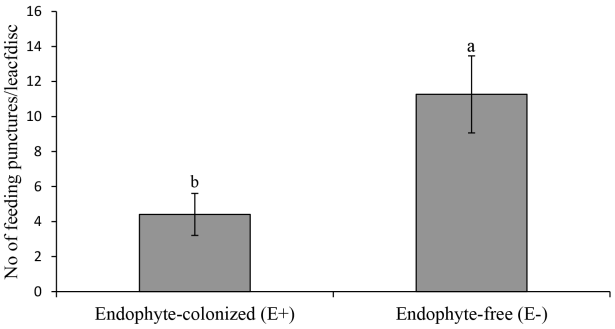

FIGURE 6 | Effect of endophyte-inoculated and endophyte-free onion leaf disks on number of feeding punctures by viruliferous *Thrips tabaci*. Bars indicate average titer levels (± SE) after a 48 h feeding period (*n = 22*). Different letters indicate a significant difference between treatments.

and bean seedlings with antagonistic properties against pests such as leaf miners and thrips (Akello, 2012; Akutse et al., 2013; Muvea et al., 2014). This study further aimed to assess the poorly understood interaction between the endophyte and the thrips-transmitted virus, IYSV. Optimization of an effective virus transmission protocol is critical in such interaction assays. Comparative evaluation of mechanical and thrips-transmission of IYSV indicated that only thrips-transmission was effective, and therefore was adopted further in the study. This is in line with the previous reports by Bulajić, 2008, Srinivasan et al. (2010), and Naveed and Pappu (2012).

Endophyte-colonization of onion plants reduced feeding activity of viruliferous *T. tabaci* by 2-fold as compared to endophyte-free (E−) onion plants. In a previous study using non-viruliferous *T. tabaci*, feeding activities were reduced on endophyte-colonized onion plants and it was speculated that antibiosis and/or antixenosis repellency of thrips could have played a key role (Muvea et al., 2014, 2015). Similar observations have also been made in other endophyte-pest interactions such as *Neotyphodium*-aphid interaction in New Zealand tall fescue (Guy and Davis, 2002) and *Hypocrea/Beauveria*-leafminer interaction in Faba bean (Akutse et al., 2013). Overall, our findings highlight the utility of endophytic colonization in reducing thrips feeding damage on onions.

This study further indicates that replication of IYSV in *H. lixii*-colonized onion plants was significantly reduced as compared to endophyte free plants. There was a significant reduction in IYSV titer levels by 4 weeks after virus infection in whole plant and leaf disk assays. In the first week of sampling, difference in titer levels of IYSV between E+ and E− plants were not significant. However, the differences became apparent from the second week onwards and increased up to 2.8-fold in E− plants by the fourth week. The virus replication in the E+ plant was maintained significantly low as compared to the non-colonized controls. This could be explained due to a combination of lower feeding by viruliferous thrips on E+ plants and the reduced ability of the virus to replicate in the E+ plants. Inoculation of *Pinus halepensis* Mill seedlings with fungal endophytes including *Trichoderma* spp. significantly reduced leaf necrosis length caused by a plant pathogen, *Gremmeniella abietina* (Lagerberg) Morelet (Romeralo et al., 2015). Similarly, three endophytic isolates of *Lecanicillium* sp. suppressed production of *Podosphaera fuliginea* (Schlecht.) Pollacci spores, a plant pathogen that causes powdery mildew (Kim et al., 2007). Whereas there are numerous reports on the ability of endophytes to reduce plant pathogens (Ownley et al., 2008), to our knowledge, this is among the first report to reveal fungal endophytes from Ascomycota (*H. lixii*) as endophytic colonizers with ability to reduce tospovirus replication. The negative interaction of *H. lixii* to IYSV is closely similar to reports on reduction of incidence and severity of aphid-borne potyvirus, ZYMV in *B. bassiana*-colonized plants as compared to the non-colonized plants (Jaber and Salem, 2014). Our results are also concordant with those reported by Lehtonen et al. (2006) on meadow ryegrass using *Neotyphodium uncinatum* Gams, Petrini & Schmidt, endophytes. The authors reported reduced transmission and replication of aphid-transmitted Luteovirus, BYDV on endophyte-colonized as compared to endophyte-free plants. The authors speculated that the effect on the feeding activities of aphids on endophyte-colonized plants was the reason for the lower virus infection frequency in endophyte-colonized meadow ryegrass.

The mechanism underlying these negative effects on the vectors and the plant virus are not well understood. This could likely be due to endophytic fungi triggering gene expression in defense pathways thus increasing production of plant defensive compounds (Jung et al., 2012; Pieterse et al., 2014). The impact of fungal secondary metabolites produced *in planta* in their endophytic association could also play a role in this interaction. IYSV titre levels were lower in endophyte-colonized plants in spite of leaf samples being picked from plants with signs of feeding damage for both E+ and E−. This further adds to the speculation that a mechanism of resistance could be prevalent and which is an outcome that needs further investigation. Shrivastava et al. (2015) in a recent study reports that co-infection of endophytic *B. bassiana* and mycorrhizae in tomato plants can significantly increase terpenoid content in leaves leading to a reduction in foliar feeding by herbivores. Alkaloids released by endophytic fungi have also been reported to possess antiviral activities (Selim et al., 2012). Hence to further understand the mechanism underlying these interactions, analysis of the secondary metabolites and the head space

volatiles from endophyte colonized plants is critical. Although our laboratory and screen house experiment results cannot be extrapolated to field conditions and other onion-thrips-variety and plant-pest systems, our results suggest that this fungus may more likely enhance plant resistance to insect feeding and virus transmission when they become endophytic. Various factors in the field such as plant diversity, other insect pests and pathogens and prevailing environmental conditions are likely to influence the multitrophic interaction between the host plant-endophyte-virus-insect vectors which needs to be further investigated.

To conclude, our studies imply that endophytic colonization of onion plants may act as deterrence to feeding damage by viruliferous thrips. Moreover, endophytic colonization enhances the ineffectiveness of viruliferous thrips to transmit IYSV and has negative effects on IYSV replication in the plant. Consequently, endophytic fungi should be considered as an important component which needs to be taken into account when investigating plant-endophyte-insect interactions. This knowledge has clear implications for understanding the epidemiology of insect-transmitted plant diseases and improving their management options under integrated agricultural systems. A better understanding of the interaction between the insect, virus, endophytes and the host plant is important since biochemical and physiological factors may act in combination or independently, resulting in differences in efficacy of selected endophytes on viruliferous insects feeding on different onion varieties.

AUTHOR CONTRIBUTIONS

AM, RM, SS, H-MP, SE, and NM conceived and designed the experiments. AM, SS, and NM performed the experiments. AM, SS, and RM analyzed the data. NM, SS, and SE contributed reagents, materials, and analysis tools. AM, NM, SS, H-MP, SE, and RM contributed to the writing of the manuscript. SS, RM, NM, H-MP, SE, and RM obtained funding. All authors contributed to revisions and commented on previous versions of the manuscript.

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frontiers in Plant Science

ORIGINAL RESEARCH
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Induced Resistance Against Western Flower Thrips by the *Pseudomonas syringae*-Derived Defense Elicitors in Tomato

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Western flower thrips (WFT) *Frankliniella occidentalis* (Pergande) is a key agricultural pest of cultivated tomatoes. Induced host plant resistance by activating jasmonic acid (JA) signaling pathway constitutes a promising method for WFT control. The phytotoxin coronatine (COR), produced by *Pseudomonas syringae* pv. tomato DC3000 (*Pst*), mimics the plant hormone JA-Isoleucine and can promote resistance against herbivorous arthropods. Here we determined the effect of *Pst* and COR on tomato resistance against WFT, induction of JA and salicylic acid (SA) associated defenses, and plant chemistry. Additionally, we investigated the presence of other components in *Pst*-derived and filtered culture medium, and their interactive effect with COR on tomato resistance to WFT. Our results showed that infiltration of COR or *Pst* reduced WFT feeding damage in tomato plants. COR and *Pst* induced the expression of JA-associated gene and protein marker. COR also induced expression of a SA-related responsive gene, although at much less magnitude. Activation of JA defenses in COR and *Pst* infiltrated plants did not affect density of type VI leaf trichomes, which are defenses reported to be induced by JA. An untargeted metabolomic analysis showed that both treatments induced strong changes in infiltrated leaves, but leaf responses to COR or *Pst* slightly differed. Application of the *Pst*-derived and filtered culture medium, containing COR but not viable *Pst*, also increased tomato resistance against WFT confirming that the induction of tomato defenses does not require a living *Pst* population to be present in the plant. Infiltration of tomato plants with low concentrations of COR in diluted *Pst*-derived and filtered culture medium reduced WFT feeding damage in a greater magnitude than infiltration with an equivalent amount of pure COR indicating that other elicitors are present in the medium. This was confirmed by the fact that the medium from a COR-mutant of *Pst* also strongly reduced silver damage. In conclusion, our results indicate that induction of JA defenses by COR, *Pst* infection, the medium of *Pst* and the medium of a *Pst* COR$^-$ mutant increased resistance against WFT. This was not mediated by the reinforcement of leaf trichome densities, but rather the induction of chemical defenses.

Keywords: coronatine, *Frankliniella occidentalis*, induced plant defenses, jasmonic acid, *Pseudomonas syringae*, salicylic acid, *Solanum lycopersicum*, type VI glandular trichomes

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INTRODUCTION

The western flower thrips (WFT), *Frankliniella occidentalis* [Pergande], is one of the most serious greenhouse and field insect pests of vegetable and ornamental crops worldwide. It is a highly polyphagous insect that can feed on more than 200 wild and cultivated host species (Lewis, 1997) by piercing and sucking epidermal/mesophyll plant cells, which results in damaged areas of a silvery appearance (silver damage). WFT cause direct damage by feeding on leaves, flowers, and fruits, or indirect damage through the transmission of plant viruses (de Jager et al., 1995a,b), being the main vector of tospoviruses, such as tomato spotted wilt virus (Maris et al., 2003). Current control of WFT mainly depends on the use of pesticides and biological control. Use of pesticides leads to residue problems on marketable crops, human health risks, toxicity to non-target beneficial organisms, and environmental contamination (Bielza, 2008; Demirozer et al., 2012; Gao et al., 2012; Mouden et al., 2017). Therefore, multiple and complementary tactics are necessary in the framework of integrated pest management (IPM) programs.

Enhancement of constitutive and/or inducible host plant defenses against WFT has recently been discussed as a promising alternative for thrips control (Steenbergen et al., 2018). Plants defend themselves against herbivores by employing a plethora of physical and chemical arsenals, including trichomes, defensive enzymes, and secondary metabolites that can be present in the plant before attack or induced after detecting the presence of the attacker. Induced plant defenses against herbivory are mainly controlled by the phytohormones jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), and fine-tuned by other phytohormones such as abscisic acid, auxins, cytokinins, and gibberellins (Pieterse et al., 2012). Activation of JA-associated defenses has been reported to confer plant resistance against pierce-sucking arthropods such as spider mites and thrips (Li et al., 2002; Ament et al., 2004). In particular, WFT have been reported to be susceptible to JA-associated induced defenses in diverse plant species such as *Arabidopsis*, Chinese cabbage (*Brassica rapa*), cotton (*Aphis gossypii*), and tomato (*Solanum lycopersicum*) (Omer et al., 2001; Abe et al., 2008; Abe et al., 2009; Escobar-Bravo et al., 2017).

In tomato (*S. lycopersicum*), induction of JA-related defenses has been associated to increased levels of defensive type-VI glandular trichomes and their derived exudates, proteins such as proteinase inhibitors and polyphenol oxidases (PPO), and secondary metabolites (Boughton et al., 2005; Degenhardt et al., 2010; Escobar-Bravo et al., 2017). Type-VI trichomes are important physical and chemical defense barriers, and their absence increases tomato susceptibility against herbivory (Kang et al., 2010a,b). Overexpression of certain proteinase inhibitors has been reported to increase plant resistance against WFT (Annadana et al., 2002; Outchkourov et al., 2004), and enhanced PPO activities can confer enhanced resistance against beet armyworm (*Spodoptera exigua*), cotton bollworm (*Helicoverpa armigera*) (Bhonwong et al., 2009), and cutworm (*Spodoptera litura*) (Mahanil et al., 2008). Accordingly, application of natural or synthetic elicitors activating these JA-associated defenses can increase tomato resistance against various insects, including WFT (Thaler, 1999; Thaler et al., 2002; Escobar-Bravo et al., 2017).

The phytotoxin coronatine (COR) produced by several pathovars of *Pseudomonas syringae* acts as a virulence factor in *P. syringae* pv. tomato (*Pst*), allowing this pathogen to successfully develop high populations in the plant (Zhao et al., 2001; Uppalapati and Bender, 2005; Zheng et al., 2012). COR is a polyketide formed by the coupling of coronafacic acid (CFA) and coronamic acid (CMA) through an amide bond (Bender et al., 1993). Its structure mimics a bioactive JA conjugate (JA-Isoleucine), thus having the ability to stimulate JA-associated defense responses (reviewed by Geng et al., 2014), but also affecting ET and auxin signaling pathways (Uppalapati et al., 2005). Both JA and COR can induce chlorosis, ET emission, inhibition of root elongation, volatile production, biosynthesis of stress-associated compounds and anti-herbivore proteins (Uppalapati et al., 2005). Consequently, infiltration with COR-producing *P. syringae* or infiltration of pure COR in *Arabidopsis* enhanced plant resistance against arthropod herbivores, such as the caterpillar *Trichoplusia ni* (Cui et al., 2005). In tomato, *P. syringae* infection (López-Gresa et al., 2011) or COR application (Uppalapati et al., 2005) also induces metabolomic changes in the plant. All these studies suggest that *Pst* may potentially be used to increase resistance against WFT in tomato. Yet, the effects of *Pst* and COR infiltration on tomato defenses against herbivory may differ. Activation of defense signaling pathways in *Pst*-infected plants is not only mediated by the phytotoxin COR, but also by an array of virulence factors such as exopolysaccharides effectors secreted by the type III secretion system, and cell-wall-degrading enzymes (Zhao et al., 2003; He et al., 2004). Thus, research on the possible use of other *Pst*-derived defense elicitors for their practical application in agricultural systems is crucial, as *Pst* is a plant pathogen.

Here, we first investigated the effect of COR or *Pst* DC3000 infiltration on tomato defenses against WFT. In particular, we determined their effect on WFT-associated feeding damage, as well as variations in type-VI leaf trichome densities, leaf metabolome, expression of defense-associated genes, and tomato growth. In a further attempt to test the possible role of other *Pst* DC3000 associated defense elicitors in tomato-WFT interaction, we also studied the effect of dilution series of the *Pst*-derived filtered medium and a COR-deficient *Pst* strain on tomato resistance against WFT and activation of JA signaling pathway.

MATERIALS AND METHODS

Plants, Insect, and Bacterial Strains

The tomato cultivar "Moneymaker" (*S. lycopersicum*) was used in all experiments. Tomato seeds were germinated on filter paper soaked with MilliQ water and incubated at 20°C. Five days later, germinated seeds were planted in plastic pots (11 cm × 11 cm × 12 cm) filled with potting soil and maintained in a climate room at 20°C, 70% RH, 113.6 μmol photons $m^{-2} \cdot s^{-1}$ of photosynthetically active radiation (PAR) and L16:D8 photoperiod.

Western flower thrips, *F. occidentalis* (Pergande), were maintained on chrysanthemum flowers (cultivar Euro Sunny) in a climate room at 23°C, 60% RH, and L12:D12 photoperiod.

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000) NCPPB4369 was obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB, London, United Kingdom). *P. syringae* pv. *tomato* DB29 (*Pst* DB29, a *cmaA cfaA* double mutant of *Pst* DC3000) (Brooks et al., 2004) was kindly provided by Prof. Barbara Kunkel from Washington University in St. Louis. Both *Pst* DC3000 and *Pst* DB29 were stored in 30% glycerol at −80°C for long-term preservation.

Experimental Design

To determine the effect of COR and *Pst* DC3000 on tomato defenses against WFT (Experiment 1), 4-week-old tomato plants were infiltrated with: (1) 5 μM COR solution, (2) a 10^8 cfu ml^{-1} of *Pst* DC3000 suspension, or (3) a mock solution of sterilized MilliQ water. For this, four leaflets (two top leaflets of leaf 2 and 3 from the bottom) were pressure-infiltrated with 400 μl (100 μl for each leaflet) of one of the treatments on their abaxial leaf sides using a 1-ml needleless syringe. Seven days after infiltration, plants were sampled for type VI trichome density, metabolomics, gene expression, and polyphenol oxidase (PPO) activity analysis, or used for non-choice whole plant thrips bioassays.

With the aim to explore whether COR and other defense elicitors present in *Pst* DC3000-derived medium (without viable bacteria) increases tomato resistance against WFT, three additional experiments were conducted. First, to test if the COR present in *Pst* DC3000-derived medium can enhance tomato resistance against WFT (Experiment 2), tomato plants at four leaf-stage were infiltrated with 100 μl of (1) mock solution (MilliQ water), (2) blank medium (described in Generation of *Pst*-derived and Blank Medium below), (3) blank medium supplemented with 0.68 μM COR (blank medium + COR), (4) 10^8 cfu ml^{-1} of *Pst* DC3000 suspension (*Pst* DC3000), or (5) *Pst* DC3000-derived medium (*Pst* DC3000 medium, without viable bacteria) containing 0.68 μM COR. The COR in the *Pst* DC3000-derived medium was produced by the bacteria during 6-day cultivation, and the concentration was measured before the start of the experiment. Second, to test the existence of interactions of COR with other defense elicitors present in *Pst* DC3000-derived medium on tomato resistance against WFT (Experiment 3), 4-week-old tomato plants were infiltrated with 0.2×, 0.4×, 0.6×, 0.8×, and 1.0× concentrations of (1) blank medium, (2) 0.68 μM COR diluted with blank medium, or (3) *Pst* DC3000-derived medium containing 0.68 μM COR. Third, to confirm the effect of other defense elicitors, present in *Pst* DC3000-derived medium, on tomato resistance against WFT (Experiment 4), 4-week-old tomato plants were infiltrated with (1) blank medium, (2) 0.14 μM COR diluted with blank medium, (3) culture medium derived from *Pst* DB29, a COR^- mutant bacteria of *Pst* DC3000, diluted five fold with blank medium, or (4) five fold diluted *Pst* DB29-derived medium containing 0.14 μM COR. In these three experiments, four leaflets (two top leaflets of each of leaves 2 and 3 from the bottom) from one tomato plant were pressure-infiltrated on their abaxial leaf sides with about 400 μl in total of corresponding treatments as described above. Seven days after infiltration, parts of the plants were sampled for PPO activity measurements and the other part was used for non-choice whole plant thrips bioassays.

Pst Cultivation and Suspension Preparation

Pst DC3000 and *Pst* DB29 were cultured in a King's B medium plate (King et al., 1954) supplemented with 100 μg ml^{-1} rifampicin and grown for 2 days at 28°C prior to use (Katagiri et al., 2002). The activated *P. syringae* pv. *tomato* (*Pst*) was then transferred to King's B liquid medium supplemented with 100 μg ml^{-1} rifampicin in a shaking incubator (200 rpm) at 28°C for 8 to 12 h (Katagiri et al., 2002).

To prepare *Pst* DC3000 suspension, the obtained *Pst* DC3000 King's B liquid culture was centrifuged at 4,000 rpm for 10 min at 4°C. The supernatant was discarded, and the bacteria pellet was re-suspended in sterilized MilliQ water. The bacteria suspension was diluted with sterilized MilliQ water to reach a concentration of 10^8 colony-forming units (cfu) ml^{-1}, estimated by an optical density at 600 nm of 0.5, which was used for the experiments.

Generation of *Pst*-Derived and Blank Medium

Pst-DC3000- and *Pst*-DB29-derived medium were obtained following the protocol of Palmer and Bender (1993) with some modifications. Briefly, 100 μl of the *Pst* King's B liquid culture obtained as described above was added to 20 ml Hoitink and Sinden medium optimized for COR production (also known as HSC) (nutrients per liter with a pH = 6.8: 1.0 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7H_2O$, 4.1 g KH_2PO_4, 3.6 g $K_2HPO_4 \cdot 3H_2O$, 0.3 g KNO_3, 20 μM $FeCl_3$, 20 g glucose) supplemented with 100 μg ml^{-1} rifampicin and grown in a shaking incubator (200 rpm) at 20°C for 6 days. The *Pst* culture (20 ml) was centrifuged at 4,000 rpm for 30 min at 4°C. The supernatants were filtered using 0.22 μm regenerated cellulose (RC) filters (Sartorius AG, Göttingen, Germany) to remove *Pst* from the medium. The absence of active bacteria in the *Pst*-derived medium was confirmed by spraying the filtered *Pst*-derived medium on plates of King's B medium and culturing the plates at room temperature. No colonies were detected at 3 days after the initial culture. Blank medium used as a control in Experiment 2, 3, and 4 was generated by incubating fresh HSC medium supplemented with 100 μg ml^{-1} rifampicin in a shaking incubator (200 rpm) at 20°C for 6 days. Thereafter, the HSC culture was centrifuged and the resulting supernatant was filtered using 0.22 μm RC filters. Presence of bacteria in the blank medium was also checked as described above for *Pst*-derived medium.

Measurements of Coronatine Concentration in *Pst* DC3000-Derived Medium

Concentration of COR in the *Pst* DC3000-derived medium was determined by HPLC as described by Palmer and Bender (1993) with slight modifications. In short, 20 ml of *Pst* DC3000-derived filtered medium was adjusted to pH = 9 and extracted twice with 20 ml of ethyl acetate. The aqueous phase was adjusted

to pH = 2 and then extracted three times with 20 ml ethyl acetate. The ethyl acetate phase was dried in 45°C water bath in 50 ml tubes. The COR was recovered by re-dissolving twice with 250 μM 20% acetonitrile. Three samples were analyzed on a reverse-phase C-8 column (150 mm × 4.6 mm, 5 μm particle size, Agilent Zorbax Eclipse XDB) at 208 nm. The mobile phases, A and B, were MilliQ water and HPLC-grade acetonitrile, respectively. The flow rate was kept constant at 1 ml/min. The gradient elution was as follows: 0.00 min at 80% A, 5.00 min at 35% A, 7.00 min/10% A, 8.00 min/10% A, 8.10 min/80% A, 10.00 min/80% A. The injection volume was 50 μl, and the column temperature was 25°C. Calibration curves for quantification of COR were constructed by using dilution series of commercially available COR (Sigma-Aldrich, St. Louis, MO, United States).

Growth of *Pst* DC3000 in Infiltrated Tomato Leaves

Bacteria growth in the leaflets of *Pst* DC3000-infiltrated plants was confirmed at 7 days after infiltration. For this, one of the *Pst* DC3000-infiltrated leaflets was surface sterilized by placing it in a 70% ethanol solution for 1 min, blotted briefly on paper towels and rinsed in sterile distilled water for 1 min. Thereafter, a leaf disk (1.5 cm diameter) was punched and placed in a 3-ml microfuge tube with 100 μl sterile distilled water. The samples were ground and subsequently vortexed for 10 s; 10 μl of the leaf disk solution was plated on King's B medium supplemented with 100 $\mu g\ ml^{-1}$ rifampicin and incubated at room temperature. Number of cfu was recorded at 3 days after incubation.

Non-choice Whole Plant Thrips Bioassay

A non-choice whole plant thrips bioassay was used to test tomato resistance against WFT (Leiss et al., 2009b). For this, plants were placed inside individual WFT-proof cages consisting of transparent plastic cylinders (50 cm height, 20 cm diameter), closed at the top with displaceable lids made of nylon gauze of 120 μm mesh size. Ten adult WFT (eight females and two males) were released into each cage. Plants were maintained in a climate room with 113.6 μmol photons $m^{-2} \cdot s^{-1}$ of PAR, 16L:8D of photoperiod, 25°C and 70% RH for 7 days. WFT feeding damage (hereafter referred as "silver damage") was evaluated in all the leaves of the plant 7 days after infestation, and expressed as the damaged area in millimeter square. Evaluation of WFT-associated leaf damage in the whole plant has been proved to correlate well with resistance-associated parameters such as number of larvae, adult survival, adult abundance, and preference (De Kogel et al., 1997; Jiang et al., 2005; Badenes-Pérez and López-Pérez, 2018), and it has been used in multitude of studies determining host plant resistance to WFT (e.g., Leiss et al., 2009a,b, 2013; Mirnezhad et al., 2010; Thoen et al., 2016; Escobar-Bravo et al., 2017; Badenes-Pérez and López-Pérez, 2018; Escobar-Bravo et al., in press[1]). Silver damage symptoms caused by infestation with 10 adult WFT were very subtle at 7 days after infestation, and it did not result in significant loss of leaf tissues (see **Supplementary Figure S1**). Thus, WFT development and feeding was not limited by the available plant material in the host plant.

Measurement of PPO Activity

Polyphenol oxidase (PPO) activity was measured in one of the infiltrated leaflets belonging to the second leaf from the bottom using the protocol described in Stout et al. (1998). In short, 0.150 g of frozen and ground plant material was homogenized in a 2 ml tube with 1.25 ml ice-cold 0.1 M pH 7.0 potassium phosphate buffer containing 7% polyvinyl polypyrolidine and 0.4 ml of 10% Triton X-100. The extracts were vortexed for 2 min and centrifuged at 11,000 × *g* for 10 min at 4°C. Five microliters of the enzyme extract were added to 1 ml of 2.92 mM chlorogenic acid solution in pH 8.0 potassium phosphate buffer. The optical density (OD) at 470 nm was recorded in a spectrophotometer (UV-1800, Shimadzu) every 10 s for 1 min. PPO activity was expressed as changes in OD values per min per gram of fresh weight.

Gene Expression Analysis by RT-qPCR

Expression of the JA- and SA-associated marker genes, the *wound-inducible proteinase inhibitor II* (*WIPI-II*, also known as *PI-II)* and the *pathogenesis-related protein 6* (*PR-P6*, also known as *PR-1b*) (Alba et al., 2015), respectively, were determined in mock-, COR-, and *Pst* DC3000-treated plants at 7 days after infiltration. The two infiltrated leaflets of leaf 3 from the bottom were flash frozen, homogenized, and stored at −80°C until analysis. Around 100 mg of the leaf material was used for RNA isolation. Total RNA was extracted using a phenol/LiCl method (Verdonk et al., 2003) followed by DNase (Ambion) treatment. Single strand cDNA was synthesized from 4 μg total RNA in a 20-μl reaction using a M-MuLV Reverse Transcriptase (Fermentas) according to manufacturer's recommendations. The quantity of targeted synthesized cDNAs was analyzed with real-time quantitative reverse transcription-PCR (qRT-PCR) in CFX96™ Optics Module (BIO-RAD) using iQ™ SYBR Green Supermix (BIO-RAD). The PCR protocol was set up in 20 μl reactions containing 0.25 μM of each primer and 1 μl of cDNA. The PCR program was as follows: 50°C for 5 min, 95°C for 2 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, followed by a melting curve analysis. Four biological replicates (i.e., individual plants) for each treatment were used for qRT-PCR analysis and two technical replicates were analyzed per treatment. *Actin* was used as internal standard for the normalization of expression levels for both targeted genes. The normalized expression (NE) of both genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). To illustrate the levels of gene expressions in plot, NE values were scaled to the treatment with the lowest average NE, which was set to 1. The gene specific qRT-PCR primers are shown in **Supplementary Method S1**.

Trichome Density Measurement

Type-VI glandular trichome density was determined on non-infiltrated leaflets of mock-, COR-, and *Pst* DC3000-treated

[1]Escobar-Bravo, R., Ruijgrok, J., Kim, H. K., Grosser, K., Van Dam, N. M., Klinkhamer, P. G. L., et al. (in press). Light intensity-mediated induction of trichome-associated allelochemicals increases resistance against thrips in tomato. *Plant Cell Physiol.* doi: 10.1093/pcp/pcy166

plants at 7 days after infiltration. For this, the second terminal leaflet of the third leaf from the bottom was used. Two pictures were taken in the middle section of the leaflet, at both sides of the midrib, in the adaxial and abaxial leaf sides by using a Leica stereomicroscope (MZ16, Leica Microsystems, Wetzlar, Germany). Each picture corresponded to an area of 12 mm^2. Trichome number was counted on the pictures using the software 64-bit Fiji ImageJ[2]. The average of these two measurements was calculated for each leaflet and expressed as number of type-VI trichomes per centimeter square.

Nuclear Magnetic Resonance (NMR) Analysis

NMR metabolomic analysis was performed on mock-, COR-, or *Pst* DC3000-infiltrated leaflets at 7 days after infiltration. For this, plant material was freeze-dried and ground using a tissue lyser (Qiagen, Hilden, Germany). Twenty milligrams of fine powder were extracted with 1.5 ml of 80% methanol-*d4* in KH_2PO_4 buffer (90 mM, pH = 6.0) containing 0.02% (w/v) trimethyl silyl-3-propionic acid sodium salt-*d4* (TMSP). Plant extracts were vortexed for 1 min, ultra-sonicated for 15 min, and centrifuged at 13,000 rpm for 15 min at room temperature. Eight hundred microliters of the supernatant was transferred to the NMR tubes for analysis.

The ^{1}H NMR spectra were acquired using a 600-MHz Bruker AV-600 spectrometer equipped with cryo-probe operating at a proton NMR frequency of 600 MHz at 25°C, as described in López-Gresa et al. (2012). Deuterated methanol served as internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min acquisition time with a digital resolution of 0.25 Hz/point, a pulse angle of 30° (10.8 μs), and a recycle delay of 1.5 s per scan. A pre-saturation sequence was used to suppress the residual water signal with low power selective irradiation at the H_2O frequency during the recycle delay. Spectra were Fourier transformed with a 0.3-HZ line broadening and zero-filled to 32 K points. Phase and baseline correction of the resulting spectra were done manually, followed by a calibration to TMSP at 0.00 ppm using Topspin (version 2.1, Bruker). ^{1}H NMR spectra were then converted and saved as ASCII files using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to the intensity of the internal standard TMSP and reduced to integrated regions, referred to as buckets, of equal width (0.04 ppm) corresponding to the region of δ 10.0–0.2. The regions in the range of δ 4.92–4.70 and δ 3.33–3.28, corresponding to water and methanol, respectively, were removed prior to statistical analyses.

Statistical Analysis

All statistical analyses were performed using the SPSS software package (version 23; SPSS Inc., Chicago, IL, United States). Effect of mock-solution, COR, *Pst* DC3000, or *Pst* DC3000-derived medium infiltration on silver damage symptoms, type VI trichome density, PPO activity, and normalized expression of *WIPI-II* and *PR-P6* (Experiments 1 and 2) were analyzed by one-way ANOVA, followed by Fisher's least significant difference (LSD) *post hoc* test. Residuals of the data were first tested for normality and homogeneity of variance. Data on silver damage and *WIPI-II* and *PR-P6* expression obtained from Experiment 1 and PPO activity determined in Experiment 2 were Log transformed prior to analysis to meet ANOVA assumptions. Effect of treatments (blank medium, blank medium + COR, and *Pst* DC3000-derived medium), concentration (0.2, 0.4, 0.6, 0.8, and 1.0×), and the interaction between these two factors (Experiment 3) on silver damage symptoms, and PPO activity was determined by generalized linear models (GLM) using linear distribution and identity link functions, followed by LSD *post hoc* test. Data on silver damage were Log-transformed prior to analysis. Effect of COR, *Pst* DB29-derived medium, and their interaction (Experiment 4) on silver damage and PPO activity was analyzed by GLM using linear distribution and identity link functions, followed by LSD *post hoc* test. Data on silver damage were Log-transformed prior to analysis. Patterns of chemical shifts detected by NMR in leaf extracts of mock-, COR-, or *Pst* DC3000-treated plants were subjected to multivariate analysis using the SIMCA-P 15 software package (Umetrics, Umeå, Sweden). Supervised partial least squares discriminant analysis (PLS-DA) was applied to determine the variation in X variables (chemical shifts) modeled by the Y explanatory variable corresponding to mock, COR or *Pst* DC3000 treatments. R^2X and R^2Y is the cumulative variation explained by the PLS-DA model in variable X and Y, respectively. Q^2 is the cumulative predicted variation in Y, according to cross-validation. The final model was the one with minimum number of latent variables showing the highest value of Q^2. The chemical shifts with a variable importance in projection (VIP) > 1 were selected as the important X variables, some of which were identified and tested using a nonparametric analysis followed by non-parametric Kruskal–Wallis test. Detailed statistical results are shown in **Supplementary Table S1**.

RESULTS

Infiltration of COR or *Pst* DC3000 Increases Tomato Resistance Against WFT

Infiltration of tomato plants with COR or *Pst* DC3000 reduced silver damage by 47% and 37%, respectively, compared to the mock-treated plants (ANOVA: $P < 0.05$, **Figure 1**). Overall, this reduction was evident in both infiltrated and non-infiltrated leaves of COR- and *Pst* DC3000-treated plants (ANOVA: $P < 0.05$, **Supplementary Figure S2**).

COR and *Pst* DC3000 Induced JA-Signaling, and COR Induced Both JA and SA

To further determine the mechanism of COR and *Pst* DC3000-mediated induction of tomato defenses against WFT, expression of JA- and SA-responsive genes, as well as the activity of the JA-associated defensive protein PPO, were analyzed at 7 days after infiltration. Both COR and *Pst* DC3000 infiltration strongly induced PPO activity in infiltrated tomato leaves (ANOVA:

[2]http://fiji.sc/Fiji

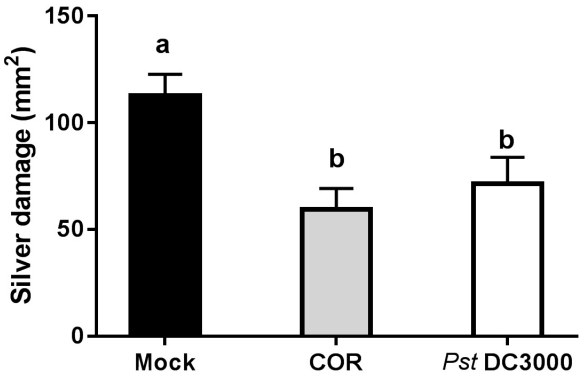

FIGURE 1 | Effect of COR and *Pst* DC3000 on tomato resistance against WFT. Silver damage symptoms (mean ± SEM, $n = 15$) in tomato plants infiltrated with mock solution (mock), coronatine (COR), or *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000). Plants were infested with western flower thrips (WFT) at 7 days after the initial treatment and evaluated 7 days after WFT infestation. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P < 0.05$.

$P < 0.05$, **Figure 2A**). Similarly, the expression of *WIPI-II*, a JA marker gene, was about 900 and 1,300 times higher in COR- and *Pst* DC3000-infiltrated plants, respectively, than in mock-treated leaves of control plants (ANOVA: $P < 0.05$, **Figure 2B**). Interestingly, for *PR-P6*, a SA marker gene, a 28-times higher expression was observed in COR-treated plants, but not in mock and *Pst* DC3000-infiltrated plants (ANOVA: $P < 0.05$, **Figure 2C**).

Infiltration of Tomato Plants With COR or *Pst* DC3000 Does Not Increase Type-VI Trichome Density

To determine whether COR and *Pst* DC3000 induce trichome-associated defenses against WFT, type-VI trichome density was determined on both adaxial and abaxial leaf sides of mock-, COR-, and *Pst* DC3000-treated plants. Surprisingly, type-VI trichome density in the adaxial leaf side was marginally decreased by COR or *Pst* DC3000 infiltration (ANOVA: $P = 0.071$, **Figure 3A**). However, type-VI trichome density on abaxial leaf sides was slightly reduced in COR-treated plants in comparison to *Pst* DC3000- and mock-treated plants (ANOVA: $P < 0.05$, **Figure 3B**).

COR and *Pst* DC3000 Induced Similar Metabolomic Changes in Infiltrated Tomato Leaves

A total of 244 signals were obtained from ^{1}H NMR measurement of the mock-, COR-, and *Pst* DC3000-treated tomato plants. The multivariate PLS-DA analysis of the NMR signal profiles resulted in a model with five latent variables (LVs) that cumulatively explained 74.6% of the total metabolomic variation and 91.1% of the elicitor agent treatments, with a 40.7% total model predictability (model statistics: $R^2X = 0.746$, $R^2Y = 0.911$, and $Q^2 = 0.407$) (**Figure 4**). The first LV explained 23.3% of the variance and separated mock – from both COR – and *Pst* DC3000-treated plants (**Figure 4A**). The second LV explained 15.8% and separated COR – from *Pst* DC3000-treated plants. The discriminated patterns among mock-, COR-, and *Pst* DC3000-treated plants were mainly explained by 80 signals with VIP scores higher than 1 (**Figure 4B** and **Supplementary Figure S3**). Among these 80 NMR signals, 22 were identified, which corresponded to 16 different compounds (**Figure 4C**), including isoleucine (δ 0.96), leucine (δ 1.00), valine (δ 1.04), alanine (δ 1.48), acetate (δ 1.92), glutamate (δ 2.04), malic acid (δ 2.48), aspartic acid (δ 2.64, 2.68, 2.80), citric acid (δ 2.72), gamma-aminobutyric acid (GABA, δ 3.00), ethanolamine (δ 3.12), sucrose (δ 5.40), chlorogenic acid (δ 6.40, 6.44, 6.88), rutin (δ 6.52, 7.00), fumaric acid (δ 6.56), and phenylalanine (δ 7.56). Both COR and *Pst* DC3000 treatments significantly increased leaf content of aspartic acid, ethanolamine and fumaric acid. However, increased GABA and rutin levels were only observed in *Pst* DC3000-treated plants (**Figures 4D–H**). For the other identified compounds, we did not find significant differences among treatments.

Pst DC3000-Derived Medium Enhances Tomato Resistance Against WFT

To confirm that *Pst* DC3000-derived compounds are responsible for the induced resistance against WFT in tomato, we infiltrated plants with the *Pst* DC3000-derived medium (containing COR) but no viable bacteria, and compared the effect on tomato resistance against WFT with those triggered by the infiltration with water mock, blank medium control, blank medium + COR, and *Pst* DC3000. Silver damage symptoms were significantly reduced in tomato plants infiltrated with COR, *Pst* DC3000-, or *Pst* DC3000-derived medium compared to water mock or blank medium-treated plants (ANOVA: $P < 0.05$, **Figure 5A**). This reduction in silver damage was stronger in infiltrated leaves, when compared to systemic leaves (i.e., non-infiltrated leaves) (**Supplementary Figure S4**). In addition, PPO activity was induced in blank medium + COR-, *Pst* DC3000-, and *Pst* DC3000-derived medium-treated tomato plants compared to water mock and blank medium controls (ANOVA: $P < 0.05$, **Figure 5B**). This confirms the role of COR on the induction of tomato defenses against WFT, and that no bacterial infection is required to elicit WFT resistance.

Existence of Other Defense Elicitors Besides COR Existed in *Pst* DC3000-Derived Medium

In the previous experiment, plants infiltrated with COR or *Pst* DC3000-derived medium (containing 0.68 μM COR as in the COR treatments) showed a similar reduction in silver damage symptoms. Yet, the effect of other defense elicitors present in *Pst* DC3000-derived medium might have been masked by the high concentration of COR in the medium. Thus, we further assessed the effect of serial dilutions of blank medium, blank medium + COR, and *Pst* DC3000-derived medium on tomato resistance against WFT and PPO induction (**Figure 6A**). *Pst*

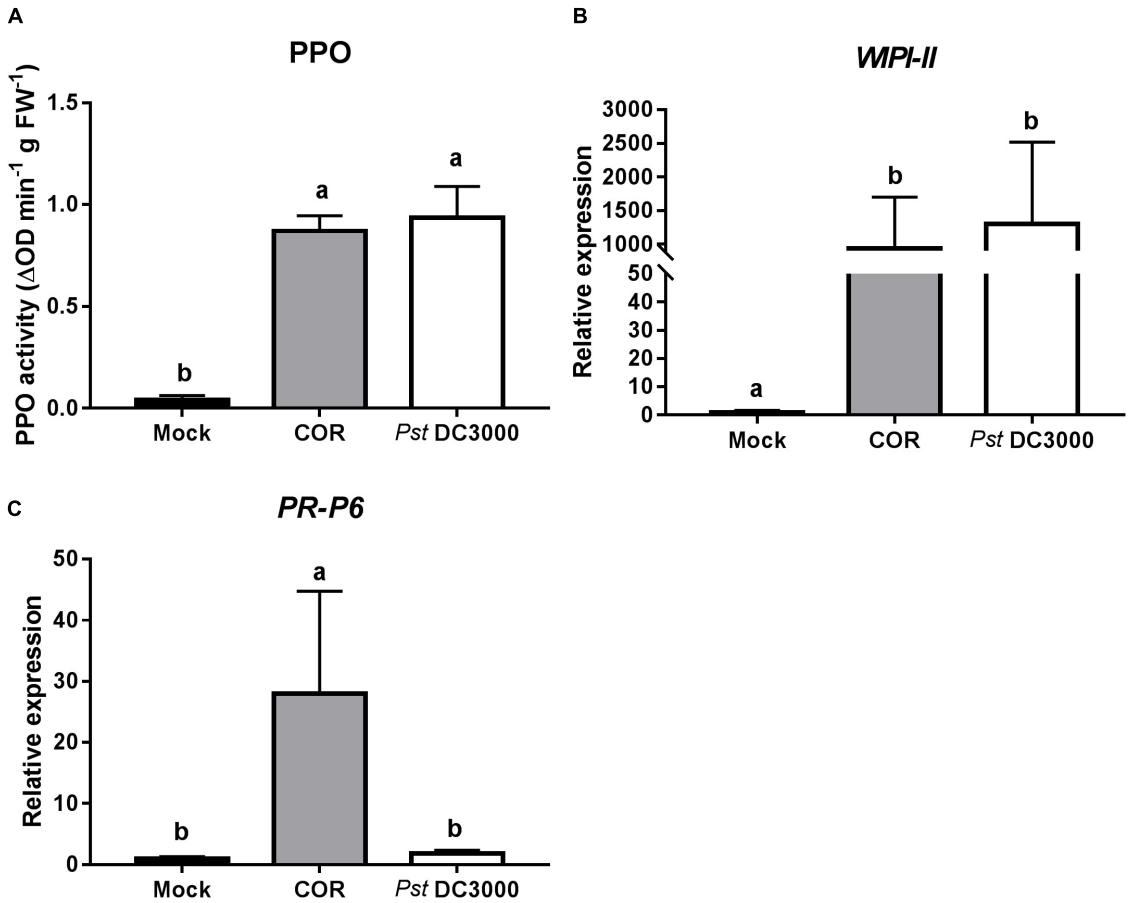

FIGURE 2 | Effect of COR and *Pst* DC3000 on jasmonic acid- and salicylic acid-associated responses. **(A)** Polyphenol oxidase (PPO) activity (mean ± SEM, $n = 5$) and relative transcript levels of **(B)** the JA-responsive gene *wound inducible proteinase inhibitor-II* (*WIPI-II*) and **(C)** the SA-responsive gene *pathogenesis related protein 6* (*PR-P6*) (mean ± SEM, $n = 5$) were measured in tomato plants at 7 days after infiltration with coronatine (COR), *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000), or a mock solution (mock). The analysis was performed on infiltrated leaflets from the bottom second/third leaf. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P < 0.05$.

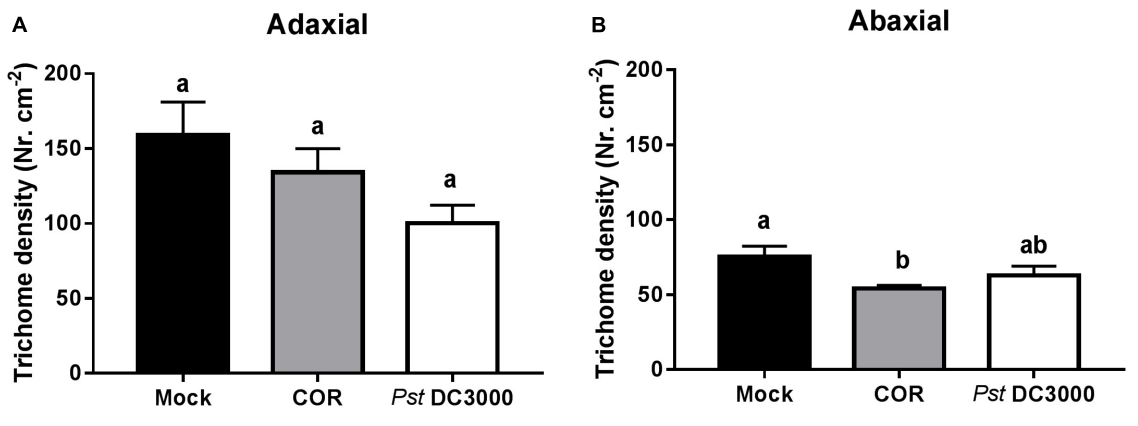

FIGURE 3 | Effect of COR and *Pst* DC3000 on type VI trichome density. Type VI trichome density (mean ± SEM, $n = 10$) on **(A)** adaxial or **(B)** abaxial leaf side was determined in tomato plants infiltrated with coronatine (COR), *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000), or a mock solution (mock) at 7 days after the initial treatments. The analysis was performed in leaflets collected from the third youngest leaf. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P < 0.05$.

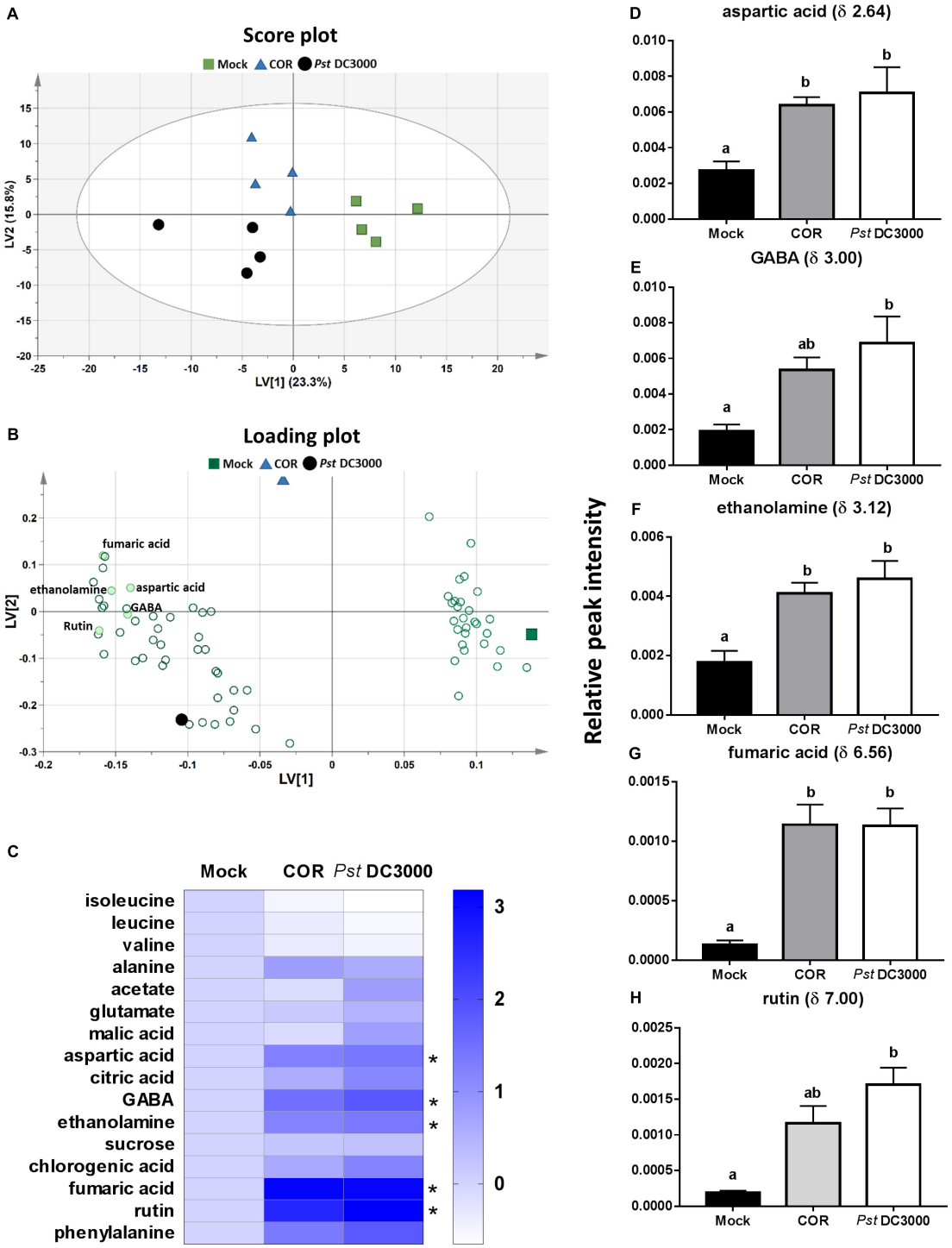

FIGURE 4 | Metabolome responses of tomato plants to COR and *Pst* DC3000 infiltration. Leaf metabolites were analyzed on tomato leaves infiltrated with coronatine (COR), *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000), or a mock solution (mock) by NMR at 7 days after the initial treatment. Partial least square-discriminant analysis (PLS-DA) was performed based on ^{1}H-NMR spectra (n = 4 individual plants), and resulted in five latent variables (LVs) that cumulatively explained 74.6% of the total metabolomic variation and 91.1% of the treatment response, with a 40.7% total model predictability. **(A)** Score plot showing the first two LVs. The ellipse represents the Hotelling T2 with 95% confidence in score plot. **(B)** Loading plot showing important metabolites contributing most to the model (VIP score > 1). **(C)** Heatmap of the identified 16 compounds. Each of the three Heatmap columns represents the $\log_2$ fold change of relative peak intensity from one of the treatments Mock, COR, or *Pst* DC3000 in comparison to Mock. Thus, all $\log_2$ fold change of compounds in mock treatment was 0 (fold change = 1) as shown in the first column. **(D–H)** Relative peak intensities (mean ± SEM, n = 4) of five metabolites (aspartic acid, GABA, ethanolamine, fumaric acid, and rutin) identified in the ^{1}H NMR spectra that significantly differed among treatments. Different letters indicate significant differences among treatments tested by Mann–Whitney U test, $P < 0.05$.

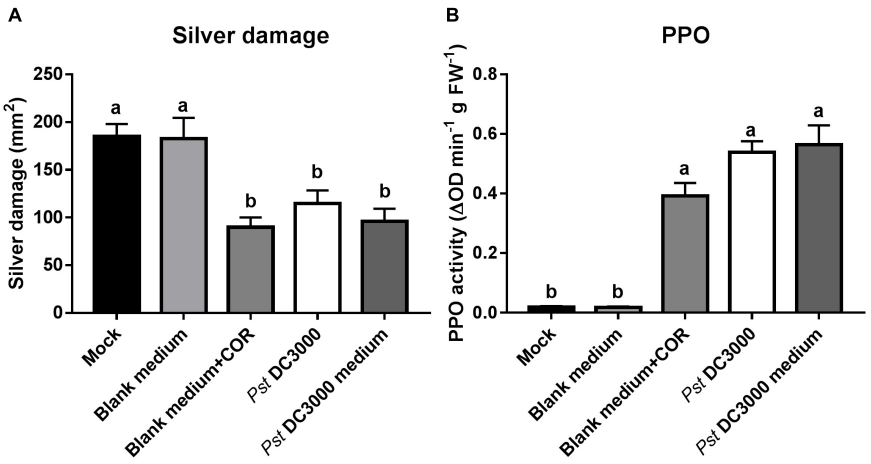

FIGURE 5 | Effect of *Pst* DC3000-derived medium on tomato resistance against WFT and JA-associated responses. **(A)** Silver damage symptoms (mean ± SEM, $n = 10$) in tomato plants infiltrated with mock solution (mock), blank medium, 0.68 μM coronatine (COR) dissolved in blank medium (blank medium + COR), *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) suspension, or *Pst* DC3000-derived medium (containing 0.68 μM of COR). Plants were infested with western flower thrips (WFT) at 7 days after the initial treatment and evaluated 7 days after WFT infestation. **(B)** Polyphenol oxidase (PPO) activity (mean ± SEM, $n = 5$) was measured in the second leaf from the bottom of tomato plants pressure infiltrated with the above described treatments at 7 days after the initial treatment. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P < 0.05$.

DC3000-derived medium- and blank + COR-treated plants showed a significant reduction in silver damage symptoms (GML: $P < 0.05$ for treatment; $P = 0.078$ for dilution; $P = 0.383$ for the interaction). Notably, a stronger reduction in silver damage symptoms was observed in tomato plants treated with 0.2× concentration of *Pst* DC3000-derived medium when compared to 0.2× blank medium and 0.2× blank medium + COR. As these differences were only found at 0.2× concentration, this might explain why the interaction factor between treatment and dilution was not statistically significant. These results suggest that there might be other plant defense elicitors in *Pst* DC3000-derived medium that, maybe in combination with COR, trigger stronger plant defense responses against WFT than COR alone (i.e., in blank medium + COR treatment). Indeed, at 0.2× concentration, induction of the PPO activity was significantly higher in *Pst* DC3000-derived medium-treated plants than in those infiltrated with blank medium + COR (GLM: $P < 0.05$ for interaction) (**Figure 6B** and **Supplementary Table S1**). No significant differences in PPO activity between *Pst* DC3000-derived medium- and blank + COR-treated plants were observed at 0.6, 0.8, and 1.0× concentration.

Confirmation of the Existence of Other Defense Elicitors in *Pst* DC3000-Derived Medium

Our previous results showed that while the effect of COR on tomato defenses against WFT was concentration-dependent, the effect of the *Pst* DC3000-derived medium was not, thus pointing out to the existence of other defense elicitors in *Pst* DC3000-derived medium. To further investigate this, we tested the effect of medium obtained from a COR defective mutant of *Pst* DC3000 (*Pst* DB29), blank medium, or both treatments supplemented with a low concentration of COR (0.14 μM) on WFT resistance and PPO activity (**Figure 7**). Silver damage symptoms did not significantly differ between plants infiltrated with blank medium and blank medium + COR (GLM: $P = 0.994$, for COR treatment) (**Figure 7A**), thus confirming our previous results. Yet, a small reduction in silver damage was observed in the infiltrated leaves of blank medium + COR (**Supplementary Figure S5**). Infiltration of plants with *Pst* DB29-derived medium without COR, however, significantly reduced silver damage symptoms when compared to blank medium and blank medium + COR treatments (GLM: $P < 0.05$ for the *Pst* DB29-derived medium; $P < 0.05$ for the interaction). This reduction was significant in both infiltrated and non-infiltrated leaves (**Supplementary Figure S5**). PPO activity was significantly induced by COR and *Pst* DB29-derived medium (GLM: $P < 0.05$ for COR treatment; $P < 0.05$ for the *Pst* DB29-derived medium). Furthermore, *Pst* DB29 + COR-treated plants showed a slight higher PPO induction when compared to *Pst* DB29-derived medium and blank medium + COR treatments (GLM: $P = 0.104$ for their interaction) (**Figure 7B**).

DISCUSSION

Activation of defense-associated signaling pathways by using natural or synthetic defense elicitors has shown to increase plant resistance against different arthropod herbivores, and it might be regarded as a valuable strategy for pest control in agriculture (Thaler, 1999) in combination with other IPM techniques, such as biological control. Here, we have shown that infiltration with COR, *Pst* DC3000, or *Pst*-derived medium increased tomato resistance against WFT through the induction of enzymatic and chemical defenses.

Our results first showed that infiltration of tomato plants with the COR-producing bacteria *Pst* DC3000 or COR alone

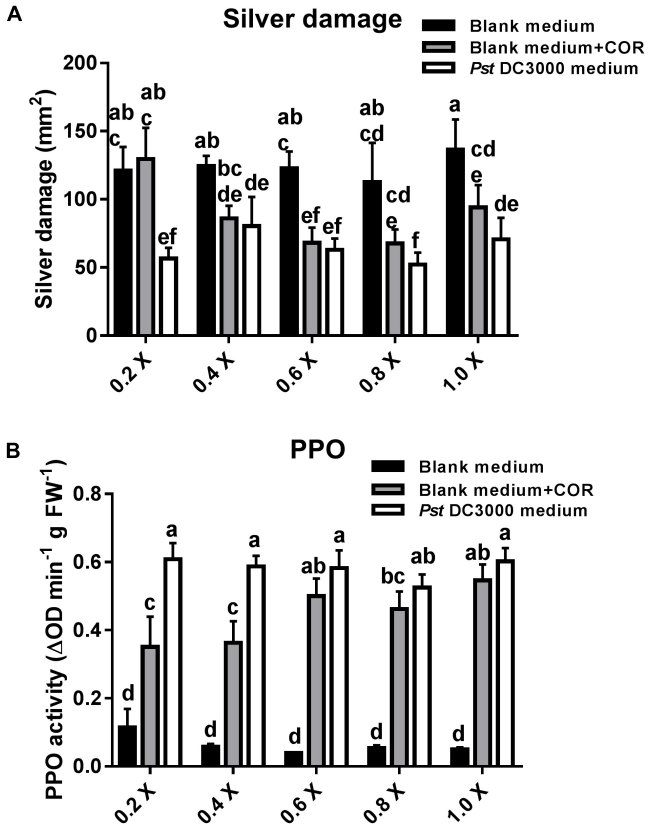

FIGURE 6 | Effect of different concentrations of COR in *Pst* DC3000-derived medium on WFT resistance and JA-associated responses. **(A)** Silver damage symptoms (mean ± SEM, $n = 7$) in tomato plants infiltrated with 0.2, 0.4, 0.6, 0.8, or 1.0× concentrations of (1) blank medium, (2) blank medium + coronatine (COR) (0.64 μM), or (3) *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000)-derived medium (no viable bacteria, containing 0.64 μM of COR). Plants were infested with western flower thrips (WFT) at 7 days after the initial treatment and evaluated 7 days after WFT infestation. **(B)** Polyphenol oxidase (PPO) activity (mean ± SEM, $n = 5$) was measured in the second leaf from the bottom of plants infiltrated with the above described treatments at 7 days after the initial treatment. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P < 0.05$.

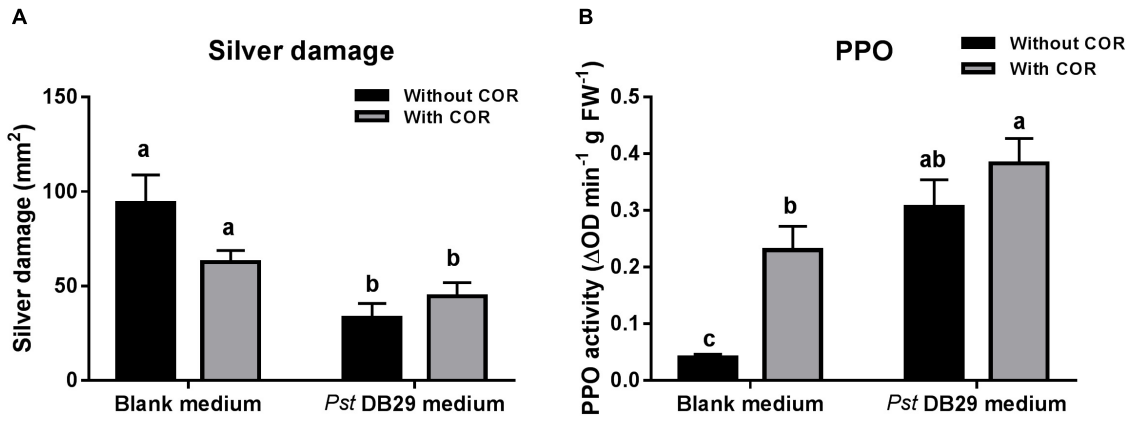

FIGURE 7 | Effect of COR and *Pst* DB29 medium on WFT resistance and JA-associated responses. **(A)** Silver damage symptoms (mean ± SEM, $n = 10$) determined in tomato plants infiltrated with blank medium, 0.14 μM coronatine (COR) in blank medium, *Pseudomonas syringae* pv. tomato DB29 (*Pst* DB29)-derived medium diluted five fold with blank medium or 0.14 μM COR in *Pst* DB29-derived medium diluted five fold with blank medium. Plants were infested with western flower thrips (WFT) at 7 days after the initial treatment and evaluated 7 days after WFT infestation. **(B)** Polyphenol oxidase (PPO) activity (mean ± SEM, $n = 5$) was measured in the second leaf from the bottom of tomato plants infiltrated with the above described treatments at 7 days after the initial treatment. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P < 0.05$.

significantly reduced WFT-associated damage in non-choice whole plant bioassays (**Figure 1**). This is in line with previous reports. Cui et al. (2005) described that the increased susceptibility to the caterpillar *Trichoplusia ni* in *Arabidopsis* plants infiltrated with virulent strains of *P. syringae* ES4326 was counteracted by COR, and that COR alone increased *Arabidopsis* resistance to this caterpillar. In tomato, Stout et al. (1999) described that infiltration with *P. syringae* pv. tomato significantly reduced *Helicoverpa Zea* larvae growth. Here we report on the effects of both *Pst* DC3000 and COR infiltration on tomato resistance against WFT. Furthermore, we show that not only COR, but that presence of other defense elicitors in *Pst*-derived medium can increase tomato resistance to WFT.

The enhancement of plant defenses against arthropod herbivores by infiltration of COR-producing *Pst* or COR itself has been explained by the strong induction of the JA-associated defense signaling pathway and suppression of the SA defense signaling (Cui et al., 2005). Analysis of the effect of COR and *Pst* DC3000 infiltration on the activation of JA and SA signaling pathways showed that both treatments strongly induced the expression of the JA-associated gene marker *WIPI-II*, which encodes for a proteinase inhibitor II protein (PI-II), and increased activity of the JA-related defensive enzyme PPO at 7 days after the infiltration. This agrees with previous results described by Stout et al. (1999), who found that *Pst* infiltration increased PI-II and PPO activities in infiltrated tomato plants. *Pst* DC3000 is reported to activate JA signaling in tomato (Zhao et al., 2003; Uppalapati et al., 2005) and *Arabidopsis* (He et al., 2004), which is proposed to be explained by the action of *Pst* DC3000-derived COR and type III effectors (He et al., 2004). Our results showed that application of COR also induced the expression of the SA-associated gene marker *PR-P6*, a pathogen defense-related gene (PR) (**Figure 2C**). Yet, the magnitude of the induction of *PR-P6* was approximately 30 times lower than that of *WIPI-II* in COR-treated plants. Both COR treatment and infection with *Pst* DC3000 lead to slight increases of SA levels in *Arabidopsis* (Uppalapati et al., 2005) and tomato (Zhao et al., 2003). In tomato, this induction has been described to be stronger in COR deficient *Pst*, and thus it was suggested to be highly suppressed by the activation of JA signaling in COR-producing *Pst* (Zhao et al., 2003). The lack of induction of *PR-P6* in *Pst* DC3000-infected tomato plants (**Figure 2B**) might be explained by our sampling time for gene expression analysis. Hence, induction of PRs has been generally observed 24 h after *Pst* infiltration (Zhao et al., 2003; Uppalapati et al., 2008; López-Gresa et al., 2011). Overall, the strong activation of JA-associated defenses by *Pst* DC3000 and COR infiltration might explain the increased tomato resistance against WFT. Previous studies have shown that induction of JA defenses can reduce WFT-associated damage in tomato and other plant species (Li et al., 2002; Abe et al., 2009; Escobar-Bravo et al., 2017). Activation of JA defense signaling is often associated with reduced plant growth (Guo et al., 2018). Interestingly, our results showed that neither COR or *Pst* DC3000 infiltration significantly affected plant dry biomass or height of tomato plants (**Supplementary Figure S6**). Additionally, we did not detect any *Pst* DC3000 colonies in systemic leaves of *Pst* DC3000-infiltrated plants, but only in the local leaves (**Supplementary Figure S7**), confirming that even localized *Pst* DC3000 infections have a great impact on tomato defenses against other biotic stressors.

Activation of JA signaling pathway through exogenous application of jasmonates, such as the volatile form of JA methyl jasmonate (MeJA) (Boughton et al., 2005; Maes and Goossens, 2010; Tian et al., 2012; Escobar-Bravo et al., 2017) is reported to increase type VI glandular trichome density in tomato leaves. We thus hypothesized that infiltration with *Pst* DC3000 or COR might induce these tomato defenses as well. Our results, however, showed that none of these treatments increased type VI trichome densities in newly formed leaves at 7 days after infiltration. This might be explained by differences in the magnitude of the induction of JA defenses when plants are treated with exogenous application of COR or *Pst* DC3000 infiltration, but also by the activation of different defense signaling pathways. Hence, although COR and MeJA application shared similar activities on tomato plants, some sets of genes are differently regulated by these two compounds (Uppalapati et al., 2005; Tsai et al., 2011). Both COR and *Pst* DC3000 are reported to induce JA, ET, and auxin signaling pathways (O'donnell et al., 2003; Cohn and Martin, 2005; Uppalapati et al., 2005), and COR slightly induced SA signaling as well. Whether the induction of these signaling pathways explains the lack of induction of trichomes in COR and *Pst* DC3000 infiltrated plants would require further research. Alternatively, COR-mediated activation of SA signaling might have attenuated JA-mediated induction of trichomes (Traw and Bergelson, 2003), as both signaling are known to interact via antagonistic crosstalk (Pieterse et al., 2012). Together, these results suggest that COR- and *Pst*-DC3000-mediated induction of tomato resistance against WFT is not explained by increased type-VI trichome densities.

An untargeted metabolomic analysis of tomato leaves infiltrated with COR or *Pst* DC3000 revealed that both treatments induced similar but not the same metabolomic changes. Both COR and *Pst* DC3000 increased the leaf content of organic acids, phenolics, and amino acids (**Figure 4**). These results are in agreement with those reported by López-Gresa et al. (2010, 2011), where higher concentrations of amino acids, organic acids, rutin, and phenylpropanoids were detected in *Pst*-infected tomato plants. However, no comparison between the effects of COR and *Pst* infiltration on plant metabolome has been performed before. Interestingly, our results showed that the levels of the amino acid aspartic acid and the non-protein amino acid GABA, as well as the phenolic rutin, were slightly higher in *Pst* DC3000-infiltrated tomato leaves. Yet, these differences did not affect the levels of resistance of tomato plants against WFT, as both COR and *Pst* DC3000 significantly reduced silver damage symptoms in infiltrated plants (**Figure 1**). The increase in some of these compounds might have influenced tomato defenses against WFT. For instance, high concentrations of the flavonoid rutin (quercetin-3-O-β-rutinoside) have been reported to deter herbivore feeding (reviewed by Simmonds, 2001). On the other hand, increases in GABA levels are reported to occur in *Pst* DC3000-infected plants (Ward et al., 2010), but also in response to other biotic and abiotic stresses (Bouché et al., 2003). Although all the functions of GABA in plants have not been completely elucidated, it is induced upon herbivory or insects crawling on

the leaf surface (Bown et al., 2002; Scholz et al., 2015), and it has a negative effect on arthropod's performance when ingested by feeding in transgenic plants with elevated GABA levels or in GABA-enriched artificial diets (Ramputh and Bown, 1996; McLean et al., 2003; Scholz et al., 2015). Yet, whether its induction might affect tomato resistance against WFT would need further research.

Our results further showed that application of *Pst* DC3000-derived medium (without viable *Pst* bacteria and containing 0.68 μM of COR), COR (0.68 μM), or *Pst* DC3000, all increased tomato resistance against WFT (**Figure 5A**). Moreover, these treatments increased PPO activities in infiltrated leaves, indicating the activation of JA signaling (**Figure 5B**). Hence, infiltration of tomato plants with ca. seven times less COR than in our initial experiments (i.e., 5 μM, see **Figure 1**) resulted in a similar reduction in silver damage symptoms. This suggested that COR has a strong impact on tomato defenses even at low concentrations, and that we might have overlooked the possible effect of other defense elicitors present in *Pst* DC3000-derived medium. This prompted us to further investigate whether infiltration with much lower concentrations of COR alone or in *Pst* DC3000-derived medium had the same effects on tomato resistance against WFT. Notably, infiltration of tomato plants with a 0.2× concentration of *Pst* DC3000-derived medium (containing 0.14 μM of COR) resulted in a stronger reduction of silver damage symptoms than application of COR (0.14 μM) alone dissolved in blank medium. Moreover, induction of PPO activity was higher in plants infiltrated with a 0.2× concentration of *Pst* DC3000-derived medium than with a 0.2× concentration of COR or blank medium. Hence, this suggests that the presence of other defense elicitors in *Pst* DC3000-derived medium might increase tomato resistance against WFT, and that this induction is also probably explained by a stronger activation of JA signaling. Indeed, further assays using a COR deficient mutant of *Pst* DC3000, *Pst* DB29, showed that tomato plants infiltrated with *Pst* DB29-derived medium displayed lower silver damage symptoms after WFT infestation and induced PPO activities as well (**Figures 7A,B**). It should be noted that *Pst* DB29 is defective in the synthesis of COR precursors, CFA and CMA, both reported to induce some JA/wound associated plant responses in tomato, but at much less magnitude than COR (Uppalapati et al., 2005). Thus, activation of tomato defenses against WFT could not be explained by the presence of CFA and CMA in the *Pst* DB29-derived medium. We hypothesize that these responses might be explained by changes in the culture medium composition in terms of (1) primary or secondary metabolites modified in the medium by the *Pst* growth, or (2) presence of *Pst*-derived effectors. For instance, He et al. (2004) described that effectors secreted by the type III secretion system of *Pst* DC3000 can augment the JA-signaling pathway to promote virulence. Nevertheless, this requires further research.

In summary, our study shows that infiltration with COR and *Pst* DC3000 increases tomato resistance against WFT by activating JA-associated defenses, but not type-VI leaf trichome densities. Our results also show that *Pst* DC3000-derived medium contains other defense elicitors that can increase resistance against WFT in infiltrated tomato plants, thus providing a potential treatment for WFT control in agriculture systems.

AUTHOR CONTRIBUTIONS

GC, RE-B, PK, and KL carried out the experimental design. GC conducted the infiltration, insect experiments, and chemical analyses. GC and RE-B performed the RT-qPCRs. GC, RE-B, PK, and KL performed the data analysis and interpretation. HK interpreted the NMR data. GC wrote the draft manuscript. All authors critically reviewed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01417/full#supplementary-material

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METHODS
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A Robust Functional Genomics Approach to Identify Effector Genes Required for Thrips (*Frankliniella occidentalis*) Reproductive Performance on Tomato Leaf Discs

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Thrips (*Frankliniella occidentalis*) is a persistent plant pest that is able to vector pathogenic viruses. Natural plant resistance to thrips has become a prominent breeding target in commercial crops. The main reason for this is the shift toward banning key pesticides used for controlling thrips infestations and the lack of effective alternatives. Despite this urgent need for crop plants that are resistant, or tolerant, to thrips infestation, the toolbox for studying genetic resistance to this insect is still underdeveloped. Essentially, there is a lack of robust protocols for the screening and identification of thrips genes relevant to its performance on crop plants. To bridge this gap, we have developed a functional analysis screening method. Our approach relies on the, *Agrobacterium tumefaciens*-mediated, homogeneous, and transient ectopic expression of thrips genes in large tomato leaf discs followed by the assessment of thrips reproductive performance. The setup is designed to maintain gene expression during the course of the assay, where GFP signal in the control treatment is used as a reporter of expression. The screen is conducted in a climate box under controlled settings. As a result, multiple genes can be screened for their effect on thrips reproductive performance in a single experiment and in a relatively small space, without the need for generating stable transgenic plants. The method also eliminates the need for a greenhouse equipped to accommodate the combination of *A. tumefaciens*-infiltrations and thrips infestations. It is not only flexible and convenient for screening genes encoding putative thrips effectors but also for plant resistance genes or effector-targets of host plants and can be adapted for other crop plants, or other herbivorous arthropods.

Keywords: thrips bioassay, resistance, effectors, functional-genomics, tomato

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INTRODUCTION

Western flower thrips, *Frankliniella occidentalis* (Pergande), is a piercing-sucking pest which puncture plant cells with a specialized mandible and then ingest the cell content (Hunter and Ullman, 1989, 1992). Damaged cells die and form a phenotype visible on the surface of plant leaves and fruits, commonly termed as "silvery damage." If the damaged cells are in developing organs like young leaves, small fruits or flower pods, then infestation usually results in the deformation

of these organs. On top of this, thrips is a successful vector in transmitting several economically important plant viruses, like the tomato spotted wilt virus (TSWV), from infected to healthy plants (Steenbergen et al., 2018). The search for genetic resistance against thrips has been ongoing for more than two decades (Fery and Schalk, 1991; Douglas, 2018). An increasingly used strategy to breed resistant plants or to understand the arms race between plants and their attackers is to identify effector proteins released by the attacker during the interaction with the plant. This has been extensively done for microbial plant pathogens. Subsequently, the identified effectors are either used to directly identify resistance genes, usually in wild germplasm, or to identify their targets in the plant (Vleeshouwers and Oliver, 2014; Khan et al., 2018). The latter approach can provide valuable information to understand the effector-mediated mechanism of disease and thus explore novel strategies for resistance-breeding programs. Recently it has been shown that aphids and spider mites produce effector proteins in their saliva to manipulate plant immunity (Bos et al., 2010; Mugford et al., 2016; Villarroel et al., 2016). Interestingly, an effector from aphid (*Myzus persicae*) was found to target a protein involved in endomembrane vesicle trafficking in *Nicotiana benthamiana*, demonstrating that herbivorous insects utilize effectors to target host proteins and to increase their virulence on the host (Rodriguez et al., 2017).

The recent banning of key chemicals used for controlling thrips infestations and the emergence of heritable pesticide resistance in thrips (Kirk and Terry, 2003) has emphasized the importance of identifying genetic plant resistance against thrips. However, the toolbox for studying genetic resistance to this insect is still very limited. The main reason for this is that studying the contribution of individual genetic factors in crop plants often require the generation of stable transgenic lines. Moreover, the small size and fast movement of thrips, especially adults, make them champions in escaping from experimental bioassay setups and hence the results of experiments may suffer from bias. Although screens which rely on transient gene expression in model plants has been used to identify effectors from other herbivorous arthropods (Bos et al., 2010; Villarroel et al., 2016), the application of this methodology for thrips might face a number of challenges. First, the use of model plants, although ensuring efficient gene expression, might be suboptimal as host and thus less suitable for evaluating insect performance. As a consequence, this can lead to a failure in correctly measuring the response caused by the treatment and may cause inconsistencies during verification in host plants. Second, suitable hosts are often not optimal for transient gene expression by the traditional pressure-based agro-infiltration of leaves. This is either due to the low transformation competence or the induction of necrosis, like in tomato leaves when pressure-infiltrated with the *Agrobacterium tumefaciens* strain GV3101 (Wroblewski et al., 2005). In that case, necrosis is visible at the infiltration sites in response to the used bacterial strain and the wounding caused by the infiltration. It is therefore often difficult to obtain a uniform expression in the host tissue which leads to an increase in treatment-independent variations in the final bioassay results. To overcome these issues, we have optimized a functional analysis method to screen for genes required for thrips reproductive performance in large tomato leaf discs. We demonstrate the feasibility of this approach by the transient ectopic expression of a set of effector candidate-genes from thrips and then evaluating thrips reproductive performance. The expression of enhanced GFP (eGFP) is used as negative control to which thrips reproductive performance in the treatments is compared with and to simultaneously monitor the level of GFP expression throughout the experiment. This protocol provides several advantages among which: (a) it allows for necrosis-free, transient expression in tomato leaf tissue due to the use of *A. tumefaciens* strain 1D1249 (Wroblewski et al., 2005); (b) it establishes protein expression for as long as 12 days post-infiltration with peak expression at day 5. This time frame exceeds the total time required for the screen including the development of nymphs which is 8 days; (c) it increases the homogeneity of protein expression in the tissue due to efficient vacuum infiltration of *A. tumefaciens* followed by a recovery-from-infiltration procedure; (d) it provides efficient containment of adult thrips and their progeny during the course of the experiment which eliminates the need for a greenhouse equipped to accommodate the combination of agro-infiltrations and thrips infestations; (e) it is conducted in 6-well plates, thus requiring a relatively small space, and is therefore conducted under controlled conditions in a climate box which increases reproducibility between experiments; (f) due to compactness of the setup, one can screen multiple genes with enough replicates for statistical analysis; (g) different plant species can be used for screening either effectors or plant genes to identify their significance to thrips reproductive performance and, finally, (h) the method can be used for screens with other small insect pests or microbes.

MATERIALS AND METHODS

Isolation and Cloning of Thrips Effector Candidate-Genes

RNA was extracted from *F. occidentalis* samples, anesthetized by incubation on ice for 1 min then flash frozen in liquid nitrogen, stored in liquid nitrogen, according to the Trizol procedure (TRI Reagent, T9424, Sigma) followed by Turbo DNAse treatment (TURBO DNAse, AM1907, Thermo Scientific) and first strand cDNA synthesis using 5 μg RNA and the RevertAid H Minus Reverse Transcriptase (Thermo Scientific, K1631). Initial thrips candidate effectors were selected by proteome prediction from cDNA sequences that we have generated from sequences obtained from the public Sequence Read Archives (SRA) for thrips whole body and salivary gland transcript sequences (SRX457725, SRR1826954, SRR1826955 and SRR1826956). We selected cDNAs that encode relatively small proteins (50–300 amino acid residues), predicted to have signal peptides by SignalP4.1 (Petersen et al., 2011), lack transmembrane domains (using TM-HMM, DTU Bioinformatics) and predicted to be secreted outside the cell by WolfPSort (Horton et al., 2007). Primers were designed to contain the Gateway (Thermo Scientific) attB sites fused to ATG at the start codon and the cDNA-specific sequence encoding the mature protein. Similarly,

the gene encoding eGFP was amplified and the generated expression construct used as a negative control during the screen. PCR amplification from the generated cDNA was conducted using Phusion polymerase (Thermo Scientific, F530S) according to the manufacturers' protocol and then directly purified from reaction (Thermo Scientific, K0702). BP reactions were conducted using pDONR221 as recipient vector with BP clonase and the reactions were transformed to electro-competent *Escherichia coli* DH5α cells. Positive colonies were selected on growth medium supplemented with 50 μg/ml kanamycin and verified by colony-based PCR using the plasmid M13 forward primer and a gene-specific reverse primer. Plasmids were isolated (Thermo Scientific, K0503) from two independent clones and the insert was confirmed by sequencing (Macrogen). Verified entry plasmids were used for LR reaction with either the *in planta* expression vectors pGWB512 (Nakagawa et al., 2007) for expression in tomato or pTRBO-FLAG-GW (**Figure S2**) for expression in *N. benthamiana*. pTRBO-FLAG-GW was modified from Lindbo (2007) by incorporating the FLAG peptide (Hopp et al., 1988) and the Gateway cassette, containing the R1 and R2 recombination sequences, at the PacI and NotI restriction sites and verified by sequencing. Both vectors add an N-terminal FLAG tag to the mature protein. The LR reaction products were transformed to *E. coli* DH5α cells and positive clones, growing on 30 μg/ml spectinomycin, were selected and verified by colony-based PCR using a plasmid forward primer and a gene-specific reverse primer. Subsequently, plasmids were isolated and used to transform electro-competent *A. tumefaciens* strain 1D1249 for expression in tomato (Wroblewski et al., 2005).

Thrips Rearing

The thrips colony was maintained in 1 L plastic containers with a side opening, or a cover containing an opening, that had been sealed with thrips mesh (SEFAR NITEX, 03-80/37, **Figure 1A**). Each box was regularly supplemented (after the formation of L2 nymphs) with fresh, cleaned, common bean pods and typha (*Typha latifolia*) pollen. The boxes were maintained in a closed climate box (ECD01, Snijders Labs) at 21°C, 16 h light and 65% RH. Under these conditions, a complete cycle of a developmental-stage synchronized thrips colony, from oviposition to the production of adults, takes 3.5–4 weeks (Note 1). First, ∼300 female adult thrips were collected and allowed to lay their eggs for 24 h into the surface of fresh common bean pods, to which typha pollen were added. Next, the adults were removed and the beans were further incubated for 5 days until the emergence of the nymphs, which were fed with pollen twice a week until they developed into adults after 2 more weeks.

Preparation of *A. tumefaciens* Inoculum

A. tumefaciens strains carrying expression constructs for eGFP or genes to be screened were inoculated from overnight pre-cultures in a shaking incubator (28°C and 225 rpm, Innova 4330, Brunswick Scientific) so that the optical density ($O.D._{600}$, OD), obtained the next day when harvesting the cells, is between 0.8 and 2.0 (Note 2). This can be done by adding X microl of a pre-culture that was grown overnight to 10 ml of sterile YEP medium (Bacto-Trypton, 10 g/L; yeast extract, 10 g/ L; NaCl, 5 g/ L; pH 7.5) in 50 ml sterile tubes and incubating at 28°C and 225 rpm (Note 3), where X = (Z/OD)/10), Z = 8,0000/(2^ (ΔT)/2), and ΔT = the time between medium inoculation and harvesting the cells (Note 4). On the day of infiltration, the OD of the bacterial cultures was determined and cells were harvested by centrifugation at 3,600 g and 21°C (Hettich Rotina 420R, Sigma) after which supernatant was discarded and each pellet was resuspended in a volume of infiltration medium (20 g/L sucrose, 5 g/L MS basal salt mixture without vitamins, 1.95 g/L MES, pH = 5.6 and 1 ml/L 200 mM acetosyringone) to reach the final infiltration OD = 0.3 for each treatment. To vacuum-infiltrate 12 leaf discs, ∼30 ml of this infiltration-ready culture is required.

Plant Material and Preparation of Leaf Discs

On the day of the vacuum infiltration with *A. tumefaciens,* leaf discs, 3 cm ø, were prepared using a cork borer from the third and fourth, fully stretched, true leaves of 3.5 to 4-week-old tomato (Moneymaker, growing in 15 cm ø pots at 21°C, 16 h light and 65% relative humidity) or 4-week-old *N. benthamiana* plants by cutting leaflets, excluding the older terminal leaflets, on thick Whatman paper using a sharpened cork borer. The leaf discs were collected into a 1 L beaker filled with tap water to wash the leaf discs and remove cell debris at the leaf disc circumference. Once all leaf discs have been prepared, the water was refreshed twice to wash and reduce damage components. Just before the vacuum infiltration with *A. tumefaciens*, the leaf discs were surface dried on a clean tissue and placed as two stacks of 6 leaf discs in preparation for vacuum infiltration.

Infiltration and Recovery of Plant Leaf Discs

Per treatment, a 50 ml syringe (BD Plastipak, 300865) was converted into a vacuum infiltration device by either melting or blocking the syringe tip using hot glue (11 mm ø, Mascot Europe BV) and making a small ventilation hole (1 mm ø) in the syringe barrel at the 30 ml mark (**Figure 1B**).

Two groups of the previously prepared leaf discs (6 stacked leaf discs per group) were gently placed into the syringe barrel, after removing the syringe plunger, one after another and by gently striking against a hard surface until they almost reach the bottom (Note 5). Subsequently, the barrel was filled with the infiltration culture until just below the ventilation hole. At that point, the leaf discs should be submerged in the bacterial culture. The plunger is placed back into the barrel in such a way that air, which is trapped above the surface of the culture, is allowed to escape via the ventilation hole. After air removal, the ventilation hole was closed using a gloved finger and the plunger was simultaneously pulled out from the barrel to generate a negative pressure. At that point air bubbles were formed, indicative for the extraction of air trapped in the intercellular space of the plant tissue. The extracted air was allowed to leave the liquid and reach the seal of the plunger by holding the syringe vertically and gently striking two times against a hard surface. Releasing the finger from the ventilation hole or, alternatively, pushing the plunger back into the barrel while keeping the ventilation

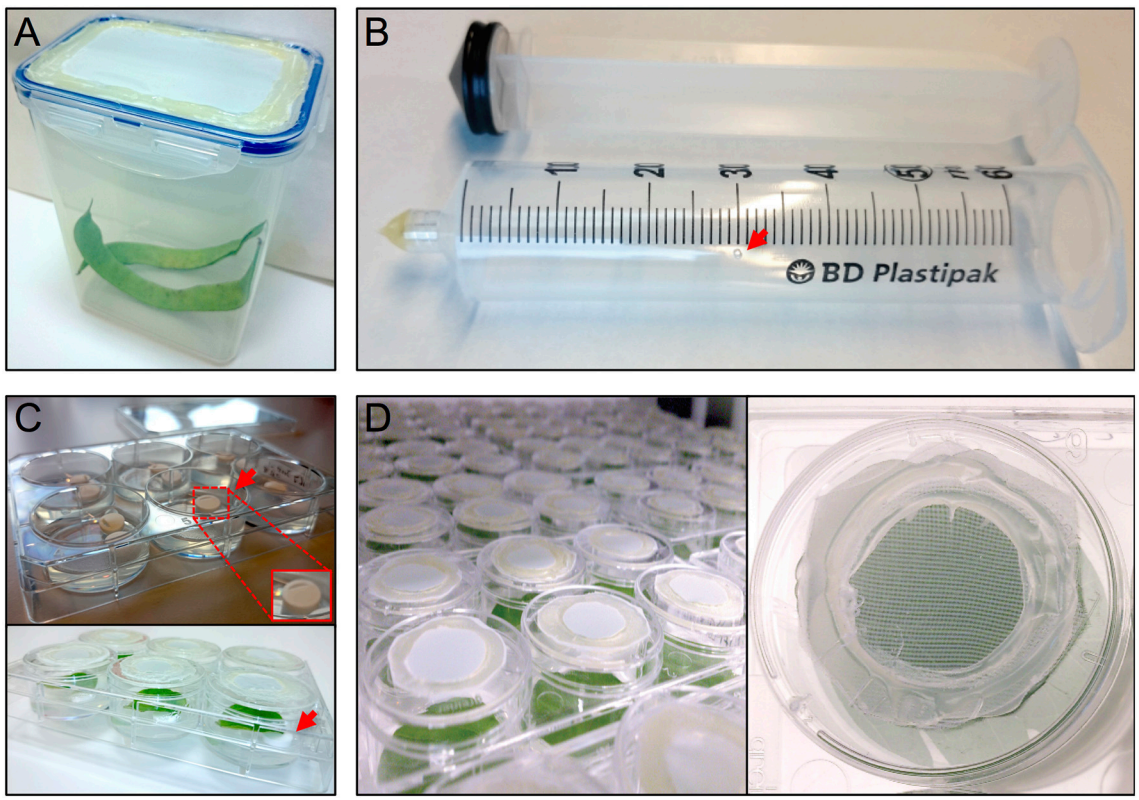

FIGURE 1 | Setup thrips rearing, vacuum infiltration and plate assay. **(A)** thrips rearing box with an open lid that has been sealed with mesh, **(B)** a 50 ml syringe converted to a vacuum infiltration device with a ventilation hole (arrow), **(C)** leaf discs are lifted away from the agar from one side using tube coders (arrows), and **(D)** modified 3.5 cm ø 6-well plate caps with an open top sealed with mesh.

hole closed neutralized the negative pressure and caused the infiltration of the leaf tissue after which the leaf discs appeared as being water soaked. Subsequently, the culture was discarded and the leaf discs were removed from the barrel by striking the barrel opening against a thick filter paper positioned on a hard surface. The surface of the leaf discs was dried using clean tissue and the leaf discs were placed, with abaxial side pointing upwards, on a shallow cover of a large Petri dish (Greiner, 145 mm, Z652539) containing wet sterilized chromatography paper (Whatman, 3MM CHR, 3030-917) and allowed to recover from the infiltration in a down flow cabinet for 45 min and up to 1 h (Note 6).

After recovery, each leaf disc was positioned with adaxial side up in a single well of a 6-well plate (Greiner, 657160) containing 4 ml of 0.75% sterile water agar (Daishin agar, Duchefa, D1004.1000), prepared using ultrapure water (MQ, Milli-Q System, Millipore Corporation). Leaf discs were positioned in their wells so that the thick side of the midrib (previously the closest to the leaf base) is in contact with the agar. The opposite side of the midrib was resting on two surface sterilized lifters (**Figure 1C**) (Nalgene cryogenic vial coders, Thermo Scientific, 5045-0000) (Note 7). After loading a plate with leaf discs, their contact points with the agar surface were pressed slightly into the agar using tweezers to ensure sufficient but shallow contact (Note 8). Subsequently, the wells were closed using individual lids (Thermo Scientific, Nunc, 150350) equipped with thrips mesh to prevent thrips from escaping the wells and allow the exchange of air and humidity during incubation inside the climate box (ECD01, Snijders Labs, **Figure 1D**). For this, each lid was modified by making a 2 cm ø hole at the center and then sealing the created opening with thrips mesh (**Figure 1D**) using hot glue. Subsequently, leaf discs were allowed to further recover in the climate box for 1 day. At this point, the plates were not covered with the original rectangular plate-cover in order to allow a better ventilation and thus facilitate the leaf disc recovery.

Thrips Starvation

One day before infesting the plant leaf discs with thrips, adult age-synchronized female thrips were collected from the rearing boxes, excluding dead thrips and pollen, by sucking individual adults into a 50 ml disposable tube. Adults were collected from the original rearing box by mouth-aid air suction, using a transparent, small size plastic hose covered at one end with a filter consisting of a small piece of mesh and jammed into a 1 ml pipette tip of which the end has been cut off to create a larger opening (**Figure 2A**) (Note 9). Although 5 female adult thrips are required for each well, ∼40% more were collected to compensate for loss during starvation and contamination with male adult

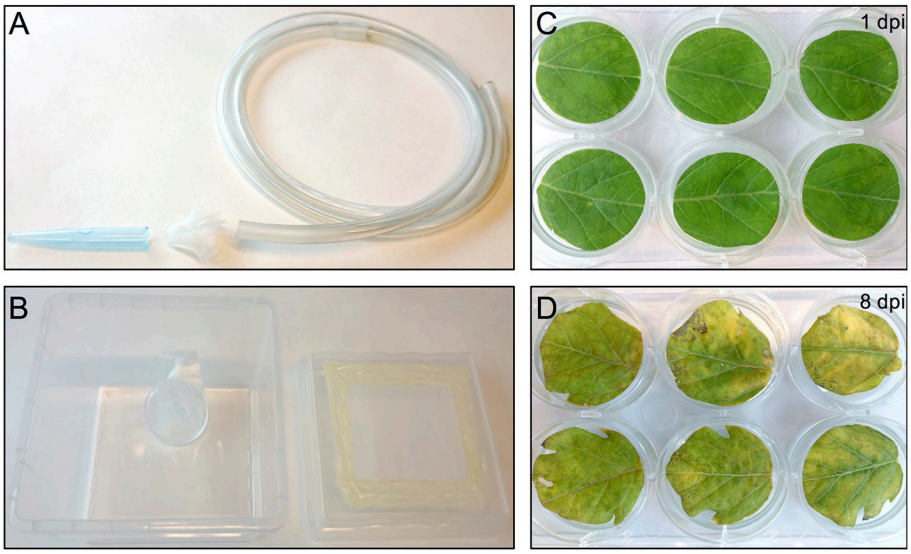

FIGURE 2 | Thrips collection and starvation setup and the effect of agro-infiltration on leaf-disc phenotype. **(A)** Small size plastic hose covered at one end with mesh and a 1 ml pipette tip used to capture thrips by sucking. **(B)** Thrips starvation setup consisting of a plastic box in which a small water container has been placed, covered with a stretched layer of Parafilm. The lid of the box contains an opening that has been covered with mesh. **(C)** Tomato leaf discs infiltrated with *A. tumefaciens* strain 1D1249 carrying an *in planta* expression construct at 1 dpi or at 8 dpi **(D)**.

thrips. Subsequently, a second round of cleaning was applied by releasing the collected adult thrips into the center of a large clean shallow box and collecting adults that migrate away from the center. Water was offered to the double-cleaned adult thrips in a water container which is made by filling a cover of small cell culture plate (Thermo Scientific, Nunc, 150350) with tap water and covering the top with a stretched layer of Parafilm (Parafilm M, Sigma, P7793-1EA) (Note 10). The water plate was fixed by pressing the excess of the Parafilm to the side of a plastic box so that it is resting on the bottom of the box at a 45° angle which allows air to go to the top and thus facilitate the utilization of water via the Parafilm by thrips (**Figure 2B**). Finally, the box was covered with mesh and starvation was sustained for 24 h in the climate box.

Thrips Bioassay

After 1 day recovery of the leaf discs in the climatebox, for each well 5 starved female adult thrips were collected into 1.5 ml microtubes. Once all tubes were filled, they were randomized and placed in groups of 12, sufficient for infesting 2 plates (12 leaf discs) for each treatment. Infestation and oviposition were started by removing the adjusted lids from the wells of two plates, each containing 6 recovered leaf discs, and placing the two plates and 12 thrips tubes on ice for 1 min (Note 11). Directly after, the adults were added to each plate by tapping each tube content in each well and checking if all 5 adults have been transferred to the well. This process was conducted quickly to avoid thrips from gaining consciousness. Once adults were added to all wells in one plate, the wells were quickly closed with the adjusted individual lids. The same was done for the second plate. Next, the two plates were closed with the original rectangular plate-cover and transferred to the climate box for a 24 h infestation.

After infestation, adult thrips were scored per well (dead/alive) for each treatment before being removed. The thrips-free wells were closed with both the adjusted individual lids and plate cover and further incubated in the climate box for 5 days. On day 8 post-infiltration, the nymphs in each well were counted using a stereo microscope (M3, Wild-Heerbrugg).

Statistical Analysis

A single measure parametric analysis was conducted using InVivoStat (Bate and Clark, 2014) on the data from each screen using a one-way ANOVA analysis. When significant differences with the negative control are encountered, the analysis is followed by comparisons of the predicted means of the treatment factor (the over-expressed gene) back to the control (eGFP) group mean using the Least Significant Difference (LSD) procedure.

GUS Assay

Leaf discs were agro-infiltrated as described above using *A. tumefaciens* strain 1D1249 carrying the plasmid pGWB512-GUS and GUS activity was measured at different time points post-agro-infiltration. For each time point, three leaf discs were vacuum-infiltrated in 40 ml GUS buffer supplemented with X-gluc (5-Bromo-4-chloro-1*H*-indol-3-yl β-D-glucopyranosiduronic acid, Thermo Scientific, R0851), using a modified syringe as the one used for agro-infiltration. First 500 ml GUS buffer (50 ml of 1 M phosphate buffer, pH 7.5, 5 ml Triton X-100, 5 ml DMSO, 10 ml of 0.5 M EDTA, pH 8 plus 635 ml demineralized water) were prepared without the addition of the GUS substrate and stored at 4°C. Directly before infiltration, 40 ml of the GUS buffer were allowed to reach

room temperature and then 200 µl of the X-gluc stock (100 mM in DMSO) were added and mixed thoroughly. After vacuum infiltration, leaf discs and the infiltration buffer were incubated at 37°C for 24 h. The blue product that is formed due to the cleavage of the substrate X-gluc by GUS activity is diffused into the buffer, as tissue fixation was omitted, and subsequently measured per ml buffer/leaf disc (∼100 mg tissue) by absorbance at 460 nm. The obtained values reflect the difference in GUS activity and thus the level of *GUS* gene expression among the samples. A list of all used equipment is provided in **Supplementary Material**.

Notes

1. The use of a synchronized thrips colony makes it easier to distinguish female adult thrips from male adult thrips, as the latter are slightly smaller in size, and thus reduces the variations in nymph counts among treatments.
2. It is important to maintain this OD to ensure that the cells are in the logarithmic growth phase and thus reduce variability between treatments and increase reproducibility.
3. *A. tumefaciens* strain 1D1249 grown as a liquid culture can form aggregates. We found that growing this culture at 28°C in a shaker set to a value between 225 and 250 rpm reduced aggregate formation without affecting the final expression levels after agro-infiltration.
4. If the OD on the day of infiltration is still too low then the value of ΔT in the formula can be reduced with 1 or 2 h without adjusting the actual number of hours used for the incubation. Similarly, if the OD appears to be higher than desired on the day of infiltration, the value of ΔT can be increased with 1 or 2 h.
5. It is important to avoid causing any damage to the leaf discs during the whole procedure to avoid triggering wound responses. This is also why the leaf discs should be cut using a cork borer with a sharp edge to minimize tissue damage.
6. The cover of a Petri dish is used as it is shallow and when placed near to the air stream in the down flow, it allows air to circulate around the leaf discs and thus improve water evaporation from the intracellular space. This rapid recovery step is meant to evaporate the excess of water and remove the water soaking phenotype from the infiltrated leaf discs in a short time but without allowing the them to dry out. The recovery from infiltration is necessary to prevent hypoxia which can otherwise lead to tissue necrosis. Although the leaf discs are not sterile, we use clean or sterilized material to reduce the chance of developing fungal infection on the leaf discs during the course of the experiment.
7. The use of the lifters allows further recovery of the leaf discs from infiltration during incubation, maintains better humidity around the leaf disc and allow the adult thrips and the nymphs to move freely around the leaf disc. The cavity of the lifters should be facing the agar surface. This prevents trapping humidity if they were facing the leaf tissue which increases the chance of fungal infection.
8. A shallow contact with the agar surface is important to reduce tissue necrosis at this region of the leaf disc due to hypoxia.
9. It is important to avoid collecting dead thrips to which pollen are usually stuck or collect the pollen themselves which will disturb the starvation process.

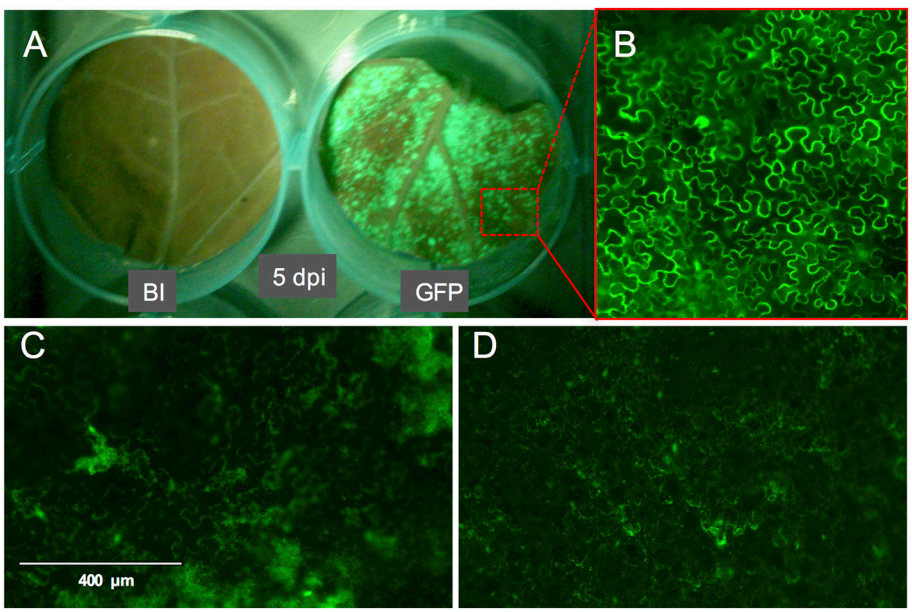

FIGURE 3 | GFP fluorescence signal on whole leaf discs of *Nicotiana benthamiana* at 5 dpi and the phenotype of the GFP signal. **(A)** *N. benthamiana* leaf disc infiltrated with buffer (left) or with *A. tumefaciens* strain GV3101 carrying pTRBO-FLAG-GW (right) at 5 dpi. **(B)** Microscopic image of GFP signal from the leaf disc in **(A)**. **(C,D)** GFP signal phenotype observed as a result of hypoxia when the leaf discs were not sufficiently recovered from the vacuum infiltration of *A. tumefaciens* in *N. benthamiana* **(C)** or tomato **(D)**.

10. Avoid stretching the Parafilm more than one time its length to prevent possible water leakage and drowning the thrips.
11. Depending on the temperature of the surrounding, the incubation time on ice for thrips can be decreased as long it will remain anesthetized. Do not exceed the 1 min as it can lead to mortality.

RESULTS

Vacuum Infiltration and Leaf Disc Recovery

Compared to pressure infiltration or the infiltration using a conventional vacuum pomp, our infiltration procedure allowed us to obtain complete tissue infiltration where the water soaking phenotype was observed on the whole tissue. This is despite that we have used large leaf discs which are usually more difficult to infiltrate. Also, the leaf discs did not suffer from wounding as that caused by pressure infiltration. We found that tomato leaf discs of the cultivar Moneymaker recover slightly faster (45 min to 1 h after infiltration) from the water-soaking phenotype, caused by the infiltration, than *N. benthamiana* leaf discs (1 h after infiltration). We found it to be essential that the infiltrated leaf discs reach a near-complete recovery before being transferred to the agar plates, otherwise tissue necrosis could appear on them 2–3 days later and then they become more vulnerable to fungal infection. Although protein expression was often still visible in these non-recovered regions, it was somewhat diffused as observed after the expression of eGFP (**Figures 3C,D**). Notably, the recovery of the leaf discs from the infiltration needed to be continued on agar. Immersing a large part of the leaf disc into the agar or having direct contact of the complete leaf disc surface with the agar often resulted in reoccurrence of the water soaking phenotype and necrosis often developed at these contact regions. To solve this, the leaf discs were lifted from the agar from one side using clean plastic micro-tube coders (see materials) while the thick side of the midrib remained slightly inserted into the agar. Failing to use these settings caused a prolonged presence of water soaked tissue and the development of necrosis at the positions where the leaf disc was contacting the agar. Also, thrips benefit

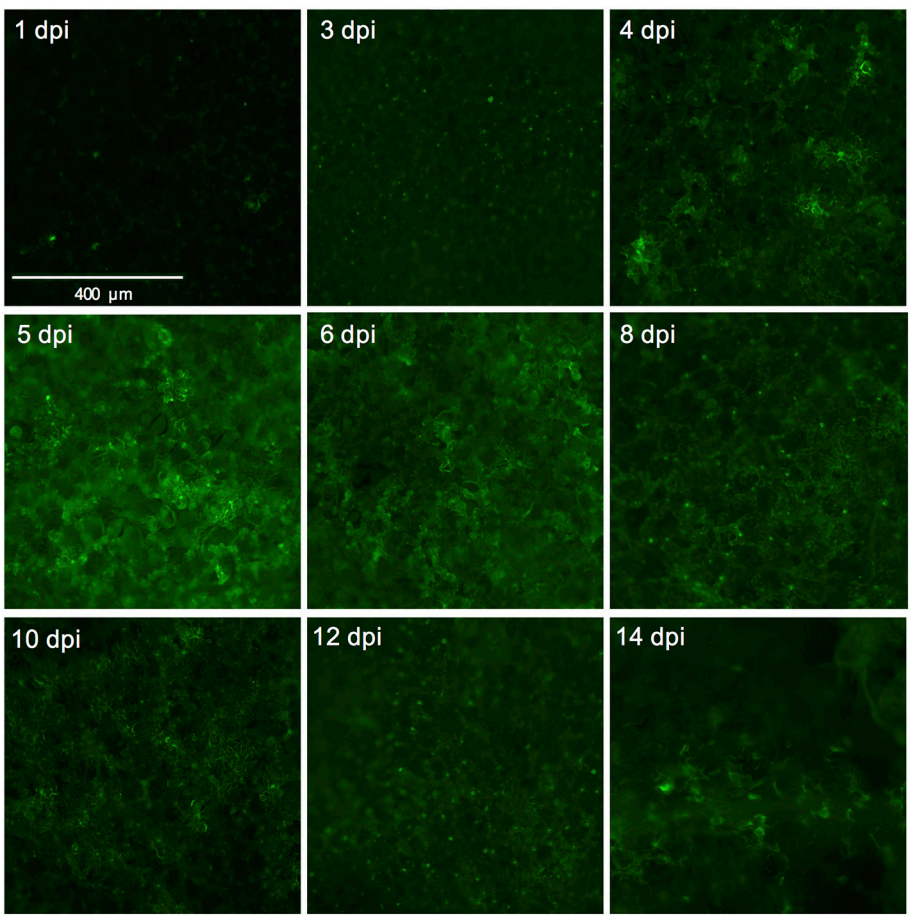

FIGURE 4 | Successful expression of GFP in tomato leaf discs after vacuum infiltration with *Agrobacterium tumefaciens* and recovery from infiltration. Leaf discs prepared from 3.5 to 4-week-old tomato plants of the cultivar Moneymaker were vacuum-infiltrated with the *Agrobacterium tumefaciens* strain 1D1249 carrying the plasmid pGWB512 in which the eGFP is expressed under the control of the 35S promoter of cauliflower mosaic virus. Leaf discs were allowed to recover from the infiltration for 1 h under an air stream and then placed on water agar in 6-well plates. Pictures were taken using a wide-field fluorescence microscope at the indicated time points and magnification.

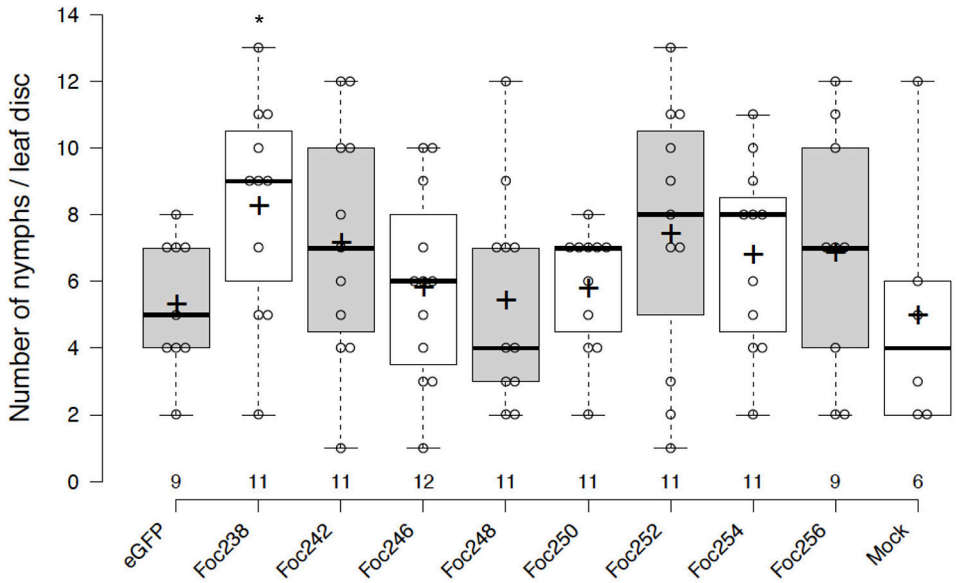

FIGURE 5 | Effect of transient expression of thrips effector-candidate genes on thrips reproductive performance. The box plot represents the results from an effector screen, showing the number of nymphs scored at 8 days post-agro-infiltration to transiently express eGFP (control) or thrips effector candidate genes in tomato leaf discs. Oviposition by adult female thrips was started at 2 dpi and continued for 24 h. Mock represents leaf discs infiltrated with only infiltration medium. The box center-lines represent the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; outliers are represented by detached dots; crosses represent sample means; data points are plotted as open circles; n is between 6 and 12 leaf discs. *LSD, $P = 0.07$.

from this as adults, and later, nymphs are often encountered at the abaxial side of the leaf disc which becomes more accessible due to lifting the leaf discs.

Protein Expression

We used the eGFP control as an indicator for protein expression during the course of the experiment by monitoring GFP fluorescence. The GFP signal was visible at 4 dpi and continued to increase at 5- and 6 dpi after which it started to decline at 8 dpi. At 14 dpi the signal was much weaker and was often confined to the areas adjacent to the veins (**Figures 3A,B**, **4**).

Containment of Thrips and Bioassay Optimization

Counting the number of the adult female thrips after infestation and oviposition showed that no escapes occurred, which reflected efficient containment of thrips in the utilized experimental setup. Moreover, a low mortality rate (1–2%) was observed which indicates that adult thrips can cope with the defenses existing on the treated tomato leaf discs. This is different from the mortality rate that we observed in treated *N. benthamiana* leaf discs and that was up to 35% (data not shown), indicating the high toxicity of that species for thrips.

After several trials, we found that it is important to optimize the number of adult female thrips used for infestation and oviposition, the contact time with the plant leaf discs and the incubation time of the leaf discs before counting the nymphs. This was particularly important to reduce sample to sample variation and increase the reproducibility of the results. Accordingly, we found that it is optimal to use 5–6 female adults per leaf disc (3 cm ø). This ensures obtaining a reasonable number of nymphs that can be counted concurrently in 1 day for a single screen containing 15 treatments, including the controls. The infestation and oviposition was allowed for 24 h in the climate box. We found this to be important for generating a synchronized emergence of the progeny so that most eggs would have hatched and could be counted in the scoring day. Under these experimental settings, the optimum incubation time that allowed most eggs to hatch was between 7 (in the summer) and 8 (in the winter) days post-oviposition. This incubation time might differ slightly depending on the tested plant species as thrips reproductive performance is known to vary among plant species (Baez et al., 2011).

Identifying Genes That Influence Thrips Reproductive Performance

The plotted nymph counts showed variations within the treatments, but also between some treatments and the negative control (eGFP). None of the thrips effector-candidates that were tested here showed statically significant difference at the 5% level on nymph counts, compared with the control (**Figure 5**). However, expression of the effector candidate Foc238 led to a near significant ($P = 0.07$) enhancement of thrips reproductive

performance as the number of nymphs was higher than in the negative control. Thus, Foc238 is a putative effector that can now be further tested with increased sample sizes. Foc238 is predicted to contain a complete open reading frame, which encodes a small protein (163 AA) with a signal peptide and two repeat domains. It is highly expressed in the salivary glands of thrips and has no significant similarity with known sequences from other organisms. We are currently using this screening method to study a large set of putative effector candidates from thrips in a medium throughput setup to find those that have a stronger effect on the reproductive performance of the thrips.

DISCUSSION

The tremendous increase in sequence-based genetic information from plants and their herbivores and the quest for plant genetic resistance requires rapid genetic screening procedures to identify resistance-related traits. Our screening procedure addresses this by eliminating the need for generating stable transgenic plants and to be able to screen with herbivorous insects for up to, at least, 8 days. It also allows conducting the screen under strictly controlled conditions and in a relatively small space which increases the reproducibility of the results. Another benefit is the cost reduction due to omitting the occupation of a large greenhouse space equipped to accommodate the combination of genetic screens with genetically modified organisms and insect bioassays. Furthermore, the procedure allows a necrosis-free, transient protein expression in tomato leaf tissue. This is accomplished by the use of *A. tumefaciens* strain 1D1249 in a setup that results in prolonging the duration of protein expression to encompass the time required for finalizing the thrips bioassay. Although infiltration of the tomato leaf discs with the *A. tumefaciens* strain 1D1249 does not induce necrosis on the leaf discs, we observed chlorosis which was especially most pronounced at the end of the screen, starting from day 8 (**Figures 2C,D**). The observed chlorosis was similar to that formed when infiltrating leaves attached to whole plants with this strain (data not shown). However, this did not prevent protein expression until the end of the thrips bioassay as observed in the eGFP control treatments (**Figure 4**) and on the whole leaf-disc level after expression of the *GUS* gene (**Figure S1**).

The utilized vacuum infiltration method, followed by a recovery-from-infiltration procedure, increases the homogeneity of protein expression in the tissue. This is important to reduce the score variations among samples of the same treatment and increases the reproducibility of the results. The use of an age-synchronized thrips colony and the efficient containment of adult thrips and their progeny during the course of the experiment also contributes to reducing variations. It is possible to use this method for other plant species to screen either single effectors or plant genes or to screen wild accessions to identify their significance to thrips reproductive performance. In the latter, the screen becomes even faster as the vacuum infiltration and recovery steps are not required. Similarly, rapid genetic functional analysis techniques like virus-induced gene silencing can also be combined with this screen, where silenced plants are used to generate leaf discs that are directly used without the need for agro-infiltration and recovery. Despite that one of the aims of this procedure is to reduce the within-treatment variations, we still see them. This may have to do with the complexity of the involved biological system which includes plant, bacteria and thrips. Therefore, treatments that cause small effect on thrips performance might not directly show statistically significant difference. A possible solution for this could be to increase the sample size, although this will limit the number of genes that can be tested in one screen. Thrips effectors that have positive or negative effect on thrips reproductive performance are very useful to include in the screen. However, there are no thrips effectors known to date. Instead we expressed several known aphid effectors (Mp10, MpC002, and Mp42) (Bos et al., 2010) and we also cloned and tested a thrips homolog of one of these aphid effectors, Mp10 (Mugford et al., 2016) and evaluated thrips performance. However, the expression of none of these genes affected thrips reproductive performance in our assay.

Finally, this method can be used to identify genes that play a role in the plant interaction with other small pests or microbes as long as the screen can be conducted within the time frame of expression. However, we think that in the future there is room for further optimization of this method by using more potent expression vectors and *A. tumefaciens* strains selected for high transformation efficiency, especially on the studied plant species. The desired improvements would be extending the duration of maintaining plant tissue in a healthier state while obtaining sufficient levels of protein expression. This will allow using this approach for studying other organisms for which the screen requires a longer time frame.

AUTHOR CONTRIBUTIONS

AA designed the experiments. AA and SH conducted the experiments. AA, SH, and RS edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01852/full#supplementary-material

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frontiers in Plant Science

ORIGINAL RESEARCH
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An Integrated System for the Automated Recording and Analysis of Insect Behavior in T-maze Arrays

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Host-plant resistance to insects like thrips and aphids is a complex trait that is difficult to phenotype quickly and reliably. Here, we introduce novel hardware and software to facilitate insect choice assays and automate the acquisition and analysis of movement tracks. The hardware consists of an array of individual T-mazes allowing simultaneous release of up to 90 insect individuals from their individual cage below each T-maze with choice of two leaf disks under a video camera. Insect movement tracks are acquired with computer vision software (EthoVision) and analyzed with EthoAnalysis, a novel software package that allows for automated reporting of highly detailed behavior parameters and statistical analysis. To validate the benefits of the system we contrasted two *Arabidopsis* accessions that were previously analyzed for differential resistance to western flower thrips. Results of two trials with 40 T-mazes are reported and we show how we arrived at optimized settings for the different filters and statistics. The statistics are reported in terms of frequency, duration, distance and speed of behavior events, both as sum totals and event averages, and both for the total trial period and in time bins of 1 h. Also included are higher level analyses with subcategories like short-medium-long events and slow-medium-fast events. The time bins showed how some behavior elements are more descriptive of differences between the genotypes during the first hours, whereas others are constant or become more relevant at the end of an 8 h recording. The three overarching behavior categories, i.e., choice, movement, and halting, were automatically corrected for the percentage of time thrips were detected and 24 out of 38 statistics of behavior parameters differed by a factor 2–6 between the accessions. The analysis resulted in much larger contrasts in behavior traits than reported previously. Compared to leaf damage assays on whole plants or detached leaves that take a week or more to complete, results were obtained in 8 h, with more detail, fewer individuals and higher significance. The potential value of the new integrated system, named EntoLab, for discovery of genetic traits in plants and insects by high throughput screening of large populations is discussed.

Keywords: video tracking, insect, plant, EntoLab, EthoAnalysis, *Frankliniella occidentalis*, *Arabidopsis*

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INTRODUCTION

Breeding for host-plant resistance has gained much interest in recent years due to the increasing bans on the use of chemical pesticides. A crucial element in the breeding process is the accurate estimation of the resistance level of large populations of plant accessions (Eigenbrode and Trumble, 1994). This requires robust phenotyping systems that can accurately screen many different plant lines in a high-throughput manner (Kloth et al., 2012; Goggin et al., 2015).

Current phenotyping methods mainly focus on costly, labor-intensive and time-consuming end-point measurements of feeding damage or insect performance (reproduction and mortality). Clip-on cage techniques are not effective with thrips as they easily escape. Conventional visual rating systems that score feeding damage often do not allow precise quantification and are sensitive to subjectivity and inconsistency of the scoring process, although recently a more objective method was reported (Visschers, 2018). Usually experiments are, therefore, done in greenhouses with an open choice situation that suffer from environmental effects. An alternative, automated high-throughput and controlled process to phenotype host-plant resistance to thrips would therefore greatly aid research and the breeding process for thrips-resistant cultivars.

We have recently demonstrated the value of automated video tracking of peach aphid (*Myzus persicae*) (Kloth et al., 2015) and Western flower thrips (*F. occidentalis*) to establish host-plant resistance levels in *Arabidopsis* (*Arabidopsis thaliana)* (Thoen et al., 2016) and locate associated genes in Genome Wide Association Study (GWAS) populations (Kloth et al., 2016, 2017; Thoen et al., 2017). Those studies were, however, done in microtiter plates that were not suitable for T-maze applications, nor for the study of whole leaves, and suffered from poor detection due to condensation of water droplets on the inside of the plastic cover after a few hours. In thrips choice experiments, movement and halting in a two-choice setting was only reported using the sum total time or speed of all movement or halting events, and not the event averages which would have given more detailed and differentiating information of the nature of halting or movement behavior (Thoen et al., 2016). All studies so far were further sensitive to differences in detection percentages (tracking efficiency). Extraction, of more detailed behavior categories, that could distinguish short and long or fast and slow moving or halting events, were only reported in the aphid studies, as this required a large amount of extra offline manual processing and *ad hoc* programming work. Not analyzing the more detailed behavior information contained in the thrips tracks, however, neglects important details of animal behavior that can be far more informative than sum totals (Benjamini et al., 2010; Kloth et al., 2017). The goal of the development of the EntoLab™ phenotyping system presented here was to overcome these shortcomings, and automate the data extraction and statistical analysis. Here, we validate its utility for the study of thrips in a T-maze setting. It involves a camera, camera stand, illumination, and EthoVision® software, plus newly developed T-maze array hardware, and EthoAnalysis™ software for automated data extraction and statistical analysis. This system is shown with fewer insects to deliver larger contrasts that are more accurate and significant compared to the previous report (Thoen et al., 2016). Furthermore, it provides more detailed insight in the behavior of insects like thrips on different plant genotypes. With two accessions the system is operated using the following workflow:

1. T-maze array experiment: video recording of thrips behavior in a fully controlled environment. Leaf discs of two accessions are placed into each of the parallel T-maze arenas. In each arena, one insect is placed and behavior is video-recorded over a time span of 8 h.
2. Extracting video tracks: EthoVision video-tracking software is used to determine the position, zone, and velocity of each insect in each video frame during the complete run of the experiment.
3. Analysis of behavior statistics: EthoAnalysis is used to convert raw tracking data exported from EthoVision into zone-specific movement and halting events, and to generate higher level behavior statistics, that are less sensitive to differences in tracking efficiency (detection) between genotypes. The statistics include zone preference, average velocity, total time moving/halting, short/medium/long moving and halting duration and slow/medium/fast moving events, mostly also per hourly time bin. For these choice assays, the differences between the behavior statistics in the two zones containing the genotypes are modeled in order to establish differences in insect preference between two genotypes.

For validation of the system, we used two wild *A. thaliana* accessions, Cur-3 and Rmx-A180. In the previous study Cur-3 was shown to be much more resistant to thrips in choice settings with Rmx-A180 as susceptible reference genotype (Thoen et al., 2016). In that study, the behavior of Western flower thrips (*F. occidentalis*) was recorded in 88 parallel arenas the size of a single 6 mm microtiter-plate well filled with two half leaf discs. The results were found to be consistent with choice assays with whole plants and detached leaves in which damage levels were assessed.

In this follow up study, we introduce a novel parallel arena plate with T-maze type designs. The two leaf discs each occupied their own 6 mm well and were separated by a central well from which simultaneous access by a single thrips individual was possible after shifting a gate plate. EthoAnalysis subsequently provides a number of filters to automatically remove entire records or specific events based on various quality criteria. Setting these filters and tuning their parameters generally allows navigating between data quality and data quantity. Additionally, for some behavior statistics, EthoAnalysis requires insect specific parameters, like time intervals for probing vs. feeding to be set. We discuss the potential contribution of the EntoLab system to studies in the field of plant-insect science and resistance breeding.

MATERIALS AND METHODS

Plants

We used *Arabidopsis thaliana* as host plant species. Accessions Rmx-A180 (CS76220, collected by J. Bergelson, latitude 42,036,

longitude −86,511, Michigan, USA) and Cur-3 (CS76115, collected by F. Roux, latitude 45,000, longitude 1,750, France) were used for this study. For insect assays, plants were grown from seeds in small plastic pots (5 cm diameter) on pasteurized soil (4 h at 80°C; Lentse potgrond, Lent, The Netherlands) in a climate room (21 ± 1°C, 50–70% relative humidity; 8 h:16 h L:D photoperiod; light intensity 200 μmol m^{-2} s^{-1}). For both reported experiments, ten 5-weeks-old plants were used. From each plant the top 4 youngest leaves large enough to produce 4 leaf discs of 6 mm were harvested. Leaf discs of both genotypes were subsequently randomly combined but their leaf position was recorded in the software to allow analysis of such aspects if desired.

Insects

The Western flower thrips [*Frankliniella occidentalis* (Pergande)] used in this study were collected from chrysanthemum flowers in a climate chamber (25 ± 1°C, L:D 16:8). In the experiments non-synchronized adult females were used, that were starved overnight in Perspex tubular cages closed on one side with gauze and on the other side with two layers of stretched sheets of Parafilm containing a droplet of water to enable drinking. Thrips were anesthetized with CO_2 and placed on ice just prior to experiments.

T-maze Array Plate

A novel T-maze array plate was designed to allow easier and controlled high throughput T-maze behavior studies with insects using leaf discs. The T-maze array setup was created by stacking multiple layers of micro-machined and laser cut Perspex in a holder (**Figure 1**). In the bottom cage plate cold-anesthetized insects are placed row by row and retained there by covering the cages with a gate plate. The gate plate prevents the insects from entering the top arena plate until it is pushed into the stack by about ∼1 cm at the start of the trial, so that the holes in the gate plate simultaneously create access to all 90 arenas of the arena plate. Each arena consists of three separate circular zones of 6 mm diameter, arranged in a row. The zones are connected through 2 mm wide and deep tunnels. The two outer zones are 4 mm deep, while the center zone cuts through the plate, and is used to release the insects into the arena from the cage below. Leaf discs are placed on 20 μl water in the two outer zones. To prevent condensation, the glass cover plate was coated with indium tin oxide and heated to 27–30°C by applying a voltage. The coating did not affect the transparency of the glass. The whole plate stack was placed in a holder and mounted for video recording. In this study only 40 of the 90 available parallel T-maze arenas were used, because for a simple comparison of just two genotypes 40 replications are more than enough. A blue color filter with excised holes in the positions of the leaf discs was placed on top of the arena plate below the cover plate to equalize the light intensities from areas with and without leaves (**Figures 1B**, **2B**). This improved the tracking success when the insects were moving between zones with a high contrast of direct and leaf disc filtered backlight (data not shown).

Video-Tracking Setup and Experiment

Thrips behavior was recorded with a digital video camera (Basler acA2040-25gc, 1″ CMOS sensor, Kowa LM35HC lens, 35 mm/F1.4). A backlight unit (LED panel 30 × 30 cm, 24 V and 18 W, 5,000 K) was used to illuminate the arenas. The arena plate with T-maze arenas was mounted ∼1 cm above the backlight unit, with ventilation in between to prevent heating. Room temperature was kept constant at 21–22°C. Videos were recorded at the maximum resolution of 2,046 × 2,046 pixels at 10 video frames per second using Debut Video Capture software (version1.88, http://nchsoftware.com/capture/index.html). Eight-hour recordings of 40 parallel two-choice assays were run (**Figure 2B**). For each arena, leaf discs of 6 mm from both accessions (Cur-3 and Rmx-A180) were placed on 20 μl of water in alternating arms of the T-maze. A section of 4 × 10 arenas was filmed representing a size of 108 × 100 mm, implying a resolution of ∼20 pixels/mm or 50 micron per pixel.

EthoVision Video-Tracking Settings and Export

We tracked thrips behavior with EthoVision® XT 11.5 video tracking and analysis software (Noldus Information Technology BV, Wageningen, The Netherlands, www.noldus.com/ethovision) at 3.33 frames/s. Each leaf disc was assigned to a specific zone (Z1 and Z2); in addition, there was a neutral zone that did not contain leaf material (Z3). Dynamic subtraction and center-point detection were used as detection methods, with a dark contrast of 8–255. Subject size detection was limited to the range of 40–385 pixels. Pixel smoothing was set to medium. Moving thresholds were not set because EthoVision was only used for tracking and not for event analysis. Raw data files with genotypes in a "Genotype" column separated by a "$" sign were exported per subject for analysis in EthoAnalysis as .txt files for each arena (ca 1 Mb per arena per hour).

EthoAnalysis Extraction of Behavior Events From Tracking Data

The video tracking software EthoVision produces series of track samples (set at 3.33 samples/s, 10800/h) for all insects/arenas as described before (Thoen et al., 2016). Each track sample contains an (x,y)-coordinate, a velocity, and an indication of the current zone in which the insect resides unless the tracking is not successful because EthoVision could not detect the insect, in which case the sample is recorded as not detected. These track files were imported by EthoAnalysis, a software package developed by Wageningen Plant Research.

Supplementary Data File 1 provides screenshots of how the software processes the data in steps based on consecutive tabs. In the "Project" tab with a new project a dialogue box is given where the experiment should be named and the location of the data indicated. A summary of genotypes and experiments (arenas) is given once data are added in the other tabs. In the "Input data" tab (**Supplementary Data File 1**) trials and experiments can be added or removed. Crucial are the import settings which require a value for the look-ahead window, and the velocity threshold.

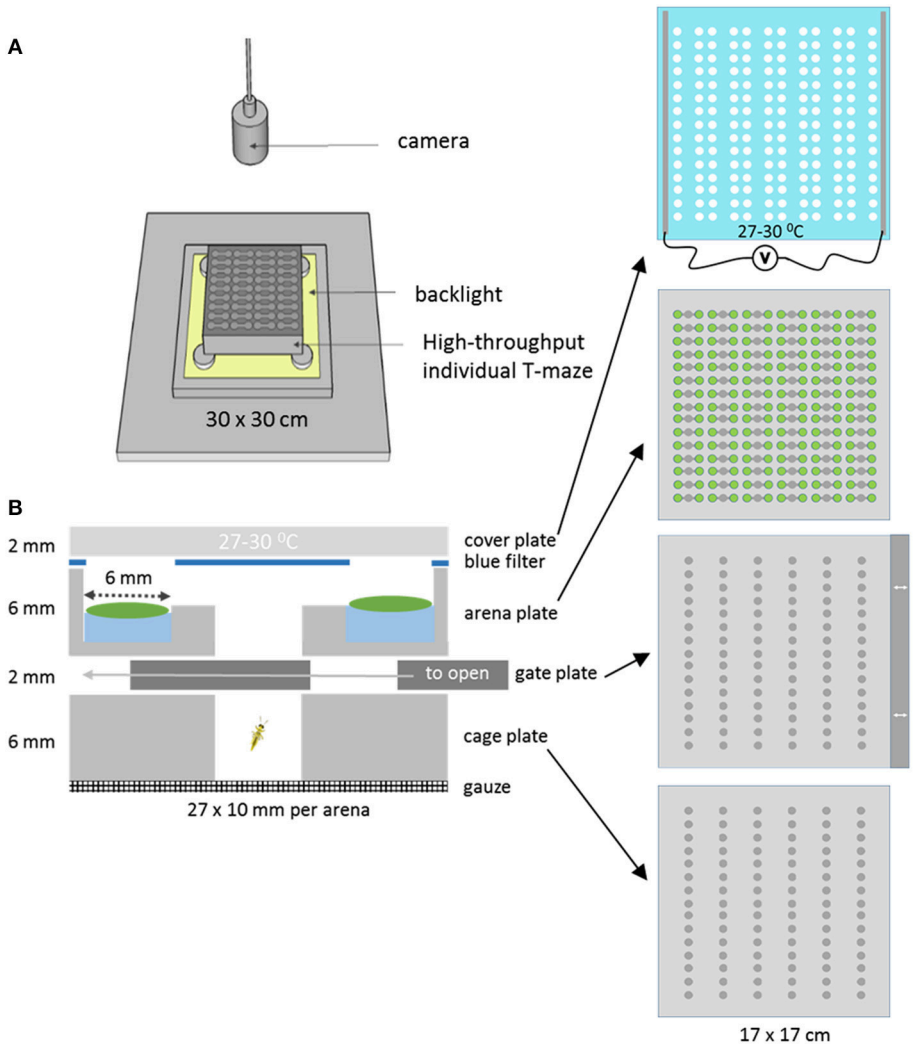

FIGURE 1 | The T-maze array set up. **(A)** The T-maze array consisted of stacked layers of Perspex plates topped with a heated glass cover. It was lit from below with a backlight and monitored from above with a camera. **(B)** Cross section of one single T-maze arena of the complete array of 90. The bottom compartment of a cage plate closed with a gate plate, that could create access to the arena plate by sliding it to the left. The T-maze arena consisted of two leaf disk zones with disks placed on 20 μl water, a blue filter with holes in the position of the leaf discs, and a heated cover plate made of glass.

The look-ahead window exists to ignore minor drops below the velocity threshold within a movement event, or single spikes above the velocity threshold while halting and is an important tool to accurately follow the unique behavior of specific insects. Based on these settings EthoAnalysis software translates these raw track data into series of zone-specific behavior events of three types: halting, moving, and not-detected (events that do not contribute to the calculation of behavior statistics) using the following procedure:

- Start at the first track sample at the beginning of the trial with a *movement state unknown* and process the track file sample by sample in time.
- Iterate over the sample records using the following decision rules:

A. Determine the *movement state* of the current sample based on these rules:

1. Current state is *moving* if either of the following two conditions is met:
   - **Start moving:** The previous state is not *moving* **and** the current velocity is greater than or equal to the velocity threshold **and** any of the next *n* samples (the look-ahead window) has a velocity greater than or equal to the velocity threshold. This protects both the moving and halting states from short halting or movement spikes.
   - **Remain moving:** The previous state is *moving* **and** any velocity in the current or the next *n* samples is greater than or equal to the velocity threshold.

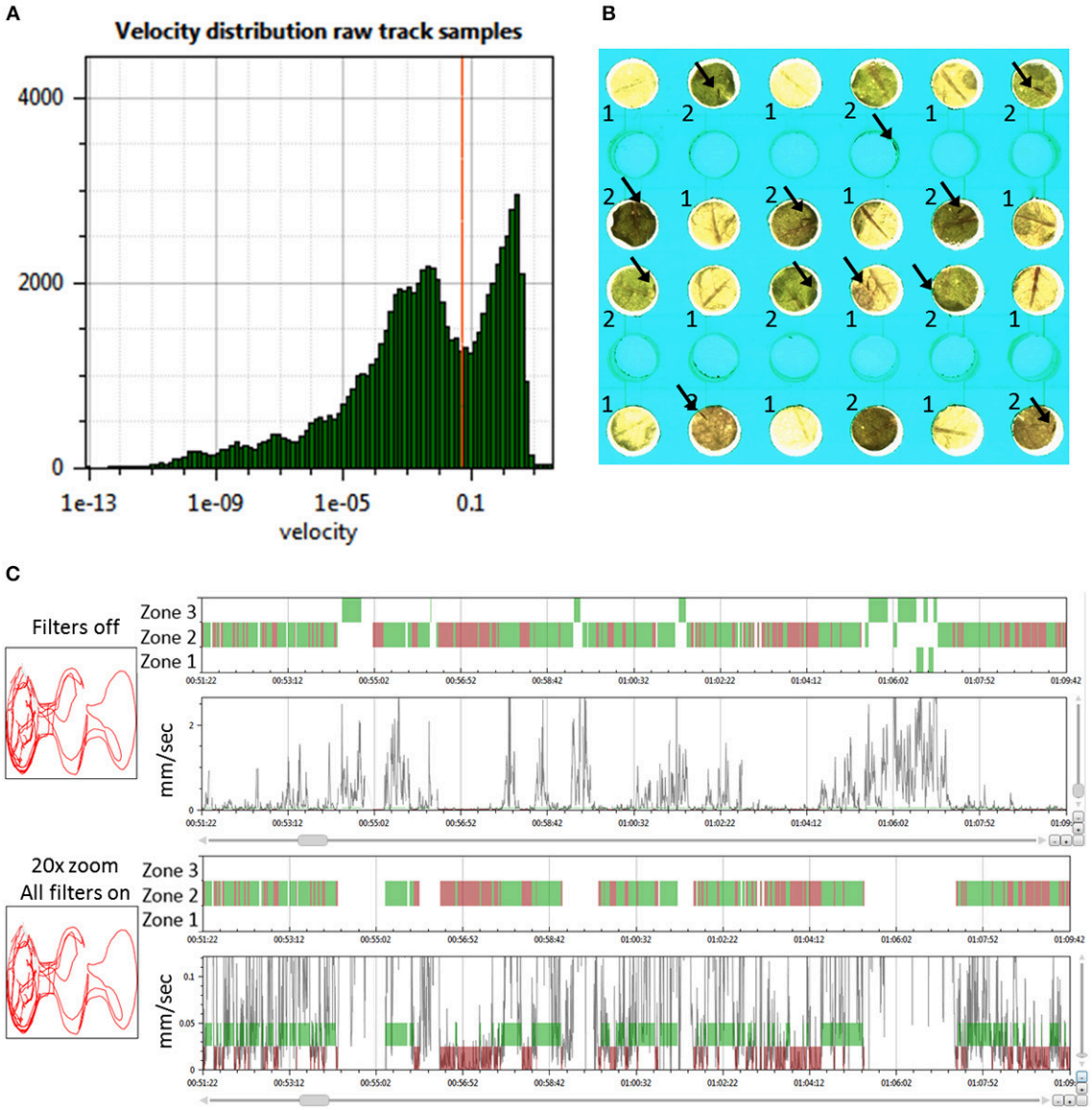

FIGURE 2 | Determining the behavior state and zone position of thrips. **(A)** Histogram of the velocity distribution of one thrips in a selected arena to aid in selecting proper thresholds for the determination of movement and halting events (velocity in mm/s); **(B)** Zoom of 12 arenas of the T-maze array with the two *Arabidopsis* accessions Cur-3 (1) and Rmx-A180 (2) in alternating positions. Arrows indicate the position of thrips individuals. The central empty well provided the access route of thrips from the cage plate below; **(C)** View on the left of the 2D arena shape position trace (red line) and on the right the 1D thrips zone position (zones 1–3 for Cur-3, Rmx-A180 and other) and velocity traces (black line) and movement state (green band for moving and red for halting) for a selected time interval (ca 18 min): thrips per arena are assigned to either a movement or halting state based on the velocity threshold and look-ahead window settings. The second panel is a Y-zoom of the velocity trace. It shows the effects of the velocity threshold (0.05 mm/s) and filters. All movement events in zone 1 and 3 are removed by applying all filters because in those zones they do not start and end with halts within the zone.

2. Else, if the current state is not recognized as *moving*, the current state is *halting* if either of the following two conditions is met:
   - **Start halting:** The previous state is not halting and the current sample has a positive detection.
   - **Remain halting:** The previous state is halting and any sample in the current or the next *n* samples has a positive detection.
3. Otherwise, if the current state is not recognized as *moving* or *halting*, the current state is set to *not-detected*.

B. If the current *movement state* is not equal to the previous *movement state* **or** the current zone is not equal to the previous zone, then add a new event for the previous state with its start time, end-time, and, if moving, the distance moved during this event.

From these series of events, the various behavior statistics are extracted (see section below about the calculation of the behavior statistics). An optional feature is to check "Recover halting from non-detected events." If checked, then halting events interrupted by a period of non-detection (due to tracking failure) are recovered and merged into a single halting event. More specifically, for each event-triplet of halting, non-detect, and halting, the start-position of the second halt event should lie within a given radius of the end-position of the first halt event for this merging to occur, otherwise merging cannot take place. The radius-size is defined by the total displacement (i.e., distance between start-point and end-point) within the first halting event. This option is checked by default in order to obtain more reliable data with less non-detect time [not detected (n.d.) was in the range of 10% in these experiments]. It is essential to check the "multiple zones" option to process the data in the format of the T-maze nature of the experiment in which zones can be contrasted against each other.

For various reasons, it may also be necessary to exclude certain types of events for analysis. Minor insect displacements on the zone boundary may cause artificial consecutive halting and moving events. Similarly, movement events containing extreme (unrealistic) velocities can be caused by video tracking hick ups, e.g., when a dirt particle or an optical reflection similar in size with the insect is confused with the insect. EthoAnalysis contains a number of "event filters" which change such events to non-detect events and therewith exclude them from analysis (**Figure 2C**). The effects of the filters are discussed below.

EthoAnalysis Filtering of Records

To obtain high-quality data it is important to remove records with insects that are either dead or obviously less active compared to the rest. For this purpose "Record filters" are used to automatically exclude records based on inactivity, detection percentage, and event count (**Supplementary Data File 1**, Filters tab). When applying these criteria all records are immediately updated and the table shows which records are deleted on which grounds. In the case of this experiment, the following criteria for record removal were applied: >3,600 s inactivity, <50% detection and <1,000 events. Three records were removed based on these settings in trial 3, all on the basis of event count. In experiment 3 there were on average 1,161 halts and 1,569 moves. More moves than halts can occur when insects move from one zone to another: that splits one movement into two parts. Once records are imported it is possible to inspect their quality using a velocity histogram (**Figure 1A**). This can also serve to guide modifications to the velocity threshold setting. Changing settings requires recalculation which took about 30 s for this experiment with 120 records and 8 h of recording.

EthoAnalysis Calculation of Behavior Statistics

The behavior statistics (**Supplementary Data File 1**, Behavior statistics tab) are extracted from the series of behavior events. The software contains a number of behavior statistics that can be extracted for all events of the complete trial, but some of these statistics are also of interest when looking at the statistics per zone, per hour, per zone/hour, or per type of behavior event, e.g., event duration category (short/medium/long) or movement velocity (slow/medium/fast). For the analysis, the robust zone-specified behavior events were selected.

EthoAnalysis Statistical Analysis

In the experiments, the two genotypes Cur-3 and Rmx-A180 were assessed in multiple trials, where each trial contains multiple choice arenas. Each arena consists of a single leaf of a plant from Cur-3 in one zone, and a single leaf from a Rmx-A180 plant in the other zone. In each trial 4 leafs of 10 plants of either genotype were used and the positioning of leafs in arenas is completely at random.

Suppose that the underlying means of some behavior statistic, such as the duration detected per zone, are μ_A and μ_B for Cur-3 and Rmx-A180, respectively, and the observed values in an arena are y_A and y_B. Choice experiments, like the one described here, are commonly analyzed by modeling the log ratio $\log\left(\frac{y_A}{y_B}\right)$ using normal errors, see, e.g., Elston et al. (1996). However, the disadvantage of the log ratio analysis is that it cannot properly handle zero observations. Moreover, observations very close to zero frequently popup as outliers in such analyses. Alternatively, a (conditional) logit model can be used, see, e.g., Hauber et al. (2016). In this approach, the log ratio is rewritten as $\text{logit}(\pi_A)$, where $\pi_A = \frac{\mu_A}{(\mu_A+\mu_B)}$, i.e., the mean for Cur-3 relative to the sum of the means. For behavior statistics, such as duration detected per zone, π_A can be interpreted as the probability of being in the zone with Cur-3. Information about this parameter is contained in the conditional distribution of y_A given the sum $y_A + y_B$, for which it is natural to assume a quasi-binomial distribution resulting in logistic regression.

Both the log ratio analysis model, which is a linear mixed model (LMM), and the logit analysis model, which is a generalized linear mixed model (GLMM), are included in EthoAnalysis. The logit model is implemented by means of an iterative re-weighted restricted maximum likelihood (IRREML) algorithm as proposed by Schall (1991). The LMM within this iterative procedure is fitted using the lmer function in the R package lme4 (Bates et al., 2015).

For the analyses in this paper, the logit model was applied to all behavior statistics to generate predictions of the ratios between means. Confidence intervals of these ratios and p-values were constructed using Satterthwaite's degrees of freedom approximation method [implemented in the R package lmerTest (Kuznetsova et al., 2017)]. To account for differences between plants, random effects for plants of Cur-3 and for plants of Rmx-A180 were added to the model. For analyses over multiple trials, a random effect for trial was included as well. The analysis was done using the EthoAnalysis software (version 1.3.0.6) which internally makes use of the lm function of a local installation of R (version 3.1.2) (**Supplementary Data File 1**, Analysis and Output tabs).

RESULTS

Optimizing EthoAnalysis Input and Filter Settings

We performed a sensitivity analysis on various input and filter settings in EthoAnalysis to test how these settings affected the

contrasts between both genotypes for the 38 different traits extracted from the tracks (**Tables 1**, **2**). At the basis of each comparison was the optimized F1 analysis. The robustness of that analysis was explored by altering settings one by one and usually in steps of two.

Effects of the Velocity Threshold and Look-Ahead Window Settings

The look-ahead window and the velocity thresholds that are applied to the velocity trace of each arena jointly determine the classification into moving and halting events. For a first estimation EthoAnalysis provides frequency histograms of all measured velocities per individual insect (**Figure 2A**; **Supplementary Data File 1**). From the camel shape of most histograms the velocity separating halting and moving behavior can roughly be deduced (**Figure 2A**). Most commonly at the dip of the camel shape we observed a velocity of around 0.05 mm/s. To explore the sensitivity of the system we also evaluated thresholds that were both higher (0.1 mm/s) and lower (0.025 and 0.012 mm/s) in steps of 2. The velocity threshold setting was also affecting the number of selected records (**Table 1**). Inspection showed that at least three velocity tracks were indeed poor and correctly deleted from the analysis. The table contains 38 parameters related to the recorded movement and halting events. The results of the statistical analysis are given in the table with the red/yellow color indicating various degrees of significance [$\log_{10}(p)$]. The table shows how the velocity threshold affects the significance of certain parameters more or less strongly. Often less significance in one parameter is complemented with more significance in another one. The most significant p-values ($\sim 10^{-10}$) were found at velocity threshold 0.025 mm/s for average movement distance and duration (F1 vs. F2–4).

The setting of the look-ahead window around 4 was subsequently explored by also testing 1, 2 and 8 frames-ahead of an original one (time window of 0.3–2.4 s). Both the longer and shorter look-ahead windows yielded results quite similar to a look-ahead window of 4 frames but on average 4 frames was optimal (**Table 1**, F1 vs. F6, F7).

Effect of Event Categories on Movement and Halting Duration, Frequency and Velocity

Kloth et al. (2017) have shown with aphids that it is insightful to subdivide halt events into event categories of different duration to specifically investigate the more frequent test probing phase that is interrupted (<3 min) separate from the less frequent succesful probing phase that continues into sustained feeding (>25 min). In EthoAnalysis this type of detailed analysis of behavior is accessible for both halting and moving events. Short, medium and long intervals can be defined by setting two time thresholds that separate short and medium and medium and long and which can be evaluated in terms of average and total duration and frequency. In the case of Western flower thrips, which is a frequently moving species, 2 and 10 s for halts and 2 and 5 s for movements appeared optimal for creating the most significant behavior distinctions between both accessions. Velocity categories were tested and found to yield relevant differences at the thresholds of 0.025 mm/s and 0.075 mm/s (**Table 2**, F1 vs. F8-F10).

Effect of Event Filters

Due to the strict assignment of all events to specific zones in two-choice assays, every moving/halting event terminates when the subject leaves a designated zone even though the movement continues. This leads to about 30–40% more moves than stops in the F1 settings of this experiment. The first event filter we applied was a filter for extreme velocity events that result from tracking artifacts. This removed 10% of all moves in experiment 3. **Table 2** (F11 vs. F1) shows that filtering out those events improved the statistics related to movements a little bit. Filtering out consecutive halts and movements due to zone boundary effects made little overall difference, but strongly reduced significance of the best statistic of average movement duration (F12 vs. F1). This can be explained by the fact that the highest speeds and longest distances and moves to other zones are generated on the most resistant genotype. Removing those zone transition data actually creates a bias toward "normal" behavior to accept the genotype for feeding and strongly reduces the number of events on the resistant genotype with resultant decreases in significance of the contrast. A similar and even larger effect is observed by the option to filter out "incomplete events" (F13 vs. F1), which are events that are interrupted by a non-detect state.

Different Behavior Statistics in a Choice Arena

Using the optimized settings above we compared two independent experiments in **Figure 3** for an impression of the variation between genotypes and between experiments in the values of different statistics and their significance.

Average Movement Duration, Velocity and Distance

The average duration of movement events is the most distinctive feature of thrips behavior between these two genotypes with highly significant scores of $P = 10^{-10}$ in the best experiment 3 with 39 samples (**Tables 1**, **2**). Comparing this with the independent experiment 2 (using the same F3 settings for both experiments) here also average movement duration yielded the best significance score. Interestingly the contrasts for this movement duration trait differed less than a factor 2: apparently the frequent measurement at every move creates a very reliable average for each arena (**Figure 3**). In experiment 3 the significant differences appeared to reside mostly in the >5 s moves which were dominant in the resistant Cur-3 genotype, but this relationship was not visible in experiment 2. This may be due to the fact that arena averages were based on a very high number of ca 200 movement events per hour which are obviously reduced when the data are split into the three subcategories (**Figure 3**). The average distance of movement events was also a highly significant feature of thrips behavior. Movement duration and distance are a function of velocity, but the average velocity differed less strongly between the genotypes (30–50%) than duration and distance. Yet, it is interesting to note that the insects were moving faster on average on the resistant genotype.

TABLE 1 | EthoAnalysis settings to evaluate the effect of velocity threshold and look-ahead window on the significance of the statistics [$\log_{10}(p)$].

Settings and statistics	Effect of velocity threshold				Effect of lookahead window			
ANALYSIS NUMBER	**F2**	**F1**	**F3**	**F4**	**F5**	**F6**	**F1**	**F7**
Lookahead window (frames)	4	4	4	4	1	2	4	8
Velocity threshold (mm.sec)	0.0125	0.025	0.05	0.1	0.025	0.025	0.025	0.025
Recover halting from non detect events	yes	yes	yes	yes	yes	yes	yes	yes
Mulitple zones	yes	yes	yes	yes	yes	yes	yes	yes
Include arena/neutral zone	no	no	no	no	no	no	no	no
Trial	3	3	3	3	3	3	3	3
Filter inactivity	3600	3600	3600	3600	3600	3600	3600	3600
Filter detection percentage	50	50	50	50	50	50	50	50
Filter by event count	1000	1000	1000	1000	1000	1000	1000	1000
Auto-selected arenas from 40 total	39	39	37	36	39	39	39	37
Filter consec. halts d.t. zone boundary effects	no	no	no	no	no	no	no	no
Filter consec. movements d.t. zone boundary effects	no	no	no	no	no	no	no	no
Filter events of the first x seconds	no	no	no	no	no	no	no	no
Filter extreme velocity events	8	8	8	8	8	8	8	8
Filter incomplete events	no	no	no	no	no	no	no	no
Filter long halts (duration > x seconds)	no	no	no	no	no	no	no	no
Filter short halts (duration < x seconds)	no	no	no	no	no	no	no	no
Event velocity categories (µm/sec) A-B	12-37	25-75	50-150	100-300	25-75	25-75	25-75	25-75
Halt duration categories (seconds) R-S	2-10	2-10	2-10	2-10	2-10	2-10	2-10	2-10
Movement duration categories (seconds) X-Y	2-5	2-5	2-5	2-5	2-5	2-5	2-5	2-5
BEHAVIOUR STATISTIC	**p**	**p**	**p**	**p**	**p**	**p**	**p**	**p**
Average halting duration	-1.64	-2.30	-0.85	-0.36	-1.64	-1.99	-2.30	-2.81
Average halting duration < R	-0.12	-0.05	-1.23	-2.46	-0.12	-0.30	-0.05	-0.08
Average halting duration R <= duration < S	-0.65	-0.05	-2.52	-2.43	-0.65	-0.13	-0.05	-0.60
Average halting duration >= S	-1.68	-3.09	-1.37	-0.21	-1.68	-3.27	-3.09	-3.48
Average movement distance	-4.02	-4.45	-4.31	-3.56	-4.02	-4.31	-4.45	-4.50
Average movement duration	-6.31	-10.59	-5.64	-4.27	-6.31	-4.45	-10.59	-6.69
Average movement duration < X	-0.54	-1.15	-0.31	-0.09	-0.54	-0.74	-1.15	-0.82
Average movement duration X <= duration < Y	-1.29	-0.30	-0.71	-2.77	-1.29	-0.41	-0.30	-1.44
Average movement duration >= Y	-9.35	-10.31	-5.33	-3.02	-9.35	-4.54	-10.31	-7.80
Average velocity	-3.48	-3.34	-2.97	-2.16	-3.48	-3.14	-3.34	-3.52
Estimated distance moved	-1.07	-0.93	-0.61	-0.29	-1.07	-0.78	-0.93	-1.03
Estimated duration halting	-2.19	-2.32	-2.78	-2.89	-2.19	-2.45	-2.32	-2.19
Estimated duration halting duration < R	-4.49	-4.90	-3.85	-2.82	-4.49	-4.64	-4.90	-4.24
Estimated duration halting R <= duration < S	-4.12	-4.57	-4.57	-3.63	-4.12	-4.72	-4.57	-4.18
Estimated duration halting duration >= S	-1.57	-1.89	-2.43	-2.75	-1.57	-1.98	-1.89	-1.76
Estimated duration moving	-3.63	-3.39	-2.65	-2.06	-3.63	-3.14	-3.39	-3.65
Estimated duration moving duration < X	-2.69	-3.56	-4.58	-4.09	-2.69	-4.39	-3.56	-2.95
Estimated duration moving X <= duration < Y	-4.41	-4.59		-3.57	-4.41	-4.82	-4.59	-3.63
Estimated duration moving duration >= Y	-3.31	-2.56	-1.41	-0.23	-3.31	-1.55	-2.56	-3.36
Estimated duration moving velocity < A	-2.88	-3.61	-4.03	-3.18	-4.15	-3.79	-3.61	-3.33
Estimated duration moving A <= velocity < B	-3.88	-4.23	-3.11	-2.16	-4.37	-3.97	-4.23	-4.45
Estimated duration moving velocity >= B	-2.79	-1.99	-0.90	-0.12	-1.94	-2.03	-1.99	-1.97
Halt frequency	-3.26	-4.11	-4.33	-3.57	-3.26	-4.43	-4.11	-3.56
Halt frequency duration < R	-4.72	-5.09	-4.19	-3.23	-4.72	-4.69	-5.09	-4.70
Halt frequency R <= duration < S	-4.38	-4.62	-4.41	-3.46	-4.38	-4.69	-4.62	-4.34
Halt frequency duration >= S	-1.75	-2.38	-3.71	-3.53	-1.75	-2.57	-2.38	-2.21
Movement frequency	-3.50	-4.22	-4.31	-3.57	-3.50	-4.46	-4.22	-3.79
Movement frequency duration < X	-2.69	-3.47	-4.51	-4.14	-2.69	-4.28	-3.47	-2.82
Movement frequency X <= duration < Y	-4.38	-4.57	-4.72	-3.69	-4.38	-4.87	-4.57	-3.58
Movement frequency duration >= Y	-4.45	-3.89	-2.90	-1.71	-4.45	-2.95	-3.89	-4.39
Movement frequency velocity < A	-2.90	-4.24	-4.07	-3.40	-3.58	-3.72	-4.24	-3.87
Movement frequency A <= velocity < B	-4.06	-4.12	-4.38	-3.52	-4.29	-4.35	-4.12	-3.77
Movement frequency velocity >= B	-4.49	-4.45	-3.78	-2.52	-4.34	-4.16	-4.45	-4.16
Ratio detection to total trial duration	-2.75	-2.76	-2.97	-2.98	-2.75	-2.80	-2.76	-2.75
Ratio halting to detection duration	-0.74	-1.54	-2.42	-2.67	-0.74	-1.90	-1.54	-0.97
Ratio halting to total trial duration	-2.47	-3.55	-2.81	-2.83	-2.47	-2.65	-3.55	-3.38
Ratio movement to detection duration	-0.60	-2.47	-3.19	-3.42	-0.60	-2.59	-2.47	-0.91
Ratio movement to halting duration	-1.78	-2.74	-3.25	-3.22	-1.78	-3.06	-2.74	-2.00
AVERAGE	**-3.03**	**-3.48**	**-3.14**	**-2.65**	**-3.07**	**-3.15**	**-3.48**	**-3.15**

Marked beige to red are significant [$\log_{10}(p) < -1.3 \sim p < 0.05$], marked gray are not significant [$\log_{10}(p) > -1.3 \sim p > 0.05$]. F1-F7 are different analyses on the same data. Marked blue are the settings that were tested.

TABLE 2 | EthoAnalysis settings to evaluate the effect of event categories and event filtering on the significance of the statistics [$\log_{10}(p)$] in trial 3.

Settings and statistics	Effect of event category				Effect of filtering events			
ANALYSIS NUMBER	**F8**	**F1**	**F9**	**F10**	**F11**	**F1**	**F12**	**F13**
Lookahead window (frames)	4	4	4	4	4	4	4	4
Velocity threshold (mm.sec)	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Recover halting from non detect events	yes	yes	yes	yes	yes	yes	yes	yes
Mulitple zones	yes	yes	yes	yes	yes	yes	yes	yes
Include arena/neutral zone	no	no	no	no	no	no	no	no
Trial	3	3	3	3	3	3	3	3
Filter inactivity	3600	3600	3600	3600	3600	3600	3600	3600
Filter detection percentage	50	50	50	50	50	50	50	50
Filter by event count	1000	1000	1000	1000	1000	1000	1000	1000
Auto-selected arenas from 40 total	39	39	39	39	39	39	39	39
Filter consec. halts d.t. zone boundary effects	no	no	no	no	no	no	yes	no
Filter consec. movements d.t. zone boundary effects	no	no	no	no	no	no	yes	no
Filter events of the first x seconds	no	no	no	no	no	no	no	no
Filter extreme velocity events	8	8	8	8	no	8	8	8
Filter incomplete events	no	no	no	no	no	no	no	yes
Filter long halts (duration > x seconds)	no	no	no	no	no	no	no	no
Filter short halts (duration < x seconds)	no	no	no	no	no	no	no	no
Event velocity categories (µm/sec) A-B	25-75	25-75	50-150	100-300	25-75	25-75	25-75	25-75
Halt duration categories (seconds) R-S	1-5	2-10	4-20	8-40	2-10	2-10	2-10	2-10
Movement duration categories (seconds) X-Y	1-2	2-5	4-10	8-20	2-5	2-5	2-5	2-5
BEHAVIOUR STATISTIC	**p**	**p**	**p**	**p**	**p**	**p**	**p**	**p**
Average halting duration	-2.30	-2.30	-2.30	-2.30	-2.30	-2.30	-2.34	-1.74
Average halting duration < R	-0.11	-0.05	-0.09	-0.23	-0.05	-0.05		-0.66
Average halting duration R <= duration < S	-0.09	-0.05	-0.72	-2.56	-0.05	-0.05	-0.05	-0.53
Average halting duration >= S	-2.69	-3.09	-2.03	-1.14	-3.09	-3.09	-3.09	-2.52
Average movement distance	-4.45	-4.45	-4.45	-4.45	-4.16	-4.45	-3.75	-2.43
Average movement duration	-10.59	-10.59	-10.59	-10.59	-10.17	-10.59	-6.96	-3.29
Average movement duration < X	-0.88	-1.15	-1.09	-0.12	-1.87	-1.15	-1.61	-1.11
Average movement duration X <= duration < Y	-1.47	-0.30	-5.21	-2.67	-0.24	-0.30	-0.75	-0.35
Average movement duration >= Y	-11.99	-10.31	-9.43	-3.54	-10.15	-10.31	-10.31	-3.76
Average velocity	-3.34	-3.34	-3.34	-3.34	-2.74	-3.34	-2.45	-2.07
Estimated distance moved	-0.93	-0.93	-0.93	-0.93	-1.22	-0.93	-3.29	-1.46
Estimated duration halting	-2.32	-2.32	-2.32	-2.32	-2.32	-2.32	-2.32	-3.51
Estimated duration halting duration < R	-4.88	-4.90	-4.77	-4.64	-4.90	-4.90	-4.90	-4.62
Estimated duration halting R <= duration < S	-4.76	-4.57	-3.61	-2.59	-4.57	-4.57	-4.57	-4.49
Estimated duration halting duration >= S	-2.15	-1.89	-1.54	-1.01	-1.89	-1.89	-1.89	-1.75
Estimated duration moving	-3.39	-3.39	-3.39	-3.39	-3.35	-3.39	-3.91	-3.74
Estimated duration moving duration < X	-3.26	-3.56	-4.18	-4.58	-3.54	-3.56	-3.40	-4.60
Estimated duration moving X <= duration < Y	-3.69	-4.59	-4.54	-3.93	-4.49	-4.59	-4.60	-4.29
Estimated duration moving duration >= Y	-3.17	-2.56	-1.50	-0.03	-2.54	-2.56	-3.19	-2.66
Estimated duration moving velocity < A	-3.61	-3.61	-4.22	-3.99	-3.61	-3.61	-3.60	-4.48
Estimated duration moving A <= velocity < B	-4.23	-4.23	-3.08	-2.08	-4.23	-4.23	-4.23	-4.17
Estimated duration moving velocity >= B	-1.99	-1.99	-0.64	-0.36	-1.99	-1.99	-2.75	-1.43
Halt frequency	-4.11	-4.11	-4.11	-4.11	-4.11	-4.11	-4.11	-3.89
Halt frequency duration < R	-4.65	-5.09	-4.89	-4.83	-5.09	-5.09	-5.08	-4.62
Halt frequency R <= duration < S	-4.78	-4.62	-3.93	-2.89	-4.62	-4.62	-4.62	-4.54
Halt frequency duration >= S	-3.03	-2.38	-1.87	-1.26	-2.38	-2.38	-2.39	-2.27
Movement frequency	-4.22	-4.22	-4.22	-4.22	-4.18	-4.22	-4.23	-3.58
Movement frequency duration < X	-3.27	-3.47	-3.84	-4.13	-3.47	-3.47	-3.32	-4.48
Movement frequency X <= duration < Y	-3.62	-4.57	-4.60	-4.09	-4.47	-4.57	-4.56	-4.23
Movement frequency duration >= Y	-4.50	-3.89	-2.85	-0.49	-3.83	-3.89	-4.31	-3.66
Movement frequency velocity < A	-4.24	-4.24	-3.65	-3.89	-4.24	-4.24	-4.20	-4.23
Movement frequency A <= velocity < B	-4.12	-4.12	-4.63	-4.26	-4.12	-4.12	-4.12	-3.93
Movement frequency velocity >= B	-4.45	-4.45	-3.82	-2.69	-4.17	-4.45	-4.62	-3.98
Ratio detection to total trial duration	-2.76	-2.76	-2.76	-2.76	-2.90	-2.76	-2.91	-3.84
Ratio halting to detection duration	-1.54	-1.54	-1.54	-1.54	-1.54	-1.54	-1.55	-1.76
Ratio halting to total trial duration	-3.55	-3.55	-3.55	-3.55	-3.55	-3.55	-3.56	-3.55
Ratio movement to detection duration	-2.47	-2.47	-2.47	-2.47	-2.48	-2.47	-1.57	-0.68
Ratio movement to halting duration	-2.74	-2.74	-2.74	-2.74	-2.81	-2.74	-0.90	-1.30
AVERAGE	**-3.54**	**-3.48**	**-3.41**	**-2.91**	**-3.46**	**-3.48**	**-3.51**	**-3.00**

Marked beige and red are significant [$\log_{10}(p) < -1.3$ equivalent to $p < 0.05$], marked gray are not significant [$\log_{10}(p) > -1.3$ equivalent to $p > 0.05$]. F1, F8–F13 are different analyses on the same data. Marked blue are the settings that were tested.

FIGURE 3 | Effect of independent experimental replication (experiments 2 and 3) on the log2 of RMX180/Cur3 value ratios and the value ratios across experiments per genotype and on the significance of the statistics ($N = 39$–40). Marked in red are significant [$\log_{10}(p) < -1.3 \sim p < 0.05$], marked gray are not significant [$\log_{10}(p) > -1.3 \sim p > 0.05$]; the settings of analysis F3 (**Tables 1**, **2**) were used to compare both experiments.

Estimated Duration Moving

By contrast the total estimated duration moving (corrected for detection) has less significant results for the aggregated results compared to some of the subcategories (frequency, velocity and distance) of this moving duration statistic (**Figure 3**). The aggregated total duration moving is less than a factor 2 different between both genotypes whereas the subcategories with the <2 and 2–5 s intervals are in the range of a factor 2–5 (**Figure 3**). The likely reason for this higher significance in these subcategories is that the durations are not averaged but totalled, and thus also reflect the choice between genotypes. Apparently, then, the moves of <5 s, which are associated with feeding on the preferred genotype, and not related to searching, are the most significant. This is also reflected in the statistic "estimated duration moving (at a certain) velocity (threshold)." Here the moving events are not totalled on the basis of their duration but their velocity instead. This yields almost a 6-fold difference between the genotypes for the slowest speeds of <0.05. These speeds are clearly associated with movements between cells during feeding: 50 μm per second is exactly in the range of the size of an epidermal cell. Most events were <5 s, based on the other statistics, so the range of movement was not more than 250 μm much less than the length of the insect (2 mm).

Estimated Distance Moved

Despite the fact that average movement distance differed significantly between both genotypes, the estimated distance

moved did not. The *average* distance moved on the resistant Cur-3 genotype was much longer but more *total* distance was covered on the susceptible genotype simply because more time was spent there.

Movement Frequency Duration and Velocity

The movement frequency is a way of describing movement events not in terms of duration or velocity, but simply in terms of the number of events. It represents just a subtle variation on the other statistics but assumes neutrality to the values and provides similar or more significant values. Also here the subcategories of frequency in terms of the duration and velocity of the events are given in **Figure 3**. The frequency of movements of short duration (<5 s) and low speed (<0.15 mm/s) are most significantly different.

Average and Estimated Halting Duration and Frequency

In strong contrast to average movement duration, the average halting duration, also when split into duration categories, is hardly or not significantly different between both genotypes (**Figure 3**). In the T-maze the thrips can choose either genotype for halting/feeding. Feeding on the resistant Cur-3 accession apparently follows the same behavior pattern except that a lower proportion of the total time is spent on feeding there (3- to 4-fold less, **Figure 3**). This effect of choice and uniformity of halting behavior on both genotypes is visible as well in the halting frequency for different durations: 3- to 4-fold differences are seen with similar significances and ratios for all categories in both experimental replications (**Figure 3**).

Ratio Detection and Halting to Total Trial Duration

The ratio of detection of the thrips individual relative to total trial duration represents the choice for activities (moving and halting) on either accession and a conventional way of studying relative resistance looking at preference. The ratio of detection is 2-fold more on the susceptible accession and the significance is reasonable but not as good as reported by the more specific statistics (**Figure 3**). Taking out only the component of halting (excluding moving) does not improve the result (**Figure 3**).

Ratio Halting and Movement to Detection Duration

Halting and movement to detection duration split up the comparison of time detected inside a zone into either halting or moving. The statistics show that on the susceptible genotype Rmx-A180 a slightly higher proportion of time is spent on halting and a slightly lower proportion on moving (**Figure 3**).

Ratio Movement to Halting Duration

Potentially this statistic ratio of movement and halting amplifies the differences between two tested genotypes and indeed compared to movement to detection and halting to detection the differences are combined and larger (around a factor 2). However, the significance of the difference is not better compared to the individual statistics so that this ratio statistic is also not as useful as some of the others offered above (**Figure 3**).

Western Flower Thrips Behavior in Time

For 14 of the statistics above the EthoAnalysis software also offers a time course analysis in time bins of 1 h. This feature is very useful for a quick insight into how the trait values and significance change in time. **Figure 4** shows that most of the 14 statistics have 95% confidence intervals (not taking experimental design into account) that do not overlap at some or all points in time during the experiment. Some obvious trends are that the average movement duration and distance decrease in time for both genotypes. The estimated duration spent halting and detection and halting to total trial duration on the other hand clearly diverge. Apparently, thrips develop a growing preference for Rmx-A180 over time.

Correlation of Behavior Statistics Within Experiments

Many of the EthoAnalysis behavior statistics are likely dependent on each other to some degree, and the purpose of looking at many is to determine which one across independent experiments reproducibly best describes the genotype differences. Both dependence and independence may have causes in genes (both plant and insect) or environment (assay quality, insect age) or assay set up (choice vs. no-choice). To test this, the software automatically generates Spearman correlation tests. We are showing the results for statistics obtained for Zone 2 (Rmx-A180) (**Figure 5**) in the EthoAnalysis software. This diagram of the correlation between the statistics of all reported traits for the entire 8 h of the recording shows how specific thrips behavior parameters correlate negatively (red boxes), positively (blue boxes), or not (white boxes), within zone 2.

Immediately obvious is the large blue square of positively correlated parameters of estimated movement and halt frequency duration and velocity. Exceptions are the estimated duration at high velocity which correlates only with long distance moved and the frequency of long halts which correlates with long duration and correlates negatively with the ratio movement to halting duration. Average velocity appears to correlate negatively with most traits except average movement distance and long fast moves.

The effect of assay set up on correlations found should be considered as well. The fact that the insects can choose their host has a strong effect on some of the ratios found depending on whether they represent average or cumulative/frequency data. The choice for the susceptible genotype will increase both the total movement and halting time on that genotype but not necessarily the average time of the events.

DISCUSSION

We have developed and evaluated a T-maze array in the novel EntoLab video tracking and behavior analysis system. The system makes use of dedicated hardware consisting of 90 parallel T-mazes to which individual insects like thrips can be admitted simultaneously by opening a gate plate. The heated cover plate was essential to allow prolonged recordings without the appearance of condensation droplets. We

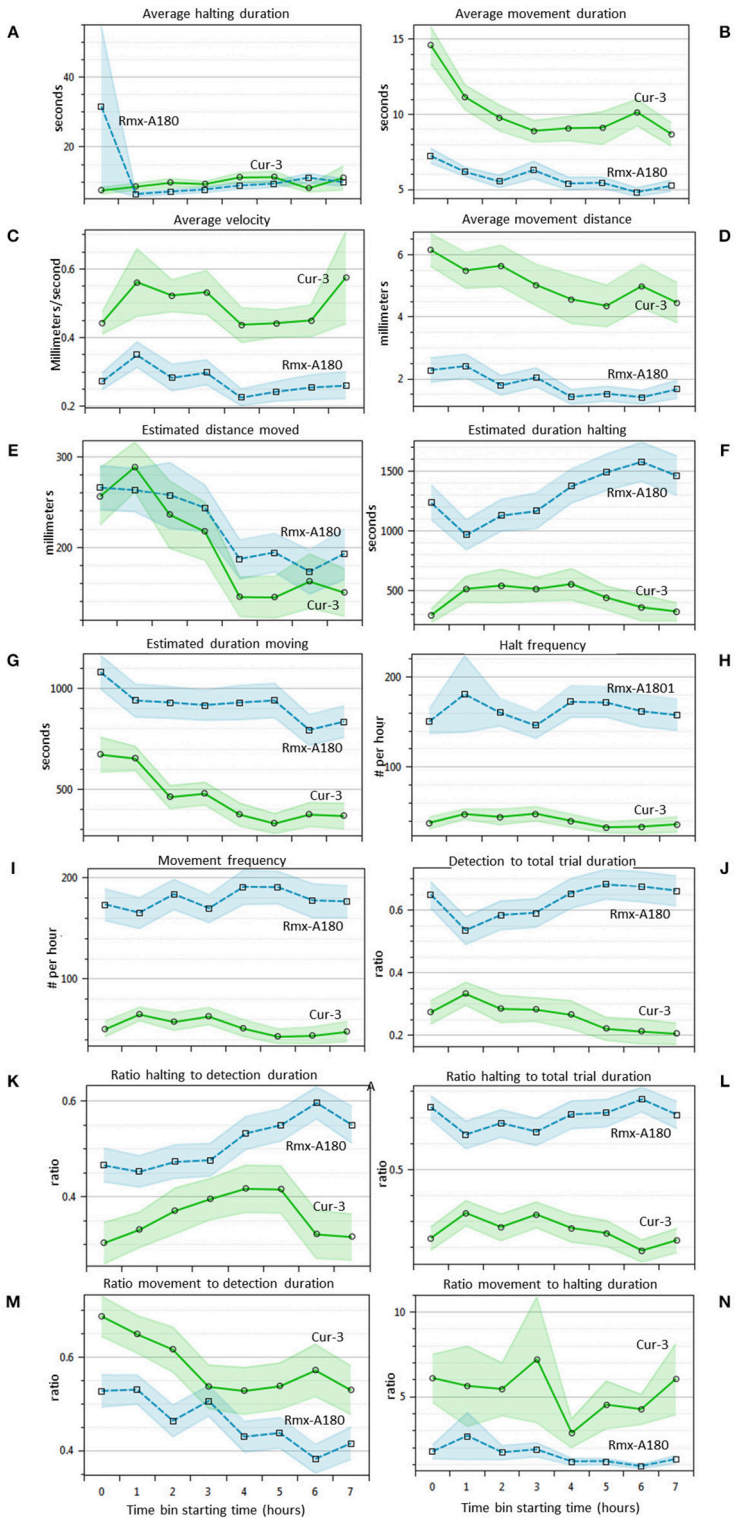

FIGURE 4 | Thrips choice behavior statistics in time **(A–N)**. Overview of 14 different statistics that are automatically indexed per hour by EthoAnalysis. The 95% confidence intervals are indicated by blue and green shading (excluding effects of experimental design). The results are given for experiment 3 (analysis F1) and are based on 39 arenas. Estimated values are per hour, corrected for the percentage detection (except frequency), and log-transformed for averaging. The average detection level was ∼90% in this experiment.

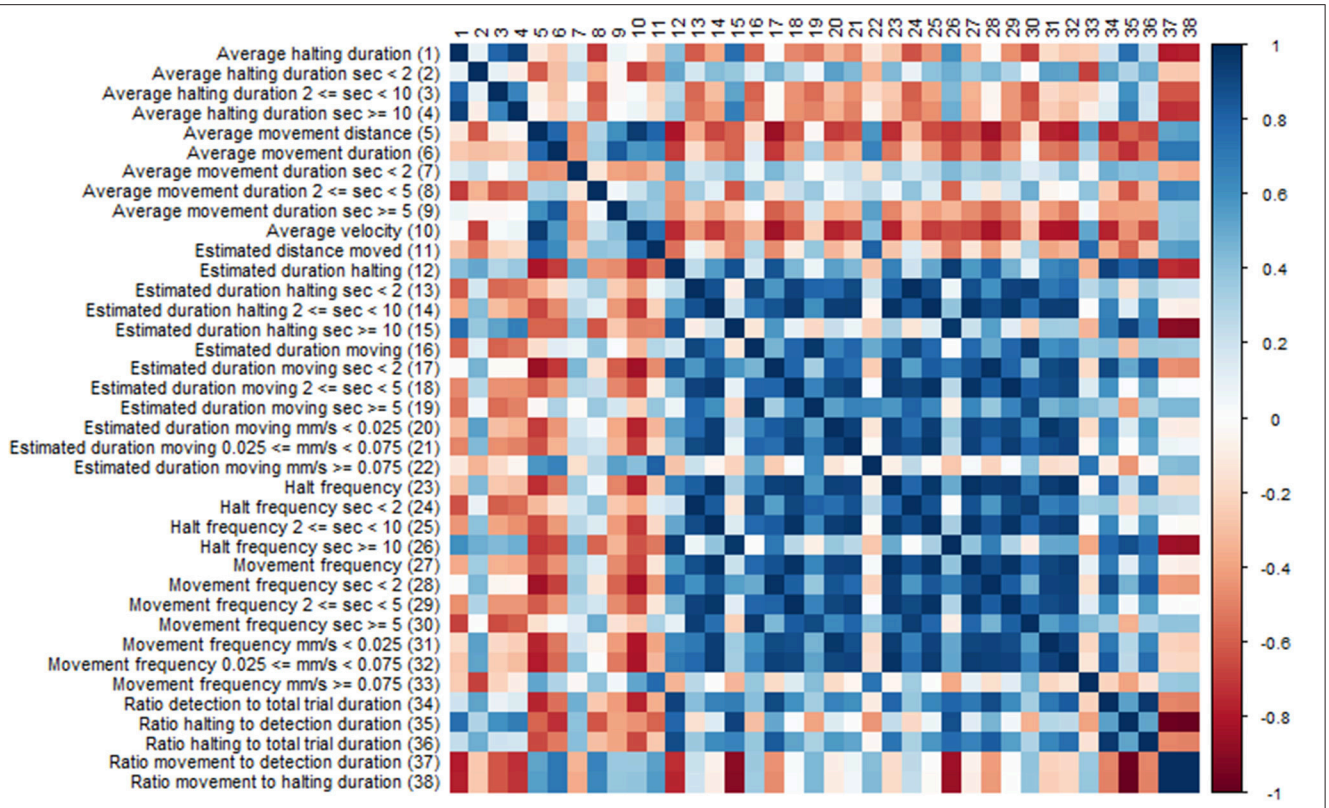

FIGURE 5 | Correlation diagram of behavior statistics. Colors indicate spearman *Rho* correlation values, where blue boxes indicate positive correlations and red boxes negative correlations between traits.

recorded the behavior of the insects on video and subsequently performed video tracking and computation of position and speed using EthoVision software. Exported tracks were analyzed by EthoAnalysis (**Supplementary Data File 1**). Both the hardware and software represent a large improvement relative to the use of microtiter plates and the previous time-consuming data extraction and manual statistical analysis (Thoen et al., 2016). In the new set up, with some experience, a powerful statistical analysis of multiple experiments including some optimization steps can now be done in 10–20 min whereas before *ad hoc* R scripts were written taking months to learn and carry out to obtain a similarly detailed output that we deem relevant to describe insect behavior (**Table 3**). With different insect species it will be easy to modify the size of the T-mazes to properly accommodate the size of the insects tested as was recently described for parasitic wasps for example (De Bruijn et al., 2018).

EthoAnalysis significantly extends the capabilities of EthoVision software for plant phenotyping studies (**Table 3**). Firstly, it also analyzes behavior statistics at the event level. These cannot be obtained directly from EthoVision, but are shown here to be the most reliable indicators of plant resistance to thrips. Secondly, filters can be applied in EthoAnalysis that automatically remove entire records or events with poor data, so that more reliable records remain. EthoVision only allows manual filtering of entire tracks based on visual inspection of deviant records. EthoAnalysis can also redefine input thresholds without the need to re-run the entire recording, as with EthoVision. This saves much time when settings must be adjusted to find the optimum. Post-recording 1D and 2D track and zone visualizations are available including the event assignments at any desired zoom level providing feedback on the effects of specific statistic settings on the assigned behavior in the recorded track (**Table 3**). We extracted 38 statistics in three overarching behavior categories of choice, halting behavior and movement behavior. In the given example these behavior parameters related to host-plant acceptance, and comprised of both dependent and independent statistics with different qualities to describe the difference between the genotypes. Eighteen of these statistics are available in time bins of 1 h as well (**Table 3**). EthoAnalysis also includes a built-in software package based on R, that properly transforms data of multiple experiments and applies the appropriate statistical models to evaluate behavior parameters, which are visualized in graphs (**Supplementary Data File 2**, **Table 3**).

A relevant question is whether one needs 38 partly dependent statistics to describe the behavior on these two plant genotypes. We think it is very helpful for two reasons: First of all, from these statistics a highly detailed time-resolved view emerges of how thrips is behaving in the T-maze on the combination of genotypes. Secondly, the most powerful statistics can be used

TABLE 3 | Features of EthoAnalysis for analyzing insect behavior.

Event determination	Post-analysis • adjusting event thresholds is done on raw track data, this takes 10–30 s for an 8 h movie depending the total number of arenas
Behavior parameters	• Choice (=detection/total trial duration) • Velocity • Halting duration + 3 subcategories duration • Halting frequency + 3 subcategories duration • Moving duration + 6 subcategories velocity and duration • Moving frequency + 6 subcategories velocity and duration • Moving distance • Event duration halting + 3 subcategories duration • Event duration moving + 3 subcategories duration • Event distance moved • Halting/total trial duration • Halting/detection duration • Movement/halting duration • Movement/detection duration 38 statistics (14 in time bins)
Data filter	• Automatic track filtering on inactivity period, detection percentage, and event count with threshold settings • Broad variety of event filters. Allows event filters based on duration of events, consecutive events due to zone transitions and velocity.
Detection dependency	Robust statistics that are corrected for the percentage of detection per subject
Statistics	Built-in statistical package and default transformations on data
Graphical visualization of data	Summary tables, graphs per trait, visualization of statistical models used
Post-recording video/track interaction	Fully interactive with a 2D visual tracking trace corresponding to the arena shape for the selected time period and with a corresponding 1D velocity trace with velocity threshold and a color coded assignment of movement, halting events and zone position. All with immediate feedback of applied filtering and detection success (red or gray).

to analyze large populations much more efficiently. In a high-throughput setting one would wish to reduce the number of replications and also the observation time (for example only 2 h and 10 insects per genotype). In such a setting it becomes critical to have access to the most informative statistic. In some cases such statistic information may be obtained in advance for crossing populations by pretesting the parents, but in the case of GWAS populations and also many crossing populations it will generally be a trial and error testing of all statistics to find which one best generates a particular QTL. This may require multiple testing corrections of the associated gene found, if all traits are given equal weight in quantitative genetic studies. One way to successfully overcome this with multivariate phenotypes like insect behavior, would be to map summary statistics or values generated through dimensional reduction methods, like principle component analyses or discriminant analyses (Horton et al., 2014).

We can evaluate the way in which the event-based analysis provides access to the detailed feeding behavior of thrips. A single feeding event of thrips can be divided in five consecutive steps: (1) Placing the tip of the mouth cone on the cell surface; (2) Thrusting the mandible through the plant surface layers; (3) Inserting the maxillary stylets into the cavity created by the mandible intrusion; (4) Sucking of the contents of punctured cells; (5) Retracting stylets and lifting the mouth cone (Kindt et al., 2003, 2006). Step 3, in which maxillary stylets are inserted into the created cavity is considered the start of a probing event, where cell contents are evaluated by the thrips. Only if this test probe is satisfactory, step 4 (the sustained sucking of contents) will follow. Feeding events where this last step does not follow, are thus likely not real feeding events, but just probing events. These "test probes" might occur more frequently when plant material is of suboptimal quality for thrips. Given that these probes were reported to generally take <10 s (Lewis, 1997), we thought that the relative duration of short and long probes could serve as a proxy for host plant suitability. However, in this choice assay the halting event durations were not very different and only the frequencies were strongly different due to the preference difference. Apparently, the free choice situation created "normal" feeding behavior (halting time) on both accessions. We expect, though, that this could be different in non-choice assays, because in that case the total time on each genotype is identical.

The selected settings were used to characterize two experiments with the *Arabidopsis* accessions Cur-3 and Rmx-A180. The value ratios of the two replicated experiments on different days delivered qualitatively very similar results, but quantitatively exhibited differences in both the absolute values and value ratios. Apparently, there are environmental batch effects between consecutive experimental days that can lead to such differences, and it will be crucial in complex experiments to deal with that. In an application of the EntoLab system for association genetics by screening large populations, proper assay design and mathematical treatment of these variations in values and ratios will be crucial for high quality results. High frequent alleles in a plant panel will to some extent compensate for experimental variation, but obviously it will always be important that the experimental design and analysis method should minimize these effects (Kloth et al., 2017). Future studies for application in plant genetics should, therefore, concentrate on best practices to minimize or correct for such variation. One approach could be to achieve complete block design on each plate. The current set up can contain 90 genotypes once and 10 plates could be tested to obtain 10 replications per accession for example. Yet with larger populations incomplete block design will be necessary. In choice assays such modeling approaches need to take into account genotype $\times$ genotype interactions, however, that can deal with a situation in which the reference genotype can both be relatively "susceptible" and "resistant" compared to the contrasted reference.

Heritability is essential in quantitative genetic studies. If biological variation arises from genetic or environmental effects, stochastic effects are classified as environmental because they are not passed on to offspring. But non-heritable effects can be subdivided into those which can be predicted from measurable variables, and those that cannot, e.g., stochastic effects (Honegger and De Bivort, 2018). Reducing the amount of stochasticity in spontaneous behavior might greatly increase data quality. Data output from EntoLab can be subsequently analyzed in dynamic

models that can accurately capture stochasticity, non-linearity and non-stationary behavior transitions from active to non-active (Melanson et al., 2017).

Potentially, long recordings also contain information on changes in thrips preference over time, due to resistance mechanisms that take a few hours to establish their effects on thrips, or resistance mechanisms that require induction by herbivory before defense pathways are activated. An example of "slow-acting" defense compounds are protease inhibitors, which can take at least 4 h to result in a significant effect on thrips choice behavior (Outchkourov et al., 2004). Thrips induced defenses are mediated by jasmonic acid and trigger the metabolism of a wide array of defensive compounds, but these can take 24 h to fully establish (De Vos et al., 2005; Abe et al., 2008, 2009). If such time-resolved data will be mapped onto plant genomes one would expect the associated QTLs to change in the course of hours, providing additional insight into the resistance mechanisms at work. Alternatively in screenings this induction period could be mimicked with a prior treatment with jasmonic acid so that data relevant to induced plants are obtained.

CONCLUDING REMARKS

EntoLab is a promising new instrument for high-throughput phenotyping insect behavior on plants and other substrates. We have so far validated the setup in either choice or no-choice assays for different species of thrips, aphids and whitefly (*Frankliniella occidentalis*, *Thrips tabaci, Myzus persicae, Brevicoryne brassicae, Nasonovia ribisnigri, Aphis gossypii, and Bemisia tabaci)* in various combinations on pepper, tomato, water melon, chrysanthemum, white cabbage, lily, lettuce, and bitter gourd. A paper describing results with no-choice assays of *N. ribisnigri* on whole leaves of lettuce is in preparation. We expect the potential use of the EntoLab system to extend beyond the assay of plant material and herbivores. Different arena designs, insect sizes and test samples can be easily implemented to fit the universal arena plate holder as was recently shown in a case study on learning and memory retention in parasitic wasps (De Bruijn et al., 2018). We hope that the unlocking of behavioral details that now often go unnoticed will in the future lead to more insight into the environmental and genetic mechanisms that control it.

DATA AVAILABILITY STATEMENT

The raw and processed data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher. The EthoVision and EthoAnalysis software can be obtained with a time-limited trial license. The entire EntoLab set up can be obtained from Noldus Information Technology BV (www.noldus.com/entolab).

AUTHOR CONTRIBUTIONS

MJ and MT wrote the paper. MJ designed the hardware and supervised the research. LP and GW carried out the experiments. PG designed the method for the statistical analysis. MD and LN supervised the research and cooperation. JK wrote the EthoAnalysis software. All authors have read and approved the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00020/full#supplementary-material

Supplementary Data File 1 | EthoAnalysis screen capture of all tabs.

Supplementary Data File 2 | Automatically generated statistical analysis by the EthoAnalysis software using the F1 settings. This represents the most extensive format. Optional are reports with only the main results.

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Conflict of Interest Statement: The hardware and software of the EntoLab system will be marketed jointly by Noldus Information Technology BV and Wageningen Plant Research. This will promote the distribution and use of the system, but does present a potential commercial and financial conflict of interest.

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PERSPECTIVE
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Herbivore-Associated Bacteria as Potential Mediators and Modifiers of Induced Plant Defense Against Spider Mites and Thrips

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Induced plant defense, comprising contact with exogenous stimuli, production of endogenous signals alerting the plant, associated biochemical cascades, and local and/or systemic expression of the defense mechanisms, critically depends on the nature of the inducing agents. At large, bio-trophic pathogenic microorganisms and viruses usually trigger the salicylate (SA)-mediated pathway, whereas necro-trophic pathogens and herbivores usually trigger the jasmonate (JA)-mediated pathway in plants. The SA- and JA-mediated pathways do not operate independently but commonly interfere with each other. Several recent studies revealed abnormal plant responses upon herbivore attack in diverse plant-herbivore systems. Observed abnormalities range from suppression of the common JA-pathway, induction of the SA-pathway to no response, yet the underlying proximate causes and ultimate consequences of these variations are elusive. Strikingly, some studies provide compelling evidence that anti-herbivore plant responses may decisively depend on bacteria associated with the herbivore attacking the plant (HAB for herbivore-associated bacteria). HAB may influence herbivore recognition by the plant and alter the biochemical cascades inside plants. Here, I report cases in point of HAB manipulating induced anti-herbivore plant responses, suggest spatial and temporal categorization of HAB, and point at proximate and ultimate aspects of plant defense manipulation by HAB. Following, I overview the diversity of HAB of spider mites and herbivorous thrips, argue that, considering recently reported phenomena of abnormal plant responses upon spider mite attack, some of these HAB could represent important, but hitherto largely neglected, mediators/modifiers of induced plant defense against spider mites and thrips, and conclude with suggestions for future research.

Keywords: induced plant response, bacteria, spider mites, thrips, endosymbionts, gut

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BACKGROUND

Plant defense against pathogenic and/or herbivorous organisms may be constitutive, induced or a combination of both. Induced plant defense comprises four phases: (i) contact with exogenous stimuli, (ii) production of endogenous signals alerting the plant, (iii) associated biochemical cascades, and (iv) local and/or systemic expression of the defense mechanisms. All phases

critically depend on the nature of the inducing exogenous agents (Karban and Baldwin, 1997; Agrawal et al., 1999; Mithöfer and Boland, 2012). At large and from a generalizing/categorizing viewpoint, bio-trophic pathogenic microorganisms and viruses usually trigger the salicylate (SA)-mediated pathway, whereas necro-trophic pathogens and herbivores usually trigger the jasmonate (JA)-mediated pathway in plants. The SA- and JA-mediated pathways do not operate independently but influence, and commonly interfere with, each other (e.g., Thaler et al., 1999, 2012). Mechanistically, herbivore-attacked plants may defend themselves directly by changes in chemistry, such as the production of toxic substances (e.g., digestibility reducers) or proteinase inhibitors, and/or by morphological alterations, such as cell wall lignification, and/or indirectly by recruiting and fostering third trophic level natural enemies (predators and parasitoids) of the herbivores via emitting attractive volatiles or increasing the availability of alternative food. In detail and from a more sophisticated viewpoint, the above associations between defense-inducing agents and the SA- and JA-mediated pathways of plant defense are not that clear-cut. Induced plant responses to herbivores may vary between individuals, lines and populations of the same herbivore species. Observed abnormalities in plant response to herbivore attack range from suppression of the common JA-pathway, induction of the SA-pathway to no response, yet the underlying proximate causes of these variations and ultimate consequences for multi-trophic plant–herbivore–carnivore interactions are elusive. Strikingly, a number of recent studies provide compelling evidence that the response of plants to herbivore attack may be decisively influenced by microorganisms associated with the herbivore attacking the plant. Suggestions of herbivore-associated microorganisms possibly playing important roles in plant–herbivore interactions have been made decades ago (Berenbaum, 1988; Barbosa et al., 1991), yet rigorous experimental assessment has gained momentum only recently. Notably, studies on beetle larvae and whiteflies show that herbivore-associated microorganisms may fundamentally change the plant response to herbivore attack (Chung et al., 2013a,b; Su et al., 2015). Microorganisms playing decisive roles in induced plant defense may also be true for spider mites (Tetranychidae) and herbivorous thrips (Thripidae), many species of which are globally distributed and tremendously important as both agricultural pests and model organisms in plant-animal interaction research. These two families include two of the most destructive agricultural plant pests worldwide, i.e., the two-spotted spider mite *Tetranychus urticae* and western flower thrips *Frankliniella occidentalis*. Both species are highly polyphagous with >1000 host plant species reported for *T. urticae* (Bolland et al., 1998) and >250 host plant species reported for *F. occidentalis* (CABI, 2018). Spider mites feed on their host plants by piercing parenchyma cells and sucking out the cell contents; thrips feed on their host plants by rasping open the epidermal cells and sucking out the cell contents; thrips are also feared vectors of plant-pathogenic viruses (Rotenberg et al., 2015). Species of both families may be associated with a host of microorganisms, and phenomena akin to those observed in beetles and whiteflies, such as specific individuals, lines or populations eliciting abnormal plant responses upon attack, have been described. For example, Abe et al. (2012) observed a beneficial effect of tomato spotted wilt virus-infection in *Arabidopsis* on *F. occidentalis*' performance by upregulating SA-mediated defense interfering with JA-mediated pathways. Kant et al. (2008) observed variation regarding JA-mediated defense induction and susceptibility in *T. urticae* on tomato *Lycopersicon esculentum*, among others with one non-inducing but still susceptible line; Sarmento et al. (2011) and Alba et al. (2015) described a line of *Tetranychus evansi* (an invasive spider mite species primarily occurring on solanaceous host plants) that suppresses both SA- and JA-mediated defense pathways in tomato. Whether or not microorganisms are at play in the spider mites under investigation has been largely untested (but see Staudacher et al., 2017).

This article focuses on potential implications of herbivore-associated microorganisms in modulation of plant responses induced by spider mites (Tetranychidae) and thrips (Thripidae). Due to the lack of knowledge for herbivore-associated viruses and fungi, except for transmission of plant-pathogenic viruses by various thrips species (Rotenberg et al., 2015) and the spider mite *Petrobia latens* (Robertson and Carroll, 1988), this article is primarily concerned with gut/saliva bacteria and facultative intracellular endosymbionts (i.e., secondary symbionts). Primary symbionts influencing herbivore–plant interactions, such as *Buchnera* sp. (see Engel and Moran, 2013 for review), are left out for no primary symbionts have been described in spider mites and thrips. For up-to-date treatises of diverse phenomena of microorganisms affecting plant–herbivore interactions across herbivore taxa, see Frago et al. (2012); Hansen and Moran (2014), Giron et al. (2017), and Shikano et al. (2017). The aims of this article are reporting cases in point of herbivore-associated bacteria (HAB) fundamentally changing induced plant defense against herbivores, overviewing the diversity of bacteria associated with spider mites and thrips, discussing possible roles of those bacteria in mediating induced plant defense against herbivores, and providing suggestions for pertinent future research using spider mites and thrips.

CATEGORIES OF BACTERIA POTENTIALLY MEDIATING PLANT RESPONSE TO HERBIVORE ATTACK

Bacteria mediating the plant response to herbivore attack can be allocated to three, mutually non-exclusive, major groups, based on their location relative to the herbivore body (Hansen and Moran, 2014): environmental (or external), digestive system (internal extracellular), and endosymbionts (internal intracellular) (**Figure 1A**). Environmental (external) bacteria may reside on the outside of the herbivore, on the mouthparts or other body parts, or on the plant surface (Redford et al., 2010). Regarding environmental microorganisms much more is known about fungi than bacteria (Hansen and Moran, 2014) and experimental studies addressing the functions of environmental bacteria in plant–herbivore interactions are rare. Internal extracellular are mainly bacteria in the digestive system, i.e., in the gut and/or in the salivary glands (Dillon and Dillon, 2004;

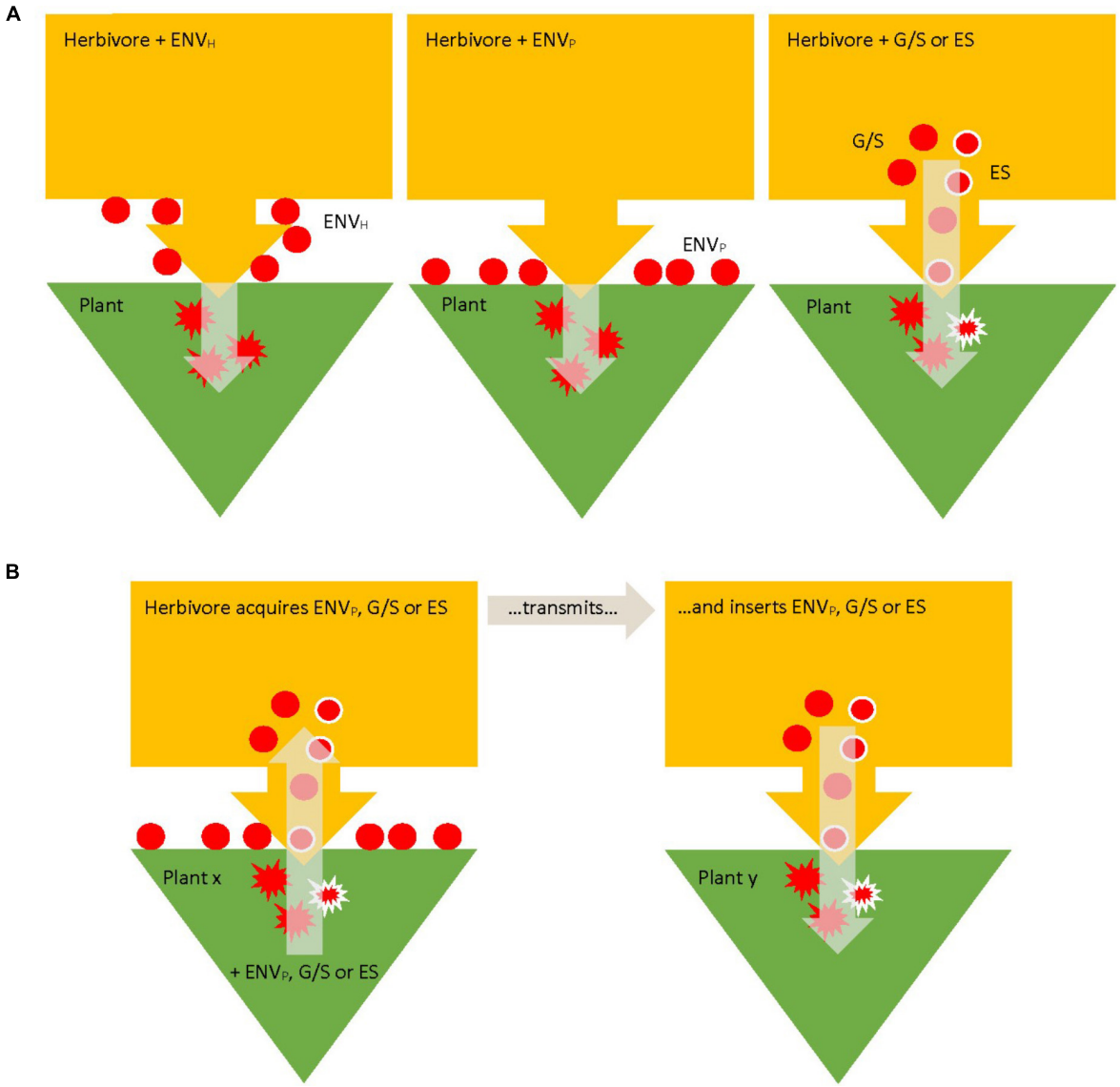

FIGURE 1 | Pathways of transmission of herbivore (yellow)-associated bacteria (HAB; red) to their host plants (green). HAB may be environmental ($ENV_{H,P}$; on the outside of the herbivore or plant), in the gut/saliva (G/S), or intracellular endosymbionts (ES; red with white margin), based on their location relative to the herbivore body. Circles symbolize HAB possibly involved in induced plant defense; splashes symbolize HAB activity in induced plant defense inside plant tissue. HAB may be intrinsically associated with the herbivores, either at the herbivore/plant interface or inside the herbivores **(A)** or may be acquired from plants and transmitted to other plants **(B)**.

Engel and Moran, 2013); internal intracellular are mainly reproductive and other endosymbionts (Werren, 1997; Weeks et al., 2003; Engelstädter and Hurst, 2009). In any case, bacteria of all three location categories can be transmitted from herbivores to inner plant tissues by feeding activity (albeit with varying likelihood and reliability) and have the potential to change the defensive response of the plant to the herbivores (**Figure 1A**). HAB may further be allocated along the continuum from permanence to transience regarding their association with the herbivores. On the close end of this continuum are endosymbionts and bacteria in the digestive system reliably transmitted vertically or horizontally among conspecific individuals; on the distant end of this continuum are plant pathogens or other miscellaneous microorganisms present in the phyllosphere, which are just vectored by feeding activity from the outside to the inside of the plant or from one plant to another. Bacteria in the gut and/or salivary glands and intracellular endosymbionts influencing plant response may be transient if picked up from one plant by feeding and inserted or deposited in feces on another plant (**Figure 1B**), such as plant-pathogenic bacteria transmitted by spider mites (Choi et al., 2016) or may be permanently, or close to permanently, associated with herbivores such as secondary extra- and intracellular endosymbionts. In any case, acquisition of bacteria from plants may also lead to

novel more permanent individual associations such as described for plant-mediated transmission of secondary endosymbionts (Caspi-Fluger et al., 2012 for *Rickettsia*; Gonella et al., 2015 for *Cardinium*; Li et al., 2017 for *Wolbachia*).

EVIDENCE OF HAB MEDIATING PLANT RESPONSE TO HERBIVORE ATTACK

As in animals in general, herbivorous mites and insects house a host of different microorganisms in their gut and salivary glands, with the bacteriomes often being dominated by a small number of species, but high inter-taxon variability (Engel and Moran, 2013; Jones et al., 2013). Feeding herbivores commonly transmit gut/saliva bacteria to the plant. For example, Bansal et al. (2011) determined that up to 70% of the bacterial genera found in Hessian fly larvae (*Mayetiola destructor*) were also found in fly-infested wheat, indicating that the bacteria are transmitted to the host plant via feeding. Striking examples of gut/saliva HAB manipulating plant response come from Colorado potato beetle, *Leptinotarsa decemlineata* on tomato, *L. esculentum* (Chung et al., 2013a,b). Bacteria such as *Enterobacter*, *Stenotrophomonas*, and *Pseudomonas* in the beetle saliva induce plant defense against pathogens, i.e., the SA-dependent pathway, which in turn downregulates the JA-dependent pathway due to negative cross-talk (Thaler et al., 1999, 2012), altogether promoting growth of the beetle larvae (Chung et al., 2013a,b). Similar suppression of JA-mediated plant defense occurs in various *Solanum* sp. although different bacterial communities in the beetle saliva are involved (Chung et al., 2017). Also, in the false potato beetle *Leptinotarsa juncta*, bacteria in the regurgitate may suppress anti-herbivore plant defense in tomato and horsenettle, with host-plant-specific bacterial effects (Wang et al., 2016). Host plant specificity has also been shown for bacteria (e.g., *Pantoea*) in the saliva of fall armyworms *Spodoptera frugiperda*, which suppress anti-herbivore defense in tomato, but induce such defense in maize (Acevedo et al., 2017). In *Arabidopsis*, greenhouse whiteflies *Trialeurodes vaporariorum* induce the SA-dependent pathway but suppress JA-dependent anti-herbivore defense (Zarate et al., 2007); however, it is as yet unknown whether this is due to herbivore-associated microorganisms or intrinsic (endogenous) herbivore traits.

Tobacco whiteflies *Bemisia tabaci* harboring the facultatively endosymbiotic bacteria *Hamiltonella defensa* in their saliva suppress JA-dependent defenses compared to whiteflies free of the bacteria (Su et al., 2015). In the tomato psyllid, *Bactericerca cockerelli*, the endosymbiont Candidatus Liberibacter psyllaurous (which can also be plant-pathogenic) downregulates both JA- and SA-dependent defense responses of tomato plants (Casteel et al., 2012). *Wolbachia*, *Cardinium*, and *Spiroplasma* are widespread, primarily maternally transmitted symbionts manipulating reproduction in many arthropods (for review, see Engelstädter and Hurst, 2009). Among the secondary symbionts, *Wolbachia* is the most widely documented and the most prominent reproductive endosymbiont, infecting a large proportion of arthropod species (Werren et al., 2008). *Wolbachia* is best known for reproductive manipulation of their hosts to enhance its own spread while lowering host fitness. Recent studies provide a growing body of evidence for *Wolbachia*−associated fitness benefits (for review, see Zug and Hammerstein, 2015). Also, other bacterial endosymbionts, such as *Cardinium* (Weeks et al., 2003), *Spiroplasma* (Cisak et al., 2015), or *Rickettsia* (Weinert et al., 2009), ranked at decreasing prevalence in arthropods (Duron et al., 2010), are similarly able to manipulate reproduction of their hosts (Engelstädter and Hurst, 2009). These endosymbionts have been previously thought to be primarily vertically transmitted from mother to offspring but recent experimental evidence suggests that horizontal transmission via feeding on prey/hosts (Le Clec'h et al., 2013; Ahmed et al., 2015), mating (Moran and Dunbar, 2006), sharing hosts (Huigens et al., 2004; Duron et al., 2010) and/or feeding on plants (Caspi-Fluger et al., 2012 for *Rickettsia*; Gonella et al., 2015 for *Cardinium*; Li et al., 2017 for *Wolbachia*) is probably more common than previously anticipated. *Wolbachia* has been shown to persist in plant tissue for more than 50 days at unknown temperature (Li et al., 2017). *Wolbachia* is often also present in the salivary glands of their hosts (Dobson et al., 1999), where it may change the composition of the saliva, which in turn may influence plant defense. Contrasting evidence exists for *Wolbachia* mediating induced plant defense against larvae of the western corn rootworm *Diabrotica virgifera virgifera* in maize. While Barr et al. (2010) concluded that *Wolbachia*-infected but not uninfected weevil larvae suppress anti-herbivore defense in maize roots, Robert et al. (2013) did not find any evidence for differences in the plant response to *Wolbachia*-infected and uninfected weevil larvae. Possible reasons for these contrasting results include antibiotic treatments likely removing other microorganisms, including gut/saliva bacteria, from the weevils and/or the bacteriomes differing between the weevils used by Barr et al. (2010) and Robert et al. (2013).

PROXIMATE AND ULTIMATE CONSIDERATIONS

Proximate questions are whether the substances abnormally modulating plant defense are produced by the herbivores themselves (and thus represent herbivore-associated-molecular patterns, HAMPs) or constitute metabolites of the bacteria in their saliva, the regurgitate or on the outside of their mouthparts (and thus represent microbe-associated-molecular patterns, MAMPs) (Maffei et al., 2012). Alternative underlying mechanisms include substances produced by the bacteria and the herbivores masking each other, or the herbivores are lacking substances that are normally needed by the plants to recognize the herbivores, such as linolenic acid in caterpillars of *Heliothis subflexa* (De Moraes and Mescher, 2014). HAB and/or their products may have direct effects on the plants or indirect via affecting (inducing or changing) the expression of elicitors, effectors or HAMPs in the herbivores' saliva or regurgitate. For example, gut bacteria induce the expression of an elicitor in *Helicoverpa zea* triggering JA-dependent plant defense in tomato (Wang et al., 2017). In contrast, Acevedo et al. (2017) found that *Pantoea* did change the plant response to fall armyworms

but did not change the expression of salivary proteins or known elicitors in the caterpillar's saliva, indicating MAMPs. Abnormal plant responses upon herbivore attack could simply constitute recognition errors by the plant.

One may argue that herbivores evolved associations with specific microorganisms to disguise themselves from proper recognition by the plants and thus trick plants into activating biochemical cascades and defense against non-existent threats usually posed by pathogenic microorganisms. However, important ultimate questions to be addressed case by case are whether these phenomena truly represent counter-adaptations in the arms race between plants and herbivores targeted to disguise herbivore identity, deceive plants into incorrect recognition, and/or switch off or circumvent the defense response against herbivores, or whether it is side effects of accidental associations with microorganisms, or epiphenomena of co-evolved associations for other reasons than manipulating plant defense, or whether it is simply recognition errors by the plant. Answering those questions requires additionally assessing both direct and indirect fitness consequences of bacterial associates for the herbivores and considering the transience-permanence continuum of the bacteria–herbivore association. HAB providing direct fitness benefits to their host (Zug and Hammerstein, 2015) and additionally manipulating plant defense to the benefit of their hosts and promoting their own spread are clear indications of bacteria-herbivore co-evolution. However, even HAB lowering herbivore fitness in a direct way, may indirectly promote herbivore fitness if suppressing anti-herbivore plant defenses. Herbivores may yield net fitness benefits through provision of enemy-free space – because attacked plants do not cry for help the herbivores remain cryptic to their natural enemies (see De Moraes and Mescher, 2014) – or through the prevention of production of digestion inhibitors or other toxins (Boone et al., 2013; Douglas, 2013).

SUSPICIOUS BACTERIA ASSOCIATED WITH SPIDER MITES AND THRIPS

Overall, regarding gut/saliva and intracellular endosymbiotic bacteria more studies are available for spider mites than thrips. Accordingly, there is a much wider spectrum and diversity of bacteria reported for spider mites than thrips (Supplementary Tables S1, S2) but this does not necessarily mean that spider mites are more prone to harbor bacteria than thrips because of differences in detection efforts. In any case, members of both families may harbor bacteria that have been found to manipulate plant defense in other arthropods (Supplementary Tables S1, S2). Among Tetranychidae, two-spotted spider mites *T. urticae* and *T. evansi* are especially well studied, with a large number and diversity of gut/saliva and endosymbiotic bacteria (e.g., Yoon et al., 2010; Knegt et al., 2017; Staudacher et al., 2017 for gut bacteria; e.g., Zélé et al., 2018 for endosymbionts). Spider mite species are especially likely to harbor single or multiple reproductive symbionts such as *Wolbachia*, *Cardinium*, or *Spiroplasma* (Supplementary Tables S1, S2). Notably, Staudacher et al. (2017) detected *Wolbachia* and *Cardinium* in *T. urticae* and Zélé et al. (2018) in *T. evansi*, lines of which have been described to abnormally manipulate plant defense against herbivores (Sarmento et al., 2011; Alba et al., 2015). The two most prominent thrips pests, western flower thrips *F. occidentalis* and onion thrips *Thrips tabaci* do not harbor reproductive endosymbionts but are stably associated with Enterobacteriaceae such as *Erwinia* and *Pantoea* (de Vries et al., 2001, 2008; Chanbusarakum and Ullman, 2008; Facey et al., 2015; Dutta et al., 2016a). Endosymbionts such as *Wolbachia* or *Cardinium* have been found in many other herbivorous thrips species such as *Thrips palmi* (Saurav et al., 2016). Virtually nothing is known about fungi, fungal spores, or fungal metabolites in the saliva of thrips and mites.

MIND THE BACTERIA OF SPIDER MITES AND THRIPS IN INDUCED PLANT DEFENSE

Similar to other herbivores, for spider mites, there exist a number of studies reporting unusual plant responses upon attack (suppression of JA-mediated defense and/or induction of the SA-mediated pathway) (e.g., Kant et al., 2008; Sarmento et al., 2011; Alba et al., 2015). However, studies aiming at deciphering and suggesting potential involvement of microorganisms in modulation of abnormal plant responses are scarce and restricted to two-spotted spider mites *T. urticae* (Ueda et al., 2010; Staudacher et al., 2017). Both studies report that removal of microorganisms from the spider mites by antibiotics changes the response of tomato and bean plants to their attack. Most notably, among other differences in plant response induction among doubly infected, singly-infected and non-infected mites, Staudacher et al. (2017) observed downregulation of JA-precursors but upregulation of SA following removal of *Wolbachia* but conservation of *Spiroplasma*. Some lines of *T. evansi* and *T. urticae* may suppress both SA- and JA-dependent plant defenses (Sarmento et al., 2011; Alba et al., 2015), which has been associated with altered expression of genes responsible for the production of effector proteins in the spider mite saliva (Jonckheere et al., 2016; Villarroel et al., 2016). Nonetheless, HAB influence and altered gene expression are rather inter-related than mutually exclusive explanations for induction of abnormal plant responses. HAB may both influence gene expression of their hosts, as documented in the fruit fly *Drosophila melanogaster* (Broderick et al., 2014; Combe et al., 2014), and alter the occurrence and composition of salivary proteins, as documented for *Wolbachia* and *Spiroplasma* in the spider mite *Tetranychus truncatus* (Zhu et al., 2018). Abnormal plant responses could also be due to HAB influence in the evolutionary past if genes coding for enzymes involved in mite-plant interactions stem from horizontal gene transfers between HAB and spider mites (Wybouw et al., 2018). Additional or alternative explanations on the plant side include multiple signals masking each other or reflecting a conflict in the response of the plant to herbivore attack because of simultaneously perceiving endogenous spider mite signals (normally inducing the JA pathway) and bacteria-related signals (normally inducing the SA pathway), together resulting in no or suppressed plant response.

Overall, it cannot be excluded that intraspecific variations in induction of plant responses observed in different spider mite species such as *Tetranychus kanzawai* (Matsushima et al., 2006), *T. evansi* (Sarmento et al., 2011), and *T. urticae* (Kant et al., 2008), which correlate with genetic differences (Yano et al., 2003; Villarroel et al., 2016), may be co-determined by differences in the bacteriome (Yoon et al., 2010; Knegt et al., 2017; Zélé et al., 2018). Genetic intraspecific differences such as resistance to acaricides may covary with differences in the quantity and diversity of HAB (Yoon et al., 2010). In addition to reproductive endosymbionts, Yoon et al. (2010) detected also *Pantoea*, *Enterobacter*, and *Pseudomonas* in the gut of *T. urticae*; in the absence of reproductive endosymbionts, Knegt et al. (2017) detected *Pseudomonas* and *Stenotrophomonas* in the gut of *T. evansi*. These gut bacteria have potential to change and manipulate anti-herbivore plant response, as shown for Colorado potato beetles (Chung et al., 2013a,b), false potato beetles (Wang et al., 2016), and fall armyworms (Acevedo et al., 2017).

Compared to spider mites, only little is known about induced plant responses to thrips attack (for review, see Steenbergen et al., 2018), not to speak of plant responses manipulated by thrips-associated bacteria. Thrips are well known for vectoring plant-pathogenic viruses such as tospoviruses (Rotenberg et al., 2015). Abe et al. (2012) observed that virus transmission may alter the anti-herbivore plant response to the benefit of thrips, i.e., upregulate the SA-dependent pathway and downregulate the JA-dependent pathway. This trade-off is unsurprising and readily comprehensible given that the plant must simultaneously deal with two types of threats. Nonetheless, even the few gut and endosymbiotic bacteria found in association with herbivorous thrips (Supplementary Tables S1, S2) indicate potential for manipulation of anti-thrips plant response. For example, western flower thrips and onion thrips are associated with *Erwinia* (de Vries et al., 2001, 2008) and *Pantoea* or *Pantoea*-like bacteria (Facey et al., 2015; Dutta et al., 2016a), which could influence anti-herbivore plant responses. Dutta et al. (2016b) reported changes in plant response to thrips *Frankliniella fusca* upon deposition of feces containing *Pantoea ananatis* on the plant surface but not upon contact with salivary secretions.

CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

Given the widespread occurrence and diversity of gut/saliva and endosymbiotic bacteria found in spider mites and thrips, which have been shown for their ability to manipulate anti-herbivore plant defenses in other arthropod taxa, it is more than plausible to anticipate that these bacteria play a role in anti-spider mite and/or anti-thrips plant response. Current methodological standard is comparing the plant response to untreated herbivores harboring microorganisms to that of herbivores treated with broad spectrum antibiotics, such as tetracycline, and thus being free, or below detectability, or having strongly reduced titers, of bacteria. However, broad spectrum antibiotic treatment eliminates or reduces both gut/saliva and endosymbiotic bacteria in the herbivores or changes the bacterial composition due to varying susceptibility (see Staudacher et al., 2017). Thus, broad spectrum antibiotic treatments may blur cause and effect of HAB. Studies comparing the response to herbivores treated with broad spectrum antibiotics and left untreated as the only experimental way of assessing the effect of HAB remain correlational, but do not provide stringent proof of cause and effect. Such studies are important and certainly needed to obtain indications of microorganisms at play but do not allow concluding on one species or operational taxonomic unit (OTU) of eliminated/changed microorganism to be the causative agent of an observed effect. Use of antibiotics is also suitable to disentangle expression of HAB-transferred genes (Wybouw et al., 2018) versus endogenous HAB genes. In an ideal case, future studies should strive to achieve selective elimination of single bacteria taxa, to pinpoint which bacteria are responsible for manipulating plant defense. If possible, supplemental experiments should examine plant responses to cultured OTUs and their metabolites and test for masking, synergism, or interference among MAMPs and HAMPs, elicitors, and effectors, in the saliva and regurgitates of herbivores. Multiple bacterial infections may turn negative direct effects of each bacterial taxon alone into direct positive fitness effects for their host (see Zhang et al., 2018 for *T. truncatus* doubly infected with *Wolbachia* and *Spiroplasma*); the same may apply to the direct effects of multiple versus single HAB on induced plant response (Staudacher et al., 2017), indirectly affecting fitness of the herbivores. Clearly, investigating the role of HAB of spider mites and thrips in induced plant defense will provide a highly exciting, promising and fruitful avenue of future research.

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