BIOLOGICAL MECHANISMS OF PLANT INTERACTIONS WITH A COMBINATION OF BIOTIC AND ABIOTIC STRESSES

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BIOLOGICAL MECHANISMS OF PLANT INTERACTIONS WITH A COMBINATION OF BIOTIC AND ABIOTIC STRESSES

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REVIEW
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Cross-Talk Signaling in Rice During Combined Drought and Bacterial Blight Stress

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Due to climatic changes, rice crop is affected by moisture deficit stress and pathogens. Tissue water limitation besides reducing growth rates, also renders the crop susceptible to the infection by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) that causes bacterial leaf blight. Independently, both drought adaptation and *Xoo* resistance have been extensively studied. Though the cross-talk between drought and *Xoo* stress responses have been explored from individual stress studies, examining the combinatorial stress response is limited in rice. Recently published combined stress studies showed that under the combined stress, maintenance of carbon assimilation is hindered and such response is regulated by overlapping cellular mechanisms that are different from either of the individual stresses. Several receptors, MAP kinases, transcription factors, and ribosomal proteins, are predicted for playing a role in cellular homeostasis and protects cells from combined stress effects. Here we provide a critical analysis of these aspects using information from the recently published combined stress literature. This review is useful for researchers to comprehend combinatorial stress response of rice plants to drought and *Xoo*.

Keywords: combined stress, rice, *Xanthomonas*, drought, QTL, protein synthesis

INTRODUCTION

Plants are simultaneously exposed to diverse biotic and abiotic stresses that result in reduced yields in many crops (Atkinson et al., 2013; Narsai et al., 2013; Prasch and Sonnewald, 2013; Suzuki et al., 2014; Pandey et al., 2015b; Ramegowda and Senthil-kumar, 2015; Bahuguna et al., 2018). Rice is generally grown under puddled conditions, however, due to shortage of water availability, water saving technologies have been adapted for crop production (Lampayan et al., 2004). The unexpected drought has a significant impact on nearly 23 million hectares of rain-fed rice growing area in Southeast Asia. During these situations, many bacterial pathogens infect plants and further reduce the yield. A combined effect of bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and drought situations in dry season cause significant yield losses in South Asia and

South Africa (Ogawa, 1993; Verdier et al., 2012; Dixit et al., 2014). However, highest yield losses were reported in drought stress followed by temperature, weeds, and diseases (Pantuwan et al., 2000; Savary et al., 2012; Singh et al., 2012; Aghamolki et al., 2014; Ghadirnezhad and Fallah, 2014).

Water limitation with its effect on tissue water relations besides reducing growth rates also renders the crop susceptible to the infection by *Xanthomonas*. Both drought adaptation and *Xoo* resistance have been extensively studied with significant leads. Understanding the response of the crop to a combination of drought and BB is a relevant topic. The major premise emerged from the fact that some mechanisms leading to stress adaptation could have a common link through protecting plant metabolic efficiency under these stresses (Ramegowda and Senthil-kumar, 2015).

The combined simultaneous occurrences of abiotic and biotic stresses depend on the host resistance or susceptibility and also on the race of pathogens (Tippmann et al., 2006). The multiple stress occurrence and microclimate of plant-microbe interactions also influence the response of the host plant. Overlapping plant responses to drought and bacterial stress have been reported in Arabidopsis, rice, chickpea, and sunflower (Atkinson et al., 2013; Prasch and Sonnewald, 2013; Choudhary et al., 2016; Vemanna et al., 2016). There are several common changes in morphological, physiological traits and biochemical responses of plants to drought and pathogen stresses (Pandey et al., 2017). Leaf wilting, decrease in tiller number and biomass are common processes affected in both drought and bacterial infections in rice. However, increased root growth and reduced leaf expansion, stem elongation and leaf number are observed only under drought and localized lesions, patchy brown spots or pale yellow leaves were observed upon bacterial infection in rice. There are common and unique plant responses observed in response to both stresses when exposed independently. These symptoms could be common, which may serve as morphological observations to identify the combined stress response in rice. ABA and ethylene increases in plants with concomitant reduction of photosynthetic ability under combined stresses (Grimmer et al., 2012; Zhang and Sonnewald, 2017). In these conditions, antioxidant enzymes are accumulated to scavenge the ROS generated under stress. However, ROS accumulation under pathogen infection is the cause for a hypersensitive response suggesting that ROS play similar and opposite complex functions in plant adaptation under combined stresses. Sugars and polyamines are also accumulated for stress protection under combined stresses. All these mechanisms have relevance in imparting combined stress tolerance.

The stress tolerance mechanisms adapted by rice under combined stresses is diverse that include some common/shared and unique responses. The common visible effects include wilting, reduction in tiller number due to the blockage in xylem that reduces the water flow, which affects photosynthetic machinery (Fatima and Senthil-Kumar, 2017). Drought-induced low tissue water potential and lesions caused by bacterial infection further decreases the photosynthesis and reduce yield.

TRANSCRIPTIONAL RESPONSES TO INDIVIDUAL AND COMBINED STRESS OVERLAP

A comprehensive understanding of crosstalk or regulatory networks involved in unique or shared responses for either individual or multiple stresses is much-needed (Pandey et al., 2015a). A deluge in omics data has provided greater insight into the diverse aspects of spatiotemporal responses of stresses in plants. Only a limited amount of data is available in public domain for combined stresses, especially, for drought and *Xanthomonas* infection. The meta-analysis studies using transcriptome data from different plant species have identified shared genes which acts simultaneously or independently under different stress conditions (Shaik and Ramakrishna, 2013, 2014; Vemanna et al., 2016). Meta-analysis of eight different viruses infecting Arabidopsis revealed several regulatory genes which are competently connected to the plant defense response (Rodrigo et al., 2012). These meta-analysis data help in understanding the crosstalk of specific genes between stress conditions.

Rice plants have evolved common molecular responses, which exhibit cross-talk between different hormones such as ABA, ethylene, salicylic acid, jasmonic acid, cytokinin, and brassinosteroid. The data from a few transcriptome analysis indicates the existence of crosstalk mechanisms between signaling networks under drought and pathogen stress (**Figure 1**) (Schenk et al., 2000; Cheong et al., 2002; Seki et al., 2002). Hormones play a crucial role as central regulators of many downstream responsive transcription factors (TFs) and functional proteins. The receptors for abscisic acid (ABA), brassinosteroids (BRs) and many pathogens triggered elicitors have been identified. Some are *PYR1/PYL/RCAR, BAK1,* and *LRR kinases* which act as *R* genes for many pathogens and also acts as key receptors in abiotic stress signaling (**Figure 1**). The signals received by these elicitors activates or phosphorylate the downstream protein kinases cascade to activate several TFs. The members of WRKY, NAC, AP2/ERF, bZIP, and MYC family TFs showed altered responses to both biotic and abiotic stresses (Babitha et al., 2013, 2015a,b; Xiao et al., 2013; Zhang et al., 2016; Ku et al., 2018) and played a major role in combined stresses. The reactive oxygen species (ROS) generated under oxidative stress showed unique responses to bacterial and drought stresses that trigger downstream stress responses (Apel and Hirt, 2004; Narusaka et al., 2004; Torres and Dangl, 2005). The calcium signaling is considered as a central hub in concurrent biotic and abiotic stress responses (Ranty et al., 2016; Ku et al., 2018). The signals of Ca^{2+}, inositol-3-phosphate and protein kinases and other kinases also have a significant role in combined stresses. Thus crosstalk between biotic and abiotic stress signaling pathways regulate many cellular processes.

The individual transcriptome data from biotic and abiotic stress have identified antagonistic and overlapping responses (Narsai et al., 2013). The computational comparison of the

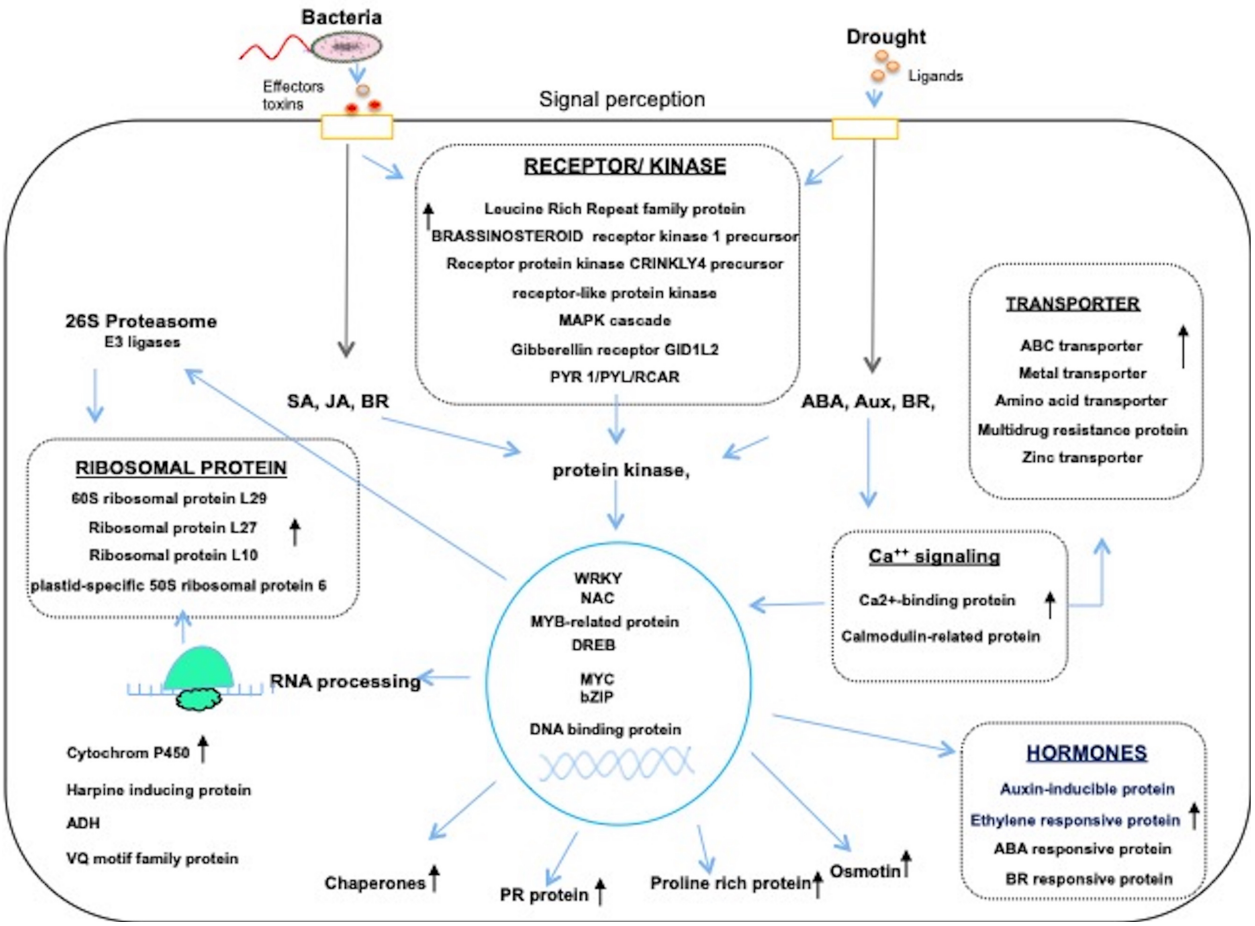

FIGURE 1 | The molecular responses under drought and *Xoo* infection in Rice. The stress signals from biotic and abiotic stresses are perceived by specific receptors and cascade of signaling processes such as protien Kinases, TFs, transporters, ribosomal protiens, and many hormone responsive genes are differentially co-regulated in both drought and pathogen stress. The transcriptional regulators which play central role enhances the transcripts of diverse functional genes and ribosomal proteins translate the message in to protein to maintain cellular homeostasis under combined stress Many of these genes were upregulatcd under combined stress have relevance in improving stress adaptation (Narsai et al., 2013; Huang et al., 2014; Zhang et al., 2016).

expression profile between abiotic and biotic stresses has revealed unique genes that showed similar response across multiple stresses (Jain et al., 2007; Ribot et al., 2008; Swarbrick et al., 2008; Hu et al., 2009; Marcel et al., 2010; Zhou et al., 2010; Narsai et al., 2013). The studies suggest that the plants respond to combined multiple stresses by crosstalk of several hormonal signaling pathways (Sharma et al., 2013; Bahuguna et al., 2018; Ku et al., 2018). In response to drought and *Xoo* infection, 2276 genes showed overlapping differential expression profiles (Narsai et al., 2013). In another, meta-analysis study between drought and bacterial stress in rice 5084 DEGs have been identified by combining the data sets. Among 1214 common genes, 565 genes were upregulated and 309 downregulated in both drought and bacterial stresses (Shaik and Ramakrishna, 2013). In a comparative study of drought and *Xoo*, transcriptome analysis of the resistant rice introgressed line H471 when compared with the recurrent parent HHZ and 306 and 840 DEGs were identified and amongst them 178 genes were common for both stresses (Zhang et al., 2016).

In combined drought and *Xoo* infection, many components of the multiple pathways responded similarly. Several genes showed opposite roles in response to pathogens and abiotic stress tolerance (Xiong and Yang, 2003; Asano et al., 2012). The *OsCPK12* acts as a negative regulator for blast resistance but positively regulates salt, drought, and cold stress tolerance in rice (Asano et al., 2012). The broad-spectrum disease resistance through *PR* genes is negatively regulated by *OsMAPK5* (Xiong and Yang, 2003). In resistant introgressed lines H471, two genes (*LOC_Os04g56000* and *LOC_Os12g43410*), were strikingly up and down-regulated in combined drought and *Xoo* infection. The studies suggest that the drought tolerance and BB resistance mechanisms are shared in resistance genotypes. Further, phosphate/phosphorus metabolic process, phosphotransferase activity, and kinase activity associated genes and peptidase/endopeptidase/enzyme inhibitor activity genes

were highly represented in up-regulated genes. The TFs *WRKY* and *NAC* showed a conserved response between abiotic and biotic stress (Seki et al., 2002; Narsai et al., 2013). Under both stress conditions, 10 DEGs encoding receptor kinases *LOC_Os08g07760 (OsBAK1),* protein kinases *LOC_Os11g31530* (brassinosteroid signaling pathway) were strongly up-regulated and *LOC_Os03g46910* (pyruvate kinase) was down-regulated in H471 introgression line which is resistant to *Xoo* and drought as compared with Huang-Hua-Zhan (HHZ) (Zhang et al., 2016).

In rice, the introgressed line H471, three DEGs involved in phytohormone signaling pathways, the BR pathway (*OsBAK1*) and gibberellin (GA) pathway genes were upregulated in both drought and *Xoo* stress. The *GA20 oxidase* an essential gene involved in GA biosynthesis that catalyzes the conversion of GA53 to GA20 was downregulated (Zhang et al., 2016). The GA response related to plant height was evident, and the response could be associated with *GA20 oxidase* expression levels (Dossa et al., 2016) The antagonistic reaction from GA with JA has been reported that they are involved in development and immunity of plants through DELLA proteins (Yang et al., 2013).

The stress-responsive signaling genes are differentially regulated in response to combined stresses. The ABC transporters such as multidrug resistance function encoding proteins, universal stress protein (*LOC_Os5g28740*), Q-rich domain-containing protein (*LOC_Os06g04240*) and lineage-specific genes (*LOC_Os12g32610*) were highly expressed in both stresses in rice signifying the importance of these transporters in combined stresses. In *Xoo* resistant genotypes, cell wall-associated genes were downregulated in 24 h of infection and significantly upregulated in 96 h. The phenylpropanoid metabolism genes UDP glucosyl/glucoronyl transferases, two genes encoding cytochrome 450 72A1 were significantly up-regulated in *Xoo* resistant type. There are receptor kinases such as *OsWAK (OsWAK127)*, a lectin-like receptor kinase, a phytosulfokine receptor precursor and a serine/ threonine kinase-like protein, an NBS-LRR type putative disease resistance protein (*LOC_Os02g30150*) and resistance protein *LR10* (*LOC_Os04g11780*) were up-regulated in response to *Xoo* infection, several of kinases were down-regulated in drought stress suggesting that there are specific receptor kinases exclusively responsive to individual stresses suggesting the unique signaling pathways may operate for stress adaptation by regulating downstream TFs.

The functional roles of some TFs have been elucidated in response to bacterial infection and drought stress. The TFs such as *WRKY28, MYB4, AP2/EREBP- DREB,* and *HSF4* were differentially regulated that control several functional genes involved in multiple stresses. The *C3H12 zinc finger* TF in downregulated at 96 h in response to bacterial *Xoo* infection (Narsai et al., 2013) and knock-out lines showed partially increased susceptibility in Zhonghu 11 genotype (Deng et al., 2012). Three *WRKY* TFs were up-regulated and had been shown to result in altered resistance. Overexpression of *WRKY71* resulted in enhanced resistance to *Xoo* bacterial infection (Liu et al., 2007). In contrast, over-expression of *WRKY45* showed increased susceptibility to *Xoo* (Tao et al., 2009). The *NAC TFs* that were upregulated in both abiotic and biotic stress also had a developmental role in plants (Hu et al., 2006; Mao et al., 2007; Tao et al., 2009; Jeong et al., 2010; Takasaki et al., 2010). Some *NAC TFs* were induced in response to *Xoo* infection and drought stress, amongst them *NAC10* showed 53-fold induction in drought stress (Jain et al., 2007) and overexpression resulted in root enlargement and improved drought stress tolerance (Jeong et al., 2010). The *bHLH* (LOC_Os01g72370), *B3* (LOC_Os03g42280), and M-type (LOC_Os04g31804) TFs were up-regulated and CO-like (LOC_Os09g06464) TF was down-regulated under drought and *Xoo* infections in resistant H471 rice genotype (Zhang et al., 2016). The *VQ* genes (VQ -FxxxVQxLTG motif) were shown to interact with WRKY TFs and were induced upon *Xoo* infection, ABA and drought stress conditions (Kim et al., 2013). The differential expression of TFs may regulate diverse functional genes, which have specific mechanisms under both stress conditions.

The cytochrome P450 monooxygenase family *CYP71P1* encoding tryptamine 5-hydroxylase function involved in cell wall biosynthesis was highly upregulated in response to *Xoo* infection as well as fungus *Magnaporthe oryzae* causing Sekiguchi lesion (SL) (Fujiwara et al., 2010; Delteil et al., 2012). The *SPL7* and *BiP3* chaperons were up-regulated in resistant genotypes in response to *Xoo* infection and drought. Suppression of SPL7 resulted in increased resistance to infection (Yamanouchi et al., 2002) and *Xa21* mediated immunity to *Xoo* infection was compromised due to overexpression of *BiP3* (Park et al., 2010). Binding proteins (BiP) play chaperone functions in endoplasmic reticulum-mediated unfolded protein response, improves cellular tolerance mechanisms by maintaining the protein quality control. The genes encoding cell cycle isomerases were differentially expressed (Yao et al., 2001) in the resistant genotype when infected with bacteria and among them, eight of them were highly upregulated in response to bacteria (Narsai et al., 2013). The pathogenesis-related *PR10* was induced in roots upon drought, salt stress, JA and blast fungus (Hashimoto et al., 2004).

The genes involved in protein degradation showed differential expression in combined stresses. The ubiquitin E3 complex and subtilizes were down-regulated in the resistant genotype in response to bacterial infection and drought. The speckle-type POZ protein (LOC_Os10g29220.1) a subunit of E3 ubiquitin complex and a subtilisin-like protease precursor (LOC_Os04g02970.1), deaminase (LOC_Os07g46630.1) involved in nucleotide degradation were downregulated in a bacterial infection in the drought-resistant cultivar. These E3 ligases are components of the 26S proteasome system are targeted by bacterial effector proteins and modulate their mechanisms against host defenses. The reduced expression of these genes in drought-resistant genotypes could be an adaptive strategy that plants have evolved to fight against *Xoo* infection.

REGULATORY NETWORKS UNDER COMBINED STRESS

Considerable crosstalk signaling mechanisms exist in response to combined bacterial and drought stress (**Figure 2**). In

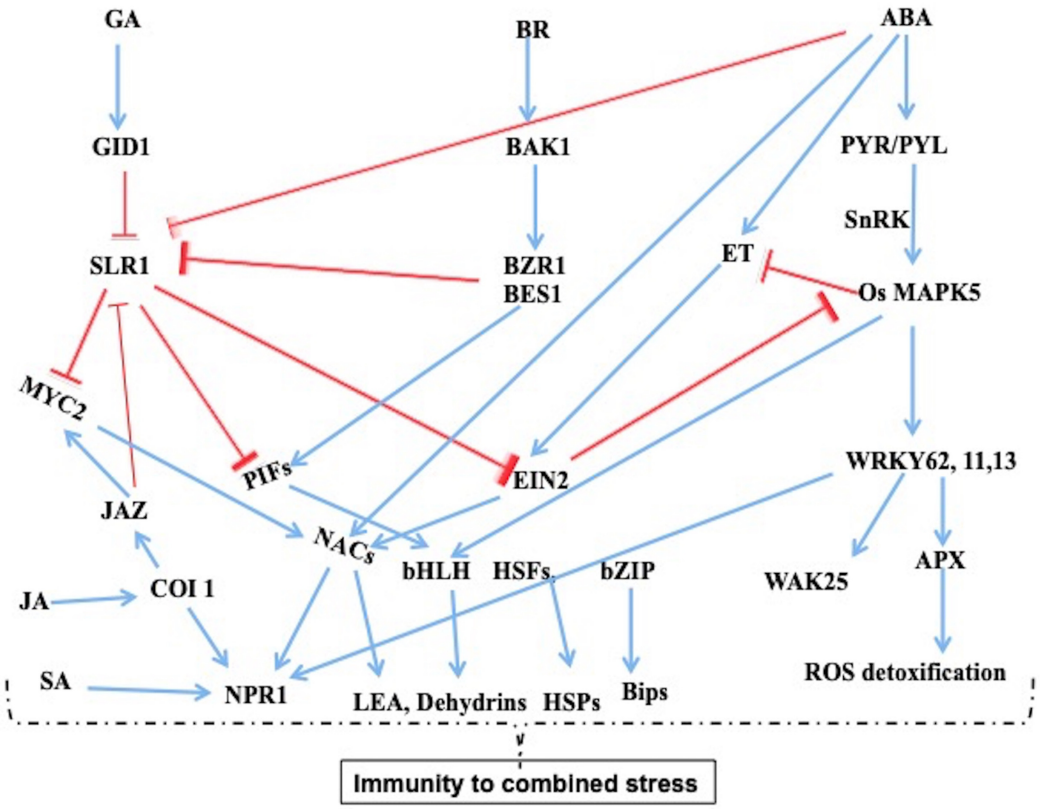

FIGURE 2 | Regulatory networks under combined stress of drought and *Xoo* infection in nee. The data taken from the combined stress transcriptomic studies and a model arrived from the knowledge from diverse studies. The phytohormones play crucial role in crosstalk signaling mechanisms. Some of the key genes play antogonistic, overlapping, and opposite role with response to combined stress which depends on intensity of stress and cellular homeostasis with regards protein turnover or stability. The LEAs. dchydrins, HSPs, Bips provide stability to proteins (Narsai et al., 2013; Sharma et al., 2013).

comparison with resistant rice introgressed line H471 and its recurrent parent HHZ, 178 common DEGs exists, among which 39 genes were found to be co-regulated in a complex network. Majority of proteins enriched belonged to stress signal perception and transduction such as *RLK, LRR, receptor kinases, protein kinases,* and the proteins were related to *BR (OsBAK1)* and *GA* (*GID1L2*) pathways (Zhang et al., 2016). There is convincing evidence to show that certain *RLKs* and *LRRs* are predicted to be involved in BR signaling process, which suggests that brassinosteroids act as a central regulatory hormone in crosstalk mechanisms with other hormone signaling process under combined studies. Several protein kinases involved in phosphorylation have been identified in both BB and drought tolerance, which further activates the TFs. However, several genes showed opposite roles in response to different stresses in rice (Xiong and Yang, 2003; Tao et al., 2009; Asano et al., 2012). The *Mitogen-Activated Protein Kinase 5 (OsMPK5), wall-associated kinase 25 (WAK25), WAK-like (WAKL), sucrose non-fermenting-1-related protein kinase-1 (SnRK1)* and *SUB1A* binding protein 23 (*SAB23*) are involved in cross-talk signaling in both abiotic and biotic interactions (He et al., 1998; Kohorn and Kohorn, 2012; Sharma et al., 2013). Suppression of *OsMPK5* reduced the ABA sensitivity and increased ethylene levels, PR protein expression, hence resulted in resistance to fungus *M. oryzae*, which causes rice blast disease (Xiong and Yang, 2003; Bailey et al., 2009). *SnRK1* has been identified as a central hub for signal integration for many pathways in the cross-talk mechanisms (Seo et al., 2011; Cho et al., 2012).

Downstream to these kinases several TFs are found to be commonly upregulated in both bacterial and drought stress which include HLH-type TF identified from combined stress (Zhang et al., 2016). Several TFs like *NAC, WRKY, MYB, MYC, bZIP, HSFs,* and *CO* identified were known to be upregulated in both stresses and induce immune responsive genes. Overexpression of *OsWRKY13* regulated SA-dependent immunity and several other physiological pathways including JA response. The *SNAC1* TF involved in abiotic stress response showed improved tolerance in rice (Qiu et al., 2007). Similarly other WRKY family TFs *OsWRKY45-1, OsWRKY62, 71,* and *76* have an interface of the biotic and abiotic stress interactomes (Qiu and Yu, 2009; Seo et al., 2011). A few ribosomal protein-encoding genes, which include metal ion transport-related genes, PR protein osmotin, and GA associated genes, were co-regulated in resistant rice H471 type (Zhang et al., 2016).

Several genes involved in photosynthesis, dehydrins and late embryogenesis abundant (LEA) proteins involved in protein stability that are implicated in desiccation tolerance are also co-regulated in bacterial stress (Hand et al., 2011; Shaik and Ramakrishna, 2013). ABA contributes to adaptation to osmotic stress and also involved in defense response by regulating plant physiological process which acts as a barrier for pathogen entry (Asselbergh et al., 2008; Ton et al., 2009; Kaundal et al., 2017). The *BAK1 and DELLA* proteins appears to be central regulators in abiotic and biotic stresses that positively affects ROS detoxification by activating many antioxidant proteins (Achard et al., 2008; Divi et al., 2010; Albrecht et al., 2012; Belkhadir et al., 2012; Sharma et al., 2013). In Arabidopsis, it was shown that the BR receptor *BAK1* is a primary signaling receptor that modulates the interaction of GA, BR, JA, and SA signaling pathways (**Figure 2**) (Yamada et al., 2015; Li et al., 2016; Nolan et al., 2017). DELLAs also sensitize JA signaling at the expense of SA mediated defense and enhances resistance to necrotrophic pathogens (Navarro et al., 2008). These studies demonstrate that the complex overlapping co-regulatory network of many pathways and genes contribute for adaptation to the combined stress conditions (**Figure 2**).

RIBOSOMAL PROTEIN-ENCODING GENES ARE DIFFERENTIALLY REGULATED UNDER COMBINED STRESS

Many omics reports over-representing genes associated with translational mechanisms such as TFs, RNA processing, RNA binding, ribosomal proteins, protein synthesis, and folding were differentially expressed. Recent studies show that the ribosomal protein-encoding genes have extra-ribosomal functions and they are involved in specific mechanisms (Nagaraj et al., 2016). Ribosomal proteins are critical for the translation of diverse proteins and involved in the overall fitness of the cell under stress conditions. From this context response of genes encoding ribosomal proteins were specifically looked in the combined stress conditions. However, the functional relevance of many of these genes with response to either individual or combined stresses is still needed. In response to bacterial infection, 50 genes encoding ribosomal proteins were up-regulated in the resistant rice genotype, and 46 of them were also up-regulated under fungal infection (*M. grisea*). However, in drought stress, 46 ribosomal proteins were downregulated (Narsai et al., 2013). The differential responses of all the ribosomal proteins including small and large subunit encoding proteins have been studied using genome-wide studies in rice with response to multiple stress condition (Moin et al., 2016; Saha et al., 2017). The ribosomal large subunit protein encoding genes were differentially expressed in response to abiotic stresses, and amongst them, 34 genes showed significant changes. Out of which, 6 of them were *RPL12, 28, 30,* 36, 44, and 51 that showed down-regulation in response to *Xoo*. *RPL38* was unchanged, and the remaining genes like *RPL10, 11, 15, 24a, 26, 27, 37* were activated more than 10-fold (Moin et al., 2016). The qRT-PCR analysis of small subunit encoding ribosomal protein genes revealed 14 genes downregulated, and others were upregulated in response to biotic and abiotic stresses. The *RPS6a, RPS9, RPS10a,* and *RPS4* showed high upregulation in both biotic and abiotic stresses (Saha et al., 2017). It was observed that out of 50, 32 ribosomal protein promoters have TELOBOX elements (Narsai et al., 2013). The differential regulation of these genes suggests that, unlike there role in protein synthesis, they also possess extra-ribosomal functions. The precise function of each ribosomal proteins with response to combined or individual stresses need to be identified.

INTROGRESSION OF QTLs FOR DROUGHT AND *Xa* GENES IMPROVES COMBINED STRESS TOLERANCE

Considerable progress has been made in identifying QTLs for drought tolerance (Prince et al., 2015). Similarly, around fifty genes /QTLs for resistance against *Xanthomonas* bacteria have been identified in rice (Dossa et al., 2016). Most of the genes are targeted to *Xoo* that causes BB and are referred to as *Xa* genes (Khan et al., 2014; Zhang et al., 2014). The *Xa21* being a major gene conferred resistance against bacterial infections, and subsequently, introgression of different *Xa* genes (*Xa5, Xa13, and Xa21*) provided broad-spectrum resistance in different rice cultivars (Pradhan et al., 2015). Drought stress influences plant response to pathogens through a gene for gene interaction and depends on the severity of stress. The combined interactive effect of bacterial disease and drought QTLs are dependent on QTLs or genes associated with specific traits. However, the combined stress response depends on soil water content and genotypes having different *Xa* genes (Wright and Beattie, 2004; Dossa et al., 2016). To date, no QTLs have been identified for combined bacterial and drought stress tolerance in rice. A few studies have shown genotype dependent BB pathogen infection in rice plants under drought-induced conditions (Dossa et al., 2016). Though many genotypes have different *Xa* genes, increased lesions were visible under mild drought stress conditions, indicating under combined stress, rice plants are affected and bacterial virulence enhanced.

The combined stress effect at vegetative stage showed different lesion length upon BB infection under drought stress which depends on genotypes having different *Xa* genes. The genotype containing single *Xa*4 gene and drought QTL - DTY2.2 did not show any significant BB induced lesion in either control or at severe drought conditions (Dossa et al., 2016). The susceptible IR24, IR64, and other two *Xa* gene introgressed lines had reduced lesion lengths under moderate drought stress. The multiplication and spread of *Xoo* were increased in rice genotypes under mild drought

stress even though *Xa4* gene was expressed. However, under both compatible and incompatible interactions, BB disease infection was reduced under drought stress when drought severity was increased. The lesions in the single *Xa4* gene containing genotypes were larger than the genotypes having *Xa7* gene when inoculated with virulent PXO145 (avr*Xa4* + avr*Xa5* + avr*Xa7*) indicating that the genotypes with suitable *Xa* gene may still provide resistance against the pathogen under drought stress conditions. Rice genotypes having different *Xa7, Xa4* + *Xa7, Xa4* + *Xa5* + *Xa7*, and *Xa4*/ qDTY2.2 showed less disease development under drought stress. However, the genotypes having *Xa4, Xa4*/ qDTY2.2 + qDTY4.1 were less effective to combined stresses. Severe drought stress reduces the bacterial multiplication due to higher leaf water loss. The rice genotype with *Xa7* showed reduced bacterial multiplication under severe drought stress and was dependent on the tissue water status. Under severe drought stress, the leaf water loss is more which influences bacterial multiplication (Freeman and Beattie, 2009).

Single *Xa* gene is not sufficient to provide resistance. The rice genotype carrying single *Xa4* gene showed increased BB severity under drought stress. The compromised resistance response was also observed in rice genotype having *Xa4* gene under high temperature (Webb et al., 2010) and drought stress (Dossa et al., 2016). Similar reports were found in the combined stress of high temperature and BB, drought stress and BB at the seedling stage. From this context, the QTLs associated with drought tolerance with multiple *Xa* genes at the seedling level may improve tolerance for combined stress. From this context, introgression of *Xa* genes with drought QTLs signifies that the drought-tolerant genotypes with specific QTLs could be beneficial for BB disease development. The drought tolerant genotypes that can maintain water loss by regulating stomata or by deep roots with specific *Xa* genes could contribute to BB tolerance and improve combined stress tolerance. Higher stomata and root hydraulic conductivity under drought showed inhibition of BB in rice genotypes having three different *Xa* genes (Yu et al., 2008, 2013; Henry et al., 2015). Studies demonstrated that, when two major *R* genes (*Xa4 and Xa7*) are present in a genotype, combined stress tolerance is enhanced. In a recent study to improve the multiple stress tolerance, four BB resistance genes (*Xa 4*, *xa5*, *xa13*, *Xa21*) were pyramided with submergence (*Sub1*), salinity (*Saltol*), blast (*Pi2*, *Pi9*) and gall midge (*Gm1*, *Gm4*) improved Tapaswini an elite rice cultivar successfully that showed multiple stress tolerance (Das et al., 2018). The enhanced rice resistance to combined stress can be achieved by introgression of multiple drought QTLs and multiple R genes in a single elite genotype.

GENETIC MANIPULATION FOR COMBINED STRESS TOLERANCE

Several genes have been identified and functionally characterized for their role in specific pathways and stress responses. However, most of these studies were limited to the single type of stresses and this could be considered as a major limitation in transgenic research aimed for product development, as the plants were not evaluated under combined stress conditions that occur in the natural environmental conditions. Here, we list a few genes that are tested for multiple individual stresses that showed differential roles (**Supplementary Table 1**). However, none of these genes were tested for combined stress response, and hence the data presented here is only a speculation that these genes may provide tolerance.

Overexpression of *MoHrip1 and MoHrip2* from *M. oryzae* in rice enhanced the resistance to bacterial disease caused by *Xoo* and drought stress. In transgenic plants, higher expression of two JA/ethylene biosynthesis-related genes *OsLOX2* and *OsAOS2* and SA signal-related genes *OsEDS1, OsPAL1, OsNH1, OsPR-1a,* and *OsPR-10a* was observed in response to bacterial pathogen and abiotic stress responsive genes *OsbZIP23, OsZEP1, OsNCED2,* and *OsNCED3* were highly upregulated under drought conditions (Wang et al., 2017). A few TFs from *NAC*, *WRKY*, *bHLH*, *AP2,* and *bZIP* family have been shown to be induced upon both drought and bacterial stresses (Nakashima et al., 2007; Xiao et al., 2013; Jisha et al., 2015). The *WRKY45-2* TF showed broad-spectrum disease resistance to *M. oryzae,* bacterial pathogens *Xoo* and *Xanthomonas oryzae* pv. *oryzicola.* However, this TF had been shown to act as a negative regulator of salt, cold, and drought stresses in rice (Tao et al., 2009, 2011). Similarly, overexpression of *WRKY13* enhanced rice resistance to *Xoo* and *M. oryzae* and reduced resistance to cold and salt stresses by influencing the transcription of more than 500 genes (Qiu et al., 2007, 2008). Transcriptional repressor *WRKY13* suppresses the expression of two important genes *SNAC1* and *WRKY45-1* by binding to sequence-specific W-like-type *cis*-elements on the promoters of these genes under abiotic and biotic stress. The autoregulation of *WRKY13* is associated with balancing its function when the rice plants experience different stress environments (Xiao et al., 2013). Ectopic expression of *OsWRKY11* resulted in up-regulation of defense-associated genes and drought-responsive genes that improve stress tolerance. *OsWRKY11* play positive regulator function in plant defense to drought and *Xoo* (Lee et al., 2018). Overexpression and suppression of a few specific genes resulted in resistance to combined biotic and abiotic stresses (Zhang et al., 2016). These studies demonstrate that the transcriptional regulators play a crucial role in improving multiple stress tolerance in rice. However, their response to combined stress needs to be assessed to gain more insight into their role in enhancing adaptation to natural environmental stresses. The genes that showed tolerance to both drought and *Xoo* could be attractive targets for genetic manipulation of rice for combined stress.

CONCLUSION AND PERSPECTIVE

1. The plant responses under combined drought and bacterial infection need further understanding and studies using simultaneous stress imposition are much needed.

2. The existing transcriptome studies suggest combined stress responses are complex and sophisticated and hence detailed understanding of unique and shared signaling mechanisms is important.
3. Prospecting the candidate genes and functional validation using diverse approaches may lead to developing durable, resistant rice for combined bacterial and drought stress.
4. Understanding the regulatory networks involved in combined stress responses may provide an option to manipulate the signaling mechanisms which serve as a key for adaptation by using novel approaches such as genome editing tools.
5. Ribosomal protein-encoding genes seem to be attractive candidates for gene manipulation. However, the functional relevance in combined stress needs to be explored.
6. Combining drought QTLs and *Xa* genes could be a better strategy due to their success in the drought-prone areas. However, there is a need to identify QTLs at different stages of crop growth and develop introgressed lines, which may provide an option to improve rice for combined stress tolerance.
7. Pyramiding multiple genes/QTLs associated with multiple stresses in the elite background may provide durable resistance to combined stress.
8. Transgenics using candidate genes, which provide combined stress tolerance, are the best option because of their precise molecular mechanisms. However, more concerted efforts are needed to explore the candidate genes.
9. The alternate strategies like discovery of novel small molecules or dsRNA-mediated approaches can be employed to improve combined stress tolerance.

AUTHOR CONTRIBUTIONS

RV conceived the concept and wrote a review. RV, RB, and PB drafted the manuscript. MK edited the pathogen-related and SS edited the drought-related aspects in manuscript. MS-K, UM, and RV edited and finalized 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.2019.00193/full#supplementary-material

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The Effects of Combined Abiotic and Pathogen Stress in Plants: Insights From Salinity and *Pseudomonas syringae* pv *lachrymans* Interaction in Cucumber

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Plants are often challenged by abiotic and biotic stresses acting in combination and the response to combinatorial stress differs from that triggered by each factor individually. Although salinity and pathogens are major stressors limiting plant growth and productivity worldwide, their interaction is poorly understood. The reactions to pathogens overlap with those to abiotic stresses, and reactive oxygen species (ROS) and stress hormones represent central nodes in the interacting signaling pathways. Usually, abiotic stress negatively affects plant susceptibility to disease. Specific focus of this review is on cucumber plants exposed to salt stress and thereafter infected with *Pseudomonas syringae* pv *lachrymans* (*Psl*). We addressed this problem by discussing the changes in photochemistry, the antioxidant system, primary carbon metabolism, salicylic acid (SA) and abscisic acid (ABA) contents. Salt-treated plants were more prone to infection and this effect was determined by changes in the hormonal and redox balance as well as the carboxylate metabolism and activities of some NADPH-generating enzymes. Our detailed understanding of the interactive effects of biotic and abiotic stresses is fundamental to achieve enhanced tolerance to combination stress in agronomically important crops.

Keywords: cucumber, *P. syringae*, salt stress, stress interactions, redox signaling, abscisic acid, salicylic acid, carboxylate metabolism

INTERACTIONS OF ABIOTIC STRESSES AND PATHOGENS: POSSIBLE SCENARIOS

Multifactorial stresses affecting plants are common to many agricultural areas worldwide, and they represent one of the most pressing threats in the field. The well-recognized combinations are those between abiotic stresses (Bandurska and Cieślak, 2013; Rasmussen et al., 2013; Sewelam et al., 2014). With the global climate change, complex stress combinations are expected to occur, and one of the major threats is the establishment of new plant-pathogen interactions due to species migration

(Chakraborty, 2005). Our knowledge on how the adverse abiotic conditions can modulate plant-pathogen interactions is limited, except for the plant's interaction with simultaneous drought and pathogen (Ramegowda and Senthil-Kumar, 2015; Choudhury et al., 2017).

Salinity, affecting many agricultural areas of the globe, and plant pathogens represent an excellent example of abiotic and biotic stresses which co-occur in the field and their interaction may severely influence food quality and safety (Munns and Gilliham, 2015). Salinity (Naliwajski and Skłodowska, 2014; Forieri et al., 2016) and pathogenic bacteria (Gao et al., 2013; Yang et al., 2015) were extensively studied as individual stresses, but their combined impact on crops is not well recognized, although evidence confirming their co-occurrence is still growing (Dileo et al., 2010; Nostar et al., 2013; Nejat and Mantri, 2017; Zhang and Sonnewald, 2017). As to cucumber, the fourth most important vegetable crop worldwide (Lv et al., 2012), 17% of the plants grown in salinated soils in Uzbekistan showed symptoms of *Fusarium solani*-induced diseases (Egamberdieva et al., 2011), and increasing salinity of irrigation water from 0.01 to 5 dS m^{-1} increased the incidence of pythium damping-off of cucumber from 40 to 93% (Al-Sadi et al., 2010).

Reports on combined abiotic and biotic stresses, describe synergistic effects showing that abiotic stress influences the plant-pathogen interaction both positively and negatively, thereby enhancing or decreasing the severity of disease. Most studies with plants simultaneously exposed to drought/heat and biotic stress combinations indicate the dominant role of abiotic factors which facilitates plant diseases (Luo et al., 2005; Kiraly et al., 2008), especially those caused by weakly aggressive facultative pathogens (Desprez-Loustau et al., 2006). Salinity favored disease development caused by *Oidium neolycopersici* in tomato (Kissoudis et al., 2014), increased tomato susceptibility to *Phytophthora infestans* and *Pseudomonas syringae* (Thaler and Bostock, 2004), while it enhanced resistance against *Botrytis cinerea* (Achuo et al., 2006). The comparison of *P. syringae* growth in knockout *Arabidopsis* mutants showed interactions among pathogen growth and physiology and salinity tolerance genes at the gene level (Saleem et al., 2017), but the detailed knowledge of how plant immunity and salt stress tolerance are connected is lacking. Episodic abiotic stress occurring prior to infection was also shown to predispose the plant to disease (Bostock et al., 2014), indicating that responses induced in plants recovering from abiotic stress may conflict with those for resisting pathogens (Boyer, 1995). The increased susceptibility to pathogens under stress may be related to the changed hormonal balance, reduced defense genes expression and to primary metabolism down-regulation which was observed as a general response to multiple stress (Mohr and Cahill, 2003; Prasch and Sonnewald, 2013).

For some interactions, however, the phenomenon of cross-tolerance between abiotic and biotic stresses has been described (Sharma et al., 1996; Achuo et al., 2006; Foyer et al., 2016). It confirms that abiotic and biotic stresses share signals, responsive genes and products and drought improved tomato defense against the *Botrytis cinerea* (Achuo et al., 2006). In a broader context, this phenomenon is linked to defense priming by abiotic factors. The primed plants show relatively little defense expression, but they respond more effectively to the subsequent biotic stress than the non-primed ones due to defense signaling activation (Rejeb et al., 2014). Priming for enhanced defense is interpreted as a defense mechanism with limited fitness costs (Vos et al., 2013). In natural systems, however, the fitness consequences of infection in plants exposed to abiotic stress may vary, as the activation of plant defense can have subsequent effects on the entire plant-associated microbial community, including the non-pathogenic species competing the disease-inducing ones (Barrett et al., 2009; Vos et al., 2013).

CONVERGENCE POINTS AND MASTER REGULATORS OF THE INTERACTION BETWEEN ABIOTIC FACTORS AND PATHOGENS

Stress signaling in plants constitutes a complex network (Miller et al., 2009; Baxter et al., 2014; Gilroy et al., 2014), however, the main crosstalk nodes between abiotic and biotic signaling pathways have been identified. They are represented by signaling components shared between abiotic and biotic stress responses, such as ROS and redox compounds, calcium ions, phytohormones as well as signal-response coupling factors, e.g., protein kinases and transcription factors (Fujita et al., 2006).

At the sites of stress perception, the information of the nature of the stimulus is encoded by the spatiotemporal dynamics in ROS and calcium [Ca^{2+}] cellular changes (Fujita et al., 2006). These ROS and calcium signatures can be decoded by different sensors, leading to stimulus-specific hormonal and metabolic response at the site of stress action (Fujita et al., 2006; Tippmann et al., 2006). This stress-specific response is usually transmitted throughout the entire organism to elicit an integrated whole plant reaction. Stresses, including salinity and pathogen infection, can be signaled by cell-to-cell autopropagating, vascular ROS and calcium waves which are integrated by the respiratory burst oxidase homolog (Rboh) protein, and by the activation of stress-specific metabolic cues (Jiang et al., 2012; Gilroy et al., 2014).

The ROS signature is shaped by the antioxidant system, with the ascorbate-glutathione (AA-GSH) cycle being the major ROS-processing mechanism which links the protection against ROS to redox-regulated defense (Kuźniak, 2010; Foyer and Noctor, 2011; Shigeoka and Maruta, 2014). However, there are only some reports on the involvement of AA-GSH cycle components in plant tolerance to concurrent abiotic and biotic stresses refer mainly to example ascorbate peroxidase (APX, Satapathy et al., 2012; Nenova and Bogoeva, 2014) or APX and glutathione reductase activities under salt stress and fungal infection (Nostar et al., 2013).

Similarly to ROS, phytohormones are important players in orchestrating signaling pathways as well as transcriptional and metabolic responses shared between abiotic and biotic stresses (Robert-Seilaniantz et al., 2011). SA, jasmonic acid (JA) and ethylene (ET) are mainly known to control plant defense against pathogens (Pieterse et al., 2009; Verma et al., 2016). ABA

primarily regulates plant responses to drought, low temperature and salinity (Sah et al., 2016), but it also mediates defense against pathogens. Treatment with ABA increases plant susceptibility to bacterial and fungal pathogens, and inhibition of ABA signaling improves plant defense against pathogens (Audenaert et al., 2002; Mohr and Cahill, 2003; Anderson et al., 2004; Asselbergh et al., 2007). ABA usually antagonizes SA and JA/ET defense signaling thereby interfering with plant responses to biotrophic and necrotrophic pathogens, respectively (Ton et al., 2009; Lievens et al., 2017). At the pre-invasion stage, however, ABA can have a positive effect on defense, as it increases penetration resistance by inducing stomatal closure (Ton et al., 2009).

Under combined stress, ABA is recognized as the master hormonal switch prioritizing the abiotic or biotic responses, depending on the nature of individual stresses and the harmful effects they can induce in plants. Two ABA signaling components, the transcription factor ATAF1 (*Arabidopsis* NAC domain containing 1) and the proline oxidase ERD15 (EARLY RESPONSIVE TO DEHYDRATION 15) act as switches which activate ABA-dependent, post-penetration resistant responses at the expense of abiotic stress tolerance (Ton et al., 2009; Atkinson and Urwin, 2012; Suzuki et al., 2014).

The close relationship of ABA with SA and JA/ET-mediated defense signaling may have critical consequences for the outcome of plant-pathogen interaction in the field, as any abiotic stress which leads to ABA accumulation is likely to suppress disease resistance mechanisms (Sivakumaran et al., 2016). The outcome of plant-pathogen interaction can be also modulated by crosstalk between ROS and ABA signaling. Salinity predisposing plants to disease has been found to increase ROS content by impairing the ROS-scavenging system (Jiang and Deyholos, 2006), and ABA-regulated genes are also induced by oxidative stress (Cho et al., 2009).

In the multistress environment, the interactions between the signaling pathways provide the plant with powerful regulatory capacity and are likely to reduce the metabolic costs of plant defense (Vos et al., 2013). Crosstalk between abiotic and biotic stresses leads to changes in the primary metabolism which is shifted from growth and biomass production programs to defensive processes (Rojas et al., 2014). By understanding these cross-regulation mechanisms, we could predict the outcome of plant-pathogen interactions under abiotic stress conditions. It is especially important in the context of global climate changes, when the abiotic and biotic stresses are expected to increase.

CASE STUDY: CUCUMBER RESPONSE TO SEQUENTIAL SALT STRESS AND BACTERIAL PATHOGEN INFECTION

As the interaction between salinity and pathogens is still poorly recognized, we discuss here how salt stress influences the plant response to diseases, taking leads from studies on cucumber sequentially exposed to salt stress and angular leaf spot (ALS), the second most severe cucumber disease, caused by *Psl* (Olczak-Woltman et al., 2009).

In cucumber exposed to 7-day salt stress and thereafter inoculated with *Psl* the combinatorial stress intensified the negative impact of NaCl on plant growth, confirming results on the additive effect of stress factors. Salt-treated cucumber was more prone to ALS as shown by enhanced bacteria growth and disease symptom development in the NaCl-treated plants simultaneously with the recovery from salt stress (Chojak-Koźniewska et al., 2017). Salt stress and recovery are mediated by shared signaling components, including ROS and redox elements, phytohormones, and C metabolites which might also contribute to pathogen defense (Tang et al., 2015).

In plants, stress sensing is primarily reflected in PSII photochemistry imbalance, and maximum PSII quantum yield (Fv/Fm) and NPQ are recognized indicators of plant stress (Kuźniak et al., 2010; Qu et al., 2012). Changes in the photosynthetic apparatus also play an important regulatory role via retrograde signaling (Gollan et al., 2015). In cucumber sequentially exposed to NaCl and *Psl*, Fv/Fm and photochemical quenching coefficient (qP) which quantify the photochemical efficiency, decreased, and the single stress scenarios of perturbations of PSII were changed under combined stress, e.g., qP was significantly decreased only in plants exposed to NaCl and Psl (Chojak-Koźniewska et al., 2018). The interaction of NaCl and *Psl* strongly reduced the cucumber capacity to recover leaf photochemistry after salt stress alleviation when compared to plants subjected to single stress (**Figure 1**). This could be caused by impaired photosynthetic biochemistry (Ennahli and Earl, 2005) or decreased CO_2 availability due to sustained stomata closure (Galmés et al., 2007). The salt stress-induced stomata closure signaled by root-produced ABA was intensified after infection, likely via SA/H_2O_2-related mechanism, and could contribute to the prolonged inhibition of PSII under combined stress (Chojak-Koźniewska et al., 2017).

In cucumber, salt stress, *Psl* and their combination induced NPQ which protects plants from damaging effects of ROS and integrates into the defense responses against biotic stress (Chojak-Koźniewska et al., 2018). In *Arabidopsis*, increased photooxidative stress at PSII decreased resistance to *Sclerotinia sclerotiorum* (Zhou et al., 2015) and plants impaired in NPQ were suggested to be constantly primed to pathogen attack (Barczak-Brzyżek et al., 2017). Many authors reported infection-induced inhibition of photosynthesis, but its role in pathogenesis remains unclear as it could reflect the attack strategy of the pathogen and a defense response relying on reprogramming plant metabolism from growth to defense (Cheng et al., 2016; Dong et al., 2016).

In cucumber coping with combinatorial stress, the infection-induced oxidative stress, manifested by H_2O_2 accumulation and lipid peroxidation, was stronger than in plants exposed to salinity and *Psl* individually (Chojak et al., 2012). The non-halo lesion type similar to the hypersensitive response is an important component of cucumber resistance to ALS and chlorotic halo is typical for susceptibility (Słomnicka et al., 2018). Thus, ROS generated in the infected plants contributed to susceptibility to ALS. The antioxidant response was organ and stimulus-specific, except for proline accumulation (**Figure 1**) which is a common defense response in plants grown under stress (Nostar et al., 2013; Pandey et al., 2015). Other studies also showed

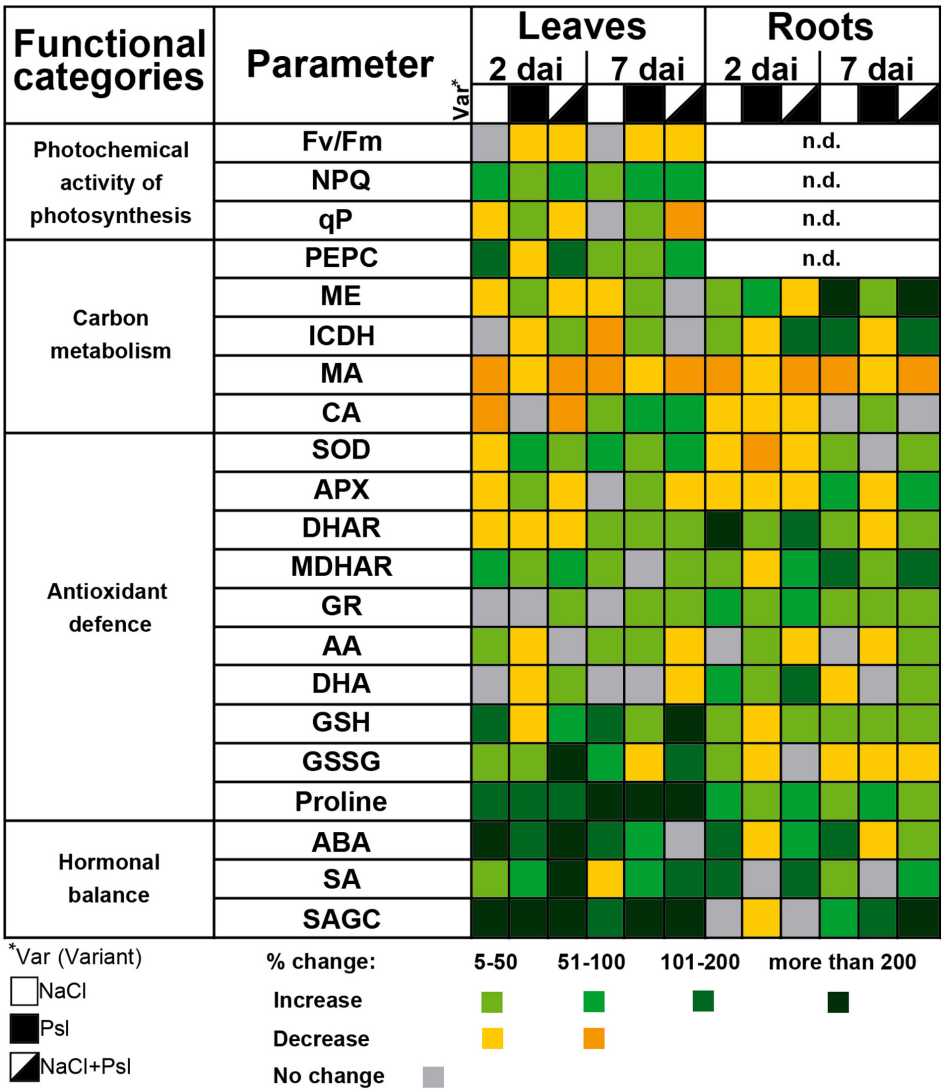

FIGURE 1 | The effect of salt stress and *Pseudomonas syringae* pv *lachrymans* (Psl) infection applied individually and in combination on photochemical activity of photosynthesis, carbon metabolism, antioxidant defense and hormonal balance in leaves and roots of cucumber plants (Chojak et al., 2012; Chojak-Koźniewska, 2017; Chojak-Koźniewska et al., 2017, 2018). Plants were pretreated for 7 days with 100 mM NaCl and then infected with *Psl*. Analyses were performed 2 and 7 days after inoculation (dai). Changes in contents/activities are *color coded,* relative to control set as 100%. Fv/Fm, maximum PSII quantum yield; NPQ, non-photochemical quenching; qP, photochemical quenching coefficient; PEPC, phosphoenolpyruvate carboxylase; ME, NADP-malic enzyme; ICDH, NADP-isocitrate dehydrogenase; MA, malic acid; CA, citric acid; SOD, superoxide dismutase; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; MDHAR, monodehydroascorbate reductase; GR, glutathione reductase; AA, ascorbic acid (reduced); DHA, dehydroascorbate; GSH, glutathione (reduced); GSSG, glutathione disulphide; ABA, abscisic acid; SA, free salicylic acid; SAGC, SA conjugates with glucose.

proline accumulation after salt treatment and bacterial pathogen infection, correlated with disease severity (El-Hendawy, 1999; Tiwari et al., 2010). In roots, the activities of superoxide dismutase (SOD) and the AA-GSH cycle in plants sequentially exposed to salt stress and *Psl*, closely resembled those induced by salinity, confirming abiotic stress dominant impact of. In leaves, the combinatorial stress-induced changes showed that plants previously exposed to salt stress required specific antioxidant protection after infection, as exemplified by the induction of chloroplastic FeSOD found exclusively in plants under combined stress (Chojak-Koźniewska et al., 2017). 7 days after infection, during the salt stress recovery phase (**Figure 1**), the AA-GSH cycle-related antioxidant profile in the leaves of plants exposed to NaCl and *Psl* was characterized by significantly decreased APX activity and ascorbic acid (AA) content, accompanied by glutathione (GSH) accumulation (Chojak-Koźniewska, 2017). This indicated a substantial uncoupling of ascorbate and glutathione redox pairs under combined stress. Contrary, NaCl treatment and bacterial infection applied individually induced parallel changes in ascorbate and glutathione pools. In other studies, NaCl led to a decrease in ascorbate and glutathione levels in cucumber seedlings and *P. syringae* infection increased

the contents of these antioxidants in *Arabidopsis* (Großkinsky et al., 2012; Shu et al., 2013). As redox signals are early warnings controlling the adjustment of energy production to consumption in the leaf (Foyer and Noctor, 2009), these changes could regulate the execution of plant metabolic reprogramming under combined stress. The ascorbate pool and the related components of the AA-GSH cycle were affected stronger than glutathione (Chojak-Koźniewska, 2017). Ascorbate plays a critical regulatory role in the network of photosynthesis, respiratory electron transport and tricarboxylic acid cycle (Szarka et al., 2013), thus these changes could have negative consequences for plant resistance to *Psl*.

Besides the redox modifications, salt stress through ABA upregulation had antagonistic effects on SA-mediated signaling and compromised the defense against the pathogen. The changed ABA/SA equilibrium in leaves hindered *PR1* gene expression (pathogenesis-related 1), known to regulate SA-mediated defense to pathogens (Rivas-San Vicente and Plasencia, 2011; Chojak-Koźniewska et al., 2017; Zhang and Sonnewald, 2017).

Stress defense responses and recovery require energy inputs and diversion of carbon metabolites and reducing equivalents to anabolic pathways. NADPH-generating enzymes, such as NADP-isocitrate dehydrogenase (ICDH) and NADP-malic enzyme (ME), provide NADPH which is required for growth and detoxification, participates in the equilibrium of cellar redox homeostasis, supports the AA-GSH cycle and the NADPH oxidase in the apoplast (Noctor, 2006; Leterrier et al., 2012). Abiotic stress, which affected plants stronger than infection, was the dominant factor shaping the response of carbon metabolism to combinatorial stress at both the biochemical and transcriptomic levels (Chojak-Koźniewska et al., 2018). In roots, salt stress intensified the activities of ME and ICDH, indicating the need for increased detoxification. Although in other studies similar results were also reported for leaves of salt-stressed cucumber (Hýsková et al., 2017), we observed that the NADPH-generating enzyme activities tended to decrease (**Figure 1**). This, combined with the reduced contents of malic and citric acids which are involved in energy-producing pathways (López-Millán et al., 2000) and compensate for salt-stress induced ionic imbalance (Sanchez et al., 2008), could represent changes in the leaf metabolic environment predisposing plants to infection. Decreased contents of malic and citric acids in salt-stressed cucumber were also described by Zhong et al. (2016). As to the biotic stress, high level of malic acid was suggested to be a pre-selection criterion for resistance to ascochyta blight in chickpea (Çağirgan et al., 2011).

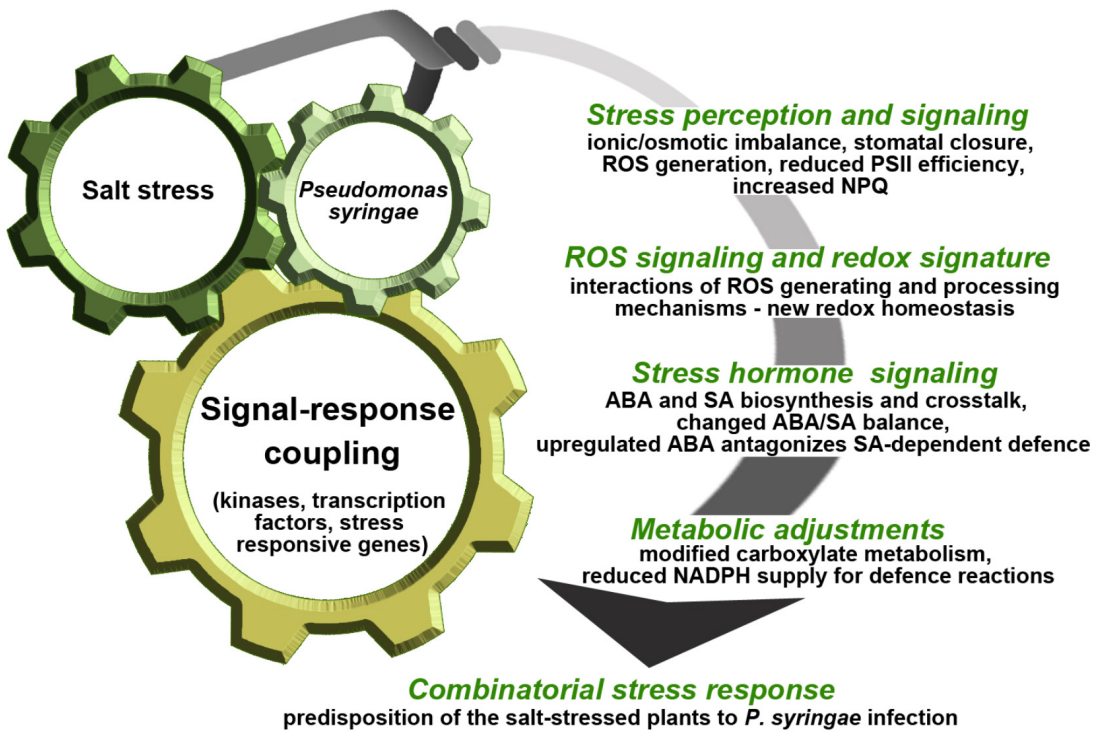

FIGURE 2 | Schematic illustration of cucumber plant response to sequential application of salt stress and *P. syringae*. Salt stress and *P. syringae* infection differ in severity of impact on cucumber plants and the combinatorial stress response is dominated by the abiotic factor. Episodic salt stress occurring prior to infection may predispose cucumber plants to *P. syringae* by weakening SA-mediated defense as well as by shaping the hormonal, ROS/redox and metabolic signals induced under combinatorial stress. These signals are transduced by specific kinases and transcription factors activating or suppressing functional genes, finally resulting in higher susceptibility of the salt-stressed plants to *P. syringae* infection. This model is based on data presented in Chojak-Koźniewska (2017) and Chojak-Koźniewska et al. (2017, 2018). PSII, photosystem II; NPQ, non-photochemical quenching; ROS, reactive oxygen species; ABA, abscisic acid; SA, salicylic acid; NADPH, nicotinamide adenine dinucleotide phosphate.

The integration of stress response at the whole-plant level requires long distance signals, including ROS, hormones and metabolites which communicate the information of the status of roots to leaves and vice versa (Mittler et al., 2011; Gilroy et al., 2014; Ko and Helariutta, 2017). Biotic stress in leaves initiated rootward signaling to induce whole-plant stress response as shown by specific changes in the ABA/SA balance, carbon metabolism and the profiles of antioxidants in roots of plants which leaves were infected with *Psl*. Similarly, abiotic stress sensed in roots prior to infection changed the status of leaves during recovery, making them more prone to infection (**Figure 1**).

In cucumber, salinity and bacterial pathogen applied sequentially generated prolonged inhibition of PSII, and unique redox signature as a result of ROS overproduction and novel interactions between the AA-GSH cycle components. This implies specific adjustments to other signaling components, especially ABA and SA. Salt stress-induced ABA accumulation compromised the SA-mediated defense response. This was favored by modifications in the carboxylate metabolism and could lead to insufficient energy and reducing power supply to defense reactions (Chojak-Koźniewska, 2017; Chojak-Koźniewska et al., 2017, 2018). This example supports the role of ABA in predisposition, and illustrates biochemical mechanisms underpinning this phenomenon in the context of whole-plant response (**Figure 2**). The case study, supported by additional data, demonstrated that under combined stress most single stress responses were maintained, although differentially regulated. The functional analysis of the enhanced or reduced components of the stress responses may be a hint to what processes and in what stress scenarios could increase plant tolerance.

CONCLUDING REMARKS

Stress factors acting in combination lead to decreases in crop yield that exceed single stresses. Plant acclimations to combinatorial stress vary widely depending on the type, sequence of application, and intensity of the individual stresses implied. Many studies have shown that combined stress triggers specific transcriptomic response. Yet, little is known about the stress combination-unique response elicited by the partially overlapping stressors at the physiological and metabolic levels. The response to a combination of abiotic and biotic stresses is usually dominated by abiotic stress, at the expense of resistance to pathogens. However, our current understanding of mechanisms predisposing plants affected by abiotic stress to infectious diseases is still limited.

The knowledge of how abiotic environmental factors influence plant resistance to pathogens and of processes specifically involved in response to combined abiotic and biotic stress has implications for disease management and is important with respect to the breeding programs aimed at improving multiple stress tolerance in plants.

AUTHOR CONTRIBUTIONS

JC-K, EK, and JZ took responsibility for the integrity of the work as a whole.

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Exploring Combined Effect of Abiotic (Soil Moisture) and Biotic (*Sclerotium rolfsii* Sacc.) Stress on Collar Rot Development in Chickpea

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Plants being sessile are under constant threat of multiple abiotic and biotic stresses within its natural habitat. A combined stress involving an abiotic and a biotic factor reportedly increases susceptibility of the plants to pathogens. The emerging threat, collar rot disease of chickpea (caused by *Sclerotium rolfsii* Sacc.) is reported to be influenced by soil moisture condition (SMC). Hence, we studied the influence of differential SMC *viz.* upper optimum (100%), optimum (80%), lower optimum (60%), and limiting (40%) soil moisture conditions on colonization and collar rot development over the course of infection in two chickpea cultivars, Annigeri (susceptible to collar rot) and ICCV 05530 (moderately resistant to collar rot). Disease incidence was found to be directly proportional to increase in soil moisture ($R^2 = 0.794$). Maximum incidence was observed at 80% SMC, followed by 100 and 60% SMC. Expression of genes (qPCR analysis) associated with host cell wall binding (lectin) and degradation *viz.* endopolygalacturonase-2, endoglucosidase, and cellobiohydrolase during collar rot development in chickpea were relatively less at limiting soil moisture condition (40%) as compared to optimum soil moisture condition (80%). As compared to individual stress, the expression of defense response genes in chickpea seedlings were highly up-regulated in seedlings challenged with combined stress. Our qPCR results indicated that the expression of defense-related genes in chickpea during interaction with *S. rolfsii* at low SMC was primarily responsible for delayed disease reaction. Involvement of moisture and biotic stress-related genes in combined stress showed a tailored defense mechanism.

Keywords: chickpea, collar rot, gene expression, *Sclerotium rolfsii*, soil moisture

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the most important and essential legumes crops for semi-arid tropical area. India is the largest producer of chickpea, accounts 70.9 % of the world cultivated area and produces 67.1 % of the total world production (FAOSTAT, 2013). The vulnerability of chickpea to biotic and abiotic stresses is major constraint for reduced yields. The on-going changes in climatic conditions such as increase in CO_2 emissions, unpredicted rainfall patterns, temperature rise, decrease/increase in relative humidity, and low soil moisture stress (Zhao and Running, 2010) are likely to influence the plant diseases establishment, its distribution and epidemiology (Graham and Vance, 2003). Consequently, the evidences suggest major

shift in the chickpea soil-borne diseases like dry root rot (*Rhizoctonia bataticola*), Fusarium wilt (*Fusarium oxysporum* f. sp. *ciceris*), collar rot (*Sclerotium rolfsii*), wet root rot (*Rhizoctonia solani*) and black root rot (*Fusarium solani*) in the semi-arid tropic (SAT) regions.

As we know, plants in fields are always exposed to multiple biotic and abiotic stresses where plants exhibit certain unique and convergent physiological and molecular responses that interact and impact each other to withstand the combined effect of these stresses (Choi et al., 2013; Padaria et al., 2015; Pandey et al., 2015). These combined stress interactions in plants may either have direct or indirect effect on pathogens through other community interactions (Sharma and Ghosh, 2017) leading to either positive or negative effects on plant responses (Ramegowda et al., 2013). In a combined stress scenario, drought can affect the pathogen infection either positively or negatively (Graham and Vance, 2003). Previous reports have shown that susceptibility of plants to bacterial pathogens tend to increase under low soil moisture stress (Mohr and Cahill, 2003; Choi et al., 2013). In chickpea, low soil moisture stress significantly increased the incidence of dry root rot caused by *Rhizoctonia bataticola* (Sharma and Pande, 2013). Conversely, reports also indicated that low soil moisture stress improves the defense response of plants against pathogens (Ramegowda et al., 2013; Hatmi et al., 2015; Sinha et al., 2016). On the other hand, it has also been found that not only low but high soil moisture condition also favors some diseases (Blaker and MacDonald, 1981; Ferraz et al., 1999). Therefore, it is crucial to understand the effect of combined stress and the respective defensive strategies adopted by the plants to overcome the synchronous onslaught of low soil moisture stress and pathogen. The molecular responses of different crops against several pathogen infection and combined low soil moisture stress have been reported (Choi et al., 2013; Ramegowda et al., 2013; Hatmi et al., 2015). However, so far no attempt has been made to understand the molecular responses of chickpea to combined soil moisture stress with soil borne fungal infections. Among soil borne diseases in chickpea, dry root rot and collar rot are predisposed by low and high soil moisture respectively. There are some reports of effects of soil moisture and temperature on dry root rot of chickpea but minimal work has been done with respect to collar rot.

Collar rot is an emerging soil-borne disease of chickpea that may incite 55–95 % mortality of chickpea seedlings under favorable environmental conditions like heavy rainfall and high soil temperature (25–30°C) (Sharma and Ghosh, 2017). Moreover, collar rot management is quite challenging owing to the pathogens wide host range including at least 500 species coming under 100 families commonly in legumes, crucifers, and cucurbits (Aycock, 1966). *Sclerotium rolfsii* survives in the form of mycelium in the infected tissues and plant debris and as sclerotial structures in the soil or in association with plant debris and usually attacks the collar region of plants. Because of high competitive saprophytic survival ability, in recent years, *S. rolfsii* is becoming more prevalent in agricultural areas where sudden rainfall increases soil moisture for longer periods combined with warm temperatures. With the availability of such a large range of natural hosts, *S. rolfsii* could even survive in dry climatic regions and continue to persist in the soil for prolonged periods even after several crop rotations. Lack of sufficient information regarding the factors affecting collar rot development have made its control quite difficult.

The present study was therefore aimed to understand the influence of differential soil moisture stress on the severities of *S. rolfsii* infection in chickpea. Attempts have been made to study the differences in the net impact of combined stress compared to the respective individual stresses at molecular level including differential gene expression. To the best of our knowledge this is the first report showing the molecular responses of chickpea during individual and combined biotic (*S. rolfsii*) and abiotic (soil moisture) stress interactions.

MATERIALS AND METHODS

Plant Material and Growth Conditions

For the present investigation, we have considered two chickpea cultivars Annigeri and ICCV 05530 based on their resistance and susceptibility reaction to collar rot (*S. rolfsii*). Annigeri is highly susceptible and ICCV 05530 is moderately resistant to collar rot. The latter is also found to be resistant/moderatly resistant to other soil borne diseases-*Fusarium* wilt and dry root rot in chickpea (Tarafdar et al., 2017). Apparently healthy seeds of both cultivars were surface sterilized with 2% sodium hypochloride (NaOCl) for 2 min followed by two times washing with sterile deionized water. Seven seeds per pot were sown in 15 cm plastic pots containing sterilized vertisol and sand (3:1) and kept in a growth chamber with controlled environment having a diurnal cycle of 14 h light/10 h dark with 28 ± 1°C and 50–60% relative humidity.

Fungal Isolate and Inoculum Preparation

A pathogenic isolate of *S. rolfsii* viz. Sr 1, isolated from infected chickpea plant from experimental field of ICRISAT, Patancheru, Telangana, India was used in the experiments (Tarafdar et al., 2017). For mass multiplication of *S. rolfsii* inoculum, 100 g seeds of sorghum were soaked in water for 2 days and 2 ml of 2% sucrose solution was added prior to autoclave. Four plugs of actively growing 4-day-old *S. rolfsii* culture on PDA were inoculated into the autoclaved sorghum grains and incubated at 28 ± 1°C for 7 days.

Soil Moisture Stress Imposition

Effect of different soil moisture conditions on the development of collar rot disease in chickpea was studied. Seven-day-old chickpea seedlings grown in pre-weighted pots were divided into four different sets with three replications and pots were maintained in four different soil moisture conditions(SMC), i.e., upper optimum soil moisture (100%), optimum soil moisture (80%), lower optimum soil moisture (60%), and limiting soil moisture (40%) conditions (**Table 1**). The SMC was maintained by withholding the water supply and determined by means of gravimetric method on oven dry basis as described earlier (Sharma and Pande, 2013). To obtain all the four moisture condition at same time, water withdrawal for each moisture level was done in batches. For imposing 40% soil moisture

TABLE 1 | Details of experimental set up used to study *Sclerotium rolfsii* and chickpea interaction and summary of the observation.

	Parameter		Remarks
Soil moisture conditions (%)	**Description**	**Pathogen**	
40	Limiting soil moisture	Inoculated	i.*S. rolfsii* colonization initiation at 24 hpi. Low pathogen growth even at 48 hpi due to low soil moisture condition. ii.Physiological wilting occurred due to purely abiotic stress in plant. iii.No plant mortality. iv.Not considered for gene expression study.
		Non-inoculated	i.Purely abiotic stress for plant ii.Considered for plant defence gene expression study w.r.t abiotic stress (48 h)[†]
60	Lower optimum soil moisture	Inoculated	i.Delayed *S. rolfsii* colonization initiation at 24 hpi due to lower optimum soil moisture condition and moderate disease incidence at 48 hpi. ii.Considered as combined (both biotic and abiotic) stress for plant and abiotic stress for pathogen growth. iii.Considered for gene expression studies for plant defense-related genes w.r.t combined stress and pathogenicity-causing genes w.r.t abiotic stress.
		Non-inoculated	i.Lower optimum soil moisture condition for plant. ii.Not considered for gene expression study.
80	Optimum soil moisture	Inoculated	i.Early initiation of *S. rolfsii* colonization at 12 hpi. Progressive colonization and high disease incidence due to optimum growth condition for both chickpea and *S. rolfsii.* ii.Purely biotic stress for plant growth iii.Optimum condition for studying host-pathogen interaction w.r.t plant defence genes and its expression. iv.Considered for study biotic stress.
		Non-inoculated	i.Optimum soil moisture for plant. ii.Considered as experimental control condition for plant growth. iii.Taken as control to normalize the gene expression profiles of plant defence-related genes.
100	Upper optimum soil moisture	Inoculated	i.Delayed *S. rolfsii* colonization initiation at 24 hpi due to high moisture condition, but fast growth of pathogen after the initial colonization led to disease incidence at par with optimum soil moisture condition at 48 hpi. ii.Upper optimum soil moisture for plant growth as well as pathogen growth. iii.Not considered for gene expression study.
		Non-inoculated	i.Upper optimum soil moisture for plant growth. ii.Not considered for gene expression study.

[†] *From the moisture stress point of view, the sampling time of 6–24 h is very short for inducing any significant abiotic stress in plants for gene expression studies, hence we have consider only 48 h sample in gene expression study w.r.t abiotic stress.*

stress in chickpea seedlings, water withdrawal in pots was started 5 days prior inoculation, including 5 days of acclimatization. Similarly, for 60% SMC, water withdrawal was started 4 days before inoculation and for 80% SMC 2 days before inoculation.

Combined Soil Moisture and Pathogen Stress Imposition

Twelve days old chickpea seedlings maintained at different SMC were inoculated with *S. rolfsii* to understand the impact of SMC on development of collar rot disease in chickpea. *S. rolfsii*-infested sorghum grains were placed near the collar region of chickpea seedlings. Plants inoculated with sterile sorghum seeds served as mock. Different SMC were maintained in pots (described in above section) by regularly weighing each pot for the moisture deficit and replacing it by adding de-ionized water till the end of an experiment. The experiment was conducted in completely randomized design (CRD) and the disease incidence was recorded every day till the mortality of plants. Disease incidence was calculated by following formula: Disease incidence (%) = Total number of infected plants/total number of plants × 100. Correlation between disease incidence and SMC was observed by establishing the regression model, calculated at 1% level of significance at different moisture conditions.

Quantification of *S. rolfsii* Colonization

For quantification of *S. rolfsii* colonization, the samples (the shoot region up to 1 cm immediately adjacent to the infected collar

region) were harvested from cv. Annigeri at 6, 12, 24, and 48 h post-inoculation (hpi), quick freezed in liquid N_2, and preserved at −80°C for downstream experiments.

Genomic DNA was extracted from infected plant samples (cv. Annigeri) grown in different SMC and SR 1 isolate using PureLink Plant Total DNA Purification kit (Invitrogen, USA) as per the manufacturer's protocol. 100 mg of the harvested tissue was finely ground using liquid N_2 and resuspended in 250 μL Resuspension buffer supplied in the kit. The resuspended tissue was vigorously vortexed until the samples homogenized completely. To lyse the tissues and avoid the RNA contamination, about 15 μL each of 20 % SDS and RNase (20 mg/ mL) was added to the tissue resuspension mixture and incubated for15 min at 55°C. The total gDNA from the sample was eluted by adding 50 μL of Elution buffer and stored at −20°C (Ghosh et al., 2017). Purified DNA was checked on 0.8% agarose gel and the extracted DNA was stored at −20°C for further use.

The absolute quantification of *S. rolfsii* DNA was measured through qPCR. The primers, qSR_5.8S (F), and qSR_5.8S (R) were designed from conserved region of the 5.8S sequences (Soeta et al., 2009) using IDT Primer Quest software (eu.idtdna.com/Primerquest/Home/Index;) (**Table S1**). DNA isolated from infected plants was used as template in qPCR to quantify fungal colonization. qPCR was carried out in Eppendorf Realplex Master Cycler (Eppendorf, Hamburg, Germany) using 10 μL reaction mixture consisting of 5 μL 2X KAPA SYBR Green PCR master mix (KAPA Biosystems, USA), 500 nM of each primer (qSR_F and qSR_R) and 1 ng of each template DNA. The PCR thermal cycling conditions were as follows: 95°C for 3 min followed by 40 cycles of 95°C for 10 s (denaturation), and 62°C for 30 s (annealing and extension) at which the fluorescence was measured and subsequently a melting curve was constructed by measuring continuous fluorescence at 60–95°C with increase of 0.5°C per second.

The standard curve (a plot of the Ct value vs. log DNA concentration) was prepared by following the protocol of Sharma et al. (2015) for quantifying the fungal DNA. The pure DNA of *S. rolfsii* was 10-fold serially diluted ranging from 10 ng to 0.01 pg and threshold cycle (Ct) for amplification of each diluted DNA was determined in qPCR under the same reaction conditions described above. Each sample amplification was conducted in triplicates in every experiment. The statistical significance of the difference in pathogen relative quantification at different soil moisture conditions was calculated by two-way ANOVA using Genstat 18.

Real-Time Quantitative Analysis of Gene Expression

For validation of gene(s) expression, total plant RNA was isolated from harvested plant samples (cv. Annigeri and ICCV 5530) at different hpi (6, 12, 24, and 48 hpi using GSure Tissue RNA kit (GCC Biotech, Kolakata, India) by following manufacturer's instruction. About 25–30 mg of tissue sample was ground in liquid nitrogen (N_2) and suspended 250 μL of Buffer GRT1. The suspension was incubated at 70°C for 15 min and vortexed it every 2 min interval. In final step total RNA was eluted in 50 μL of nuclease-free water. Purified RNA was quantified in Nanodrop spectrophotometer and evaluated on 1% agarose gel. The RNA was stored at −20°C for further downstream process.

A total of 1 μg of RNA in 20 μl reaction mixture was used for cDNA synthesis following the manufacture's protocol of Super Script III cDNA synthesis kit (Thermo Fisher, USA) following manufacturer's instruction. The expression profiling of genes causing pathogenicity in *S. rolfsii* and defense-related genes in chickpea were assessed. The 5.8s gene of *S. rolfsii* and actin of chickpea were used as endogenous reference genes for normalizing the gene expression (James et al., 2015). The primers used in expression analysis of qPCR assays are shown in **Table S1**. All the qPCR was carried out in 10 μL reaction mixtures as described above. Expression profile of each gene was determined by averaging of Ct value of three technical replicates from three biological replications. The primer specificity was confirmed using melting curve analysis. Relative expression of the genes was calculated by the 2^{-DDCT} method using Ct value (Livak and Schmittgenm, 2001). The statistical significance of the difference in relative expression at different soil moisture conditions in different chickpea cultivars was calculated by three-way ANOVA using Genstat 18.

RESULTS

Assessment of Fungal Pathogenicity and Combined Stress Imposition

At 48 hpi, the infected chickpea plants exposed to lower optimum (60%), optimum (80%), and upper optimum (100%) conditions revealed cellular degradation with tissue maceration and rotting of the collar region ultimately resulting in death of the plants. There was significant delay in the disease progression in ICCV 05530 as compared to Annigeri ($p < 0.05$; **Table S2**). At 48 hpi, the disease incidence was 9.5 and 4.8% in ICCV 05530 under optimum and upper optimum SMC, whereas, in Annigeri, the incidence was 76.2 and 71.4% respectively. Similar trend of disease development was observed up to 96 hpi in both the cultivars. However, disease incidence was at par in both the cultivars at 144 hpi. Collar rot incidence was lower during the combined application of low soil moisture and pathogen. There was a positive relationship ($R^2 = 0.794$) between soil moisture (independent variable) and disease incidence (dependent variable) where, disease incidence (y) = (– 44.047) + 1.488x. However, it was observed that once disease appeared, the disease progression was faster in upper optimum soil moisture condition as compared to optimum soil moisture condition and no significant effect of soil moisture on collar rot incidence was observed after a certain period of inoculation (48 and 72 hpi; **Figure 1**). Under limiting soil moisture (40%) condition, no disease symptoms were observed in both chickpea cultivars even after 8 days of inoculation. Further, upon prolonged stress conditions (40% SMC), both inoculated and un-inoculated plants showed physiological wilting, indicating that longer exposure to insufficient moisture condition inhibited the normal growth of plants. No symptoms were recorded in un-inoculated control plants. On the other hand, it was observed that chickpea grown

in upper optimum soil moisture condition had significantly taller stature with higher root and shoot biomass as compared to those from lower moisture condition.

Fungal Biomass vs. Time-Course of Infection Progression

In order to determine the infection and colonization pattern of *S. rolfsii* in chickpea plants as affected by different soil moisture conditions, the fungal biomass within infected plant tissue (shoot) of cv. Annigeri was quantified using real-time qPCR assay. Absolute quantity of *S. rolfsii* DNA in infected chickpea plants was measured by assaying the *S. rolfsii* 5.8s gene using standard curve generated by 10-fold over 7-log range from 100 to 1×10^{-3} ng/ μL serially diluted *S. rolfsii* DNA (**Figure S1**). The slope of linear regression curve and correlation coefficient (R^2) were −3.278 and 0.998 respectively, demonstrating the PCR efficiency of 101.87%. The calculated value for the limit of detection (LOD) at 95% level 2.9, which indicate heterogeneity in biological replication. For normalization of gDNA, the coefficient of variation (CV) was determined using gene copy number analysis. The CV of gene copy number was 33.13. The quantity of fungal DNA was recorded to exponentially increase in the host tissues over time, at all the soil moisture conditions except at limiting soil moisture condition At all the soil moisture conditions, no detectable amount of *S. rolfsii* biomass was measured within the host tissue during early stages of infection (at 6 hpi). However, the early initiation of *S. rolfsii* colonization was noted in 12 hpi at optimum soil moisture condition. Conversely, at 48 hpi, highest colonization of *S. rolfsii* from shoot tissue was recorded at all the soil moisture conditions. After 24 hpi the colonization of the fungus at lower optimum soil moisture condition was comparatively low with respect to succeeding higher moisture conditions (**Figure 2**). This correlated with the corresponding disease incidence at the respective time point. The highest fungal biomass of nearly 9-fold increase from limiting soil moisture condition and up to 2-fold increase from lower optimum soil moisture condition was recorded at optimum soil moisture condition closely followed by upper optimum soil moisture condition at 48 hpi. Even though there was successful infection at limiting soil moisture condition at 48 hpi, the colonization was not sufficient enough to lead to plant mortality (**Figure 2**).

Expression Analysis of Selected Pathogenicity-Causing Genes of *S. rolfsii* in Chickpea

The expression pattern of pathogen virulence-related genes *viz.* lectin and three genes related to plant cell wall degradation, endo β-1,4-glucanase (*EG*), cellobiohydrolase (*CBH*), and endopolygalacturinase-2 (*PG*-2) were studied in chickpea grown at optimum and lower optimum soil moisture condition. These genes were evaluated at 6, 12, 24, and 48 hpi by using real time qPCR, where only two genes (lectin and *PG*-2) were found to be highly expressed throughout the complete time-scale of infection. The trend of expression profile for the pathogenicity-causing genes was almost similar in both cultivars, Annigeri and ICCV 05530 (**Figure 3**). Among the four genes, lectin was highly up-regulated and showed higher transcript levels at early stages (6–12 hpi) of infection at optimum soil moisture condition. At lower optimum moisture condition, a delayed expression of lectin gene occurred which in turn was found to be much less compared to that of optimum soil moisture condition (9 and 36-fold in cvs. Annegeri and ICCV 05530, respectively). During initial stage of infection at 6 hpi, no detectable amount of fungal DNA was measured indicating insignificant colonization, but higher expression of lectin genes was recorded at the same time point, which aid in the initial attachment of the *S. rolfsii*

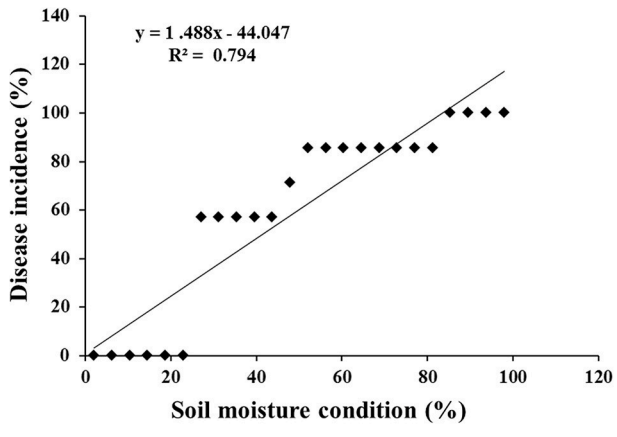

FIGURE 1 | Scatter plot with corresponding regression line and regression equation for the relationship between the dependent variable disease incidence (%) and independent variable soil moisture condition (%).

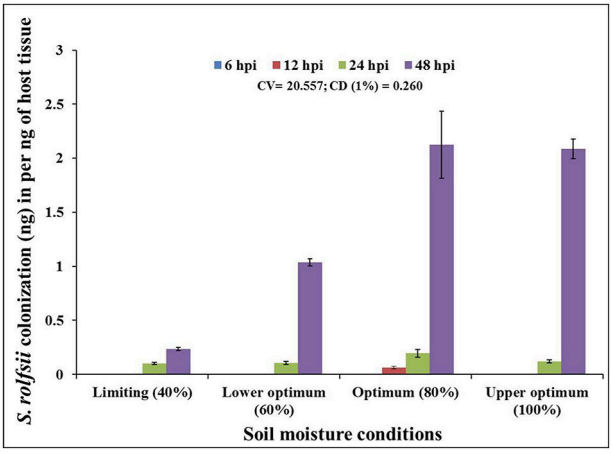

FIGURE 2 | Chronological colonization profile of *S. rolfsii* in shoot tissues of inoculated chickpea (cv. Annigeri) grown in different soil moisture conditions. Absolute quantification of fungal DNA was determined in real-time PCR assay using sequences of 5.8S rDNA. Error bar represents the standard error of three biological replications at 95% confidence interval. The statistical significance between *S. rolfsii* growth and different soil moisture conditions was calculated by factorial ANOVA.

to the plant surface. Once adhered during successful infection, the lectin gene expression was found to be downregulated at 12 hpi and further over the time period, while there was a simultaneous increase of *EG* and *CBH* gene expression at optimum soil moisture condition, where the soil moisture favored early disease establishment as conveyed by the *S. rolfsii* colonization profile (**Figure 2**). *EG* and *CBH* acts synergistically to breakdown the polymers of cell wall components by cleaving the internal bonds in the cellulose chain leading to growth and colonization of *S. rolfsii* (**Figure S2**). In lower optimum soil moisture condition, the expression of such genes was delayed owing to unfavorable soil moisture condition for *S. rolfsii* growth. The *PG*-2 gene expression was gradually up-regulated since initial time points and after successful colonization at 24 hpi, the maximum expression of *PG*-2 gene was found in optimum soil moisture condition, while the same was delayed up to 48 hpi in lower optimum soil moisture condition. The *PG*-2 gene has initially resulted in cell separation and maceration of the plant tissues which further allowed higher colonization of *S. rolfsii* in chickpea.

Differential Expression of Moisture Stress and Biotic Stress-Related Defense Genes

To understand the possible underlying molecular mechanism during stress interaction, the expression profiling of 21 pathogen defense-related genes and five moisture-responsive genes (**Table S1**) in chickpea was conducted for three circumstances viz. biotic (inoculated; optimum soil moisture condition), abiotic (non-inoculated; limiting soil moisture condition) and combined pathogen and moisture stress (inoculated; lower optimum soil moisture condition) conditions (**Table 1**). We grouped the plant defense-related genes into three main categories which were (i) 12 pathogenesis-related (PR) genes comprising of PR-2 (β-1,3-endoglucanase), PR-4, PR-3-type chitinase (*CHI* I,*CHI* II, *CHI* III, *CHI* IV, and *CHI* V), PR-5 (thaumatin-like), PR-12 (defensin), narborin, endochitinase and germin; (ii) five phenylpropanoid pathway genes involved in phytoalexin biosynthesis comprising of phenylalanine ammonia-lyase (*PAL*-1), chalcone synthase (*CHS*), flavonoid 3'-monooxygenase (*Flav* 1), flavonoid 3' hydroxylase (*Flav* 2) and myeloblastosis family transcription factor (*MYB-Tf*); and (iii) four genes involved in reactive oxygen species (ROS) metabolism and stress-related categories comprising of lipoxygenase (*LOX*), catalase (*CAT*), peroxidase, and superoxide dismutase (*SOD*). The moisture stress responsive genes used were late embryogenesis abundant genes (*LEA*-1, *LEA*-2, and *LEA*-4), 9-cis epoxycarotenoid dioxygenase (*NCED*), and dehydration responsive element binding protein-2A (*DREB*-2A).

Differential Expression of PR Genes

The temporal expression of PR genes showed dual (similar and differential) expression pattern within the two chickpea cultivars and also under the three different stress conditions. All PR genes except *CHI* V and narborin were found to be highly up-regulated in combined stress condition, while the expression pattern of *CHI* V gene was found to be similar in both biotic and combined stress condition. In addition, the gene for *CHI* V was specifically found to be highly expressed in cultivar ICCV 05530 at 24 hpi and 48 hpi in both combined (60% SMC) and biotic (80% SMC) stress condition. But in case of narborin gene, the cv. ICCV 05530 showed higher expression under biotic stress condition, while cv. Annigeri in combined stress. Further the genes for PR-4 and *CHI* II were over expressed in cv. Annigeri as compared to cv. ICCV 05530, while the expression pattern of *CHI* II, *CHI* IV, and PR-2 genes were found to be at par in both cultivars. The rest of the PR genes were highly expressed in cv. ICCV 05530 (**Figure 4**).

In abiotic stress condition (48 hpi), the *CHI* IV gene was up-regulated in Annegiri, whereas *CHI* II and *CHI* III genes were up-regulated only in ICCV 05530. On the contrary, the gene for PR-4 was expressed in both cultivars. The expression of rest of the PR genes was not found to be significant (**Figure 4**).

According to the time points in biotic stress condition, it was observed that at initial time point (6 hpi) there was no significant higher expression of any of the PR genes. At 12 hpi, only a single gene PR-4, was highly expressed in both cultivars. Among the expression of chitinase genes, *CHI* II was higher at 24 hpi in both cultivars. *CHI* I gene was observed to be gradually up-regulated throughout the time points in both cultivars, whereas in Annigeri higher expression of *CHI* V was found at 12 hpi. *CHI* III gene displayed a gradual increase in case of ICCV 05530 up to 24 hpi, but increase in expression was significantly higher at 48 hpi, while the expression in Annigeri remained at par in both biotic as well as combined stress throughout the time period. The genes codifying for defensin and germin showed highest expression in ICCV 05530 at 24 hpi, while the same was true for Annigeri at 48 hpi. In case of the gene for thaumatin, an opposite trend was observed to that of defensin and germin. In both cultivars the genes for narborin and glucanase exhibited highest expression at 24 hpi.

During combined stress condition at initial time point (6 hpi), *CHI* II and narborin genes showed highest expression in Annigeri, however for ICCV 05530, the highest expression was realized only at 24 hpi for the former, while the expression pattern remained at par for narborin gene throughout the time points. Also it was observed that the trend in expression pattern for PR-4 gene had gradually decreased over the time points for both cultivars. The expression pattern of genes for thaumatin, germin and endochitinase were similar, as the highest upregulation in ICCV 05530 was observed for all three at 12 hpi and the same for Annigeri at 24 hpi, except the gene for endochitinase, where the peak was observed at 48 hpi in ICCV 05530. The genes for glucanase and defensin were found to exhibit maximum expression in both ICCV 05530 and Annigeri at 12 and 24 hpi respectively. At 24 hpi, *CHI* IV gene produced a higher expression pattern in ICCV 05530 while the same for Annigeri was true at 6 hpi. A gradual increase in the expression of *CHI* III gene was observed in ICCV 05530 until the maximum was realized at 48 hpi. Only the gene for *CHI* I, produced an expression pattern that was at par throughout all the time period in both the cultivars. The only exception was at 48 hpi, where the expression of the gene was significantly high in ICCV 05530 (**Figure 4**).

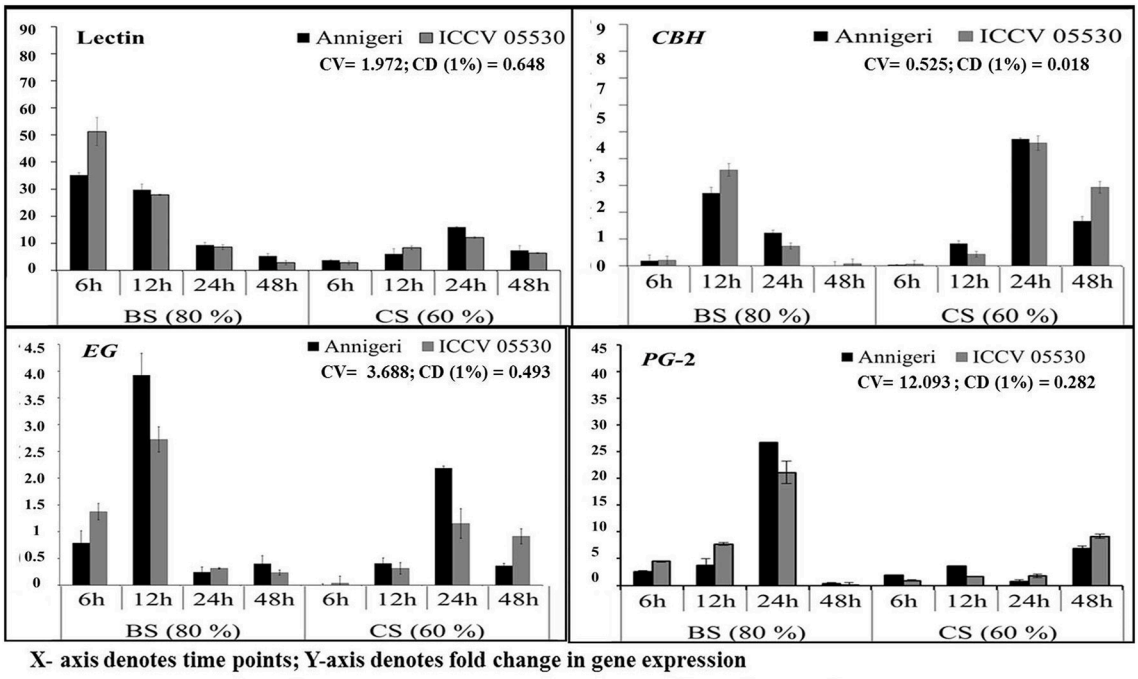

FIGURE 3 | RT-qPCR analysis of different pathogenicity-causing genes of *S. rolfsii* differentially expressed at lower optimum (60%) and optimum (80%) soil moisture conditions in course of infection to chickpea. Time (in hours) and fold change in gene expression are shown on X and Y axis, respectively. Standard error of three biological replications was calculated and represented as error bar. BS and CS denote biotic stress and combined stress respectively. The statistical significance of gene expression in between the control and treated seedlings was calculated by factorial ANOVA.

Differential Expression of Phenylpropanoid Pathway Genes

The upregulation of genes involved in phenylpropanoid pathway was found to be high in combined stress than in biotic stress condition. During abiotic stress (48 h) *CHS* gene was up-regulated only in ICCV 05530 while expression of *PAL* 1 gene was at par in both cultivars. No significant expression was found for *MYB Tf*, *Flav* 1, and *Flav* 2 genes in both cultivars, while genes coding for MYB Tf, FLAV 2 were downregulated in abiotic stress than control in cv. Annigeri (**Figure 5**).

During biotic stress, very low expression of *Flav* 1 and *Flav* 2 genes were found throughout infection period in both cultivars. *PAL* 1 gene expression was observed to be at par in all the time points as well as for both cultivars. For the gene codifying for *CHS*, no significant expression was found during biotic stress except 24 hpi where the maximum upregulation of 2-fold over control was observed.

In combined stress, expression pattern of *Flav* 1 gene was similar in both cultivars and at par in all the time points of up to 4-fold than control. In Annigeri, *Flav* 2 was found to be downregulated at all the time points except 48 hpi, where as in ICCV 05530, the expression level was very low up to 2.5-fold at par throughout the time points. The maximum expression of *PAL* 1 gene of up to 6-fold was observed at 48 hpi in Annigeri while in ICCV 05530 the maximum expression was 4-fold over control. In ICCV 05530, the maximum expression of *CHS* gene of up to 5-fold was found at 12 hpi and declined during later stages. Also, in Annigeri, there was a gradual increase in the CHS gene expression of up to 4.5-fold till 24 hpi after which a decline was observed (**Figure 5**).

Differential Expression of ROS Metabolism Pathway Genes

During combined stress, the expression of genes with the exception of catalase involved in ROS metabolism pathway was higher than in biotic stress condition. The expression of gene for catalase was found to be similar in both stress conditions. Apart from this, in abiotic stress (48 h) condition, only lipoxygenase gene expression was found to be significant in cv. ICCV 05530. Among the two cultivars, all the genes displayed maximum expression in ICCV 05530 than Annigeri. Among the different time points in biotic stress condition, no gene expression was observed at 6 hpi. In Annigeri, no significant expression of SOD and catalase genes was found throughout the infection period except at 12 hpi, where catalase gene was found to be expressed 2-folds than control. In ICCV 05530, maximum expression of catalase and SOD genes was found at 12 and 24 hpi respectively. In combined stress condition, maximum expression of catalase gene was found in both cultivars, where the gene coding for lipoxygenase expressed maximum only in ICCV 05530 at 48 hpi. The maximum upregulation of SOD gene was found at 12 hpi after which a decline was observed (**Figure 6**).

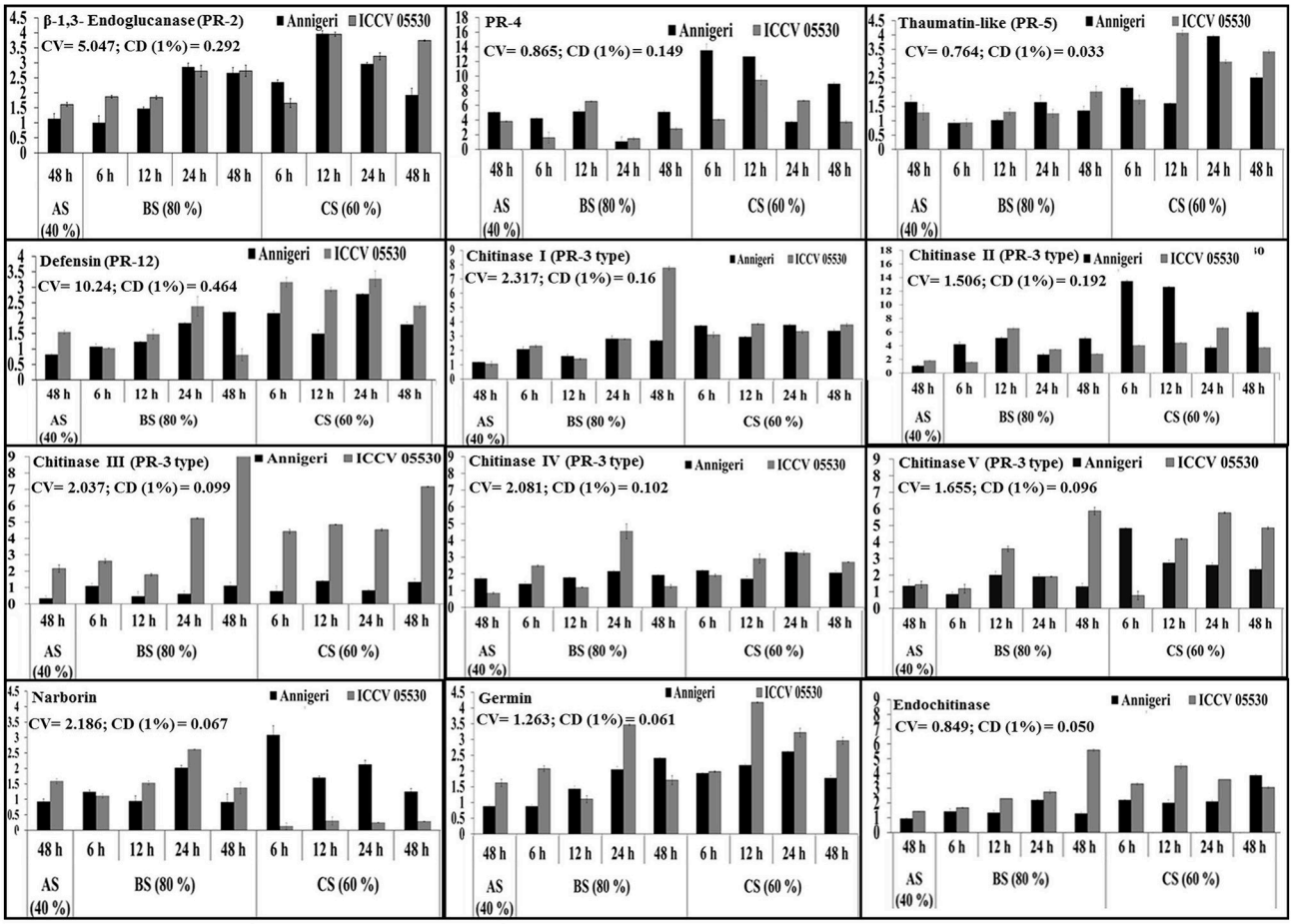

FIGURE 4 | RT-qPCR analysis of different pathogenesis-related genes expressed differentially in chickpea during interaction with *S. rolfsii* at limiting (40%), lower optimum (60%) and optimum (80%) soil moisture conditions. Time (in hours) and fold change in gene expression are shown on X and Y axis, respectively. Standard error of three biological replications was calculated and represented as error bar. AS, BS, and CS denote abiotic stress, biotic stress, and combined stress respectively. The statistical significance of gene expression in between the control and treated seedlings was calculated by factorial ANOVA.

Differential Expression of Moisture Stress Responsive Genes

According to the profile, the cv. ICCV 05530 resulted in more expression of such genes except *NCED* in all three circumstances than the cv. Annigeri. Among the different stress conditions studied, for both cultivars, expression of those genes at combined stress was found to be more than that of the individual stress. Within the *LEA* genes, expression of *LEA* 1 was high as compared to *LEA* 2 and *LEA* 4 in both cultivars under all three stresses. In ICCV 05530, the maximum expression of *LEA* 1 gene of up to 56-fold was detected at 24 hpi, while in Annigeri the same of up to 51-fold was detected at 48 h. In both the cultivars there was a gradual increase in the expression of *NCED* and *DREB* 2A genes for up to 24 hpi, after which a decline was observed (**Figure 7**).

DISCUSSION

Being one of the most extensively fluctuating and acutely affecting climatic variables among other environmental factors, soil moisture has a profound influence over the soil pH, aeration and availability of nutrients, thereby affecting the growth and population dynamics of the soil microorganisms such as bacteria, oomycetes, fungi etc. (Rousk and Bååth, 2011). Despite its importance, moisture relations of soil microorganisms are a less ventured aspect and even more in phytopathogenic fungi. The existing data on disease severity and incidence caused by soil fungi in relation to soil moisture content are often time consuming. In the case of emerging collar rot disease of chickpea, high disease incidence in field level is often reported to be associated with high soil moisture condition (Sharma and Ghosh, 2017). Currently, there is limited information about molecular basis of chickpea and *S. rolfsii* interaction with soil moisture. Hence in this study, we reviewed molecular response of both chickpea and *S. rolfsii* during collar rot development under different soil moisture conditions.

In our *in vitro* study with collar rot in chickpea, a higher percentage of collar rot incidence in chickpea was observed at high soil moisture condition (80–100% SMC). The development

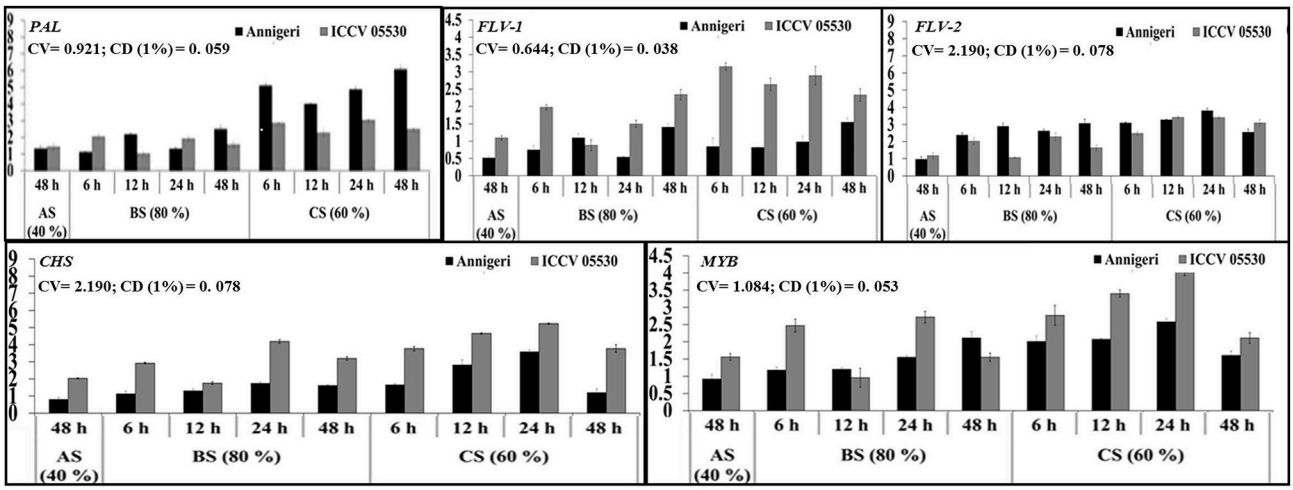

FIGURE 5 | RT-qPCR analysis of differentially expressed genes involved in phenylpropanoid pathway in chickpea during interaction with *S. rolfsii* at limiting (40%), lower optimum (60%), and optimum (80%) soil moisture conditions. Time (in hours) and fold change in gene expression are shown on X and Y axis, respectively. Standard error of three biological replications was calculated and represented as error bar. AS, BS, and CS denote abiotic stress, biotic stress, and combined stress respectively. The statistical significance of gene expression in between the control and treated seedlings was calculated by factorial ANOVA.

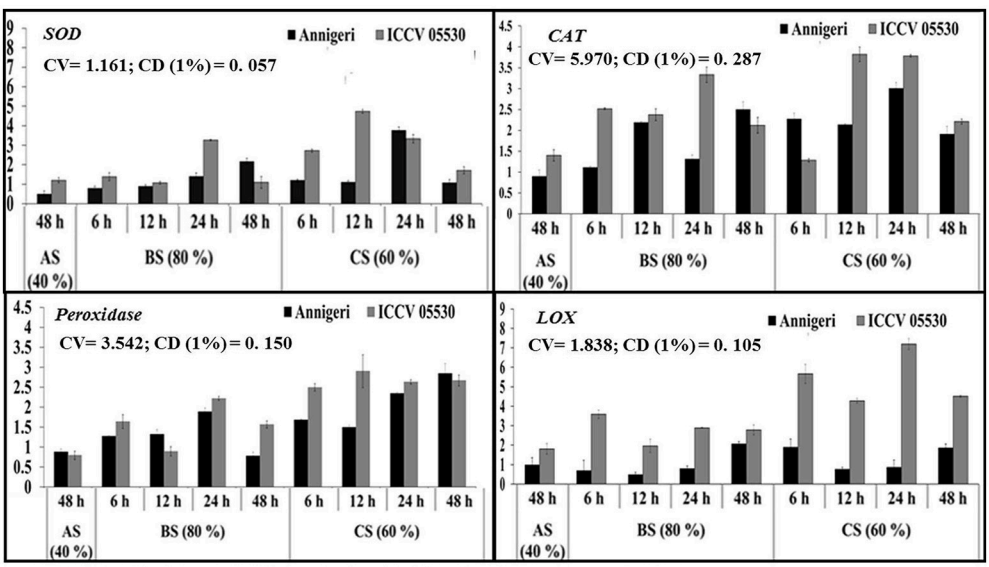

FIGURE 6 | RT-qPCR analysis of differentially expressed genes involved in ROS metabolism pathway in chickpea during interaction with *S. rolfsii* at limiting (40%), lower optimum (60%), and optimum (80%) soil moisture conditions. Time (in hours) and fold change in gene expression are shown on X and Y axis, respectively. Standard error of three biological replications was calculated and represented as error bar. AS, BS, and CS denote abiotic stress, biotic stress, and combined stress respectively. The statistical significance of gene expression in between the control and treated seedlings was calculated by factorial ANOVA.

of collar rot in the field was correlated with growth and colonization. *S. rolfsii* colonization was found to be maximum toward higher SMC (80 and 100% SMC) whereas, the pathogen growth was found to decline in lower SMC (60%) and the least growth was found in limiting SMC (40%). Prasad and Saifulla (2012) observed a decrease in growth and population of *F. udum* causing pigeonpea wilt at 25 to 50% SMC while 75% SMC was favorable for the same.

During infection, phytopathogenic fungi produce lectins and an array of digestive enzymes to degrade the plant cell wall and establish pathogenicity (Sharma et al., 2016). Lectins are carbohydrate binding proteins highly specific for sugar moieties

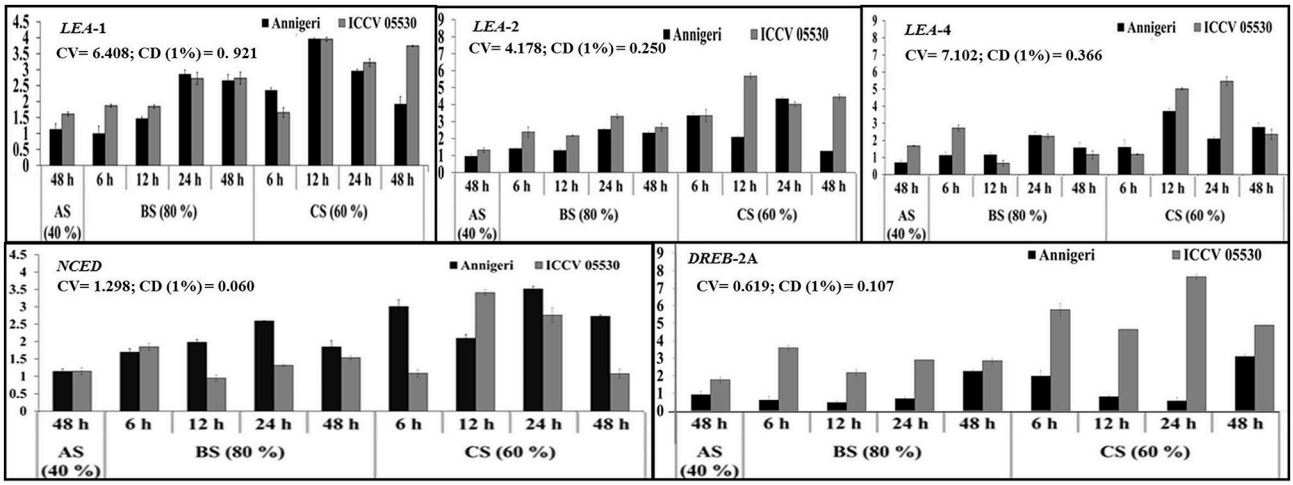

FIGURE 7 | RT-qPCR analysis of different moisture stress responsive genes differentially expressed in chickpea during interaction with *S. rolfsii* at limiting (40%), lower optimum (60%), and optimum (80%) soil moisture conditions. Time (in hours) and fold change in gene expression are shown on X and Y axis, respectively. Standard error of three biological replications was calculated and represented as error bar. AS, BS, and CS denote abiotic stress, biotic stress, and combined stress respectively. The statistical significance of gene expression in between the control and treated seedlings was calculated by factorial ANOVA.

and helps pathogen to bind the host surface (Rutishauser and Sachs, 1975). The enzymes EG, CBH and PG-2 work on different polysaccharides like cellulose, hemicellulose and pectin to loosen the cell wall integrity in plants and help the fungi for nutrient acquisition. EG is an enzyme that catalyzes the hydrolysis reaction to breakdown the glucosidic bonds in polysaccharide, e.g., glucan present in plant cell wall. Post-catalytic reaction of EG on cellulose, the enzyme CBH catalyzes it into cellobiose and glucose through breakdown the 1,4-β-D-glycosidic linkages (Polizeli et al., 2016), and the enzyme endopolygalacturinase-2 hydrolyzes the α-1,4-glycosidic bonds between galacturonic acid residues, a significant component of the pectin network comprising plant cell wall (**Figure S2**).

In our study, at the time of infection, lectin gene showed high level of expression at 6 hpi in optimum SMC, indicating the early adhesion of *S. rolfsii* in chickpea at initial stage of collar rot development. In lower optimum SMC, delayed expression of lectin gene, resulted in the infection to be adjourned up to 24 hpi. After attachment of *S. rolfsii* on host plant, at 12 hpi, the *EG* and *CBH* genes expressed synergistically which led to breakdown of cellulose present in cell wall by serial cleaving of chemical bonds which produced simple organic sugars and it acquires in the form of nutrients by *S. rolfsii* and helps it to successful colonization within the host tissue. The gene for PG-2 expressed in early stage of infection in optimum SMC, and gradually increased with the disease progression, in accordance with the results reported previously in other plant-fungal interactions (Shieh et al., 1997). On the contrary, in lower optimum SMC, the expression of *PG*-2 gene was detected only at the later stages (24 and 48 hpi). These expressions in late stages suggests attempt of the pathogen to acquire nutrients in growth limiting environment. The expression of PGs has earlier been reported under nutrient-deprived conditions and also in the presence of pectin (Yao et al., 1999). The expression patterns of the candidate pathogenicity-causing genes analyzed in this study correlate well with colonization pattern of *S. rolfsii* in chickpea plants.

Plants have their own defense mechanisms to defend against multiple stresses (biotic and abiotic) and they may modify it according to occurrence of stress present in its immediate surroundings (Mickelbart et al., 2015; Padaria et al., 2015). In a condition when the multiple stresses occur at once in plants, the net impact of each stress during their interaction and the corresponding responses to combined stress is quite different when compared to individual stresses alone (Choi et al., 2013; Ramegowda et al., 2013; Ramegowda and Senthil-Kumar, 2015). In present study, we have compared the molecular response of several defense-related as well as moisture stress responsive genes in chickpea during combined stress of *S. rolfsii* and soil moisture. The present study reports the expression profiling of defense-related transcripts during *S. rolfsii* interactions with chickpea host system, along with the comparison of transcriptional response during soil moisture stress or/and dual stress of soil moisture and pathogen. Jogi et al. (2016) also conducted similar research in the identification of differentially expressed genes during early interactions between the stem rot causing *S. rolfsii* and peanut (*Arachis hypogea*) cultivars with increasing disease resistance levels.

Principal observations from this study is on expression profiling of defense-related transcripts during *S. rolfsii* interactions with the host system, along with the comparison of transcriptional response during soil moisture stress alone or dual stress with pathogen. During collar rot development in chickpea plants at optimum SMC, the usual expression of the most defense response genes (PR-2, PR-5, PR-12, *CHI* I, *CHI* III, narborin,

defensin, germin, and endochitinase) was not significant at early stages of infection. Moreover, their expression was up-regulated more during combined stress condition involving soil moisture (60% SMC) and pathogen, thereby delaying the collar rot development. Even at 48 hpi, there was negligible expression of *CHI* II and *CHI* IV genes apart from the above genes in chickpea plants maintained at limiting (40%) SMC. The reason might be because 48 h duration of stress to chickpea plants at limiting SMC was not sufficient for induction of genes related to low soil moisture stress. At optimum (80 %) SMC some of the PR-3 type genes (*CHI* I, *CHI* III and *CHI* V) and gene for endochitinase had significantly over expressed up to 48 hpi in ICCV 05530 as compared to Annigeri, supporting initial resistance in the cultivar. High constitutive levels of chitinases, PR-2 and defensin in chickpea during combined stress was observed which may signify its role in releasing fungal cell wall elicitors at the onset of infection Previous reports showed that degradation products of fungal chitin and glucan functions as elicitors and trigger the hypersensitive response (HR) in host plants (Jones and Dangl, 2006). In earlier studies, Gupta et al. (2016) also observed similar findings in accordance to our results in *Arabidopsis thaliana* during soil moisture and pathogen interaction, and Boominathan et al. (2004) with chickpea and soil moisture interaction. Chitinases from plants can inhibit fungal growth by degrading chitin present in the fungal cell walls; also, the resultant chitinolytic breakdown products are able to further elicit other defense reactions within the plants (**Figure S2**). Antifungal properties of defensins induced its expression upon fungal attack and have been reported (Penninckx et al., 1996). Germin like proteins possessing strong oxalate oxidase activity has reported to be involved in several biotic and abiotic stress-related processes (Woo et al., 2000). Increased expression of endochitinase during biotic stress and combined stress indicates involvement of ethylene signaling pathway. Over-expression of endochitinase gene in *Nicotiana tabacum* and *Brassica napus* has reduced *R. solani* symptoms (Broglie et al., 1991). Induction of pathogen defense responsive genes under varied drought stress and vice-versa has been noted which is well in concurrence with the previous reports Liu et al., 2013; Ramegowda et al., 2013.

Phenylalanine ammonia-lyase (PAL) has an important role in plant systemic resistance through its biosynthesis of salicylic acid and active involvement in phenylpropanoid metabolic network (Chaman et al., 2003). Different classes of phenylproponoids are synthesized and accumulated in response to pathogen infection in leguminous plants (Gurjar et al., 2012). In flavonoid biosynthetic pathway, CHS plays an important catalytic role during the initial stage (**Figure S2**). *CHS* gene expression is reported to be induced in plants under various biotic and abiotic stress conditions. CHS expression results in accumulation of several flavonoid compounds, thereby inducing the salicylic acid defense pathway (Dao et al., 2011). In our study, the significant expression of the four key enzymes (phenylalanine ammonia-lyase, chalcone synthase, flavonoid 3'-monooxygenase and flavonoid 3' hydroxylase) in chickpea plants with combined stress positively correlates with earlier reports.

ROS accumulation is associated with plant defense against pathogens (Hückelhoven and Kogel, 2003). However, ROS resulting from biotic and abiotic stresses can also result in cause severe cellular damage and is therefore tightly regulated and detoxified by complex enzymatic and non-enzymatic mechanisms (**Figure S2**). Antioxidant enzymes such as POD, SOD, and CAT are activated to scavenge the redundant ROS and play a crucial role in the antioxidant systems to protect plant cells from damage (Naya et al., 2007). In our study, a pronounced increase in the POD and SOD activities were observed in collar rot infected chickpea plants, especially, the plants maintained at lower optimum SMC. The results indicated that chickpea plants at lower optimum SMC had gained, to some extent, more ability to withstand against disease than the plants maintained at optimum SMC.

Gene encoding a chaperon protein like LEA, involved in preventing water stress as well as inducing an aggregation of sensitive proteins (Olvera-Carrillo et al., 2010) is also expressed during biotic and combined stress apart from soil moisture stress (**Figure S2**). Another soil moisture stress specific *NCED* 3, an abscisic acid (ABA) biosynthesis gene have been shown to be up-regulated during soil moisture and combined stress leading to accumulation of ABA (Iuchi et al., 2001; Padaria et al., 2016). DREBs are an important group of plant transcription factors responsible for regulating the expression of many stress-inducible genes generally in an ABA-independent manner. They also play a critical role in improving the abiotic stress tolerance ability of plants Lata and Prasad, 2011). Similarly, the significantly higher expression of few soil moisture stress inductive genes (*LEA* family, *NCED* 3, and *DREB*) was observed in collar rot infected chickpea plants maintained at lower optimum SMC compared optimum SMC.

Our study indicates that the combined stress imposition initiated a much stronger and earlier response than when only pathogen inoculation or moisture stresses were applied individually. Soil moisture stress alone incited a slower response compared to pathogen inoculation and the combined stresses. Although differences in the transcript expression were noted following the individual pathogen and soil moisture stress as well as, in combined pathogen and soil moisture stress, there was a substantial degree of overlap recorded. Many genes reported to be pathogen-induced were also influenced by the soil moisture stress, although the response was lower compared to inoculation of pathogen alone. These findings provide evidence of induction in basal defenses as a contributory factor for the enhanced resistance response.

CONCLUSION

The present study demonstrates that, soil moisture stress (limiting and upper optimum) reduces the multiplication of *S. rolfsii in planta*. Delayed and significantly lower expression of pathogenicity-causing genes of *S. rolfsii* was observed in the infected chickpea plants exposed to lower SMC irrespective of the cultivars. Inversely, more expression of defense-related genes at lower SMC had additional effects on inhibition of fungal growth and in decisive decrease of collar rot severity. Therefore, the net effect of soil moisture stress on gene expression in

both systems *viz.* host and pathogen could lead to adjourned disease establishment and symptom development. The true explanation of these findings could be correlated with collar rot disease occurrence in chickpea seedlings at optimum and upper optimum soil moisture conditions, where few defense related genes like chitinase and endochitinase were over-expressed. The functional validation of these genes will assist in further understanding the chickpea defense system against *S. rolfsii*.

AUTHOR CONTRIBUTIONS

AT and TR in consultation with MS conceived, designed and initiated the study. AT and TR contributed equally and were responsible for analysing and interpretation of results and initial drafting of the manuscript. UC helped in setting up of experiments. TR conducted validation studies, RG, and AT contributed in further improvement of analysis and provided inputs in drafting the manuscript. DC helped in analysis and manuscript writing. MS provided critical inputs at various stages of the study and edited 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.2018.01154/full#supplementary-material

Figure S1 | Calibration of qPCR for quantification of *S. rolfsii* Standard curve showing the correlation between the log10 DNA quantity (ng) vs. the Ct values for 10-fold dilution of *S rolfsii* pure genomic DNA. The single peak of targeted amplicon at melting temperature (Tm) 81.5°C indicates the specificity of the qPCR primers to *S. rolfsii*. No contaminating product was detected in PCR reaction.

Figure S2 | Schematic diagram of molecular interaction between chickpea and *S. rolfsii* under low soil moisture condition. (Courtesy of diagram of plant cell wall: www.thoughtco.com/cell-wall-373613).

Table S1 | Primer sequences, correspondence sequence ID used for real-time PCR and gene expression profile in chickpea.

Table S2 | Collar rot disease incidence in chickpea cultivars.

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Cultivar-Dependent Responses of Eggplant (*Solanum melongena* L.) to Simultaneous *Verticillium dahliae* Infection and Drought

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Several studies regarding the imposition of stresses simultaneously in plants have shown that plant responses are different under individual and combined stress. Pathogen infection in combination with drought can act both additively and antagonistically, suggesting a tailored-made plant response to these stresses. The aforementioned combination of stresses can be considered as one of the most important factors affecting global crop production. In the present research we studied eggplant responses to simultaneous *Verticillium dahliae* infection and drought with respect to the application of the individual stresses alone and investigated the extent to which these responses were cultivar dependent. Two eggplant cultivars (Skoutari and EMI) with intermediate resistance to *V. dahliae* were subjected to combined stress for a 3-week period. Significant differences in plant growth, several physiological and biochemical parameters (photosynthesis rate, leaf gas exchanges, Malondialdehyde, Proline) and gene expression, were found between plants subjected to combined and individual stresses. Furthermore, plant growth and molecular (lipid peroxidation, hydrogen peroxide, gene expression levels) changes highlight a clear discrimination between the two cultivars in response to simultaneous *V. dahliae* infection and drought. Our results showed that combined stress affects significantly plants responses compared to the application of individual stresses alone and that these responses are cultivar dependent.

Keywords: eggplant, *Verticillium dahliae*, water stress, combined stress, cultivar, plant growth, plant physiological, molecular responses

INTRODUCTION

Plants in their natural habitats are constantly and most of the time simultaneously exposed to a diverse range of biotic (fungi, bacteria, nematodes, insects, and non cellular pathogens such as viruses and viroids) and abiotic (drought, heat, cold, salinity, heavy metals) stresses. Being sessile, plants have developed specific mechanisms to overcome these challenges and survive. These mechanisms involve overlapping regulatory networks functioning principally at the cellular level and include molecular components that allow plants to perceive environmental changes and pathogen recognition; biochemical changes (such as inorganic ion fluxes, alteration

of the redox status and metabolite homeostasis); plant hormones and activated/deactivated protein cascades. These networks participate in stress-signaling mediation as well as in the regulation of local defense responses such as, changes in cell wall and membrane composition and properties, callose deposition, production of reactive oxygen species (ROS), secondary metabolites and osmoprotectants and increase of soluble solutes (Fujita et al., 2006; Atkinson and Urwin, 2012; Kissoudis et al., 2014; Ramegowda and Senthil-Kumar, 2015). These mechanisms also involve systemic signals that prime plant defense to counter subsequent infection attempts and abiotic stresses (Prime-A-Plant Group et al., 2006; Conrath et al., 2015; Nejat and Mantri, 2017) and initiate adaptive responses to abiotic stresses through plant morphological and physiological changes such as changes in root system architecture, cuticular wax composition and leaf water potential (Xiong and Yang, 2003; Enright and Cipollini, 2011; Matus et al., 2014).

Previous evidence from laboratory studies regarding molecular and -omics data analyses (Ma et al., 2006; Seo et al., 2011; Rasmussen et al., 2013; Shaik and Ramakrishna, 2014) as well as field studies (Rizhsky et al., 2004; Mittler, 2006; Mittler and Blumwald, 2010), has shown that there is a synergistic or antagonistic crosstalk between plants responses to combined stress, and that plants respond to a specific combination of stresses in a tailored-made fashion rather than in an additive effect manner. A sequential occurrence of a certain combination of biotic and abiotic stresses may have different impacts, either positive or negative on the plant's responses regarding pathogen resistance, abiotic stress tolerance, plant performance and fitness costs (Atkinson and Urwin, 2012; Kissoudis et al., 2014; Ramegowda and Senthil-Kumar, 2015; Nejat and Mantri, 2017; Pandey et al., 2017; Zhang and Sonnewald, 2017). These responses do not only depend on the nature of the stresses *per se* (type of stress, combination of stress types), but should be studied in relation to stress intensity, the order of occurrence (i.e., pathogen infection prior to an abiotic challenge or acclimation response followed by biotic stress), and the species genetic background (Kissoudis et al., 2014; Nejat and Mantri, 2017). The challenge to study the combinatory effect of multiple environmental stresses on plants performance becomes more evident, taking into consideration the effects of a climate change scenario on future agriculture needs. Under such environmental conditions plant–pathogen–abiotic stress interactions are drastically affected.

Drought stress is undoubtedly one of the most important of several, environmental factors that can greatly influence the productivity of crop plants around the world (Reddy et al., 2004). Plants respond to drought through multiple mechanisms such as altering shoot/root ratio (Bootraa et al., 2010), reducing plant growth rates (Reddy et al., 2004) and increasing water use efficiency (WUE) (Sivamani et al., 2000; Bootraa et al., 2010). On the other hand, plant pathogenic fungi are responsible for serious yield losses on an annual basis (Savary et al., 2012). *Verticillium dahliae* Kleb., is a widely distributed soilborne fungus, causing destructive vascular wilt to a wide range of herbaceous and woody plant hosts and severe yield losses (Pegg and Brady, 2002; Daayf, 2015). Since there are no effective chemical treatments to control the disease, management strategies encounter preventive measures (Blok et al., 2000), biological control regimes (Mercado-Blanco, 2012; Angelopoulou et al., 2014; Markakis et al., 2016) and use of cultivars that tolerate the disease to a certain extent (López-Escudero et al., 2004; Zhang et al., 2015). Many biochemical responses induced by drought are similar to those induced by pathogen attack, including increases in proline content (Farooq et al., 2009; Verslues and Sharma, 2010; Liang et al., 2013), ROS formation (Borden and Higgins, 2002; Degara et al., 2003), and upregulation of gene expression (Ramirez et al., 2009; Asano et al., 2012).

Previous publications show that in the cases of combined stresses, there is either a positive or negative effect of the preceding stress (i.e., drought or a specific pathogen attack) on plant responses toward the succeeding stress. In general drought influences negatively the plants tolerance to pathogen infection, causing detrimental effects to plants pathogen resistance (Mayek-Perez et al., 2002; Wang et al., 2009). On the other hand, increased resistance of barley plants to barley powdery mildew under drought conditions was observed through shared defense responses (Wiese et al., 2004). Additionally, tomato plants growth under drought conditions showed enhanced resistance to *Botrytis cinerea* and *Oidium neolycopersici* (Achuo et al., 2006). Pathogen infection often results in lower photosynthetic rates and reduction of WUE (Grimmer et al., 2012) and increases the susceptibility to drought stress (Audebert et al., 2000). Nevertheless, *Arabidopsis* plants infected with *V. dahliae* showed increased drought tolerance due to new xylem formation (Reusche et al., 2012). Furthermore, beneficial microbes such as arbuscular mycorrhizae and certain types of bacteria were shown to affect positively drought tolerance (Aroca et al., 2008; Kohler et al., 2008).

Eggplant (*Solanum melongena* L.) is one of the most important vegetable crops, especially for the Mediterranean basin, after potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) (Rotino et al., 2014). Eggplants are very susceptible to many abiotic and biotic limiting factors throughout their entire biological cycle. They are very sensitive to water deficit, as they need substantial amount of water for their growth and development (Fu et al., 2013) and are susceptible to important fungal pathogens, such as the *Fusarium oxysporum* and *V. dahliae* (Collonnier et al., 2001), which cause significant crop damage. Therefore, one of the main objectives of eggplant breeding programs is the improvement of resistance to biotic and abiotic stresses. As eggplant is one of the most important vegetables for the Mediterranean region, and climate change will enforce the frequency as well as the intensity of combined stresses, it is of great importance to address the issues of climate-resilient vegetables.

In the present work we studied eggplant responses to the combined application of two main stress factors that affect its cultivation (*V. dahliae* and drought), compared to the application of the individual stresses alone. In addition, we investigated the extent to which these responses to combined stress are cultivar dependent. We showed that combined stress affects significantly the plants physiological and biochemical responses compared

to application of individual stresses alone and that cultivar dependent plant growth significant differences are also affected by the type and combination of stresses applied. Furthermore, we showed that the expression of specific eggplant abiotic and biotic stress marker genes is induced by both drought and *V. dahliae* infection and that combined stress strongly enhances the expression of biotic stress marker genes in the leaves and roots of both eggplant cultivars.

MATERIALS AND METHODS

Plant Material and Treatments

Two of the most common Greek eggplant cultivars, "Skoutari" and "EMI" with intermediate resistance to *V. dahliae* (Bletsos, unpublished results) were selected for this study. The cultivars were provided by the Agricultural Research Center of Northern Greece, ELGO-Demetra. Seeds were surface sterilized in 1.25% sodium hypochlorite for 20 min, rinsed three times in sterile distilled water, sown in sterile Klasmann-TS2 soil (Klasmann-Deilmann, Geeste, Germany) distributed in 50-ml QP Standard plastic pot trays (Agrohoum, Athens, Greece) and allowed to germinate in darkness. Plantlets were grown in a glasshouse under controlled conditions of 24°C, 70–80% RH, 16 h light – 8 h dark photoperiod, maximum light intensity 1110 μmol photons m^{-2} s^{-1}. The eggplant seedlings were transplanted to 10 cm diameter pots when they were 7 weeks old at the stage of four true leaves. The selected seedlings were artificially inoculated either with a 10^6 mL^{-1} *V. dahliae* conidial suspension or with water for mock controls, just before transplantation, according to the dip-root method reported by Cappelli et al. (1995). The virulent BPIC 2681 *V. dahliae* isolate from eggplant, maintained in the BPI official collection of fungi, was used for inoculation. The seedlings were then watered and fertilized with Nutri-Leaf 20:20:20 (Miller Chemical & Fertilizer, LLC, Hanover, PA, United States) every week. Pots were arranged in a complete randomized design. Drought stress regimes were initiated the day the first wilting symptoms appeared, which was 17 days post inoculation (DPI) with the pathogen. For a time-interval of 3 weeks, four groups of plants were formed, each subjected to a specific treatment: (a) plants watered up to 100% of soil field capacity (FC) that served as a control, (b) plants infected with *V. dahliae* and watered up to 100% of FC, (c) plants watered up to 25% of FC, and (d) plants infected with *V. dahliae* and watered up to 25% of FC.

Leaf and Root Material Sampling

For all measurements and analyses performed, each plant constituted a single biological replicate. Leaf and root material sampling was performed during the last day of the treatments applied. For the determination of the H_2O_2, Malondialdehyde (MDA), and Proline concentrations and for RNA extraction, leaf and root material were collected from individual plants for each of the different treatments. For the leaf material the first, third, and forth fully developed leaves from the shoot apex were collected. The whole root system was collected after washing out the soil from the rhizosphere for a time interval of up to 3 min and using tap water previously acclimated at glasshouse controlled temperature conditions. Samples were briefly dried, immediately frozen in liquid nitrogen and stored at −80°C before subsequent use.

Plant Growth Parameters

The length of the stems from the base to the tip and the number of leaves were measured in each plant at an interval of 3 days through the duration of the experiment. Relative Growth Rate (RGR) respect to stem elongation was estimated as follows: RGR = [(T2−T1)/t]·1/T2, where T1 and T2 represent the stem length at the beginning and at the end of a time (t), respectively. Root length was measured at the end of the experiment. Fresh leaves, stems and roots were harvested, and oven dried at 85°C for 48 h to determine the dry weights.

Leaf Gas Exchange Parameters

Net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration (Ci), were measured twice per plant per treatment, using both the third and forth fully developed leaves from the shoot apex. The measurements were carried out with the Li-6400XT (Li-COR, Lincoln, NE, United States) portable photosynthesis measuring system under steady light intensity (800 μmol m^{-2} s^{-1}) and CO_2 concentration (400 mg l^{-1}), while leaf temperature ranged between 27.5 and 29.8°C. WUE was computed as the ratio between P_N and E of each one measurement. Similarly, the carbon dioxide use efficiency and the ratio between net photosynthetic rate and stomatal conductance were calculated by dividing the P_N values with Ci or g_s values of the recorded at each one measurement, respectively.

Lipid Peroxidation and Hydrogen Peroxide

Leaf tissue (250 mg) was homogenized in 10 ml 0.1% trichloracetic acid (TCA) at 4°C. After centrifugation at 4000 rpm for 15 min, the supernatant was used for the determination of both lipid peroxidation level and H_2O_2 concentration. Lipid peroxidation was measured as MDA content determined by reaction with 0.5% 2-thiobarbituric acid (TBA) in 20% TCA (w/v). The concentration of MDA was calculated from the difference of the absorbance at 532 and 600 nm using the extinction coefficient of 155 $mmol^{-1}$ cm^{-1} (Heath and Packer, 1968). Hydrogen peroxide was also measured spectrophotometrically, after reaction with potassium iodide (KI). The reaction mixture consisted of 0.5 mL 0.1% trichloroacetic acid (TCA), leaf extract supernatant, 0.5 mL of 0.1 M potassium-phosphate buffer (pH 7) and 1 mL 1 M KI (w/v) reagent. The reaction color was developed for 45 min in darkness and absorbance was measured at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve prepared with eight known concentrations of H_2O_2.

Proline Concentration

Proline content in root and leaf tissues was measured via reaction with ninhydrin (Bates et al., 1973). For colorimetric

determinations, a solution of proline, ninhydrin acid and glacial acetic acid (1:1:1) was incubated at 90°C for 1 h. The reaction was then cooled in an ice bath. The chromophore was extracted using 2 ml of toluene and its absorbance at 520 nm was determined by a BioMate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States).

Primer Sequences

Solanum melongena EST sequences were retrieved after homology search from GenBank and Sol Genomics Network. Primers (**Table 1**) for quantitative real time PCR experiments were designed using the OligoPerfect^{TM} Designer (Thermo Fisher Scientific, Waltham, MA, United States), following standard primer design criteria. Primers were tested in end-point and real time PCR reactions using 10-fold dilutions of *S. melongena* genomic DNA ranging from 50 ng to 50 fg.

cDNA Synthesis and Relative Gene Expression Study

Total RNA was extracted using the TRI Reagent (Sigma-Aldrich, St. Louis, MI, United States) reagent following the instructions of the manufacturers. cDNA was synthesized using the PrimeScript^{TM} RT reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan) and 1 μg of total RNA according to the manufacturer's instructions. The Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, United States) was used for real time PCR assays. Samples were prepared using the KAPA SYBR FAST qPCR kit 2X master mix (KAPA Biosystems, Inc., Boston, MA, United States), 200 nM of each primer and either 1 μl of cDNA template (1/20th of cDNA reaction volume) or RNase free-H_2O for non-template controls, in a final reaction volume of 15 μl. Four individual biological replicates for each of the different treatments were assayed per RT-PCR run, in two individual runs considered as technical replicates. Non-reverse transcribed samples were also assayed in real time PCR runs prior to sample analyses. For all target genes assayed the PCR amplification thermal profile used consisted of one initial denaturation cycle of 3 min at 95°C followed by 40 cycles of 3 s at 95°C and 20 s at 60°C. Melting curves were programmed as 15 s at 95°C, 15 s at 60°C, 20 min slow ramp, and 15 s at 95°C. The data for the dissociation curve were captured during this slow ramp, and the melt curve was visualized using the ABI PRISM 7900 software. The relative gene expression was determined with the comparative Ct method (Livak and Schmittgen, 2001), calculating the mean threshold cycle (Ct) values of the target and endogenous control genes for four individual biological replicates of both technical replicate runs.

Statistical Analysis

Analysis of Variance (three-way ANOVA) was carried out with *V. dahliae* infection, water stress level and cultivars as fixed factors (sample size: 24 or 32 plants). Differences between means were assessed using Fisher's Protected least significant difference (LSD) ($P < 0.05$). The statistical package used for analyzing the data was GENSTAT 10th edition. Normality was checked by examining residual plots produced which provide a way of checking the normality and the equal variance assumptions of the ANOVA and the Shapiro–Wilk test. The data was found to be normally distributed. In addition, Analysis of Variance (one-way ANOVA) was carried out for the parameters presented in **Figures 2**, **3** to test differences between treatments within each cultivar. Differences between means were assessed using Student Newman Keuls test ($P < 0.05$).

RESULTS

Verticillium dahliae, Drought Stress and Eggplant Cultivars Double and Triple Interactions Significantly Affect the Plant Relative Growth Rate and the Shoot to Root Fresh Weight Ratio

The significance of the pathogen, water stress and cultivars main effects and their double and triple interactions on RGR, root

TABLE 1 | Primers sequences used for the gene expression study.

Primer name	Sequence 5′ to 3′	Gene target	GenBank/Sol Genomics Network ID	Amplicon length (bp)	Reference
Sm-AREB-F	GGGATGGTTGGTATCGCTGA	*AREB*	Sme2.5_12232.1_g00002.1	100	This study
Sm-AREB-R	CTCCAGCCCCTAAACCTACC				
Sm-CBF1-F	TGGGTTTGCGAAGTCAGAGA	*CBF*1	Sme2.5_16389.1_g00001.1	121	This study
Sm-CBF1-R	CAGAACGGCCCCTTAATGCT				
Sm-NAC-F	GAGCACCTTCCTCCTGGATT	*NAC*	Sme2.5_15135.1_g00001.1	147	This study
Sm-NAC-R	AGGCAATTCCCAAGGGTCG				
Sm-PR1-F	GTGGGTCGATGAGAAGCAAT	*PR*1	AB222697.1	92	This study
Sm-PR1-R	TACGCCACACCACCTGAGTA				
Sm-PR5-F	CAAACACCCTGGCTGAATACG	*PR*5	Sme2.5_30700.1_g00001.1	113	This study
Sm-PR5-R	ACTAGGATTGGTCGGTGCAA				
Sm-LOX-F	GGAGGGATCAAACTTCCTCA	*LOX*	AB244527.1	101	This study
Sm-LOX-R	ATTCCTTCACCGTCTGTTCG				
Sm-Actin-F	ACCACAGCTGAGCGAGAAAT	*ACTIN*	JX524155.1	133	Zhou et al., 2014
Sm-Actin-R	GACCATCGGGAAGCTCATAG				

to stem length ratio and shoot to root fresh and dry weight ratios, are presented in **Tables 2** and **3**. Triple interactions are further presented in **Figure 1** by creating separate plots which illustrate the interaction of cultivar and *V. dahliae* stress (Vd−/+) at normally irrigated (No DS) and water stressed (DS) plants. In the graphs, the mean value of each parameter assesses/measured and the LSD values are presented.

Drought stress and cultivars as single factors had significant effect on RGR (**Table 2**). *V. dahliae*, water stress and cultivars imposed together had also significant effect on RGR as indicated in all double and triple interactions, presented in **Table 2** and **Figures 1A,B**. As presented in **Table 3**, for the *V. dahliae* and drought stress combination (irrespective of the cultivar) RGR values for all plant treatments were significantly reduced compared to the control (0.025 cm day^{-1} cm^{-1}). Plants under combined stress had an intermediate RGR value (0.019 cm day^{-1} cm^{-1}) with respect to *V. dahliae* infected (0.022 cm day^{-1} cm^{-1}) and drought-stressed (0.015 cm day^{-1} cm^{-1}) alone.

Cultivars was the only significant main factor to affect the plants root to stem length ratio (mean values of 0.91 and 1.04 for Skoutari and EMI cultivars, respectively). Triple interaction of cultivar with the two stress types were also found to be statistically significant (**Table 2**). All three factors affected significantly the shoot to root fresh weight ratio, as main factors and in their double and triple interactions except the interaction between *V. dahliae* and cultivars (mean values and significance levels are presented in **Table 2** and **Figures 1C,D**). As presented in **Table 3** for the *V. dahliae* and water stress interaction, the ratio was much lower in infected plants (8.10) respect to the control (25.70) and was reduced further in drought stressed plants (5.31) and plants under combined stress (5.20). The cultivars significant effect with the *V. dahliae* and drought stress interaction on the shoot to root fresh weight ratio is presented in **Figures 1E,F**. The shoot to root dry weight ratio was affected significantly only by drought stress (mean values of 8.29 and 6.86 for control and stressed plants, respectively, **Table 2**). The values of the dry weight ratio for the *V. dahliae* infected and drought stressed plants were higher respect to their corresponding fresh weight ratio values.

Similar Reduction in Photosynthesis Rate and Leaf Gas Exchange Parameters in Plants Under Drought and Combined Stress

Photosynthesis rate (P_N), and leaf gas exchange parameters (g_s, *E*, and *C*i), were reduced in both cultivars in all stresses applied respect to the untreated control (**Figures 2A–D**). Furthermore, the same pattern regarding the reduction levels was observed in between stress treatments. In both cultivars, all parameters measured were affected more (with respect to the mean values) by drought stress and less by *V. dahliae* infection, whereas the combined stress resulted to intermediate mean values respect to those of drought stressed and pathogen infected plants alone (**Figures 2A–D**). For the Skoutari cultivar, statistically significant differences for the three out of four parameters tested

TABLE 2 | Effect of, and interactions between, *Verticillium dahliae*, drought stress and cultivars on developmental, physiological and biochemical parameters eggplants (3-factor ANOVA).

Factors		RGR (cm·day^{-1} cm^{-1})	Root/Stem length ratio	Shoot/Root fresh weight ratio	Shoot/Root dry weight ratio	WUE (μmol CO_2·mmol H_2O^{-1})	P_N/g_s (μmol CO_2·mol H_2O^{-1})	H_2O_2 (μmol·25 g^{-1})	MDA (μmol g^{-1})	Proline (mg·100 g^{-1})
Verticillium dahliae	No	0.020	1.01	**15.51**	7.83	5.67	132.11	**42.9**	57.1	194.5
(*n* = 24 or 32)	Yes	0.020	0.95	**6.65**	7.32	6.03	146.24	**83.0**	61.1	222.1
Drought stress	No	**0.023**	0.98	**16.90**	**8.29**	**4.28**	**97.96**	**37.3**	**33.2**	**168.3**
(*n* = 24 or 32)	Yes	**0.017**	0.98	**5.26**	**6.86**	**7.41**	**180.39**	**88.6**	**84.9**	**248.2**
Cultivars	Skoutari	**0.024**	**0.91**	**11.78**	7.86	5.72	139.12	**70.3**	61.4	**188.3**
(*n* = 24 or 32)	EMI	**0.016**	**1.04**	**10.38**	7.30	5.99	139.23	**55.6**	56.7	**228.3**
ANOVA results (*p*-value)										
Verticillium dahliae (Vd)		NS	NS	**<0.001**	NS	NS	NS	**<0.001**	NS	NS
Drought stress (DS)		**<0.001**	NS	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Cultivars (C)		**<0.001**	**<0.001**	**0.017**	NS	NS	NS	**0.015**	**NS**	**0.022**
Vd x DS		**<0.001**	NS	**<0.001**	NS	**<0.001**	**<0.001**	NS	**<0.001**	**<0.001**
Vd x C		**0.002**	NS	NS	NS	NS	NS	NS	NS	NS
DS x C		**0.047**	NS	**0.016**	NS	NS	NS	**<0.001**	NS	**0.005**
Vd x DS x C		**0.019**	**0.033**	**<0.001**	NS	NS	NS	**0.005**	**0.006**	**<0.001**

The values represent means; NS, not significant; RGR, Relative Growth Rate; WUE, Water Use Efficiency; P_N, Photosynthesis rate; g_s Stomatal conductivity; MDA, Malondialdehyde. Bold values indicate statistically significant differences.

TABLE 3 | Effect of *Verticillium dahliae* infection and drought stress on eggplant developmental, physiological and biochemical parameters.

	RGR ($cm \cdot day^{-1} \cdot cm^{-1}$)	Shoot/Root fresh weight ratio	WUE ($\mu mol\ CO_2 \cdot mmol\ H_2O^{-1}$)	P_N/g_s ($\mu mol\ CO_2 \cdot mol\ H_2O^{-1}$)	MDA ($\mu mol\ g^{-1}$)	Proline ($mg \cdot 100\ g^{-1}$)
no DS – no Vd	0.025 d	25.70 c	3.46 a	74.25 a	12.6 a	26.1 a
DS – no Vd	0.015 a	5.31 a	7.87 c	189.98 c	101.6 c	362.9 d
no DS – Vd	0.022 c	8.10 b	5.11 b	121.66 b	53.57 b	310.6 c
DS – Vd	0.019 b	5.20 a	6.96 c	170.81 c	68.2 b	133.6 b
p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
LSD	0.0022	1.61	1.02	23.34	16.73	47.51

The values represent means; RGR, Relative Growth Rate; WUE, Water Use Efficiency; PN, Photosynthesis rate; gs Stomatal conductivity; MDA, Malondialdehyde; DS, Drought Stress; Vd, Verticillium dahlia. Bold values indicate statistically significant differences.

were observed between treated plants (irrespective the type of stress) and untreated controls. For the EMI cultivar, statistically significant differences were observed for all parameters tested between plants treated either with drought or combined stress in comparison to control or *V. dahliae* infected plants (**Figures 2A–D**).

A statistical analysis of the pathogen, drought stress and cultivar main effects as individual factors and the significance of their double and triple interactions, on WUE and Photosynthesis rate/Conductivity ratio (P_N/g_s) are presented in **Tables 2**,**3**. Drought stress had a significant effect on both ratios, while *V. dahliae* and cultivars had no significant effect on either ratio (**Table 2**). With respect to *V. dahliae*, drought stress and cultivar double interactions, combined stress (irrespective the cultivar) was the only one to affect significantly both ratios (**Tables 2**,**3**). Specifically, WUE increased in *V. dahliae* infected plants (5.11 $\mu mol\ CO_2 \cdot mmol\ H_2O^{-1}$) compared to the control (3.46 $\mu mol\ CO_2 \cdot mmol\ H_2O^{-1}$) and doubled in plants under drought and combined stresses (7.87 and 6.96 $\mu mol\ CO_2 \cdot mmol\ H_2O^{-1}$, respectively) (**Table 3**). The P_N/g_s ratio followed the same pattern as WUE and increased in *V. dahliae* infected plants (121.66 $\mu mol\ CO_2\ mol\ H_2O^{-1}$) with respect to the control (74.25 $\mu mol\ CO_2 \cdot mol\ H_2O^{-1}$) and was more than double in plants under drought and combined stresses (189.98 and 170.81 $\mu mol\ CO_2 \cdot mol\ H_2O^{-1}$, respectively) (**Table 3**). The combination of cultivar, *V. dahliae* and drought stress had no significant effect on WUE and P_N/g_s ratio as presented in **Table 2**.

Determination of H_2O_2, MDA, and Proline Concentrations in Leaves of Plants Treated With Individual and Combined Stresses

The concentration of H_2O_2 in leaves increased after application of either individual or combined stresses with respect to the untreated control plants, however, with a different mode for the two cultivars (**Figure 3A**). The highest H_2O_2 concentration (138.81 $\mu mol \cdot 25\ g^{-1}$ of tissue fresh weight) was observed in Skoutari plants under combined stress. In the same cultivar, H_2O_2 mean levels in individually drought stressed or *V. dahliae* infected plants were at 85.00 and 47.91 $\mu mol \cdot 25\ g^{-1}$, respectively, higher than the untreated control (9.36 $\mu mol \cdot 25\ g^{-1}$). For Skoutari plants statistical differences were observed between all treatments and the control. On the other hand, H_2O_2 in leaves of EMI treated plants was similar with mean values of 68.81, 61.90, and 76.45 $\mu mol \cdot 25\ g^{-1}$, for plants under combined stress, drought stress or *V. dahliae* infection, respectively. They all are statistically different from the untreated control (15.36 $\mu mol \cdot 25\ g^{-1}$).

Malondialdehyde concentrations varied between treatments in both cultivars (different statistical significance in each cultivar), as seen in **Figure 3B**. The highest mean values were observed in drought stressed plants of both cultivars (100.38 and 102.83 $\mu mol\ g^{-1}$ for Skoutari and EMI, respectively), whereas, *V. dahliae* infected plants showed lower MDA mean values (42.71 and 64.77 $\mu mol\ g^{-1}$ for Skoutari and EMI, respectively) compared to drought stressed plants. In plants subjected to combined stress, MDA ranged at the same levels as drought stressed plants (mean value of 85.03 $\mu mol\ g^{-1}$) for Skoutari, however, was lower (mean value of 51.35 $\mu mol\ g^{-1}$) for EMI. In untreated controls MDA mean values were 17.54 and 7.74 $\mu mol\ g^{-1}$ for Skoutari and EMI, respectively.

As presented in **Figure 3C**, proline concentration was increased in all treated samples. A high increase was observed respect to the controls (mean values of 2.7 $mg \cdot 100\ g^{-1}$ and 49.5 $mg \cdot 100\ g^{-1}$ for Skoutari and EMI, respectively) in drought stressed (362.1 $mg \cdot 100\ g^{-1}$ and 363.7 $mg \cdot 100\ g^{-1}$ for Skoutari and EMI, respectively) and *V. dahliae* infected plants (344.4 $mg \cdot 100\ g^{-1}$ and 276.7 $mg \cdot 100\ g^{-1}$ for Skoutari and EMI, respectively). It was, however, reduced in plants subjected to combined stress (43.9 $mg \cdot 100\ g^{-1}$ for Skoutari and 223.3 $mg \cdot 100\ g^{-1}$ for EMI). Although, a similar pattern of the plant's response to the stresses applied is observed for both cultivars, statistically significant differences vary in between stress types for each cultivar.

The statistical analysis of the pathogen, drought stress and cultivar main effects as individual factors and the significance of their double and triple interactions, on H_2O_2, MDA, and Proline concentrations are presented in **Tables 2**, **3**. *V. dahliae* had a significant effect on H_2O_2 only, while drought stress influenced all three parameters tested. Cultivar as single factor had a significant effect on both H_2O_2 and Proline (**Table 2**). The combined *V. dahliae* and drought stress (irrespective the cultivar) had significant effect on MDA and Proline concentrations as indicated in **Tables 2**, **3**. Specifically, MDA concentrations were significantly increased in all treated samples respect to the control, with the highest mean value observed (**Table 3**) in

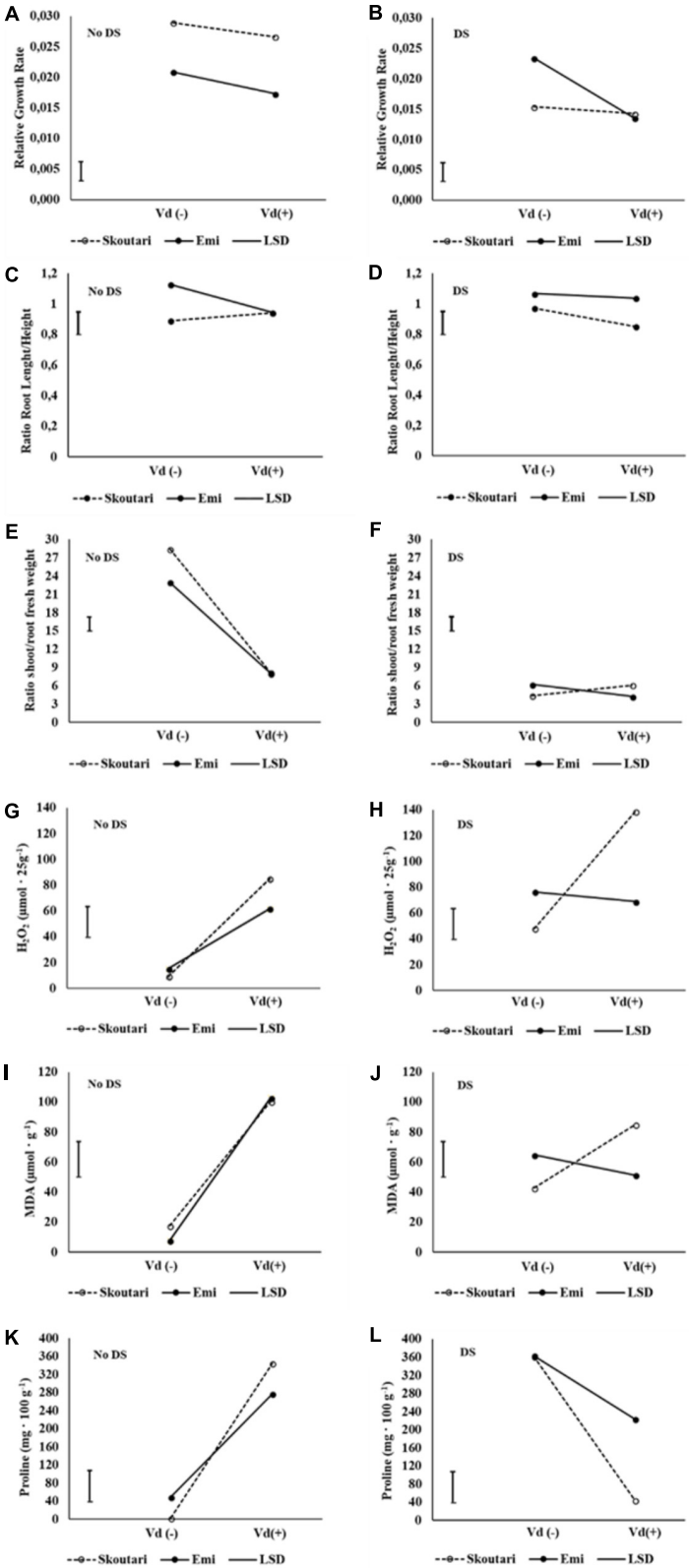

FIGURE 1 | Three-way interaction plots of RGR **(A,B)**, root to stem length ratio **(C,D)**, shoot to root fresh weight ratio **(E,F)**, H_2O_2 **(G,H)**, MDA **(I,J)** and Proline **(K,L)** concentrations, on two eggplant cultivars (Skoutari and Emi), at *V. dahliae* infection and drought stress. Vd (±): *Verticillium dahliae*, DS: drought stress.

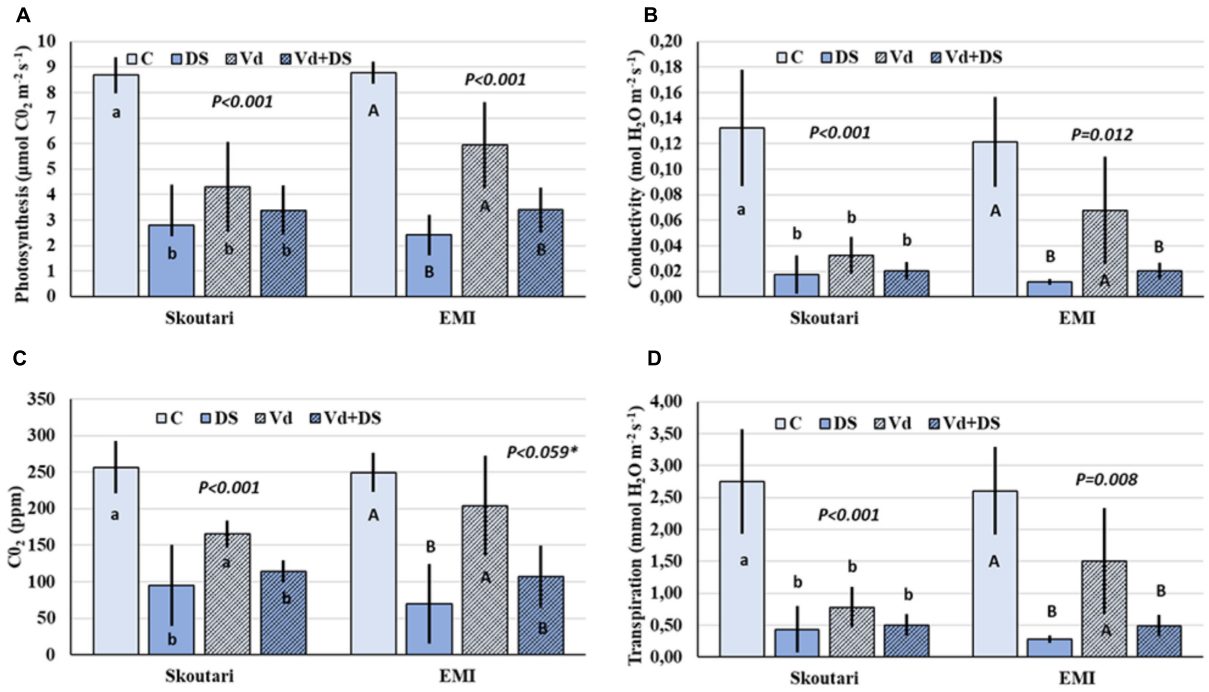

FIGURE 2 | Photosynthesis rate and leaf gas exchange parameters in eggplant cultivars subjected to abiotic, biotic and combined stresses. The net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (*Ci*) and transpiration rate (*E*) (**A–D**, respectively), were measured in eggplant cultivars under individual and combined stress. C, control; DS, drought stress; Vd, *Verticillium dahliae*; Vd+DS, Combined stress. Bars represent standard deviation. Means within the same cultivar with different lower (Skoutari) or upper (EMI)-case letters are significantly different according to Student Newman Keuls test ($P < 0.05$ or $P < 0.1$*).

drought stressed plants (101.6 μmol g^{-1}). MDA concentration was reduced to nearly half in *V. dahliae* infected plants (53.57 μmol g^{-1}) and had an intermediate value (68.2 μmol g^{-1}) for plants under combined stress, though not statistically different (LSD, 16.73) from the former. Proline concentrations (**Table 3**) were significantly increased in plants subjected to individual stress (mean values of 362.9 and 310.0 mg·100 g^{-1} for drought stressed and *V. dahliae* infected plants, respectively), respect to the control (26.1 mg·100 g^{-1}). Plants under combined stress had an intermediate mean value (133.6 mg·100 g^{-1}) though statistically different from the control (LSD, 47.51). No significant interaction of *V. dahliae* and variety was observed for all three parameters tested, while the drought stress and variety interaction were only significant for H_2O_2 and Proline (**Table 2**). The cultivar, *V. dahliae* and drought stress triple interaction had a combined effect on H_2O_2, MDA, and Proline (**Table 2**) with significant differences between plants of the two cultivars observed under combined stress (**Figures 1G–L**).

Combined *V. dahliae* and Drought Stress Enhances the Expression of Biotic Stress Marker Genes

Figure 4 shows the gene expression fold differences of selected marker genes for the two cultivars subjected under individual and combined stresses. For the *PR*1 and *PR*5 biotic stress marker genes a basal level of expression was detected, as for the abiotic stress marker genes, in untreated controls in all cases. Expression of the two genes was induced in *V. dahliae* infected plants as expected and in plants under drought stress, in leaves and roots of both cultivars. A strong upregulation of gene expression was observed in plants under combined stress compared to plants either under biotic or drought stress alone, with the highest values (fold changes respect to untreated controls) observed in leaves with respect to roots of both cultivars for the *PR*1 gene (76-fold and 94-fold for the Skoutari and EMI cultivars, respectively) and in roots with respect to leaves for the *PR*5 gene (102-fold and 87-fold for the Skoutari and EMI cultivars, respectively). The expression of the *LOX* gene did not vary significantly between the different treatments and the control for both cultivars and organ types.

The *NAC* marker gene was significantly induced in drought treated plants showing the highest values (fold changes respect to untreated controls) in both tissues of the Skoutari cultivar (298-fold and 237-fold, respectively). Significant induction was also observed in plants subjected to combined stress, however, fold changes ranged at the same levels as of drought induced plants for the EMI cultivar in both leaves and roots (29-fold and 37-fold for leaves and 17-fold and 19-fold for roots, respectively), but where significantly downregulated compared to drought induced plants for the Skoutari cultivar (48-fold

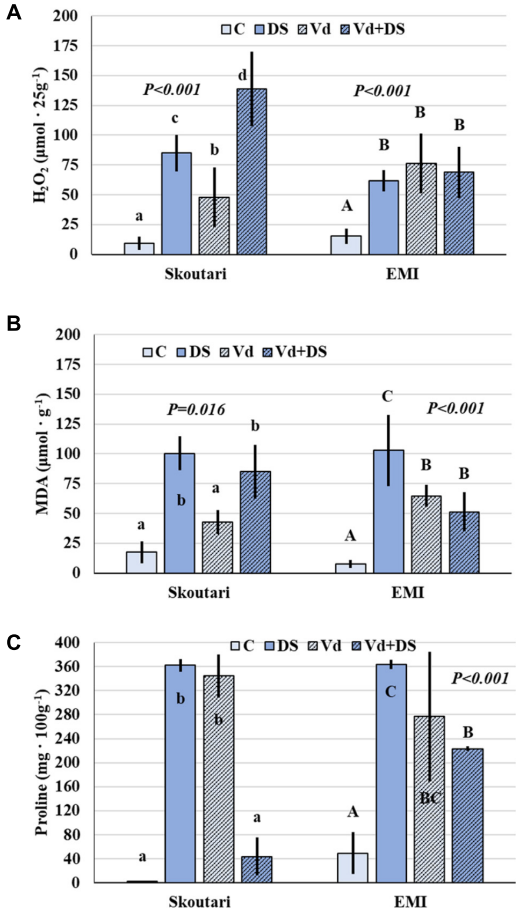

FIGURE 3 | H_2O_2, MDA, and Proline concentration alterations in eggplant cultivars subjected to abiotic, biotic and combined stresses. The H_2O_2, MDA, and Proline concentration (**A–C**, respectively) were measured in eggplant cultivars under individual and combined stress. C, control, DS, drought stress, Vd, *Verticillium dahliae*, Vd+DS, Combined stress. Bars represent standard deviation. Means within the same cultivar with different lower (Skoutari) or upper (EMI)-case letters are significantly different according to Student Newman Keuls test ($P < 0.05$).

and 298-fold for leaves and 8-fold and 237-fold for roots, respectively). For the *AREB* marker gene significant induction of expression was observed in plants treated with drought and plants under combined stress. In both cases the levels of fold differences did not vary between the two types of treatment irrespectively the cultivar or type of organ with the highest values observed in Skoutari roots (88-fold and 65-fold for drought and combined stress, respectively). Significant gene expression induced by biotic stress alone was observed only for *NAC* in Skoutari leaves (69-fold respect to untreated control). Significant differences in the expression of *CBF*1 were observed only in Skoutari leaves. Expression was induced by all types of treatments with the highest fold differences observed in drought and combined stresses (15-fold and 10-fold compared to untreated control, respectively).

DISCUSSION

A limited number of studies regarding the imposition of stresses simultaneously in plants revealed that plants respond in a different manner under individual and combined stress (Xu et al., 2008; Atkinson et al., 2013; Prasch and Sonnewald, 2015; Gupta et al., 2016). The combination of two stresses (abiotic–abiotic or abiotic–biotic) does not always have a negative effect on plants. Reports have shown that drought in combination with pathogen infection can act both additively and antagonistically. This combination can be considered as one of the most important stress combinations affecting global crop production, given the number of reports of plant diseases caused by drought stress and the frequency of water stress incidences. Pathogens that induce wilt, such as *V. dahliae*, impose a physiological drought stress effect in plants by blocking the xylem (Genin, 2010). When wilt disease coincides with drought, a new net effect of combined stress could be expected that is different from the individual stresses (Abd El-Rahim et al., 1998; Choi et al., 2013; Sinha et al., 2016). To study this assumption, the impact of the combined occurrence of *V. dahliae* and drought was compared with the impact of the two stressors imposed separately, by measuring several morphological, physiological and biochemical parameters. The simultaneous imposition of drought and a vascular pathogen is often found to reduce several morphological and physiological parameters such as the plant height, total leaf area and transpiration (Pennypacker et al., 1991; Abd El-Rahim et al., 1998; Choi et al., 2013; Sinha et al., 2017).

Our results indicate that the concurrent effect of pathogen and drought stresses is different in each cultivar compared to the net effect of drought stress and *V. dahliae* applied individually. Similarly, the effect of drought and *Verticillium* wilt on eggplant cultivars had been studied under field conditions (Bletsos et al., 1999) and showed that different irrigation regimes in combination with *Verticillium* wilt significantly affected fruit quantitative characteristics of different eggplant cultivars.

Specifically, when drought and pathogen stress occurred simultaneously, they caused a significant decrease in RGR. Plant growth reduction can be the outcome of a pathogen infection (Johnstone et al., 2005). Results by Buhtz et al. (2017) demonstrated that *V. dahliae* infection greatly compromised plant growth of tomato plants. Nevertheless, this reduction can limit the water requirements of the plant thus improving plant survival during the simultaneous imposition of drought as shown in other studies (Xu et al., 2008). Interestingly our results indicate further that significant interactions of cultivar, drought and *V. dahliae* stresses were also obtained. One of the most common suite of traits that plants utilize to circumvent the stressful environments is the reduced stem/root ratio, thus optimizing the use of their available resources (Chapin et al., 1993; Gargallo-Garriga et al., 2014; Koevoets et al., 2016; Buhtz et al., 2017; Pandey et al., 2017). Even though, this was not shown in the double interaction, there was a significant difference in the cultivars effect (as single factor) and in the triple interaction. Our results demonstrated a significantly higher root/shoot length ratio of EMI plants compared to Skoutari plants during the stress,

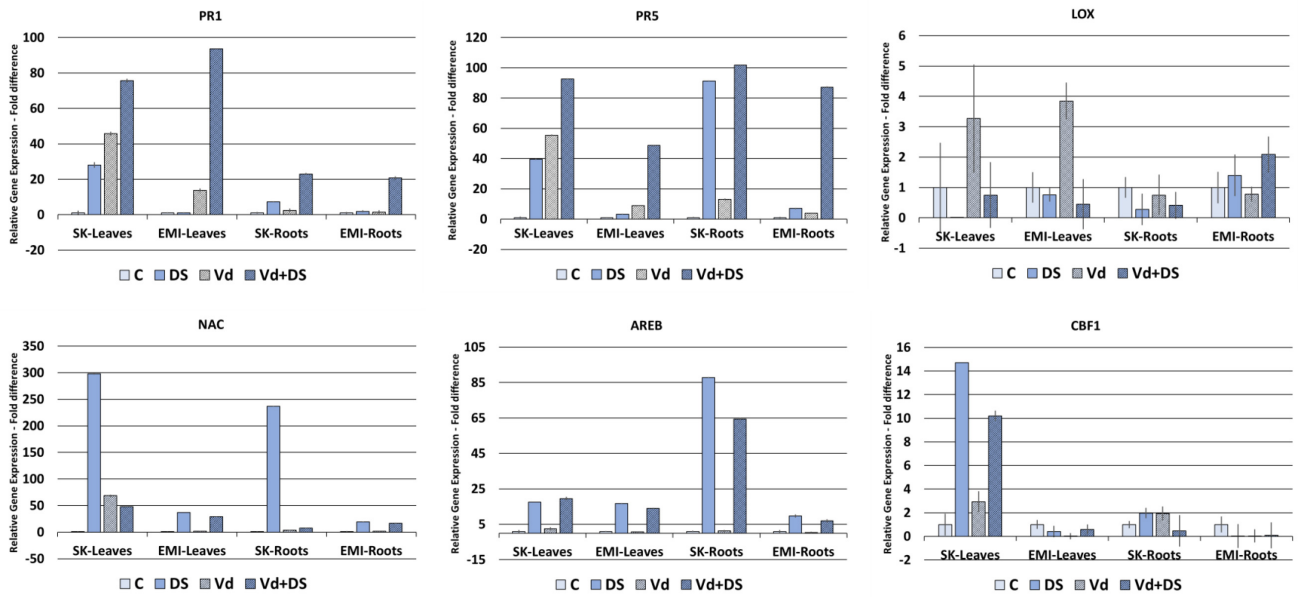

FIGURE 4 | Relative gene expression of the *PR*1, *PR*5, *LOX*, *NAC*, *AREB*, and *CBF*1 marker genes in leaves and roots of eggplant plants subjected to abiotic, biotic, and combined stresses. The relative expression of selected marker genes was measured in eggplant cultivars under individual and combined stress. C, control; DS, drought stress; Vd, *Verticillium dahliae*; Vd+DS, Combined stress. Bars represent standard error.

indicating that EMI alters its morphological traits in order to circumvent more efficiently the combination of stressors.

Stressful environments, including drought, pathogen infection and their combination, greatly influence the process of photosynthesis in most plants by altering several photosynthetic parameters such as WUE and stomatal regulation (Ashraf and Harris, 2013; Pandey et al., 2017). One of the key mechanisms of plants in order to adapt to multiple stresses is stomatal regulation by allowing plants to make the optimum CO_2 assimilation toward evapotranspiration. In several cases of combined stresses (either abiotic-abiotic or abiotic-biotic) photosynthesis rate (P_N), and leaf gas exchange parameters (g_s, *E*, and *C*i) dramatically decreased under such conditions compared to single stress (Choi et al., 2013; Suzuki et al., 2014). In our study, the highest reduction of the photosynthetic rate and of leaf gas exchange parameters (**Figure 2**) was shown when plants were subjected either to severe drought or to combined stress, suggesting that the decline of photosynthesis rate is due to stomatal closure. Additionally, WUE and P_N/g_s ratios were mainly dependent on water stress alone and to combined stress. Xu et al. (2008) demonstrated that when virus infection preceded water stress, plants responded better to the two stressors due to partial stomatal closure and lower transpiration rates. Our results highlighted that both varieties maintained their photosynthetic activity during the combined stress. Moreover, WUE and Pn/g were higher under combined stress than biotic stress alone (**Table 3**) The maintenance of plants photosynthetic activity is very important for their acclimation to a combination of pathogen and drought stress (Pandey et al., 2015).

During abiotic and/or biotic stress interactions, plants produce rapidly and transiently ROS, functioning as signaling molecules (i.e., induce production of ROS scavengers) and being one of their first defense responses against stresses. Likewise, fungi infection would result in oxidative stress in plants, and any concomitant stress could create inevitably additional production of ROS (Dikilitas et al., 2016). Continuous stress can cause an accumulation of ROS at the plasma membrane, producing an oxidative stress that leads to increased membrane damage and MDA content (Hasanuzzaman et al., 2013; Schieber and Chandel, 2014; Choudhury et al., 2017). Oxidative stress is often associated with excessive concentrations of H_2O_2 that is considered the central ROS signaling molecule (Sies, 2017). Interestingly, significant increase in H_2O_2 levels was monitored in all treatments compared to controls (**Tables 2** and **3**) and significant differences were observed between the two cultivars for plants under combined stress and controls (**Figures 1G,H**). The increased H_2O_2 levels at the end of the experiment indicated an additive effect of combined stress on H_2O_2 formation and a prolonged oxidative stress for the plants of Skoutari cultivar (**Figure 3**). Similarly, the triple interaction of the cultivar, *V. dahliae* and water stress had a significant combined effect on MDA one of the most well studied markers of lipid peroxidation and oxidative stress (**Figures 1I,J**). It is noteworthy that researchers have associated powerful oxidative stress with significant decrease of root growth as well (Miller et al., 2009). It should be noted that EMI cultivar exhibited better membrane homoeostasis and lower H_2O_2 accumulation under stress combination suggesting that may have a stronger capacity for membrane lipid replacement than Skoutari (**Figure 1**). In the review by Suzuki et al. (2014) it is clearly highlighted that the inhibition of oxidative stress can at least partially ensure plants protection against stress combination.

On the other hand, proline has been reported to be the most abundant osmolyte accumulated in response to multiple environmental stresses including water stress and pathogen infection, which acts as an ROS scavenger as well (De Ronde et al., 2000; Hanci and Cebeci, 2014; Rodziewicz et al., 2014). Recent findings have demonstrated that water stressed eggplants accumulated osmoprotectants like proline, as well as glucose and fructose and this phenomenon has been correlated with increased tolerance toward water deficit and prevention from wilting (Mibei et al., 2018). Our results showed significant difference between control plants and water stressed or *V. dahliae* infected plants in proline concentration, however, no significant difference was observed between control plants and plants under combined stress for the Skoutari cultivar. On the contrary, significant difference was observed between control plants and plants under combined stress for the EMI cultivar. This indicates that other osmoprotectants could be up-regulated during concomitant imposition of *V. dahliae* and water stress in certain eggplant cultivars.

Though the availability of genome sequences for other members of the *Solanaceae* plant family such as tomato and potato enabled gene expression and transcriptome studies for these species, no such genome sequence facilities were available for eggplant until the publication of the draft eggplant genome sequence (Hirakawa et al., 2014) and a pre-publication version from the Eggplant Genome Project[1]. Since then, various publications on comparative transcriptome analyses regarding the phylogenetic relations of eggplant and closely related wild species (Yang et al., 2014, 2015), the determination of molecular markers (Gramazio et al., 2016), characterization of genes and gene families (Barbierato et al., 2016; Li et al., 2016), gene function (Na et al., 2016), or miRNAs (Yang et al., 2013), have been made. To study the combined effect of water stress and *V. dahliae* infection at gene transcriptional level, we have selected specific marker genes, after homology search in GenBank and Sol Genomics Network and designed suitable primers for quantitative real-time polymerase chain reaction (RT-qPCR). Pathogenesis related (*PR*1 and *PR*5) and lipoxygenase (*LOX*) genes had been shown to be induced by Salicylic acid (SA) and Jasmonic acid (JA), respectively, and are commonly used as biotic stress markers in gene expression studies (Aime et al., 2008; Kissoudis et al., 2017; Mahesh et al., 2017), *AREB*, and *CBF*1 are water and cold stress related markers (Hsieh et al., 2002; Wang et al., 2016), while *NAC* genes are reported to have roles in both abiotic and biotic stresses (Tweneboah and Oh, 2017). Previous studies on the validation of eggplant reference genes for RT-qPCR assays, have indicated appropriate candidates for use in quantitative gene expression studies (Gantasala et al., 2013; Zhou et al., 2014). For our study we have selected *ACTIN* as reference gene (Zhou et al., 2014).

Our gene expression study results (**Figure 4**) showed a strong upregulation of *PR*1 and *PR*5 genes by *V. dahliae* infection as well as by water stress. Interestingly, their expression was further enhanced when combined stress was applied. These results taken together with the fact that *V. dahliae* infection preceded the water stress applied, suggest a crosstalk between biotic and abiotic stress biochemical regulatory pathways and a strong influence of water stress on biotic stress plant responses at the transcriptional level. Furthermore, the relative gene expression fold changes observed in plants under combined stress compared to plants under single stress, and particularly in the EMI cultivar, suggest that the regulation of gene expression does not necessarily fall in a mode of an additive pattern, but supports a cultivar dependent tailored made regulation. The enhanced expression of *PR*1 and *PR*5 under combined stress appears tissue dependent as well. *PR*1 is strongly induced in leaves respect to roots of both cultivars, while *PR*5 has a higher induction in roots, indicating possibly a different involvement in tissue-specific gene expression in the overall plants responses to the stresses. The low relative expression levels of *LOX* in all treatments compared to the untreated control in both cultivars, in conjunction with the strong upregulation of *PR*1 and *PR*5, indicate that the plants responses to all types of stresses applied are regulated principally *via* SA. Our results also show that the high expression of *NAC* in leaves of Skoutari water stressed plants is reduced in plants under combined stress, however, not in the EMI cultivar. This supports further a crosstalk between the biochemical pathways regulating the responses to biotic and abiotic stress and indicates a cultivar dependent mechanism that masks or suppresses the *NAC* expression. The expression patterns for *AREB* and *CBF*1 suggest that these genes are principally induced/regulated by water stress, since no differences are observed in plants under combined stress.

Our study showed that Skoutari and EMI responses to combined stress are cultivar dependent. Both cultivars were also subjected to milder drought stress (50% of FW) and we found clear difference in their responses, with EMI being the more tolerant to drought stress (data not shown). It can be speculated that the morphological adaptations, the stronger capacity for membrane lipid replacement and the higher osmoprotectant accumulation of EMI enhanced its tolerance to the combined stress. On the other hand, the increased plant growth and plant tolerance to stressful environments act in an antagonistic manner. This is the case with our cultivars as well. Skoutari cultivar exhibited the higher RGR in all conditions. However, it showed less osmoprotectant accumulation and more lipid peroxidation of membranes. Consequently, improving traits such as stability of leaf gas exchange parameters, membrane homeostasis and ROS regulation in cultivars that produce higher biomass should be further explored to gain better adaptation to higher stress environments.

It is of our interest to define further, cultivar dependent molecular responses of eggplant to either *V. dahliae*, drought stress and their possible combinations by profound transcriptomic and proteomic studies. These would allow us to dissect specific molecular pathways and define eggplant key regulatory genes for theses stresses. Recently, a characterization of an indigenous eggplant cultivars and landraces collection has been performed (Ganopoulos et al., 2015). It would be interesting

[1] www.eggplantgenome.org, 2017

to define further their responses to combined *V. dahliae* and drought stress and utilize them in future breeding projects.

AUTHOR CONTRIBUTIONS

ET and DK were involved in the experimental design of the work, performed experiments, analyzed, and interpreted the data and wrote the paper. EM performed and interpreted statistical analysis. IP, DT, GL, DM, and IK performed experimental work. All authors approved the final version of the manuscript to be submitted for publication and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

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Osmotic Stress and ABA Affect Immune Response and Susceptibility of Grapevine Berries to Gray Mold by Priming Polyamine Accumulation

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Abiotic factors inducing osmotic stress can affect plant immunity and resistance against pathogen attack. Although a number of studies have characterized grapevine responses to various forms of biotic and abiotic stresses, the relationships between osmotic stress response and susceptibility of mature berries to *Botrytis cinerea* still remain unknown. In this study, we investigated the effects of osmotic stress and abscisic acid (ABA) on defense responses of mature grapevine berries before and after *B. cinerea* infection. We focused on the possible involvement of polyamines in the interaction between osmotic stress response and susceptibility to *B. cinerea*. We showed that osmotic stress induced by PEG or sucrose, and exogenous ABA induce transient but low defense responses, including weak expression of *PR* genes and phytoalexin synthesis in mature berries. This was accompanied by an upregulation of *NCED2* involved in ABA biosynthesis and a large production of free polyamines. However, osmotic stress followed by *B. cinerea* infection primed berries for enhanced accumulation of polyamines, but slowed down the defense responses and increased susceptibility to the pathogen. A weak increase of diamine- and polyamine-oxidase activities was also recorded in stressed berries, but declined after pathogen infection. The pretreatment of stressed berries with appropriate inhibitors of diamine- and polyamine-oxidases further increased polyamine level and greatly lowered defense responses, leading to higher susceptibility to *B. cinerea*. These results suggest that increased polyamine titer through low activation of their oxidative degradation in grape berries may contribute at least in part to the weakening of defense responses and subsequent disease susceptibility.

Keywords: *Botrytis cinerea*, immune response, osmotic stress, polyamines, *Vitis vinifera*

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INTRODUCTION

Drought inducing osmotic stress is among the most serious abiotic constraints for global agriculture that may intensify during the next few decades (Zhao and Running, 2010). Emerging evidences suggest that abiotic stress can strongly modulate plant–pathogen interactions resulting in plant susceptibility or resistance to diseases (Sinha et al., 2016). These effects involve crosstalk between different responses during successive or combined biotic and abiotic stresses (Anderson et al., 2004; Atkinson et al., 2013; Seifi et al., 2013). Contrasting findings suggest that water deficit might differentially affect

plant immunity and microbial pathogenesis in different pathosystems (Bidzinski et al., 2016; Sinha et al., 2016; Gupta et al., 2017). This type of stress can weaken plant immune responses or other metabolic pathways occurring during water stress then resulting in a predisposition of plants to pathogen infection. Recent evidences show that drought stress can suppress pathogen associated molecular pattern-triggered immunity and effector-triggered immunity in rice, which becomes highly susceptible to the blast fungus *Magnaporthe oryzae* infection (Bidzinski et al., 2016). The susceptibility could be attributed to increased levels of abscisic acid (ABA) in drought stressed plants which can interfere with pathogen-induced immune signaling pathways and thereby reduce the expression of defense-related genes (Fujita et al., 2006; Ton et al., 2009; Pieterse et al., 2012; Atkinson et al., 2013). However, successive abiotic and pathogen stresses may also lead to a priming state of plants for enhanced basal defenses resulting in plant resistance to pathogen. Some studies showed that drought-stressed Arabidopsis, chickpea, and tomato plants are more resistant to bacterial pathogens, *Pst* DC3000, *Pseudomonas syringae* pv. *phaseolicola*, and the fungus *Botrytis cinerea*, respectively (Achuo et al., 2006; Gupta et al., 2016; Sinha et al., 2016). Similarly, osmotic stress enhanced barley resistance to powdery mildew caused by *Blumeria graminis* through the primed formation of papillae (Wiese et al., 2004).

The overall response of plants to the successive or combined abiotic and biotic stress is generally governed by plant hormones, salicylate (SA), jasmonate (JA), and ethylene (ET) as key regulators of plant immunity (Pieterse et al., 2012). SA is generally required for plant defense against biotrophic pathogens, and JA and ET are considered to play their major role in plant defense against necrotrophic pathogens (Glazebrook, 2005; Pieterse et al., 2012). Furthermore, as for ABA, the SA and JA pathways are also reported to play a key role in mediating drought or salt tolerance and resistance to pathogens (Borsani et al., 2001; Seo et al., 2011; Ismail et al., 2012). Given their involvement in regulating both biotic and abiotic stress responses, a crosstalk between these signaling pathways has often been postulated. ABA can act as a negative regulator of defense responses mediated by SA and enhance plant susceptibility to pathogens (Mohr and Cahill, 2003). In addition to their antagonistic regulation, a positive crosstalk between SA and ABA pathways has also been reported. This synergistic interaction can result in stomatal closure contributing to drought tolerance (Miura et al., 2013).

Polyamines and their metabolic pathways constitute also integral parts of both adaptive responses to abiotic stress (Aziz et al., 1999; Bouchereau et al., 1999; Cuevas et al., 2008; Alcázar et al., 2010) and immune system upon plant–microbe interactions (Marina et al., 2008; Gonzalez et al., 2011; Nambeesan et al., 2012). Through their oxidative pathways, polyamines can generate oxidative burst mediating the hypersensitive response and the expression of defense genes (Cona et al., 2006; Yoda et al., 2006). Otherwise, polyamines were shown to interact with JA and ET signaling, thereby enhancing plant susceptibility to pathogens (Anderson et al., 2004; Nambeesan et al., 2012). Numerous biological functions have further been attributed to polyamines due in part to their polycationic nature, since they can interact with most of negatively charged molecules and stabilize their structure under various conditions (Alcázar et al., 2010). In plants, the major polyamines are putrescine, spermidine, and spermine. Putrescine can be synthesized from arginine or ornithine through arginine decarboxylase or ornithine decarboxylase, respectively (Bouchereau et al., 1999). Polyamine homeostasis involves also their oxidative degradation through copper amine oxidase (CuAO) and polyamine-oxidase (PAO). Some PAO isoforms are also involved in back-conversion of spermine to spermidine or putrescine (Moschou et al., 2008). These oxidative pathways may affect polyamine homeostasis and modulate hormone signaling and redox status, thereby conferring a protective role under stress conditions (Aziz et al., 1999; Moschou et al., 2008; Pál et al., 2015). Polyamine homeostasis can also be affected by an imbalance between activation of biosynthetic and oxidative pathways in stressed conditions, thus leading to an excess or deficiency of polyamines and conferring susceptibility to stress (Aziz et al., 1999; Nambeesan et al., 2012; Hatmi et al., 2015). This is consistent with the suggestion that the higher polyamine level is not always the better, but its regulatory role and the fine tuning of their level seem more important in stress responses (Pál et al., 2015).

Polyamine oxidation is also intrinsic to signaling mechanisms in both abiotic stress adaptation and disease resistance response (Cona et al., 2006; Moschou et al., 2008; Hatmi et al., 2014; Pal and Janda, 2017). The generation of H_2O_2 through the oxidative pathways of polyamines is intimately linked to improved resistance against pathogens and tolerance to abiotic stress (Takahashi et al., 2004; Cona et al., 2006; Moschou et al., 2008; Yoda et al., 2009). The generated H_2O_2 can induce the expression of various defense or stress responsive genes and inhibit pathogen growth. H_2O_2 is also involved in programmed cell death as well as in stomata opening (Yoda et al., 2006), and used in cross-linking and maturation of the cell wall by peroxidases (Takahashi et al., 2004; Cona et al., 2006; Yoda et al., 2009). Furthermore, polyamine pathways are connected to other signaling molecules such as nitric oxide, ET, and γ-aminobutyric acid (GABA) which are part of plant adaptation and immune response to abiotic stress and pathogen attack (Cona et al., 2006; Cuevas et al., 2008). It has been shown that the over accumulation of spermidine in transgenic tomato was associated to a weakened ET-induced defense responses, therefore increasing the susceptibility of fruit to *B. cinerea* (Nambeesan et al., 2012).

Although a number of studies have characterized grapevine responses to various forms of biotic and abiotic stresses, transcriptomic studies have revealed a weak expression of immune response in mature berries during early infection with *B. cinerea* (Bézier et al., 2002; Kelloniemi et al., 2015). More recently, it has been shown that osmotic stress attenuated defense responses in grapevine leaves after *B. cinerea* challenge and enhanced their susceptibility to the fungal pathogen (Hatmi et al., 2014). Other study using grapevine varieties

with contrasting tolerance to drought stress illustrated that polyamine oxidation may affect the signaling network in grapevine leaves resulting in improved defense response (Hatmi et al., 2015). However, the relationships between osmotic stress response and susceptibility of mature grapevine berries to *B. cinerea* still remain unknown. The role of polyamines in the interaction between osmotic stress response and triggered immune defense after pathogen attack has not yet explored in grape berries.

The present study aimed to understand the relationships between osmotic stress and the susceptibility of mature grapevine berries to *B. cinerea*, and to unravel mechanisms mediating interactions between grapevine berry responses to osmotic stress and pathogen infection. Here, we first investigated the effects of osmotic stress and ABA on defense responses of detached berries before and after *B. cinerea* inoculation. We further focused on the role of polyamines in the interaction between osmotic stress response and immune response. We also used pharmacological approach to assess whether oxidative pathways of polyamines may affect defense responses induced by osmotic stress and the susceptibility of berries to *B. cinerea*.

MATERIALS AND METHODS

Plant Material and Stress Treatments

Grapevine berries (*Vitis vinifera* cv. Chardonnay) were collected from vineyards at full ripening. Detached berries with pedicels were washed three times with sterilized water and the pedicels were dipped in 12.5 mM MES buffer containing 7.5 mM KCl and 5 mM $CaCl_2$, pH 6 as a control medium. Osmotic stress was applied using 400 g L^{-1} PEG 6000 or 600 mM sucrose to the control medium. ABA was also added to the control medium at 100 μM in 0.02% ethanol. ABA concentration used in this study approximates ABA concentration of berries prior to the onset of ripening (Giribaldi et al., 2010). Samples were put in growth chamber with a 16/8 h photoperiod, at a photosynthetic photon flux density of 60 μmol m^{-2} s^{-1} and a 25°C/22°C day/night, respectively. Berries were harvested at 0, 3, 7, and 12 days of osmotic stress or ABA treatment. On each sampling time berries were deseeded, frozen, and ground to fine powder in liquid nitrogen. Three replicates were used from independent batches of 12–15 berries.

For the priming state, berries were first treated by dipping the pedicels in control medium supplemented with 400 g L^{-1} PEG 6000 or 600 mM sucrose or 100 μM ABA for 72 h, then single berries were drop-inoculated with *B. cinerea* and harvested at 7 days post-infection (dpi).

Inhibitor Treatments

Two pharmacological chemicals were used in this study, aminoguanidine (AG, Sigma) and guazatine (GUA, Sigma) as specific and competitive inhibitors of CuAO (Xing et al., 2007) and PAO, respectively (Yoda et al., 2006). Single berries were pretreated at the level of the pedicel with 2 mM AG or 100 μM GUA for 12 h, then transferred to control or stressing medium with 400 g L^{-1} PEG 6000 or 600 mM sucrose for 3 days under the same conditions described above. Berries were then used for analysis of defense- or stress-responsive genes and metabolites before and after pathogen infection.

Fungal Inoculation and Disease Assay

Fungal culture and preparation of conidial spores were as described previously (Aziz et al., 2003). After pretreatment of berries with inhibitors or with osmotic agents for 3 days, one needle prick wound was applied in each single berry and covered with 5 μL of a conidial suspension of *B. cinerea* (5×10^5 conidia mL^{-1}). Disease symptoms were measured on 20–25 berries from three independent experiments at 7 or 10 days post-inoculation. Disease rating was assessed as the fraction of berries falling in different classes: (I) spreading lesion with less than the 20% of the berry surface; (II) spreading lesion with the 21–35% of the berry surface; (III) spreading lesion with the 36–50% of the berry surface; (IV) spreading lesion with more than 50% of the berry surface.

Polyamine Analysis

Berries were sampled at different times of treatment, frozen and powdered with liquid nitrogen. Free polyamines were extracted with cold 1 M HCl (2:1, w/v) on ice, as described by Aziz et al. (2001). The homogenate were kept for 1 h at 4°C and then centrifuged for 20 min at 24,000 *g*. The supernatants were used for the dansylation. The aliquots of 250 μL from the supernatants were mixed with sodium carbonate and dansyl chloride as described in Hatmi et al. (2014). The mixture was incubated at room temperature for 16 h. The reaction was stopped with 300 μL of proline solution (100 mg mL^{-1}). Derivatized polyamines were extracted into 2 mL of ethyl acetate. After the organic phase was evaporated under nitrogen stream, the residue was solubilized with 1 mL of methanol, filtered through 0.22 μm PTFE filters and dansyl polyamines were analyzed using Acquity UPLC system (Waters), and a BEH C18, 1.7 μm, 2.1 mm × 100 mm column heated at 30°C. Dansyl polyamines were eluted with acetonitrile: water solvent gradient and detected by an Acquity fluorimeter (Waters) with an excitation wavelength of 365 nm and an emission wavelength of 510 nm as described by Hatmi et al. (2014). Polyamines were quantified after calibration with external standards (Sigma).

Phytoalexin Analysis

Stilbenic phytoalexins were extracted from 1.5 g of freeze-dried powder with 2 mL of methanol 100% in the dark at room temperature. Tubes were placed in shaker for 1 h and then centrifuged for 10 min at 8000 *g* (Hatmi et al., 2014). The supernatants were dried under vacuum and residues were solubilized with 1 mL of methanol then filtered through 0.22 μm PTFE filters. Resveratrol and ε-viniferin were analyzed using an Acquity UPLC system (Waters, Milford, United States) with an Acquity UPLC BEH C18, 1.7 μm, 2.1 mm × 100 mm, heated at 40°C and a gradient from 10 to 90% acetonitrile as described in Hatmi et al. (2014). Phytoalexins

were detected with an Acquity fluorimeter (Waters) at an excitation wavelength of 330 nm with an emission wavelength of 375 nm. Resveratrol and ε-viniferin were identified and quantified with reference of retention time and calibration with external standards.

RNA Extraction and qRT-PCR

Total RNA was isolated from deseeded berries using the Extract-All reagent (Eurobio) and 150 ng was used for reverse-transcription using the Verso cDNA Synthesis kit (Thermo Electron) according to the manufacturer's instructions. The transcript levels were determined by real-time PCR using the CFX96 system (Bio-Rad) and absolute blue qPCR SYBR Green as recommended by the manufacturer (Thermo Electron). PCRs were performed using a 10-fold cDNA dilution in duplicates as template in 96-well plates in a 15-μL final volume containing 1× SYBR Green I mix (including Taq polymerase, dNTPs, SYBR Green dye) and 280 nM forward and reverse primers. Cycling parameters were 15 min of Taq polymerase activation at 95°C, followed by 40 two-step cycles composed of 10 s of denaturation at 95°C and 45 s of annealing and elongation at 60°C. The EF1 gene was used as a reference gene and experiments were repeated three times. The specific primers of analyzed genes are listed in Supplementary Table S1. Relative gene expression was determined with the formula fold induction: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta$Ct = [*Ct* TG (US) − *Ct* RG (US)] − [*Ct* TG (RS) − *Ct* RG (RS)], where Ct is cycle threshold, Ct value is based on the threshold crossing point of individual fluorescence traces of each sample, TG is target gene, RG is reference gene, US is unknown sample, and RS is reference sample. Integration of the formula was performed by the CFX Manager 3.0 software (Bio-Rad). The reference sample is the control sample at each sampling time point chosen to represent 1× expression of the target gene.

Amine-Oxidase Activity

Copper amine oxidase and PAO assays were performed as described in Hatmi et al. (2015). Briefly, enzymes were extracted on ice from 250 mg of powdered plant material with 1 mL of 100 mM potassium phosphate buffer, pH 6.5 for PAO or pH 7.0 for CuAO. Extracts were centrifuged at 12,000 *g* and 4°C for 10 min, then CuAO and PAO activity was measured with spectrophotometer using 2 mM putrescine or 2 mM spermidine as the substrates. The formation of pink adduct resulting from the oxidation and condensation of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid (Sigma-Aldrich) catalyzed by horseradish peroxidase (Cona et al., 2006) was quantified (ε515 = 2.6 × 104 M^{-1} cm^{-1}) at 25°C.

Statistical Analysis

Data are averages of three independent experiments and are shown as means ± SDs. Statistical analyses were carried out using the SigmaStat 3.5 software. Data were analyzed using two-way analysis of variance (ANOVA). For treatment effect, mean values were compared by Tukey's test ($P < 0.05$).

RESULTS

Osmotic Stress and ABA Induce Transient, but Low Defense Responses in Berries

We investigated whether exposure of detached berries to PEG or sucrose inducing osmotic stress or ABA treatment can affect immune response. Using quantitative real-time PCR (qRT-PCR) and specific primers (Supplementary Table S1), we examined the expression levels of some defense-related genes, including *STS1* (a stilbene synthase), *PR2* (a β-1,3-glucanase), *PR3* (an acidic chitinase IV), and *PR5* (a thaumatin-like protein), as well as *NCED2* encoding 9-*cis*-epoxycarotenoid dioxygenase involved in ABA biosynthesis. Berries were collected at different time points after PEG, sucrose, or ABA treatment. We showed that most of the targeted genes were transiently upregulated in berries during osmotic stress or ABA treatment (**Figure 1A**). The steady state levels of mRNA for *STS1*, *PR2*, and *PR3* moderately increased at day 3 in response to osmotic stress and ABA compared to control. The highest expression of *STS1* (12-fold) and *PR3* (6-fold) was observed in response to PEG and sucrose, while *PR2* was more expressed with PEG (11-fold) and ABA (12-fold). The transcript levels were subsequently returned to their basal values after 12 days of osmotic stress and ABA treatment. The expression of *PR5* gene is only slightly increased at 3 or 7 days post-treatment with PEG and ABA. The expression of *NCED2* was also transiently upregulated in berries, albeit to much lower extent during the exposure to both osmotic stress and ABA (**Figure 1A**). The expression being maximal at day 3, it reached 5.2-fold, compared with control.

We also analyzed the main stilbenic phytoalexins, *trans*-resveratrol (*trans*-3,5,4′-trihydroxy-*trans*-stilbene) and its dimer *trans*-ε-viniferin as one of the most important responses of basal and induced immunity. As expected, ripe berries showed high basal levels of *trans*-resveratrol (**Figure 1B**) and *trans*-ε-viniferin (**Figure 1C**) in control conditions. However, when they were exposed to osmotic stress or ABA, berries exhibited different responses with respect to stilbene accumulation. The amount of resveratrol did not change during exposure of berries to PEG and sucrose compared to control (**Figure 1B**). However, resveratrol level increased in ABA-treated berries by about 2.5- to 3.5-fold at days 3 and 12. ε-Viniferin was also present to similar extents compared to resveratrol in control berries (**Figure 1C**). However, its amounts significantly lowered in response to osmotic stress, and the decrease was prominent with sucrose than with PEG at days 7 and 12. Again, as for resveratrol, ε-viniferin amount increased albeit to a much lower level in response to ABA, and peaked at day 3 and 7 of treatment.

Osmotic Stress and ABA Induce Changes in Free Polyamine Titers in Berries

To investigate the role of polyamines in berries following exposure to stress, we first quantified the diamine putrescine, the polyamines spermidine and spermine and their oxidation

A

Treatment	Gene	Days of stress 0	3	7	12
PEG	*STS1*	1.1±0.1 a	12.7±3.1 c	3.5±2.2 b	1.8±0.3 a
SUC		0.9±0.2 a	10.6±2.3 c	6.9±3.2 bc	2.4±0.9 ab
ABA		1.2±0.1 a	5.9±2.5 b	3.3±0.9 b	2.4±1.0 ab
PEG	*PR2*	0.9±0.1 a	10.9±2.9 c	3.0±0.3 ab	1.1±0.4 a
SUC		1.1±0.2 a	6.2±2.4 b	0.9±0.4 a	0.5±0.1 a
ABA		1.1±0.2 a	11.8±0.9 c	5.2±1.8 b	1.3±0.2 a
PEG	*PR3*	1.1±0.1 a	5.9±1.2 c	2.9±0.6 b	0.5±0.1 a
SUC		1.0±0.1 a	6.1±2.5 c	2.8±1.0 b	1.4±0.1 a
ABA		0.8±0.1 a	1.3±0.4 a	4.3±1.4 bc	0.7±0.3 a
PEG	*PR5*	0.8±0.1 a	2.1±0.7 b	2.1±0.2 b	1.7±0.1 ab
SUC		0.9±0.2 a	1.2±0.7 a	1.4±0.4 a	1.1±0.1 a
ABA		1.0±0.1 a	1.6±0.1 ab	2.3±0.8 b	0.8±0.1 a
PEG	*NCED2*	1.1±0.2 a	5.2±1.6 c	2.8±1.2 b	2.2±0.3 ab
SUC		1.0±0.0 a	4.9±1.3 c	2.4±1.0 b	2.8±0.1 b
ABA		1.2±0.2 a	4.2±1.1 c	2.1±0.6 ab	2.5±0.3 b

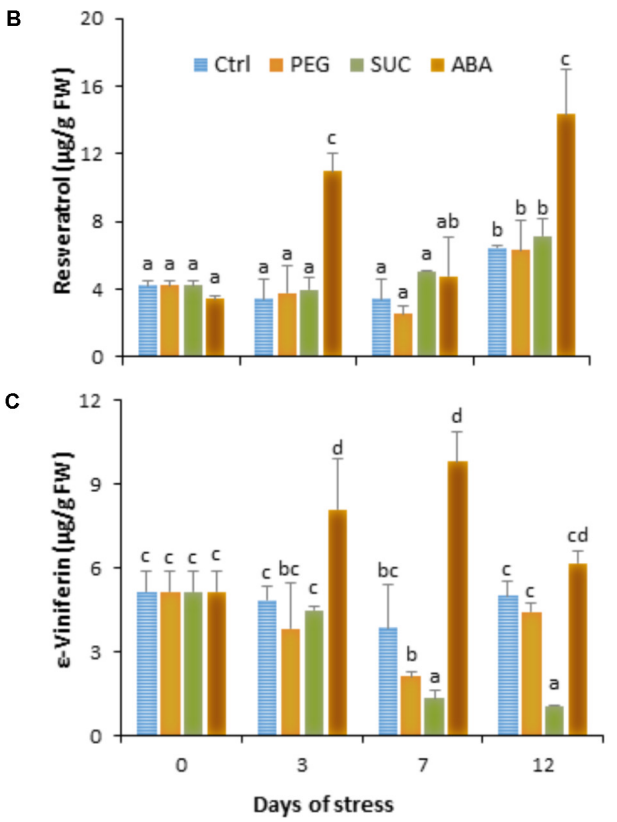

FIGURE 1 | Expression of defense- or stress-related genes and phytoalexin accumulation in grapevine berries in response to osmotic stress and ABA treatment. Single berries were treated by immersing the pedicel in control medium (Ctrl) containing 400 g L^{-1} PEG 6000 (PEG) or 600 mM sucrose (SUC) or 100 µM ABA and harvested at the indicated times. Values of gene expression **(A)** are expressed as the fold increase in transcript level relative to the corresponding control at each sampling time. *trans*-Resveratrol **(B)** and ε-viniferin **(C)** concentrations were determined in control, osmotic stressed- and ABA-treated berries. Data are means ± SD representative of three experiments. Different letters indicate significant differences (Duncan's multiple range test, $P < 0.05$).

product 1,3-diaminopropane (Dap) during osmotic stress or ABA treatment. Data (**Figure 2**) showed that putrescine (**Figure 2A**) and spermidine (**Figure 2B**) are the most abundant polyamines in ripe berries. The amounts of polyamines did not change in PEG-treated berries during the first days, while they significantly increased from the third day in both sucrose- and ABA-treated berries (**Figure 2**). Thereafter, the level of the four polyamines became important after a 12 days in response to osmotic stress and ABA, excepted for Dap which significantly decreased in ABA-treated berries (**Figure 2D**). The putrescine content increased by about two- to threefold and those of spermidine and spermine increased by about three to five times in the osmotically stressed and ABA-treated berries. However, the amount of Dap slightly increased in response to PEG and sucrose, but remained unchanged at 12 days in ABA-treated berries. These results suggest that both osmotic stress and ABA treatment upregulate polyamine biosynthetic pathways over than their oxidation.

Osmotic Stress Potentiate Free Polyamine Accumulation but Not CuAO and PAO Activity After Pathogen Infection

In order to investigate if the induced change in free polyamines and defense responses by osmotic stress or ABA are influenced by *B. cinerea* infection, berries were first exposed to osmotic stress or ABA for 3 days then inoculated with *B. cinerea*. Data showed that infection of control berries with *B. cinerea* resulted in enhanced level of putrescine (**Figure 3A**), spermidine (**Figure 3B**), and spermine (**Figure 3C**), while the amount of Dap decreased (**Figure 3D**). In most cases, pretreatment of detached berries with osmotic agents maintained putrescine (**Figure 3A**) to a higher level after pathogen challenge, while the amounts of spermidine (**Figure 3B**), spermine (**Figure 3C**), and Dap (**Figure 3D**) were significantly increased compared to non-infected or control infected berries. Similarly, in berries pretreated with ABA then challenged with *B. cinerea* the level of putrescine remained unchanged, whereas spermidine and spermine contents increased, but to a lower extent compared to osmotically stress berries. In the same condition Dap content increased after pathogen infection. This suggests that both osmotic stress and ABA potentiate free polyamine accumulation probably by activating their biosynthesis more than their oxidation after *B. cinerea* infection.

Copper amine oxidase and PAO activity was also assayed to determine whether polyamine levels could be related to extent of their oxidative degradation in osmotically stressed berries after pathogen challenge. Data showed that CuAO (**Figure 3E**) activity was slightly enhanced by osmotic stress (by about 1.2-fold in mock berries), while the effects of *B. cinerea* infection resulted in a significant reduction of CuAO activity in both control, and stressed berries. Similarly, PAO activity (**Figure 3F**) was stimulated to a lower (but not significant) extent in PEG- and sucrose-stressed berries. However, no clear difference of PAO activity was observed between mock and *B. cinerea*-infected berries. The low or reduced activity of CuAO and PAO in

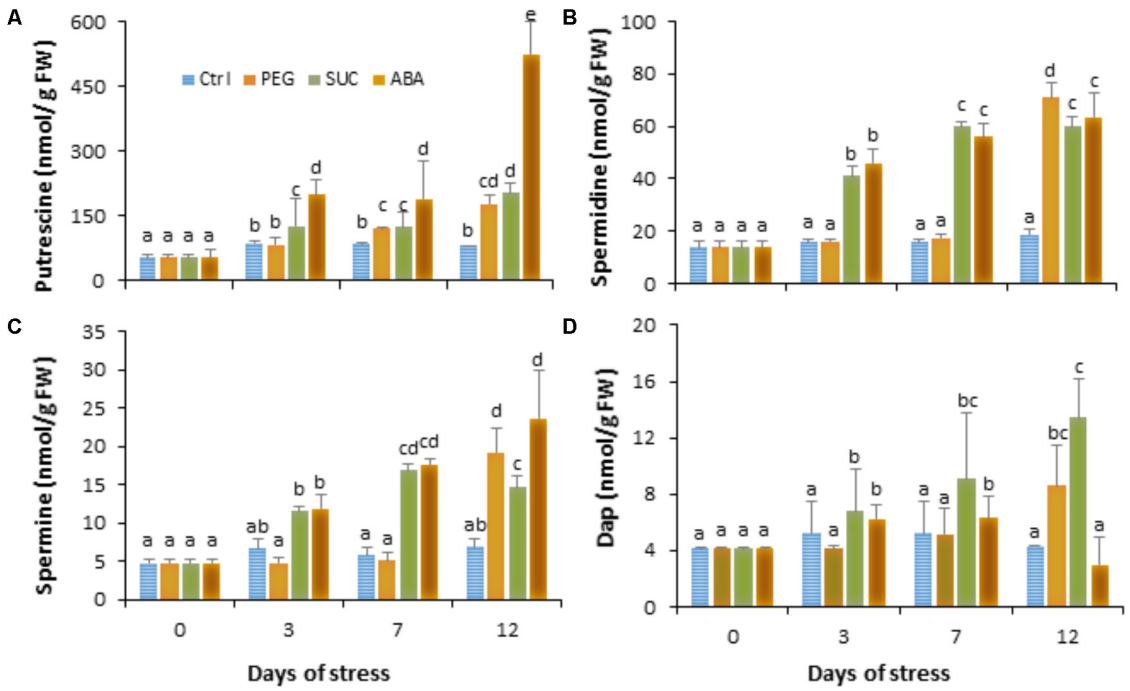

FIGURE 2 | Free polyamine levels in single berries in response to osmotic stress and ABA treatment. Detached berries were treated by dipping the pedicel in control medium (Ctrl) amended with 400 g L^{-1} PEG 6000 (PEG) or 600 mM sucrose (SUC), or 100 μM ABA and harvested at the indicated times. Putrescine **(A)**, spermidine **(B)**, spermine **(C)**, and 1,3-diaminopropane **(D)** concentrations were determined in control, osmotic stressed and ABA-treated berries. Values are means ± SD of three experiments. Different letters indicate significant differences (Duncan's multiple range test, $P < 0.05$).

infected berries is consistent with the primed accumulation of free polyamines after *B. cinerea* challenge.

Osmotic Stress and ABA Slow Down Phytoalexin Accumulation After Infection and Increase Susceptibility to *B. cinerea*

To determine whether osmotic stress or exogenous ABA can affect defense responses after *B. cinerea* infection, the main stilbenic phytoalexins were analyzed in both mock and infected berries. As shown previously, the amounts of phytoalexins, resveratrol (**Figure 4A**) and ε-viniferin (**Figure 4B**) in osmotically stressed berries (mock) were comparable to that of control, while the amount of both stilbenes increased in response to ABA (**Figure 4**). In *B. cinerea*-infected control the amount of resveratrol and ε-viniferin increased (**Figures 4A,B**). However, the amounts of phytoalexins was not primed by PEG or sucrose after pathogen infection (**Figures 4A,B**), and only a potentiated accumulation of resveratrol, but not of ε-viniferin was observed with ABA after infection (**Figure 4A**). Our data indicate that osmotic stress has no consistent priming effect on phytoalexin accumulation in berries after pathogen challenge.

Disease incidence experiments were conducted to determine whether the effects of osmotic stress or ABA benefit *B. cinerea* development in mature berries. Seven days after infection, large control berries developed necrosis with less than 20% (**Figure 4C**). Pretreatment of berries with PEG or with sucrose significantly enhanced disease incidence caused by the pathogen. Within 7 dpi, approximately 40% of osmotically stressed berries were infected at more than 35% (**Figure 4C**). Similarly, more than 50% of ABA-treated berries showed heavy gray mold symptoms (**Figure 4C**). After 10 dpi, osmotically stressed berries showed a dramatic increase of gray mold disease (**Figure 4D**), with approximately 60 and 50% of berries heavily infected with PEG and sucrose, respectively. In the case of ABA, the number of heavily infected berries increased and reached more than 75% of the berry area.

Inhibitors of Polyamine Oxidation Strongly Increase Polyamine Levels, and Reduce Defense Responses in Osmotically Stressed Berries

The above reported data indicated that osmotic stress and ABA increased polyamine levels, and susceptibility of grapevine berries to *B. cinerea*, but poorly induced some defense responses. We hypothesized that increased amounts of polyamines may be linked at least in part to low activation of polyamine oxidation during osmotic stress or pathogen infection. To assess whether oxidative pathways of polyamines may affect defense responses induced by osmotic stress and the susceptibility of berries to *B. cinerea* we used two inhibitors; AG and GUA as specific and competitive inhibitors of CuAO and PAO, respectively. The effect of AG and GUA was first evaluated on the polyamine levels in the osmotically stressed berries. As shown in **Figure 5**, although putrescine content showed no significant differences

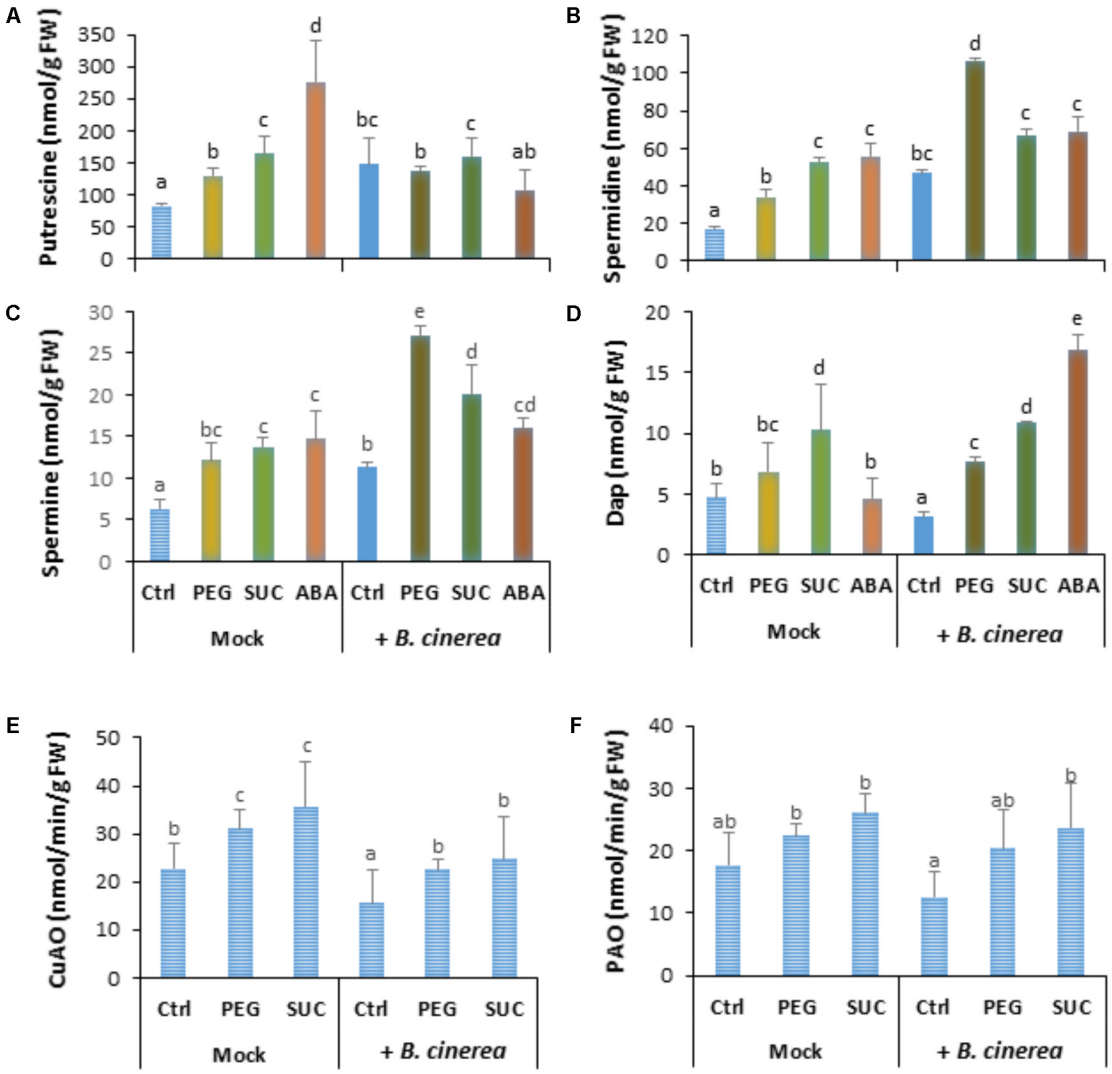

FIGURE 3 | Polyamine accumulation and CuAO and PAO activity in osmotically stressed or ABA-treated grapevine berries after *B. cinerea* infection. Berries were first treated by dipping the pedicel in control medium (Ctrl) supplemented with 400 g L^{-1} PEG 6000 (PEG) or 600 mM sucrose (SUC) or 100 μM ABA for 72 h, then drop-inoculated with *B. cinerea* and harvested at 7 dpi. Values of putrescine **(A)**, spermidine **(B)**, spermine **(C)**, 1,3-diaminopropane **(D)**, and activity of CuAO **(E)** and PAO **(F)** are means ± SD of three experiments. Different letters indicate significant differences (Duncan's multiple range test, $P < 0.05$).

between control and pretreated berries with AG (**Figure 5A**), the amounts of spermidine (**Figure 5B**), spermine (**Figure 5C**), and Dap (**Figure 5D**) increased significantly with AG. The AG effect resulted also in enhanced levels of spermidine, spermine, and Dap in osmotically stressed berries. This effect was more apparent with sucrose than PEG. GUA, however, slightly increased the level of putrescine (**Figure 5A**) in control berries, but strongly enhanced the levels of spermidine (**Figure 5B**) and spermine (**Figure 5C**). In the same conditions Dap content decreased by about twofold compared to the control (**Figure 5D**). The contents of the four polyamines also increased in PEG- and sucrose-stressed berries pretreated with GUA. The CuAO and PAO activity was also examined in the presence of AG and GUA. It was shown that both inhibitors reduced the enzymatic activity over than 50 and 60% in both control and stressed berries (data not shown). These results emphasize the contribution of the CuAO and PAO pathways in polyamine homeostasis in both control and stressed conditions.

The steady state level of defense responses was then examined in the presence of inhibitors. Data (**Figure 6A**) showed that AG without osmotic stress did not affect the basal level of defense gene transcripts, except for *PR2* which increased by about 2.6-fold compared to control. However, AG treatment followed by osmotic stress strongly lowered the osmo-induced expression of all studied genes. The expression of *STS*, *PR3*, and *PR5* was even repressed to a lower level with AG after sucrose stress. Similar results were obtained with GUA (data not shown). The expression of *NCED2* involved in ABA synthesis followed the same trends after AG and/or osmotic stress application (**Figure 6A**). Data support the idea that polyamine oxidation may play a critical

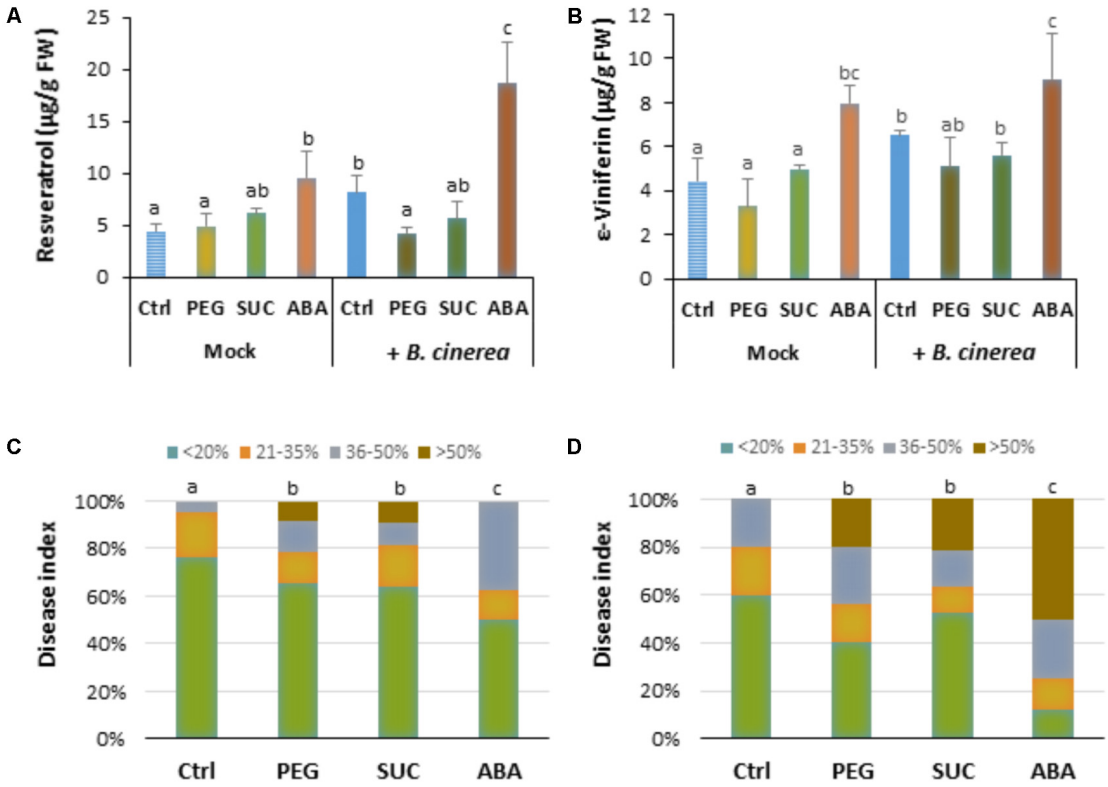

FIGURE 4 | Phytoalexin accumulation and susceptibility of osmotically stressed or ABA-treated grapevine berries to *B. cinerea*. Berries were first treated by dipping the pedicel in control medium supplemented with 400 g L^{-1} PEG 6000 (PEG), 600 mM sucrose (SUC) or 100 µM ABA for 72 h, then drop-inoculated with *B. cinerea* and harvested at 7 dpi. Values of resveratrol **(A)** and ε-viniferin **(B)** are means ± SD of three experiments. Disease rating was evaluated at 7 **(C)** and 10 days post-inoculation **(D)** as the fraction of berries falling into the following classes: (I) spreading lesion of less than 20% of the berry area; (II) spreading lesion of 21–35% of the berry area; (III) spreading lesion of 36–50% of the berry area; (IV) spreading lesion of more than 50% of the berry area. Values are means of the average disease index from 20 to 30 berries from three batches. Different letters indicate significant differences (Duncan's multiple range test, $P < 0.05$).

role in immune response, involving ABA synthesis under osmotic stress condition.

Similarly, AG or GUA alone did not affect the production of resveratrol (**Figure 6B**), but significantly reduced the ε-viniferin level in control berries (**Figure 6C**). However, the AG-treated berries exhibited a significant accumulation of both resveratrol and ε-viniferin after osmotic stress, especially with sucrose. While GUA had only a slight effect on phytoalexin content of sucrose-stressed berries, those stress with PEG showed only enhanced level of ε-viniferin, suggesting that phytoalexin response was at least partly under the control of polyamine oxidation under osmotic stress.

Inhibitors of Polyamine Oxidation Increase the Susceptibility of Osmotically Stressed Berries to *B. cinerea*

The relationship between polyamine oxidation and the susceptibility to *B. cinerea* was then examined by treating berries with AG or GUA prior exposure to sucrose- or PEG-induced osmotic stress and pathogen infection. The percentage of diseased berries was determined at 7 dpi. As shown previously, osmotically stressed berries were highly susceptible to *B. cinerea*, and showed more extensive necrosis than non-stressed ones (**Figure 7**). Pretreatment of berries with AG followed by osmotic stress resulted in a marked amplification of gray mold symptoms with a large proportion of berries heavily infected (**Figure 7**). The disease rating was dramatically increased with approximately more than 60 and 75% of berries heavily infected beyond 35% after sucrose and PEG stress, respectively. Similar results were obtained when berries were pretreated with GUA prior to osmotic stress (data not shown). In the same conditions, levels of polyamines were greatly increased, suggesting that the weakened polyamine oxidation in mature berries under osmotic stress can greatly enhance their susceptibility to the necrotrophic fungus *B. cinerea*.

DISCUSSION

Grapevine (*V. vinifera*) varieties are highly susceptible to various environmental factors and pathogenic fungi, especially at the ripening stages. Up to now research on grapevine has focused on responses to individual stresses, but the interactions between abiotic and biotic stresses and their impact on susceptibility

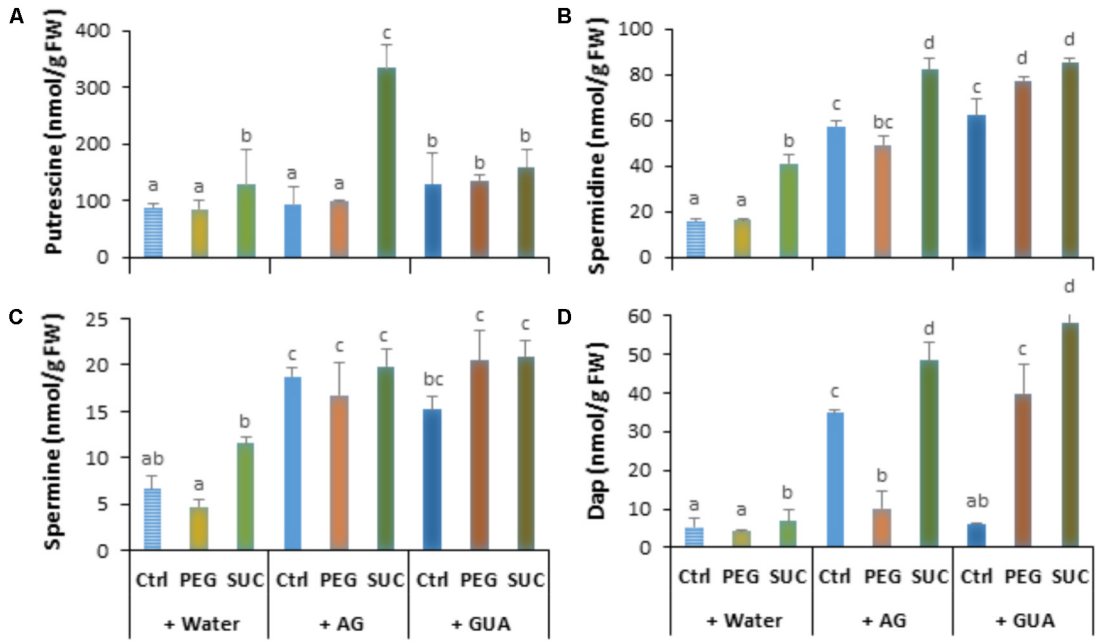

FIGURE 5 | Free polyamine levels in osmotically stressed detached berries pretreated with aminoguanidine (AG) or guazatine (GUA) as inhibitors of CuAO and PAO. Detached berries were pretreated at the level of the pedicel with water, 2 mM AG or 100 μM GUA for 12 h, then transferred to a new medium containing 400 g L^{-1} PEG 6000 (PEG) or 600 mM sucrose (SUC), and harvested at day 3 post-stress. Values of putrescine **(A)**, spermidine **(B)**, spermine **(C)**, and 1,3-diaminopropane **(D)** are means ± SD of three experiments. Different letters indicate significant differences (Duncan's multiple range test, $P < 0.05$).

or resistance to pathogens still remain unknown. Therefore, understanding the mechanisms involved in adaptive response of the grapevine berries and its cross-link with the susceptibility to gray mold is of great interest for the future sustainable viticulture. We previously reported that osmotic stress or ABA attenuated defense responses triggered by *B. cinerea* in grapevine leaves (Hatmi et al., 2014). A close connection between water stress tolerance and the ability of grapevine cultivars to express high defense responses in leaves and then to resist better to the pathogen *B. cinerea* was also reported (Hatmi et al., 2015). These reports emphasize the importance of ABA and polyamine homeostasis in grapevine leaves under osmotic stress as a part of the host-signaling network that affects immune response and disease susceptibility. Using ripe berries, we showed that osmotic stress and ABA potentiate polyamine accumulation but slow down defense responses in ripe berries, especially after *B. cinerea* infection. Pharmacological experiments also provided evidence that inhibition of polyamine oxidation greatly reduced the expression of *PR* genes and *NCED2*, a key gene involved in ABA biosynthesis, and to lesser extent phytoalexin accumulation in osmotically stressed berries.

Osmotic Stress and ABA Induce a Low Expression of Defense Responses in Grapevine Berries

We showed that both osmotic stress and ABA induced a transient but low expression of *PR2* and *PR3* genes, which were responsive to SA in grapevine (Dufour et al., 2013), and *STS1* gene encoding a stilbene synthase, as well as a low accumulation of resveratrol and ε-viniferin. However, the expression of *PR5* encoding a thaumatin-like protein present I a large quantity in mature berries (Monteiro et al., 2003), remained unchanged. This emphasizes the importance of osmotic status in regulating immune response in ripe berries. Osmotic stress and ABA also increased the transcript level of *NCED2* involved in ABA biosynthesis. This is in accordance with other studies showing that exogenous ABA triggered its own biosynthesis and increased ABA concentration in grapevine berries (Giribaldi et al., 2010). This also suggests that, apart from its role in mediating plant responses to osmotic stress, ABA may also have an important role in regulating defense responses in ripened berries and under abiotic stress. It has been shown that ABA can negatively regulate the production of phytoalexins that play a role in plant defense in various plants, and interact with JA, ET, and SA signaling pathways (Audenaert et al., 2002; Anderson et al., 2004) as well as with polyamine metabolism (Alcázar et al., 2010). All these compounds have been associated with plant immune response to pathogens (Glazebrook, 2005; Ton et al., 2009; Hatmi et al., 2014).

Osmotic Stress and ABA Induce Polyamine Accumulation in Grapevine Berries

In the present work, we provide evidence that both osmotic stress and ABA play a key role in the polyamine homeostasis in ripe

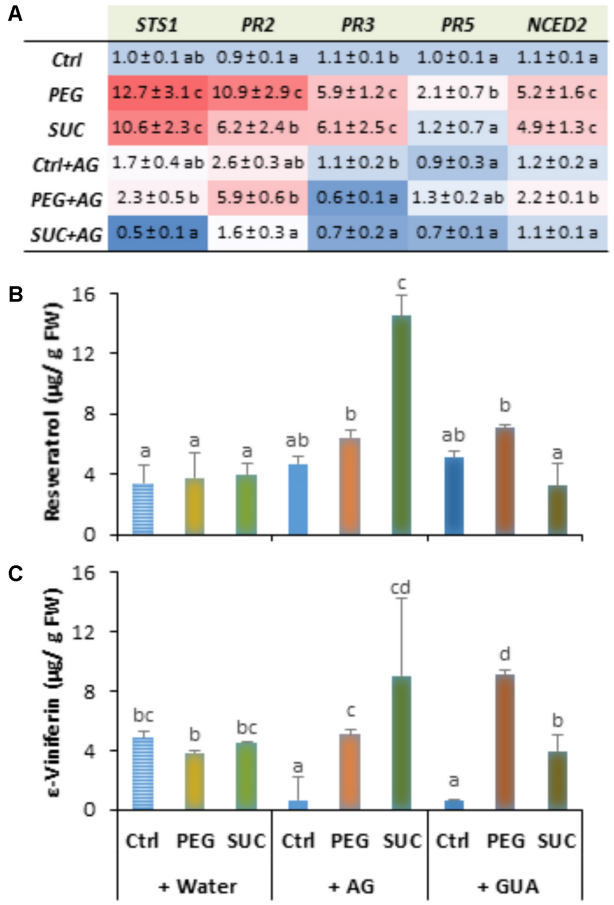

FIGURE 6 | Expression of defense- or stress-related genes and phytoalexin accumulation in osmotically stressed grapevine berries pretreated with aminoguanidine (AG), or guazatine (GUA) as inhibitors of CuAO and PAO, respectively. Detached berries were pretreated at the pedicel level with water, 2 mM AG or 100 µM GUA for 12 h, then transferred to a new medium containing 400 g L^{-1} PEG 6000 (PEG) or 600 mM sucrose (SUC), and harvested at day 3 post-stress. Gene expression **(A)** is expressed as the fold increase in transcript level relative to the corresponding control at 3 days post-stress. *trans*-Resveratrol **(B)** and ε-viniferin **(C)**. Data are means ± SD representative of three experiments. Different letters indicate significant differences (Duncan's multiple range test, $P < 0.05$).

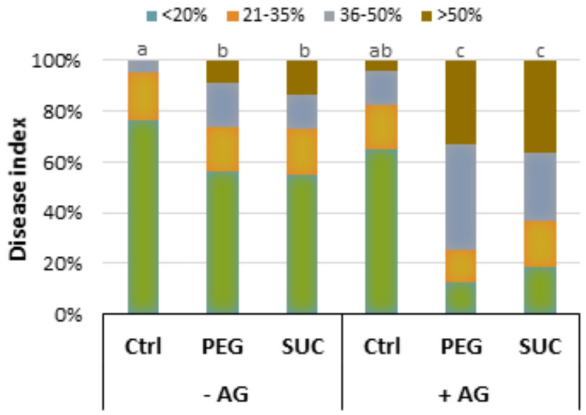

FIGURE 7 | Effect of aminoguanidine (AG), an inhibitor of CuAO, on the osmotic stress-induced susceptibility of grapevine berries to *B. cinerea*. Detached berries were pretreated at the pedicel level with water (–AG) or 2 mM AG (+AG) for 12 h, then transferred to a new medium containing 400 g L^{-1} PEG 6000 (PEG) or 600 mM sucrose (SUC) for 72 h, and inoculated with *B. cinerea*. Disease rating was evaluated at 7 days post-inoculation as the fraction of berries falling into the following classes: (I) spreading lesion of less than 20% of the berry area; (II) spreading lesion of 21–35% of the berry area; (III) spreading lesion of 36–50% of the berry area; (IV) spreading lesion of more than 50% of the berry area. Values are means of the average disease index from 20 to 30 berries from three batches. Different letters indicate significant differences (Duncan's multiple range test, $P < 0.05$).

berries. We showed that the levels of free polyamines greatly enhanced after exposure to osmotic stress induced by PEG or sucrose, or to ABA. The concentration of 1,3-Dap, as an oxidation product of spermidine and spermine, slightly increased in response to PEG and sucrose, but significantly decreased in ABA-treated berries. The level of conjugated polyamines remained unchanged under osmotic stress in ripe berries (data not shown). Despite differential responses of polyamines, these results suggest that both osmotic stress and ABA induced free polyamine accumulation probably by activating their biosynthesis more than their oxidation, which seems poorly activated in stressed berries. However, we cannot exclude the possibility that the levels of polyamines in berries can be regulated by the back-conversion through PAO (Moschou et al., 2008). Similar effects have been observed in *B. cinerea*-infected berries which showed a significant increase of free polyamine titers. This fits well with the observation that polyamine oxidation through CuAO and PAO may play a regulatory role in abiotic stress and immune response (Cona et al., 2006; Yoda et al., 2006; Hatmi et al., 2015). Polyamine oxidation can generate H_2O_2 as a signal molecule in plant tissue mediating the hypersensitive response and the expression of defense genes (Cona et al., 2006; Yoda et al., 2006; Pál et al., 2015). Polyamine pathways are also linked to other important signaling pathways involved in either abiotic stress or disease tolerance, including ET, GABA, nitric oxide, and ABA (Cona et al., 2006; Cuevas et al., 2008).

Osmotic Stress and ABA Potentiate Polyamine Accumulation but Weaken Defense Responses After Infection With *B. cinerea*

We also showed that osmotic stress followed by *B. cinerea* infection primed spermidine and spermine accumulation, but lowered the production of phytoalexins in berries. The potentiated polyamine level could be related to an increase of polyamine synthesis and/or low activation of their oxidation after pathogen challenge. In this study, a weak increase of diamine- and polyamine-oxidase activities was recorded in stressed berries, but declined after pathogen infection. This is consistent with increased level of polyamines in stressed berries after pathogen infection. The potentiated accumulation of polyamines in berries is associated with the weak

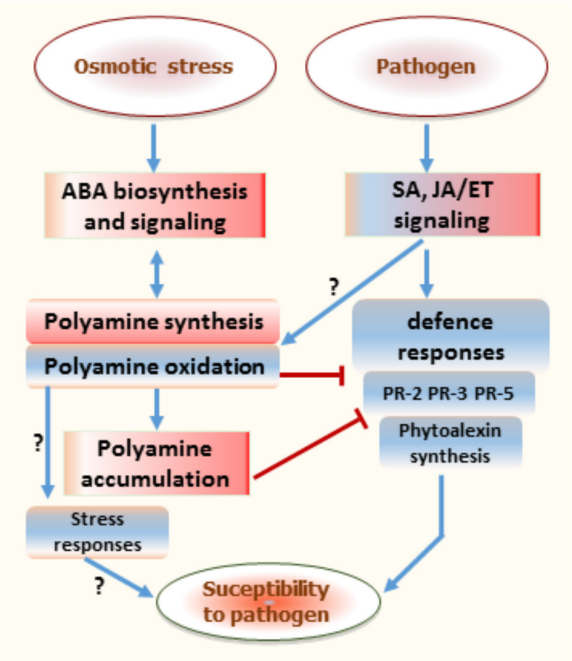

FIGURE 8 | Proposed schematic diagram illustrating the role of polyamine status in regulating the interaction between osmotic stress and subsequent pathogen stress responses. Red color boxes indicate upregulation and blue boxes show downregulation. Blue arrows show induction or positive regulation, while red bars show inhibition or repression of response. ABA, abscisic acid; ET, ethylene; JA, jasmonic acid; SA, salicylic acid; PR, pathogenesis-related.

expression of defense responses after infection, and enhanced susceptibility to *B. cinerea*. These results suggest that a high accumulation of polyamines in osmotically stressed or ABA-treated berries may not be in favor for berries to resist to the necrotrophic fungus. This emphasizes the importance of polyamine level as a critical regulator of both biotic and abiotic stress responses. Accumulation of polyamines can be toxic to berries, while their catabolism through CuAO and PAO could contribute to the adjustment of polyamine levels and thus to disease resistance. It is so conceivable that ABA-induced osmotic stress could be a primary factor predisposing grapevine berries to gray mold (Hatmi et al., 2014). It has also been shown that drought stress or ABA treatment can increase the susceptibility of Arabidopsis to an avirulent strain of *P. syringae*, while in tomato ABA increased susceptibility to *B. cinerea* and *Erwinia chrysanthemi* (Audenaert et al., 2002; Mohr and Cahill, 2003; Seifi et al., 2013). By contrast, ABA has been shown to be necessary for defense against some biotrophic pathogens, and prevent pathogen infection by inducing stomatal closure (Ton and Mauch-Mani, 2004; Adie et al., 2007). Like ABA, polyamines were also shown to interact with JA and ET signaling, thereby enhancing plant susceptibility to pathogens (Anderson et al., 2004). The over accumulation of spermidine in transgenic lines of tomato was shown to be associated with impaired function of ET in induced defense responses, thereby increasing the susceptibility of fruit to *B. cinerea* (Nambeesan et al., 2012).

Inhibition of Polyamine Oxidation Attenuates Defense Responses and Increases Susceptibility of Berries to *B. cinerea*

Pharmacological experiments with appropriate CuAO and PAO inhibitors prior to osmotic stress showed that inhibition of polyamine oxidation greatly enhanced polyamine levels and reduced the expression of defense genes and *NCED2*, as well as the amounts of resveratrol and ε-viniferin to different extents. These effects fit with the observations that the high level of free polyamines induced by osmotic stress in berries is correlated with high sensitivity to *B. cinerea* infection. Different studies showed that upregulation of polyamine oxidation is an important site of metabolic regulation involved in water stress tolerance or in resistance mechanisms toward pathogens (Marina et al., 2008; Moschou et al., 2008, 2012; Alcázar et al., 2010). Our data support the idea that both CuAO and PAO pathways could be required for an optimal level of polyamines, and subsequently for immune response in ripe berries. This is also in accordance with other previous findings (Takahashi et al., 2004; Marina et al., 2008), showing that inhibition of CuAO and PAO repressed the expression of defense-related genes.

The reduced expression of *NCED2* involved in ABA synthesis by CuAO inhibitor and/or osmotic stress suggests a relationship between polyamine oxidation and ABA synthesis under osmotic stress conditions. This is consistent with the amplification of symptoms of gray mold in berries by CuAO and PAO inhibitors prior to osmotic stress. These data highlight the role of polyamine titers in controlling defense responses and susceptibility of ripe berries to *B. cinerea*. Hydrogen peroxide derived from polyamine oxidation (Rea et al., 2002; Walters, 2003) and the balance between polyamine and ET synthesis (Nambeesan et al., 2012) may also be involved a major regulators of defense process.

Overall, exposure of grapevine berries to osmotic stress prior pathogen infection may result in a complex interaction leading to enhanced disease susceptibility (**Figure 8**). Data from this study may suggest that osmotic stress or ABA slow down the expression of defense responses and then facilitate gray mold in ripe berries. The combined osmotic stress and *B. cinerea* challenge primed berries for enhanced polyamine accumulation, which is in part related to weak activation of their oxidation. This emphasizes the importance of polyamine level or their oxidative pathways as critical regulators of both osmotic stress signaling and triggered defense responses against pathogen. Under osmotic stress polyamines can interact with ABA synthesis and interfere with defense responses in grapevine berries, especially after pathogen infection. It is hypothesized that polyamine oxidation can benefit grapevine fitness by providing the trade-off between osmotic stress response and disease susceptibility.

SIGNIFICANCE STATEMENT

Osmotic stress and ABA affect immune response in grape berries, while polyamine level may have a role in weakening plant defense and resistance to the necrotrophic pathogen *B. cinerea*.

AUTHOR CONTRIBUTIONS

SH and SV performed most of the experiments. PT-A provided substantial help in experiments. AA and SH designed the research, wrote the manuscript with contributions, and discussion from all the co-authors. All authors read 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.2018.01010/full#supplementary-material

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GhSNAP33, a t-SNARE Protein From *Gossypium hirsutum*, Mediates Resistance to *Verticillium dahliae* Infection and Tolerance to Drought Stress

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Soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins mediate membrane fusion and deliver cargo to specific cellular locations through vesicle trafficking. Synaptosome-associated protein of 25 kDa (SNAP25) is a target membrane SNARE that drives exocytosis by fusing plasma and vesicular membranes. In this study, we isolated *GhSNAP33*, a gene from cotton (*Gossypium hirsutum*), encoding a SNAP25-type protein containing glutamine (Q)b- and Qc-SNARE motifs connected by a linker. *GhSNAP33* expression was induced by H_2O_2, salicylic acid, abscisic acid, and polyethylene glycol 6000 treatment and *Verticillium dahliae* inoculation. Ectopic expression of *GhSNAP33* enhanced the tolerance of yeast cells to oxidative and osmotic stresses. Virus-induced gene silencing of *GhSNAP33* induced spontaneous cell death and reactive oxygen species accumulation in true leaves at a later stage of cotton development. *GhSNAP33*-deficient cotton was susceptible to *V. dahliae* infection, which resulted in severe wilt on leaves, an elevated disease index, enhanced vascular browning and thylose accumulation. Conversely, *Arabidopsis* plants overexpressing *GhSNAP33* showed significant resistance to *V. dahliae*, with reduced disease index and fungal biomass and elevated expression of *PR1* and *PR5*. Leaves from *GhSNAP33*-transgenic plants showed increased callose deposition and reduced mycelia growth. Moreover, *GhSNAP33* overexpression enhanced drought tolerance in *Arabidopsis*, accompanied with reduced water loss rate and enhanced expression of *DERB2A* and *RD29A* during dehydration. Thus, GhSNAP33 positively mediates plant defense against stress conditions and *V. dahliae* infection, rendering it a candidate for the generation of stress-resistant engineered cotton.

Keywords: cotton (*Gossypium hirsutum*), GhSNAP33, *Verticillium dahliae*, drought stress, transgenic *Arabidopsis*, resistance

INTRODUCTION

Eukaryotic cells including those of plants contain membrane-enclosed organelles that communicate through vesicle trafficking and exchange (Steegmaier et al., 1998; Jahn and Scheller, 2006). This process delivers cargo to specific locations in the cell via a membrane fusion event such as exo- or endocytosis, and is critical for cell growth and division and for maintaining the spatial organization of biochemical reactions (McNew et al., 2000; Bonifacino and Glick, 2004). Soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins mediate membrane fusion at each step of the secretory pathway (Bock et al., 2001; Jahn and Scheller, 2006). Selective membrane fusion is achieved through interactions between SNAREs located on vesicles and on target membranes (v- and t-SNAREs, respectively) (McNew et al., 2000). A typical SNARE complex contains glutamine (Q)a-, Qb-, and Qc- and arginine (R)-SNARE motifs that forms a tetrameric bundle of coiled helices (Ernst and Brunger, 2003; Bassham and Blatt, 2008).

Synaptosome-associated protein of 25 kDa (SNAP25)-type proteins are the best known isoform of the t-SNARE subfamily and mediate the fusion of vesicles with the plasma membrane during exocytosis (Fukuda et al., 2000). These proteins contain Qb and Qc SNARE domains connected via an anti-parallel linker (Steegmaier et al., 1998; Wang et al., 2008). SNAP25-type proteins play important roles in various organisms. The yeast homologs Sec9 and Spo20 function in secretion and sporulation, respectively (Fukuda et al., 2000; Strop et al., 2008). Of the four mammalian SNAPs (SNAP23, SNAP25, SNAP29, and SNAP47) (Holt et al., 2006), SNAP25 is a component of the synaptic SNARE complex that mediates synaptic vesicle fusion and exocytosis (Nagy et al., 2008). SNAP23 regulates phagosome formation and maturation in macrophages, with its loss delaying maturation of and reducing uptake by phagosomes (Sakurai et al., 2012). Loss of SNAP29 impairs endocytic recycling and cell motility, resulting in cerebral dysgenesis, neuropathy, ichthyosis, and keratoderma syndrome (Rapaport et al., 2010). SNAP47 is widely distributed on intracellular membranes of neurons and can replace SNAP-25 in SNARE complex formation (Holt et al., 2006). PtSNAP, the *Paramecium* homolog of metazoan SNAP-25, shows several divergent features including resistance to cleavage by botulinum neurotoxins (Schilde et al., 2008). SNAP25 depletion in the Gulf Coast tick impaired feeding and engorgement and prevented oviposition (Browning and Karim, 2013). AtSNAP33 is the first characterized SNAP25-type protein in plants and regulates cytokinesis in *Arabidopsis* via interaction with KNOLLE (Heese et al., 2001), and is also involved in the response to pathogens and mechanical stimulation (Wick et al., 2003). The SNARE family proteins penetration (PEN)1, SNAP33, and vesicle-associated membrane protein (VAMP)721/722 constitute an immune secretory pathway in plant defense that modulates immune responses through focal secretion (Collins et al., 2003; Kwon et al., 2008b; Yun et al., 2016; Yun and Kwon, 2017). *OsSNAP32* is involved in the response to polyethylene glycol (PEG) 6000 and low temperature stress and may enhance rice resistance against blast fungus (Bao et al., 2008b; Luo et al., 2016). HvSNAP34, a SNAP25-type protein in barley, associates with syntaxin (ROR2) and contributes to powdery mildew resistance (Collins et al., 2003). *Cynanchum komarovii CkSNAP33* enhances *Arabidopsis* disease resistance to *Verticillium dahliae* (Wang et al., 2017), and *Glycine soja GsSNAP33* increases tolerance to salt and drought stress in transgenic *Arabidopsis* (Nisa et al., 2017).

Cotton is a widely grown agricultural and industrial crop of considerable economic value in the textile industry (Sunilkumar et al., 2006; Gao et al., 2011). Significant effort has been expended to increase the sustainable yield and quality of cotton by improving plant cultivars and cultivation practices (Hill et al., 1999; Wang et al., 2011a; Zhang et al., 2012; Zhao et al., 2012). The recent availability of genome sequences not only provides genetic resources to study complex genome evolution, but also allows the exploitation of genetic resources for improvement of cotton agricultural performance under conditions of biotic and abiotic stress (Wang et al., 2012; Li et al., 2014, 2015; Zhang T. et al., 2015). Additionally, the development of *Agrobacterium*-mediated virus-induced gene silencing (VIGS) has facilitated the investigation of cotton gene function and has contributed to the dissection of the stress response in cotton (Gao et al., 2011; Gao X. et al., 2013; Cox et al., 2017).

Verticillium wilt caused by the soil-borne fungal pathogen *V. dahliae* Kleb is among the most prevalent and lethal diseases in cotton (Daayf et al., 1995; Fradin and Thomma, 2006; Klosterman et al., 2009). *V. dahliae* colonizes the plant through young, uninjured roots or puncture wounds to the xylem and causes browning of the vasculature, leaf discoloration, wilting, and defoliation (Garas et al., 1986). Verticillium wilt is difficult to control due to the viability and persistence of *V. dahliae* microsclerotia in soil (Fradin and Thomma, 2006) and shortage of resistance cotton germplasms (Yang et al., 2008). It has been reported that cotton phenylpropanoid pathway, terpenoid pathway, salicylic acid, reactive oxygen species and jasmonic acid signaling pathways are important contributors to *V. dahliae* response (Ashraf et al., 2018). In addition, many other cotton genes were shown to be required for resistance to *V. dahliae*, such as *GhHb1* (Qu et al., 2005), *GhNDR1*, *GhMKK2* (Gao et al., 2011), *GhPGIP* (Liu et al., 2017), *GhPMEI3* (Liu et al., 2018), and receptor like protein or kinase genes *GbaVd1* and *GbaVd2* (Chen et al., 2017), *GhBAK1* (Gao X. et al., 2013), *Gh-LYK1* and *Gh-LYK2* (Gu et al., 2017). Among different abiotic stresses, drought stress is a major factor affecting cotton production. Many studies have examined the genetic basis of the drought stress response (Li et al., 2017; Ma et al., 2017) and ways to enhance cotton drought tolerance (Guo et al., 2017; Mishra et al., 2017; Zahoor et al., 2017). Meanwhile, Several drought-related genes including transcription factors *GhWRKY59* (Li et al., 2017), *GhDERB2* (Li et al., 2017), *GhERF38* (Ma et al., 2017), *GhNAC79* (Guo et al., 2017) and *GhABF* (Kerr et al., 2017) and *GhAnn1* (Zhang F.et al., 2015) have been reported in cotton. However, none of vesicle trafficking related genes has been characterized in cotton so far and their contributions to cotton stress and disease defense responses remains elusive.

To this end, in the present study we isolated *GhSNAP33*—the first SNARE family gene identified in cotton (*Gossypium hirsutum*)—encoding a SNAP25-type protein. *GhSNAP33*

expression in response to various types of stress was characterized in cotton and via ectopic expression in yeast (*Saccharomyces cerevisiae*). The VIGS assay was used to evaluate the function of *GhSNAP33* in cotton development and defense against *V. dahliae* infection. We also evaluated the role of *GhSNAP33* in disease resistance and drought tolerance in transgenic *Arabidopsis* plants. These findings advance our understanding of the function plant SNAP25-type protein in both biotic and abiotic stress and may facilitate the development cotton with improved adaptability to different environment.

MATERIALS AND METHODS

Plant, Yeast, and *V. dahliae* Cultivation

Gossypium hirsutum L. cultivar Zhongzhiming 2 (Verticillium wilt-resistant upland cotton) seeds were provided by the Cotton Research Institute, Chinese Academy of Agricultural Sciences and germinated in pots filled with a mixture of soil and vermiculite (2:1, w/w) in a growth chamber under 16-h light (25°C)/8-h dark (22°C) conditions. *Arabidopsis* seeds (Columbia ecotype) were used in this study. After vernalization for 3 days at 4°C, the seeds were geminated in pots containing a mixture of soil and vermiculite (1:1, w/w) in a chamber under 16-h light (22°C)/8-h dark (20°C) conditions. *S. cerevisiae* strain INVSC1 (genotype MATα-ahis3Δ1 leu2 trp1-289, ura3-52) was used as the yeast host cell. The highly aggressive defoliating isolate Vd991 of *V. dahliae* was cultured on potato dextrose agar at 25°C for 7 days, and then inoculated in Czapek liquid medium. After 7 days, the suspension was harvested by filtration through four layers of cheesecloth and adjusted to a concentration of 10^6 conidia per mL for inoculation.

Gene Cloning and Sequence Analyses

Total RNA was isolated from cotton seedlings using a kit (Promega, Madison, WI, United States) according to the manufacturer's instructions. The PolyATract mRNA Isolation System (Promega) was used to generate polyadenylated mRNA. The cDNA library was prepared as previously described (Wang et al., 2011b; Liu et al., 2017) and propagated on 140 mm plates to obtain about 10^6 clones. The conserved region of SNAP33 (Heese et al., 2001; Wang et al., 2017) was used as a probe to screen for positive clones (Liu et al., 2016; Wang et al., 2017).

The theoretical isoelectric point (pI) and molecular mass were calculated with ProtParam[1]. A transmembrane hidden Markov model (TMHMM) analysis of the transmembrane domain was performed using the TMHMM online tool[2]. Multiple amino acid sequence alignment was performed with Clustal Omega[3], and the multiple alignment file was shaded with BoxShade[4]. A motif analysis was performed using the National Center for Biotechnology Information (NCBI) conserved domain search program[5]. A phylogenetic tree was constructed with the neighbor-joining method using MEGA 6, with bootstrap values from 1000 replicates indicated at the nodes.

Analysis of *GhSNAP33* Expression

Two-week-old cotton seedlings were gently uprooted from soil and cleaned with water for treatment. The seedlings were placed in 10% (w/v) PEG, 100 μM abscisic acid (ABA), 1 mM salicylic acid (SA), or 10 mM H_2O_2 solution or inoculated with *V. dahliae*. For pathogen treatment, seedling roots were inoculated with a *V. dahliae* conidial suspension for 3 min and the seedlings were transplanted into pots with fresh soil. Control samples were treated with sterile water. Three plants were combined for RNA isolation at each time point of each treatment condition. The experiment was repeated three times.

Real-time PCR was performed to detect transcript levels of *GhSNAP33* using SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara Bio, Dalian, China) on an ABI 7500 thermocycler (Applied Biosystems, Foster City, CA, United States) under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. A pair of primers (qGhSNAP33-F and qGhSNAP33-R) was designed to amplify a fragment of the *GhSNAP33* gene, and *GhUBQ7* (DQ116441) was used as an internal reference gene that was amplified with primers qUBQ-F/qUBQ-R [26]. The relative transcript level of *GhSNAP33* was calculated with the comparative $2^{-\Delta\Delta Ct}$ method.

The Generation of Transgenic Plants

The full-length *GhSNAP33* was cloned using primers ZW33-F/ZW33-R with *Xba*I/*Sal*I restriction sites at the 5′ and 3′ ends, respectively. The resultant PCR fragment was inserted into a modified pCAMBIA 1300 vector harboring a hygromycin phosphotransferase (*hptII*) gene and the green fluorescent protein (GFP) gene under the control of the super promoter (Wang et al., 2011b). The vector was introduced into *Arabidopsis* Columbia ecotype via *Agrobacterium tumefaciens* (strain GV3101)-mediated transformation. Transgenic *Arabidopsis* seeds were screened on MS plates containing 25 μg/mL hygromycin B and the genomic DNA was extracted for verification by PCR using the vector-specific primers 1300-F/1300-R. Semi-quantitative RT-PCR was performed to confirm *GhSNAP33* expression. The primers are listed in Supplementary Table S1.

Yeast Transformation and Stress Tolerance Assays

The *GhSNAP33* gene was amplified by PCR with primers pYES-GhSNAP33-F/pYES-GhSNAP33-R and introduced into the pYES2.0 vector between the *Eco*RI/*Xho*I restriction sites. *S. cerevisiae* strain INVSC1 was transformed with the pYES2.0 and pYES-GhSNAP33 plasmids using the lithium acetate method (Lee et al., 1999; Bao et al., 2008a; An et al., 2011). Total RNA was isolated from the cells and *GhSNAP33* expression was evaluated by semi-quantitative RT-PCR. The growth rate of transformed

[1]http://web.expasy.org/protparam/

[2]http://www.cbs.dtu.dk/services/TMHMM-2.0

[3]http://www.ebi.ac.uk/Tools/msa/clustalo/

[4]http://www.ch.embnet.org/software/BOX_form.html

[5]https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

yeast cells was monitored under various conditions as previously described (An et al., 2011). Briefly, the cells were cultured overnight and the medium was changed to SC-ura plus 2% galactose for 24 h. The optical density at 600 nm was adjusted at 0.4 with medium containing 100 mM H_2O_2 or 0.75 M mannitol, followed by culturing for 24 h. 3 μL serial diluted cells were spotted onto SC-ura agar medium. The assay was repeated at least three times with similar results.

Agrobacterium-Mediated VIGS

Total RNA was isolated from cotton seedlings using EASYspin Fast Plant RNA kit (Biomed, Beijing, China) according the manufacturer's instructions. First-strand cDNA was synthesized using the FastQuant RT kit (Tiangen, Beijing, China). Fragments of *GhCLA1* and *GhSNAP33* were amplified from cotton cDNA and inserted into the pTRV2 vector by ligation-independent cloning (Dong et al., 2007). The ligation product was transformed into *Escherichia coli* DH5α cells. Plasmids from positive transformants were tested by PCR analysis and sequencing.

The pTRV1 (pYL192), pTRV2-*GhCLA1*, and pTRV2-*GhSNAP33* plasmids were transformed into *A. tumefaciens* strain GV310 by heat shock. *Agrobacterium* clones positive for pTRV1, pTRV2-*GhCLA1*, or pTRV2-*GhSNAP33* were inoculated in Luria–Bertani (LB) broth supplemented with 50 mg/L kanamycin and 50 mg/L rifampicin and cultured overnight at 28°C. The culture was expanded in fresh LB medium containing 50 mg/L kanamycin, 50 mg/L rifamycin, 10 mM 2-(*N*-morpholino) ethanesulfonic acid, and 20 μM acetosyringone. Bacterial collection, pretreatment, and infiltration were carried out as previously described (Gao et al., 2011).

The efficiency of *GhSNAP33* and *GhCLA1* silencing was evaluated by RT-PCR. Two weeks after *Agrobacterium* infiltration—i.e., when *GhCLA1*-silenced plants showed signs of albinism—the second true leaf of each plant was harvested for RNA isolation. The vGh33F/R and vGhCLA1 F/R primers were used for RT-PCR; *GhUBQ7* from cotton was amplified as an internal control with primers qUBQ-F and qUBQ-R. The experiment included three biological repeats.

Histochemical Analysis of H_2O_2 Production, Cell Death and Callose Deposition

3,3′-Diaminobenzidine (DAB) was used to detect H_2O_2 in cotton leaf tissue (Xiao et al., 2003; Gao X. et al., 2013). Detached leaves were incubated in 1 mg/mL DAB-HCl (pH 3.8) (Sigma-Aldrich, St. Louis, MO, United States) in the dark for 8 h. The leaves were fixed and cleared by boiling in alcoholic lactophenol (95% ethanol: lactophenol, 2:1 v/v) for 20 min. Trypan blue staining was performed as previously described to detect cell death in leaves (Gao X. et al., 2013; Liu et al., 2017). Briefly, detached leaves were stained by boiling in lactophenol-Trypan Blue (10 mL lactic acid, 10 mL glycerol, 10 g phenol, and 40 mg trypan blue dissolved in 10 mL distilled water) and then immersed in choral hydrate solution (250% w/v) to remove chlorophyll. The stained leaves were observed and images of cotton leaves were acquired with a Nikon digital camera. Callose deposition on *Arabidopsis* was stained by aniline blue as previous (Meyer et al., 2009). For *Arabidopsis* leaves, cell death and mycelia growth were observed and imaged under an optical microscope (Nikon ECLIPSE Ti, Tokyo, Japan) and callose deposition were imaged on fluorescence microscopy (Nikon C1). At least eight leaves from each VIGS cotton or *Arabidopsis* plants were evaluated, and the experiment was repeated at least three times.

Inoculation of *V. dahliae* and Disease Detection

Three weeks after *Agrobacterium*-mediated VIGS, true leaves of *GhCLA1*-silenced cotton plants showed clear signs of albinism. The plants were inoculated with *V. dahliae* as previously reported (Gao W. et al., 2013; Zhang et al., 2017). Briefly, the seedlings were uprooted from the soil and their roots were immersed in the *V. dahliae* spore suspension (1×10^6 conidia/mL) for 3 min. The seedlings were then replanted in soil and cultured in a moist growth chamber. The disease index was calculated based on three repeats, each comprising at least 15 plants. Two weeks after inoculation, slices of fresh stems approximately 1 cm below the cotyledon were collected and examined under an optical microscope (ECLIPSE Ti; Nikon).

The disease phenotype of transgenic *Arabidopsis* plants was examined. Three-week-old transgenic and wild-type (WT) *Arabidopsis* plants were inoculated with *V. dahliae* spores as previously described (Gao X. et al., 2013). The disease index and fungal biomass were calculated (Wang et al., 2017). The primers used for the quantification of *V. dahliae* were qVd-F/qVd-R. Real-time PCR was performed as previous to determine the transcription level of the genes pathogenesis-related protein 1 (*PR1*) and pathogenesis-related protein 5 (*PR5*) in *V. dahliae* infected *Arabidopsis* at 6 dpi. The specific primers for *PR1* and *PR5* were qPR1-F/qPR1-R and qPR5-F/qPR5-R, respectively. *AtEF1-α* (NM_100666.3) was used as the endogenous control and was detected using the primer pair AtEF1α-F/AtEF1α-R (the sequences of the primers mentioned here are listed in Supplementary Table S1).

The fungal filtrate assay was performed as reported (Thatcher et al., 2009; Chen et al., 2016), with minor modification. Leaves detached from 3-week-old *Arabidopsis* plants were placed adaxial side up on moist filter paper in Petri dishes and 5 μL of *V. dahliae* spores suspension (10^7 conidia/mL) were applied to each leaf. The dishes were sealed with Parafilm and incubated at 25°C in a moist chamber. Trypan Blue staining was performed to visualize *V. dahliae* mycelia and assess cell death at 6 dpi. Callose deposition was detected at 24 h post inoculation.

Transmission Electron Microscopy (TEM)

For TEM observation of thylose accumulation, the *V. dahliae*-infected control and *GhSNAP33*-silenced cotton roots were washed with distilled water and sliced vertically into less than 1 mm thick. These slices were fixed immediately in 2.5% glutaraldehyde, washed with 0.1 M PBS (pH 7.4) buffer and post-fixed with 1% osmium tetroxide. After dehydration with a graded acetone series (30, 50, 80, 90, and 100%), the slices were embedded in Spurr's resin mixture. Ultrathin serial sections

were cut from resin blocks, followed by uranyl acetate and lead citrate staining. The samples were sectioned for TEM analysis with JEM-1230 electron microscope (JEOL, Ltd., Japan).

Plant Drought Treatment

Arabidopsis drought treatment was carried out as previously described (Shi et al., 2013; Li et al., 2017). Briefly, water was withheld from 3-week-old *Arabidopsis* plants for 20 days, followed by watering for 2 days; the survival rate was then recorded. The experiment was repeated three times with 18 plants each.

For the water loss test, detached aerial parts of 3-week-old *Arabidopsis* seedlings were placed on filter paper on a bench in the growth chamber. The decrease in fresh weight was monitored at indicated times (Liu et al., 2013; Li et al., 2017). The DREB2A and RD29A transcript level was testes by real-time PCR in *Arabidopsis* plants at 1 h after dehydration. The assay was repeated with at least three different batches, with each line containing 10 plants as one set.

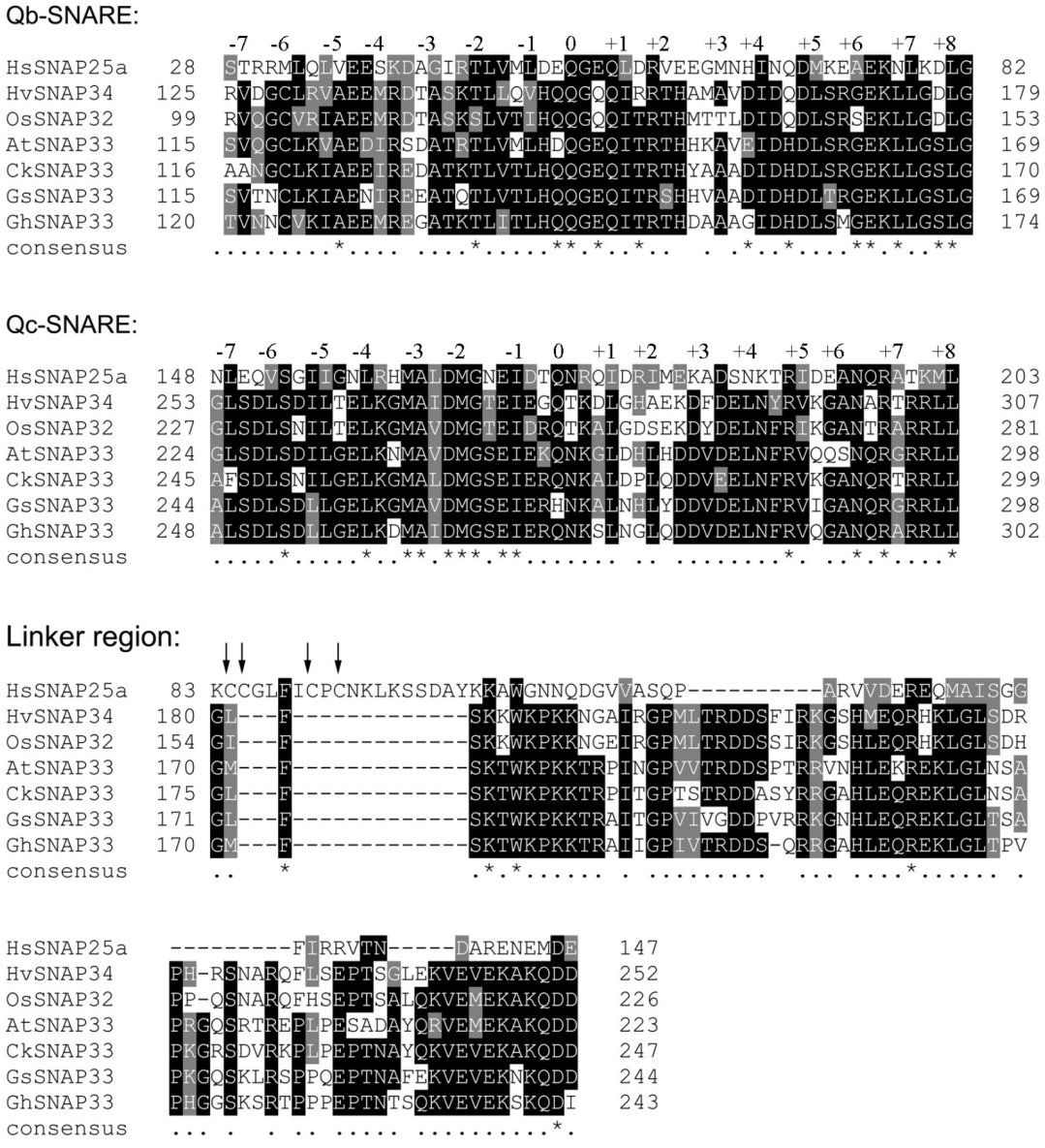

FIGURE 1 | Alignment of conserved domains of GhSNAP33 and other SNAP25-type t-SNARE proteins. HsSNAP25a (AAH10647.1) from *Homo sapiens*, OsSNAP32 (AAW82752.1) from *Oryza sativa*, HvSNAP34 (AAP79417.1) from *Hordeum vulgare*, AtSNAP33 (Q9S7P9.1) from *Arabidopsis thaliana*, *CkSNAP33* (KR011961) from *Cynanchum komarovii* and GsSNAP33 from *Glycine soja*. Conserved and similar residues are shaded in black and gray, respectively. The well conserved heptad repeat layers –7 to +8 are indicated. The four cysteine residues involved in palmitoylation of SNAP25 are indicated using arrow. The asterisks (*) and dots (.) under the sequence are indicated the completely conserved and highly conserved amino acids, respectively. Multiple amino acid sequence analyses were performed using Clustal Omega and the multiple alignment file was shaded using the BoxShade program.

RESULTS

Characterization of GhSNAP33

A synaptosome-associated protein was isolated from *G. hirsutum* by colony *in situ* hybridization and named GhSNAP33 (GenBank accession number: KR011955). The DNA sequence of GhSNAP33 was 2281 bp with five exons and four introns (Supplementary Figure S1A) and a 915 bp open reading frame encoding a 305 amino acid protein (Supplementary Figure S1B) with a theoretical pI of 6.27 and molecular weight of 33.75 kDa. GhSNAP33 had no transmembrane domain or signal peptide. An analysis of the functional domains of GhSNAP33 with other known SNAP25-type proteins revealed that a conserved Qb-SNARE was located at amino acids 120–174 and Qc-SNARE domain was located at amino acids 248–302 of the C terminus and a relatively conserved linker region among all the SNAP25 protein from plants (**Figure 1**). Q145 and Q273 of GhSNAP33 contributed to the formation of the zero ionic layer and heptad repeat layers engaged in a hydrophobic interaction with Qa- and R-SNARE (**Figure 1**). Different from the HsSNAP25a, neither of these plant SNAP25 contains palmitoylation sites (**Figure 1**).

A phylogenic analysis of several functional SNAP25-type proteins showed that GhSNAP33 formed a cluster with GsSNAP33 and had a close genetic relationship to CkSNAP33 (**Figure 2**) indicating that they may have similar function. A conserved domain analysis confirmed that all the SNAP25-type homolog contains tandem Qb- and Qc-SNARE motifs comprising 64–65 and 59 amino acids, respectively. The linker region is different on the account of species, plant SNAP25-type protein linker region is comprising of 70–71 amino acids; linker of Sosec9 and Scspo20p from yeast is 89 and 81 amino acids; however, the linker region in HsSNAp25 is shorter with 60 amino acids (**Figure 2**). The N-terminal region of HsSNAP25 is much shorter and that of the two proteins from yeast is than those of plant SNAP25 homologs (**Figure 2**).

GhSNAP33 Transcription Is Activated by Various Stresses

GhSNAP33 was most highly expressed in cotton leaf, with higher levels observed in the root than in the stem (**Figure 3A**). The change in *GhSNAP33* expression in response to various stresses was investigated. *GhSNAP33* transcription was induced with PEG6000, ABA, SA, or H_2O_2 treatment and plants were infected with *V. dahliae* at different time points. Treatment with 10% (w/v) PEG6000 markedly increased gene expression at 1 and 3 h (**Figure 3B**). *GhSNAP33* expression was upregulated at 24 and 72 h after *V. dahliae* infection (**Figure 3C**). *GhSNAP33* transcription was slightly increased in the presence of 10 mM H_2O_2 at 0.5 h and then declined to a normal level before increasing at 30 h (**Figure 3D**). *GhSNAP33* level was highly increased at 9 and 24 h of treatment with 100 μM ABA (**Figure 3E**), whereas 1 mM SA caused *GhSNAP33* expression to increase at 12 h, with a maximum level observed at 30 h (**Figure 3F**).

GhSNAP33 Transformed Yeast Exhibits Stress Tolerance

GhSNAP33 expression was confirmed in transformed yeast cells by semi-quantitative RT-PCR; cells transformed with the empty vector pYES2.0 served as a control (**Figure 4A**). Serially diluted cultures showed no differences in colony density between *GhSNAP33*-transformed and control cells (**Figure 4B**). Cells expressing *GhSNAP33* showed an increase in colony density following treatment with 1 mM H_2O_2 as compared to the control (**Figure 4C**). In the presence of 0.5 M mannitol, *GhSNAP33* transformants had a higher colony density (**Figure 4D**). These results demonstrate that ectopic *GhSNAP33* expression enhances yeast tolerance to H_2O_2 and mannitol, suggesting that GhSNAP33 enables yeast cells to adapt to oxidative and osmotic conditions.

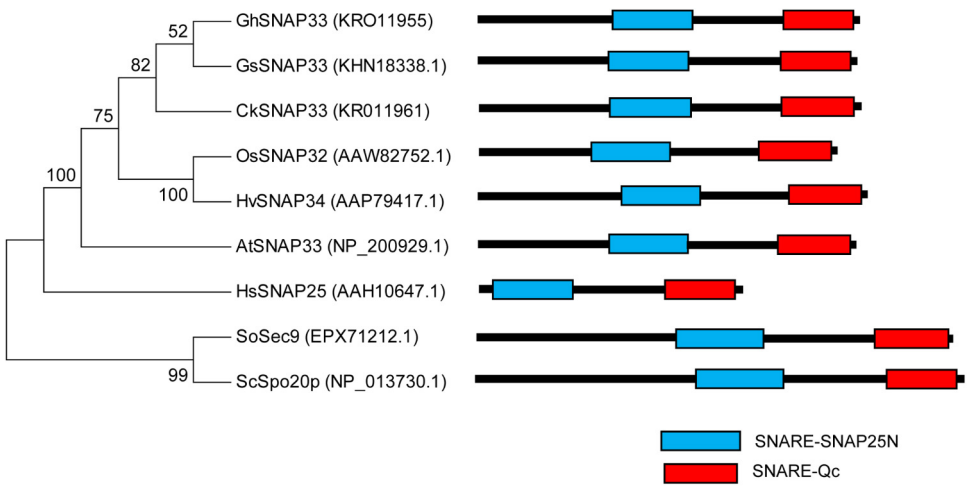

FIGURE 2 | Phylogenetic analysis and domain structures of GhSNAP33 and known SNAP25-type proteins. A phylogenetic tree was constructed using the neighbor-joining method in MEGA v.6.06 and bootstrap values from 1,000 replicates are indicated at the nodes. The conserved SNARE domain was predicted via NCBI CDD search.

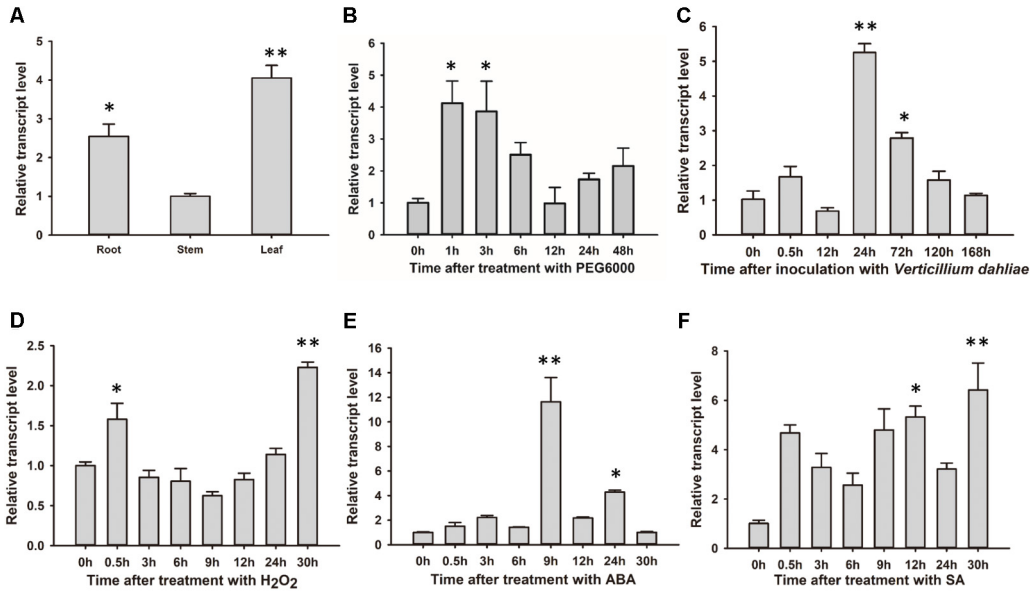

FIGURE 3 | *GhSNAP33* mediates crosstalk between various stress responses in cotton. **(A)** Tissue-specific expression of GhSNAP33 in cotton. **(B–F)** GhSNAP33 expression in the presence of 10% (w/v) PEG 6000 **(B)**, following *V. dahliae* inoculation **(C)**, and upon treatment with 10 mM H_2O_2 **(D)**, 100 μM ABA **(E)**, or 1 mM salicylic acid **(F)**. Results were pooled from three independent biological replicates. Data are shown as mean ± standard error of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ vs. control.

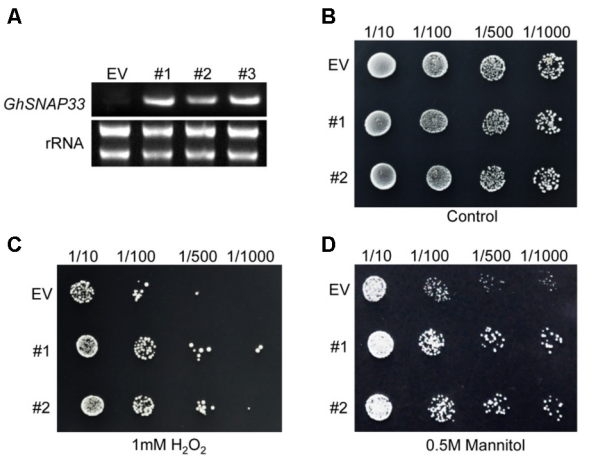

FIGURE 4 | Stress tolerance in yeast cells overexpressing *GhSNAP33*. **(A)** RT-PCR analysis of GhSNAP33 expression in yeast cells. EV, yeast cells transformed with the pYES2.0 empty vector; #1, #2, and #3, yeast cell lines transformed with the pYES2.0-GhSNAP33 vector. **(B)** Growth serially diluted yeast cells (1/10, 1/100, 1/500, and 1/1000 dilutions). **(C)** Growth of yeast cells (1/10, 1/100, 1/500, and 1/1000 dilutions) after 1 mM H_2O_2 treatment. **(D)** Growth of yeast cells (1/10, 1/100, 1/500, and 1/1000 dilutions) after 0.5 M mannitol treatment. These assays were repeated three times with similar results.

GhSNAP33 Is Involved in Cotton Development

To clarify the role of GhSNAP33 in cotton development, we generated VIGS-*GhSNAP33* cotton seedlings. Expression of *CLOROPLASTOS ALTERADOS* (*GhCLA1*), a gene involved in chloroplast development (Gao et al., 2011), was used as a visual marker to monitor VIGS efficiency. The semi-quantitative RT-PCR confirmed that transcription of *GhSNAP33* and *GhCLA1* was reduced at 2 weeks after VIGS (**Figure 5B**).

At 2 weeks post infiltration, *GhSNAP33*-slienced plants were smaller than controls (**Figure 5A**) and senior true leaves became abnormally curved and had black spots at 4 weeks (**Figure 5C**). Trypan blue staining revealed an increase in the number of blue dots in second true leaves of *GhSNAP33*-inoculated cotton, while nearly no dots were observed on the leaves of control plant, indicated that *GhSNAP33* silencing triggered leaf cell death (**Figure 5D**). In addition, the second and third true leaves of *GhSNAP33*-deficient plants showed elevated reactive oxygen species (ROS) levels compared to control leaves, as determined by DAB staining (**Figure 5E**).

GhSNAP33-Deficient Cotton Plants Are Susceptible to *V. dahliae* Infection

To analyze the role of *GhSNAP33* in the defense response of cotton against *V. dahliae*, *GhSNAP33*-silenced seedlings were inoculated with *V. dahliae*. Loss of *GhSNAP33* resulted in exacerbation of wilting relative to control plants (**Figure 6A**); the leaves became wilting along the edge of leaves at 12 day post inoculation (dpi) (**Figure 6B**). The disease index was also increased in these plants (**Figure 6C**). Additionally, deepened vascular browning (**Figure 6D**) and increased thylose accumulation (**Figure 6E**)

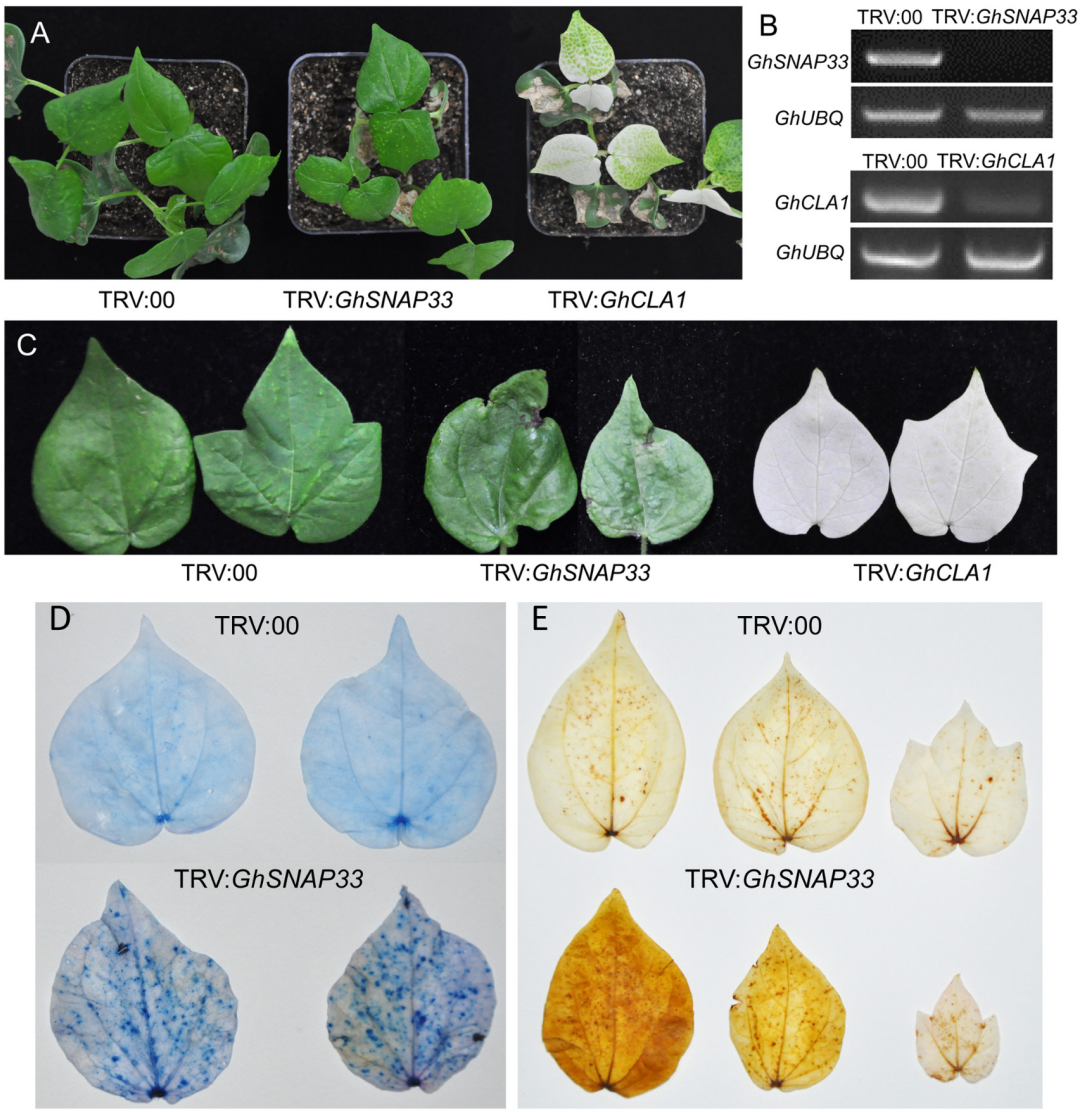

FIGURE 5 | Cell death and ROS production induced by *GhSNAP33* silencing. **(A)** Growth of cotton plants 2 weeks after *Agrobacterium*-mediated VIGS. **(B)** Evaluation of *GhSNAP33* knockdown efficiency by semi-quantitative RT-PCR. GhUBQ was served as a control. **(C)** Cotton leaves from VIGS-deficient plants 4 weeks after *Agrobacterium* infiltration. **(D)** Cell death in true leaves induced by *GhSNAP33* silencing, as determined by trypan blue staining. Leaves were detached and stained 4 weeks after VIGS. **(E)** GhSNAP33 silencing induces ROS production, as detected by DAB staining. Leaves were detached and stained 4 weeks after VIGS.

in *GhSNAP33*-deficient plants confirmed their susceptibility to *V. dahliae* infection.

GhSNAP33 Overexpression Enhances *Arabidopsis* Resistance to *V. dahliae*

Hygromycin-resistant *GhSNAP33*-overexpressing *Arabidopsis* lines were identified by genomic PCR analysis (Supplementary Figure S3A). Homozygous transgenic (T3 generation) lines of L1 and L3 were selected for subsequent experiments based on semi-quantitative RT-PCR analysis (Supplementary Figure S3B).

To investigate the contribution of GhSNAP33 to disease resistance in plant, *GhSNAP33*-transgenic plants were inoculated with *V. dahliae* spores by the root dipping method. 3-week-old *GhSNAP33*-expressing *Arabidopsis* plants infected with *V. dahliae* showed apparently less wilt, yellowish and necrosis as compared to the WT at 20 dpi (**Figure 7A**); this was associated with a lower disease index (**Figure 7B**). An analysis of fungal biomass confirmed that there was less fungus in the two transgenic lines than in WT plants (**Figure 7C**) and the expression of *PR1* and *PR5* was increased in infected transgenic plants compared with infected WT plants (**Figures 7D,E**).

Detached leaves of the *Arabidopsis* lines were inoculated with *V. dahliae* to evaluate the defense response. Chlorosis symptoms were milder in *V. dahliae*-infected leaves from transgenic as compared to WT plants at 6 dpi (**Figure 8A**). The fungal lesion

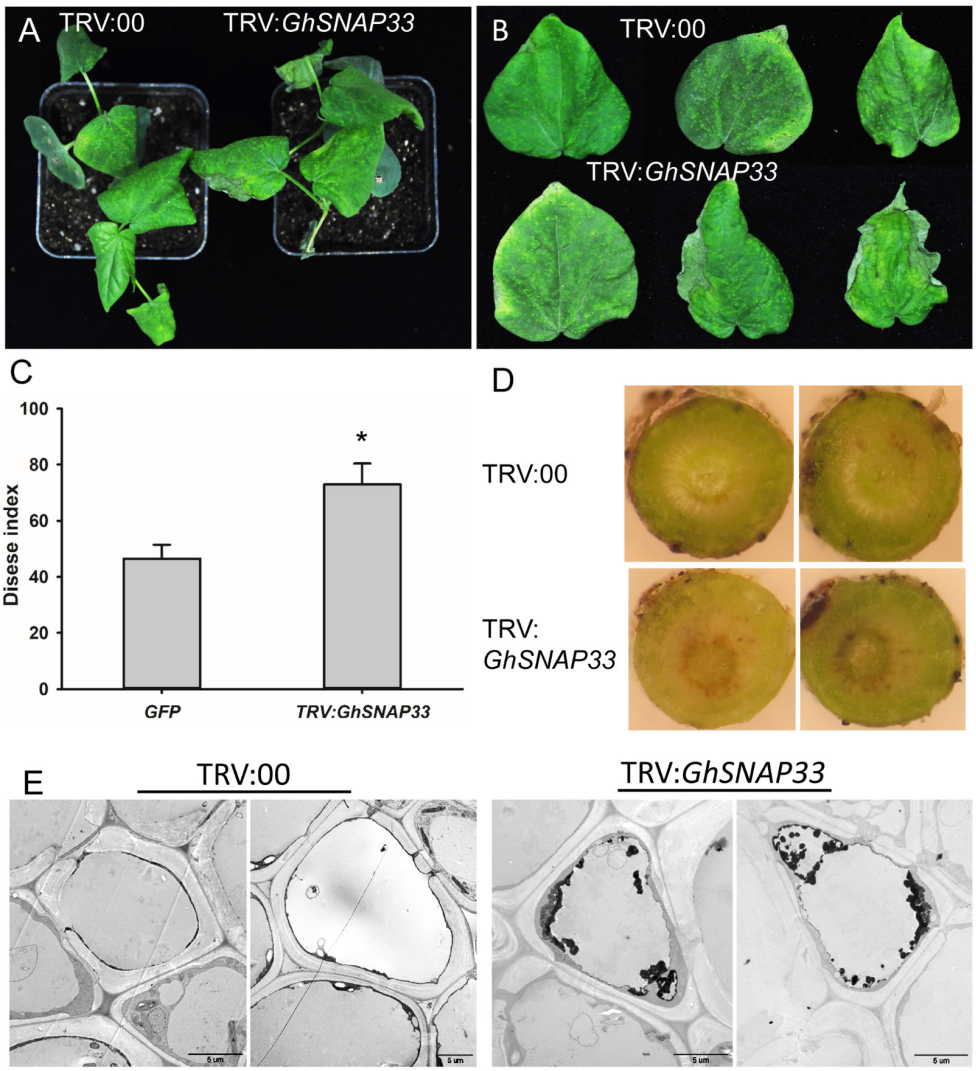

FIGURE 6 | Susceptibility of *GhSNAP33*-deficient cotton plants to *V. dahliae* infection. **(A)** Disease symptoms of VIGS plants 12 dpi. **(B)** Representative leaves from plants at 12 dpi. **(C)** Disease index at 14 dpi. **(D)** Vascular browning in stems of plants infected with fungus. **(E)** The thylose in root cell of infected plants at 14 dpi. Data represent mean ± standard error of three independent repeats (n = 3) with at least 16 plants each. *P < 0.05 vs. control.

area was smaller in the two transgenic line (**Figure 8B**). Trypan blue staining revealed that *V. dahliae* proliferated more quickly in leaves of WT plant with more mycelia growth extending the lesion area compared with transgenic plants L1 and L3 indicating *GhSNAP33* expression in *Arabidopsis* hindered *V. dahliae* mycelia growth (**Figure 8C**). Aniline blue staining showed enhanced callose deposition in *GhSNAP33*-transgenic plants (**Figure 8D**).

GhSNAP33 Overexpression Increases Plant Tolerance to Drought

Based on our observation that ABA and PEG600 induced *GhSNAP33* expression, we investigated the drought tolerance of *GhSNAP33*-transgenic *Arabidopsis* plants. The plants were in relatively good condition relative to their WT counterparts after 20 days without irrigation (**Figure 9A**). Most of the *GhSNAP33*-expressing plants recovered from drought after 2 days of re-watering (**Figure 9A**), accompanied with a higher survival rate than in WT (**Figure 9B**). Consistent with these findings, the dehydration assay showed that *GhSNAP33* overexpression decreased the rate of water loss in *Arabidopsis* (**Figure 9C**) and the expression of *DERB2A* and *RD29A* was increased in the both overexpression lines (**Figures 9D,E**).

DISCUSSION

SNAREs are essential for membrane fusion during vesicular transport in eukaryotic cells. SNAP25-type proteins regulate fusion between vesicles and plasma membrane during exo- and endocytosis in yeast and mammals. Of the 54 SNARE genes in the *Arabidopsis* genome, three encode SNAP25-type

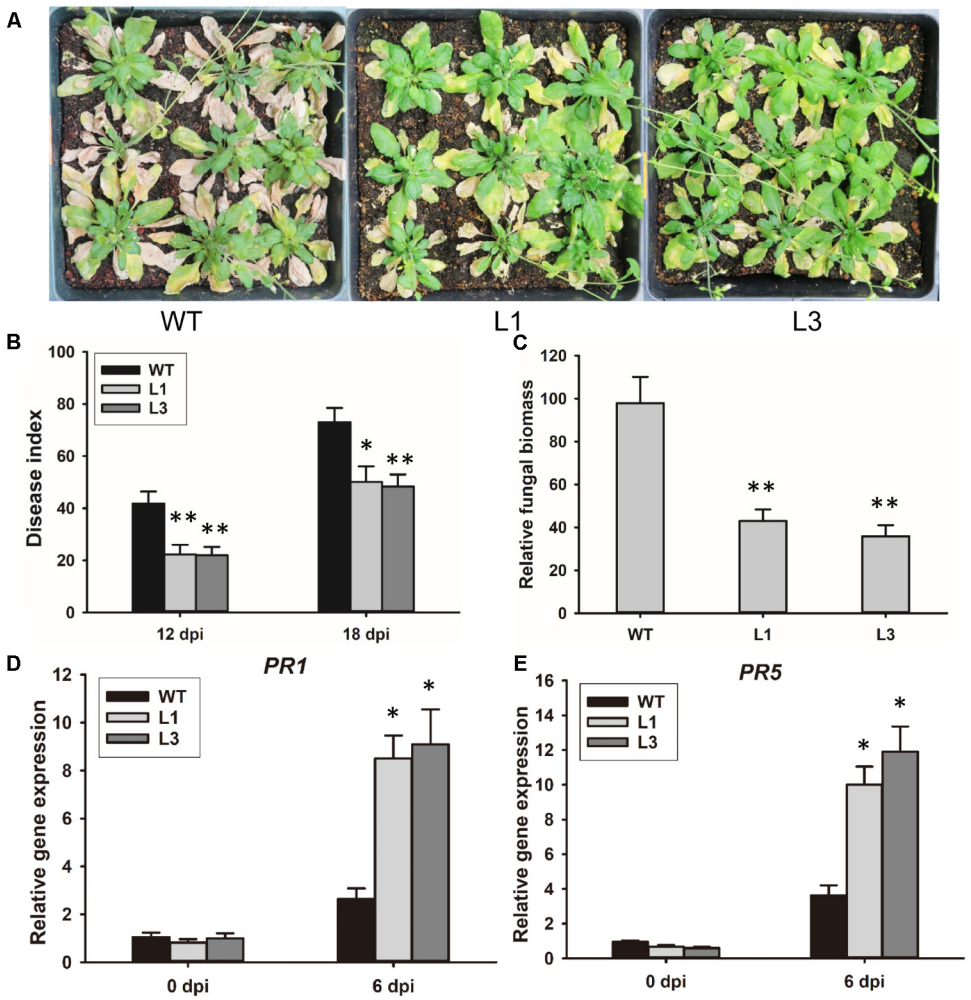

FIGURE 7 | Resistance to *V. dahliae* infection in *Arabidopsis* conferred by *GhSNAP33* overexpression. **(A)** *Arabidopsis* plants were inoculated with *V. dahliae* by root dipping. Images were taken at 20 dpi. The assay was repeated three times. **(B)** Disease index of WT and transgenic plants at 12 and 18 dpi. **(C)** Biomass of *V. dahliae* in infected plants at 20 dpi. **(D)** The expression of *PR1* in infected plants. **(E)** The expression of *PR5* in infected plants. Data represent mean ± standard error of three independent repeats ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. WT.

proteins. One of these, AtSNAP33, localizes to the plasma membrane and functions in exocytosis during cell division and in the plant defense response (Sanderfoot et al., 2000; Uemura et al., 2004). Although several SNAP25-type genes have been cloned in plants, there have been no reports on cotton SNARE family proteins. In the present study, we characterized *GhSNAP33*, a SNAP25-type t-SNARE gene, from cotton. GhSNAP33 contains C- and N-terminal Qb- and Qc-SNARE motifs, respectively, which are evolutionarily conserved stretches of 60–70 amino acids arranged as heptad repeats (Jahn and Scheller, 2006). These motifs are connected by an anti-parallel linker that anchors the protein to the cell membrane and contributes to exocytosis in mammals (Gonzalo et al., 1999; Nagy et al., 2008). The GhSNAP33-GFP protein in transgenic *Arabidopsis* was expressed at the plasma membrane (Supplementary Figure S2), consistent with the subcellular localization of AtSNAP33 (Heese et al., 2001), OsSNAP32 (Bao et al., 2008b), and CkSNAP33 (Wang et al., 2017). The amino acid sequence of GhSNAP33 shared high sequence identity with other SNAP25-type proteins, including CkSNAP33 from *C. komarovii* (69.51%), GsSNAP33 (67.54%) *A. thaliana* AtSNAP33 (63.61%), *Oryza sativa* L. OsSNAP32 (53.44%), and *Hordeum vulgare* L. HvSNAP34 (52.38%), indicating they may have similar functions in plant development and defense response to abiotic and biotic stress.

SNAP25-type proteins are essential for growth and development in all organisms. In mammals, loss of function of a key SNAP25-type protein leads to physical defects and disease (Rapaport et al., 2010; Browning and Karim, 2013). AtSNAP33 is ubiquitously expressed in roots, stems, leaves, and flowers of *Arabidopsis* and the *atsnap33* mutant developed large necrotic lesions on cotyledons and rosette leaves, and died before flowering (Heese et al., 2001). *StSNAP33*-deficient potato showed high levels of free SA at 3 weeks and exhibited spontaneous

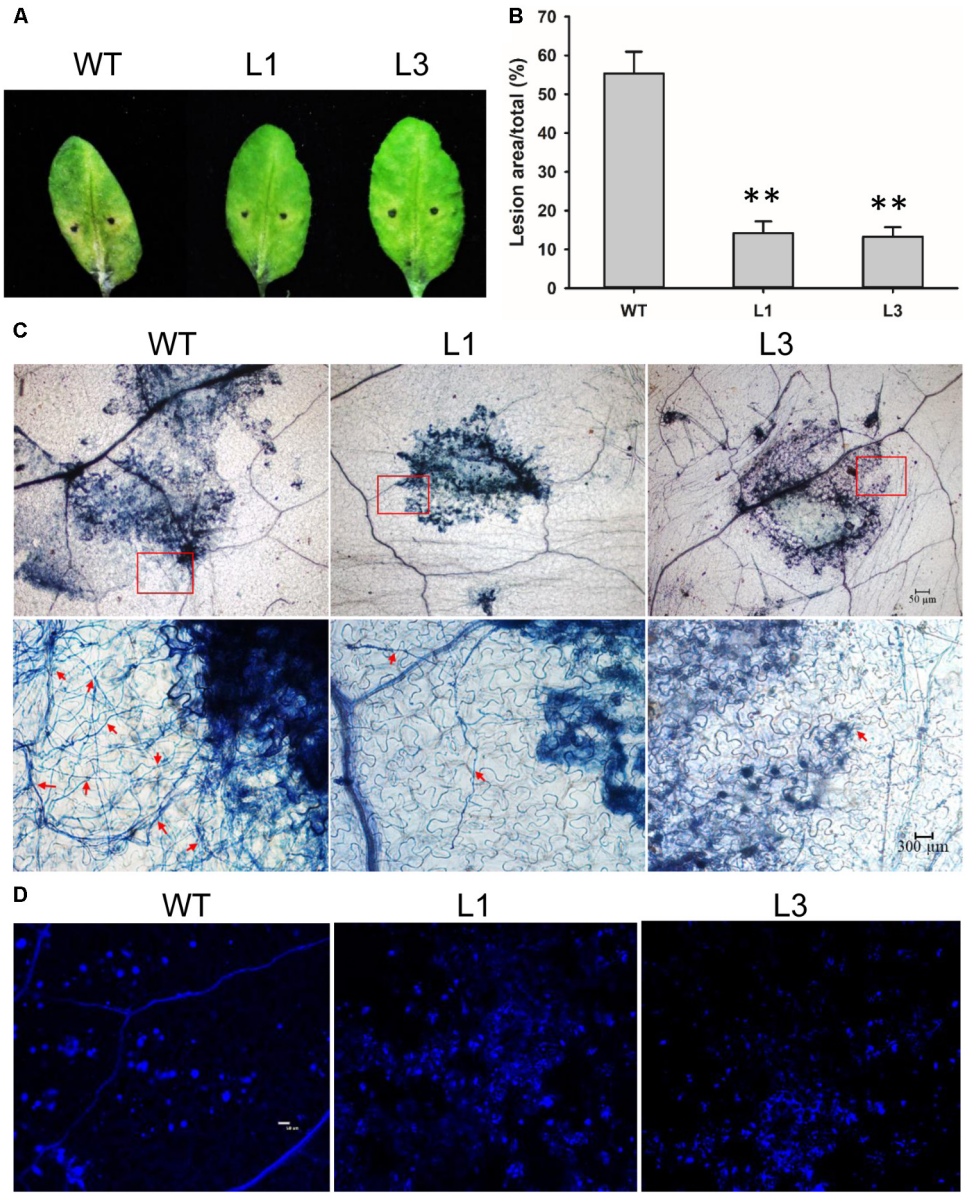

FIGURE 8 | Elevated callose deposition and reduced mycelia growth in *GhSNAP33* transgenic *Arabidopsis*. **(A)** *V. dahliae* spores suspension was applied to detached WT and *GhSNAP33* transgenic plants; representative leaves are shown at 6 dpi. **(B)** Lesion area of infected plants at 6 dpi. **(C)** The mycelia growth and cell death on infected leaves at 6 dpi stained with trypan blue. Top panel: Trypan blue staining of leaves. Lower panel: Closeups from corresponding top panel. The fungal mycelia are indicated by red arrows. **(D)** Callose deposition on the infected leaves stained with aniline blue at 24 h after inoculation. Similar results were obtained in independent experiments. Data represent mean ± standard error of three independent repeats ($n = 3$). $^{**}P < 0.01$ vs. WT.

necrosis and chlorosis at later stages (Eschen-Lippold et al., 2012). A tissue-specific analysis of *GhSNAP33* revealed higher expression in roots and leaves than in the stem of cotton plants. *GhSNAP33* knockdown yielded smaller plants and spontaneous lesion on the senior true leaf, which was associated with increased cell death and ROS accumulation. These results suggest that *GhSNAP33* is critical for cotton development, although further study is needed to determine whether the phenotypic defect is related to compromised cytokinesis resulting from the absence of *GhSNAP33*.

The SNARE-mediated secretory pathway delivers cellular defense factors to infection sites during exocytosis-associated immune responses in plants (Kwon et al., 2008a). SNAP25-type proteins have been shown to contribute to disease resistance in plants: for example, HvSNAP34 against powdery mildew (*Blumeria graminis* f. sp. *Hordei*) in barley (Collins et al., 2003), OsSNAP32 against blast fungus (Luo et al., 2016), and CkSNAP33 against *V. dahliae* infection (Wang et al., 2017). *GhSNAP33* transcription was upregulated in response to ABA, SA and H_2O_2 treatment and *V. dahliae* infection, and *GhSNAP33*-deficient

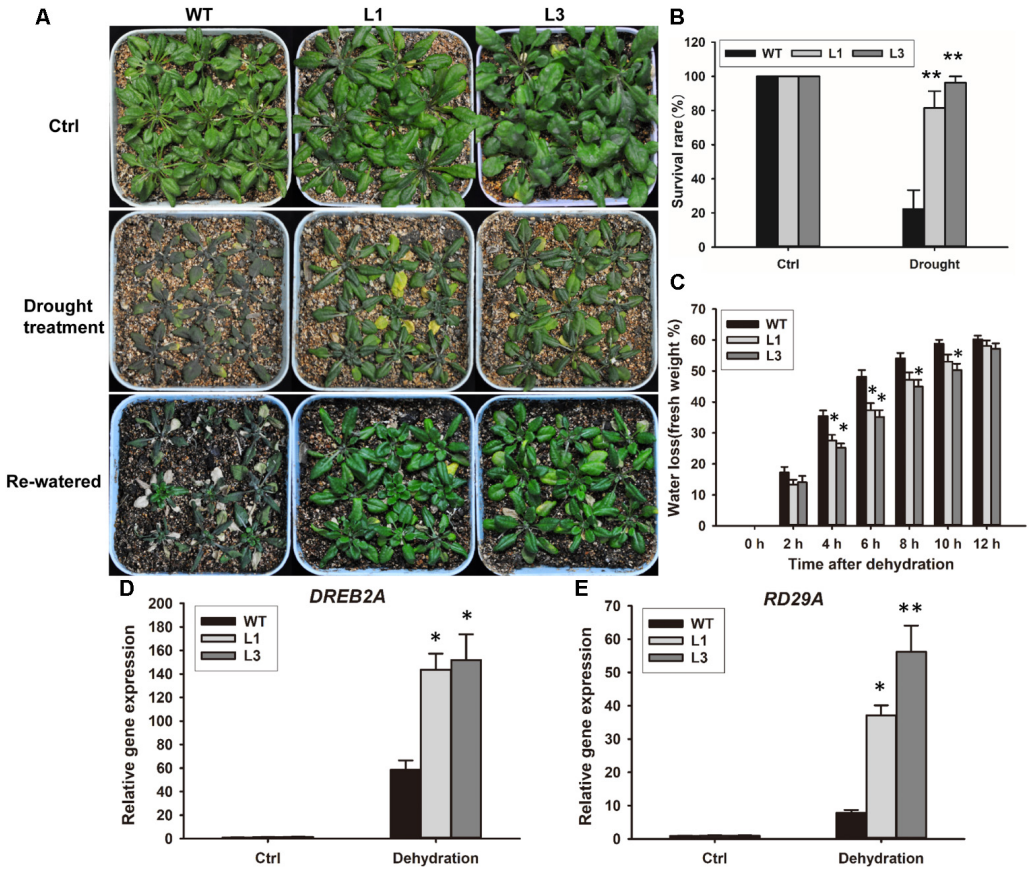

FIGURE 9 | Enhanced plant drought tolerance in *Arabidopsis* by ectopic expression of *GhSNAP33*. **(A)** *GhSNAP33* overexpression reduced plant susceptibility to drought. Top panel: *Arabidopsis* plants under normal conditions. Middle panel: 3-week-old plants were subjected to water withholding for 20 days. Lower panel: plants after drought treatment were re-watered for 2 days. Experiments were repeated three times. **(B)** Survival rate of WT and *GhSNAP33*-overexpressing plants after a 2-day recovery from drought stress. **(C)** Reduced water loss in *GhSNAP33*-overexpressing plants. The weight of detached leaves was measured at indicated time points. **(D)** The expression of *DREB2A* in *Arabidopsis* plants after dehydration. **(E)** The expression of *RD29A* in *Arabidopsis* plants after dehydration. Data represent mean ± standard error of three independent repeats ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. WT.

plants were more susceptible to *V. dahliae*, as evidenced by the greater severity of disease symptoms, elevated disease index, deepened vascular browning and increased thylose accumulation. On the other hand, *GhSNAP33* overexpression in *Arabidopsis* enhanced disease resistance relative to WT plants, accompanied with elevated expression of *PR1* and *PR5*; the results of the fungal filtrate assay revealed that leaves from transgenic plants had less chlorosis and fungal mycelia, and more callose deposition providing further evidence for the involvement of GhSNAP33 in disease resistance against *V. dahliae*.

With the changing of global climate, drought stress is becoming a major environmental problem affecting crop growth, development, and production (Ahuja et al., 2010). GsSNAP33, the *G. soja* homolog of GhSNAP33, is involved in plant tolerance to drought and salt stress in *Arabidopsis* (Nisa et al., 2017). We found that *GhSNAP33* overexpression was enhanced by ABA and PEG6000 treatments and ectopic expression of *GhSNAP33* increased yeast tolerance to mannitol. Importantly, transgenic *Arabidopsis* plants expressing *GhSNAP33* showed heightened tolerance to drought as compared to the WT with high survival rate after drought treatment. These results were supported by the observation that transgenic plants exhibited reduced rates of water loss and elevated expression of drought-responsive genes, *DREB2A* and *RD29A*, under conditions of dehydration.

The upregulation of *GhSNAP33* expression upon ABA and SA treatments indicates that GhSNAP33 may be involved in the cotton hormone-mediated signaling pathways. It has been reported that the systemic induction of *AtSNAP33* is SA dependent (Wick et al., 2003) indicating the implication of SNAP25-type protein in SA signaling pathway. SNAREs have been implicated in ABA-mediated responses to abiotic stress (Carter et al., 2004) and to pathogen resistance (Collins et al., 2003). Therefore, GhSNAP33 may implicate in ABA-mediated drought responses and ABA-dependent callose deposition after *V. dahliae* infection. In view of the antagonism of SA-dependent resistance by ABA in plant–pathogen interaction (Ton et al., 2009), further work is needed to make sense of the effect of GhSNAP33 on SA and ABA signaling pathway and interplay between ABA- and SA-dependent defense pathway in cotton stress response.

In pathogen–plant interactions, the SNARE complex mediates immune responses through focal secretion (Bednarek et al., 2010; Yun and Kwon, 2012). In addition to pathogenesis-related (PR) proteins, secondary cell wall components and/or enzymes may be transported to achieve plant immunity (Collins et al., 2003; Assaad et al., 2004; Kalde et al., 2007; Yun and Kwon, 2017). SNAP25-type proteins catalyze vesicle exocytosis by forming a ternary SNARE complex with other two other SNARE family members containing Qa- and R-SNARE motifs. The PEN1/SYNTAXIN OF PLANTS (SYP) 122/SYP132–SNAP33–VAMP721/722 pathway is a default secretory pathway essential for growth and development and the defense response in plants (Bednarek et al., 2010). SNAP33, as the only SNAP25-type protein in these processes, is essential for the focal secretion. GhSNAP33 may play similar roles in the defense response. The enhanced callose deposition in *GhSNAP33* transgenic plants suggested that GhSNAP33 may also be involved in the directional delivery of callose precursors and/or the callose synthase-like protein to infection sites, since the secretory PEN1–SNAP33–VAMP721/722 complex is essential for the penetration resistance of cell wall at early time points in *Arabidopsis* (Kwon et al., 2008a; Bednarek et al., 2010). NbSYP132 has been reported was implicated in the exocytosis of vesicles containing antimicrobial PR1 (Kalde et al., 2007). The elevated expression of *PR1* and *PR5* in *GhSNAP33* transgenic plants may be related to the exocytosis mediated by cognate SNARE complex. SNARE proteins also participate in tip-focused membrane trafficking for root hair tip growth in *Arabidopsis* (Ichikawa et al., 2014) and in trafficking of plasma membrane Aquaporin for the modulation of cell membrane water permeability (Besserer et al., 2012; Hachez et al., 2014). It is possible that GhSNAP33 is involved in a SNARE complex that transports similar factors and affects the drought-related maker genes including *DREB2A* and *RD29A* to mediate drought tolerance in cotton.

In summary, our functional analysis of GhSNAP33, a synaptosome-associated t-SNARE protein, revealed a potential role in vesicle trafficking in cotton development and defense responses. Loss of *GhSNAP33* expression resulted in cell death and increased ROS production in cotton leaves, and compromised plant resistance against *V. dahliae* infection. Conversely, resistance to *V. dahliae* was enhanced by *GhSNAP33* overexpression. Ectopic expression of *GhSNAP33* increased tolerance to osmotic stress in yeast and drought tolerance in *Arabidopsis*. Thus, GhSNAP33 is not only essential for the development of cotton plants but is critical for plant drought tolerance and resistance to *V. dahliae*. These findings provide a basis for developing strategies to improve drought tolerance and disease resistance in cotton plants to meet emerging environmental challenges. However, additional research is needed to clarify the role of GhSNAP33 in the specific secret pathway via membrane fusion in cotton.

AUTHOR CONTRIBUTIONS

PW, FL, and YH conceived and designed the study. PW, YS, and YP conducted the experiments. PW, XL, and XZ performed the data analysis. PW and YH drafted the manuscript. All authors were reviewed and revised the manuscript and figures.

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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.2018.00896/full#supplementary-material

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frontiers in Plant Science

MINI REVIEW
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The Role of Tomato *WRKY* Genes in Plant Responses to Combined Abiotic and Biotic Stresses

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In the field, plants constantly face a plethora of abiotic and biotic stresses that can impart detrimental effects on plants. In response to multiple stresses, plants can rapidly reprogram their transcriptome through a tightly regulated and highly dynamic regulatory network where WRKY transcription factors can act as activators or repressors. WRKY transcription factors have diverse biological functions in plants, but most notably are key players in plant responses to biotic and abiotic stresses. In tomato there are 83 *WRKY* genes identified. Here we review recent progress on functions of these tomato *WRKY* genes and their homologs in other plant species, such as Arabidopsis and rice, with a special focus on their involvement in responses to abiotic and biotic stresses. In particular, we highlight *WRKY* genes that play a role in plant responses to a combination of abiotic and biotic stresses.

Keywords: abiotic stress, biotic stress, combined stresses, disease resistance, effector-triggered immunity (ETI), PAMP-triggered immunity (PTI)

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INTRODUCTION

WRKY transcription factors (WRKYs) are a large family of transcriptional regulators, which are defined by the highly conserved WRKY domain (the WRKYGQK motif at the end of the N-terminal and a zinc-finger-like motif at the C-terminus) (Rushton et al., 2010). WRKYs are categorized into three groups (Rushton et al., 2010; Rinerson et al., 2015). Group I (with two WRKY domains) and Group II (with one WRKY domain) contain the zinc-finger-like motif C_2–H_2 (C–X_{4-5}–C–X_{22-23}–H–X_1–H). Group III contains one WRKY domain and a C_2–HC zinc-finger-like motif (C–X_7–C–X_{23}–H–X_1–C) (Eulgem et al., 2000). Based on the primary amino acid sequences, Group II can be further divided into three subgroups (Zhang and Wang, 2005).

Through the binding of the WRKY domain to the W-box *cis*-acting element (consensus sequence: (T)(T)TGAC(C/T)) in the promoters of their target genes, WRKYs can act as transcriptional activators or repressors in regulatory cascades (Rushton et al., 2010; Yokotani et al., 2013; Bakshi and Oelmuller, 2014). The functional specificity of WRKYs is defined by many factors including the W-box (Yan et al., 2013), the WRKY domain (Cheng et al., 2015), interactions with other proteins (Brand et al., 2013; Franco-Zorrilla et al., 2014), and post-translational modifications (Lai et al., 2011).

Many WRKYs have been identified in the plant kingdom (**Supplementary Table S1**). Numerous expression and functional studies have given insight in the involvement of WRKYs in different aspects of plant biology (Van Esse et al., 2009; Rushton et al., 2010; Ishihama and Yoshioka, 2012; Hu et al., 2013; Bakshi and Oelmuller, 2014; Yang et al., 2016). Tomato (*Solanum lycopersicum*) has 83 *SlWRKY* genes (Huang et al., 2012; Karkute et al., 2018). This review focuses on tomato *SlWRKY* genes with regard

to their roles in plant responses to biotic and abiotic stresses. The nomenclature of the *SlWRKY* genes follows that of Huang et al. (2012) and Karkute et al. (2018). For *SlWRKY* genes that have not been studied in detail yet, we propose potential roles in response to (a)biotic stresses by looking at their homologs in other plant species (**Supplementary Figure S1**). We paid special attention to the role of *WRKY* genes in the complex regulatory process of plant responses to combined stresses.

BIOTIC STRESS-RELATED WRKYs

Plants have developed two layers of induced defense responses (Jones and Dangl, 2006), in which WRKYs are shown to function as either positive or negative regulators (e.g., Bakshi and Oelmuller, 2014; Sarris et al., 2015). The first layer, termed PAMP-triggered immunity (PTI), is activated by the recognition between pathogen-associated molecular patterns (PAMPs) and plant's pattern recognition receptors. Adapted pathogens can express effector proteins to suppress PTI. The second layer [named effector-triggered immunity (ETI)] is triggered by the recognition of pathogen effectors by plant resistance (R) proteins. Plant R proteins usually comprise nucleotide binding-leucine rich repeat (NB-LRR). PTI and ETI induce both local and systemic acquired resistance responses through the production of reactive oxygen species (ROS) and activation of an integrated signaling network including MAP kinases and hormonal signaling pathways (Dodds and Rathjen, 2010). Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the classical immunity-related hormones.

WRKYs are involved in PTI and ETI at different regulatory levels (Bakshi and Oelmuller, 2014). Firstly, WRKYs can interact (in)directly with PAMPs or effector proteins to activate or repress both PTI and ETI. In barley (*Hordeum vulgare*), *HvWRKY1* and *HvWRKY2* were activated by flg22 (a MAMP) and acted as repressors of PTI against the powdery mildew fungus *Blumeria graminis* f.sp. *hordei*. In addition, the fungal effector AVR_{A10} activated a specific association between the R protein MLA10 and *HvWRKY1/2* leading to inactivation of the repressor function of *HvWRKY1/2* (Shen et al., 2007). In Arabidopsis, *AtWRKY18*, *AtWRKY40*, and *AtWRKY60*, homologs of *HvWRKY1* and *HvWRKY2* (Shen et al., 2007), showed redundant function in negatively regulating PTI to *Pseudomonas syringae* (Xu et al., 2006) and the powdery mildew fungus *Golovinomyces orontii* (Shen et al., 2007). Activation of defense-related genes was observed in *wrky18 wrky40* and *wrky18 wrky60* double mutants and the *wrky18 wrky40 wrky60* triple mutants (Xu et al., 2006; Shen et al., 2007). Similarly, the rice (*Oryza sativa*) *OsWRKY62* gene functions as a negative regulator of both PTI and ETI (conferred by the *Xa21* gene) to *Xanthomonas oryzae* (Peng et al., 2008). These WRKYs are members of the WRKY II-a subfamily and the results above suggest that members of this subfamily may have a conserved negative regulatory function in plant defense. However, overexpression of the WRKY II-a subfamily member *OsWRKY71* enhanced resistance to *Xoo* in rice (Liu et al., 2007). Secondly, WRKYs can be regulated by mitogen-activated protein kinases (MAPKs) (Pandey and Somssich, 2009; Ishihama and Yoshioka, 2012). In *Nicotiana benthamiana*, *NtWRKY7, NtWRKY8, NtWRKY9*, and *NtWRKY11*, phosphorylated by pathogen-responsive MAPKs, were able to bind to the W-box in the promoter of the *RBOHB* gene leading to ROS burst (Ishihama and Yoshioka, 2012; Adachi et al., 2015). *AtWRKY33* interacted with MPK4 and MAP kinase 4 substrate 1 (MKS1) (Andreasson et al., 2005). Upon being challenged with *P. syringae* or upon elicitation by the MAMP flg22, *AtWRKY33* was released from this trimeric complex and subsequently bound to the promotor region of Phytoalexin Deficient3 (PAD3) facilitating the synthesis of antimicrobial camalexin (Qiu et al., 2008; Mao et al., 2011; Ishihama and Yoshioka, 2012). Thirdly, WRKYs regulate hormonal signaling pathways. For example, overexpression of *AtWRKY18* and *AtWRKY70* led to induced expression of defense-related genes, including SA-induced *PR1* (Li et al., 2004). The increased susceptibility to *Botrytis cinerea* of the *atwrky33* Arabidopsis mutant was associated with SA-mediated repression of the JA pathway (Birkenbihl et al., 2012). In addition, WRKYs can contribute to plant immunity by modulating small RNAs (smRNAs), by epigenetic mechanisms through histone methylation, as well as by proteasome-mediated degradation and inter-organelle retrograde signaling (Bakshi and Oelmuller, 2014; Phukan et al., 2016).

In tomato, WRKYs are studied for their roles in plant defense by either overexpression and/or silencing them (**Supplementary Table S2** and **Figures 1**, **2**). Many tomato WRKYs function as positive regulators of plant responses to biotic stresses. *SlWRKY31* (named *SlDRW1* in Liu et al., 2014) and *SlWRKY33* (named *SlWRKY33B* and *SlWRKY33A* in Zhou et al., 2015), homologs of *AtWRKY33*, were able to complement the compromised tolerance to *B. cinerea* of the *atwrky33* mutant (Zheng et al., 2006). Additionally, overexpression of the *Solanum pimpinellifolium* allele of *SlWRKY33* (named *SpWRKY1* in Li et al., 2015a,b) resulted in resistance to the hemi-biotrophic oomycetes *Phytophthora nicotianae* in tobacco and *Phytophthora infestans* in tomato. The *SlWRKY39* gene, homolog of *AtWRKY40*, was significantly upregulated in tomato upon being challenged with *P. syringae* (Huang et al., 2012) and tomato lines over-expressing *SlWRKY39* showed enhanced resistance to this pathogen (Sun et al., 2015). Overexpression of *SlWRKY45*, another homolog of *AtWRKY40*, enhanced tomato susceptibility to the root-knot nematode *Meloidogyne javanica*, which was associated with decreased expression of JA- and SA marker genes (Chinnapandi et al., 2017). *SlWRKY72*, *SlWRKY73*, or *SlWRKY74* (*SlWRKY72a* or *SlWRKY72b* in Bhattarai et al., 2010) contributed positively to both PTI and *Mi-1*-mediated ETI against root-knot nematodes (*M. javanica*) and potato aphids (*Macrosiphum euphorbiae*) (Bhattarai et al., 2010). Also, *SlWRKY80* (*SlWRKY70* in Atamian et al., 2012) was required for *Mi-1*-mediated resistance against potato aphids and nematodes.

Upon infection of pathogens, altered expression was reported for several tomato WRKYs, including *SlWRKY23* (homolog of *AtWRKY23*), *SlWRKY46* (homolog of *AtWRKY40*), *SlWRKY53/54* (homolog of *AtWRKY23*), *SlWRKY80* and *SlWRKY81* (homologs of *AtWRKY38* and *AtWRKY62*) (Huang et al., 2012, 2016; Du et al., 2015; Lucioli et al., 2016; Rezzonico et al., 2017). Their homologs in Arabidopsis act as negative regulators of plant defense: *AtWRKY38*, *AtWRKY48*, and

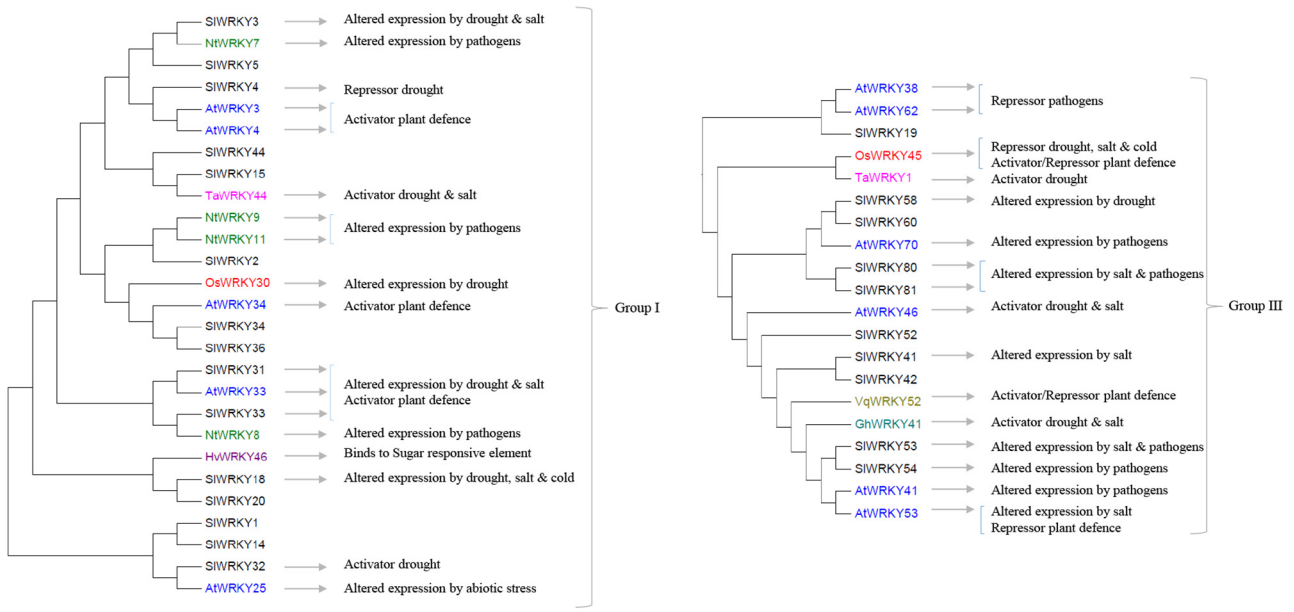

FIGURE 1 | The involvements of Group I and III tomato *SlWRKY* genes and their homologs (highlighted in different colors) in plant responses to biotic and abiotic stresses. The phylogenetic relations of tomato SlWRKYs and their homologs in Arabidopsis (AtWRKYs), rice (OsWRKYs), tobacco (NtWRKY), wheat (TaWRKY), barley (HvWRKY), cotton (GhWRKY), and grape (VqWKRY) are based on the phylogenetic tree presented in **Supplementary Figure S1**.

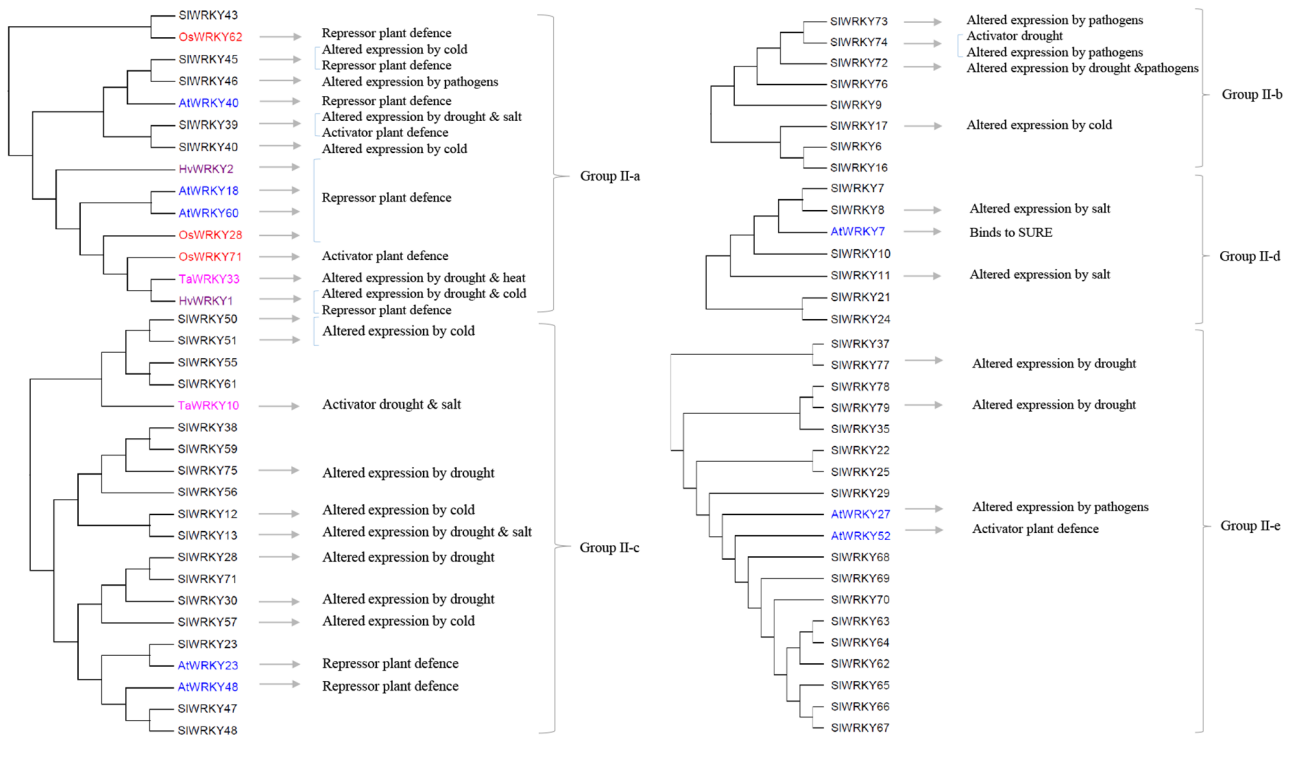

FIGURE 2 | The involvements of the Group II tomato *SlWRKY* genes and their homologs (highlighted in different colors) in plant responses to biotic and abiotic stresses. The phylogenetic relations of tomato SlWRKYs and their homologs in Arabidopsis (AtWRKYs), rice (OsWRKYs), wheat (TaWRKY), and barley (HvWRKY) are based on the phylogenetic tree presented in **Supplementary Figure S1**.

AtWRKY62 in the response to *P. syringae* (Xu et al., 2006; Kim et al., 2008; Xing et al., 2008), *AtWRKY23* in response to the nematode *Heterodera schachtii* (Grunewald et al., 2008), and *AtWRKY27* and *AtWRKY53* in response to *Ralstonia solanacearum* (Murray et al., 2007; Mukhtar et al., 2008). Interestingly, overexpression of the grape (*Vitis quinquangularis*) *VqWRKY52* gene in Arabidopsis, a homolog of *AtWRKY53* and *SlWRKY53/54*, enhanced resistance to *Golovinomyces cichoracearum* and *P. syringae*, but increased susceptibility to *B. cinerea*, which was associated with increased expression of SA-pathway related genes and enhanced cell death (Wang et al., 2017). Therefore, further functional analysis of these tomato WRKY genes is needed to confirm their role in either enhanced resistance or increased susceptibility to certain pathogens.

ABIOTIC STRESS-RELATED WRKYs

A number of studies demonstrate that WRKYs are involved in plant responses to abiotic stresses, such as drought and salinity (**Supplementary Table S2** and **Figures 1**, **2**). Expression of genes responsive to the signaling hormone ABA was altered in *AtWRKY40* and *AtWRKY40/AtWRKY18* knockout lines. Overexpression of wheat (*Triticum aestivum*) *TaWRKY1* and *TaWRKY33* (a homolog of *AtWRKY40*) in Arabidopsis enhanced drought tolerance through an ABA-dependent pathway (He et al., 2016). The *SlWRKY39* gene, homolog of *AtWRKY40*, was induced by salt, drought, ABA, SA, JA, and *P. syringae* (Huang et al., 2012; Sun et al., 2015). The *SlWRKY45* gene, another homolog of *AtWRKY40*, was upregulated by cold treatment (Chen et al., 2015). *AtWRKY46* was shown to regulate stress tolerance and hormonal response via ABA signaling and auxin homeostasis (Ding et al., 2015).

Overexpression studies of *TaWRKY10* and *TaWRKY44* in tobacco showed that these genes acted as enhancers of drought and salt stress tolerance through regulation of osmotic balance and ROS scavenging (Wang et al., 2013, 2015). Overexpression of the Chrysanthemum *DgWRKY5* gene enhanced tolerance to salt stress by augmenting ROS scavenging and osmotic adjustment (Liang et al., 2017). The rice *OsWRKY30* was involved in drought tolerance in rice via MAPK activation (Rushton et al., 2010; Shen et al., 2012). *DgWRKY5*, *AtWRKY25*, *TaWRKY44*, and *OsWRKY30* are all members of the WRKY family Group I (Liang et al., 2017).

The *AtWRKY46* gene enhances drought and salt stress tolerance, and regulates stomatal closure (Ding et al., 2015). One of its tomato homologs, *SlWRKY41*, was upregulated under salt stress, in addition to *SlWRKY53*, *SlWKRY80*, and *SlWRKY81* (Huang et al., 2012). *SlWRKY58* was upregulated under drought stress (Karkute et al., 2018). Overexpression of the cotton (*Gossypium hirsutum*) *GhWRKY41* gene, the closest homolog of *SlWRKY58*, in tobacco resulted tolerance to drought and salt stress through enhanced stomatal closure as well as by regulating ROS scavenging (Chu et al., 2015).

In addition, altered expression was observed for many other *SlWRKY* genes in tomato, including induction of *SlWRKY23*, *SlWRKY33*, and *SlWRKY57* under salt stress (Huang et al., 2012), upregulation of *SlWRKY12*, *SlWRKY13*, *SlWRKY23*, *SlWRKY50*, and *SlWRKY51* under cold stress (Chen et al., 2015), up-regulated *SlWRKY31* by drought and salt stress (Huang et al., 2012). Under drought stress, *SlWRKY32* and *SlWRKY74* were significantly upregulated (Huang et al., 2012), while *SlWRKY4* was downregulated (Karkute et al., 2015). The possible positive or negative roles of these *SlWKRY* genes in plant responses to abiotic stresses still need to be further verified by functional analyses.

WRKYs IN CROSSTALK BETWEEN ABIOTIC- AND BIOTIC-STRESS TOLERANCE

Several of the aforementioned WRKYs are active at crossroads of plant responses to both biotic and abiotic stresses. In Group I (**Figure 1**), *AtWRKY33* and its two tomato homologs *SlWRKY31* and *SlWRKY33* are activators of plant defense to several pathogens (Zheng et al., 2006; Lippok et al., 2007; Liu et al., 2014; Li et al., 2015a). In addition, induction of *SlWRKY31* and *SlWRKY33* was observed under drought and/or salt stresses (Huang et al., 2012). In Group II-a (**Figure 2**), *HvWRKY1* (also designated *HvWRKY38* in Mare et al., 2004), *AtWRKY40* and its tomato homologs *SlWRKY39* and *SlWRKY45* are involved in the response to the infection of pathogens and several abiotic stresses (Xu et al., 2006; Shen et al., 2007; Huang et al., 2012; Chen et al., 2015; Sun et al., 2015; Chinnapandi et al., 2017). Similarly, several WRKYs in Group II-b (**Figure 2**, *SlWRKY72* and *SlWRKY74*) and Group-III (**Figure 1**, *OsWRKY45* and *TaWRKY1*, *SlWRKY80*, and *SlWRKY81*, as well as *SlWRKY53* and *AtWRKY53*) can increase plant tolerance to multiple stresses (Murray et al., 2007; Mukhtar et al., 2008; Qiu and Yu, 2009; Tao et al., 2009, 2011; Bhattarai et al., 2010; Atamian et al., 2012; Huang et al., 2012; Wang et al., 2013, 2015; Marques de Carvalho et al., 2015; He et al., 2016). It is worthwhile to note that WRKYs have been studied for their responses to a single stress at the time. Therefore, further functional analyses of these WRKYs are needed to verify whether the responses to individual stresses remain the same when the plants are exposed to combination(s) of those stress factors. A role for WKRY genes in the interaction of response pathways was obvious in tomato plants in which *SlWRKY23* was silenced (Kissoudis, 2016). These plants exhibited increased resistance to tomato powdery caused by *Oidium neolycopersici*, but this resistance was compromised under salt stress. This example clearly indicates a role for WRKY transcription factors in the crosstalk between biotic and abiotic stress responses, and demonstrates that the responses to individual stresses may not be additive when the plants have to deal with combinatorial stresses.

Tomato is a host for more than 200 species of pathogens, some of which can be controlled by R genes derived from wild tomato relatives (Bai et al., 2018). Evidence is accumulating that plant resistances to pathogens can be attenuated or enhanced by abiotic stresses (Suzuki et al., 2014; Kissoudis et al., 2017). For example, the *Mi-1*-mediated nematode resistance was compromised under heat stress (Marques de Carvalho et al., 2015). Four tomato WRKYs were shown to contribute to the *Mi-1*-mediated nematode resistance [*SlWRKY72* to *SlWRKY74*

(Bhattarai et al., 2010) and *SlWRKY80* (Atamian et al., 2012)]. The intriguing question is whether these WRKYs are involved in the instability of the *Mi-1*-mediated resistance under heat stress, or, more generally, do WRKYs play a role in the (in)stability of plant R genes-mediated resistance associated with different molecular mechanisms (Kissoudis et al., 2016).

A (WKRY) gene that confers resistance or tolerance to multiple stresses would be highly useful for breeding. However, *WRKY* genes can also have opposite effects on abiotic and biotic stress tolerance since complex interactions among signaling networks can lead to both synergistic and antagonistic effects on regulation of plant responses to different stresses (Phukan et al., 2016; Bai et al., 2018). For example, *OsWRKY45* that positively mediates broad-spectrum disease resistance while inhibiting adaptation to abiotic stresses (Qiu and Yu, 2009; Tao et al., 2009, 2011), and *OsWRKY75* that increases susceptibility to rice blast fungus while improving tolerance to cold stress (Yokotani et al., 2013). Similarly, other transcription factors have also been shown to play an antagonistic role in modulating responses to abiotic and biotic stresses, such as tomato stress-responsive factor TSRF1 (Zhang et al., 2007), Arabidopsis DEAR1 (DREB (dehydration-responsive element binding protein 1) and EAR (ethylene response factor-associated amphiphilic repression) motif protein 1) (Tsutsui et al., 2009). The regulation of plant responses to multiple stresses relies on tightly regulated and highly dynamic regulatory networks where WRKYs can function as activators or repressors (Eulgem and Somssich, 2007; Bakshi and Oelmuller, 2014; Phukan et al., 2016). Therefore, it is necessary that the roles of WRKYs in a plant's tolerance to biotic and abiotic stresses should be studied under individual stresses as well as combination(s) of the studied stress factors.

It is important to note that some WRKYs were shown to function in a cluster (Cheng et al., 2015; Phukan et al., 2016), such as the *AtWRKY18-40-60* cluster (Yan et al., 2013). These three WRKYs form both homomeric and heteromeric complexes to modulate downstream target genes and cross-regulate each other, leading to a variety of responses to stresses and during development. It can be difficult to make use of such WRKY-clusters for crop improvement since multiple responses can lead to unwanted traits along with beneficial effects (Phukan et al., 2016). In tomato, five *SlWRKY* genes are close homologs of these three *AtWRKY* genes in Group II-a and shown to be responsive to both abiotic and biotic stresses (**Figure 1**). Further studies are needed to verify whether they also function in clusters and to identify other *SlWRKY* clusters. In this review, we tried to infer functions of unstudied *SlWRKY* genes via their homologs in other plant species. However, it should be stressed that slight changes in the DNA-binding domain may have an important effect on the binding specificity, and sequence homologs may be highly similar yet have different functions (Tao et al., 2009, 2011; Du et al., 2014). For example, the close tomato homologs *SlWKRY3* and *SlWRKY4* are predicted to interact with the W-box DNA through a different motif, RKYGQK, and WRKYGQK, respectively (Lai et al., 2008; Aamir et al., 2017). There is evidence that motifs outside the WRKY domain may provide binding specificity to WRKYs (Phukan et al., 2016). Also, WRKYs have been shown to bind non-W-box elements, including the sugar-responsive element by HvWRKY46, Calmodulin (CaM)-binding domain and the VQ proteins (Phukan et al., 2016). Identification of motifs associated with functions of tomato WRKYs will contribute to the understanding of their regulatory networks under combined stresses.

AUTHOR CONTRIBUTIONS

YB designed the outline of the manuscript. YB, SS, and CK contributed to writing and revisions of the manuscript. RV and CvdL contributed to revisions of the manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00801/full#supplementary-material

FIGURE S1 | The phylogenetic tree of tomato WRKYs and their homologs in Arabidopsis, rice, tobacco, wheat, barley, and grape. WRKYs of tomato (SlWRKYs), Arabidopsis (AtWRKYs), rice (OsWRKYs), tobacco (NtWRKY), wheat (TaWRKY), barley (HvWRKY), cotton (GhWRKY), and grape (VqWKRY) are colored in black, blue, red, green, fuchsia, purple, teal, and olive, respectively. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992) and 500 bootstrap (Felsenstein, 1985). The percentages of bootstrap value higher than 50% are indicated on the nodes.

TABLE S1 | WRKY transcription factors discovered in different plant species.

TABLE S2 | The involvements of tomato *SlWRKY* genes and their homologs in plant responses to biotic and abiotic stresses.

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Interactions Between Drought and Plant Genotype Change Epidemiological Traits of *Cauliflower mosaic virus*

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Plants suffer from a broad range of abiotic and biotic stresses that do not occur in isolation but often simultaneously. Productivity of natural and agricultural systems is frequently constrained by water limitation, and the frequency and duration of drought periods will likely increase due to global climate change. In addition, phytoviruses represent highly prevalent biotic threat in wild and cultivated plant species. Several hints support a modification of epidemiological parameters of plant viruses in response to environmental changes but a clear quantification of plant–virus interactions under abiotic stresses is still lacking. Here we report the effects of a water deficit on epidemiological parameters of *Cauliflower mosaic virus* (CaMV), a non-circulative virus transmitted by aphid vectors, in nine natural accessions of *Arabidopsis thaliana* with known contrasted responses to water deficit. Plant growth-related traits and virus epidemiological parameters were evaluated in PHENOPSIS, an automated high throughput phenotyping platform. Water deficit had contrasted effects on CaMV transmission rate and viral load among *A. thaliana* accessions. Under well-watered conditions, transmission rate tended to increase with viral load and with CaMV virulence across accessions. Under water deficit, transmission rate and virulence were negatively correlated. Changes in the rate of transmission under water deficit were not related to changes in viral load. Our results support the idea that optimal virulence of a given virus, as hypothesized under the transmission-virulence trade-off, is highly dependent on the environment and growth traits of the host.

Keywords: plant growth traits, viral load, viral transmission, virulence, tolerance, plant–virus interactions, water deficit

INTRODUCTION

Under field conditions, plants are exposed to various biotic and abiotic stresses that can impact their performance and their population dynamics and ecology (Suzuki et al., 2014; Pandey et al., 2015; Prasch and Sonnewald, 2015; Ramegowda and Senthil-Kumar, 2015; Aou-ouad et al., 2017). In particular, soil water deficit (WD) and plant virus diseases are major abiotic and biotic constraints impacting plant physiology and growth as well as agricultural

productivity worldwide (Zhang and Sonnewald, 2017). The relationships between host plants and viruses appear more complex than previously described, particularly regarding the role of perturbing environmental factors. To date, environmental conditions and plant physiological mechanisms that can lead to contrasted relationship with viruses, from mutualism to increased pathogenicity, remain poorly studied (Roossinck, 2015). Few recent investigations combining abiotic and biotic stresses clearly demonstrate that in addition to factors related to the virus and plant genotype the fate of plant–virus interactions also depends on the abiotic environment (Prasch and Sonnewald, 2013; Ramegowda and Senthil-Kumar, 2015; Fraile and García-Arenal, 2016; Aguilar et al., 2017; Carr, 2017). A growing body of data is uncovering the intimate entanglement of the plant physiological pathways involved in responses to various abiotic stresses and in defense against pathogens and herbivores (Bostock, 2005; Pandey et al., 2015; Nejat et al., 2016). For instance, antiviral plant immune responses may interact with responses to additional environmental changes through cross-talks among hormonal pathways (Alazem and Lin, 2015; Nejat et al., 2016).

On the one hand, it has been shown that heat, drought or salt stress enhance plant susceptibility to pathogens (Atkinson et al., 2013; Kissoudis et al., 2015). On the another hand, viruses can enhance the ability of plants to counteract abiotic stresses by inducing drought or cold tolerance (Xu et al., 2008; Hily et al., 2016). Although the mechanisms involved are not clear, the authors evoked the increase of potential osmoprotectants in virus-infected plants (Xu et al., 2008). In the case of *Cucumber mosaic virus* (CMV), drought tolerance in *Arabidopsis thaliana* is triggered by the 2b viral RNA silencing suppressor protein, a viral protein interfering with abscisic acid-mediated plant signaling (Westwood et al., 2013). It has been speculated that this effect of 2b may ultimately serve viruses by aiding host plants to survive periods of environmental stress (Westwood et al., 2013).

Virus transmission is a key epidemiological parameter for which most plant viruses rely on arthropods vectors (Bragard et al., 2013). Impacts of abiotic stresses on virus spread have long focused on the vector biology (e.g., developmental time, longevity, fecundity, migration) and ecology (Nancarrow et al., 2014; Davis et al., 2015). While most of these studies speculated on a possible impact of environmental changes on the rate of virus transmission, direct experimental support was only brought very recently (Dáder et al., 2016; Nachappa et al., 2016; van Munster et al., 2017; Yvon et al., 2017). Because some studies suggest that transmission should be positively correlated to virulence, due to their shared relationship with viral accumulation (Alizon et al., 2009; Froissart et al., 2010) and that the environment may change viral transmission rate, abiotic stresses may also affect the relationship between these epidemiological parameters. Unfortunately, the plant's surrounding environment is most often ignored when studying such epidemiological correlations (Fraile and García-Arenal, 2016).

In the present study, we monitored the effect of WD on various important viral life traits, such as viral accumulation, virulence and transmission. In order to explore the genetic variability of these traits and their relationships we selected nine wild accessions of *A. thaliana* (L.) Heynh (*Brassicaceae*) with known contrasted responses to WD (Rymaszewski et al., 2017). All accessions were infected with the *Cauliflower mosaic virus* (CaMV; *Caulimoviridae*), a non-circulative virus transmitted by aphids, and grown under strictly controlled environmental conditions in the high throughput phenotyping platform PHENOPSIS (Granier et al., 2006). Interestingly, our results suggest that the perturbing effects of WD on plant growth traits can change the trade-off between virus accumulation, virulence and transmission rate.

MATERIALS AND METHODS

Plant Material and Growth Conditions

We selected nine natural accessions of *A. thaliana* (Mr-0, Col-0, Ct-1, Sha, Cvi-0, Mt-0, Bay-0, Ler-1 and Est-1) based on their contrasted responses to drought (Rymaszewski et al., 2017). Three to five seeds were sown at soil surface in 225 ml pots filled with a 30:70 (v/v) mixture of clay and organic compost (Substrate SP 15% KLASMANN). Soil water content was estimated for each pot before sowing, as previously described (Granier et al., 2006). Subsequent changes in pot weight were attributed to change in water status. Soil surface was moistened with a modified one-tenth strength Hoagland solution, and pots were placed in the PHENOPSIS growth chamber (Granier et al., 2006) in the dark for 2 days at 12°C air temperature and 70% air relative humidity. Pots were dampened with sprayed deionized water three times a day until germination. During germination phase (7 days), plants were cultivated under 8 h day length (200 μmol m^{-2} s^{-1} photosynthetic photon flux density, at plant height), air temperature was set to 20°C, and air relative humidity was adjusted in order to maintain constant water vapor pressure deficit (VPD) at 0.6 kPa. Then, plants were thinned to one plant per pot and grown at 21/18°C day/night while VPD was set at 0.75 kPa. Each pot was daily weighed and watered with the modified Hoagland solution to reach the target soil relative water content (RWC_{soil}). RWC_{soil} was maintained at 1.6 g H_2O g^{-1} dry soil until application of the WD treatment. One week before the application of the WD treatment, watering was done with deionized water until the end of the experiment (**Figure 1A**).

Virus Purification and Plant Inoculation

The CaMV isolate Cabb B-JI (Delseny and Hull, 1983) efficiently transmitted following a non-circulative strategy by the aphid species *Myzus persicae*, was used in this study. Virus particles were purified from CaMV-infected *Brassica rapa* cv. "Just Right" (turnip) plants according to Hull and Shepherd (1976). The quality and the quantity of purified virus were assessed by polyacrylamide gel electrophoresis under denaturing conditions (12% SDS-PAGE) and by spectrometric measurements at 230, 260, and 280 nm (NanoDrop 2000 spectrophotometer). Virus concentration was estimated by spectrometry using the formula described by Hull and Shepherd (1976).

One-month-old *A. thaliana* plants (20 plants per accession per treatment) were mechanically inoculated as previously described

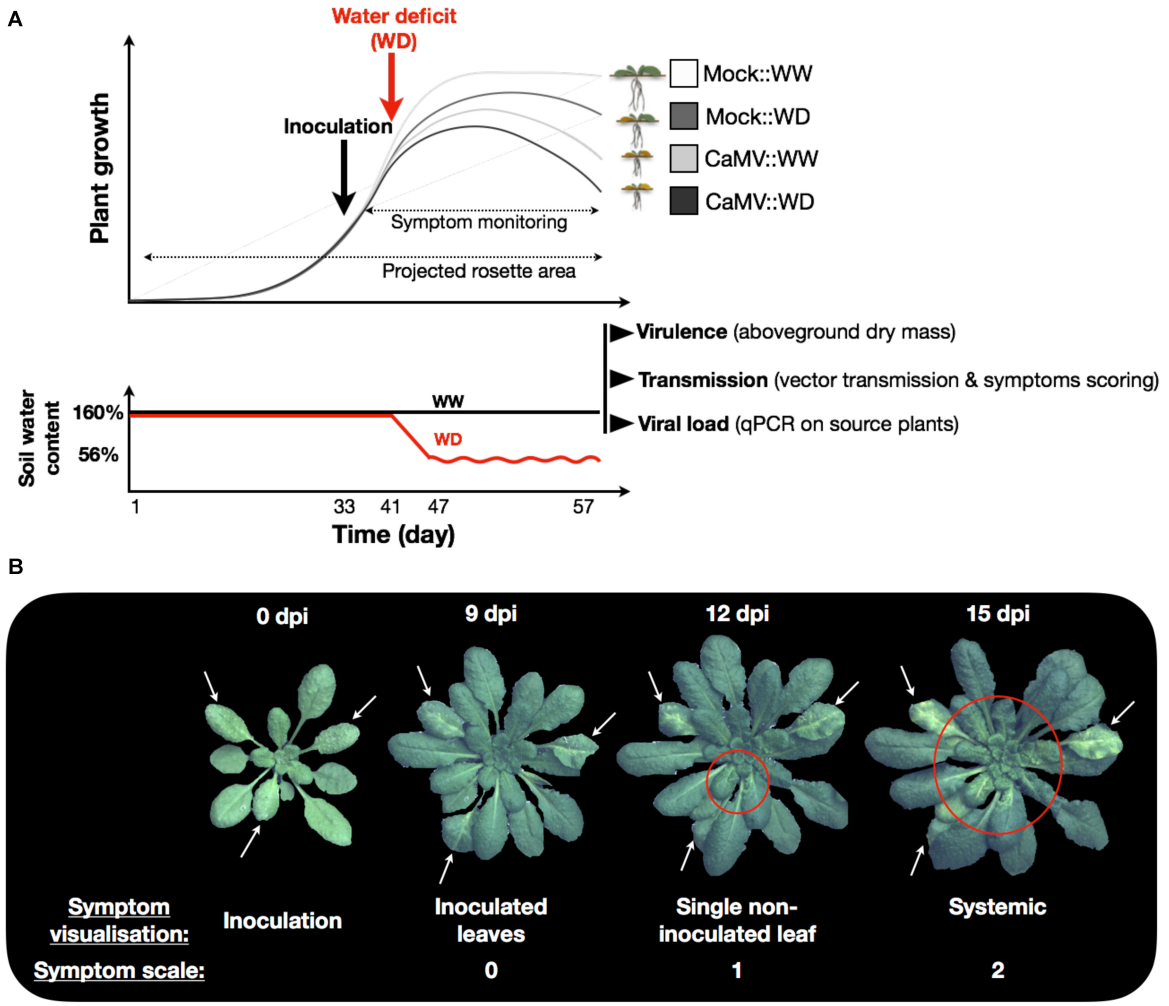

FIGURE 1 | Schematic representation of the experiment and timing of measurements. **(A)** Plant inoculation (mock- or CaMV; black arrow) was realized 33 days after germination and corresponds to the beginning of symptom scoring. One week later, soil water deficit treatment (WD; red arrow) was applied by stopping watering. Mean soil water content of individual pots during the course of the experiment is represented in the second panel. Soil relative water content was maintained at 160% in the control treatment (WW; black line) while stopping irrigation in the WD treatment brought the soil water content to 56% after 7 days and was maintained to this value by an adequate water supply (WD; red line). At the end of the experiment (day 57), viral traits (virulence, transmission efficiency and viral accumulation in source plants) were estimated. **(B)** Symptom monitoring was performed daily (from 9 to 25 dpi) on CaMV-infected:WW ($n = 20$) and CaMV-infected:WD ($n = 20$) plants. Symptoms were scaled 0, 1, or 2 for no symptom (symptom on inoculated leaves), presence of symptoms on a single non-inoculated leaf, or systemic symptoms, respectively.

(Blanc et al., 1993) with a mix containing CaMV-infected turnip extract enriched with the virus purification. Briefly, CaMV-infected turnip extract was prepared from 1 g of infected leaf material [leaves presenting systemic symptoms collected at 21 days post inoculation (dpi)] grinded in 1 ml of distilled water. Purified CaMV particles were then added to this mix at a final concentration of 0.2 mg ml^{-1} to optimize infection success. For each inoculated plant, three leaves of median rank in the rosette were rubbed with a small pestle soaked in the solution described above. Control group (eight plants per accession and per watering treatment) was mock-inoculated in a similar way to mimic the wound induced by mechanical inoculation. Mock-inoculation was performed with a mix containing non-infected plant extract and the buffer used for virus purification (100 mM Tris-HCl, 2.5 mM $MgCl_2$, pH 7). All plants were inoculated in a random order, independent of their accession and of the watering regime. In summary, four conditions representing mock-inoculated:WW ($n = 8$), mock-inoculated:WD ($n = 8$), CaMV-infected:WW ($n = 20$) and CaMV-infected:WD ($n = 20$) plants per accession were analyzed.

Water Deficit Treatment

One week after inoculation, corresponding to the approximate time of appearance of the first symptoms, irrigation of half of the CaMV- and mock-inoculated plants was stopped to reach the WD treatment at 0.56 H_2O g^{-1} dry soil (this value was reached after 7 days of water deprivation). RWC_{soil} was then maintained to this value until the end of the experiment. In the well-watered

treatment (WW) RWC_{soil} was maintained at 1.6 g H_2O g^{-1} dry soil (**Figure 1A**).

Measurement of Plant Traits and Symptoms Development

Projected rosette area (7–10 plants per accession and treatment) was estimated from automated daily pictures using a semi-automatic procedure developed in the image analysis environment Image J (Research Services National Institute of Mental Health, Bethesda, MD, United States) and downloadable on the PHENOPSIS web site (Fabre et al., 2011). For each accession and watering treatment (WW and WD), four mock-inoculated and ten CaMV-infected individual plants were harvested 25 dpi and individual aboveground dry mass was determined after 5 days at 60°C.

Symptom monitoring was performed daily (from 9 to 25 dpi) on CaMV-infected:WW (n = 20) and CaMV-infected:WD (n = 20) plants for each accession. The symptoms were scaled 0, 1, or 2 for no symptom, presence of symptom on a single non-inoculated leaf, or systemic symptoms, respectively (see illustration on **Figure 1B**). Time of symptoms appearance, rate of systemic spread, and maximum proportion of infected plants were then calculated from logistic regressions fitted to these observations.

Aphid Rearing

The colony of the aphid-vector species *M. persicae*, collected over 30 years ago in the south of France was maintained on eggplants (*Solanum melongena*) in insect-proof cages, in a growth chamber at 23/18°C with a photoperiod of 14/10 h (day/night), in conditions ensuring clonal reproduction. Aphids were transferred to new cages and to new non-infested host plants (*Solanum melongena)* every 2 weeks, in order to avoid overcrowding and induction of the development of winged morphs.

Aphid Transmission Assays

Transmission efficiency of CaMV was assessed at 25 dpi (Supplementary Figure S1). Batches of 20 *M. persicae* larvae (L2-L4 instars) were starved for 1 h before being transferred at the rosette center of a source plant for virus acquisition. Ten symptomatic source plants were used per accession and watering treatment. When aphids stopped walking and inserted their stylets into the leaf surface, they were allowed to feed for a short 2-min period. Viruliferous aphids were then immediately collected in a Petri dish and individually transferred to 1-month-old Col-0 plantlets (test plants) grown under non-stressing conditions (one aphid per test plant; nine test plants per source plant). After an inoculation period of 3 h, aphids were eliminated by insecticide spray (0.2% Pirimor G). Test plants were then placed in a growth chamber with the same conditions of air humidity, temperature and light as source plants and maintained under non-stressing conditions. Symptoms of virus infection were recorded 21 days later by visual inspection on test plants, as previously reported (Doumayrou et al., 2013; van Munster et al., 2017) and virus transmission rate was then calculated. After transmission assays, three leaves were randomly collected on each source plants and stored at −80°C for further nucleic acid extraction and quantification of the virus accumulation.

Plant DNA Extraction

Total DNA from CaMV-infected leaf samples (pool of the three leaves collected per plant) was extracted according to a modified Edwards protocol (Edwards et al., 1991) with an additional washing step with 70% ethanol (10 biological replicates per accession and treatment). DNA was resuspended in 50 µl of distilled water, and 10-fold dilutions were used as qPCR templates. The quality and quantity of the extracted total nucleic acid were assessed by spectroscopic measurements at 230, 260, and 280 nm (NanoDrop 2000 spectrophotometer).

DNA Quantification by qPCR

DNA quantification (10 biological replicates per accession and treatment, Supplementary Figure S1) was performed as duplicated qPCR in 384-well optical plates using the LightCycler FastStart DNA Master Plus SYBRGreen I kit (Roche) in a LightCycler 480 (Roche) thermocycler according to the manufacturer's instructions. Specific primers designed for the quantification of CaMV genome (Ca4443-F: 5′-GACCTA AAAGTCATCAAGCCCA-3′ and Ca4557-R: 5′-TAGCTTT GTAGTTGACTACCATACG-3′) and *A. thaliana* ubiquitin-conjugating enzyme 21 gene (UBC21; UBC21_At_F: 5′-TGCA ACCTCCTCAAGTTCGA-3′ and UBC21_At_R: 5′-GCAGG ACTCCAAGCATTCTT-3′) were used at a final concentration of 0.3 µM. All qPCR reactions were performed with 40 cycles (95°C for 15 s, 62°C for 15 s and 72°C for 15 s) after an initial step at 95°C for 10 min. The qPCR data were analyzed with the LinReg PCR program to account for the efficiency of every single PCR reactions (Ruijter et al., 2009). The absolute initial viral concentration in *A. thaliana* plants, expressed in arbitrary fluorescence units (N_0 CaMV) was divided by that of *A. thaliana UBC21* gene (N_0 UBC21; Genbank accession DQ027035), in order to normalize the amount of plant material analyzed in all samples.

Data Analyses

For each accession, the effects of the treatments on aboveground dry mass, transmission rate and viral load were analyzed by non-parametric Kruskal–Wallis tests. Time of symptoms appearance, rate of systemic spread, and maximum proportion of infected plants were extracted for each accession and watering treatment from logistic regression using the equation $A/(1 + \exp((4 * \mu / A) * (\lambda - t) + 2))$, where A is the maximum rate of infection, λ is the time necessary for the appearance of a systemic symptom on a non-inoculated leaf and μ is the time necessary to detect systemic symptoms on the full plant. The effect of watering on transmission rate was tested in a generalized linear model (glm) model with the binomial link function. Response ratios of aboveground plant dry mass (the ratios of mean outcome in the experimental group to that in the control group) were used to quantify the response of each genotype to watering and viral infection (i.e., virulence). We tested the significance of the relationships between

epidemiological parameters with the Spearman's rank correlation test.

All analyzes were performed in the programming environment R (R Core Team, 2017). Kruskal–Wallis tests were performed using the corresponding function in AGRICOLAE package. Bootstrapped 95% confidence intervals (CI) of mean trait values were computed following the *mean_cl_boot* procedure of the HMISC package. Non-linear models were fitted using the *nls* function and 95% confidence intervals for the parameters of fitted models were computed with *confint* function of the package MASS. Generalized linear models were tested using the *glm* function of the STAT package. Mean response ratios and corresponding 95% confidence intervals were calculated using *sci.ratio* of the MRATIO package.

RESULTS

Systemic Spread Varies Between *A. thaliana* Accessions and, in Some Accessions, It Is Conditioned by Watering Treatment

CaMV isolate Cabb B-JI successfully infected plants of all *A. thaliana* accessions selected. Symptoms, i.e., chlorotic lesions and vein-clearing of rosette leaves, were similar across accessions though their timing of appearance and intensity was greatly variable as detailed below. The proportion of CaMV-inoculated plants showing characteristic virus symptoms 25 dpi varied from 96 to 100% across accessions whatever the soil watering treatment (**Figure 2**). In WW condition, the mean time of systemic symptoms appearance on the first non-inoculated leaf (±95% CI) was 12.3 (±1.9) dpi (**Figure 3A**). However, lag time of systemic symptoms varied significantly between accessions from 10 (±0.3) dpi in Bay-0 to 14.7 (±0.2) dpi in Sha. Lag time did not change in response to WD in five accessions whereas it was significantly lower (i.e., faster appearance of firsts symptoms) in three accessions and higher in one accession (**Figure 3A**). For all accessions, the rate of systemic spread was lower under WD than in WW (**Figure 3B**). In particular, it was significantly reduced for Mr-0, Col-0, Ct-1, Sha and Cvi-0 (**Figure 3B**).

Plant Growth Response to Water Deficit and Viral Infection Vary Across Natural Accessions

We selected nine natural accessions of *A. thaliana* (Mr-0, Col-0, Ct-1, Sha, Cvi-0, Mt-0, Bay-0, Ler-1 and Est-1) based on their contrasted responses to drought (**Figure 4A**). Plant aboveground dry mass (±SD) ranged from 0.21 (±0.02) g (Ler-1) to 0.46 (± 0.04) g (Mr-0) under WW condition (**Figure 4B**). As determined 19 days after the start of the treatment, WD reduced aboveground dry mass production (20–25% reduction) in the four accessions the less tolerant to WD: Mr-0, Col-0, Ct-1 and Sha. Growth reduction due to WD was marginally significant in Cvi-0 ($P = 0.083$), and not significant in Mt-0, Bay-0, Ler-1 and Est-1, which are accessions the most tolerant to WD (**Figure 4B**).

As determined at 25 dpi, CaMV virulence under WW, calculated as CaMV-infected:WW/mock-inoculated:WW for aboveground biomass, varied significantly among accessions. CaMV infection significantly reduced aboveground dry mass (10–21% reduction) in all accessions but Ct-1, Sha and Est-1 (**Figure 4B**). In particular, aboveground dry mass of CaMV-infected plants was significantly reduced in Bay-0 ($P = 0.0072$), Col-0 ($P = 0.048$) and Mt-0 ($P = 0.047$).

In general, the combination of WD and viral infection tended to be more deleterious than each of the two stresses taken separately. However, plant responses differed widely between accessions. For example, WD and CaMV infection combination did not have a significant effect on aboveground mass of Est-1 and Ler-1 compared to the mock-inoculated:WW condition, whereas a 13–40% significant reduction was found in five other accessions ($P < 0.05$; **Figure 4B**). However, in two accessions, Sha and Ct-1, stress combination was less severe than the effect of WD alone although it was no significant (Sha: $P = 0.15$ and Ct-1: $P = 0.20$; **Figure 4B**). Similar trends were observed for the projected rosette area since this trait was highly significantly correlated to aboveground dry mass ($R^2 = 0.68$, $P < 0.001$; Supplementary Figure S2). Response ratios of aboveground biomass of each accession and for the different combinations of treatment are presented in Supplementary Figure S3.

Variation of Viral Load and Transmission Rate Under WD Is Dependent on the Accession

Preliminary experiments showed that CaMV transmission rate (from a virus donor infected plant) did not vary significantly according to the identity of the accession used as test plant (receptor plant) in transmission experiments (data not shown). For practical reasons, we therefore used Col-0 as test plants in subsequent transmission assays. Whatever the watering treatment and accession, transmission rate varied from 30 to 57% (**Figure 5A**). Surprisingly, while in most accessions WD did not significantly affect the transmission rate or marginally reduced it (Ler-1, from 55 to 38%, $P < 0.10$), we observed a significant increase of transmission rate in Mr-0 and Sha (**Figure 5A**). In these two latter accessions transmission rate increased from 35–51% to 38–57%, respectively ($P < 0.05$). Noteworthy, a similar trend was observed for Ct-1 and Est-1 (**Figure 5A**).

Viral load, i.e., CaMV accumulation in source plants, significantly decreased when plants were grown under WD compared to WW condition for Mr-0, Ct-1, and Sha (10–25% reduction, $P < 0.05$), and tended to decrease in Col-0, Cvi-0, Mt-0 and Ler-1 (**Figure 5B**). No correlation was found between viral load and transmission rate across accessions whatever the watering condition (Spearman's $r = 0.40$, $P = 0.29$ in WW; Spearman's $r = 0.40$, $P = 0.17$ in WD).

Water Deficit Alters the Relationship Between Transmission Rate and Virulence

A significant positive correlation between CaMV virulence (i.e., CaMV-infected:WW/mock-inoculated:WW response ratio

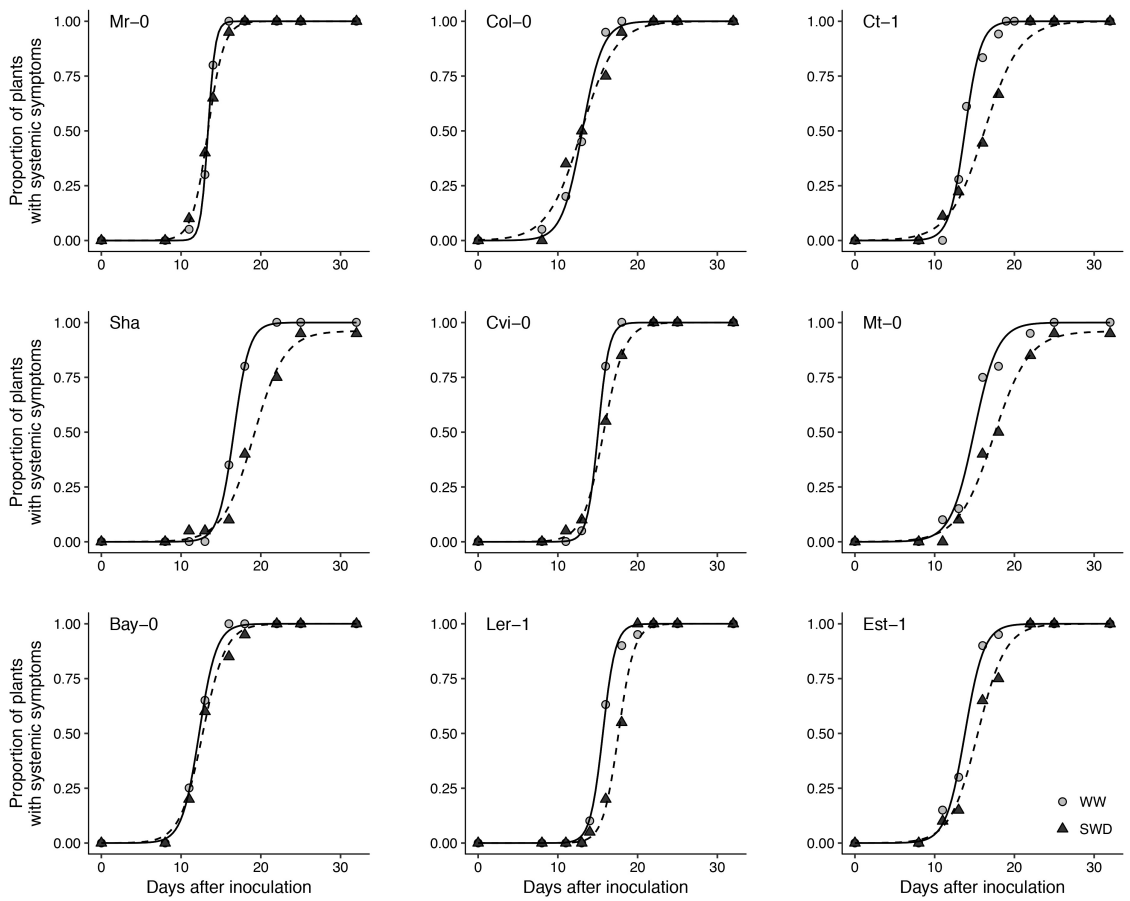

FIGURE 2 | Symptom dynamics in nine *A. thaliana* accessions inoculated with CaMV and grown under two watering conditions. Each panel represents one accession grown under well-watered (gray circles) and water deficit (black circles), respectively. Points are means of the proportion of plants with systemic symptoms ($n = 20$ plants per accession and watering treatment). Curves are logistic fitting following equation A/(1 + exp((4*μ/A)*($\lambda - t$) + 2)), where t is the number of days after inoculation, A is the maximum rate of infection, λ is the time necessary for the appearance of symptoms on a non-inoculated leaf and μ is the time required to visualize systemic symptoms.

of aboveground dry mass) and transmission rate was found under WW (Spearman's $r = 0.68$, $P < 0.05$; **Figure 6**). In other words, transmission rate was higher in accessions the most susceptible to CaMV infection in terms of aboveground mass. Under WD, transmission rate increased in accessions that were the most tolerant to CaMV while the reverse trend was observed in accessions more susceptible to CaMV. As a result, a significant negative correlation between transmission rate under WD and CaMV virulence was found (Spearman's $r = -0.75$, $P < 0.02$; **Figure 6**). The relationship between virulence and transmission was distorted regardless of the response ratio calculated for virulence (Supplementary Figures S4, S5).

DISCUSSION

We investigated plant responses to simultaneous exposure to WD and infection with one isolate of the CaMV in nine wild accessions of *A. thaliana* (L.) Heynh. We tested the hypothesis that perturbing effects of WD on plant growth traits would lead to changes in key epidemiological parameters such as systemic spread, virulence, viral load and transmission rate. Our results showed that under WD, viral infection spread into the plant was slower compared to WW treatment. Furthermore, WD had contrasted effects on CaMV transmission rate and viral load among *A. thaliana* accessions. Under WW, transmission rate tended to increase with the susceptibility of the accession to CaMV. Under WD, transmission rate and susceptibility were negatively correlated.

Most plant viruses move in a systemic way within the host plant (Matthews, 1991; Leisner et al., 1993). After inoculation by an aphid vector or by mechanical inoculation like in the present study, CaMV re-initiate infections in different and distant tissues, and such viral spread guarantees its survival (Schoelz et al., 2015). Virus spread within the host plant happens through cell-to-cell movement (via plasmodesmata) and long-distance movement mainly through phloem vessels (Matthews, 1991; Stavolone et al., 2005). It is known that the time at which viruses move out of the inoculated leaf into the rest of the plant varies widely depending on factors such as host and virus species,

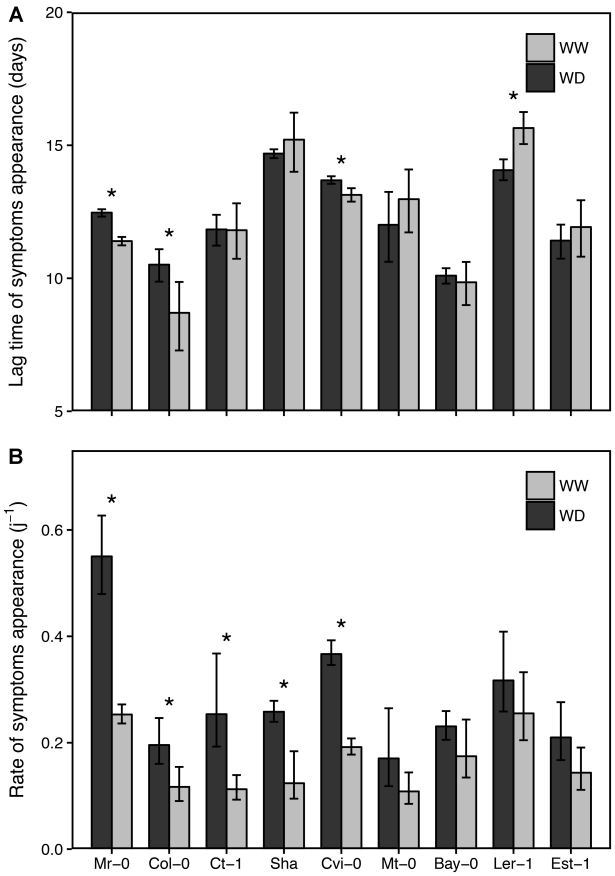

FIGURE 3 | Lag time and rate of CaMV symptoms appearance in nine *A. thaliana* accessions grown under two watering conditions. Bars and error bars are means and bootstrapped 95% confidence intervals of lag time **(A)** and rate **(B)** of symptoms appearance extracted from sigmoidal curve fitting of symptom dynamics (see **Figure 1**) under well-watered (gray bars) and water deficit (black bars), respectively. Stars indicate significant difference between watering treatment for each accession (nonparametric Kruskal–Wallis tests; $P < 0.05$). Accessions are ordered according to increasing tolerance to WD.

age of the host, method of inoculation and abiotic constraints (Jensen, 1973; Ismail and Milner, 1988). In this study, we showed that the lag time to symptoms appearance (the mean time for symptoms to appear in the first non-inoculated leaf) and rate of systemic spread (the time required for all the plants of an accession to exhibit systemic symptoms) of CaMV was affected by accession identity and WD. Previous studies have already shown that inherent differences in development, particularly flowering phenology, and growth, can affect the dynamics of viral infection across accessions (Leisner et al., 1993). Virus spread is also influenced by the flow of metabolites in the plant (Bennett, 1940). Since WD may affect the relationship between carbon availability and sink organ growth (Muller et al., 2011), it can be assumed that long-distance transport of viral particles through phloem will also be affected, leading to slower systemic movement (Córdoba et al., 1991; Leisner et al., 1993; Leisner and Howell, 1993). Here, correlated with the negative effects of WD on plant growth and development we observed a significant reduction in the rate of systemic symptoms appearance in the most WD-sensitive accessions such as Mr-0 and Ct-1. This reinforces the idea of a strong interaction between growth, phenology and viral infection dynamics. These observations are confirmed by the negative correlation between time or rate of systemic spread and tolerance to WD but also with the time of appearance of flowering buds (i.e., bolting) among accessions (not shown).

Regarding the impact of each independent stress on growth, CaMV infection had a generally lower negative effect on plant growth than WD since the virulence of the viral strain Cabb B-JI on vegetative growth was not too deleterious for most accessions studied. The effect of double stress was even more detrimental to rosette growth compared to control conditions. There was no correlation between tolerance to WD and tolerance to CaMV infection. Despite several reports on virus capacity to improve plant tolerance to abiotic stresses (Xu et al., 2008; Westwood et al., 2013), we could not find any significant positive effect of virus infection on plant tolerance to WD.

The success of transmission from infected to healthy host plants is crucial for the survival of all plant viruses. Interestingly, we showed that a WD triggered a significant increase of transmission rate in Sha and Mr-0, two accessions among the most sensitive to WD as previously described (Rymaszewski et al., 2017). These results are consistent with the earlier report of increased transmission of CaMV and *Turnip mosaic virus* (TuMV) from *B. rapa* plants submitted to WD (van Munster et al., 2017). However, the variation of transmission rate under abiotic stress depends on the plant-virus-vector pathosystem and on the type of abiotic stress (Dáder et al., 2016; Nachappa et al., 2016; Yvon et al., 2017). It has been anticipated that abiotic stresses can impact multiple steps of the intricate plant–virus–vector interactions and so modify the transmission rates in many different ways (Mauck et al., 2012; Blanc and Michalakis, 2016). For example, it was demonstrated that the CaMV can 'sense' the aphid feeding activity and immediately produce transmissible morphs, and that this viral "behavior" is also triggered by some abiotic stresses (Martinière et al., 2013). This remarkable phenomenon has been designated 'transmission activation' (Martinière et al., 2013). It is most probably triggered by activation of plant defense pathways against aphid attacks, and that some abiotic stresses induce similar effects is likely due to partially overlapping pathways (Kiegle et al., 2000; Fujita et al., 2006; Suzuki et al., 2014; Pandey et al., 2015). In our study, we revealed that most WD-sensitive accessions have an increased CaMV transmission rate under WD, despite a reduced virus accumulation. This surprising observation might reflect a higher induction of signaling/defense pathways than in others accessions, and thus potentially, a stronger 'transmission activation' of the CaMV.

Virus accumulation in source plants was evaluated as a potential explanatory factor of the altered transmission efficiency. Indeed, it has been reported that abiotic stresses modify viral load of several viruses, such as *Tobacco mosaic virus* and *Potato virus A*, within stressed host plants (Dorokhov et al., 2012; Suntio and Mäkinen, 2012). Moreover, a positive correlation between virus load and transmission efficiency has also been reported in

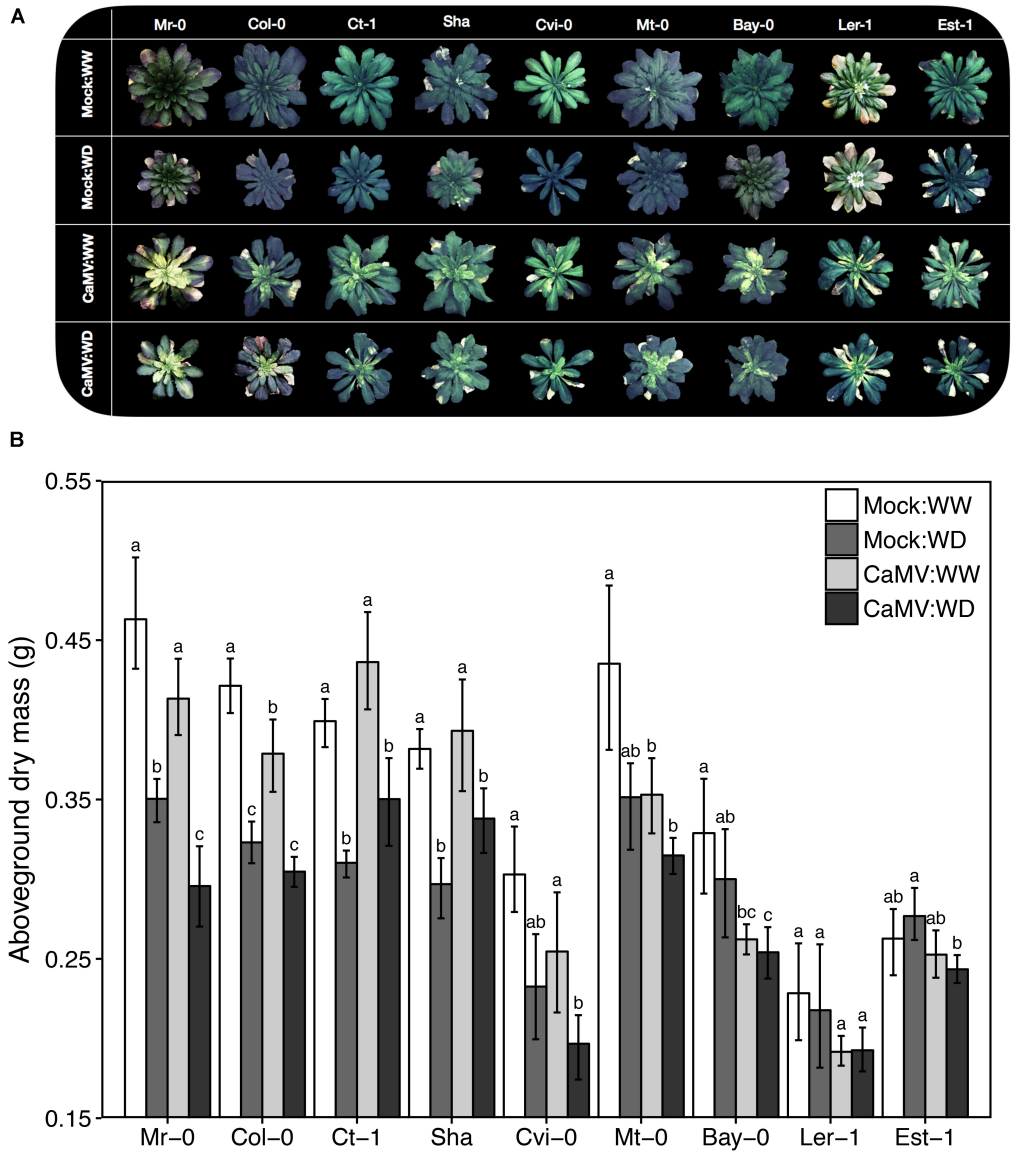

FIGURE 4 | Illustrative photographs of the rosettes and aboveground dry mass of nine *A. thaliana* accessions inoculated with CaMV and grown under two watering conditions. **(A)** Illustrative photographs of the rosettes of nine *A. thaliana* accessions (Mr-0, Col-0, Ct-1, Sha, Cvi-0, Mt-0, Bay-0, Ler-1 and Est-1) inoculated with a mock treatment or with CaMV and grown under well-watered (WW) or water deficit (WD) conditions. All photographs were taken 24 days postinoculation (dpi), 1 day before the transmission test. **(B)** Aboveground dry mass of nine *A. thaliana* accessions. Bars and error bars are means ± bootstrapped 95% confidence intervals of aboveground dry mass 25 dpi and 19 days after the start of water deficit treatment (WD). Mock-inoculated:WW (white bars, $n = 8$), mock-inoculated:WD (dark gray bars, $n = 8$), CaMV-infected:WW (light gray bars, $n = 20$) and CaMV-infected:WD (black bars, $n = 20$) conditions. Different letters indicate a significant difference between treatments for each accession (nonparametric Kruskal–Wallis tests; $P < 0.05$). Error bars were not used to interpret the results. Accessions are ordered according to increasing tolerance to WD.

some instances (Froissart et al., 2010; Doumayrou et al., 2013). In our study, we could not find any significant correlation between these two traits, i.e., a higher viral particles accumulation in the plant did not invariably correlate with a higher transmission. In addition to the phenomenon of the transmission activation discussed above, we also emphasize that many other unknown factors may be responsible for the observed altered transmission under water deficit condition. For example, feeding behavior of the aphid vector may differ on WD infected plants as a consequence of change in plant quality such as changes in carbon-to-nitrogen ratio (Gutiérrez et al., 2013; Prasch and Sonnewald, 2013; Trębicki et al., 2016) and/or morpho-anatomical changes (Carmo-Sousa et al., 2014; Dáder et al., 2017).

In our CaMV-Arabidopsis pathosystem, virulence, as measured by the response ratio of aboveground dry mass, was significantly positively and linearly correlated to transmission rate under WW. The positive relationship between basal virulence and transmission is in accordance with evolutionary

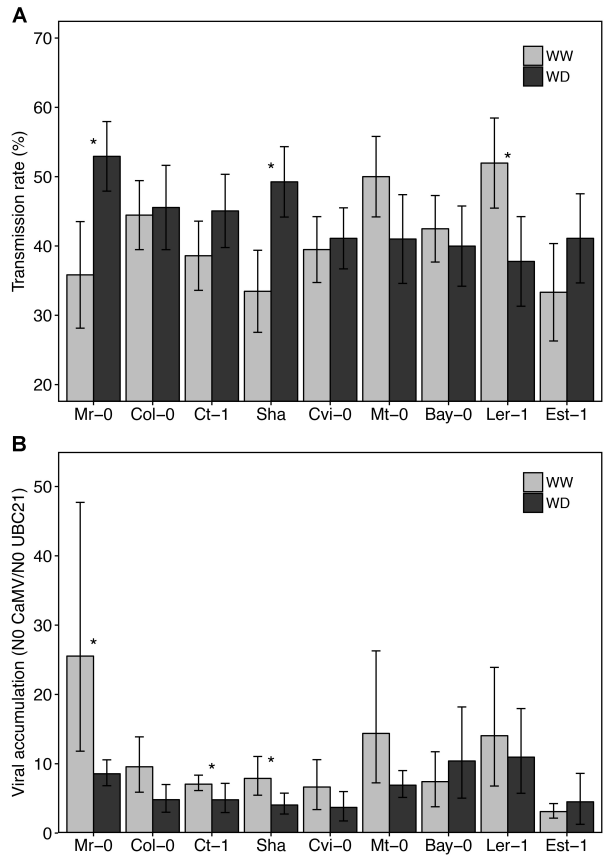

FIGURE 5 | CaMV transmission rate and accumulation in nine *A. thaliana* accessions grown under two watering conditions. Transmission rate **(A)**, i.e., mean proportion of infected test plants ($n = 9$ per source plant) and viral load **(B)** of source plants ($n = 10$) of each accession grown under well-watered (gray bars) and water deficit (black bars), 25 dpi and 19 days after the start of water deficit treatment, respectively. Stars indicate a statistically significant difference between watering treatment for each accession (nonparametric Kruskal–Wallis tests; $P < 0.05$). Error bars represent bootstrapped 95% confidence intervals but were not used to interpret the results. Accessions are ordered according to increasing tolerance to WD.

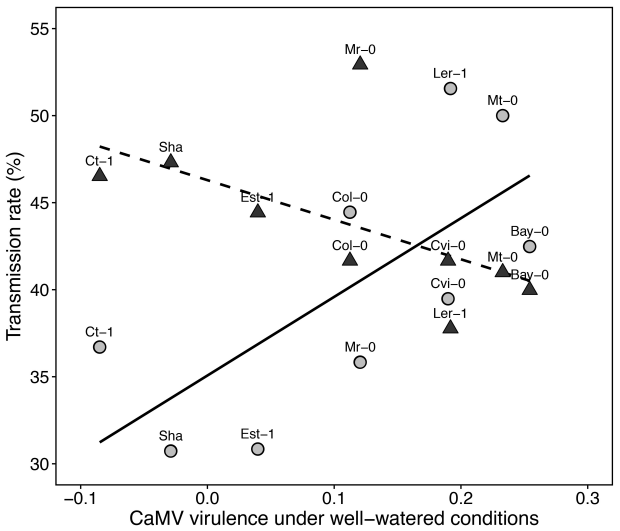

FIGURE 6 | Relationship between virulence and transmission rate of CaMV in nine *A. thaliana* accessions. Each labeled point represents CaMV virulence under well-watered and transmission rate under well-watered (gray circles) and water deficit (black triangles) for each accession. Lines represent linear regressions under well-watered (solid line; Spearman's $r = 0.68$, $P = 0.05$) and water deficit (dashed line; Spearman's $r = -0.75$, $P = 0.019$), respectively.

expectations as well as other empirical observations (Asplen et al., 2012; Doumayrou et al., 2013; Alizon and Michalakis, 2015). The 'trade-off hypothesis' postulates that increased virulence of a parasite is a viable evolutionary strategy if and only if its costs (mortality of the host) are counterbalanced by an increased transmission efficiency (Pagán et al., 2007). For instance, a positive relationship but with a saturating trend between these two traits was found in the CaMV-*Brassica rapa* pathosystem by Doumayrou et al. (2013) when testing different natural CaMV isolates on one cultivar of *B. rapa*. Surprisingly, we showed that WD had a significant reversing effect on this relationship. Indeed, CaMV transmission rate from the most virus-tolerant *A. thaliana* accessions (Sha, Ct-1, Est-1) significantly increased under WD while it decreased or did not change in other accessions. To our knowledge, this is the first study showing an alteration of the relationship (here reversed) between viral tradeoffs due to a change in abiotic conditions (Fraile and García-Arenal, 2016).

CONCLUSION

Our results support the idea that optimal virulence of a given virus, as hypothesized under the transmission-virulence trade-off, is highly dependent on the environment and growth traits of the host. The multi-faceted relationships between virulence, viral load and transmissibility according to the environmental conditions experienced by the host will require further theoretical and experimental investigations. In particular, investigations concerning the behavior of the aphid (alteration of its behavior by the environment) but also on the implication of plant and virus genetic diversity. Especially if these relationships have to be incorporated into models of virus epidemiology under scenarios of climate changes.

AUTHOR CONTRIBUTIONS

SEB, MvM, SB, and DV conceptualized the study. SEB, MvM, MD, AB, CV-R, GR, MY, and DV performed the experiments. SEB, MvM, and DV analyzed the data and wrote the original draft. All coauthors edited and reviewed the final version of 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.2018.00703/full#supplementary-material

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Abscisic Acid as a Dominant Signal in Tomato During Salt Stress Predisposition to Phytophthora Root and Crown Rot

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Salt stress predisposes plants to Phytophthora root and crown rot in an abscisic acid (ABA)-dependent manner. We used the tomato–*Phytophthora capsici* interaction to examine zoospore chemoattraction and assessed expression of pathogenesis-related (PR) genes regulated by salicylic acid (SA) and jasmonic acid (JA) following a salt-stress episode. Although salt treatment enhances chemoattraction of tomato roots to zoospores, exudates from salt-stressed roots of ABA-deficient mutants, which do not display the predisposition phenotype, have a similar chemoattraction as exudates from salt-stressed, wild-type roots. This suggests that ABA action during predisposing stress enhances disease through effects on plant responses occurring after initial contact and during ingress by the pathogen. The expression of *NCED1* (ABA synthesis) and *TAS14* (ABA response) in roots generally corresponded to previously reported changes in root ABA levels during salt stress onset and recovery in a pattern that was not altered by infection by *P. capsici*. The PR genes, *P4* and *PI-2*, hallmarks in tomato for SA and JA action, respectively, were induced in non-stressed roots during infection and strongly suppressed in infected roots exposed to salt-stress prior to inoculation. However, there was a similar proportional increase in pathogen colonization observed in salt-stressed plants relative to non-stressed plants in both wild-type and a SA-deficient *nahG* line. Unlike the other tomato cultivars used in this study that showed a strong predisposition phenotype, the processing tomato cv. 'Castlemart' and its JA mutants were not predisposed by salt. Salt stress predisposition to crown and root rot caused by *P. capsici* appears to be strongly conditioned by ABA-driven mechanisms in tomato, with the stress compromising SA-and JA-mediated defense-related gene expression during *P. capsici* infection.

Keywords: abiotic stress, induced resistance, induced susceptibility, jasmonic acid, *Phytophthora capsici*, salicylic acid

INTRODUCTION

Plants rely on an array of phytohormones to coordinate and finely regulate response networks to biotic and abiotic stresses (Grant and Jones, 2009; Bostock et al., 2014). Studies of phytohormone regulation of defense responses in plant–microbe interactions generally have focused on salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). In *Arabidopsis*, SA-mediated defenses

are considered deterrents to biotrophic pathogens, whereas JA/ET-mediated defenses deter necrotrophic pathogens (Thomma et al., 2001; Glazebrook, 2005). However, this dichotomy with respect to parasitic strategy may be less clear in other host species (Thaler et al., 2004). While studies of SA and JA/ET signaling have shaped our current understanding of induced resistance mechanisms, consideration of other phytohormones is gaining traction in terms of how multiple stress response pathways overlap as non-linear networks to coordinate plant responses to diverse biotic challengers (Grant and Jones, 2009; Eyles et al., 2010; Bostock et al., 2014). These interactions can be synergistic or antagonistic, or phenotypically neutral if the disease assays cannot discern subtle differences. For example, SA and JA can be antagonistic in certain contexts leading to trade-offs in defense against different attackers (Bostock, 2005; Koornneef and Pieterse, 2008). Similarly, there is substantial evidence that elevated levels of the phytohormone, abscisic acid (ABA), can diminish host resistance (Henfling et al., 1980; Audenaert et al., 2002; de Torres-Zabala et al., 2007; Asselbergh et al., 2008). Nonetheless, ABA enhances resistance in some biotrophic and other interactions (Achuo et al., 2006; Ton et al., 2009b). This parasitic context dependency of ABA action illustrates the challenge in identifying a unifying mechanism to explain ABA's effects in plant–microbe interactions.

Various root stresses reduce plant water potential and induce ABA accumulation to trigger adaptive biochemical and physiological changes that enable plants to maintain water balance (Taiz and Zeiger, 2010). However, episodic root stresses, even those from which plants fully recover, occur routinely in agricultural and natural systems, transiently elevating cellular ABA concentrations to levels that predispose plants to inoculum densities they would normally resist (Boyer, 1995; Dileo et al., 2010). Disease predisposition from abiotic stress has long been recognized in the plant pathology literature (Yarwood, 1959), and is particularly well-documented in classic studies of root and crown diseases caused by *Phytophthora* spp., where episodes of waterlogging, soil salinity, and drought are important factors in disease development (Duniway, 1977; MacDonald, 1982). Although a role for ABA in conditioning the increased susceptibility during and following stress episodes is recognized, the underlying mechanisms and impacts on host defenses are unresolved (Asselbergh et al., 2008). Furthermore, relatively little attention has been directed at defense-related phytohormone signaling in root–pathogen interactions where the predisposing stresses of water deficit, hypoxia and soil salinity are encountered most directly (Dileo et al., 2010).

Salicylic acid is involved in multiple physiological processes (Vlot et al., 2009), but is perhaps most studied for its role in systemic acquired resistance (SAR) and as a strong inducer of pathogenesis-related (PR) proteins (van Loon et al., 2006). SA biosynthesis in plants occurs by two pathways, one via isochorismate synthase (ICS), and the other via phenylalanine ammonia lyase (Rippert et al., 2009; Vlot et al., 2009). Knockout mutants in the ICS pathway (Catinot et al., 2008) and transgenic plants carrying *nahG* encoding a bacterial salicylate hydroxylase (Gaffney et al., 1993) have reduced SA levels, are highly susceptible to pathogens, have severely reduced levels of PR-proteins, and fail to develop local and systemic resistance (Métraux et al., 1990; Lawton et al., 1995; Seskar et al., 1998; Nawrath and Métraux, 1999; Audenaert et al., 2002). ABA appears to antagonize SA action in defense (Yasuda et al., 2008; Fan et al., 2009).

Jasmonic acid is an oxylipin involved in defense responses against necrotrophic pathogens and insect herbivores, and has been found to have positive or negative interactions with SA depending on the specific host-parasite/pest context (Moons et al., 1997). JA also acts synergistically with the phytohormone ET, and either synergistically or antagonistically with ABA (Robert-Seilaniantz et al., 2007). In soybean leaves, JA levels increase with ABA during dehydration, and a few studies have shown ABA signaling necessary for JA biosynthesis and elaboration of defense responses (Creelman and Mullet, 1995; Adie et al., 2007). In *Arabidopsis*, application of ABA suppresses some JA/ET activated genes such as *PDF1.2*, while JA/ET responsive genes are up-regulated in ABA-deficient mutants such as *aba1* and *aba2* (Anderson et al., 2004). Several JA synthesis mutants are available in tomato, including *defenseless-1* (*def1*), blocked in the conversion of 13-hydroperoxylinoleic acid to 12-oxophytodienoic acid, and *acx1*, a mutant defective in acyl-CoA oxidase (Howe et al., 1996; Li et al., 2005). These mutations result in reduced JA accumulation and pathogen-related transcripts (Schilmiller et al., 2007).

Phytophthora capsici is a broad host-range pathogen that can cause significant economic losses in vegetable crops in the Cucurbitaceae, Solanaceae, and Leguminosae families (Lamour et al., 2012). Similar to other soilborne *Phytophthora* species, *P. capsici* causes extensive root and crown rots that are exacerbated by predisposing stresses such as waterlogging and salinity. In a previous study, we imposed acute levels of salt stress on hydroponically grown tomato seedlings prior to inoculation with *P. capsici* to show that predisposition in roots and stems occurred in an ABA-dependent and ET-independent manner (Dileo et al., 2010). In a related study (Pye et al., 2013), we showed that plant activators that engage SA-mediated defenses in tomato induce resistance to the bacterial speck pathogen, *Pseudomonas syringae* pv. *tomato* (*Pst*), both in non-stressed and salt-stressed plants, but not in the case of *P. capsici* where plants exposed to these same treatment regimes displayed similar severity of root and crown rot. The objective of this study was to further assess the impact of salt stress on the infection and colonization of tomato roots by *P. capsici* and to determine if there is discernible interaction between ABA and SA or JA during salinity-induced predisposition. We examined the impact of salt stress on *P. capsici* zoospore attraction and early infection and colonization in tomato roots in wild-type and ABA-deficient mutants. Since ABA can alter the action of SA and JA (Robert-Seilaniantz et al., 2011), we evaluated SA- and JA-deficient tomato plants for altered predisposition phenotypes. In addition, we profiled the expression of hallmark genes for stress adaptation and defense during predisposition onset and recovery and *P. capsici* infection.

MATERIALS AND METHODS

Plant Material and Hydroponic Cultivation

Tomato plants (*Solanum lycopersicum*) of cultivars 'New Yorker,' 'Rheinlands Ruhm,' or 'Castlemart' and mutant or transgenic lines within these backgrounds were used in experiments. 'New Yorker' and 'Rheinlands Ruhm' are determinate and indeterminate cultivars, respectively, used primarily for fresh market consumption, and 'Castlemart' is a determinate, processing cultivar that was bred for the arid growing conditions of California and other regions. In our experimental format, all three cultivars are susceptible to *P. capsici*. 'New Yorker' seeds were obtained from a commercial source (Totally Tomatoes, Randolph, WI, United States). The homozygous ABA-deficient mutants *sitiens* and *flacca* were compared with their isogenic, wild-type background, 'Rheinlands Ruhm,' and seeds for these were obtained from the C. M. Rick Tomato Genetics Resource Center, University of California, Davis. 'Rheinlands Ruhm,' *sitiens*, and *flacca* plants were grown for seed production in the greenhouse. NahG transgenic plants were generated in the 'New Yorker' background, similar to the method used by Gaffney et al. (1993). The *nahG* construct containing the transgene salicylate hydroxylase under control of the CaMV 35S promoter in the binary vector pCIB200 was a gift of Syngenta Crop Protection, Inc. SA deficiency of our transgenic line was confirmed previously (Pye et al., 2013). The *acx1* and *def1* mutants in the cv. 'Castlemart' background were a gift of Gregg Howe, Michigan State University. Seeds of 'Castlemart' were obtained from the C. M. Rick Tomato Genetics Resource Center. Four-week-old plants with two or three true leaves were grown hydroponically as described previously (Dileo et al., 2010; Pye et al., 2013). Experiments were conducted in a growth chamber (150 μmol m^{-2} s^{-1}, 16-h photoperiod, 22°C, 70% RH).

Pathogen Isolates and Culture

A pepper isolate of *P. capsici* (designated "Yolo-1," from Yolo County, CA, United States; also pathogenic on tomato) was used for most experiments. A *P. capsici* isolate transformed with the green fluorescent protein (GFP) was a gift of Christine Smart and William Fry, Cornell University (Dunn et al., 2013). Wild-type and transformant *P. capsici* strains were maintained on V8 juice agar plates or V8 juice amended with 100 mg/L geneticin (G418; Gibco), respectively. Zoospore inoculum was prepared using methods described previously (Dileo et al., 2010).

Salinity Stress Treatment and Inoculation

The salt stress regime selected for these experiments was based on prior studies of root stress predisposition (MacDonald, 1982; Bostock et al., 1990; Dileo et al., 2010). The impact of salinity stress differs from other osmotic dehydration stresses primarily in that salt-stressed plants are additionally exposed to abnormally high extracellular concentration of ions such as sodium and chloride (Bartels and Sunkar, 2005). The inclusion of calcium helps to mitigate the confounding toxicity caused by sodium and emphasizes the osmotic facet of salinity stress, which is likely applicable to other dehydration stresses (Cramer, 2002). Plants were subjected to salt stress by replacing the 0.5X Hoagland's solution with 0.2 M NaCl and 0.02 M $CaCl_2$ for 18 h. Plants were returned to 0.5X Hoagland's solution, allowed to regain turgor and recover for 2 h, and then inoculated with zoospores of *P. capsici* (10^4 or 10^5 ml^{-1}, as indicated).

Zoospore Attraction

To determine whether there was an effect on zoospore motility and chemotaxis, a microcapillary swim-in assay similar to that described by Morris and Ward (1992) was used with exudates collected from tomato roots. Following 18 h salt stress, tomato roots of uniform volume were rinsed in deionized H_2O and transferred to tubes containing 2 ml of deionized water. Exudates were allowed to accumulate for 2 h, tomatoes were then removed, and the exudates were vortexed and immediately loaded into 1 μl microcapillary tubes (Drummond Scientific, Broomall, PA, United States). Exudate-loaded microcapillaries were placed into 15 cm petri dishes with one end submerged in a 500 μl droplet of 5×10^5 zoospores ml^{-1}. Microcapillaries were photographed under a dissecting microscope 15 min after being placed into the zoospore suspension. Zoospore attraction was determined as the proportion of the microcapillary's inside diameter blocked by encysted zoospores and scored on a 0–5 rating scale (zoospore attraction rating scale, ZARS; **Figure 2**).

Confocal Microscopy

The *P. capsici*-GFP transformant was visualized 24 and 48 hours post inoculation (hpi) in tomato roots using a Leica TCS SPE confocal system (Leica Microsystems GmBH, Germany). Following infection and just prior to microscopy, roots were dipped into a 10 μg/ml solution of propidium iodide (PI, Sigma) for 30 s and rinsed in sterile water before mounting on microscope slides (Huang et al., 1986). GFP was excited at 488 nm and emission was collected between 510 and 550 nm. PI was excited at 534 nm and emission was collected between 600 and 650 nm. Laser power was set to 50% with a gain of 800–900 for both the 488 nm and 534 nm channels. Final images were composites of five Z steps through root tissues approximately 40 μm in depth.

Pathogen DNA Quantitation and Gene Expression Profiling in Infected Host Tissue

To estimate the progression of *P. capsici* colonization in tomato seedlings by qPCR, *nahG, def1,* and *acx1* plants and wild-type plants of their corresponding backgrounds (cvs. 'New Yorker' and 'Castlemart') were frozen in liquid N_2 at 48 hpi, and stored at −80°C until extraction and analysis. Samples for quantitation of *P. capsici* DNA were extracted and analyzed using the method described in Dileo et al. (2010). For gene expression analyses, RNA was extracted from tomato seedlings using RNeasy Plant Mini kits according to the manufacturer's instructions (Qiagen, Valencia, CA, United States). Samples were obtained from roots pooled from five plants, with three samples for each treatment in each experiment. Extracts were

treated with Dnase I (Fermentas) to remove genomic DNA contaminants. Intact 25s and 18s ribosomal RNA bands were visualized by gel electrophoresis (D'Ambrosio et al., 2004). cDNA stock solutions were prepared with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, United States). A complete list of target genes and primers can be found in **Table 1**. Gene expression was quantified with a 7500 FAST Real-time PCR thermocycler (Applied Biosystems, Foster City, CA, United States), using SsoFAST EvaGreen Supermix with low Rox (Bio-Rad, Hercules, CA, United States). Relative quantities were determined using the $\Delta\Delta$CT method, normalizing against cyclophilin (*Cyp*, M55019.1) and uridylate kinase (*UK*, SGN-U566261).

JA-Treatment Experiments

Jasmonic acid was generated by base hydrolysis of methyl jasmonate [3-oxo-2-(2-pentenyl)cyclopentaneacetic acid, methyl ester, 95% purity; Sigma-Aldrich] according to the procedure of Farmer et al. (1992). The experimental treatment sequence was as follows. Roots of hydroponically grown tomato seedlings (cv. 'New Yorker') were immersed for 72 h in a solution of JA (25 μM in 0.5X Hoaglands, final concentration of immersive solution). Seedlings were removed from the JA solution and transferred to fresh 0.5X Hoaglands for 48 h, and then exposed to salt stress for 18 h as described above. After a 2 h recovery in 0.5X Hoaglands, the roots were inoculated with 1×10^4 zoospores/ml of *P. capsici*. Roots were then collected at 24 hpi for gene expression analyses as described above, with samples obtained from roots pooled from five plants and three samples analyzed for each treatment. JA at 25 μM was selected because higher concentrations (50–100 μM) were slightly phytotoxic in our experimental format. Appropriate controls (i.e., no JA, no salt, no inoculation, and various combinations thereof, as indicated) were included.

TABLE 1 | Real-time qPCR primers used in this study.

Name	Sequence	Product length
Cyp	5′GGCCAATTCTGGACCTAACA′3 5′CATGTTCCATAGAGCGGACA′3	134 bp
UK	5′GCTGTTTTTGCCCCATCTAA′3 5′CATCGTTTTGCTGCTGAAGA′3	154 bp
Phytophthora capsici target (for quantifying colonization)	5′TTTAGTTGGGGGTCTTGTACC3′ 5′CCTCCACAACCAGCAACA3′	452 bp
TAS14	5′AGATGGCACAATACGGCAAT′3 5′ACCAGTACCCATGCCTTGAG′3	174 bp
NCED1	5′CTGCTTCTTCCCAAGCATTC′3 5′ACCTGTTCCACCACAAGGAC′3	176 bp
P4	5′AGGTGACACTATAGAATAAACA ATGGGTGGTGGTTCAT′3 5′GTACGACTCACTATAGGGATAG CAACATGTCAGAAATAGACGA′3	143 bp
PI-2	5′CCCACGTTCAGAAGGAAGTC′3 5′TGAACGGGGACATCTTGAAT′3	142 bp
13-LOX (TomLOXD)	5′TTGTGCCTGAAAAAGCAGTG′3 5′GTTCTAGCGCGACATTCCTC′3	141 bp
13-AOS (LeAOS1)	5′GGGGCTAAACTCCACAGTCA′3 5′TGCTACCGGAGGTTCAATTC′3	147 bp

For each pair, the forward primer is listed first followed by the reverse primer. See text for further description.

Statistical Analyses

Disease assays in 'New Yorker,' 'Rheinlands Ruhm,' and 'Castlemart' backgrounds were performed three times, with five replicates for each treatment within each experiment. For ordinal data and for qPCR data that typically did not satisfy the analysis of variance (ANOVA) criterion for normality, the Wilcoxon rank sums or Kruskal–Wallis tests were used for means comparisons. Gene expression time courses were performed twice. When data satisfied the criterion for normality, ANOVA and the Dunnett's test or Student's *T*-test were used for means comparisons. Analyses were performed with JMP Pro software (SAS, Inc.).

RESULTS

P. capsici Zoospore Attraction and Infection in Predisposed Tomato Roots

A brief episode of salt stress applied prior to inoculation of tomato seedlings with zoospores of *P. capsici* results in infections of greater severity and a classic predisposition phenotype (**Supplementary Figure S1**). Previously, increased zoospore attraction was observed in salt-stressed chrysanthemum roots relative to non-stressed roots (MacDonald, 1982). To determine if salt-stress enhances the attraction of tomato roots to zoospores and whether ABA influences this, we used a quantitative chemotaxis choice assay to compare exudates from non-stressed and salt-stressed tomato roots. Exudates collected from ABA-deficient *flacca* and *sitiens* mutants and their background wild-type 'Rheinlands Ruhm' roots following salt stress were significantly more attractive to *P. capsici* zoospores than exudates collected from non-stressed roots. However, exudates from the ABA-deficient mutants, *sitiens* and *flacca*, were equally attractive as those collected from 'Rheinlands Ruhm' (**Figure 1**). ABA alone was not a chemoattractant in this assay, having a ZARS value of 0, the same as deionized water.

We used confocal microscopy to further characterize root infections under our experimental regime to determine if salt stress of the host prior to inoculation causes *P. capsici* to change its infection and colonization strategy. Examination of roots inoculated with a *P. capsici*-GFP strain 24 hpi revealed haustoria in host cells deep within the root tissue (**Supplementary Figure S2**). Haustoria were observed in both salt-stressed and non-stressed roots, with the only apparent microscopic distinction between the treatments during the course of observation being the greater extent of colonization in salt-stressed roots. Propidium iodide (PI), which stains nuclei in dead or dying cells, was used as a vital stain to assess root cell viability under the various treatments. Non-inoculated roots in the non-stressed and salt-stressed treatments were similar in appearance, with occasional PI-staining of nuclei (**Figures 2A,B**). There was non-specific staining of plant cell walls by PI in all treatments, which is common due to the exclusion of the dye from membranes of living cells that makes outlines of the cells visible. Inoculated, non-stressed roots were mostly intact with limited instances

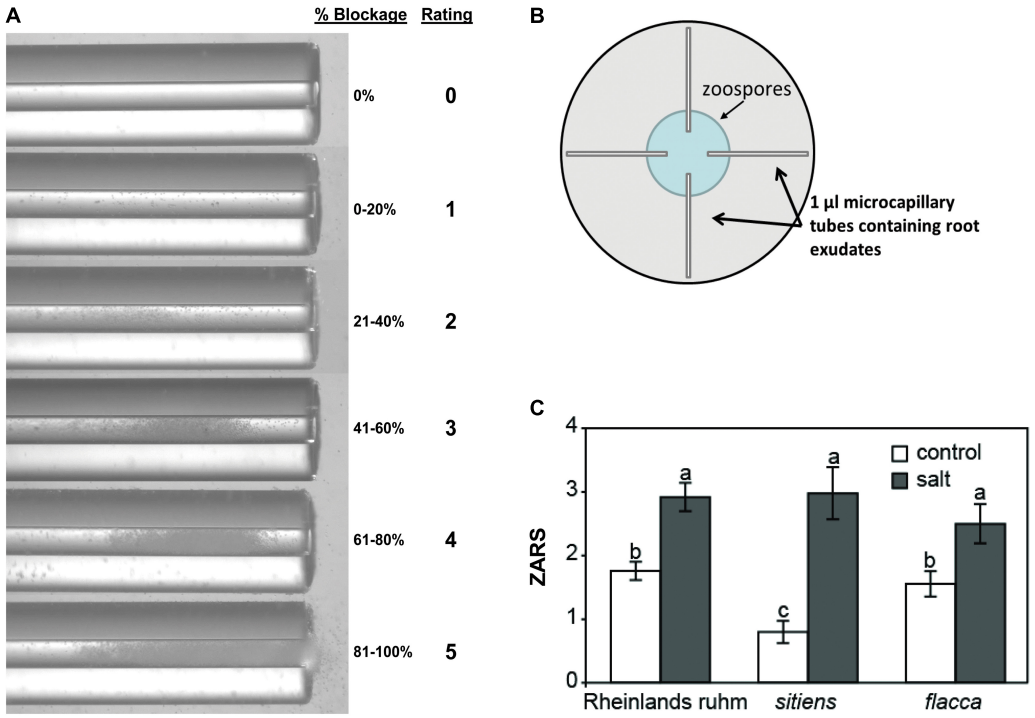

FIGURE 1 | Attraction of *P. capsici* zoospores to the root exudates of tomatoes. Root exudates were collected from non-stressed (control) and salt-stressed (0.2 M NaCl/0.02 $CaCl_2$) roots of wild-type 'Rheinlands Ruhm' (RR) and the ABA-deficient mutants, *sitiens* and *flacca.* **(A)** Microcapillary tubes with root exudates showing varying degrees of blockage of the tube by zoospores. The zoospore attraction rating scale (ZARS) is indicated on the right. **(B)** One microliter microcapillary tubes were filled with exudates and then one end submerged in a droplet of *P. capsici* zoospores (5×10^5 ml^{-1}) in a 15 cm diameter petri dish as shown. Results were scored 15 min later. **(C)** ZARS values for each treatment and tomato genotype. Values are the means ± SE from three experiments, with five samples each from a separate seedling for each treatment within an experiment (n = 15). Letters indicate significant differences among treatment means at P = 0.05 by the Kruskal–Wallis test as performed in JMP Pro 13.0. There is also a significant difference in attraction between the salt and the control treatments of 'Rheinlands Ruhm' and *sitiens*.

of PI staining of nuclei (**Figure 2C**), while inoculated, salt-stressed roots contained numerous PI-stained nuclei (**Figure 2D**). In both treatments, root tips and the bases of lateral roots were the most colonized regions.

ABA-Related Gene Expression During Predisposing Salt Stress and *P. capsici* Infection

In a previous study, we found that ABA levels in tomato roots increase rapidly following exposure to salt stress and during the onset of predisposition, and then decline to near pre-stress levels (Dileo et al., 2010). To determine if the expression of genes associated with ABA synthesis and response follows a similar course during stress onset and recovery, *NCED* and *TAS14* were monitored by qPCR in tomato roots. *NCED* encodes the 9-*cis*-epoxycarotenoid-dioxygenase (EC 1.13.11.51), a critical step in ABA biosynthesis and generally considered to be rate-limiting (Qin and Zeevaart, 1999; Thompson et al., 2007). *TAS14* (X51904.1) is a tomato dehydrin gene that is induced by salt stress and ABA, but not by cold or wounding, and serves as a salt stress-induced marker of ABA responses in tomato (Godoy et al., 1990). *NCED1* expression increased rapidly in tomato roots following salt exposure in a manner that generally corresponded with ABA measurements reported previously (see Figure 5 in Dileo et al., 2010), and returned to pre-stress levels similar to ABA (**Figure 3A**). Salt challenge of tomato roots induced *TAS14* within 3 h after immersion of the roots in the salt solution, with maximum expression as much as ∼4,000-fold above the initial basal expression (**Figure 3B**). *NCED1* gene expression levels returned to basal levels 24 h following removal of the roots from the salt treatment (**Figure 4A**), whereas *TAS14* gene expression levels from the same plants returned to pre-stress values within 12 h of salt removal (**Figure 4B**). The changes in TAS14 expression were limited to salt-stressed roots, as baseline expression in non-stressed roots was at or below the sensitivity of our analytical platform. *P. capsici* infection in either salt-stressed or non-stressed plants did not appear to influence *NCED1*and *TAS14* expression.

Defense-Related Gene Expression Following Salt Stress and Disease Onset

In tomato, *P4* (M69247.1), a *PR-1* ortholog, serves as a marker for induction of the SA pathway (Fidantsef et al., 1999; Uehara et al., 2010). *P4* transcript accumulation was measured in non-stressed and salt-stressed 'New Yorker' tomato roots following inoculation with *P. capsici*. *P4* was induced only in plants inoculated with *P. capsici* (**Figure 5A**). Plants that had been

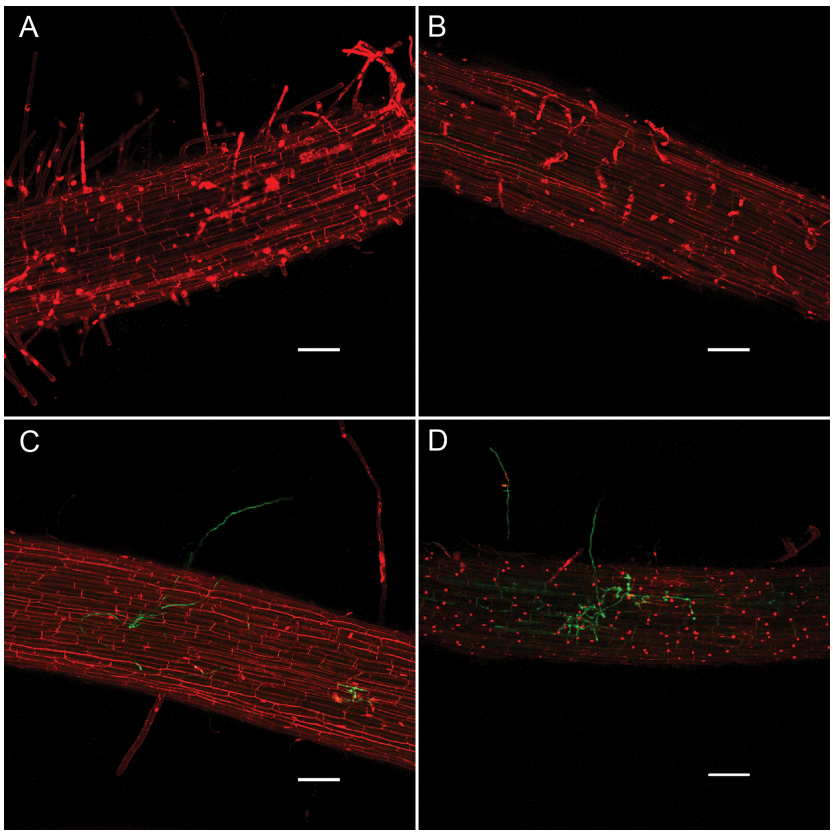

FIGURE 2 | Infection of 'New Yorker' tomato roots by *P. capsici*-GFP (green fluorescence) visualized by confocal microscopy with propidium iodide staining (red fluorescence), 24 hpi. **(A)** Non-stressed non-inoculated tomato roots. **(B)** Salt-stressed (0.2 M NaCl/0.02 $CaCl_2$), non-inoculated tomato roots. **(C)** Non-stressed, inoculated tomato roots (*P. capsici* at 10^4 zoospores ml^{-1}). **(D)** Salt-stressed (0.2 M NaCl/0.02 $CaCl_2$) inoculated tomato roots (*P. capsici* at 10^4 zoospores ml^{-1}). Bars indicate 100 μm.

salt-stressed prior to inoculation had significantly lower levels of *P4* transcripts relative to non-stressed, inoculated plants (**Figure 5A**). *P4* expression remained suppressed even at 48 hpi in salt-stressed, inoculated plants.

In tomato, proteinase inhibitor II (*PI-2,* K03291.1) is a wound- and pathogen-inducible marker of JA responses (Farmer and Ryan, 1992; Hondo et al., 2007). *PI-2* showed a similar pattern of expression as *P4* in our experimental regime and was induced only in *P. capsici*-inoculated plants (**Figure 5B**). Prior salt stress resulted in significantly reduced *PI-2* gene expression throughout the period of observation (48 hpi; **Figure 5B**). Salt stress alone did not induce *P4* or *PI-2* expression.

Assessment of Predisposition to Phytophthora Root and Crown Rot in SA and JA-Modified Tomato Plants

To determine if SA and JA influence the severity of disease susceptibility induced by salt-stress, tomato plants altered in SA levels (*nahG* transgenic) and JA synthesis (*acx1* and *def1* mutants) were evaluated in the predisposition assay. NahG and WT (cv. 'New Yorker') tomatoes both displayed enhanced susceptibility following salt stress, but NahG plants had significantly higher basal susceptibility to *P. capsici* even without salt stress (**Figure 6**). Nonetheless, the proportional increase in *P. capsici* colonization in salt-treated plants relative to non-salted plants was similar in both the WT (3.2-fold increase) and NahG (3.1-fold increase) tomato genotypes.

'Castlemart' tomatoes, and the *acx1* and *def1* mutants within this genetic background, unlike other tomato genotypes we have used in predisposition studies, did not display a predisposition phenotype under our treatment regime (**Supplementary Figure S3**). Colonization of these plants by *P. capsici* trended less in the salt-treated seedlings, and significantly less ($P = 0.032$) in salt-treated *acx1* seedlings compared to non-salted plants (**Supplementary Figure S3B**). This was unexpected, rendering results with the *def1* and *acx1* mutants inconclusive relative to the issue of JA action in predisposition.

Without suitable JA-deficient mutants available to this study, we then sought to determine whether exogenous JA could alter or override the salt stress inhibition of *PI-2* gene expression using 'New Yorker' seedlings, which display a consistent and clear predisposition phenotype. Treatment of roots with exogenous JA (25 μM) strongly induced *PI-2* transcripts, with salt treatment reducing transcript accumulation (**Figure 7A**). The *PI-2* expression pattern was similar in the inoculated

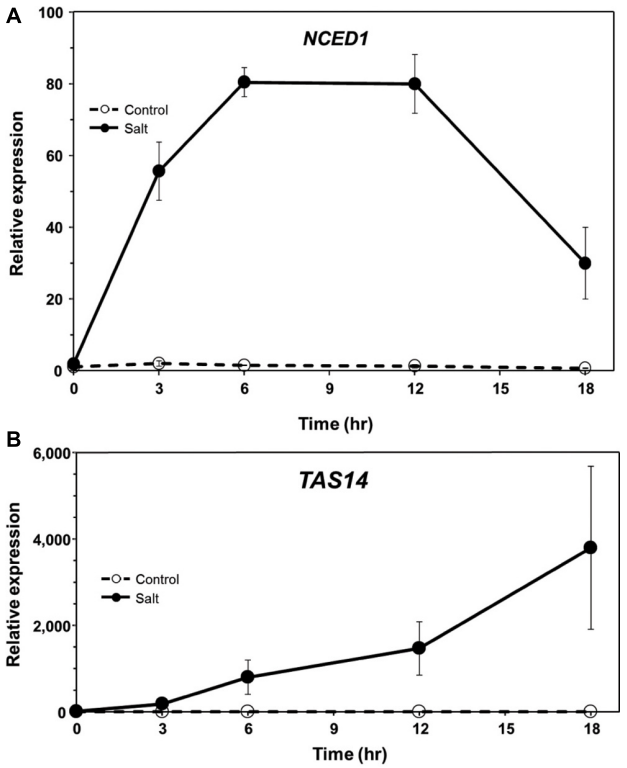

FIGURE 3 | ABA-related gene expression in 'New Yorker' tomato seedling roots during the onset of salt stress. **(A)** Time course of *NCED1* and **(B)** *TAS14* expression in non-stressed (control) and salt-stressed tomato roots (0.2 M NaCl/0.02 $CaCl_2$). *NCED1* and *TAS14* expression was normalized against *Cyp* and *UK*. Values are the means ± SE from two experiments, with roots from five plants pooled per sample and three samples analyzed for each treatment mean per time point.

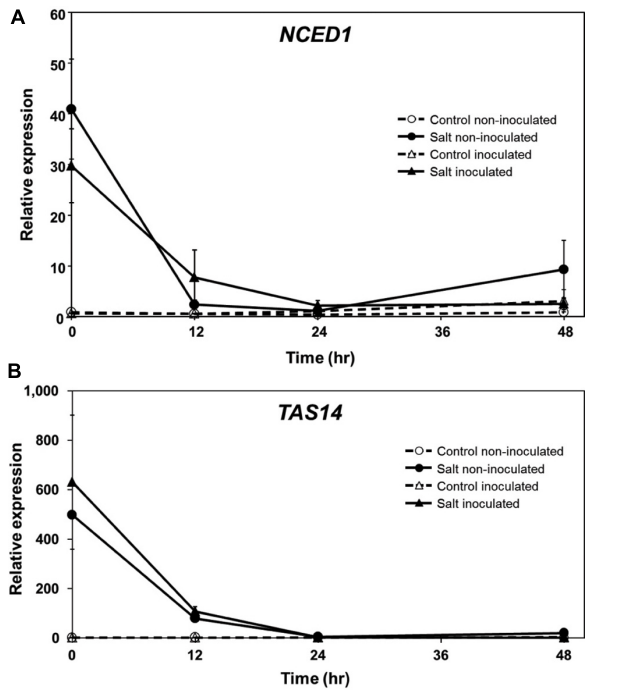

FIGURE 4 | ABA-related gene expression in inoculated 'New Yorker' tomato seedling roots following an episode of salt stress. **(A)** *NCED1* and **(B)** *TAS14* expression in non-stressed (control) roots and in roots after 18 h exposure to 0.2 M NaCl/0.02 $CaCl_2$, with (triangles) and without (circles) inoculum (*P. capsici* at 2 ml of 10^4 zoospores ml^{-1}). *NCED1* and *TAS14* expression was normalized against *Cyp* and *UK*. Values are the means ± SE from two experiments, with roots from five plants pooled per sample and six samples analyzed for each treatment mean per time point. Note that seedlings were removed from the salt stress and returned to 0.5X Hoaglands during the course of analysis.

seedlings pretreated with JA and/or salt. The tomato *13-LOX* and *13-AOS* genes encode key enzymes in JA biosynthesis (Mosblech et al., 2009). *13-LOX* expression at the time of sampling was not significantly affected by any treatment (**Figure 7B**). Although *AOS* transcript levels were relatively low in all treatment combinations, salt stress reduced *AOS* expression by more than half in both non-inoculated and inoculated seedling roots (**Figure 7C**). This reduction was partially offset by JA pre-treatment. *P4* expression was not induced by JA, salt or their combination; however, inoculation with *P. capsici* following JA treatment resulted in a strong induction of *P4* transcripts (**Figure 7D**).

DISCUSSION

Previous research in our laboratory demonstrated that tomato seedling roots and crowns became highly susceptible to *P. capsici* following a brief exposure of the roots to salt stress (Dileo et al., 2010) (**Supplementary Figure S1**). These plants generally regained turgor during the course of the stress treatment, but remained in a predisposed state in the absence of visible stress symptoms for up to 24 h following removal from the salt. The salt stress effect on disease appears to operate through an ABA-dependent mechanism, as evidenced by the loss of predisposition in ABA-deficient mutants and partial complementation with exogenous ABA to restore the predisposition phenotype (Dileo et al., 2010). Salinity stress also has been shown to make roots more attractive to *Phytophthora* zoospores (MacDonald, 1982). In the present study, chemoattraction of *P. capsici* zoospores to exudates from salt-stressed roots was significantly greater than to exudates from non-stressed roots. However, exudates from salt-stressed roots of wild-type tomato plants and ABA-deficient mutants were equally attractive (**Figure 1**). Thus, differences in root attraction to zoospores cannot explain the differences in disease severity between wild-type and ABA-deficient plants. These results reinforce our view that the determinative effects of stress-induced ABA in predisposition occur during infection, invasion and colonization, rather than during pre-infection events related to root exudation, zoospore attraction and initial contact with the root (Swiecki and MacDonald, 1988). Our results also affirm an earlier study on salinity-induced susceptibility to Phytophthora root rot that pointed to a strong effect of the stress on host defenses (MacDonald, 1984).

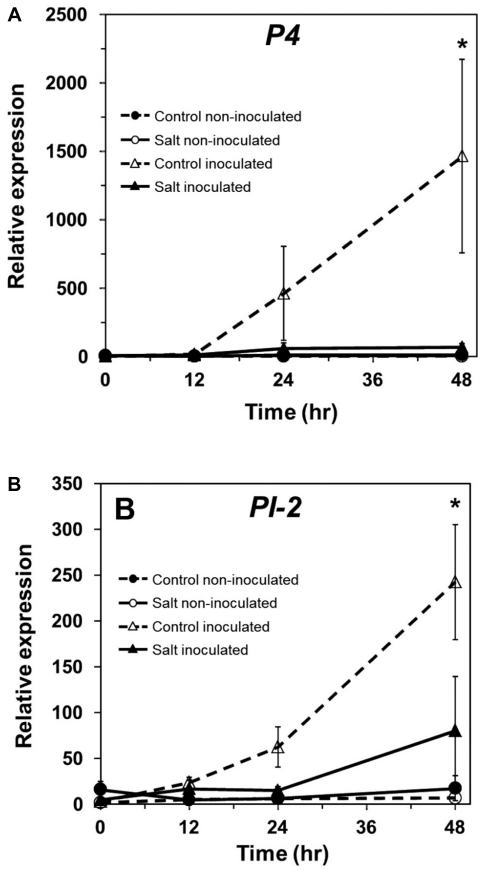

FIGURE 5 | Pathogenesis-related protein gene expression in inoculated and salt-stressed 'New Yorker' tomato seedling roots. **(A)** Time course of *P4*, and **(B)**, *PI-2* expression in non-stressed (control) and 18 h salt stressed (0.2 M NaCl/0.02 $CaCl_2$) roots not inoculated or inoculated with *P. capsici* at 10^4 zoospores ml^{-1}. *P4* and *PI-2* expression was normalized against *Cyp* and *UK*. Values are the means ± SE from two experiments, with roots from five plants pooled per sample and six samples analyzed for each treatment mean per time point. Asterisks indicate significant differences among treatment means by the Wilcoxon rank sums test. For *P4*, $\chi^2 = 14.06$, $P = 0.003$; for *PI-2*, $\chi^2 = 12.19$, $P = 0.007$.

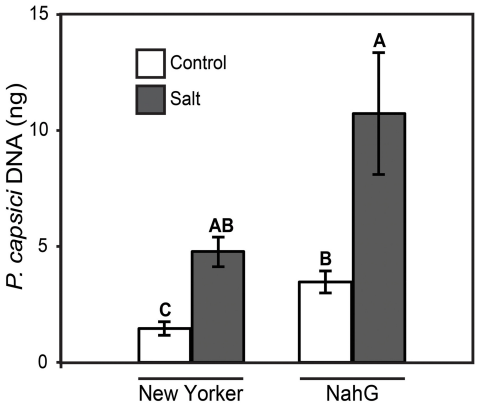

FIGURE 6 | *P. capsici* colonization 48 hpi on 'New Yorker' (WT) and NahG (in "New Yorker' background) tomato seedlings non-stressed (control) or salt stressed with 0.2 M NaCl/0.02 M $CaCl_2$ for 18 h. Colonization estimated by qPCR of pathogen DNA. Letters indicate significant differences at $P = 0.05$ (*T*-test). Values are the means ± SE from three experiments, with five samples, each from a separate seedling, for each treatment within each experiment ($n = 15$).

P. capsici is a hemibiotroph, establishing haustoria in host cells during the early stages of infection, and then necrotizing host tissue as the infection progresses (Lamour et al., 2012). Confocal imaging revealed the presence of haustoria in infected tomato roots that appeared as simple protrusions into root cells (**Supplementary Figure S2**), closely resembling those described in the literature for *Phytophthora* haustoria (Hwang et al., 1989; Lee et al., 2000). After reviewing dozens of *P. capsici* infections in non-stressed and salt-stressed roots, we concluded that haustoria are present in both treatments. Therefore, it does not appear that *P. capsici* alters its fundamental infection strategy in salt-stressed tomato roots. The only clear distinction apparent between treatments was the increased rate of colonization, as reflected in greater abundance of hyphae in the salt-stressed roots relative to the controls. While the pathogen's infection strategy does not appear to change, based on microscopic examination, it is possible that *P. capsici* alters its strategy in other ways, such as the timing or pattern of display of effectors. We attempted to measure expression of putative and known *P. capsici* effector genes believed to correspond to the switch from biotrophy to necrotrophy (Kelley et al., 2010). Pathogen RNA proved difficult to recover during early infection and later as plant tissues died, and so we were unable to detect alterations in effector expression as a function of treatment. Transcriptome analyses using deep sequencing as reported in a study of *P. capsici* on tomato leaves may prove to be better able to address this question (Jupe et al., 2013).

Endogenous ABA levels are tightly regulated in the plant by balancing biosynthesis, catabolism and conjugation (Tian et al., 2004; Nambara and Marion-Poll, 2005; Szepesi et al., 2009). *NCED1* expression in roots during the 18 h salt stress treatment (**Figure 3A**) generally corresponded with salt-induced ABA accumulation that we reported in our previous study (Dileo et al., 2010). Similar findings in *Phaseolus vulgaris* showed stress-induced expression of *NCED*, with accumulation of NCED protein and ABA occurring within a 2 h window (Qin and Zeevaart, 1999). While stimuli have been described that up-regulate *NCED1* gene expression, relatively little information is available regarding mechanisms for its down regulation. In drought-stressed *Arabidopsis*, ABA production and expression of *NCED3* (homologous to tomato *NCED1*) is correlated with the level of available carotenoid substrates (Tian et al., 2004). *NCED1* expression in tomato roots may diminish as ABA levels decline or as external stresses are removed. Possible post-transcriptional and/or post-translational regulation of *NCED1*/NCED cannot be ruled out, as suggested for regulation of *AAO* (abscisic aldehyde oxidase), the terminal step in ABA synthesis (Xiong et al., 2001; Seo and Koshiba, 2002). Following an episode of salt stress and inoculation with *P. capsici*, *NCED1* transcript levels returned to

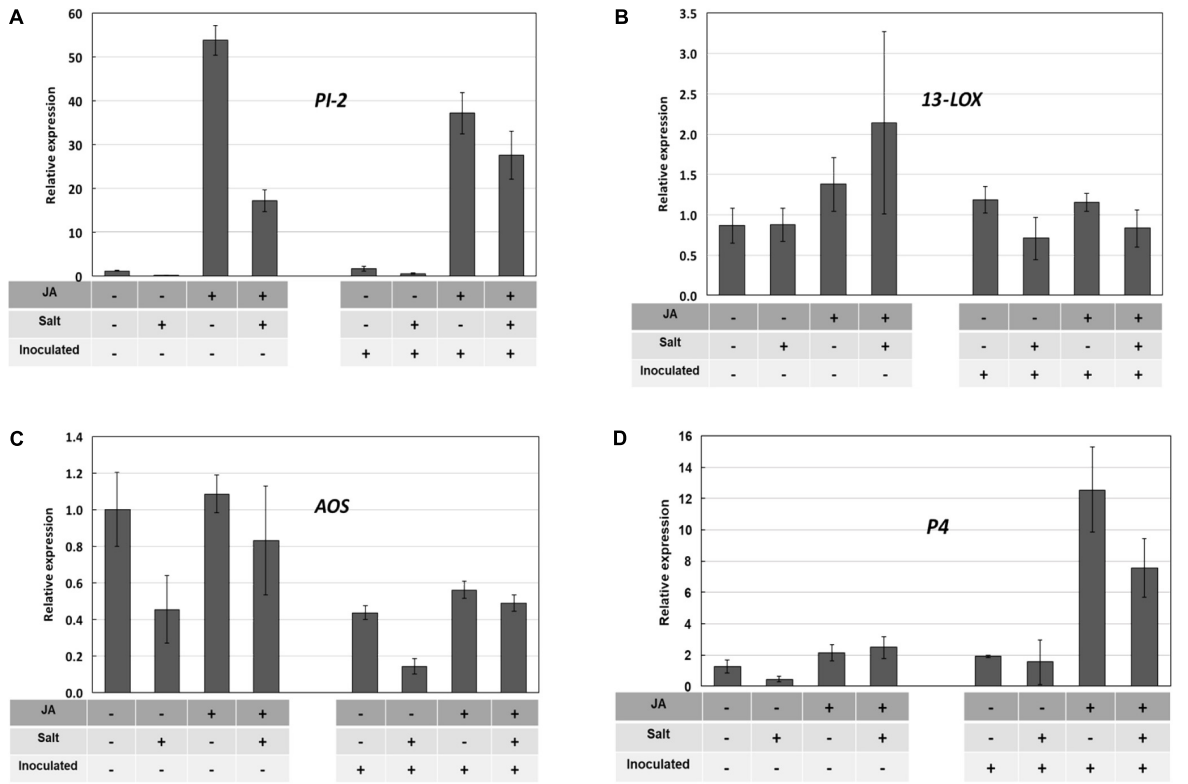

FIGURE 7 | Pathogenesis-related (PR) and JA-synthesis gene expression in 'New Yorker' tomato roots in response to JA, salt stress, and inoculation with *P. capsici*. Treatment combinations are indicated by "+" and "–" below each bar. Bars indicate relative gene expression determined by RT-qPCR (means and SE from three determinations are indicated; selected means were compared by Wilcoxon rank sums test). The treatment sequence was: JA or water (72 h) recovery (48 h) salt or no salt (18 h) recovery (2 h) inoculated or non-inoculated (24 h) collect roots for RT-qPCR. See Section "Materials and Methods" for additional detail. **(A)** *PI-2* expression; JA strongly induced *PI-2* expression ($\chi^2 = 15.6$, $P < 0.0001$), salt suppressed the JA induction ($\chi^2 = 3.15$, $P = 0.076$) **(B)** *13-LOX* expression; **(C)** *13-AOS* expression; salt reduces AOS expression ($\chi^2 = 4.03$, $P = 0.045$) **(D)** *P4* expression; JA potentiates *P4* expression in inoculated roots ($\chi^2 = 3.00$, $P = 0.083$).

pre-stress levels in tomato roots and remained at basal levels in all treatments throughout the 48 h infection time course (**Figure 4A**). However, we saw no evidence for *NCED1* induction or ABA accumulation during infection with *P. capsici*. This is in contrast to *Arabidopsis* infected by *Pst*, which induces *AtNCED3* and ABA accumulation in leaves (de Torres-Zabala et al., 2007).

Expression of *TAS14*, which encodes a tomato dehydrin, is triggered by osmotic stress and ABA (Godoy et al., 1990). When overexpressed in tomato, *TAS14* confers partial drought and salinity tolerance (Muñoz-Mayor et al., 2012). In our study, *TAS14* increased rapidly after salt stress onset and remained elevated throughout the course of the stress treatment. Similar to *NCED1*, *TAS14* did not show altered expression following *P. capsici* infection, and in the case of salt treatment, *TAS14* expression returned to basal levels within 24 hpi (**Figure 4B**).

The possibility of *P. capsici*-derived ABA was of interest because some plant pathogenic fungi produce ABA (Dorffling et al., 1984; Crocoll et al., 1991), and some stramenopiles such as the malarial pathogen, *Plasmodium falciparum*, are capable of ABA synthesis (Tonhosolo et al., 2009). However, we did not detect ABA in *P. capsici* culture filtrates or mycelium by immunoassay (Pye et al., 2013), and genes encoding the necessary biosynthetic enzymes are not evident in oomycete genomes (Tyler et al., 2006). Furthermore, we found no evidence that *P. capsici* infection further engages the pathway as part of its infection strategy, either in non-stressed or salt-stressed tomato plants. These results indicate that salt stress, but not *Phytophthora* infection, strongly engages the ABA pathway in tomato roots – *NCED1* and *TAS14* gene expression, and ABA synthesis and accumulation.

The SA-induced tomato PR protein, P4, is homologous to PR-1 in tobacco and *Arabidopsis*. *P4* gene expression is induced in tomato leaves by plant activators (SA-mimics), pathogens, including *Phytophthora infestans*, and the oomycete elicitor arachidonic acid (Joosten et al., 1990; Van Kan et al., 1992; Fidantsef et al., 1999). We found that infection of tomato roots by *P. capsici* strongly induces *P4*, but exposure of the roots to salt prior to inoculation essentially abolished *P4* expression relative to non-stressed, inoculated plants (**Figure 5**). Similarly, expression of the JA-induced *PI-2* was significantly reduced in infected plants that had been previously salt-stressed. Our findings that salt stress prevents pathogen-induced SA- and JA-regulated gene expression are consistent with results in other plant–microbe

interactions that demonstrate ABA-mediated suppression of SA and JA defense responses (Anderson et al., 2004; Yasuda et al., 2008).

Tomato plants suppressed in SA accumulation by the *nahG* transgene are more susceptible to *P. capsici* than the wild-type control plants in both non-stressed and salt-stressed assay formats (**Figure 6**). This suggests a role for SA-mediated responses in partially limiting *P. capsici* colonization. However, the proportional increase in pathogen colonization observed in salt-stressed plants relative to non-stressed plants is the same in both WT and NahG backgrounds. Impairment of SA action by salt stress may contribute to increased pathogen colonization; however, we did not see a compounding effect of the SA-deficiency in NahG plants on stress-induced disease severity.

Salicylic acid's role in tomato resistance to *P. capsici* is complex. In a study using chemical activators that mimic SA action to induce resistance, we found these activators when applied to roots induced systemic protection of tomato leaves against the bacterial speck pathogen (*Pst*), with and without predisposing salt stress (Pye et al., 2013). However, these same plant activator treatments afforded no protection against *P. capsici*, with or without the salt stress treatment. *Pst* and *P. capsici* are quite different in their infection strategies and requirements, as well as the organs they attack in the plant, so interpreting differences in disease outcomes following different treatments is a speculative exercise, at best. *P. capsici* may simply be a more aggressive pathogen relative to *Pst*, and our experimental format is highly conducive to root and crown rot disease. So *P. capsici* attack overwhelms any chemically induced resistance that is otherwise capable of withstanding *Pst* challenge. It is also possible that there is subfunctionalization within the SA response network in tomato. NahG expression may impair a set of SA-mediated defenses that are effective against *P. capsici*, but differ from a subset, induced by chemical activators, that are insufficient to resist this pathogen.

The JA-deficient tomato mutants *acx1* and *def1* in the 'Castlemart' background are compromised in defense against insects and pathogens (Ament et al., 2004; Bhattarai et al., 2008). Although severity of the predisposition phenotype can vary among tomato cultivars, we were astonished that 'Castlemart' and its JA mutants were not predisposed by salt, strongly trending instead toward enhanced resistance (**Supplementary Figure S3**). This suggests a stress response in 'Castlemart' that is different from other tomato genotypes we have examined in predisposition studies. The reason for this is unclear, and limited resources precluded our further examining predisposition in this cultivar. Unlike the other genotypes used in our study, 'Castlemart' is a processing variety with a pedigree that may have incorporated different stress tolerances. It is a determinate variety that was bred for arid climates, and arid zone soils are more commonly associated with salinity (R. Chetelat, personal communication). 'Castlemart' has been reported to accumulate proteinase inhibitors in response to high salinity (Dombrowski, 2003).

Jasmonic acid and its methyl ester when applied to leaves can induce resistance in tomato to *P. infestans* (Cohen et al., 1993). *Arabidopsis* mutants in JA perception (Staswick et al., 1998) and synthesis (Savchenko et al., 2010) are more susceptible to oomycete pathogens. Studies with other oomycete diseases also illustrate JA's importance in resistance (Guerreiro et al., 2016). We found that exogenous JA enabled tomato roots to respond in a manner that partially offset the salt stress impairment of PR-protein gene expression (**Figures 7A,D**). The induction of *P4* only during infection of JA-treated plants is reminiscent of the reported sensitization by methyl jasmonate of the plant's response to eicosapolyenoic acid elicitors released during infection by *Phytophthora* species (Il'inskaya et al., 2000) and potentiation of JA signaling by the plant activator β-aminobutryic acid (Hamiduzzaman et al., 2005).

Our results with the tomato genotypes and treatments used in this and previous studies (Dileo et al., 2010; Pye et al., 2013) affirms ABA's dominant effect relative to the salt-induced impacts on SA and JA action during predisposition to Phytophthora root and crown rot. ABA appears to be necessary to predispose tomato seedlings to this disease following acute salt stress. However, results presented here and previously (Pye et al., 2013) indicate that priming through chemical activation (Ton et al., 2009a) of the SA and JA response networks may partially offset the stress-induced impairment of defense-related gene expression and the increased susceptibility in tomato to certain pathogens. We recognize that the response pathways modulated by ABA, JA and SA during episodic root stress may interact in subtle ways beyond the resolution afforded by the pathosystem and treatments we selected (Moeder et al., 2010). Comparative transcriptomics, proteomics and metabolomics of plants under predisposing stress should help identify key regulatory features (Bostock et al., 2014). Studies with additional mutants as well as salt- and drought-tolerant genotypes also may reveal additional variation that could be useful to refine our understanding of the abiotic-biotic stress 'interactome' (Pandey et al., 2015). This information could suggest novel targets to mitigate the impact of root stresses that increase severity of soilborne diseases.

AUTHOR CONTRIBUTIONS

Research conceived and planned by MP, JM, and RB, and executed by MP. Data were analyzed by MP and RB. Experiments presented in **Figure 7** were planned by RSR, SD, and RB, performed by RSR, and analyzed by RSR, SD, and RB. The manuscript was written by MP and RB. All authors 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.2018.00525/full#supplementary-material

FIGURE S1 | The salinity-induced predisposition phenotype in 4-week-old hydroponically grown 'New Yorker' tomato seedlings 48 hpi with *Phytophthora capsici* (10^4 zoospores ml^{-1}).

FIGURE S2 | Confocal visualization of hyphae and haustoria (small arrow) of *P. capsici*-GFP in 'New Yorker' tomato root cells and nucleus (large arrowhead) stained with propidium iodide (PI), 48 hpi. Bars indicate 10 μm.

FIGURE S3 | (A) *P. capsici* colonization 48 hpi on 'Castlemart' and JA- deficient *def1* (in 'Castlemart' background) tomato seedlings non-stressed (control) or salt stressed with 0.2 M NaCl/0.02 M $CaCl_2$ for 18 h prior to inoculation. Colonization estimated by qPCR of pathogen DNA. Values are the means ± SE from three experiments, with five samples, each from a separate seedling, for each treatment within each experiment (n = 15). Differences are not significant (Wilcoxon rank sums test, χ^2 = 3.08, P = 0.379). **(B)** *P. capsici* colonization 48 hpi on 'Castlemart' and JA-deficient *acx1* (in 'Castlemart' background) tomato seedlings non-stressed (control) or salt stressed with 0.2 M NaCl/0.02 M $CaCl_2$ for 18 h prior to inoculation. Colonization estimated by qPCR of pathogen DNA. Values are the means ± SE from three experiments (n = 15) as in **(A)**. Asterisk indicates a significant difference between the *acx1* control and salt treatments (T-test, P = 0.032).

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MINI REVIEW
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Coordination Between ROS Regulatory Systems and Other Pathways Under Heat Stress and Pathogen Attack

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Regulatory systems of reactive oxygen species (ROS) are known to be integrated with other pathways involving Ca^{2+} signaling, protein kinases, hormones and programmed cell death (PCD) pathways to regulate defense mechanisms in plants. Coordination between ROS regulatory systems and other pathways needs to be flexibly modulated to finely tune the mechanisms underlying responses of different types of tissues to heat stress, biotic stresses and their combinations during different growth stages. Especially, modulation of the delicate balance between ROS-scavenging and producing systems in reproductive tissues could be essential, because ROS-dependent PCD is required for the proper fertilization, despite the necessity of ROS scavenging to prevent the damage on cells under heat stress and biotic stresses. In this review, we will update the recent findings associated with coordination between multiple pathways under heat stress, pathogen attack and their combinations. In addition, possible integrations between different signals function in different tissues via ROS-dependent long-distance signals will be proposed.

Keywords: abscisic acid (ABA), Ca^{2+} signaling, growth stages, programmed cell death (PCD), reactive oxygen species (ROS), respiratory burst oxidase homologues (RBOHs)

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INTRODUCTION

Plants are exposed to multiple abiotic and biotic stresses that may simultaneously occur in the natural environments. Although necessity of the researches deciphering the molecular mechanisms underlying response of plants to stress combinations has been proposed in many reviews, such studies are still scarce (Suzuki et al., 2014; Pandey et al., 2015). In addition, several transcriptome studies demonstrated that unique mechanisms that govern the responses of plants to stress combinations could not be easily predicted from the studies focusing on individual stress factors (Atkinson et al., 2013; Prasch and Sonnewald, 2013; Rasmussen et al., 2013). Under the natural environment, effects of pathogens on plants can be altered by abiotic factors (Pandey et al., 2015). Indeed, mechanisms that regulate defense responses in plants were shown to be different under different temperatures (Aoun et al., 2017). For example, heat stress, high temperature that negatively impacts plant growth, enhanced sensitivity of plants to pathogens in many cases (Pandey et al., 2015). In addition, the level of crosstalk between heat responses and defense responses might

depend on several factors such as plant species, tissues, developmental stage, stress intensity, and timing (Nejat and Mantri, 2017).

A recent review proposed that strategies of plants to adapt to stress combinations consists of both shared and unique mechanisms, and shared mechanisms constitute a considerable portion of plants' responses to both individual and combined stresses (Pandey et al., 2015). Reactive oxygen species (ROS) regulatory systems might be one of the most essential shared mechanisms to modulate the machineries governing defense and acclimatory responses to minimize the damage on cells (Suzuki et al., 2012; Rejeb et al., 2014; Pandey et al., 2015). Although ROS have long been known as harmful compounds under stress conditions, several lines of evidences indicated the significance of ROS as signaling molecules in the regulation of various biological processes (Choudhury et al., 2013; Mittler, 2017). ROS might activate the mechanisms that attenuate the effects of oxidative stress caused by abiotic stress, as well as programmed cell death (PCD) under biotic stresses (Kissoudis et al., 2014). Furthermore, ROS as universal signals might integrate with other pathways such as Ca^{2+} signaling, kinase cascades, and hormone signaling to tailor the specific mechanisms to adapt to different types of biotic and abiotic stresses (Mittler et al., 2011; Suzuki et al., 2013a).

Taken together, we can hypothesize that plants might possess the ability to flexibly coordinate the ROS regulatory systems and other integrating pathways to tailor the cellular homeostasis depending on the types of biotic and abiotic stresses individually or simultaneously occurred, and depending on the growth stages and types of tissues. In this review, we will not address detailed mechanisms underlying the response of plants to combinations of biotic and abiotic stresses, which have been discussed in previous reviews (Atkinson and Urwin, 2012; Kissoudis et al., 2014; Zhang and Sonnewald, 2017), but focus on coordination between ROS regulatory systems and other pathways under heat stress and pathogen infections. Especially, coordination of signals depending on different growth stages and tissues, and links between different signals function in different tissues will be suggested.

SIGNALING PATHWAYS INVOLVED IN HEAT RESPONSE, DEFENSE RESPONSE AND PCD IN DIFFERENT GROWTH STAGES AND TISSUES

Pathways involved in heat responses have been extensively studied using seedlings and vegetative tissues. More than decade ago, Larkindale et al. (2005) analyzed heat tolerance in seedlings of Arabidopsis mutants deficient in different signals, and demonstrated the significance of various pathways such as ROS regulatory systems and hormone signaling including abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and ethylene signaling as well as heat shock protein (HSP)-dependent pathways in the heat response of plants. In addition, Arabidopsis plants deficient in *S*-nitrosoglutathione reductase (GSNOR), which metabolizes the nitric oxide (NO) adduct *S*-nitrosoglutathione, were more sensitive to heat stress compared with WT plants (Lee et al., 2008), suggesting the involvement of GSNOR-dependent NO metabolism in heat tolerance of plants. Recent studies using vegetative tissues of Arabidopsis plants identified the four putative heat sensors localized in different subcellular components. They include a calcium channel on the plasma membrane, a histone sensor in the nucleus, and two unfolded protein sensors in the endoplasmic reticulum (ER) and the cytosol (Sugio et al., 2009; Che et al., 2010; Kumar and Wigge, 2010; Finka et al., 2012). The deficiency in one of the putative heat sensors, cyclic nucleotide-gated channel 2 (CNGC2) resulted in enhanced heat tolerance in seedlings accompanied by enhanced accumulation of HSPs as well as increased cytosolic Ca^{2+} level (Finka et al., 2012). Furthermore, heat sensing mechanisms involving unfolded protein responses in the ER and the cytosol might be linked via ROS regulatory systems during vegetative stages (Sun and Guo, 2016; Kataoka et al., 2017). More recently, phytochrome B was shown to be another heat sensor that mediates the switching of cellular status between growth-promoting mode and heat-acclimation mode (Jung et al., 2016; Quint et al., 2016). Some of these pathways implicated in heat responses in seedlings and vegetative tissues were also shown to contribute to defense responses. For example, CNGC2, also known as defense no death 1 (DND1) is required for the activation of PCD under pathogen attack (Clough et al., 2000). In addition, phytochrome B was shown to regulate the defense pathway involving lipoxygenase and mitogen-activated protein kinases 3 and 6 (Zhao et al., 2014). Furthermore, involvement of key players of heat responses such as ROS regulatory systems, Ca^{2+} signaling, kinases and various hormones in defense responses has been demonstrated in previous studies (Zhang et al., 2016, 2017; Luo et al., 2017). For example, a lectin receptor-like kinase (LecRK-IX.2) was shown to be required for the defense mechanisms triggered by pathogen recognition receptors. LecRK-IX.2 might induce phosphorylation of a ROS producing NADPH oxidase, respiratory burst oxidase homologue D (RBOHD) by recruiting Ca^{2+}-dependent protein kinases to trigger ROS production (Luo et al., 2017). In addition, overexpression of ethylene response factor 014 (ERF014) resulted in enhanced resistance of Arabidopsis to pathogens accompanied by enhanced expression of SA response genes and oxidative burst that was shown to be induced by RBOHD (Zhang et al., 2016), suggesting the roles of ERF014 in the regulation of SA signaling and ROS production in defense responses.

High sensitivity of reproductive tissues especially male reproductive tissues to heat stress, has been addressed in many studies (Zinn et al., 2010; Giorno et al., 2013). It should be noted that processes regulating reproductive development and fertilization share common features with mechanisms underlying defense responses, such as increase in RBOH-dependent ROS production and cytosolic Ca^{2+} (Chen et al., 2015) (**Figure 1**). For example, appropriate timing of tapetal degeneration involving PCD regulated by RbohE-mediated proper temporal ROS regulation was shown to be essential in pollen maturation and fertility (Xie et al., 2014; Kurusu and Kuchitsu, 2017)

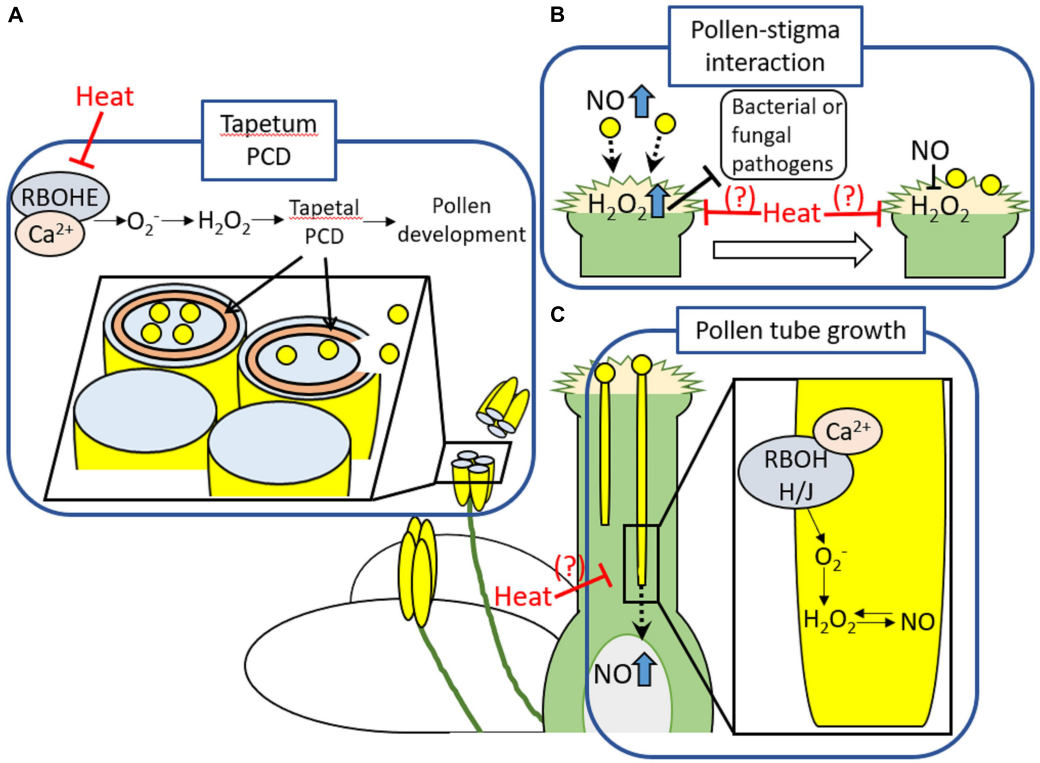

FIGURE 1 | Developmental processes in reproductive tissues involving ROS production or PCD. **(A)** RBOHE that might be activated by Ca^{2+} produces ROS to enhance tapetal PCD. This process occurred in the appropriate timing might be required for the proper development of pollens. **(B)** High level of NO and H_2O_2 accumulation in pollens and papilla cells, respectively, might be essential for the recognition of pollens by papilla cells followed by the attachment of pollens to the top of stigma. In addition, level of H_2O_2 could be sufficient to protect papilla cells against pathogens, but, not as much as causing damage (Hiscock and Allen, 2008). Following the attachment of pollen on the stigma, NO derived from pollen might function to decrease the level of H_2O_2 accumulation in stigma. The same tendency in ROS and NO level at the arrival of pollens to pistils was commonly observed among the different species (Zafra et al., 2010). These findings suggest that dramatic shift of the ROS-NO redox state could be required for initiation of pollen germination. However, significance of the decrease in H_2O_2 level in papilla cells in fertilization is still not clear. **(C)** RBOHH and RBOHJ that might be activated by Ca^{2+} produce ROS to regulate pollen tube growth. Appropriate level of RBOHH/J-dependent ROS production might be maintained by NO signals. Production of NO in ovule might be also involved in guidance of pollen tube growth (Serrano et al., 2015). These developmental processes required for fertilizations are sensitive to heat stress. Indeed, tapetal PCD was shown to be inhibited by heat stress (De Storme and Geelen, 2014). Effects of heat stress on ROS signaling involved in other developmental processes in reproductive tissues should be elucidated in more detail.

(**Figure 1A**). In addition, ROS and NO also play an important role in pollen-pistil interactions, as well as pathogen defense (Hiscock and Allen, 2008; Traverso et al., 2013; Serrano et al., 2015) (**Figure 1B**). Prior to and during arrival of pollens to pistils, high level of NO and H_2O_2 in pollens and papilla cells, respectively, might be required for the recognition of pollens by papilla cells. Following the arrival of pollen grains on a tip of stigma, NO derived from pollens might function to decrease the level of H_2O_2 accumulation in papilla cells (Zafra et al., 2010; Traverso et al., 2013; Serrano et al., 2015). Furthermore, RBOHH- and RBOHJ-dependent ROS production integrated with Ca^{2+} and NO signaling might be required for the pollen tube elongation (Domingos et al., 2015) (**Figure 1C**). During this process, NO-ROS redox signal might navigate pollen tube growth to the proper direction (Prado et al., 2008; Traverso et al., 2013; Serrano et al., 2015). These processes implicated in reproductive development and fertilization were shown to be sensitive to heat stress (Zinn et al., 2010; De Storme and Geelen, 2014). Despite the significance of RBOHs-dependent ROS production and PCD in pollen development and fertilization, pollen PCD might be also one of the main cause of damages on plant reproduction during heat stress (De Storme and Geelen, 2014). Pollen and tapetal cells were known to possess great number of mitochondria, one of the main source of ROS in plant cells (Muller and Rieu, 2016). Imbalance between mitochondrial ROS production and countering scavenging pathways could lead to the dysregulation of PCD during heat stress (Muller and Rieu, 2016). These findings therefore indicate the significance of cellular ROS homeostasis in the protection of reproductive tissues against heat stress and abiotic stresses.

Heat stress can also alter carbohydrate distribution in source and sink tissues, and resulted in impaired production of seeds and fruits (Sato et al., 2006). This heat-induced imbalance in carbohydrate distribution could be further exacerbated by pathogens (Zhang and Sonnewald, 2017). Dysregulation of carbohydrate distribution under heat stress accompanied by pathogen infection could affect the mechanisms underlying the PCD, and probably ROS signaling, because tapetal PCD was

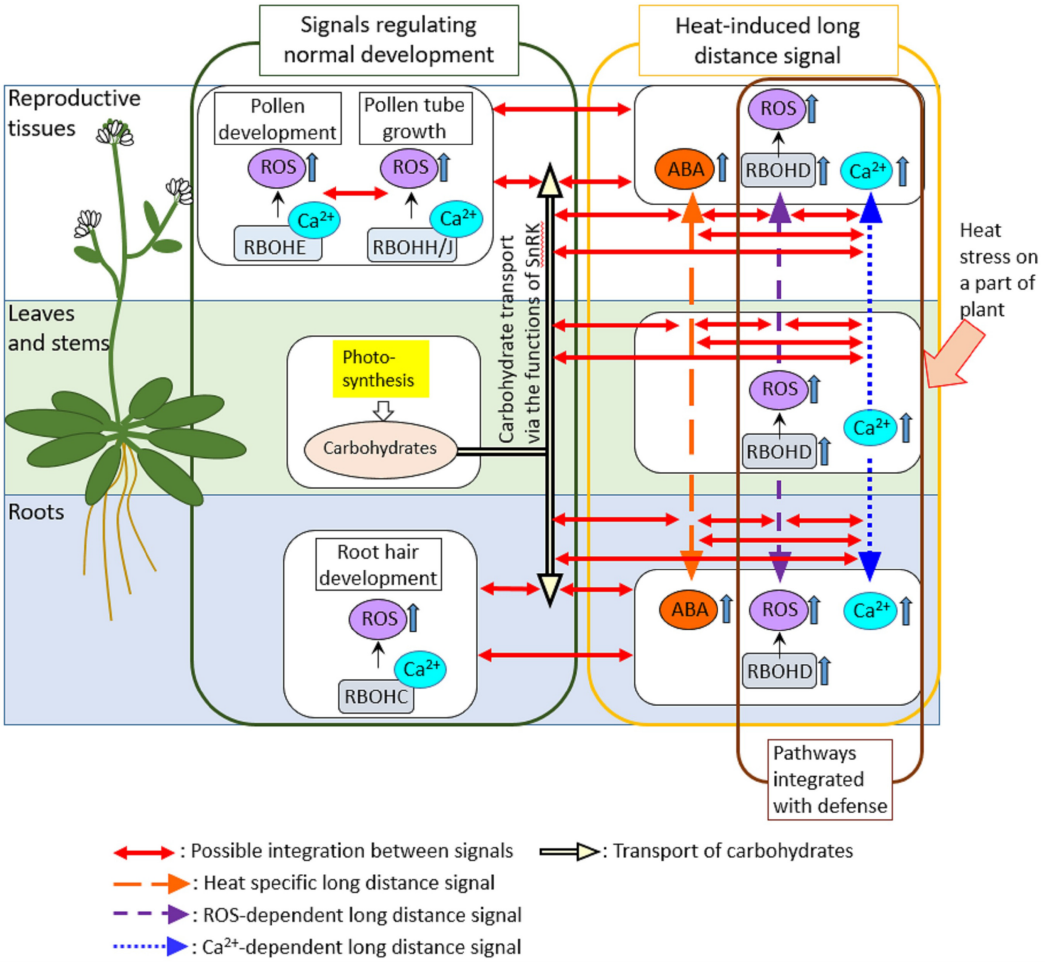

FIGURE 2 | Possible integrations of signals activated in different tissues. In this review, we suggest the possible integration between different signals that regulate development, heat response and pathogen defense in different tissues via ROS-dependent long-distance signaling. Long-distance signals that can be activated by heat stress and pathogens might involve Ca^{2+} signals as well as RBOHD-dependent ROS signals. Therefore, other RBOH proteins that function in different tissues to regulate developmental processes could be activated by Ca^{2+} signals propagated from the different part of the plant. In addition, carbohydrate transport that might be regulated by ABA signals could be also integrated with ROS- and Ca^{2+}-dependent long-distance signals. Indeed, ABA was shown to function together with RBOHD-dependent ROS signals to activate heat-induced long-distance signaling (Suzuki et al., 2013a). Furthermore, tapetal PCD was also shown to be required for the delivery of carbohydrates necessary for pollen development, suggesting the integration between PCD pathways and mechanisms that regulate carbohydrate distributions in plants.

also shown to be required for the delivery of carbohydrates and other compounds necessary for pollen development (Muller and Rieu, 2016). In addition, ABA that might switch positive and negative effects of abiotic stimuli on defense responses in plants (Rejeb et al., 2014), could be also involved in the regulation of carbohydrate distribution. A previous study demonstrated that sucrose non-fermenting-1-related protein kinase (SnRK1), a regulator of ABA signaling (Tsai and Gazzarrini, 2014; Zhang and Sonnewald, 2017) was shown to be required for sugar sensing (Li and Sheen, 2016) and communication between sink and source under various abiotic stresses (Lin et al., 2014).

Taken together these findings suggest that ROS regulatory systems and other pathways such as PCD pathways, Ca^{2+} signaling and hormone signaling needs to be strictly coordinated in plants depending on the different growth stages and tissues to finely tune the mechanisms underlying proper development, and responses to heat stress and pathogen attack.

POSSIBLE LINKS BETWEEN SIGNALS ACTIVATED IN DIFFERENT TISSUES

ROS-dependent heat response mechanisms might be differently modulated in vegetative and reproductive tissues. For example, expression pattern of genes regulated by HSFA2 that might be involved in H_2O_2 sensing was shown to diverge between leaves and anthers under heat stress (Muller and Rieu, 2016). In addition, Arabidopsis plants deficient in a cytosolic ROS scavenging enzyme, ascorbate peroxidase 2 (APX2) showed enhanced heat sensitivity in seedlings, while enhanced heat

tolerance in seed production (Suzuki et al., 2013b). Conversely, deficiency in CNGC2 in Arabidopsis resulted in enhanced heat tolerance in seedlings, but, enhanced heat sensitivity in seed production (Katano et al., 2017). The difference in heat tolerance between seedlings and reproductive tissues in plants deficient in CNGC2 might be due to the differences in ROS regulatory systems. These findings indicate that effects of heat stress on ROS-dependent pathogen defense could be also different depending on growth stages and tissues. Indeed, expression of CNGC2 was shown to be altered depending on the growth stages (Finka et al., 2012; Katano et al., 2017). Cross talk between CNGC2-dependent heat response and defense response can be also supported by the fact that CNGC2 is also known as DND1 that is involved in activation of PCD during pathogen attack (Clough et al., 2000). In addition, infection of tomato yellow leaf curl virus mitigated the heat response via inhibiting HSFA2 and APX2 (Anfoka et al., 2016).

We cannot ignore the possibility that long-distance signaling might link the mechanisms activated in different tissues (**Figure 2**). In Arabidopsis, each different RBOH protein might function in different tissues and play key roles in the regulation of different biological processes (Suzuki et al., 2011). For example, RbohE plays key roles in the regulation of PCD in tapetal cells that is required for proper development of pollens (Xie et al., 2014). On the other hand, RBOHC was shown to be required for development of root hairs (Knight, 2007). In addition, RBOHD is required for long-distance signaling that can be propagated through entire plant when part of a plant is subjected to pathogen attack or heat stress (Dubiella et al., 2013; Suzuki et al., 2013a). It should be interesting to address if RBOHD-dependent long-distance signals integrate with signals activated by other RBOHs. Integration of different RBOHs via long-distance signals could be also hypothesized by the fact that Ca^{2+} signaling which might be required for the activation of RBOH proteins is also propagated through entire plant in response to biotic or abiotic stresses on part of a plant (Gilroy et al., 2016). In addition, it might be also necessary to investigate how long-distance signaling and its integration with other pathways are coordinated in response to heat stress, pathogen attack and their combinations. A previous study provided a good example showing the activation of long-distance signaling by the local application of abiotic stress (high light), leading to the activation of defense mechanisms in the distal leaves (Karpinski et al., 2013).

It could be also interesting to study integration of long-distance signaling induced by heat stress or pathogens with carbon transport between sink and source tissues, because of the certain level of overlaps in the mechanisms between these processes (**Figure 2**). ROS-dependent signal might be integrated with heat-specific long-distance signal that might enhance ABA synthesis, but decrease the intermediate of sugar metabolism in leaves not directly exposed to heat stress when part of a plant was subjected to heat stress (Suzuki et al., 2013a). Ca^{2+} signaling that might integrate with ABA- and SnRK-dependent pathways (Wasilewska et al., 2008) was also shown to be integrated with ROS-dependent long-distance signaling in response to local application of abiotic and biotic stimuli (Gilroy et al., 2016). These findings suggest that the mechanisms to balance the assimilate use for plant development, and responses to abiotic and biotic stress rely on integration between several pathways, including ROS production, Ca^{2+} signaling, metabolite sensing and hormone balance.

CROSS TALK BETWEEN ROS-DEPENDENT SIGNALS AND OTHER PATHWAYS UNDER COMBINATIONS OF HEAT STRESS AND PATHOGEN ATTACK

Defense responses might be modulated depending on temperature. In many cases, abiotic stresses negatively impact on disease resistance of plants (Huot and Castroverde, 2017). For example, ornamental plant roots directly exposed to high soil temperatures increased severity of *Phytophthora infestans* (Pandey et al., 2015). The heat-dependent suppression of disease resistance might be due to the inhibition of hypersensitive response and R-gene mediated defense responses (Pandey et al., 2015; Huot and Castroverde, 2017). Nevertheless, several studies demonstrated the enhanced disease resistance by abiotic stresses. Enhancement of rust pathogen resistance of wheat regulated by Yr36 gene, which was accompanied higher accumulation of ROS and activated HR was observed especially under high temperature (Li et al., 2016).

Comprehensive expression analysis of HSPs in wheat revealed that different types of HSPs can be enhanced in response to different types of pathogens (Muthusamy et al., 2017). NO and ROS regulatory systems might be involved in the regulation of HSP- and HSF-dependent pathways during heat stress and pathogen infection. For example, compounds that produce or scavenge NO and an inhibitor of ROS producing NADPH oxidases affected HSP70 accumulation under heat stress and a combination of heat stress and pathogen infection (Piterkova et al., 2013). In addition, mitochondrial uncoupling protein in tomato (LeUCP) might also play key roles in the regulation of heat tolerance and pathogen defense (Chen et al., 2013). Overexpression of LeUCP resulted in enhanced tolerance of tomato plants to heat stress as well as *Botrytis cinerea* accompanied by lower accumulation of ROS. In this case, cross talk between heat response and defense response might be strictly regulated by fine-tuned mechanisms to balance between ROS scavenging to prevent oxidative damage caused by heat stress and ROS production necessary for the defense responses. This finding therefore suggests that mitochondrial ROS regulatory systems might play key roles in the ROS-dependent cross talks between heat response and pathogen defense. It should be also interesting how UCP-dependent mechanisms might modulate pathogen defense and PCD under heat stress in pollens that contain many mitochondria (Muller and Rieu, 2016).

A recent study demonstrated that Xa7-mediated pathogen resistance in rice might function better under high temperature (Cohen et al., 2017). This Xa7-mediated pathogen resistance might be regulated by inhibition of ABA signaling, but not by activation of SA signaling. Roles of ABA in the cross talk between

biotic and abiotic stresses are still controversial. Although ABA plays key role in the stomatal closure a common entry point for pathogen (Lim et al., 2015), ABA might antagonistically interact with pathogen response involving SA signaling. This dual effect indicates the role of ABA as a switch to modulate positive and negative effects of heat stress on defense mechanisms (Rejeb et al., 2014). Furthermore, ABA was shown to play pivotal role to tailor the response of plants to various stress combinations via integration with other hormones and ROS regulatory systems (Suzuki, 2016).

CONCLUSION

Based on the previous findings, we propose that coordination between ROS regulatory systems and other pathways such as Ca^{2+} signaling and hormone signaling should be finely tuned in plants under heat stress, pathogen attack and their combination. Such coordination between multiple pathways might be modulated depending on the growth stages and type of tissues. Although appropriate ROS scavenging is essential to prevent damage on cells under heat stress (Chen et al., 2013), ROS-dependent PCD is also essential to regulate pathogen defense in plants (Kissoudis et al., 2014). In this context of the balance between ROS scavenging and PCD, it might be urgent to elucidate the mechanisms underlying the response of reproductive tissues to heat stress, pathogen attack and their combinations, because PCD is essential for the appropriate development of pollens and fertilization (Kurusu and Kuchitsu, 2017).

ABA signaling and carbohydrate distributions that might be integrated with ROS regulatory systems and Ca^{2+} signaling are shown to be modulated through the entire plants over the long distance. Furthermore, different RBOH proteins were shown to be specifically expressed in different tissues. Therefore, it should be necessary to address how heat acclimation and defense mechanisms in different tissues are linked via long-distance signaling under heat stress, pathogen infection and their combinations.

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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Dehydration Stress Contributes to the Enhancement of Plant Defense Response and Mite Performance on Barley

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Under natural conditions, plants suffer different stresses simultaneously or in a sequential way. At present, the combined effect of biotic and abiotic stressors is one of the most important threats to crop production. Understanding how plants deal with the panoply of potential stresses affecting them is crucial to develop biotechnological tools to protect plants. As well as for drought stress, the economic importance of the spider mite on agriculture is expected to increase due to climate change. Barley is a host of the polyphagous spider mite *Tetranychus urticae* and drought produces important yield losses. To obtain insights on the combined effect of drought and mite stresses on the defensive response of this cereal, we have analyzed the transcriptomic responses of barley plants subjected to dehydration (water-deficit) treatment, spider mite attack, or to the combined dehydration-spider mite stress. The expression patterns of mite-induced responsive genes included many jasmonic acid responsive genes and were quickly induced. In contrast, genes related to dehydration tolerance were later up-regulated. Besides, a higher up-regulation of mite-induced defenses was showed by the combined dehydration and mite treatment than by the individual mite stress. On the other hand, the performance of the mite in dehydration stressed and well-watered plants was tested. Despite the stronger defensive response in plants that suffer dehydration and mite stresses, the spider mite demonstrates a better performance under dehydration condition than in well-watered plants. These results highlight the complexity of the regulatory events leading to the response to a combination of stresses and emphasize the difficulties to predict their consequences on crop production.

Keywords: plant-biotic-abiotic interaction, *Hordeum vulgare*, *Tetranychus urticae*, dehydration stress, differential gene expression

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INTRODUCTION

One of the most important threats to crop production is the attack of combined biotic and abiotic stressors. Under natural conditions, plants may suffer different stresses simultaneously or in a sequential way. Current knowledge on plant responses to different biotic and abiotic combined stresses has been recently reviewed (Zhang and Sonnewald, 2017). Previous studies have been focused in the impact of simultaneous or sequential exposure to pathogens/pests and abiotic stresses (reviewed in Atkinson and Urwin, 2012; Ramegowda and Senthil-Kumar, 2015). In many

cases, exposure of plants to pathogens/pests enhances abiotic stress responses, while abiotic stress weakens disease resistance. Thus, the activation of the responsive mechanisms of the plant is determined not only by the current stress/es, but also by the stress/es suffered previously, and plant response to combined stresses cannot be predicted from the responses to individual stresses (Zhang and Sonnewald, 2017). The understanding of how plants deal with the whole potential stresses affecting them will provide new breakthroughs that could be used in the development of biotechnological tools to protect plants.

At present, drought is one of the most important environmental stresses in agriculture (Fahad et al., 2017). Growing water demand, diminishing water supply and the increasing rainfall variability associated to climate change are expected to increase water deficit impact on crop yield (Lobell et al., 2011). Currently, drought causes yield reductions through negative impacts on plant growth and physiology in several crop species (Barnabás et al., 2008; Daryanto et al., 2016). The plant response to drought includes stomata closure, photosynthesis inhibition, accumulation of osmotically active compounds and protective proteins, changes in the sink/source allocation of several compounds, and triggering of phytohormones-related signaling pathways, mainly abscisic acid (Golldack et al., 2011; Nakashima et al., 2014). These protective mechanisms usually lead to a reduction on crop yield (Farooq et al., 2009; Praba et al., 2009). Besides, *Tetranychus urticae* is a cosmopolitan agricultural acari pest feeding on over 1,100 different plants, many of them of agricultural importance (Migeon and Dorkeld, 2006–2017). Due to high fecundity, inbreeding, and short generation time, Tetranychidae populations quickly develop resistance to acaricides (Van Leeuwen and Dermauw, 2016). Furthermore, increased temperatures and drought stress associated to climate change favor spider mite development. The spider mite life cycle is shortened; more generations are produced per year; and the pest appears earlier and with a broad range of hosts (DeLucia et al., 2012; Ximénez-Embún et al., 2017a). *T. urticae* pierces parenchymatic plant cells using stylets to suck their nutrients, and cause severe chlorosis leading to a reduction in crop yield (Bensoussan et al., 2016). Due to the *T. urticae* impact in agriculture, research on plant responses to spider mite infestation has been performed on crops as well as in model plants (Maserti et al., 2011; Agut et al., 2015; Díaz-Riquelme et al., 2016). Although jasmonic acid is the main hormone involved in the establishment of spider mite-induced defense responses, salicylic acid signaling pathway is also trigger by mite feeding (Ament et al., 2004; Zhurov et al., 2014; Martel et al., 2015; Santamaría et al., 2017).

The effect of drought on the performance of herbivores is uncertain and variable. Meta-analyses have pointed out that water stress tended to decrease the fecundity of chewing and sucking insects, with the strongest negative effects on sap-phloem insects (Koricheva et al., 1998; Huberty and Denno, 2004). In contrast, aphid performance was found to be highest in *Brassica* plants subjected to moderate dehydration stress (Tariq et al., 2013), and the leaf-chewing herbivore *Pieris brassicae* performed better on dehydration-stressed than on well-watered *Alliaria petiolata* plants (Gutbrodt et al., 2011). Regarding herbivore acari, the spider mites *T. urticae* and *T. evansi* appeared to benefit from drought stress in tomato plants because of the improved nutritional value of the leaves (Ximénez-Embún et al., 2016, 2017a). Likewise, the tomato russet mite *Aculops lycopersici* grows faster and causes more damage on drought-stressed tomato plants (Ximénez-Embún et al., 2017b). These data further illustrate the complexity of biotic-abiotic crosstalk under variable environmental conditions and demonstrate potential difficulties in predicting herbivore pest status under changing environments (Foyer et al., 2016).

Based on its wide environmental range, barley (*Hordeum vulgare*) has been proposed as an excellent model for understanding adaptation to climate change (Dawson et al., 2015). Drought is an impact factor for yield losses of this cereal (Wehner et al., 2016). The existence of barley genotypes tolerant and sensitive to drought has been largely used to analyze global gene expression changes after drought stress (Guo et al., 2009; Svoboda et al., 2016; Zeng et al., 2016; Cantalapiedra et al., 2017). Disparities in the results are likely due to differences in experimental set up and plant material (Cantalapiedra et al., 2017). In contrast, transcriptomic analyses on differential gene expression after herbivore attack are scarce. Only transcriptional responses have been characterized upon interaction with aphid species (Delp et al., 2009; Escudero-Martinez et al., 2017) and, as far as we know, no transcriptomic analysis has been performed in barley combining biotic and abiotic stresses.

Barley is a host of the spider mite *T. urticae* (Diaz-Mendoza et al., 2017). As well as for drought stress, the economic importance of the spider mite on agriculture is expected to increase due to climate change. To obtain insights on the combined effect of drought and mite stresses on the defensive response of the plant, in this work we have analyzed the transcriptomic responses of barley plants subjected to dehydration treatment, spider mite attack, or to the combined dehydration-spider mite stress. Besides, the performance of the mite in water stressed and well-watered plants has been analyzed. This knowledge could assist to the development of biotechnological tools for integrated abiotic/biotic management with a practical applicability in the context of climate change.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Grains of the drought-susceptible cultivar "Golden Promise" of barley (*H. vulgare* L.) were germinated in a mixture of soil and vermiculite (3:1) and grown at 23°C under a 16 h light/8 h darkness photoperiod for 7 days in Sanyo MLR-350-H chambers. At this point, plants were subjected to spider mite infestation (M), dehydration stress (D) or the combination of both stresses (DM). Controls (C) were always grown in parallel.

Spider Mite Material and Growth Conditions

A colony of the two-spotted spider mite *T. urticae* London strain (Acari: Tetranychidae), provided by Dr. Miodrag Grbic

(UWO, Canada), was maintained on beans in a Sanyo MLR-350-H growth chamber at 25°C under a 16 h light/8 h darkness photoperiod. This colony was transferred to barley where it was maintained under the same conditions for more than 30 generations to ensure mite adaptation to the host.

Mite Infestations and Dehydration Treatments

For spider mite infestation, each plant was infested with 20 adult female mites adapted to barley. The infestation was performed by placing a barley leaf with the mites on the experimental plant leaves. A falcon tube with holes was used to help maintaining the leaves together. Barley plants were confined in pots with plastic cylinders covered on top by nylon nets to avoid dispersion of mites. The same isolation system was applied to control and dehydration stressed plants. For dehydration treatments, pots were placed over Petri plastic plates to individualize watering. Plants were subjected to dehydration stress imposed by continuous water deprivation. Controls were watered to maintain soil moisture at 70% (considered as optimal watering). In addition, both stresses were combined at the same time in order to study the effect of mixing biotic and abiotic stresses.

Barley phenotypes were monitored at different time points in control plants and in plants subjected to mites, dehydration or the combination of both stresses. Leaves were harvested after 4, 7, 10, and 13 days. Samples were imaged and scanned, or frozen into liquid nitrogen and stored at −80°C for further analysis. Three independent experiments were performed.

Physiological and Biochemical Parameters

The effect of spider mite infestation, dehydration and the combination of both stresses was studied 7 days post treatment by determining several physiological parameters, always done at the same time of the day to avoid circadian effects. Aerial plant biomass and soil mass were determined by weighting (Precisa XB 2200 C) at the end of each treatment (fresh weight), and after drying in a stove at 70°C (dry weight). Aerial plant water content (PWC) and soil water content (SWC) were obtained from these measurements (fresh weight minus dry weight). As growth indicators the number of leaves and the length (cm) of the longest leaf were also quantified. In addition, the quantum yield (QY) of the photosystem II and the continuous fluorescence yield (FT) were estimated as indicators of the photosynthesis efficiency using a FluorPen FP 100 (PSI, Drasov, Czechia). Both parameters were measured on the oldest and the youngest leaves. Nine plants from three independent experiments were used per treatment.

RNA Isolation, cDNA Library Construction and Illumina Sequencing

Total RNA was extracted from frozen barley leaves by the phenol/chloroform method, followed by precipitation with 8 M LiCl (Oñate-Sánchez and Vicente-Carbajosa, 2008) and digested with DNase (Promega). Using poly-T oligo-attached magnetic beads, mRNAs were purified from the total RNA. Then, the mRNAs were fragmented and cDNA was synthesized using random hexamer-primers, DNA polymerase I and RNase H. The double-stranded cDNAs were purified with magnetic beads and ligated to adaptors for Illumina sequencing. The quality and quantity of the library was verified using an Agilent 2100 Bioanalyzer and an ABI StepOnePlus Real-Time PCR system, respectively. The cDNA libraries were sequenced using the Illumina HiSeq2000 platform by the Beijing Genomics Institute (BGI). More than 10M single-end reads were obtained for each sample (three biological replicates). Raw reads in fastq format were firstly filtered and reads with adaptor sequences and low quality reads were removed. RNA-seq data are available on ArrayExpress database at EMBL-EBI[1] under accession number E-MTAB-6565.

Sequence Data Analysis and Annotation

The gene and genome sequences of *H. vulgare* retrieved from the PGSB/MIPS PlantsDB website[2] (Nussbaumer et al., 2013) were used as the reference databases (Mayer et al., 2012). Clean reads were pseudoaligned to the reference High Confidence genes using Kallisto (Bray et al., 2016). The transcript abundance was quantified as TPM (transcripts per million) and 100 bootstrap samples were performed. Differential expressed genes (DEGs) between groups were obtained using the RNA-seq 2G portal[3]. From the 25 methods available in this portal, five methods were selected, which do not use log-transformed data as input and has its own normalization procedure (Zhang et al., unpublished). They differ in the distribution assumed for the data (Poisson-like for PoissonSeq, negative binomial for edgeR, DESeq2 and EBSeq, and non-parametric for NOISeq) and the statistical test used by the method (Poisson goodness-of-fit for PoissonSeq, exact/likelihood ratio for edgeR, generalized linear model for DESeq2, empirical Bayesian for EBSeq and NOISeq). DEGs were obtained from a meta-analysis of the results from the previously five single selected methods. The Simes method was used, and genes with a combined *P*-value lower than 0.05 and a logFC (from edgeR method) higher than 1 or lower than −1 were considered as DEGs. Euler diagram was created in the VennDiagrams.net website[4]. Clustering and heatmap were performed by the ClustVis web tool[5]. Gene enrichment analyses were performed with the Fischer's exact test using topGO package in R[6] and the GO file retrieved from the PGSB/MIPS PlantsDB website.

Comparative Analysis of DEGs

For Arabidopsis and tomato species, DEGs after mite attack were taken from Supplementary Material of corresponding publications (Zhurov et al., 2014; Martel et al., 2015). EnsemblPlants BioMart tool was used to obtain InterPro IDs and descriptions from the barley, Arabidopsis and tomato

[1]https://www.ebi.ac.uk/arrayexpress/

[2]http://pgsb.helmholtz-muenchen.de/plant/barley/index.jsp

[3]http://52.90.192.24:3838/rnaseq2g/

[4]http://www.venndiagrams.net/

[5]http://biit.cs.ut.ee/clustvis/

[6]http://bioconductor.org/packages/release/bioc/html/topGO.html

DEGs. Venn diagrams were created using the Venny 2.1 tool[7]. InterPro IDs network was constructed using Cytoscape v3.5.1[8].

Real-Time Reverse Transcription Quantitative PCR Analyses (RT-qPCR)

For real-time RT-qPCR studies, total RNA was isolated from barley leaves as described above. cDNAs were synthesized from 2 μg of RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions. RT-qPCR analyses were performed for triplicated samples by means of a Light Cycler 480 real-time system (Roche) using the SYBR Green detection system (Roche). mRNA quantification was expressed as relative expression levels ($2^{-\Delta Ct}$) (Livak and Schmittgen, 2001) and standardized to barley cyclophilin (*HvCycl* gene) mRNA levels (Diaz-Mendoza et al., 2014). Expression levels of the *T. urticae* Ribosomal Protein 49 (*TuRP49*) mRNA (Santamaria et al., 2015) were quantified as relative expression levels ($2^{-\Delta Ct}$) by subtracting the ΔCt value of the barley cyclophilin from the ΔCt value of the mite probe to normalize for the amount of barley tissue present in each sample. To normalize for the amount of barley and mite tissue present in each sample, expression levels of the *T. urticae* Vitellogenin (*TuVTG*) and Autophagy related protein (*TuATG13*) were quantified as fold change levels ($2^{-\Delta\Delta Ct}$) normalized to both, the barley cyclophilin and the *T. urticae* Ribosomal Protein 49. The primers used are shown in Supplementary Table 1. Efficiency of the primers was determined based on the slope of a standard curve and was between 97.5 and 102.5% for all primer pairs tested. Three plants from the three independent experiments were used per treatment.

Statistical Analysis

Data related to physiological and biochemical characterization experiments were compared between control and stressed plants and analyzed by One-Way ANOVA followed by a many-to-one Dunnett's *post hoc* test. In figures, one or three asterisks indicated significant differences (Dunnett's test, $P < 0.05$ and $P < 0.001$, respectively). Expression analyses of mite performance were performed by One-Way ANOVA, followed by Student Newman–Keuls (SNK) *post hoc* test. In figures, different letters indicated significant differences (SNK test, $P < 0.05$).

RESULTS

Combined Water Deprivation and Spider Mite Infestation Strengthen Alterations in the Plant Physiological Status

To establish a time point to further analyze the effects of dehydration and mites, 7-day-old barley plants were subjected to water deprivation, mite infestation or combined stresses for 4, 7, 10, and 13 days (Supplementary Figure 1). Phenotypic observations showed progressive deleterious effects caused by both treatments. Mite feeding produced chlorotic spots and induced yellowish of the apical part of the leaves. Dehydration induced a delay in plant growth and a progressive whiter of the plant, which was reinforced when combined with mites stress. As at 10 days of dehydration and mites combined stresses plants were too damaged to guarantee a complete molecular analysis, 7 days was selected as the time point to analyze the effects caused by mites and dehydration.

After 7 days of treatment, plant phenotypes were analyzed and physiological and biochemical parameters studied. Plant images corroborated the deleterious effects of both stresses and detailed images highlight the chlorotic spots caused by spider mite infestation (**Figure 1A**). As expected, SWC was significantly lower in dehydration stressed samples (**Figure 1B**). Dehydration effects were potentiated by mite stress. Combined stresses lead to a higher reduction in PWC, leaf length and leaf number than that observed with only water deprivation (**Figures 1B,C**). When mites were included in the treatment, a significant reduction in the QY and in the continuous FT was observed in the oldest leaf (**Figures 1D,E**).

Single or Multiple Stresses Differentially Affect the Expression of Barley Genes

To obtain more information on the changes in gene expression associated to the response of the barley plant to the different stresses, a RNA-seq analysis was performed in plants subjected to spider mite infestation (M), dehydration (D), mite infestation and dehydration (DM), or maintained under control conditions (C) during 7 days. In order to reduce the impact of intrinsic disparity between analytical methods and gene length bias, differential expressed genes (DEGs) were obtained in the RNA-seq 2G platform using a meta-analysis of the results from five selected methods (Supplementary Data Sheet 1).

When compared with control plants, the highest number of DEGs was found in the plants subjected to simultaneous mite and dehydration treatments (284 genes), and the lowest in the dehydration stressed plants (29 genes). **Figure 2** summarizes the overlaps in the DEGs in response to the different treatments. From the 284 DEGs between C and DM conditions, 186 were not differentially expressed in response to mites or dehydration. On the contrary, 92 of the 109 DEGs in response to mites and 16 of the 29 DEGs in response to dehydration were shared by DM treatment (a complete list of DEGs for subgroups is compiled in Supplementary Data Sheet 2). Whereas most DEGs were up-regulated after mite (90.8%) or mite/dehydration (86.3%) treatments, a similar number of genes were up- or down-regulated after dehydration stress (55.2 and 44.8%) (Supplementary Figure 2). All shared DEGs were regulated in the same direction with the exception of a glucan endo-1,3-beta glucosidase and an undescribed protein that were down-regulated in the dehydration treatment and up-regulated in the M and DM treatments, respectively. Many up-regulated genes in both, the mites and dehydration and mites treated plants, were involved in the jasmonic acid pathway. Several genes are related to jasmonate biosynthesis (allene oxide synthase, allene oxide cyclase, lipoxygenases), and a higher number was related to

[7]http://bioinfogp.cnb.csic.es/tools/venny/

[8]http://www.cytoscape.org/

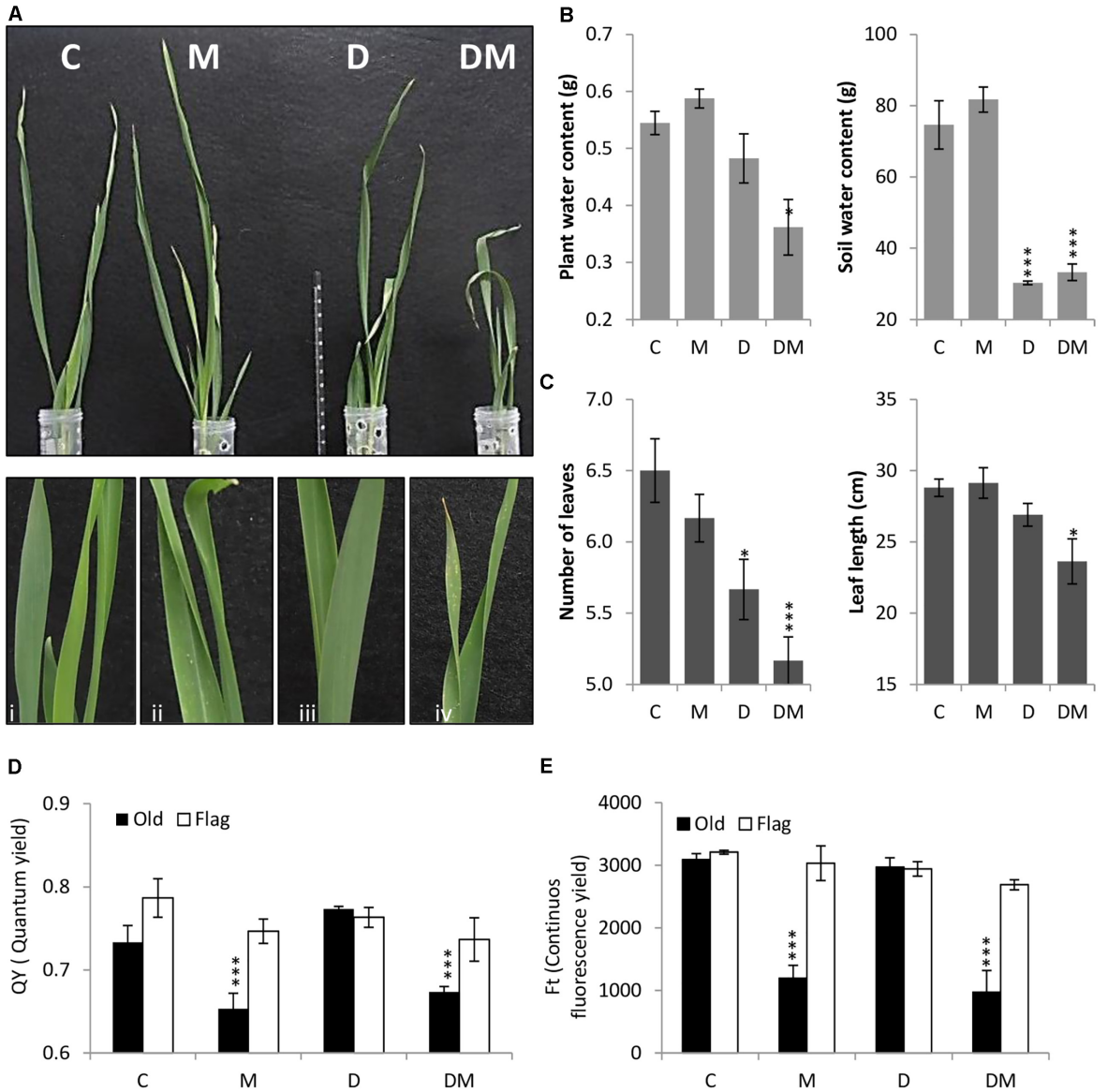

FIGURE 1 | Phenotypical, physiological and biochemical parameters of barley plants 7 days post treatment. **(A)** Phenotype of barley plants 7 days post spider mite infestation (M), dehydration (D), spider mite infestation + dehydration (DM) or control conditions (C) after taking out plastic cylinders to avoid mite dispersion. Plant details are showed in i, ii, iii, and iv. **(B)** Plant/Soil water content (g). **(C)** Number of leaves and leaf length (cm). **(D)** Quantum yield of the photosystem II (QY). **(E)** Continuous fluorescence yield (FT). Data represent the mean ± SE of $n = 3$ replicates. Asterisks indicate significant differences among control plants and each treatment determined by a One-way ANOVA test ($P < 0.05$, Dunnett's *post hoc* test).

jasmonate response (protease inhibitors, thionins, not specified jasmonate-induced proteins). Ten genes were differentially expressed after the three (M, D, and DM) treatments. All of these shared DEGs were up-regulated and included genes of the defense-related thionin family.

When we focused in the relationships of DEGs with specific biological processes using enrichment Gene Ontology (GO) analysis, several findings were observed (Supplementary Table 2). In all comparisons, with the exception of M/DM, an enrichment of the biological categories related to defense response, response to (biotic) stress or response to (biotic) stimulus were reported. When DEGs between M/DM treatments were compared, an enrichment of GO terms related with the response to abiotic (dehydration) stimuli was found. In addition, an enrichment of DEGs related to oxidation-reduction processes was present in the C/DM comparison. The two/three GO enriched terms with the lowest *p*-values for the control/treatment comparisons and for the overlapping subgroups are included in **Figure 2** (a complete list of enriched GO terms for subgroups is compiled in Supplementary Data Sheet 2).

To further analyze the expression of the DEGs, a heatmap was performed. This heatmap shows the relative expression

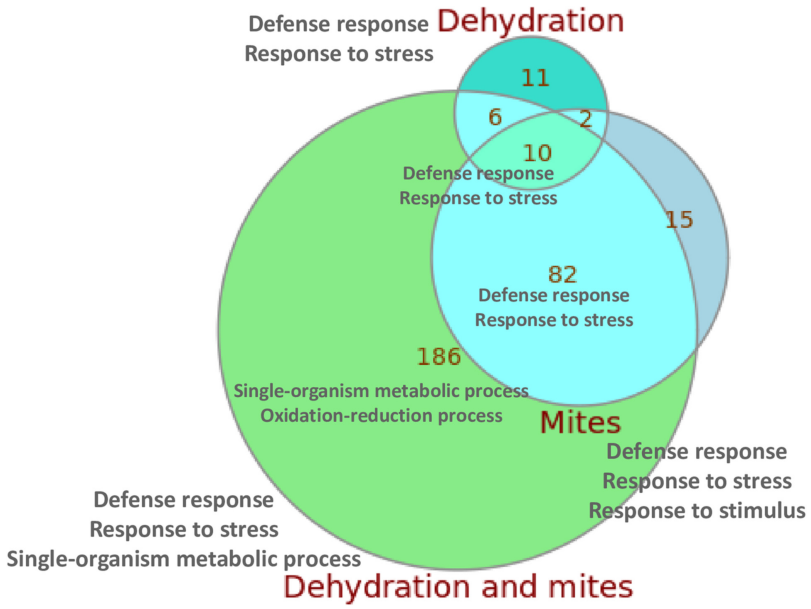

FIGURE 2 | Euler diagram showing the number of specific and shared DEGs detected after the different treatments. The main enriched GO categories for each group/subgroup are indicated.

values for the 350 DEGs obtained from at least one treatment comparison (**Figure 3A**). Globally, the heatmap highlights the greater changes in gene expression following mite infestation, which are reinforced by dehydration. Control and dehydration samples were grouped together, as well as mites and dehydration and mites samples. Clustering showed the presence of groups of genes similarly regulated (**Figure 3B** and Supplementary Data Sheet 3). A high number of genes are grouped in cluster 3. These genes are hugely induced by the DM combined stress. Whereas most of them are also induced by mite single stress (3.1 cluster), a small subset showed lower expression in mites and control than in dehydration treatment (3.2 cluster). Many GO terms related to defense and stress responses are enriched in this group. Cluster 1 includes genes induced by mites and repressed by dehydration, with enrichment in genes related to the response to oxidative stress. Cluster 2 is formed by genes more induced after mite stress than after DM stress and includes several genes related to carbohydrate metabolic processes. The rest of clusters did not present any enrichment GO term but present interesting features. For example, cluster 6 comprises genes repressed by any stress, and clusters 4 and 9 are formed by genes induced after dehydration and mites and dehydration treatments or only by water-deficit stress, respectively.

Protein Domains From Differentially Expressed Genes in Response to Mites Are Quite Conserved in Barley, Arabidopsis and Tomato

The response of barley to mite stress was compared with the response to mites previously reported for Arabidopsis and tomato (Zhurov et al., 2014; Martel et al., 2015). The functional domains present in the proteins encoded by DEGs were retrieved from the Interpro annotations in the EnsemblPlants database. The higher number of DEGs reported in Arabidopsis and tomato in response to *T. urticae* correlated with a higher number of Interpro annotated domains. Whereas 1606 and 1787 different Interpro identifiers were obtained from Arabidopsis and tomato, respectively, 113 were identified in barley. When Interpro domain identifiers were compared (**Figure 4A**), Arabidopsis and tomato only shared with barley the 60.4% and a 54.3% of identifiers, respectively. On the contrary, a 75.2% of the barley identifiers were shared by the three species, and an 89.4% was shared by barley and Arabidopsis or tomato. A schematic network highlighting the Interpro identifiers not shared by the three species is shown in Supplementary Figure 3. Supplementary Data sheet 4 includes the Interpro identifiers for each comparison involving barley data.

To obtain more information on the meaning of the 12 Interpro identifiers specific for barley, the functional domains present in the proteins encoded by DEGs after the DM combined treatment were added to the comparison (**Figure 4B**). Eleven of the 12 identifiers were also detected in proteins encoded by DEGs from the combined stresses, suggesting they are domains involved in the response of barley to mites. Further analysis of this comparison indicated that 226 of 254 Interpro identifiers from the DEGs after combined stresses were also identified after mite treatment of barley, Arabidopsis or tomato. The rest 28 identifiers were only recorded after combined treatment and could be involved in the response to dehydration stress.

To check this hypothesis, Interpro identifiers found in the proteins encoded by DEGs after dehydration treatment were compared to those coming from barley dehydration and mites treatment, and Arabidopsis and tomato mite infestation

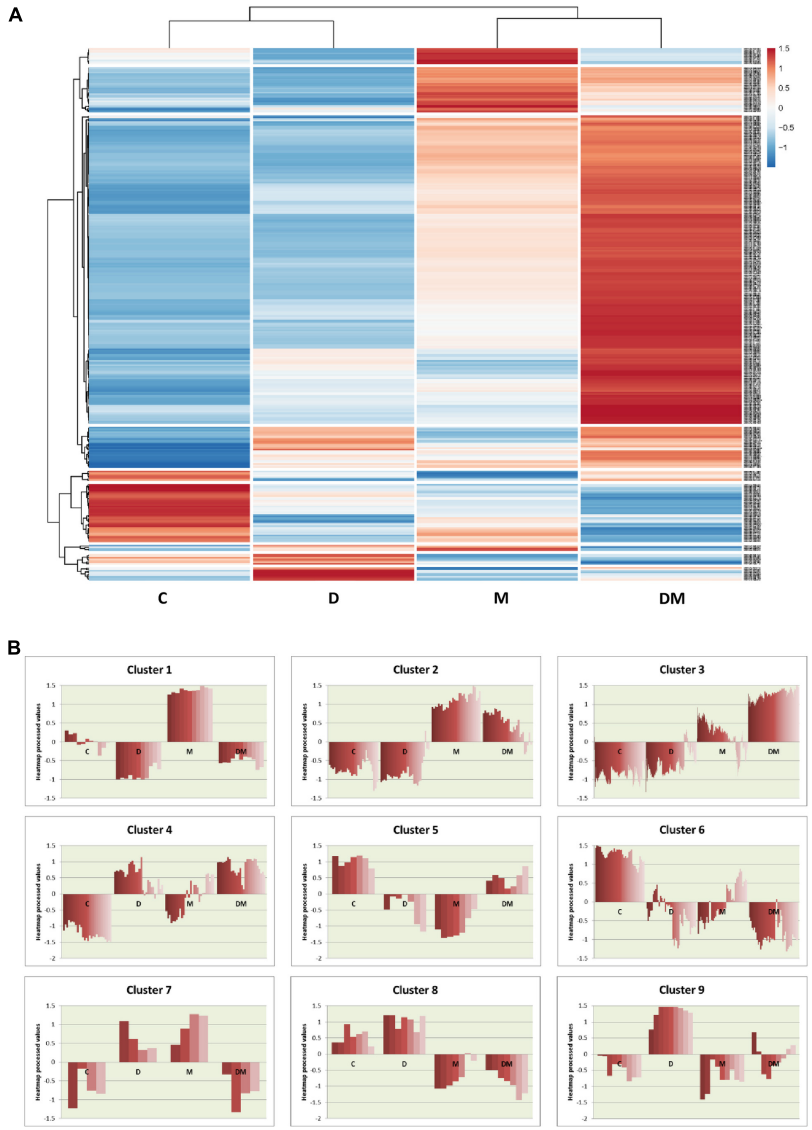

FIGURE 3 | Hierarchical clustering of differentially expressed genes among the different treatments. **(A)** Heatmap showing normalized color intensity from the expression values of the 350 DEGs. **(B)** Main clusters identified from hierarchical gene tree cluster analysis showing heatmap expression processed values for every gene after the different treatments. C, control; D, dehydration; M, mites; DM, dehydration and mites.

(**Figure 4C**). Twenty of the 26 barley identifiers were also detected after mite treatment of Arabidopsis or tomato. Three of the other six identifiers were common to barley D, M, and DM categories (domains corresponding to ribosomal proteins). Only three identifiers were specific from D and DM categories, which are present in adenylate kinase proteins, and could be assigned specifically to the dehydration stress response.

Combined Biotic-Abiotic Stresses Influences the Time Course Response of Mites and Dehydration Induced Genes

DEGs analyses point to a quicker response to mite stress than that observed after dehydration stress. This response is reinforced in water deprivation conditions. To further characterize the time course of these responses the expression levels of several DEGs was quantified at different time points upon stress conditions (**Figure 5**). Twelve genes were selected in function of their expression patterns upon the stress treatments (Supplementary Figure 4). Most genes were from cluster 3, with the highest expression after the dehydration and mites treatment. Six of these genes were selected from the large subset of genes with a higher expression in mites than in dehydration or control categories (3.1 cluster), and correspond with genes from families largely known to be involved in plant defense (a protease inhibitor, a lipoxygenase and two thionins), a gene encoding a protein with a lectin (jacalin) domain and a dirigent domain, with homologs previously related to plant defense (Weidenbach et al., 2016),

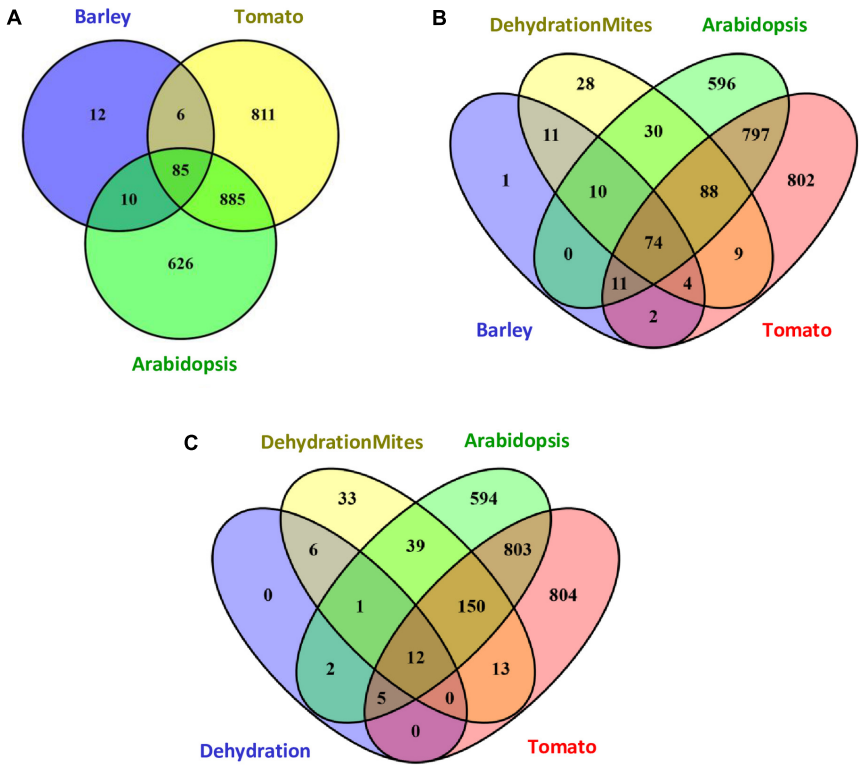

FIGURE 4 | Venn diagrams showing the number of specific and shared Interpro identifiers. **(A)** Interpro identifiers from DEGs after mites treatment in Arabidopsis, tomato and barley. **(B)** Interpro identifiers from DEGs after mites treatment in Arabidopsis, tomato and barley, including the overlap with the dehydration and mites treatment in barley. **(C)** Interpro identifiers from DEGs after dehydration and dehydration and mites treatments in barley including the overlap with the mites treatment in Arabidopsis and tomato.

and a protein with a laccase domain, which is involved in lignification and resistance to pathogens and pests (Hu et al., 2017). Four genes belong to the small subset of genes with lower expression in mites and control than in dehydration categories (3.2 cluster) and correspond with genes encoding enzymes previously related to pathways involved in drought response in plants, such are aldo/keto reductases, polyamine oxydases, sterol C4-methyl oxidases, and gamma glutamyl phosphate reductases (Liang et al., 2013; Hatmi et al., 2015; Kumar et al., 2015; Sengupta et al., 2015). One gene was selected from cluster 2. This gene encodes a protein combining the known defensive carbohydrate-bound protein lectin domain with a serine/threonine kinase signaling domain and was previously related to plant defense (Bouwmeester et al., 2011). The last one (encoding a lipid transfer protein) belongs to cluster 4, formed by genes induced by dehydration and dehydration and mites treatments. This family of proteins has been associated to drought tolerance (Guo et al., 2013).

Results pointed out interesting features (**Figure 5**). All genes putatively induced by mites (clusters 2 and 3.1) were more expressed after the combined dehydration and mite treatments than after the single mite treatment. Besides, whereas their expression always peaks after 10 or 13 days of MD treatment, the expression of some genes peaks after 7 days of M treatment. The dehydration-related genes (clusters 3.2 and 4) showed peaks of expression at 10 or 13 days after both, D and DM treatments. However, most genes were induced earlier after combined MD treatment than after single dehydration treatment.

Mite Population Development Depends on the Water Availability for the Plant

The expression of plant genes in response to mite infestation is affected by dehydration or well-watered conditions. To check its consequences on the mite population dynamics, the presence of the mite at different time points of infestation was analyzed by quantifying *T. urticae* Ribosomal Protein 49 (*TuRP49*) mRNA levels (**Figure 6A**). Total mite population increased from 4 to 7 days after infestation, growth that was stronger in water deprivation condition. Then, the mite population decreased markedly from 7 to 10 days in both conditions. Finally, mites increased again from 10 to 13 days, achieving a higher level in dehydration than in well-watered conditions.

To further analyze this timely up and down behavior, an approximation to the ratio of females/males was performed by quantifying the expression of a gene encoding a vitellogenin, which is mainly expressed in females (Cabrera et al., 2009) (**Figure 6B**). When plants were placed in dehydration conditions, the estimated amount of females was already high after 4 days of infestation and presented a marked peak at 10 days. On

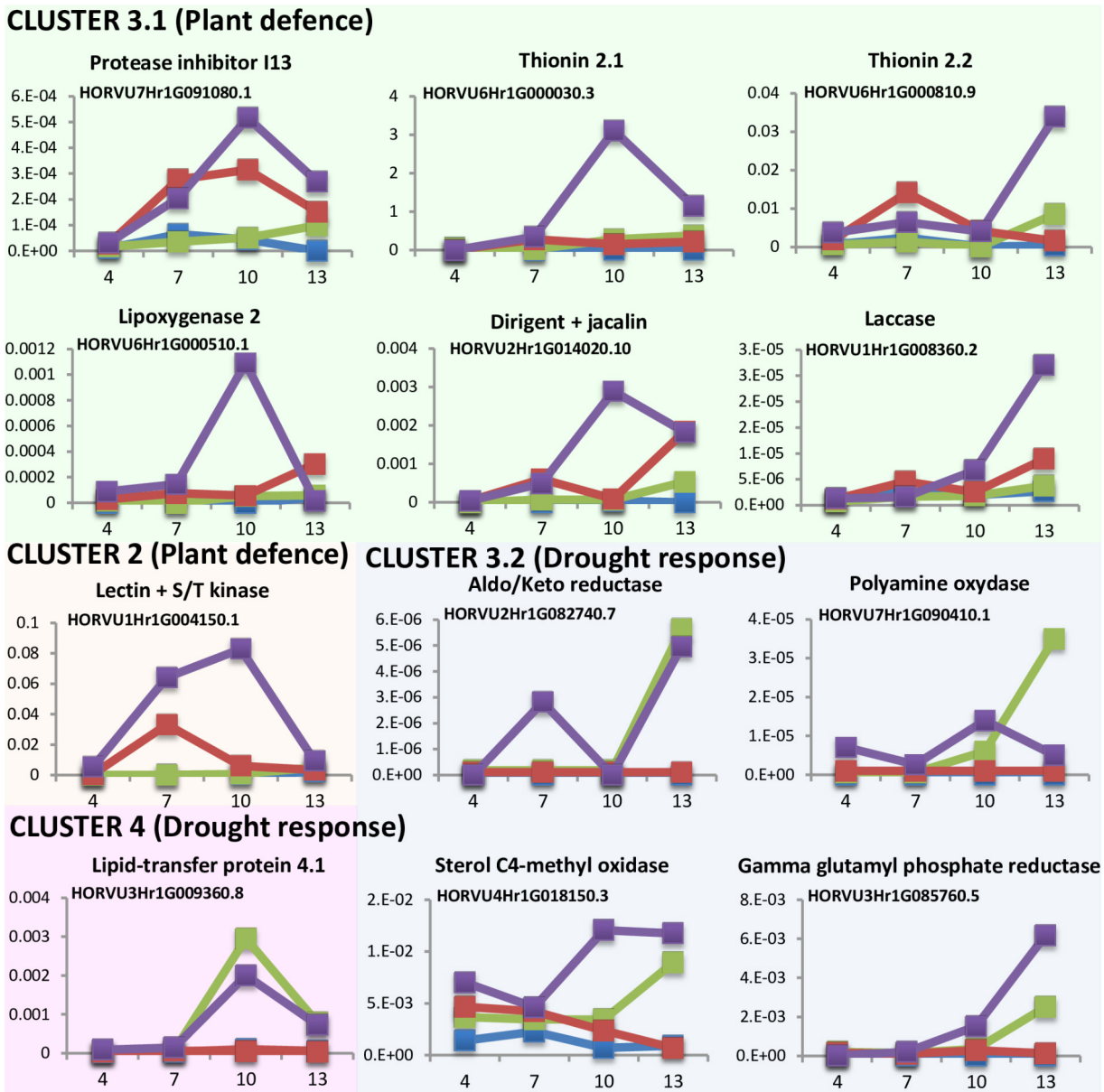

FIGURE 5 | Messenger expression levels of selected differentially expressed barley genes after 4, 7, 10, and 13 days of stress treatment assayed by RT-qPCR. Total RNA was extracted from leaves after dehydration treatment (green), mites treatment (red), dehydration and mites treatment (purple) and non-treated leaves (blue). Data were expressed as relative mRNA levels normalized to barley *cyclophilin* mRNA content. Genes from the same cluster identified in the hierarchical analysis have the same background color.

the contrary, when water was present, the estimated amount of females was scarce at 4 and 10 days, and peaks at 7 days of treatment. Likewise, the prevalence of egg stage was assessed by quantifying the expression of a gene encoding an autophagy related protein predominantly expressed in eggs (ORCAE *T. urticae* website[9]) (**Figure 6C**). Whereas in water deprivation the estimated amount of eggs showed two peaks, at 7 and 13 days of treatment, the amount of eggs was scarce in full water treatment and was only detected after 13 days of infestation.

DISCUSSION

Understanding how herbivores behave in field conditions is crucial to establish accurate integrated pest management actions. Since field conditions are complex and variable, analysis of small pieces of this natural scenario may help to understand the whole system. As drought and spider mites are two

[9]http://bioinformatics.psb.ugent.be/orcae/overview/Tetur

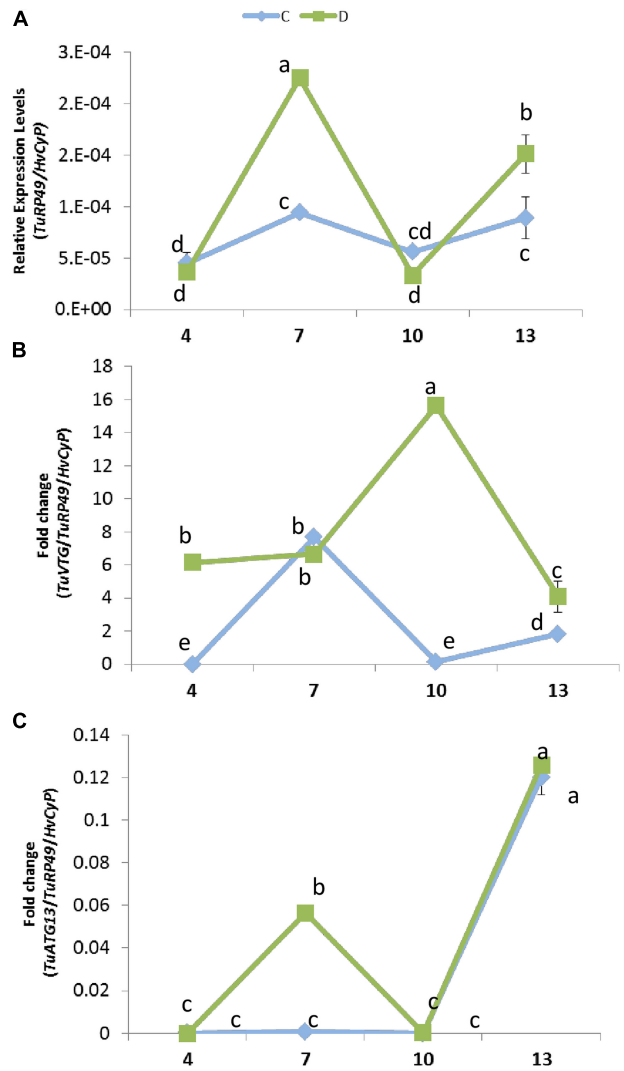

FIGURE 6 | Effect of barley dehydration on *T. urticae* performance. **(A)** Quantification of *T. urticae* Ribosomal Protein 49 (*TuRP49*) mRNA expression. **(B)** *TuVTG* (Vitellogenin) mRNA expression normalized to the spider mite population (*TuRP49*). **(C)** *TuATG13* (Autophagy related protein) mRNA expression normalized to the spider mite population (*TuRP49*). Expression levels were quantified 4, 7, 10, and 13 days post infestation under control or dehydration stress conditions. Different letters indicate significant differences determined by a One-way ANOVA test ($P < 0.05$, Student Newman–Keuls *post hoc* test).

abiotic/biotic stresses predicted to increase crop losses under current climate change, the knowledge of plant responses to a combination of both stresses could help future crop production strategies.

The outcome of stress experiments depends on the relative severity and duration of the stresses applied, and the developmental stage of the plant (Zhang and Sonnewald, 2017). In our experimental conditions, water deprivation produces stronger phenotypical effects than mite stress at 10–13 days of treatment, with a remarkable reduction in plant size and an advanced withered state. However, at 7 days of treatment, mild phenotypical impact was observed. While dehydration provokes a delay in growth, with a lower number of leaves, mite feeding produced a decrease in photosynthesis efficiency. Water deficit consequences were exacerbated when combined stresses were applied. Thus, transcriptomic responses were expected for all stress combinations at this time point. Early responses are common across many different treatments. Changes in Ca_2^+ signatures, sugar signals, reactive oxygen species (ROS) and phytohormone levels are triggered by most biotic and abiotic stresses (Atkinson and Urwin, 2012; Sham et al., 2015; Nguyen et al., 2016b). However, depending on the environmental stimuli, specific subsets of genes respond. In order to guarantee survival and reproductive success, this fine tuning permits to prioritize resources toward growth or defense when plants are subjected to different stresses (Denancé et al., 2013; Huot et al., 2014).

Intriguingly, mite attack induced stronger transcriptomic changes than water deprivation. Whereas the expression of 109 genes was modified by mite treatment, only 29 responded to mild dehydration. These data suggest that changes in the expression of a reduced number of genes are enough to retard plant growth, but a broader remodeling of gene expression is needed to establish appropriate defensive programs against mites. Since interactions between abiotic and biotic stress occur at multiple levels in plants, it is difficult to predict overlaps between signaling pathways induced by single and combined stresses. In fact, molecular changes triggered by the application of simultaneous or sequential stresses differ from those induced by the individual stress (Atkinson et al., 2013; Prasch and Sonnewald, 2013; Rasmussen et al., 2013; Suzuki et al., 2014; Coolen et al., 2016; Davila Olivas et al., 2016). Furthermore, the number of regulated features usually increased with the complexity of the stresses applied. For example, the number of regulated features in individual drought and virus stress in Arabidopsis plants was 518 and 682, respectively, but rose to 1,744 when both stresses were applied in combination (Prasch and Sonnewald, 2013).

In our experimental conditions, the number of DEGs reached 284 when both dehydration and mites were applied. Jasmonic acid is the main hormone involved in the establishment of spider mite-induced defense responses in plant species (Ament et al., 2004; Zhurov et al., 2014; Martel et al., 2015; Santamaría et al., 2017). As expected, many up-regulated genes in both, the mites and dehydration and mites treated plants, were involved in the jasmonic acid pathway. At least, a half of the fifty most up-regulated genes were associated to jasmonic acid response. Many of them encode defensive proteins such as protease inhibitors and thionins with putative deterrent effects on herbivores. The actual role of some of these genes in the defensive mechanism triggered by jasmonic acid is also supported by their up-regulation in lines over-expressing the lipoxygenase involved in jasmonate biosynthesis (Losvik et al., 2017). Furthermore, the DEGs specific for the dehydration and mites treatment were enriched in functional categories related to both, biotic and abiotic responses. These data suggest an extensive remodeling of metabolic processes when both stresses are combined. This synergistic response overtakes gene expression adjustment triggered by individual stresses. Besides, many DEGs suffered a higher

induction of their expression when mite infestation was done in dehydration conditions than in well-watered plants. This feature was previously described (Nguyen et al., 2016a). The expression of DEGs was higher in *Solanum dulcamara* plants infested with the caterpillar *Spodoptera exigua* in drought conditions. Authors pointed out a role for secondary defensive metabolites in controlling other physiological processes. In our dataset, the defensive molecules thionins were also up-regulated after water deprivation. If thionins could have a role in dehydration tolerance or their induction by water deprivation is related to reinforce cell walls against possible pathogen/pest attacks remains to be elucidated. The temporal expression patterns of several genes induced by mites confirmed the stronger gene induction caused by the synergistic effect of mites and dehydration, which roughly starts at the same time than that following individual mite stress, but is maintained at higher levels during more time. On the contrary, dehydration gene regulation seems to start earlier in the combined treatment but reaches similar expression levels than that triggered by the individual dehydration stress.

As the synergistic behavior of dehydration and mites stresses leads to a broader and higher differential expression than that caused by individual stresses, the assignment of specific DEGs to dehydration or mite response was not possible by comparing individual and combined transcriptomic results. As an alternative, previous characterization of tomato and Arabidopsis responses to *T. urticae* were explored (Zhurov et al., 2014; Martel et al., 2015). Interpro identifiers were used to establish if common domains were carried out by the induced/repressed proteins after mites' infestation, and then, to discover dehydration-related domains by comparisons using the DEGs after dehydration and dehydration and mites treatments. Although profound divergences were found in the spider mite-induced responses between tomato and Arabidopsis (Martel et al., 2015), most barley domains present in proteins regulated by mite infestation were shared with those reported from tomato and Arabidopsis. The only barley-specific domain previously related to plant defense was the Bowman–Birk protease inhibitory domain, which was absent in the proteins encoded by the Arabidopsis and tomato genomes, MEROPS database (Rawlings et al., 2016). When dehydration and dehydration and mites results were added, a set of 28 Interpro identifiers were not shared with those from barley, Arabidopsis or tomato mite-treated plants. Six of them were regulated after both, dehydration and dehydration and mites treatments. This set includes the adenylate kinase domain, previously related to drought response in tomato (Gong et al., 2010). From the other 22 Interpro identifiers, only found after the dehydration and mites treatment, the cystatin domain from cysteine protease inhibitor proteins was reported to be differentially expressed in response to both, water deprivation and herbivore attack (Diaz-Mendoza et al., 2016; Martinez et al., 2016); the glutathione synthase domain is involved in the production of glutathione, which is an anti-oxidant protective compound with a critical role in plant resistance to biotic and abiotic stresses (Noctor et al., 2012);

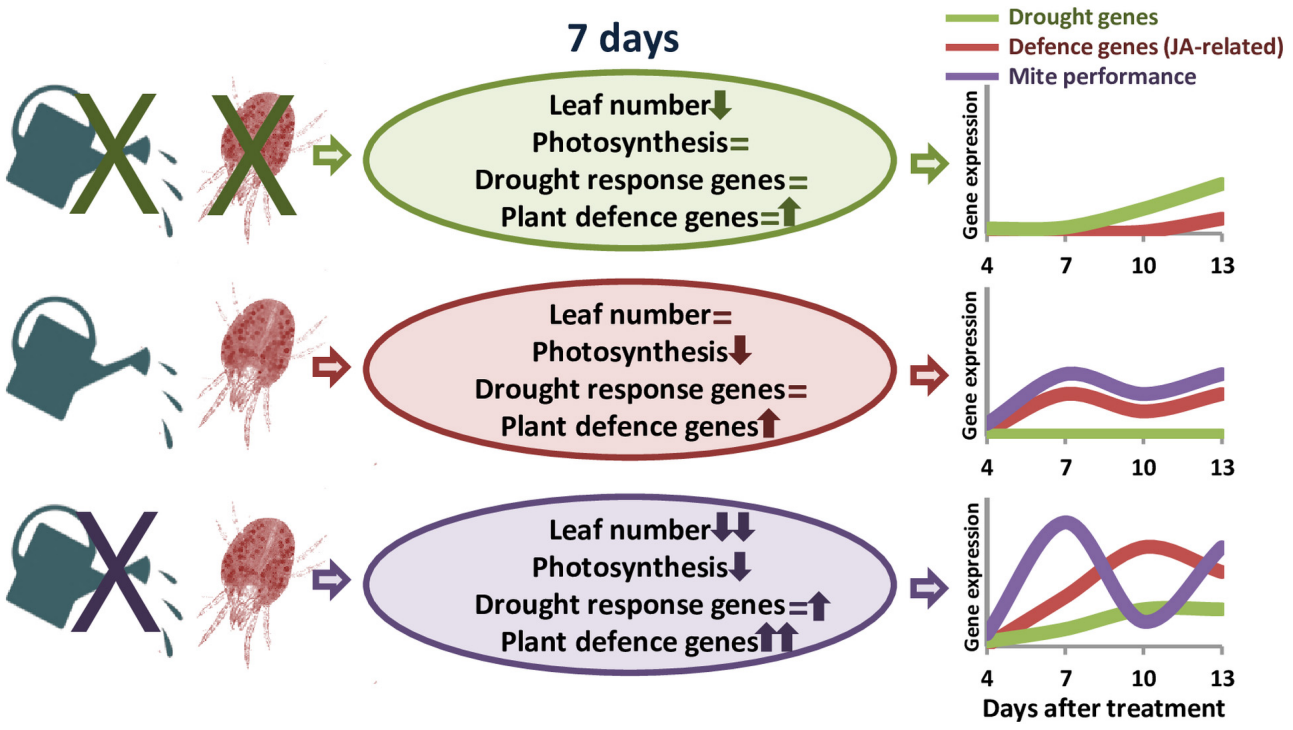

FIGURE 7 | Schematic model summarizing the main effects of dehydration and spider mite treatments. Ovals include information on the variations found in plant phenotype features and gene expression. Up arrows, down arrows and equal signs mean positive effect, negative effect or no-effect, respectively. Line drawings show the predicted time course of the expression of defense and drought-related genes, and the performance of the mite throughout the different treatments.

and the gamma-glutamyl phosphate reductase domain catalyzes the second step in the biosynthesis of the amino acid proline from glutamate. Proline is an osmoprotectant and signaling molecule largely accumulated in response to abiotic stresses, but also with a role in the response to biotic stresses (Szabados and Savouré, 2010). These ambiguities remark the difficulties to assign the Interpro identifiers from the DEGs after combined stresses specifically to the dehydration or the mite response.

Transcriptomic analyses in barley revealed a stronger defensive response to the aphid *Myzus persicae* than to the aphid *Rhopalosiphum padi*, which was correlated to the fact that barley is a good-host of *R. padi* but a poor-host of *M. persicae* (Escudero-Martinez et al., 2017). Likewise, *S. exigua* performed less well on *S. dulcamara* drought-stressed plants than on well-watered plants, which was correlated to stronger induction of plant herbivore-induced processes in drought conditions (Nguyen et al., 2016a). Thus, we will expect a worse performance of *T. urticae* in dehydration-stressed plants, since the defensive response of the plant is considerably higher than in well-watered conditions. However, mite performance was higher in water-stressed barley plants, as was previously reported for mites feeding on drought-stressed tomato plants (Ximénez-Embún et al., 2016, 2017a,b). Different hypothesis have been postulated to establish the consequences of drought stress on herbivore performance. In particular, the "plant stress hypothesis" states that drought causes the plant to have a higher nutritional value for herbivores, and the "plant vigor hypothesis" associates a reduction in growth and an increase in defense compounds caused by drought to a lower suitability for herbivores' feeding (White, 2009). In maize, simultaneous soil drought and *T. urticae* infestation elevates the amount of proteins that enable maize to maintain the efficiency of photosynthesis and metabolism, as well as to protect its cells against metabolic injuries (Dworak et al., 2016). Besides, drought increases the improved nutritional value of tomato leaves by accumulating free amino acid and sugars, and altering hormonal balance (Ximénez-Embún et al., 2016, 2017b). In transgenic barley plants overexpressing the cysteine protease HvPap-1, a higher susceptibility to *T. urticae* correlated with a higher induction of protease inhibitors (Diaz-Mendoza et al., 2017). These findings suggest a mixed scenario where mite performance depends on an optimal balance of nutrients in the plant and its adaptation to plant defense compounds (Gutbrodt et al., 2011). In barley, drought effects on nutrients availability could be stimulating mite feeding, which would provoke a strong induction of plant defenses. However, the accumulation of proteins potentially required to resist the biotic attack would not be enough to increase plant resistance. Population dynamics supports this hypothesis, as total mite population showed temporal variations caused by the developmental cycle. In *T. urticae*, local mate competition conditioned sex-allocation strategies and favors female-biased sex ratios (Macke et al., 2011). Under dehydration conditions, higher mite populations are correlated to an elevated ratio of eggs, which are mostly fertilized to produce females to favor a quick increment of the population to take advantage of higher nutrient availability. In well-watered conditions, peaks of females and eggs ratios are temporally more distant, suggesting a larger developmental cycle and a slower increment of the mite population. **Figure 7** summarizes the main findings of this work. These findings highlight the striking differential behavior of both, the barley plant and the spider mite, depending on the watered state of the plant.

CONCLUSION

Results demonstrate the enhanced performance of spider mite in barley under water deficit conditions. Unexpectedly, this behavior was concomitant to an apparent higher up-regulation of mite-induced defenses, which expression patterns were earlier modified than that related to dehydration tolerance signaling pathways. Thus, a stronger transcriptomic effort to manage an herbivore-stress should not be directly correlated to a higher plant resistance. Complex signaling networks leading to metabolic alterations affected by combined stresses should be in the focus to really understand and predict the consequences of actual stresses in crop production.

AUTHOR CONTRIBUTIONS

ID and MM conceived the research. MS performed most of the experimental research. MS, ID, and MM participated in the design, acquisition, analysis, and interpretation of data for the work. All authors contributed to 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.2018.00458/full#supplementary-material

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