DEVELOPMENTAL MODIFICATION UNDER BIOTIC INTERACTIONS IN PLANTS

EDITED BY: Shinichiro Sawa, Bruno Favery and Masa H. Sato

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DEVELOPMENTAL MODIFICATION UNDER BIOTIC INTERACTIONS IN PLANTS

Topic Editors:
Shinichiro Sawa, Kumamoto University, Japan
Bruno Favery, Institut National de la Recherche Agronomique (INRA), France
Masa H. Sato, Kyoto Prefectural University, Japan

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Editorial: Developmental Modification Under Biotic Interactions in Plants

Shinichiro Sawa[1]*, *Masa H. Sato*[2] and *Bruno Favery*[3]

[1] Graduate School of Science and Technology, Kumamoto University, Kumamoto, Japan, [2] Laboratory of Cellular Dynamics, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan, [3] INRAE, Université Côte d'Azur, CNRS, ISA, Sophia Antipolis, France

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Editorial on the Research Topic

Developmental Modification Under Biotic Interactions in Plants

In many ecosystems, plants represent the center of biological interactions. As the Earth entered the Cenozoic era, gymnosperm, and fern populations declined while flowering angiosperms became the dominant plant species. Plant pollination and seed dispersal mechanisms then evolved and diversified as animals began to feed on plant nectars and fruits. It is believed that these interactions drove the evolutions and diversities of both plants and other organisms. Even though many plant interspecific interactions have been studied, the molecular mechanisms regulating these interactions have not been characterized extensively. In many cases, plants secret various metabolites into the environment to act as signaling molecules detected by other organisms. Conversely, interactions with other organisms may also cause modifications in plant development, such as the generation of novel cell types and organs (Favery et al., 2020).

The first step of biological interaction between plants and other organisms, particularly parasites and pathogens, likely involves chemotaxis. Molecules secreted by plants often can act as guidance cues. Many sugars, organic acids, phenolics, amines, and phytohormones secreted by plants were shown to possess attractive properties to various animals and microorganisms, and some of their cognate receptors have also been identified (Tsai et al., 2019; Oota et al., 2020; Tsai et al.). Attractants are thought to consist of unique or unusual compounds that may explain parasite/pathogen's host range. However, it appears that most plant parasites and pathogens are in fact attracted to common compounds such as plant metabolites and/or plant hormones. Therefore, it seems the identities of attractants alone is not sufficient to explain plant parasite/pathogen's host range. Instead, the specific interactions between the host-secreted attractants and the parasite receptors may help to better understand how these interactions drove the evolution of both plants and their interacting partners (Tsai et al.).

After parasites infiltrate their plant hosts, they may modify the host's signaling pathways to aid their own infection. Plant-parasitic nematodes (PPN), which include cyst nematodes and gall-inducing root-knot nematodes, are both scourges for agriculture and interesting case studies for such host manipulations. These nematodes spend the majority of their life cycles in plant roots, where they induce the formation of specific feeding structures inhabiting specialized feeding cells. These feeding cells known as giant cells and syncytia are multinucleate, hypertrophied, and hypermetabolic (Favery et al., 2020). Transcriptomic analysis have shown that the induction of these feeding cells involved an extensive reprogramming of gene expression. Regulators of gene expression, such as the small non-coding microRNAs, were shown to be essential for PPN

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Edited by:
Jeremy Astier, INRA UMR1347 Agroécologie, France

Reviewed by:
M. Estrella Santamaria, Polytechnic University of Madrid, Spain

***Correspondence:**
Shinichiro Sawa
sawa@kumamoto-u.ac.jp

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feeding site formation. These microRNAs are likely to be master regulators expression re-programming during PPN feeding structure formation (Jaubert-Possamai et al.). PPN have been documented to inject molecules, named effectors, to induce the differentiation of feeding cells (Mejias et al.), and to suppress the host plant's immune response (Sato et al.). These effectors, associate with specific host proteins, enabling them to hijack important processes for cell morphogenesis and physiology and/or immunity. Some PPN-resistant plants have evolved intracellular nucleotide-binding domain leucine-rich repeat (NLR)-type receptors, which would recognize PPN effectors and induce NLR-triggered immunity (Sato et al.).

Aside from nematodes, insects are the other major multicellular animal taxon that interact with plants. The feeding of plant tissues from within plants (endophagy) is common among insects. Nutrition appears to be the main driver for the evolution of endophagy, however competition reduction, water conservation, and predation avoidance may have played significant roles (Tooker and Giron). Meanwhile, plant signaling pathways upon insect herbivory, a cascade of events including phosphorylation of a subset of transcription factors (e.g., ERF13) by calcium-dependent protein kinases (CRK2 and CRK3), have also recently been characterized in Arabidopsis (Miyamoto et al.). These phytohormone-responsive CRKs may thus play major roles in the coordination of plant defense responses during insect herbivory. On the other hand, many social aphids are known to form elaborate galls on aerial plant organs, where up to thousands of individuals may occupy for up to over a year. Insect-induced galls are unique organs that provide both shelter and nutrients to plant-parasitic insects. In addition, aphids were

FIGURE 1 | Word cloud summarizing this Research Topic on developmental modification under biotic interactions in plants. This visual display was made with tagxedo (http://www.tagxedo.com/) using the words of this editorial article.

also found to utilize the gall vasculature to remove wastes from the colony (Kutsukake et al.), suggesting the utilities of insect galls are indeed more complex than expected. Transcriptomic analysis of Chinese sumac (*Rhus javanica*) horned galls induced by aphids (*Schlechtendalia chinensis*) revealed that meristem, flower, and fruit development master regulators as well as biotic and abiotic stress-responsive genes were highly upregulated in aphid galls (Hirano et al.).

Not all plant parasites are animals. Limitations in soil fertility have influenced the diversification of nutrient acquisition strategies in plants, driven certain species to parasitize on other plants (Zemunik et al., 2015). About 4,000 species of parasitic plants have been documented from all regions of the world, which consists of ∼1% of all flowering plants (Nickrent, 2002). Many parasitic plants are known to have wide host ranges and can infect many economically important crop plants (Lanini and Kogan, 2005). In particular, members of the Orobanchaceae family such as *Striga*, *Orobanche*, and *Phelipanche* are important pest worldwide. Some parasitic plants, such as those from the genus *Cuscuta* (family Convolvulaceae), develop disc-like structures known as holdfasts in response to light and tactile cues, which are used to adhere to host plants (Shimizu and Aoki). Most parasitic plants then develop the root-like haustoria from the inner cortex, and invade the apoplastic space of the host's root. Once the haustorium reaches the host's vascular tissues, the parasite connects its own vasculature to the host's to acquire nutrients (Yoshida et al., 2016). Haustorium development is induced by host-derived signal molecules, collectively called haustorium-inducing factors, which include several cell wall-derived quinones and phenolics. In addition, the plant hormone cytokinins were also shown to trigger haustorium formation (Goyet et al.). Transcriptomic analyses using an *in vitro Cuscuta campestris* haustorium induction system revealed that genes involved in vascular stem cell development and proliferation were up-regulated in the haustoria in the absence of host, whereas genes required for xylem vessel cell differentiation were up-regulated only after the haustoria made contact with the host xylem. These results suggest host-derived signals and physical contact with the host vasculature are likely required to initiate haustoria xylem differentiation through transcriptional regulation (Kaga et al.).

Plant interspecific interactions are not limited to herbivory and parasitism. One of the best-characterized symbiotic plant interspecific interactions is formed between leguminous plants and nitrogen-fixing rhizobia bacteria. These interactions take place in root nodules, which are unique root organs formed specifically to house the rhizobia. The host plants receive organic nitrogen from the rhizobia, in exchange for providing the rhizobia with carbohydrates as nutrients and nodules as accommodation. This mutualistic interaction has evolved sophisticated signaling networks that regulate rhizobia recognition, colonization, differentiation, and nodule formation. Cysteine-rich peptides, reactive oxygen/nitrogen species and toxin–antitoxin modules have all been documented to contribute to the regulation of legume-rhizobia symbiosis (Syska et al.). Interestingly, these molecules are typically known to be involved in anti-microbial immune responses, yet here they have evolved to accommodate rhizobia colonization by escaping the host plant's innate immunity (Syska et al.). Furthermore, two plant growth-promoting rhizobacteria (PGPR) strains, *Bacillus* sp. (12D6) and *Enterobacter* sp. (16i) were shown to be capable of rapidly colonizing the rhizosphere (Jochum et al.). Interestingly, inoculation of these PGPR strains to wheat (*Triticum aestivum*) and maize (*Zea mays*) rhizospheres significantly delays the onset of drought symptoms, which are likely due to root system architecture modifications induced by the PGPR (Jochum et al.).

The diverse collection of articles in this Research Topic highlights the vibrant and rapidly-changing research in the field of plant developmental modification during biotic interactions (**Figure 1**). These new findings contribute to the understanding of how species interactions influence the evolution and diversity across ecosystems.

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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ORIGINAL RESEARCH
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Tyrosine Kinase-Dependent Defense Responses Against Herbivory in Arabidopsis

*Takumi Miyamoto[1], Takuya Uemura[1], Keiichirou Nemoto[2], Maho Daito[1], Akira Nozawa[3], Tatsuya Sawasaki[3] and Gen-ichiro Arimura[1]**

[1] Department of Biological Science and Technology, Faculty of Industrial Science and Technology, Tokyo University of Science, Tokyo, Japan, [2] Iwate Biotechnology Research Center, Iwate, Japan, [3] Proteo-Science Center, Ehime University, Matsuyama, Japan

Tyrosine (Tyr) phosphorylation (TP) is important for promotion of plants' signaling. Arabidopsis calcium-dependent protein kinase related protein kinases (CRK2 and CRK3) phosphorylate Tyr residues of a subset of transcription factors (TFs), including herbivory-responsive ethylene response factor 13 (ERF13), but the *in vivo* functions of these kinases in plant defense responses and development remain to be clarified. We show that when CRKs were coexpressed with ERF13 in Arabidopsis leaf protoplasts, the transcription activity regulated via ERF13 was elevated by CRK2 but not CRK3 or their kinase-dead form mutants. Moreover, this elevation was abolished when a Tyr-phosphorylation mutant of ERF was coexpressed with CRK2, indicating that CRK2 serves as an effector of ERF13 mediated by Tyr-phosphorylation. Moreover, CRK2 and CRK3 acted as effectors of RAP2.6 and WRKY14, respectively. CRK-overexpressing lines and knockout mutants of Arabidopsis plants showed increased and decreased expression levels of the defensin gene *PDF1.2* in leaves, respectively, conferring on the plants modulated defense properties against the generalist herbivore *Spodoptera litura*. However, these lines did not show any obvious developmental defects, indicating that CRKs play a role in defense responses but not in the ordinary growth or development of plants. Transcription of both *CRK2* and *CRK3* was positively regulated by jasmonate signaling and abscisic acid (ABA) signaling upon herbivory. Our findings suggest that these phytohormone-responsive CRKs work coordinately for plant defense responses via Tyr phosphorylation of herbivory-responsive regulators.

Keywords: *Arabidopsis thaliana*, calcium-dependent protein kinase related protein kinase (CRK), defense response, *Spodoptera litura*, tyrosine kinase

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Shinichiro Sawa, Kumamoto University, Japan

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Kazunori Okada, The University of Tokyo, Japan
Hirokazu Ueda, National Agriculture and Food Research Organization (NARO), Japan

****Correspondence:***
Gen-ichiro Arimura
garimura@rs.tus.ac.jp

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INTRODUCTION

Tyrosine (Tyr) phosphorylation (TP) is a notable regulator of signal transduction in eukaryotic cells (Blume-Jensen and Hunter, 2001). It has been estimated that in *Arabidopsis thaliana* (Arabidopsis) and rice, 4% of phosphopeptides are Tyr-phosphorylated peptides, which is similar to the proportion in humans (Nakagami et al., 2010). Given the fact that TP is involved in abscisic acid (ABA) signaling (Ghelis et al., 2008), gibberellin responses (Fu et al., 2002), cold stress (Sangwan et al., 2001), and sugar responses (Ritsema et al., 2009), TP is considered to be multiply involved in not only plant growth and development but also defense responses to biotic and abiotic stresses.

Notably, Nemoto et al. (2015) recently reported that Arabidopsis calcium-dependent protein kinase (CPK)-related protein kinases [CRK2 (At3g19100) and CRK3 (At2g46700)] phosphorylate Tyr residues of beta-tubulin and an array of transcription factors (TFs), including ethylene response factor 13 (ERF13) (At2g44840), WRKY DNA-binding protein 14 (WRKY14) (At1g30650), ERF subfamily B-4 member ERF/AP2 transcription factor 2.6 (RAP2.6) (At1g43160), and cryptochrome-interacting basic-helix-loop-helix 5 (CIB5) (At1g26260). The transcript level of ERF13 in Arabidopsis leaves is responsive to exogenous ABA and jasmonate (JA) application, suggesting that ERF13 is relevant to plant stress responses (Lee et al., 2010; Schweizer et al., 2013). RAP2.6, another member of the ERF family, has also been shown to function in plant defense responses to nematodes, ABA, salt and osmotic stresses (Zhu et al., 2010; Ali et al., 2013; Guo and Sun, 2017). The same holds true for the WRKY gene family, which plays key roles in plant stress responses, including toward biotic stress (Eulgem et al., 2000; Chen et al., 2012; Birkenbihl et al., 2018). Notably, in *Coptis japonica*, TP has been proposed to enhance the nuclear localization, DNA-binding activity and transactivation of a WRKY involved in regulating the biosynthesis of the defensive products benzylisoquinoline alkaloids (Yamada and Sato, 2016). It is therefore clear that TP plays central roles in cellular signaling of plant stress responses. CRK2 and CRK3 share 57.7% amino acid identity, and they share a serine (Ser)/threonine (Thr) kinase domain and a degenerate calcium-binding EF-hand motif. In spite of the structural similarity of CRKs to typical Ser/Thr-type protein kinases, CRK2 and CRK3 preferentially phosphorylate tyrosine residues in the absence of calcium (Nemoto et al., 2015).

On the other hand, Ser/Thr phosphorylation has been classically focused on regarding its relevance to plants' stress responses. For instance, it has been elucidated that CPK2 (NtCDPK2) modulates the activation level of stress-induced mitogen-activated protein kinases (MAPKs), leading to increased levels of the defense-associated phytohormones JA, 12-oxo-phytodienoic acid, and ethylene in tobacco (Ludwig et al., 2005). Moreover, Arabidopsis AtCPK3 and AtCPK13 have been reported to activate a heat shock TF (HsfB2a) involved in activation of the defense gene *PDF1.2* in Arabidopsis plants infested by larvae of the generalist herbivore *Spodoptera litura* (Nagamangala Kanchiswamy et al., 2010). In contrast to these Ser/Thr kinases, however, the nature of Tyr kinases that act in plant defense responses remains obscure. We therefore focused on CRKs involved in the phosphorylation of defense-associated TFs. Here we show that CRK2 and CRK3 play a central role in eliciting defense responses of Arabidopsis host plants against the generalist herbivore *S. litura*. Moreover, since CRKs are also known to be involved in gibberellin signaling through the phosphorylation of GARU (gibberellin receptor RING E3 ubiquitin ligase), leading to ubiquitin-dependent degradation of gibberellin receptor (GID1) in Arabidopsis seedlings (Nemoto et al., 2017), phenotypic analyses were carried out using CRK overexpression and mutant lines.

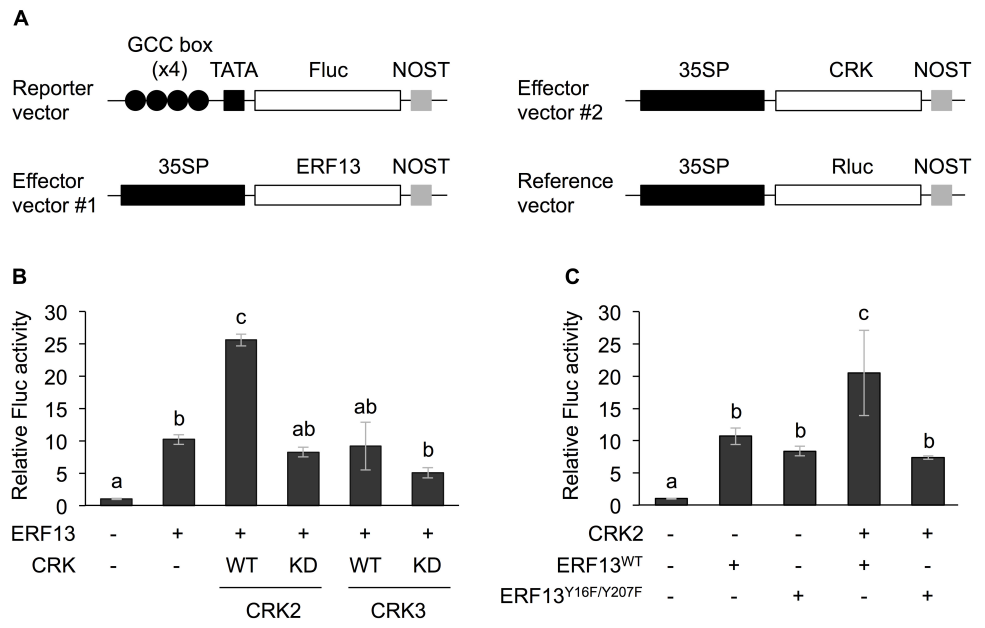

FIGURE 1 | Dual luciferase (LUC) activity mediated through CRK-activated ERF13. **(A)** Schematic diagram of the reporter and effector vectors used in dual LUC assays. **(B)** Four inverted repeats of GCC-box fused to a minimal TATA-box and a firefly LUC (Fluc) reporter gene. Transient activation of the reporter gene according to co-expression with (+) or without (-) ERF13, wild-type (WT) and kinase dead-mutant (KD) of CRK2 or CRK3 in Arabidopsis protoplasts was assessed. **(C)** Likewise, transient activation of the reporter gene according to co-expressed effector(s), CRK2, WT of ERF13 ($ERF13^{WT}$), or ERF13 mutant deficient in CRK-phosphorylated sites ($ERF13^{Y16F/Y207F}$) in Arabidopsis protoplast cells was assessed. Data represent the mean and standard error ($n = 3$). Renilla luciferase (Rluc) activity was used to normalize for the efficiency of transformation. Means indicated by different small letters are significantly different, based on a one-way ANOVA with *post hoc* Tukey's HSD ($P < 0.05$). NOST, nopaline synthase terminator; 35SP, cauliflower mosaic virus 35S promoter; and TATA, TATA-box.

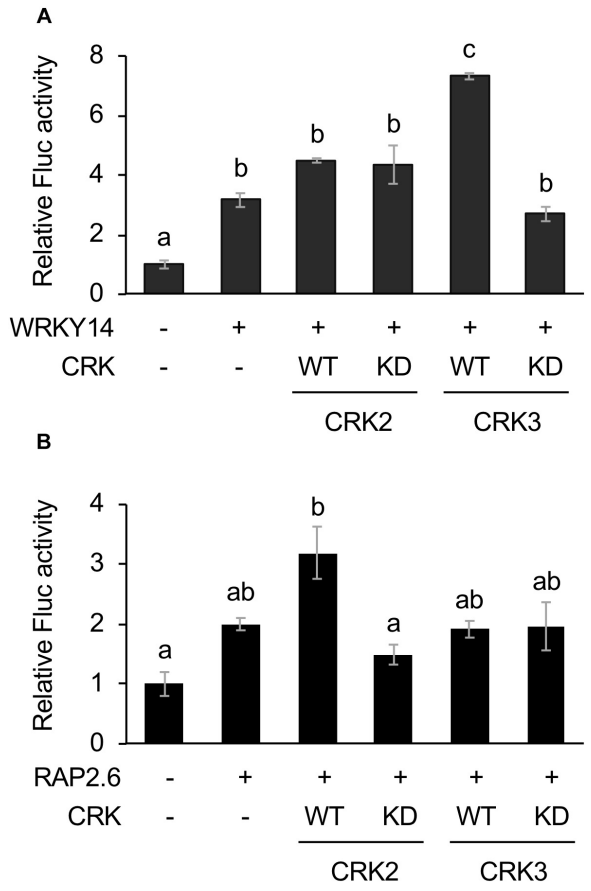

FIGURE 2 | Dual luciferase (LUC) activity mediated through CRK-activated WRKY14 or RAP2.6. Four inverted repeats of W-box or GCC-box fragment fused to a minimal TATA-box and a firefly LUC (Fluc) reporter gene. Transient activation of the reporter gene according to co-expression with (+) or without (-) WRKY14 **(A)** or RAP2.6 **(B)**, WT or kinase dead-mutant (KD) of CRK2 or CRK3 in Arabidopsis protoplast cells was assessed. Renilla luciferase (Rluc) activity was used to normalize for the efficiency of transformation. Data represent the mean and standard error (n = 3). Means indicated by different small letters are significantly different, based on a one-way ANOVA with *post hoc* Tukey's HSD ($P < 0.05$).

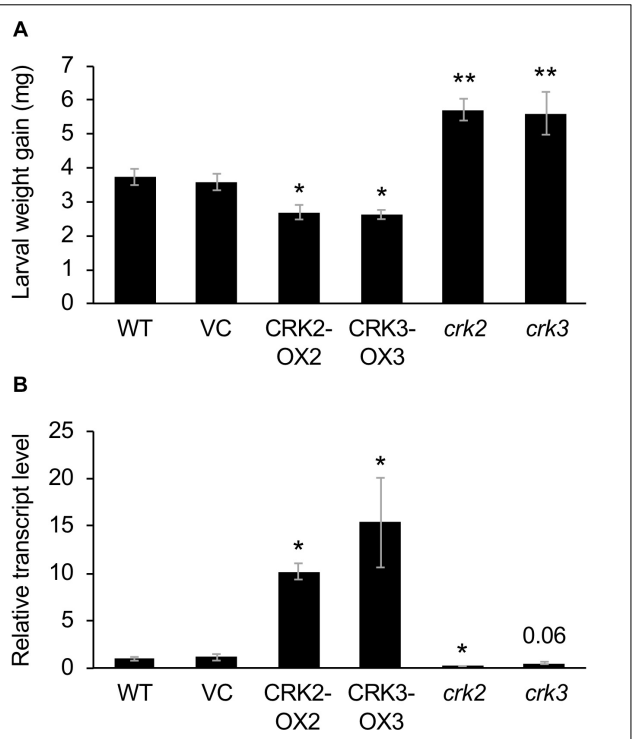

FIGURE 3 | Defense property of wild type (WT), *crk* mutants, and CRK-overexpressing lines. **(A)** The net body weight that *Spodoptera litura* larvae gained during 3 days after they had been placed on potted plants of WT, *crk2*, or *crk3* mutants, CRK-overexpressing lines (CRK2-OX2 and CRK3-OX3) and their vector control (VC) lines. Data represent the mean and standard error (n = 16–22). **(B)** Transcript levels of a defensin gene *PDF1.2* in the leaves of WT, *crk* mutants, CRK-overexpressing plants, and VC plants. Transcript levels of genes were measured by RT-qPCR and normalized by those of *ACT8*. Data represent the mean and standard error (n = 4 for VC, CRK2-OX2, and CRK3-OX3; n = 7 for WT, *crk2*, and *crk3*). Data marked with an asterisk are significantly different from those of WT **(A)** or VC **(B)**, based on a one-way ANOVA with Holm's sequential Bonferroni *post hoc* test (**$P < 0.01$, *$0.01 \leq P < 0.05$). Otherwise, the mean followed by P value is marginally different from the control value.

MATERIALS AND METHODS

Plants

Wild-type (WT) Arabidopsis ecotype Col-0 plants, CRK T-DNA insertion mutants [*crk2* [Salk_090938C], and *crk3* [Salk_128719C] (Nemoto et al., 2015)], ABA INSENSITIVE 1 mutant [*abi1-1* (Allen et al., 1999)], ETHYLENE INSENSITIVE 2 mutant [*ein2* (Solano et al., 1998)], and transgenic plants overexpressing *CRK2* or *CRK3* (see below) were grown in plastic pots for 4–5 weeks in a growth chamber at 22 ± 1°C with a photoperiod of 14 h (80 μE m^{-2} s^{-1}). WT of Arabidopsis ecotype Landsberg erecta (Ler) and its *erf13* mutant (CS26912) were grown in these same growth conditions. The CORONATINE INSENSITIVE1 (COI1) mutant (*coi1-1*; Col-0 background) seeds were germinated on 1/2 Murashige and Skoog (MS) medium supplemented with 2% sucrose, 0.8% agar, and 50 μM methyl jasmonate (MeJA, Wako Pure Chemical Industrials, Ltd., Osaka, Japan) to screen the individuals showing normal root growth for 2 weeks (Xie et al., 1998). The screened plants were transferred and grown in plastic pots for an additional 3 weeks.

Chemical and Herbivore Treatments

S. litura were reared on an artificial diet (Insecta LF, Nihon Nosan Nogyo Ltd., Tokyo, Japan) in the laboratory at 24 ± 1°C with a photoperiod of 16 h. For herbivore treatment, four third-instar larvae per plant were released on potted Arabidopsis plants in a growth chamber for 24 h. After chemical and herbivore treatment, all the plants were incubated at 22 ± 1°C (14 h photoperiod at a light intensity of 80 μE m^{-2} s^{-1}).

Arabidopsis plants were evenly sprayed with 1 mL of aqueous solutions (0.1% (v/v) ethanol) of MeJA (Wako Pure Chemical industrials; 0.2 mM), 1-aminocyclopropane-1-carboxylic acid (ACC, Wako Pure Chemical Industries; 0.01 mM) or ABA

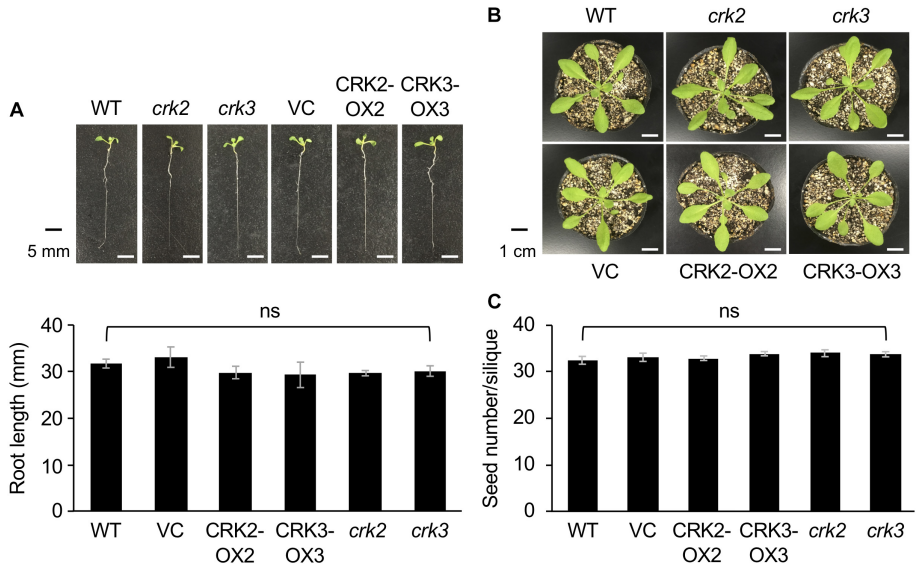

FIGURE 4 | The phenotype and root length of seedlings **(A)** and rosette plants **(B)**, and the numbers of seeds **(C)** of wild type (WT), *crk2*, and *crk3* mutants, CRK-overexpressing lines (CRK2-OX2 and CRK3-OX3), and their VC line. Plant seedlings were grown on medium for 14 days, and rosette plants and plants during the harvest time were grown on soil for 4 weeks and ∼8 weeks, respectively. Means and standard errors of root lengths were determined using 12 individual seedlings. Means and standard errors of the numbers of seeds were determined using five pods from eight individual plants. ns, not significant based on a one-way ANOVA.

(Tokyo Chemical Industry Co., Ltd., Tokyo, Japan; 0.1 mM) and incubated in a growth chamber for up to 24 h.

Primers

Primers used for all the polymerase chain reactions (PCRs) in this study are listed in **Supplementary Table S1**.

Protoplast Preparation and Transfection

The full-length coding region of *CRK2*, *CRK3*, or *ERF13* was cloned into the p35SΩ-GW-NOST vector [cauliflower mosaic virus 35S promoter (35SP)::Ω sequence (translation enhancer)::the Gateway cassette (GW) region::Nopaline synthase terminator (NOST) (Nemoto et al., 2015)] using the Gateway cloning system (Thermo Fisher Scientific, Waltham, MA, United States). The kinase dead (*KD*) form mutants of CRK2 (Lys_{176} to Arg [$CRK2^{KD}$]) and CRK3 (Lys_{175} to Arg [$CRK3^{KD}$]), and ERF13 mutants (Tyr_{16} to Phe and Tyr_{207} to Phe [$ERF13^{Y16F/Y207F}$]) were generated using a PrimeSTAR Mutagenesis Basal Kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions (Nemoto et al., 2015). These cDNAs were also inserted into the GW region of p35SΩ-GW-NOST vector. Four repeat sequence of a GCC-box (AGCCGCC) fragment or a W-box (TTTGACC) fragment was fused to a minimal TATA box::a firefly luciferase (Fluc) reporter gene::NOST in the pMA cloning vector (Thermo Fisher Scientific, Waltham, MA, United States). The map of the representative vectors used is shown in **Figure 1**.

Protoplast isolation from Arabidopsis leaves was performed as previously described (Wu et al., 2009). The peeled leaves (4-5-week-old plants), still adhering to the tape, were transferred to a Petri dish containing 10 ml of enzyme solution (2% (w/v) cellulase "Onozuka" R10 [Yakult Pharmaceutical Industry, Tokyo, Japan], 0.3% (w/v) macerozyme "Onozuka" R10 [Yakult Pharmaceutical Industry], 0.4 M mannitol, 10 mM $CaCl_2$, and 5 mM MES [pH 5.7]). The leaf tissues were incubated at room temperature for 1 h until the protoplasts were sufficiently released into the solution. The protoplasts isolated were diluted with an equal volume of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES [pH 5.7]) and filtered with filter paper to remove undigested leaf tissues. The protoplast suspension was centrifuged at 100 *g* for 2 min and re-suspended with W5 solution to adjust it to 2×10^5 cells ml^{-1}. The protoplast suspension was centrifuged again and finally resuspended in an equal volume of modified MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, and 5 mM MES [pH 5.7]).

Polyethylene glycol-mediated DNA transfection was performed as previously described (Yoo et al., 2007). The protoplast suspension (100 μl) was supplemented with a mixture of vectors carrying 35SP::CRK ($CRK2^{WT}$, $CRK2^{KD}$, $CRK3^{WT}$, or $CRK3^{KD}$)::NOST, 35SP::TF ($ERF13^{WT}$, $ERF13^{Y16F/Y207F}$, WRKY14, or RAP2.6)::NOST, GCC-box or W-box::TATA::Fluc::NOST and reference (35SP::Renilla luciferase [Rluc]::NOST) vector at a ratio of 4:5:5:1 to protoplast suspension with 110 μl PEG solution [40% (w/v) polyethylene glycerol, 0.4 M mannitol, and 0.1 M $Ca(NO_3)_24H_2O$]. The transfection was carried out at room temperature for 5 min and stopped by adding 400 μl of W5 solution. The protoplasts were collected by centrifugation at 100 *g* for 2 min and resuspended with 500 μl of WI solution (5 mM MES [pH 5.7], 0.4 M mannitol, and 20 mM KCl) and incubated in a 12-well tissue culture plate at room temperature overnight.

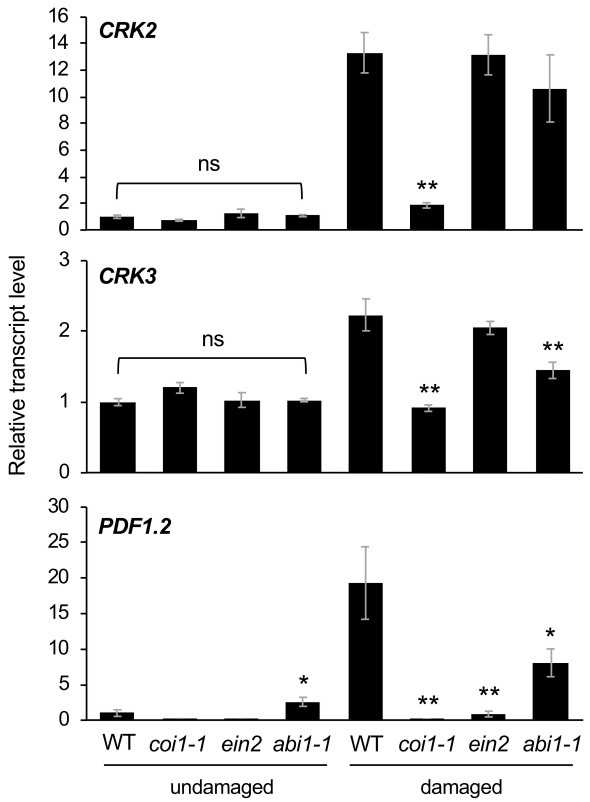

FIGURE 5 | Transcriptional regulation of *CRKs* in infested leaves. Transcript levels of *CRK2*, *CRK3*, and *PDF1.2* in leaves of Arabidopsis wild-type (WT), *coi1-1*, *abi1-1*, and *ein2* plants damaged or not with *S. litura* larvae for 24 h. Transcript levels of genes were measured by RT-qPCR and normalized by those of *ACT8*. Data represent the mean and standard error (n = 5–8). Data marked with an asterisk are significantly different from those of undamaged or damaged WT plants, based on a one-way ANOVA with Holm's sequential Bonferroni *post hoc* test (**$0.001 \leq P < 0.01$, *$0.01 \leq P < 0.05$). ns, not significant.

Luciferase (LUC) Assay

The LUC assay was performed as previously described (Luehrsen et al., 1992). The protoplasts were collected by centrifugation at 100 *g* for 2 min, and re-suspended with 100 μl of EX buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, and 0.5% (v/v) Triton X-100). The protoplasts were again centrifuged at 20,000 *g* for 10 min at 4°C, and 10 μl of supernatant was used for a LUC assay. LUC activity was measured with a 1420 Luminescence Counter ARVO Light (Perkin Elmer, Waltham, MA, United States) using the Dual-Luciferase® Reporter assay system (Promega, Madison, WI, United States). Fluc activity produced due to the transfected reporter construct was expressed as the value normalized by the Rluc activity produced due to the co-transfected reference vector. Replicate analyses were conducted with 3 independent samples.

Generation of Transgenic Arabidopsis Plants

The full-length coding region of *CRK2* or *CRK3* was inserted into binary vector pMDC32 (2x 35SP::GW::NOST) using the Gateway cloning system (see above). The resulting vector, pMDC32-*CRK2*, pMDC32-*CRK3* or pMDC32 [vector control (VC)], was transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation. WT Arabidopsis plants that had been grown for about 6–7 weeks were transformed via the floral-dip transformation method (Clough and Bent, 1998). Transgenic T1 seeds from each transformant were tested for germination on 1/2 MS medium supplemented with 30 mg l^{-1} hygromycin. T2 seeds harvested from each individual T0 plant that showed ca. 3:1 segregation ratio was tested for hygromycin-resistance again. T3 homozygous plant lines were used for further analyses.

RNA Extraction, cDNA Synthesis and Quantitative PCR (qPCR)

RNA extraction, first-strand cDNA synthesis and quantitative PCR were performed according to the method described previously (Ali et al., 2019).

Herbivore Assay

We performed assays to assess the growth of *S. litura* larvae at 22 ± 1°C (14 h photoperiod at 80 μE m^{-2} s^{-1}). Third-instar larvae were initially weighed (1.7–2.1 mg), and each larva was released onto a potted plant for 3 days. The net body weight that *S. litura* larvae gained each of the following 3 days was determined. When a larva died or was lost during the assay, we excluded that sample, and final replicate analyses were conducted with 16–22 independent samples.

Root Length Measurement

Plant seedlings (14 days old) were grown on 1/2 MS medium. Root lengths were determined using ImageJ software [version 1.50i; (Schneider et al., 2012)].

Statistical Analysis

We performed *t*-tests for pairwise analysis and one-way ANOVA with Holm's sequential Bonferroni *post hoc* test or Tukey's HSD test using the program[1] for comparing multiple samples.

RESULTS

In vivo Function of CRK2 in Transactivation of ERF13

Both CRK2 and CRK3 phosphorylate ERF13 at two Tyr residues (Y16 and Y207) (Nemoto et al., 2015). To investigate the roles of CRK-promoted TP in ERF13 transactivation, CRKs were expressed together with ERF13 as an activator of a reporter (firefly LUC [Fluc]) gene coexpressed under the control of a chimeric promoter that consisted of four inverted repeats of GCC-box [ERF-binding *cis*-element (Fujimoto et al., 2000)] fused to a minimal TATA-box, in Arabidopsis mesophyll protoplasts (**Figure 1A**). Expression of ERF13 caused a 10-fold increase of Fluc activity in comparison to the activity in the absence of ERF13 (**Figure 1B**). When WT CRK2 ($CRK2^{WT}$) was

[1]http://astatsa.com/OneWay_Anova_with_TukeyHSD/

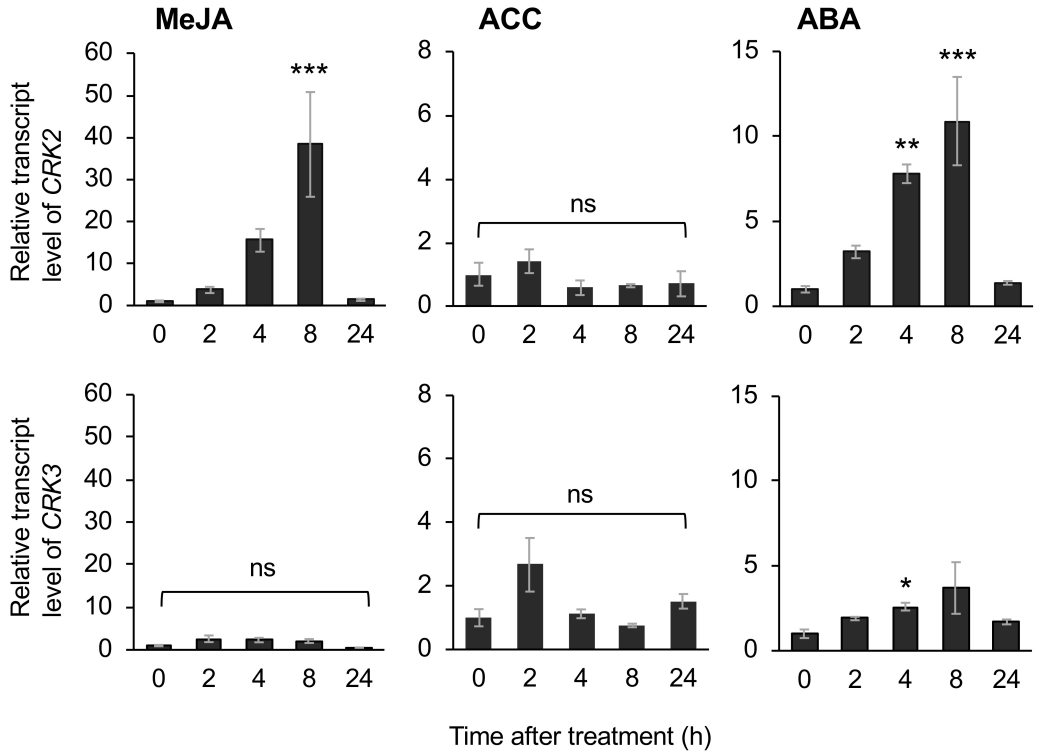

FIGURE 6 | Phytohormone-induced regulation of CRK expression. Transcript levels of *CRK2* and *CRK3* in leaves of Arabidopsis wild-type plants in response to exogenous application of methyl jasmonate (MeJA), an ethylene precursor [1-aminocyclopropane-1-carboxylic acid (ACC)], abscisic acid (ABA) for up to 24 h. Transcript levels of genes were measured by RT-qPCR and normalized by those of *ACT8*. Data represent the mean and standard error ($n = 6$). Data marked with an asterisk are significantly different from those of undamaged or damaged WT plants, based on a one-way ANOVA with Holm's sequential Bonferroni *post hoc* test (***$P < 0.001$, **$0.001 \leq P < 0.01$; *$0.01 \leq P < 0.05$). ns, not significant.

coexpressed with ERF13, an additional 2.5-fold increase of Fluc activity was detected. However, transactivation of ERF13 was not caused by either CRK3 or kinase domain-mutant KD CRK2 ($CRK2^{KD}$, whose lack of kinase activity has been shown previously; Nemoto et al., 2015, 2017) when they were concomitantly expressed in the cells. Moreover, when an ERF13 mutant deficient in CRK-phosphorylated sites ($ERF13^{Y16F/Y207F}$), instead of WT ERF13 ($ERF13^{WT}$), was co-expressed with CRK2, the Fluc activity declined to the basal level achieved by the expression of $ERF13^{WT}$ alone (**Figure 1C**).

Transactivation of WRKY14 and RAP2.6 by CRK

CRK2 and CRK3 are also able to phosphorylate WRKY14 (Nemoto et al., 2015), one of the WRKY members involved in an array of plant defense responses (Skibbe et al., 2008; Bakshi and Oelmuller, 2014; Li et al., 2015). Moreover, RAP2.6, another AP2/ERF protein member involved in plant stress responses (Krishnaswamy et al., 2011; Ali et al., 2013), has been shown to be phosphorylated by CRK2 but not CRK3 (Nemoto et al., 2015). We therefore explored those two TFs as substrate targets for CRKs, utilizing the transient Fluc expression system in protoplast cells, using the two *cis*-elements, i.e., a W-box and a GCC-box for WRKY14 (Chen et al., 2012) and RAP2.6 (Zhu et al., 2010), respectively.

The Fluc activity was increased by WRKY14 expression. This activity marginally tended to be elevated by coexpression of WT CRK3 ($CRK3^{WT}$) (**Figure 2A**). However, this transactivation was achieved by neither $CRK3^{KD}$ nor CRK2 ($CRK2^{WT}$ or $CRK2^{KD}$). In contrast, Fluc activity, which was only marginally increased by expression of RAP2.6, was elevated by coexpression of $CRK2^{WT}$ (**Figure 2B**). Again, this transactivation was not achieved by coexpression of $CRK2^{KD}$ or CRK3 ($CRK3^{WT}$ or $CRK3^{KD}$).

Defense Ability and Growth/Development of CRK Mutants and Overexpressing Lines

We obtained two lines and three lines of CRK2- or CRK3-overexpressing plants, respectively. Two respective representative lines (CRK2-OX2 and CRK3-OX3) exhibited 170-fold and 70-fold increased levels of *CRK2* and *CRK3* expression under the constitutive 35SP, respectively, compared to the levels in leaves of the VC line (**Supplementary Figure S1**).

These transgenic lines exhibited lower development of larvae of the generalist herbivore *S. litura* hosted on the potted plants for 3 days, compared to that on VC plants (**Figure 3A**). This was in agreement with the constitutively elevated expression levels

of the JA-inducible plant defensin gene *PDF1.2* (Manners et al., 1998) in leaves of these two transgenic lines (**Figure 3B**). In contrast, *crk2* and *crk3* knockdown mutants (Nemoto et al., 2015) exhibited enhanced development of larvae on the potted plants during 3 days, in accord with the constitutively lower expression level of *PDF1.2* in their leaves (**Figures 3A,B**). The GCC-box located at −255 to −261 in the *PDF1.2* promoter has been shown to play a key role in conferring JA responsiveness to *PDF1.2* expression (Brown et al., 2003). Putative W-boxes (TGACC/T) are also located at −388 to −384, −773 to −768, and −828 to −823 in the *PDF1.2* promoter upstream region.

None of the transgenic lines or mutants of CRKs showed any marked differences in plant growth, development or morphology, including root growth, vegetative stage development, or seed number (**Figure 4**).

Transcriptional Regulation of *CRK2* and *CRK3* via Phytohormone Signaling for Defense Responses

Finally, Arabidopsis mutant plants defective in JA signaling (*coi1-1*) (Xie et al., 1998), ethylene signaling (*ein2*) (Ju and Chang, 2015), and ABA signaling (*abi1-1*) (Pei et al., 1997) were assessed to evaluate the involvement of hormone signaling in *CRK* activation during damage by *S. litura* (**Figure 5**). In comparison to the induction of transcripts of *CRK2* and *CRK3* in WT leaves, *coi1-1* plants exhibited defective elevation of these transcript levels in leaves upon herbivory, as did *PDF1.2* plants. *ein2* and *abi1-1* plants did not show defective elevation of transcript levels of *CRK2* upon herbivory. However, *abi1-1* plants showed defective elevation of the *CRK3* transcript level upon herbivory. Although *abi1-1* leaves showed slightly higher expression of *PDF1.2* compared to WT in undamaged leaves, this issue was not further explored because it was outside the focus of the present study.

The application of exogenous phytohormone solutions to WT plants resulted in induced expression of *CRK2* in leaves treated with the methyl form of JA (MeJA) or ABA but not an ethylene precursor (ACC) (**Figure 6**). Moreover, expression of *CRK3* was elicited at 4 h in leaves treated with ABA but not MeJA or ACC for up to 24 h.

DISCUSSION

Tyr phosphorylation mediated by Arabidopsis CRKs appeared to modulate the activities of TFs including ERF13, WRKY14, and RAP2.6 (**Figures 1**, **2**). Although ERF13 and WRKY14 were previously shown to be phosphorylated by both CRK2 and CRK3 using an *in vitro* phosphorylation system (Nemoto et al., 2015), the transactivation of ERF13 and WRKY14 was achieved by CRK2 and CRK3 alone, respectively (**Figures 1**, **2**). However, these findings are not surprising because *in vitro* phosphorylation activity does not always accord with the *in vivo* functions (Delom and Chevet, 2006). This may be because the concentrated kinase protein and/or possibly contaminating kinases from the eukaryotic protein synthesis system cause non-specific phosphorylation of substrate targets in *in vitro* assays. Moreover, it is known that phosphorylation modification of TFs can be responsible not only for their transactivation but also for their nuclear translocation (Liu et al., 2017) as well as enhancement of their binding to the particular *cis*-element of the respective promoter region (Gao et al., 2013).

According to our phenotypic characterization of loss and gain of CRK functions, it appeared that both CRK2 and CRK3 are involved in plant defense responses to *S. litura* damage (**Figure 3**). However, it is important to note that the *erf13* mutant did not fully modulate the herbivore performance or the *PDF1.2* transcript in leaves, compared to those in WT (**Supplementary Figure S2**), in accord with previous findings (Schweizer et al., 2013). All these facts lead us to propose a model in which multiple CRK substrates, including not only ERF13 but also WRKY14, RAP2.6 and unknown TF substrates, may individually and/or synergistically coordinate the upregulation of defense genes such as *PDF1.2* (**Figure 7**). In addition, CRKs may control various regulatory molecules besides TFs in cellular signaling. For example, CRK2 phosphorylates GARU, a protein involved in ubiquitin-dependent degradation of the gibberellin receptor GID1 in gibberellin signaling of Arabidopsis seedlings (Nemoto et al., 2017). Both *garu* mutant and CRK2-OX plants enhance GID1 stabilization and DELLA degradation, indicating that CRK2 is positively involved in gibberellin signaling through the

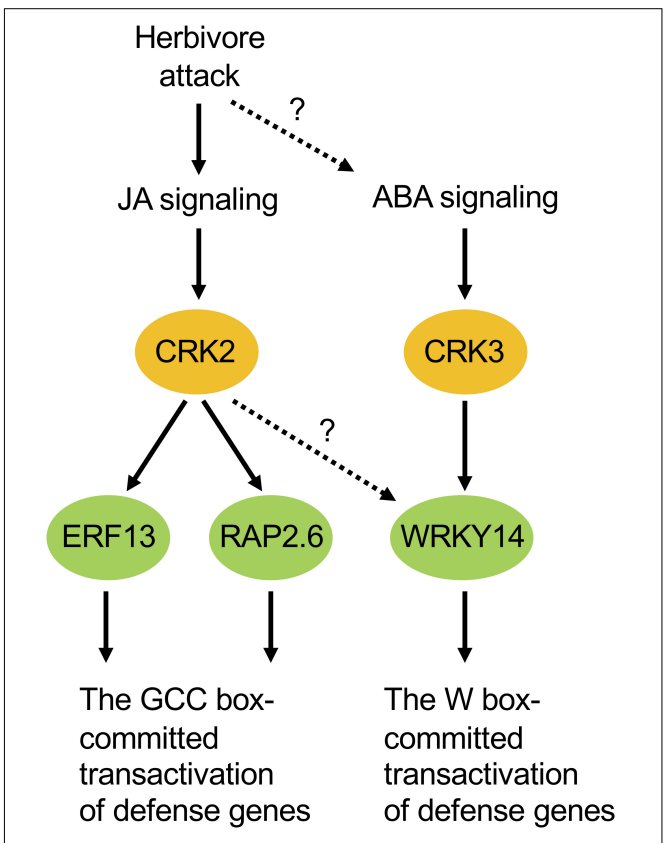

FIGURE 7 | Possible model of cellular signaling mediated by CRKs under herbivore attack. ABA, abscisic acid; JA, jasmonate.

CRK2-mediated Tyr phosphorylation of GARU in Arabidopsis seedlings (Nemoto et al., 2017).

Moreover, ABA-responsive CRK3 (**Figure 5**) may be involved in *in planta* responses to not only herbivory but also pathogenesis and multiple environmental stresses. For example, WRKY, a substrate of CRK3, should especially function in gene regulation for environmental stress tolerance (Chen et al., 2012), and TP has been shown to be involved in ABA signaling (Ghelis et al., 2008), as described above. On the other hand, given the transcriptional profile of *CRK2* in the leaves of MeJA-treated WT plants and infested *coi1-1* plants, there is no doubt that JA is a master switch for *CRK2* activation in the infested leaves (**Figures 5**, **6**, **7**). In contrast to this, although ABA application activates *CRK2* expression, ABA is not likely to contribute to herbivory-response signaling, considering the data observed using *abi1-1*. In contrast, *CRK3* expression was not responsive to JA, but JA is likely involved in herbivory-response signaling according to data observed using *coi1-1*. Regarding this, we presume that *CRK3* is not directly activated by JA signaling, but probably concomitant effects from other signaling pathways such as ABA signaling, in concert with defense-signaling cross-talk (Erb et al., 2012), might affect *CRK3* expression in *coi1-1* leaves during herbivory.

Finally, it should be remarked that neither CRK-OX nor mutant lines show any phenotypic defects in plant growth, development or morphology in comparison to WT plants (**Figure 4**). Thus, CRKs are not likely to be relevant to plant development in the normal growth condition, although this seems to be paradoxical to the above-described possible involvement of CRK2 in gibberellin signaling. We therefore propose a possible model that CRK2 does not play a significant role in the GID1/GARU system under the normal condition, in which a low threshold level of endogenous gibberellin is maintained. However, when plants suffer from threats such as a lack of nutrients or biotic/abiotic stresses, plants switch to reduced endogenous gibberellin levels (Wild et al., 2012; Colebrook et al., 2014), and then CRK2 is recruited to play a primary role in gibberellin signaling. In other words, CRKs do serve under certain conditions for plants' defense responses to environmental threats and plant growth/development, mediated through the assistance of phytohormone signaling.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because data has not yet been linked to any public still domains. Requests to access the datasets should be directed to G-iA, garimura@rs.tus.ac.jp.

AUTHOR CONTRIBUTIONS

TM, TU, KN, MD, TS, and G-iA contributed to the conception and design the study. TM, TU, KN, MD, and AN performed the experiments. G-iA wrote the first draft of the manuscript. TM, TU, KN, and G-iA wrote sections 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.2019.00776/full#supplementary-material

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Plant Manipulation by Gall-Forming Social Aphids for Waste Management

Mayako Kutsukake[1], Keigo Uematsu[2] and Takema Fukatsu[1]*

[1]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan, [2]Department of General Systems Studies, University of Tokyo, Tokyo, Japan

Many social aphids form spectacular galls on their host plants, in which hundreds to thousands of aphids thrive for several months or even for over a year. Here, in addition to colony defense against natural enemies, waste disposal is an important task for the gall dwellers to sustain their social life. In open galls, soldier nymphs actively clean colony wastes such as honeydew droplets, cast-off skins, and cadavers by pushing them with their head out of the gall opening. In the gall, the excreted honeydew is coated with aphid-derived powdery wax to form "honeydew balls," which prevents the aphids from wetting and drowning with their own excretion. How the aphids deal with the accumulated honeydew in closed galls has been a mystery. Here, we report a novel gall-cleaning mechanism: the gall inner surface absorbs and removes the liquid waste through the plant vascular system. Such a plant-mediated water-absorbing property is commonly found in aphids forming closed galls, which must have evolved at least three times independently. By contrast, the inner surface of open galls is wax-coated and water-repelling, and in some cases, the inner surface is covered with dense trichomes, which further enhance the water repellency. In conclusion, gall-forming aphids induce novel plant phenotypes to manage the waste problems by manipulating plant morphogenesis and physiology for their own sake. This review describes our recent studies on waste management strategies by gall-forming social aphids and discusses future directions of this research topic.

Keywords: social aphid, gall, manipulation, waste management, plant cuticle, trichome

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****Correspondence:***
Mayako Kutsukake
m-kutsukake@aist.go.jp

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INTRODUCTION

Aphids, exclusively living on plant phloem sap, embrace approximately 5,000 species in the world (Blackman and Eastop, 2000). Most of them form open colonies on their specific host plants, whereas no more than 10% of the aphids induce conspicuous galls on their host plants, whose morphology is quite characteristic and diverse (**Figures 1A,D**; Wool, 2005). Since the gall founder, called fundatrix or stem mother, forms a unique-shaped gall in a species-specific manner, the galling aphid species can usually be identified solely based on the gall morphology. This means that the morphological characteristics of the galls are mainly determined by aphid-derived genetic components rather than plant-derived ones, and for this reason such morphological traits of the galls are often regarded as "extended phenotypes" of the inducer insects (Stern, 1995; Inbar et al., 2004).

Most of the gall-forming aphids are restricted to the two subfamilies Eriosomatinae and Hormaphidinae in the family Aphididae (Wool, 2005). Their typical life history is complicated,

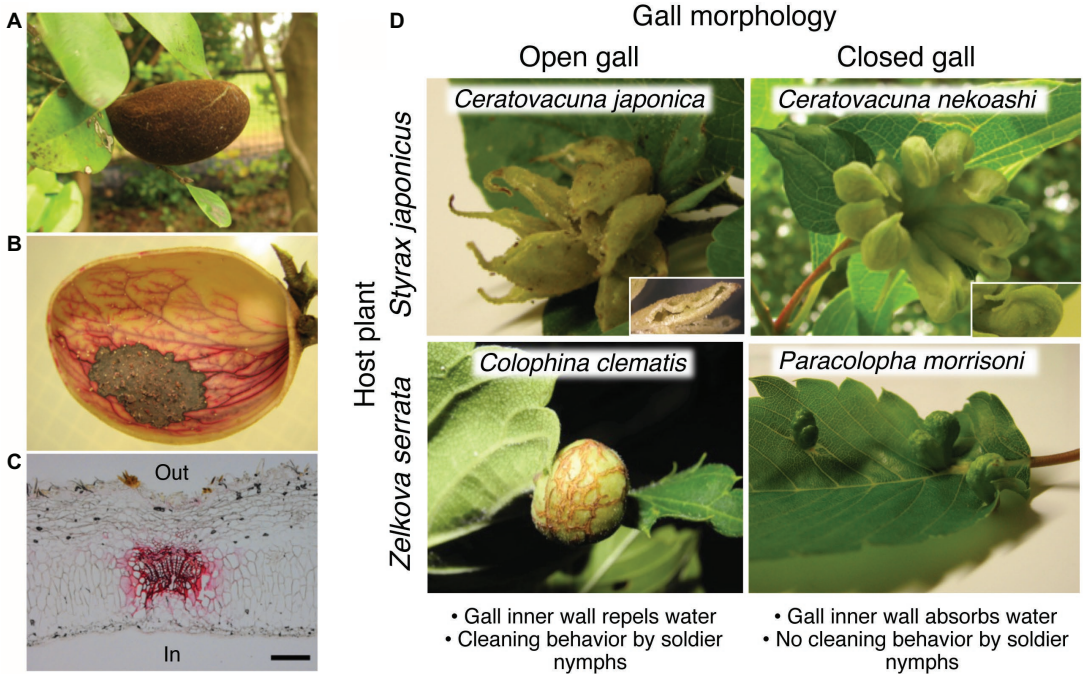

FIGURE 1 | Open and closed galls of various aphids and water-absorbing property. **(A–C)** Closed galls of *N. monzeni* showing water-absorbing property. **(A)** A gall of *N. monzeni* on *D. racemosum*. **(B)** Safranin staining of the gall tissue, showing transported routes of absorbed water in red. **(C)** A histological section of the safranin-stained gall, in which the vascular bundle is conspicuously stained in red. Scale bar, 0.2 mm. **(D)** Waste management strategies in open and closed galls. Galls of *C. japonica* and *C. nekoashi* on *S. japonicus*, and *C. clematis* and *P. morrisoni* on *Z. serrata* are shown. A subgall of *C. japonica* with a slit opening and a subgall of *C. nekoashi* with no opening are also shown. Figures were modified from Kutsukake et al. (2012) and Uematsu et al. (2018). In, inner side of the gall; Out, outer side of the gall.

where they have both sexual and parthenogenetic generations and alternate their host plants seasonally (Wool, 2005; Aoki and Kurosu, 2010). Briefly, a fundatrix appears from a fertilized egg in spring, induces a gall on the primary host plant, and produces offspring parthenogenetically in the gall, where the aphid colony experiences several parthenogenetic generations. Typically in early summer, winged adults appear and migrate to a different plant, namely the secondary host plant, where they also spend several generations parthenogenetically. Then, winged adults of a different type, called sexuparae, appear and return to the primary host plant to produce sexual females and males, where they mate and lay fertilized overwintering eggs that are to be fundatrices in next spring. Note that some hormaphidine species have multi-year life cycles, where they develop galls that last for over a year and thereby attain large colony sizes (Kurosu and Aoki, 2009; Aoki and Kurosu, 2010; Uematsu and Shibao, 2014). In addition to these morphs, many, if not all, gall-forming aphids are known to be social with altruistic morphs called "soldiers," which are typically first- or second-instar nymphs specialized for colony defense (Stern and Foster, 1996; Abbot and Chapman, 2017). Soldier nymphs of some species also perform labors for nest maintenance including gall cleaning and gall repair (Aoki, 1980; Aoki and Kurosu, 1989; Kurosu and Aoki, 1991; Benton and Foster, 1992; Kurosu et al., 2003; Shibao and Fukatsu, 2003; Pike and Foster, 2004; Kutsukake et al., 2009, 2019; Lawson et al., 2014). Considering that all social species form galls at some point in their life cycle, gall formation is considered as one of the important ecological factors that have promoted social evolution in aphids (Aoki, 1987; Foster and Northcott, 1994; Stern and Foster, 1996; Pike and Foster, 2008).

For animals, especially those living in a nest, waste disposal is an essential issue to sustain a long-term survival. Aphid galls contain hundreds to thousands of insects and often continue for several months, or in some species even for over a year. Aphids suck plant phloem sap continuously and excrete plenty of sugar-rich honeydew. Accumulated honeydew within the gall would be fatal for inhabiting aphids due to contamination or drowning. How do the gall-forming aphids deal with the liquid wastes and sustain long-term social life? This review describes our recent findings of novel and unexpected biological solutions to the waste problems in aphid galls, in which aphids manipulate plant morphogenesis and physiology for their own sake to keep their social life healthy and safe. We also discuss future directions on this research topic.

GALL CLEANING AND WAX PRODUCTION BY APHIDS IN OPEN GALLS

Gall morphology can be classified into two types, namely open galls and closed galls. The open galls possess opening(s) on the underside of the gall, so that aphids are able to dispose

colony wastes through the openings. Previous studies reported that soldier nymphs in the open galls perform cleaning behavior by pushing or rolling honeydew balls, cast-off skins, and cadavers out of the openings (Aoki, 1980; Aoki and Kurosu, 1989; Benton and Foster, 1992; Uematsu et al., 2018). Inhibition of the waste disposal by turning the gall orientation upside down (the openings to be upward) resulted in high mortality of the aphids inside, indicating that gall cleaning is indispensable for survival of the gall inhabitants (Benton and Foster, 1992). Besides, aphids produce large amounts of powdery wax, which coats the excreted honeydew to form unsticky "marbles" or "honeydew balls" (Pike et al., 2002; Kutsukake et al., 2012; Uematsu et al., 2018). The wax-coated honeydew balls are repelled by the gall inner wall so that the aphids can easily push them without being wet or contaminated.

WATER ABSORPTION AND HONEYDEW REMOVAL BY PLANT TISSUES IN CLOSED GALLS

Waste disposal is impossible for aphids that form closed galls without openings. For a long time, it had been an enigma why the aphids living in completely closed galls can survive for a long period until the galls mature and finally form an exit for emigration. The answer to the mystery was presented in our research on a social aphid, *Nipponaphis monzeni*, that forms completely closed galls on the tree *Distylium racemosum* (**Figure 1A**; Kutsukake et al., 2012). *N. monzeni* is known to form an extremely long-lasting gall (taking some 2.5 years to maturity) that contains a large number of insects (over 2,000 aphids in mature galls) (Kurosu and Aoki, 2009). Despite the large colony size, we found no honeydew droplets accumulating within the galls, but only some powdery wax and cast-off skins. The possibility that aphids excreted little honeydew was rejected because, when the aphids were reared on an artificial feeding system (Shibao et al., 2002), we observed honeydew excretion. These observations led us to a hypothesis that the honeydew may be somehow removed from the inner cavity of the closed galls. In an attempt to verify the hypothesis, we injected 1 ml of food dye solution into natural galls of *N. monzeni* in the field and subsequently cut the galls to inspect the gall contents. Strikingly, no dye solution remained in the inner cavity of the galls 1 day after injection. Safranin solution injected into the gall cavity clearly stained the vascular bundles in red, indicating that the solution was absorbed by the plant tissues and removed through the vascular system (**Figures 1B,C**; Kutsukake et al., 2012). We also investigated whether the galls of *N. monzeni* are able to absorb sucrose solution, because aphid honeydew contains a large amount of sucrose. As the sucrose concentrations were elevated, the absorption efficiencies reduced: almost 100% absorption in 15 of 16 galls for 0% sucrose water; over 90% absorption in 6 of 10 galls for 2% sucrose water; 35–90% absorption in 8 of 10 galls for 4% sucrose water; and less than 40% absorption in all 11 galls for 8% sucrose water. Interestingly, the honeydew excreted by *N. monzeni* exhibited a low sugar content (less than 0.5% glucose), suggesting the possibility that the aphids may control their physiology to produce low-sugar honeydew that is easier for absorption by the gall inner surface. Considering that *N. monzeni* aphids produce plenty of powdery wax from their dorsal wax plates, we suggest the possibility that, although speculative, the aphids consume much sugar for massive production of the excreted wax, which might be relevant to the low sugar content in the honeydew of *N. monzeni*. As for possible mechanisms of water absorption of the plant side, we initially suspected two mechanisms, the passive water transportation driven by water potential of the plant tissue, and the active water transportation through water channels like aquaporins. From our data, the latter mechanism was unlikely because mercury chloride solution, an aquaporin inhibitor, did not affect the water absorption efficiency (Kutsukake et al., 2012). Plausibly, the passive water transportation mechanism driven by osmotic pressure-related water potential is involved in the water absorption property of the closed galls.

EVOLUTION OF WATER-REPELLING/ ABSORPTION PROPERTIES IN APHID GALLS

Thus far, the water absorption property of closed galls was observed not only in *N. monzeni* but also in other aphid species (**Figure 2A**). In the subfamily Hormaphidinae, the water-absorbing closed galls were estimated to have evolved twice in the tribes Nipponaphidini and Cerataphidini independently. In the Nipponaphidini, *N. monzeni* and its allied species *Nipponaphis distyliicola* form water-absorbing closed galls (Kutsukake et al., 2012). Other allied nipponaphidine aphids also form closed galls in which no honeydew balls were detected, suggesting that their galls may absorb liquid wastes as well. In the Cerataphidini, *Ceratovacuna nekoashi* forms water-absorbing closed galls, whereas *Ceratovacuna japonica* and other Cerataphidini species form water-repelling open galls on *Styrax* trees (Kutsukake et al., 2012). In the subfamily Eriosomatinae, the water-absorbing closed galls have evolved at least once in the tribe Eriosomatini. *Paracolopha morrisoni* forms water-absorbing closed galls on *Zelkova serrata* leaves, whereas many other species, including *Colophina clematis*, form water-repelling open galls on the same *Zelkova* leaves (Uematsu et al., 2018). Taken together, water-absorbing property in the closed galls has evolved at least three times in the evolutionary history of the gall-forming aphids (**Figure 2A**).

MANIPULATION OF STRUCTURE AND HYDROPHOBICITY OF GALL INNER SURFACE BY APHIDS

Here we focus on two congenic aphid species, *C. japonica* that forms water-repelling open galls and *C. nekoashi* that forms water-absorbing closed galls on the same tree *Styrax japonicus* (**Figure 1D**). *C. nekoashi* and *C. japonica* are both social species, whose life cycle and gall shape are quite similar. Their

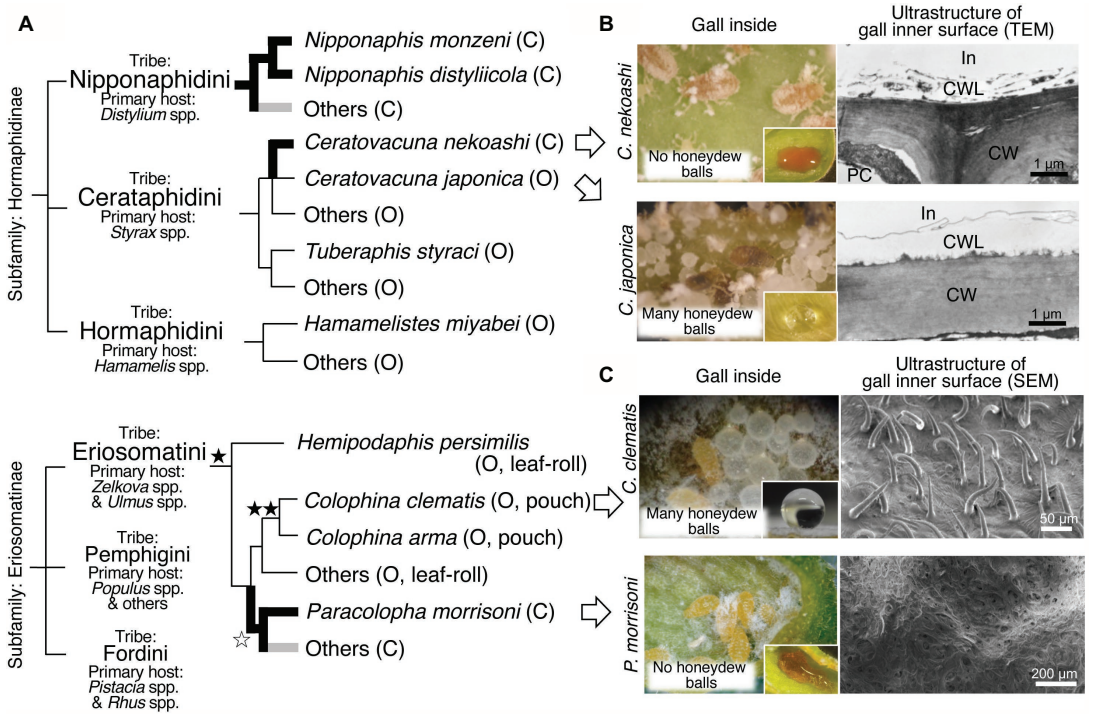

FIGURE 2 | Evolution and plant surface structures of water-absorbing closed galls and water-repelling open galls. **(A)** A schematic phylogeny of the gall-forming social aphids and the evolution of water-absorbing/repelling properties. The occurrences of the water-absorbing closed galls are indicated in bold branches. The water-absorbing closed galls reported in Kutsukake et al. (2012) and Uematsu et al. (2018) are indicated in black, whereas others (potential water-absorbing galls) are indicated in gray. The evolution of dense trichomes on gall inner surface is indicated by black stars. High-density trichomes are indicated by a single star and very high-density trichomes are indicated by double stars. A white star indicates loss of trichomes. Closed (C) or open (O) galls are indicated in brackets. The phylogenetic relationship of gall-forming social aphids is based on Sano and Akimoto (2011) and Kutsukake et al. (2012). **(B)** Gall inside views and transmission electron micrographs of inner wall surface in galls of *C. nekoashi* and *C. japonica*. Hydrophobicity of the gall inner surface on which was placed a drop of food dye solution or water is also shown. **(C)** Gall inside views and scanning electron micrographs of the inner wall surface of galls of *C. clematis* and *P. morrisoni*. Note that aphid-derived wax was removed during the fixation procedure before the observation using a scanning electron microscope. Figures were modified from Kutsukake et al. (2012) and Uematsu et al. (2018). C, closed gall; CW, cell wall; CWL, cuticle wax layer; In, inner side of the gall; O, open gall; PC, plant cell.

banana-bundle-shaped galls, that are transformed from axillary buds of a shoot by fundatrices, consist of approximately 10 subgalls with 50–100 insects per subgall (Kurosu and Aoki, 1988, 1990, 1994). Notably, however, *C. japonica* forms open galls wherein soldier nymphs actively clean wastes, whereas *C. nekoashi* forms closed galls wherein soldier nymphs do not clean (Kurosu and Aoki, 1988; Kurosu et al., 1990). Kutsukake et al. (2012) reported that no honeydew balls were found in the galls of *C. nekoashi* (**Figure 2B**), and food dye solution artificially introduced into the gall cavity was completely absorbed by the gall inner surface. The inner surface was hydrophilic on which the introduced dye solution rapidly spread (**Figure 2B**). By contrast, the gall inner surface of *C. japonica* was hydrophobic on which the introduced water was repelled and formed a sphere (**Figure 2B**). Ultrastructural observations revealed that the gall inner surface of *C. nekoashi* was covered with a reticular and spongy plant cuticle layer, whereas the gall inner surface of *C. japonica* was covered with a thick and dense cuticle layer (**Figure 2B**). These observations indicate that the cuticle wax structure determines the hydrophobicity and water-absorbing/repelling properties of the gall inner surface, which is determined by the galling aphids rather than by the host plant.

TRICHOME DEVELOPMENT AND HIGH WATER REPELLENCY IN SOME OPEN GALLS

Recently, another intriguing phenomenon on insect-induced plant surface structure was discovered in galls of the wooly aphid *Colophina clematis*. This aphid forms pouch-shaped open galls on leaves of *Z. serrata* (**Figure 1D**). Uematsu et al. (2018) found that the gall inner surface was covered with a number of trichomes (**Figure 2C**), whose density was about 30 times higher than that on the non-galling area of the same leaf. The gall inner surface was covered with not only dense trichomes, but also aphid-derived hydrophobic wax particulates. Water droplets placed on the inner surface were highly repelled with contact angles of around 150°, whereas the water droplets on the wax-removed inner surface (trichomes only) were less repelled with contact angles of around 130°. The water droplets placed on normal non-galling leaf areas (with neither trichomes nor aphid wax) were not repelled with contact angles of less than 90°. Thus, the hydrophobicity of the gall inner surface of *C. clematis* was remarkably enhanced by the co-existence of the trichomes and aphid-derived hydrophobic wax, by which

the aphids are able to clean the honeydew balls efficiently. Such microscale hierarchical structures on the organismal surface often contribute to water repellency of the surface, which is known as "lotus effect" observed on the surface of lotus leaves and other plant and animal surface (Barthlott and Neinhuis, 1997). Such massive trichomes were also found in pouch-shaped open galls of *Colophina arma*, an allied species of *C. clematis,* whereas no trichomes were detected in water-absorbing closed galls of *P. morrisoni* (**Figures 1D,C, 2A**). In leaf-rolling open galls of *Hemipodaphis persimilis*, the trichome densities were around a half of those in *C. clematis* galls (Uematsu et al., 2018). Thus, the trichomes developed in the open galls of the Eriosomatini species are regarded as another example of an extended phenotype of gall-forming social aphids.

CONCLUSION AND FUTURE DIRECTIONS

For waste management, gall-forming aphids employ either of the following strategies. In open galls, soldier nymphs dispose honeydew balls from gall openings, where the gall inner surface is hydrophobic, being covered with thick plant cuticle layer and sprinkled with aphid-derived powdery wax. In closed gall, liquid wastes are absorbed by hydrophilic gall inner surface, which is composed of plant-derived reticular and spongy layer, and removed through the vascular bundle system of the host plant. The two ecological traits, gall openness and waste removal strategies, seem to be tightly linked to each other among the gall-forming aphids, whereby colony defense and colony hygiene are harmoniously realized not only in open galls but also in closed galls. In a sense, water absorption in the closed galls can be regarded as "plant-mediated indirect social behavior" by the inducer aphids. While the water-absorbing closed galls have been identified in three gall-forming aphid lineages (**Figure 2A**), wider surveys of diverse gall-forming aphids (e.g., members of the tribes Pemphigini and Fordini) are needed to clarify the whole picture on this issue.

Another fascinating question on this research topic is molecular mechanisms underlying water-absorbing cuticle formation and water-repelling trichome development in the gall-forming aphids and the host plants. Upon gall formation, the fundatrix injects saliva into the plant tissue through the stylet (needle-like mouthpart). In this process, some bioactive molecules in the saliva may promote plant cell growth and cell division, hijack the plant developmental programs, and manipulate the plant morphologenesis and physiology for their own sake (Stone and Schönrogge, 2003; Raman et al., 2005). Such molecules may be effectors produced in the aphid salivary glands, although little is known about how insect effectors manipulate the plant morphogenesis and physiology to form the gall (Giron et al., 2016). In addition, phytohormones have been long believed to play a role in hypertrophy and hyperplausia of the plant cells in the gall tissues. Some studies detected high levels of phytohormones, such as auxin and cytokine, in the body or salivary glands of gall-forming insects, suggesting the involvement of phytohormones in the gall formation (Mapes and Davies, 2001; Tooker and De Moraes, 2011a,b; Yamaguchi et al., 2012). Hence, analyses of effectors and phytohormones in the salivary glands of the gall-forming aphids would be of great interest for further investigation. In addition, plant cuticle formation and trichome development have been well studied using the model plants, mainly *Arabidopsis thaliana* (Kunst and Samuels, 2009; Yeats and Rose, 2013; Pattanaik et al., 2014). In our study using non-model plants and insects, a candidate gene approach would be applicable, and the model plant researches will help unveil a molecular basis of the aphid-induced plant phenotypes described in this paper. Expectedly and hopefully, comparison between *C. japonica* vs. *C. nekoashi,* or *C. clematis* vs. *P. morrisoni*, which form open and closed galls on the same host plant, respectively, would unveil molecular components of the aphids and plants that are involved in the induction of water absorption/repellency of the galls.

AUTHOR CONTRIBUTIONS

MK wrote the manuscript. TF and KU critically revised the manuscript. All authors approved the final version of the manuscript.

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Plant Proteins and Processes Targeted by Parasitic Nematode Effectors

*Joffrey Mejias, Nhat My Truong†, Pierre Abad, Bruno Favery and Michaël Quentin**

Centre National de la Recherche Scientifique, Institut National de la Recherche Agronomique, Institut Sophia Agrobiotech, Université Côte d'Azur, Sophia Antipolis, France

Sedentary endoparasitic nematodes, such as root-knot nematodes (RKN; *Meloidogyne* spp.) and cyst nematodes (CN; *Heterodera* spp. and *Globodera* spp.) cause considerable damage to agricultural crops. RKN and CN spend most of their life cycle in plant roots, in which they induce the formation of multinucleate hypertrophied feeding cells, called "giant cells" and "syncytia," respectively. The giant cells result from nuclear divisions of vascular cells without cytokinesis. They are surrounded by small dividing cells and they form a new organ within the root known as a root knot or gall. CN infection leads to the fusion of several root cells into a unique syncytium. These dramatically modified host cells act as metabolic sinks from which the nematode withdraws nutrients throughout its life, and they are thus essential for nematode development. Both RKN and CN secrete effector proteins that are synthesized in the oesophageal glands and delivered to the appropriate cell in the host plant via a syringe-like stylet, triggering the ontogenesis of the feeding structures. Within the plant cell or in the apoplast, effectors associate with specific host proteins, enabling them to hijack important processes for cell morphogenesis and physiology or immunity. Here, we review recent findings on the identification and functional characterization of plant targets of RKN and CN effectors. A better understanding of the molecular determinants of these biotrophic relationships would enable us to improve the yields of crops infected with parasitic nematodes and to expand our comprehension of root development.

Keywords: root-knot nematodes, cyst nematodes, galls, syncytium, effectors

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Ghent University, Belgium
Valerie Moroz Williamson,
University of California, Davis, United States

***Correspondence:**
Michaël Quentin
michael.quentin@inra.fr

†Present address:
Nhat My Truong,
Faculty of Advanced Science and Technology, Kumamoto University, Kumamoto, Japan

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INTRODUCTION

Plant-parasitic nematodes (PPN) are microscopic worms that withdraw nutrients from the cytoplasm of cells in the aerial or below-ground parts of plants. Root-knot nematodes (RKN) and cyst nematodes (CN) are the most widely studied PPN, as these two groups are the most damaging to crop plants (Singh et al., 2013). RKN from the *Meloidogyne* genus are found throughout the world and are extremely polyphagous, infecting thousands of plant species, including both monocotyledons and eudicotyledons (Blok et al., 2008). By contrast, CN tend to specialize on a particular crop and form two common genera: *Globodera* spp. (potato CN) and *Heterodera* spp. (sugar beet, soybean, or cereal CN), each of which causes huge yield losses on its host.

Both CN and RKN are sedentary endoparasites and obligate biotrophs. Mobile preparasitic juveniles (J2) penetrate the host root, traveling toward the vascular cylinder, where they become

sedentary, triggering the formation of an unusual feeding site. The RKN feeding site consists of so-called "giant cells" (**Figure 1A**). These cells are produced from about half a dozen vascular root cells, which undergo repeated nuclear divisions without cell division. These cells become polynucleate and may be more than 300 times larger than normal cells. Giant cells are surrounded by dividing cells, the hyperplasia and hypertrophy of which lead to the formation of a novel organ called a gall (Kyndt et al., 2013; Favery et al., 2016; Palomares-Rius et al., 2017). By contrast, CN induce the formation of a different type of feeding site called a syncytium. Syncytium formation involves partial dissolution of the root cell wall and protoplast fusion, leading to an iterative process of fusion of the first CN-infested vascular cell with its neighbors (**Figure 1B**; Sobczak and Golinowski, 2011; Palomares-Rius et al., 2017). Some mature syncytia are the result of fusions of more than 200 cells. Giant cells and syncytia have a number of features in common, including a fully expanded endoplasmic reticulum, a fragmented vacuole, a reorganized cytoskeleton, thickened cell walls with local ingrowths, a large mitochondrial network and endoreduplicated nuclei (Kyndt et al., 2013; Rodiuc et al., 2014). These specialized feeding cells supply the nematodes with nutrients throughout the sedentary part of their life cycle. Female RKN lay their eggs in a gelatinous matrix generally on the root surface, whereas the cyst of CN consists of a dead and hardened female containing eggs.

Root-knot nematodes and CN secrete molecules called "effectors," to facilitate invasion of the host root, avoid plant defense responses and reprogram root cells to form specialized feeding cells. These effectors are produced principally in three oesophageal salivary glands and are then injected into plant cells via the syringe-like stylet. The activity of the oesophageal glands is developmentally regulated. The two subventral glands (SvG) secrete effectors allowing J2 penetration and migration in the root while proteins secreted during parasitism are produced by SvG and particularly by the dorsal gland (DG) (Nguyen et al., 2018). Some effectors may also be produced in other secretory organs, such as chemosensory amphids, or directly secreted through the PPN cuticle. Molecular dialog studies have focused mostly on secreted proteinaceous effectors (Hewezi and Baum, 2013; Mitchum et al., 2013; Quentin et al., 2013; Hewezi, 2015; Ali et al., 2017; Vieira and Gleason, 2019) even though other secreted molecules, such as phytohormones, have been shown to favor these interactions (Siddique and Grundler, 2018).

Various approaches have been used to characterize nematode effector repertoires. Proteomic analysis has directly identified about 500 proteins secreted by *M. incognita* preparasitic J2s or females (Bellafiore et al., 2008; Wang et al., 2012). Effector identification has greatly benefited from advances in sequencing technologies. Complete genome sequences are now available for four RKN – *M. incognita*, *M. hapla*, *M. javanica*, and *M. arenaria* (Abad et al., 2008; Opperman et al., 2008; Blanc-Mathieu et al., 2017) – and three CN: *G. pallida*, *G. rostochiensis*, and *H. glycines* (Cotton et al., 2014; Eves-van den Akker et al., 2016; Masonbrink et al., 2019). Bioinformatic methods for identifying genes encoding putative secreted proteins, which are based on the presence of a signal peptide for secretion but absence of transmembrane domains, remain the most convenient approach to identify candidate effector genes. *Cis*-regulatory sequences called "DOG boxes" were recently identified within the promoters of *G. rostochiensis* and *H. glycines* genes encoding effectors specifically expressed within the DG of the CN (Eves-van den Akker et al., 2016; Masonbrink et al., 2019). This discovery opens up new possibilities for effector prediction and implies that effector production in the DG is synchronized by master regulators, such as key transcription factors (Eves-van den Akker and Birch, 2016). Finally, transcriptomics has made it possible to compare different stages of nematode development and to identify RKN (Li et al., 2016; Petitot et al., 2016; Nguyen et al., 2018; Shukla et al., 2018) and CN (Cotton et al., 2014; Kumar et al., 2014; Yang et al., 2017; Gardner et al., 2018) genes upregulated in plants. *In situ* hybridisation (ISH) has generally been used for the initial validation of candidate effector gene expression within secretory organs (**Figure 1C** and **Table 1**). Remarkably, the secretion of a few effectors has been demonstrated *in planta*, by immunolocalisation (**Table 1**). Delivery to the host apoplast has been demonstrated for several effectors (Jaubert et al., 2005; Vieira et al., 2011; Iberkleid et al., 2013; Eves-van den Akker et al., 2014; Zhuo et al., 2019), but few demonstrations of translocation into the host feeding cell have been reported (Jaouannet et al., 2012; Lin et al., 2012; Chen et al., 2017; Lilley et al., 2018; Naalden et al., 2018). These studies have expanded the repertoire of putative effectors considerably, with hundreds of ISH-validated effectors now known (Truong et al., 2015; Gardner et al., 2016). However, the vast majority of these proteins are pioneer proteins with no known functional domains. As a result, the functions of only a few RKN and CN effectors have been deciphered. Cell wall-degrading effectors have been reported to help nematodes to penetrate and migrate within the root, and effectors suppressing plant defenses have been described (Quentin et al., 2013; Goverse and Smant, 2014), but only a few effectors have been shown to contribute to the *de novo* organogenesis and maintenance of feeding sites. Functional analyses of PPN effectors have clearly benefited from the identification of the host targets of these molecules, mostly through yeast two-hybrid approaches (**Table 1**). We review here the most recent advances in our understanding of RKN and CN effector functions, focusing on those for which the plant processes targeted have been identified.

PARASITISM REQUIRES THE MANIPULATION OF DIVERSE HOST FUNCTIONS

Nematode effectors target the apoplast and different subcellular compartments, including the nuclei, reflecting the diversity of host cell processes manipulated to promote infection and feeding site formation (**Table 1**). Many of the members of the PPN effector repertoire have been shown to suppress plant immunity (Goverse and Smant, 2014; Favery et al., 2016; Ali et al., 2017). However, their precise mode of action remains largely unknown and only a few of their direct targets in plants

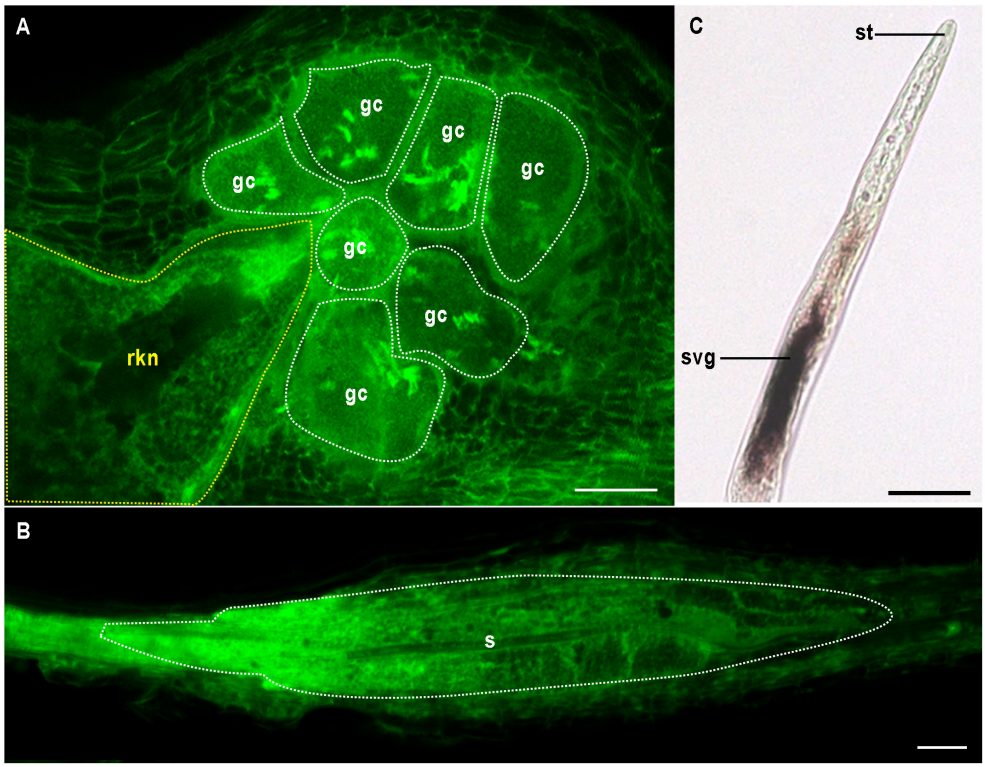

FIGURE 1 | Multinucleate and hypertrophied feeding cells induced by endoparasitic plant nematodes. **(A)** Giant cells (gc and white outline) induced by the root-knot nematode *Meloidogyne incognita* (rkn and yellow outline) in *Arabidopsis thaliana*. **(B)** Syncytium (s and white outline) formed by the cyst nematode *Heterodera schachtii* in *A. thaliana*. **(A,B)** confocal images were obtained by visualizing glutaraldehyde fixative auto-fluorescence after BABB clearing as described in Cabrera et al. (2018). **(C)** *In situ* hybridisation of the pioneer *M. incognita* effector gene *Minc16401* encoding a predicted peptide of 69 amino acids (Abad et al., 2008) was performed as described in Jaouannet et al. (2018). *Minc16401* expression was localized in subventral glands (svg), suggesting the effector could be secreted *in planta* via the stylet (st). Bars = 50 μm.

have been identified. PPN effectors may interact with host proteins to scavenge reactive oxygen species (ROS) accumulating during the oxidative burst following the induction of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). *M. javanica* MjTTL5 scavenges ROS by interacting with a thioredoxin reductase catalytic subunit (AtFTRc) in the plant (Lin et al., 2016). *H. schachtii* Hs10A06 has been shown to interact with a spermidine synthase (AtSPDS2), thereby enhancing spermidine production and inducing ROS-scavenging activity when the spermidine is oxidized by polyamine oxidase (Hewezi et al., 2010). Various pathogenesis-related (PR) proteins involved in the production of antimicrobial proteins by plants in response to pathogen attack have also been identified as direct targets of nematode effectors. *H. glycines* Hg30C02 targets a beta-1,3-endoglucanase (AtPR2), the inactivation of which in a mutant *Arabidopsis* line increases susceptibility to cyst nematode infection (Hamamouch et al., 2012). *M. graminicola* MgMO237 has been shown to suppress PTI by interacting with multiple host PR proteins, a 1,3-beta-glucan synthase (OsGSC), the cysteine-rich repeat secretory protein 55 (OsCRRSP55) and a pathogenesis-related Bet v I family protein (OsBetvI) (Chen et al., 2018). The GrVAP-1 effector from *G. rostochiensis* targets an apoplastic papain-like cysteine protein (PLCP) called RCR3^{Pim}, to subvert immunity. GrVAP-1 is also recognized by a plant immune receptor called Cf-2 that can trigger effector-triggered immunity (ETI) followed by a hypersensitive response (Lozano-Torres et al., 2012).

Like other classes of plant pathogens that have to overcome host defenses, PPNs produce effectors that converge on evolutionarily conserved host targets called "hubs" (Carella et al., 2018). The *M. incognita* "Passe-Muraille" peptide effector, for example, interacts with subunit 5 of the COP9 signalosome (CSN5) (Bournaud et al., 2018), a hub targeted by bacterial, fungal and viral effectors (Mukhtar et al., 2011; Weßling et al., 2014). The function of CSN5 in RKN parasitism remains unknown, but this target protein is known to be involved in plant salicylic acid-mediated defense (Kazan and Lyons, 2014). Similarly, the *H. schachtii* Hs25A01 effector interacts with eIF-2bs, a member of the eIFs family of translation initiation factors including known host targets of fungi, bacteria and viruses, and a role for this target in parasitism was demonstrated by the observation of changes in susceptibility to nematodes in eIF-2bs knockout mutants (Pogorelko et al., 2016). A third striking example is provided by PLCPs, which constitute key hubs in plant immunity (Misas-Villamil et al., 2016). PLCPs are targeted by *M. chitwoodi* Mc01194 (Davies et al., 2015) and

TABLE 1 | Nematode effectors and their identified plant targets.

Nematode effectors			Plant targets					References
Name	**Annotation**	***In planta* localisation[a]**	**Plant species, annotation, and name**	**Y2H[c]**	**Co-IP or other[c]**	**BiFC[c]**	**Evidence for a role in parasitism**	
Root-knot nematodes, *Meloidogyne incognita* (*Mi*), *M. javanica* (*Mj*), *M. graminicola* (*Mg*), *M. chitwoodi* (*Mc*)								
Mi8D05	Unknown	nd	Tomato aquaporin tonoplast intrinsic protein 2 (SlTIP2)	✓			nd	Xue et al., 2013
Mi16D10	CLE-like peptide	nd	Tomato and Arabidopsis SCARECROW-like transcription factors (SCL)	✓	✓		nd	Huang et al., 2003, 2006, 2007; Yang et al., 2013
MiPFN3	Profilin	Actin filaments (TE)	Actin monomers		✓		nd	Leelarasamee et al., 2018
MiPM	Passe-Muraille	Nucleus (TE)	Soybean fifth subunit of the COP9 signalosome (GmCSN5)		✓		nd	Bournaud et al., 2018
MiMIF-2	Macrophage migration inhibitory Factor	Cytoplasmic (IL)	Arabidopsis annexin 1 and 4 (AnnAt1 and AnnAt4)		✓	✓	*Ann1At1* or *AnnAt4* At OE less susceptible to *Mi*, At KO more susceptible to *Mi*	Zhao et al., 2019
MjTTL5	Transthyretin-like protein	nd	Arabidopsis ferredoxin-thioredoxin reductase catalytic subunit (AtFTRc)	✓	✓		nd	Lin et al., 2016
MgMO237	Unknown	Cytoplasmic and nuclear (TE)	Rice 1,3-beta-glucan synthase (OsGSC), cysteine–rich repeat secretory protein 55 (OsCRRSP55), pathogenesis–related Bet v I family protein (OsBetvI)	✓	✓		nd	Chen et al., 2018
Mg16820	Unknown	Cytoplasmic and nuclear (TE)	Rice dehydration-stress inducible protein 1 (OsDIP1)	✓		✓	nd	Naalden et al., 2018
MC01194	Unknown	nd	Arabidopsis papain-like cysteine protease responsive to dehydration (RD21A)	✓		✓	*rd21-1 At* KO more susceptible to *Mc*	Davies et al., 2015
Cyst Nematodes, *Heterodera glycines* (Hg), *H. schachtii* (Hs) and *H. avenae* (Ha)								
HgCLE1 HgCLE2	CLE-like peptide	Syncytia cytoplasm (IL)	Soybean CLAVATA (CLV1A, CLV2A, and CLV2B), LRR-RLP proteins (RPK2A and RPK2B)		✓		*CLV2*, *CLV2-RPK2* and *CLV2-CLV1-RPK2* soybean RNAi lines less susceptible *to Hg*	Wang et al., 2010; Guo et al., 2015, 2017
Hg30C02	Unknown	nd	Arabidopsis beta-1,3-endoglucanase (AtPR2)	✓		✓	*AtPR2 At* OE lines less susceptible to Hs, *At* KO more susceptible to *Hs*	Hamamouch et al., 2012
HgSLP1	SNARE-Like Protein	nd	Soybean resistance protein α-SNAP (RHG1)		✓		Soybean resistance protein	Bekal et al., 2015
HgGLAND4	Gr-3E10-like	Nucleus (TE)	Arabidopsis DNA[b]: repressor of two lipid transfer protein (LTP) gene expression	✓			*LTP At* OE lines less susceptible to *Hs* and *Pst*	Noon et al., 2015; Barnes et al., 2018
HsCLE2	CLE-like peptide	nd	Soybean CLAVATA (CLV1A, CLV2A, and CLV2B), LRR-RLP proteins (RPK2A and RPK2B)		✓		*clv1*, *clv2* and *rpk2* At KO less susceptible to *Hs*	Replogle et al., 2011; Guo et al., 2015
Hs10A06	Unknown	Cytoplasmic (TE)	Arabidopsis spermidine synthase 2 (AtSPDS2)	✓		✓	*SPDS2 At* OE lines more susceptible to *Hs*, KO lines susceptibility to *Hs* unchanged	Hewezi et al., 2010

(Continued)

TABLE 1 | Continued

Nematode effectors			Plant targets				References
Name	**Annotation**	***In planta* localisation[(a)]**	**Plant species, annotation, and name**	**Y2H[(c)]**	**Co-IP or other[(c)]**	**BiFC[(c)]**	**Evidence for a role in parasitism**
Hs10A07	Unknown	Cytoplasmic and nuclear (TE)	Arabidopsis plant kinase (IPK) and transcription factor (IAA16)	✓		✓	*IPK or IAA16 At* OE lines more susceptible to *Hs, At* KO lines are less susceptible to *Hs*	Hewezi et al., 2015
Hs19C07	Unknown	Cytoplasmic (TE)	Arabidopsis auxin influx transporter (LAX3)	✓		✓	*AUX/LAX* double and quadruple mutants less susceptible to *Hs*	Lee et al., 2011
Hs25A10	Unknown	Cytoplasmic (TE)	Arabidopsis F-box containing protein (AtAUF1), chalcone synthase (AtCHS), translation initiation factor (AteIF-2bs)	✓	✓	✓	*eif-2bs At* KO more susceptible to *Hs, chs* and *auf1* KO susceptibility to *Hs* unchanged	Pogorelko et al., 2016
Hs30D08	Unknown	Nucleus (TE)	Arabidopsis suppressor of mec-8 and unc-52 spliceosomal protein homolog 2 (SMU2)	✓	✓	✓	*smu2-1 At* KO are less susceptible to *Hs*	Verma et al., 2018
Hs32E03	Unknown	Nucleus (TE)	Arabidopsis histone deacetylase (HDT1) and histone Chaperone (FKBP53)	✓	✓		*hdt1* KO susceptibility to *Hs* unchanged	Vijayapalani et al., 2018
HsCBP	Cellulose-binding protein	Cytoplasmic (TE)	Arabidopsis pectin methylesterase (PME3)	✓		✓	*PME3 At* OE lines more susceptible to *Hs, At* KO less susceptible to *Hs*	Hewezi et al., 2008
Hs4E02	Unknown	Nucleus (TE)	Arabidopsis papain-like cysteine protease responsive to dehydration (RD21A)	✓	✓	✓	nd	Pogorelko et al., 2019
Hs4F01	Annexin-like	nd	Arabidopsis 2OG-Fe (II) oxygenase/oxidoreductase family (JOX2)	✓			nd	Patel et al., 2010
HaVAP2	Venom allergen-like protein	Nucleus (TE)	Barley CYPRO4-like protein (HvCLP)	✓		✓	nd	Luo et al., 2019
HaGLAND5	Unknown	cytoplasm and nucleoplasm (TE)	Arabidopsis PYRUVATE DEHYDROGENASE SUBUNIt (AtEMB3003)	✓		✓	nd	Yang et al., 2019
Cyst Nematodes, *Globodera pallida* (Gp) and *G. rostochiensis* (Gr)								
GpRBP1.	SPRYSEC	Cytoplasmic (TE)	Potato CC-NB-LRR resistance protein (GPA-2)			✓	Potato resistance protein	Jones et al., 2009; Sacco et al., 2009
GpSPRY414-2	SPRYSEC	Cytoplasm and nucleoplasm (TE)	Potato cytoplasmic linker protein (CLIP)-associated protein (StCLASP)	✓		✓	*At clasp1* KO susceptibility to *Hs* unchanged	Mei et al., 2018
GrVAP1	Venom allergen-like protein	nd	Tomato apoplastic papain-like cystein protease (SlRCR3)	✓			*rcr3-3 Sl* mutant susceptibility to *Gr* unchanged; *RCR3* necessary for *Cf-2*-mediated resistance	Lozano-Torres et al., 2012
GrSPRYSEC-19	SPRYSEC	nd	Tomato CC-NB-LRR resistance protein (SWF-5)			✓	Tomato resistance protein	Rehman et al., 2009; Postma et al., 2012
GrCLE1	CLE-like peptide	nd	Arabidopsis and potato CLAVATA2 (CLV2), Arabidopsis receptor-like kinase LRR-RLKs (BAM1 and BAM2)		✓		*StCLV2* RNAi lines less susceptible to *Gr*	Lu et al., 2009; Guo et al., 2011; Chen et al., 2014

[(a)]Effectors expressed in subventral glands (in blue), in dorsal glands (in green), and in hypodermis (in yellow); nd, not determined; IL, immunolocalisation; TE, transient expression in Nicotiana benthamiana leaves or Arabidopsis protoplasts. [(b)]DNA Binding effector. [(c)]Approaches used for target identification and validation (Y2H, yeast two hybrid; Co-IP, co-immunoprecipitation; pull-down or in vitro binding assay; BiFC, Bimolecular Fluorescence complementation or Luciferase Complementation). At, Arabidopsis thaliana; Pst, Pseudomonas subtilis DC3000; Mc, Meloidogyne chitwoodi; Sl, Solanum lycopersicum; OE lines, overexpressing lines; KO, T-DNA knockout line.

CN *G. rostochiensis* GrVAP-1 and *H. schachtii* Hs4E02 (Lozano-Torres et al., 2012; Pogorelko et al., 2019) effectors in diverse host plants. Mc01194 and Hs4E02 target the same *Arabidopsis* PLCP, "Responsive to Dehydration 21A" (RD21A), to promote parasitism (Davies et al., 2015; Pogorelko et al., 2019). The expression of *G. rostochiensis* VAP1, which targets $RCR3^{Pim}$ in tomato, promotes susceptibility to *G. rostochiensis* and to the leaf mold *Cladosporium fulvum* (Lozano-Torres et al., 2012). It seems likely that other such molecular hubs are targeted by nematode effectors.

Other host functions targeted by RKN and CN effectors may be more related to the *de novo* formation and functioning of the specialized feeding site. The formation of feeding cells induced by RKN and CN requires a major reorganization of cytoskeletal networks (de Almeida Engler and Favery, 2011). RKN have been reported to secrete cytoskeleton components, such as actin or tubulin (Bellafiore et al., 2008), or associated proteins. A *M. incognita* profilin-like effector, MiPFN3, was recently shown to bind actin, altering its filament structure to favor parasitism (Leelarasamee et al., 2018). The *G. pallida* GpSPRY-414-2 effector, a CN-specific secreted SPRY domain-containing protein (SPRYSEC), has been shown to bind a potato microtubule-associated protein, CLASP (for cytoplasmic linker protein-associated protein) (Mei et al., 2018). CLASP proteins are involved in both cell division and cell expansion (Ambrose et al., 2007). GpSRY-414-2 therefore probably modulates the microtubule network in syncytia. New specific screens should identify new effectors targeting this key process for cell morphogenesis and pathogen response.

Finally, several RKN and CN effectors have been characterized that mimic and/or interfere with plant hormone peptide pathways (recently reviewed by Gheysen and Mitchum, 2019). Several CN effectors resemble the CLAVATA3 (CLV3)/ESR (CLE) hormones involved in controlling cell proliferation and differentiation. *In vitro* binding assays have confirmed the interaction of these CLE-like effectors with known receptors of plant CLE-peptides, such as CLAVATA2 (CLV2). The secretion of such hormone-mimicking peptides enables PPN to modulate root cell hormonal balance to promote feeding site formation. Additional effectors, such as the *H. schachtii* Hs19C07 and Hs10A07, have been shown to modulate auxin signaling, by interacting with the auxin transporter LAX3 (Lee et al., 2011), and by affecting the expression of auxin-responsive factors (ARFs) (Hewezi et al., 2015; see below), respectively, to facilitate feeding site formation.

HOST CELL REPROGRAMMING THROUGH THE MODULATION OF GENE EXPRESSION

The morphological, structural and metabolic changes associated with the ontogenesis of nematode feeding cells require the extensive reprogramming of plant gene expression (Szakasits et al., 2009; Favery et al., 2016). Gene expression is regulated principally in the nucleus, and several effectors are thought to target the nuclei of the cells destined to become feeding cells, as they have predicted plant-like nuclear or nucleolar localisation signals, and some have been detected in the nucleus following ectopic expression in *Nicotiana benthamiana* leaves. Nuclear translocation in host cells has been demonstrated by immunolocalisation for only three RKN effectors: the *M. incognita* MiEFF1 (Jaouannet et al., 2012) and the *M. javanica* MjNULG1a (Lin et al., 2012) of unknown functions, and the *M. graminicola* MgGPP involved in plant defense suppression (Chen et al., 2017). All three were localized to giant cell nuclei. However, the targets of these effectors have yet to be characterized. Interestingly, some RKN and CN effectors have been shown to target key regulatory processes, including the epigenetic modification of histones, transcriptional regulation and mRNA splicing.

The Hs32E03 effector of *H. schachtii* alters the acetylation of histones by interacting with the *Arabidopsis* histone deacetylase (HDAC) HDT1 and FK506 binding protein, FKBP53 (Vijayapalani et al., 2018) in the nucleus. HDT1 and FKBP53 repress the transcription of rRNA genes, with HDT1 deacetylating histone H3 at Lys-9. Hs32E03 has been shown to inhibit HDAC, and an assessment of histone modifications in Hs32E03-expressing *Arabidopsis* lines based on chromatin immunoprecipitation revealed that these lines had abnormally high levels of acetylation in rDNA regions. As expected, rRNA levels were high in the line showing a low expression of Hs32E03 and displaying higher levels of CN infection. Interestingly, lower levels of rRNA were detected in the line highly expressing Hs32E03, due to the hypermethylation of rDNA promoters, resulting in an inhibition of nematode development. These findings highlight the importance of rRNA levels for syncytium formation, as protein overproduction is required, which in turn necessitates the synthesis of additional ribosomes. Hs32E03 is the first nematode effector for which a role has been reported in the epigenetic regulation of plant gene expression to promote parasitism.

Several other nuclear effectors have been shown to target transcription factors directly. The *M. incognita* Mi16D10 effector, which has a C-terminal CLE-like domain, interacts with SCARECROW-like transcription factors from both tomato and *Arabidopsis* (Huang et al., 2006). SCARECROW transcription factors are involved in root radial patterning, particularly in endoderm differentiation, and they act in concert with a short root transcription (SHR) factor (Hirsch and Oldroyd, 2009). Plants overexpressing Mi16D10 have larger root systems, implicating this effector in the modulation of root development. Another example is provided by the *H. schachtii* effector Hs10A07, which is secreted into the cytoplasm and then phosphorylated by an *Arabidopsis* kinase. This phosphorylation leads to its translocation into the nucleus, where it interacts with a second protein, IAA16, an Aux/IAA transcription factor, to modulate ARF expression (Hewezi et al., 2015).

Other effectors may modulate gene transcription directly by binding to DNA. Examples include *H. glycines* HgGLAND4 (Barnes et al., 2018) and the *M. incognita* 7H08 effector (Zhang et al., 2015). HgGLAND4 has been shown to bind specifically to the promoters of LTP genes implicated in plant defense, suppressing their expression (Barnes et al., 2018). Mi7H08

has been shown to be imported into the nucleus, and to activate the transcription of a reporter gene *in planta*, but the host genes regulated by this effector have yet to be identified (Zhang et al., 2015).

Finally, a *H. schachtii* effector, Hs30D08, has been shown to interfere with mRNA splicing, thereby altering gene expression in feeding sites (Verma et al., 2018). RNA splicing is required to remove introns from pre-mRNA and to join the protein-coding sequences (exons) together during the translation of mRNA into protein. Alternative splicing (AS) may occur, and this represents another way of regulating gene expression and increasing protein diversity. In *Arabidopsis*, 70% of genes may be alternatively spliced, and AS has been shown to play a significant role in plant development, and in responses to abiotic and biotic stresses (Reddy et al., 2013; Yang et al., 2014). Hs30D08 has been shown to interact with an actor of the spliceosome machinery, the auxiliary spliceosomal protein SMU2, in *Arabidopsis* (Verma et al., 2018). Transcriptomic analyses of *Arabidopsis* lines expressing the Hs30D08 confirmed its function in modulating AS and gene expression. Future investigations will shed light on the role of splicing and AS in feeding cell formation and plant responses to CN and RKN.

CONCLUSION AND PERSPECTIVES

The repertoire of putative RKN and CN effectors is extremely large, and proteinaceous effectors have been shown to target diverse compartments, manipulating many host plant functions to orchestrate the suppression of plant defenses, the formation of feeding sites and the promotion of nematode survival and reproduction. Moreover, the arsenal of plant pathogens is not restricted to proteinaceous effectors. They also secrete other molecules, such as secondary metabolites, glycolipids, hormones analogs, or small RNAs, to alter plant functions (Weiberg et al., 2013; Manosalva et al., 2015; Collemare et al., 2019). However, few data are available concerning the functions of effectors and the plant processes they target. The elucidation of effector function and the identification of host targets during parasitism thus remain major challenges. The large-scale identification of effector targets, particularly in crops, would be an important breakthrough potentially leading to the discovery of new processes involved in plant-nematode dialog. Comparison of RKN- and CN-targets will shed light on processes involved in their specific parasitic strategies and host ranges.

Functional analyses of effector targets may lead to the identification of susceptibility genes with potential for use in resistance breeding (De Almeida Engler et al., 2005; van Schie and Takken, 2014). In addition, "hubs," susceptibility factors frequently targeted by different pathogens, may constitute ideal candidates for the design of broad-host range resistance in plants. However, these susceptibility genes are often crucial for plant physiology and development. Interfering with host protein recognition by pathogen effectors may be an interesting way of preserving important plant functions whilst breaking the susceptibility of the plant to pathogens. The breeding of new crops harboring point mutations that are less susceptible to diseases may be achieved with new technologies, such as the TILLING and CRISPR/Cas9 technologies, which are increasingly widely used (Engelhardt et al., 2018; Zaidi et al., 2018). Improvements in our understanding of effector/target functions are required if we are to block plant–microbe compatible interactions and engineer durable disease resistance.

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Haustorium Inducing Factors for Parasitic Orobanchaceae

Vincent Goyet[1†], Syogo Wada[2†], Songkui Cui[3], Takanori Wakatake[4], Ken Shirasu[4,5], Gregory Montiel[1], Philippe Simier[1] and Satoko Yoshida[2,3]*

[1] Laboratoire de Biologie et Pathologie Végétales, Université de Nantes, Nantes, France, [2] Division of Biological Science, Graduate School of Science and Technology, Nara Institute of Science and Technology, Ikoma, Nara, Japan, [3] Institute for Research Initiatives, Division for Research Strategy, Nara Institute of Science and Technology, Ikoma, Nara, Japan, [4] RIKEN Center for Sustainable Resource Science, Yokohama, Japan, [5] Graduate School of Science, The University of Tokyo, Tokyo, Japan

Parasitic plants in the Orobanchaceae family include devastating weed species, such as *Striga*, *Orobanche*, and *Phelipanche*, which infest important crops and cause economic losses of over a billion US dollars worldwide, yet the molecular and cellular processes responsible for such parasitic relationships remain largely unknown. Parasitic species of the Orobanchaceae family form specialized invasion organs called haustoria on their roots to enable the invasion of host root tissues. The process of forming haustoria can be divided into two steps, prehaustorium formation and haustorium maturation, the processes occurring before and after host attachment, respectively. Prehaustorium formation is provoked by host-derived signal molecules, collectively called haustorium-inducing factors (HIFs). Cell wall-related quinones and phenolics have been known for a long time to induce haustoria in many Orobanchaceae species. Although such phenolics are widely produced in plants, structural specificities exist among these molecules that modulate their competency to induce haustoria in different parasitic plant species. In addition, the plant hormone cytokinins, structurally distinct from phenolic compounds, also trigger prehaustorium formation in Orobanchaceae. Recent findings demonstrate their involvement as rhizopsheric HIFs for *Orobanche* and *Phelipanche* species and thus address new activities for cytokinins in haustorium formation in Orobanchaceae, as well as in rhizospheric signaling. This review highlights haustorium-inducing signals in the Orobanchaceae family in the context of their host origin, action mechanisms, and species specificity.

Keywords: haustorium, haustorium-inducing factor, Orobanchaceae, parasitic plants, Striga, quinone, cytokinin, lignin

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****Correspondence:***
Satoko Yoshida
Satokoy@bs.naist.jp

†These authors have contributed equally to this work.

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INTRODUCTION

Limitations in soil fertility have influenced the diversification of nutrient acquisition strategies in plants (Zemunik et al., 2015). Parasitic plants develop haustoria (singular haustorium), specialized organs for nutrient acquisition from host plants (Yoshida et al., 2016). Although the term haustoria is commonly used in biotrophic plant-pathogenic fungi or oomycetes and both fungi and parasitic plant haustoria have functions for nutrient withdrawing, the morphology and organization of haustoria in these organisms are quite different. The fungal haustoria represent unicellular

hyphae surrounded by host-derived extrahaustorial membrane, whereas haustoria of parasitic plants are multicellular organs that consist of different cell types and invade host tissues intercellularly (Yoshida et al., 2016). Among angiosperms, there are approximately 4,500 species of parasitic plants that have the ability to form haustoria and connect their vasculatures with those of their hosts to obtain water and nutrition. Parasitic plants have evolved independently at least 12 times and can be classified into two groups, root parasites and stem parasites, depending on which host tissue is parasitized (Westwood et al., 2010). The Orobanchaceae family contains the largest number of parasitic plants and consists of root parasites with various degrees of host dependency and photosynthetic activity, i.e., facultative parasites, obligate hemiparasites, and obligate holoparasites. Facultative parasites include species that have the ability for an autotrophic lifestyle and opportunistically parasitize host plants. Obligate parasites include species that cannot complete their life cycles without host plants; those who have photosynthetic activity are called hemiparasites, and those who have lost photosynthetic ability are called holoparasites (Westwood et al., 2010). Some obligate parasitic plants in the Orobanchaceae family, especially in the genera of *Striga*, *Orobanche*, and *Phelipanche*, parasitize crop species and cause devastating yield losses. *Striga* species infect maize, sorghum, and upland rice especially in sub-Saharan Africa with the economic damage caused by *Striga* estimated to be about 1 billion US dollars per year (Spallek et al., 2013). *Orobanche and Phelipanche* species infect economically important crops including pea, faba bean, sunflower, and oilseed rape (Fernández-Aparicio et al., 2016a). Germination of these obligate parasites depends on stimulating compounds including strigolactones and strigolactone-like compounds exuded by the roots of host plants (Yoneyama et al., 2010). Signaling components involved in strigolactone perception in Orobanchaceae are found to be associated with their germination (Lechat et al., 2015; Brun et al., 2018; Brun et al., 2019). In comparison, the molecular basis underlying haustorium formation remains mostly unknown. Nevertheless, recent studies have identified several haustorium-inducing chemicals for various Orobanchaceae members and have characterized their structural commonality and species specificity. This review will focus on the early developmental processes for haustorium formation in the Orobanchaceae family.

HAUSTORIUM DEVELOPMENTAL PROCESSES

According to the parasite species, two kinds of haustoria can be observed in the Orobanchaceae family: one is a "terminal haustorium" that develops on the radicle tip, and the other is a "lateral haustorium" that develops as a lateral extension of primary and lateral roots of parasitic plants (Joel, 2013; Yoshida et al., 2016). Most facultative parasites in the Orobanchaceae form lateral haustoria, while terminal haustoria are characteristic of several obligate holo- and hemiparasite clades, such as *Orobanche*, *Phelipanche*, *Striga*, and *Alectra* (Joel, 2013). Despite the structural variation among haustoria, haustorial developmental processes seem largely conserved among species.

Haustorium formation processes can be divided into two phases: prehaustorium formation induced by haustorium-inducing factors (HIFs) and haustorium maturation upon host infection. Except for some species that can form self-haustoria (Xiang et al., 2018), the initial step of haustorium formation is provoked by host-derived small compounds, collectively called HIFs. Several hours after recognition of HIFs, parasite roots or radicles start to show morphological changes including cell expansion, cell division, and differentiation of haustorial hairs in hemiparasites or papillae in holoparasitic genera *Orobanche* and *Phelipanche* spp., and semi-spherically shaped prehaustorial organs are formed within a few days (Riopel and Musselman, 1979; Baird and Riopel, 1985; Cui et al., 2016; Ishida et al., 2016; Goyet et al., 2017). The resultant organ is defined as "prehaustorium" or an "early haustorial structure" due to its premature architecture. Haustorium maturation occurs only after host attachment and is not solely promoted by HIFs, indicating the requirement of additional host signal(s) other than HIFs for structural maturation. Once the haustorium reaches host tissues, the epidermal cells of the parasite haustorium apex differentiate into intrusive cells, the specialized cells for host invasion with characteristic elongated shapes (**Figure 1**) (Stephens, 1912; Olivier et al., 1991). Intrusive cells grow inside host tissues toward host vasculatures. Upon reaching the host vasculature, some of the adjacent parasite cells differentiate into tracheary elements and form a xylem connection between the host and the parasite (Dorr, 1997), a structure called xylem bridge. Cell lineage analysis using the facultative parasite *Phtheirospermum japonicum* revealed dynamic cell fate transitions during haustorium formation (Wakatake et al., 2018). Intrusive cells originate from epidermal cells, and the central part of the haustorium has small cells with procambium-like cell identity as indicated by marker gene expression (Wakatake et al., 2018). Notably, development of such internal structures is only observed concomitant with host interaction; some incompatible hosts can interfere with the formation of intrusive cells and the subsequent xylem bridge (**Figure 1**) (Olivier et al., 1991; Yoshida and Shirasu, 2009), rendering these cells minimal prerequisites for establishing parasitism. Transcriptome analysis of *P. japonicum* revealed that *YUCCA3*, a gene encoding a key enzyme for auxin biosynthesis, is induced by HIF treatment in the epidermal cell layer of the haustorium-forming site (Ishida et al., 2016). Artificial induction of *YUCCA3* expression within epidermal cells of *P. japonicum* roots can induce haustorium-like semi-spherical structures with haustorial hair proliferation (Ishida et al., 2016), indicating the involvement of auxin biosynthesis in organogenesis of the prehaustorium.

HIFS FOR HEMIPARASITES

Induction of prehaustoria by host-derived HIFs is an initial step for haustorium formation. Isolation of HIFs has been accomplished mainly using photosynthetic hemiparasitic species whose prehaustoria are easy to observe due to their characteristic shape and their proliferation of haustorial hairs. HIF activities have been recognized in plant exudates since the

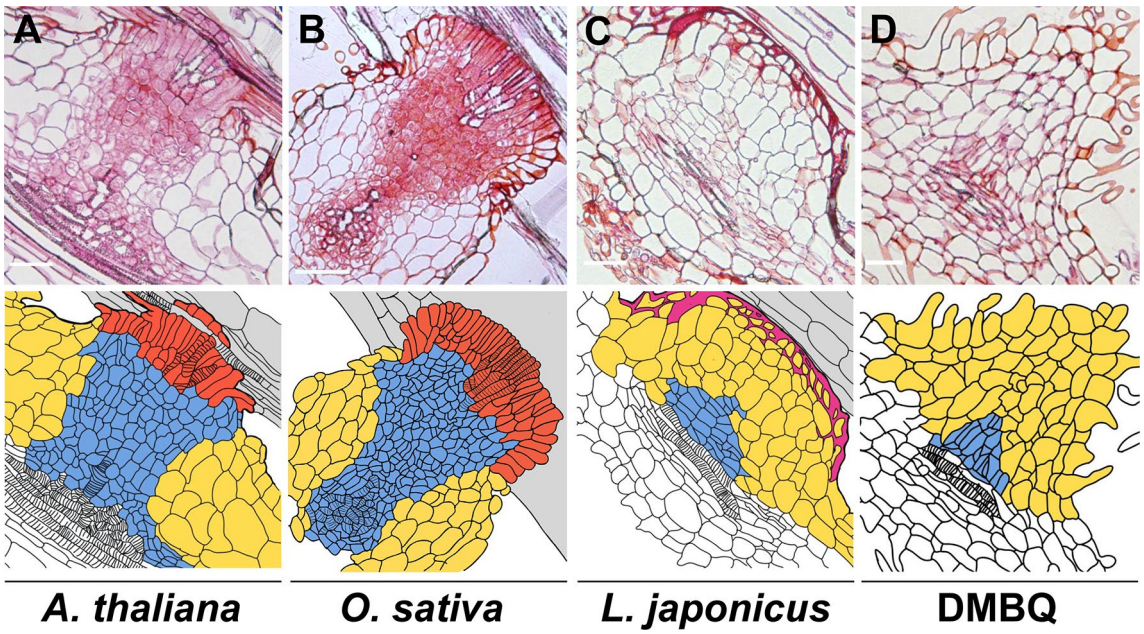

FIGURE 1 | Haustorial cell structures formed upon infection of various plants or induced by DMBQ. The upper photos show the longitudinal sections of *P. japonicum* haustoria after infection of susceptible plants (*Arabidopsis thaliana* **(A)** and *Oryza sativa* **(B)**) and a resistant plant (*Lotus japonicus* **(C)**) or induction by DMBQ treatment **(D)**. The lower drawings show the cell outlines of the upper sections. The colors denote cell types. Red, blue, yellow, purple, and gray indicate intrusive cells, small (procambium-like) cells, large cells, lignin accumulation, and host root cells, respectively. Stripes mark the location of xylem cells. Bars = 100 μm.

1970s from studies showing that root exudates of numerous plant species were able to induce prehaustoria in the facultative parasites *Agalinis purpurae* and *Castilleja exserta* (previously known as *Orthocarpus purpurascensi*) (Atsatt et al., 1978; Riopel and Musselman, 1979). Several small molecules were afterwards reported to be HIFs (**Figure 2**). Two flavonoids, named xenognosin A and B, were isolated from gum tragacanth, an exudate of *Astragalus* spp., as HIFs for *Agalinis* (Lynn et al., 1981; Steffens et al., 1982). Further attempts to identify HIFs yielded a terpenoid soyasapogenol B from root exudates of *Lespedeza sericea* (Fabaceae), a host of *Agalinis* (Steffens et al., 1983); however, the activity was not sufficiently high to account for the entire host exudate activity (Lynn, 1985; Steffens et al., 1986). A few years later, a quinone 2,6-dimethoxy-1,4-benzoquinone (DMBQ) was isolated from root extracts of sorghum, a natural host for *Striga*, and found to be a HIF for *Striga asiatica* and *Agalinis* (Chang and Lynn, 1986). As DMBQ could not be detected from a healthy root exudate in that report, it has remained questionable whether DMBQ is a naturally occurring HIF. Nevertheless, DMBQ has the highest activity and widest parasite range among currently known HIFs. For example, DMBQ is able to induce haustoria in the obligate hemiparasite *Striga* spp., as well as in the facultative parasites *P. japonicum, Triphysaria* spp., and *Agalinis* spp. (Chang and Lynn, 1986; Ishida et al., 2011; Tomilov et al., 2004). Compounds with structures similar to DMBQ, such as phenolic acids (including syringic acid, vanillic acid, and ferulic acid), aldehydes (including syringaldehyde), and flavonoids (including peonidin), were reported also to induce haustoria in *Triphysaria versicolor*, *P. japonicum*, and *Striga hermonthica* (Albrecht et al., 1999; Cui et al., 2018). *Castilleja tenuiflora* was reported to react with vanillic acid as well as catechin and hydrogen peroxide (H_2O_2) (Salcedo-Morales et al., 2013), although H_2O_2 alone does not induce haustoria in *S. hermonthica* (Wada et al., 2019).

Because highly active HIFs in host exudates have yet to be identified, localization of HIF production and mode of action of HIFs remain largely unknown. Although some reports suggest that HIF activity is related to the pectin fraction of host cell walls (Keyes et al., 2000), the structures of HIFs are more closely related with those of lignin monomers. Lignin is a complex polymer abundant in the secondary cell walls of vascular plants and is composed of aromatic monolignols, i.e., *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, derived from the phenylpropanoid pathway (Vanholme et al., 2010). Polymerization of monolignols results in three major types of generic lignin units, p-hydroxyphenyl (H), guaiacyl (G), and syringil (S) units, which respectively contain zero, one, and two methoxy groups on the aromatic rings. A series of lignin precursors and lignin-derived phenolics were tested for their ability to induce haustoria in *S. hermonthica* and *P. japonicum* (Cui et al., 2018). Interestingly, S-type compounds that have two methoxy groups at the 3 and 5 positions and a hydroxyl group at position 4 of the aromatic ring, e.g., sinapaldehyde, synapyl alcohol, syringic acid, and acetosyringone, can induce haustoria in both *S. hermonthica* and *P. japonicum* (Cui et al., 2018). In contrast, G-type compounds that have one methoxy group at the 3 or 5 position and a hydroxyl group at position 4, including ferulic acid, vanillic acid, vanillin, and apocynin,

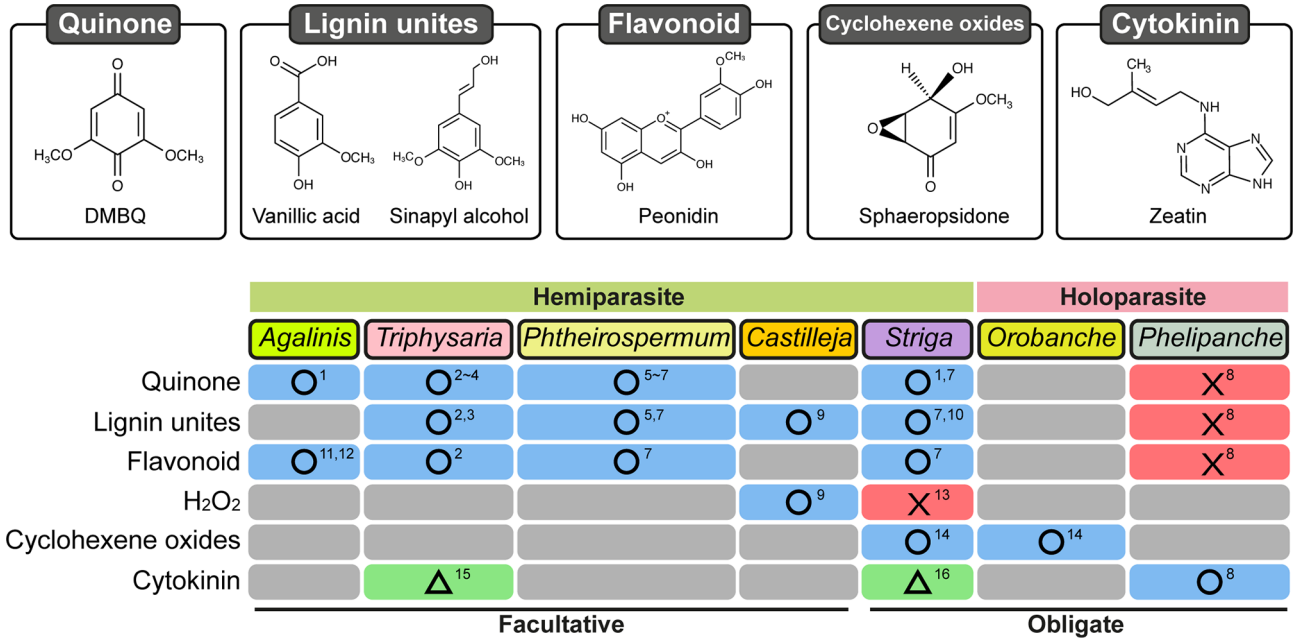

FIGURE 2 | Response of Orobanchaceae parasitic plants treated with various chemicals for haustorium induction. Chemical categories previously reported as HIFs are shown on the left. Blue boxes with circles: at least one chemical was reported in the corresponding species. Red boxes with crosses: tested chemical(s) did not induce haustoria. Green boxes with triangles: haustoria-like structures were reported. Gray boxes: not reported. Numbers indicate references: 1, Chang and Lynn (1986); 2, Albrecht et al. (1999); 3, Bandaranayake et al. (2010); 4, Bandaranayake et al. (2012); 5, Cui et al. (2016); 6, Ishida et al. (2016); 7, Cui et al. (2018); 8, Goyet et al. (2017); 9, Salcedo-Morales et al. (2013); 10, Lynn and Chang (1990); 11, Lynn et al. (1981); 12, Steffens et al. (1982); 13, Wada et al. (2019); 14, Fernández-Aparicio et al. (2016b); 15, Wrobel and Yoder (2001); 16, Keyes et al. (2000).

have a high capacity for inducing haustoria in *S. hermonthica* but not in *P. japonicum*. H-type compounds that do not have a methoxy group generally do not show haustorium-inducing activity for either species (Cui et al., 2018). These results suggest that there are certain species specificities for HIF recognition. Consistently, purified natural lignin polymers as well as artificially synthesized lignin polymers composed of only G-units can induce haustoria only in *S. hermonthica*, and those containing G and S units can induce haustoria in both *P. japonicum* and *S. hermonthica* (Cui et al., 2018). Because the prehaustorium-inducing activity of lignin polymers is greatly enhanced by application of white rot fungi-derived laccase, an enzyme that can produce monolignols and quinones *via* oxidative lignin depolymerization (Pollegioni et al., 2015), monomeric phenolics or quinones are more likely to be the active compounds *in vivo* (Cui et al., 2018). These results could also explain the high HIF activity for *Striga* in the pectin fraction from sorghum cell walls (Keyes et al., 2000). The primary cell wall (pectin-rich) fraction from Poaceae species is known to contain abundant amounts of ester-linked ferulic acid, which acts as a HIF for *Striga* (Harris and Trethewey, 2010). Alteration of the lignin monomer composition in a host using genetic modification affects the haustorium-induction activity of *P. japonicum* and *S. hermonthica* (Cui et al., 2018), indicating that HIFs naturally produced in hosts originate at least partly from lignin biosynthesis or degradation pathways, although details of the origin of HIFs need to be further investigated in the future.

HIFS FOR HOLOPARASITES

HIFs have been less studied in holoparasitic Orobanchaceae partly because their prehaustorial structures are less apparent than hemiparasites that proliferate haustorial hairs. Prehaustorium induction in *Orobanche* and *Phelipanche* is nevertheless characterized by radicle growth arrest, radicle tip swelling, and extension of epidermal cells to form secretory papillae bearing a host-attachment function similar to that of haustorial hairs in hemiparasites (Baird and Riopel, 1985; Joel and Losner-Goshen, 1994; Fernández-Aparicio et al., 2016a; Fernández-Aparicio et al., 2016b; Goyet et al., 2017). Because a study reported *in vitro* host-independent prehaustorium induction in the species *Orobanche cumana* and *Phelipanche aegyptiaca* (Joel and Losner-Goshen, 1994), prehaustorium induction in holoparasites has been considered for a long time as a host-independent process. However, recent findings in the species *Phelipanche ramosa* demonstrate that root exudates collected from healthy oilseed rape plants induce prehaustorium formation *in vitro* (Goyet et al., 2017). A similar observation has been made for the species *O. cumana* in response to sunflower root exudates (Montiel & Simier, personal communication). These recent findings therefore imply that prehaustorium induction by host-derived chemical signals or molecules occurring in the rhizosphere is a common process in both hemiparasitic and holoparasitic Orobanchaceae.

However, the currently known HIFs for hemiparasites, including DMBQ, syringic acid, and vanillic acids, are inefficient

for inducing prehaustoria in *P. ramosa* (Goyet et al., 2017). In addition, DMBQ is also inactive towards *P. aegyptiaca* (Westwood et al., 2010). Recently, the mycotoxins sphaeropsidone and epi-sphaeropsidone, which are cyclohexene oxides isolated from *Diplodia cupressi* (**Figure 2**), the causal agent for cypress (*Cupressus sempervirens* L.) canker (Evidente et al., 1998), were shown to bear HIF activity *in vitro* towards *Orobanche crenata* and *O. cumana* (Fernández-Aparicio et al., 2016a; Fernández-Aparicio et al., 2016b). Moreover, according to structure-activity relationship analyses, structural modifications of sphaeropsidone and epi-sphaeropsidone affect differently prehaustorium induction in *O. crenata* and in *O. cumana* (Fernández-Aparicio et al., 2016b). As reported in the hemiparasitic Orobanchaceae, these findings suggest that certain species specificities for HIF recognition also occur in the holoparasite *Orobanche*. Nevertheless, these compounds are also active *in vitro* towards *Striga* while being structurally different from the currently known HIFs for hemiparasites. Although activity towards *Striga* could be possibly due to the conversion of sphaeropsidone and its derivatives to active 3-methoxyquinones (Fernández-Aparicio et al., 2016b), this eventually suggests that *Orobanche* may have a more strict specificity for HIF compounds than *Striga*.

CYTOKININS AS HIFS

Cytokinins are known to be important phytohormones acting in a vast array of plant development processes including root development as well as nodule formation during plant-bacteria symbiosis (Pacifici et al., 2015). Cytokinins were detected in the rhizosphere of many plants including maize, rice, and tomato (Davey and van Staden, 1976; van Staden and Dimalla, 1976; Soejima et al., 1992; Yang et al., 2002; Kirwa et al., 2018), and interestingly, cytokinins are also effective *in vitro* for inducing prehaustorium-like structures in hemiparasites and prehaustoria in holoparasites (**Figure 2**). Kinetin, a synthetic cytokinin, and 6-benzylaminopurine (BAP) were indeed reported to induce prehaustoria-like structures in *S. asiatica* and in *T. versicolor*, respectively (Keyes et al., 2000; Wrobel and Yoder, 2001). However, some morphological differences between phenolic HIF-induced and cytokinin-induced prehaustoria were underlined; e.g., in *T. versicolor*, cytokinin-induced prehaustoria have smaller swelled structures than those induced by DMBQ. All these findings made questionable the involvement of cytokinins in cooperation with phenolic HIFs as prehaustorium inducers for these hemiparasitic species (Estabrook and Yoder, 1998).

However, recent analyses on prehaustorium formation in the holoparasite *P. ramosa* parasitizing oilseed rape showed clear evidences of the existence of a signal carrying both cytokinin and HIF activities in oilseed rape rhizosphere (Goyet et al., 2017). Bioguided ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC-ESI(+)-/MS/MS) analyses showed that oilseed rape root constitutively exudes a cytokinin with dihydrozeatin characteristics. In parallel, cytokinins at physiological levels (10^{-7}–10^{-8} M) were effective for prehaustorium induction in *P. ramosa*. Moreover, as expected from its antagonist activity toward cytokinins, auxin treatment prevented prehaustorium formation in response to cytokinins or root exudates. Gene expression analysis after treatment with oilseed rape root exudates or exogenous t/zeatine confirmed that plant hormone-related genes, including cytokinin-related genes (notably *PrRR5*, *PrCKX2* and *PrCKX4*), were up-regulated during prehaustoria induction (Goyet et al., 2017). These findings highlight cytokinins as *bona fide* rhizosphere signals for holoparasites and that the possible roles for hemiparasite prehaustorium induction need to be re-investigated.

ACTION MECHANISMS OF HIFS IN PREHAUSTORIUM INDUCTION

Most of the currently known phenolic HIFs for hemiparasites contain an aromatic ring with a hydroxyl group at position 4 and one or two methoxy groups at positions 3 and/or 5 (**Figure 2**). Variation of the functional group at position 1 could affect the haustorium induction activity (Cui et al., 2018), probably due to modification of the redox range of the molecules (Smith et al., 1996), but a structural requirement at position 1 is not apparent. Lignin-related HIFs, syringic acid and syringaldehyde, were reported to be oxidized to produce DMBQ in the presence of peroxidase or laccase *in vitro* (Frick et al., 1996; Ibrahim et al., 2013). Kim et al. (1998) identified peroxidase activity in a *Striga* cell wall fraction that is able to convert syringic acid to DMBQ. Furthermore, quinone oxidoreductase (QR) genes were upregulated in the parasite after DMBQ treatment and/or host infection. Two types of QR (QR1 and QR2) were isolated from *T. versicolor*, *P. japonicum*, and *S. hermonthica*. QR1 reduces one electron from quinone and converts it to a semiquinone, whereas QR2 is thought to reduce two electrons from quinone to hydroquinone (Bandaranayake et al., 2010; Ishida et al., 2017). In *T. versicolor*, *QR1* expression is upregulated by DMBQ and host exudates, whereas *QR2* is induced by DMBQ but not by host exudates (Matvienko et al., 2001). In *P. japonicum* and *S. hermonthica*, *QR2* but not *QR1* expression is upregulated upon both DMBQ treatment and host infection (Ishida et al., 2017). Knockdown of *QR1* expression in *T. versicolor* and *QR2* expression in *P. japonicum* resulted in reduced haustoria formation (Bandaranayake et al., 2010; Ishida et al., 2017). Although it is uncertain why the different types of QRs are involved in haustoria formation in different plant species, the likely scenario is that electron transfer by a QR function is crucial for haustorium initiation.

Oxidation-reduction cycles produce reactive oxygen species (ROS), which may act as signals for haustorium induction (Bandaranayake et al., 2010). To investigate the roles of ROS in haustorium formation, pharmacological analyses were conducted. Application of catalase, a scavenger of H_2O_2, reduced haustorium induction of *S. asiatica* by syringic acid but not DMBQ, suggesting that H_2O_2 is important for conversion of syringic acid to DMBQ (Keyes et al., 2000). However, a similar experiment using *S. hermonthica* showed that catalase reduces haustorium induction by both DMBQ and syringic acid (Wada et al., 2019), indicating that ROS are also necessary downstream of DMBQ. Application of a series of ROS inhibitors and scavengers revealed that reduced nicotinamide adenine

dinucleotide phosphate (NADPH) oxidase inhibitors, especially diphenyleneiodonium (DPI), could efficiently reduce haustorium formation in *S. hermonthica* (Wada et al., 2019). NADPH oxidase is an enzyme that produces O^{2-}, which is further converted to H_2O_2 by the superoxide dismutase (SOD) enzyme. An inhibitor of SOD also reduced haustorium formation, indicating that H_2O_2 production plays a pivotal role for prehaustorium induction in response to DMBQ. Furthermore, peroxidase inhibitors also reduced the haustorium formation rates, whereas exogenous application of peroxidase increased the haustorium formation rates (Wada et al., 2019). These results suggest that ROS modulation *via* peroxidase may regulate HIF-derived signaling, but how ROS and peroxidases interact with HIF perception and signal transduction in the context of haustorium initiation remains to be elucidated.

In the holoparasite *P. ramosa*, transcriptomic analysis focusing on time points surrounding prehaustorium formation in response to HIF-containing host root exudates did not reveal any over-representation of ROS-related Gene Ontology (GO) terms. Therefore, involvement of ROS in prehaustorium induction in holoparasites remains obscure.

ROLES OF HIFS IN HOST-PARASITIC PLANT INTERACTIONS AFTER PREHAUSTORIUM INDUCTION

The roles of HIFs in other functions during the host plant-parasitic plant interaction besides prehaustorium induction have not been well studied yet. Cytokinins may have roles during invasion by *P. ramosa* because pre-treatment of germinated seeds of *P. ramosa* with cytokinins increased the number of parasites invading host tissues. Furthermore, pre-treatment with PI-55, an inhibitor of cytokinin signaling (Spíchal et al., 2009), reduced the number of invading parasites (Goyet et al., 2017). Increased successful invasion rates, regarded as *P. ramosa* aggressiveness, upon cytokinin treatment could be due to either modification of prehaustoria formation rates or modification of haustorium functioning. In addition, cytokinins have been reported to manipulate host physiology after successful host penetration (Spallek et al., 2017). Upon infection, *trans*-zeatin-type cytokinins accumulate in both *P. japonicum* and *Arabidopsis thaliana*. Analysis of *Arabidopsis* mutants defective in cytokinin biosynthesis indicated that the accumulated cytokinins in host plants were delivered by the parasites (Spallek et al., 2017). Cytokinin accumulation in the host causes hypertrophy of the infected area of the host and is characterized by an increase in vascular diameter and cell number (Spallek et al., 2017). These morphological and physiological changes of host vascular would lead to increased sink strength at haustorium attachment site, which could contribute to nutrient uptake by the parasitic plant.

Many phenolic HIFs are lignin precursors and, therefore, can be incorporated into lignin polymers in the secondary cell walls of hosts or parasitic plants. Lignin is known to be involved in physical defense against various pathogens including bacteria, fungi, plant-parasitic nematodes, and parasitic plants (Goldwasser et al., 1999; Bhuiyan et al., 2009; Miedes et al., 2014; Khanam et al., 2018; Mutuku et al., 2019). Lignin is deposited at the *Striga* infection site in a *Striga*-resistant rice cultivar, and lignin modification in the rice host affects resistance against *Striga* (Mutuku et al., 2019). Thus, phenolic HIFs may be used by hosts for forming a physical barrier against parasitic plants after parasite attack.

Redox cycling between quinones and hydroquinones occurs in the rhizosphere with a great impact on plant-microbe interactions (Taran et al., 2019). Such redox cycling is known to be used as a lignocellulolytic agent by wood-decaying brown rot fungi. A variation of DMBQ, 2,5-dimethoxy-*p*-benzoquinone (2,5-DMBQ) and its reduced form 2,5-dimethoxy-*p*-hydroquinone (DMHQ) were detected in wafers of aspen wood colonized by the brown rot fungus *Serpula lacrymans* (Korripally et al., 2013). Oxidation of DMHQ to 2,5-DMBQ drives a Fenton reaction: $H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + \cdot OH$ (Suzuki et al., 2006; Korripally et al., 2013). The resulting hydroxyl radical (·OH) is highly active and non-enzymatically deconstructs the lignocellulose structure (Cragg et al., 2015). During intrusion, parasitic members of the Orobanchaceae family need to pass through lignified endodermal cell layers (Yoshida and Shirasu, 2009), and intrusive cells in some species, such as *Striga* spp., can penetrate into lignified host xylem vessels (Dorr, 1997). It is tempting to hypothesize that parasitic plants may employ the Fenton reaction to depolymerize host lignin during invasion, and quinone-type HIFs may act as driving forces of the reaction. Understanding the physiological roles of HIF molecules beyond prehaustorium induction will help us to know why parasitic plants use these molecules as host signals.

CONCLUSIONS

Evidences collected on hemi- and holoparasites indicate a commonality and specificity of HIFs in parasitic members of the Orobanchaceae family. In addition to cytokinins, ROS-producing quinones or phenolics are commonly recognized as HIFs. For a long time, redox cycling has been suggested to manipulate prehaustorium induction, but the detailed mechanisms of this activity are yet to be discovered. Future studies to understand the common and specific signaling pathways from different types of HIFs should reveal how parasitic plants sense the presence of a host and begin their parasitic lifestyles.

CONTRIBUTION TO THE FIELD STATEMENT

Parasitic plants in the Orobanchaceae family are among world's most devastating weed pests, yet the molecular processes underlying their parasitism have not been well understood. Parasitic plants develop haustoria, specialized invasive organs, upon recognition of host-derived chemical signals, the so-called haustorium-inducing factors (HIFs). This review summarizes past work and recent advances in the research of HIFs for parasitic plants in the Orobanchaceae family. Recent research

efforts have begun to reveal the origins, species specificity, and action mechanisms of HIFs.

AUTHOR CONTRIBUTIONS

VG, SW, and SY conceived the paper and drafted the manuscript. TW and SC drew the figures. SC, TW, KS, GM, PS, and SY edited and finalized the manuscript.

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Bioprospecting Plant Growth-Promoting Rhizobacteria That Mitigate Drought Stress in Grasses

*Michael D. Jochum[1], Kelsey L. McWilliams[1], Eli J. Borrego[1,2], Mike V. Kolomiets[1], Genhua Niu[3], Elizabeth A. Pierson[1,4] and Young-Ki Jo[1]**

[1] Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX, United States, [2] Thomas H. Gosnell School of Life Sciences, Rochester Institute of Technology, Rochester, NY, United States, [3] Texas A&M AgriLife Research and Extension Center, El Paso, TX, United States, [4] Department of Horticultural Sciences, Texas A&M University, College Station, TX, United States

This study reports the application of a novel bioprospecting procedure designed to screen plant growth-promoting rhizobacteria (PGPR) capable of rapidly colonizing the rhizosphere and mitigating drought stress in multiple hosts. Two PGPR strains were isolated by this bioprospecting screening assay and identified as *Bacillus* sp. (12D6) and *Enterobacter* sp. (16i). When inoculated into the rhizospheres of wheat (*Triticum aestivum*) and maize (*Zea mays*) seedlings, these PGPR resulted in delays in the onset of plant drought symptoms. The plant phenotype responding to drought stress was associated with alterations in root system architecture. In wheat, both PGPR isolates significantly increased root branching, and *Bacillus* sp. (12D6), in particular, increased root length, when compared to the control. In maize, both PGPR isolates significantly increased root length, root surface area and number of tips when compared to the control. *Enterobacter* sp. (16i) exhibited greater effects in root length, diameter and branching when compared to *Bacillus* sp. (12D6) or the control. *In vitro* phytohormone profiling of PGPR pellets and filtrates using LC/MS demonstrated that both PGPR strains produced and excreted indole-3-acetic acid (IAA) and salicylic acid (SA) when compared to other phytohormones. The positive effects of PGPR inoculation occurred concurrently with the onset of water deficit, demonstrating the potential of the PGPR identified from this bioprospecting pipeline for use in crop production systems under drought stress.

Keywords: PGPR, drought, bioprospecting, plant, growth-promoting, rhizobacteria, wheat

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****Correspondence:***
Young-Ki Jo
ykjo@tamu.edu

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INTRODUCTION

Drought is a major abiotic stress threatening agricultural production worldwide. In the last 40 years, drought stress has reduced yields in cereals by as much as 10% (Lesk et al., 2016) and is forecasted to affect production on over 50% of the arable land by 2050 (Vinocur and Altman, 2005). In order to address this global challenge in agriculture, research has focused on improving germplasm and developing crop management practices to increase water use efficiency (Passioura, 2007; Ngumbi and Kloepper, 2016). However, recent attention has turned to the application of beneficial microorganisms that mediate drought tolerance and improve plant water-use efficiency and these

efforts have been augmented due to technological advances in next generation sequencing and microbiomics (Dimkpa et al., 2009; Marulanda et al., 2009; Yang et al., 2009; Ngumbi and Kloepper, 2016; Vurukonda et al., 2016).

The application of plant growth-promoting rhizobacteria (PGPR) is considered a sustainable synergistic biological approach to cope with water deficiency in crop production. PGPR readily colonize the root rhizosphere and establish both free-living and intimate associations with host plants. Often, these interactions lead to enhancement of crop productivity and mitigation of biotic and abiotic stresses through a variety of mechanisms (Mayak et al., 2004; Berg, 2009; Dimkpa et al., 2009; Liu et al., 2013; Mendes et al., 2013; Vacheron et al., 2013; Porcel et al., 2014; Gontia-Mishra et al., 2016; Ngumbi and Kloepper, 2016; Vurukonda et al., 2016; Barnawal et al., 2017; Forni et al., 2017). PGPR may play critical roles as suppressors of plant disease, biofertilizers, alleviators of abiotic stress and remediators of toxins from the soil (Mayak et al., 2004; Naveed et al., 2014; Timmusk et al., 2014). Mechanisms associated with PGPR-derived drought tolerance include alterations in host root system architecture, osmoregulation, management of oxidative stress via the biosynthesis and metabolism of phytohormones or the production of antioxidants for scavenging reactive oxygen species (ROS), the production of large chain extracellular polysaccharide (EPS) that may serve as humectant, and transcriptional regulation of host stress response genes (Dimkpa et al., 2009; Liu et al., 2013; Vacheron et al., 2013; Osakabe et al., 2014; Timmusk et al., 2014; Gontia-Mishra et al., 2016; Ngumbi and Kloepper, 2016; Vurukonda et al., 2016; Barnawal et al., 2017; Forni et al., 2017).

The objective of this study was to design and implement a bioprospecting screen to isolate PGPR capable of rapidly colonizing seedling rhizospheres and mediating drought stress in multiple cereal hosts. For this purpose, the screening method was developed that emphasized the following: (a) A selection of a likely source containing PGPR, (b) A pre-screening process focused on desired plant phenotypes, and (c) A final screening process focused on candidates likely to provide desired outcomes under practical production practices on both wheat and maize.

The original source of PGPR were the rhizospheres of perennial grasses collected from El Paso, TX, where the semi-arid environment provides a strong selective pressure for survival under nearly constant water deficit. The rationale for choosing the starting material was that perennial grasses growing vigorously under pervasive water stress conditions were likely to foster a microbiome capable of mitigating drought stress. The pre-screening process focused on the desired host phenotype, rather than bacterial phenotypes. The host phenotype used for screening was the delayed of onset of drought stress symptoms in seedlings, since seedling establishment is often the most vulnerable stage and may have large impacts on crop stand and yield (Pessarakli, 1999). The final selection process focused on the identification of PGPR that are most likely to have applications in existing commercial production systems. Given current limitations in "seed space" for new growth stimulating products combined with the difficulties in reliable formulation of application-friendly seed treatments, the focus of this study was on identifying isolates that could be applied as needed prior to the onset of water stress conditions. The screening protocol was designed to specifically select isolates that could rapidly colonize and provide benefits to the host, e.g., if inoculated at the onset of water deficit conditions. In this manner, this screen provides the unique ability to select strains that can be added as needed, as compared to current seed coating applications. Isolated candidate PGPR strains demonstrating robust effectiveness were validated on two different grass hosts, wheat (*T. aestivum*) and maize (*Z. mays*).

MATERIALS AND METHODS

Rhizobacteria Sampling and Screening

Twenty-five bermudagrass (*Cynodon* spp.) thatch core samples (10 cm diameter and 15 cm depth) were collected in the summer of 2015 and 2016 in El Paso, TX, United States. Sampling sites included medians, parks, roadsides and ranches. Intact core samples were immediately shipped upon removal under ambient temperatures to the lab in College Station, TX. Each sample core was then subdivided into 5 cm diameter cores, transferred to a round plastic pot (10 cm diameter, 12 cm height) with holes in the bottom, filled-in with autoclaved potting mix (Metro-Mix 900, Sun Gro Horticulture, Agawam, MA, United States), and grown in a greenhouse for 14 days. Grasses were exposed to three different levels of watering: non-stressed (watering up to the field capacity every other day), moderate stress (watering once a week), and severe stress (no watering). The onset of drought symptoms was daily monitored and recorded based on phenotype: leaf wilting, curling, tip burning, and plant lodging. The five cores containing plants for which drought symptoms were most delayed under both the moderate and severe watering regimes were used for the next step: bacterial isolation and preservation for screening trials. By conducting this pre-screening of grass samples in a controlled setting, we mitigate the possibility of sampling habitats of compensation that demonstrated drought resistant phenotypes due to source-sink effects (Leibold et al., 2004).

Rhizosphere samples for bacterial isolation were obtained from one gram of root tissue, excised from the grasses in each of the selected cores. Root tissue samples were first washed in sterile dH_2O to remove detritus and non-root adherent soil, suspended in 10 ml of 0.1 M phosphate buffered saline (1 min), and macerated using a drill homogenizer (115V Bio-Gen PRO200 homogenizer unit, 5 × 75 mm generator probe). PBS suspensions were serially diluted and plated on Luria-Bertani (LB) agar amended with 5 mg L^{-1} cycloheximide and 10% sorbitol (Kavamura et al., 2013). Plates were maintained 25°C and inspected daily for bacterial growth. Morphologically distinct colonies were re-isolated to obtain axenic cultures and then grown separately overnight in LB broth (25°C, 120 rpm agitation) and stored in 40% glycerol at −80°C.

PGPR Screening

Wheat (*T. aestivum* subsp. *aestivum* cultivar TAM111) and maize (*Z. mays* cultivar B73) seeds were surface sterilized in 10%

NaOCl for 10 min, followed by 10 subsequent rinses in sterile dH_2O. Seeds were germinated on sterile filter paper 24 h at 37°C for wheat and 25°C for maize. Germinated seeds were planted separately in pots (10 cm diameter, 12 cm height) with holes containing 400 g sterilized Metro-Mix 900. Seedlings were watered to field capacity every day, determined by water leaching through the bottom of the pot, and cultivated in a growth chamber for 7 days (30°C, using fluorescent bulbs emitting 300 μmol m^{-2} s^{-1}, 12:12 h light and dark cycle). Plants were inoculated 7 days post germination with test strains, followed by withholding water for the next 7 days. For the bacterial inoculum, overnight cultures were grown in LB at 25°C, collected via centrifugation (2,500×*g*, 5 min) and re-suspended in an equal volume of 0.1 M PBS. 80 μl of resuspended inoculum was applied to the soil at the base of each seedling. Inoculation with 0.1 M PBS was used as a no-inoculum control. For PGPR isolates that showed positive activity, in subsequent trials, inoculum densities were regulated to insure populations of approximately 10^7 colony-forming unit (CFU) ml^{-1} via optical density (600 nm) measurements. Growth curves comparing colony counts and optical density were used to determine the optical densities that provided the desired population densities.

Drought Tolerance Phenotyping

At the end of the 7-day water stress treatment (14 days post planting), inoculated and non-inoculated plants were examined for drought symptoms such as wilting, leaf curling and marginal leaf necrosis. Plants were then removed from the soil, with special care to preserve the intact root system. Roots were washed to remove soil and detritus via spraying with dH_2O against a 0.5 mm mesh sieve. Harvested root and shoot tissues were saturated with dH_2O via storage in wet germination paper at 4°C overnight, in preparation for downstream analysis (Himmelbauer et al., 2004). Washed roots were separated from above ground tissue, submerged in dH_2O and spread out to prevent overlap in a root positioning tray (20 × 30 cm) with three roots per tray. Roots were scanned using a flatbed scanner (EPSON, Perfection V-750). Root image data obtained by scanning were analyzed using WinRHIZO Arabidopsis 2017a (WinRHIZO, RRID:SCR_017120), generating estimates of total root length, root surface area, average root diameter, number of root tips, and root branching as previously described (Arsenault et al., 1995; Himmelbauer et al., 2004). For plants that exhibited delayed drought stress symptoms relative to control plants, bacterial population sizes were determined via serial dilution plating. In all experiments, root population sizes were 10^6–10^7 CFU g^{-1} of rhizosphere, defined as root and root adherent soil. Bacteria were re-isolated from root rhizosphere on LB amended with cycloheximide and stored as before.

The experiment for evaluating drought tolerance phenotypes by PGPR was conducted in a completely randomized block design with five replications (plants). The experiment was repeated once. Plant phenotype data from WinRHIZO and LC-MS results were analyzed using an analysis of variance (ANOVA) (Statistical Analysis System, RRID:SCR_008567). Pairwise comparisons between the treatments were conducted using Fischer's least significant difference (LSD) test at $P = 0.05$. Root scans and statistical analysis scripts can be found at the https://github.tamu.edu/jochum00/04_16_2019_SAS.

Isolate Sequencing

For bacterial strains of interest, taxonomic information was obtained via sequencing of the 16S and 23S ribosomal RNA subunit and ITS regions (Stackebrandt and Goebel, 1994; Dinesh et al., 2015). Genomic DNA from each strain was extracted using the CTAB protocol (William et al., 2012). Polymerase chain reaction (PCR) was used to amplify the target region with the following primers: 16S region forward 8F/pA (5′-GAGTTTGATCCTGGCTCAG-3′) and 23s reverse p23SR01 (5′-GCTGCTTCTAAGCCAAC-3′) (Stackebrandt and Goebel, 1994; Dinesh et al., 2015). PCR was performed in a thermocycler (Applied Biosystems Thermocycler 2720) with the following reaction conditions: 1 min 95°C; 35 cycles of 1 min 95°C, 1 min 52.7°C, and 1.5 min 72°C; 1 cycle 10 min 72°C; maintain at 4°C until retrieval. PCR amplicons were gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega, RRID:SCR_006724), and sequenced (Eton Bioscience, RRID:SCR_003533) with the aforementioned PCR primers and sequencing primers 1542R/pHr (5′-TGCGGCTGGATCACCTCCTT-3′) and 1542R/pH (5′-AAGGAGGTGATCCAGCCGCA-3′). The reads were aligned using MAFFT algorithm in Benchling (Benchling, RRID:SCR_013955). Consensus alignments were taxonomically identified at the genus level via NCBI nucleotide Basic Local Alignment Search Tool (BLASTN, RRID:SCR_001598).

Phytohormone Profiling

Ten milliliters of LB overnight cultures from each strain were pelleted via centrifugation at 10,000 rpm for 10 min. Supernatants were decanted into a Nalgene® Rapid-Flow™ sterilization filter unit containing a 0.2 μm nitrate cellulose membrane and filtered via vacuum filtration. Pellet and filtrate samples were lyophilized for 24 h., followed by resuspension in 500 μl extraction buffer consisting of n-propanol, H_2O and HCl (2:1:0.002 by volume) spiked with 500 nM of following deuterated internal standards: d-ABA ([$^2H_6$] (+)-*cis*,*trans*-abscisic acid; Olchemlm cat# 0342721), d-ACC (1-Aminocyclopropane-2,2,3,3-d4-carboxylic acid; Sigma cat#736260), d-*trans*-Cinnamic acid (d7- cinnamic acid; Sigma cat#513954), d-IAA([2H5] indole-3-acetic acid; Olchemlm cat# 0311 531), d-JA (2,4,4-d3; acetyl-2,2-d2 jasmonic acid; CDN Isotopes cat# D-6936), and d-SA (d6- salicylic acid; Sigma cat#616796). Following resuspension, we conducted phase separation via the addition of dicholormethane (CH_2Cl_2) for 30 min at 4°C, followed by centrifugation at 14,000 rpm for 10 min. The organic phase was removed, evaporated under N_2 gas in a glass vial, followed by re-solubilization in 150 μl methanol precipitation and incubated overnight in −20°C. Samples were then centrifuged at 14,000 rpm for 5 min. After centrifugation, 10 μl of supernatant from each sample were injected into a C18 analytical column for liquid chromatography analyte separation, followed by detection via triple quadruple mass spectrometry. Samples were quantified for phytohormones and oxylipins via comparison against the internal deuterated

standards as previously described (Stumpe et al., 2005; Strauch et al., 2015).

RESULTS

Out of 200 isolates tested, soil inoculation by two PGPR strains, 12D6 and 16i, significantly alleviated drought stress symptoms in both wheat (**Figure 1**) and maize (**Figure 2**) seedlings. Qualitative assessment of plant performance across replicate experiments suggested strain 12D6 was somewhat more effective in mediating a delay in the onset of drought symptoms in wheat, whereas strain 16i was more effective in mediating this effect in maize. Results from the NCBI BLASTN query based on rRNA sequence identified strain 12D6 (accession no. MH678658 and MH683042) as *Bacillus* sp. (ident = 99%) and 16i (accession no. MH678659 and MH683043) as *Enterobacter* sp. (ident = 99%).

Results from a two-way ANOVA (host × bacterial treatment) revealed that given the larger size of the maize root system compared to the wheat root system, all maize root system dependent variables were statistically larger than those of wheat ($P < 0.0001$). Consequently, the ANOVA was performed separately for each host (**Table 1**).

In wheat, the root systems of seedlings (**Figure 3**) treated with either bacterial inoculum were more branched than those of the non-inoculated seedlings. Treatment of seedlings with *Bacillus* sp. (12D6) contributed to greater total root length compared to the control treatment (**Table 2**).

In maize, the root systems of seedlings (**Figure 4**) treated with either bacterial inoculum were larger in terms of total root length and surface area and had more root tips than non-inoculated seedlings (**Table 2**). Some differences between the treatments in other metrics were observed. The seedlings treated with *Enterobacter* sp. (16i) had longer total root length, more branching and smaller average root diameter compared with those treated with *Bacillus* sp. (12D6) or the controls (**Table 2**).

Targeted analyte LC/MS based phytohormone profiling of PGPR strains grown *in vitro* revealed that both strains produced indole-3-acetic acid (IAA) and salicylic acid (SA) (**Supplementary Figure S1**) in relatively high amounts ($P < 0.0005$) compared to the other phytohormones

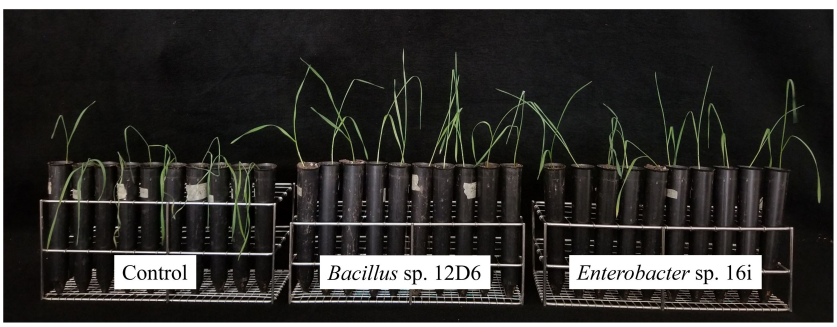

FIGURE 1 | Wheat seedlings treated with plant growth-promoting rhizobacteria (PGPR). *Bacillus* sp. 12D6 **(middle)** and *Enterobacter* sp. 16i **(right)** demonstrated the delayed onset of drought symptoms versus control **(left)** in wheat seedlings after exposure to 7 days of continuous water deficit.

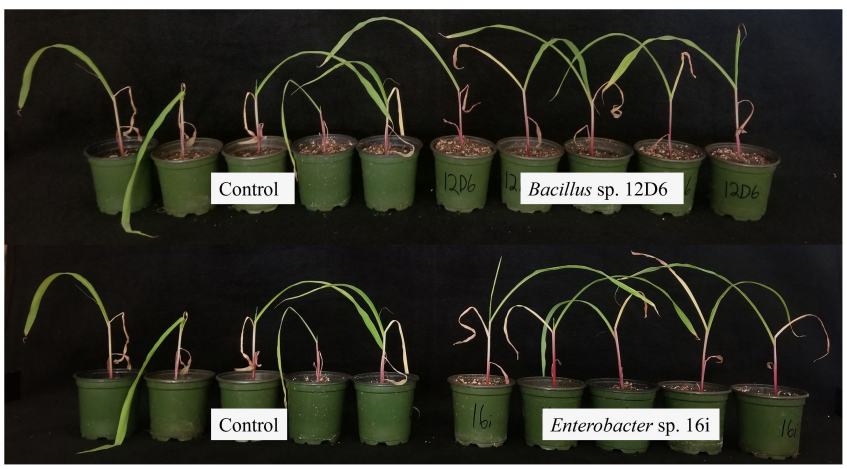

FIGURE 2 | Maize seedlings treated with plant growth-promoting rhizobacteria (PGPR). *Bacillus* sp. 12D6 **(top right)** and *Enterobacter* sp. 16i **(bottom right)** demonstrated the delayed onset of drought symptoms in maize seedlings versus control **(top left** and **bottom left)** after exposure to 7 days of continuous water deficit.

TABLE 1 | Analysis of variance (ANOVA) for the effect of plant growth-promoting rhizobacteria (PGPR) treatment on wheat and maize root systems following a 7-day water deficit.

Dependent variable	Wheat				Maize			
	df	Mean squared	*F*	*P*	*df*	Mean squared	*F*	*P*
Root length	2	4512.80756	3.13	0.0599	2	26904.8926	13.89	<0.0001
Root surface area	2	23.16365397	1.93	0.1653	2	147.012501	4.48	0.0198
Average diameter	2	0.00067100	1.29	0.2929	2	0.00723170	8.82	0.0010
Root tips	2	69630.700	1.42	0.2596	2	207948.394	5.02	0.0132
Root branching	2	110906.8000	4.91	0.0152	2	512832.212	8.72	0.0010

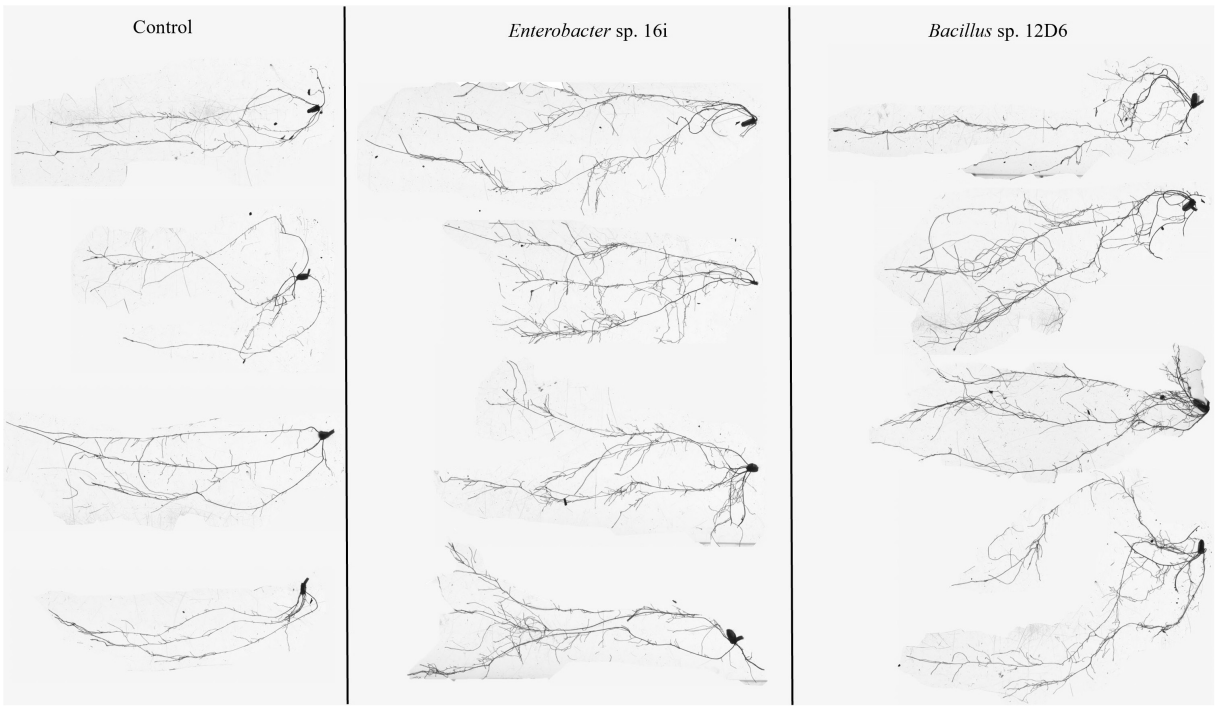

FIGURE 3 | Root system architecture in wheat seedlings treated with the control **(left)**, *Enterobacter* sp. 16i **(center)** and *Bacillus* sp. 12D6 **(right)** after exposure to 7 days of continuous water deficit.

TABLE 2 | Pairwise comparisons using Fischer's LSD test (n = 10) of wheat and maize root system architecture with and without plant growth-promoting rhizobacteria (PGPR) inoculation, analyzed using WinRHIZO software.

Host plant	Treatment	Root length (cm)	Root surface area (cm^2)	Average diameter (mm)	Number of root tips	Number of root branching
Wheat	*Bacillus* sp. 12D6	165.40A	11.88	0.248	676.2	604.6A
	Enterobacter sp. 16i	161.49AB	12.08	0.236	628.3	544.8A
	Control	126.81B	9.35	0.233	513.8	399.8B
Maize	*Bacillus* sp. 12D6	323.94B	40.49A	0.399B	1149.8A	1299.6B
	Enterobacter sp. 16i	370.16A	42.55A	0.367A	1098.2A	1600.4A
	Control	271.31C	35.44B	0.417B	890.1B	1181.6B

Means in the same column of each host plant with the same letter are not significantly different at P = 0.05.

profiled and the LB control ($P < 0.0001$). The analytes were found both in the pelleted cells and the filtrate compared to the LB control, indicating both PGPR strains may secrete both compounds.

DISCUSSION

This study reports the development and use of a bioprospecting pipeline to effectively screen PGPR for the ability to rapidly

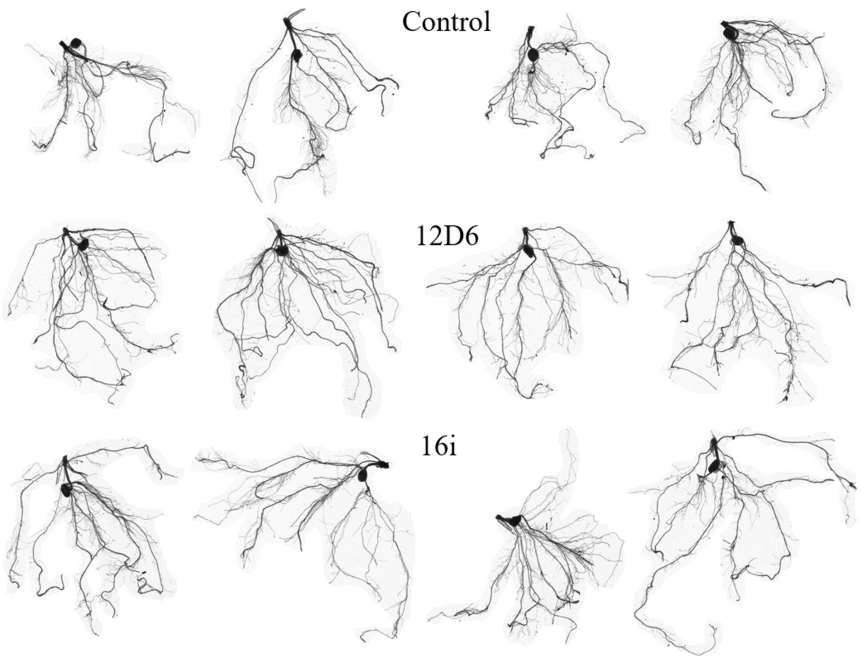

FIGURE 4 | Root system architecture in maize seedlings treated with the control **(top)**, *Bacillus* sp. (12D6) **(center)** and *Enterobacter* sp. (16i) **(bottom)** after exposure to 7 days of continuous water deficit.

mitigate plant drought stress symptoms in multiple cereal hosts when applied to plants at the onset of water deficit conditions. By starting with samples of perennial grasses (bermudagrass) that appeared healthy under constant water deficit conditions in the semi-arid environment of El Paso, TX, we attempted to focus on rhizosphere microbiomes that may be selected for and adapted to mitigating drought tolerance to grasses under these conditions. The pre-screening approach was based on selection of PGPR that mediated the desired seedling phenotype of delayed onset and severity of drought symptoms. This screening procedure succeeded in selecting specific PGPR capable of producing these results rapidly and under water stress conditions.

Using this pipeline, two PGPR strains were identified as *Bacillus* sp. (12D6) and *Enterobacter* sp. (16i). Both wheat and maize seedlings experienced a delay in the onset of drought symptoms when treated with either isolate, although visual assessment of plant performance suggested strain 12D6 was somewhat more effective in mitigating drought symptoms in wheat, whereas strain 16i was more effective in maize. These phenotypic differences in seedling tolerance of drought stress were associated with changes in root system architecture, although there were some differences between hosts in response to the PGRP strains. For instance, in wheat, although both strains had a significant effect on root system architecture, producing more branched roots than non-inoculated seedlings, 12D6-treated seedlings also produced larger root systems in terms of total root length than 16i-treated seedlings or the controls. In maize, both strains produced larger root systems in terms of total root length and surface area and had more root tips, compared with non-inoculated seedlings. However, the root systems of 16i-treated seedlings also had greater total root length, more branches, and smaller average root diameters than those of 12D6-treated seedlings or the controls.

The production of greater linear root length, surface area, and more root tips has been correlated previously with better water stress tolerance and overall improvements in maintaining plant productivity under drought (Comas et al., 2013). Root system length and surface area contribute to better soil exploration, whereas the proliferation of higher order roots resulting in more root tips are important for root water uptake capacity (Vardharajula et al., 2011; Naseem and Bano, 2014; Ngumbi and Kloepper, 2016; Barnawal et al., 2017). Previous research demonstrates that reductions in root diameter may enable faster relative growth rates and rapid resource acquisition through expansion of the root system coupled with lower investment in dry biomass (Garnier, 1992; Wahl and Ryser, 2000; Birouste et al., 2014). Although hosts differed somewhat in how their root systems responded to PGPR treatment, in general these results suggest that water stress tolerance resulted in part from bacterially mediated changes in root system architecture that may have led to enhanced avoidance of drought stress symptoms.

Previous research suggests that host-specific selection of and response to PGPR are complex (Kloepper, 1996; Smith and Goodman, 1999; Drogue et al., 2012). For example, differences in the response of spring wheat to *Bacillus* sp. at the cultivar level have been observed (Chanway et al., 1988). At the molecular level, plant-microbe specificity may be driven by plant and microbial signals important for host-microbe

perception, microbial recruitment, and microbial initiation of host response to symbiosis traits (Smith and Goodman, 1999). In the case of drought tolerance-mitigating PGPR, bacterial adaptation to water stress (e.g., EPS production), and host specific responses to drought stress (e.g., root system architecture, stomatal closure) also may be important. Success in mediating water stress tolerance by PGPR ultimately depends on effective root colonization, reliable expression of microbial traits important for PGPR activity, and cultivar specific differences in mechanisms of adaptation to drought stress (Kloepper, 1996; Drogue et al., 2012). Although both strains successfully colonized the rhizosphere at concentrations of at least 10^6 CFU g^{-1} sample (root and rhizosphere soil), any of these other factors may have contributed to the observed differences in the effectiveness 12D6 and 16i in mitigating drought stress in maize and wheat.

Production and secretion of bacterial compounds that may serve as stimulators of plant growth and development or signals within whole-plant signaling pathways (e.g., phytohormones) have been reported to be involved in bacterially mediated drought tolerance in plants (Dodd et al., 2010; Bakker et al., 2014; Ngumbi and Kloepper, 2016). Our LC-MS phytohormone profiling of bacterially produced compounds demonstrated that both 12D6 and 16i bacterial strains produced IAA and SA in cellular components and supernatant fractions when grown in LB liquid overnight (**Supplementary Figure S1**).

Bacteria have multiple pathways for IAA biosynthesis, which may function in tryptophan storage, and regulation of tryptophan-dependent IAA biosynthesis may have wide-spread effects on bacterial gene expression patterns (Spaepen et al., 2007; Spaepen and Vanderleyden, 2011; Duca et al., 2014). Research has shown that that bacterially produced IAA may function in microbe–microbe signaling and is important for establishing symbiotic relationships with plants, such as during nodule or tumor formation (Spaepen et al., 2007). It is presumed that over 80% of all bacteria isolated from the rhizosphere can produce IAA (Patten and Glick, 1996; Duca et al., 2014). In plants, endogenously produced IAA serves as a phytohormone involved in the regulation of plant growth and development, including the root system. Exogenous application of IAA causes alterations in root system architecture that appear to depend on IAA concentration. For example, low concentrations of IAA generally stimulate primary root elongation, whereas high IAA levels may diminish primary root growth and stimulate the formation of lateral roots and root hairs (Patten and Glick, 2002; Vacheron et al., 2013). The application of IAA-producing PGPR has been shown to produce similar root system responses, which have been linked to plant drought stress tolerance (Marulanda et al., 2009; Bresson et al., 2013; Ngumbi and Kloepper, 2016). Moreover, the specific role of IAA in mediating these phenotypes was demonstrated via comparison of growth promoting activity by auxin-producing PGPR and auxin-deficient mutants (Patten and Glick, 2002; Vacheron et al., 2013). For example, canola seedlings treated with the auxin-producing PGPR *Pseudomonas putida* GR12-2 produced longer roots compared to seedlings treated with an auxin-deficient mutant or the untreated control. Cell-free supernatants of the wild type also enhanced the proliferation of adventitious roots on mung bean cuttings compared to supernatants of the mutant or the control (Patten and Glick, 2002; Vacheron et al., 2013). In contrast, bacterial production of IAA at high concentrations may have inhibitory on root growth and elongation, as demonstrated by the application of IAA overexpression derivatives (Sarwar and Kremer, 1995; Xie et al., 1996). In the present study, the alterations in root system architecture of both wheat and maize seedlings associated with the application of either strain are consistent with the hypothesis that bacterially produced IAA may have contributed to these phenotypes, and this hypothesis merits further investigation.

Production of SA among rhizosphere-colonizing bacteria has been shown to be widespread and some strains can produce significant amounts when cultivated *in vitro*. For example, there are reported cases of *Pseudomonas fluorescens* biocontrol SA "super-producers" that can synthesize concentrations of SA up to 55 μg per ml *in vitro* (Bakker et al., 2014). SA production may be significantly increased under water stress, as observed for PGPR strains *Achromobacter xylosoxidans*, *B. pumilus* SF3, and *B. pumilus* SF4 (Forchetti et al., 2010). In plants, endogenously produced SA serves as a phytohormone involved in stress response. Although primarily studied for its involvement in activating systemic acquired resistance SAR in defense of biotic stresses, SA has also been shown to aid in abiotic stress tolerance, including drought (Wituszynska et al., 2013; Khan et al., 2018). Both phytohormones SA and abscisic acid (ABA) have been proposed to increase drought tolerance through the accumulation of induced ROS and induced signaling of stomatal closure (Daszkowska-Golec and Szarejko, 2013). By eliciting stomatal closure, these phytohormones can reduce transpirational water loss and allow for increasing water storage in the above ground tissue during drought conditions. It is therefore intriguing to speculate that bacterial production of SA may be involved in abiotic stress tolerance via its contribution to the endogenously produced plant SA pools and SA signaling pathways. However, despite the numerous examples of PGPR that produce SA and induce biotic or abiotic stress tolerance, there is very little evidence for the direct role of bacterially produced SA in these processes (Bakker et al., 2014). As Bakker et al. (2014) argue in a 2014 review of rhizobacterial salicylate production, although many root-inhabiting bacteria produce SA *in vitro*, in the rhizosphere they most likely excrete SA primarily as SA-based siderophores under iron limiting conditions or as an adaptation to high temperature conditions when other siderophore molecules are no longer functioning. In contrast to the lack of effect on plants, bacterially produced SA has been shown to be involved in the regulation of key bacterial traits necessary for rhizosphere survival and thus may be important for regulating bacterial community dynamics under drought stress conditions (Bakker et al., 2014). The production of SA by both strains selected for root colonization under drought stress conditions via our bioprospecting pipeline would seem to support this hypothesis.

In summary, the development and application of a novel bioprospecting pipeline effectively screened PGPR for the capacity to rapidly mitigate seedling drought stress symptoms. The screen isolated and identified two PGPR candidates of *Bacillus* sp. (12D6) and *Enterobacter* sp. (16i). Compared to untreated controls, both wheat and maize seedlings treated with either strain were significantly more vigorous following a 7-day water deficit and displayed alterations in root system architecture that likely facilitated the drought avoidance phenotype. The ability of both strains to survive and rapidly protect both wheat and maize seedlings when applied at the onset of drought is a positive indicator of their potential for mitigating seedling drought stress in cereal cropping systems, which will be tested in future research.

DATA AVAILABILITY

The data that support the findings in this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

MJ conceived the study, prepared the figures, and wrote the manuscript. GN collected the samples. MJ, EB, and KM performed laboratory assays and data analysis. Y-KJ, MK, and EP conceived the study and contributed resources. All authors edited the manuscript and approved the final draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02106/full#supplementary-material

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frontiers in Plant Science

REVIEW
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Plant Immune Responses to Parasitic Nematodes

Kazuki Sato [1], Yasuhiro Kadota [1] and Ken Shirasu [1,2]**

[1] *RIKEN Center for Sustainable Resource Science, Yokohama, Japan,* [2] *Graduate School of Science, University of Tokyo, Bunkyo, Japan*

Plant-parasitic nematodes (PPNs), such as root-knot nematodes (RKNs) and cyst nematodes (CNs), are among the most devastating pests in agriculture. RKNs and CNs induce redifferentiation of root cells into feeding cells, which provide water and nutrients to these nematodes. Plants trigger immune responses to PPN infection by recognizing PPN invasion through several different but complementary systems. Plants recognize pathogen-associated molecular patterns (PAMPs) derived from PPNs by cell surface–localized pattern recognition receptors (PRRs), leading to pattern-triggered immunity (PTI). Plants can also recognize tissue and cellular damage caused by invasion or migration of PPNs through PRR-based recognition of damage-associated molecular patterns (DAMPs). Resistant plants have the added ability to recognize PPN effectors *via* intracellular nucleotide-binding domain leucine-rich repeat (NLR)-type immune receptors, leading to NLR-triggered immunity. Some PRRs may also recognize apoplastic PPN effectors and induce PTI. Plant immune responses against PPNs include the secretion of anti-nematode enzymes, the production of anti-nematode compounds, cell wall reinforcement, production of reactive oxygen species and nitric oxide, and hypersensitive response–mediated cell death. In this review, we summarize the recognition mechanisms for PPN infection and what is known about PPN-induced immune responses in plants.

Keywords: pattern-triggered immunity, NLR-triggered immunity, anti-nematode enzymes, anti-nematode compounds, cell wall reinforcement, reactive oxygen species, nitric oxide, hypersensitive response cell death

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****Correspondence:***
Yasuhiro Kadota
yasuhiro.kadota@riken.jp
Ken Shirasu
ken.shirasu@riken.jp

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INTRODUCTION

Plant-parasitic nematodes (PPNs) are among the most devastating agricultural pests worldwide with an annual global crop loss estimated at about 80 billion USD (Jones et al., 2013). PPNs infect a broad host range of commercially important crop families such as the Solanaceae (tomato, potato, pepper), Fabaceae (soybean), Malvaceae (cotton), Amaranthaceae (sugar beet), and Poaceae (syn. Gramineae; rice, wheat, maize). In general, the economically important PPNs have a broad host range and are highly virulent. PPNs may possess sophisticated virulent strategy as they can infect many plants without inducing strong immune responses (Warmerdam et al., 2018). This characteristic feature makes it difficult to isolate mutants of *Arabidopsis thaliana* that are defective

Abbreviations: BABA, β-aminobutyric acid; CC, coiled-coil; CN, Cyst nematode; FTR, ferredoxin:thioredoxin reductase; HR, hypersensitive response; JA, jasmonic acid; LRR, leucine-rich repeat; NLR, nucleotide-binding domain leucine-rich repeat; NO, nitric oxide; OG, oligogalacturonides; PAMP, pathogen-associated molecular pattern; PG, polygalacturonase; PGIP, polygalacturonase inhibitor proteins; PLCP, papain-like cysteine protease; PPN, plant parasitic nematode; PRR, pattern recognition receptor; PTI, pattern-triggered immunity; RBOH, respiratory burst oxidase homolog; RKN, root-knot nematode; RLK, receptor-like kinase; ROS, reactive oxygen species; SNP, sodium nitroprusside; SPRYSEC, secreted SP1a and ryanodine receptor (SPRY) domain; TIR, toll-interleukin 1 receptor; TRX, thioredoxin.

in immunity against PPNs. However, recent progress in plant and nematode genomics has opened a way to understanding the plant's mechanisms for recognizing PPN infection. There is now a large body of work surrounding the immune, tolerance, and susceptible responses of plant species to nematode infection (summarized in **Supplementary Table 1**). In this review, we summarize the known plant recognition mechanisms for PPN infection, and the host immune responses to PPN. In addition, we discuss how different recognition systems activate different immune responses.

PPN LIFE CYCLES

PPNs are divided into three major groups according to feeding behavior: ectoparasitic, semi-endoparasitic, and endoparasitic (Decraemer and Hunt, 2013; Palomares-Rius et al., 2017; Smant et al., 2018). Ectoparasitic nematodes spend their entire life cycle outside of the host, with the only physical contact being the insertion of a long and rigid feeding stylet (**Figure 1A**). Semi-endoparasitic nematodes penetrate roots to feed, with its posterior part remaining in the soil. Endoparasitic nematodes completely enter the root and feed on internal tissues. Each of these feeding types is further divided into either migratory or sedentary lifestyles. For example, migratory endoparasites (e.g., the root-lesion nematodes *Pratylenchus* spp., and the burrowing nematodes *Radopholus* spp.) migrate through root tissues to feed on plant cells, causing damage to tissues as they migrate (**Figure 1B**), whereas sedentary endoparasites move into the vascular cylinder and induce redifferentiation of host cells into multinucleate and hypertrophic feeding cells. The two main PPNs in the sedentary group are the root-knot nematodes (RKNs) in the genus *Meloidogyne*, and the cyst nematodes (CNs) including the genera *Globodera* and *Heterodera* (**Figures 1C**, **D**).

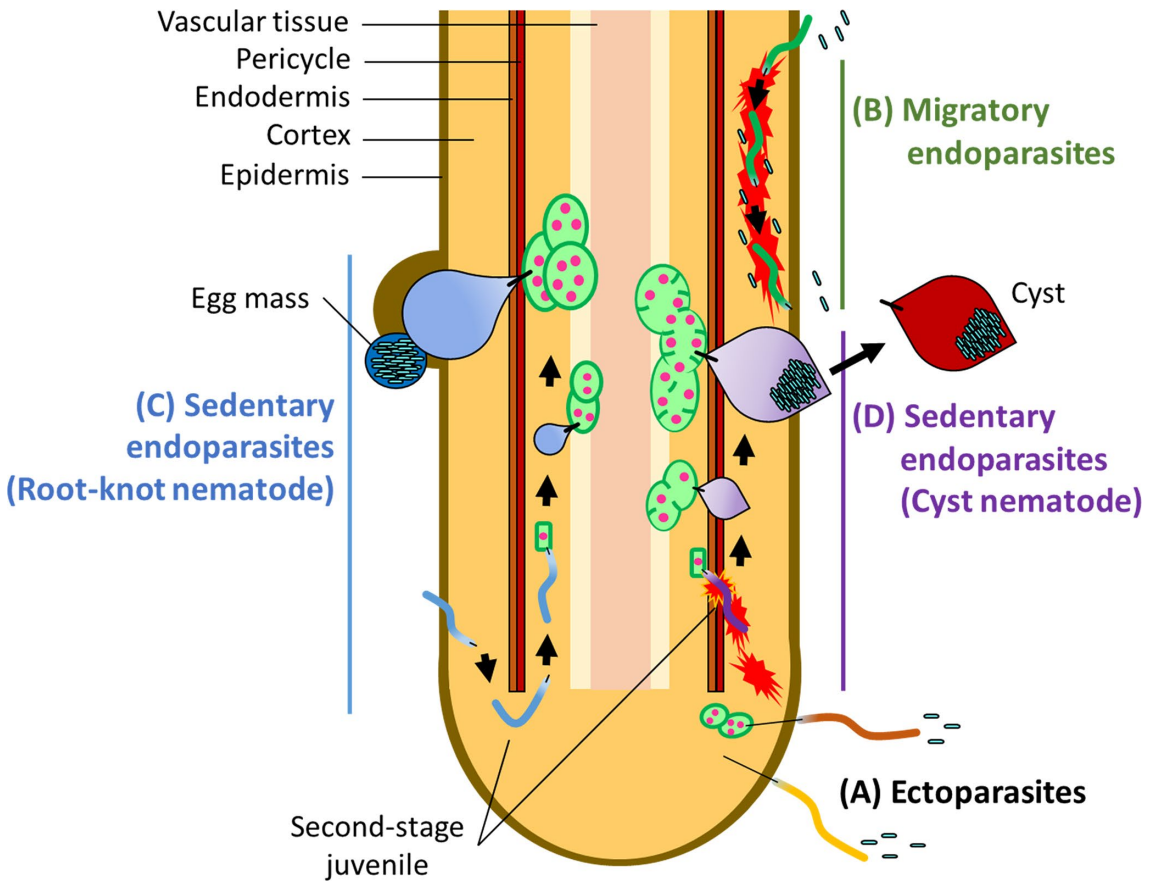

FIGURE 1 | Infection strategies of PPNs **(A)** Ectoparasites take up nutrients from plant cells without invading the plant root. Some ectoparasites such as needle nematodes (*Longidorus* spp.) and dagger nematodes (*Xiphinema* spp.) induce the formation of nurse cells which extends the period of feeding. **(B)** Migratory endoparasites move through inside of the root tissues causing destruction *en route* and feed on plant tissues. Sedentary endoparasites include the root-knot nematodes (RKNs), *Meloidogyne* spp. and the cyst nematodes (CNs), including *Globodera* spp. and *Heterodera* spp. **(C)** Second-stage RKN juveniles enter the root near the root-tip then migrate intercellularly to the vascular cylinder where they reprogram root tissues into giant cells. After establishment of giant cells, RKN juveniles become sedentary and take up nutrients and water through a feeding stylet. Adult RKN females form an egg mass on or below the root surface. **(D)** Second-stage juveniles of the CNs move inside of the root intracellularly, causing destruction of plant tissues as they go, and establish syncytia in the vascular tissues as feeding cells. CN juveniles also become sedentary and start feeding from syncytia. Adult CN females retain eggs inside of the body, which forms a cyst after death.

RKNs and CNs are the most devastating nematodes in the world (Jones et al., 2013).

Both RKNs and CNs induce host-cell redifferentiation to establish feeding cells for own development and reproduction, but in two different ways. Infective RKN juveniles enter near the root-tip and migrate intercellularly to the vascular cylinder where feeding cells are formed. Once RKNs enter a favorable location, they induce the redifferentiation of plant cells into multinucleate giant cells by repeated nuclear divisions without cytoplasmic division (Abad et al., 2009; Escobar et al., 2015). About 4–6 weeks after infection, the pear-shaped mature adult RKN female lays eggs in a gelatinous egg mass on or below the surface of the root (Abad et al., 2009; Escobar et al., 2015). RKNs exhibit variable reproduction modes such as amphimixis, facultative parthenogenesis and obligate parthenogenesis. In particular, the most devastating RKN species, *Meloidogyne incognita*, *Meloidogyne arenaria*, and *Meloidogyne javanica*, reproduce by obligate parthenogenesis and males appear to have no role in reproduction (Castagnone-Sereno, 2006). CN juveniles enter the root and move intracellularly into the vascular cylinder where, unlike RKNs, they induce syncytia through the local dissolution of cell walls and protoplast fusion of neighboring plant cells. Hundreds of eggs are produced inside of the female body after mating. When the female dies, its body forms a cyst, which can protect the eggs for many years in the soil (Bohlmann and Sobczak, 2014; Bohlmann, 2015). Both RKNs and CNs secrete virulence effectors through a stylet to manipulate host cells for establishing feeding cells. PPNs secrete effectors include cell wall degrading enzymes, inhibitors of anti-nematodal plant enzymes, plant immune signaling suppressors, and proteins required for the establishment of feeding cells (Davis et al., 2008; Gheysen and Mitchum, 2011; Hewezi and Baum, 2013; Goverse and Smant, 2014; Smant et al., 2018; Mejias et al., 2019).

RECOGNITION OF PPNS

In general, pathogens are perceived by several different recognition systems in plants (Jones and Dangl, 2006; Dodds and Rathjen, 2010). The first recognition system is mediated by the perception of pathogen-associated molecular patterns (PAMPs) (e.g., bacterial flagellin, fungal chitin) and damage-associated molecular patterns (DAMPs) released by the disrupted host plant tissues. PAMPs and DAMPs are perceived by cell surface–localized pattern recognition receptors (PRRs), leading to pattern-triggered immunity (PTI) (Boutrot and Zipfel, 2017; Hou et al., 2019). Plant PRRs are usually either receptor-like kinases (RLKs) or receptor-like proteins (Boutrot and Zipfel, 2017). Successful pathogens secrete effector proteins into host apoplast and cytoplasm to interfere with recognition and immune signaling. In resistant plants, however, these effectors are often recognized by intracellular nucleotide-binding domain leucine-rich repeat (NLR)-type immune sensors, leading to NLR-triggered immunity (Cui et al., 2015). The N-terminus of NLR proteins usually contains a toll-interleukin 1 receptor (TIR) domain or coiled coil (CC), which are used to classify NLR proteins into two subgroups TIR-NLRs and CC-NLRs. In addition, some PRRs in resistant plants also recognize apoplastic effectors to induce PTI.

PPNs are known to induce PTI in plants. For example, ascaroside, an evolutionarily conserved nematode pheromone, is the first and only nematode PAMP identified so far (Manosalva et al., 2015). Ascr#18, the most abundant ascaroside in PPNs, activates typical plant immune responses, such as mitogen-activated protein kinases, PTI-marker gene expression, and salicylic acid– and jasmonic acid (JA)–mediated defense signaling pathways. Importantly, treatment with Ascr#18 increases resistance to both RKNs and CNs in *Arabidopsis*. Moreover, Ascr#18 is also recognized by tomato, potato, and barley, suggesting that the recognition of Ascr#18 is well conserved in both monocots and dicots. However, the corresponding PRR for recognizing Ascr#18 has not yet been identified. The first identified PRR involved in the induction of PTI in response to a PPN-derived molecule is a leucine-rich repeat (LRR)-RLK encoded by *Arabidopsis Nilr1* (*nematode-induced LRR-RLK 1*) (Mendy et al., 2017). NILR1 was isolated as an essential component for recognizing "NemaWater," an aqueous solution incubated with infective-stage juveniles of CN (*Heterodera schachtii*) and RKN (*M. incognita*) as PTI inducers. Interestingly, the extracellular receptor domain of NILR1 is widely conserved among dicots and monocots, which is consistent with the fact that NemaWater activates immune responses in tomato, sugar beet, tobacco, and rice. However, the corresponding PAMP molecule recognized by NILR1 has not been identified. The importance of PTI in immunity against PPNs has also been demonstrated in *Arabidopsis* PTI-deficient mutants (Teixeira et al., 2016; Mendy et al., 2017). The susceptibility of *Arabidopsis* to RKNs was enhanced in *bak1–5* and *bik1* mutants (Teixeira et al., 2016). BAK1 is a co-receptor for many PRRs inducing PTI, and in the BIK1 mutant, it is a required receptor-like cytoplasmic kinase for PTI signaling. *bak1–5* and *bak1–5 bkk1* (BKK1 is the closest homolog of BAK1) mutants are more susceptible to CNs (Mendy et al., 2017). Importantly, RKNs and CNs have multiple virulence effectors that are able to suppress PTI responses (Chen et al., 2013; Jaouannet et al., 2013; Lin et al., 2016; Chen et al., 2018; Naalden et al., 2018; Kud et al., 2019; Yang et al., 2019).

PPN infections induce host-cell damage, thus they likely produce DAMP(s), which results in PTI induction. For example, CNs migrate intracellularly, thus their migration results in the release of oligogalacturonides (OGs) from plant cell walls. Fungal pathogens produce cell wall degrading enzymes like polygalacturonase (PG) to digest plant cell wall materials (D'Ovidio et al., 2004), and most plants have polygalacturonase inhibitor proteins (PGIPs) that attenuate pectin degradation by PGs, resulting in OG release. The released long-chain OGs activate PTI (Bishop et al., 1981; Hahn et al., 1981; Nothnagel et al., 1983; Benedetti et al., 2015). *Arabidopsis* has two PGIPs, PGIP1, and PGIP2, both of which are rapidly expressed during the migratory stage of CNs. A genetic study showed that PGIP1 activates plant camalexin and indole-glucosinolate pathways, thus attenuating CN infection (Shah et al., 2017). In addition, exogenous treatment with OGs enhances resistance against CNs. These results suggest that upon CN infection, *Arabidopsis* PGIP1 releases OGs, triggering PTI (Shah et al., 2017). Furthermore, CN infection induces ethylene production by the host, a signaling step that delays establishment of the syncytial-phase, indicating

that damage-induced ethylene responses contribute to immunity against CNs (Marhavý et al., 2019). In contrast, there is as yet no clear evidence for damage-induced immunity against RKNs, which migrate intercellularly and are thus less-destructive than CNs. For example, neither PGIP1 nor PGIP2 are induced during the migratory stages of RKNs, and PGIP-mediated DAMP responses are not required for resistance against RKNs (Shah et al., 2017). Similarly, the loss of other DAMP receptors, PEPR1 and PEPR2 for plant elicitor peptides or DORN1 for extracellular ATP, fails to affect susceptibility to RKNs (Teixeira et al., 2016). However, it is possible that unknown DAMPs might be important for inducing immunity against RKNs, as PTI activation by exogenous application of known DAMPs is quite effective for suppressing the reproduction of RKNs (Lee et al., 2018).

NLR proteins also play critical roles in recognizing PPNs. NLRs involved in PPN recognition are mostly encoded by resistance (*R*) genes (Kaloshian et al., 2011). Well-studied *R* genes include tomato *Mi-1.2*, *Mi-9*, and *Hero-A*; potato *Gpa2* and *Gro1–4*; pepper *CaMi*; and prune *Ma* (Milligan et al., 1998; van der Vossen et al., 2000; Ernst et al., 2002; Paal et al., 2004; Chen et al., 2007; Jablonska et al., 2007; Claverie et al., 2011). *Mi-1.2*, *Mi-9*, *CaMi*, and *Ma* confer resistance against RKNs, while *Hero-A*, *Gpa2*, and *Gro1–4* provide resistance against CNs. *Gro1–4* and *Ma* encode TIR-NLRs, whereas the others encode CC-NLRs. Interestingly, Ma protein has a large and highly polymorphic C-terminal post-LRR region that is thought to be important for the recognition of PPNs (Claverie et al., 2011). Few examples of PPN avirulence factors recognized by NLRs are known. Gp-RBP-1, one of the secreted SP1a and RYanodine receptor (SPRY) domain (SPRYSEC) proteins from CN *Globodera pallida*, is an effector that induces hypersensitive response (HR)–cell death in the presence of GPA2 and Ran

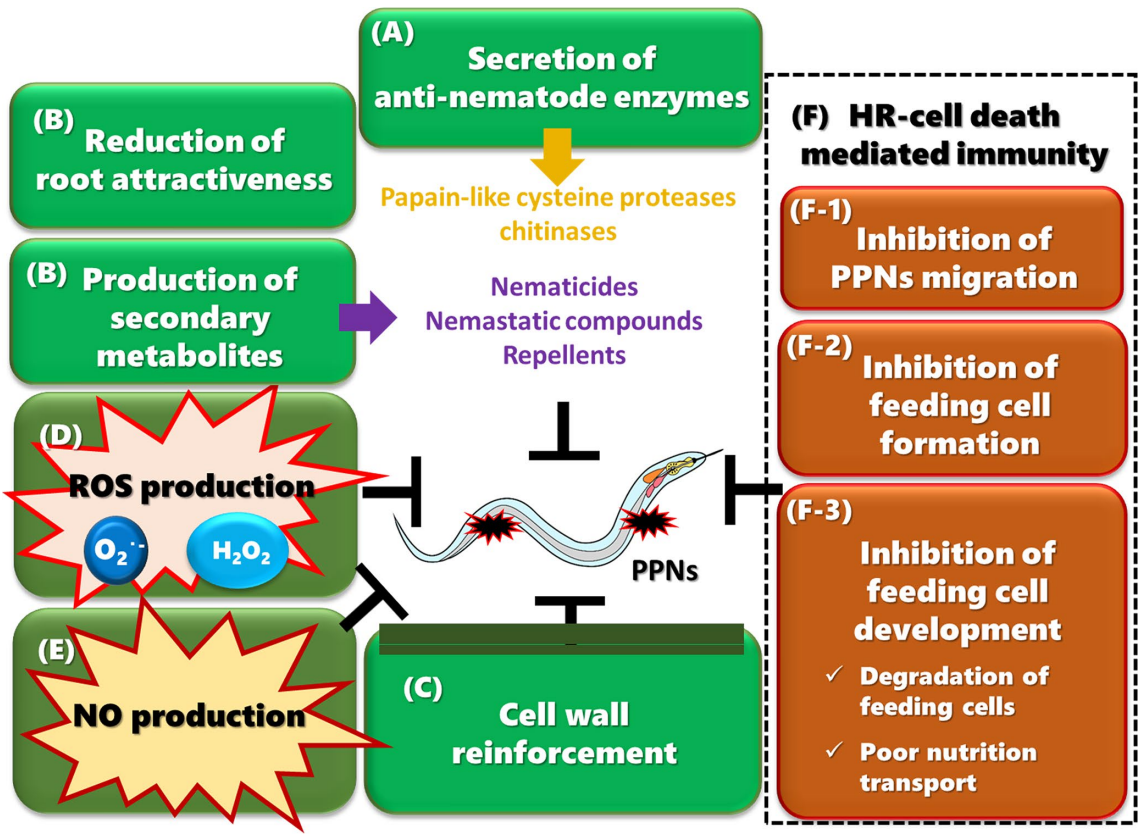

FIGURE 2 | Multiple plant immune responses against PPNs **(A)** Plants secrete anti-nematode enzymes such as papain-like cysteine proteases (PLCPs) and chitinases into the apoplast to attack PPNs. **(B)** Resistant plants produce a wide range of secondary metabolites in response to PPN infection. Some metabolites inhibit egg hatching, suppress the motility of migrating PPNs, arrest growth and development, or kill nematodes. Plants may also reduce chemoattraction by secreting less amounts of attractants or more repellents. **(C)** Plants reinforce their cell walls by accumulating lignin, suberin, and callose, which strengthen the physical barrier to PPNs. **(D)** PPN infection induces the production of ROS, which may be directly toxic to PPNs. Hydrogen peroxide plays a role in cell wall cross-linking. ROS may also work as a transducing signal to activate immune responses and to control HR-cell death. **(E)** NO production is induced upon PPN infection and may play a role in JA-mediated defense responses, possibly through the production of protease inhibitor 2. **(F)** HR-cell death is crucial for limiting PPN movement and completing the life cycle. **(F-1)** HR-cell death occurs during penetration and migration of PPNs in cortical and epidermal tissues, contributing to inhibition of migration. **(F-2)** HR-cell death is induced in cells infected by RKNs or CNs, which inhibit the formation of feeding cells. **(F-3)** HR-cell death is also induced in cells surrounding feeding cells, often resulting in degeneration of feeding cells. Even if some feeding cells survive, the nutrient transport from surrounding tissues to the feeding cells is limited, causing a reduction in the number of eggs, and production of relatively more males. Some resistant plants induce the deterioration of feeding cells without any HR-cell death of surrounding cells.

GTPase-activating protein 2 (RanGAP2) (Blanchard et al., 2005; Sacco et al., 2009). The proline residue at position 187 in the SPRY domain of Gp-RBP-1 is required for recognition by GPA2, whereas the virulent type Gp-RBP-1 variant allele has a mutation at this position, allowing it to avoid host recognition. Moreover, RanGAP2 interacts with the CC domain of GPA2 (Tameling and Baulcombe, 2007), suggesting that the RanGAP2-GPA2 complex is required for the recognition of the SPRY domain of Gp-RBP-1. Other example of an avirulence factor recognized by plants is Cg-1 in *M. javanica,* an RKN. The *Cg-1* gene is present in an *Mi-1.2*-avirulent population, but virulent RKN strains carry a deletion of *Cg-1* (Gleason et al., 2008; Gross and Williamson, 2011). Moreover, silencing of *Cg-1* in an avirulent strain increased virulence on *Mi-1.2*-containing tomato, suggesting a possible role for Cg-1 as a factor recognized by R protein Mi-1.2, although its signal transduction mechanism is unclear.

Surface-localized PRRs are also known to recognize PPN effectors. Venom allergen–like protein Gr-VAP1 from the CN *Globodera rostochiensis* interacts with apoplastic papain-like cysteine protease (PLCP) RCR3^{pim} in tomato to suppress host immunity (Lozano-Torres et al., 2012). However, Cf-2, a plasma membrane-localized receptor-like protein with extracellular LRRs, recognizes the interaction of Gr-VAP1 with RCR3^{pim}, triggering HR-cell death in resistant hosts. Notably, *Cf-2* was originally identified as a resistance gene against the fungal pathogen *Cladosporium fulvum* (Rooney et al., 2005). Similar to Gr-VAP1, *C. fulvum* secretes AVR2, which interacts with and inhibits RCR3^{pim}, and this interaction is recognized by Cf-2 protein. Thus, Cf-2 recognizes both fungal and nematode pathogens by monitoring RCR3^{pim}.

SECRETION OF ANTI-NEMATODE ENZYMES INTO THE APOPLAST

The fact that the PPN effector Gr-VAP1 inhibits RCR3^{pim}, a PLCP, implies that its enzymatic activity is important in immunity against PPNs (**Figure 2A**). Indeed, the absence of RCR3^{pim} homologs in *Arabidopsis* results in enhanced susceptibility to CN (Lozano-Torres et al., 2014). In addition to Gr-VAP1, Mc1194, an effector of RKN *Meloidogyne chitwoodi* targets another PLCP, RD21A in *Arabidopsis* (Davies et al., 2015b). Lack of RD21A leads to hyper-susceptibility to *M. chitwoodi*, showing that this PLCP also plays a positive role in immunity against RKN. However, it is not yet known how these PLCPs inhibit PPN infection.

Chitinases are also potentially important apoplastic enzymes in immunity against PPNs (**Figure 2A**). Upon fungal infection, plants often secrete chitinases, which degrade chitin in the fungal cell walls (Kumar et al., 2018; Pusztahelyi, 2018). In nematodes, chitin is the main component of the egg shell (Clarke et al., 1967; McClure and Bird, 1976; Perry and Trett, 1986) and makes up part of the pharyngeal lumen walls of *Caenorhabditis elegans* (Zhang et al., 2005), suggesting that chitinases may have anti-nematodal activity and thus contribute to immunity against PPNs. Consistent with this idea, chitinase activity and transcript levels are upregulated after PPN infection in resistant plants (Qiu et al., 1997; de-Deus Barbosa et al., 2009; Bagnaresi et al., 2013). However, there is currently no genetic evidence connecting plant chitinases to resistance against PPNs.

PRODUCTION OF ANTI-NEMATODE COMPOUNDS

Plants produce secondary metabolites in response to PPN invasion (**Figure 2B**). For instance, chlorogenic acid, a phenolic compound, is produced in various plants including solanaceous plants (Milne et al., 1965; Hung and Rohde, 1973; Pegard et al., 2005), carrots (Knypl et al., 1975), and rice (Plowright et al., 1996), suggesting a common defense response against PPN infection. Although the production of chlorogenic acid is well-correlated with PPN resistance levels, chlorogenic acid itself is only weakly nematicidal for *M. incognita* (Mahajan et al., 1985; D'Addabbo et al., 2013) with moderate activity against *Nacobbus aberrans*, a false root-knot nematode (López-Martínez et al., 2011). One possible explanation for this lack of correlation between response and effectiveness is that metabolized products of chlorogenic acid have higher nematicidal activity in the target organism, but those compounds may be unstable or highly toxic in plants. Chlorogenic acid can be hydrolyzed to quinic acid and caffeic acid, with the latter being further oxidized to orthoquinone, which is toxic to PPNs (Mahajan et al., 1985). However, the roles of caffeic acid and orthoquinone in resistance against PPNs need to be further established.

Another phenolic compound, phenylphenalenone anigorufone accumulates at the infection sites of the burrowing nematode *Radopholus similis* in a resistant banana cultivar (*Musa* sp.) (Dhakshinamoorthy et al., 2014; Hölscher et al., 2014). Anigorufone has high nematicidal activity because of the formation of large lipid–anigorufone complexes in the bodies of *R. similis*. Anigorufone is also known as an antifungal phytoalexin, and its synthesis is activated by infection with the pathogenic fungus *Fusarium oxysporum* (Luis et al., 1995). Interestingly, anigorufone also kills the human protozoan parasite *Leishmania* through the inhibition of succinate dehydrogenase in the mitochondrial respiratory complex II (Luque-Ortega et al., 2004). However, the toxic mechanism of anigorufone in PPNs and its relationship to the formation of large lipid–anigorufone complexes remains to be determined.

Flavonoids constitute a large class of secondary metabolites in plants. Some flavonoids play important roles in PPN resistance by functioning as nematicides, nemastatic compounds (which do not kill but inhibit their movement), repellents, or inhibitors of egg hatching (Chin et al., 2018). These flavonoids that have anti-nematodal activity mostly belong to the classes of flavonols (e.g., kaempferol, quercetin, myricetin), isoflavonoids, and pterocarpans (e.g., medicarpin, glyceollin). Kaempferol inhibits egg hatching of *R. similis* (Wuyts et al., 2006b). Kaempferol, quercetin, and myricetin are repellents and nemastatic to *M. incognita* juveniles (Wuyts et al., 2006b), and medicarpin also inhibits the motility of *Pratylenchus penetrans* in a concentration-dependent manner (Baldridge et al., 1998). Similarly, patuletin, patulitrin,

quercetin, and rutin are nematicidal for infective juveniles of *Heterodera zeae*, a CN (Faizi et al., 2011). The synthesis of some flavonoids is also induced during infection in resistant plants. For instance, *M. incognita*–resistant soybean cultivars accumulate glyceollins, a group of soybean-specific prenylated pterocarpan phytoalexins that are expressed upon infection (Kaplan et al., 1980). Interestingly, glyceollin inhibits the motility of *M. incognita* (Kaplan et al., 1979; Kaplan et al., 1980). Glyceollin accumulation is also higher in CN-resistant soybean cultivars than in susceptible ones. One of the glyceollin isomers, glyceollin I accumulates in tissues adjacent to the head of the CN in resistant soybean roots (Huang and Barker, 1991), suggesting accumulation of glyceollin is spacio-temporally specific to the infection site.

Apart from phenolic compounds, other nematicidal chemicals are produced by several nematode-antagonistic plants, such as marigold and asparagus, which have been used for reducing nematode populations in soil. Marigold roots secrete α-terthienyl (Gommers and Bakker, 1988; Wang et al., 2007; Faizi et al., 2011), an oxidative stress-inducing chemical that effectively penetrates the nematode hypodermis and exerts nematicidal activity (Nivsarkar et al., 2001; Hamaguchi et al., 2019). Similarly, asparagus produces asparagusic acid, which inhibits hatching of two important CNs, *Heterodera glycines* and *G. rostochiensis* (Takasugi et al., 1975).

In Brassicaceae family plants, the broad spectrum antimicrobial isothiocyanates and indole glucosinolates are considered as anti-PPN compounds. Isothiocyanates effectively inhibit hatching of CNs and RKNs (Brown et al., 1997; Yu et al., 2005) and also have toxicity to RKNs and the semi-endoparasitic nematode *Tylenchulus semipenetrans* (Zasada and Ferris, 2003). In *Arabidopsis*, the synthesis of camalexin, an indole alkaloid glucosinolate-type phytoalexin, is catalyzed by three cytochrome P450-dependent monooxygenases, CYP79B2, CYP79B3 (Hull et al., 2000; Mikkelsen et al., 2000; Bak et al., 2001; Mikkelsen et al., 2004), and PAD3 (phytoalexin-deficient 3, CYP71B15). Double mutants *cyp79b2/b3* which do not accumulate indolic glucosinolates are more susceptible to CNs (Shah et al., 2017), while *pad3,* camalexin-deficient mutants are more susceptible to RKNs than wild type (Teixeira et al., 2016). These results suggest that some indole glucosinolates including camalexin have some inhibitory effects on PPNs, but there have so far been no reports of direct toxicity of indolic glucosinolates on PPNs.

In addition to nematicides and nemastatic compounds, interruption of PPN chemotaxis may also be an effective plant response for inhibiting or limiting PPN infection. Ethylene, which is normally produced after wounding as well as during pathogen invasion, reduces PPN attraction to the root (Booker and DeLong, 2015; Guan et al., 2015; Marhavý et al., 2019). An ethylene-overproducing *Arabidopsis* mutant is less attractive for PPNs, and attractiveness is greater in plants treated with ethylene-synthesis inhibitors or in ethylene-insensitive mutants (Fudali et al., 2013; Hu et al., 2017). These results suggest that PPN infection induces ethylene production, which possibly prevents secondary PPN invasion by reducing attractiveness. The reduced attractiveness could be due to a reduction in attractant secretion or an increase in repellents. However, the molecular basis of the attractiveness for PPNs is still largely unknown. Several groups have tried to identify RKN attractants from root tips (Čepulytė et al., 2018) and seed-coat mucilage (Tsai et al., 2019). The identification of chemoattractants and chemorepellents may offer some insight into how plants respond to nematodes in the rhizosphere both before and during PPN infection.

REINFORCEMENT OF CELL WALL AS A PHYSICAL BARRIER

Since all PPNs must penetrate the cell wall for feeding, reinforcement of cell wall structure has been implicated as an effective defense as a physical barrier (**Figure 2C**). For instance, PPN infection often induces accumulation of lignin in resistant plants (Balhadère and Evans, 1995a; Balhadère and Evans, 1995b; Andres et al., 2001; Dhakshinamoorthy et al., 2014). Moreover, *Arabidopsis* mutants with increased levels of syringyl lignin have reduced *M. incognita* reproduction rates (Wuyts et al., 2006a). These results suggest that lignin accumulation in roots is an effective antagonist to PPN infection.

The effectiveness of lignin accumulation for suppressing nematode infection is also supported by plant immune inducers such as β-aminobutyric acid (BABA), thiamine, and sclareol. BABA, a non-protein amino acid, has broad efficacy against viruses, bacteria, fungi, and oomycetes in various plants (Alexandersson et al., 2016; Cohen et al., 2016). Treatment with BABA inhibits RKN invasion, delays giant cell formation, and retards RKN development. Interestingly, BABA induces lignin accumulation in roots, and callose accumulation in galls (Ji et al., 2015). Thiamine (vitamin B1) treatment also induces lignin accumulation in roots; enhances the expression of phenylalanine ammonia-lyase, a key enzyme of the phenylpropanoid biosynthesis pathway; reduces PPN penetration; and delays PPN development (Huang et al., 2016). An inhibitor of phenylalanine ammonia-lyase suppresses thiamin-mediated immunity, indicating that activation of the phenylpropanoid pathway with subsequent lignin accumulation is important for thiamin-mediated immunity against nematodes. Treatment with sclareol, an antimicrobial compound with activity against some plant-pathogenic bacteria and fungi (Bailey et al., 1975; Kennedy et al., 1992; Seo et al., 2012), also induces lignin accumulation and suppresses RKN penetration (Fujimoto et al., 2015). Importantly, an *Arabidopsis* mutant of *cinnamoyl-coA reductase* (*ccr2*) defective in lignin accumulation cannot induce sclareol-mediated suppression of RKN penetration, suggesting that lignin accumulation is important for the sclareol-mediated immunity.

Similar to lignin accumulation, callose deposition and suberin accumulation may also reinforce cell walls and contribute to immunity against PPNs. The RKN *Meloidogyne naasi* induces callose deposition at an early infection stage, and suberin accumulation at a later stage in the resistant grass plant *Aegilops variabilis* (Balhadère and Evans, 1995a; Balhadère and Evans,

1995b). Infection of *Arabidopsis* by RKN or CN also induces transcriptional activation of suberin biosynthesis genes at the site of infection (Holbein et al., 2019). Overexpression of the transcription factor RAP2.6 in *Arabidopsis* leads to enhanced callose deposition at syncytia and results in higher resistance to CN (Ali et al., 2013). RAP2.6 is strongly downregulated in syncytia compared to uninfected root; therefore, it is possible that CN suppresses RAP2.6 expression to inhibit callose deposition within syncytia.

Lignin and suberin in suberin lamellae and casparian strips at the endodermis are also important basal physical barriers to RKNs. RKNs are not able to directly cross the endodermis because of the reinforcement of cell walls by suberin lamellae and casparian strips (Wyss et al., 1992; Abad et al., 2009). Indeed, *Arabidopsis* mutants defective in casparian strips are more susceptible to RKNs (Holbein et al., 2019).

REACTIVE OXYGEN SPECIES (ROS)

The rapid production of ROS, such as superoxide anion and hydrogen peroxide, is a conserved signaling response across kingdoms, and in plants, it is induced at an early stage of PPN infection (**Figure 2D**). ROS have direct antimicrobial properties but also serve as signaling molecules to activate additional and complementary immune outputs such as strengthening cell walls by cross-linking polymers, amplifying and propagating intra- and intercellular defense signals, and regulating HR-cell death (Torres et al., 2006; Kadota et al., 2015). Resistant tomato plants carrying the *Mi-1.2* gene respond to RKN infection with a strong and prolonged induction of ROS. On the other hand, susceptible tomato plants have weak and transient ROS induction in response to nematode infection (Melillo et al., 2006; Melillo et al., 2011; Zhou et al., 2018). Similarly, strong ROS production is induced in *Arabidopsis* roots during incompatible interactions with the soybean CN *H. glycines* (Waetzig et al., 1999). Histochemical studies showed that hydrogen peroxide accumulates in the apoplast after infection of the avirulent RKNs or CNs (Waetzig et al., 1999; Melillo et al., 2006).

The plasma membrane-bound NADPH oxidase respiratory burst oxidase homologs (RBOHs) are important for the production of apoplastic ROS (Kadota et al., 2015). In tomato, whitefly-induced 1 (WFI1), an RBOH homolog, is required for Mi-1.2-mediated ROS accumulation during RKN infection. Consistently, HSFA1, a class-A heat-shock factor that regulates *Wfi1* transcription by binding to the *Wfi1* promoter, is also critical for *Mi-1.2*-mediated ROS production (Zhou et al., 2018). In *Arabidopsis*, which has 10 RBOHs, RBOHD is the primary source of ROS production during PTI and NLR-triggered immunity. RBOHF may also work redundantly with RBOHD in some responses, because the *rbohD rbohF* double mutant has a stronger defense response phenotype against bacterial pathogens (Torres and Dangl, 2005; Torres et al., 2006). Similarly, *rbohD rbohF* produces more galls after RKN infection than the wild type (Teixeira et al., 2016), indicating a positive role for RBOHD and RBOHF ROS production in immunity against RKNs. Interestingly, fewer CNs develop in *rbohD rbohF* double mutant, suggesting that CNs require a different level of ROS control by RBOH for successful establishment of infection. Furthermore, *rbohD rbohF* exhibits larger regions of HR-cell death and less syncytium formation upon CN infection, suggesting that CNs utilize RBOHD- and RBOHF-mediated ROS to suppress HR-cell death in the host (Siddique et al., 2014).

To protect themselves from the toxicity of produced ROS by the host, endoparasitic nematodes may have evolved a number of antioxidant enzymes on their surface and in the hypodermis (Henkle-Dührsen and Kampkötter, 2001). For example, both CNs and RKNs produce peroxiredoxins; some of the most abundant detoxifying antioxidant enzymes, which remove hydrogen peroxides from the apoplast of host plants by thioredoxin (TRX) cysteine thiol-disulfide exchange (Robertson et al., 2000; Henkle-Dührsen and Kampkötter, 2001; Dubreuil et al., 2011). PRX2.1, a clade B peroxiredoxin in *M. incognita*, is expressed upon infection, and knock-down of the gene reduces resistance against oxidative stress, resulting in fewer galls. This interaction suggests a critical role for PRX2.1 in infection. CNs also secrete GPX-1, a glutathione peroxidase variant, from the hypodermis to scavenge host-derived ROS, thereby protecting external cell membranes from oxidation (Jones et al., 2004). *M. incognita* glutathione-S-transferases are delivered into the host apoplast to detoxify the products of oxidative stress (Dubreuil et al., 2007). Indeed, freshly hatched infective juveniles of *M. incognita* are much more resistant to exogenous treatment with hydrogen peroxide than *C. elegans* (Isermann et al., 2004).

Another PPN strategy for protection against host ROS is to activate the host ROS-scavenging system by the secretion of virulence effectors. For example, CN effector 10A06 interacts with host spermidine synthase 2 and increases spermidine content in infected tissues (Hewezi et al., 2010). Spermidine in higher concentrations functions as a ROS scavenger, and in lower concentrations, it indirectly decreases oxidative stress by activating cellular antioxidant systems (Kasukabe et al., 2004). Indeed, ectopic expression of 10A06 in *Arabidopsis* increases the expression of several genes encoding antioxidant enzymes. Similarly, MjTTL5, a virulence effector from *M. javanica* interacts with the *Arabidopsis* ferredoxin:TRX reductase (FTR) catalytic subunit (FTRc) in plastids (Lin et al., 2016). FTR activates TRXs in chloroplasts or plastids by receiving reducing equivalents from reduced ferredoxin (Balmer et al., 2006; Kirchsteiger et al., 2012). The interaction of MjTTL5 with FTRc drastically increases host ROS-scavenging activity, thus modulating the plant immune reaction. Because peroxiredoxins use TRX to reduce hydrogen peroxide (Broin et al., 2002; Kotze, 2003), it is possible that FTRc works in part with peroxiredoxins by providing reduced TRX to lower ROS production in plants.

NITRIC OXIDE (NO) AND PROTEASE INHIBITOR-BASED IMMUNITY

NO is an essential signaling molecule that has multiple functions in plants (Delledonne et al., 1998; Torres et al., 2006; Bellin et al., 2013; Mur et al., 2013; Scheler et al., 2013) (**Figure 2E**). After infection with *M. incognita,* resistant tomato plants carrying

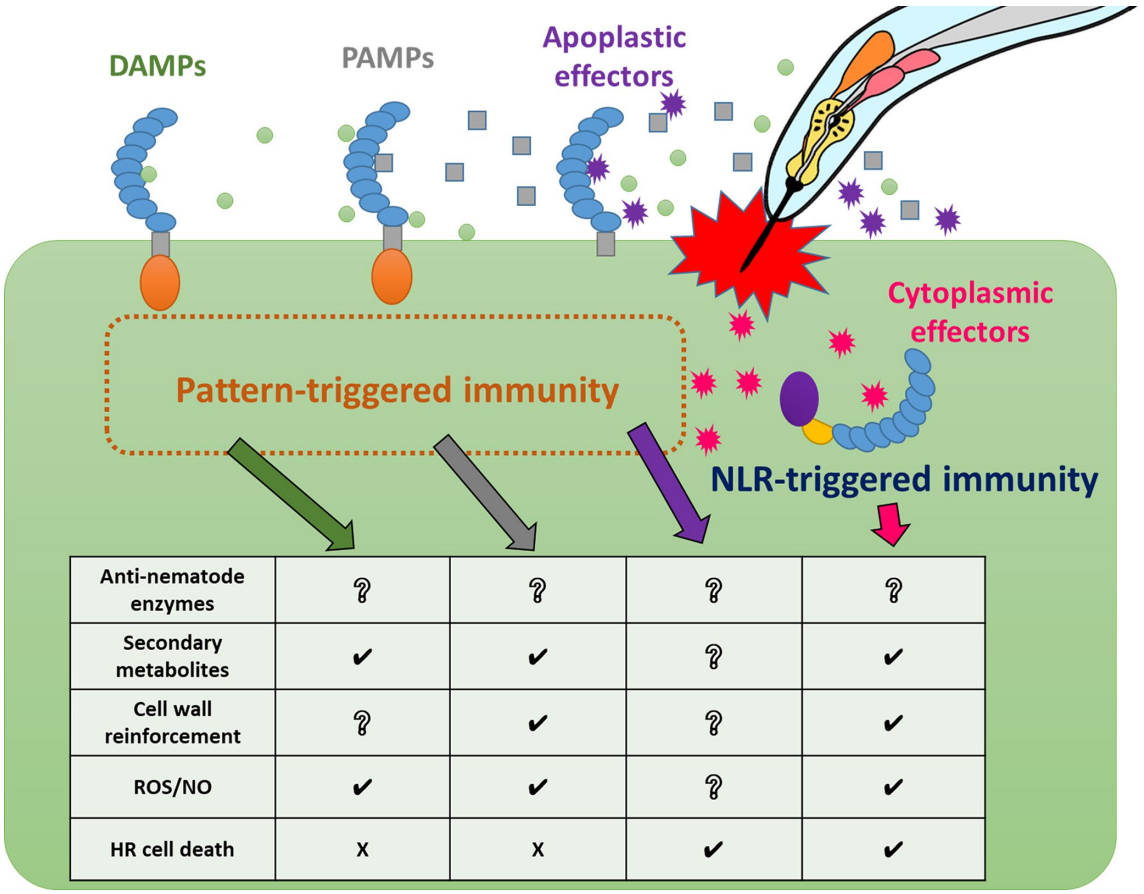

FIGURE 3 | Relationships between nematode recognition and immune responses Plants activate pattern-triggered immunity and NLR-triggered immunity against PPN infection using different immune receptors. These receptors trigger a variety of defense responses. Some immune responses, such as ROS/NO production, are induced in common by some immune receptors with different kinetics, while other responses, such as cell death, are induced by specific immune receptors.

Mi-1.2 produce more NO than susceptible cultivars (Melillo et al., 2011). Application of an exogenous NO donor, sodium nitroprusside (SNP), to susceptible tomato plants significantly enhances immunity against RKNs (Zhou et al., 2015). Treatment with SNP reduces the number of egg masses and restores the growth inhibition associated with PPNs, suggesting that NO plays a positive role in immunity. NO may be involved in the JA-dependent RKN defense pathway, as an NO scavenger partially inhibitis JA-induced RKN defense responses. Moreover, the inhibition of JA biosynthesis by chemical inhibitors significantly increased susceptibility to RKNs, but resistance was effectively restored by exogenous SNP application. Because both JA- and SNP-induced RKN defense responses are compromised by silencing *protease inhibitor 2* (*PI2*), the NO- and JA-pathways likely converge to induce immunity against PPNs (Zhou et al., 2015). However, it remains unclear which proteases PI2 inhibits. Since PPNs use a variety of proteases for their virulence and for their development (Urwin et al., 1997; Neveu et al., 2003), these activities can be inhibited by PI2. Interestingly, heterologous expression of various protease inhibitors, including trypsin inhibitors and cysteine protease inhibitors, confer resistance against PPNs, showing the effectiveness of protease inhibitor-based immunity against PPNs (Hepher and Atkinson, 1992; Urwin et al., 2000; Urwin et al., 2003).

HR-CELL DEATH-BASED INHIBITION OF NEMATODE DEVELOPMENT

HR-cell death, a type of programmed cell death that is induced after the invasion of avirulent pathogens to prevent the spread of biotrophic pathogens (Huysmans et al., 2017), also plays a crucial role in PPN immunity (**Figure 2F**). HR-cell death has been observed at three different phases of PPN infection in resistant plants: (1) in the cortex and epidermis during PPN penetration and migration (Hung and Rohde, 1973; Thomason et al., 1976; Finetti Sialer, 1990; Balhadère and Evans, 1995b; Pegard et al., 2005; Proite et al., 2008; Albuquerque et al., 2010; Khallouk et al., 2011; Cabasan et al., 2014; Davies et al., 2015a), (2) in vascular tissues during the initiation of feeding cell formation (Paulson and Webster, 1972; Melillo et al., 2006), and (3) in cells adjacent to developing feeding cells (Kim et al., 1987; Rice et al., 1987;

Sobczak et al., 2005; Kim et al., 2010; Kim et al., 2012; Cabasan et al., 2014; Seo et al., 2014; Ye et al., 2017).

During PPN penetration and migration, cell death is also often observed in susceptible plants, but it is less rapid and less frequent than in resistant varieties (Endo and Veech, 1970; Thomason et al., 1976; Sobczak et al., 2005). HR-cell death may inhibit nematode migration, but it is not clear if HR-cell death stops PPN movement directly, or indirectly by releasing nemastatic or nematicidal chemicals or DAMPs to activate other immune responses. HR-cell death is also induced during the initiation of feeding cell development. For instance, *Mi-1.2*-resistant tomato plants induce HR-cell death during the RKN induction of giant cells, thus inhibiting the development of feeding cells (Paulson and Webster, 1972; Melillo et al., 2006). Another possible function of HR-cell death is to create a physical gap between feeding cells and surrounding cells to block nutrient and water supplies. For example, in resistant tomato lines carrying the *Hero* gene, potato CN (*G. rostochiensis*) makes syncytia, but HR-cell death is induced in surrounding cells, which resulted in the separation of the syncytium from stelar conductive tissues (Sobczak et al., 2005). Disconnection of feeding cells from surrounding tissue also occurs in resistant plants after infection with RKNs (Seo et al., 2014; Ye et al., 2017). Disassociation of surrounding tissue leads to poor nutrient supply, thereby inhibiting growth or causing the death of feeding cells, reducing fecundity in females, and increasing male development (Acedo et al., 1984; Rice et al., 1987; Kouassi et al., 2004; Sobczak et al., 2005). Increased male development coincides with a reduced number of females, resulting in the reduction of PPN eggs. In some resistant plants, death of feeding cells is also induced without HR-cell death of surrounding cells. For example, death of syncytia is induced in resistant soybeans (Yan and Baidoo, 2018), and deterioration of giant cells is induced in resistant cowpea carrying *Rk* gene without typical HR-cell death in surrounding cells (Das et al., 2008). These differences in HR-cell death initiation site may depend on the specific expression pattern of host *R* genes (Yan and Baidoo, 2018) and PPN effectors.

The importance of HR-cell death is supported by the observation that both RKNs and CNs have effectors that suppress HR-cell death. The *M. incognita* effector MiISE5, a zinc-finger protein, suppresses HR-cell death induced by the non-host bacterial pathogen, *Burkholderia glumae* in *N. benthamiana*, possibly through reprogramming of the host transcriptome (Shi et al., 2018). The RKN effector MeTCTP from *Meloidogyne enterolobii* also suppresses HR-cell death triggered by the mouse pro-apoptotic protein, Bcl2 associated X protein (Zhuo et al., 2017). CNs also have HR-cell death suppression effectors such as SPRYSEC effectors (Ali et al., 2015b), RHA1B, an E3 ubiquitin ligase (Kud et al., 2019), and GrEXPB2, an expansin-like protein (Ali et al., 2015a). However, these CN effectors do not specifically inhibit HR-cell death but also inhibit other defense responses.

CONCLUSIONS AND FUTURE DIRECTIONS

As a result of the identification of several NLR-type and PRR-type receptors involved in immunity against PPNs, we have gradually begun to understand how plants recognize and respond to nematode infection at the molecular level. However, PPN effectors and PAMPs are still largely unknown, and the corresponding receptors remain unidentified. Similarly, various immune responses against nematodes in a wide range of resistant crop and model plants have been recognized (**Figure 2** and **Supplementary Table 1**), but there is still much that is unknown between the phenomena of PPN recognition and the triggering of specific immune responses (**Figure 3**). Thus, significant challenges for future research in the field of plant and nematode interactions would be to identify immune receptor-ligands pairs (PAMPs, DAMPs, and effectors), to clarify the molecular bases of signaling pathways leading to individual immune responses, to understand the interactions of these components and signaling pathways in PPN immunity, and to identify the molecular components that define host specificity. Loss of significant agricultural productivity in a burgeoning global population goes beyond monetary losses. The absence of truly effective strategies for controlling nematode populations and infection has serious and worsening consequences for sustainable agriculture. Understanding the molecular mechanisms of PPN recognition and immune signaling networks will provide a knowledge base for much-needed PPN disease control strategies in the future.

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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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01165/full#supplementary-material

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MicroRNAs, New Players in the Plant–Nematode Interaction

Stéphanie Jaubert-Possamai†, Yara Noureddine† and Bruno Favery*

ISA, INRA, Université Côte d'Azur, CNRS, Sophia Antipolis, France

Plant-parasitic root-knot and cyst nematodes are microscopic worms that cause severe damage to crops and induce major agricultural losses worldwide. These parasites penetrate into host roots and induce the formation of specialized feeding structures, which supply the resources required for nematode development. Root-knot nematodes induce the redifferentiation of five to seven root cells into giant multinucleate feeding cells, whereas cyst nematodes induce the formation of a multinucleate syncytium by targeting a single root cell. Transcriptomic analyses have shown that the induction of these feeding cells by nematodes involves an extensive reprogramming of gene expression within the targeted root cells. MicroRNAs are small noncoding RNAs that act as key regulators of gene expression in eukaryotes by inducing the posttranscriptional silencing of protein coding genes, including many genes encoding transcription factors. A number of microRNAs (miRNAs) displaying changes in expression in root cells in response to nematode infection have recently been identified in various plant species. Modules consisting of miRNAs and the transcription factors they target were recently shown to be required for correct feeding site formation. Examples include miR396 and *GRF* in soybean syncytia and miR159 and MYB33 in *Arabidopsis* giant cells. Moreover, some conserved miRNA/target modules seem to have similar functions in feeding site formation in different plant species. These miRNAs may be master regulators of the reprogramming of expression occurring during feeding site formation. This review summarizes current knowledge about the role of these plant miRNAs in plant–nematode interactions.

Keywords: root-knot nematodes, cyst nematodes, galls, syncytium, microRNAs, siRNAs

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****Correspondence:***
Stéphanie Jaubert-Possamai
stephanie.jaubert@inra.fr

†These authors have contributed equally to this work

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INTRODUCTION

Sedentary endoparasitic nematodes are the most damaging plant-parasitic nematodes (PPNs) that cause massive crop yield losses worldwide (Blok et al., 2008). There are two main groups of PPNs: the root-knot nematodes (RKNs) of the genus *Meloidogyne* and the cyst nematodes (CNs) of the genera *Heterodera* and *Globodera* (Jones et al., 2013). After penetrating the root and migrating to the vascular cylinder, mobile second-stage juvenile (J2) selects one (CNs) or a few (RKNs) initial root cells, into which it injects a cocktail of secretions that transform these cells into hypertrophied multinucleate feeding cells that supply nutrients required for nematode development: the giant cells induced by RKNs (**Figure 1A**) or the syncytium induced by CNs (**Figure 1B**).

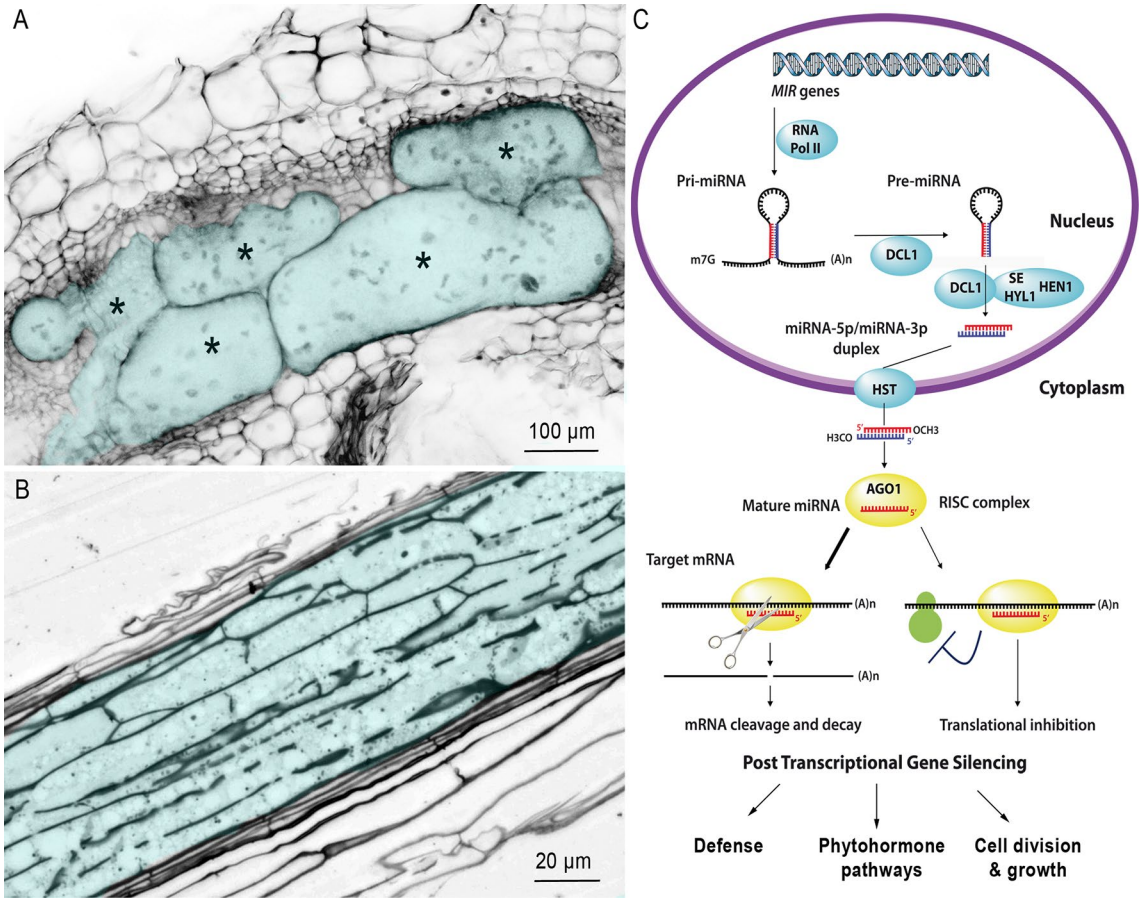

FIGURE 1 | Multinucleate and hypertrophied feeding cells induced by RKN and CN. **(A)** Confocal section of a gall induced by *M. incognita* in *Nicotiana benthamiana*. Galls were fixed and cleared with the BABB method described by Cabrera et al. (2018). Giant cells are colored in blue and marked with an asterisk to differentiate them from surrounding cells of normal size. Bar = 100 µm. **(B)** Longitudinal section of a syncytium induced by the CN *H. schachtii* in Arabidopsis roots, 10 days after inoculation. The syncytium is colored in blue. Bar = 20 µm. **(C)** Simplified biogenesis and mechanism of action of miRNAs in plants. The *MIR* genes are transcribed by RNA polymerase II (RNA Pol II) to generate single-stranded hairpin-containing primary transcripts (pri-miRNA). The pri-miRNA is then cleaved, in the nucleus, by Dicer-like 1 (DCL1), in association with hyponastic leaves 1 (HYL1) and serrate (SE), to produce a precursor miRNA (pre-miRNA). The pre-miRNA is, in turn, cleaved by DCL1 and its cofactors, thus generating a duplex composed of the mature miRNA and its complementary strand. The HUA ENHANCER 1 protein (HEN1) then adds a methyl group to the OH end of each strand of the miRNA duplex, to protect against degradation. The miRNA duplex is then actively transported from the nucleus to the cytosol through interaction with the hasty (HST) exportin. One of the two strands of the duplex is then loaded onto the argonaute 1 (AGO1) protein, the main constituent of the multiprotein RNA-induced silencing complex (RISC). The AGO1-associated strand guides the RISC to target mRNAs by sequence complementarity, resulting in target cleavage or the inhibition of protein synthesis (reviewed by Yu et al., 2017).

Common and Specific Processes Involved in Feeding Site Formation

Both hypertrophied and multinucleate feeding cells are highly active metabolically and have a dense cytoplasm, with a large number of organelles and invaginated cell wall (**Figure 1A**, **B**) (Grundler et al., 1998; Sobczak and Golinowski, 2011; Favery et al., 2016). They accumulate sugars and amino acids (Hofmann et al., 2010; Baldacci-Cresp et al., 2012). The nuclei and nucleoli of both giant cells and syncytia are larger than normal root cells, due to endoreduplication (de Almeida Engler and Gheysen, 2013). However, these two feeding structures have very different ontogenies. RKN J2 selects five to seven parenchyma cells and induces their dedifferentiation into giant cells through successive mitosis without cytokinesis (Caillaud et al., 2008b). Expansion of giant cells by isotropic growth (Cabrera et al., 2015) together with hyperplasia of the root cells surrounding the giant cells results in a swelling of the root, known as a gall, the characteristic symptom of RKN infection. By contrast, CN J2 targets a single initial root cell. This cell expands within the vascular tissue by progressive cell wall dissolution and incorporation into the syncytium of adjacent cells *via* cytoplasm fusion (Golinowski et al., 1996; Grundler et al., 1998).

Studies of the feeding site formation have greatly benefited from whole-transcriptome analyses. Such analyses were initially developed in the model host plant *Arabidopsis thaliana* and were then extended to various crop species (Escobar et al., 2011; Favery et al., 2016; Yamaguchi et al., 2017). All these analyses showed that feeding site formation involves an extensive reprogramming of gene expression within the root

cells targeted by the nematodes. These analyses suggested that CNs and RKNs establish feeding sites by recruiting and/or manipulating several plant functions, including plant defense and phytohormone pathways (Gheysen and Mitchum, 2019), cell wall modification (Sobczak and Golinowski, 2011), cytoskeleton (Caillaud et al., 2008a), and the cell cycle (de Almeida Engler and Gheysen, 2013). These analyses also revealed the conservation of some nematode-responsive genes within the plant kingdom (Portillo et al., 2013).

MicroRNAs Are Key Regulators of Gene Expression

Plant miRNAs are 20- to 22-nucleotide-long noncoding RNAs (Bartel, 2004) that regulate gene expression through posttranscriptional gene silencing. Plant miRNA precursors are produced from *MIR* genes and are processed by several proteins, including Dicer-like 1 (DCL1), to generate a mature miRNA duplex. One strand of the duplex is loaded into the RNA-induced silencing complex (RISC), in which its sequence complementarity directs gene silencing (**Figure 1C**) (Yu et al., 2017). Perfect miRNA/mRNA complementarity generally induces cleavage of the mRNA at nucleotide position 10 or 11 (Franco-Zorrilla et al., 2007; Bartel, 2009). However, in some cases, such as the miR172/*APETALA2* module in *Arabidopsis*, the miRNA inhibits mRNA translation (Chen, 2004; Zhang and Li, 2013). Interestingly, the miRNA target may activate the expression of its regulator miRNA, e.g. CUC2 and *MIR164a* (Nikovics et al., 2006). Therefore, regulation of genes by miRNA does not always imply a negative correlated expression between mature miRNA and the targeted transcripts. Plant *MIR* genes are often organized into multigene families in which the sequences of the precursors differ, but the mature sequences are almost identical, suggesting that they share some target mRNAs (Palatnik et al., 2007). Moreover, many *MIR* families are conserved between evolutionarily distant plant species, either targeting conserved genes or having different targets in different plant species (Jones-Rhoades, 2012). Small regulatory RNAs are major regulators of gene expression in plant development and in responses to various microorganisms such as beneficial mycorrhizal fungi (Bazin et al., 2013) and fungal (Park et al., 2014) or bacterial pathogens (Navarro et al., 2006). Plant miRNA may regulate the plant defense or the neoformation of specific structures during plant–microbe interactions (Combier et al., 2006; Park et al., 2014; Lee et al., 2015). Plant-parasitic nematodes induce the neoformation of feeding structures within host roots by inducing an extensive reprogramming of gene expression in the targeted root cells. The role of small noncoding RNAs in the plant–nematode interaction was established with the increased resistance to RKN and CN of *A. thaliana* mutants disrupted for miRNA or siRNA pathway (Hewezi et al., 2008; Medina et al., 2017; Ruiz-Ferrer et al., 2018). The development of sequencing technologies has made it possible to initiate studies of the role of plant miRNAs in this process in various plant species. This review provides an overview of current knowledge about of the conserved and species-specific plant miRNAs involved in responses to RKNs and CNs.

Plant MicroRNAs Responding to RKNs

The identification of novel and differentially expressed (DE) miRNAs involved in plant response to nematodes is based principally on the sequencing of small RNAs (< 35 nt) from infected and uninfected root tissues. If three independent replicates *per* sample are available, the comparison can be performed directly, by digital expression profiling. Otherwise, sequencing identifies the miRNAs expressed in the samples analyzed, and the levels of these miRNAs are then compared between samples by reverse transcriptase–quantitative polymerase chain reaction (RT-qPCR). The miRNAs involved in the gall formation induced by RKN have been investigated in *Arabidopsis* dissected galls and uninfected roots, 3 (Cabrera et al., 2016), 7, and 14 dpi (Medina et al., 2017). This approach identified 62 miRNAs as DE in galls induced by *Meloidogyne javanica* at 3 dpi, and 24 miRNAs as DE in galls induced by *Meloidogyne incognita* at 7 and/or 14 dpi. Only two DE miRNAs with the same expression profile were common to these three stages of gall formation: miR390, which is upregulated in galls, and miR319, which is repressed in galls. Using RT-qPCR, identified 17 miRNAs as DE in tomato galls at one or more of the five developmental stages analyzed (Kaur et al., 2017), while Pan et al. (2019) identified 16 miRNAs as DE in whole cotton roots infected by *M. incognita* at 10 dpi (**Table 1**). A comparison of susceptible and resistant tomato cultivars identified five RKN-responsive miRNAs in the WT and/or the jasmonic acid–deficient *spr2* mutant at 3 dpi (Zhao et al., 2015). Some conserved miRNA families present similar expression profiles in galls from different plant species at similar time points. For example, the evolutionarily conserved miR159 is upregulated in *Arabidopsis*, tomato, and cotton galls at 10 to 14 dpi, and miR172 is upregulated in *A. thaliana* and tomato at 3 to 4 dpi (**Table 1**). The genes targeted by miRNAs have been identified by *in silico* prediction (Zhao et al., 2015; Cabrera et al., 2016; Pan et al., 2019) or by 5′ RNA ligase-mediated (RLM)–rapid amplification of cDNA ends (RACE) sequencing (Kaur et al., 2017). The expression profiles of genes predicted or known to be targeted by miRNAs were analyzed by transcriptomic analysis or RT-qPCR. A negative correlation between the levels of several DE miRNAs and their targeted transcripts, for miR156/SPB or miR159/MYB, for example, was observed in galls from *Arabidopsis*, tomato, and cotton (Zhao et al., 2015; Cabrera et al., 2016; Pan et al., 2019).

Multiple miRNAs have been shown to be DE, but the functions of only four plant miRNAs in plant-RKN interactions have been validated to date. Functional validation involves the characterization of expression profile, often with reporter gene lines or by *in situ* hybridization, and analyses of the infection status of plants with modified expression or functions for either miRNAs (e.g. overexpression, KO or buffering "target mimicry" lines) or their targets (e.g. overexpression of a miRNA-resistant form, with a mutation in the miRNA target site or knockout lines). For example, miR319 is upregulated in tomato galls at 3 dpi, whereas its target, *TCP4* (*TEOSINTE BRANCHED 1/CYCLOIDEA/PROLIFERATING FACTOR 4*), is downregulated (Zhao et al., 2015). Tomato plants overexpressing a miR319-resistant *TCP4* have fewer galls and higher levels of endogenous JA, whereas the opposite effect is observed in lines overexpressing *Ath-MIR319*. These results

TABLE 1 | List of functionally validated miRNAs differentially expressed in response to RKN and/or CN.

miRNA	Host plant	Infected material	Nematode species[a]	miRNA regulation[b]					References
				3 or 4	7	10	14	27-30	
miR159	*Arabidopsis*	Galls	*M. javanica*						Cabrera et al., 2016
		Galls	*M. incognita*						Medina et al., 2017
	Tomato	Roots							Zhao et al., 2015
		Roots							Kaur et al., 2017
		Roots	*G. rostochiensis*						Koter et al., 2018; Święcicka et al., 2017
	Cotton	Roots	*M. incognita*						Pan et al., 2019
miR172	*Arabidopsis*	Galls	*M. javanica*						Díaz-Manzano et al., 2018 (pre-miRNA)
		Galls	*M. javanica*						Cabrera et al., 2016 (mature)
		Roots	*H. schachtii*	*172c*	*172c*				Hewezi et al., 2008
		Roots	*H. schachtii*		*172a*				Hewezi et al., 2008
	Tomato	Galls	*M. javanica*						Díaz-Manzano et al., 2018
		Galls	*M. incognita*						Kaur et al., 2017
		Roots	*G. rostochiensis*						Koter et al., 2018
	Pea	Galls	*M. javanica*						Díaz-Manzano et al., 2018
miR319	*Arabidopsis*	Galls	*M. javanica*						Cabrera et al., 2016
		Galls	*M. incognita*						Medina et al., 2017
	Tomato	Roots	*M. incognita*						Zhao et al., 2015
		Roots	*G. rostochiensis*						Koter et al., 2018
	Cotton	Roots	*M. incognita*						Pan et al., 2019
miR390	*Arabidopsis*	Galls	*M. javanica*						Cabrera et al., 2016
		Galls	*M. incognita*						Cabrera et al., 2016
	Cotton	Roots	*M. incognita*						Pan et al., 2019
	Tomato and pea	Galls	*M. incognita*						Díaz-Manzano et al., 2018
miR396	*Arabidopsis*	Roots	*H. schachtii*	*396a*	*396a*				Hewezi et al., 2008; Hewezi et al., 2012
		Roots	*H. schachtii*	*396b*	*396b*				Hewezi et al., 2008; Hewezi et al., 2012
	Tomato	Roots	*M. incognita*						Zhao et al., 2015; Kaur et al., 2017
		Roots	*G. rostochiensis*						Święcicka et al., 2017
	Cotton	Roots	*M. incognita*						Pan et al., 2019
	Soybean		*H. glycines*						Noon et al., 2019
miR827	*Arabidopsis*	Roots	*H. schachtii*						Hewezi et al., 2016
	Cotton	Roots	*M. incognita*						Pan et al., 2019
miR858	*Arabidopsis*	Galls	*H. schachtii*						Piya et al., 2017

[a]*nematodes species: RKN in yellow, CN in pink.*

[b]*expression pattern between 3 and 27-30 dpi; up-regulated in infected material in red; down-regulated in infected material in green.*

suggest that the miR319/TCP4 module is essential in tomato galls by modulating the JA biosynthesis induced by RKN invasion (Zhao et al., 2015). miR159 is a conserved family of miRNAs upregulated in *Arabidopsis* galls at 14 dpi (Medina et al., 2017). Studies on transgenic GUS lines demonstrated the posttranscriptional regulation of MYB33, the main target of miR159, in *Arabidopsis* galls at 14 dpi. The *mir159abc* triple loss-of-function mutant displays enhanced resistance to RKN, with decreased numbers of galls and egg masses, demonstrating the role of the miR159 family in the response of *Arabidopsis* to *M. incognita*, probably through the regulation of MYB33. Furthermore, *in situ* hybridization has shown that *miR159* is also expressed in tomato giant cells (Medina et al., 2017) and a conserved upregulation of miR159 associated with a downregulation of MYB transcription factors has also been observed in galls from tomato (3 dpi and 13-15 dpi; Zhao et al., 2015; Kaur et al., 2017) and cotton (10 dpi; Pan et al., 2019). These results suggest that the function of the miR159/MYB module may be conserved in the galls *Arabidopsis*, tomato and cotton (Medina et al., 2017). The conserved auxin-responsive miR390 family is overexpressed in *A. thaliana* galls at 3, 7, and 14 dpi (Cabrera et al., 2016; Medina et al., 2017). In *Arabidopsis*, the cleavage of *TAS3* transcripts by miR390 generates secondary siRNAs (tasiRNAs) that induce post-transcriptional repression of the auxin-responsive transcription factors *ARF2*, *ARF3*, and *ARF4* (Marin et al., 2010). Cabrera et al. (2016) demonstrated the coexpression of *MIR390A* and *TAS3* in galls and giant cells at 3 dpi and the post-transcriptional regulation of *ARF3* by tasiRNAs in galls, in experiments comparing *ARF3* sensor lines sensitive or resistant to cleavage by tasiRNAs. Studies of *miR390a* and *tas3* loss-of-function mutants reported the production of fewer galls, suggesting that the miR390/TAS3/ARF3 regulatory module is required for correct gall formation (Cabrera et al., 2016). Finally, a role for the regulatory gene module composed by miR172 and the two transcription factors TOE1 (target of early activation tagged 1) and FT (flowering locus T) has been demonstrated in root galls during the formation of giant cells in *Arabidopsis* (Díaz-Manzano et al., 2018). The role for the miR172/TOE1/FT module has been first described during *Arabidopsis* flowering (Aukerman and Sakai, 2003). In *Arabidopsis* root, the 3′ strand of mature miR172 has been shown to be downregulated in galls at 3 dpi, whereas the

pri-miR172 precursor is induced, and its target *TOE1* repressed, according to transcriptome data for microdissected *A. thaliana* giant cells at the same time point (Barcala et al., 2010). Consistent with the negative regulation of *FT* by TOE1, an induction of *FT* was observed in galls at 3 dpi. *Arabidopsis* plants expressing miR172-resistant *TOE1* or KO for *FT* were less susceptible to RKNs and had smaller galls and giant cells. Like miR390, miR172 is an auxin responsive microRNA. Auxin is a crucial signal for feeding site formation and parasitism. An enhanced auxin response has been observed in RKN feeding sites (Hutangura et al., 1999) and auxin has been identified in the secretion of RKNS (De Meutter et al., 2005). The function of miR390 and miR172 in the feeding site is probably a part of the auxin response.

Plant Small Noncoding RNAs Responding to CNs

The identification and analysis of miRNAs involved in plant-CN interaction are based on the same approaches that the ones described above. Sequencing identified 30 mature DE miRNAs in *Arabidopsis* syncytia induced by *Heterodera schachtii* at 4 and 7 dpi, and qPCR analyses revealed inverse expression profiles for six miRNAs and their targets (Hewezi et al., 2008). A recent analysis of syncytia from tomato plants infected with *Globodera rostochiensis,* performed at 3, 7, and 10 dpi, identified between 200 and 300 miRNAs at each stage as DE (Koter et al., 2018). Reverse transcriptase–qPCR analyses revealed inversely correlated expression patterns for six miRNAs and their targets (Koter et al., 2018). Moreover, the expression of eight tomato miRNAs regulating defense-related proteins was specifically analyzed by qPCR at 3 and 7 dpi; an inverse correlation between the expression of these miRNAs and their targets in response to CN infection was observed (Święcicka et al., 2017). Finally, several studies have analyzed expression of soybean miRNAs in response to infection with *Heterodera glycines* by comparing expression levels in resistant and susceptible cultivars (Li et al., 2012; Xu et al., 2014; Tian et al., 2017). Tian et al. (2017) identified 60 miRNAs from 25 miRNA families as DE relative to uninfected roots in susceptible and/or resistant cultivars and validated the expression profiles of most of these miRNAs by qPCR. While most of the miRNAs identified by Tian et al. (2017) are upregulated in resistant lines relative to susceptible lines, the majority of miRNAs were downregulated in the study performed by Li et al. (2012). These discrepancies may reflect differences in resistance between these soybean cultivars or a technical bias related to the number of replicates analyzed in these two studies. A comparison of the expression profiles of conserved miRNAs in response to CN infection identified some miRNAs as DE, with the same expression profile, in several plant species. miR396b and the miR167 family were downregulated in *Arabidopsis* roots infected by *H. schachtii* at 4 and 7 dpi (Hewezi et al., 2008) and in tomato syncytia induced by *G. rostochiensis* at 3 and 7 dpi (Święcicka et al., 2017) (**Table 1**).

Three miRNAs DE in syncytia were validated by functional approaches. In *Arabidopsis,* miR396 was repressed at the onset of syncytium formation in roots infested with *H. schachtii* and upregulated at later stages, whereas its target transcription factors, the growth-regulating factors (GRF) *GRF1*, *GRF3*, and *GRF8*, displayed the opposite pattern (Hewezi et al., 2012). *Arabidopsis thaliana* mutants overexpressing miR396 have smaller syncytia and greater resistance to CN. These results suggest that the coordinated regulation of miR396 and *GRF1* and *GRF3* is required for correct syncytium development in *Arabidopsis*. Interestingly, a repression of the miR396 family associated with an upregulation of soybean *GRF* genes was observed in soybean syncytia induced by *H. glycines* at 8 dpi (Noon et al., 2019). A combination of 5′ RLM-RACE and a reporter gene approach demonstrated that the *GRF6* and *GRF9* genes were targeted by miR396 in syncytia. Transgenic soybean lines overexpressing pre-miR396 and GRF9 RNAi lines displayed similar decreases in the number of *H. glycines* females per root, reflecting an increase in resistance to CN. These results indicate that the miR396/GRF module is essential for *H. glycines* infection, and this role is conserved in *Arabidopsis* and soybean. Furthermore, the use of a reporter gene strategy made it possible to demonstrate an inverse correlation in the expression profiles of the conserved miR827 and its known target *NLA* (nitrogen limitation adaptation) during syncytium development in *Arabidopsis* (Hewezi et al., 2016). The overexpression of miR827 increased susceptibility to *H. schachtii,* whereas the expression of a miR827-resistant *NLA* decreased plant susceptibility. These results show that miR827 downregulates *Arabidopsis* immunity to *H. schachtii* by repressing *NLA* activity in the syncytium (Hewezi et al., 2016). Finally, a role for the miR858/MYB83 module has been established in *Arabidopsis* syncytia induced by *H. schachtii*, in which an inverse correlation of transcript levels was observed between miR858 and its target MYB83 at 7, 10, and 14 dpi (Piya et al., 2017). Modulation of the expression of these genes through gain- and loss-of-function approaches altered the *Arabidopsis* response to nematode infection, demonstrating a role for this module in syncytium formation.

Conclusions and Perspectives

The results presented provide the first insigths into the function of miRNAs in the plant response to nematode infection. Except for miR390, expression profile of most miRNAs in feeding site shows heterogeneity (**Table 1**), with different expression profiles according to the type of feeding structures, the plant species, and/or the phase of development. Difference of expression in giant cell and syncytia may be explained by their distinct ontogenesis. Whether these variations of expression of plant miRNAs are directly induced by the nematode or are the results of modification of plant hormonal balance is a question that still needs to be investigated. The identification of the targets of these DE miRNAs and the biological pathways they regulate would improve our understanding of feeding cell development. Moreover, resistance genes of the nucleotide binding site-leucine-rich repeat (NBS-LRR) family genes are known to be targeted by miRNAs and phased siRNAs (reviewed by Fei et al., 2016). An inverse correlation on several tomato NB-LRR transcripts and their miRNA regulators has been evidenced after infection by CN (Święcicka et al., 2017).

A better understanding of the role of miRNA in PPN feeding sites may lead to new methods of control for these organisms.

Most studies to date have focused on miRNAs, but few studies investigating the siRNAs expressed in roots infected with PPNs in *Arabidopsis* (Hewezi et al., 2017; Medina et al., 2018; Ruiz-Ferrer et al., 2018) have highlighted an overrepresentation in galls of 24 nt siRNAs known to be associated with RNA-directed DNA methylation. Two first studies of changes in DNA methylation have been performed in *A. thaliana* and soybean plants infected with CN (Rambani et al., 2015; Hewezi et al., 2017). These studies support a role for changes in DNA methylation in plant responses to PPN infection. Future combined studies of small RNAs, methylome and transcriptome should result in an integrative understanding of the epigenetic regulation of feeding site formation. Several intriguing questions remain unanswered: i) How do PPNs modify the expression of small RNA genes in the plant genome? ii) Do the small RNAs produced by nematodes play a role in the plant and vice versa? Genomes of several PPN species are now available (Cotton et al., 2014; Eves-van den Akker et al., 2016; Blanc-Mathieu et al., 2017; Masonbrink et al., 2019) and should be used to investigate the small RNAs produced by the nematode during parasitism. Finally, cross-kingdom RNAi (reviewed by Weiberg and Jin, 2015) probably also occurs during interactions between plants and PPNs. Integrative analyses of the small RNAs from both side of the interactions should shed light on this molecular dialog.

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YN, BF, and SJ-P organized the study and wrote this manuscript. All authors have read and approved the manuscript.

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frontiers in Plant Science

REVIEW
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Development of Parasitic Organs of a Stem Holoparasitic Plant in Genus *Cuscuta*

*Kohki Shimizu and Koh Aoki**

Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Japan

Parasitic plants infect a broad range of plant species including economically important crops. They survive by absorbing water, minerals, and photosynthates from their hosts. To support their way of life, parasitic plants generally establish parasitic organs that allow them to attach to their hosts and to efficiently absorb substances from the vascular system of the host. Here, we summarize the recent progress in understanding the mechanisms underlying the formation of these parasitic organs, focusing on the process depicted in the stem holoparasitic genus, *Cuscuta*. An attachment structure called "holdfast" on the stem surface is induced by the light and contact stimuli. Concomitantly with holdfast formation, development of an intrusive structure called haustorium initiates in the inner cortex of the *Cuscuta* stem, and it elongates through apoplastic space of the host tissue. When haustoria reaches to host vascular tissues, they begin to form vascular conductive elements to connect vascular tissue of *Cuscuta* stem to those of host. Recent studies have shown parasite-host interaction in the interfacial cell wall, and regulation of development of these parasitic structures in molecular level. We also briefly summarize the role of host receptor in the control of compatibility between *Cuscuta* and hosts, on which occurrence of attachment structure depends, and the role of plant-to-plant transfer of long-distance signals after the establishment of conductive structure.

Keywords: attachment cells, conductive cells, *Cuscuta*, haustorium, host factors, intrusive cells, parasitic organs, parasitic plants

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Masa H. Sato,
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Satoko Yoshida,
Nara Institute of Science and Technology (NAIST), Japan
Takashi Ishida,
Kumamoto University, Japan

****Correspondence:***
Koh Aoki
kaoki@plant.osakafu-u.ac.jp

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INTRODUCTION

A group of plants called "parasitic plants" have been reported to consist of 4000 or more species, which is equivalent to approximately 1% of flowering plants, and are found all over the world (Nickrent, 2002). In many cases, the host range of a parasitic plant is wide, infesting many plant species including economically important crops (Lanini and Kogan, 2005). Thus, parasitic plants cause serious damage to crop production.

Parasitic plants can be classified into two classes: hemiparasites that retain the ability to perform photosynthesis, and holoparasites that have little or no photosynthetic capability. Consequently, holoparasites need to live a heterotrophic lifestyle by depriving nutrients and water from host plants Heide-Jørgensen, 2008). Parasitic plants belonging to the genus *Cuscuta*, a member of the family Convolvulaceae, infest a broad range of hosts and have been used as a model for the study of stem parasitic plants. The genus *Cuscuta* has been reported to consist of more than 150 species (Yuncker, 1932), and belong to the holoparasitic class with degenerated leaves and roots, and, as they do not perform photosynthesis, depend entirely on host plants for nutrients and water. To

understand *Cuscuta* at genetic level and to prevent damage to crop production, the whole genomes of *Cuscuta australis* (Sun et al., 2018) and *C. campestris* (Vogel et al., 2018) have been recently sequenced.

After germination, *Cuscuta* extends a thread-like shoot. During shoot extension, the extending stem performs a swinging movement to increase the probability of contact with the host plant (Tada et al., 1996). It has been reported that *Cuscuta* perceives volatiles emitted from the host and extends toward it (Runyon et al., 2006). If *Cuscuta* cannot find a host plant, it will die in about 2 weeks after germination.

After contact with the host, the stem of *Cuscuta* forms a counterclockwise coil around the stem of the host (**Figure 1A**). The coiling behavior has been shown to be induced by the cooperative effects of far-red/blue light and tactile stimuli (Lane and Kasperbauer, 1965; Tada et al., 1996; Furuhashi et al., 1997). Effect of far-red light on the coiling of *C. japonica* was canceled by red light, suggesting the involvement of phytochrome (Furuhashi et al., 1997). Coiling and projection of haustoria of *C. japonica* can be induced by placing the stem between two glass plates to apply contact pressure under far-red or blue light, but was not induced under red- or white light, suggesting the cooperative effect of light and tactile stimuli (Tada et al., 1996).

After coiling on the host stem, a series of organogenesis occurs to establish a parasitic connection, including formation of an adhesive disc-like organ, referred to as a "holdfast" on the surface of the *Cuscuta* stem in contact with the host stem, and the development of a "haustorium" that intrudes into the host stem and finally makes vascular connection to the xylem vessels and phloem sieve tubes of the host (Yoshida et al., 2016). In this review, we describe the mechanisms underlying the formation of these parasitic organs, and propose hypotheses for the involvement

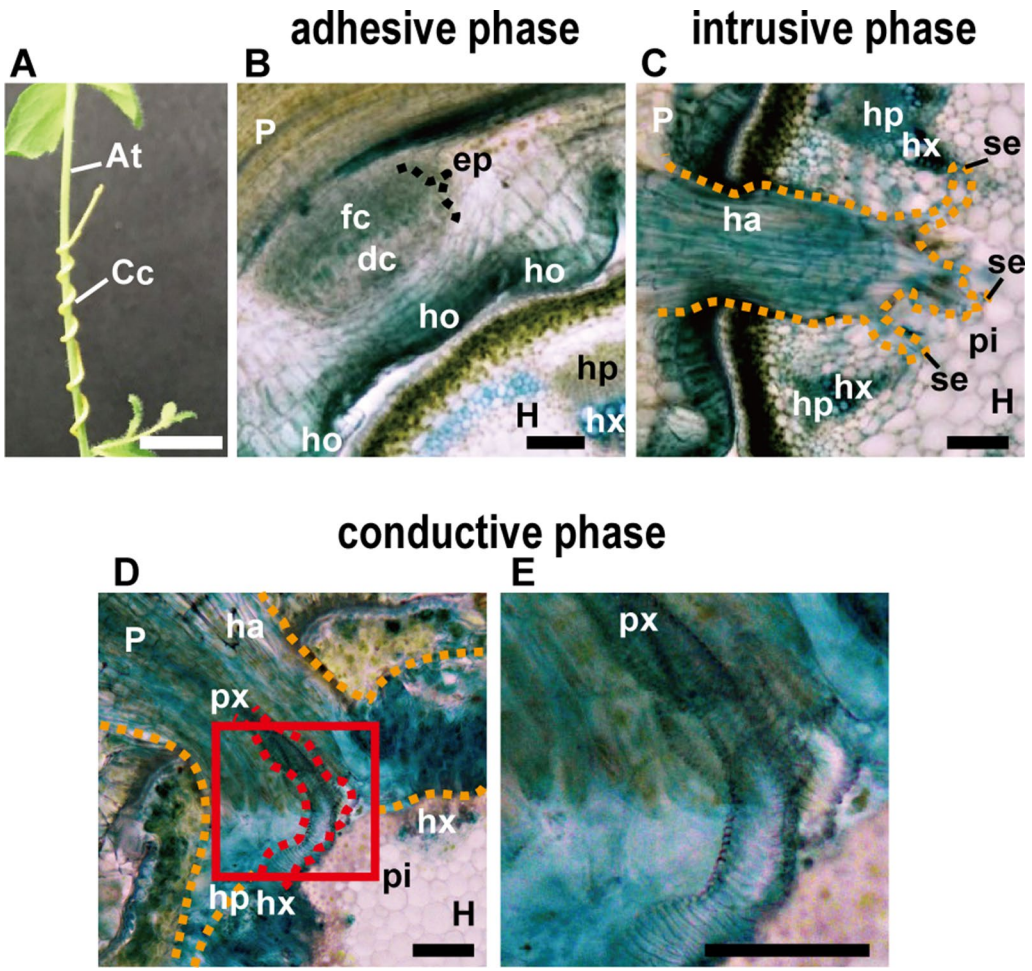

FIGURE 1 | (A) Appearance of parasitic site formed between *Cuscuta campestris* (Cc) and *Arabidopsis thaliana* (At) from the outside. *C. campestris* coils around the inflorescence stem of Arabidopsis. Scale bar, 1 cm. **(B–E)** Transverse sections of the three phases of parasitic processes of *Cuscuta*. Scale bars, 200 μm. **(B)** Adhesive phase. Holdfast (ho) is formed on the host-attaching surface of *C. campestris*. Prehaustorium develops in the inner cortex of the stem right behind holdfast. In the endophyte primordium (ep), digitate cells (dc) and file cells (fc) differentiate and start to elongate. **(C)** Intrusive phase. Haustorium (ha) intrudes in the cortex of the host stem. It sometimes reaches to the pith (pi). **(D)** Conductive phase. **(E)** Area in the red square in **(D)** is magnified. Vascular conductive elements (px) are formed in the haustorim. P, parasite; H, host; ha, haustorium; hp, host phloem; hx, host xylem; px, parasite xylem; pi, pith; se, searching hypha; orange dotted line, outline of haustorium; red dotted line, outline of parasite xylem. In all panels, 200-μm-thick micro-slicer sections were stained with toluidine blue.

of putative host factors. Comparison of *Cuscuta* with other well-studied root parasites belonging to Orobanchaceae that are taxonomically distant from *Cuscuta* highlight diversity with respect to the structure and function of the parasitic organs. We also briefly summarize the role of host receptor in the control of compatibility between *Cuscuta* and hosts, and the role of plant-to-plant transfer of long-distance signals after the establishment of conductive structure.

ORGANOGENESIS ASSOCIATED WITH PARASITIC CONNECTION

The parasitic processes of *Cuscuta* can be classified into three phases; the adhesive, intrusive, and conductive phases (**Figures 1A–E**) (Heide-Jørgensen, 2008). In the adhesive phase, a specialized adhesive organ called the holdfast is formed in the *Cuscuta* stem in contact with the stem of the host plant. Holdfast is formed essentially by the elongation of cells in the epidermal and cortical layers of *Cuscuta* stem, and characterized by the presence of secretory cells that secrete adhesive compounds (Heide-Jørgensen, 2008). In the intrusive phase, *Cuscuta* develops a specialized intrusive organ called the haustorium. When the haustorium reaches the vascular tissues of the host, a specific group of haustorial cells differentiate into vascular conductive cells and *Cuscuta* proceeds into the conductive phase. In the conductive phase, *Cuscuta* exchanges various information molecules with the host, as well as absorbs water and nutrients.

Adhesive Phase

After coiling (**Figure 1A**), epidermal cells of the *Cuscuta* stem in contact with the host elongate toward the contacting surface of the host epidermis and divide anticlinally to become digitate in form (**Figure 1B**; Vaughn, 2002). Tight adhesion between *Cuscuta* and the host can be achieved by secretion of adhesive substances and elongation of cells toward the host surface. The divided epidermal cells of *Cuscuta campestris* (synonymous with *Cuscuta pentagona*, Costea et al., 2015) secrete pectin-rich adhesive (cement) to make a tight adhesion (Vaughn, 2002). Homogalacturonan, which constitutes up to 65% of cell wall pectin, is synthesized in a methyl-esterified form (Ridley et al., 2001). Methyl esters are removed enzymatically by pectin methylesterases (PMEs) from homogalacturonan (Micheli, 2001; Pelloux et al., 2007). Several studies using Arabidopsis have shown that low-esterified pectin is responsible for the organ adhesion (Sieber et al., 2000; Sala et al., 2019). In the epidermal layer of *Cuscuta* holdfast, immunolabeling of cell wall using antibodies against low-esterified homogalacturonan, such as JIM5 and LM19, is relatively stronger than that using antibodies against high-esterified homogalacturonan, such as JIM7 and LM20 (Vaughn, 2002; Johnsen et al., 2015; Hozumi et al., 2017). These result suggested that low-esterified homogalacturonan is responsible for the adhesion of *Cuscuta* to the hosts (**Figure 2A**).

Arabinogalactan proteins (AGPs) have been reported to be found in common in many adhesion-based mechanisms (Bowling and Vaughn, 2008; Huang et al., 2016). Implication for the involvement of AGPs in *Cuscuta* adhesion to the host was obtained by accumulation of AGP in the surface of the holdfast (**Figure 2A**). Staining with LM2 antibody which recognizes carbohydrate moiety of AGPs demonstrate that AGPs accumulate in epidermal cells on the surface of holdfasts of *Cuscuta reflexa* (Striberny and Krause, 2015) and *C. campestris* (Hozumi et al., 2017). Staining with Yariv reagents and LM6 antibody further support AGPs accumulation in epidermal cells of holdfasts of *C. campestris* (Hozumi et al., 2017). Accumulation of AGPs are due to the cell type-specific expression of a subset of fasciclin-like family member genes, *CcFLA7*, *16* and *17*. Accumulation of AGP on the contacting surface was also reported for host plants (Albert et al., 2006; Striberny and Krause, 2015). Contact of *Cuscuta reflexa* to the surface of tomato stem induces the expression of *attAGP* in tomato (Albert et al., 2006). Expression levels of tomato *attAGP* was positively correlated with the force of attachment. This result suggests a positive contribution of AGPs to parasite-host attachment (Albert et al., 2006). However, exact role of AGP in parasite-host attachment is still unknown.

To contact tightly to the host surface, divided epidermal cells of holdfast elongate toward the host surface (**Figures 1B** and **2A**). Outgrowth of the epidermal cells of the holdfast contributes to tightening of the adhesion by accommodating the surface of the host plant (Vaughn, 2002). The surface of the holdfast, which was in a pointed fingerlike extension form, becomes flat or rounded (**Figure 2A**). This malleability of the holdfast epidermis facilitates the formation of tight seal with the host surface (Vaughn, 2002). Identity of the elongating cell was referred to as a secretary trichome which contains a large number of secretary vesicles (Vaughn, 2002). Epidermal cells of the *Cuscuta* holdfast likely to share common developmental mechanisms with root hair (Ishida et al., 2008) or leaf trichome (Wang et al., 2019), although the expression of marker genes for these types of cells have not been demonstrated yet.

Initiation of Intrusive Phase

The intrusive phase is characterized by the development of a haustorium (**Figure 1C**). To be accurate, primordia of haustoria have already been initiated in the adhesive phase. When *Cuscuta* develops holdfasts after contact to the host's stem, the precursor of mature haustorium, or so-called prehaustorium, is differentiated in the cortex near the vascular cylinders right behind the holdfast (**Figure 1B**).

Initiation of the haustorium development appears to be a host-independent process. Development of haustoria in *Cuscuta* species can be induced even when *Cuscuta* coiled to non-biological object (Tada et al., 1996; Heide-Jørgensen, 2008; Hong et al., 2011). Microscopic studies have shown that meristem cells of haustorium develop simultaneously with the development of holdfast (Lee and Lee, 1989; Lee, 2007; Heide-Jørgensen, 2008). Initiation of haustorium development requires far-red light, and also blue light even though the effect is weaker than far-red light (Furuhashi et al., 1995), and by contact stimuli concomitantly applied with light (Tada et al., 1996; Furuhashi et al., 1997). Red or white light did not induce haustorium, and haustorium induction by far-red light can be cancelled by the

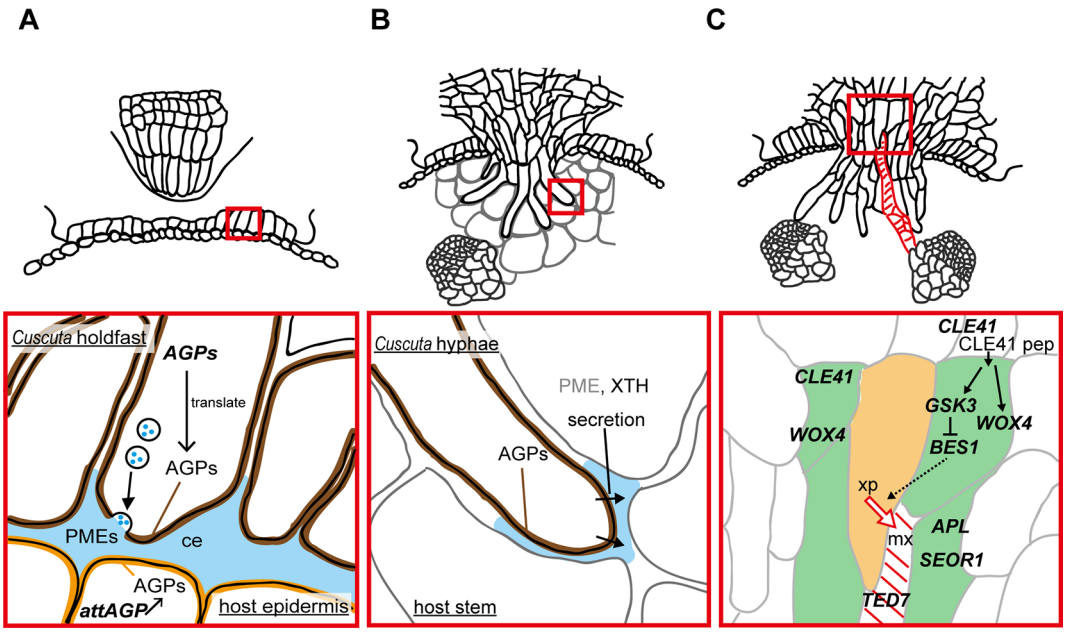

FIGURE 2 | Functions of enzymes and genes associated with the parasitic processes. Panels in the bottom show magnified views of the areas in red squares in panels on the top. **(A)** Putative function of cell wall-modifying enzymes secreted from holdfast in the adhesive phase. Holdfast cells tighten the adhesion by pectin-rich cement (ce, blue). It has been shown that holdfast cells of *Cuscuta campestris* contain numerous secretion vesicles containing the components of cell-wall-loosening complexes. Pectin methylesterases (PMEs) are probably secreted to tighten the adhesion of *Cuscuta* to host. Specific members of genes encoding arabinogalactan proteins (*AGPs*) are expressed in searching hyphae, and accumulate AGP proteins (brown). AGP also have roles in host cell surface (orange) in the adhesion of parasite (Albert et al., 2006). **(B)** Secretion of cell wall-modifying enzymes to the cell walls adjacent to searching hyphae in the intrusive phase. Xyloglucan endotransglucosylation (XET) activity of XTH was detected in interface (blue) at the tip of haustoria of *C. reflexa* (Olsen and Krause, 2017). In *C. campestris,* searching hyphae-specific expression of *FASCICLIN-LIKE* genes causes the accumulation of AGPs in the interfacial cell walls surrounding searching hypha cells (brown) (Hozumi et al., 2017) Exact role of AGPs in the intrusive phase is still unknown. **(C)** Expression of genes associated with differentiation of vascular elements during the transition from intrusive phase to conductive phase in haustorim of *Cuscuta japonica*. Green, procambium/phloem region, orange, xylem precursor (xp), red diagonal lines, mature xylem vessel (mx). *WOX4*, *WUSCHEL RELATED HOMEOBOX 4*; *CLE41*, *CLAVATA3/EMBRYO SURROUNDING REGION-RELATED 41*; *GSK3*, GLYCOGEN SYNTHASE KINASE 3; *BES1*, *BRI1-EMS-SUPPRESSOR 1; TED7*, *TRACHEARY ELEMENT DIFFERENTIATION-RELATED 7*; *APL*, *ALTERED PHLOEM DEVELOPMENT*; *SEOR1*, *SIEVE ELEMENT OCCLUSION-RELATED 1*.

following red light, suggesting the involvement of phytochrome in the regulation of haustorium development (Tada et al., 1996; Furuhashi et al., 1997). Cryptochrome is involved in blue light perception (Cashmore et al., 1999), and mechanosensitive ion channels are likely to be involved in the perception of contact stimuli (Hamilton et al., 2015; however, primary receptors for these stimuli have not been identified yet in *Cuscuta*.

Cytokinin has been reported to induce haustorium of *Cuscuta reflexa* in the absence of the host (Ramasubramanian et al., 1988), and in the dark (Haidar et al., 1998). These results imply that cytokinin may be a downstream signal of light and contact stimuli.

Genetic networks involved in the initiation of haustorium development have not yet been elucidated. Haustoria of *Cuscuta* develop as lateral protrusion of parasite stems, thus classified as "lateral haustoria" (Joel, 2013; Yoshida et al., 2016). Mechanisms involved in the formation of lateral organs, such as lateral roots and adventitious roots, have been studied in detail in Arabidopsis (Hu and Xu, 2016; Liu et al., 2018; Ibáñez et al., 2019; Lee et al., 2019), which may serve as a reference model for the initiation of *Cuscuta* haustorium.

Development of Haustorium

Initial cells of *Cuscuta* haustorium are formed in the stem inner cortex, which then divide anticlinally and periclinally to give rise meristem cells (Lee and Lee, 1989; Lee, 2007). Meristem cells are then organized in "endophyte primordium" consisting of two cell types; elongate digitate cells and smaller file cells, before intruding into the host (**Figure 1B**; Lee, 2007). These cells become intrusive and force their ways through the stem cortex cells in front of them, the epidermal layer of its own stem and the epidermal layer and the cortex of the host. Cells in between the meristem and the stele also divide and form tabular cells, which are added to the file cell layer. This morphological observation suggests that the intrusive cells of *Cuscuta* originate from the cortex. This is different from the case of *Phtheirospermum japonicum*, a root hemiparasitic plant belonging to Orobancheceae, whose intrusive cells have been shown to originate from the root epidermal cells (Wakatake et al., 2018) (**Figures 3A, B**). During intrusive growth in the host's cortex, intrusive cells advance in the apoplastic space by pushing the cells. At the front of haustorial intrusive part, the elongate digitate cells search for the host's vascular tissues, and, thus are called "searching hyphae" (**Figure 1C**; Vaughn, 2003).

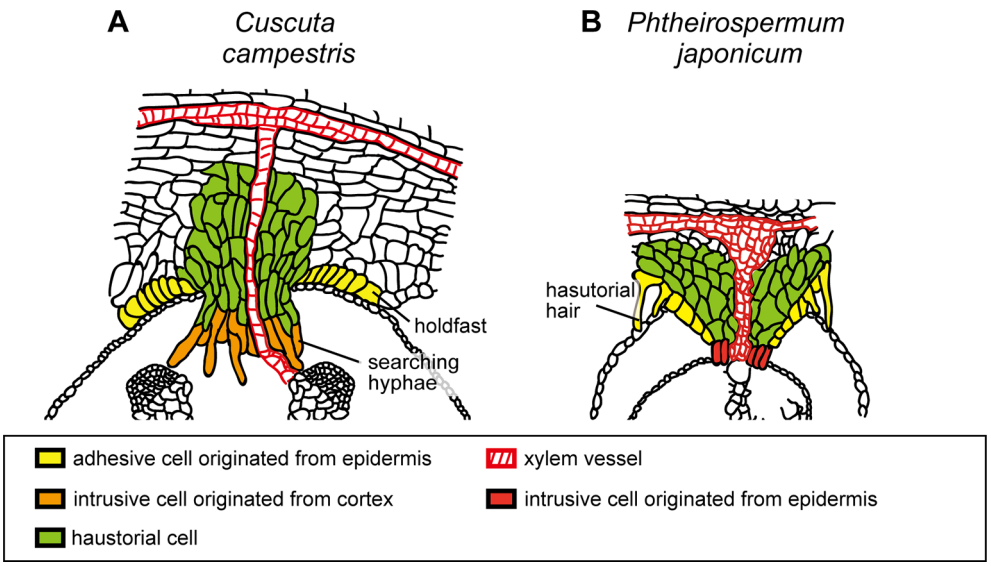

FIGURE 3 | Schematic illustration of the structures of haustoria of *Cuscuta campestris* and *Phtheirospermum japonicum*. **(A)** *Cuscuta campestris*, a holoparasitic plant belonging to Convolvulaceae, develops lateral haustoria. **(B)** *Phtheirospermum japonicum*, a hemiparasitic plant belonging to Orobanchaceae, develops lateral haustoria. Holdfast of *C. campestris* and haustorial hair of *P. japonicum* are likely to be analogous that develop from epidermal cells and contribute to the adhesion of parasite to host. Intrusive cells of *C. campestris*, searching hyphae, develop from digitate cells which have been differentiated from the cortex or endodermal cells of the stem. On the other hand, intrusive cells of *P. japonicum* are shown to be differentiated from the epidermal cells.

Intrusive cells grow through apoplastic space by pushing host cells aside, rather than by crushing them. *Cuscuta* secretes enzymes to the interfacial cell walls to loosen the cell wall and aid the elongation of intrusive cells in the apoplastic space. In *Cuscuta reflexa*, haustorium-specific expression of gene encoding a cysteine protease, namely cuscutain, were reported (Bleischwitz et al., 2010). In the parasitic interface of *C. japonica* and the host, *Glycine max*, expression of *C. japonica* genes encoding cell wall degrading- and modifying- enzymes, such as PME, pectate lyase, polygalacturonase, and xyloglucan endotransglucosylase/hydrolase (XTH) were up-regulated (Ikeue et al., 2015). In the far-red light-induced haustoria of *C. reflexa* and *C. gronovii,* two *XTH* genes have shown to be up-regulated (Olsen et al., 2016b). One of the two enzymatic activities of XTH, xyloglucan endotransglucosylation (XET), were secreted from haustoria, and localized at the host-parasite border of the endophytically growing haustoria of *C. reflexa*, *C. campestris* and *C. platyloba* (Olsen and Krause, 2017). Because XET activity of XTH grafts the reducing end of the cleaved xyloglucan onto an acceptor xyloglucan chain (Rose et al., 2002; Olsen et al., 2016a), these results indicate that *Cuscuta* XTHs play a role in invading growth of haustoria (**Figure 2B**).

In addition to cell wall modifying enzymes, searching hyphae of *C. campestris* and *C. japonica*, which develop on the haustorial tip, accumulate AGPs in the cell surface (**Figure 2B**). In *C. campestris*, hyphal AGP accumulation is accompanied by the expression of hyphae-specific *FASCICLIN-LIKE* family members (Hozumi et al., 2017). However, roles of hyphal AGP in intrusive growth is still unclear.

Transition From Intrusive Phase to Conductive Phase

Once searching hyphae reach the host's vascular tissues, the invasion process is almost complete. Searching hyphae acquire identities as xylem- and phloem-conductive elements (**Figures 1D, E**; Vaughn, 2006; Shimizu et al., 2018), which is concomitantly associated by the differentiation of vascular conducting elements in the center of haustorium (**Figure 2C**). Cells that have a procambium-attribute, from which vascular elements are differentiated, have emerged before contact with the host's vascular elements. Cells with a procambium-attribute can be identified by the expression of *WUSCHEL RELATED HOMEOBOX 4* (*WOX4*) (Hirakawa et al., 2010). Expression of *C. japonica WOX4*, *CjWOX4*, was detected in the central region of the basal haustorium, and in cells surrounding the precursor cells which later differentiate into xylem vessels (**Figure 2C**; Shimizu et al., 2018).

Differentiation of searching hyphae into xylem starts near the tip. Searching hyphae penetrate into host xylem vessels through the pits, and starts a series of changes to differentiate xylem vessels (Vaughn, 2006). Xylem differentiation in haustoria of *C. japonica* include many processes in common with those elucidated in vascular tissues of model plants (Ito et al., 2006; Hirakawa et al., 2008). Before the onset of xylem differentiation, high expression of *C. japonica CLAVATA3/EMBRYO SURROUNDING REGION-RELATED 41* (*CjCLE41*), and CLE41 peptide is likely to be secreted to repress the differentiation of the procambium-like cells into tracheary elements. Expression of *CjCLE41* begins to decrease upon the onset of xylem differentiation, which probably down-regulates the kinase activity of GLYCOGEN SYNTHASE

KINASE 3 (GSK3) protein. Down-regulation of GSK3 releases the expression of *BRI1-EMS-SUPPRESSOR 1* (*CjBES1*) from the deactivated state. Consequently, activated *CjBES1* expression induces the xylem differentiation processes (Shimizu et al., 2018). Expression of the gene specific to developing xylem vessels, *TRACHEARY ELEMENT DIFFERENTIATION-RELATED 7* (*CjTED7*), is under the detection limit before the onset of xylem differentiation, whereas up-regulated with xylem vessel formation (Shimizu et al., 2018).

Compared to xylem, differentiation of phloem in haustoria has been rather controversial and appears to differ from species to species. In *C. japonica*, marker genes of phloem companion cell, *ALTERED PHLOEM DEVELOPMENT* (*CjAPL*; Bonke et al., 2003), and of developing sieve elements, *SIEVE ELEMENT OCCLUSION-RELATED 1* (*CjSEOR1*; Knoblauch et al., 2014), were detected in the intruding haustoria (**Figure 2C**; Shimizu et al., 2018). Substances from the host's sieve tube to *Cuscuta* translocate in distinct arrays of conductive cells (Birschwilks et al., 2006; Shimizu et al., 2018), indicating that phloem conductive cells develop in haustoria and are symplastically separated from surrounding cells. However, *in situ* hybridization for *CjCLE41*, whose Arabidopsis ortholog was expressed in phloem cells and adjacent pericycles (Hirakawa et al., 2008), demonstrated that it is expressed in cells overlapping with the region where *CjWOX4* is expressed (Shimizu et al., 2018). This incomplete compartmentalization implies immaturity of haustorial phloem relative to that in the conventional vascular bundles.

Differentiation processes of vascular cells in *Cuscuta* haustoria contain common and different processes compared to those in other parasitic plants. In *Phtheirospermum japonicum*, expression of procambium-specific genes, *PjWOX4*, *HOMEOBOX PROTEIN 8* (*PjHB8*) and *PjHB1*, were detected before the formation of xylem vessels, (Wakatake et al., 2018). This demonstrates that the development of procambium-like cells precedes the differentiation of haustorial vascular cells, as seen in *C. japonica*. On the other hand, organization of haustorial vascular cells appears to be different from that of *C. japonica*. Although the presence of xylem vessels are apparent, absence of *AtAPL* promoter activity, which is expressed in phloem, in haustoria suggest that phloem does not develops in *P. japonicum* haustoria (Spallek et al., 2017; Wakatake et al., 2018). Similarly, immaturity of the phloem is also shown in the haustoria of root holoparasitic plant, *Phelipanche aegyptiaca*, which also belongs to Orobanchaceae (Ekawa and Aoki, 2017). On the other hand, formation of mature sieve elements in haustoria has been reported for *Orobanche crenata* and *O. cumana* (Dörr and Kollmann, 1995; Krupp et al., 2019). These results, together with haustorial development of *Cuscuta* described in this section, suggest that development of procambium-like cells and haustorial xylem vessels are observed in common, on the other hand, development of phloem is different between different parasitic plants species. Mechanisms that bring diversity to phloem development have not been elucidated yet.

Conductive Phase

Cuscuta becomes a strong sink after the establishment of the haustorial bridge and competes with sink organs of the host itself for assimilates. Searching hypha cells that contact to host xylem vessels invade vessels through the pits in the cell wall (Heide-Jørgensen, 2008). Then, the ends of hypha cell wall become thin and perforated, finally forms an open connection with host xylem vessels (**Figure 4A**). Vaughn (2006) mentioned that the nature of the opening between host xylem and hyphal xylem appears to be dependent on the angle and orientation of hyphae with respect to host xylem. The open connection allows the translocation of

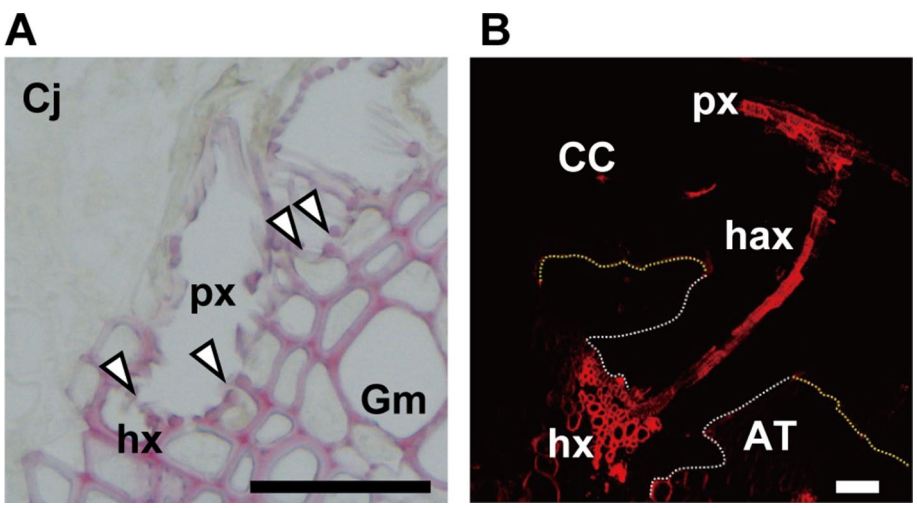

FIGURE 4 | (A) Open connection (arrowheads) between xylem vessels of parasite (px) and host (hx) in the parasitic interface of *Cuscuta japonica* (Cj) with *Glycine max* (Gm). Scale bar, 50 μm. A 20-μm-thick paraffin-embedded section was stained with phloroglucinol. **(B)** Transfer of 5-carboxytetramethylrhodamine (TMR) 10-kDa dextran (red) from host xylem vessel to haustorial xylem vessels, and then to *Cuscuta* stem xylem. White dotted line; outline of haustorium, yellow dotted line; outline of attachment boundary between *Cuscuta* and host *Arabidopsis*. CC, *Cuscuta campestris*; AT, *Arabidopsis thaliana*; px, parasite xylem vessel; hax, haustorial xylem vessel; hx, host xylem vessel. Scale bar, 200 μm.

xylem-mobile dyes, for example, fluorescently labeled 10-kDa dextran (**Figure 4B**).

The nature of phloem connection has been controversial. Ultrastructural studies demonstrated that phloem continuity is achieved by a contacting searching hyphae which split in finger-formed elongation, and the plasmodesmata and sieve pores are absent between the searching hypha and host sieve tube, suggesting an apoplastic transfer of xylem solutes *via* transfer-type cells (Heide-Jørgensen, 2008). On the other hand, *Cuscuta* has been known as a vector for transmission of virus and phytoplasma (Hosford, 1967; Heintz, 1989), and the transport requires a symplastic connection. Finally, evidence for the presence of symplastic connection was given by the translocation of GFP from sieve tubes of hosts to *Cuscuta* (Haupt et al., 2001). Various phloem-mobile compounds, including sucrose, amino acids, plant hormones, and xenobiotics have been shown to translocate from the host to parasite (Birschwilks et al., 2006). The transport rate does not show any selectivity with respect to the compounds, suggesting that phloem-mobile compounds are transported through an open symplastic connection.

Flow of water from hosts *via* xylem to *Cuscuta* is probably driven by the gradient of water potential between the host and the parasite. In Orobanchaceae, *Orobanche cernua* accumulates a higher level of potassium than the host (Hibberd et al., 1999), and *Striga hermonthica* and *Phelipanche ramosa* accumulates mannitol (Robert et al., 1999). On the other hand, the direction of transport *via* phloem can occur from the parasite to host, and, thus, is bi-directional. The bi-directional nature of phloem transport lays foundations for mutual control between the parasite and the host.

INTERACTION WITH HOST

Host Receptor for Immune Response Against *Cuscuta*

Cuscuta spp. have a broad host range, but there are a few plants that are resistant to *Cuscuta* (Kaiser et al., 2015). Interestingly, cultivated tomato species, *Solanum lycopersicum*, is resistant to *Cuscuta reflexa* (Ihl et al., 1988; Albert et al., 2004; Runyon et al., 2010; Kaiser et al., 2015), while a wild relative of tomato, *Solanum pennellii*, is susceptible (Hegenauer et al., 2016; Krause et al., 2018). At the end of the attachment phase, epidermal cells of resistant *S. lycopersicum* die following a hypersensitive-type response, and hypodermal cells are modified to protect intrusion from haustoria (Ihl et al., 1988). Cuscuta factor (CuF), a 2-kDa peptide with *O*-esterified modification, was identified to trigger defense response of the host plant including production of reactive oxygen species and ethylene (Hegenauer et al., 2016). Analysis of introgression lines of *S. lycopersicum* × *S. pennellii* (Eshed and Zamir, 1995) lead to the identification of a gene for tomato receptor of CuF, *CuRe1*, which encodes a leucine-rich repeat receptor like protein (LRR-RLP) (*S. lycopersicum* allele, Solyc08g016270) (Hegenauer et al., 2016). Stable introduction of *S. lycopersicum CuRe1* into susceptible *S. pennellii,* and *N. benthamiana* confers responsiveness to the CuF and increased resistance to *C. reflexa* (Hegenauer et al., 2016). These results suggest that defense response, likely pattern-triggered immunity (PTI) response, of incompatible tomato species could be induced by the perception of the CuF by the receptor CuRe1, although either the molecular identity of CuF or direct binding of CuF to CuRe1 have not been demonstrated yet (Hegenauer et al., 2016). The presence of additional CuRe1-like receptors is also suggested, and the identification of their ligand will pave the way to investigate parasite-host recognition and its relation to plant immunity (Fürst et al., 2016).

Involvement of Host Factors for Parasitic Organ Development

Host-derived signal substances, or "host factors," control the organ development processes of parasites. A well-known example of the host factors are strigolactones, that are exuded from host root, that trigger germination of seeds of Orobancaceae plants (for reviews, see Xie et al., 2010; Lumba et al., 2017). In the case of *Cuscuta*, volatiles emitted from the host is known to mediate host location by *Cuscuta* (Runyon et al., 2006). On the other hand, haustoria can be induced in a host-independent manner (Furuhashi et al., 1995; Tada et al., 1996). Although haustorium initiation can occur host independently, the latter steps, such as elongation of searching hyphae and their differentiation to conductive cells, may require host factors (**Figure 5**).

First, elongation of searching hyphae should be initiated by host factors. The rationale for this is that, although elongation of endophyte primodium of *Cuscuta* initiated by attaching to non-biological substances, such as acryl rod and bamboo stick, develops file cells and digitate cells, they do not show further elongation or development of searching haypha cells (Heide-Jørgensen, 2008; Hong et al., 2011). Host factors involved in this elongation process have not yet been identified.

Second, host factors may be involved in the differentiation of searching hypha cells into xylem and phloem conductive elements (Vaughn, 2006; Krupp et al., 2019). Upon contacting xylem vessels or phloem sieve tubes of the host, the hyphal cells of haustorium starts to differentiate into respective conductive elements, implying that hyphal cells recognize the type of host conductive elements they hit in order to differentiate into the correct elements. Although it is not clear whether this process happens in all cases or not, establishing the right connection between right elements must be essential for the survival of parasitic plants. This raises questions; what the cues of hyphal differentiation are, and whether hyphal cells have multipotency or not. Further study is needed to answer these questions.

We mention that host factors inducing haustorium, or "haustorium inducing factors (HIFs)" are well characterized for Orobancaceae root parasitic plants. Cytokinin (Goyet et al., 2017), 2,6-dimethoxy-1,4-benzoquinone (DMBQ) (Lynn et al., 1981) and lignin-related compounds (Cui et al., 2018) have been shown to have HIF activity. Orobancaceae root parasitic plants may also require host factor(s) for the elongation of intrusive cells because the elongation does not happen when prehaustorium is induced solely by HIFs (Estabrook and Yoder, 1998).

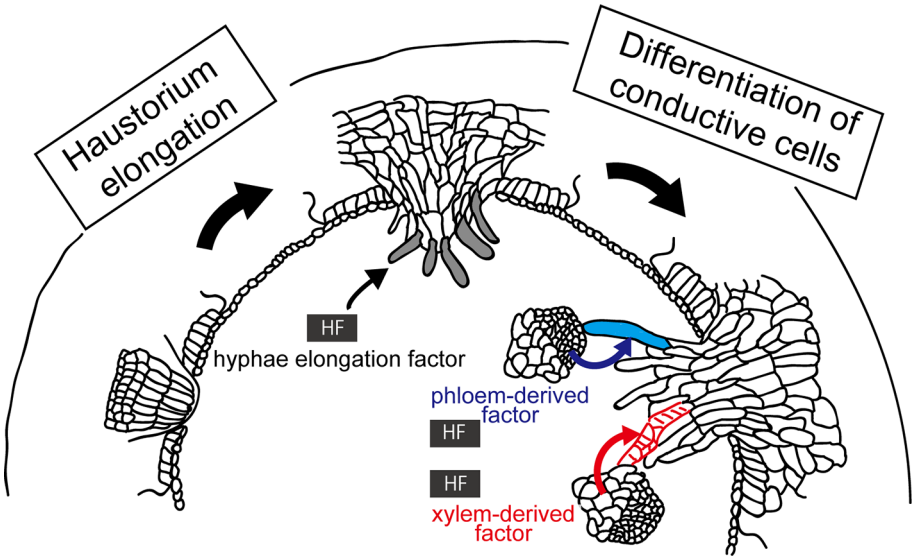

FIGURE 5 | Involvement of host factors (HFs) in the elongation and differentiation of searching hyphae. (Left) HF inducing elongation of searching hyphae (left) has been hypothesized because digitate cells or file cells of *C. campestris* initiated in a host-independent manner do not show further development of searching hyphae without host. (Right) HF has been implied in the differentiation of searching hypha cells into xylem (red lines) and phloem conductive elements (blue), because upon contacting xylem vessels or phloem sieve tubes of the host, the hyphal cells starts to differentiate into respective conductive elements. These HFs have not been identified yet.

PARASITIC PLANTS MODULATE ORGANS OF THE HOST?

We so far focused on the organ formation in parasitic plants. On the contrary, modulation of organ morphology by the parasite also occurs in the host plants. Parasitization often causes the swelling of host tissues, which is called "hypertrophy" (Heide-Jørgensen, 2008). In the recent study on the parasitic complex of *Phtheirospermum japonicum* and the host Arabidopsis, thickening of Arabidopsis roots is reported to be induced by the cytokinin produced in *P. japonicum* (Spallek et al., 2017).

In the host plants parasitized by *Cuscuta*, induction of new vascular elements in the host was previously reported (Dawson et al., 1994). However, in *Impatiens balsaminea* parasitized by *Cuscuta pentagona*, little or no new growth of host vascular elements were observed (Vaughn, 2006). In *Glycine max* parasitized by *Cuscuta japonica*, changes in the expression levels were observed for genes responsible for vascular development and cell proliferation, although apparent increase of cell number was not observed in the area adjacent to the invading haustoria (Ikeue et al., 2015). Further study needs to clarify whether invasion of *Cuscuta* affects the morphology of host organ or not.

TRANSFER OF LONG-DISTANCE SIGNALS AFTER CONDUCTIVE PHASE

RNA Movement

Translocation of mRNAs and small RNAs between *Cuscuta* and the host plant have been shown (Alakonya et al., 2012; LeBlanc et al., 2013) and selectivity of the mobility or uptake of RNA has also been suggested (LeBlanc et al., 2013). A recent study using high-throughput RNA sequencing technology revealed that mRNAs representing more than 8000 genes of the parasite *Cuscuta campestris* and those representing more than 9000 genes of the host Arabidopsis move to the parasitic partner (Kim et al., 2014). Although an unexpectedly large number of RNAs were shown to move from plant to plant, biological relevance and necessity of the movement of mRNAs in the establishment of parasitic relationship are still unclear.

Trans-species movement of small RNAs (sRNA) has been documented for artificially induced short interfering RNA. Trans-silencing of a target gene was employed to demonstrate the role of *SHOOT MERISTEMLESS-LIKE 1* in haustorium development in *C. campestris* (Alakonya et al., 2012). Recently, induction of microRNA (miRNA) was demonstrated in *C. campestris* in the parasitic interface with the host Arabidopsis (Shahid et al., 2018). The miRNAs target transcripts encoding defense-related proteins, such as *AtSEOR1*, *BOTRYTIS-INDUCED KINASE 1* (*AtBIK1*), and members of the *TRANSPORT INHIBITOR RESPONSE 1* (*AtTIR1*)/*AUXIN SIGNALING F-BOX 2* and *3* (*AtAFB2*/*AtAFB3*) family, and accumulation of these transcripts were reduced during parasitization. Although direct evidence for the enhancement of vigor of the parasite has not yet been obtained, the biomass of *C. campestris* on Arabidopsis loss-of-function mutants, *seor1* and *afb3-4*, increased, suggesting that repression of these defense-related genes by miRNAs from the parasite may have biological significance (Shahid et al., 2018).

Trans-species movement of mRNA likely recruit the mechanisms for long-distance movement *via* phloem. Although experimental mRNA mobility can be explained by abundance and half-life of transcripts (Calderwood et al., 2016), presence

of sequence motifs such as tRNA-like motifs have been reported selective long-distance movement of mRNA through graft union (Thieme et al., 2015; Zhang et al., 2016). It will be of interest whether the same motifs are functional in the trans-specific movement or not. The mechanisms involved in sRNA transfer needs to be elucidated as well.

Signals in Response to Herbivory Feeding

Responses to herbivory-feeding in one host plant can transfer to the second host plant connected by the bridging *Cuscuta australis* (Hettenhausen et al., 2017), indicating the feeding signals transfer from host to parasite on the first host, and the other way round on the second host. Feeding by green pea aphid, *Myzus persicae*, induces a local response to *C. australis*, and the signal moves to the soybean host and induces the expression of the herbivory response (Zhuang et al., 2018). These results demonstrate that *Cuscuta* can transmit and receive the systemic signal for herbivory response, although the systemic signal has not been identified yet.

CONCLUSION AND FUTURE PERSPECTIVES

Elucidation of cellular and molecular processes involved in the formation of parasitic organs of *Cuscuta* has unveiled mechanisms hidden in the parasitic interface tissues. *Cuscuta* probably recruits genetic networks shared by other vascular plants, such as the genetic network for protrusive outgrowth of the epidermal cells and for formation of vascular tissues. They use the set of genes in a non-canonical way, though, as seen in the patterning of procambium, xylem, and phloem cells in haustorium. In addition to the formation of parasitic organs, trans-species trafficking of macromolecules, such as RNAs, through parasitic interface suggests a possibility of bi-directional control of biological processes between host and parasite. Understanding of *Cuscuta* will suggest parallels with other multi-organism processes, such as grafting, nematode infection, and formation of insect galls (Melnyk and Meyerowitz, 2015; Viera and Gleason, 2019). Comparative analyses of these processes will reveal the fundamental roles of extracellular and intracellular communication in multi-organism complexes.

AUTHOR CONTRIBUTIONS

KS and KA conceived and wrote the manuscript. KS prepared figures. All authors read and approved the final manuscript.

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MINI REVIEW
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Molecular Weapons Contribute to Intracellular Rhizobia Accommodation Within Legume Host Cell

*Camille Syska[1], Renaud Brouquisse[1], Geneviève Alloing[1], Nicolas Pauly[2], Pierre Frendo[1], Marc Bosseno[1], Laurence Dupont[1] and Alexandre Boscari[1]**

[1] Université Côte d'Azur, INRA, CNRS, ISA, Sophia Antipolis, France, [2] Laboratoire des Interactions Plantes-Microorganismes, INRA, CNRS, Université de Toulouse, Castanet-Tolosan, France

The interaction between legumes and bacteria of rhizobia type results in a beneficial symbiotic relationship characterized by the formation of new root organs, called nodules. Within these nodules the bacteria, released in plant cells, differentiate into bacteroids and fix atmospheric nitrogen through the nitrogenase activity. This mutualistic interaction has evolved sophisticated signaling networks to allow rhizobia entry, colonization, bacteroid differentiation and persistence in nodules. Nodule cysteine rich (NCR) peptides, reactive oxygen species (ROS), reactive nitrogen species (RNS), and toxin–antitoxin (TA) modules produced by the host plants or bacterial microsymbionts have a major role in the control of the symbiotic interaction. These molecules described as weapons in pathogenic interactions have evolved to participate to the intracellular bacteroid accommodation by escaping control of plant innate immunity and adapt the functioning of the nitrogen-fixation to environmental signalling cues.

Keywords: legumes, symbiosis, bacteroid, reactive oxygen species, nitric oxide, nitrogen- fixation, nodule-specific cysteine rich peptides, toxin–antitoxin

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Jesus Montiel,
Aarhus University, Denmark

****Correspondence:***
Alexandre Boscari
alexandre.boscari@inra.fr

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INTRODUCTION

The nitrogen-fixing symbiosis (NFS) results from the relationship between plants of the legume family and soil bacteria referred to as rhizobia. After a recognition step, bacteria infect legume roots, induce the formation of specialized root organs, the nodules, and colonize nodule cells by endocytosis to form structures called symbiosomes (Ferguson et al., 2010). Inside symbiosomes, bacteria differentiate into bacteroids that can convert atmospheric dinitrogen (N_2) into ammonia (NH_4^+), *via* the nitrogenase activity. NH_4^+ is then transferred to the whole plant through either amino acids or ureide compounds (Oldroyd and Downie, 2008; Masson-Boivin et al., 2009). NFS provides substantial agronomic and environmental benefits such as the substitution to nitrogen (N) fertilizer inputs to increase the plant yields (Vitousek et al., 2013).

The setting of NFS depends on a signal exchange. An initial plant defense response is observed during the first hours of the interaction with the rhizobium, and then is actively suppressed after the recognition. How rhizobia are recognized as symbionts rather than pathogens by the host plant is well described (Jones et al., 2007; Soto et al., 2009), and the strategies of the plants to adjust their own defense systems to enable rhizobia entry, colonization, and differentiation are detailed in several reviews (Oldroyd et al., 2011; Oldroyd, 2013). Recent reports support the hypothesis

that the regulation of immune response does not end at the recognition stage, but rather continue to allow rhizobial long-term accommodation inside the plant cells (Cao et al., 2017; Zipfel and Oldroyd, 2017; Berrabah et al., 2018; Yu et al., 2019). Multiple compounds such as nodule-specific cysteine rich (NCR) peptides, reactive oxygen species (ROS), reactive nitrogen species (RNS) and toxin–antitoxin (TA) modules have been shown to control the setup and the functioning of the interaction between the two partners The purpose of the present review is to provide an overview of the role of these compounds, described as weapons in pathogenic interactions, in the intracellular bacteroid accommodation (rhizobial colonization, differentiation, and control of plant innate immunity for persistence) and the adjustment of the nitrogen-fixation activity to environmental signalling cues.

Reactive Oxygen Species (ROS) in Bacterial Colonization of the Plant Cell and Bacteroid Persistence in the Nodule

ROS are involved in adaptation to environmental perturbations (Apel and Hirt, 2004; Waszczak et al., 2018). They are also essential for promoting normal cellular processes in bacteria and plants (Mittler, 2017). The level of ROS in cells depends on the tight regulation of a complex array of ROS generating systems and detoxification mechanisms, and antioxidant metabolites like glutathione and ascorbate. The balance between ROS production and detoxification regulates the cellular redox homeostasis in plants as well as in bacteria (Apel and Hirt, 2004).

In plants, the respiratory burst oxidase homologs (RBOH) proteins (also called NADPH oxidase) emerged as the major sources of apoplastic ROS (**Figure 1**) and key players in the redox signaling during pathogen infection and other processes (Kadota et al., 2015; Liu and He, 2016; Montiel et al., 2016). Some members of this multigenic family are differentially expressed in *Medicago truncatula* nodule tissues and play different roles from the establishment of the symbiotic interaction to the functioning of mature nodule (Marino et al., 2011; Montiel et al., 2018). The reduction in the N_2-fixation capacity in transgenic roots knocked-down for *MtRbohA* was the first evidence of a RBOH involvement in nodule functioning (Marino et al., 2011)(**Table 1**). Authors suggested that MtRBOHA activity contributes to the communication between the plant and the microsymbiont. Hydrogen peroxide production was visualized in *M. truncatula* infection zone and regulates genes involved in the nodulation process (Andrio et al., 2013). Similar results were obtain in *Phaseolus vulgaris* using knocked-down *PvRbohA* gene (Arthikala et al., 2017). Moreover, the roots overexpression of *PvRbohB* increases the number of bacteroids in the symbiosomes and improves biological N_2-fixation in *P. vulgaris* (Arthikala et al., 2014). In contrast, mutations of *NAD1* gene (Nodules with Activated Defence) in *M. truncatula* activate a strong defence response after rhizobia are released from infection threads into plant cells, leading to necrotic cell death of symbiotic cells (Wang et al., 2016). The knock-out of either *MtRbohB*, *MtRbohC,* or *MtRbohD* in the *nad1* mutant reverts this cell death phenotype indicating that nodule innate immunity is notably mediated by RBOH activity (Yu et al., 2018; Yu et al., 2019). These data provide evidences that MtRBOH-mediated ROS production has positive and negative functions in the reception of the microsymbionts in the nodule cells.

To cope with the plant ROS production, the microsymbiont contains a number of antioxidants and ROS-scavenging enzymes to preserve the bacteroids against ROS damages (Puppo et al., 2005; Becana et al., 2010). Analysis of bacterial mutants deficient in glutathione synthetase (*gshB*), thioredoxin (*trxL)*, glutaredoxins (*grx1*, or *grx2*), superoxide dismutase (*sodA*), and catalases (double mutants *katA/katC* or *katB/katC*) showed that the alteration in antioxidant pools as well as the mutation of ROS detoxification enzymes impact the formation of nodules, decrease the N_2-fixing capacity and induce a premature nodule senescence (**Table 1**)(Santos et al., 2000; Jamet et al., 2003; Harrison et al., 2005; Castro-Sowinski et al., 2007; Benyamina et al., 2013). Besides, nodules induced by *a Sinorhizobium meliloti* deletion mutant of *lsrB*, which encodes a LysR transcription factor acting as a ROS regulator, showed premature senescence with impaired bacteroid differentiation (Luo et al., 2005; Tang et al., 2013). LsrB was found to induce the expression of the *lrp3-lpsCDE* operon involved in lipopolysaccharide biosynthesis required for infection or bacteroid survival in host cells (**Figure 1**)(Tang et al., 2014) and that of γ-glutamylcysteine synthetase, involved in glutathione synthesis (Tang et al., 2017).

Nodule-Specific Cysteine Rich (NCR) Peptides and Terminal Bacteroid Differentiation

NCR peptides have been specifically found in the Inverted Repeat-Lacking Clade (IRLC) legumes such as *Medicago* spp., and in Dalbergoid legumes such as *Aeschynomene* spp., where bacteria are terminally differentiated to polyploïd non-dividing bacteroids (Mergaert et al., 2003; Mergaert et al., 2006; Alunni and Gourion, 2016). They encode highly divergent peptides, which resemble defensin-type antimicrobial peptides involved in plant and animal innate immunity (Mergaert et al., 2003). Indeed, some NCR peptides have a strong *in vitro* antimicrobial activity when applied to free-living bacteria (Van de Velde et al., 2010; Maróti and Kondorosi, 2014; Farkas et al., 2017).

Almost all NCR genes are exclusively expressed in the infected cells of nodules and their products are targeted to the symbiosome through the endoplasmic reticulum secretory system (**Figure 1**) (Wang et al., 2010; Guefrachi et al., 2014). Challenge of cultured bacteria with synthetic NCR peptides and ectopic expression of NCR peptides in legumes devoid of NCR genes cause features of bacteroid differentiation, demonstrating that these NCR peptides are sufficient to induce the irreversible differentiation (Van de Velde et al., 2010). The number of NCR genes is remarkably variable (from 7 in *Glycyrrhiza uralensis* to over 700 members in *M. truncatula*), and a positive correlation was found between the size of the NCR peptide family in the plant genome and the degree of bacteroid elongation (Montiel et al., 2017). Despite the large size of NCR peptide family in *M. truncatula* suggesting an extensive redundancy, NCR169, NCR211, and NFS1 are essential and the corresponding plant mutants are unable to

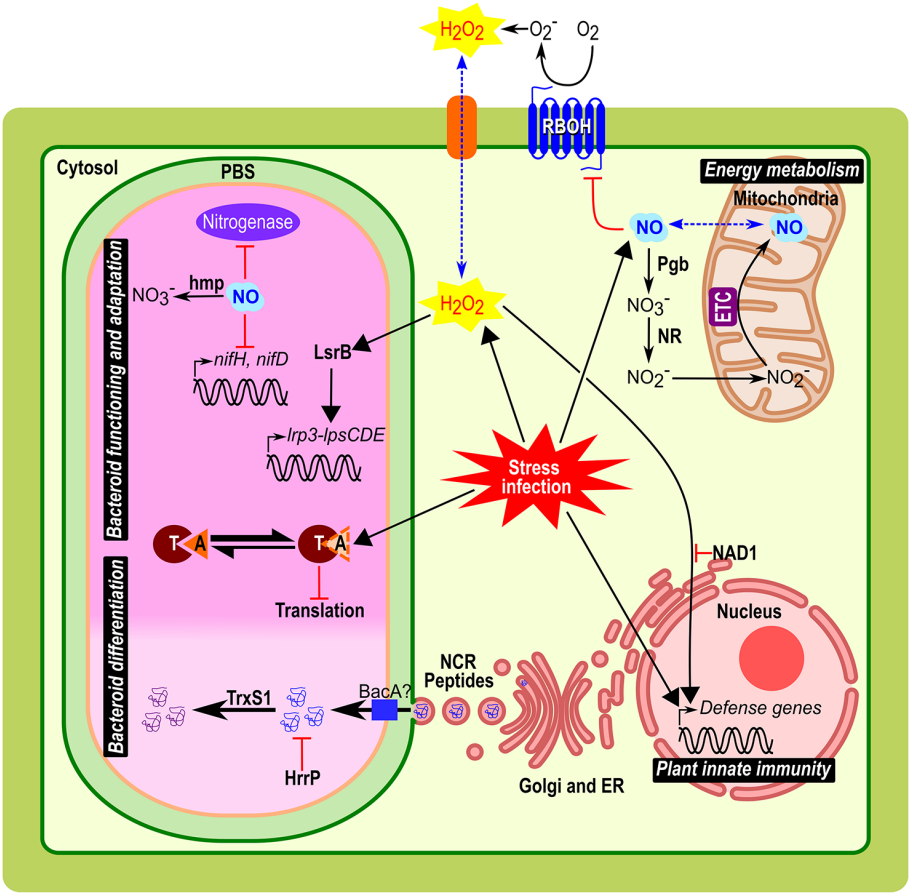

FIGURE 1 | Implication and connection of ROS, NO, NCR peptides, and TA modules in symbiosomes from *Medicago* root nodules. Biological role of these compounds during bacteroid differentiation, nodule functioning and adaptation, plant innate immunity, and energy metabolism are represented. Plant host cells infected by bacteria/bacteroid, implied various stress responses such as oxidative/nitrosative stress, acidic pH, microoxia, and exposure to NCRs. In the symbiosome, the clear part corresponds to the infection zone and the dark pink to the fixation zone with bacteria differentiated in bacteroid. Black arrows indicate metabolism reaction or downstream signal transduction pathways; red arrows indicate regulation mechanism (activation with arrowhead or repression with bar-headed lines). Blue dotted arrow indicates a diffusion through the membrane. Abbreviations: PBS, peribacteroid space; NR, nitrate reductase; Pgb, Phytoglobin; RBOH, respiratory burst oxidase homologs; $O_2^{\cdot-}$, superoxide radical; H_2O_2, hydrogen peroxide; ETC, electron transfer chain; NO, nitric oxide; ER; endoplasmic reticulum; NCR peptides, nodule-specific cysteine-rich peptides; Hmp, flavohemoglobin; NAD1, Nodules with Activated Defence 1; TrxS1, Thioredoxine S1; HrrP, Host-range restriction peptidase; LsrB, LysR transcription factor; T, toxin; A, antitoxin.

establish a functional NFS (**Table 1**) (Horváth et al., 2015; Kim et al., 2015; Yang et al., 2017). Both NFS1 and NCR211 exemplify NCR peptides that control the survival of fully differentiated bacteroids instead of triggering the terminal differentiation of bacteroids. NFS1 controls the discrimination mechanisms against incompatible microsymbionts (Yang et al., 2017), provoking bacterial cell death and early nodule senescence in an allele-specific and rhizobial strain-specific manner, while NCR211 is required for bacteroid persistence inside symbiotic cells (Kim et al., 2015).

To survive exposure to toxic NCR peptides *S. meliloti* requires the integrity of the BacA ABC-transporter. A *bacA* mutant strain is unable to differentiate and rapidly dies after its release from infection threads (**Figure 1**) (Haag et al., 2011). Similarly, the BacA-like peptide transporter BclA of *Bradyrhizobium japonicum* is essential for bacteroid differentiation and survival in *Aeschynomene* nodule, which suggests that the NCR peptides uptake may be a common mechanism used by different rhizobia to counteract the toxic effect of peptides (Guefrachi et al., 2015). In the symbiosis between *Sinorhizobium freedi* and *G. uralensis* alternatively, bacteroid differentiation occurs *via* a *bacA*-independent pathway and is rather associated with LPS modification of the bacteroid outer membrane (Crespo-Rivas et al., 2016). Additionally, a *S. meliloti* natural strain can escape the control of NCR peptides and proliferate in nodules using the plasmid encoded host-range restriction peptidase Hrrp, which is able to digest NCR peptides *in vitro* (**Figure 1** and **Table 1**) (Price et al., 2015). The expression of *hrrp* increases the fitness of rhizobial strains while inhibiting N_2-fixation in some plant ecotypes (Price et al., 2015).

Another layer of regulation may come from posttranslational modifications of NCR peptides (Marx et al., 2016). In particular,

TABLE 1 | Non-exhaustive summary of genes involved in ROS, NCR, NO, and TA modules pathways within legume nodule cells.

	Mutant/transgenic line	Origin	Proteic activity	Symbiotic function	Reference
ROS	*nad1*	*M. truncatula*	Nodule activated defense protein -uncharacterized	Nodule innate immunity, Bacteroid differenciation/ survival, N_2-fixation	Wang et al. (2016)
	RbohA: RNAi			N_2-fixation	Marino et al. (2011)
	RbohA: RNAi	*P. vulgaris*	Respiratory burst oxidase homolog - ROS production	Bacterial infection, Nodule formation, Bacteroid survival, N_2-fixation	Arthikala et al. (2017)
	RbohB: RNAi			Bacterial infection, Nodule formation, N_2-fixation	Montiel et al. (2012)
	RbohB: OE			Bacterial infection, Nodule formation, Bacteroid differenciation, N_2-fixation	Arthikala et al. (2014)
	gshB	*S. meliloti*	ROS detoxification enzymes	Nodule formation, N_2-fixation	Harrison et al. (2005)
	trxL			N_2-fixation	Castro-Sowinski et al. (2007)
	grx1			N_2-fixation	Benyamina et al. (2013)
	grx2			Nodule formation, N_2-fixation	
	sodA			N_2-fixation, Bacteroid differenciation	Santos et al. (2000)
	katA/katC; katB/katC			Nodule formation, Infection, N_2-fixation, Bacteroid differenciation	Jamet et al. (2003)
	lsrB		LysR transcription factor	Infection, Bacteroid differenciation/survival, N_2-fixation	Luo et al. (2005); Tang et al. (2013); Tang et al., (2014); Tang et al., (2017)
NCR	*dnf7-2* deletion mutant	*M. truncatula*	Antimicrobial peptide NCR169	Bacteroid survival/ persistence	Horváth et al. (2015)
	dnf4 deletion mutant	*M. truncatula*	Antimicrobial peptide NCR211- Symbiont Specificity	Bacteroid survival/ persistence	Kim et al. (2015)
	*NFS1-/- (NCR*α-β)	*M. truncatula*	Antimicrobial peptide - Symbiont Specificity	Bacteroid survival, Senescence	Yang et al. (2017)
	Trx s1: RNAi Trx s1: OE	*M. truncatula*	Thioredoxin-NCR reduction	Bacteroid differenciation	Ribeiro et al. (2017)
	bacA	*S. meliloti*	ABC transporter- Symbiont protection against NCRs	Bacteroid differenciation	Haag et al. (2011)
	bclA	*B. japonicum*		Bacteroid differenciation/ survival	Guefrachi et al. (2015)
	hrrP	*S. meliloti*	M16A family metallopeptidase- Escape NCR control	Bacteroid fitness	Price et al. (2015)
NO	Hb1: RNAi	*L. japonicus*	Leghemoglobin- degradation of nitric oxide	N_2-fixation	Ott et al. (2005)
	Hb1: OE		Phytoglobin- degradation of nitric oxide	N_2-fixation	Shimoda et al. (2009)
	Hb1: OE	*A. firma*			
	hmp	*S. meliloti*	Flavohemoprotein- NO degradation	Bacteroid survival, N_2-fixation, Senesence	Cam et al. (2012); Meilhoc et al. (2013); Blanquet et al. (2015)
	hmp++				
	norB *nnrS1*				Blanquet et al. (2015)
TA modules	*vapC-4* (*ntrR*)	*S. meliloti*	VapB (antidote), VapC (site-specific RNase)	Nodule formation	Dusha et al. (1989)
				N_2-fixation, Senescence	Oláh et al. (2001)
	vapB-5			Nodule formation, Bacteroid differenciation	Lipuma et al. (2014)
	vapC-5			N_2-fixation, Bacteroid survival, Senescence	
	bat/bto = vapBC	*B. japonicum*		Nodule formation, N_2-fixation	Miclea et al. (2010)

Genes studied have a rhizobial (orange) or a plant (light green) origin. The nitrogen-fixing phenotype of the mutant or transgenic line is depicted in green if defective or in pink if improved in the column symbiotic function.

a nodule-specific thioredoxin, TrxS1, capable to reduce NCR peptides and targeted to symbiosomes, has been shown to be required for bacteroid differentiation, suggesting that NCR redox state is important *in planta* (**Figure 1** and **Table 1**)(Ribeiro et al., 2017). In this context, the redox control of the bacteroid differentiation probably occurs through the NCR peptide activity suggesting a crosstalk between the different regulators described in this review.

Considered together, these data indicate that the symbiosis efficiency of terminally differentiated bacteria is the outcome of a tight balance between the effects of NCR peptides and the ability of rhizobia to resist them. The rupture of this balance can lead to the activation of the plant innate immunity (Yu et al., 2019).

Nitric Oxide (NO) in Functional Nodules: Microoxia, Energetic Metabolism

NO production was observed in functional nodules of *Lotus japonicus* and *M. truncatula*, mainly in the N_2-fixation zone (Baudouin et al., 2006; Shimoda et al., 2009), and in the nodule senescence zone (Cam et al., 2012; Fukudome et al., 2018). Although the origin and the biological significance of NO production in nodules has been thoroughly analyzed over the last few years (Boscari et al., 2013; Hichri et al., 2015; Berger et al., 2019), there are still many questions to be clarified concerning the relative importance of the signaling/metabolic functions of NO versus its toxic action on host plant and symbiont.

Functional nodules are characterized by a microoxic environment to protect the bacterial nitrogenase from irreversible denaturation by oxygen (O_2) which requires the setup of an O_2 barrier in the outer cell layers of the nodule and the synthesis of leghemoglobin (Lb) (Appleby, 1992). In plant roots submitted to hypoxia, a "Phytoglobin-NO respiration" has been shown to use nitrite as a final electron acceptor instead of O_2 to be reduced to NO by the mitochondrial electron transfer chain (ETC), which allows cell energy status retention (**Figure 1**) (Igamberdiev and Hill, 2009; Gupta and Igamberdiev, 2011). Accumulated data support the existence of such a Phytoglobin-NO respiration in *M. truncatula* and *Medicago sativa* nodules, in which both nitrate reductase (NR) and ETC are involved in NO production and in the maintenance of the nodule energy state (Horchani et al., 2011; Berger et al., 2018).

Despite its role in acclimation to microoxic environment, NO is also a potent inhibitor of nitrogenase activity (Trinchant and Rigaud, 1982; Sasakura et al., 2006; Kato et al., 2010). In nodules of soybean plants subjected to flooding, the increase in NO production is associated with the repression of bacterial *nifH* and *nifD* (**Figure 1**), and this inhibition is partially reversed by the application of the NO scavenger cPTIO, which illustrates the inhibitory role of NO on the expression of nitrogenase genes (Sánchez et al., 2010). Furthermore, using both pharmacological approach, with NO-donors and scavengers, and molecular approach with transgenic plants with modified NO levels, several studies report that NO inhibits *in vivo* N_2-fixing activity in soybean, *L. japonicus*, and *M. truncatula* nodules (**Table 1**) (Shimoda et al., 2009; Kato et al., 2010; Cam et al., 2012).

The biological activity of NO is mediated through redox-dependent protein modifications such as metal-nitrosylation, S-nitrosation and Tyr-nitration (Stamler et al., 2001; Besson-Bard et al., 2008). In *M. truncatula* mature nodules, 80 proteins have been reported to be S-nitrosated, most of them involved in primary metabolism, energy regeneration and nitrogen assimilation (Puppo et al., 2013). In this context, *M. truncatula* glutathione peroxidase 1 and glutamine synthetase 1a were shown to be regulated by NO through S-nitrosation and Tyr-nitration modifications (Melo et al., 2011; Castella et al., 2017).

Beside the nodule metabolism regulation, a participation of NO to the life-time of the symbiotic interaction was also observed (Cam et al., 2012). Increased NO level in nodule obtained either by using *S. meliloti* mutant strains deficient in the degradation of NO (*hmp*, *norB*, *nnrS1*)(**Table 1**), or by treating nodules with NO donors (Cam et al., 2012; Meilhoc et al., 2013; Blanquet et al., 2015) leads to premature nodule senescence. Conversely, by using *S. meliloti* mutant strains that over-expressed *hmp*, a decrease in NO level was observed correlated to a delay of nodule senescence (**Table 1**)(Cam et al., 2012). Therefore, NO concentration should be tightly controlled, in time and space, in both partners to avoid its toxic effects and to fulfil its signaling and metabolic functions during nodule functioning and under environmental stresses (Berger et al., 2019).

Toxin-Antitoxin (TA) Systems in Bacteroid Adaptation in Infected Plant Cells

TA systems are key players of intracellular survival of invading bacteria during eukaryote interactions (Lobato-Márquez et al., 2016). TA genes encode a stable toxin and its cognate antitoxin. Depending on the antitoxin nature (RNA or protein) and its mode of action, TA modules are classified into six different types (I–VI). The type II, where both toxin and antitoxin are small proteins forming a stable complex, is the most abundant type in pathogens, particularly exposed to diverse micro-environments during host interaction (Ramage et al., 2009; Georgiades and Raoult, 2011). Due to the self-poisoning effect of the toxin, TA modules could be considered as intracellular molecular timebombs. TA expression is tightly regulated to allow either growth arrest and bacterial adaptation or cell death (Hayes and Kędzierska, 2014). Under various stress conditions, the antitoxin is degraded by bacterial proteases leading to the deregulation of the TA operon and delivery of the toxin which targets specific cellular functions (DNA replication, translation) (Gerdes et al., 2005). In phytopathogenic bacteria, TA have been recently demonstrated as involved in virulence and biofilm formation during plant infection (Shidore and Triplett, 2017; Martins et al., 2018).

Among the 29 chromosomal type II TA systems of *S. meliloti*, eleven belong to the VapBC family; VapB being the antitoxin and VapC the toxin, acting as a site-specific RNase (**Table 1**). The importance of two *vapBC* operons, *vapBC-4* (*ntrPR*) and *vapBC-5*, has been shown in *S. meliloti* during symbiotic interaction with *Medicago* sp. (Dusha et al., 1989; Lipuma et al., 2014). NtrPR was identified on the capacity of the toxin *ntrR* mutant (for nitrogen regulator) to form more nodules on alfalfa roots in the presence

of exogenous ammonium (Dusha et al., 1989). This suggests that NtrR toxin is involved in the nodulation efficiency depending on the level of nitrogen supply. This module plays also a role in mature nodules in a nitrogen-tolerant manner, as *ntrR*-induced nodules have an enhanced N_2-fixation capacity and an increased plant yield (Oláh et al., 2001). Regarding VapBC-5 module, the *vapC-5* toxin mutant improves the symbiotic interaction with alfalfa (increase in N_2-fixation capacity and plant yield) associated to a delay in nodule senescence (Lipuma et al., 2014).

These *vapC* mutants have no free-living phenotypes. Therefore, TA modules might play a role in the bacterial adaptation to infection stresses (metabolic shifting, acidic pH, microoxia, ROS, antimicrobial peptides, stresses known to activate pathogen TA modules (Lobato-Márquez et al., 2016)) (**Figure 1**). Thus, in a wild-type context, NtrPR and VapBC-5 modules likely limit the symbiotic interaction upon specific plant signals and/or contribute to the nodule senescence onset. The high number of TA systems in *S. meliloti* genome could be due to functional redundancy or to different roles independent of the NFS. Indeed, Milunovic et al. (2014) showed that the deletion of four TA operons from the pSyma and pSymb plasmids induces a cell toxicity phenotype in free living, with no symbiotic effect during alfalfa interaction (Milunovic et al., 2014). In contrast, in *B. japonicum* USDA110, the complete deletion of the *bat/bto* TA resulted in a limited production of soybean nodules associated to a reduced plant yield (Miclea et al., 2010). Such a phenotype suggests that this system might play a positive role on the symbiotic interaction with soybean, although this could also be linked to the pleiotropic effects observed for this deletion mutant in free-living conditions.

Concluding Remarks

The evidences presented in this review show the importance of ROS, NO, NCR peptides, and TA modules in the intracellular bacteroid accommodation and the N_2-fixation activity regulation. These molecules, considered in certain situations as cellular weapons, are necessary not only in the nodule functioning, but also in the rupture of the symbiosis under unfavourable conditions such as deficient bacterial symbionts, adverse environmental conditions or cellular aging. The importance of these regulatory elements is now clearly demonstrated, but their mode of action still remains to be fully deciphered. Identification of the molecular pathways involved in the regulation of the bacterial intracellular life during NFS will be helpful to dissect the crosstalk between these different regulatory elements. Evidences exist of the connection between ROS, NO, and NCR in plant cells to balance the plant immune response, to regulate the rhizobial differentiation and control the switch from bacteroid persistence to cell death. Among these recent findings it can be noted the involvement of three RBOH in the activation of immunity in *Medicago* nodules and the regulation of bacteroid differentiation *via* TrxS1-dependent redox regulation of some NCRs *in planta* (Ribeiro et al., 2017) (**Figure 1**). Furthermore, it was previously shown that NO could inhibit NADPH oxidase activity by post-translational modification (**Figure 1**)(Yun et al., 2011).

Similarly, the connection between TA, NO, ROS, and NCR produced by both partners represents a field of future interest to identify the signals involved in TA activation. The delayed senescence of nodules induced by the *vapC-5* toxin mutants conducted to higher the expression of NCR001 gene compared to control *Rhizobium* strain (Lipuma et al., 2014). Finally, a better understanding of these regulatory processes may give promising strategies to improve the NFS and reduce the use of fertilizers.

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CS and AB conceived the idea of the review. All the authors were involved in the manuscript writing.

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Interspecific Signaling Between the Parasitic Plant and the Host Plants Regulate Xylem Vessel Cell Differentiation in Haustoria of *Cuscuta campestris*

Yuki Kaga[1], Ryusuke Yokoyama[1], Ryosuke Sano[2], Misato Ohtani[2,3], Taku Demura[2], Takeshi Kuroha[1†], Naoki Shinohara[4] and Kazuhiko Nishitani[1,4]*

[1] Graduate School of Life Sciences, Tohoku University, Sendai, Japan, [2] Graduate School of Science and Technology, Nara Institute of Science and Technology, Ikoma, Japan, [3] Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Japan, [4] Faculty of Science, Kanagawa University, Hiratsuka, Kanagawa, Japan

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***Correspondence:**
Kazuhiko Nishitani
nishitani@kanagawa-u.ac.jp

†Present address:
Takeshi Kuroha,
Division of Applied Genetics, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization (NARO), Tsukuba, Japan

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The genus *Cuscuta* is stem parasitic angiosperms that parasitize a wide range of vascular plants via de novo formation of a distinctive parasitic organ called a haustorium. In the developing haustorium, meristematic cells, which are initiated from the stem cortical tissue, differentiate into haustorial parenchyma cells, which elongate, penetrate into the host tissues, and finally connect with the host vasculature. This interspecific vasculature connection allows the parasite to uptake water and nutrients from the host plant. Although histological aspects of haustorium development have been studied extensively, the molecular mechanisms underlying vasculature development and the interspecific connection with the host vasculature remain largely unknown. To gain insights into the interspecific cell-to-cell interactions involved in haustorium development, we established an *in vitro* haustorium induction system for *Cuscuta campestris* using *Arabidopsis thaliana* rosette leaves as the host plant tissue. The *in vitro* induction system was used to show that interaction with host tissue was required for the differentiation of parasite haustorial cells into xylem vessel cells. To further characterize the molecular events occurring during host-dependent xylem vessel cell differentiation in *C. campestris*, we performed a transcriptome analysis using samples from the *in vitro* induction system. The results showed that orthologs of genes involved in development and proliferation of vascular stem cells were up-regulated even in the absence of host tissue, whereas orthologs of genes required for xylem vessel cell differentiation were up-regulated only after some haustorial cells had elongated and contacted the host xylem. Consistent results were obtained by another transcriptome analysis of the haustorium development in *C. campestris* undergoing parasitization of an intact host plant. These findings suggest the

involvement of host-derived signals in the regulation of non-autonomous xylem vessel differentiation and suggest that its connection to the host xylem during the haustorium development activates a set of key genes for differentiation into xylem vessel cells.

Keywords: stem parasitic plant, plant-plant interspecific interaction, haustorium development, xylem vessel differentiation, transcriptome analysis

INTRODUCTION

Although land plants originated as autotrophic organisms, some angiosperms have evolved parasitism. Parasitic angiosperms have acquired the ability to absorb water and nutrients from host plants through an invasive organ called a haustorium (Westwood et al., 2010). Parasitic angiosperms are categorized by the degree of their dependency on the host plant for nutrients. Hemiparasitic angiosperms have photosynthetic capacity and rely only partly on the host plant, while holoparasitic angiosperms lack this capacity and cannot survive without parasitizing the host plant (Heide-Jørgensen, 2008). The genus *Cuscuta* or dodder plants, which are classified in the family Convolvulaceae, lack roots and true leaves, and are considered to be holoparasitic angiosperms (Dawson et al., 1994).

After a *Cuscuta* stem coils around the stem of a host plant, the cortical tissue on the concave side of the *Cuscuta* stem, in contact with stem surface of the host plant, begins to proliferate and expand to form a haustorial meristem (Dawson et al., 1994). Two types of cell differentiate within the meristem: tip cells (apical side) and file cells (proximal side) (Hong et al., 2011). As haustorium development proceeds, tip cells and file cells grow into search hyphae and axial cells, respectively (Hong et al., 2011), and the haustorium begins to penetrate into the host epidermal tissues. Penetration is facilitated by enzymatic cell-wall degradation and driven by the force generated by cell division and cell elongation in the axial cell region (Nagar et al., 1984; Dawson et al., 1994). After the penetration event, search hyphae begin to elongate extensively by tip growth in the host tissue (Dawson et al., 1994), and intrude into the host xylem, where they differentiate into xylem vessel cells (also termed xylem hyphae) (Hong et al., 2011). Connections between host and parasite xylems have also been observed in mature haustoria (Birschwilks et al., 2007). However, it remains unclear how xylem differentiation is regulated and how the xylem connection is established between the host plant and the parasitic plant.

During vasculature development in angiosperms, xylem vessel cell formation is initiated by differentiation of vascular stem cells under the regulation of MONOPTEROS (MP), which belongs to a family of auxin-responsive factors (ARFs). MP directly activates the expression of *ARABIDOPSIS THALIANA HOMEOBOX8 (ATHB8)* (Schlereth et al., 2010), which encodes a transcription factor that induces the expression of the *PIN-FORMED 1* (*PIN1*) gene and activates the development of pre-procambial cells (Scarpella et al., 2006). Additionally, MP directly activates the expression of *TARGET OF MONOPTEROS* and *TMO5-LIKE1* (*TMO5* and *T5L1*) (Scarpella et al., 2006). A heterodimeric complex of TMO5/T5L1 and LONESOME HIGHWAY (LHW) promotes cytokinin biosynthesis in cells surrounding xylem precursor cells by triggering the transcription of *LONELY GUY3* and *LONELY GUY4* (*LOG3* and *LOG4*), resulting in the regulation of cell division and patterning in vascular tissues (De Rybel et al., 2013; Ohashi-Ito et al., 2014). Phytohormones including auxins and cytokinins are involved in xylem vessel formation. Brassinosteroids also play a role in xylem vessel formation by promoting the transcription of HD-ZIP III transcription factor family genes, which are involved in establishing vascular patterning and determining cell fate (Ohashi-Ito et al., 2002; Fukuda, 2004). After the determination of cell fate in vascular tissue, the VASCULAR-RELATED NAC-DOMAIN (VND) family of transcription factors activates the expression of a set of genes required for xylem vessel cell differentiation (Kubo et al., 2005; Tan et al., 2018). The final process in xylem vessel differentiation is formation of patterned secondary cell walls (SCWs) and programmed cell death (PCD) (Fukuda, 2004).

Although the formation of xylem vessels in angiosperms is well understood, relatively little is known regarding haustorium and xylem development in parasitic plant genera such as *Cuscuta*. In an attempt to identify key genes responsible for the development of haustoria, transcriptome analyses have been performed that compared the expression profiles of different developmental stages of the *Cuscuta* haustoria. Genes involved in response to stimulus, transport activity, and cell wall functions exhibited high expression during haustorial development (Ranjan et al., 2014; Ikeue et al., 2015; Olsen et al., 2016). Despite these extensive studies, the molecular mechanisms regulating xylem differentiation during haustorium formation are still poorly understood.

Given that haustorium development is a cell-non-autonomous process that is influenced by interspecific cell-to-cell interactions, it is necessary to distinguish the contributions of the parasite and host to understand haustorium development. Accordingly, to investigate the effect of host factors on the development of the parasitic haustorium and its penetration into host tissues, an *in vitro* system was developed to enable separation of host and parasitic factors. This *in vitro* parasitization system was used to analyze the effect of host tissues on the transcriptional regulation of haustorium development in *C. campestris* by comparing expression in the absence and presence of host tissue. The results showed that elongation of search hyphae was initiated irrespective of host-derived biological factors but that host-derived factors were required for further differentiation of search hyphae in the haustorium and for final differentiation into xylem vessel cells

and connection to the host vasculature to complete the parasitic linkage.

MATERIALS AND METHODS

Plant Materials

Seeds of *Arabidopsis thaliana* (L.) Heynh. accession Col-0 were sown on mineral wool (Rockwool B.V., Grodan) moistened with MGRL liquid medium (Tsukaya et al., 1991) and grown under continuous white light (45 μmol m^{-2} s^{-1}) in a growth chamber at 22°C (Nippon Medical and Chemical Instruments, Co., Ltd.). Seeds of *Cuscuta campestris* Yuncker were soaked in concentrated sulfuric acid for 25 min at 22°C, washed with distilled water at 22°C five times, and placed on a filter paper (No.5A 90 mm, Toyo Roshi Kaisha, Ltd.) immersed in tap water for germination under continuous white light (45 μmol m^{-2} s^{-1}) in the growth chamber at 22°C.

Induction of Parasitism

After germination, 5-day-old seedlings of *C. campestris* were placed in a position to attach to the inflorescence stems of 4–5-week-old *A. thaliana* plants. Parasitism was induced under blue light (wavelength peak = 444 nm, 7 μmol m^{-2} s^{-1}) in a growth chamber at 25°C for 2 days, after which plants were grown under continuous white light (45 μmol m^{-2} s^{-1}) at 22°C.

Parasitism was also induced using excised lateral shoots from mature *C. campestris* plants. Lateral shoots (3 cm in length) with the apex attached were cut from mature *C. campestris* plants that had parasitized a host plant. Shoot segments were then attached to new inflorescence stems of 4–5-week-old *A. thaliana* using surgical tape (Micropore™ Surgical Tape, 3M Company) and parasitism induced under blue light at 25°C as for seedlings. The process of parasitism was recorded by time-lapse imaging (TLC200, Brinno). The time at which coiling of *C. campestris* around the host plant was complete was designated as 0 hours after coiling (hac).

In vitro Haustorium Induction

Lateral shoots of *C. campestris* stem were cut 3 cm below the apex. Shoot segments were placed on 3% agarose gel containing 0.1% Plant Preservation Mixture™ (Plant Cell Technology, Inc.), weighted with a stack of glass slides (S1225, Matsunami Glass Ind., Ltd.), and incubated under blue light irradiation at 25°C (**Figure 1A**). To induce differentiation of search hyphae into xylem hyphae, a 3-cm-long lateral shoot segment of *C. campestris* was overlaid with a fresh rosette leaf of 4–5-week-old *A. thaliana*, and was weighted with a stack of glass slides (**Figure 2A**). Haustoria were classified into two types: haustoria protruding search hyphae were designated as true haustoria, while conical-shaped ones were designated as pseudo haustoria according to Hong et al. (2011). The numbers of true and pseudo haustoria were counted under a stereomicroscope (M205 FA, Leica).

Phytohormone Treatment of Search Hyphae

C. campestris lateral shoot sections that had been pressed for 54 hours to induce haustoria were placed on 3% agarose media containing different phytohormone compositions. Sections were placed so that search hyphae were in contact with the medium and were incubated in a growth chamber for 48 hours. Shoot segments were incubated under the same conditions as for the *in vitro* haustorium induction system. Phytohormone compositions in the media were as followed: (1) 1 μM brassinolide (BL) and 10 mM H_3BO_3; (2) 0.1 mg/L naphthaleneacetic acid (NAA) and 0.2 mg/L benzyladenine (BA); (3) 1.25 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25 mg/L kinetin and 10 μM Bikinin; and (4) 50 ng/ml kinetin, 500 ng/ml 2,4-D and 1 mM BL.

Histological Staining and Microscopy

Lateral shoots with induced haustoria were embedded in 5% agarose gel and sectioned transversely or longitudinally at a thickness of 60 μm using a vibratome (VT1200S, Leica). Sections were fixed with FAA solution (4% paraformaldehyde, 20 mM sodium cacodylate buffer) and stored at 4°C. Sections were cleared with an ethanol series (50%, 60%, 70%, 80%, 90%, and 95%) and washed three times with phosphate buffered saline (PBS). The cleared sections were stained with a solution containing 0.002% Fluostain I (Sigma-Aldrich) and 0.2% propidium iodide (Fujifilm Wako Pure Chemical) for 1 hour followed by washing three times with PBS. The stained sections were immersed in 50% 1 × PBS/glycerol solution, and Z-serial optical sections were obtained under a laser scanning confocal microscope (FV1000-D, Olympus). Digital accumulation of Z-serial optical sections was performed using ImageJ (ver. 2.0.0).

Images of *C. campestris* shoots with induced haustoria were obtained using a stereomicroscope (for **Figures 1B** and **2B**). Tissues from which RNA samples for RNA-sequencing (RNA-seq) analysis were prepared were visualized using a stereomicroscope (for **Figures 4A–C**), or a light microscope (DM RXP, Leica) after transverse (for **Figure 4D,** control) or longitudinal (**Figure 4D**, 57 and 87 hours after induction) sectioning.

Transcriptome analysis of *In Vitro* Haustorium Development

Tissues for RNA extraction were manually excised from control and *in vitro* induced-haustorium shoots under a stereomicroscope. Control samples were excised from epidermal and cortical tissues obtained at 0 hours after induction (hai) from 3-cm shoot sections that had not been pressed by glass slides or placed in contact with host tissue [0 hai (−/−)]. For induced samples, haustorium development was induced in 3-cm lateral shoot sections of *C. campestris* as described above. Sections were pressed under a stack of glass slides with or without contact with host leaf tissue for 57 or 87 hours after induction (**Figure 4**). Pressed samples with host contact were designated 57 hai (+/+) and 87 hai (+/+), and pressed samples without host contact were designated 57 hai (+/−) and 87 hai (+/−). Haustoria for RNA extraction were manually excised from shoot segments, with minimal host tissue included for the 57 hai (+/+) and 87 hai (+/+) samples.

Tissue samples were immediately frozen in liquid nitrogen, and total RNAs were isolated using an RNeasy Plant Mini Kit

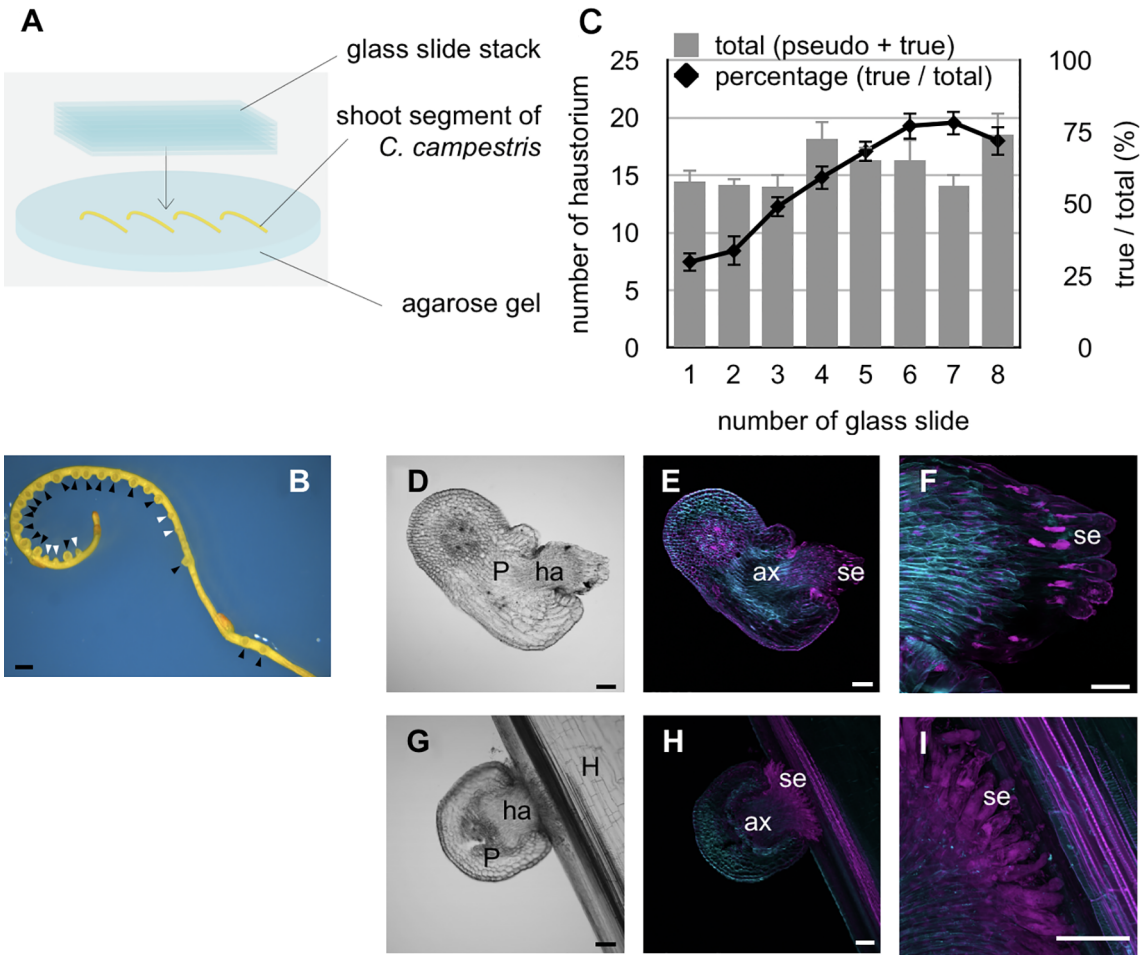

FIGURE 1 | Physical pressure and blue light irradiation induce the protrusion of search hyphae during haustorium development. **(A)** Schematic of the *in vitro* haustorium induction procedure. **(B)** Formation of haustoria in an excised *C. campestris* lateral shoot that was pressed with a stack of glass slides under blue light (444 nm) irradiation at the dosage of 7 μmol m^{-2} s^{-1}, and incubated at 25°C for 72 hours. Black arrowheads indicate true haustoria, while white arrowheads indicate pseudo haustoria. **(C)** Excised lateral shoots were placed on 3% agarose gel and pressure applied with a varying number of glass slides followed by incubation at 25°C for 72 hours. Total numbers of haustoria produced in each of the 3-cm long lateral shoot segments produced by the *in vitro* induction system, and percentage of true haustoria among the total haustoria, are shown as a function of the number of glass slides applied. Mean values from 11 biological replicates are shown with standard errors (SE) as vertical lines. **(D–F)** Images of a haustorium produced by the *in vitro* induction system at the stage of 72 hours after induction. **(D)** A bright-field image of a longitudinal section of the haustorium **(E, F)** Digital accumulation of fluorescence images of Z-serial optical sections of the same longitudinal section as for **(D)**. The section was double stained with Fluostain I and propidium iodide. **(F)** A high-magnification image of **(E)**. **(G–I)** Images of haustoria from *C. campestris* parasitizing an intact inflorescence stem of *A. thaliana* at the stage of 42 hours after coiling. **(G)** A bright-field image of a longitudinal section of the haustorium. **(H, I)** Digital accumulation fluorescence images of Z-serial optical sections of the same longitudinal section as for **(G)**, which was double stained with Fluostain I and propidium iodide. **(I)** High-magnification images of **(H)**. Scale bars: **(B)** 1 mm; **(D–I)** 100 μm. P, parasitic plant; ha, haustorium; ax, axial cell; se, search hypha; H, host plant.

(Qiagen Inc.) with RNase-Free DNase (Qiagen Inc.) according to the manufacturer's protocol. Extracted RNA was quantified with a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific). RNA quality was assessed with an Agilent 2100 Bioanalyzer using an RNA 6000 Nano Kit (Agilent Technolozies, Inc.).

RNA-seq was performed using the BGISEQ-500 platform (BGI), and 100 bp pair-end reads for each library were mapped independently to the references described below using the HISAT2 (ver. 2.1.0) alignment program (Kim et al., 2015). Annotated reference genome sequences for *C. campestris* were downloaded from plaBiPD (https://www.plabipd.de) (Vogel et al., 2018). Three biological repeats were used for reference genome mapping. One of the three 87 hai (+/+) treatment libraries was an outlier according to hierarchical clustering and was therefore excluded from differential expression analysis. Transcript expression levels and differentially expressed genes (DEGs) were determined using the StringTie (ver. 1.3.6; Pertea et al., 2015) and TCC (Sun et al., 2013) packages, respectively. Transcript expression levels were normalized to transcripts per million (TPM), and genes with q-value < 0.01 were regarded as DEGs.

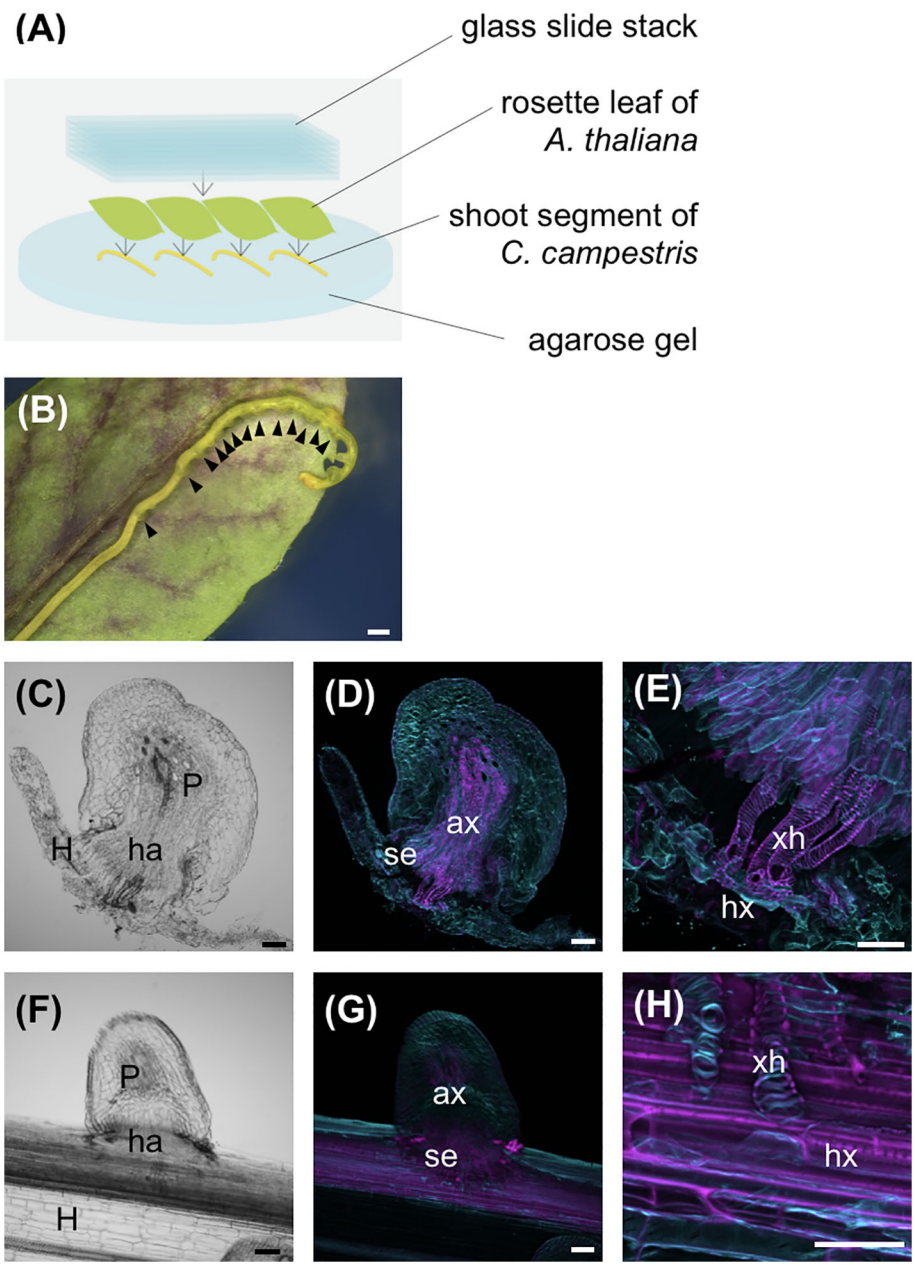

FIGURE 2 | Host xylem is necessary for search hyphae to differentiate into xylem vessel cells. **(A)** Schematic of the *in vitro* haustorium induction procedure with *A. thaliana* rosette leaves used as the host. Excised *C. campestris* lateral shoots were covered with *A. thaliana* rosette leaves and pressure applied with a stack of glass slides. **(B)** Formation of haustoria in an excised *C. campestris* lateral shoot under an *A. thaliana* rosette leaf and a stack of glass slides. Black arrowheads indicate haustoria. **(C–E)** Images of a haustorium produced by the *in vitro* induction system with host rosette leaf at the stage of 96 hours after induction. **(C)** A bright-field image of a longitudinal section of the haustorium. **(D, E)** Digital accumulation fluorescence images of Z-serial optical sections of the same longitudinal section as for **(D)**, which was stained with Fluostain I and propidium iodide. **(E)** A high-magnification image of **(D)**. **(F–H)** Images of a haustorium from *C. campestris* parasitizing an intact inflorescence stem of *A. thaliana* at the stage of 66 hours after coiling. **(F)** A bright-field image of a longitudinal section. **(G, H)** Digital accumulation fluorescence images of Z-serial optical sections of the same longitudinal section as for (F), which was double stained with Fluostain I and propidium iodide. **(H)** A high-magnification image of **(G)**. Scale bars: **(B)** 1 mm; **(D–H)** 100 μm. P, parasitic plant; ha, haustorium; ax, axial cell; se, search hypha; xh, xylem hypha; hx, host xylem; H, host plant.

Transcriptome Analysis of Haustorium Development in *C. campestris* Parasitizing Intact *A. thaliana*

Coiling regions of *C. campestris* lateral shoots parasitizing an intact *A. thaliana* inflorescence stem were harvested 0, 12, 42, and 54 hac. The harvested tissues obtained at 0 hac consisted of epidermis and cortex from the concave region of the parasite stem. Harvested tissue at 12 hac contained prehaustoria and those obtained at 42 hac and 54 hac contained haustoria. Tissue samples were transverse sectioned (100 μm) to the host stem axis using a vibratome. Haustorial regions were excised from transverse sections by laser microdissection using a PALM MicroBeam (Carl Zeiss Microscopy GmbH) (**Supplementary Figure 3**). Control samples were derived from *C. campestris* lateral shoots that were irradiated with blue light for 24 hours, but which did not coil around the host stem. Control samples consisted of the epidermis and cortex and were harvested, sectioned and subjected to the laser micro-dissection as coiled samples.

Excised tissue samples were immersed in RNAlater Solution (Thermo Fisher Scientific) and stored at 4°C. Total RNAs were isolated using an RNeasy Plant Mini Kit with RNase-Free DNase according to the manufacturer's protocol. Extracted RNA was quantified with and a NanoDrop spectrophotometer. RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technolozies, Inc.) using an RNA 6000 Pico Kit (Agilent Technolozies, Inc.). For screening, cDNA libraries were constructed using an NEBNext RNA Library Prep Kit for Illumina (NEW ENGLAND BioLabs), according to the manufacturer's protocol. After ligation of indexed adaptors (**Supplementary Table 1**), products were purified using Agenocourt AMPure XP Beads (Beckman Coulter) and amplified by PCR with KAPA Hifi HotStart ReadyMix (KAPA Biosynthesis). The cDNA libraries were separated by 2% agarose gel electrophoresis, extracted using a QIAquick Gel Extraction Kit (QIAGEN), and finally quantified using a Library Quantification Kit (Takara, Japan). In total, 15 cDNA libraries consisting of three biological replicates of five experimental conditions (0 hac, 12 hac, 42 hac, 54 hac, and control) were pooled in equal amounts (18 pM and 20 pM) for multiplexing. Libraries were sequenced using a Genome Analyzer IIx instrument (Illumina), and the 33 nt single-end reads from each library were mapped independently to the references described above using the HISAT2 (ver. 2.1.0) alignment program (Kim et al., 2015). Three biological replicates were used for reference genome mapping. Transcript expression levels and DEGs were determined using the StringTie (ver. 1.3.6; Pertea et al., 2015) and TCC (Sun et al., 2013) package, respectively. Transcript expression levels were normalized to TPM, and genes with q-value < 0.01 were regarded as DEGs.

Phylogenetic Analysis

Similarity searches were performed against The Arabidopsis Information Resource 10 database (TAIR10; ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR10_genome_release/TAIR10_gff3/TAIR10_GFF3_genes.gff; Lamesch et al., 2012) using BLASTP. Collected protein sequences were aligned using MAFFT (ver. 7.427) (Kato and Standley, 2013) then visually inspected and manual refined. Gaps and ambiguous sites were removed from the alignment. Phylogenetic trees were constructed with a maximum likelihood method using MEGA7 (Kumar et al., 2016) with bootstrap replication of 1,000.

Clustering Analysis

Soft clustering was performed on gene sets that were defined as DEGs using Mfuzz (Futschik and Carlisle, 2005) based on TPM. Functional annotations of DEGs and clustered gene sets were produced from the reference annotation information.

Enrichment Analysis

Enrichment was determined using the hypergeometric distribution (Johnson et al., 1992) and Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

RESULTS

Establishment of an *In Vitro* System for Induction of Haustorium Development in *C. campestris*

An *in vitro* system for inducing haustorium development outside an intact host was developed and used to examine the host-dependent formation of haustoria in *C. campestris*. Previous studies reported that tactile stimuli induced the formation of haustoria under far-red light irradiation (Tada et al., 1996; Olsen et al., 2016), and that blue light irradiation promoted parasitism in *Cuscuta* seedlings (Lane and Kasperbauer, 1965). Accordingly, in this study, the relationship between haustorium formation and a mechanical stimulus was investigated by pressing lateral shoot segments of *C. campestris* with a stack of glass slides under blue light irradiation for 72 hours (**Figure 1A**). Two types of haustoria were induced using this experimental system. One is those protruding search hyphae, which we termed true haustoria, and the other is conical-shaped one, which we termed pseudo haustoria (Hong et al., 2011) (**Figure 1B**). True haustoria accounted for approximately 30% of observed haustoria when one glass slide was used to apply pressure to the lateral shoot sections (equivalent to approximately 20.74 kPa), whereas about 75% of haustoria protruded search hyphae when seven glass slides were used to apply pressure of ~145.20 kPa (**Figure 1C**). However, the number of slides used to apply pressure had no significant effect on the overall number of haustorium produced by the lateral shoots (**Figure 1C**). Under these conditions, when *C. campestris* shoot segment did not attach to the host, elongation of axial cells and search hyphae was observed in the true haustoria, search hyphae did not differentiate into xylem hyphae (**Figures 1D–F**). These true haustoria were similar to those observed just after penetration into host inflorescence stems (**Figures 1G–I**).

Next, to investigate the involvement of host-derived phytohormones in haustorium development, excised shoots with induced true haustoria were placed on solid agarose

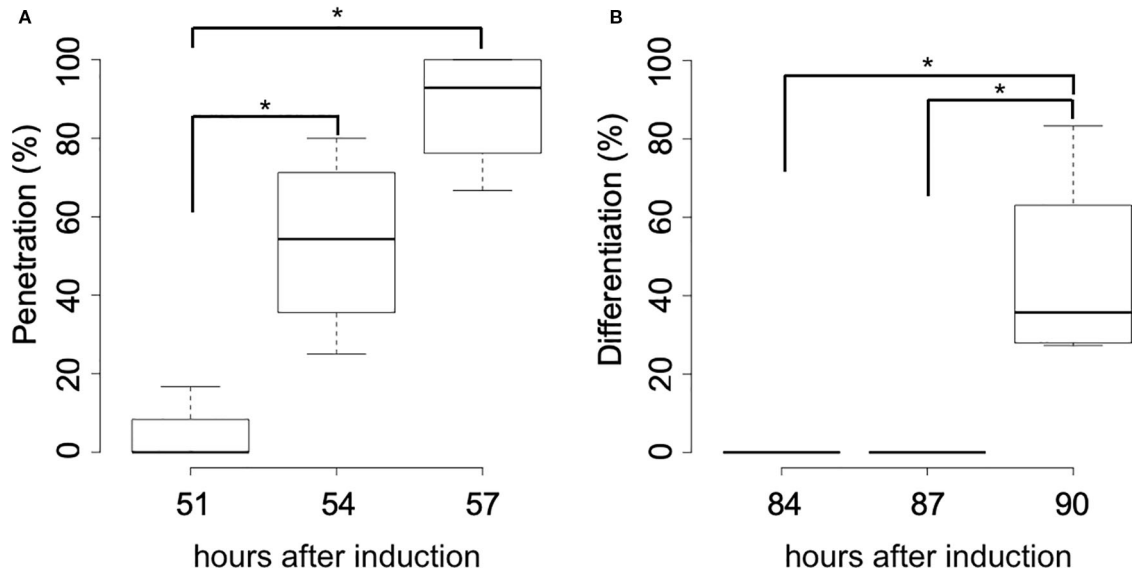

FIGURE 3 | Time-course of the haustorial penetration and differentiation of search hyphae. **(A)** Percentage of haustoria that penetrated *A. thaliana* rosette leaves at the stage of 51, 54, and 57 hours after induction. **(B)** Percentage of haustoria whose search hyphae differentiated into xylem hyphae at the stage of 84, 87, and 90 hours after induction. Significance was determined by the Wilcoxon rank-sum test (n = 4). Asterisks indicate significant differences in pairwise comparisons ($p < 0.05$).

media containing four different phytohormone or chemical mixtures, that reportedly induced xylem vessel differentiation in other angiosperms (Demura et al., 2002; Kubo et al., 2005; Kondo et al., 2016; Tan et al., 2018). Shoots were placed to ensure that search hyphae were in contact with the agarose medium. No visible alterations in haustorium development were observed after exposure to the phytohormone mixtures under the conditions we examined (**Supplementary Figure 1**).

As discussed above, although haustorium development in *C. campestris* proceeded to the host-penetration stage upon application of pressure and exposure to blue light irradiation, search hyphae did not differentiate into xylem hyphae. Three typical phytohormones that were previously shown to play essential roles in xylem vessel formation in other angiosperms were not effective in inducing differentiation of search hyphae into xylem hyphae. These results suggest that the inability to induce differentiation in the absence of host tissue is not due to a lack of phytohormones derived from the host plants, implying that host-derived factors other than auxins, cytokinins, or brassinosteroids are needed for xylem vessel differentiation of *Cuscuta* haustorial cells.

Differentiation of Search Hyphae Into Xylem Hyphae is Induced Upon Contact With Host Xylem

To determine whether search hyphae produced by the *in vitro* haustorium induction system had the potential to differentiate into xylem hyphae upon contact with host xylem, lateral shoot segments of *C. campestris* were overlaid with fresh *A. thaliana* rosette leaves, and then pressed with a stack of glass slides (**Figure 2A**). Haustorium invaded the host tissue in the presence of host leaves. Helical-patterned SCW differentiation was observed where the search hyphae came into contact with the host xylem (**Figures 2B–E**). The SCW deposition pattern of xylem hyphae observed with the *in vitro* induction system was comparable to the that observed during *C. campestris* parasitization of intact host plant stems (Dawson et al., 1994) (**Figures 2F–H**). These results indicate that the search hyphae produced by the *in vitro* induction system have the same potential for differentiation as the cells that penetrate intact host stems and eventually differentiate into xylem vessel cells during parasite-host interactions.

Subsequent time-course observation of the *in vitro* induction process revealed that most haustoria had penetrated the host rosette leaf by 57 hai (**Figure 3A**), and that differentiation of search hyphae into xylem hyphae had occurred by 90 hai (**Figure 3B**). Thirty hours after penetration, xylem vessel cells had formed in the induced haustoria.

Transcriptional Regulation During Haustorium Penetration of Host Tissue

RNA-seq was used to examine the transcriptional regulation of haustorium development during penetration into host tissue, RNA-seq libraries were prepared from tissue samples taken at specific time points determined through time-course observation of haustorium development (**Figure 3**). Tissue samples were derived from haustoria that penetrated into the host tissue at 57 hai (+/+) and at 87 hai (+/+), haustroria that did not contact host tissue at 57 hai (+/−) and at 87 hai (+/−), and epidermal and cortical cells of *C. campestris* at 0 hai (−/−) as a no-haustorium control (**Figure 4**). Sequenced read pairs

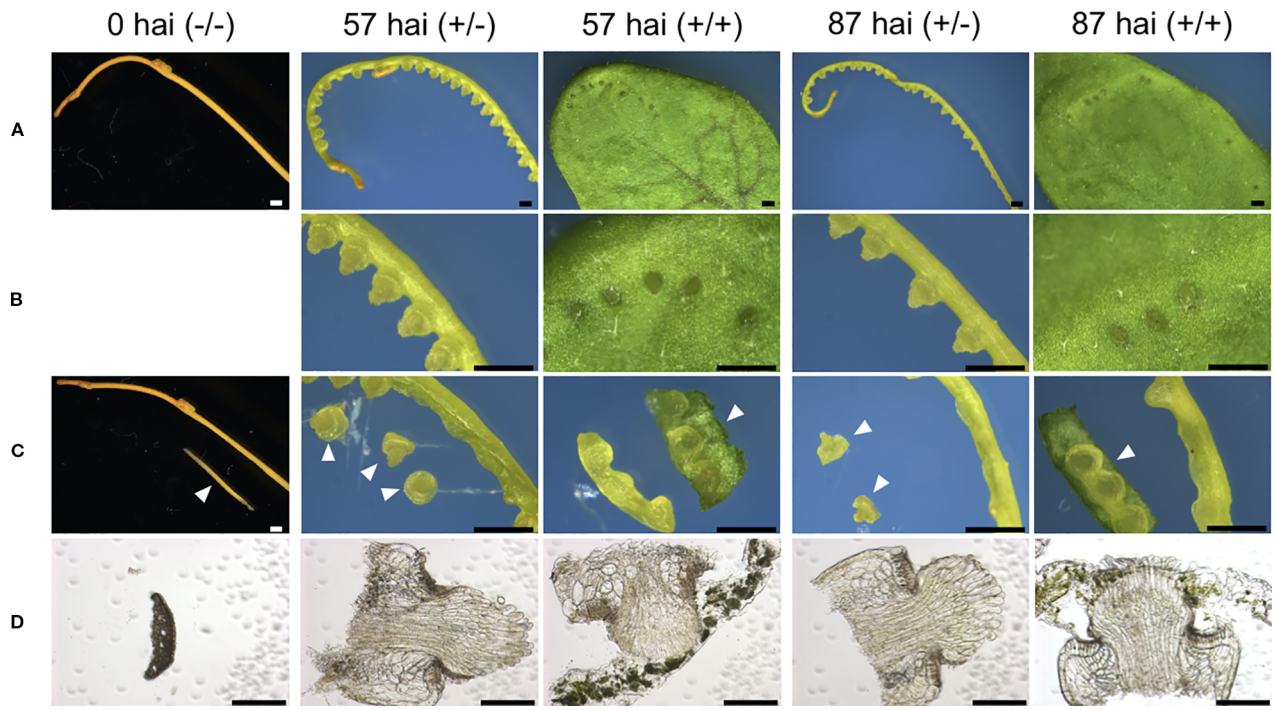

FIGURE 4 | Tissue sampling at different stages of *in vitro* haustorium development for RNA-seq libraries. **(A)** Control sample of intact lateral shoot of *C. campestris* at the onset of the induction is designated here as 0 hai (–/–). Three-centimeter long segments prepared from the lateral shoots were subjected to *in vitro* haustorium induction under pressure from a stack of glass slides under blue light (440 nm) irradiation at the dosage of 7 µmol m^{-2} s^{-1}, in the absence (+/–) or presence (+/+) of an *A. thaliana* rosette leaf for 57 or 87 hours after induction (hai). These samples are designated here as 57 hai (+/–), 57 hai (+/+), 87 hai (+/–), and 87 hai (+/+), respectively. **(B)** Magnified images of **(A)**. **(C)** Control sample, 0 hai (–/–), consisted of epidermal and cortical cells isolated from lateral shoot segments. Samples of 57 hai (+/–) and 87 hai (+/–) consisted of haustoria excised from shoot segments, while samples of 57 hai (+/+) and 87 hai (+/+) consisted of shoot segments containing haustoria with minimal host leaf included. Arrowheads show samples used for individual RNA-seq libraries. **(D)** Bright-field images of a transverse section for 0 hai (–/–), and longitudinal sections for the other four samples used for RNA-seq. Scale bars: **(A–C)** 1 mm; **(D)** 200 µm. hai, hours after induction.

were mapped against the *C. campestris* genome. The mapping rate of the 57 hai (+/+) and 87 hai (+/+) sequence reads was 16.6% lower than reads from the other libraries (**Supplementary Figure 2**). The 57 hai (+/+) and 87 hai (+/+) libraries contained reads derived from host tissues, suggesting that the lower mapping rate was due to the proportion of reads that did not map to the *C. campestris* reference genome. Differential expression analysis using a false discovery rate (FDR) < 0.01 produced 15,277 DEGs in the haustorium compared with the epidermal and cortical cells (**Supplementary Data 1–4**). Of these 4,239 DEGs, 1,721 of which were functionally annotated, were shared among the four haustorium conditions (**Figure 5**). Consitent with previous gene expression studies of the genus *Cuscuta* (Ranjan et al., 2014; Olsen et al., 2016), genes encoding functionally annotated proteins for carbohydrate metabolism, cell wall, and solute transport, as well as phytohormones, protein degradation, and RNA biosynthesis (q-value < 0.01, **Figure 5**) were up-regulated in the haustorium. DEGs were also compared among all five conditions (including the no-haustorium control), using an FDR < 0.01, and 28,958 DEGs were identified. After normalizing the count data to TPM, DEGs were soft-clustered, and clusters were analyzed for enriched functional annotations (**Figure 6**, **Table 1**, and **Supplementary Data 5**). Of the 28,958 DEGs, 11,802 genes were functionally annotated in reference annotation data of *C. campestris*.

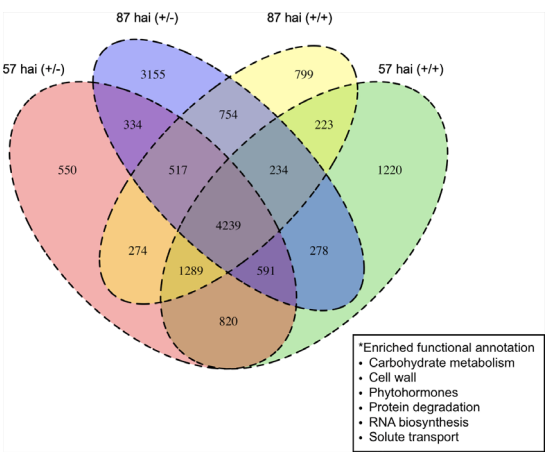

FIGURE 5 | Numbers of differentially expressed genes up-regulated in the haustorium at 57 or 87 hours after induction (hai) when subjected to pressure in the absence (+/–) or presence (+/+) of host tissue compared with control tissue containing epidermal and cortical cells at 0 hai (–/–). Enriched functional annotations for all four haustorial conditions are shown in the box. hai, hours after induction.

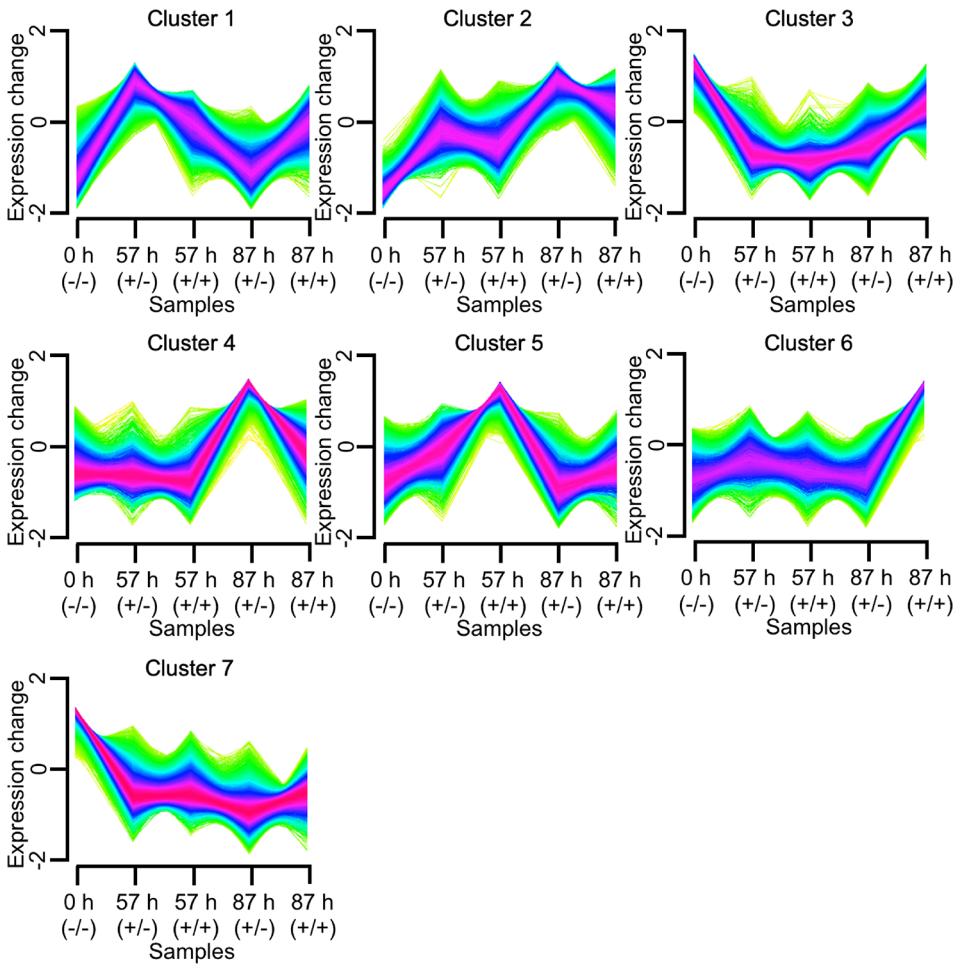

FIGURE 6 | Clustering analysis of 28,958 differentially expressed genes (false discovery rate < 0.01) using Mfuzz. Tissue samples were taken 57 or 87 hours after induction with pressure in the absence (+/–) or presence (+/+) of an *A. thaliana* rosette leaf, and designated here as 57 h (+/–), 57 h (+/+), 87 h (+/–), and 87 h (+/+), respectively. For control, samples containing epidermal and cortical cells of *C. campestris* stem were taken at the onset of the induction and designated here as 0 h (–/–). h, hours after induction.

At 57 hai (+/+), genes encoding proteins for cell wall, phytohormones, protein modification, and secondary metabolism were significantly enriched (q-value < 0.01, **Figure 6**, **Table 1**, and **Supplementary Data 5**). At 87 hai (+/+), phytohormones, polyamine metabolism, and RNA biosynthesis were up-regulated (q-value < 0.01, **Figure 6** and **Table 1**, **Supplementary Data 5**). These results indicate that gene expression is dynamically regulated by penetration into host tissue as well as during haustorium formation.

Transcriptional Regulation of Xylem Cell Differentiation in Haustoria

Contact with host xylems was necessary for differentiation of search hyphae into xylem hyphae, and we therefore focused on relevant genes whose expression profiles correlated to vascular development during the penetration of host tissue. RNA-seq data for orthologous genes reported to be involved in the development and proliferation of vascular stem cells were examined. At 57 hai (+/–), Cc*MP* (Cc035111), *Cc*TMO5 and *CcT5L1* (Cc004934 and Cc032564), *CcLHW* (Cc010690 and Cc026768), *CcLOG3* and *CcLOG4* (Cc028025 and Cc016389), and *CcHB8* (Cc027108 and Cc003079) were all up-regulated (**Figure 7**), indicating that haustoria acquired the potential for differentiation into xylem cells in the absence of penetration. The results indicate that vascular stem cells and xylem precursor cells can differentiate within the *Cuscuta* haustorial tissue without penetration into the host tissue.

It should also be noted that some members of the type-A *ARABIDOPSIS RESPONSE REGULATORs* (Cc008302, Cc009786, Cc001355, and Cc046889), which might negatively regulate cytokinin signaling, are up-regulated at 57 hai (+/+). This might suggest that the vascular stem cell proliferation is repressed *via* cytokinin signaling in the haustorium after the penetration into the host tissue (**Figure 7**).

CcVND7 (Cc010187), the orthologous gene to *VND7*, was up-regulated at 87 hai (+/+), but no ortholog of *VND6* was identified

TABLE 1 | Enriched functional annotation in each of the seven clusters of DEGs.

Cluster No.	Functional annotation	q-value
1	Cell wall	8.99E-03
1	Lipid metabolism	2.32E-03
1	Nutrient uptake	4.32E-03
1	Protein modification	7.84E-08
1	Solute transport	1.78E-04
2	Protein degradation	1.37E-05
2	RNA biosynthesis	3.25E-08
2	Solute transport	7.86E-03
3	Amino acid metabolism	1.53E-05
3	Cell cycle	1.09E-27
3	DNA damage response	2.46E-04
3	Environmental stimuli response	1.11E-03
3	Nucleotide metabolism	1.57E-04
3	Photosynthesis	4.17E-10
3	Protein biosynthesis	8.41E-73
3	Protein translocation	8.24E-21
3	RNA processing	6.78E-36
4	Carbohydrate metabolism	3.86E-03
4	Coenzyme metabolism	8.47E-05
4	Photosynthesis	1.18E-32
4	Secondary metabolism	3.85E-03
5	Cell wall	4.56E-12
5	Phytohormones	3.73E-03
5	Protein modification	3.48E-06
5	Secondary metabolism	5.05E-04
6	Phytohormones	3.22E-04
6	Polyamine metabolism	3.22E-04
6	RNA biosynthesis	1.82E-09
7	Cell wall	4.45E-06
7	Cellular respiration	3.53E-25
7	Cytoskeleton	1.76E-04
7	Lipid metabolism	2.21E-09
7	Membrane vesicle trafficking	2.18E-07
7	Protein biosynthesis	1.48E-18

Significance was determined by the hypergeometric distribution (p-value < 0.01) and Benjamini-Hochberg procedure (q-value < 0.01).

(**Figure 7**). Genes active downstream of *VND7,* namely, *MYB46*, *MYB83* (Cc016476 and Cc000889), *CELLULOSE SYNTHASE A4/IRREGULAR XYLEM 5* (*CESA4/IRX5)* (Cc037502), and *CESA7/IRX3* (Cc020329 and Cc026519), exhibited the same expression pattern as *CcVND7* (Kubo et al., 2005) (**Figure 7**). In *A. thaliana,* CESA4 and CESA7 are involved in synthesis of SCWs (Taylor et al., 2000; Taylor et al., 2003), with two functionally redundant MYB transcription factors, MYB46 and MYB83, acting as master regulators of SCW biosynthesis (Zhong et al., 2007; McCarthy et al., 2009). Here, two lignin biosynthesis-related genes, four genes encoding cysteine peptides, and eleven genes encoding serine peptidase were identified in Cluster 6 (**Figure 6**).

On the other hand, at 87 hai (+/−), the expressions of *BIFUNCTIONAL NUCLEASE 1* (Cc007021 and Cc006655), *CcARR10* and *CcARR12* (Cc015843 and Cc24708) were up-regulated, but activation of genes encoding proteins involved in promotion of xylem vessel cell formation was not found (**Figure 7**).

Importantly, a set of genes for xylem vessel cell formation whose expression were up-regulated at the stage of 87 hai (+/+) in the *in vitro* system were also up-regulated in the haustorium produced in *C. campestris* shoot 54 hours after coiling around an intact inflorescence stem of *A. thaliana,* when search hyphae contacted the host xylem (**Figure 8** and **Supplementary Figure 3**). Thus, the expression patterns of these genes in *in vitro* system were consistent with those in the haustorium development in *C. campestris* shoot parasitizing an intact host. These findings indicate that contact of search hypha with the host xylem triggers the up-regulation of a *VND7* ortholog in *C. campestris* and induces the formation of xylem vessel cells in the haustorium.

DISCUSSION

In this study, an *in vitro* system for inducing *C. campestris* haustorium formation through application of pressure in the presence or absence of host tissue was developed used to analyze host-dependent transcriptional regulation during haustorium development. Two types of haustoria, true haustorium and pseudo haustorium, were induced in *C. campestris* lateral shoots in the absence of host plants. The ratio of true haustorium to pseudo haustorium was dependent on the pressure applied to the *C. campestris* shoots. Application of a force of 145.20 kPa to a single 3-cm segment of *C. campestris* lateral shoot was optimum for the effective formation of true haustoria.

Our findings are consistent with previous research showing that *Cuscuta* plants promote haustorium development by sensing the pressure generated by coiling around the stem of a host plant (Lee, 2009). Our results suggest that the coiling of *C. campestris* around the host plant might exert pressure at a load of more than 100 kPa.

The *in vitro* pressure-based system was sufficient to induce elongation of search hyphae and axial cells, but was not sufficient to promote differentiation into xylem vessel cells. These results suggest that additional signaling derived from the host plant is necessary for xylem differentiation in *C. campestris* haustoria.

Next, *in vitro*-induced haustoria were cultivated on solid agarose media containing phytohormones that were previously shown to induce differentiation into xylem vessel cells (Demura et al., 2002; Kubo et al., 2005; Kondo et al., 2016; Tan et al., 2018); however, this exposure did not stimulate the haustorial cell differentiation into xylem vessel cells in *C. campestris*. These results suggest that the host-derived signaling factors that trigger differentiation of search hyphae into the xylem cells are not phytohormones.

Auxin activates MP transcription factor during vascular development in angiosperms. MP enhances ATHB8 expression and cytokinin biosynthesis, which promote vascular stem cell development and proliferation (Scarpella et al., 2006; Ohashi-Ito et al., 2014). In addition, brassinosteroids promote the transcription of HD-ZIP III transcription factor family genes, which play key roles in the establishment of vascular patterning in *Zinnia elegans* (Ohashi-Ito et al., 2002; Carlsbecker and Helariutta, 2005). This study examined transcriptional regulation of haustorium development in *C. campestris* and found that *MP* and downstream genes related to vascular stem cell specification and proliferation were up-regulated in the

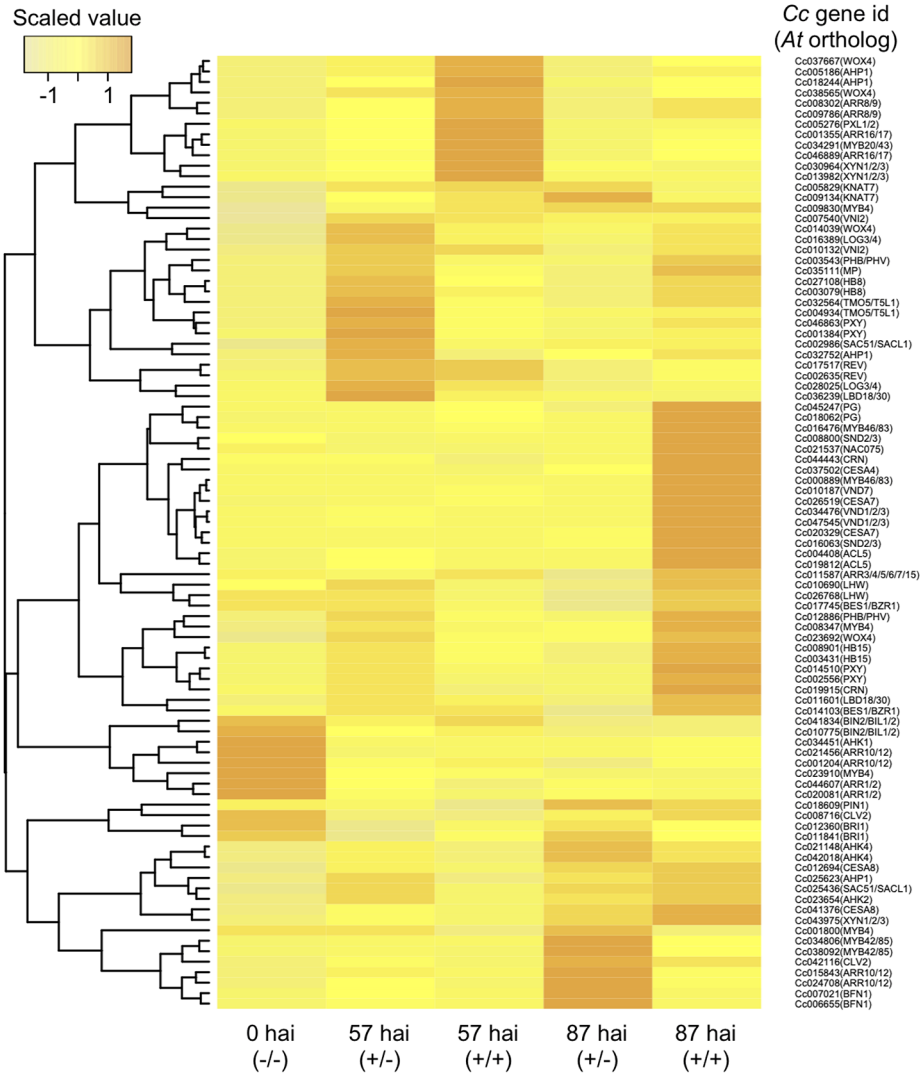

FIGURE 7 | Hierarchical clustering of *C. campestris* orthologous genes with biological functions putatively related to vascular development in *A. thaliana*. Transcript per million data from RNA-seq results of the *in vitro* induction system were normalized, and relative gene expression levels among the five samples are visualized in a heat map image according to the color scale shown in the left-hand panel. Samples were taken 57 or 87 hours after induction (hai) with pressure in the absence (+/–) or presence (+/+) of an *A. thaliana* rosette leaf and designated here as 57 hai (+/–), 57 hai (+/+), 87 hai (+/–), and 87 hai (+/+), respectively. For control, samples containing epidermal and cortical cells of *C. campestris* stem were taken at the onset of the induction and designated as 0 hai (–/–). hai, hours after induction.

haustorium, even in the absence of host plant tissue. It is therefore likely that the host-derived signaling factor(s) capable of triggering differentiation from search hyphae into xylem hyphae is those that activate the process of xylem vessel cell differentiation after vascular stem cell fate determination.

Previous morphological analyses showed that search hyphae penetrating into the host xylem differentiated into xylem hyphae (Hong et al., 2011). This suggests that contact between search hypha and the host xylem is required for the differentiation of search hyphae into xylem hyphae and also suggests that search hyphae might receive signals as a result of contact with the host xylem. The *C. campestris* orthologs of *VND7*, *MYB46*, and *MYB83* were expressed after search hyphae contacted the host xylem. These transcription factors are master regulators of xylem vessel cell differentiation and SCW biosynthesis (Kubo et al., 2005; Zhong et al., 2007; McCarthy et al., 2009). These data suggest that search hyphae receive host-derived signals that activates transcription of *CcVND7* and stimulate differentiation

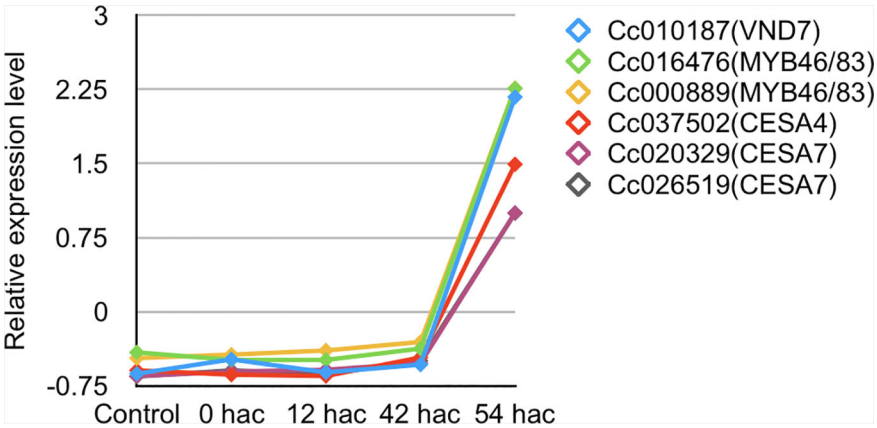

FIGURE 8 | Expression patterns of *C. campestris* orthologous genes related to xylem vessel cell differentiation in *A. thaliana*. Transcripts per million data from RNA-seq analysis of haustorium development in *C. campestris* parasitizing an intact host were normalized. Samples for RNA-seq analysis were collected from transverse sections of haustoria at a thickness of 100 μm using laser microdissection. Control, tissue region containing epidermal and cortical cells of *C. campestris* after irradiation with blue light for 24 hours without contact with the host; 0 hac, tissue region consisting of epidermal and cortical cells of the contact site with the host inflorescence stem just after coiling; 12 hac, prehaustorium at the stage of 12 hours after coiling; 42 hac and 54 hac, haustoria penetrating into the host plant at the stage of 42 and 54 hours after coiling. hac, hours after coiling.

into xylem hyphae in a non-cell-autonomous manner. Furthermore, the transcriptome data showed that the *NAC DOMAIN CONTAINING PROTEIN 75* (*NAC075*) ortholog in *C. campestris* was also up-regulated. NAC075 is thought to regulate the expression of *VND7* in *A. thaliana* (Endo et al., 2015), and the parasitic plant might therefore recognize signals from the host plant that promote *CcNAC075* expression.

This study characterized the dynamics of transcriptional regulation during the differentiation of xylem vessel cells in haustorium development in *C. campestris*. The results suggest that haustoria have acquired the potential for differentiation into xylem vessel cells without penetration into host tissue, probably through activation of genes involved in vascular stem development and proliferation. However, the expression of genes needed for xylem vessel cell differentiation appears to require contact between search hypha and the host xylem. This contact might be critical for efficient establishment of the xylem connection between the host plant and the parasitic plant. Signals derived from the host xylem appear to trigger the differentiation into xylem hyphae, possibly through the expression of *CcVND7*, regulated by CcNAC075, in the search hyphae. Further research is needed to identify host-derived signaling factors and signal transduction pathways that regulate expression of *CcNAC075* and *CcVND7* during parasitic invasion.

DATA AVAILABILITY STATEMENT

The RNA-seq data presented in this study have been deposited with links to accession number DRA009453 in the DDBJ database (Sequence Read Archive: https://trace.ddbj.nig.ac.jp/dra/index_e.html).

AUTHOR CONTRIBUTIONS

YK performed the experiments and data acquisition with occasional discussions with RY, RS, MO, TD, TK, and NS under supervision and organization of KN. The manuscript was written by YK under supervision of KN with discussion with YR, RS, TD, MO, NS, and TK. Deposition of sequence data was supported by RS.

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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.2020.00193/full#supplementary-material

SUPPLEMENTARY TABLE 1 | Sequences of indexing primers.

SUPPLEMENTARY FIGURE 1 | Haustoria produced by the *in vitro* induction system were further incubated on a solid agarose medium containing phytohormones/chemicals mixtures for 48 hours. Digital accumulation images of Z-serial optical sections of the haustorium. Scale bars; 100 μm. BL, brassinolide; NAA, naphthaleneacetic acid; BA, benzyladenine; 2,4D, 2,4-dichlorophenoxyacetic acid.

SUPPLEMENTARY FIGURE 2 | Mapping counts of reads for each library against the *C. campestris* genome.

SUPPLEMENTARY FIGURE 3 | Tissue samples from haustorium development in an intact host for RNA-sequencing libraries. Samples for each stage of the haustorium development were collected from transverse sections of 100 μm thickness using laser microdissection. Outside of the green circle showed the collected region for the control sample. Inside of the green circles showed the collected regions at 0, 12, 42, and 54 hours after coiling (hac). control, epidermal and cortical cells of *C. campestris* shoot irradiated with blue light for 24 hours; 0 hac, epidermal and cortical cells of the contact site with the host inflorescence stem just after the coiling; 12 hac, prehaustorium; 42 hac, haustorium just after penetration into the host; 54 hac, haustorium contact with the host xylem, Scale bars, 300 μm.

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Reprogramming of the Developmental Program of *Rhus javanica* During Initial Stage of Gall Induction by *Schlechtendalia chinensis*

*Tomoko Hirano[1], Seisuke Kimura[2], Tomoaki Sakamoto[2], Ayaka Okamoto[1], Takumi Nakayama[1], Takakazu Matsuura[3], Yoko Ikeda[3], Seiji Takeda[4], Yoshihito Suzuki[5], Issei Ohshima[6] and Masa H. Sato[1]**

[1] Laboratory of Cellular Dynamics, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan, [2] Faculty of Life Sciences, Kyoto Sangyo University, Kyoto, Japan, [3] Institute of Plant Science and Resources, Okayama University, Okayama, Japan, [4] Laboratory of Cell and Genome Biology, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan, [5] Department of Food and Life Sciences, College of Agriculture, Ibaraki University, Ibaraki, Japan, [6] Laboratory of Applied Entomology, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan

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***Correspondence:**
Masa H. Sato
mhsato@kpu.ac.jp

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Insect galls are unique organs that provide shelter and nutrients to the gall-inducing insects. Although insect galls are fascinating structures for their unique shapes and functions, the process by which gall-inducing insects induce such complex structures is not well understood. Here, we performed RNA-sequencing-based comparative transcriptomic analysis of the early developmental stage of horned gall to elucidate the early gall-inducing process carried out by the aphid, *Schlechtendalia chinensis,* in the Chinese sumac, *Rhus javanica*. There was no clear similarity in the global gene expression profiles between the gall tissue and other tissues, and the expression profiles of various biological categories such as phytohormone metabolism and signaling, stress-response pathways, secondary metabolic pathways, photosynthetic reaction, and floral organ development were dramatically altered. Particularly, master transcription factors that regulate meristem, flower, and fruit development, and biotic and abiotic stress-responsive genes were highly upregulated, whereas the expression of genes related to photosynthesis strongly decreased in the early stage of the gall development. In addition, we found that the expression of class-1 *KNOX* genes, whose ectopic overexpression is known to lead to the formation of *de novo* meristematic structures in leaf, was increased in the early development stage of gall tissue. These results strengthen the hypothesis that gall-inducing insects convert source tissues into fruit-like sink tissues by regulating the gene expression of host plants and demonstrate that such manipulation begins from the initial process of gall induction.

Keywords: *Rhus javanica*, floral organ development, gall formation, *Schlechtendalia chinensis*, RNA-seq analysis

INTRODUCTION

Galls are plant tissues or organs formed by hyperplasia (increased cell number) and/or hypertrophy (increased cell size) induced by parasitic or pathogenic organisms including viruses, fungi, bacteria, nematodes, mites, and insects (Mani, 1964). Among galls formed by various organisms, insect galls are extraordinarily complex and highly organized structures comprised of several specialized tissue types (Stone and Schönrogge, 2003; Giron et al., 2016). Insect galls range in complexity from relatively simple mine-galls (Guiguet et al., 2018), open- or folded-type galls such as pit galls, blister galls, and roll galls to complex structures in which the gall-inducing insects are entirely enclosed by plant tissues to form covering galls or mark galls in leaf, stem, and bud (Dreger-Jauffret and Shorthouse, 1992; Guiguet et al., 2019).

The most complex gall structures are generated by gall wasps, gall midges, and gall-inducing aphids in which the galls have extra-floral nectarines, and a coating of hair, spines, and sticky resins (Price et al., 1987; Stone and Schönrogge, 2003; Wool, 2004). The complex insect galls consist of various tissues such as nutritive and protective tissues. The nutritive tissues consist of callus cells and vascular cells, which transport nutrients to the callus; the tissues are ingested by gall-inducing insects. The protective tissues (sclerenchyma) are composed of lignified cells arranged as a layer on the outside of the nutritive tissues and function as a physical shelter against natural enemies and outside environment.

Several lines of evidence indicate that many gall-inducing insects have the potential to precisely secrete effectors into plant tissues using their mouthparts or ovipositors, and such effectors are likely to play a central role in gall induction (Sopow et al., 2003; Matsukura et al., 2009; Stuart et al., 2012; Giron et al., 2016). Thus, gall-inducing insects are believed to manipulate plant developmental programs to generate complex gall structures by secretion of certain chemical compounds in plants (Miles, 1968), and this idea has been supported by histological observations and physiological analyses of insect galls (for a review Giron et al., 2016). The most important characteristic of insect galls is their function as a sink for insect nutrition (Rohfritsch and Shorthouse, 1982). The existence of insect galls near the source organs redirects the flow of plant resources such as carbohydrates, lipids, proteins from the original sink organs to the induced galls. Thus, gall formation results in development of a stronger sink of nutrients for gall-inducing insects than the original sink organs such as buds, flowers, fruits, and storage roots (Weis and Kapelinski, 1984; McCrea et al., 1985; Burstein et al., 1994; Larson and Whitham, 1997).

Darwin (1868) pointed out that the shapes of some complex insect galls resemble flowers or fruits. Indeed, many remarkable flower- and fruit-like traits are observed in insect galls, in particular those that are induced by gall midges and cynipids on various plant species (Rohfritsch and Shorthouse, 1982), suggesting that the formation of gall tissues is similar to the development of flowers or fruits (Kurosu and Aoki, 1990; Ferreira and Isaias, 2014). Recently, Schultz et al. (2018) reported that the gene expression pattern during Phylloxera leaf-gall development is similar to that during carpel development. These results indicate that the parasite may, at least partly, hijack the processes of flower development during gall formation (Schultz et al., 2018), supporting the hypothesis that flower- or fruit-like galls are generated by manipulation of flower development, although the initial process of induction of gall tissues in vegetative tissues is still largely unknown.

Aphids are small phloem sap-feeding insects belonging to the super family Aphidoidea, which embraces approximately 5,000 species in nature (Blackman and Eastop, 2007). Of these, no more than 10% of the aphid species can induce apparent galls on their host plants (Wool, 2004). Like other aphids, the gall-inducing aphids have complicated life cycles, in which a fundatrix or stem mother emerges from a fertilized egg in spring, and initiates the induction of a gall on the primary host plant. Then, the fundatrix parthenogenetically produces offspring inside the gall, and this parthenogenetic production is continued over several generations in particular aphid taxa. In summer or early autumn, winged adults appear and exit from the gall for migrating to the secondary host plant, where they spend several generations in autumn and winter (Wool, 2004; Aoki and Kurosu, 2010). A gall-inducing aphid, *Schlechtendalia chinensis*, induces large, single-chamber galls called horned galls on the leaf wings of several *Rhus* species (Anacardiaceae) in China, Korea, Taiwan, Malaysia, and Japan (Blackman and Eastop, 2007). Galls are first induced when the fundatrix of *S. chinensis* feeds on the adaxial side of the leaf wings. After the fundatrix is enclosed in the gall, the gall is enlarged quickly to form large horned galls with forked structures. During gall development, drastic morphological rearrangement occurs in the leaf wing tissues, in which the palisade tissues of the galled leaf wings are reorganized and replaced by parenchyma cells, and galled zones connect to non-galled zones by newly formed vascular bundles (Liu et al., 2014). Such complexity both in the developmental process and in the structure of *S. chinensis* galls implies that modified but well-organized host-plant gene networks could be incorporated in the process of gall development. However, the underlying molecular mechanisms contributing to the gall formation are largely unknown.

In this study, we performed RNA-sequencing-based comparative transcriptomics of a host plant, *R. javanica*, to understand the molecular characteristics of the early phase of gall development induced by *S. chinensis*. We found that there was no clear similarity in the global gene expression profiles between the gall tissue and other tissues. The genes involved in the phytohormone metabolic and signaling pathways, abiotic and biotic stress responses, and organ development were significantly upregulated, whereas photosynthetic genes were dramatically downregulated. These results imply that the gall-inducing aphid manipulates the plant reproductive programs to convert source tissues into fruit-like sink tissues during the initial process of gall induction.

MATERIALS AND METHODS

Plant Materials

The phase 4 of developmental stage of galls (Liu et al., 2014) (collected in May 22, 2017, **Figure 1a**), young leaves (collected in May 22, 2017), flowers (collected in September 9, 2017), and fruits (collected in September 28, 2017) (**Figure 1b**) of *R. javanica* were collected from a natural plantation located in the Kyoto Prefecture of Japan (35°06′00.83″N 135°72′86.94″E).

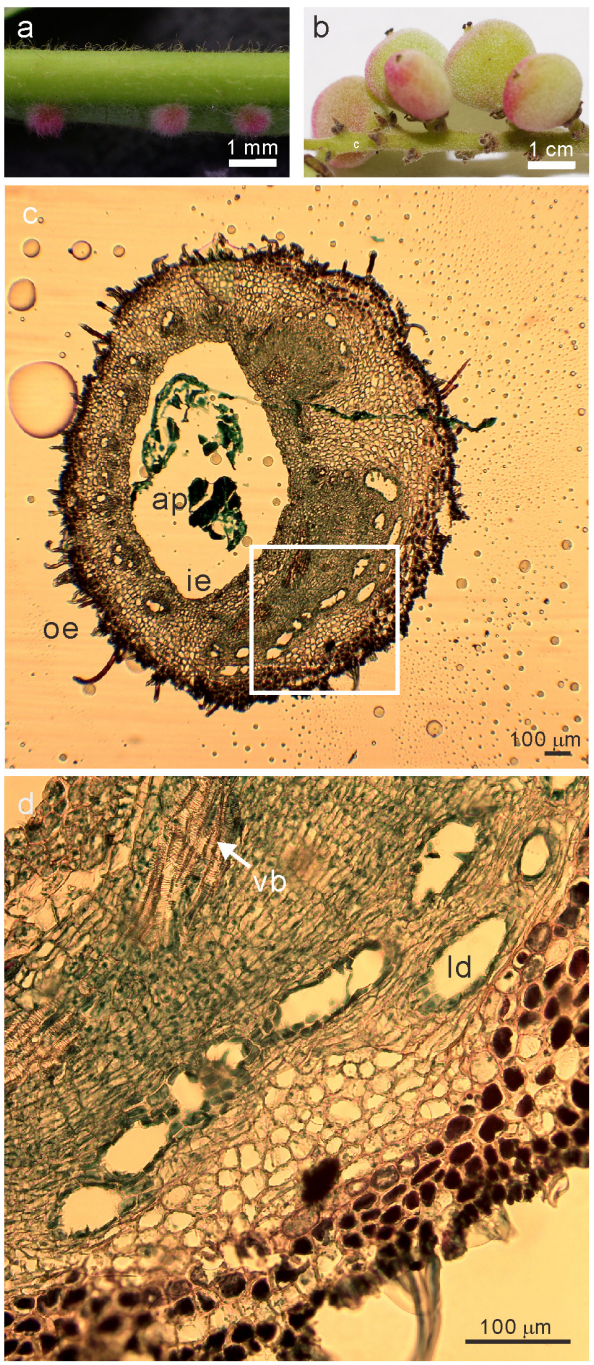

FIGURE 1 | Images of the sampled **(a)** galls at the early developmental stage of (phase 4) and **(b)** fruits **(c)** transverse section of stage 4 galls **(d)** the magnified image (white square in **c**) of *R. javanica*. Abbreviations: ap; aphid; ie, inner epidermis; oe, outer epidermis; ld, latex duct; vb, vascular bundles.

RNA Extraction, Library Construction, and RNA-Seq Analysis

Total RNA was extracted from the young leaves, female flowers, fruits, and gall tissues with an RNeasy Plant Mini Kit (QIAGEN). RNA-seq libraries were prepared using the Illumina TruSeq Stranded RNA LT Kit (Illumina, CA, United States) according to the manufacturer's instructions. Three independent RNA samples for each tissue were used for the analysis. The qualities of the prepared libraries were checked using the QuantiFluor dsDNA System and Agilent High Sensitivity DNA Assay (Agilent, CA, United States). The pooled libraries were sequenced on the NextSeq500 sequencing platform (Illumina, CA, United States) and paired-end reads were obtained. Then, the obtained reads were assembled into transcriptome contigs using Trinity with the default settings. Blastx searches of the obtained contigs against non-redundant protein sequences from GenPept, SwissProt, PIR, PDF, PDB, and NCBI RefSeq (nr) databases using the DIAMOND software (Buchfink et al., 2015) were conducted to find similar protein sequences. Each contig was classified into a taxon group based on the top hits of the blastx results and NCBI taxonomy lineage data. Finally, the contigs classified into the Viridiplantae (plant) kingdom were extracted as *R. javanica* reference transcript contigs to exclude contigs from aphids or other contaminants. RNA-seq analysis of *R. javanica* tissues was biologically repeated at least three times per each tissue sample (**Supplementary Table S1**).

Gene Expression Profiling With RNA-Seq Data

The obtained reads were mapped to the *R. javanica* reference transcript contigs using the Burrows-Wheeler alignment tool (BWA)[1]. The count data were subjected to the trimmed mean of *M*-values normalization in EdgeR. Multi-dimensional scaling was performed by calculating the log-fold changes between the accessions and by using differentially expressed genes (DEGs) to compute distances in EdgeR with the plotMDS function. Transcript expression profiles and DEGs were defined with the EdgeR general linear models approach (Robinson et al., 2010). The threshold for DEGs was a log-fold change of >2 and a false discovery rate of <0.01.

Cloning of cDNAs From *R. javanica* Tissues and Quantitative Reverse Transcription PCR Analysis

The gall, young leaf, flower, and fruit samples were frozen in liquid nitrogen. The total RNA was isolated using the NucleoSpin RNA Plant and Fungi Kit (Takara), and the cDNA library construction was performed using the ReverTra Ace qPCR RT Master Mix (TOYOBO) as per the manufacturer's instructions.

[1]http://bio-bwa.sourceforge.net

The same amount of cDNA was used as a template for the qPCR, which was performed with the THUNDERBIRD SYBR qPCR Mix (TOYOBO) and gene-specific primers. UBQ10 was used as an internal control for normalization. The primers used in this study are listed in **Supplementary Table S2**.

Quantitative Analysis of Indole-3-Acetic Acid and Cytokinins

The endogenous levels of the indole-3-acetic acid (IAA) and cytokinins (CKs) in the whole *S. chinensis* bodies were quantitatively analyzed according to Tanaka et al. (2013). Briefly, the endogenous levels of IAA and cytokinins in the aphids were analyzed by extracting the samples that were spiked with stable isotope-labeled internal standards ([$^2H_5$]tZ, [$^2H_5$]tZR, [$^2H_6$]iP, [$^2H_6$]iPR, and [$^{13}C_6$]IAA), pre-purifying them with solid-phase extractions, and quantifying them by liquid chromatography/tandem mass spectrometry (3200 QTrap, AB Sciex).

tZ, iP, IAA contents in *R. javanica* leaves and galls were determined according to Yamane et al. (2019) with minor modifications. Briefly, leaf and gall samples (approximately 100 -200 mg per sample) were collected and frozen in liquid nitrogen, and the weight of each tissue was measured. Then the samples were ground and subjected to extraction in 80% acetonitrile and 1% acetic acid containing stable isotope-labeled compounds for internal standards [D_5-tZ, D_6-iP, $_{13}C_6$-IAA] (OlChemim, Czech Republic). After sample purification by HLB and MCX columns (Waters), Phytohormones were analyzed with a 6410 Triple Quad LC/MS System (Agilent Technologies Inc., United States) equipped with a ZORBAX Eclipse XDB-C18 column and an XDB-C8 Guard column (Agilent Technologies Inc.), and peak areas were determined using MassHunter Workstation software (vB.04.00; Agilent Technologies). Four independent samples were analyzed for calculation of averages and standard deviations.

Histological Analysis and RNA *in situ* Hybridization

Young gall tissues were fixed in the fixative solution consisting of 4% (w/v) paraformaldehyde in 1X PBS under vacuum condition until the samples were drawn to the bottom of the tube. After fixation, the sample were dehydrated through a graded ethanol series, and then followed by a D-Limonene series, and embedded in Paraplast Plus (Sherwood Medical). Microtome sections (4 μm) were deparaffinized in D-Limonene, and rehydrated through a graded ethanol series. In the case of histological observation, the sections were stained with Hematoxylin-Safranin-Fast Green FCF.

Full-length cDNA of RjKNAT6 (KNAT6 of *Rhus javanica*) was cloned into the pENTR vector, and then was subcloned into pGEM11 vector by the Gateway system. Labeled RNA probes were synthesized using *in vitro* transcription in the presence of Digoxigenin-11-UTP by RNA polymerases T7 or SP6 (DIG RNA labeling Mix, Roche). Samples were washed twice in PBST (1X PBS plus 0.1% (v/v) Tween-20) for 10 min and then incubated with 1 μg ml^{-1} proteinase K (Roche) for 15 min. Digestion was stopped by incubating the samples in 1X PBS plus 0.2% glycine for 5 min and then washing them twice in PBST for 10 min. Samples were washed twice in PBST for 10 min and once in the hybridization solution 50% (v/v) formamide in 2X SSC (20X SSC: 3 M NaCl, 300 mM sodium citrate, pH 7.0 with 1 M HCl) for 10 min, and then preincubated in the same solution for 1 h at 50°C. The hybridization step was performed overnight at 42°C by incubating samples in supplemented hybridization solution containing a cocktail of denatured (80°C for 2 min) labeled RNA probes (20–100 ng per ml of the hybridization solution). Samples were washed: three times (10, 60, and 20 min) in a solution of 50% (v/v) formamide. Thereafter samples were incubated with a mixture of the selected primary antibodies (Chicken anti-digoxigenin, Immunology Consultant Laboratory) diluted (1:100) in (PBST + BSA), for 2 h at RT under gentle shaking. Subsequently, samples were washed three times for 10 min in PBST, once for 30 min in PBST plus BSA and then incubated with a mixture of the secondary antibodies (Alexa Fluor dyes 555 Goat Anti-Chicken, INVITROGEN) diluted (1:100) in PBST plus BSA overnight at RT in the dark. After incubation samples were washed twice for 15 min in PBST under gentle shaking in the dark. Fluorescence and differential interference contrast (DIC) images were obtained using Leica TCS SP8 laser scanning confocal microscope. The captured images were processed using Leica LAS X.

RESULTS

Transcription Factors Involved in Meristem Formation and Flower Development Were Expressed in Gall Tissues

Liu et al. (2014) reported the histological analysis of the developmental process of *R. chinensis* gall; they categorized the developmental process of the gall into six different phases. To investigate the changes in the gene expression profile during the early phase of gall development, we isolated total RNA from various tissues including entire galls in the phase 4 of development (about 1 mm diameter) in which the gall is completely closed (**Figure 1a** and **Supplementary Figure S1a**), young leaves (**Supplementary Figure S1b**), female flowers (**Supplementary Figure S1c**), and fruits (**Figure 1b**). The cross-section of the phase 4 gall revealed that the inner and outer epidermis had two to three layers and the parenchyma cells were well developed between the outer and inner epidermal cell layers with vascular bundles and latex ducts (**Figures 1c,d**). Phase 4 galls enlarged slowly and contained 1–2 aphids inside of a gall, then in phase 5, the size of the gall increased quickly from August to late September, and finally the horn-like or fork shaped galls were formed (Liu et al., 2014). According to the enlarging gall size, the number of aphids inside of galls increases exponentially (**Supplementary Figure S2**).

Paired-end reads for the gall, young leaf, flower, and fruit tissues were obtained by RNA-seq (**Supplementary Table S1**). *De novo* assembly of all the reads yielded 265,145 transcript contigs

by Trinity with N50 and average lengths of 1842 and 905.3 nt, respectively. The reference transcript contigs for *R. javanica* were extracted from the raw assembled contigs based on the blastx results against known protein databases, and their N50 and average lengths were 2267 nucleotides and 1331 nucleotides, respectively. Based on the N50 length, which is an indicator of assembly quality, we confirmed that the quality of the *de novo* assembly was sufficient for the subsequent analyzes (**Supplementary Table S2**).

We aligned all single reads to the contigs and compared the number of DEGs in the galls, flowers, and fruits to those in the young leaves. First, we performed a principal component analysis to compare the gene expression profiles of the gall, leaf, flower, and fruit tissues of *R. javanica*. The eigen values of the two components were greater than 1, and the first component and second component explained 41.4 and 34.6% of the variation, respectively (**Supplementary Figure S3a**). The factorial map of the principal component analysis showed that the four dots corresponding to each tissue were widely distributed in the graph (**Supplementary Figure S2b**). These results suggested that there was no clear similarity in the global gene expression profiles between the gall tissue and other tissues.

Compared with the transcripts for young leaves, the transcripts for the gall, flower, and fruit tissues showed upregulation of 1829, 1330, and 2583 DEGs, and downregulation of 1879, 1554, and 4409 DEGs, respectively (log-fold change of >2 and a false discovery rate of <0.01) (**Figure 2** and **Supplementary Tables S3, S4**). As the *R. javanica* genome has not yet been read and no functional annotation exists, we assigned *R. javanica* transcripts to *Arabidopsis thaliana* orthologs using functional annotations from The *Arabidopsis* Information Resource (TIAR). To assess the similarity between the *R. javanica* and *Arabidopsis* orthologs, we cloned and sequenced several *R. javanica* full-length transcripts (*AG, AP1, CLE41/44), SEP2, CYCD4;1, and SEOR1)* according to the deduced sequences assembled by Trinity, and then compared them with the full-length sequences of the *Arabidopsis* counterparts. The cloned transcripts have considerable similarity with the *Arabidopsis* counterparts (**Supplementary Figure S4**), implying that most of the *R. javanica* transcripts could be correctly assigned to the *Arabidopsis* orthologs. To evaluate the DEGs identified by RNA-seq, we measured the differences in the expression levels of several of the upregulated genes by quantitative real-time PCR and found that *SHP1, KNAT6, CLE44, AG, AP1, KNAT1, HEC1, VND7,* and *CYCD4;1* were considerably upregulated in the gall tissue (**Figure 3**).

Genes Involved in Meristem Formation and Floral Organ Development Were Upregulated in the Gall, Flower, and Fruit Tissues

When comparing the genes upregulated in the galls with those upregulated in the flowers and fruits, we found that expression of 337 genes was increased in the gall, and in the floral and reproductive organs. Gene ontology (GO) term enrichment analysis of these upregulated genes revealed that the genes assigned to the GO categories of the floral organ development (GO: 0048481, 0048440, 0048437, and 0090567) were enriched by over 5-fold (**Figure 4**). Of these, several genes encoding transcription factors involved in the regulation of floral organ morphogenesis were upregulated in the early development stage of gall tissue. For example, the upregulated genes included a floral integrator (*LFY*), class-1 *KNOX* genes (*KNAT1/2/6, and STM*), and MADS-box-type transcription factors (*SEP1, SEP2, SEP3, AP1, AP3 AG, TT16*, *FUL*, and *SHP1*) (**Supplementary Table S5**). *In situ* hybridization analysis revealed that *KNAT6* was predominantly expressed in the parenchyma cells of developing gall tissues (**Figure 5**).

Phytohormone Metabolic and Signaling Pathways Were Activated in the Gall Tissue

In the *R. javanica* gall tissue, genes involved in the auxin- (*IAA17, PILS1, GH3.1, GH3.3, WRKY23*, and *PBP1*), ethylene- (*ERF017, ERF022, ERF13, ERF72*, and *ERF109*), and abscisic acid- (*NHL6*, *MAPK3, AHG1, CBF4, ABR1*, and *RDUF2*) response pathways were significantly upregulated (**Supplementary Tables S5, S7**), whereas genes belonging to the GO category "response to cytokinin (GO:0009735)" were downregulated (**Supplementary Table S6**), suggesting that several phytohormone signaling pathways may be activated by the actions of the gall-inducing aphid. Since active phytohormones such as indole-3-acetic acid (IAA), abscisic acid, and cytokinins (CKs) have been identified in several gall-inducing insects (Mapes et al., 2001a,b; Dorchin et al., 2009; Yamaguchi et al., 2012; Tanaka et al., 2013), we reasoned that *S. chinensis* would also produce phytohormones for gall induction on *R. javanica* leaves. Therefore, we measured the contents of IAA and CKs in the whole aphid bodies, and identified a considerable amount of IAA (718.9 $\pm$ 269.0 ng/g FW) and CKs, particularly iP (5.106 $\pm$ 1.503 ng/g FW), iPA (7.726 $\pm$ 1.451 ng/g FW), and tZR (7.726 $\pm$ 1.451 ng/g FW) in the whole aphid body (**Table 1**). In contrast, the concentrations of these phytohormones in gall and leaf tissues were lower than those in the aphid body (**Supplementary Figure S5**).

Genes Involved in the Secondary Cell Walls, Vascular Tissue, and Callus Formation Were Highly Upregulated in the Gall Tissue

During the gall growth process, the latex ducts and vascular elements become denser in the inner gall layer, the outer epidermal cell layer hardens due to the construction of a lignified secondary cell walls, and the palisade tissue of the galled leaf wings is reorganized and replaced by parenchyma cells (Liu et al., 2014). In this study, we observed that the latex ducts and vascular bundles emerged, and the outer epidermal cell layer began to harden by lignification during the early developmental phase of the galls (**Figures 1c,d**). In this stage, the genes involved in cell wall synthesis (e.g., *CSLD2/6*, *CESA8*, *XTH4*, and *CEL2*), production of lignin and suberin deposition (e.g., *CAD5, PRX25, CYP84A1, PRX72*, *MYB58*, *FAR4*, and *CER9*), and vascular tissue morphogenesis (e.g., *CLE44, SEOR, VND7*, and *TDR*) were

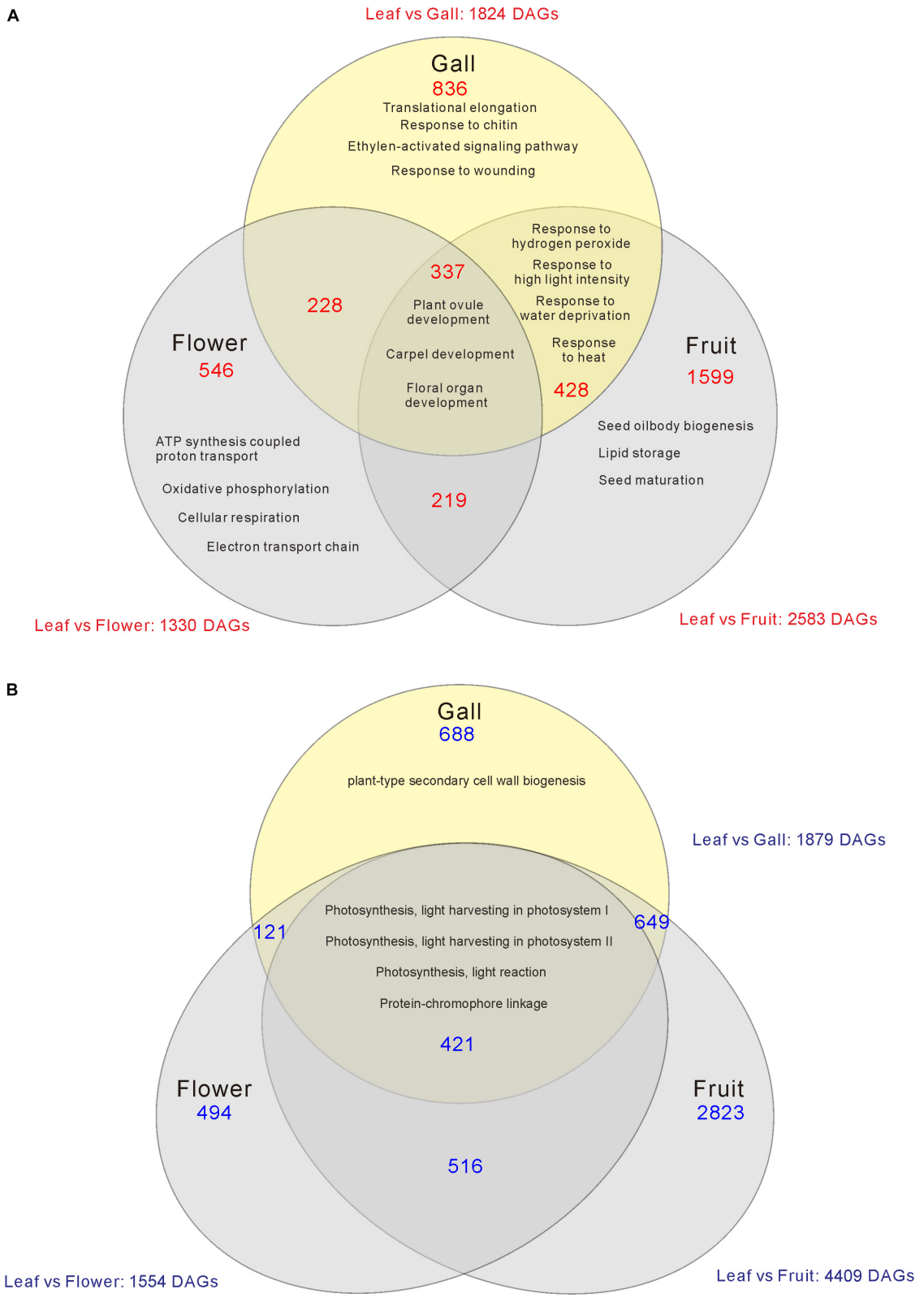

FIGURE 2 | Venn diagram analysis of the number of increased **(A)** or decreased **(B)** differentially expressed genes (DEGs) in the gall, flower, and fruit tissue compared with the young leaves. The numbers in each region indicate the DEGs in each tissue. Overlapping regions of the Venn diagram indicates shared DEGs among corresponding groups. Descriptions in each region indicate typically enriched gene ontology categories of the corresponding groups.

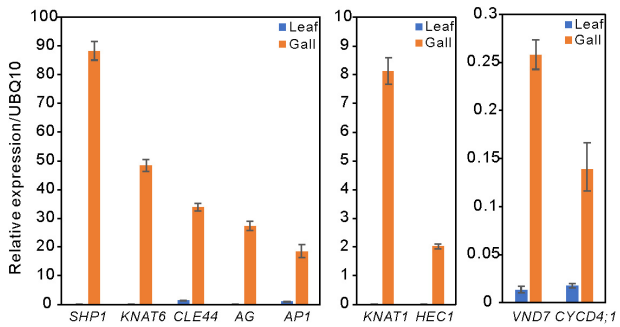

FIGURE 3 | Relative expression levels of several upregulated DEGs in the leaf and gall tissues. The gene expression levels were analyzed by quantitative reverse transcription PCR. UBQ10 served as the internal expression control. Experiments were repeated three times using three biologically distinct samples, and gave similar results. The data is represented as the mean ± standard deviation (Four technical replicates).

upregulated (**Supplementary Table S7**). The changes in these genes might reflect the morphological changes in the early stage of gall development.

Abiotic and Biotic Stress-Response Pathways Were Activated in the Gall Tissues

We identified that a significant number of genes related to "the jasmonic acid metabolic process" (GO: 0009694) and "response to jasmonic acid" (GO: 0009753), "response to chitin" (GO: 0010200), "response to hydrogen peroxide" (GO: 0042542), "response to wounding" (GO: 0009611), and "response to salicylic acid" (GO: 0009751) were considerably enriched only in the developing gall tissues (**Figure 5**). After a detailed analysis of these genes, we found that the expressions of *AOC3/4*, *JAZ1/2/8/10*, *CYP94C1*, and *JOX2* were dramatically upregulated in the developing gall tissues. Also, a significant number of genes involved in plant–pathogen interactions were upregulated in the gall tissues. In particular, transcription factors related to PAMP-triggered immunity (e.g., *WRKY33, WRKY40, MYB51*, and *TGA9*) were highly expressed in the gall tissues (**Supplementary Table S7**). Additionally, the expression of a significant number of abiotic stress-responsive genes, including those that respond to water deprivation (GO: 0009414) and heat (GO: 0009408), was increased in the gall tissues (**Figure 4**). Most of these genes (e.g., *RD17, LTI45, ERD14, HSFB2A, DDF1, LSR3*, and *ERD7*) respond to drought and heat stresses.

The Changes in Expression of Photosynthetic Genes and Transporter Genes Between Leaf and Gall Tissues

We next categorized the 3809 downregulated genes and found that the genes belonged to GO categories related to photosynthesis (GO: 0097868, 0018298, 009769, 0015977, and 0015995) (**Supplementary Tables S4, S6**). For instance, the components of photosystem I (*PsaD, PsaE, PsaF, PsaG, PsaH, PsaK, PsaN*, and *PsaO*), photosystem II (*PsbO, PsbP, PsbR*, and *PsbW*), and carbon fixation (*GAPA2*, *RBCS1A*, *SBPASE*, and *CFBBP1*) were dramatically downregulated (**Figures 2**, **6** and **Supplementary Table S4**). In contrast, the transcripts of various transporters including amino acid transporters (*UMAMIT14*, and *AAP3/4*), sugar transporters (*SUC2*, *SWEET7*), metal transporters (*AMT2*, *ZIP1*), and water transporters (*TIP1;3*, *PIP1;2*) were increased in the gall tissues (**Figure 6** and **Supplementary Table S7**).

DISCUSSION

Transformation From Source to Sink Tissues During Gall Formation in the Leaf Wings

One of important characteristics of galls is their function as sinks for insect nutrients (Rohfritsch and Shorthouse, 1982). The existence of insect galls near the source organs changes the flow of plant resources by partially blocking and redirecting the resources from the original sink organs to the galls. Thus, gall formation results in making a stronger sink than plant sink organs such as buds, flowers, and fruit (Weis and Kapelinski, 1984; McCrea et al., 1985; Burstein et al., 1994; Larson and Whitham, 1997). In *R. javanica*, galls start to develop on the leaf wing when the fundatrix of *S. chinensis* feeds on the surface of the leaf wing. In the early stage of gall development, the feeding site on the leaf wing tissue grows abnormally to form hyperplastic tissues, in which the outer epidermal layer of the gall is covered with denser trichomes and is lignified to form a rigid structure. The palisade tissues of the leaf wing are reorganized and replaced by dedifferentiated parenchyma cells, and the latex ducts and vascular elements become denser in the inner gall layer and close to the gall cavity (Liu et al., 2014). Throughout this process, a galling aphid such as *S. chinensis* creates horned galls as a novel source organ on the leaf wing.

In the case of the grape gall formation by phylloxera, the expression of genes associated with "light harvesting and photosynthetic carbon assimilation" strongly decreased, whereas that of the transcripts associated with "sucrose mobilization" and "glycolysis and fermentation" considerably increased in gall tissues compared to that in ungalled leaf tissues (Nabity et al., 2013). In this study, we revealed that the photosynthesis-related genes involved in the photosystem I (GO: 0009768), photosystem II (GO: 0009769), and carbon fixation pathway (GO: 0015977) were dramatically downregulated during the early gall development in *R. chinensis*. In contrast, the expression of the genes involved in the translational elongation process (GO: 0006414) increased, suggesting that genes related to *de novo* protein synthesis necessary for secondary metabolites in gall tissues are activated during gall formation. Through a detailed analysis of the upregulated genes involved in this process, we found that a significant number of molecular chaperons, ribosomal proteins, and various transporter genes such as sugar and amino acid transporters were highly expressed in the gall tissues (**Supplementary Tables S5, S7**).

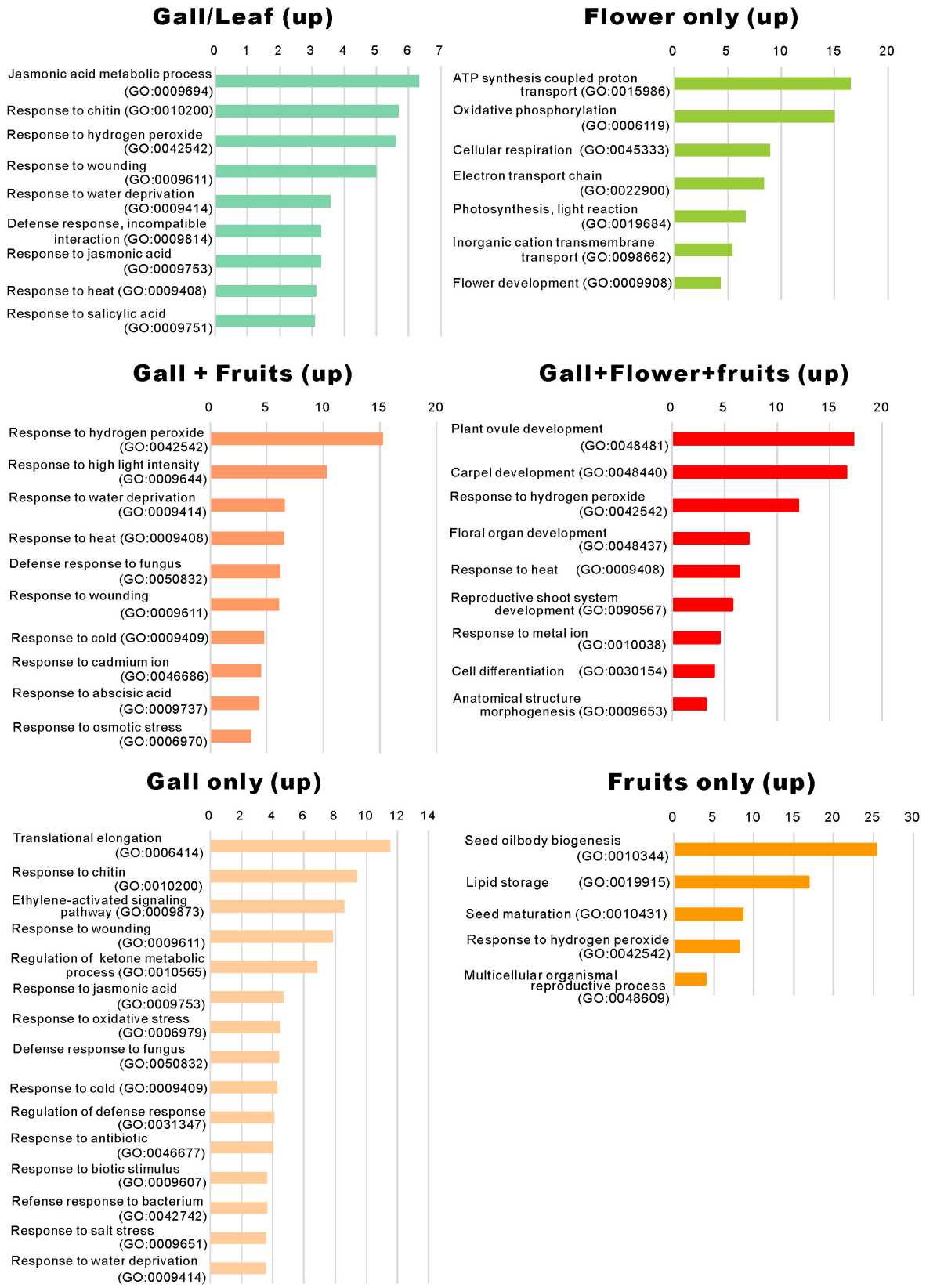

FIGURE 4 | Gene ontology term enrichment of the upregulated genes. The upregulated differentially expressed genes in **Figure 2A** were subjected to the enrichment analysis (Mi et al., 2019) using the TAIR website (https://www.arabidopsis.org/tools/go_term_enrichment.jsp).

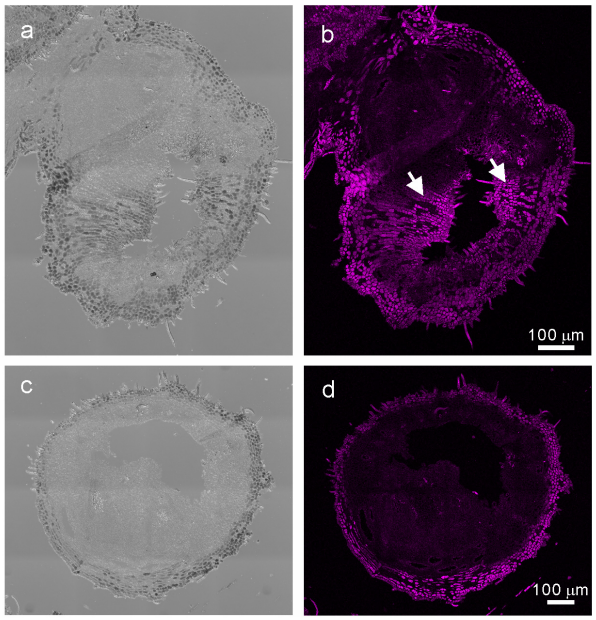

FIGURE 5 | *In situ* hybridization analysis of *R. javanica* KNAT6 in the early developing stage of gall. **(a)** Differential interference contrast (DIC) image of cross-section of the developing gall hybridized with RjKNAT6 anti-sence probe. **(b)** Fluorescent image of the developing gall hybridized with RjKNAT6 anti-sence probe. **(c)** Differential interference contrast (DIC) image of cross-section of the developing gall hybridized with RjKNAT6 sence probe. Arrows indicate the positive signals of the anti-sense probe. **(d)** Fluorescent image of the developing gall hybridized with RjKNAT6 sense probe. The images were taken at least three biologically distinct samples and gave the same results. Scale bars: 200 μm.

TABLE 1 | Endogenous phytohormone contents in *S. chinensis*.

Plant hormone	Average (ng/g)
IAA	718.9 ± 269.0
iP	5.106 ± 1.503
iPA	7.726 ± 1.451
tZ	0.0220 ± 0.0118
tZR	6.832 ± 0.8960

The results are given as the mean ± standard deviation from five replicates. Abbreviations: IAA, indole-3-acetic acid; iP, isopentenyladenine; iPA, isopentenyladenosine; tZ, trans-zeatin; tZR, trans-zeatin riboside. Experiments were repeated at least three times using three biologically distinct samples.

Pathogens acquire glucose from their hosts, thereby hijacking host sugar efflux systems (Sutton et al., 1999; Voegele et al., 2001). In particular, SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS (SWEETs) sugar transporters have been reported to be utilized by pathogens for the acquisition of sugars. For instance, the rice homologs SWEET11 and SWEET14 are specifically exploited by bacterial symbionts and fungal and bacterial pathogens, indicating that the sugar efflux function of SWEET transporters is probably targeted by pathogens and symbionts for nutritional gain (Chen et al., 2010). *Plasmodiophora brassicae* is the causal agent of clubroot, a severe disease of Brassica crops. The pathogen lives inside roots, and hijacks nutrient sink in infected roots to trigger active sugar translocation between the sugar producing tissues and the clubbed tissues recruiting the SWEET sucrose transporters within developing galls (Li et al., 2018; Walerowski et al., 2018). In this study, we found that the expression of *Arabidopsis* SWEET7 homolog is increased in the early development stage of gall tissues, indicating that the expression of SWEET sugar transporter gene of *R. javanica* is likely activated by the feeding action of *S. chinensis*.

Collectively, the changes in the expression profile during gall formation imply that the cells of the palisade tissues in the leaf wing were reorganized to be de-differentiated into parenchyma cells, thereby losing their photosynthetic and reconstruction cellular functions, to convert the tissues architecture of the leaf wing from source to sink tissues during the gall development process.

Expression of Abiotic and Biotic Stress-Related Genes in the Gall Tissues

Plants respond to herbivory with the induction of a combination of defense responses such as salicylic acid, jasmonic acid, and ethylene signaling pathways to produce toxins and defensive proteins that target physiological processes in insects (Agrawal et al., 1999; De Vos et al., 2005). Aphid feeding is perceived by plants as pathogenic and herbivory; hence, on sensing the phytopathogens and mechanical damage caused by stylet probing, the plants elicit a defense response that involves both salicylic and jasmonic acid pathways (Kaloshian and Walling, 2005; Thompson and Goggin, 2006; Gao et al., 2007). It has been reported that the other known minor pathways including ethylene, abscisic acid, gibberellic acid, nitric oxide, and auxin are also activated in response to aphid feeding (Smith and Boyko, 2007).

An investigation on the molecular response of gall formation by the gall-inducing aphid *S. chinensis* on *R. chinensis* by comparing expression profiles of leaves and 100-day grown galls has demonstrated that the genes involved in the biosynthesis of secondary metabolites, plant–aphid interactions, and plant hormone signal transduction were highly expressed in galls (Wang et al., 2017). In this study, we compared the expression profile of the early developmental galls with that of the early developmental leaves, and found that the salicylic acid-, jasmonic acid-, and ethylene-response pathways were considerably activated in the gall tissues. In particular, genes related to the wounding response and PAMP-triggered immunity (**Supplementary Table S7**) were highly expressed in the gall tissues. These defense responses were likely induced by sensing the feeding stress of gall-inducing aphid for protecting the plant body from the aphid's invasion of plant signaling pathways driven by jasmonic acid, salicylic acid, ethylene, abscisic acid, and gibberellic acid. However, the growth and proliferation of *S. chinensis* inside the gall seemed to be unaffected, which was probably because the aphids adapted to, and overcame the host's defense systems, suggesting that the gall-growing leaves or plants to become more tolerant against other pathogenic organisms than those are not induced gall.

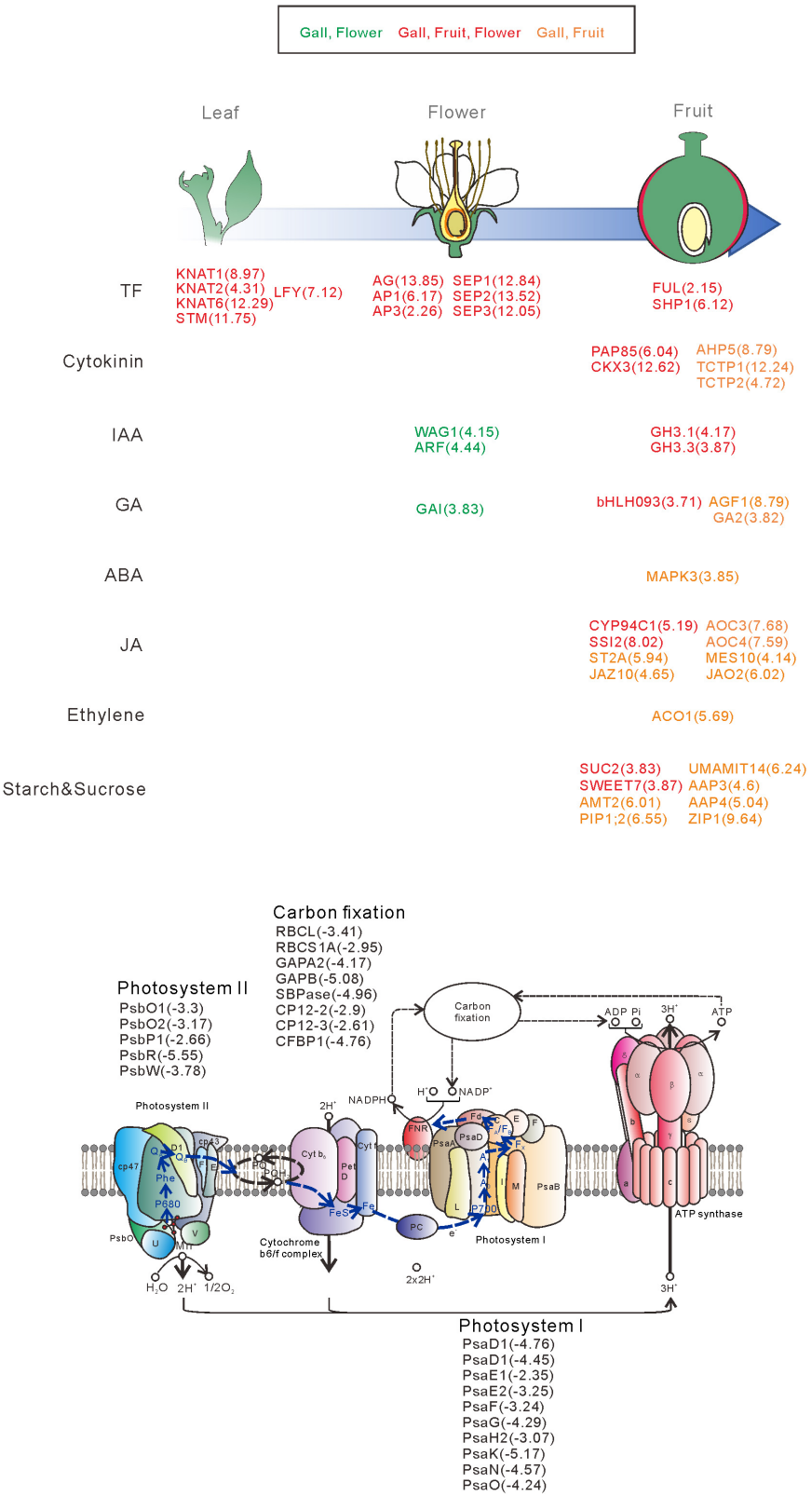

FIGURE 6 | Overview of the representative genes expressed in the galls, flowers, and fruit. The genes are categorized by the developmental stage, the role of the transcription factors, different hormonal signals, and transporters. The numbers in parentheses represent the log-fold change values of the differentially expressed genes compared with the young leaves. Upregulatd genes in gall and flower (Green), gall, fruit, and flower (red), gall and fruit (orange).

Putative Action of Phytohormones of the Gall-Inducing Aphid

During the growing process of the horned gall, caused by the gall-inducing aphid *S. chinensis*, a combination of cell division (hyperplasia) and growth (hypertrophy) occurs in several layers of tissues, inducing the formation of nutritive and protective gall tissues (Liu et al., 2014). It has long been hypothesized that phytohormones produced by gall-inducing insects play a key role in gall formation (Tooker and Helms, 2014). Indeed, active phytohormones such as IAA and CKs have been identified in several gall-inducing insects at various concentrations (e.g., IAA = 60–9000 ng g fw^{-1}, iP = 3–350 ng g fw^{-1}, iPR = 8–190 ng g fw^{-1}, tZ = 2–1300 ng g fw^{-1}, and tZR = 0.4–70 ng g fw^{-1}) (Mapes et al., 2001a,b; Dorchin et al., 2009; Yamaguchi et al., 2012; Tanaka et al., 2013), and the application of exogenous phytohormones led to induction of gall-like structures in plants (Bartlett and Connor, 2014). We found that the concentrations of IAA and CKs in *S. chinensis* fell within the ranges of those in the gall-inducing insects. The contents of IAA and CKs were considerably higher than those in all and leaf tissues, suggesting that the aphids that may control gall-inducing process on *R. javanica* leaves by producing phytohormones such as IAA and CKs themselves. Given that IAA and CKs are involved in abnormal cell division, cellular enlargement, and differentiation, our results suggest that the phytohormones secreted from the aphids may be involved in the gall formation reported here, although we could not completely rule out the possibility that the phytophormones in aphid are derived from the host plant tissues.

Gene Expression Similarities Between Gall Formation and Floral Organ Development

Some of insect galls are believed to resemble flowers or fruits in their morphology (Darwin, 1868), suggesting that the developmental program of the reproductive organs of plants may be hijacked and exploited by gall-inducing insects. Recently, Schultz et al. (2018) reported that reproductive genes involved in floral organ development were significantly enriched in the developing galls of wild grapevine that were induced by *Phylloxera*, suggesting that a galling insect utilizes plant reproductive programs during gall development. In the present study, we also found that a significant number of transcription factors involved in floral organ development were upregulated during *R. javanica* gall development. Of these genes, *LFY* is an important master regulator for the transition from vegetative to reproductive phase during meristem development (Blázquez et al., 1997). Constitutive expression of *LFY* under the 35S promoter causes the conversion of indeterminate lateral meristems into flowers and the conversion of the inflorescence meristem into a flower (Weigel and Nilsson, 1995; Siriwardana and Lamb, 2012). *AP1*, *AP2*, and *AG* determine the floral organ identity, and A-, B-, and C-type floral MADS-box genes (Alvarez-Buylla et al., 2010) in combination with the *SEP1/2/3* MADS-box subfamily being required for specifying the "floral state" (Alvarez-Buylla et al., 2010). The *FUL* and *SHP1* genes are also members of the MADS-box transcription factors, and are involved in valve development and differentiation of both the lignified layer and separation layer of the valve margin in the developing ovaries, respectively (Gu et al., 1998; Liljegren and Bowman, 2000).

In addition to these genes, in the present study, we found that the class-1 *KNOTTED1-like homeobox* (*KNOX*) genes (*KNAT1/2/6*, and *STM*) were highly expressed in the developing gall tissues. The *KNOX* genes are found in all higher plant species and encode homeodomain transcription factors similar to those that regulate development in animals (Scofield and Murray, 2006; Hay and Tsiantis, 2010). The class-1 *KNOX* genes in *Arabidopsis* are expressed in the shoot apical meristem (SAM) but not in the lateral organs (Lincoln et al., 1994; Pautot et al., 2001; Lenhard et al., 2002; Dean et al., 2004). It has been reported that the ectopic overexpression of *STM* or *KNAT1* leads to the formation of ectopic knot-like meristematic structures, which results in formation of lobed leaves on the adaxial surface of the leaf (Chuck et al., 1996; Brand et al., 2002; Lenhard et al., 2002). When gall formation begins, the development of ectopic meristematic structures could be made from leaf wing tissues. Similarities in organ structure between the lobed leaves caused by *STM* or *KNAT1* overexpression and the initiation stage of the gall structure imply that the ectopic overexpression of the class-1 *KNOT* genes induced by gall-inducing insects may initiate de-differentiation of the leaf wing cells to generate a meristematic region followed by the formation of an initial gall structure on the leaf wing.

From these results, we propose the following molecular mechanisms of the early stage of gall formation in *R. javanica*: (i) an ectopic meristematic structure is generated by the overexpression of class-1 *KNOX* genes, (ii) the ectopic meristem is converted to floral-like meristem by the expression of *LFY*, (iii) the floral-like meristem develops to form fruit-like gall structures induced by expression of floral regulatory genes, and (iv) during the transformation from leaf to gall tissues, many photosynthetic genes are downregulated, while transporter and secondary metabolic genes are upregulated to change the tissue functions (**Figure 6**).

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI using the following link: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJDB8441.

AUTHOR CONTRIBUTIONS

TH, MS, and IO conceived and designed the study. TH, ST, and AO collected samples and extracted RNA. TH and TN performed qRT-PCR analysis. YS performed a quantitative analysis of indole-3-acetic acid and cytokinins in *S. chinensis*. TM and YI measured the content of various phytohormones in *R. javanica* tissues. TS and SK constructed library, and performed RNA-sequencing. TS, SK, TH, ST, and MS analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00471/full#supplementary-material

FIGURE S1 | Images of the sampled **(a)** *R. javanica* leaf attaching several phase 4 stage galls at the wing region, **(b)** young leaf **(c)** female flowers.

FIGURE S2 | Sizes and numbers of aphids inside of various stages of galls. **(a)** The number of aphids inside of the developmental galls. **(b–e)** Images of the developmental galls corresponding to the plots in **(a)**.

FIGURE S3 | Principal component analysis of the gene expression profile. **(a)** Eigenvalues and the cumulative contribution ratio (8%). Bars and open circles represent the eigenvalues and cumulative contribution ratio, respectively. **(b)** The global expression profile of each transcript as principal components 1 and 2.

FIGURE S4 | Amino acid sequence alignment of several orthologous genes of *A. thaliana* and *R. javanica*. AP1, APETALA1 **(a)**; SEP2, SEPALLATA2; CLE41, CLAVATA3/ESR-RELATED 41 **(b)**; CYCD4;1, CYCLIND4; 1 **(c)**.

FIGURE S5 | Phytohormone contents in *R. javanica gall and leaf tissues*. The results are given as the mean ± standard deviation from five replicates. Abbreviations: tZ, trans-zeatin iP, isopentenyladenine; IAA, indole-3-acetic acid. Experiments were repeated at least three times using three biologically distinct samples.

TABLE S1 | Results of RNA-seq analyzes of various *R. javanica* tissues.

TABLE S2 | List of primers used for qRT-PCR.

TABLE S3 | Transcriptome sequencing and summary statics of *de novo* assembly.

TABLE S4 | List of upregulated genes in gall, flower, fruit of *R. javanica*.

TABLE S5 | List of downregulated genes in gall, flower, fruit of *R. javanica*.

TABLE S6 | Result of GO enrichment analysis of up-regulated gens in gall of *R. javanica*.

TABLE S7 | Result of GO enrichment analysis of down-regulated gens in gall of *R. javanica*.

TABLE S8 | List of upregulated genes categorized by the biological processes.

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Chemotactic Host-Finding Strategies of Plant Endoparasites and Endophytes

Allen Yi-Lun Tsai[*†‡], *Morihiro Oota*[‡] *and Shinichiro Sawa*

Graduate School of Science and Technology, Kumamoto University, Kumamoto, Japan

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Edited by:
Yusuke Saijo,
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***Correspondence:**
Allen Yi-Lun Tsai
yi-lun.tsai@riken.jp

†Present address:
Allen Yi-Lun Tsai,
Dormancy and Adaptation Research Unit, RIKEN Center for Sustainable Resource Science, Yokohama, Japan

‡*These authors have contributed equally to this work*

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Plants interact with microorganisms in the environment during all stages of their development and in most of their organs. These interactions can be either beneficial or detrimental for the plant and may be transient or long-term. In extreme cases, microorganisms become endoparastic or endophytic and permanently reside within a plant, while the host plant undergoes developmental reprogramming and produces new tissues or organs as a response to the invasion. Events at the cellular and molecular level following infection have been extensively described, however the mechanisms of how these microorganisms locate their plant hosts *via* chemotaxis remain largely unknown. In this review, we summarize recent findings concerning the signalling molecules that regulate chemotaxis of endoparasitic/endophytic bacteria, fungi, and nematodes. In particular, we will focus on the molecules secreted by plants that are most likely to act as guidance cues for microorganisms. These compounds are found in a wide range of plant species and show a variety of secondary effects. Interestingly, these compounds show different attraction potencies depending on the species of the invading organism, suggesting that cues perceived in the soil may be more complex than anticipated. However, what the cognate receptors are for these attractants, as well as the mechanism of how these attractants influence these organisms, remain important outstanding questions. Host-targeting marks the first step of plant—microorganism interactions, therefore understanding the signalling molecules involved in this step plays a key role in understanding these interactions as a whole.

Keywords: chemotaxis, endophytes, endoparasites, gall-forming bacteria, arbuscular mycorrhizal fungi, plant pathogenic nematode

INTRODUCTION

Plants do not live in solitary isolation but instead are constantly interacting with other organisms in their environment. Organisms known to interact with plants include herbivores, commensals, symbionts, and pathogens from multiple kingdoms. These organisms can interact with essentially any plant organ throughout all stages of plant development. Certain plant parasites and symbionts infect host-plant tissues and spend the majority of their lives within their host (Compant et al., 2010; Hassani et al., 2018). Such manipulation of host development has evolved independently several

times and can be found in multiple classes of organisms, including bacteria, fungi, nematodes, mites, and insects (Barash and Manulis-Sasson, 2009; Dodueva et al., 2020).

Plant endoparasite/endophyte-induced structures can have profound effects in agriculture. Colonization by symbionts usually grants certain advantages to the host plant, such as enhanced nutrient acquisition, and is thus generally preferred (if not required) in agriculture (Khare et al., 2018). On the other hand, parasite-induced ectopic structures are typically signs of disease that reduce crop performance, and can sometimes be fatal. However, what remains unclear is how these organisms locate their hosts. Despite plants being sessile, endoparasites and endophytes nevertheless need to make an effort to locate their host plants. Some endoparasites and endophytes have very specific host ranges, while for others plant hosts are obligatory to complete their life cycles. As such, host-seeking is clearly a vital behavior in plant endoparasites and endophytes and one that requires intricate regulation. It is generally accepted that to locate host plants, endosymbionts and endoparasites sense attractants secreted by these plants. However, the mechanisms by which these attractants are perceived and identified generally remain unclear.

This review aims to explore the current status the chemotactic behaviors of plant endoparasite/endophyte, particularly those that induce host-plant structural remodeling. The chemotactic behavior and chemosensory mechanisms of bacteria, fungi, and nematodes toward plants will be introduced, summarizing chemotactic signaling systems established in these respective taxa using model organisms. The chemotactic mechanisms and known attractants for plant-infecting members of each taxon will then be discussed.

ENDOPARASITIC AND ENDOPHYTIC BACTERIA

The best-characterized examples of plant-infecting organism-induced plant developmental remodeling are caused by bacteria. *Rhizobium radiobacter*, the causative agent of crown gall disease, stimulates tumor formation on the shoots and roots of many plant species, while various rhizobia species colonize plant roots and form nodules to provide organic nitrogen in exchange for carbohydrates (Escobar and Dandekar, 2003; Poole et al., 2018). Multiple species of bacteria have been shown to migrate toward root exudates, and the rhizosphere is indeed known to be colonized by many species of microorganisms (Walker et al., 2003; Berendsen et al., 2012). However, the specific components within root exudates that soil bacteria respond to largely remain to be deciphered. In addition, exudate compositions also vary among root regions, adding temporal and spatial variations to bacterial behavior in the rhizosphere (Scharf et al., 2016). Lastly, root exudates can mediate bacterial colonization not only through chemotaxis but also through other means, such as promoting nodulation or inducing flagellin expression (Kierul et al., 2015; Li et al., 2016).

Chemotaxis has been well-characterized in the model organism *Escherichia coli*. The perception of chemotactic signals in *E. coli* is mediated by the core complex, which consists of four methyl-accepting chemotaxis proteins (MCPs) that act as chemoreceptors, and redox receptor Aer, histidine kinase CheA and adaptor protein CheW (Yang and Briegel, 2020). The core complexes in turn form large hexagonal clusters on the plasma membrane, known as the chemosensory array, and are responsible for phosphorylating downstream signalling modules upon chemoattractant binding (Yang and Briegel, 2020). Downstream targets of the core complex include CheB, which mediates sensory adaptation and inhibits the MCPs as a negative feedback signal, and CheY, which controls flagella-mediated locomotion (Wadhams and Armitage, 2004). By favoring long-flagella-mediated propulsion in the presence of chemoattractants, the bacterial cell gradually moves closer to the attractant.

The number of chemoreceptors and the tertiary structures of the core complex show great diversity among bacterial taxa, although in general the chemotactic machinery seen in *E. coli* is well-conserved among bacteria and serves as a suitable model system (**Table 1**). Currently, 19 bacterial chemotaxis systems have been identified; 17 based on the *E. coli* Che system, with two other unique systems known as type IV pili motility (Tfp) and alternative cellular function (ACF) (Wuichet and Zhulin, 2010). More than half of the motile bacteria possess multiple chemosensory systems, highlighting the importance of processing and fine-tuning chemosensory signalling and responses (Wuichet and Zhulin, 2010). Expectedly, the number and diversity of MCPs expressed in a given taxon correlate with its lifestyle and metabolism complexity (Lacal et al., 2010). Several species of soil bacteria have been documented to be attracted by organic acids, for which the cognate chemoreceptors have been identified in many species (Sampedro et al., 2015; **Table 2**). Other common bacterial chemoattractants include sugars and sugar alcohols (Bowra and Dilworth, 1981; Burg et al., 1982; Alexandre et al., 2000; Meier et al., 2007; Miller et al., 2007; **Table 2**).

Rhizobium radiobacter (formerly known as *Agrobactetrium tumefaciens*), the causative agent of crown gall disease, is perhaps the best-known endoparasitic organism that manipulates plant development. *R. radiobacter* probably targets molecules specifically released by wounding, since it infiltrates plant tissues *via* wound sites. As such, *R. radiobacter* has been shown to be attracted to various sugars, amino acids, opines, and phenolics (Ashby et al., 1987; Ashby et al., 1988; Loake et al., 1988; Kim and Farrand, 1998). One of the chemoreceptors, ChvE, has been shown to be essential for host-finding and shares structural homology with *E. coli* proteins known to bind galactose and glucose, suggesting ChvE may similarly function as a chemoreceptor for sugars (Cangelosi et al., 1990). Interestingly, *R. radiobacter* expresses two CheW homologues, both of which are required for chemotaxis towards plant tissue, yet neither is encoded in the Che operon (Huang et al., 2018).

Rhizobium leguminosarum is one of the best characterized rhizobia and is related to *R. radiobacter*; they both belong to the Rhizobiaceae family. *R. leguminosarum* forms nodules in the roots of legumes, such as peas, clovers, and various beans, and is

TABLE 1 | Chemotactic genes of endoparasites and endophytes discussed in this review.

Endoparasite, endophyte	Chemotactic gene	Model organism orthologues	Predicted functions	Reference
Rhizobium radiobacter (bacteria)	ChvE	*E. coli* galactose/glucose-binding protein (GBP)	Putative sugar chemoreceptor	Cangelosi et al., 1990
	$CheW_1$, $CheW_2$	*E. coli* CheW	Scaffold protein binding chemoreceptor and histidine kinase CheA	Huang et al., 2018
Rhizobium leguminosarum (bacteria)	McpB, McpC	*E. coli* MCPs	Chemoreceptors with unknown ligands	Yost et al., 1998
Sinorhizobium meliloti (bacteria)	McpE, McpS, McpT, McpU, McpV, McpW, McpX, McpY, McpZ	*E. coli* MCPs	Chemoreceptors for sugars, amino acids and organic acids	Meier et al., 2007
	CheY1, CheY2	*E. coli* CheY	Binds and changes the rotation direction of flagellar motor,	Sourjik and Schmitt, 1996; Sourjik and Schmitt, 1998
	CheD	*E. coli* CheD	Deaminase that regulates chemoreceptor activities	Scharf et al., 2016
	CheS	N/A	Regulates phosphorylation of CheY1	Dogra et al., 2012
	CheT	N/A	Required for chemotaxis, function unknown	Scharf et al., 2016
Fusarium oxysporum (fungi)	STE2	*S. cerevisiae* Ste2	Chemoreceptor for unknown host signal	Turrà et al., 2015
	Fmk1	*S. cerevisiae* Fus3 and Kss1	MAP kinase for chemotropism signaling,	Di Pietro et al., 2001
Meloidogyne incognita (nematode)	Mi-odr-1	*C. elegans* odr-1	Membrane-bound guanylyl cyclase that produces cGMP secondary messenger	Shivakumara et al., 2019
	Mi-odr-3	*C. elegans* odr-3	Gα protein that regulates cyclic nucleotide metabolism	Shivakumara et al., 2019
	Mi-tax-2, Mi-tax-4	*C. elegans* tax-2 and tax-4	Subunits of cyclic nucleotide-gated cation channel involved in G-protein-mediated signalling	Shivakumara et al., 2019

an important contributor to nitrogen fixation. Its genome contains two chemotaxis operons, where Che1 is likely to be the main driver mediating chemotaxis toward sugars and is essential for host-finding and nodulation (Miller et al., 2007). *R. leguminosarum* has been shown to be attracted to amino acids and flavonoids (Armitage et al., 1988). In addition, two of its chemoreceptors, McpB and McpC, are known to positively regulate nodulation, but their ligands remain unknown (Yost et al., 1998). The importance of these two receptors may be more relevant depending on the host species and competing soil microbiota (Yost et al., 1998).

Sinorhizobium meliloti is another well-characterized member of the Rhizobiaceae family. *S. meliloti* has been shown to colonize specific regions of alfalfa roots, confirming their preference for cues from specific parts of the roots (Gulash et al., 1984). The *S. meliloti* genome contains nine chemoreceptors, all of which were shown to be required for chemotaxis toward sugars, amino acids, and organic acids (Meier et al., 2007). Two CheY homologues are also present, with CheY2 controlling the unidirectional flagella motor speed, while CheY1 terminates the chemotaxis signal (Sourjik and Schmitt, 1996; Platzer et al., 1997; Sourjik and Schmitt, 1998; Attmannspacher et al., 2005). The chemotactic machineries are encoded in two operons (Meier et al., 2007; Meier and Scharf, 2009). The Che1 operon of *S. meliloti* contains the CheD deamidase that modulates chemoreceptor activities (Scharf et al., 2016). *S. meliloti* also expresses CheS, a novel protein that complexes with CheA to facilitate dephosphorylation of CheY1 (Dogra et al., 2012). CheT is another novel protein in *S. meliloti* Che1 operon required for chemotaxis, though its function is currently unknown (Scharf et al., 2016). *S. meliloti* has been documented to be attracted to luteolin, 4',7-dihydroxyflavone, 4',7-dihydroxyflavanone, and 4,4'-dihydroxy-2-methoxychalcone, all of which are found in root exudates (Caetano-Anollés et al., 1988; Dharmatilake and Bauer, 1992). In addition, *S. meliloti* has been shown to be attracted to amino acids in alfalfa seed exudates, which is mediated by McpU, as well as to common sugars (Götz et al., 1982; Malek, 1989; Meier et al., 2007; Webb et al., 2014; Webb et al., 2017a). Other known *S. meliloti* attractants include quaternary ammonium compounds (betonicine, choline, glycine betaine, stachydrine, and trigonelline), which are recognized by McpX (Webb et al., 2017b).

ENDOPARASITIC AND ENDOPHYTIC FUNGI

The other prominent class of organisms known to invade plant tissues is the fungi. Unlike bacteria, fungi are immobile and under most circumstances are not chemotactic. Nevertheless, plant-symbiotic and parasitic fungi make deliberate efforts to mediate hyphae growth toward potential hosts *via* chemotropism. Hyphae chemotropism towards plants was first described in *Uromyces appendiculatus* growing towards soybean leaf stomata, with the tips of hyphae recognized as the area responsible for sensing chemical cues and processing chemotropism (Turrà and Di Pietro, 2015; Turrà et al., 2016). By 1905, it was noted that the constituents of host exudates dictated the type of fungi attracted, which consolidates the importance of chemotaxis in plant parasitism (Massee, 1905).

TABLE 2 | Chemoattractants of endoparasites and endophytes discussed in this review.

Attractant class	Perceived by	Attractants	Notes	References
Sugars and alcohols	*Rhizobium radiobacter* (bacteria)	Sucrose, glucose, fructose, maltose, lactulose, galactose, raffinose, stachyose, arabinose	May be perceived by chemoreceptor ChvE	Loake et al., 1988; Cangelosi et al., 1990
	Rhizobium leguminosarum (bacteria)	Mannitol, galactose	Perception requires the Che1 chemotaxis operon	Miller et al., 2007
	Sinorhizobium meliloti (bacteria)	Fructose, galactose, maltose, mannitol, sucrose	Perception requires all 9 chemoreceptors McpE, McpS-McpZ	Meier et al., 2007
		Sucrose, glucose, arabinose, galactose		Malek, 1989
	Meloidogyne incognita (nematode)	Mannitol	Signal transduction may require *Mi-odr-1*, *Mi-odr-3*, *Mi-tax-2* and *Mi-tax-4*	Fleming et al., 2017; Shivakumara et al., 2019
Organic acids	*Rhizobium leguminosarum* (bacteria)	Pyruvate, succinate	Perception requires the Che1 chemotaxis operon	Miller et al., 2007
	Sinorhizobium meliloti (bacteria)	Citrate, fumarate, malate, succinate	Perception requires all 9 chemoreceptors McpE, McpS-McpZ	Meier et al., 2007
	Meloidogyne incognita (nematode)	Vanillic acid, lauric acid	Signal transduction may require *Mi-odr-1*, *Mi-odr-3*, *Mi-tax-2* and *Mi-tax-4*.	Dong et al., 2014; Fleming et al., 2017; Shivakumara et al., 2019
Amino acids	*Rhizobium radiobacter* (bacteria)	Valine, arginine		Loake et al., 1988
	Rhizobium leguminosarum (bacteria)	Homoserine		Armitage et al., 1988
	Sinorhizobium meliloti (bacteria)	All standard amino acids	Perception requires all 9 chemoreceptors McpE, McpS-McpZ	Götz et al., 1982; Malek, 1989; Meier et al., 2007; Webb et al., 2014; Webb et al., 2017a
		Citrulline, γ-aminobutyric acid, ornithine	Perception requires chemoreceptor McpU	Webb et al., 2017a
		Homoserine		Götz et al., 1982
	Meloidogyne incognita (nematode)	Argenine, lysine	Signal transduction may require *Mi-odr-1*, *Mi-odr-3*, *Mi-tax-2* and *Mi-tax-4*	Fleming et al., 2017; Shivakumara et al., 2019
Phenolics	*Rhizobium radiobacter* (bacteria)	Acetosyringone, sinapinic acid, syringic acid		Ashby et al., 1987; Ashby et al., 1988
	Meloidogyne incognita (nematode)	Tannic acid	Signal transduction may require *Mi-odr-1*, *Mi-odr-3*, *Mi-tax-2* and *Mi-tax-4*	Fleming et al., 2017; Shivakumara et al., 2019
Flavonoids	*Rhizobium leguminosarum* (bacteria)	Apigenin, naringenin, kaempferol		Armitage et al., 1988
	Sinorhizobium meliloti (bacteria)	Luteolin, 4',7-dihydroxyflavone, 4',7-dihydroxyflavanone, and 4,4'-dihydroxy-2-methoxychalcone		Caetano-Anollés et al., 1988; Dharmatilake and Bauer, 1992
Phyto-hormones	*Gigaspora margarita* (fungi)	Strigolactone	Likely perceived by novel receptors not conserved in plants.	Akiyama et al., 2005; Akiyama and Hayashi, 2006; Gutjahr, 2014; Boyer et al., 2014
	Meloidogyne incognita (nematode)	6-Dimethylallylaminopurine, salicylic acid, gibberellic acid, Indole-3-acetic acid	Signal transduction may require *Mi-odr-1*, *Mi-odr-3*, *Mi-tax-2* and *Mi-tax-4*	Fleming et al., 2017; Shivakumara et al., 2019
Organic amines	*Sinorhizobium meliloti* (bacteria)	Betonicine, choline, glycine betaine, stachydrine, trigonelline	Perception requires chemoreceptor McpX	Webb et al., 2017b
	Meloidogyne incognita (nematode)	Cadaverine, 1,3-diaminopropane, putrescine		Oota et al., 2019
Opines	*Rhizobium radiobacter* (bacteria)	Octopine, nopaline, mannopine, agrocinopines A+B		Kim and Farrand, 1998
Others	*Rhizobium leguminosarum* (bacteria)	Unknown host signal	Perception requires chemoreceptors McpB and McpC	Yost et al., 1998
	Fusarium oxysporum (fungi)	Unknown host signal	Perception requires α-STE2 chemoreceptor and Fmk1 MAPK kinase, signal requires peroxidase activity from host	Turrà et al., 2015
	Trichoderma harzianum (fungi)	Unknown host signal	requires stress, peroxidase and oxylipin activities from host	Lombardi et al., 2018
	Meloidogyne incognita (nematode)	Calcium chloride		Wang et al., 2018

In a similar way to how *E. coli* serves as a model for bacterial chemotaxis, studies using *Saccharomyces cerevisiae* and *Neurospora crassa* have provided invaluable insights into fungal chemotropism (**Table 1**). *S. cerevisiae* cells develop mating projections known as shmoos in the presence of the opposite mating type, by detecting secreted mating peptide pheromone a or α. These pheromones are perceived by seven transmembrane G-protein-coupled receptors; MATa cells express Ste2, which binds the α- pheromone, while MATα cells express Ste3, which binds the a-pheromone (Hagen et al., 1986; Blumer et al., 1988). The receptors function as guanine exchange factors and activates the Gα subunit (GPA1) upon pheromone-binding, which promotes the dissociation of the Gβγ subunits (STE4 and STE18) from the complex (Schrick et al., 1997). This then initiates a signalling cascade mediated by Fus3 and Kss1 (MAPK), leading to transcriptional regulation, cell cycle arrest, cell shape alternation, and ultimately shmoo development toward the mating partner (Arkowitz, 2009). In an analogous case, female hyphae of *Neurospora crassa* (trichogyne) grow towards male spores *via* chemotropism. This process is mediated by the spore pheromone peptides MFA-1 and CCG-4, which are perceived by the receptors PRE-1 and PRE-2 (orthologues of Ste2 and Ste3), respectively (Kim and Borkovich, 2004; Kim and Borkovich, 2006). Pheromone perception in *N. crassa* initiates a similar MAPK signalling cascade mediated by heterotrimeric G-proteins (Dettmann et al., 2014). Another case of chemotropism in *N. crassa* is anastomosis, where hyphae from cells of an identical genotype (sometimes the same cell) are attracted towards each other, followed by fusion (Leeder et al., 2011). The anastomosis chemotropism signal is similarly transduced by a MAPK cascade using orthologues of Fus3 and Kss1 (Read et al., 2009). The *N. crassa* anastomosis signal may be a peptide pheromone (Roca et al., 2005), and it has been hypothesized that both parties use the same signalling molecule, which positively regulates itself (Read et al., 2012). Lastly, hyphae repellants may also play a role in chemotropism, and the direction of hyphae growth is likely to be a balance between attraction and repulsion (Leeder et al., 2011).

Hyphal chemotropism in response to plants is well-characterized in pathogens of the genus *Fusarium*, which are ubiquitous, filamentous ascomycete fungi. *Fusarium oxysporum* spores respond to host cues in order to germinate, and its hyphae elongate toward host roots using chemotropism. Although *F. oxysporum* does not manipulate the host's developmental program, it nevertheless serves as a good model to decipher how yeast chemotropism has been specialized for pathogenesis. *F. oxysporum* requires the α-STE2 signalling module and Fmk1 (an orthologue of Fus3 and Kss1) for infection (Di Pietro et al., 2001; Turrà et al., 2015). Considering *F. oxysporum* does not undergo sexual reproduction, the conserved mating pheromone chemotropism pathway may have evolved to detect host signals (Turrà et al., 2015). *F. oxysporum* has been shown to be able to distinguish between live and dead cells, suggesting it is likely to be able to perceive certain live cell-exclusive signals (van der Does et al., 2008). Furthermore, *F. oxysporum* root-targeting behavior has been shown to require the secretion of a haem-containing peroxidase released from root wounds (Turrà et al., 2015), suggesting the product of this peroxidase may be a potential chemoattractant, in addition to nutrients such as amino acids and sugars. On the other hand, the biocontrol agent *Trichoderma harzianum* has been shown to be preferentially attracted to root exudate secreted by tomato plants under stress; peroxidase and oxylipins are required in the exudate for this attraction to occur (Lombardi et al., 2018). Interestingly, stress did not enhance the attraction of tomato root exudate to *F. oxysporum*, even though peroxidase has been shown to be an important element in *F. oxysporum* chemotropism (Turrà et al., 2015; Lombardi et al., 2018). The specific identities of the peroxidase-dependent attractants for *F. oxysporum* may be more complicated than expected.

The fungal counterparts of the bacterial rhizobia are the arbuscular mycorrhizal fungi (AMF). AMF include the Glomeromycetes, obligate symbionts that form highly branched structures known as arbuscules to mediate nutrient exchange with their host root's cortical cells. AMF provide their plant hosts with various nutrients, predominantly inorganic phosphate, while receiving photosynthetic products such as hexoses and fatty acids from their host (Jiang et al., 2017; Luginbuehl et al., 2017). AMF have been estimated to colonize ~80% of all land plants, while fossil records suggest plant—AMF symbioses occurred as early as 460 million years ago, coinciding with the colonization of land by plants (Martin et al., 2017; Strullu-Derrien et al., 2018). These lines of evidence suggest AMF may be a key factor in plant terrestrial adaptation. AMF probably locate their host plants by recognizing molecules from root exudates, as root exudates have been shown to promote AMF spore germination and hyphal branching. The phytohormone strigolactone (SL) has been shown to promote hyphal branching in *Gigaspora margarita* (Akiyama et al., 2005; Akiyama and Hayashi, 2006; **Table 2**), while pea plants deficient in SL synthesis show reduced AMF colonization (Gómez-Roldán et al., 2008). Specifically, SL treatment stimulates AMF mitochondria proliferation and shape change, and increases metabolism (Besserer et al., 2006; Besserer et al., 2008; Besserer et al., 2009). SL also induces spore germination in AMF (Besserer et al., 2006; Besserer et al., 2008). Together, these lines of evidence confirm that secreted SL is indeed a vital positive regulator of AMF colonization. No fungal receptor of SL has yet been identified, but it is likely to be different from the plant SL receptor, since *G. margarita* perceives different forms of SL than plants do (Gutjahr, 2014; Boyer et al., 2014), and the *Rhizophagus irregularis* genome does not appear to contain orthologues of plant SL receptors (Tisserant et al., 2012).

On the other hand, SL is probably not the only molecule that AMF target for host-localization. Plants deficient in SL synthesis show a reduction in, but not the abolishment of, AMF colonization (Gómez-Roldán et al., 2008), while AMF non-host plants have also been shown secrete SL from their roots, albeit at lower levels (Goldwasser et al., 2008; Yoneyama et al., 2008). It seems plausible that SL-insensitive AMF can still colonize roots if encountered by chance, and SL merely functions to enhance

host-guidance but is not essential for colonization. AMF species including *Gigaspora gigantean* and *Glomus mosseae*, and ectomycorrhizal fungal species including *Pisolithus tinctorius* and *Paxillus involutus* have been shown to prefer host roots over non-hosts or dead plants (Koske, 1982; Horan and Chilvers, 1990; Sbrana and Giovannetti, 2005). Since SL appears to be ubiquitously found in all plants, the presence of SL alone is not sufficient to dictate AMF colonization. Other root-derived AMF branching factors probably exist, but the situation is complicated since different compounds may have different effects on the same AMF, while the same compound may have different effects on different AMF (Nagahashi and Douds, 2000; Nagahashi and Douds, 2007). Different forms of SL may also have different attracting strengths and activities.

ENDOPARASITIC NEMATODES

Another class of endoparasitic plant pathogens known to cause novel organ formation and developmental reprogramming of the host is the nematodes. The major nematode plant pathogens comprise the root-knot nematodes (RKNs, genus *Meloidogyne*), the cyst nematodes (CN, genera *Heterdera* and *Globodera*) and the pine-wilt nematodes (PWN, *Bursaphelenchus xylophilus*). Although RKNs and CNs appear to have evolved independently, both use infection mechanisms that have much in common. In both cases, second-instar juveniles (J2) roam freely in the soil searching for the roots of appropriate host plants. Once a suitable root has been identified, the J2s infect the root and inject effectors that reprogram the host's vascular cells to form specialized feeding organs (Bartlem et al., 2014; Favery et al., 2016). RKNs stimulate host cells to undergo endoreduplication and form multi-nucleated giant cells, while CNs merge multiple host cells together to form syncytia (Siddique and Grundler, 2018). The nematodes then feed on these specialized organs and develop to maturity, whereupon females emerge from the roots to lay eggs and release the next generation to the environment.

J2 host-targeting behavior is therefore critical in plant parasitic nematode biology, and chemotaxis towards plant exudates has been associated with this behavior. Soybean, pea, potato, tomato, and rice root exudates have all been shown to attract J2s of various plant pathogenic nematodes (Papademetriou and Bone, 1983; Zhao et al., 2000; Reynolds et al., 2011; Xu et al., 2015; Yang et al., 2016; Čepulytė et al., 2018). Specifically, phenolics, flavonoids, glycoside, fatty acids, and diamines in exudates and volatiles from roots have been shown to act as nematode attractants (Chitwood, 2002; Zhao et al., 2007; Ohri and Pannu, 2010; Ali et al., 2011; Oota et al., 2019; **Table 2**). In addition, Arabidopsis seeds were also shown to attract RKN, suggesting RKN may interact with plant seeds as well aside from roots (Tsai et al., 2019). Furthermore, it was revealed that nematode attractants and repellents are produced not only by plants but also by nematodes themselves. Many plant-parasitic nematodes have been shown to produce ascarosides, a class of glycolipid-based signaling molecules synthesized almost exclusively by nematodes (Manosalva et al., 2015). Depending on the types and compositions, ascarosides can regulate the aggregation/dispersion of conspecifics or even other nematodes (Manohar et al., 2020). On the other hand, other compounds have also been documented to influence nematode behavior such as carbon dioxide; the amino acids arginine and lysine; phenolic acids; the plant hormones salicylic acid and gibberellic acid; the growth supplement ethephon; 6-dimethylallylaminopurine; and nitrate analogues (Pline and Dusenbery, 1987; Wang et al., 2009; Wang et al., 2010; Fleming et al., 2017; Hosoi et al., 2017; **Table 2**).

Caenorhabditis elegans has been established as a model organism for nematodes, and its genome, cell development pathway and nervous system have been extensively characterized. By examining elements conserved among *C. elegans* and plant pathogenic nematodes it may be possible to further expand our knowledge of pathogenic nematode behavior. Chemotaxis in nematodes is regulated by the amphid and phasmid sensory organs in their head and tail, respectively. In *C. elegans*, a pair of amphids acts as the main sensory organs, which contain twelve types of sensory neurons. By using laser ablation of individual or combinations of neurons, the corresponding stimulant signals being transmitted by each neuron can be identified (Mori, 1999; Rengarajan and Hallem, 2016). Despite the fact that the neural structures of plant-parasitic nematodes are at least somewhat conserved with *C.elegans*, molecular evidence suggests plant-parasitic nematodes likely evolved from fungivorous ancestors, which are likely evolutionarily distant from bacterivorous *C. elegans* (Quist et al., 2015). Cautions should be applied when inferring homology relationships between plant-parasitic nematodes and *C. elegans* to account for their evolutionary divergence and different foraging preferences.

Olfactory receptors are highly expressed in the sensory neurons and play important roles in sensing specific signals. For example, the AWA neuron expresses the ODR-10 receptor, which is responsible for diacetyl detection, and consequently *odr-10* mutants fail to detect diacetyl compounds (Sengupta et al., 1996). Currently, 194 putative olfactory receptor genes have been identified in the *C. elegans* genome (Taniguchi et al., 2014). Therefore, we performed homology searches to look for orthologues of *C. elegans* olfactory receptors in the genomes of plant pathogenic nematodes, including the RKNs *Meloidogyne incognita* and *Meloidogyne arenaria*, the CNs *Heterodera glycines* and *Globodera rostochiensis*, and the PWN *B. xylophilus* (**Table 3**). Interestingly, the majority of *C. elegans* olfactory receptors are not conserved among plant pathogenic nematodes, although the few receptor orthologues that are present may be informative in determining their chemotactic behaviors. The *B. xylophilus* genome contains orthologues of SRV-11 (pentanedione avoidance), SRV-12 (benzaldehyde attraction), SRSX-26 (butanone attraction), SRSX-32 (pyrazine attraction), SRSX-33 (pentanedione and pyrazine attraction), SRSX-37 (pentanedione attraction), SRT-18 and SRT-25 (diacethyl avoidance). Meanwhile, the *M. incognita* and *M. arenaria* genomes contain orthologues of SRG-37 (pyrazine attraction). It would be interesting to determine whether the functions of these receptors are conserved among pathogenic nematodes and similarly regulate

TABLE 3 | *C. elegans* olfactory receptor orthologues present in plant pathogenic nematodes, their predicted functions, and E-values of DNA sequence similarities.

C. elegans GPCR	Predicted function	Species	Orthologue	E-value
SRV-11	Pentanedione avoidance	*B. xylophilus*	BXY_0066100	3.00E-11
		B. xylophilus	BXY_1231200	4.00E-10
		B. xylophilus	BXY_0069300	2.00E-09
SRV-12	Benzaldehyde attraction	*B. xylophilus*	BXY_0066100	5.00E-10
		B. xylophilus	BXY_1231200	3.00E-07
		B. xylophilus	BXY_0069300	1.00E-06
SRSX-26	Butanone attraction	*B. xylophilus*	BXY_1070000	4.00E-10
		B. xylophilus	BXY_1013200	1.00E-07
SRSX-32	Pyrazine attraction	*B. xylophilus*	BXY_1070000	3.00E-10
		B. xylophilus	BXY_1013200	2.00E-07
		B. xylophilus	BXY_1013400	4.00E-05
		B. xylophilus	BXY_0557500	6.00E-04
		B. xylophilus	BXY_0027800	9.00E-04
SRSX-33	Pentanedione and pyrazine attraction	*B. xylophilus*	BXY_1070000	2.00E-06
		B. xylophilus	BXY_0809300	3.00E-06
		B. xylophilus	BXY_1013400	3.00E-05
		B. xylophilus	BXY_0557500	9.00E-05
		B. xylophilus	BXY_0027800	2.00E-04
SRSX-37	Pentanedione attraction	*B. xylophilus*	BXY_1013400	3.00E-09
SRT-18	Diacethyl avoidance	*B. xylophilus*	BXY_1024600	5.00E-20
SRT-25	Diacethyl avoidance	*B. xylophilus*	BXY_1024600	9.00E-20
SRG-37	Pyrazine attraction	*M. incognita*	Minc3s00775g17185	2.30E-02
		M. arenaria	tig00002579.g60974	5.90E-02

Homologies were determined using Protein BLAST from WormBase ParaSite (https://parasite.wormbase.org/index.html) with the protein sequences of the 194 C. elegans chemoreceptors described by Taniguchi et al. (2014) against the following proteome databases: Bursaphelenchus_xylophilus_prjea64437, Meloidogyne_arenaria_prjna438575 and Meloidogyne_incognita_prjeb8714, Globodera_rostochiensis_prjeb13504, and Heterodera_glycines_prjna381081 using the default setting.

chemotaxis. On the other hand, *H. glycines* and *G. rostochiensis* genomes contain no orthologues of *C. elegans* olfactory receptors. This surprisingly low level of conservation of olfactory receptors suggests plant pathogenic nematodes have independently evolved unique signalling pathways to detect chemical signals. Meanwhile, four *C. elegans* chemosensory gene orthologues were identified in *M. incognita* as *Mi-odr-1*, *Mi-odr-3*, *Mi-tax-2* and *Mi-tax-4*, where knockdown mutants showed defects in their attraction to root exudates, volatile compounds (alcohols, ketones, aromatic compounds, esters, thiazole, and pyrazine), non-volatile compounds (carbohydrates, phytohormones, organic acids, amino acids, and phenolics), as well as ascaroside signalling (Shivakumara et al., 2019; **Table 1**). These putative *M. incognita*-specific chemosensory signaling modules are likely to play important roles in host-targeting, and the identification of the corresponding olfactory receptors for these pathways may help identify specific RKN chemoattractants.

OUTSTANDING CHALLENGES AND FUTURE PERSPECTIVES

Aside from microorganisms, many arthropod species are also known to be endoparasitic and can manipulate their host plant's developmental program during infection. Insects from the orders Hemiptera and Hymenoptera and mites from the superfamily Eriophyoidea include endoparasitic members that form galls. Similar to the ectopic organs formed by endoparasitic/endophytic microbes, galls induced by endoparasitic arthropods function as feeding organs and/or physical barriers for protection. Mechanisms that mediate arthropod-mediated galling through phytohormone manipulation have been characterized in great detail (Tooker and Helms, 2014; de Lillo et al., 2018). However, how arthropod parasites locate their host plants has been relatively poorly investigated, for various reasons. First, arthropods may not rely heavily on chemotaxis to find hosts. Endoparasitic arthropods typically have poor mobility and rely on random forces for locomotion, such as wind (Nault and Styer, 1969; Sabelis and Bruin, 1996). Other endoparasitic arthropods specialize in infecting a single long-lived host plant, where progenies can continue to infect the same host as their parents (Lindquist and Oldfield, 1996; Manson and Oldfield, 1996). Second, arthropod-induced galls are among the most structurally diverse, with 13,000 insect species documented to form plant galls. Galling behavior appears to have evolved in arthropods multiple times, possibly through horizontal gene transfer from symbiotic bacteria or fungi (Gullan et al., 2005; Raman et al., 2005). Therefore, no single model organism system may be sufficient to represent the molecular signalling mechanisms for chemotaxis in endoparasitic arthropods, and these behaviors may have to be addressed in a case-by-case fashion.

Another major challenge in the characterization of plant endoparasites and endophytes are tri-trophic and other interactions that involve more parties. In nature, it is likely that plants will simultaneously encounter several of the endoparasites and endophytes discussed above, considering the same chemicals may attract organisms from multiple taxons. The outcome of these complex interactions will not be easy to predict under controlled laboratory conditions. For example, *Fusarium solani*, a plant fungal pathogen related to *F. oxysporum*, has been shown to induce virulence genes in response to the

isoflavanoid pisatin in host roots, which is made by plants during stress (Straney et al., 1994; Straney et al., 2002). Similarly, the plant pathogenic nematode *M. incognita* can be attracted to polyamines from plant root exudates, which are also known to be produced in stressed plants (Oota et al., 2019). It appears that pathogens from multiple taxa tend to favor stressed plants, making simultaneous infection or colonization very likely scenarios in nature. On the other hand, SL has been shown to not only promote hyphal branching in AMF but also the germination of parasitic plants of the genera *Striga* and *Orobanche* (Cook et al., 1966; Cardoso et al., 2011). The plant SL production levels fluctuate during the course of AMF infection, with SL-synthesis genes up-regulated during early infection, and down-regulated during later infection stages (López-Ráez et al., 2015; Kobae et al., 2018). Furthermore, host plants utilize overlapping signalling components in response to both AMF and rhizobia infections, suggesting the two processes may have evolved together (Hirsch and Kapulnik, 1998; Guinel and Geil, 2002; Vierheilig and Piché, 2002; Parniske, 2008). Plants inoculated with rhizobia also show reduced *Orobanche* infection (Mabrouk et al., 2007a; Mabrouk et al., 2007b; Mabrouk et al., 2007c), while SL-synthesis genes are up-regulated during rhizobia colonization (Breakspear et al., 2014; van Zeijl et al., 2015). These lines of evidence suggest the interactions between host plants, rhizobia, AMF, and parasitic plants mediated by SL require more elaborate analysis to decipher.

In general, it appears that most known attractants of plant endoparasites and endophytes consist of common compounds such as metabolites and plant hormones, instead of unique or unusual compounds. Currently it remains very difficult to use chemotactic behavior alone to explain endoparasites'/endophytes' host range. The more likely explanation may be that soil microorganisms sense and respond to multiple chemoattractant simultaneously. Plants may also produce chemoattractants that are toxic to attracted microorganisms. Lauric acid has been shown to have different effects on *M. incognita* depending on concentrations (Dong et al., 2014). Abiotic environmental factors may also influence the behavior of soil microorganisms. Factors such as pH, ions and temperature, redox potential, chelating compounds, and electrical potential have been documented to affect the behavior of plant parasitic nematodes (Rasmann et al., 2012). Therefore, endoparasite/endophyte host-targeting behavior is likely to be complex, involving both biotic and abiotic factors. Nevertheless, the identification and characterization of chemoattractants can have practical applications in agriculture. These chemoattractants or repellants may be applied in fields directly to manipulate the microorganisms behaviors, and ultimately improve the growth of crop plants. The chemotactic behaviors of different organisms may even be combined, as *C. elegans* has been shown to be capable of carrying rhizobia bacteria to plant hosts through phoresis (Horiuchi et al., 2005). With the identification of more chemoattractants, more sophisticated agricultural application strategies may eventually be designed and implemented in the future.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

AY-LT and SS conceptualized the work. AY-LT, MO, and SS wrote the article.

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REVIEW
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The Evolution of Endophagy in Herbivorous Insects

John F. Tooker[1] and David Giron[2]*

[1] Department of Entomology, The Pennsylvania State University, University Park, PA, United States, [2] Institut de Recherche sur la Biologie de l'Insecte, UMR 7261, CNRS/Université de Tours, Parc Grandmont, Tours, France

Herbivorous feeding inside plant tissues, or endophagy, is a common lifestyle across Insecta, and occurs in insect taxa that bore, roll, tie, mine, gall, or otherwise modify plant tissues so that the tissues surround the insects while they are feeding. Some researchers have developed hypotheses to explain the adaptive significance of certain endophytic lifestyles (e.g., miners or gallers), but we are unaware of previous efforts to broadly characterize the adaptive significance of endophagy more generally. To fill this knowledge gap, we characterized the limited set of evolutionary selection pressures that could have encouraged phytophagous insects to feed inside plants, and then consider how these factors align with evidence for endophagy in the evolutionary history of orders of herbivorous insects. Reviewing the occurrence of endophytic taxa of various feeding guilds reveals that the pattern of evolution of endophagy varies strongly among insect orders, in some cases being an ancestral trait (e.g., Coleoptera and Lepidoptera) while being more derived in others (e.g., Diptera). Despite the large diversity of endophagous lifestyles and evolutionary trajectories that have led to endophagy in insects, our consideration of selection pressures leads us to hypothesize that nutritionally based factors may have had a stronger influence on evolution of endophagy than other factors, but that competition, water conservation, and natural enemies may have played significant roles in the development of endophagy.

Keywords: Coleoptera, Diptera, gall-inducing insect, Hemiptera, Hymenoptera, leaf-mining insect, Lepidoptera, Thysanoptera

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****Correspondence:***
John F. Tooker
tooker@psu.edu

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INTRODUCTION

Among insects, feeding within plant tissue, or endophagy, has evolved numerous times and is one of the major feeding strategies for herbivorous insects. Guilds of endophytic feeders include borers, miners, and gall inducers and inquilines, but allied taxa, such as leaf tiers and leaf rollers, also tend to be included in the guild because they all have concealed feeding habits associated with plants. Endophytic associations of insects and their host plants can be millions of years old and are evident in the fossil record. For example, wood boring, leaf mining and insect galls have all been recorded from Carboniferous deposits and may have even evolved earlier (~300 million years ago; Chaloner et al., 1991; Labandeira and Phillips, 1996; Feng et al., 2017). Additionally, there are even some extant endophytic taxa evident in fossils, with good examples provided by the lepidopterans *Ectodemia* and *Stigmella*, and the aphid *Melaphis rhois*, suggesting that the interactions of these taxa with their host plants are 97- and 48-million years old, respectively (Moran, 1989; Labandeira et al., 1994).

In some taxa, feeding within plant tissue appears to have been an ancestral state, whereas in others endophagy appears to be derived. In still others, specialized endophagy has developed even further, into an extremely sophisticated form of feeding, occasionally involving mutualistic symbionts. For example, galls and mines represent extended phenotypes of the insect species that induce or form them; these structures result from complex interactions among genomes of the host plant, insect, and, sometimes, their symbionts (Giron et al., 2016).

Despite the ubiquity of endophagy within Insecta, we are unaware of any previous effort in the ecological or systematics literature to broadly delimit the guild across taxa and characterize the limited set of selective forces that could have facilitated evolution and diversification of endophytic feeding habits. Certainly some publications have address a single taxon (e.g., flies; Labandeira, 2005) or characterized the adaptive significance of a particular form of endophagy (e.g., leaf mining and gall inducing; Price et al., 1987; Connor and Taverner, 1997; Stone and Schönrogge, 2003), but these publications did not generally consider endophagy beyond these specific guilds, nor did they consider the evolution of endophagy in a comparative framework.

From just reading classical literature that often forms the basis of ecological courses, one could easily get the impression that most taxa have followed a simple progression as typified by some model systems, from exposed leaf feeding to endophagy to more specialized forms of endophagy like mining and/or galling (Price et al., 1987; Price, 1992; Nyman et al., 1998, 2000). However, the systematics literature, which is often not closely tracked by ecologists, reveals that evolution of endophagy varies greatly by taxa, so a diversity of selection pressures must have been involved in its evolution and diversification. Highlighting the presumed evolutionary sequence leading to endophagy in each insect order can provide insights on selection pressures that could have played a role on its evolution. And comparing evolutionary pathways in these various endophagous feeding guilds can provide evidence about which of these selective forces may have played major evolutionary roles, allowing us to formulate hypotheses that could be tested with quantitative methods.

Our goal in this paper is to consider via existing literature the selection pressures that could have played a role in evolution and diversification of endophagy within Insecta. We will begin by defining endophagy and generally describing its occurrence across Insecta. We then will discuss the selection pressures that could have encouraged various groups of insects to develop a concealed feeding habit and subsequently diversify. The six selection pressures we selected are drawn from previous literature with herbivorous insects generally (Strong et al., 1984) and gall insects and leaf miners, more specifically (Price et al., 1987; Connor and Taverner, 1997). They have support from particular taxa, but have not been discussed previously in terms of general endophagy. By considering endophagy generally, we aim to stimulate hypotheses that can be tested in specific groups or through comparative studies that seek to clarify selection pressures associated with the adaptive significance of various forms of endophagy. In considering evolutionary selection pressures that could have facilitated endophagy, we do not address neutral evolutionary processes (e.g., genetic drift), which can influence patterns of evolution in some insect taxa but would require a quantitative analysis of taxa and their feeding styles (Peterson et al., 2016), which is beyond the scope this paper. We also do not aim to comment on the latest developments from a molecular perspective concerning the evolution of herbivory and feeding specialization (Groen and Whiteman, 2016). Rather, we use published phylogenies to illustrate the diversity of patterns of endophagy among insect orders and how they can diverge from the simplistic views held by ecologists. After considering the possible selection pressures, we will broadly characterize occurrence of endophagy across orders of herbivorous insects and discuss which selection pressures could have been active for these taxa. We finish by considering which selection pressures may have been most relevant for evolution of the endophytic habitat generally.

ENDOPHAGY

The definition of "endophagy" or "endophytic feeding" that we will use in this paper is "insect feeding on plant or fungal tissues that occurs within tissue of a living plant, whether the specific plant tissue is live or dead." This definition allows us to include insects that feed upon non-living portions of living plants, such as bark, heartwood, and pith, but excludes insects that mostly feed upon dead or decaying plants (i.e., decomposers or detritivores, like termites; Weesner, 1960) or those that live inside plants but do not eat them (e.g., Edwards et al., 2009). Consistent with previous assessments (Labandeira, 2005), we also include seed feeders (i.e., seed predators) whose endophytic larvae consume seeds prior to seed dispersal (Janzen, 1971). The definition is not perfect because some taxa, particularly in Coleoptera (e.g., Cerambycidae), contain species that feed in live plants while others that feed in dead plants. In cases like this where the taxon falls into some gray area near our definition, we try to include them to provide appropriate context and acknowledge that biological continuums can be difficult to divide into perfect bins. Lastly, to be considered an endophagous feeder, a taxon needs just one life stage to feed endophagously. Most commonly, larvae or nymphs are the concealed feeders but adults of some taxa are also endophytic (e.g., bark beetles [Curculionidae: Scolytinae], or aphids [Aphididae] or thrips [Thysanoptera] that develop in galls). We are not aware of any taxa in which immature stages are not endophytic but adults are.

We avoid the term "endophytophagy" because others have accepted the term "phytophagy" to mean "feeding on living tissue of higher plants" ("higher plants" being a synonym for "vascular plants"; Strong et al., 1984; Mitter et al., 1988) and we want to include in our discussion insects that have found a way to live in and feed upon any plant or fungal tissue of a live plant. By focusing on plant or fungal feeding, our definition includes mutualisms between insects and fungi (e.g., ambrosia galls) in which the insect indirectly feeds upon plants by eating fungi, which consume the plant; these often-symbiotic fungi have facilitated endophytic lifestyles for some taxa (e.g., Hymenoptera, Coleoptera, Diptera; Bissett and Borkent, 1988; Hanson, 1995;

Farrell, 1998; Heath and Stireman, 2010). Our definition excludes predation or parasitoidism, animal-animal interactions which can occur within plant tissue but are obviously not plant feeding.

This definition will permit us to consider a full range of herbivorous insects with concealed lifestyles, including borers, miners, gall inducers, inquilines, and leaf rollers, tiers, and webbers. Borers and miners are similar and appear to be informally distinguished from one another based on their depth away from plant surfaces or tissue layers, with miners being close to the surface and borers being deeper. More formally, mines have been defined as "feeding channels caused by insect larvae inside the parenchyma or epidermis of plants, in which its outer wall remains undamaged, thus shutting off the mine activity from outside" (Hering, 1951). Mining can occur in bark, cambium, flowers, fruits, leaves, and stems (Powell et al., 1998), and comes in different shapes and sizes (e.g., linear, digitate, blotch or tentiform mines, among others) that tend to be species specific (Eiseman, 2020). Borers (sometimes known as tunnelers) can feed upon tissues of live trees, such as cambium, pith or wood in trunks, branches, shoots, stems, and roots, but borers can also attack flowers, fruits, and seeds (Solomon, 1995; Powell et al., 1998). Broadly speaking, gall inducers can also attack a range of plant tissues, but as a group they typically oviposit into, or feed upon, meristematically active tissues to force production of their galls (Raman et al., 2005). Some gall insects can even induce meristematically active tissues (Ananthakrishnan, 1992), which is an impressive accomplishment without an obvious mechanism. At the species level, many endophytic insects, particularly gallers and leaf miners, are monophagous and attack specific plant tissues at a specific plant-developmental stages (Connor and Taverner, 1997; Raman et al., 2005; Giron et al., 2016). Though they may appear outwardly similar, leaf-roller species take one of two approaches to hide: those that use silk to roll the leaf and others that induce tissue proliferation ("roll galls") by feeding upon one side of the leaf, leading to rolling (Dreger-Jauffret and Shorthouse, 1992).

Endophytic insects are concentrated in six of the largest orders of Insecta: Thysanoptera, Hemiptera, Hymenoptera, Coleoptera, Lepidoptera, and Diptera (**Table 1**). Of all phytophagous orders, Orthoptera and Phasmatodea do not appear to have any endophagous taxa. We will briefly address in phylogenetic order the occurrence of endophagy in these large orders (Grimaldi and Engel, 2005; Peters et al., 2014), and later we will return to these taxa to consider specific selection pressures that likely influenced evolution of endophagy in these groups of animals. The routes to endophagy for some orders are similar, but others took different paths (**Figure 1**).

The hemipteroid orders Thysanoptera and Hemiptera have evolved limited forms of endophagy. For both groups, plant-fluid feeding was a key innovation that appears to contributed to their success (Johnson et al., 2018), but this mode of feeding must have limited their ability to evolve different modes of endophagy—sucking mouthparts facilitate injection of effectors stimulating the proliferation of new plant tissues around the insect but may also restrict their ability to enter plant tissue. As a result, the only recorded endophytic species in these orders feed between attached leaves, induce galls, or are inquilines that exploit these

TABLE 1 | Taxa of plant-feeding insects that include significant endophagous species.

Order	Percent herbivorous spp.	Types of endophytic feeders	Notable taxa with significant endophytic species
Thysanoptera	68	Gall inducers	Phlaeothripidae[G]
		Gall inquilines	
Hemiptera	78	Gall inducers	Aphidoidea
		Gall inquilines	Aphididae[G]
			Coccoidea
			Asterolecaniidae[G]
			Beesoniidae[G]
			Eriococcidae[G]
			Phylloxeroidea
			Phyllorxeridae[G]
			Adelgidae[G]
			Psylloidea
			Calophyidae[G]
			Phacopteronidae[G]
			Psyllidae[G]
			Tingoidea
			Tingidae[G]
Hymenoptera	7	Borers	**Symphyta**
		Leaf folders	Pamphilioidea
		Leaf rollers	Pamphiliidae
		Leaf miners	Tenthredinoidea
		Gall inducers	Tenthridinidae[G]
		Gall inquilines	Siricoidea
			Siricidae
			Cephoidea
			Cephidae
			Apocrita
			Ichneumonoidea
			Braconidae[G]
			Chalcidoidea
			Agaonidae[G]
			Eulophidae[G]
			Eurytomidae[G]
			Pteromalidae[G]
			Tanaostigmatidae[G]
			Torymidae[G]
			Cynipoidea
			Cynipidae[G]
Coleoptera	26	Borers	Buprestoidea
		Miners	Buprestidae[L,G]
		Gall inducers	Elateroidea
		Gall inquilines	Elateridae
			Lycidae
			Bostrichoidea
			Bostrichidae
			Anobiidae
			Tenebrionoidea
			Mordellidae
			Tenebrionidae

(Continued)

TABLE 1 | Continued

Order	Percent herbivorous spp.	Types of endophytic feeders	Notable taxa with significant endophytic species
			Chrysomeloidea
			Cerambyciidae[G]
			Chrysomelidae[G,L]
			Curculionoidea
			Anthribidae
			Attelabidae[L]
			Brentidae[G]
			Curculionidae[G]
Lepidoptera	100	Borers	Nepticuloidea
		Leaf folders	Nepticulidae[G,L]
		Leaf rollers	Gracillarioidea
		Leaf tiers	Gracillariidae[G,L]
		Leaf miners	Yponomeutoidea
		Gall inducers	Glyphipterigidae[G,L]
		Gall inquilines	Gelechioidea
		Leaf mine-gallers	Cosmopterigidae[G,L]
		Leaf Mine-rollers	Depressariidae
			Elachistidae[G,L]
			Gelechiidae[G,L]
			Sesioidea
			Sesiidae[G]
			Cossoidea
			Cossidae
			Tortricoidea
			Tortricidae[G]
			Pterophoroidea
			Pterophoridae[G,L]
			Pyraloidea
			Crambidae[G]
			Pyralidae
			Thyridoidea
			Thyrididae[G]
			Noctuoidea
			Noctuidae
Diptera	28	Borers	**Nematocera**
		Leaf miners	Sciaroidea
		Gall inducers	Sciaridae[L]
		Gall inquilines	Cecidomyiidae[G,L]
			Chironomoidea
			Chironomidae[L]
			Ceratopogonidae[L]
			Brachycera
			Stratiomyoidea
			Pantophthalmidae
			Xylomyidae
			Asiloidea
			Asilidae
			Empidoidea
			Dolichopodidae[L]
			Platypezoidea
			Phoridae[L]
			Syrphoidea
			Syrphidae[L]
			Schizophora-Acalyptratae
			Diopsoidea
			Psilidae[L]
			Tephritoidea
			Lonchaeidae[G]
			Tephritidae[G,L]
			Opomyzoiea
			Agromyzidae[G,L]
			Fergusoninidae[G]
			Lauxanioidea
			Lauxaniidae[G,L]
			Ephydroidea
			Drosophilidae[L]
			Ephydridae[L]
			Chloropidae[G,L]
			Schizophora-Calyptratae
			Muscoidea
			Anthomyiidae[G,L]
			Scathophagidae[L]

In most taxa, nymphs or larvae are the endophytic life stage. Orders with fewer endophytic taxa have most of their endophytic families listed, whereas space limitations prevents listing of all endophytic families. Endophytic designations based on various edited volumes (McAlpine et al., 1981, 1987; Solomon, 1995; Powell et al., 1998; Arnett and Thomas, 2000; Arnett et al., 2002; Labandeira, 2005; Raman et al., 2005). Groups marked with a superscript G or L include gall-inducing or leaf-mining species. Percent herbivorous species is taken from Wiens et al. (2015).

feeding sites (**Table 2**; Ananthakrishnan, 1992; Burckhardt, 2005; Gullan et al., 2005; Mound and Morris, 2005). Members of these two orders, of course, have incomplete metamorphosis; therefore, immature stages have the same form of feeding as adults. Compared to holometabolous taxa, in which larvae and adults have often evolved different forms of feeding, hemimetabolous metamorphosis may have in part constrained the forms of endophytic feeding that could have evolved in these two orders.

In contrast to hemimetabolous groups, holometabolous groups have benefited from the diets and feeding styles that can evolve differently in larvae and adults. Indeed, endophagy among holometabolous groups occurs mainly in larval stages, and evolution of complete metamorphosis may be tied to concealed feeding niches (Grimaldi and Engel, 2005). Current evidence suggests that holometabolous insects may have evolved from an ancestor with an orthognathous head and chewing mouthparts that fed externally on plants or fungi (Peters et al., 2014). Moreover, the common ancestor of Coleoptera, Lepidoptera, and Diptera (among other taxa in

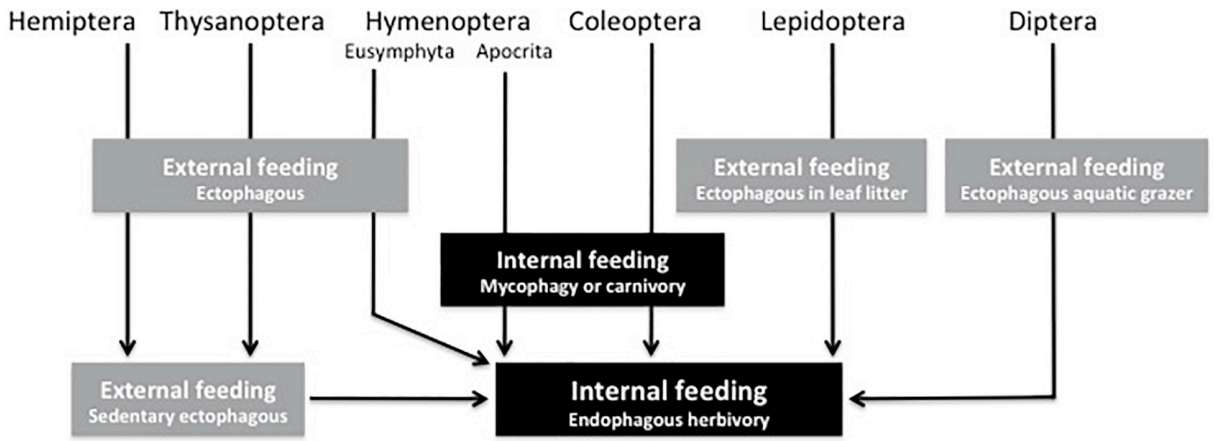

FIGURE 1 | The ancestral feeding modes of six herbivorous insect orders and how they led to endophagy.

TABLE 2 | Endophagous feeding modes that evolved in each herbivorous order.

Taxa	Derived feeding modes					
	Lfld	Lfrl	Lfmn	Bori	Gall	Inqu
Thysanoptera					●	●
Hemiptera					●	●
Hymenoptera Eusymphyta	●	●	●	●	●	
Hymenoptera Apocrita					●	●
Coleoptera			●	●	●	●
Lepidoptera	●	●	●	●	●	●
Diptera			●	●	●	●

Abbreviations for derived feeding mode are: Lfld, leaf folding; Lfrl, leaf rolling; Lfmn, leaf mining; Bori, boring; Gall, galling; Inqu, inquilines.

Aparaglossata) may have had prognathous heads, which would have facilitated burrowing into substrates (Peters et al., 2014). Chewing mouthparts on orthognathus or prognathous heads may have been key innovations in the evolution of endophagy in Hymenoptera, Coleoptera, Lepidoptera and Diptera, allowing them to chew into plant tissue and evolve a diversity of endophytic feeding modes (Labandeira, 1997). Generally, it seems that evolution of mouthparts is key in evolution of feeding habits, allowing transitions from ecto- to endophagy but also the diversification of endophagous feeding modes (Body et al., 2015; Guiguet et al., 2019).

Endophagy of Hymenoptera and Coleoptera may have been initially facilitated by fungi (Sharkey, 2007; Massini et al., 2012), but endophytic feeding in both groups extends well beyond fungus feeding and they contain wide varieties of endophytic feeders (**Table 2**). The evolutionary trajectory of two major clades of Hymenoptera, Eusymphyta and Apocrita, has resulted in different diversities of endophytic species in the two groups (**Table 2**). Coleoptera has a limited range of endophytic taxa compared to Hymenoptera and Lepidoptera, but the abundance of endophagy in Coleoptera is remarkable; larvae of particularly speciose taxa, like Curculionidae and Cerambycidae, are almost exclusively endophytic (Turnbow and Thomas, 2002; Oberprieler et al., 2007). Lepidoptera appears to host the most diverse array of endophytic habits, in part because endophagy developed early in the evolution of the group (Powell et al., 1998). Diptera has evolved a diversity of endophytic habits, and endophagy is particularly important for flies because it is practically the only form of herbivory within the group (Labandeira, 2005).

SELECTION PRESSURES LEADING TO EVOLUTION OF ENDOPHYTIC FEEDING

Most evidence suggests that endophagy has evolved repeatedly in most of the dominant orders of herbivorous insects (see below for details). In some orders (Hemiptera, Thysanoptera), diversification of endophagy has been limited to few modes of feeding and relatively few families contain endophagous members, while in others (Lepidoptera, Coleoptera, Diptera, and Hymenoptera) modes of endophagy are more diverse and there appear to be more abundant taxa that have evolved endophagy (**Table 1**). Obviously, endophagy can be a successful method of feeding on plants even if in some respects it can constrain diversification (Powell et al., 1998). The question we want to address is "why did it evolve so frequently?" In other words, what are the advantages of endophagy, and what active selection pressures could have facilitated its evolution?

Over evolutionary time, insect herbivores have had to overcome several challenges to use plants as food sources. Four primary challenges that have been proposed are attachment (i.e., remaining on plants), desiccation, nourishment, and plant defenses (Strong et al., 1984), all of which could have been selection pressures that encouraged the evolution of endophagy. To this list of challenges that needed to be overcome for herbivores to be successful, we add two more, natural enemies

and competition (Price et al., 1987; Denno et al., 1995); therefore, we will consider a total of six challenges that may have played a role in encouraging insects to feed within plant tissue. Three of these factors (desiccation, nutrition, and natural enemies) have previously been identified as selection pressures that likely contributed to the evolution of galling and leaf mining; these three factors have been formulated into hypotheses known as the microenvironment, nutrition, and natural enemy hypotheses (or something similar; Price et al., 1987; Connor and Taverner, 1997; Stone and Schönrogge, 2003). In addition to being involved in evolution of gall induction and leaf miners, these three selection pressures are also relevant to the broader topic of the evolution of endophagy. We will relate each of these six factors to endophagy, and consider how endophytic feeding may have mitigated some of the challenges of herbivory. Of these factors, we first address attachment, desiccation and natural enemies, all of which deal with mortality external to plants. Next, we address nourishment and plant defenses together because these two intertwined issues relate to food intake. Lastly, we address competition, which appears to have been largely overlooked as a potential factor that could have facilitated endophagy or its diversification.

Attachment

Staying attached to their host plants is a challenge faced by external-feeding herbivores. Plants surfaces can be hairy, spiny, or waxy, making it difficult for herbivores to keep hold of plants. Insects, however, have evolved various adaptations for grasping plants, including abdominal prolegs, crochets, empodia, and various setae (Strong et al., 1984). In contrast, many endophytic insects, particularly borers and gall inducers, face minimal challenges of attachment because the parts of the plants that they attack (e.g., roots, stems, and branches) are usually well integrated into the plant. Moreover, even eggs and immature insects of many endophytic taxa face little risk of falling off the plant because their mothers insert eggs into plant tissue, then upon hatching the insects begin feeding endophytically, with little or no exposure to the external environment (e.g., Whiteman et al., 2011). We do not mean to imply that boring into plants is easier than holding on to the outside of them, but key morphological adaptations and specific traits (e.g., chewing mouthparts, plant-penetrating ovipositor) could have facilitated evolution and diversification of endophagy (Body et al., 2015; Pelaez et al., 2020), decreasing the challenge of attachment.

As evidence that selection pressures can encourage some insects to remain attached to plants, consider leaf miners and leaf gallers. These guilds of insects face the risk of abscission should their host shed leaves prematurely (Williams and Whitham, 1986; Stiling and Simberloff, 1989; Connor and Taverner, 1997). To counteract this risk, some leaf miners have evolved an ability to prevent leaf abscission by modulating phytohormone levels (Zhang et al., 2016), while others can maintain the photosynthetic activity of their host leaves, which could mitigate some effects of premature leaf drop (Giron et al., 2007; Kaiser et al., 2010).

Desiccation/Microenvironment

Desiccation is a general concern for insects, but it is particularly relevant for species that feed externally on plants because they are exposed to wind and solar radiation, which can dry them out quickly. To combat desiccation, insect species have evolved methods to counteract or minimize water loss, including actively drinking water or positioning themselves on parts of plants with the highest humidity (Strong et al., 1984). Other insect taxa have evolved tactics that modify their immediate surroundings by folding or rolling leaves, living within plant tissue (i.e., mining or boring) or creating new tissues to live in (i.e., galling; Strong et al., 1984), but the role of desiccation prevention in evolution of these endophagous traits is not clear. Nevertheless, endophytic insects and their eggs, which are often embedded in plant tissues, are likely to benefit from being encased in water-filled plant tissue that likely protects them from the drying effects of sun and wind. Being surrounded by water-filled tissue would be particularly important for small, immature stages, which are most vulnerable to water loss (Strong et al., 1984). Moreover, endophagous larvae can benefit physiologically from associating with water-rich tissues, which can simultaneously increase O_2 and decrease CO_2 concentrations near larvae, preventing risks of hypoxia or hypercarbia (Pincebourde and Casas, 2016). As mentioned elsewhere in this paper, herbivory in Diptera has evolved almost exclusively in moist, endophytic situations (Dempewolf, 2005), with its taxa likely thriving due to their intimate association with moist tissues or habitats.

The importance of internal feeding for tolerance of desiccation is supported by patterns of galling that show that there are more galls in hotter and drier parts of the world (Price et al., 1987; Fernandes and Price, 1988; Ananthakrishnan, 1992). Gall diversity is also found to be higher in hotter and/or drier environments, like deserts or the upper canopy of Amazonian forests, where leaf temperatures can reach lethal limits (Price et al., 1998; Julião et al., 2014). Similar surveys seem to be lacking for most other endophytic taxa. For leaf miners, some studies have found no association between abundance of leaf-miner species and rainfall, whereas others have found more leaf miners in xeric sites (Sinclair and Hughes, 2010). Experimental evidence, however, indicates that temperatures inside mines are up to 8°C cooler than those on the exposed leaf surface, and can differ from atmospheric temperature by up to 13°C (Pincebourde and Casas, 2006; Pincebourde et al., 2007). Such data suggest that insects in mines would experience lower temperatures, which should relate to lower rates of water loss, but other advantages related to mines preventing desiccation have not emerged (Connor and Taverner, 1997).

Natural Enemies

Feeding inside plant tissues appears to provide some protection from natural enemies simply because, compared to ectophytic species, endophytic insects appear harder to find and access. From an evolutionary perspective, the first insects that found their ways inside plant tissues likely had selective advantages within populations if they suffered less mortality from predators, parasitoids, and pathogens, possibly facilitating evolution of endophagy. Natural enemies have previously been hypothesized as factors that may have selected for endophytic lifestyles (e.g., leaf mining and galling; Price et al., 1987; Connor and Taverner, 1997; Stone and Schönrogge, 2003). While support for these

hypotheses has not been uniform across taxa, endophytic life styles generally appear to be less susceptible to natural-enemy-induced mortality (Cornell and Hawkins, 1995). Analyses of life tables have revealed that some endophytic life stages or groups of insects tend to be attacked less by natural enemies than external feeding species (Cornell and Hawkins, 1995). In particular, eggs of endophytic insects, which tend to be inserted into plant tissues, are killed significantly less often by predators than eggs of ectophytic insect taxa, which tend to be deposited on plant surfaces (Hawkins et al., 1997). (The lower egg mortality rates of endophytic insects may also arise because internal-feeding species tend to lay small and inconspicuous eggs while external feeders often lay eggs in clusters; Connor and Taverner, 1997). Similarly, borers, root feeders, and gallers generally appear to suffer significantly less mortality from predators and pathogens than exophytic species, while also gaining some protection from parasitoids by being hidden inside tissue (Hawkins et al., 1997; see below for exceptions associated with parasitoids). Moreover, at least one group of gall inducers shows strong support for the benefit of endophagy for protection against natural enemies. The mean number of parasitoids attacking nematine sawflies decreased steadily from those that attack external feeders to leaf gallers and finally to shoot gallers, suggesting that more concealed insects suffer less mortality (Price and Pschorn-Walcher, 1988). Compared to external feeders, leaf miners also appear to gain some protection from feeding within plant tissue because they appear to suffer very little mortality from pathogens and significantly less mortality from predators, likely because miners are not usually exposed to the external environment (Connor and Taverner, 1997; Hawkins et al., 1997).

Generally concealed feeders gain protection from natural enemies, but notable exceptions emerge when considering mortality from hymenopteran parasitoids, which tend to have specialized ovipositors that can reach hosts hidden in plant tissues. Compared to leaf rollers, borers, and root feeders, leaf-mining larvae suffer significantly higher mortality from parasitoids (Connor and Taverner, 1997; Hawkins et al., 1997). Moreover, classical biological control programs have been successful against exotic leaf-mining species, indicating parasitoids can severely limit leaf-miner success (Sinclair and Hughes, 2010). Similar to leaf miners, some gall-insect taxa tend to suffer similar mortality from parasitoids as exposed-feeding taxa (Hawkins et al., 1997; Stone and Schönrogge, 2003). This higher mortality of miners and gallers may be driven in part by visual cues associated with most leaf mines and galls, which tend to be obvious (at least to some visual systems), perhaps facilitating their location by parasitoids. Moreover, parasitoids can generally learn to associate rewards with shapes (Wäckers and Lewis, 1999) and some parasitoid species preferentially land on mined leaves (Godfray, 1994). Parasitoids, of course, can also use vibratory and chemical cues to find their hosts. In some endophytic systems, these types of cues can attract parasitoid wasps or help parasitoids localize the host in its hidden microhabitat (Djemai et al., 2001, 2004; Tooker and Hanks, 2006), but in other systems such cues may not be available (Tooker and De Moraes, 2007; Tooker et al., 2008; Hall et al., 2017). Therefore, it may be that cues associated with other endophytic guilds are more challenging for parasitoids to exploit than cues from mines and galls.

Nourishment and Plant Defenses

Endophagous organisms are peculiar for several reasons. First, most display high levels of fidelity to specific organs of particular host-plant species, although a few appear to have some flexibility across related plant species (Hering, 1951; Raman et al., 2005). This evolved selectivity may have allowed insects to consume the optimal food from among the available plant species in their environment. Feeding inside plant tissues also appears to provide some nutritional advantages simply because endophytic insects can avoid highly defended, outer layers of plant tissue and access nutritionally rich inner plant tissues. Many endophagous insects only consume certain tissues or cell types and reject others. This provides them with the unique scenario of consuming high-quality tissues in an otherwise low-quality plant or plant organ, thus aligning their nutritional intakes with their energetic requirements. Specifically, endophagous insects tend to avoid, or encounter lower amounts of, chemical and/or structural plant defenses that tend to concentrate in the cuticle and epidermis (Cornell, 1989). Many leaf-mining species, for example, consume nutrient-rich, internal mesophyll cells and do not eat epidermis and/or vascular tissues (Hering, 1951; Kimmerer and Potter, 1987; Body et al., 2015). Avoiding plant defenses and feeding on the most nutritious layers led to higher feeding efficiencies and higher performance of internal feeders compared to external feeders (Connor and Taverner, 1997; Giron et al., 2016).

Second, some endophagous larvae have also evolved specific morphological adaptations to cope with their confined nutritional niche and optimize their nutrition (Body et al., 2015). Hypermetamorphosis has been described in several lepidopteran leaf-miner species (e.g., Gracillariidae) and can be defined as a strong modification of larval morphology from one instar to the next associated with changes in feeding mode (Snodgrass, 1935). Evolution of this feeding strategy allows larvae to exploit over time different nutritional resources; therefore, early and late larval instars can occupy different feeding niches, providing superior nutrition by partitioning limited feeding resources within a confined nutritional space. Morphological adaptations, along with behavioral strategies, associated with hypermetamorphosis may also allow endophagous insects to avoid triggering plant defenses. Precise larval feeding may circumvent plant defenses that a clumsier feeding style might induce. For example, inconspicuous feeding targeting one or a few cell types (Djemai et al., 2001) may induce limited and/or transient plant defensive responses that have limited effects on herbivores, but this hypothesis still needs to be explicitly tested.

Beyond feeding styles, long-lasting interactions and intimate associations associated with endophagy are likely to have facilitated biochemical and hormonal crosstalk between internal-feeding insects and plants, setting the groundwork for host-plant manipulation by insects. Plant manipulation appears to provide an nutritional advantage because plant-manipulating insects are somehow able to concentrate nutrients and lower plant defenses in their food source, leading to higher insect performance and supporting the nutrition hypothesis for the

adaptive nature of galls (Diamond et al., 2008; Stuart et al., 2012). Moreover, the manipulative ability of some endophagous insects may have facilitated various adaptive radiations of endophagy. By working from within plant tissue, some endophagous insects, particularly gall inducers and some leaf miners, are able to somehow 'reprogram' expression of the plant genome to force production of specialized nutritional resources that benefit the insect at the expense of plant growth and reproduction (Mothes and Engelbrecht, 1961; Giron et al., 2007; Diamond et al., 2008; Saltzmann et al., 2008; Kaiser et al., 2010; Giron et al., 2016). In fact, recent evidence suggests that gall-inducing species might be able to accomplish this reprogramming by synthesizing plant hormones, which alter host-plant physiology, including gene expression and host-plant defenses (Tooker and De Moraes, 2011a,b; Yamaguchi et al., 2012; Giron et al., 2016; Cambier et al., 2019). Conceivably, such manipulative traits may have played a role in adaptive radiations.

Notably, plant manipulation is not only restricted to gall inducers and leaf miners, as commonly assumed, but is shared by other endophagous insects (Stone and Schönrogge, 2003; Gutzwiller et al., 2015; Giron et al., 2016), and perhaps even ectophagous species (Andreas et al., 2020). Because endophagous insects secure their nutrition (and shelter) via their feeding habit, they also must evolve feeding strategies allowing them to meet their energetic and nutrient requirements, face variation in food and nutrient composition, and counteract plant defensive mechanisms. For example, larvae of European corn borer, *Ostrinia nubilalis*, can promote significant protein accumulation and elevated sugar and fatty-acid levels at their feeding site most likely due to effectors secreted by larvae (Dafoe et al., 2013). Contrary to gall-specific nutritive tissues where plant defenses are lowered (Stone and Schönrogge, 2003), stem borers appear to trigger plant-defense responses. However, increased levels of nutrients can override negative effects of plant chemical defenses (Dafoe et al., 2013) or larvae can potentially evolve effective tolerance or detoxification mechanisms against plant-produced defensive compounds. The intimate association between *O. nubilalis* and its host plant, including its nutritional limitations, may have selected for individuals that could alter nutritional resources while circumventing plant defenses.

These cases of endophytic species altering nutritional quality and/or defenses of host plants provide evidence that some insect species have evolved to exploit host-plant species for the nutrition that individuals need even if plants do not typical provide it, or enough of it. Should such an innovation arise, it is easy to imagine that selection would favor the trait, allowing it to spread across populations and perhaps lineages. Plant manipulation for nutritional purposes may thus have played a role in the evolution and diversification of endophagy.

Competition

Competition is a key force that structures plant and animal communities; those individuals that gain competitive advantages for access to resources should succeed and reproduce. Despite some older ecological theory to the contrary (Hairston et al., 1960), competition is common among phytophagous insect species, including some endophytic species (Denno et al., 1995; Kaplan and Denno, 2007). However, little attention has focused on the potential role of competition for selecting for lineages to evolve internal feeding.

At first glance, one may not expect competition to influence internal feeders any differently than other sorts of herbivorous arthropods, but endophytic species, which are somewhat sessile, may be expected to compete even more strongly for their restricted resource than ectophytic species, which can often move to other food sources if they encounter competition (Denno et al., 1995). And a recent study found this to be the case; in particular, endophytic species appear to compete strongly with sap feeders (Bird et al., 2019). Therefore, once a lineage evolved an ability to be surrounded by plant tissue (e.g., boring, galling, mining, etc.), competition with some ectophytic species may have given endophytic species an advantage that may have first allowed the lineage to succeed and then to diversify. Indeed, competition among endophytic species appears to be quite common (Denno et al., 1995), suggesting that selection pressures may force endophytic species to partition resources to minimize competition (Bird et al., 2019). There is evidence of competition between free-living folivores and internal feeders (Denno et al., 1995; Kaplan and Denno, 2007; Bird et al., 2019), perhaps providing a glimpse of competitive interactions that may have encouraged endophagy, but such conclusions would be premature. In some of these interactions, the external feeder appears to have the competitive advantage, whereas in others the internal feeder does, making generalizations difficult (e.g., West, 1985; Fisher et al., 1999; Bird et al., 2019). We are unaware of any ecological evidence that suggests competition encouraged some taxa to adopt endophagy, which is an outcome over evolutionary time that seems plausible. Such scenarios may have to be inferred from phylogenies, but this would be challenging. Based on phylogenetic analyses, competition has been invoked as a factor that may have played a role in the shift from external feeding to internal feeding, including gall induction (Nyman et al., 2006) and in the transition from leaf rolling to gall induction (Guiguet, 2019), but the exact role of competition in these systems may be difficult to clarify.

While evidence for the role of competition in the evolution of endophagy may be scarce, some research supports competition as a force that could have increased the intimacy of interactions that some endophytic species have with their host-plant species. Some endophytic species (leaf-mining and stem-boring species) appear to have evolved an ability to manipulate their host plants to improve the local nutritional environment (Giron et al., 2007; Dafoe et al., 2013). Gall-inducing species, however, have evolved more intimate associations with their host plants and often can manipulate various aspects of plant morphology, chemistry, and physiology to improve their own success (Fay et al., 1993; Nyman and Julkunen-Tiitto, 2000; Stone and Schönrogge, 2003). Some of these manipulations appear to improve protection for gall inhabitants against invaders, whereas others decrease plant chemical defenses and/or improve nutritional quality (Nyman and Julkunen-Tiitto, 2000; Stone and Schönrogge, 2003; Tooker and De Moraes, 2007, 2009, 2011a,b; Tooker et al., 2008). Some evidence indicates that leaf miners and gall inducers can share the same host plant

with other herbivores and avoid competitive exclusion by having different lifestyles. Indeed, even though gall-inducing and leaf-mining insects in early instars can both exploit the same resource, in later instars they can diverge to occupy different ecological niches within the same host plant (Guiguet, 2019), suggesting that niche partitioning to avoid competition may have been a strong evolutionary force leading to either form of endophagy.

Still other manipulations appear to give gall-inducing species advantages in competitive interactions with other herbivorous species. Often phenotypic changes associated with gall induction, such as altered plant physiology or chemistry, can extend beyond the gall to adjacent plant tissue, or may even extend to distant portions of the host plant, with effects that decrease the success of the other herbivorous species, but benefit the gall inducer (Schultz, 1992; Inbar et al., 1995; Foss and Rieske, 2004; Pascual-Alvarado et al., 2008; Prior and Hellmann, 2010; Rostás et al., 2013). For example, development of invasive gall wasp larvae on oaks negatively influenced foliar quality, which reduced performance of a native caterpillar species (Prior and Hellmann, 2010). Remarkably, gall-induced volatiles also can repel browsing mammals (Rostás et al., 2013). There are also examples of gall insects that have little influence on other herbivores on the same plant or even gall insects that facilitate more herbivory by other species (e.g., Fritz and Price, 1990; Nakamura et al., 2003), but the key to competitive advantage may relate to the manipulative capacity of the insect and associated sink strength.

The more resources that gall inducers tend to require from their host plants, the stronger the resource sink that they are likely to induce. Similarly, sink strength can potentially increase with more individuals feeding within a gall, or even more individuals infesting the same tissue. Competitive interactions between nutrient sinks have been largely overlooked. If demonstrated, this would be highly relevant for understanding the adaptive success of some endophytic strategies that can group tens of individuals on a single leaf (e.g., the horse-chestnut leafminer *Cameraria ohridella*) or in a single gall (e.g., gall-inducing social aphids or thrips). It may also shed light on evolution of sociality in endophytic insects as a way to optimize plant-nutrient interception against competition with plant and insect-induced sinks (Larson and Whitham, 1991, 1997). The strength of resource sinks appears to relate to the success of the gall inducer in competitive interactions with other herbivore species (Burstein et al., 1994; Inbar et al., 1995) or with plant sinks (Larson and Whitham, 1997). Further, it is logical then to expect that the stronger the resource needs of any gall-inducing species, the more likely it will have evolved manipulative tactics that give it an advantage in competitive interactions with other species. These tactics could involve altering host-plant chemistry, physiology, or morphology to negatively influence other herbivorous species. We propose that when the influence of gall insects reaches farther from the local vicinity of the gall that competition becomes increasingly relevant as a selective force that can shape the strength and direction of interactions with other herbivorous species. It is likely that revisiting nutrient allocation between various sinks through mass spectrometry imaging (Kaspar et al., 2011), tracing experiments, and manipulating sink strength with transplantation experiments and killing (Guiguet et al., 2018) will provide insight on the role of competition in the ecology and evolution of the endophagous lifestyle.

ENDOPHYTIC TAXA

Now that we have summarized some of the selection pressures that could have encouraged evolution of endophagy, we will consider the variety of endophagous feeding habitats that have evolved in six orders of herbivorous insects. For each taxon, we will then discuss which selection pressures that were likely to have played a role in the evolution and diversification of its endophytic groups. Because of similarities between selection pressures for Thysanoptera and Hemiptera, we discuss them together in one section, but treat the remaining taxa separately.

Thysanoptera and Hemiptera

With their unique sucking mouthparts, thrips are not capable of burrowing into plant tissues to become endophytic (**Figure 1** and **Tables 1**, **2**). Nevertheless, endophagy has evolved multiple times within Thysanoptera, usually via tactics that allow thrips to attach leaves together or trigger plant responses that surround thrips in plant tissue (Mound and Morris, 2005). Ancestral families of thrips appear to be mycophagous, and this feeding habitat appears to be plesiomorphic (Grimaldi and Engel, 2005). Other groups of thrips feed upon flowers or leaves, and endophagy appears to have evolved, possibly multiple times, in each of these three lineages of thrips (Mound and Morris, 2005). Some endophytic thrips species feed within domiciles that they form by gluing together phyllodes, modified petioles that act as leaves, whereas other endophytic species are inquilines in galls induced by other thrips species (Morris et al., 1999).

Most endophytic thrips, however, are gregarious gall inducers, with many individuals contributing to gall induction (Ananthakrishnan, 1992). Lineages that include gall-inducing species also tend to include species whose feeding induces leaf crinkling, rolling, or folding, which are thought to be intermediate endophytic steps on the path to gall induction (Mound and Morris, 2005). Gall-inducing species tend to be monophagous on woody plant species in hot, dry portions of the Old World tropics, including Australia and Indo-Malaysia (Ananthakrishnan, 1992); thus, it seems likely that endophagy, and gall induction in particular, in many thrips species evolved as an adaptation to a persistent resource in challenging environments (Crespi et al., 1997; Grimaldi and Engel, 2005). Some gall-inducing taxa have also evolved advanced forms of sociality, including species that have soldier morphs (Crespi et al., 1997).

Ancestral hemipterans were plant feeders that ingested fluids (Grimaldi and Engel, 2005). Because of their characteristic sucking mouthparts, Hemiptera, similar to Thysanoptera, are unable to bore into plant tissue; thus, to feed endophytically various Hemiptera taxa have evolved feeding tactics that alter the structure of host-plant tissues and encase the feeding insect

(**Figure 1**). Most endophytic Hemiptera tend to be gall-inducing species (**Tables 1**, **2**). Galling is usually considered a derived feeding tactic, but within Hemiptera galling is also an ancient characteristic because it is represented in the most primitive group of psyllids (Burckhardt, 2005). Within some groups (e.g., psyllids and scale insects), the ability to induce galls appears to have evolved separately in multiple lineages (Burckhardt, 2005; Gullan et al., 2005).

Hemipteran gallers induce a diversity of galls, ranging from simple pit galls, to leaf-roll or fold galls to more complex covering galls, which may reflect degrees of evolutionary advancement (**Figure 1**; Yang and Mitter, 1994; Burckhardt, 2005; Gullan et al., 2005). Endophytic hemipterans can also be inquilines (Miller, 2005). Most species tend to be host-plant specific, often monophagous, but some gall-inducing scales insects are oligophagous or even polyphagous, though complex galls appear to be induced by species with more restricted host ranges (Gullan et al., 2005). In some cases oviposition can initiate galls, but typically nymph hemipterans induce galls and continued gall growth tends to require continuing nymphal feeding (Burckhardt, 2005). Endophytic feeding is uncommon in most hemipteran taxa. In aphids (Aphididae), for example, less than 10% of species are confirmed gall inducers (Wool, 2005).

As indicated above, Thysanoptera and Hemiptera have limited forms of endophagy. Most herbivorous species in these orders are external feeders and endophagy, has evolved a limited number of times, mostly as gall induction (**Table 1**). Of the three hypotheses that have been proposed to explain the adaptive significance of gall induction, the nutrition hypothesis has some of the strongest support for these two taxa, particularly for aphid and thrips galls (Stone and Schönrogge, 2003). For example, compared to ungalled leaves, aphid galls can provide increased concentrations of essential amino acids, which improve aphid performance (Koyama et al., 2004). Other hemipteran galls provide such a high quality diet that the gall-inducing inhabitants do not require bacterial endosymbionts, which help most hemipteran species process ingested food to satisfy dietary needs (Overholt et al., 2015). Similarly, in Australian thrips galls, galls with many internal folds have convergently evolved to provide superior nutrients to "hyperfecund" foundresses, suggesting that nutritional-based selection pressures have encouraged induction of resources that satisfy the nutrient needs of thrips females capable of producing high numbers of progeny (Crespi and Worobey, 1998).

Despite evidence supporting the importance of nutrition in evolution of gall induction for Thysanoptera and Hemiptera, other forces are likely to also have been at play. As mentioned above, the many thrips galls found in arid portions of Australia and Indo-Malaysia support the microenvironment hypothesis, suggesting that living inside plants may have decreased water stress that could prevent exterior feeders from thriving in hot dry environments (Ananthakrishnan, 1992; Crespi et al., 1997; Grimaldi and Engel, 2005). And for at least for some aphid species, competition may have contributed to evolution of the manipulative control that some galling aphids have over their host plant species. Galling aphids reap benefits of inducing strong nutrient sinks because they can extract the resources they need from their host plants while simultaneously depriving competing herbivores of resources they need (Burstein et al., 1994; Inbar et al., 1995).

Hymenoptera

Endophagy has been key to the evolution of groups within Hymenoptera, but it is unclear whether the earliest hymenopterans were endophagous or ectophagous herbivores (**Figure 2**; Sharkey, 2007; Peters et al., 2017). If they were ectophagous, among their first food sources could have been sporophylls of gymnosperms, as eaten by the extant family Xylidae (Grimaldi and Engel, 2005). Relatives of these early external feeders appear to have led to a radiation of ectophagous sawflies (i.e., Eusymphyta; **Figures 1**, **2**), which includes the superfamilies Pamphilioidea and Tenthredinoidea (Peters et al., 2017). These groups include taxa that secondarily evolved a range of endophytic habits, including leaf miners, folders, and rollers and gall inducers (**Table 2**; Price, 1992; Connor and Taverner, 1997; Nyman et al., 1998). In particular, the family Tenthridinidae includes lineages that appear to have followed an evolutionary path from ectophagous leaf feeders to endophagous leaf folders and then gall inducers (Nyman et al., 1998, 2000). Within sawflies, the ability to induce a gall appears to have evolved independently six to ten times (Roininen et al., 2005). Some other early phytophagous hymenopterans (Xiphydriidae and Siricidae) also evolved endophagy as borers in dying or dead trees (**Figure 2**), and their lifestyle was facilitated by symbiotic fungi, which digest wood providing the wasp larvae more nutritious diets (Hanson, 1995; Solomon, 1995; Sharkey, 2007).

Despite some evidence that early hymenopterans were ectophagous, recent analyses raise the possibility that the common ancestor of symphytans and the remaining Hymenopterans (Eusymphyta + Unicalcarida) may have been an endophytic herbivore (Peters et al., 2017). Notably, once it evolved, the endophagous habit may have contributed to the diversification of the huge suborder of Apocrita (**Figures 1**, **2**); endophytic taxa gave rise to carnivorous species that attacked other wood-boring Hymenoptera and Coleoptera, setting the path toward evolution of parasitoidism, which may have contributed to the success of Apocrita (Grimaldi and Engel, 2005; Sharkey, 2007).

Apocrita are largely carnivorous, but many apocritans have reverted to herbivory. None of these secondarily herbivorous taxa are external leaf feeders; they are all endophytic, feeding on nutritious plant tissues such as seeds, pollen, or gall tissue (including gallers and inquilines) or fungal tissue inside galls (**Tables 1**, **2**; Hanson, 1995; La Salle, 2005; Wharton and Hanson, 2005). Some of these reversions to herbivory may have occurred via an intermediate step of entomophytophagous feeding, in which parasitoid species begin development by feeding upon their arthropod host and finish development by feeding upon plant tissue (La Salle, 2005). The next evolutionary step, of course, would be wasp species that feed only upon plant tissue. Other hymenopteran endophagous taxa appear to have evolved directly from phytophagous predecessors (Roskam, 1992; La Salle, 2005). Regardless of the path (**Table 2**), it is clear that endophagy, and more specifically gall induction, evolved many times in various

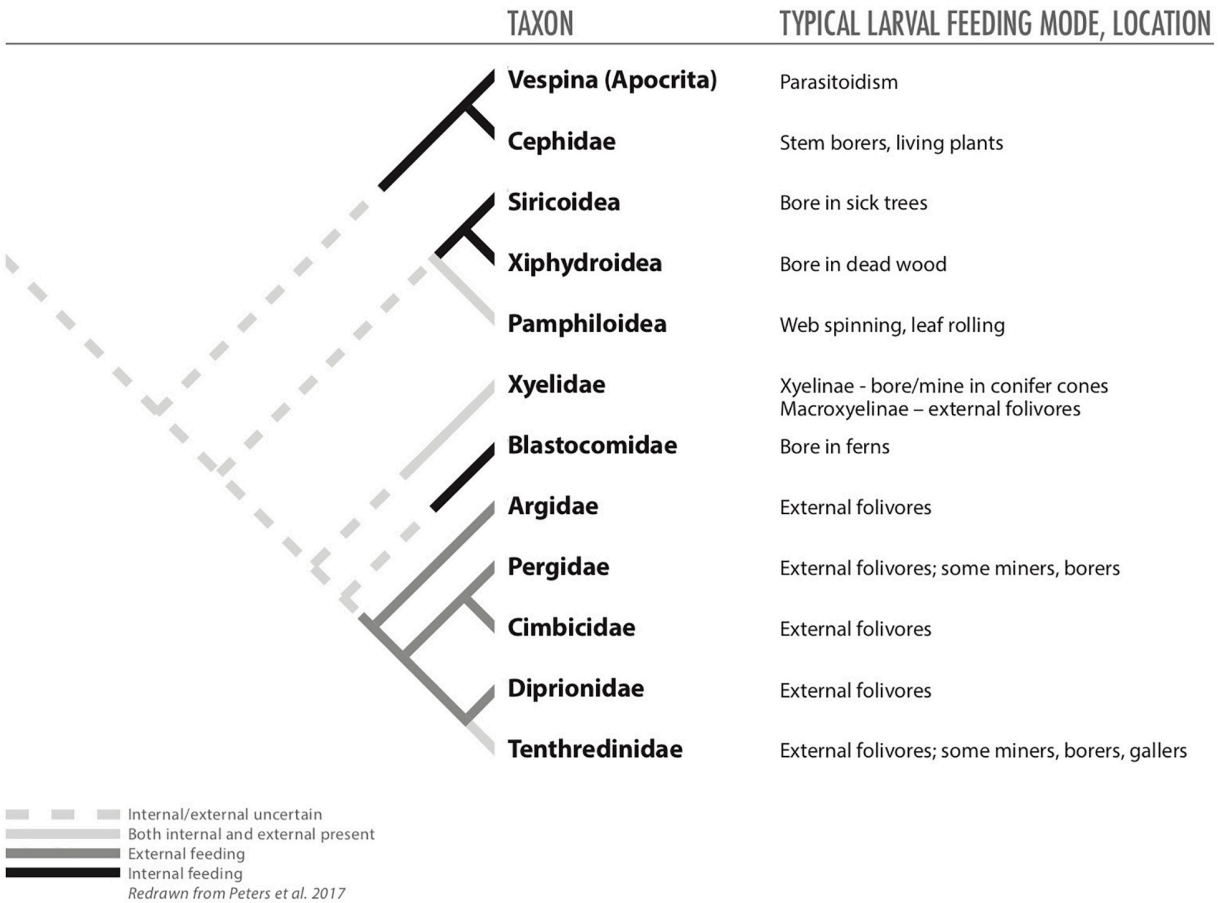

FIGURE 2 | Phylogeny of basal Hymenoptera (from Peters et al., 2017) redrawn to illustrate hypothesized distributions of internal and external feeding among taxa.

apocritan taxa (Hanson, 1995; La Salle, 2005; Wharton and Hanson, 2005).

For Hymenoptera, ancestral species may have been endophytic, but this detail is unclear (Peters et al., 2017). Nevertheless, an endophytic lifestyle was established early in the evolution of Hymenoptera (Grimaldi and Engel, 2005). This internal feeding habit (i.e., boring in decaying wood) seems likely to have evolved because of nutritional benefits that could have been facilitated initially by symbiotic fungi, and later other types of symbiotic microbes, which provided access to previously inaccessible food resources (Hanson, 1995; Solomon, 1995; Sharkey, 2007). Moreover, the endophytic habit also appears to have been key for the evolution of parasitoidism, and for some endophytic Apocrita, like Agaonidae and Cynipidae (La Salle, 2005). Considering the closest non-herbivorous relatives of these taxa may provide insight on selective forces that led to their reversion to endophytic herbivory. Predecessors of both these endophagous groups (and others like Tanaostigmatidae) appear to be parasitoids (Peters et al., 2017); thus, prior to feeding upon plant tissue their relatives were already spending much of their lives inside hosts, which were embedded within plant tissues. As mentioned above, the switch to herbivory, therefore, could have been facilitated by entomophytophagous feeding, with fully herbivorous species evolving in a later step (La Salle, 2005). Because these groups are largely gall inducers, they likely later evolved their ability to manipulate their hosts to produce galls that provided even better nutritional resources (for agaonids, the enlarged endosperm of their galls; for cynipids, the nutritive tissues lining their galls; Weiblen, 2002; Csóka et al., 2005).

Of course other non-nutritional factors are likely to have been in play during evolution of endophagy in Hymenoptera. Some sawflies (e.g., Tenthredinoidea) appear to have followed a path in which ectophytic feeding appears to be ancestral and various forms of endophagy evolved later (Nyman et al., 2006). All the selection pressures involved in these transitions are not clear, but the natural-enemy hypothesis helps explain patterns of mortality documented within a gradation of external- to internal-feeding sawflies (Price and Pschorn-Walcher, 1988). Other studies with endophagous hymenopterans have found support for the microenvironment hypothesis (Miller et al., 2009), and benefits of endophagy for competition (Foss and Rieske, 2004).

Coleoptera

Within Coleoptera, endophytic feeding achieves a diversity that exceeds that of Thysanoptera, Hemiptera, and Hymenoptera. Coleopterans can be borers, miners, gallers, and inquilines,

and these endophytic species are most evident in the large superfamilies Chrysomeloidea and Curculionoidea (**Tables 1**, **2**). This diversity of habits and abundance of species may be attributable in part to evolution of larvae with prognathous heads and chewing mouthparts, which would have allowed them to eat their way into plant tissue (Labandeira, 1997).

In beetles, the endophytic habit appears to be derived from the ancestral state of boring in wood or other decaying tissues. Archostemata, one of the most basal suborders of Coleoptera, comprise families of specialized wood borers (**Figures 1**, **3**; Grimaldi and Engel, 2005; McKenna et al., 2019). Larvae from early Permian beetles appear to have been associated with wood, similar to the extant families Ommatidae and Cupedidae, which are within Archostemata (Young, 2001; McKenna et al., 2019). The earliest fossil records of coleopteran wood boring are from the mid- to upper Permian (∼250 million years ago) in fungus-decayed wood, indicating that wood boring evolved early within Coleoptera (Feng et al., 2017; McKenna et al., 2019). Diversification of wood boring in beetles appears to have been facilitated by cellulolytic fungi that decomposed wood and ancestral beetle larvae appear to have fed upon the fungi, similar to modern ambrosia beetles, which independently converged on mycophagy (Massini et al., 2012; Feng et al., 2017; Hulcr and Stelinski, 2017). Saprophytic fungi, therefore, may have facilitated the transition of ancient beetles, or their predecessors, from feeding upon saprophytic fungi in leaf litter to borers feeding on similar fungi in decaying wood (Farrell, 1998; Grimaldi and Engel, 2005; Feng et al., 2017). Eventually endophytic beetle taxa evolved capacities to feed directly on wood and other tissues of living trees with its digestion facilitated by symbionts (**Figure 3**; Martin, 1991; Feng et al., 2017; Lieutier et al., 2017).

Recent evidence suggests that plant cell wall-degrading enzymes (PCWDE), which were acquired via horizontal gene transfer from bacteria and fungi that originated in detritus or insect guts, were a key innovation that facilitated success of beetles, particularly lineages whose larvae feed endophytically (McKenna et al., 2019). In Curculionoidea and Chrysomeloidea, for example, endophagy, apparently facilitated by PCWDE, may have been a key innovation that drove their diversification, allowing them to radiate inside a diversity of plant tissue and occupy novel niches (Farrell and Sequeira, 2004; Oberprieler et al., 2007; McKenna et al., 2019). Moreover, the abundance of endophagous species within these and other taxa may be explained in part by constraints imposed by morphological and behavioral traits associated with endophytic feeding; these traits may limit switches to other types of plant tissue, canalizing evolutionary trajectories (Farrell and Sequeira, 2004).

As larvae, the majority of endophytic Coleoptera taxa are associated with decaying, dying, or healthy plants, feeding within virtually all tissues (**Table 1**). Some taxa bore largely in herbaceous stems (e.g., Mordellidae; Jackman and Lu, 2002) or are specialized seed feeders (Chrysomelidae: Bruchinae; Kingsolver, 2002). Comparatively few major coleopteran taxa have evolved leaf-mining or gall-inducing habits (**Table 1**), which are often considered more derived endophytic feeding habits (Hering, 1951; Korotyaev et al., 2005). Among chrysomelids, however, seed boring by bruchine beetles appears to be the youngest or most derived endophagous habit, and seems to have evolved in a progression from stem feeding to gall inducing to seed boring (Farrell and Sequeira, 2004).

Because the diets of ancestral Coleoptera taxa may have been facilitated by fungi, microbial symbionts, or PCWDE (Martin, 1991; Farrell, 1998; Grimaldi and Engel, 2005; Feng et al., 2017; McKenna et al., 2019), the paths to endophagy may have been driven by nutritional selection pressures. The more derived taxa of Chrysomeloidea and Curculionoidea maintain this feeding habitat, which appear to be at least partly responsible for their success and diversification (Marvaldi et al., 2002). Beyond nutritional selection pressures, endophytic coleopteran populations must benefit from lower mortality from natural enemies associated with internal feeding (Hawkins et al., 1997) and likely gain advantages from being buffered from heat or moisture stress by being hidden within plant tissue, but we are unaware of explicit tests of these sorts of hypotheses with beetles.

Lepidoptera

Lepidoptera represents the largest diversification of herbivorous insects, and perhaps not surprisingly, also contains the highest diversity of endophagous habits, including many types of borers, concealed leaf feeders, leaf miners, gall inducers and inquilines (**Table 2**; Powell et al., 1998). Endophagy arose early in the evolution of Lepidoptera and may have fostered their subsequent radiation (**Figure 4**; Powell et al., 1998; Menken et al., 2010). Larvae of Micropterigidae appear to have fed on decaying tissue or live plants on the forest floor, but other basal lepidopterans adopted endophagy early in the evolution of Lepidoptera (**Figures 1**, **4**; Regier et al., 2015). For example, larvae of Agathiphagidae are seed borers in pines of Araucariaceae, while larvae of Heterobathmiidae and Eriocraniidae mine leaves of tree species of Fagales (Kristensen et al., 2007; Regier et al., 2015).

Endophagy, therefore, was an early innovation in Lepidoptera that influenced the feeding habits of many non-ditrysian lineages (Regier et al., 2015). The endophagous habit further diversified onto angiosperms when they became available (Wiegmann et al., 2000; Menken et al., 2010) and specialized internal feeders begat larger insect taxa that fed as concealed external feeders (e.g., leaf rolling or similar), followed by radiations of fully exposed external feeders that achieved even larger sizes (Regier et al., 2013). In fact, the transition in Lepidoptera from endophagy to ectophagy may have been an "adaptive escape" from negative consequences of internal feeding, such as limits on body size, number of generations per year, access to alternative hosts, and leaf abscission (Powell et al., 1998). Notably, some extant taxa provide evidence of apparently "transitional" traits that combine endophytic and ectophytic habits. For example, some species of Adelidae and Incurvariidae feed internally in seeds and then switch to external feeding on fallen leaves (Powell et al., 1998). Species in the genus *Buccalatrix* (Bucculatricidae) move from leaf miners to external leaf feeders, while some gracilariids combine two different endophytic habits (*Caloptilia*, *Parornix*), feeding as miners for the first few instars and then become leaf folders (Hering, 1951; Nakadai and Kawakita, 2016). Other gracilariids first feed as leaf miners and then become gall inducers, and this

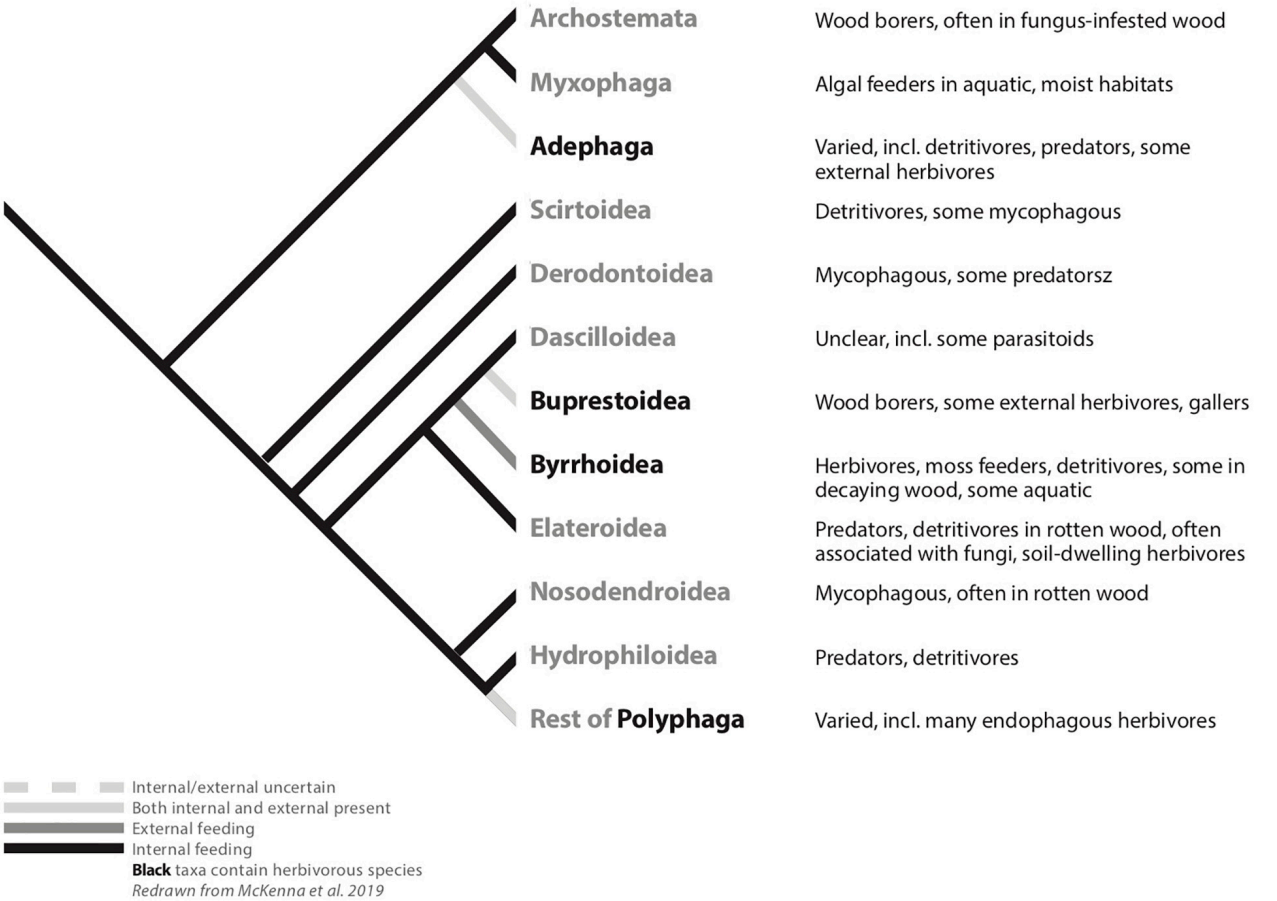

FIGURE 3 | Phylogeny of basal Coleoptera (from McKenna et al., 2019) redrawn to illustrate hypothesized distributions of internal and external feeding among taxa.

transition involves hypermetamorphosis of mouthparts (Guiguet et al., 2018, 2019; Guiguet, 2019).

Because endophagy appears to have evolved early among lepidopterans, many taxa have had long associations with their host-plant taxa, allowing them to become specialized internal feeders. Indeed, some lepidopteran families, such as Nepticulidae, Gracillariidae, Cosmopterigidae, and Sesiidae, among others, are dominated by internal feeders (Powell et al., 1998). The long associations that many endophytic taxa have had with their host-plant species appears likely to contribute to most groups evolving some species capable of inducing galls (**Table 1**), which is considered a derived trait (Miller, 2005).

For Lepidoptera, the adoption of endophagy early in their evolution led to the large majority of non-ditrysian lineages taxa feeding inside plant tissue (Regier et al., 2015). Thus, nutrition, and perhaps exploiting empty feeding niches, may have been a primary factor in the success of early taxa. Moreover, recent evidence has demonstrated the high quality of endophagous tissue eaten by caterpillars, suggesting that internal feeding can give herbivores access to better sources of food (Diamond et al., 2008; Tooker and De Moraes, 2009; Giron et al., 2016). Nevertheless, these taxa likely gained other benefits from being inside plant tissues. Lepidopteran leaf miners appear to gain some protection from pathogens and predators by being hidden within plant tissue, but seem just as susceptible to parasitoid wasps as external feeders, perhaps discounting the value of the natural-enemies hypothesis for explaining the success of leaf mining within Lepidoptera (Connor and Taverner, 1997). We are not familiar with explicit tests of some of the other selection pressures that we have considered.

Diptera

Unlike its role in the evolution of the three other large groups of holometabolous insects (i.e., Hymenoptera, Coleoptera, and Lepidoptera), herbivory appears to have played a smaller role driving the basal patterns of evolution of Diptera (Grimaldi and Engel, 2005; Bertone et al., 2008; Wiegmann et al., 2011). The larvae of the most basal fly families are aquatic grazers, as it seems were ancestral dipterans with many species feeding upon algae (**Figures 1**, **5**; Courtney, 1990; Wiegmann et al., 2011). Slightly more derived taxa are semi-aquatic and saprophagous

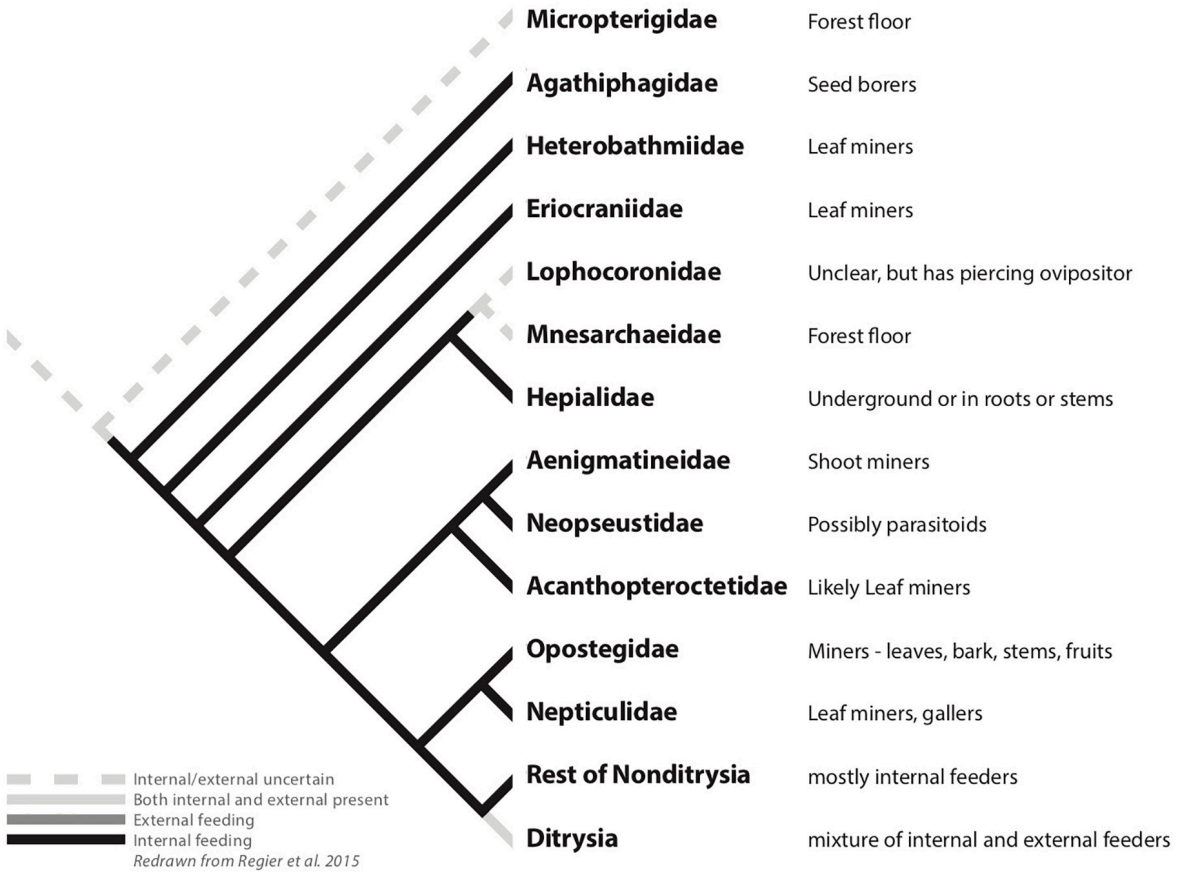

FIGURE 4 | Phylogeny of basal Lepidoptera (from Regier et al., 2015) redrawn to illustrate hypothesized distributions of internal and external feeding among taxa.

(or even bacteriophagous) or mycophagous (**Figure 5**; Courtney, 1990; Dempewolf, 2005; Grimaldi and Engel, 2005; Wiegmann et al., 2011).

Nevertheless, herbivory in Diptera evolved at least 26 times, likely more than in any other order (Mitter et al., 1988), and is a dominant, derived feeding strategy within the order (Wiegmann et al., 2011). Remarkably, there are very few records of ectophagous herbivores within Diptera (possibly only in Tipulidae); therefore, plant feeding within flies appears to be synonymous with endophagy, with taxa that include borers (including seed feeders), miners, gallers, and inquilines (Labandeira, 2005; **Table 1**). These endophytic habits appear to have facilitated colonization of nutritional food sources, allowing fly larvae to remain in moist environments (i.e., avoid desiccation), eating liquid, or near liquid, diets, and allowing them to access nutrients despite having mouthparts poorly suited to chewing (Dempewolf, 2005). For some taxa, large radiations occurred across pteridophytes, gymnosperms, and angiosperms, resulting in ecologically and economically important groups, like Agromyzidae and Cecidomyiidae, which are dominated by mining and gall-inducing species, respectively, and are the most speciose taxa of endophytic Diptera (Labandeira, 2005). Beyond miners and gallers, Diptera contains relatively few borers, but they occur in all major clades of the order and appear to represent opportunistic exploitation of niches rather than an evolutionary radiation (Labandeira, 2005).

Dipteran miners are particularly notable for being a diverse guild that is well distributed taxonomically across the order from lower nemotocern dipterans (e.g., Culicomorpha: Chironomidae, Ceratopogonidae) to higher cyclorrhaphous flies (Muscoidea: Scathophagidae, Anthomyiidae; Labandeira, 2005). Mining appears to have evolved independently at least 25 times (Labandeira, 2005). Mining may even have been the initial entry into herbivory for Diptera (Dempewolf, 2005), but the fossil record appears unclear on this point because galling by flies is currently known from older deposits than mining by flies (Labandeira, 2005). Moreover, in some cases, mining appears to have been a predecessor to galling, but thus far there is limited evidence for this path to galling within Diptera (Dempewolf, 2005).

Diptera are also notable for containing one of the most unusual endophytic taxa, the Fergusoninidae. On their myrtaceous host plant species, these acalypterate flies have

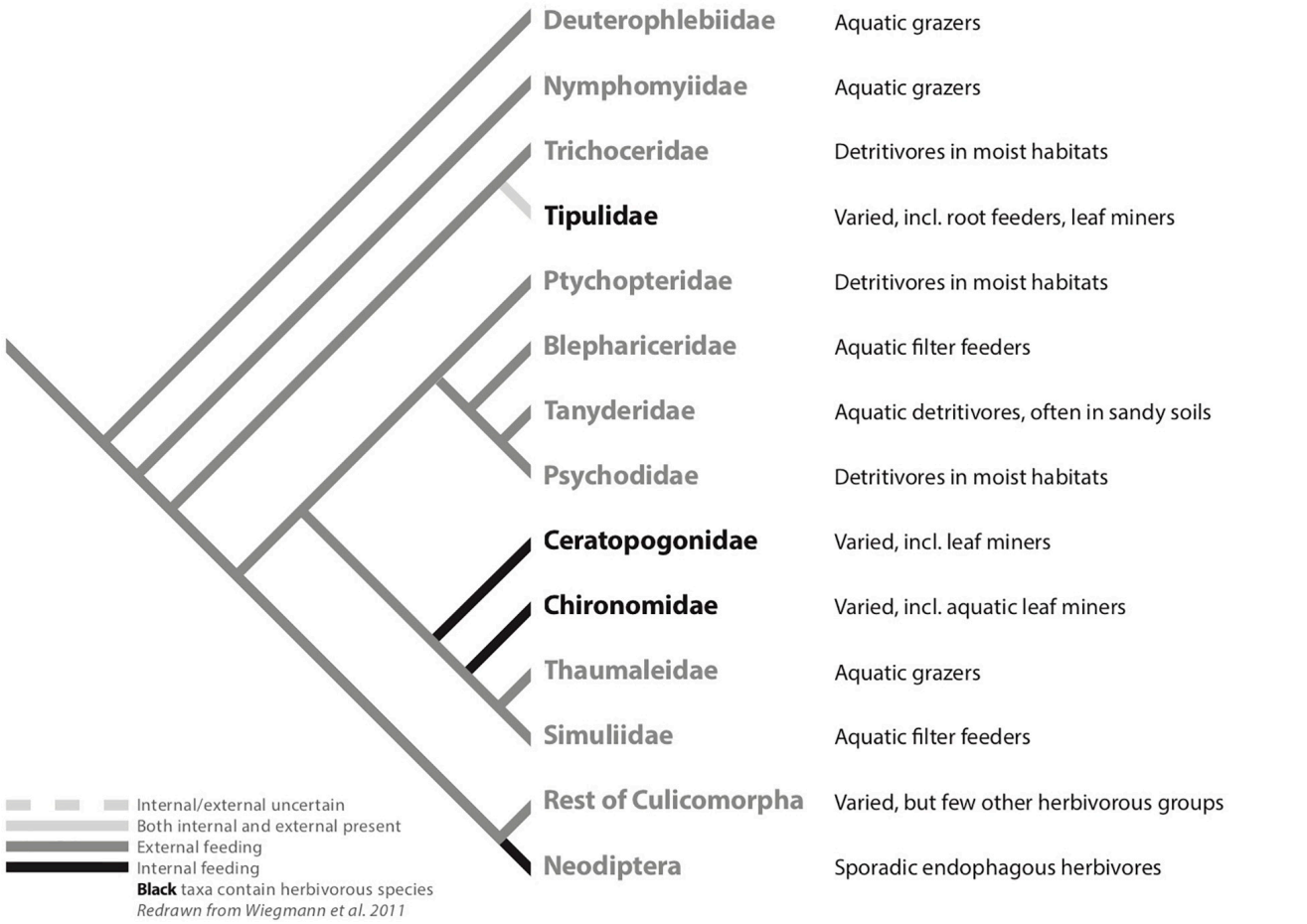

FIGURE 5 | Phylogeny of basal Diptera (from Wiegmann et al., 2011) redrawn to illustrate hypothesized distributions of internal and external feeding among taxa.

evolved complex, co-evolved lifecycles with mutualistic nematodes, which are deposited into meristematic tissue along with fly eggs and induce the galls in which maggots develop (Taylor et al., 2005). The maggot and nematodes feed together on plant tissue within the gall, then the worms enter female maggots where they become parasitic, eventually colonizing fly oviducts so they can be oviposited with the fly egg (Taylor et al., 2005). This mutualistic interaction is similar to relationships that other fly taxa (e.g., Cecidomyiidae) have with symbiotic fungi, which in some cases induce galls and then are feed upon by immature flies. In other cases, fungi occur in galls but only provide protection and do not appear to induce the gall or provide food (Gagné, 1989). Notably, plant feeding in cecidomyiids may have initially evolved from mycophagous ancestors (Roskam, 1992).

For Diptera, ancestral larval flies, and likely their progenitors, were aquatic grazers, and larvae of lower flies have remained faithful to aquatic or semi-aquatic habitats (Bertone et al., 2008). Even taxa that are largely saprophagous feed within liquid, or at least moist, habitats (e.g., decaying plant material in temporary pools, rotten wood; Bertone et al., 2008). Significantly, for each of the 26 times that herbivory has evolved within Diptera, the larval habitat has been endophagous; therefore, even among derived herbivorous fly taxa, species appear to be tied to moist environments inside plants. For hypothesizing which selection pressures played prominent roles in evolution of endophagy among Diptera, a parsimonious evolutionary explanation could be based on moist microenvironments (i.e., the microenvironment hypothesis), but a nutrition-based explanation could be just as likely because larval diets of flies are liquid, semi-liquid, or moist, as necessitated by the morphology of larval mouthparts (Labandeira, 2005). Importantly, once herbivory arose in dipteran taxa, how larvae fed upon plants and the nutrients they gained appears to have translated well to other tissues on the same plant or tissue of nearby plants, whether plant taxa were closely related or not, accounting in part for some of the species-level diversity in some fly taxa (Labandeira, 2005). Selection pressures associated with natural enemies seem less important because, as mentioned previously leaf-mining flies suffer high

mortality from parasitoids wasps (Connor and Taverner, 1997), and gall flies do not necessarily gain more protection from larger galls (Waring and Price, 1989; Rossi et al., 1992; Abrahamson and Weis, 1997).

Conclusion

After having considered endophagy in a much broader range of taxa than has been considered previously, we hypothesize that nutritional selection pressures played a primary role in the evolution of endophagy across orders of herbivorous insects. Given the general importance of nutritional resources to the success of animals, this hypothesis may not be surprising, but recurring support for it across orders is notable, as is the lack of consistent evidence supporting the other possible selection pressures. We must note, however, that nutritional hypotheses may just have received more attention in the literature rather than being more important for endophagy than the other factors we considered; further testing of the other explanatory hypotheses for the evolution of endophagy may reveal other patterns.

Because of its strong association with access to nutritional resources, competition imposed by the sedentary lifestyle of endophytic insects emerged from our analysis as a possible selective force in evolution of endophagy, and subsequent diversification and niche partitioning. This detail is noteworthy because we are unaware of previous consideration of competition as a selection pressure that encouraged endophagy in any form.

If nutritional selection pressures tend to be primary, then it seems reasonable to hypothesize that benefits associated with the other factors (e.g., microenvironment, attachment, natural enemies, and competition) would tend to be secondary, providing stronger or weaker advantages for certain insect taxa under some conditions. For example, under challenging environmental conditions it seems likely that endophagy is likely to provide benefits for water conservation. As mentioned above, galling tends to be more common in drier or hotter environments (Price et al., 1998), but similar analyses appear to be lacking for most other endophytic taxa. It would seem profitable, therefore, for future research to explore global patterns of endophagy to gain insight on the potential role of endophagy to limit heat and water stress. Testing these newly proposed hypotheses directly seems challenging, so it may be more feasible to test them indirectly in phylogenetic contexts, perhaps by characterizing water budgets in a range of taxa and feeding styles.

If a nutrition hypothesis best explains why so many insect taxa feed endophagously, it aligns well with the evidence available to explain the adaptive significance of more specialized forms of endophagy (Connor and Taverner, 1997; Stone and Schönrogge, 2003). As mentioned above, three hypotheses, nutrition, microenvironment, and natural enemies, have been proposed to explain the adaptive significance of leaf mining and insect galls. For leaf mining, it seems that the nutrition and microenvironment hypotheses best explain the advantages derived from mining (Connor and Taverner, 1997). However, as discussed above, analyses of Diptera revealed the dominance of endophagy across phytophagous groups, revealing that fly larvae are almost always associated with moist food sources, which aligns well with the capacity of their mouthparts (Dempewolf, 2005; Labandeira, 2005). These results appear to give more support to the nutritional hypothesis for helping to explain the adaptive significance of leaf mining, but we cannot overlook the potential interaction with microenvironment because fly larvae undoubtedly benefit from being surrounded by water filled tissue, and as a result may have been poorly adapted for external feeding. For insect gallers, the majority of the evidence also appears to support the role of nutrition and microenvironments for evolution of galling, and perhaps natural enemies have played a role in the morphological diversification of gall shapes and external features (Stone and Schönrogge, 2003). Given the nutritional support of endophagy provided by our review, we could also hypothesize that nutrition is the primary adaptive significance of galling and mining, and the other benefits are secondary, but further research will have to explore this sort of ranking.

As mentioned above, there is a tendency in ecological literature to believe that the progression of feeding habits in herbivorous insects started with external feeding and moved toward internal feeding. This belief is based on well-known theoretical and experimental work with sawflies (Price et al., 1987; Price and Pschorn-Walcher, 1988), but it may be the exception. Our review revealed that the evolutionary story is far more complicated, and varies by taxa (**Table 2** and **Figures 1–5**). For some taxa, endophagy is an ancestral trait that has been around for hundreds of millions of years. For others, endophagy evolved more recently. The evidence we reviewed appears to indicate that nutritional benefits could underlie much of the evolution and diversification of endophagy across the orders of herbivorous insects. It is our hope that other researchers will now bring various research techniques to bear on these hypotheses to help clarify the evolutionary selection pressures involved in evolution of internal feeding.

AUTHOR CONTRIBUTIONS

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

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