ADVANCES IN PLANT-HEMIPTERAN INTERACTIONS

EDITED BY: Jyoti Shah and Linda Walling

PUBLISHED IN: Frontiers in Plant Science and Frontiers in Microbiology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-321-4
DOI 10.3389/978-2-88945-321-4

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ADVANCES IN PLANT-HEMIPTERAN INTERACTIONS

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Green peach aphid on *Arabidopsis thaliana.*

Image: Dr. Vamsi Nalam and Dr. Sujon Sarowar.

Hemipterans encompass a large group of insect pests of plants that utilize mouthparts which are modified for piercing and consuming fluids from plants. In addition, hemipterans vector viral and bacterial diseases of plants. This book brings together a set of reviews and research papers that showcase the the range of activities being undertaken to advance our understanding of the multi-organismal interaction between plants, hemipterans and microbes.

Citation: Shah, J., Walling, L., eds. (2017). Advances in Plant-Hemipteran Interactions. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-321-4

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frontiers in Plant Science

EDITORIAL
published: 21 September 2017
doi: 10.3389/fpls.2017.01652

Editorial: Advances in Plant-Hemipteran Interactions

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Keywords: effectors, piercing-sucking insects, resistance genes, saliva, small RNA, organismal interactions, plant defense, insect vectors

Editorial on the Research Topic

Advances in Plant-Hemipteran Interactions

HEMIPTERANS

Hemipterans (e.g., aphids, whiteflies, stinkbugs, leafhoppers, and planthoppers) encompass a large group of insects with mouthparts specially modified for piercing and consuming fluids from the host (Capinera, 2008). Many hemipterans are important pests of plants and vector viral and bacterial diseases. Plant defenses against hemipterans include mechanisms that physically hinder insect feeding, as well as mechanisms that interfere with insect physiology and behavior (Painter, 1951; Kogan and Ortman, 1978; Smith, 2005). In some cases plants can alter their physiology to tolerate infestation without any detrimental effect on growth and development. Endosymbionts and phytopathogens present in the hemiptera impose an additional layer of organismal complexity to plant-hemipteran interactions. Considering the multiple organismal interactions involved, plant-hemipteran interaction studies have been conducted at different levels. This Research Topic brings together 16 manuscripts, which include a blend of reviews and research papers that address the physiology and molecular biology of plant-hemipteran interactions at these different levels.

PLANT-HEMIPTERAN INTERACTIONS: WHAT DETERMINES RESISTANCE VERSUS SUSCEPTIBILITY?

Host-plant resistance is a heritable trait that has been employed in breeding programs to control diseases and insect infestation. Recognition of the pest is the first step in engaging the downstream defense machinery, which in many cases involves plant hormones. In plant-pathogen interaction the involvement of *Resistance* (*R*) genes in recognition of pest-derived factors or factors produced in response to infection is well known. Several resistance (*R*) genes conferring resistance to pathogens have been cloned. However, very few *R* genes conferring resistance against hemipterans have been identified. *Vat* (virus aphid transmission), which confers resistance to cotton-melon aphid (*Aphis gossypii*) in melon, *Mi-1* which confers resistance against potato aphid (*Macrosiphum euphorbiae*) in tomato, and rice *Bph14* and *Bph26* that confer resistance to brown planthopper (*Nilaparvata lugens*) are a few that have been described. Some of these genes are unique in that they confer resistance against more than just hemipterans. For example, *Mi-1* confers resistance against potato aphid, root-knot nematode (*Meloidogyne incognita*) and whitefly (*Bemisia tabaci*) (Milligan et al., 1998; Rossi et al., 1998; Vos et al., 1998; Nombela et al., 2003) and *Vat* confers resistance against *A. gossypii*, as well as cucumber mosaic virus transmitted by *A. gossypii*, but not by other vectors (Dogimont et al., 2014). Boissot et al. review the history of the discovery of the *Vat* locus, its effect and durability against aphids, and *Vat*-conferred resistance against viruses.

OPEN ACCESS

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Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *25 August 2017*
Accepted: *08 September 2017*
Published: *21 September 2017*

Citation:
Shah J and Walling L (2017) Editorial: Advances in Plant-Hemipteran Interactions. Front. Plant Sci. 8:1652. doi: 10.3389/fpls.2017.01652

A complex relationship between hormones, both cooperative as well as antagonisitic interactions, further fine-tunes defenses. However, some pests have evolved to exploit these interactions between plant hormones for their benefit to facilitate infestation. Sanchez et al. studied the relationship of hormone signaling in host specialization by pea aphid (*Acyrthosiphon pisum*, a legume specialist). They show that pea aphids perform better on their native hosts due to their ability to manipulate to their advantage the host's defense hormone pathways, in particular salicylate and jasmonate signaling.

Ji et al. have taken a genomic approach to address the question of host specialization. They compared the transcriptome of young and adult green peach aphid to that of pea aphid and found substantial changes in expression of genes involved in the metabolism and detoxification of xenobiotics between the two aphids, thus leading the authors to suggest that the ability to adapt to secondary metabolites may contribute to the host-plant adaptation by these two aphids.

Although resistance is largely viewed as the process by which plant mechanisms adversely impact pest behavior, growth, fecundity and survival, plants also have the ability to tolerate insect infestation. Unlike the classical defenses, tolerance does not adversely impact the pest. Rather, tolerance involves physiological changes in the host that alleviate the adverse impacts of herbivory on plant fitness. Koch et al. discuss the compensatory changes in plant physiology that likely contribute to tolerance, including alterations in photosynthetic rate and increase in detoxification mechanisms to counteract the damaging effects of insect infestation.

THE IMPORTANT CONTRIBUTION OF SALIVA TO PLANT-HEMIPTERAN INTERACTION

Hemipteran saliva, which contains a variety of factors including proteins, is an important component of the hemipteran, which comes in direct contact with the host cells. It is intermittently released through the stylets into the host tissue. Similar to effectors released by pathogens, some salivary components have been demonstrated to facilitate infestation, while others elicit host defenses (Elzinga and Jander, 2013; Rodriguez and Bos, 2013; Kaloshian and Walling, 2016). Thus, salivary components likely contribute to the host range of the insect. van Bel and Will review what is currently known about aphid saliva, beginning with the secretion of saliva, the types of saliva, the methods of collecting saliva, and the protein components of the saliva and their likely biochemical function and impact on plant-hemipteran interaction.

In recent years, tools for transiently delivering recombinant salivary proteins have been developed for some model plants. To study the impact of aphid saliva on host selection, these tools need to be applied to different hosts. Guy et al. describe the development of an *Agrobacterium*-based tool to deliver recombinant salivary proteins to *Medicago sativa* (alfalfa) and *Pisum sativa* (pea), two important hosts of the pea aphid (*Acyrthosiphon pisum*). These tools should facilitate studying the contribution of salivary proteins on host specialization by the related aphids.

Kettles and Kaloshian utilized transient expression tools to demonstrate the effector activity of the potato aphid salivary protein Me47, which facilitates aphid infestation in tomato (*Solanum lycopersicum*) and *Nicotiana benthamiana*. However, in *Arabidopsis thaliana*, Me47 has the opposite effect in that it adversely impacts infestation, likely by eliciting host defenses. The ability of some salivary proteins to promote infestation in one host and limit infestation in others, could potentially contribute to host specialization.

ROLE OF SMALL RNA AND EPIGENOMICS IN INFLUENCING PLANT-HEMIPTERAN INTERACTIONS

The role of non-coding small RNA (sRNA) in regulating biological processes has become more apparent in recent years. sRNAs are involved in epigenetic regulation of gene expression, post-transcriptional control of transcript abundance as well as translational control. Although, our understanding of sRNA involvement in plant-hemipteran interaction is still in its infancy, progress made to-date has uncovered the potential involvement of several sRNAs in this interaction. Sattar and Thompson review the developments in this evolving field of sRNA in plant-hemipteran interaction. They summarize the synthesis and the potential contribution of plant-derived sRNAs to plant defense. sRNAs are found in phloem and likely consumed by the hemipterans, where they could impact processes in the insect. Hemipterans also have the machinery to synthesize sRNA that could influence insect growth and development. Further, the anti-viral RNAi machinery in the host and insect could also impact the interaction between plants, hemipteran, and their viruses.

Can hemipterans deliver sRNA into the plant? That is indeed the implications of the research paper by Van Kleeff et al. who show that whitefly sRNA can be recovered from the phloem and leaf of the host plant. Potential targets of these genes in the host have been predicted, raising the interesting possibility of the involvement of hemipteran-delivered sRNA in cross-kingdom interactions.

Finally, Kim et al. review the contribution of sRNA to epigenetic regulation of gene function in microbes with reduced genomes and its potential contribution to the regulation of genes in the aphid endosymbiont *Buchnera*. Thus, a full circle of sRNA engagement at multiple levels potentially could impact the outcome of plant-hemipteran interactions.

VECTORING OF PATHOGENS BY HEMIPTERANS

The interaction between plants, viruses and hemipteran vectors has been studied extensively in recent years and covered in recent reviews (Blanc et al., 2014; Gray et al., 2014; Gilbertson et al., 2015; Whitfield et al., 2015). In comparison, the multi-organismal

interaction between plants, bacteria and hemipterans is poorly understood. Perilla-Henao and Casteel review recent progress on understanding this interaction between plants, bacteria and hemipterans, and the approaches utilized.

Phytopathogen transmission and infection is influenced by both factors in the vector and the host. Heat shock proteins (HSPs) are chaperone proteins that interact with other proteins and are involved in cellular homeostasis. HSPs also influence viral infection, which is the subject of the review by Gorovits and Czosnek, who discuss the role of HSP70 and HSP90 in plant and whitefly, respectively, on *Tomato yellow leaf curl virus* (TYLCV) life cycle and acquisition of virus by the vector. Transmission of TYLCV by whitefly is dependent on the cyclophilin CypB, the evidence for which is presented in the paper by Kanakala and Ghanim. They show that CypB interacts with TYLCV and that the transmission of TYLCV is adversely impacted when either this interaction or the activity of CypB is blocked, thus implicating an important role for CypB in transmission of TYLCV by whitefly.

INTERPLAY BETWEEN THE ENVIRONMENT AND INSECT INFESTATION OF PLANTS

The environment, including water and mineral nutrient availability, temperature and presence of other organisms are some of the factors that influence host-pest interaction. Conversely, insect infestation also influences the relationship of the plant with its immediate environment, some for the better and some for the worse. Nachappa et al. studied the effect of drought on infestation of soybean plants by soybean aphid as well as the effect of drought on *Soybean mosaic virus* transmission by the soybean aphid. They report a complex effect of drought on soybean aphid population growth, viral infection, and viral transmission by the aphid. They suggest that the effect of drought on phloem amino acid content and the defense hormones SA and JA, impacts the aphid population and viral transmission.

Guo et al. studied the effect of elevated CO_2 on the performance of whiteflies and TYLCV in tomato plants and compared the impact of the tomato *Mi1.2* gene in these two types of interactions. They observed that elevated CO_2 did not influence insect fitness or its ability to transmit virus in the resistant (*Mi1.2*) or susceptible (*mi1.2*) genotypes. In contrast, elevated CO_2 increased resistance to TYLCV in *mi1.2* plants, while it increased susceptibility to TYLCV in *Mi1-2* plants, thus suggesting that *Mi1.2* deployment under elevated CO_2 conditions might increase vulnerability to TYLCV infections.

Insect infestation of the foliage has previously been shown to alter root physiology (Nalam et al., 2013). Kong et al. further report that the root microbiome is also impacted in plants experiencing a foliar whitefly infestation. The whitefly infestation-induced alteration in microbiome included enrichment of microbial species that are detrimental to whitefly, thus suggesting that root microbiome changes could potentially benefit the host plant.

CONCLUSIONS

The range of activities being undertaken by plant-hemipteran interaction researchers to understand the physiological mechanisms and molecular factors that influence these interactions is highlighted in this Research Topic. Although still in its infancy, these studies have begun to provide insights that will have far-reaching implications at different levels, including the development of novel strategies for plant protection against hemipterans, as well as the vectored pathogens.

AUTHOR CONTRIBUTIONS

JS and LW contributed to the drafting, editing and finalizing this editorial.

ACKNOWLEDGMENTS

We thank all the authors who contributed to this research topic, and the reviewers and editorial staff at Frontiers in Plant Sciences for their time and effort in bringing this Research Topic to fruition. Research activities in plant-pest interaction in the Shah lab have been supported by the National Science Foundation, the US Department of Agriculture and the University of North Texas. Research in the Walling lab was funded by National Science Foundation and the Gates Foundation.

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Conflict of Interest Statement: The authors declare that there search was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 26 September 2016
doi: 10.3389/fpls.2016.01420

Vat, an Amazing Gene Conferring Resistance to Aphids and Viruses They Carry: From Molecular Structure to Field Effects

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We review half a century of research on *Cucumis melo* resistance to *Aphis gossypii* from molecular to field levels. The *Vat* gene is unique in conferring resistance to both *A. gossypii* and the viruses it transmits. This double phenotype is aphid clone-dependent and has been observed in 25 melon accessions, mostly from Asia. It is controlled by a cluster of genes including CC-NLR, which has been characterized in detail. Copy-number polymorphisms (for the whole gene and for a domain that stands out in the LLR region) and single-nucleotide polymorphisms have been identified in the *Vat* cluster. The role of these polymorphisms in plant/aphid interactions remains unclear. The *Vat* gene structure suggests a functioning with separate recognition and response phases. During the recognition phase, the VAT protein is thought to interact (likely indirectly) with an aphid effector introduced during cell puncture by the aphid. A few hours later, several miRNAs are upregulated in *Vat* plants. Peroxidase activity increases, and callose and lignin are deposited in the walls of the cells adjacent to the stylet path, disturbing aphid behavior. In aphids feeding on *Vat* plants, Piwi-interacting RNA-like sequences are abundant and the levels of other miRNAs are modified. At the plant level, resistance to aphids is quantitative (aphids escape the plant and display low rates of reproduction). Resistance to viruses is qualitative and local. Durability of NLR genes is highly variable. *A. gossypii* clones are adapted to *Vat* resistance, either by introducing a new effector that interferes with the deployment of plant defenses, or by adapting to the defenses it triggered. Viruses transmitted in a non-persistent manner cannot adapt to *Vat* resistance. At population level, *Vat* reduces aphid density and genetic diversity. The durability of *Vat* resistance to *A. gossypii* populations depends strongly on the agro-ecosystem, including, in particular, the presence of other cucurbit crops serving as alternative hosts for adapted clones in fall and winter. At the crop level, *Vat* resistance decreases the intensity of virus epidemics when *A. gossypii* is the main aphid vector in the crop environment.

Keywords: NLR resistance gene, durability, melon, *Cucumis melo, Aphis gossypii,* resistance deployment, resistance to insects, resistance to viruses

OPEN ACCESS

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *18 May 2016*
Accepted: *06 September 2016*
Published: *26 September 2016*

Citation:
Boissot N, Schoeny A and Vanlerberghe-Masutti F (2016) Vat, an Amazing Gene Conferring Resistance to Aphids and Viruses They Carry: From Molecular Structure to Field Effects. Front. Plant Sci. 7:1420. doi: 10.3389/fpls.2016.01420

INTRODUCTION

Host-plant resistance is an effective, environmentally friendly means of controlling insect pests, including aphids. Here, we consider plant resistance to be a heritable trait, displaying genotype-dependent variability within a plant species. Resistance to aphids has been described in several crops (Dogimont et al., 2010; Smith and Chuang, 2014). This resistance is controlled by one or several genes, which may be recessive or dominant. Resistance deters aphids from the crop, and affects their biotic potential, including their growth, development, and reproduction. So resistance is generally detected through these central aphid life history traits, rather than by a visible plant phenotype. The melon *Vat* gene is unique among the known resistance genes in that it has a pleiotropic effect as it also confers resistance to the viruses transmitted by aphids.

Melon crops are primarily colonized by only one aphid species, the melon aphid *Aphis gossypii*, a cosmopolitan aphid species. This aphid causes stunting and severe leaf-curling, and heavy colonization can result in plant death. Aphids also excrete honeydew onto the leaves and fruits. This sticky sweet substance acts as an ideal growth medium for sooty mold, which greatly decreases fruit quality. Moreover, *A. gossypii* is an efficient vector for viruses, contributing to the spread of diseases.

Resistance to *A. gossypii* in melon was first observed in the mid-20th century (Ivanoff, 1944). In 1967, an American team of entomologists and plant geneticists began a systematic study of resistance to *A. gossypii* in melon. They focused on the Indian line PI 371795, later called PI 414723, which suffers only mild attacks in the field (Kishaba et al., 1971; Bohn et al., 1972). In controlled no-choice tests, few aphids survive on this line, and the fecundity of those that do is low (Kishaba et al., 1971). This resistance is a dominant trait in PI 414723, and is controlled by a major gene and several minor genes (Kishaba et al., 1976). A French team of virologists and plant geneticists studied the resistance of the Korean line PI 161375 to *Cucumber mosaic virus* (CMV) in detail. They discovered an original phenotype of this line: complete resistance to CMV when the aphid *A. gossypii* inoculated the plant with the virus. Moreover, *A. gossypii* aphids departed from PI 161375 plants. These two phenotypes cosegregated in PI 161375 and were controlled by a single dominant gene (Pitrat and Lecoq, 1980). Complete resistance to CMV was also observed in PI 414723 when CMV was introduced into the plant by the aphid *A. gossypii* (Pitrat and Lecoq, 1982). PI 414723 and PI 161375 thus have similar features: resistance to CMV when *A. gossypii* inoculates the plant with the virus cosegregating with resistance to *A. gossypii* controlled by a single dominant gene (Pitrat and Lecoq, 1982). In both lines, the resistance to viruses is expressed only if the aphid inoculating the plant with the virus is *A. gossypii*. PI 161375 and PI 414723 plants are susceptible to viruses when other aphid species, such as *A. citricola, A. craccivora*, *A. fabae,* and *Myzus persicae,* inoculate the plant with viruses, or if viruses are introduced mechanically (Lecoq et al., 1979, 1980; Romanow et al., 1986). The resistance to viruses when *A. gossypii* inoculated the plant is also fully effective against unrelated viruses (Lecoq et al., 1980). The gene controlling this double phenotype has been named *Vat,* for 'virus aphid transmission' (Pitrat and Lecoq, 1982).

Several hundreds of accessions were tested for their effect on the aphid traits (Pitrat et al., 1996; Fergany et al., 2011). These large screenings have suggested that about 5% of accessions display resistance to colonization by *A. gossypii*. Among them, only a small number have been tested for the double phenotype characteristic of *Vat*, resistance to virus and resistance to aphids. Up to now, the double phenotype has been identified in 25 melon lines (Pitrat and Lecoq, 1980; Soria et al., 2000; Thomas et al., 2012b; Boissot et al., 2016). These melon accessions or lines originate from Asia, Africa, America, and Europe.

Two independent breeding programs were conducted early on, to transfer resistance to *A. gossypii* into cultivars, with the transfer of resistance from PI 161375 into Charentais-type melons and resistance from PI 414723 into Western Shipper–type melons. Consistent with the cosegregation of resistance to melon aphid and resistance to viruses, which were introduced by melon aphids, the inbred lines obtained in both programs also displayed resistance to viruses when the melon aphid inoculated the plant (Kishaba et al., 1992; Boissot et al., 2016). Margot became the first melon cultivar declared resistant to the melon aphid *A. gossypii* to be listed in the French catalog in 1987. Since then, 110 Charentais-type cultivars declared resistant to this aphid have been released in France (GEVES data). Melons are cultivated in the South East (SE) and South West (SW) of France, and on two islands of the Lesser Antilles (LA). Given the commercial success of some of the resistant cultivars, about 80% of the melon crops cultivated in SE France since 2000 are thought to have carried this resistance (Boissot et al., 2016).

Since these seminal studies were conducted, the molecular structure of the *Vat* gene has been elucidated, its double phenotype has been investigated at the cellular level, and its effect on the behavior and life-history traits of the aphid has been studied. Its spectrum of activity against the clonal diversity of *A. gossypii* has been studied in the laboratory, and its efficacy and the durability of these effects have been studied *in situ*. All these points will be reviewed after a short presentation of the three protagonists*: Cucumis melo*, *A. gossypii* and the viruses transmitted by *A. gossypii.*

CUCUMIS melo, *APHIS gossypii* AND THE VIRUSES IT TRANSMITS TO MELON

Cucumis melo Shares a Number of Features Specific to Cucurbits, but Is Genetically Isolated in Its Family

Cucumis melo is one of the principal species from the Cucurbitaceae family. Asia is its geographic region of origin and it belongs to the *C. melo*/*C. callosus*-*C. trigonus* complex, which diverged 3 million years ago (Mya) from an Australian sister species, *C. picrocarpus* (Sebastian et al., 2010). This clade diverged from the lineage leading to cucumber (*C. sativus*) about 10 Mya. A highly effective reproductive barrier now isolates *C. melo* from most of its relatives, with successful crosses reported only

with *C. hystrix* (Chen and Adelberg, 2000). Based on data for polymorphism at simple sequence repeat (SSR) markers, *C. melo* split into two main genetic clusters (**Table 1**), the first containing four groups (A, B, C, D) and the second containing three groups (E, F, and G; Serres-Giardi and Dogimont, 2012). These data, together with findings for chloroplast polymorphisms (Tanaka et al., 2013), suggest that there were two or three domestication events, one in Asia, another in Africa or Western Asia, and a third in Africa (Pitrat, 2013).

Cucumis melo is now found throughout the world and, like many crops, cultivated melons display extensive phenotypic polymorphism, defining botanical groups, whereas wild melons display low levels of phenotypic polymorphism (Pitrat et al., 2000). The first evidence of *C. melo* domestication date to just after 3000 BC, in China and Egypt (Pitrat, 2003). Diversification after domestication is controlled mostly by recessive traits, such as sex expression, fruit shape, vein tracts, number of placentas, a gelatinous sheath around the seeds, and white flesh color, whereas disease resistance is mostly conferred by dominant genes (Pitrat, 2013). Melon is now an important fruit crop, with 16 commercial melon types identified by the Organization for Economic Co-operation and Development (OECD) guidelines on the basis of fruit characteristics (shape, skin color and surface characteristics, color of the flesh and dehiscence of the peduncle). Twenty-five to 30 million tons of melon have been produced annually over the last 10 years, with about half of this total in China (FAOSTAT database[1]). Melon has been subject to intense selection, and its isolation in the genus *Cucumis* has led to reclaim the broad diversity present in both cultivated and wild forms (Pitrat, 2013). Twenty to 30 new melon cultivars have been added to French catalogs annually since 2000 (GEVES data[2]).

Melon is a diploid species with a relatively small genome (450 Mb) that has recently been fully sequenced (Garcia-Mas et al., 2012). It has 12 chromosomes, and, like all cucurbits, its genome displays no evidence of recent duplication since the eudicot paleotriplication event. It has a small number of resistance genes, only 81 putative NLR genes were identified (Garcia-Mas et al., 2012), possibly reflecting an unusual adaptive strategy in cucurbits potentially involving specific mechanisms of disease resistance gene regulation or the characteristic vascular structure of these plants. Cucurbits have an unusual vascular structure, with two types of phloem: the fascicular phloem is located in the main vascular bundles, and the extra-fascicular phloem is peripheral to the fascicular phloem, dispersed throughout the cortical tissue of the stems and petioles (Zhang

[1]http://faostat.fao.org/
[2]http://www.geves.fr

TABLE 1 | List of melon lines exhibiting the double phenotype, resistance to aphids and resistance to viruses when the aphids inoculate the plant, and multilocus genotypes (MLGs) of *Aphis gossypii* clones revealing that phenotype.

Characteristic of melon Accessions						Characteristics of aphid clones[c]		
Genetic groups[a]	**Botanical groups[b]**	**Asia**	**Africa**	**America**	**Europe**	**I**	**II**	**III**
(I) A	Inodorus				Anso 77		CUCU3	NM1
(I) A	Inodorus				Invernizo 8427			NM1
(I) A	Reticulatus		PI 224770					NM1
(I) B	Flexuosus		Fegouss 1					NM1
(I) B	unknown	San Ildefonso					CUCU3	NM1
(I) C	unknown	Durgapura Madhu				C9		NM1
(I) D	Makuwa	Kanro Makuwa 1				C9		NM1
(I) D	Makuwa	Kanro Makuwa 2				C9		NM1
Unknown	Momordica	AM 51				C9, CUC1, GWD	CUCU3	NM1
(II) E	Momordica	PI 414723				C9		NM1
(II) E	Wild		PI 482398			C9, GWD	CUCU3	NM1
(II) E	Wild		HSD2455				CUCU3	
(II) F	Acidulus		PI 482420			C9		NM1
(II) F	Acidulus	90625						NM1
(II) F	Acidulus	PI 164723						NM1
(II) F	Chito			Meloncillo				NM1
(II) G	Chinensis	Chenggam						NM1
(II) G	Chinensis	Miel Blanc						NM1
(II) G	Chinensis	PI 161375					CUCU3	NM1
(II) G	Chinensis	PI 255478				C9		NM1
(II) G	Chinensis	PI 266935						NM1
(II) G	Conomon	Shiro Uri Okayama						NM1
(II) G	Makuwa	K 5442				C9		NM1
(II) G	Makuwa	Ginsen Makuwa				C9		NM1
(II) G	Makuwa	Shirokawa Nashi Makuwa						NM1

[a]*According to Serres-Giardi and Dogimont (2012).* [b]*According to Pitrat et al. (2000).* [c]*According to Thomas et al. (2016).*

et al., 2010). The fascicular phloem is mostly involved in sugar transport, whereas the extra-fascicular phloem may be involved in signaling and the transport of other metabolites.

A. gossypii Glover: A Biotype Specializing on Cucurbits

The *A. gossypii* group diverged from other aphids 12 to 25 Mya, during the radiation period of its host plants (Hyojoong et al., 2011). Within this group, species diversification may have been a rapid and recent process, as suggested by phylogenetic trees based on morphological characters (Kim et al., 2010) and the inability of differentiating between species on the basis of mitochondrial DNA COI/COII (Coeur d'acier et al., 2007). The mitochondrial *Cytb* and *nuclear sodium channel para-type* (*SCP*) genes can be used to distinguish the species *A. gossypii* Glover from related species native to North America, Europe, and Asia (Carletto et al., 2009a; Hyojoong et al., 2011; Lagos-Kutz et al., 2014). Hereafter, we will use the term *A. gossypii* to refer to *A. gossypii* Glover.

Aphis gossypii is a cosmopolitan species that is extremely polyphagous, colonizing hundreds of plant species (Ebert and Cartwright, 1997). In northern areas, at latitudes above 30°N, *A. gossypii* produces sexual morphs in the fall, which produce eggs that diapause on its primary hosts (Kring, 1959; Takada, 1988; Ferrari and Nicoli, 1994). These primary hosts differ between geographic areas, with Rose of Sharon (*Hibiscus syriacus*) frequently identified as a host plant in Asia, Europe, and America. In spring and summer, *A. gossypii* becomes a pest on crops, its secondary hosts, on which it reproduces clonally. In intertropical areas, *A. gossypii* reproduces clonally all year round. Thus, depending on the area of melon production, *A. gossypii* populations may consist of a mixture of strictly clonal lineages and lineages derived from sexual reproduction or of strictly clonal lineages only.

Aphis gossypii, currently named the cotton-melon aphid, is a pest for several crops, including melon, marrow, zucchini, potato, eggplant, cotton, ornamental hibiscus, and citrus fruit trees. Like all aphids, *A. gossypii* carries the bacterium *Buchnera aphidicola* as an obligate endosymbiont providing several essential nutrients (Douglas, 2003) and the phenotypic plasticity in host plant use by *A. gossypii* may be related to the size of the *B. aphidicola* population (Zhang et al., 2016). Many other facultative endosymbionts have been detected in aphid species and shown to play a role in species ecology (Oliver et al., 2010); however, facultative endosymbionts appear to be rare in *A. gossypii* (Carletto et al., 2008).

A small number of *Cytb* sequence polymorphisms differentiate three haplotypes of *A. gossypii* collected on crops and plants from the Cucurbitaceae, Malvaceae, Solanaceae, and Rosaceae in Africa, South America, Australia, and Europe (Carletto et al., 2009a). All individuals collected from cucurbits belong to the same haplotype. A small number of mitochondrial DNA sequence polymorphisms between the *Cytb* and 16S genes distinguish two biotypes of *A. gossypii* specializing on cotton and cucurbits in North China (Wang et al., 2016). At the end of the 1990s, a set of SSR markers (SSRs) was developed to assess *A. gossypii* diversity (Vanlerberghe-Masutti et al., 1999). Several hundred multilocus genotypes (MLGs), defined on the basis of allelic combinations at eight SSR markers, have since been described. The largest set of MLGs was identified in a study of spring migrants in France and the Lesser Antilles; they formed seven genetic clusters (Thomas et al., 2012a, 2016). All individuals collected from colonies on melon shared MLGs distributed between three clusters (later named in the manuscript I, II, and III). In data analyzed with the same set of reference clones (Brévault et al., 2008; Charaabi et al., 2008; Carletto et al., 2009b; Chen et al., 2013; Thomas et al., 2016), 75 MLGs were observed in colonies collected from cucurbits in Asia, Africa, Europe, Australia, and Caribbean islands, four of which — C4, C11, C9 and NM1 — were observed in at least two geographic areas suggesting that these clusters contain individuals specializing on cucurbits.

Biological studies have been conducted at the laboratory and field levels to assess the strength of the host specialization of *A. gossypii* biotypes. The results of these studies can be related with genetic knowledge. Many laboratory host-transfer experiments have been conducted with plants from the Cucurbitaceae (cucumber), Malvaceae, (cotton, okra, and hibiscus), Solanaceae (eggplant and sweet pepper), and Rutaceae (citrus plants and Chinese prickly ash) (Guldemond et al., 1994; Liu et al., 2008; Carletto et al., 2009b; Satar et al., 2013; Wu et al., 2013; Xu et al., 2014; Wang et al., 2016). Overall, the results obtained suggest that on one hand cucurbit biotypes poorly colonize plants from other plant families, if not at all, with the exception of *H. syriacus* and on the other hand, most, if not all, biotypes specialized on other crops poorly colonize cucurbits. Host switching in the field has been inferred from molecular markers. Studies conducted in different agricultural environments in Africa and China in which cucurbits are present together with cotton, and citrus or Solanaceous crops have confirmed that lineages specializing on cucurbits cannot easily switch to other crops (Brévault et al., 2008; Charaabi et al., 2008; Wang et al., 2016).

Taking into account genetic, ecological and lab experiment data, specializing on cucurbits of a part of *A. gossypii* species is fairly clear. All clones able to colonize cucurbits form an ecological group called the Cucurbit host-race (Carletto et al., 2009b), they are assigned to the genetic clusters I, II, and III (Thomas et al., 2016). The history of co-evolution between *A. gossypii* and cucurbits merits further investigation, particularly as the role of cucurbit's distinctive phloem structure for *A. gossypii* specialization on these plants.

The Viruses Transmitted to Melon by *A. gossypii* Belong to Three Families

More than 70 virus species have been reported to attack cucurbits (Lecoq and Katis, 2014). Some cause severe epidemics in melon crops worldwide. Five of these species are transmitted by aphids, including the melon aphid. These viruses may or may not persist in the vector. Non-persistent viruses are acquired and transmitted to the plant during brief probes (lasting less than 1 min), do not require a latent period in the vector and are retained in the vector

for only short periods of time (aphids remain viruliferous for only a few hours). Persistent viruses are acquired during phloem punctures for feeding (over periods of several hours or even days); they have a latent period and are retained for long periods in the vector (aphids often remain viruliferous for life). Further information about the transmission of plant viruses by insects is available from a recent review (Fereres and Raccah, 2015).

Non-persistent viruses include the CMV, belonging to genus *Cucumovirus*, family *Bromoviridae*. This virus has a worldwide distribution, and has been observed to infect more than 1200 species from more than 100 plant families (Jacquemond, 2012). This virus may have the widest host range of any known plant virus. It can be transmitted by more than 80 aphid species. CMV causes typical mosaic symptoms on melon leaves, plant stunting, mottle or mosaic on fruits, and yield losses.

Several potyviruses (family *Potyviridae*) attacking melon crops are also non-persistent (Lecoq and Desbiez, 2012). They are transmitted by 20 to 40 aphid species. *Watermelon mosaic virus* (WMV) is observed worldwide. It can infect more than 170 plant species. On melon leaves, it induces mosaic, vein banding and deformation, such as blisters, filiformis and size reduction. On fruits, it induces severe discoloration, with slight deformation in some cases. *Zucchini yellow mosaic virus* (ZYMV) is also distributed worldwide, but has a smaller host range than CMV or WMV (only 11 families). It induces vein clearing, yellowing, with blisters and enations on leaves and severe stunting. On fruits, ZYMV induces mosaic or necrotic cracks, marbling and hardening of the flesh. Moreover isolates belonging to the pathotype F induce wilting in melons carrying the *Fn* gene (Risser et al., 1981) instead of mosaic in melons carrying the Fn^+ allele. The *Fn* gene (for *Flaccida necrosis)* is present in numerous melon accessions. *Papaya ringspot virus* (PRSV) mostly infects tropical and Mediterranean cucurbit crops. Its host range is restricted to cucurbits and a few other plant species, such as papaya. On melon, it causes severe mosaic, blistering, and malformations on leaves. Fruits may also display various degrees of discoloration and deformation.

Persistent viruses include the *Cucurbit aphid-borne yellows virus* (CABYV) a member of genus *Polerovirus*, family *Luteoviridae* (Lecoq and Desbiez, 2012). This virus infects many cucurbits, beet, lettuce and many weed species. It is transmitted by a small number of aphid species (*M. persicae* and *Macrosiphum euphorbiae* are additional vectors). It induces yellowing of the older leaves, but complete discoloration of the plant is observed with some melon cultivars. Its effect on yield is less marked than other viruses infecting melon, particularly as it has no effect on fruit quality, instead inducing flower abortions and reducing the number of fruits per plant.

Keep in Mind Some Features When Considering the Double Phenotype

The double-resistance phenotype elicited by *A. gossypii* has been identified in all seven genetic groups in *C. melo* (**Table 1**). It has been identified in wild accessions from Africa, PI 482398 and HSD2455, both of which have some cultivated characteristics, but it has not yet been identified in wild accessions from Asia. Conversely, most of the accessions and landraces displaying the double phenotype are native to Asia. The double phenotype has been observed in all botanical groups of this species in Asia. We use the term '*Vat* melon line' here to refer to any accession or line displaying this double phenotype in a study.

The aphid clones used in bioassays characterizing the double phenotype are only rarely mentioned. Molecular markers for their identification are available (Vanlerberghe-Masutti et al., 1999) but are still only rarely used to characterize the *A. gossypii* clones used in bioassays. A DNA-reference clone set, at least from clones belonging to the three cucurbit clusters, should be established by the scientific community and made available. Aphids assigned to clusters I and III are the most frequently used (**Table 1**). No data have been published concerning the capacity of clones that are not able to colonize Cucurbits, to elicit resistance to viruses in *Vat* melon.

Resistance to viruses when the melon aphid inoculates the plant with virus has been documented principally for CMV. For example, all the accessions mentioned in **Table 1** displayed the double phenotype when using this virus in the bioassays. In PI 161375 and PI 414723, the resistance to viruses is fully effective against other potyviruses, such as PRSV (formerly known as, WMV1), WMV (formerly known as, WMV2) (Lecoq et al., 1980), and ZYMV (formerly known as, MYSV) (Risser et al., 1981; Kishaba et al., 1992; Soria et al., 2000), when the melon aphid inoculates the plants. Like CMV, these viruses have a non-persistent mode of transmission. While not formally tested, it is likely that this large spectrum of resistance to viruses transmitted in a non-persistent manner is common to all accessions displaying resistance to CMV transmission from *A. gossypii*. For other virus species transmitted in a persistent manner, such as CABYV, no laboratory data have ever been published.

FROM *VAT* GENE TO *VAT* CLUSTER

The *Vat* locus was mapped to *C. melo*'s linkage group V using segregating populations from a cross between the susceptible line Védrantais and the resistant accession PI 161375 (Pitrat, 1991; Baudracco-Arnas and Pitrat, 1996). It was localized to a subtelomeric position on a saturated map combining two recombinant inbred populations resulting from crosses between Védrantais and two resistant accessions PI 161375 and PI 414723 (Périn et al., 2002). In early 2000, a map-based strategy was used to isolate the *Vat* gene. This approach involved the use of 6000 plants from a back-cross population derived from Védrantais and PI 161375. Recombination events within the terminal region of linkage group V were screened and recombinant plants were phenotyped for resistance to aphids. A physical map encompassing the *Vat* gene was obtained by screening a melon bacterial artificial chromosome (BAC) library constructed from PI 161375, and the genomic sequence spanning the *Vat* region was annotated. A comparison of molecular data and phenotypic data for resistance to melon aphid and resistance to viruses when the melon aphid inoculated the viruses showed that the *Vat* gene was a single functional locus conferring both types

of resistance (Pauquet et al., 2004). Nine of the 14 of the back-cross progeny displaying recombination in the genomic sequence spanning the *Vat* region are presented in **Figure 1**, with their phenotype. The *Vat* gene is 6-kb long, and consists of five exons and four introns. It encodes a predicted 1467-amino acid protein presumed to be located in the cytoplasm (Dogimont et al., 2014). This protein belongs to the coiled-coil (CC)-nucleotide binding site (NBS)-leucine-rich repeat (LRR) family (**Figure 2**). Only three other genes conferring resistance to aphids or other hemipterans, *Mi-1* in tomato and *Bph14* and *Bph26* in rice are known to encode proteins from the NLR family (Rossi et al., 1998; Nombela et al., 2003; Casteel et al., 2006; Du et al., 2009; Tamura et al., 2014). For confirmation of the effect of the *Vat* resistance allele, an 11-kb DNA fragment harboring *Vat*'s coding region, promoter, and 3′-flanking region, was introduced into two susceptible *C. melo* lines by *Agrobacterium*-mediated transformation (Dogimont et al., 2014). Four lines derived from independent transformation events were obtained and all lines displayed high levels of resistance to NM1 melon aphids and complete resistance to viruses when the NM1 aphids inoculate the transgenic plants with CMV, WMV, and ZYMV.

All the bioassays conducted to identify *Vat* in the melon genome to date have used NM1. This clone has been used since the early studies by the French team and has provided the most clear-cut differentiation between susceptible and resistant accessions for both resistance to melon aphid and resistance to the viruses introduced into the plant by melon aphid (Boissot et al., 2016). In a study investigating whether the *Vat* allele of PI 161375 had a specific aphid clone effect or a much broader effect, one of the transgenic lines was tested with a set of *A. gossypii* clones from the cucurbit host-race. The bioassay used assessed the resistance to viruses transmitted by the aphid. In the transgenic line, the resistance to viruses introduced by an aphid was aphid clone-specific. Surprisingly, for some clones used for inoculation purposes, resistance to the virus was expressed in the native line, PI 161375 but not in the transgenic line (**Table 2**). These remarkable differences reveal that at least one other gene is involved in the resistance elicited by some *A. gossypii* clones in PI 161375 (Boissot et al., 2016). In accordance with general rules for the naming of genes, it has been suggested that the gene isolated from PI 161375 (Dogimont et al., 2014) should be renamed *Vat-1,* and the additional gene *Vat-2*. There may be allelic series for both these loci (Boissot et al., 2016).

It is not clear from the results presented above whether *Vat-1* and *Vat-2* form a cluster. This point has been investigated indirectly. PI 161375 is a Korean line harboring *Vat-1* and *Vat-2*; it belongs to the Chinensis botanical group (**Table 1**). *Vat-1* was introgressed from this line into a Charentais line (Cantalupensis group). The process of introgression consists in a first crossing between a Charentais line and PI 161375 and after, backcrossing the aphid-resistant progeny with the Charentais line, referred to as the recurrent line. The bioassays, to select plants resistant to aphids from each back-cross progeny, used the aphid clone NM1. Remarkably, the spectrum of resistance to viruses transmitted by aphids in PI 161375 was found to be conserved in the line Margot, which was obtained after seven back-crosses, (**Table 2**)

TABLE 2 | Pattern of resistance to *Cucumber Mosaic Virus* (CMV) inoculated by six *A. gossypii* clones on PI 161375, from which *Vat-1* was isolated, TR3, the transgenic line in which it was introduced and Margot a line in which aphid resistance from PI 161375 was introduced by classical breeding.

	C6	GWD	CUC1	C9	GWD2	NM1
PI 161375	S	R	R	R	R	R
TR3	S	S	I	R	R	R
Margot	S	R	R	R	R	R

R, resistant; S, susceptible; I, intermediate.

and therefore Margot carries *Vat-2* (Boissot et al., 2016). This suggests that *Vat-1* and *Vat-2* are probably very tightly linked. We will therefore use the name '*Vat*' for the region containing *Vat-1* and *Vat-2*.

Several genomics studies have focused on the region containing *Vat*. Genes conferring resistance to various pathogens are located in the vicinity of *Vat*: resistance to *Podosphaera xanthii* (Yuste-Lisbona et al., 2001; Perchepied et al., 2005a), *Cucumber vein yellowing virus* (Ibn Oaf, 2012), the *Fn* gene (Pitrat and Lecoq, 1982) triggering plant necrosis in response to some isolates of *ZYMV* (Risser et al., 1981), and the quantitative trait loci (QTL) *FomV-2* conferring partial resistance to *Fusarium oxysporum* f. sp. *melonis* (Perchepied et al., 2005b). The density of resistance genes in melon is highest in the region containing *Vat* (Garcia-Mas et al., 2012); 28 genes of the NLR family have been identified in a 1-Mb region containing *Vat* (González et al., 2014). Characterization of four *C. melo* accessions displaying resistance to viruses when different *A. gossypii* clones inoculated the plants has identified *Vat-1*-related sequences (protein identity over 80%; **Figures 1** and **2**).

These sequences display polymorphisms within all parts of the gene (**Figure 2**). In the LRR part of *Vat*, two types of polymorphism are observed: single-nucleotide polymorphisms (SNP) and length polymorphisms. The length polymorphisms occur in a specific domain, domain D or LRR2 (**Figure 2**) (Dogimont et al., 2008a, 2014). This domain consists of near-perfect repeats of 65 amino acids. The *Vat-1*-related sequences contain two to five repeats. The repeats are 83.1–89.2% identical (Dogimont et al., 2008a). PI 161375 has a *Vat-1*-related sequence with three repeats known as *Vat*-like. *Vat*-like is located 17 kb from *Vat-1* (**Figure 1**) and is not involved in the resistance elicited by the NM1 clone (Dogimont et al., 2008b). In PI 414723, a line exhibiting resistance to several clones, four *Vat-1*-related sequences have been identified. One of these sequences has only a few SNPs relative to *Vat-1*. Two sequences have five repeats in LRR2, and both are strong candidates for the control of resistance to *P. xanthii* (Dogimont et al., 2008b). Both these *Vat*-related sequences have few SNPs relative to the sequence of *Vat-1*. The fourth *Vat-1*-related sequence has two repeats in LRR2 and more SNPs relative to *Vat-1* than the other *Vat-1*-related sequences. In 90625, a line exhibiting resistance restricted to only one clone, NM1 (Boissot et al., 2016), only one *Vat-1*-related sequence has been identified. This sequence contains four repeats in LRR2 and several SNPs. In Védrantais, a Charentais line resistant to viruses when only one aphid clone, C4, inoculate

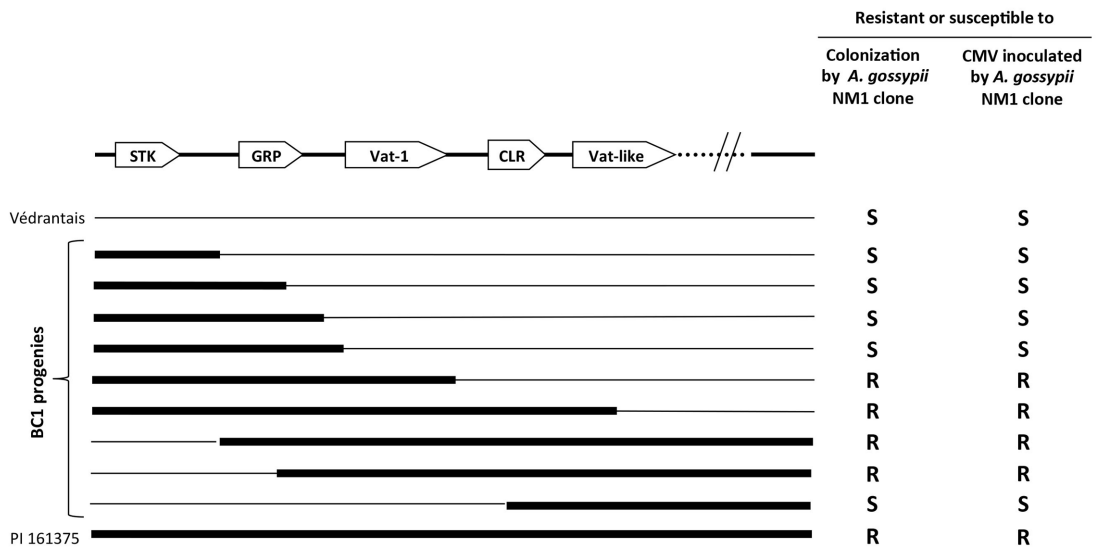

FIGURE 1 | Genomic map spanning the *Vat* region in 9 Back-Cross 1 plants derived from a cross between PI 161375 and Védrantais displaying segregation for resistance to *Aphis gossypii* and resistance to viruses transmitted by *A. gossypii*. STK, GRP, and CLR indicate serine-threonine kinase, glycine-rich protein and copia-like retroelement proteins, respectively. Adapted from (Dogimont et al., 2014).

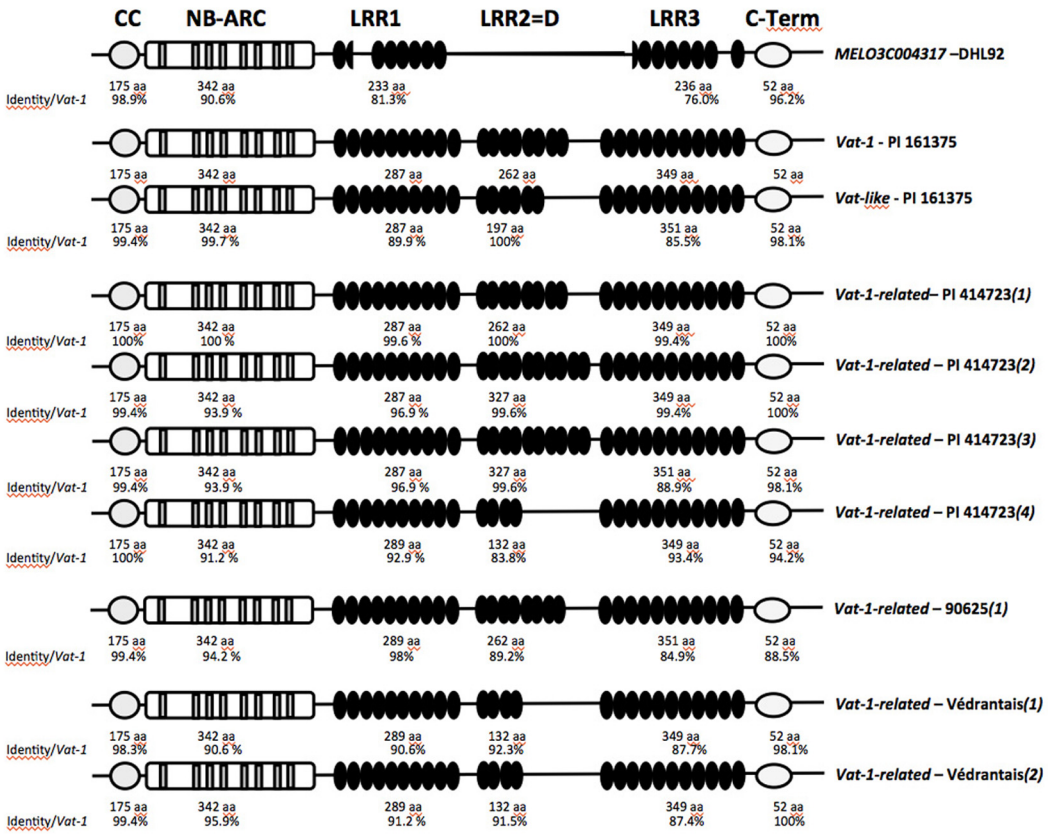

FIGURE 2 | Schematic diagrams of predicted *Vat* protein domains encoded by the *Vat-1* gene and polymorphisms detected in related sequences. Adapted from Dogimont et al. (2008a, 2014) and González et al. (2014).

the plant (Boissot et al., 2016), two *Vat-1*-related sequences with several SNPs relative to the sequence of *Vat-1* and two repeats in LRR2 have been identified. Finally, the cadre of *Vat*-like genes in each accession may not be complete, since no complete genome sequences are available for these accessions. Involvement of these *Vat-1*-related sequences in the aphid resistance has not been demonstrated. Actually, the melon reference genome was built from a line (DHL92) given as susceptible to *A. gossypii* (González et al., 2014), but accurate double-phenotypic data, with a set of characterized clones, are missing for that line. This line has a *Vat-1* homolog, MELOC004317, shorter (1038 aa) than the reference one (1467 aa), in particular the LRR2 part is fully absent (**Figure 2**) (González et al., 2014).

The presence of large numbers of NLR genes in close proximity to *Vat*, including Vat-like genes, makes assembly difficult and even unsatisfactory when sequences are obtained for small fragments. Sequencing studies of a set of accessions are required with longer fragments for an accurate assembly of the area. Comprehensive cross-comparison between molecular and phenotypic data is therefore required to obtain a full understanding of the genetic control of resistance to *A. gossypii* aphids and the viruses they transmit. The use of transgenic lines, even if difficult to obtain (Chovelon et al., 2011), will clearly help us to decipher the role of each locus in this cluster. As soon as candidates are identified, transformation with these candidates could be used to validate their roles in the resistance spectrum. This approach could provide new opportunities for genomic selection for resistance in melon.

MOLECULAR RESPONSES IN THE *VAT* MELON/*A. gossypii* APHID INTERACTION

The *Vat-1* gene belongs to the NLR gene family. According to the general framework developed for this category of resistance genes, its functioning involves separate recognition and response phases (**Figure 3A**). In this case, the recognition phase involves perception of an aphid effector by the plant's VAT protein, which may be direct or indirect. The vast majority of plant-pathogen effectors have been shown to interact indirectly with a NLR protein of their host. To date, modality of the interaction between aphid effectors and NLR proteins of their host is still unknown. This interaction should

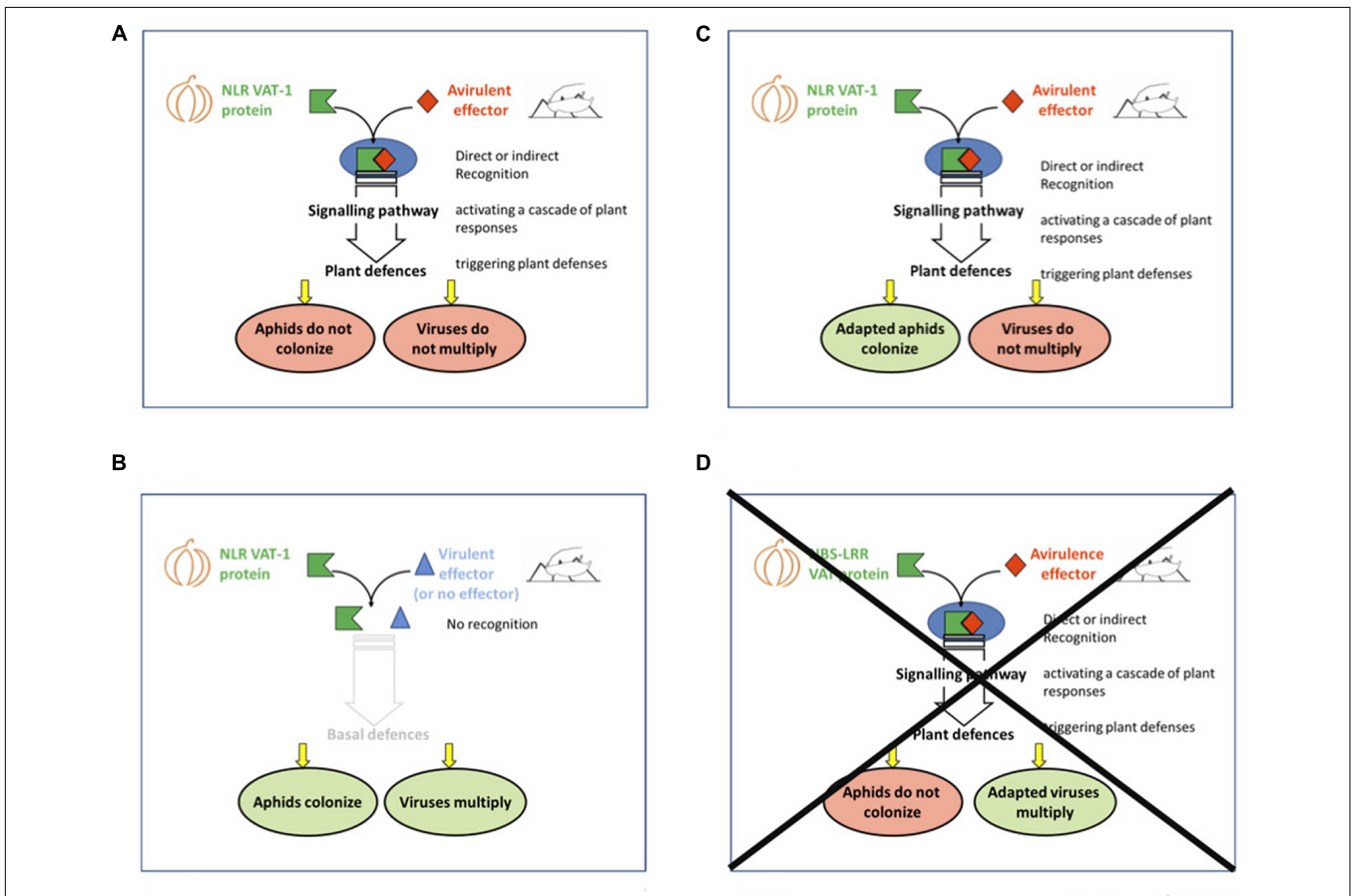

FIGURE 3 | Models of *A. gossypii*/*Vat*-melon plant interaction based on interaction (direct or not) between the VAT protein and the avirulence effector (Boissot et al., 2016). The three cases observed were: **(A)** resistance to aphids and viruses, **(B)** susceptibility to aphids and viruses, **(C)** susceptibility to aphids and resistance to viruses. **(D)** A fourth outcome, resistance to aphids and susceptibility to viruses, was not observed.

occur in the cytoplasm of cells (the predicted location of the VAT protein), and the effector molecule must therefore be delivered to the plant cell. Aphid mouthparts enclose two flexible stylets that the insect drives into the plant tissue to puncture the phloem, so that they can feed on plant sap. On their way to the phloem, the aphid stylets bend around the cells, and the salivary channel ejects a salivary gel that forms a sheath around the stylets. Along the way to their destination, the aphid stylets briefly puncture cells, into which the salivary channel ejects a watery saliva (Tjallingii, 2006). The effector is probably injected into the cells during these puncture events. No aphid effector capable of interacting with an NLR protein has yet been identified. A transcriptomic approach has been used to identify candidates involved in the virulence of *A. gossypii* on *Vat* melon (Dutartre-Fricaux et al., 2014). Genetically similar virulent and avirulent clones were used, and a head-reference transcriptome of more than 33000 contigs was generated by *de novo* assembly. This reference transcriptome has been used to search for candidate effector genes based on their differential expression and/or presenting sequence polymorphisms between virulent and avirulent aphid clones.

At the molecular plant level, the response phase is thought to involve the activation of a signaling cascade, leading to the rapid accumulation of reactive oxygen species (ROS) and defense hormones. In a *Vat* melon line, the levels of miRNAs involved in the post-transcriptional regulation of gene expression change rapidly after puncture by *A. gossypii* (Sattar et al., 2012b). Within 12 h of the infestation by melon aphids, 23 families of miRNAs display modulations. Their potential targets suggest a physiological function in disease and stress responses (5), phytohormone perception and signaling (11), miRNA biogenesis (2), and plant growth and development (Sattar et al., 2016). Ethylene, jasmonic acid and auxin have been identified as potential defense hormones in *Vat* plants infested with *A. gossypii* (Anstead et al., 2010; Sattar et al., 2016). In two *Vat* melon lines, peroxidase activity was found to increase within 10 min of aphid puncture (Sarria Villada et al., 2009). Callose, a polysaccharide usually laid down at plasmodesmata, is deposited within 20 min of aphid infestation, and lignin, a macromolecule derived from phenyl propanoids essential for cell wall thickening, is deposited 4.5 h after aphid infestation. The plasma membrane is damaged and the cells collapse. Callose and lignin are deposited in the wall of cells adjacent to the stylet sheath. These reactions do not occur in non-*Vat* plants. These data clearly illustrate the massive transcriptional reprogramming induced by *A. gossypii* infestation in *Vat*-resistant melon plants, triggering a wide range of plant defense responses (Dogimont and Boissot, 2016; Sattar et al., 2016).

At the molecular aphid level, changes in gene expression were investigated in *A. gossypii* feeding on *Vat* and non-*Vat* plants. There is an unexpectedly high abundance of 27 nt-long sRNA sequences in aphids feeding on *Vat* plants (Sattar et al., 2012a). These sRNAs belong to the Piwi-interacting RNA (piRNA) family. This class of sRNAs is absent from plants. Their biogenesis in animals is still poorly understood. They have been shown to be involved in the silencing of transposable elements exclusively in animal gonads (Vodovar and Saleh, 2012), facilitating short-term adaptation. Their role in *A. gossypii* remains unknown, but may relate to the lifting of maternal effects. Such effects are observed, for example, in aphids collected from cotton, which have low rates of reproduction in the first generation after transfer onto eggplant, but higher rates in subsequent generations (Satar et al., 2013). Eighty-one conserved microRNAs (miRNAs), 12 aphid-specific miRNAs, and nine novel candidate miRNAs have also been identified (Sattar et al., 2012a). These candidate miRNAs have been shown to be differentially regulated between aphids feeding on *Vat* and non-*Vat* plants and may affect their reproductive rates as described below.

EFFECT OF *VAT* PLANT RESPONSES ON *A. gossypii* BEHAVIOR AND BIOLOGY AND THE VIRUS THEY TRANSMIT

Aphid feeding is disrupted on *Vat* plants. Electrical-Penetration graph (EPG), in which the pathway of aphid stylets from epidermis to phloem can be followed, have been conducted on several *Vat* melon lines, with different genetic backgrounds and on melon aphid clones originating from different geographic areas (Kennedy et al., 1978; Chen et al., 1997; Klingler et al., 1998; Boissot et al., 2000; Garzo et al., 2002). The journey of the stylets through the mesophyll to the phloem takes from 90 to 140 min in non-*Vat* plants (Kennedy et al., 1978; Chen et al., 1997; Klingler et al., 1998; Boissot et al., 2000; Garzo et al., 2002), but is disrupted in *Vat* plants. The observed cellular response seems to occur after the aphid stylets have punctured plant cells rather than during the intercellular penetration of plant tissues by the stylets (Sarria Villada et al., 2009), consistent with the hypothesis that recognition occurs after the delivery of the effector to the cell. Cytological studies have shown that there are more stylet sheaths in *Vat* plants than in non-*Vat* plants (Kennedy et al., 1978), suggesting that early mesophyll cell puncture by *A. gossypii* may be more frequent in *Vat* plants. The stylets take longer to reach the phloem in *Vat* plants than in non-*Vat* plants and are less likely to reach their final destination in *Vat* plants than in non-*Vat* plants. Prior exposure of *Vat* plants to *A. gossypii* feeding does not modify the expression of this resistance (Chen et al., 1997).

Findings on *Vat* melon suggest that the plant responses elicited by short cell punctures either hinder the passage of the stylets between cells due to the deposition of callose and/or lignin in the cell walls, or deter the aphid from progressing further into the tissues, through an oxidative burst detected by aphid after brief periods of ingestion following the release of saliva into the cell. Moreover, melon aphids reaching the phloem of *Vat* plants do not remain there to feed (less than 10 min, if at all; Kennedy et al., 1978; Chen et al., 1997; Klingler et al., 1998; Boissot et al., 2000; Garzo et al., 2002). This suggests that feeding may be difficult in these plants, possibly due to phloem clogging, although this plant reaction has not yet been described in *Vat* melon. Few quantitative differences in phloem sap have been

identified between *Vat* and non-*Vat* plants that might explain the deterrence of *A. gossypii* (Chen et al., 1997). None of the studies, EPG or histological studies, investigating these aspects took into account the dual phloem structure of cucurbits and the nature of the phloem in which *A. gossypii* is able to establish feeding.

Aphids escape from *Vat* plants. In free-choice tests, winged aphids are less numerous on *Vat* plants 24 h after being offered a choice of plants (Kennedy and Kishaba, 1977), and wingless aphids are less numerous on *Vat* leaf disks from 30 min after being offered a choice of leaf disks (Garzo et al., 2002). Without choice, i.e., only one plant accession is available, wingless aphids walk away from the plant in the 2–3 days after their deposition. This behavior has been observed on several *Vat* melon lines, with aphid clones originating from different geographic areas that probably displayed marked genetic differences (Pitrat and Lecoq, 1982; Garzo et al., 2001; Boissot et al., 2010; Thomas et al., 2012b). Based on the timing of these events, we can conclude that early plant responses, occurring rapidly after cell puncture by the aphid, have an immediate effect on aphid behavior.

Aphids poorly reproduce on *Vat* plants. When wingless aphids are encaged on *Vat* plants, they display low rates of reproduction, mostly due to a longer pre-reproductive period and a smaller number of progeny (Klingler et al., 1998; Garzo et al., 2002; Boissot et al., 2010). This lower reproductive rate may be directly due to poor, disrupted feeding on the contents of phloem (Kennedy et al., 1978; Chen et al., 1997; Klingler et al., 1998; Boissot et al., 2000; Garzo et al., 2002). This hypothesis is supported indirectly by the observation that aphids produce far less honeydew when feeding on *Vat* plants than on non-*Vat* plants (Klingler et al., 1998). The resistance factor reducing the reproductive rate is not transmitted through grafting (Kennedy and Kishaba, 1977), consistent with the notion that feeding is somehow difficult, rather than with the phloem being toxic. The rate of aphid reproduction is quantitatively affected by *Vat* and QTLs (Kishaba et al., 1976; Boissot et al., 2010; Thomas et al., 2012b).

Vat plants display particularly high levels of resistance to viruses when aphids inoculated unrelated viruses transmitted in the non-persistent mode: 100% of the non-*Vat* plants displayed symptoms, whereas only 0 to a few per cent of *Vat* plants had symptoms, with this small number of plants displaying full symptoms (Lecoq et al., 1979; Kishaba et al., 1992; Boissot et al., 2016). When *Vat* resistance was first discovered, it was thought to block virus transmission, and Pitrat and Lecoq therefore named the gene responsible *Vat,* for 'virus aphid transmission' (Pitrat and Lecoq, 1982). Nevertheless, *A. gossypii* was subsequently shown to acquire the virus from *Vat* plants and to transmit it to non-*Vat* plants (Romanow et al., 1986), calling this hypothesis into question. According to the resistance to transmission hypothesis, a plant factor blocks the virus in the stylet (a molecule or a particular pH). The aphid must, therefore, first ingest material from the plant, before it egests saliva into the cells and delivers the viruses. All EPG studies have shown that, after puncturing cells, the aphid first salivates and then ingests the contents of the cell (Martin et al., 1997). Further studies have failed to demonstrate resistance to transmission due to retention of the virus in the stylets.

Vat resistance to viruses has to be considered in the general framework described for NLR resistance (Boualem et al., 2016). The VAT protein of non-*Vat* plants (i.e., carrying a 'susceptible' *Vat* allele) cannot recognize (directly or otherwise) the aphid effectors and viruses delivered to the cell by the aphid. This lack of recognition leads to systemic viral infection. In *Vat* plants, the resistant isoform of VAT recognizes an effector molecule from the aphid. This recognition induces resistance mechanisms limiting the replication and movement of the virus. The micro-oxidative burst triggered by aphid puncture in *Vat* plants (Sarria Villada et al., 2009) is thought to block the viruses in the inoculated cell or in neighboring cells. Callose deposit at plasmodesmata may help to contain virus particles in the inoculated or neighboring cells. The response is local: when a *Vat* plant is first inoculated with CMV by *A. gossypii,* CMV superinoculation with *M. persicae* on the same leaf leads to systemic infection (Mistral and Boissot, 2016). In the absence of the aphid effector, the recognition phase does not occur when the *Vat* plant is infected with viruses. In this case, the viruses replicate and move around the plant, establishing a systemic virus infection.

Virus aphid transmission (the initial name from which the Vat acronym is derived) does not provide an accurate picture of the action of the *Vat* gene product according to recent data. It would, therefore, be more appropriate to consider *Vat* to stand for 'virus aphid triggered.'

In *Vat* plants, no resistance to viruses transmitted in the persistent mode has ever been reported. As CABYV is restricted to phloem cells, the aphid must reach the phloem cells and feed for long enough to acquire virus particles. Mechanical inoculation is not possible for this virus, suggesting that effective inoculation is dependent on the delivery of the virus directly into the phloem. As *A. gossypii* rarely reaches the phloem of *Vat* plants (Kennedy et al., 1978; Chen et al., 1997; Klingler et al., 1998; Boissot et al., 2000; Garzo et al., 2002), CABYV acquisition and inoculation should be disrupted in *Vat* plants, but this point needs to be investigated.

ABILITY OF *A. gossypii* AND THE VIRUSES IT TRANSMITS TO ADAPT TO *VAT*

The LRR domain of the *Vat-1* gene is subject to diversifying selection (Dogimont et al., 2014). This selection responds to the diversifying selection acting on the avirulence gene, as frequently reported for other avirulence genes in plant pathogens (Rouxel and Balescent, 2010). This model describes the general framework for the molecular arms race between plants and pathogens. An aphid clone adapted to a given *Vat* allele would either not deliver an 'avirulent' effector to the plant (deletion) or would deliver a 'virulent' effector that is not recognized by the VAT protein. In both cases, the expected phenotype would be colonization of *Vat* plant by the clone, and susceptibility to viruses introduced into the plant by that aphid clone (**Figure 3B**).

This model has been challenged by testing transgenic lines containing the *Vat-1* gene, for resistance to both *A. gossypii* and

CMV introduced into the plant by six *A. gossypii* clones (C6, C9, CUC1, GWD, GWD2, and NM1) (Boissot et al., 2016). The phenotypes for five of the six clones were consistent with the general model (**Figures 3A,B**): the clones were either unable to fully colonize the *Vat-1* transgenic line and triggered resistance to CMV (NM1, C9, **Figure 3A**), or they fully colonized the *Vat-1* transgenic line and did not trigger resistance to CMV (C6, CUC1, GWD, **Figure 3B**). These five clones belonged to the three clusters corresponding to the cucurbit host-race (**Table 1**). The phenotypes observed with one clone (GWD2) were not consistent with the general model, with the clone triggering resistance to viruses but nevertheless being able to colonize the transgenic plants (**Figure 3C**).

Unlinking of resistance to viruses triggered by the aphid and resistance to aphids was confirmed by testing eleven *Vat* lines identified from the natural range of diversity in melon with nine clones, the six previously described and CUC6, CUC3, and C4 (Boissot et al., 2016). Only 52 of the 117 interactions characterized, considering results on transgenic lines and natural *Vat* lines, were consistent with the general model (**Figures 3A,B**). It has been hypothesized that the decoupling of the resistances to aphids and viruses (**Figure 3C**) results from aphid adaptation, making it possible for the aphid to colonize the plant even if plant defenses are elicited. The *Vat* phenotype proved to be a highly powerful tool for investigating a phenomenon never before studied for plant/pathogen interactions. This new model for adaptation to NLR resistance is revealed by the double phenotype, which can be used to follow the resistance process at two levels: recognition, and the efficacy of the plant defenses triggered.

We speculate that individuals of some clones adapted to *Vat* defenses they trigger (e.g., GWD2). If these aphids infest a *Vat* plant successfully, they must accept the *Vat* plant and reproduce at a high rate on it. EPG has revealed that *Vat* affects the exploratory behavior of the aphid on the plant, but this effect is quantitative, with some aphids reaching the phloem of *Vat* plants. Individuals of a clone adapted to *Vat* defenses probably reach the phloem more often than those of a non-adapted clone. A 'classical avenue' of research will involve comparison of the transcriptomes of adapted and non-adapted clones puncturing *Vat* plants, to track the aphid genes involved in this adaptation. We propose to explore a new avenue of research: does the dual phloem structure of cucurbits, and of melon in particular, play a role in this adaptation?

It is possible *a priori* that viruses transmitted in the non-persistent mode can overcome *Vat*-mediated resistance. In this scenario, viral variants may multiply and leave the punctured cells before the defense mechanisms are fully effective, resulting in the development of systemic infections. The expected phenotype would be 'susceptibility to viruses introduced by an aphid clone that is incapable of colonizing *Vat* plants' (**Figure 3D**). To date, this double phenotype has never been observed in a transgenic line carrying the *Vat-1* gene, suggesting that viral adaptation may not occur (Boissot et al., 2016). Consistent with these findings, experimental evolution experiments with viruses on *Vat*-plants have been unsuccessful. Sequential virus transmissions from infected *Vat* melon plants to healthy *Vat* melon plants were established with two aphid clones and three viruses, CMV, ZYMV, and WMV. None of these viruses evolved in response to the resistance triggered by these two clones, even when four sequential virus transmissions could be done (Boissot et al., 2016). These results strongly suggest that viruses transmitted in the non-persistent mode do not readily adapt to the *Vat* resistance triggered by *A. gossypii*.

EFFECT OF *VAT* ON *A. gossypii* AT THE POPULATION LEVEL AND ITS DURABILITY

Aphid density is lower on *Vat* plants than on non-*Vat* plants (Thomas et al., 2016). With the aim of quantifying the effect of *Vat* at crop level, we compiled bibliographic data for a density index for *Vat* and non-*Vat* plants grown in field experiments conducted at the same location (Schoeny et al., 2014; Thomas et al., 2016). The aphid density index was 44% lower on *Vat* plants (**Figure 4**). This index is related to aphid density per m^2 over the entire cropping period, by an exponential relationship ($y = 1463e^{0.1088x}$ with $r^2 = 0.72$, $n = 304$). Therefore, for non-*Vat* plant indexes of 90, 50, and 30, aphid density is reduced by factors of 50, 11, and 4, respectively, on *Vat* plants.

There are four key phases in the dynamics of crop infestation by aphids: visiting by winged aphids, infestation with the wingless nymphs they generate, development into colonies, and production of winged individuals for dispersal. Melon crops are visited by spring migrant aphids of numerous species. The proportion of *A. gossypii* among the visiting aphids and the genetic composition of the *A. gossypii* spring migrant population depend on geographic area (Thomas et al., 2016). Only some of the *A. gossypii* spring migrants generate progeny (**Figure 5**), mostly specializing on cucurbits (i.e., belonging to the Cucurbits I, II, and III genetic clusters). This selection leads to a significant decrease in clonal richness between the spring migrant and wingless populations on melon plants (Thomas et al., 2012a), and this decrease continues during subsequent steps, reflecting differences in fitness or competition between clones on melons (Thomas et al., 2016) (**Figure 5**).

The effect of *Vat* on the first step of infestation has never been reported at field level. However, *Vat* plants were found to be less attractive for winged aphids than non-*Vat* plants in greenhouse experiments based on artificial infestation (Kennedy and Kishaba, 1977). The effect of *Vat* on subsequent phases of infestation has been characterized in open-field melon crops under natural infestation conditions, but only in French melon production areas and in the Lesser Antilles (Thomas et al., 2016). The wingless populations on *Vat* plants have a genetic composition different from that of the populations on non-*Vat* plants, as clonal richness and clonal diversity decreased during infestation. Aphids from group III of the cucurbit host-race are eliminated in favor of aphids from group I. In French production areas, the third step, colony development, is erratic

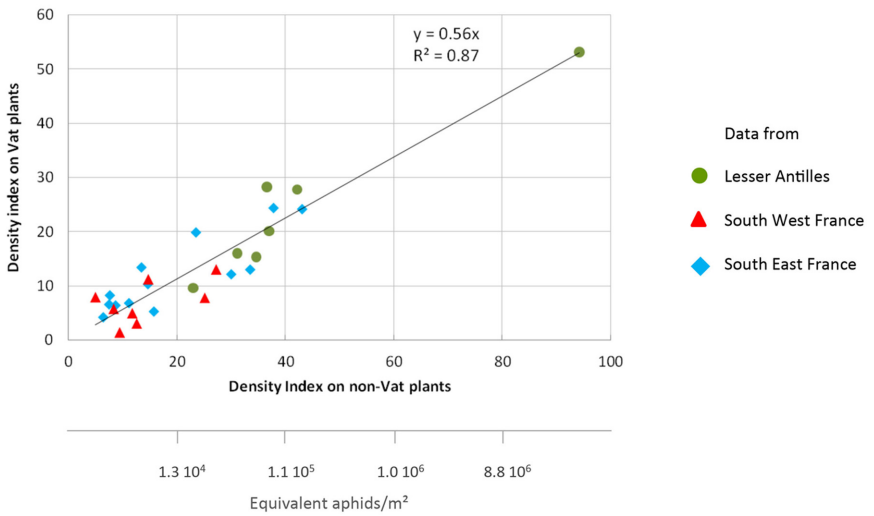

FIGURE 4 | Aphid densities on *Vat* and non-*Vat* plants grown in 28 fields in three melon production areas from 2008 to 2014. The data shown are from (Schoeny et al., 2014; Thomas et al., 2016).

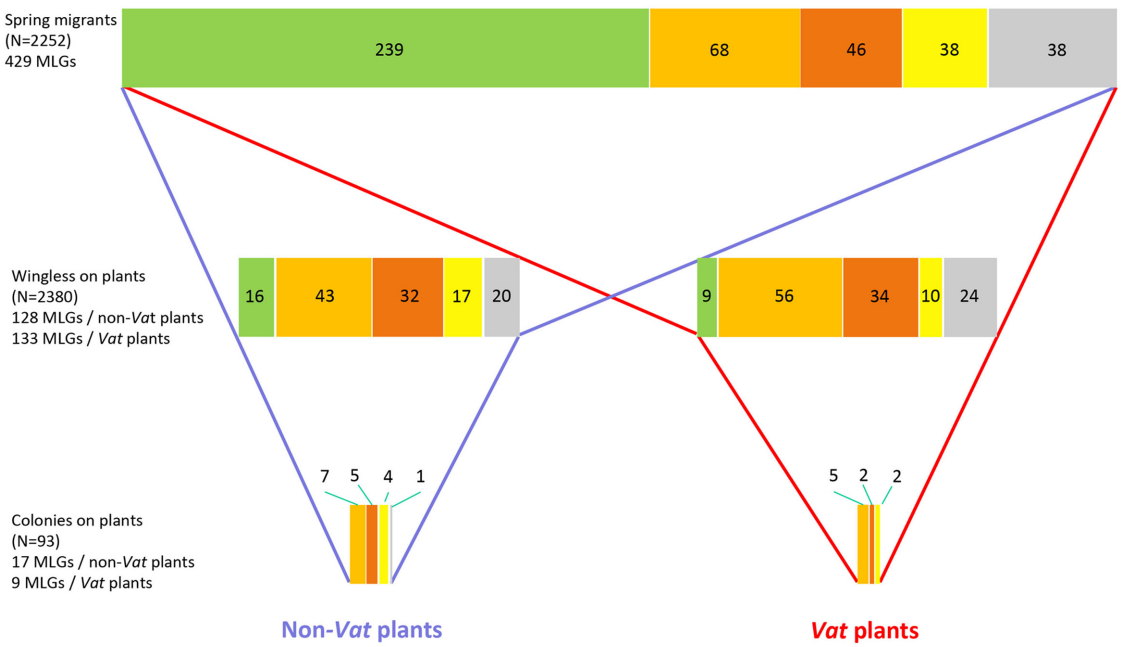

FIGURE 5 | Reduction of the clonal richness of *A. gossypii* populations during the infestation of melon crops in France. Clonal richness at each step of the infestation is represented by the length of the stripe. Colors within the stripe represent different genetic clusters of *A. gossypii* populations, with the size of each rectangle proportional to the number (indicated within) of MLGs assigned to the genetic cluster. The data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.gf54q Cucurbit I, Cucurbit II, Cucurbit III, other clusters, Not assigned at 75%.

on melon crops, and, in the presence of *Vat,* this step is very rarely observed, generally in only a few aphid genotypes from group I or II. The fourth phase, the production of winged individuals for dispersal, is density dependent (Dixon, 1985), and therefore very rare on *Vat* plants. The populations of *A. gossypii* occurring in French melon crops contain a large proportion of aphids from genetic group III, and the decrease in density and diversity on *Vat* plants probably reflects selection rather than competition. Consistent with this hypothesis, a laboratory study showed that *Vat*-mediated resistance affected the population growth of 90% of group III clones, but only 40% of those from group I or II (Lombaert et al., 2009). Only group I aphids are present in the Lesser Antilles, where the third and fourth steps are regularly reached, even on

Vat plants. The effect of *Vat* on aphid clonal richness is illustrated in **Figure 5**. Only four clones, CUC1, CUC6, GWD, and C6, have regularly been identified in colonies on *Vat* plants in field experiments (Thomas et al., 2016) and studied in laboratory experiments (Boissot et al., 2016). C6 does not trigger resistance to CMV and probably has a virulent effector not recognized by *Vat*, whereas CUC1, CUC6, and GWD trigger resistance to CMV and are adapted to plant response.

An ecological genetics analyze of melon-aphid dynamics has been applied in three different agricultural systems over the last decade, to predict the durability of *Vat* resistance to *A. gossypii* (Thomas et al., 2016). It appears that *A. gossypii* is evolving at a regional level in response to the deployment of *Vat* melon crops. For example, two different bottlenecks affect the dynamics of adapted clones in melon-producing areas, due to (i) the low levels of dispersal morph production on *Vat* melon and (ii) the winter extinction of clones. The low levels of dispersal morph production result from the containment of populations at levels of crowding below that required to induce the production of winged morphs. Winter extinction occurs due to the absence of other cucurbit crops to serve as hosts between two melon crop cycles, limiting the maintenance of *Vat*-adapted clones. In melon-producing areas without bottlenecks (such as the Lesser Antilles), resistance is predicted to be not durable. In areas in which both types of bottlenecks occur (such as South-West France), resistance is predicted to be durable. In South-East France, only one of the two bottlenecks occurs, and cucurbits are cultivated almost year-round. Moreover, in South-East France *Vat* melons have been cultivated at a large scale since 2000, and *Vat* resistance is now jeopardized by the emergence of adapted clones. These findings suggest that, for a cosmopolitan pest, such as *A. gossypii*, decisions concerning resistance deployment should take into account the genetic structure of the pest population at regional scale, the availability of winter host plants for adapted biotypes between crop cycles (Thomas et al., 2016) and the allele composition of the *Vat* cluster.

The manipulation of agricultural systems to increase the durability of *Vat* resistance through winter extinction does not appear to be feasible. However, it may be possible to increase durability by preventing the production of dispersal morphs from adapted clones. Different ways of achieving this aim have been investigated. The use of strips of flowering plants sown close to *Vat* melon crops to attract natural enemies has been investigated (Schoeny et al., 2014), but the efficacy of this approach will need to be confirmed over several years. Alternatively, QTLs decreasing the production of dispersal morphs on *Vat* plants could be sought. The identification of such QTLs is probably feasible in melon accessions displaying resistance to aphids *sensu stricto* (no elicitation of resistance to virus by *A. gossypii*). This phenotype, like classical phenotypes of resistance to aphids described in other crops, has already been observed in the natural range of melon diversity (Boissot et al., 2016). QTLs controlling this phenotype could be combined with *Vat* resistance in a melon breeding program.

EFFECT OF *VAT* ON VIRUSES AT THE POPULATION LEVEL AND DURABILITY OF RESISTANCE TO VIRUSES

The effect of *Vat* on virus epidemics is poorly documented. Field experiments were conducted in France in the late 1970s to compare the development of CMV in *Vat* accession PI 161375 and a non-*Vat* melon cultivar (Lecoq and Pitrat, 1983). CMV progression curves had the same general "S" shape, with a steep slope, but disease onset was always earlier in the susceptible plots, with symptoms observed 12–24 days later in *Vat* plots than in non-*Vat* plots. This evaluation continued into the 1980s, with the resistant cultivar Virgos (Lecoq and Pitrat, 1989). In accordance with previous results, resistance delayed the CMV epidemic development and greatly decreased the rate of disease increase. It should be borne in mind that PI 161375 and Virgos carry composite resistance to CMV: *Vat* and the oligogenic and recessive resistance to 'common' CMV strains (Guiu-Aragones et al., 2014). It is, therefore, difficult to determine the actual contribution of *Vat* to the control of CMV epidemics. These experiments were more informative for WMV, because 'resistance to common CMV strains' is not effective against WMV. In *Vat* melon plots, WMV epidemics were delayed slightly (by about 5 days), with no significant reduction of the rate of disease increase (Lecoq and Pitrat, 1989).

Recent studies in South-East France compared virus epidemic development in melon lines differing only by the presence/absence of *Vat* (Schoeny et al., 2014; Boissot et al., 2015). In most field trials, *Vat* had a significant effect on CMV epidemics, mostly by reducing the rate of disease increase. It had no effect on WMV epidemics, probably because *A. gossypii* is not the principal aphid vector of this virus.

The partial effect of *Vat* on CMV epidemics is consistent with *Vat* resistance being elicited by only a proportion of the viruliferous aphids visiting melon crops. Indeed, more than 80 aphid species are able to transmit CMV, therefore viruliferous aphids belonging to these species trigger epidemics when they visit *Vat* and non-*Vat* melon crops. Moreover within *A. gossypii*, it remains unclear whether *A. gossypii* not belonging to the Cucurbit host-race can elicit resistance to CMV resistance. Nevertheless, the partial effect of *Vat* on CMV epidemics remains significant, probably because *A. gossypii* is one of the most efficient vectors of CMV based on laboratory experiments.

Finally, the use of *Vat* to control the spread of non-persistent viruses in melon crops is dependent on the importance of viruliferous *A. gossypii* relative to other vector species in the spread of the virus in the crop. The effect of *Vat* is not sufficient for the full control of virus epidemics in crops, but the broad spectrum of this effect and the inability of viruses to adapt to it (Boissot et al., 2016) have made this type of resistance much of a 'holy grail' for plant breeders. In the postgenomic era, it may be possible to edit this resistance gene to make it possible for any aphid species to trigger resistance or for resistance to occur without the need for aphid triggering.

Concerning persistent viruses, *Vat* steadily and significantly decreases CABYV epidemics, mostly by delaying them (Schoeny et al., 2014; Boissot et al., 2015). *A. gossypii* is the principal vector of CABYV so, even though this aspect has not yet been investigated in the laboratory, it appears likely that *Vat* affects CABYV transmission by decreasing both acquisition and inoculation rates. The effect of *Vat* on CABYV population genetic diversity has not yet been documented.

Vat resistance has only a partial effect on virus epidemics in melon and is not used in that aim by growers. As a matter of fact resistance to viruses *sensu stricto* needs to be integrated in cultivars to control virus epidemics in crops. This type of resistance to viruses has been identified in *C. melo* species, but has generally been little used in plant breeding programs (Pitrat, 2016). If deployed at a large scale in melon crops, such resistance would exert a selection pressure on viruses, placing the durability of the resistance at risk. The utility of combining *Vat* with resistance to viruses *sensu stricto* has been investigated for CMV and CABYV (Boissot et al., 2015). The epidemic data obtained for *Vat* and non-*Vat* melon crops in South-East France have been integrated into a mathematical model of the evolutionary and epidemiological processes shaping the dynamics of a virus population in a landscape composed of a seasonal cultivated compartment and a reservoir compartment containing virus throughout the year (Fabre et al., 2015). Various agro-ecological systems were considered, mimicking the situation of melon crops in South-East France. The deployment of resistance to viruses *sensu stricto* combined with *Vat* would probably be beneficial for CABYV control, but the potential benefit remains uncertain (although certainly not negative) for the long-term control of CMV. Another modeling study has suggested that the maintenance of low-population aphid populations could prevent the emergence of highly virulent CMV+N-satRNA isolates (Betancourt et al., 2016).

CONCLUSION

Finally, since the description of the 'aphid side' of the pleiotropic phenotype of *Vat* in the late 1960s, each decade has contributed to improvements in our knowledge and use of this amazing gene. The 'virus side' of the pleiotropic phenotype was elucidated in the late 1970s, with the breeding and deployment of the first *Vat* cultivars in the 1980s, and mapping in the 1990s. The assignment of this gene to the NLR gene family in the first decade of the 21st century provided clues to its mode of action, which is now at least partially understood. A succession of new technologies over this period provided new insight into the pleiotropic phenotype of *Vat*. Our knowledge of the genetic diversity of *A. gossypii* has also been refined over time. *A. gossypii* genetic diversity presents a major challenge to *Vat* resistance in the field, but also provides us with opportunities to extend our knowledge of the mechanisms underlying *Vat* resistance.

The *A. gossypii*/melon interaction can be investigated within the broader *A. gossypii*/cucurbit interaction, for at least two points. First, the double phenotype conferred by *Vat* makes it possible to investigate this interaction over the subgroup of *A. gossypii* constituting the Cucurbit host-race. How diverse are the *A. gossypii* strains able to elicit resistance to viruses? The VAT protein probably interacts with ligands present in the *A. gossypii* species or in the *A. gossypii* group. Once the avirulence factor interacting with the VAT protein has been identified, it will be possible to perform genetic diversity studies on that factor. Second, the particular structure of the phloem in cucurbits may play a key role in the specialization of *A. gossypii*, an insect feeding on plant sap, on cucurbits and in adaptation to *Vat* resistance, which decreases the access of *A. gossypii* to the phloem.

The double phenotype can also be used as a tool for 'reading' the recognition phase independently of the response phase, whether this response is considered in terms of the response of the plant, or that of the aphid. Promising preliminary results have been obtained with this approach, and the double phenotype could be more extensively used for such studies. The observation that some *A. gossypii* clones trigger resistance responses in *Vat* plants and are adapted to this response provides new insight into the capacity of pests and pathogens to adapt to NLR-mediated resistance in plants. The general framework for resistance mediated by such genes is that, within a pest/pathogen species, a clone/isolate is considered to have adapted if it does not trigger NLR-mediated resistance (i.e., there is no recognition phase). The models proposed for *Vat*/*A. gossypii* interaction suggest that aphid clones are adapted to *Vat* plants either because their avirulence factors do not trigger resistance or because they can colonize the plants even if plant defenses are triggered. Does this second mechanism exist in other NLR plant resistance/pest or pathogen interactions? If so, it would complicate the identification of avirulence factors, because adapted and non-adapted pests/pathogens could have identical avirulence effectors interacting (directly or indirectly) with the protein encoded by the resistance gene. It would also call into question the validity of durability modeling approaches based exclusively on gene-for-gene interaction.

The second way in which *A. gossypii* clones can adapt, such that the *A. gossypii* clones can colonize *Vat* plants whilst eliciting resistance to viruses, appears to be the most common mechanism in *A. gossypii* populations developing colonies on *Vat* crops, raising questions about the evolutionary advantages of such a mode of adaptation. This type of adaptation increases the chances of *A. gossypii* being able to colonize a melon crop free of viruses (because the viruses transmitted by this aphid are blocked in *Vat* plants), probably leading to the production of a larger number of progeny. Further studies are required to assess the advantages of these two types of adaptation.

Many different studies of *Vat* resistance have been carried out, revealing the considerable utility of this gene for addressing research questions and the limitations of the use of resistance genes in agriculture. One key question remains: is this gene really unique among the genes conferring resistance to aphids? No other aphid resistance gene has been reported to confer resistance to viruses transmitted in the non-persistent mode. However, it

is not clear how many of the many known aphid resistance genes have been tested for effects on viruses.

AUTHOR CONTRIBUTIONS

NB conceived the review and built the figures and tables. AS contributed to the introduction', the presentation of viruses transmitted by *A. gossypii* to melon crops, to molecular features to the effect of *Vat* at the whole-plant level, *Vat* effect on viruses populations and conclusion. She revised the entire manuscript. FV-M contributed to the presentation of *A. gossypii* and the part on *Vat* effect on *A. gossypii* populations. She revised the entire manuscript.

ACKNOWLEDGMENTS

We thank LW for inviting us to contribute to this special Frontier issue. We would also like to thank Michel Pitrat, Hervé Lecoq, and Catherine Dogimont for the exciting discussions we had on *Vat*. Michel Pitrat and Véronique Chovelon also reviewed part of this manuscript, and we would like to thank them for that. We also wish to thank Gérard Febvay from the French 'Biologie adaptative des pucerons et organismes associés' (BAPOA) network for his weekly bibliographic review, which is so useful, making it easier to keep up to date with progress in this field at all times.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer IK and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 15 December 2016
doi: 10.3389/fpls.2016.01872

Modulation of Legume Defense Signaling Pathways by Native and Non-native Pea Aphid Clones

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The pea aphid (*Acyrthosiphon pisum*) is a complex of at least 15 genetically different host races that are native to specific legume plants, but can all develop on the universal host plant *Vicia faba*. Despite much research, it is still unclear why pea aphid host races (biotypes) are able to colonize their native hosts while other host races are not. All aphids penetrate the plant and salivate into plant cells when they test plant suitability. Thus plants might react differently to the various pea aphid host races. To find out whether legume species vary in their defense responses to different pea aphid host races, we measured the amounts of salicylic acid (SA), the jasmonic acid-isoleucine conjugate (JA-Ile), other jasmonate precursors and derivatives, and abscisic acid (ABA) in four different species (*Medicago sativa*, *Trifolium pratense*, *Pisum sativum*, *V. faba*) after infestation by native and non-native pea aphid clones of various host races. Additionally, we assessed the performance of the clones on the four plant species. On *M. sativa* and *T. pratense*, non-native clones that were barely able to survive or reproduce, triggered a strong SA and JA-Ile response, whereas infestation with native clones led to lower levels of both phytohormones. On *P. sativum*, non-native clones, which survived or reproduced to a certain extent, induced fluctuating SA and JA-Ile levels, whereas the native clone triggered only a weak SA and JA-Ile response. On the universal host *V. faba* all aphid clones triggered only low SA levels initially, but induced clone-specific patterns of SA and JA-Ile later on. The levels of the active JA-Ile conjugate and of the other JA-pathway metabolites measured showed in many cases similar patterns, suggesting that the reduction in JA signaling was due to an effect upstream of OPDA. ABA levels were downregulated in all aphid clone-plant combinations and were therefore probably not decisive factors for aphid-plant compatibility. Our results suggest that *A. pisum* clones manipulate plant-defense signaling to their own advantage, and perform better on their native hosts due to their ability to modulate the SA- and JA-defense signaling pathways.

Keywords: ***Acyrthosiphon pisum*, pea aphid host races, plant hormones, salicylic acid, jasmonates, abscisic acid**

OPEN ACCESS

Edited by:
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University of California, Riverside, USA

Reviewed by:
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Leibniz Institute of Plant Biochemistry, Germany
Gustavo C. MacIntosh,
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *24 June 2016*
Accepted: *28 November 2016*
Published: *15 December 2016*

Citation:
Sanchez-Arcos C, Reichelt M, Gershenzon J and Kunert G (2016) Modulation of Legume Defense Signaling Pathways by Native and Non-native Pea Aphid Clones. Front. Plant Sci. 7:1872. doi: 10.3389/fpls.2016.01872

INTRODUCTION

More than 5000 aphid species are known today (Blackman and Eastop, 2000), with at least part of the diversity due to sympatric speciation initiated by individuals that switched to new host plants (Diehl and Bush, 1984; Dres and Mallet, 2002). When aphids switch to new plants they may be confronted with new defense mechanisms (Goggin, 2007; Smith and Boyko, 2007;

Howe and Jander, 2008; Wu and Baldwin, 2010) and so may be unable to establish a compatible interaction. In most cases plants will recognize new aphid invaders on the basis of herbivore-associated molecular patterns (HAMPs) that lead to HAMP-triggered immunity (Hogenhout and Bos, 2011; Kaloshian and Walling, 2016). Among the major aphid HAMPs studied are salivary proteins, such as a 3–10 kDa protein from *Myzus persicae* that can induce a defense response in *Arabidopsis thaliana* (De Vos and Jander, 2009). Several *M. persicae* salivary HAMPs have been shown to be detrimental to aphids and reduced their fecundity on *A. thaliana* and *Nicotiana tabacum* (Bos et al., 2010; Elzinga et al., 2014) presumably because of the defense reactions they trigger. For example, HAMPs induce an influx of Ca^{2+} ions, an important second messenger in signaling actions (Wu and Baldwin, 2010). Ca^{2+} ions are associated with the production of reactive oxygen species (ROS) and other defense responses (Chen et al., 1993; Mai et al., 2013; Herrera-Vasquez et al., 2015).

The best studied defense reaction in plants is the formation of phytohormones involved in signal transduction pathways (Mauch-Mani and Mauch, 2005; Pieterse et al., 2009, 2012; Cao et al., 2011; Morkunas et al., 2011; Denance et al., 2013; Wasternack and Hause, 2013; Caarls et al., 2015), among which salicylic acid (SA) and jasmonic acid-isoleucine (JA-Ile) are the two main defense-related compounds. While the SA-defense pathway has mainly been associated with the response against biotrophic pathogens, the jasmonic acid (JA-) defense pathway, mainly activated after wounding (Howe, 2004), affects herbivorous insects and necrotrophic pathogens (Pieterse et al., 2012). Both defense pathways are, however, strongly interconnected (De Vos et al., 2005; Beckers and Spoel, 2006; Koornneef and Pieterse, 2008; Pieterse et al., 2009; Gimenez-Ibanez and Solano, 2013; Caarls et al., 2015), and it is reported that SA can negatively affect JA signaling downstream of the SCF^{COI1}-JAZ complex (Koornneef et al., 2008; Zhang et al., 2009, 2013; Van Der Does et al., 2013), and that JA can suppress the SA-defense pathway (Brooks et al., 2005; Nomura et al., 2005). Synergistic interactions between SA and JA signaling have also been detected (Schenk et al., 2000; Mur et al., 2006). Additionally the timing and the sequence of SA and JA signaling initiation (Koornneef et al., 2008; Leon-Reyes et al., 2010) as well as the levels of phytohormones seem to be important for certain defense responses (Mur et al., 2006). Other phytohormones like abscisic acid (ABA) play an important role in fine tuning the defense reponse of the plants and interfere with JA and SA signaling (Mauch-Mani and Mauch, 2005; Ton et al., 2009; Cutler et al., 2010; Cao et al., 2011; Morkunas et al., 2011; Denance et al., 2013). Initially, ABA promotes early defense responses, closing stomata and stimulating callose deposition, which blocks the intrusion of the pathogen into plant tissue. In late responses, ABA interacts with other defense pathways inhibiting the SA-dependent responses or modulating the JA-dependent pathway (Yasuda et al., 2008; Ton et al., 2009; Pieterse et al., 2012; Finkelstein, 2013). Much is still to be learned about the regulation of hormonal cross talk. Nonetheless, it is assumed that these mechanisms provide plants with an adaptable system capable of tuning defense responses to different classes of attackers (Pieterse et al., 2012) and resulting in the formation of toxic or deterrent defense compounds that prevent the colonization of the plant.

Aphids employ a range of strategies to overcome plant defense (Walling, 2008; Giordanengo et al., 2010; Kamphuis et al., 2013; Will et al., 2013; Jaouannet et al., 2014). They may detoxify defense compounds, induce nutrient sinks or sequester calcium to block phloem sealing. However, many of the effector proteins in aphid saliva may hinder activation of plant defenses and so may decrease phytohormone signaling. For example, Mp55, an effector molecule from *M. persicae* suppressed the formation of three defense compounds in *A. thaliana*: 4-methoxyindol-3-ylmethyl glucosinolate, callose and hydrogen peroxide (Elzinga et al., 2014). A structural protein of the stylet sheath, important for sealing the stylet penetration site, might prevent the influx of Ca^{2+} ions and the activation of Ca^{2+}-dependent defense signaling machinery (Abdellatef et al., 2015; Furch et al., 2015). Calcium-binding proteins in aphid saliva seem to have the same effect (Will et al., 2007). In other cases, the mode of action of salivary effectors is not known. However, effector proteins like Armet and C002 from *A. pisum* (Mutti et al., 2006, 2008; Wang et al., 2015), Me10 and Me23 from the potato aphid *Macrosiphum euphorbiae*, and PIntO1 and PIntO2 from the green peach aphid *M. persicae* enhance performance on the respective host plants (Pitino and Hogenhout, 2013), and silencing of the encoding genes by RNAi reduced aphid fecundity (Mutti et al., 2006, 2008; Bos et al., 2010; Pitino et al., 2011). These proteins may also interfere with defense-signaling pathways and so alter phytohormone levels. Thus the measurement of phytohormone levels after aphid infestation may provide excellent indications about whether these insects trigger or block defense signaling on different host plants.

One of the best studied aphid species is the pea aphid *Acyrthosiphon pisum* whose genome was the first to be completely sequenced among hemipterans (The International Aphid Genomics Consortium, 2010). The pea aphid is a legume specialist feeding on crops like lentil, bean, pea, alfalfa, and clover, as well as wild legume species. About 6200 years ago it underwent a rapid diversification, which led to the development of at least 15 different sympatric host races or biotypes specialized on certain host plants (Ferrari et al., 2006, 2008; Peccoud et al., 2009a,b, 2015). A pea aphid host race performs best on its native host plant, and has a reduced fitness or cannot survive at all on other legume species. However, all pea aphid host races can perform well, sometimes best on *Vicia faba*, the universal host plant for all pea aphid biotypes characterized to date. The mechanisms that are involved in this host specialization are mostly unknown. There were attempts to find the genomic regions associated with plant adaptation of pea aphid host races (Hawthorne and Via, 2001; Jaquiery et al., 2012; Simon et al., 2015). A genome-wide study of pea aphid host races was conducted and a few loci encoding salivary proteins were identified in regions under putative divergent selection (Jaquiery et al., 2012). Investigation of feeding behavior revealed that regardless of whether they are on their native host plant or another legume species, pea aphids start to penetrate the plant and to pierce and salivate into plant cells (Schwarzkopf et al., 2013). In order to find out what is salivated into the plant, transcriptomic analysis of salivary

glands was conducted and around 600 pea aphid salivary genes were described (Carolan et al., 2011). In addition, proteins were identified by proteomic analysis of saliva (collected from artificial diet fed by aphids) or salivary glands (Carolan et al., 2009, 2011; Vandermoten et al., 2014). These salivary proteins may suppress plant-defense responses in native host plants (Will et al., 2007; Mutti et al., 2008; Pitino and Hogenhout, 2013) or trigger defense reactions in non-host plants (Li et al., 2006; Gao et al., 2008; Hogenhout and Bos, 2011). To investigate these roles, it would be useful to determine how phytohormone levels differ among various host race-host species combinations.

The pea aphid complex has become a model system for asking questions about the origin and maintenance of feeding specialization in insect herbivores. To find out why host races can perform well on their native or the universal host plant while they are not able to colonize other plants, an important step would be to measure the defense phytohormone levels to determine whether defenses are being activated or not. The detection of differences in phytohormone levels induced by native vs. non-native host races would favor the hypothesis that native aphid races are able to manipulate plant-defense activation processes for their own benefit. So far, there is just one study investigating the phytohormone response of a native host plant (*Pisum sativum*) to pea aphid infestation (Mai et al., 2014). This study however, concentrated on changes due to aphid numbers and only used an aphid clone that was native to *P. sativum*. Thus information about how pea aphid host plants react to non-native pea aphid host races is still lacking. Therefore, in this study we investigated the phytohormone response of three native host plants of the pea aphid, *Medicago sativa*, *P. sativum*, *Trifolium pratense*, and the universal host *V. faba* over a 4-day time course after infestation with native and non-native aphid clones. We analyzed levels of the JA-Ile conjugate, SA, and ABA, and also quantified several other jasmonate metabolites to explore how aphids might manipulate hormone signaling by interfering with specific biosynthetic steps. In addition, we determined the perfomance of native and non-native aphid host races on each plant species. Although data are available in the literature on pea aphid reproduction on different hosts, this information is for plants of different ages and varieties and from different growing conditions that what was used here, and did not assess the survival and growth of adult aphids.

MATERIALS AND METHODS

Plant Material

Four legume plant species: *M. sativa* cultivar (cv.) 'Giulia' (alfalfa), *T. pratense* cv. 'Dajana' (red clover), *P. sativum* cv. 'Baccara' (pea), and *V. faba* cv. 'The Sutton' (broad bean), were grown in 7-cm diameter plastic pots with a standardized soil mixture (7:20 mixture of Klasmann Tonsubstrat and Klasmann Kultursubstrat TS1, Klasmann-Deilmann GmbH, Geeste, Germany) in climate chambers maintained at 20°C, 70 ± 10% relative humidity, and 16-h light/8-h dark photoperiod. *M. sativa* and *T. pratense* were grown three plants per pot in order to get enough plant material for phytohormone analyses (approximately 10 and 6 leaves per pot, respectively), while *P. sativum* and *V. faba* were grown individually (approximately 4 leaves per pot for each species). *M. sativa* and *T. pratense* plants were used in experiments 20 days after sowing and *P. sativum* and *V. faba* 10 days after sowing.

Aphids

Three pea aphid (*A. pisum* Harris) clones, each representing one pea aphid host race, were used in the experiments: the clone L84 representing the *Medicago* race (here called MR), the clone T3-8V1 representing the *Trifolium* race (TR), and the clone Colmar representing the *Pisum* race (PR). Aphids were initially collected from their native host plants *T. pratense*, *M. sativa,* and *P. sativum*, respectively, and genotypically assigned to their respective host race [for detailed information see Supplementary Table S1 in Peccoud et al. (2009a)]. All aphids were reared on 4-week-old broad bean plants. To synchronize the age of the aphids for the experiments, five apterous female adults were placed on a broad bean plant and were allowed to reproduce for 48 h. The nymphs were then transferred to new plants and maintained for 9 days until they reached the adult age. Several serial transfers of nymphs were done until the desired number of synchronized young adult aphids was obtained. To avoid escape of aphids, all aphid containing plants were covered with air permeable cellophane bags (18.8 cm × 39 cm, Armin Zeller, Nachf. Schütz & Co, Langenthal, Switzerland), and placed in a climate chamber under the conditions described above.

Experimental Design

To determine the performance of the three different pea aphid clones of various host races, each plant species was separately infested with each pea aphid clone resulting in 12 plant species–aphid clone combinations. To evaluate the development of the different pea aphid clones over time, plants were infested with 20 adult, apterous aphids, and performance parameters were measured 24, 48, 72, and 96 h after aphid infestation and at the start of the experiment. Survival and mean weight of adult aphids (weight of all alive adult aphids on a plant divided by the number of surviving adult aphids), and the weight of all offspring per plant were measured as performance parameters. To keep the aphids as undisturbed as possible (and to duplicate the setup used in the phytohormone experiment described below), different sets of plants and aphids were used at each time point. For this performance experiment, five replicates were used.

To evaluate the response of the plant species toward infestation with the different pea aphid clones, phytohormone levels were investigated. The experimental setup was the same as for the performance experiment with 12 plant species – aphid clone combinations sampled at four-time points. Additionally plants without aphids served as controls. Ten replicates were employed.

All experimental plants, including aphid-free control plants, were covered with air permeable cellophane bags and were placed in a climate chamber under conditions as described above.

Plant Material Sampling and Extraction

For plant sampling, the aphids were removed from the plants using a paintbrush. As a control for possible induction of phytohormones due to contact with the paintbrush, control plants were brushed in the same way as aphid-infested plants. Above-ground parts of the plant seedlings were harvested and rapidly frozen in liquid nitrogen. Frozen samples were stored overnight in 2-ml Eppendorf tubes at −80°C and then freeze-dried for 48 h. Dried plant material was homogenized into a fine powder by adding three stainless steel beads (3 mm Ø) in each tube and vigorously shaking for four min in a paint shaker (Skandex shaker SO-10 m, Fast and Fluid Management, Sassenheim, The Netherlands). Portions (10 mg) of dried plant material were extracted with 1 ml ice-cold extraction solution containing 80% methanol acidified with 0.1% formic acid with deuterated or ^{13}C-labeled phytohormones as internal standards, (40 ng ml^{-1} of jasmonic acid-d_6, SA-d_4, and ABA-d_6, and 8 ng ml^{-1} of jasmonic acid-$^{13}C_6$-isoleucine conjugate). Samples were immediately vortexed for 10 s and continuously sonicated in a water bath at room temperature (20°C) for 15 min at maximum frequency (35 kHz). After centrifugation (10 min at 4,500 *g* and −10°C), supernatants were filtered using 0.45 mm PTFE AcroPrep^{TM} 96-well filtration plates (Pall Corporation, Port Washington, NY, USA) and a vacuum filtration unit. All filtered plant extracts were stored at −80°C until LC-MS/MS analysis.

Quantification of Phytohormones by LC-MS/MS

Chromatographic separation of phytohormones was performed on an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 mm × 4.6 mm, 1.8 μm, Agilent). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0–0.5 min, 10% B; 0.5–4.0 min, 10–90% B; 4.0–4.02 min, 90–100% B; 4.02–4.50 min, 100% B, 4.50–4.51 min 100–10% B, and 4.51–7.00, 10% B. The mobile phase flow rate was 1.1 ml/min. The column temperature was maintained at 25°C. An API 5000 tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turbospray ion source was operated in negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards, where available. The ion spray voltage was maintained at −4500 eV. The turbo gas temperature was set at 700°C. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, the heating gas at 60 psi and collision gas at 7 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion fragmentations as follows: *m/z* 136.9→93.0 (collision energy (CE) −22 V; declustering potential (DP) −35 V) for SA; *m/z* 140.9→97.0 (CE −22 V; DP −35 V) for SA-d_4; *m/z* 290.9→165.1 (CE −24 V; DP −45 V) for 12-oxo phytodienoic acid (OPDA); *m/z* 209.1→59.0 (CE −24 V; DP −35 V) for JA; *m/z* 215.1→59.0 (CE −24 V; DP −35 V) for JA-d_6; *m/z* 225.1→59 (CE −24 V; DP −35 V) for the two hydroxyjasmonic acid isomers (here designated OH-JA1 and OH-JA2, respectively); *m/z* 322.2→130.1 (CE −30 V; DP −50 V) for JA-Ile; *m/z* 328.2→136.1 (CE −30 V; DP −50 V) for JA-$^{13}C_6$-Ile; *m/z* 338.1→130.1 (CE −30 V; DP −50 V) for 12-OH-JA-Ile; *m/z* 352.1→130.1 (CE −30 V; DP −50 V) for 12-carboxyjasmonic acid-isoleucine conjugate (12-COOH-JA-Ile); *m/z* 263.0→153.2 (CE −22 V; DP −35 V) for ABA; *m/z* 269.0→159.2 (CE −22 V; DP −35 V) for ABA-d_6. The hydroxyjasmonic acids include the 11- and 12-hydroxy derivatives (Miersch et al., 2008; Stitz et al., 2011), but we were unable to distinguish between them. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.6 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies was verified by analyzing dilution series of standard mixtures. Phytohormones were quantified relative to the signal of their corresponding internal standard. For quantification of OPDA and OH-JA, the internal standard JA-d_6 was used applying experimentally determined response factors of 0.5 and 1.0, respectively. These response factors were determined by analyzing a mixture of OPDA and OH-JA [both kindly provided by W. Boland, MPI for Chemical Ecology, Jena, Germany; synthesized as described in Nakamura et al. (2011) and Shabab et al. (2014)] and JA-d_6 all at the same concentration. For OH-JA-Ile and COOH-JA-Ile quantification, JA-$^{13}C_6$-Ile was used as internal standard applying a response factor of 1.0 in both cases. The response factor for OH-JA-Ile was determined by analyzing a mixture of OH-JA-Ile [kindly provided by W. Boland, MPI for Chemical Ecology, Jena, Germany; synthesized as described in Jimenez-Aleman et al. (2015)] and JA-$^{13}C_6$-Ile at the same concentration. The response factor for COOH-JA-Ile was assumed to be similar. All metabolite levels are expressed in nanograms per gram dry weight (ng g^{-1} DW).

Chemicals

The sources of the phytohormone standards were jasmonic acid-d_6 (HPC Standards GmbH, Cunnersdorf, Germany), SA-d_4 (Sigma–Aldrich), ABA-d_6 (Santa Cruz Biotechnology, Dallas, TX, USA), and jasmonic acid-$^{13}C_6$-isoleucine conjugate [synthesized as described by Kramell et al. (1988) using $^{13}C_6$-Ile (Sigma–Aldrich)].

The sources of the solvents used for the phytohormone extraction were methanol (LiChrosolv®, LC-MS grade, Merck KGaA, Germany), acetonitrile (LC-MS grade, VWR Chemicals, USA), and formic acid (LC-MS grade, Fisher Scientific, Belgium).

Statistical Analysis

All data were analyzed with R version 3.2.0 (R Development Core Team, 2015).

The percentage of surviving adults was analyzed using binomial generalized linear models (glm) with time after aphid infestation as continuous and aphid clone as categorical explanatory variables. In cases of overdispersion, standard errors were corrected using quasi-glm models. *P*-values for explanatory variables were obtained by deleting explanatory variables one after another and comparison of the most complex model with the simpler model (Zuur et al., 2009).

To make the progression of aphid weight over time comparable between the different aphid clones, the weight of

surviving adult aphids is given as a percentage of the weight at the start of the experiment, which was set as 100%. These data were analyzed using a two-way ANOVA with the time points and aphid clones as categorical explanatory variables. Models were simplified by deleting non-significant variables (Crawley, 2013). To determine differences between factor levels, pairwise *t*-tests were performed and corrected for the false discovery rate. In cases where variances were unequal, the generalized least squares method [gls from the nlme library (Pinheiro et al., 2015)] was used. First, the optimal variance structure was determined by comparing models with different variance structures and choosing the one with the smallest AIC (Akaike information criterion). Models with this variance structure were used to determine the influence of explanatory variables by subsequent removal of explanatory variables from the model and comparison of the simpler with the more complex model with a likelihood ratio test (Zuur et al., 2009). Differences between factor levels were determined by factor level reduction (Crawley, 2013).

The influence of the aphid clone and time on the offspring biomass was investigated with a two-way ANOVA. To achieve homogeneity of variances, biomass data were square root transformed. Differences between factor levels were examined by pairwise *t*-tests corrected for false discovery rate.

The influence of aphid clone and duration of aphid infestation (both used as categorical explanatory variables) on the phytohormone levels was investigated using the generalized least squares method [gls from the nlme library (Pinheiro et al., 2015)] to account for the variance heterogeneity of the residuals. The varIdent variance structure was used. Whether the different variance of aphid clones, the duration of aphid infestation or the combination of both factors should be incorporated into the model, was determined by comparing models with different variance structures with a likelihood ratio test and choosing the model with the smallest AIC. The influence (*p*-values) of the explanatory variables was determined as explained above in the analysis of adult weight.

RESULTS

Aphid Host Race Clones Performed Much Better on Their Native Host Plants

To evaluate the performance of pea aphid clones of various host races on different plants over time, we determined the survival and weight of adult aphids, and the total weight of aphid offspring.

The survival of all aphid clones on all host plants decreased over time. The strength of the decrease was, however, dependent on the plant – aphid clone combination. On their respective native host plant or the universal host plant *V. faba*, more than 80% of the aphids survived for 4 days (96 h). This survival was significantly better than the survival of non-native clones (**Figures 1A–C**). On *M. sativa* hardly any (<2) of the non-native aphids survived for 96 h (**Figure 1A**). On *T. pratense* on average only 18% of the non-native *Pisum* clone (PR) survived, whereas about 48% of the non-native *Medicago* clone (MR) survived (**Figure 1B**). The only exception from this general pattern was found for aphids on *P. sativum*. There the non-native MR survived as well as the native PR, and only the non-native *Trifolium* clone (TR) showed a strongly reduced survival (**Figure 1C**). On the universal host plant *V. faba* all aphid clones survived equally well (**Figure 1D**; **Table 1**).

Surviving adult aphids on all plants lost weight significantly during the experiment (**Figures 1E–H**; **Table 2**). In general, the aphid clones on their native host plants lost significantly less weight than non-native clones. This pattern was most pronounced on *T. pratense* plants, where the native TR lost about 20% of its initial weight over the course of the experiment, whereas both non-native clones (MR and PR) lost about 60% of their original weight (**Figure 1F**). Also, on *M. sativa* both non-native clones were significantly lighter than the native MR (**Figure 1E**). On *P. sativum,* the non-native TR lost significantly more weight than the non-native MR and the native PR (**Figure 1G**). In contrast, on the universal host *V. faba*, aphids of all clones either kept their initial weight for the first 2–3 days or even gained weight. Only after this time did they start to lose weight (**Figure 1H**).

The highest amount of aphid offspring produced during the experiment came from aphid clones on their native host plants. The total weight of these offspring increased significantly over time and was always significantly higher than the weight of offspring from non-native aphid clones (**Figures 1I–K**; **Table 3**). On *M. sativa* non-native aphids produced only a few offspring. After 96 h the total weight of their offspring added up to only one-fifth of that of native aphids (**Figure 1I**). The same was observed for the non-native PR on *T. pratense*, but there the non-native MR could produce about 40% the weight of offspring produced by the native TR (**Figure 1J**). On *P. sativum,* the weight of offspring over time increased for all aphid clones but with a significantly stronger increase for the native PR (**Figure 1K**). A significant increase in offspring weight for all aphid clones was also found on the universal host *V. faba*. On this plant, the offspring weight was always highest compared to offspring weight on other plants, but also differed between aphid clones. TR produced a significantly higher mass of offspring than the other clones. (**Figure 1L**).

Clones of Native Host Races Induced Lower Levels of SA and JA-Ile Than Clones of Non-native Races

To determine how the pea aphid clones of the various host races affected the defense response of the different plant species, we measured the amounts of three plant hormones known to be involved in defense signaling, SA, JA-Ile, and ABA, in each plant species separately infested with each of the aphid clones and in uninfested control plants.

Although SA levels in uninfested control plants changed only slightly over time, large changes were occasionally observed in aphid-infested plants (**Figures 2A–D**; **Table 4**). These changes occurred in an aphid clone-specific manner. In *T. pratense*, the SA levels after infestation with the non-native clones were always significantly higher than the ones observed after infestation with the native clone and the ones occurring in uninfested control

plants. Depending on the time point, SA levels in plants infested with the native aphid clone were higher, equal or lower than the levels in uninfested control plants (**Figure 2B**). In *M. sativa*, all aphid clones elicited a significant increase in SA levels. As in *T. pratense* this increase was significantly higher in plants infested with non-native aphid clones than in plants with the native aphid clone for the first 72 h after aphid infestation. However, after this time the SA levels in plants with the non-native aphid clones decreased whereas the levels in plants infested with the native MR clone increased to significantly higher levels (**Figure 2A**).

In *P. sativum*, the SA levels changed less over time. At most time points, SA levels in plants with the native PR clone were equivalent to levels in uninfested control plants. SA levels in plants with non-native aphid clones did not follow a consistent pattern. They were higher (TR at all time points except 72 h, MR at 24 h), lower (MR at 72 h) or similar (MR at 48 h and 96 h, TR at 72 h) than those in uninfested control plants (**Figure 2C**). In contrast, the levels of SA in the universal host *V. faba* did not change very much in the first 72 h after aphid infestation for all aphid clones. However, 96 h after aphid infestation SA levels were significantly higher in aphid infested plants than in uninfested control plants. Whereas the PR clone elicited only a minimal increase, the TR and in particular the MR clone triggered a much higher increase (**Figure 2D**).

In uninfested control plants, JA-Ile levels behaved similarly to SA levels, staying constant over time or changing only

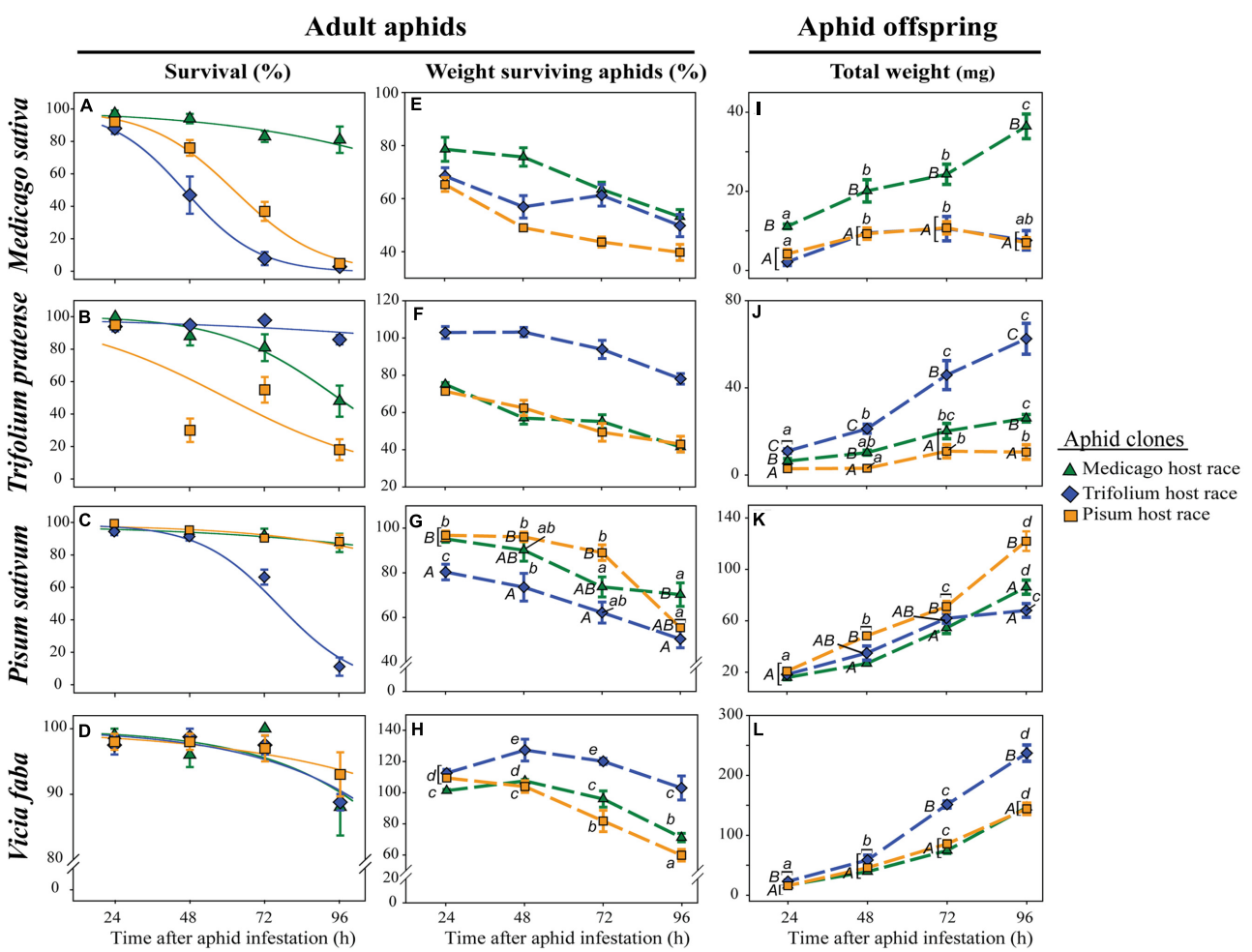

FIGURE 1 | Performance of pea aphid clones of different host races on native and non-native legume species. Survival of adult aphids **(A–D)**, mean weight of surviving adult aphids **(E–H)**, and total weight of offspring **(I–L)** are depicted for three aphid clones tested on *M. sativa*, *T. pratense*, *P. sativum*, and *V. faba* plants and measured 24, 48, 72, and 96 h after aphid infestation. The aphid clones are from the *Medicago*, *Trifolium,* and *Pisum* host races. Symbols represent means ± SE. Statistical values are given in tables 1 **(A–D)**, 2 **(E–H)**, and 3 **(I–L)**. In cases where a significant influence of the aphid clone on the weight of the surviving adults or the total weight of the offspring was dependent on the time after aphid infestation (time × race interaction), *post hoc* tests or similar methods were used to reveal differences between aphid clones at different time points. Different letters indicate significant differences ($P \leq 0.05$). Upper case letters in **(G,I–L)** indicate significant differences between aphid clones within a certain time point, while lower case letters indicate significant differences between different time points within one aphid clone. **(A–D)** Solid lines in the survival graphs are the fitted curves from the generalized linear model (glm). **(E–H)** The mean weight of surviving adult aphids is given as percentage of the weight at the start of the experiment which was set as 100%.

TABLE 1 | Statistical values for the analysis of the survival of adult aphids on different legume species according to aphid clone, time of aphid infestation, and the interaction between aphid clone and time of aphid infestation.

Plant species	Statistical test used	Factor	F/*Deviance*	*P*-value
M. sativa	glm/quasibinomial	Interaction	9.393	**<0.001**
		Clone	61.897	**<0.001**
		Time	39.620	**<0.001**
T. pratense	glm/quasibinomial	Interaction	2.201	0.121
		Clone	32.077	**<0.001**
		Time	17.905	**<0.001**
P. sativum	glm/quasibinomial	Interaction	9.402	**<0.001**
		Clone	29.848	**<0.001**
		Time	36.724	**<0.001**
V. faba	glm/binomial	Interaction	*−0.774*	0.679
		Clone	*−0.457*	0.796
		Time	*−21.990*	**<0.001**

Significant P-values are given in bold. Depending which statistical test was used F-values or Deviance are given. Deviance values are given in italics.

TABLE 2 | Statistical values for the analysis of the weight of surviving adult aphids on different legume species according to aphid clone, time of aphid infestation, and the interaction between aphid clone and time of aphid infestation.

Plant species	Statistical test used	Factor	F/*L-ratio*	*P*-value
M. sativa	ANOVA	Interaction	2.105	0.072
		Clone	30.790	**<0.001**
		Time	24.190	**<0.001**
T. pratense	ANOVA	Interaction	1.722	0.137
		Clone	152.140	**<0.001**
		Time	36.520	**<0.001**
P. sativum	ANOVA	Interaction	2.841	**0.019**
		Clone	24.307	**<0.001**
		Time	37.734	**<0.001**
V. faba	gls/varIdent error structure for each time-clone combination	Interaction	*35.768*	**<0.001**
		Clone	*24.540*	**<0.001**
		Time	*28.487*	**<0.001**

Significant P-values are given in bold. Depending which statistical test was used F-values or Likelihood ratios are given. Likelihood ratios are given in italics.

TABLE 3 | Statistical values for the analysis of the total weight of offspring produced on different legume species according to aphid clone, time of aphid infestation, and the interaction between aphid clone and time of aphid infestation.

Plant species	Transformation	Factor	*F*-value	*P*-value
M. sativa	sqrt	Interaction	3.655	**0.005**
		Clone	67.914	**<0.001**
		Time	19.396	**<0.001**
T. pratense	sqrt	Interaction	3.936	**0.003**
		Clone	84.247	**<0.001**
		Time	42.997	**<0.001**
P. sativum	sqrt	Interaction	5.113	**<0.001**
		Clone	28.904	**<0.001**
		Time	216.371	**<0.001**
V. faba	sqrt	Interaction	7.479	**<0.001**
		Clone	66.321	**<0.001**
		Time	481.858	**<0.001**

Significant P-values are given in bold.

slightly compared to changes triggered by aphid infestation. The strength of the aphid-triggered changes was aphid clone dependent (**Figures 2E–H**; **Table 4**). In *M. sativa* and *T. pratense* during the first three time points after aphid infestation, the JA-Ile concentration was significantly higher in plants with non-native clones compared to plants infested with the native clone or uninfested control plants. When infested with the native clone JA-Ile levels in *T. pratense* plants were in the same range as those in uninfested control plants, whereas JA-Ile levels in *M. sativa* were mostly significantly higher than the levels in the uninfested control plants. For both plant species the JA-Ile levels of plants infested with the native aphid clone increased after 72 h and reached similar levels as in plants infested with non-native aphids at 96 h after aphid infestation (**Figures 2E,F**).

When the native PR clone fed on *P. sativum* plants, the JA-Ile levels steadily increased starting from levels comparable with those in uninfested control plants, and ending with levels being significantly higher than in control plants, but lower than in plants infested with non-native aphid clones (MR, TR). Levels in plants infested with non-native aphid clones fluctuated over time, being as low as in control plants (TR at 24 h, MR at 72 h) or significantly higher than in control plants (TR at 48, 72, and 96 h, MR at 24, 48, and 96 h) (**Figure 2G**). In *V. faba* plants, JA-Ile levels increased in all aphid-infested plants from 24 to 48 h being always higher than levels in the control plants. Afterward JA-Ile levels triggered by aphids fluctuated in a clone specific manner over time. At 96 h after aphid infestation, JA-Ile levels in aphid-infested plants were lower (PR- and TR-infested plants), or higher (MR-infested plants) than in uninfested control plants (**Figure 2H**).

Abscisic acid levels fluctuated over time in all four plant species (**Figures 2I–L**; **Table 4**), and fluctuated depending on the aphid clone in all plant species but *V. faba*. There were no differences between native and non-native clones. ABA levels in aphid-infested plants were generally either reduced or were similar to levels in uninfested control plants (**Figures 2I–L**). Only in *M. sativa* 24 h after aphid infestation, ABA levels in aphid-infested plants were higher than in uninfested control plants (**Figure 2I**).

Clones from Native Host Races Induced Lower Levels of JA-Pathway Metabolites Than Non-native Races

To obtain information about the effect of pea aphid infestation on the formation and further metabolism of the active jasmonate, the JA-Ile conjugate, we measured the levels of its precursors the 12-oxo phytodienoic acid (OPDA), and JA, as well as its

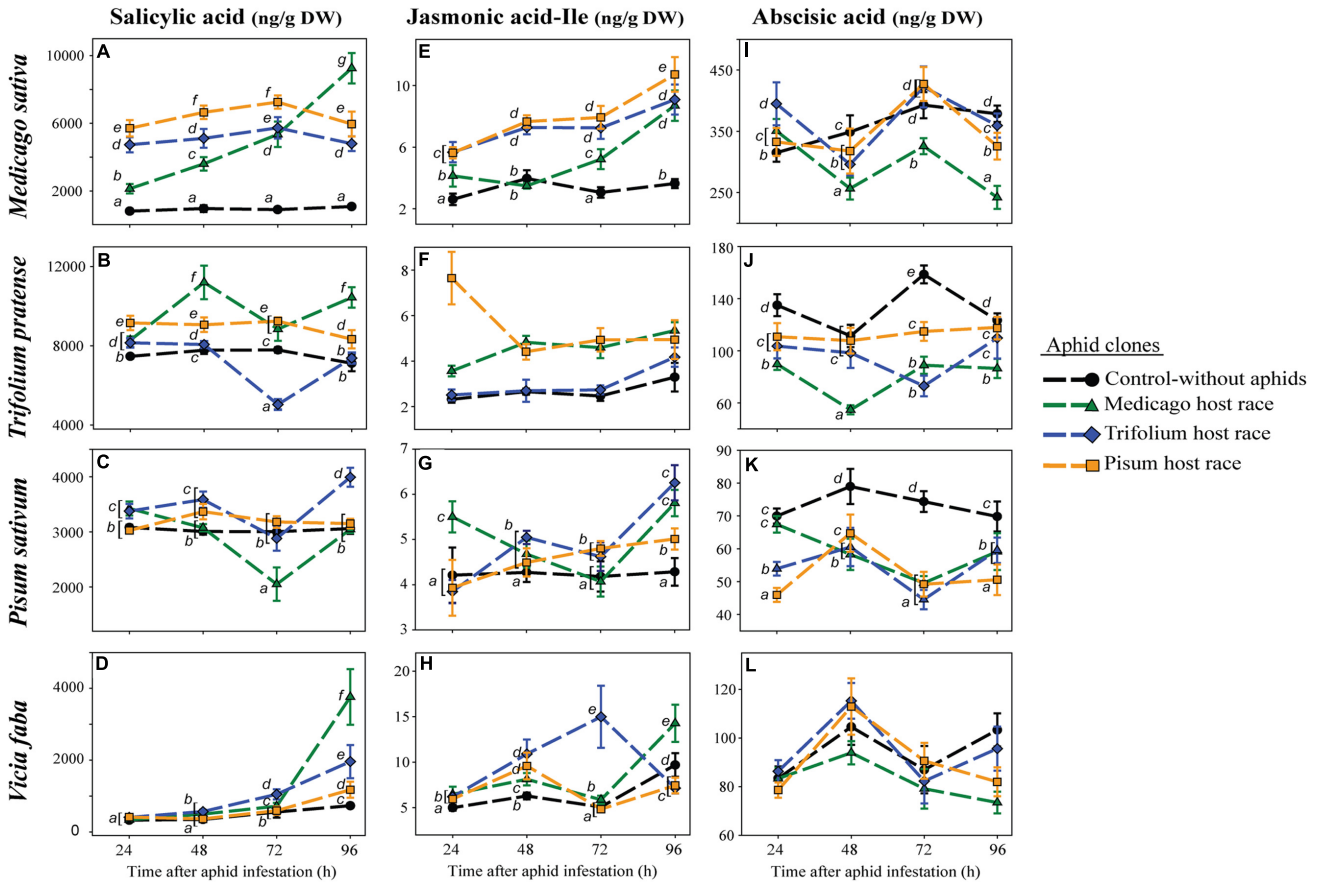

FIGURE 2 | Levels of salicylic acid (A–D), jasmonic acid-isoleucine **(E–H)** and abscisic acid **(I–L)** in legume plants after infestation with pea aphid clones of different host races. Symbols represent means ± SE. Statistical values are presented in **Table 4**. In cases where a significant influence of the aphid clone on the phytohormone level was dependent on the time after aphid infestation (interaction), significant differences ($P \leq 0.05$) between aphid clones at different time points are indicated by different letters.

metabolites, the 12-hydroxyjasmonic acid-isoleucine conjugate (12-OH-JA-Ile), the 12-carboxyjasmonic acid-isoleucine conjugate (12-COOH-JA-Ile), and two hydroxylated forms of unconjugated JA (OH-JA1 and OH-JA2).

In *M. sativa*, all measured JA-Ile precursors and further metabolites generally had significantly lower levels after infestation with the native clone MR than after the non-native clones TR and PR (**Figure 3**; **Table 4**). This pattern was especially visible for the JA-Ile precursors, OPDA and JA (**Figures 3A,B**). The levels of the hydroxylated and carboxylated forms of JA and JA-Ile were mostly lowest in plants infested with the native MR clone, similar to the levels in uninfested control plants, but increased after 72 h reaching sometimes levels comparable to the ones in plants infested with non-native aphids 96 h after aphid infestation (**Figures 3E–G**).

Equivalently in *T. pratense*, levels of the precursors of JA-Ile, OPDA, and JA were always significantly lower after infestation with the native TR clone than the non-native clones MR and PR (**Figures 4A,B**). At 24 h after aphid infestation, plants harboring the native aphid clone TR had OPDA levels even below the concentration in uninfested control plants (**Figure 4A**; Supplementary Table S2). This strong downregulation was also visible for OH-JA2 (**Figure 4E**), whereas the other metabolite of JA, OH-JA1, was not detectable in *T. pratense*. Also the levels of the hydroxylated derivatives of JA-Ile were higher in plants infested with the non-native aphid clones (**Figures 4E,F**). Levels of the carboxylated JA-Ile derivative fluctuated without evidence of a specific pattern. Of all the aphid-infested plants those infested with the native aphid clone TR showed levels most similar to the levels in uninfested control plants (**Figure 4G**).

In contrast to the other plant species, *P. sativum* did not possess detectable levels of the metabolized forms of JA or JA-Ile (**Figure 5**). Levels of both JA-Ile precursors, OPDA and JA, changed over time in a clone-specific way (**Figures 5A,B**; **Table 4**) with levels in plants infested with the native PR clone usually being most similar to levels in uninfested control plants.

In the universal host, *V. faba*, levels of the JA-Ile precursors, OPDA and JA, did change over time but in an aphid clone-specific way. At most time points both precursor levels were higher in plants infested by each of the aphid clones than

TABLE 4 | Statistical values for the analysis of phytohormone levels in different legume species according to aphid clone, time of aphid infestation, and the interaction between aphid clone and time of aphid infestation.

Phytohormone	Factor	*Medicago sativa*			*Trifolium pratense*			*Pisum sativum*			*Vicia faba*		
		Variance	*L*-ratio	*P*-value	Variance	*L*-ratio	*P*-value	Variance	*L*-ratio	*P*-value	Variance	*L*-ratio	*P*-value
SA	Interaction	Treat	39.153	**<0.001**	Treat	50.454	**<0.001**	Treat	36.765	**<0.001**	Treat	27.395	**0.001**
	Time		7.336	0.062		8.873	**0.031**		1.682	0.641		47.924	**<0.001**
	Clone		222.283	**<0.001**		52.913	**<0.001**		15.691	**0.001**		7.499	0.058
JA	Interaction	Treat	25.652	**0.002**	Treat	46.036	**<0.001**	Treat	59.924	**<0.001**	Treat	46.836	**0.001**
	Time		35.843	**<0.001**		2.629	0.452		17.347	**<0.001**		41.140	**<0.001**
	Clone		58.841	**<0.001**		84.842	**<0.001**		11.623	**0.009**		10.147	**0.017**
ABA	Interaction	Treat	29.659	**<0.001**	Race	25.066	**0.003**	Time	31.306	**<0.001**	Time	12.635	0.180
	Time		24.361	**<0.001**		21.067	**<0.001**		9.962	**0.019**		22.568	**<0.001**
	Clone		10.416	**0.015**		61.095	**<0.001**		63.165	**<0.001**		5.243	0.155
OPDA	Interaction	Treat	83.702	**<0.001**	Treat	40.459	**<0.001**	Treat	33.652	**<0.001**	Treat	61.859	**<0.001**
	Time		41.687	**<0.001**		3.666	0.300		20.965	**<0.001**		58.519	**<0.001**
	Clone		75.721	**<0.001**		51.441	**<0.001**		16.154	**0.001**		10.430	**0.015**
JA-Ile	Interaction	Treat	36.223	**<0.001**	Treat	15.788	0.071	Time	23.719	**0.005**	Treat	26.854	**0.002**
	Time		17.566	**<0.001**		19.783	**<0.001**		17.909	**<0.001**		34.264	**<0.001**
	Clone		99.135	**<0.001**		78.228	**<0.001**		11.373	0.251		8.941	**0.030**
OH-JA1	Interaction	Treat	29.956	**<0.001**							Time	37.378	**<0.001**
	Time		45.957	**<0.001**		Not detectable			Not detectable			13.691	**0.003**
	Clone		49.960	**<0.001**								11.000	**0.012**
OH-JA2	Interaction	Treat	76.052	**<0.001**	Treat	19.502	**0.021**				Treat	37.372	**<0.001**
	Time		7.005	0.072		26.260	**<0.001**		Not detectable			34.891	**<0.001**
	Clone		117.077	**<0.001**		91.853	**<0.001**					1.158	0.763
OH-JA-Ile	Interaction	Treat	82.928	**<0.001**	Race	10.144	0.339				Treat	13.692	0.134
	Time		12.537	**0.006**		2.353	0.502		Not detectable			28.695	**<0.001**
	Clone		23.852	**<0.001**		53.008	**<0.001**					9.006	**0.029**
COOH-JA-Ile	Interaction	Treat	29.033	**<0.001**	Treat	27.277	**0.001**						
	Time		4.712	0.194		7.143	0.068		Not detectable			Not detectable	
	Clone		32.150	**<0.001**		1.808	0.613						

To account for the variance heterogeneity of the residuals the varIdent variance structure was used. Under "Variance" it is specified whether the variance is controlled for each aphid clone-plant species combination (Treat), for each aphid race (Race) or for each time point (Time). Significant P-values are given in bold.

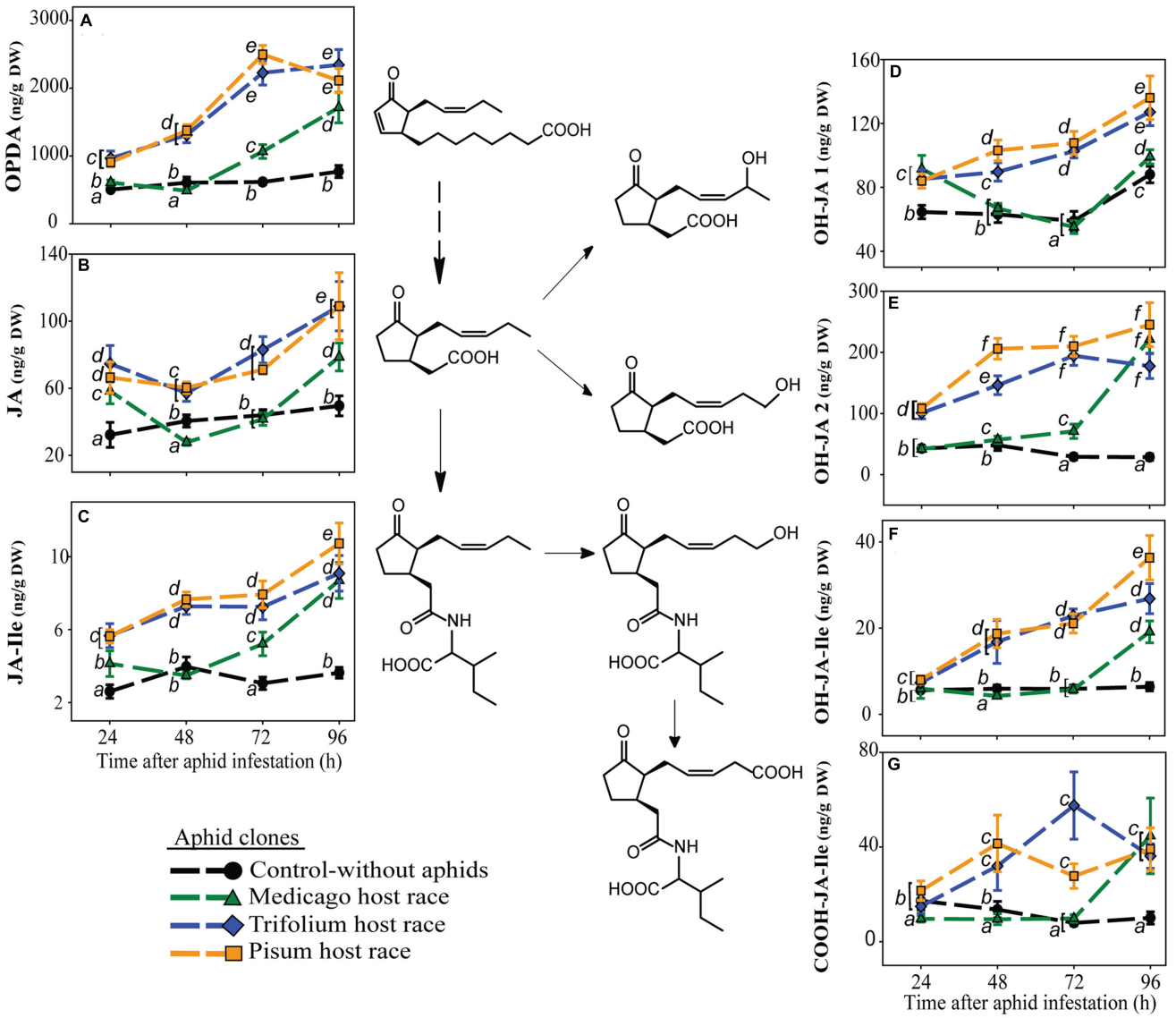

FIGURE 3 | Level of JA-pathway metabolites in *M. sativa* plants after infestation with pea aphid clones of different host races. Symbols represent means ± SE. Statistical values are presented in **Table 4**. JA-pathway metabolites are 12-oxo phytodienoic acid (OPDA) **(A)**, jasmonic acid (JA) **(B)**, JA-isoleucine conjugate (JA-Ile) **(C)**, two hydroxyjasmonic acid isomers OH-JA1 **(D)** and OH-JA2 **(E)**, 12-hydroxyjasmonic acid-isoleucine conjugate (OH-JA-Ile) **(F)**, and 12-carboxyjasmonic acid-isoleucine conjugate (COOH-JA-Ile) **(G)**. Different letters indicate significant differences between treatments ($P \leq 0.05$).

in uninfested control plants. This difference was much more pronounced for OPDA than for JA (**Figures 6A,B**). However, 96 h after aphid infestation OPDA levels were significantly lower in plants infested with the PR and TR clones than in uninfested control plants, and JA levels were similar to (for PR) or lower than (for TR) in uninfested control plants (**Figures 6A,B**). In contrast, the MR clone caused very high JA levels 96 h after aphid infestation (**Figure 6B**), and this increase carried over to the other JA metabolites detected in MR-infested *V. faba*, JA-Ile, OH-JA1, OH-JA2, and 12-OH-JA-Ile (**Figures 6C–F**). The carboxylated form of JA-Ile, 12-COOH-JA-Ile, could not be detected in *V. faba*.

For other aphid clones, levels of JA and JA-Ile metabolites were either decreased by aphid infestation (OH-JA1, **Figure 6D**) or were similar to those in uninfested control plants (OH-JA2, **Figure 6E**, and 12-OH-JA-Ile, **Figure 6F**). There were only a few significant changes in JA and JA-Ile metabolites in the control uninfested plants (e.g., **Figures 6C,D**). The ones that occurred may be ascribed to developmental changes or attempts to mimic the experimental manipulations performed on the infested plants (enclosure in an air-permeable cellophane bag to prevent aphid escape, leaf brushing to remove aphids before sampling) on the controls as well.

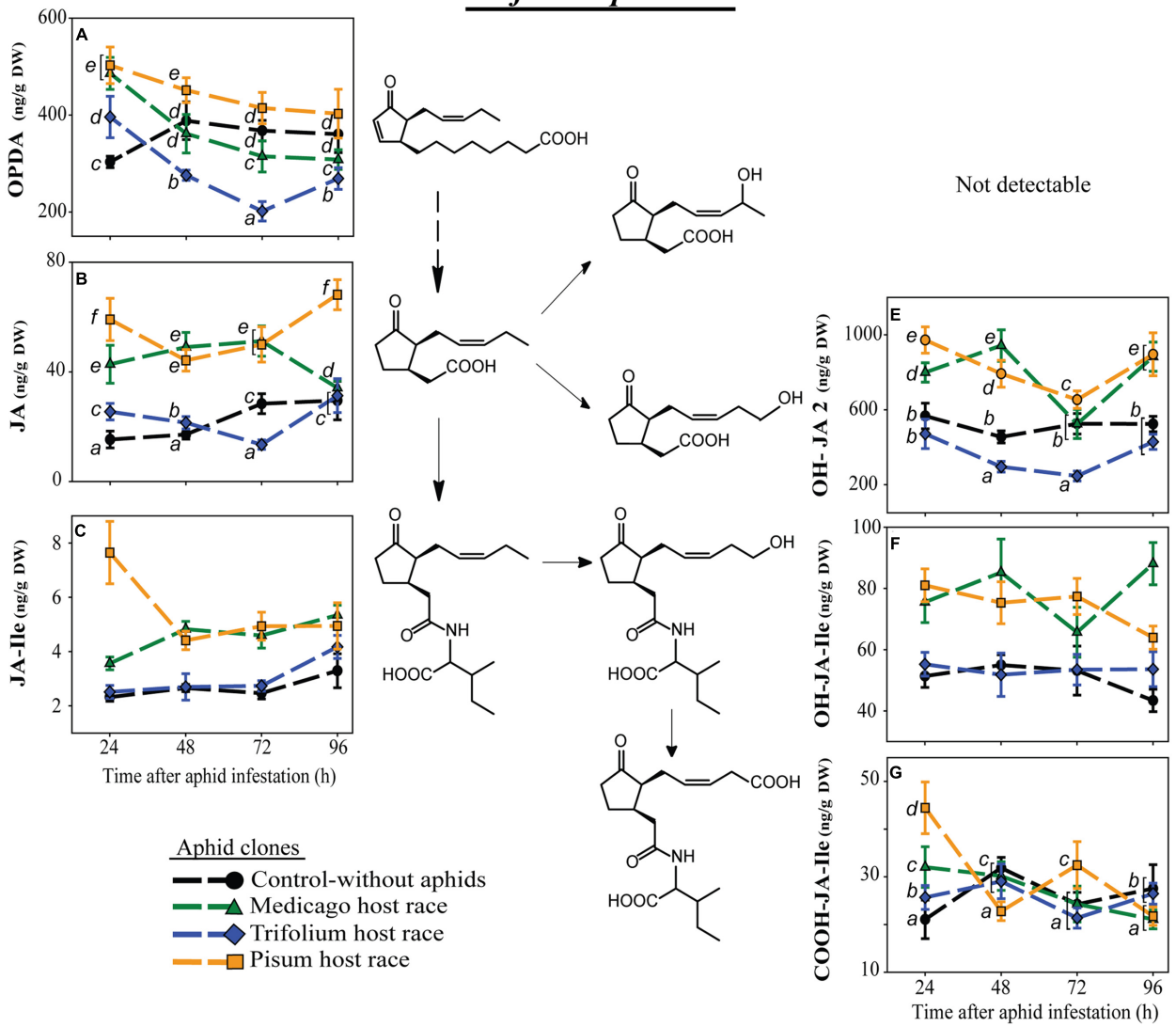

FIGURE 4 | Level of JA-pathway metabolites in *T. pratense* plants after infestation with pea aphid clones of different host races. Symbols represent means ± SE. Statistical values are presented in **Table 4**. JA-pathway metabolites are 12-oxo phytodienoic acid (OPDA) **(A)**, jasmonic acid (JA) **(B)**, JA-isoleucine conjugate (JA-Ile) **(C)**, hydroxyjasmonic acid isomer (OH-JA2) **(E)**, 12-hydroxyjasmonic acid-isoleucine conjugate (OH-JA-Ile) **(F)**, and 12-carboxyjasmonic acid-isoleucine conjugate (COOH-JA-Ile) **(G)**. In cases where a significant influence of the aphid clone on the phytohormone level was dependent on the time after aphid infestation (interaction), significant differences ($P \leq 0.05$) between aphid clones at different time points are indicated by different letters.

DISCUSSION

Infestation with Native Pea Aphid Host Races Leads to Lower Jasmonate and Salicylate Signaling

When legume plants were infested with clones of different pea aphid host races, several distinct patterns of phytohormone response were observed depending on the legume species, the pea aphid clone, the compatibility between plant and aphid, and the duration of the aphid infestation. In *T. pratense* and *M. sativa*, the concentration of the active form of the JA, JA-Ile, corresponded well with the aphid performance. Non-native aphids elicited a strong JA-Ile response whereas infestation with native aphid clones led to a much weaker induction (MR on *M. sativa*) or even to a near total absence of JA-Ile induction (TR on *T. pratense*). This weaker induction could be due to a lack of recognition of the aphid by the plant or an active suppression, which seems more likely since the JA pathway is usually activated upon wounding. When aphids initially penetrate plant tissue they regularly pierce and salivate into cells before arriving at the phloem and attempting to feed. Since aphids spend more time in this penetration phase on native than on non-native host plants (Schwarzkopf et al., 2013), they likely also pierce

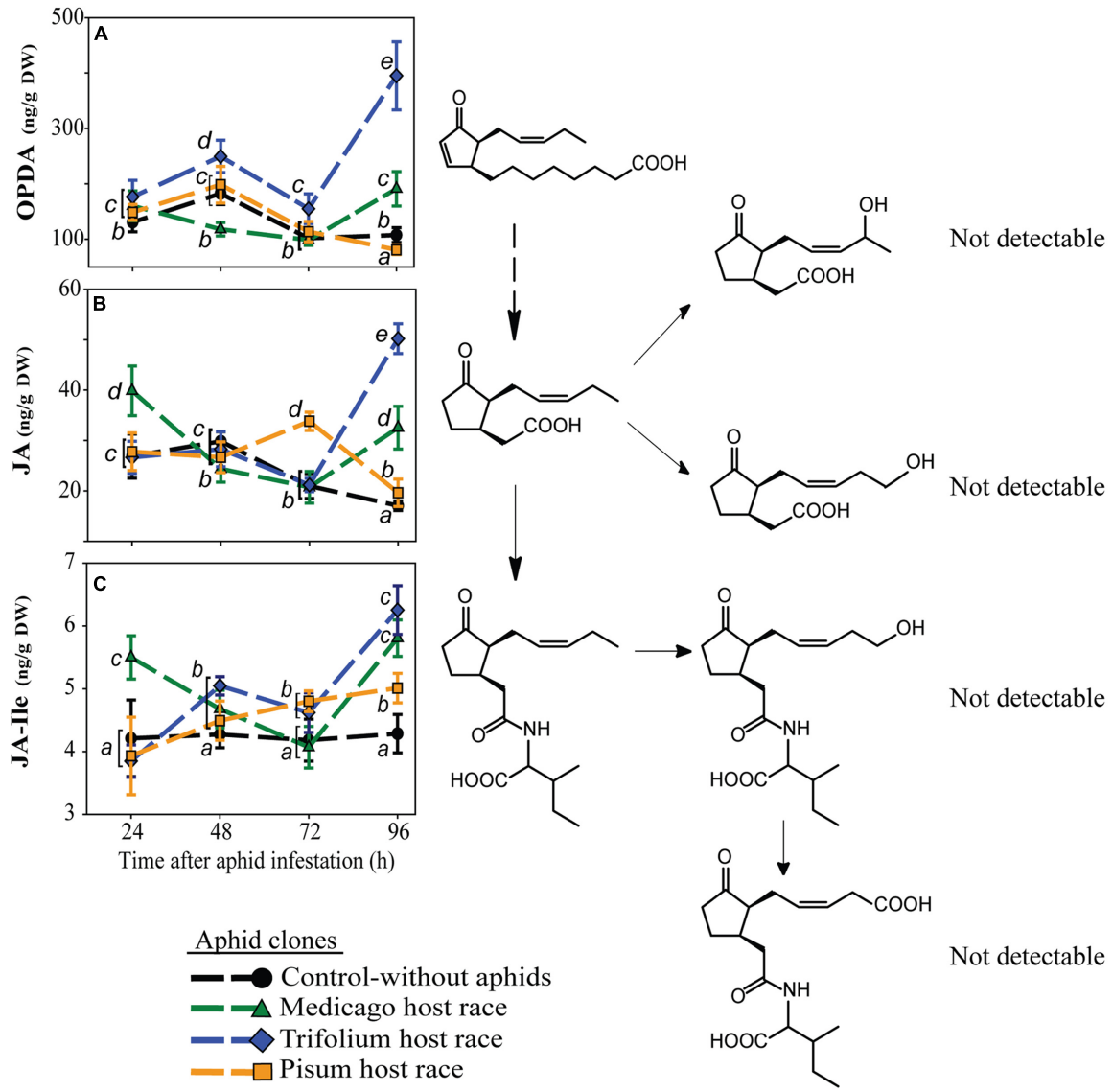

FIGURE 5 | Level of JA-pathway metabolites in *P. sativum* plants after infestation with pea aphid clones of different host races. Symbols represent means ± SE. Statistical values are presented in **Table 4**. JA-pathway metabolites are 12-oxo phytodienoic acid (OPDA) **(A)**, jasmonic acid (JA) **(B)**, and JA-isoleucine conjugate (JA-Ile) **(C)**. Different letters indicate significant differences between treatments ($P \leq 0.05$).

more cells and cause more tissue damage on native hosts. More damage should result in a stronger JA response to native aphid clones than to non-native clones, but this was not the case. Thus aphids on their native host were either able to hide the damage they caused from plant recognition systems or to actively suppress the plant-defense response. The defense suppression hypothesis is also supported from the finding that previous pea aphid infestation resulted in an increased performance of conspecific offspring (Takemoto et al., 2013). Similar effects are known from other aphid species like the soybean aphid *Aphis glycines* (Varenhorst et al., 2015). This conclusion also suggests that a strong up-regulation of JA-defense signaling is responsible for the low performance of non-adapted aphid clones.

The efficacy of JA-defense signaling has been shown in several other plant-aphid interactions. For example, in *A. thaliana* Ellis et al. (2002) recognized an enhanced resistance against *M. persicae* after the activation of the JA pathway. Genetic data also support the idea that the JA-defense pathway is the important one in plant-aphid interactions. Ten out of 13 tested genes associated with the JA pathway were induced only in *Medicago truncatula* plants resistant to *A. kondoi*, and not in susceptible *M. truncatula*, whereas all tested genes related to the SA pathway

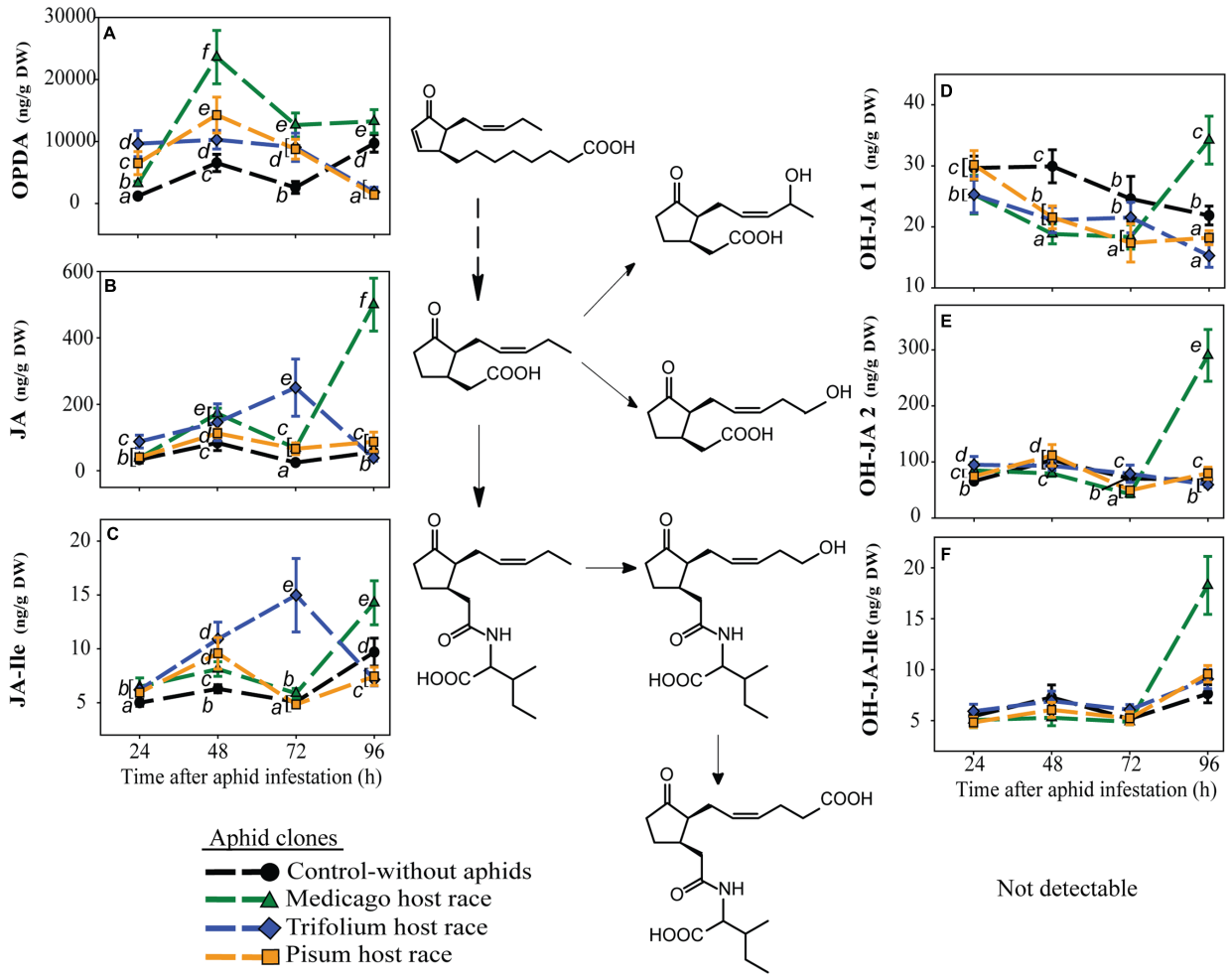

FIGURE 6 | Level of JA-pathway metabolites in *V. faba* plants after infestation with pea aphid clones of different host races. Symbols represent means ± SE. Statistical values are presented in **Table 4**. JA-pathway metabolites are 12-oxo phytodienoic acid (OPDA) **(A)**, jasmonic acid (JA) **(B)**, JA-isoleucine conjugate (JA-Ile) **(C)**, two hydroxyjasmonic acid isomers OH-JA1 **(D)** and OH-JA2 **(E)**, and 12-hydroxyjasmonic acid-isoleucine conjugate (OH-JA-Ile) **(F)**. In cases where a significant influence of the aphid clone on the phytohormone level was dependent on the time after aphid infestation (interaction), significant differences ($P \leq 0.05$) between aphid clones at different time points are indicated by different letters.

were induced independently of the susceptibility of the plant (Gao et al., 2007).

The overall negative relation between aphid performance and JA levels was only partially true for SA. For instance, after 96 h on its native host, *M. sativa*, the MR clone elicited a high SA as well as a high JA response just as high or higher than that elicited by the non-native aphid clones, but in contrast to the non-native clones MR aphids survived and reproduced well on *M. sativa*. SA levels or the expression of SA-related genes have often been reported to be upregulated due to aphid infestation (Moran and Thompson, 2001; De Vos and Jander, 2009; Mai et al., 2014; Zhang et al., 2015; Stewart et al., 2016), and so we cannot yet exclude its importance for the pea aphid. Such an SA upregulation can also be triggered by factors derived from aphid endosymbionts, which might enter the plant via insect saliva. This is known for the GroEL protein of the obligate aphid endosymbiont *Buchnera aphidicola* (Chaudhary et al., 2014), which induced SA-defense marker gene expression. Transgenic *A. thaliana* lines expressing GroEL exhibited a significant but small reduction in aphid fecundity. Thus SA-related defense triggered by endosymbionts led to a fitness cost but was not strong enough to prevent aphid increase.

Regardless of whether the JA- or SA-defense pathway was most effective against non-native aphids in our experiments, our measurements of aphid performance and phytohormone levels suggest that the native aphid clones (clone TR on *T. pratense*, clone MR on *M. sativa*) were able to suppress plant defenses on their native host plants (*T. pratense* and *M. sativa*). This suppression may not have been complete since at 96 h after aphid infestation JA-Ile levels of *M. sativa* infested by the native clone

equaled levels in most plants infested by non-native clones. Such an increase might be due to the increased number of aphids on the plant, which is known to influence the level of defense signaling (Mai et al., 2014; Stewart et al., 2016). Nevertheless the native MR clone survived and developed on its native host much better than non-native clones indicating its ability to cope with both the constitutive and any induced defense of the plant (Walling, 2008).

A different pattern of phytohormone response was observed in *P. sativum* after pea aphid infestation. This plant is the native host of the PR clone, but the other aphid clones also showed substantial survival, growth and reproduction on this plant (Figure 1, Schwarzkopf et al., 2013). The intermediate performance of non-native clones on *P. sativum* was also reflected in the SA and JA response of the plant. In contrast to the patterns for *M. sativa* and *T. pratense*, non-native aphids did not trigger a strong, consistent induction of JA-Ile and SA over the whole time course, except at 96 h after infestation when the non-native clones elicited higher JA-Ile levels than the native PR clone. Infestation with the non-native clones also caused stronger fluctuations in JA-Ile and SA profiles over time compared to infestation with the native clone. Such fluctuations were also reported for JA and JA methyl ester in *P. sativum* plants after pea aphid infestation (Mai et al., 2014). In the *A. thaliana* – *Brevicoryne brassicae* system, JA-related gene transcripts also showed fluctuations after infestation (Kusnierczyk et al., 2008). Whether these fluctuations were an expression of the intermediate ability of the aphids to deal with the plant response remains an open question. Aphid performance may be a consequence of their influence on plant-defense signaling pathways or their tolerance of defense toxins, deterrents and phloem-sealing mechanisms.

On the universal host plant *V. faba* both the JA- and the SA-regulated plant defenses seemed to be non-effective since clones of all host races performed very well in comparison to on other host plants. That pea aphids can positively influence *V. faba* for their own benefit was already reported by Takemoto et al. (2013), who observed that *A. pisum* nymphs developed faster when they could feed on *V. faba* plants previously infested by pea aphids. Since pre-infested *V. faba* produced less JA than uninfested control plants, the involvement of JA-related defenses was presumed. The pattern of phytohormone changes in this species was different than that for any other host plant. Basal SA levels were much lower than in all the other measured plant species. The levels were low for all clones until the last time point when they rose significantly with respect to those of uninfested control plants, where they reached levels also found in other plant species. Thus, SA signaling did not lead to effective defense against aphids in *V. faba*. JA-Ile levels generally rose over the whole time course, but curiously JA-Ile levels for the TR and PR clones were low at the last time point, even lower than those in the uninfested control. For these clones, the low JA-Ile levels went along with a high performance on *V. faba* at 96 h.

Abscisic acid, a phytohormone long known to regulate plant growth (Cutler et al., 2010), protect against water stress (Schroeder et al., 2001), control seed dormancy and germination (Karssen et al., 1983), and participate in source-sink communication (Yu et al., 2015), has recently been found to be a major modulator of plant defense as well (Mauch-Mani and Mauch, 2005; Ton et al., 2009; Pieterse et al., 2012). ABA has been reported to interact with the JA- and SA-defense pathways. For instance, upon wounding or herbivory ABA acts synergistically with JA on the MYC branch of the JA pathway leading to an increased resistance to herbivory (Anderson et al., 2004; Yasuda et al., 2008). On the other hand, ABA can suppress SA-dependent defenses (De Torres Zabala et al., 2009; Jiang et al., 2010; Cao et al., 2011). Concerning aphids, there are several reports that infestation induced ABA levels or ABA-regulated gene expression in *Glycine max*, *M. truncatula,* and *A. thaliana* (Studham and Macintosh, 2013; Guo et al., 2015; Sun et al., 2015; Hillwig et al., 2016). In contrast, another study showed that ABA levels in *M. truncatula* were not affected or even reduced by *A. pisum* feeding (Stewart et al., 2016) a pattern we also found in our study, where ABA levels in aphid-infested plants were generally lower or very similar than those in control plants. Since this pattern held regardless of the plant or aphid clone studied, ABA does not seem to modulate defense reactions against pea aphids in legumes. However, ABA could play other roles in plant-aphid interactions. For instance, ABA-driven stomatal closure could be advantageous for aphids under dry conditions since it maintains plant turgor and so facilitates aphid feeding (Guo et al., 2015). However, by causing reductions in photosynthetic activity, ABA-induced closure of stomata could decrease the carbohydrate supply available to aphids. Interestingly, among the plant species studied, ABA levels were quite different, ranging from about 50 ng/g DW in TR infested *P. sativum* plants (72 h after aphid infestation) to more than 400 ng/g DW in PR infested *M. sativa* plants (72 h after aphid infestation). Also basal levels of ABA varied a lot between plants which suggest that changes in phytohormone levels between treatments are more important than absolute phytohormone levels.

Native Pea Aphid Host Races May Block Specific Steps in Jasmonate Signaling or Biosynthesis

To explore the mechanism by which native aphid clones might suppress the increase of JA-Ile, we investigated the levels of JA-Ile precursors and catabolites after infestation of clones of the various host races. Lower JA-Ile levels might result from lower levels of the precursors OPDA and JA, or to increased metabolism of JA-Ile to hydroxylated and carboxylated derivatives (OH-JA-Ile, OH-JA1, OH-JA2, and COOH-JA-Ile), which could inactivate JA signaling (Miersch et al., 2008; Koo and Howe, 2012; Koo et al., 2014).

The levels of OPDA, the first metabolite in the JA pathway that we measured, were different in *M. sativa* and *T. pratense* plants depending on the infesting aphid clone. In plants infested with non-native aphid clones, levels of OPDA were higher than in plants infested with the native clone, consistent with the trends in JA-Ile concentration. In the universal host plant *V. faba* OPDA levels were generally enhanced over the 72 h following aphid infestation. However, at 96 h after infestation, the TR and PR clones suppressed OPDA formation below the levels for uninfested control plants, suggesting that aphids influence

the JA pathway prior to the formation of OPDA. The fatty acid substrate of the JA pathway is α-linolenic acid (18:3), which is produced from galactolipids of chloroplast membranes (Wasternack and Hause, 2013). Recently Kanobe et al. (2015) detected less α-linolenic acid in soybean plants (*G. max*) infested with the soybean aphid (*Aphis glycine*) than in uninfested control plants or plants infested with other soybean antagonists, the soybean cyst nematode (*Heterodera glycines*) and the brown stem rot (*Cadophora gregata*). This suggests that certain pea aphid clones might suppress one of the steps in JA signaling or biosynthesis prior to the formation of α-linolenic acid. Or, the site of suppression could follow galactolipid hydrolysis. α-Linolenic acid is converted to OPDA in three steps by the sequential action of lipoxygenase (LOX), allene oxide cyclase (AOC) and allene oxide synthase (AOS) (Wasternack and Hause, 2013). The activity of LOX increased upon aphid infestation (Mai et al., 2014), while the genes encoding LOX and AOS were upregulated more strongly in wheat infested by an incompatible biotype of the Russian wheat aphid (*Diuraphis noxia*) than in wheat infested by a compatible biotype (Liu et al., 2011). Thus compatible (native) pea aphid biotypes might suppress OPDA levels by downregulating the activities of LOX or AOS.

Aphids might also reduce JA-Ile levels by accelerating catabolism to hydroxylated and carboxylated derivatives. These metabolites might additionally contribute to a partial switch-off of JA signaling (Miersch et al., 2008). In our experiments, the abundance of JA and JA-Ile metabolites was generally correlated with that of JA and JA-Ile making it unlikely that native host races owe their suppression of JA signaling to upregulation of jasmonate catabolism. In addition, jasmonate metabolite levels were often higher in plants infested with non-adapted than adapted clones. Interestingly, among the plant species studied, there was large variation in the levels of the jasmonate metabolites. For instance, *P. sativum* did not contain JA or JA-Ile metabolites in detectable amounts, while they were highest, especially OH-JA2, in *T. pratense*. *P. sativum* might use other metabolic conversions to fine tune the JA pathway, like the methylation of JA and JA-Ile resulting in methyl-JA and methyl-JA-Ile, or glycosylation leading to JA-glucoside and JA-Ile-glucoside (Gfeller et al., 2010; Koo and Howe, 2012) – compounds which were not measured in this study. Taking the species together, when the hydroxylated and carboxylated metabolites were present, their levels were of the same magnitude as JA, whereas JA-Ile was present in levels an order of magnitude lower while OPDA was present at levels 1–2 orders of magnitude higher. However, this inter-plant variation in JA metabolites may only partially represent the true differences among the species. Other JA and JA-Ile metabolites, such as methylated or glucosylated forms of JA and JA-Ile, and other JA-amino acid conjugates are known (Gfeller et al., 2010; Koo and Howe, 2012) and might occur in legumes as well.

CONCLUSION

While plants deploy many different modes of defense against aphids (Edwards and Singh, 2006; Züst and Agrawal, 2016), aphids often feed readily on their host plants. Yet our knowledge of the mechanisms by which aphids circumvent plant defenses is still quite limited. In the pea aphid complex, we have now shown that the ability of host races to feed on their native host plants may lie in their ability to manipulate defense signaling pathways either by avoiding recognition or by suppressing JA and SA signaling much more effectively on their native hosts than on non-native plants. Strikingly, this reduced JA and SA signaling triggered by native races occurred even though plant damage on native hosts was much higher due to a greater aphid population density resulting from higher growth, survival and reproduction rates. Since lower levels of the active JA-Ile conjugate were correlated with lower levels of the other JA-pathway metabolites measured (OPDA, JA, various hydroxylated and carboxylated derivatives), native host races likely block jasmonate formation upstream of OPDA. Plant ABA concentration did not change according to the native or non-native status of the infesting aphid clone indicating that ABA does not make a large contribution to the differential ability of pea aphid host races to colonize a plant.

The low levels of JA and SA in plants infested with native pea aphid host races were combined with significantly better performance. Hence native races may be able to reduce plant defenses, such as toxins, deterrents, and phloem-sealing mechanisms. Further work is necessary to identify these defense mechanisms. Additional research is also needed to understand the cause of reduced defense signals. Previous aphid work has often focused on the salivary effector proteins that are injected into host plants and the way these modulate plant processes to facilitate feeding (Hogenhout and Bos, 2011; Pitino and Hogenhout, 2013). Since all pea aphid clones, both native and non-native, are able to begin penetrating the plant (Schwarzkopf et al., 2013), but only some are able to feed and perform well, the type and quantity of these effectors may be critical in modulating plant-defense signaling and mediating aphid success. Future work on the nature of these effectors and the differences among pea aphid host races may help identify the basis for differential defense signaling.

AUTHOR CONTRIBUTIONS

CS-A and GK conceived and designed the experiments. CS-A performed the experiments. CS-A and MR executed the phytohormone analyses. CS-A and GK analyzed data. CS-A, GK, and JG interpreted the results and wrote the manuscript. All authors critically revised and consented to the final version of the manuscript.

FUNDING

This project was supported by the Max Planck Society, Germany, and the German Academic Exchange Service (DAAD) within the framework of the PPP – Procope German-French collaboration program (project code 57049694).

ACKNOWLEDGMENTS

We thank Jean-Christophe Simon (INRA, Le Rheu, France) for providing the aphid clones, Kerstin Zipfel, Christoph Scheck, Pascal Scherreiks, and Kristina Schädel for experimental help, Andreas Weber and Elke Goschala for plant cultivation, and Ilka Vosteen and Elizabeth Pringle and two reviewers for helpful comments on previous versions of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 21 October 2016
doi: 10.3389/fpls.2016.01562

Transcriptome Analysis of Green Peach Aphid (*Myzus persicae*): Insight into Developmental Regulation and Inter-Species Divergence

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OPEN ACCESS

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *28 April 2016*
Accepted: *04 October 2016*
Published: *21 October 2016*

Citation:
Ji R, Wang Y, Cheng Y, Zhang M, Zhang H-B, Zhu L, Fang J and Zhu-Salzman K (2016) Transcriptome Analysis of Green Peach Aphid (Myzus persicae): Insight into Developmental Regulation and Inter-Species Divergence. Front. Plant Sci. 7:1562. doi: 10.3389/fpls.2016.01562

Green peach aphid (*Myzus persicae*) and pea aphid (*Acyrthosiphon pisum*) are two phylogenetically closely related agricultural pests. While pea aphid is restricted to Fabaceae, green peach aphid feeds on hundreds of plant species from more than 40 families. Transcriptome comparison could shed light on the genetic factors underlying the difference in host range between the two species. Furthermore, a large scale study contrasting gene expression between immature nymphs and fully developed adult aphids would fill a previous knowledge gap. Here, we obtained transcriptomic sequences of green peach aphid nymphs and adults, respectively, using Illumina sequencing technology. A total of 2244 genes were found to be differentially expressed between the two developmental stages, many of which were associated with detoxification, hormone production, cuticle formation, metabolism, food digestion, and absorption. When searched against publically available pea aphid mRNA sequences, 13,752 unigenes were found to have no homologous counterparts. Interestingly, many of these unigenes that could be annotated in other databases were involved in the "xenobiotics biodegradation and metabolism" pathway, suggesting the two aphids differ in their adaptation to secondary metabolites of host plants. Conversely, 3989 orthologous gene pairs between the two species were subjected to calculations of synonymous and nonsynonymous substitutions, and 148 of the genes potentially evolved in response to positive selection. Some of these genes were predicted to be associated with insect-plant interactions. Our study has revealed certain molecular events related to aphid development, and provided some insight into biological variations in two aphid species, possibly as a result of host plant adaptation.

Keywords: ***Myzus persicae*, *Acyrthosiphon pisum*, nymph and adult, transcriptome, developmental regulation, synonymous and nonsynonymous substitutions, host plant adaptation**

INTRODUCTION

Aphids (Insecta: Hemiptera), a group of economically important insect pests that consume plant phloem sap, cause substantial losses of crop yield by direct feeding on host plants and by vectoring plant viruses (Dixon, 1998). More than 450 species within Aphididae attack agricultural and horticultural plants, of which over 100 are categorized as significant and economically important pests (Blackman and Eastop, 1984). While some aphids are specific to plant species in a single taxonomic family, others have an exceptionally broad host range across many plant families. Green peach aphid (*Myzus persicae*) is a generalist with a host range comprising 40 different plant families including Brassicaceae, Solanaceae, and Fabaceae. Moreover, it is the most versatile viral vector, capable of transmitting more than 100 plant viruses (Ramsey et al., 2007). In contrast, pea aphid (*Acyrthosiphon pisum*) feeds specifically on legumes. Despite different feeding habits, they are both classified in the tribe Macrosiphini within the subfamily Aphidinae (von Dohlen et al., 2006). The close relationship between the two aphids is further supported by analysis of mitochondrial and nuclear sequences as well as transcriptomic sequence comparisons (Ramsey et al., 2007; Kim and Lee, 2008). Due to the difference in host range, green peach aphids most likely ingest toxic metabolites that pea aphids would not normally encounter, such as glucosinolates in Brassicaceae and alkaloids in Solanaceae, necessitating a more complex metabolic system (Ramsey et al., 2010).

Hemipteran immature nymphs and fully developed adults sometimes differ in their feeding behavior. *Lygus hesperus* nymphs prefer developing cotton squares, whereas adults prefer vegetative structures (Snodgrass, 1998). In three spittlebug species (*Aeneolamia varia*, *A. reducta* and *Zulia carbonaria*), foliage-feeding adults are more capable of feeding upon resistant hybrid crops than root- and stem-feeding nymphs (Cardona et al., 2010). Besides host and tissue preferences, quantity of food intake can vary (Banks and Macaulay, 1965). Profiling in nymphal and adult transcriptomes could reveal biological properties that are developmental stage-specific. In Asian citrus psyllid (*Diaphorina citri*) for instance, the transcriptome comparison revealed distinct patterns of protein and energy requirements between nymphs and adults (Vyas et al., 2015). This approach has also identified differentially expressed resistance/detoxification genes, e.g., cytochrome P450, glutathione S-transferase (GST), and ATP-binding cassette transporter genes from two developmental stages of a thiamethoxam-resistant strain of whitefly (Yang et al., 2013). Contrasting gene expression among different insect developmental stages on a large scale can not only shed light on development modulation, reproduction, and developmental stage-specific interaction with host plant, xenobiotics, and invading microbes, but can also facilitate the improvement of pest management strategies (Yang et al., 2013; Tian et al., 2015; Vyas et al., 2015). However, stage-specific gene expression in immature nymphs and fully developed adults has not yet been characterized in aphids.

While comparative genomic sequence analysis has furnished tremendous information regarding genetic factors underlying inter-species divergence (Chinwalla et al., 2002; Kaufman et al., 2002; Kirkness et al., 2003; Zdobnov and Bork, 2007; Arensburger et al., 2010; Bonasio et al., 2010; Werren et al., 2010), an increasing number of studies have applied RNA-seq for this purpose, particularly in species whose genome sequences are unavailable. For example, transcriptomic comparisons have been performed between different aphids, *A. pisum* vs. *Sitobion avenae* (Wang et al., 2014), whitefly (*Bemisia tabaci*) species complexes Middle East-Asia Minor 1 vs. Mediterranean (Wang et al., 2011), ranid frogs *Rana chensinensis* vs. *Rana kukunoris* (Yang et al., 2012), ornamental primrose species *Primula poissonii* vs. *Primula wilsonii* (Zhang L. et al., 2013), and fishes, *Erythroculter ilishaeformis* vs. *Danio rerio* (Ren et al., 2014). Comparisons among pea aphid, green peach aphid and grain aphid *(S. avenae)* have enabled investigation of the transcriptome evolution and understanding of the differences in host plant adaptation and insecticide resistance among them (Ollivier et al., 2010; Ramsey et al., 2010; Wang et al., 2014). Between grain aphid and pea aphid 340 gene orthologs are considered to be under positive selection based on the rates of nonsynonymous (Ka) and synonymous (Ks) substitutions (Wang et al., 2014). Such orthologs were also identified when Ollivier et al. (2010) compared coding sequences (CDSs) derived from the genome sequence of pea aphid and EST database derived from 5 tissues of green peach aphids reared on 5 host plants (Ramsey et al., 2007). Later, Ramsey et al. (2010) sequenced the transcriptome from mixed stages of green peach aphids using 454 pyrosequencing. Besides the reads mapped to the existing ESTs, they obtained 47,832 additional unigenes with a mean length of 160 bp, from which they identified more detoxification genes in green peach aphid than in pea aphid (Ramsey et al., 2010). However, limited transcriptomic information may not fully reflect the divergence between the two species.

In this study, we performed transcriptomic sequencing of green peach aphid nymphs and adults using Illumina RNA-seq technology, *de novo* assembled sequencing reads, and annotated the resulting unigenes. Gene expression profiling between nymphs and adults identified genes potentially involved in development modulation. Furthermore, comparative transcriptomic analyses identified genes unique to green peach aphid (relative to pea aphid) and orthologous gene pairs under positive selection. Data analysis has helped expose certain genetic factors underlying host plant adaptation by the two destructive aphid species.

MATERIALS AND METHODS

Plant Growth and Insect Rearing

Arabidopsis ecotype Col-0 plants were grown in LP5 potting medium (Sun Gro Horticulture, Agawam, WA, USA) in an environmental chamber at 23°C (day)/21°C (night), 65% relative humidity (RH), and a photosynthetic photon flux density of 88 μmol m^{-2} s^{-1} with a 12-h light/12-h dark photoperiod. The green peach aphid (a tobacco-adapted red lineage from Dr. Georg Jander, Boyce Thompson Institute for Plant Research, Cornell University) had been maintained on Col-0 for over 40 generations. Age-synchronized nymphs

and adults were subjected to RNA extraction as described below.

RNA Isolation and Transcriptome Sequencing

Neonate nymphs (within 16 h) were placed on 4-week-old Col-0 plants for 4 or 8 days respectively. Sixty 4-day-old nymphs and 60 8-day-old adults were collected, immediately frozen in liquid nitrogen, and stored at −80°C for RNA extraction. Three independent biological replicates were performed for transcriptome sequencing analysis.

Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNase-Free DNase (Qiagen, Valencia, CA, USA) was added to remove residual DNA. Samples were then further purified using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Purified total RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and qualified by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Transcriptome sequencing was performed on an Illumina HiSeq 2500 platform with 125-nucleotide (nt) paired-end reads at Texas A&M AgriLife Genomics and Bioinformatics Services (College Station, TX, USA).

Sequence Assembly and Annotation

After trimming the adaptor sequences and removing short or low-quality reads (>5% unknown nucleotides or more than 20% nts with >10% error rate), the processed reads were assembled using Trinity software (Trinity Software, Inc., Plymouth, NH, USA) and clustered with TGICL Clustering tools (The Institute for Genomic Research, Rockville, MD, USA) (Pertea et al., 2003; Grabherr et al., 2011). The publically available databases, NCBI non-redundant (Nr), NCBI non-redundant nucleotide (Nt), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Cluster of Orthologous Groups of proteins (COG) were used to perform BLAST analyses to annotate the functions of these assembled unigenes (*E*-value cutoff of 10^{-5}). Blast2GO software (http://www.geneontology.org) was used for gene ontology (GO) annotations (Conesa et al., 2005).

Differential Gene Expression and RT-qPCR Confirmation

Genes differentially expressed between nymphs and adults were identified based on Fragments Per Kilobase per Million mapped reads (FPKM) values, which adjusts the number of fragments mapped to a transcript by the total number of fragments mapped to all unigenes and the length of the transcript (Mortazavi et al., 2008; Ji et al., 2013). The false discovery rate (FDR) was used for the *P*-values in multiple tests and analyses. A FDR ≤ 0.001 and an absolute value of the $\log_2$ ratio ≥ 1 provided significance threshold for gene expression differences.

To validate the FPKM analysis, expression of 20 selected genes were measured in nymphs and adults by RT-qPCR. For each total RNA sample, 2 μg RNA was used to synthesize cDNAs with random hexamer primers (Invitrogen) and M-MuLV reverse transcriptase (New England Biolabs, Beverly, MA, USA). qPCR reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol and run on the CFX384^{TM} Real Time System (BioRad, Hercules, CA, USA). Dissociation curve analyses were performed to ensure amplification specificity. Mean fold change in gene expression was calculated as described previously (Chi et al., 2011). Primer sequences are provided in Table S1. The 18S rRNA gene of green peach aphid (Acc. No. AF487712.1) was amplified as the internal control.

Functional Analysis of Differentially Expressed Unigenes

GO enrichment analysis was performed to recognize the main biological functions of differentially expressed unigenes. The hypergeometric test was performed to find significantly enriched GO terms in differentially expressed unigenes compared to the whole reference transcriptome background (Su et al., 2012; Ji et al., 2013). The *P*-value was calculated with the formula:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

where N and n are defined as the number of genes in the transcriptome and differentially expressed genes with GO annotations, respectively. The variables *M* and *m* represent the gene number in the transcriptome annotated to a certain GO term and differentially expressed genes within the group (M-m ≥ 0), respectively. The calculated *P*-value was subjected to Bonferroni correction. GO terms with corrected *P*-value, i.e., $Q < 0.05$ were considered significantly enriched.

KEGG analyses were performed to identify significantly enriched pathways represented by differentially expressed unigenes. The hypergeometric test was used in a similar way to that for GO enrichment analysis and the terms with $Q < 0.05$ were determined as enriched pathways.

Ka and Ks Analyses

To predict CDS regions, unigenes were first aligned by BLAST analyses with *E*-value cutoff of 10^{-5} to public databases in the priority order of Nr, Swiss-Prot, KEGG, and COG. Coding regions with the best match in BLAST were considered to be the CDS. Unigenes unable to be aligned to any databases were scanned by ESTScan, which may predict some coding regions. The CDSs of pea aphid were predicted from the mRNA sequence data (https://www.aphidbase.com/aphidbase/content/download/3250/33670/file/aphidbase_2.1b_mRNA.fasta.bz2).

After filtering the redundant CDSs that may result from alternative splicing, predicted CDSs of the two aphid species were used to identify orthologous genes using OrthoMCL (Li et al., 2003). Only single-copy ortholog pairs longer than 150 bp were considered as putative orthologous gene pairs. Ka, Ks, and Ka/Ks-values were computed using the YN method implemented in the software KaKs Calculator Version 1.2 (Yang and Nielsen, 2000; Wang et al., 2011, 2014). As the sequencing errors were distributed among synonymous and non-synonymous sites at equal frequencies, they were not expected to strongly influence

the results of analyses (Tiffin and Hahn, 2002; Wang et al., 2011, 2014).

RESULTS AND DISCUSSION

Illumina Sequencing Analysis and *De novo* Assembly

High-throughput RNA-seq generated the most extensive current transcriptome for the green peach aphid. After quality checks, about 74.1, 74.0, and 74.5 million reads were obtained from the three replicates of nymphs and 74.6, 76.0, and 74.3 million reads from adults (**Table 1**). All reads were deposited in the NCBI Short Read Archive (SRA, the accession number SRP073458). The reads were assembled into 89,944, 85,416, and 82,810 contigs with mean lengths of 474, 502, and 460 nt for nymphs and 81,641, 78,710, and 87,354 contigs with mean lengths of 472, 484, and 464 nt for adults (**Table 1**). Using paired-end joining and gap-filling, these contigs were finally assembled into a total of 62,627 consensus sequences with a mean length of 1460 nt. GC contents were 39.00% for nymphs and 39.63% for adults, comparable to that of the pea aphid (38.80%) (Wang et al., 2014).

Functional Annotation and Classification of the Assembled Unigenes

Of the 62,627 unigenes, 33,543 were annotated by referencing to the Nr database (Table S2); 66.66% of the annotated sequences had very strong homology ($E < 10^{-60}$), 12.02% showed strong homology ($10^{-60} < E < 10^{-30}$) and the rest 21.32% showed homology ($10^{-30} < E < 10^{-5}$) to known sequences. With respect to species, 92.30% of the unique annotated sequences matched to pea aphid, 1.45% to *Tribolium castaneum*, 0.49% to *Bombus impatiens,* and 0.41% to *Camponotus floridana*.

GO assignments were used to classify the functions of the predicted unigenes; 14,260 sequences were categorized into 46 GO terms consisting of three domains: biological process, cellular component and molecular function (**Figure 1**). The most abundantly expressed genes in "biological process" were involved in cellular process (9028), single-organism process (7075), and metabolic process (6557). In "molecular function," genes involved in catalytic (6894), binding (6678), and transporter (1137) activities were most abundantly expressed (**Figure 1**).

To better understand the biological pathways that are active in the green peach aphid, we mapped all sequences to the canonical reference pathways in the KEGG database. As a result, 23,695 sequences were assigned to 187 insect-related KEGG pathways (Table S3), with 3286 unigenes (15.47%) being involved in metabolic pathways. These annotations could be useful for further investigation of specific processes, functions and pathways.

Comparison of Gene Expression Profiles between Nymphs and Adults

When different developmental stages were compared, 1639 genes showed higher expression in nymphs and 605 higher in adults (**Figure 2**, Table S4). We performed RT-qPCR on selected genes to validate these gene expression data. Of the 20 selected genes, 18 were in agreement with RNA-seq results, suggesting good quality of transcriptomic analysis (Table S5). To gain insight into the major biological pathways represented by the differentially expressed genes, 21 enriched insect-related pathways ($Q < 0.05$) were identified using the hypergeometric test (**Table 2**); 14 were associated with "metabolism" and 3 with "digestive system," suggesting differential metabolic and digestive activities between nymphs and adults (Banks and Macaulay, 1965; Randolph et al., 1975). The most enriched pathway being "metabolism of xenobiotics by cytochrome P450" is intriguing because it may reflect developmental stage-specific interaction with the host plant. Presumably, nymphal, and adult aphids ingest different amounts of allelochemicals, given that more detoxification genes, e.g., 16 of the 23 differential P450 genes, and all differential esterase (6) and GST (1) genes, were expressed in higher abundance in adults (**Table 3**). Developmental stage-dependent variations in expression patterns have often been observed in the detoxification genes (Harrison et al., 2001; Strode et al., 2006; Yang et al., 2013). High expression of *CYP321B1* is detected in the late larval stage of tobacco cutworm (*Spodoptera litura*) (Wang et al., 2016). In *B. tabaci*, relatively high expression of *CYP6CM1* is found in adults, correlating with the observation that specific resistance to neonicotinoid imidacloprid is largely restricted to adults (Nauen et al., 2008; Jones et al., 2011). Similarly, high expression of *CYP6P9* in adults of *Anopheles funestus,* but not in larvae, explains the adult resistance (Amenya et al., 2008). In a pyrethroid resistant strain of *Anopheles gambiae*, *CYP6Z1* is expressed in adults but undetectable in larvae or pupae (Nikou et al., 2003). Direct correlation between expression levels of detoxification genes at different developmental stages and resistance to pesticides is also exemplified by the beet webworm (*Pyrausta sticticalis*) (Leonova and Slynko, 2004) and citrus red mite (*Panonychus citri*) (Liao et al., 2013; Zhang K. et al., 2013). Banks and Macaulay (1965) reported that adult aphids have higher food consumption than nymphs. Ahmad (1982) stated that increased amounts of dietary allelochemicals due to increased food consumption may explain elevated P450-mediated metabolic activity. In parallel, green peach aphid adults likely ingest more plant materials, thus more allelochemicals from host plants, necessitating higher detoxification capacity.

The differentially expressed genes were also assigned to 20 GO enriched functional groups; ontology distributions are shown in **Figure 3**. Enriched in the "biological process" and "molecular function" include cuticle formation-related groups such as "structural constituent of cuticle," "chitin-based cuticle attachment to epithelium" and "molting cycle, chitin-based cuticle." The insect cuticle, composed of chitin and cuticle proteins, not only supports and maintains the physical structure, but also serves as a natural barrier against adverse external impacts (Andersen et al., 1995). Cuticle protein comparisons among insects at different developmental stages show that, rather than being an inert structure, the insect cuticle is developmentally modified (Chihara et al., 1982; Dombrovsky et al., 2003). Consistent with these findings, among the 81 differentially expressed transcripts of cuticular proteins and their precursors we detected, 79 were highly expressed in

TABLE 1 | Summary of transcriptome parameters of green peach aphid nymphs and adults.

	Nymph			Adult		
	1[a]	2	3	1	2	3
Number of processed reads	74,068,728	74,017,762	74,553,158	74,568,296	76,017,216	74,335,676
Number of contigs	89,944	85,416	82,810	81,641	78,710	87,354
Mean length of contigs (nt)	474	502	460	472	484	464
GC content (%)	39.11	38.89	38.06	39.58	39.69	39.44
Number of unigenes	61,186	55,776	60,271	53,928	52,829	57,758
Mean length of unigenes (nt)	1054	998	957	960	965	986

[a]*Values combined all independent biological replicates.*

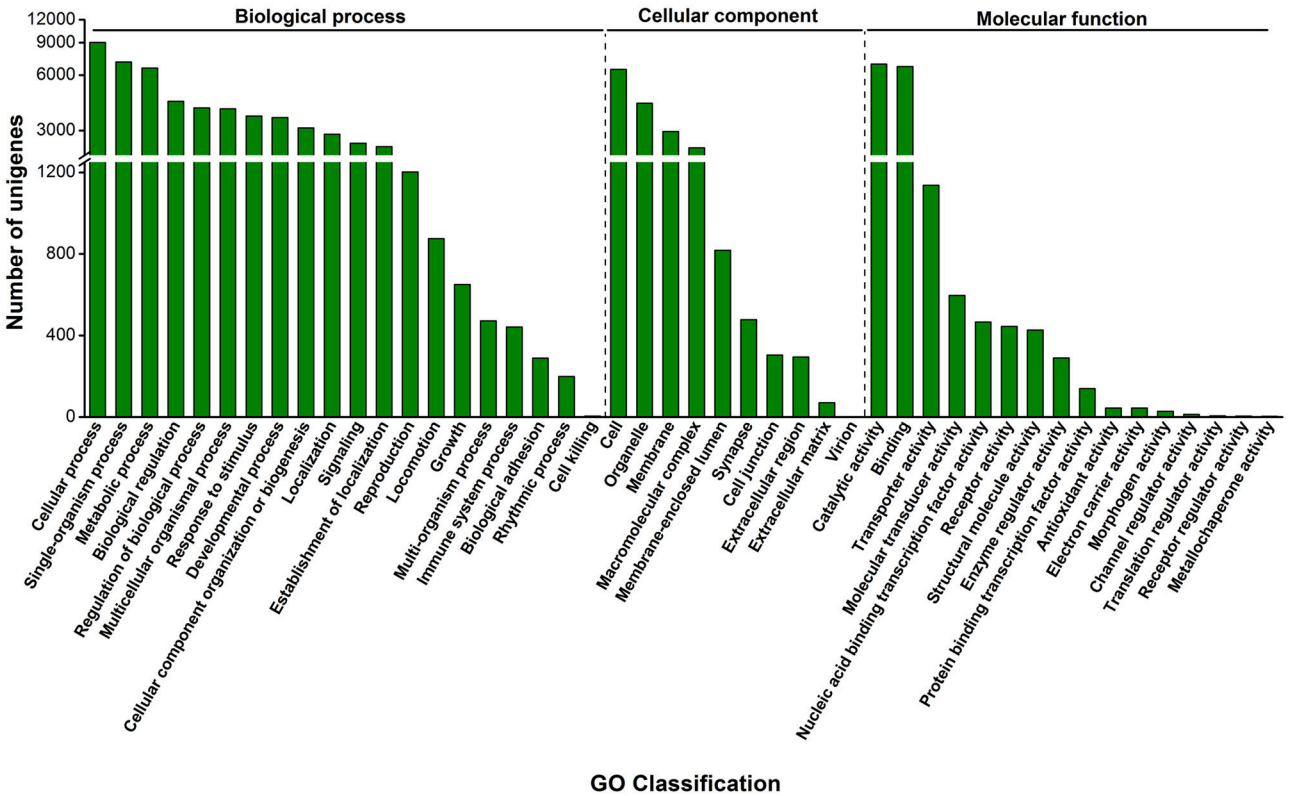

FIGURE 1 | Distribution of green peach aphid sequences by GO category. GO classification includes three domains: biological process, cellular component, and molecular function. The y-axis shows the number of matching unigenes in a category.

nymphs (**Table 3**). Insects of this developmental stage repeatedly shed their cuticles and replace them with new layers, thus their cuticle biosynthesis is likely more active. No doubt, hormones play an essential role in insect ecdysis. Enrichment of the "steroid hormone biosynthesis" pathway among the differential genes (**Table 2**) supports this notion. The major steroid hormone ecdysone plays an essential role in larval ecdysis, a process mediated by hormones, such as ecdysone and ecdysis triggering hormone (ETH) (Robbins et al., 1968; Ewer et al., 1997). Interestingly, the ETH-encoding gene *Unigene5077* was highly expressed in green peach aphid nymphs (**Table 3**).

Transcriptomic Divergences between Green Peach Aphid and Pea Aphid

Transcriptome comparisons of different aphid species could provide useful information in understanding transcriptome evolution and the genetic factors underlying the biological divergence of these species. To identify genes specific to green peach aphid (relative to pea aphid), we compared the transcriptome we obtained in this study with publically available mRNA sequence data of pea aphid. tBLASTx identified homologous pea aphid mRNAs for 41,912 of our unigenes, leaving 20,595 having no hits. After removing sequences shorter than 250 bp (too

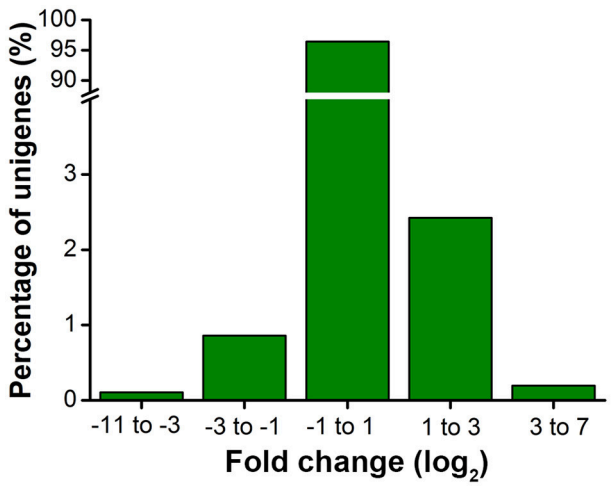

FIGURE 2 | Fold change distribution of green peach aphid unigenes between nymphs and adults. The x-axis shows the fold change (log_2 ratio) of gene expression in nymphs compared to adults. $|Log_2|$ values of 2244 unigenes are higher than 1, indicating potential importance during developmental transition.

TABLE 2 | Significantly enriched insect-related KEGG pathways represented by the genes differentially expressed between nymphs and adults.

Pathway	*Q*-value
Metabolism of xenobiotics by cytochrome P450	2.09×10^{-7}
Steroid hormone biosynthesis	1.14×10^{-6}
Retinol metabolism	1.34×10^{-6}
Pentose and glucuronate interconversions	2.76×10^{-6}
Ascorbate and aldarate metabolism	9.68×10^{-6}
Glycine, serine and threonine metabolism	9.68×10^{-6}
Circadian rhythm	2.81×10^{-5}
Pentose phosphate pathway	1.92×10^{-4}
Tyrosine metabolism	2.51×10^{-4}
Glycerophospholipid metabolism	5.00×10^{-4}
Glycerolipid metabolism	1.59×10^{-3}
Starch and sucrose metabolism	2.93×10^{-3}
Notch signaling pathway	6.75×10^{-3}
Other types of O-glycan biosynthesis	7.06×10^{-3}
RNA polymerase	7.09×10^{-3}
Fat digestion and absorption	7.09×10^{-3}
Insect hormone biosynthesis	1.99×10^{-2}
Valine, leucine and isoleucine biosynthesis	2.45×10^{-2}
Protein digestion and absorption	2.67×10^{-2}
Vitamin digestion and absorption	3.92×10^{-2}
Dorso-ventral axis formation	4.00×10^{-2}

short to be translated into polypeptides meaningful for comparisons) and BLASTn hits from pea aphid mRNAs and Nt databases, the remaining 13,752 were considered green peach aphid-specific unigenes under the rearing conditions described (Table S6).

TABLE 3 | Differentially expressed detoxification and cuticle formation-related genes in adult and nymph.

Gene ID	Fold change (log_2)*	Nr-annotation
DETOXIFICATION GENES UP-REGULATED IN ADULT		
Unigene28862	7.29	Cytochrome P450 4g15-like
Unigene5938	5.20	Cytochrome P450 4g15-like
Unigene38834	4.64	Cytochrome P450 4C1-like
Unigene12004	3.09	Cytochrome P450 6a13-like
CL1335.Contig8	2.37	Cytochrome P450
Unigene21970	2.37	Cytochrome P450 6a13-like
CL2142.Contig1	2.13	Cytochrome P450 18a1-like
Unigene8797	2.03	Cytochrome P450 6a14-like
Unigene13485	1.73	Cytochrome P450 4C1-like
Unigene17164	1.65	Cytochrome P450 6j1-like
CL4129.Contig2	1.52	Cytochrome P450 4C1-like
Unigene18192	1.35	Cytochrome P450 18a1-like
CL2142.Contig2	1.31	Cytochrome P450 18a1-like
Unigene30119	1.23	Cytochrome P450 6a2-like
CL1335.Contig7	1.12	Cytochrome P450 6a13-like
Unigene8106	1.03	Cytochrome P450 4g15-like
Unigene11947	2.98	Esterase E4-like
CL2237.Contig3	1.97	Esterase FE4-like
Unigene14425	1.89	Esterase FE4-like
Unigene30909	1.60	Esterase FE4-like
CL2237.Contig6	1.28	Esterase FE4-like
CL1600.Contig6	1.14	Carboxylesterase-6-like
Unigene8449	1.07	Glutathione S-transferase D4-like
DETOXIFICATION GENES UP-REGULATED IN NYMPH		
CL27.Contig6	−4.63	Cytochrome P450 4C1-like
Unigene12432	−3.88	Cytochrome P450 4C1-like
CL27.Contig7	−3.41	Cytochrome P450 4C1-like
CL1617.Contig5	−2.30	Cytochrome P450 6a14-like
CL3489.Contig2	−1.83	Cytochrome P450 4C1-like
Unigene13770	−1.24	Cytochrome P450 6k1-like
Unigene24402	−1.20	Cytochrome P450 6k1-like
CUTICLE FORMATION-RELATED GENES UP-REGULATED IN ADULT		
CL2631.Contig2	2.57	Cuticle protein-like precursor
Unigene31315	2.32	RR1 cuticle protein 5
CUTICLE FORMATION-RELATED GENES UP-REGULATED IN NYMPH		
Unigene2111	−4.53	Cuticle protein
CL4114.Contig1	−4.17	Cuticular protein-like precursor
Unigene17916	−4.16	Cuticular protein 11 precursor
Unigene16021	−3.72	Cuticular protein 11 precursor
Unigene7580	−3.69	Cuticular protein-like precursor
Unigene27924	−3.53	Cuticular protein 11 precursor
Unigene25191	−3.45	Cuticular protein 16 precursor
CL4114.Contig2	−3.45	Cuticular protein-like precursor
CL5082.Contig1	−3.15	Cuticular protein 21
Unigene4987	−2.93	Cuticular protein 22 precursor

(Continued)

TABLE 3 | Continued

Gene ID	Fold change (log_2)*	Nr-annotation
CL5082.Contig2	−2.85	Cuticular protein 21
Unigene21389	−2.71	Cuticular protein 21
CL6036.Contig9	−2.58	Cuticular protein 28 precursor
Unigene21306	−2.45	Cuticular protein 22 precursor
Unigene24136	−2.36	Cuticular protein 62 precursor
Unigene13474	−2.30	Cuticular protein 22 precursor
Unigene13465	−2.25	Cuticular protein 28 precursor
CL5767.Contig2	−2.21	Cuticular protein CPG12-like precursor
Unigene13461	−2.19	Cuticle protein-like
Unigene9175	−2.17	Cuticular protein 23 precursor
Unigene24655	−1.99	Cuticular protein 47 precursor
Unigene13436	−1.91	Cuticular protein 9 precursor
Unigene13449	−1.89	Cuticular protein 28 precursor
Unigene13417	−1.88	Cuticular protein 9 precursor
CL1419.Contig2	−1.87	Cuticular protein 15 precursor
Unigene8362	−1.72	Cuticular protein precursor
Unigene13478	−1.72	Cuticular protein 1 precursor
Unigene4947	−1.71	Cuticular protein 47 precursor
Unigene11489	−1.71	RR1 cuticle protein 7 precursor
Unigene13482	−1.70	Cuticular protein precursor
Unigene10882	−1.70	Cuticular protein 60 precursor
Unigene11447	−1.68	Cuticular protein CPG12-like precursor
Unigene17255	−1.67	Cuticular protein 20 precursor
CL4704.Contig1	−1.66	Cuticular protein 57 precursor
Unigene13459	−1.65	Cuticular protein 37 precursor
Unigene13431	−1.65	Cuticular protein 1 precursor
Unigene13477	−1.63	Cuticular protein 16 precursor
Unigene13435	−1.63	Cuticular protein 16 precursor
Unigene13480	−1.60	Cuticular protein 9 precursor
Unigene13432	−1.59	Cuticular protein 45 precursor
Unigene13457	−1.58	Cuticular protein 16 precursor
Unigene11389	−1.58	Cuticular protein CPG12-like precursor
CL1419.Contig4	−1.58	Cuticular protein 15 precursor
CL5117.Contig1	−1.56	RR2 cuticle protein 2
Unigene13440	−1.55	Cuticular protein 45 precursor
Unigene13416	−1.55	Cuticular protein 1 precursor
Unigene13481	−1.53	Cuticular protein precursor
Unigene13443	−1.53	Cuticular protein 37 precursor
CL1419.Contig1	−1.50	Cuticular protein 15 precursor
Unigene13479	−1.47	Cuticular protein 28 precursor
Unigene13452	−1.46	Cuticle protein-like
CL6036.Contig5	−1.45	Cuticular protein 28 precursor
Unigene13484	−1.45	Cuticular protein 1 precursor
Unigene31055	−1.42	Cuticular protein 48
CL1419.Contig3	−1.41	Cuticular protein 15 precursor
Unigene21674	−1.41	Cuticular protein 52 precursor
Unigene31334	−1.40	Cuticular protein 20 precursor
Unigene13420	−1.39	Cuticular protein 37 precursor
Unigene14603	−1.37	Cuticular protein 28 precursor
Unigene13424	−1.35	Cuticular protein 16 precursor
Unigene14604	−1.34	Cuticular protein 28 precursor
Unigene7739	−1.32	Cuticular protein analogous to peritrophins 3-D1 precursor
Unigene13438	−1.26	Cuticular protein 28 precursor
CL6036.Contig11	−1.26	Cuticular protein 28 precursor
CL6036.Contig6	−1.24	Cuticular protein 28 precursor
Unigene24750	−1.23	Cuticle protein precursor
Unigene13418	−1.21	Cuticular protein 45 precursor
CL6036.Contig10	−1.18	Cuticular protein 28 precursor
Unigene554	−1.17	Cuticular protein 31 precursor
Unigene14112	−1.14	Cuticular protein 30 precursor
Unigene13441	−1.13	Cuticular protein 9 precursor
Unigene8067	−1.09	Cuticular protein 68 precursor
Unigene13475	−1.09	Cuticular protein 28 precursor
Unigene13426	−1.08	Cuticular protein precursor
Unigene31278	−1.07	RR1 cuticle protein 1
CL6048.Contig2	−1.05	Cuticular protein precursor
Unigene1224	−1.04	Cuticle protein-like
Unigene13467	−1.03	Cuticular protein 1 precursor
Unigene24090	−1.01	Cuticular protein 58 precursor
Unigene5077	−1.55	Ecdysis triggering hormone

Log_2 (FPKM-value in adult/ FPKM-value in nymph).

Arabidopsis was selected as our host plant because it is readily consumed by green peach aphid. Its short life cycle, abundant genetic resources and well developed RNAi technique (Ramsey et al., 2007; Pitino et al., 2011; Bhatia et al., 2012; Elzinga et al., 2014) can greatly facilitate our more in-depth studies of candidate genes derived from the current study. One caveat however, is that choice of hosts may impact aphid gene expression. Few studies have been conducted to compare transcriptome profiles of the same insect species feeding on different host plants, but some information is available on differential gene expression of insect populations reared on different varieties/lines of the same host plant species (Ji et al., 2013; Bansal et al., 2014; Yu et al., 2014). It appears that the vast majority of genes are present (but likely varied in expression level) among different insect populations, and that genes solely expressed in one population are rare. Whether this observation can be extended to insect populations feeding on different host plant species is yet to be determined.

The Nr, Nt, Swiss-Prot, COG, KEGG, and GO annotations of green peach aphid-specific unigenes were then performed (Table S6). Only 4.52% were predicted to have defined functions (**Table 4**), and functions of the remaining sequences need further study in the future. Likewise, KEGG classification identified only 30 unigenes, the most predominant group being "xenobiotics biodegradation and metabolism" (13.33%) (**Figure 4**). This finding correlates well with the fact that green peach aphids feed on a wider variety of plant species, and may have to

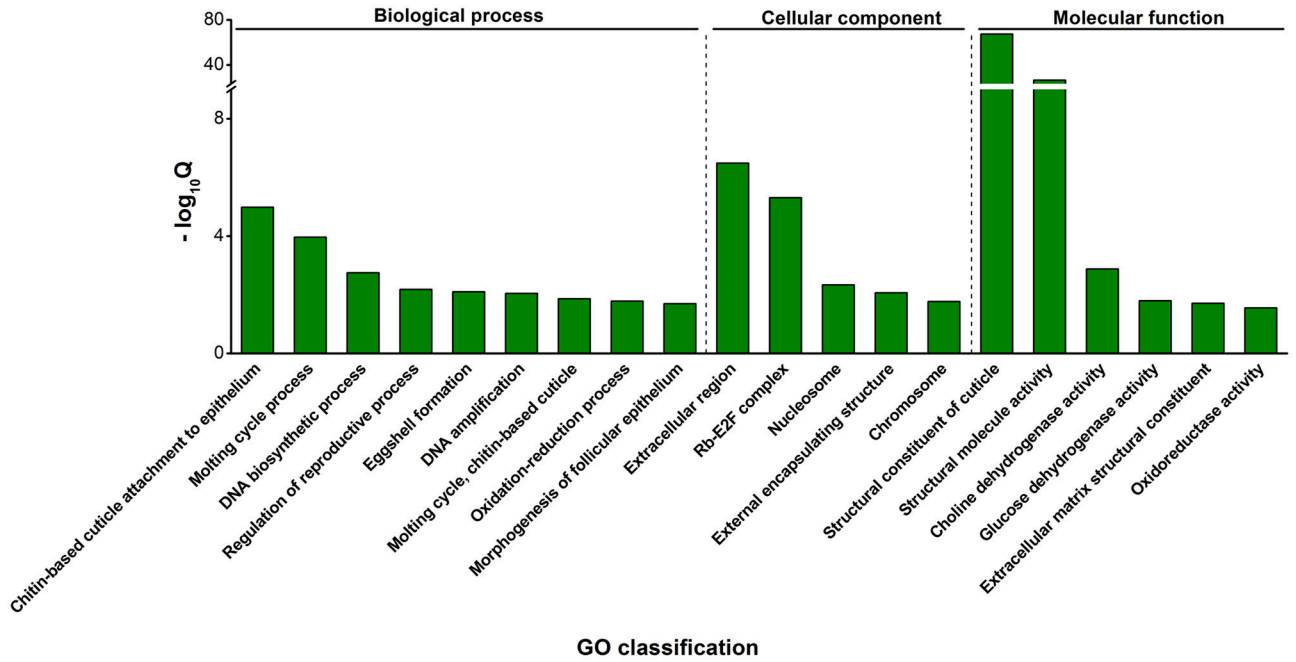

FIGURE 3 | Significantly enriched GO categories among the differentially expressed genes between nymphs and adults. GO categories with $Q < 0.05$ were considered significantly enriched. Classification consists of three domains: biological process, cellular component and molecular function. The y-axis shows the value of $-\log_{10}Q$ of the category. The GO term with highest $-\log_{10}Q$ was determined the most significant enrichment.

TABLE 4 | Annotations of green peach aphid-specific unigenes.

Public database	Number of annotated unigenes	Percentage (%)
Nr	85	0.62
Nt	562	4.09
Swiss-Prot	31	0.23
COG	9	0.07
KEGG	30	0.22
GO	10	0.07

encounter more types of toxic plant metabolites than pea aphids.

Ka and Ks Analysis between Green Peach Aphid and Pea Aphid

Contrasting with the above analysis where the focus was on genes unique to green peach aphid, here we concentrated on single-copy orthologous genes between the two aphids. From the 33,963 green peach aphid CDSs (mean length, 1275 bp) derived from our RNA-seq, 3989 that had one-to-one orthologs in pea aphid CDSs were identified, and 3824 contained both substitution types, from which Ka/Ks ratios were calculated (Table S7).

The Ka/Ks ratio provides information about the evolutionary forces operating on a particular coding gene and has been widely used to measure the intensity and mode of selection; Ka/Ks = 1 indicates a neutral evolution; Ka/Ks < 1 suggests that nonsynonymous mutations are deleterious and purged from the population; Ka/Ks>1 indicates that nonsynonymous amino acid substitutions offer fitness advantages and are fixed in the population at a higher rate than synonymous substitutions (Hurst, 2002). However, this cutoff value for positive selection has recently been adjusted to 0.5 by Swanson et al. (2004). They found that 15 of 16 genes with 0.5 < Ka/Ks < 1 showed statistical evidence for adaptive evolution (Swanson et al., 2004). Since then, this new value has been adopted for "positive selection" determination in many studies (Kelleher et al., 2007; Elmer et al., 2010; Yang et al., 2012; Zhang L. et al., 2013; Ren et al., 2014; Cheng et al., 2015; Mu et al., 2015; He et al., 2016; Pereira et al., 2016). In our study, a total of 24 pairs of orthologs had a Ka/Ks ratio greater than 1, and 124 had a Ka/Ks ratio between 0.5 and 1 (Table S8).

Relative to the earlier study by Ollivier et al. (2010), our CDS construction is more complete than that of EST-based (33,963 CDSs, 1275 bp mean length vs. 6652 CDSs, 667 bp mean length), due to improvements in sequencing technology. Nevertheless, some putative orthologs under positive selection were identified by both studies, such as C002 (Table S8). Other genes related to insect-plant interactions include those encoding mucins (Ka/Ks = 1.09 and 0.94), the essential components of peritrophic matrix. Fast-evolving mucin proteins presumably contribute to aphid adaptation to different dietary pro-oxidants, phenolic, and lipophilic xenobiotics associated with their respective host plants (Hiraishi et al., 1991; Felton and Summers, 1995; Barbehenn, 1999, 2001; Barbehenn and Stannard, 2004; Hegedus et al., 2009). Likewise, the homolog of salivary protein gene *Me17* (Ka/Ks = 0.69), identified in multiple aphid species with dissimilar plant host ranges, is thought to play important roles as the effector in suppressing defense responses in different host

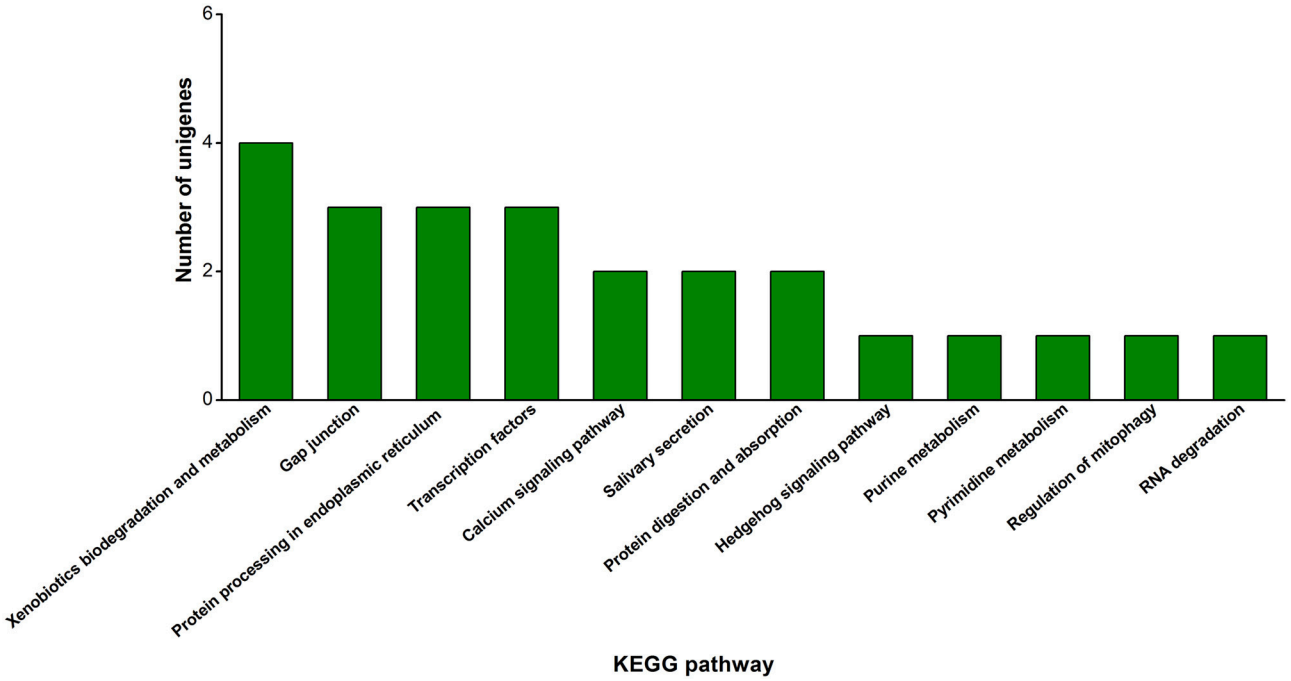

FIGURE 4 | Insect-related KEGG pathway classifications of green peach aphid-specific unigenes.

plants and in promoting aphid colonization (Atamian et al., 2013; Pitino and Hogenhout, 2013; Elzinga et al., 2014). Nicotinic acetylcholine receptors (nAChR) in insects are often the target sites for naturally occurring and synthetic insecticides (Millar and Denholm, 2007; Bass et al., 2011). A high mutation rate in the nAChR β-2 subunit (Ka/Ks = 0.55) could help green peach aphids adapt to tobacco and become resistant to nicotine, as is the strain used in this study (Devine et al., 1996; Nauen et al., 1996). Another interesting ortholog pair encode odorant-binding protein 10 (OBP10) (Ka/Ks = 0.52). Nucleotide and amino acid sequence comparisons between the two species indicated that all substitutions occurred in the predicted mature protein region, and 19 of the 62 substitutions resulted in 12 hydrophilic and hydrophobic amino acid conversions (Figure S1). Sun et al. (2012) observed that the two aphid species showed similar as well as dissimilar behavioral responses to certain tested odors. Presumably, fast evolution in OBPs could contribute to the change in their binding activity, which in turn could facilitate host shift or impact host range (Matsuo et al., 2007; Sun et al., 2012).

CONCLUSIONS

Our RNA-seq data have increased molecular resources available for the green peach aphid, a major agricultural pest as well as a biological model for insect-plant interaction studies. The transcriptomic analyses have deepened our understanding of aphid development and aphid-plant interactions. Our results have also provided useful insight into the molecular mechanisms underlying the biological variations in aphids, especially in adaptation to different host plants.

AUTHOR CONTRIBUTIONS

KZS, JF, and RJ conceived and designed the experiments. RJ performed the experiments. RJ, YW, YC, MZ, HZ, LZ, and KZS contributed to the transcriptome data analysis. RJ, JF, and KZS wrote the manuscript. All authors contributed to the discussion and approved the final manuscript.

FUNDING

This research was supported by the National Natural Science Foundation of China (31501636), the China Postdoctoral Science Foundation (2014M551529), Jiangsu Agricultural Science and Technology Independent Innovation Fund (CX(15)1055), Texas A&M Genomics Seed Grant, Texas Invasive Ant Research and Management Seed Grant, and the USDA-AFRI grant (2014-67013-21781).

ACKNOWLEDGMENTS

We would like to thank Dr. Ron Salzman for his critical review of the manuscript. We appreciate technical assistance in sample preparation for sequencing from Dr. Charles Johnson and Dr. Richard Metz.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01562

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 13 September 2016
doi: 10.3389/fpls.2016.01363

Plant Tolerance: A Unique Approach to Control Hemipteran Pests

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Plant tolerance to insect pests has been indicated to be a unique category of resistance, however, very little information is available on the mechanism of tolerance against insect pests. Tolerance is distinctive in terms of the plant's ability to withstand or recover from herbivore injury through growth and compensatory physiological processes. Because plant tolerance involves plant compensatory characteristics, the plant is able to harbor large numbers of herbivores without interfering with the insect pest's physiology or behavior. Some studies have observed that tolerant plants can compensate photosynthetically by avoiding feedback inhibition and impaired electron flow through photosystem II that occurs as a result of insect feeding. Similarly, the up-regulation of peroxidases and other oxidative enzymes during insect feeding, in conjunction with elevated levels of phytohormones can play an important role in providing plant tolerance to insect pests. Hemipteran insects comprise some of the most economically important plant pests (e.g., aphids, whiteflies), due to their ability to achieve high population growth and their potential to transmit plant viruses. In this review, results from studies on plant tolerance to hemipterans are summarized, and potential models to understand tolerance are presented.

Keywords: plant tolerance, hemipteran pests, ROS, susceptibility, inducible, constitutive, model

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *17 May 2016*
Accepted: *29 August 2016*
Published: *13 September 2016*

Citation:
Koch KG, Chapman K, Louis J, Heng-Moss T and Sarath G (2016) Plant Tolerance: A Unique Approach to Control Hemipteran Pests. Front. Plant Sci. 7:1363. doi: 10.3389/fpls.2016.01363

INTRODUCTION

Plants are constantly challenged by a diverse array of insect attackers, which can impose significant costs to plant fitness. Accordingly, plants employ multiple strategies to defend against, tolerate or avoid insect herbivory. Plant resistance can be categorized into three categories: antibiosis, antixenosis or non-preference, and tolerance. Antibiotic plant traits negatively impact a pest's biology through increases in mortality, reduced growth, longevity, and fecundity (Painter, 1951; Smith, 2005). Antixenosis, often referred to as non-preference, is a host-expressed trait that has adverse effects on insect behavior (Painter, 1951; Kogan and Ortman, 1978). In essence, insects have a non-preference for antixenotic hosts, and a preference for susceptible ones. Tolerance traits reduce the negative effects of herbivory on plant fitness after herbivory has occurred, all the while maintaining insect populations similar to those seen on susceptible plants (Painter, 1951;

Abbreviations: AUX, auxin; cpATPase, chloroplast ATP synthase; ELISA, enzyme-linked immunosorbent assay; EPG, electrical penetration graph; ET, ethylene; GPX, glutathione peroxidase; GST, glutathione transferase; HTP, high-throughput phenotyping; JA, jasmonic acid; PCD, programmed cell death; POX, peroxidase; PR, pathogenesis-related; PSII, photosystem II; RBOH, reactive burst oxidase; ROS, reactive oxygen species; RuBP, ribulose bisphosphate; SA, salicylic acid.

Panda and Khush, 1995; Smith, 2005). Because tolerance does not interfere with the insect pests' physiology or behavior, as seen in antibiotic or antixenotic resistance, selection for virulent insect populations and the threat of emerging biotypes is presumed to be limited.

Plant Tolerance to Hemipterans

When employed in integrated pest management systems, tolerance can potentially reduce yield loss caused by insect feeding and colonization (Pedigo and Rice, 2005). Insects as a group are estimated to cause anywhere from 10 to 80% loss in pre-harvest yields among the major crops grown worldwide, depending on the amount of external agronomic control measures applied (Oerke, 2006; Bruce, 2010; Ferry and Gatehouse, 2010). Among insects, the order Hemiptera account for many of the economically significant plant pests, damaging crops by feeding on phloem sap. Success of this group is due, at least in part, to their ability to rapidly reproduce and reach high population levels, as well as potentially transmit plant pathogens. Some of the most economically important hemipteran plant pests world-wide include aphids (Aphididae), whiteflies (Aleyrodidae), stinkbugs (Pentatomidae), and planthoppers (Cicadellidae), among numerous others. Insecticide resistance in many species has led to the development of insect-resistant plants (Painter, 1951; Panda and Khush, 1995). Much of the research being done on host-plant resistance as a means of managing these pests primarily concerns the integration of antibiotic or antixenotic traits through plant breeding and/or genetic engineering. However, the emergence of biotypes in these plant varieties has caused interest in other control strategies. Tolerance, treated as a resistance category of its own, has gained attention due to the plant's ability to recover from or withstand injury, without noticeable effect on the insect.

Mechanisms that Contribute to Tolerance to Hemipterans

Although tolerance to insect herbivores has received increased attention, detailed characterizations of the underlying mechanisms have remained elusive. Broadly five primary physiological mechanisms have been described by which plants may tolerate herbivory: (1) increased net photosynthetic rate after injury, (2) high relative growth rates, (3) increased branching or tillering after release of apical dominance, (4) pre-existing high levels of carbon storage in roots, and (5) ability to reallocate carbon after injury from roots to shoots (Strauss and Agrawal, 1999). To date, the most extensive research involving tolerance mechanisms to insects has involved cereal (and related grasses) resistance to hemipterans (especially aphids). Work on plant resistance to hemipterans has contributed greatly to the growing pool of knowledge regarding tolerance, and two specific physiological mechanisms have emerged as trends in tolerant plants (**Table 1**): (1) increased photosynthetic activity (Burd and Elliott, 1996; Girma et al., 1998; Haile et al., 1999; Botha et al., 2006; Heng-Moss et al., 2006; Franzen et al., 2007; Murugan et al., 2010; Luo et al., 2014; Cao et al., 2015) and (2) up-regulation of detoxification mechanisms to counteract deleterious effects of hemipteran herbivory (Heng-Moss et al., 2003b; Passardi et al., 2005; Gulsen et al., 2007, Gutsche et al., 2009; Kerchev et al., 2012; Ramm et al., 2013). As evident from published work on plant tolerance to hemipterans (**Table 1**), it is clear that underlying mechanisms that contribute to plant tolerance are largely unknown.

Photosynthetic Activity

The most commonly reported mechanism of tolerance to piercing-sucking insects has involved photosynthetic activity. Numerous studies have documented general reductions in total chlorophyll and carotenoids in susceptible plants in response to hemipteran feeding. Heng-Moss et al. (2003b) reported reductions of chlorophyll *a* and *b* and carotenoid concentrations on susceptible wheat lines in response to *Diuraphis noxia* (Russian wheat aphid) feeding, suggesting that *D. noxia* feeding possibly damages the light harvesting complex II, where chlorophylls *a* and *b* and carotenoids are important chromophores. Conversely, chlorophyll concentrations were similar between infested plants and their uninfested counterparts in the aphid-resistant isolines, suggesting that aphid feeding may have less effect on chlorophyll loss in *D. noxia* resistant wheat lines (Heng-Moss et al., 2003b). Botha et al. (2006) similarly reported a significant decrease of total chlorophyll in a susceptible wheat line when fed upon by *D. noxia*, compared to the resistant wheat. Additionally, the resistant wheat line had a significantly higher expression of *cpATPase*, relative to the susceptible wheat, indicating the potential importance of cpATPase as a compensatory mechanism to *D. noxia* injury by maintaining photosynthetic activity (Botha et al., 2006). Likewise, increased photosynthetic activity has been corroborated in many examples of tolerance to hemipterans (Burd and Elliott, 1996; Girma et al., 1998; Haile et al., 1999; Botha et al., 2006; Heng-Moss et al., 2006; Franzen et al., 2007; Murugan et al., 2010; Luo et al., 2014; Cao et al., 2015).

Gutsche et al. (2009; barley) and Franzen et al. (2007; wheat) were able to demonstrate that the rate of RuBP regeneration (as estimated from gas exchange measurements) was maintained in aphid-tolerant plants after *D. noxia* infestation, whereas susceptible plants showed accelerated declines in RuBP regeneration. Heng-Moss et al. (2006) reported photosynthetic mechanisms contributing to tolerance in buffalograss (*Buchloë dactyloides*) cultivars to the western chinch bug (*Blissus occiduus*). Notably, after prolonged exposure to chinch bugs, the susceptible buffalograss displayed reductions in photochemical quantum yield and photosynthetic electron transport rate; however, those differences were not observed in the tolerant cultivar (Heng-Moss et al., 2006). Accordingly, the tolerant buffalograss cultivar was able to enhance photosynthesis upon chinch bug attack as a compensatory mechanism to limit injury, while the susceptible cultivar was unable to maintain sufficient photosynthetic rates (Heng-Moss et al., 2006). Similarly, Haile et al. (1999) showed that the chlorophyll fluorescence yield was similar between uninfested and *D. noxia* infested leaves in a tolerant wheat line. Alternatively, susceptible and resistant (antibiosis) wheat lines, exhibited reduced chlorophyll fluorescence yield and were unable to recover, suggesting that *D. noxia* injury resulted

TABLE 1 | Plants and tolerance factors studied in response to hemipteran pests.

Plant	Insect	Plant tolerance factor measured	Reference
Aegilops tauschii	*Schizaphis graminum*	Growth, chlorophyll	Flinn et al., 2001; Smith and Starkey, 2003
Brachiaria spp.	*Aeneolamia reducta, Aeneolamia varia, Zulia carbonaria*	Growth, chlorophyll	López et al., 2009; Aguirre et al., 2013
Buchloë dactyloides	*Blissus occiduus*	Carbon exchange, chlorophyll, growth, vigor	Heng-Moss et al., 2003a, 2006; Eickhoff et al., 2008
Glycine max	*Aphis glycines*,	Yield	Pierson et al., 2010; Prochaska et al., 2013
	Pentatomidae	Yield	Souza et al., 2015
Gossypium hirsutum	*Pseudatomoscelis seriatus*	Vigor	Knutson et al., 2013
Hordeum vulgare	*Diuraphis noxia*	Chlorophyll	Burd and Elliott, 1996
		Growth, chlorophyll	Murugan et al., 2010
Lens culinaris	*Acyrthosiphon pisum*	Growth	Andarge and Westhuizen, 2007
Medicago sativa	*Empoasca fabae*	Net photosynthesis, transpiration, growth	Lamp et al., 2007
Medicago truncatula	*Therioaphis trifolii*	Growth	Kamphuis et al., 2013
Oryza sativa	*Nilaparvata lugens*	Growth	Panda and Heinrichs, 1983; Qiu et al., 2011
Panicum virgatum	*Schizaphis graminum, Sipha flava*	Growth	Koch et al., 2014
Saccharum spp.	*Mahanarva fimbriolata*	Growth, chlorophyll	Dinardo-Miranda et al., 2013
Solanum tuberosum	*Empoasca fabae*	Yield	Kaplan et al., 2008
Sorghum bicolor	*Melanaphis sacchari*	Growth, vigor	Armstrong et al., 2015
	Schizaphis graminum	Growth, chlorophyll, vigor	Dixon et al., 1990; Girma et al., 1998; Agrama et al., 2002; Nagaraj et al., 2005; Dogramaci et al., 2007
Theobroma cacao	*Sahlbergella singularis*	Survival, regrowth	N'Guessan et al., 2008
Triticum aestivum	*Diuraphis noxia*	Growth, chlorophyll, vigor	Burd and Elliott, 1996; Hawley et al., 2003; Miller et al., 2003; Randolph et al., 2005; Boyko et al., 2006; Voothuluru et al., 2006
	Schizaphis graminum	Growth, chlorophyll	Webster and Porter, 2000; Boina et al., 2005; Mojahed et al., 2012
	Sitobion avenae	Growth, photosynthetic rate, yield	Li et al., 2013; Cao et al., 2015
T. dicoccum x *Ae. tauschii* (synthetic hexaploid wheat)	*Schizaphis graminum*	Growth, chlorophyll	Lage et al., 2003
Triticum monococcum	*Sitobion avenae*	Growth	Migui and Lamb, 2004
Zoysia japonica	*Blissus occiduus*	Vigor	Eickhoff et al., 2008

in a disruption of the electron transport system reducing light absorption for photosynthesis in the susceptible but not the tolerant wheat line (Haile et al., 1999). It is likely that both mechanical (probing; removal of photosynthates) and chemical signals (aphid saliva) could be contributing to these observations.

ROS-Detoxification Mechanisms

In response to initial insect feeding, ROS have been recognized as central early signals, integrating environmental information and regulating stress tolerance (Foyer and Noctor, 2005, 2013; Kerchev et al., 2012). Normally, plants display exceptional redox control, using ROS and antioxidants, such as ascorbate and glutathione, to regulate numerous aspects of their biology including metabolism, growth, development and gene expression patterns (Apel and Hirt, 2004; Kotchoni and Gachomo, 2006; Maffei et al., 2007; Wu and Baldwin, 2010; Foyer and Noctor, 2013; Santamaria et al., 2013). Moreover, increasing evidence suggests that ROS signaling is closely related to hormone signaling, with considerable overlap occurring between ROS and the phytohormones, SA and JA pathways (Foyer and Noctor, 2005, 2013; Kwak et al., 2006; Mittler et al., 2011; Kerchev et al., 2012; Santamaria et al., 2013). Under normal conditions, ROS are rapidly detoxified, and cellular redox homeostasis is governed by the presence of enzymes and large pools of antioxidants that remove and buffer against oxidants (Foyer and Noctor, 2005; Foyer et al., 2016). However, an oxidative burst in response to environmental stresses may lead to generation of excessive ROS (Kotchoni and Gachomo, 2006). In this scenario, if the excessive accumulation of ROS is not efficiently removed, it can become toxic to plant cells, rapidly oxidizing and damaging cellular components, and ultimately leading to cell death (Foyer and Noctor, 2005; Kotchoni and Gachomo, 2006). Indeed, both ROS and antioxidants have been strongly implicated in SA signaling, regulation of PCD and the induction of PR proteins associated with systemic acquired resistance (SAR) (Foyer and Noctor, 2005; Foyer et al., 2016).

Based on these findings, a model is suggested (**Figure 1**) that integrates both the short-term (arbitrarily <5 days) and longer term (>10 days) responses that could underlie the

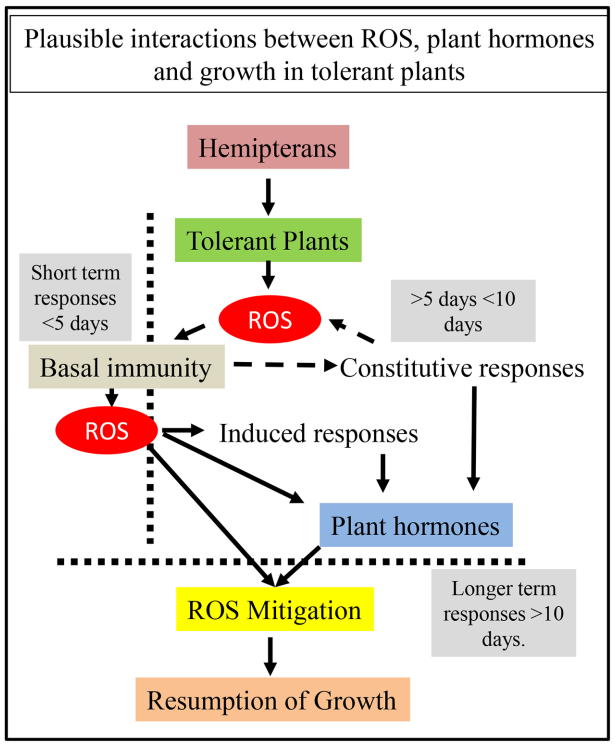

FIGURE 1 | Predicted interactions between ROS and plant hormones during the tolerance response. Initial response to herbivory is through the generation of ROS and the activation of basal immunity. Potential interactions between basal immunity and genotypic-dependent constitutive responses are represented with broken black lines ending in arrows. These events take place within a few hours to a few days. More ROS is generated during this immune response leading to interactions with both the constitutive and induced responses in the plant. Both the induced and constitutive responses result in changes in plant hormones. ROS by itself and plant hormones trigger ROS mitigation, which leads to redox rebalancing. Redox rebalancing restores growth. Changes in plant growth have been normally reported as a longer term (>10 days) response. Whereas it is possible that early responses could control tolerance, it would seem more likely that cellular networks controlling plant hormone levels and ROS mitigation are more likely to underpin the tolerance response.

tolerant response. Plants have both constitutive and inducible defenses (Mithöfer and Boland, 2012; Stout, 2013), whose interactions are likely driven by the genotype. Basal immunity (defined here as pre-existing defenses common to genetically related individuals) could be expected to be similar across genotypes within a population of plants with some variations in the strength of this response. ROS-dependent signaling, as a consequence of basal immune response, can be expected to trigger other induced responses with plant hormones as a key hub through which further signals are propagated. However, ROS are signaling molecules as well and can trigger the upregulation of the antioxidant system eventually leading to ROS mitigation. Plant hormones are central to these processes as well. How basal immunity interfaces with genotypic-dependent constitutive responses is less clear (represented with broken black lines ending in arrows in **Figure 1**). Most frequently, there is considerable overlap between the short-term and 5 to 10 day responses, but they have been separated (as depicted in **Figure 1**) to indicate that many physiological changes are noticed 5–10 days post infestation. Continued ROS mitigation appears to be a hallmark in tolerant plants, suggesting that mechanisms that permit modulation of cellular redox could be potential pathways for understanding the tolerance response. ROS mitigation appears to be linked to resumption of growth, providing another window to look for genes that both transduce and activate these pathways. It is likely that these changes do not become evident until much later (>10 days) during a plant-hemipteran interaction. Unfortunately, longer-term studies are often confounded by physiological changes that occur as plant mature that can make data interpretation more difficult. Nevertheless, detailed investigations using a range of omics strategies in well-defined tolerance systems are likely to provide significant insights about the traits controlling the tolerance response.

Over the past decade, researchers have evaluated the interrelationships between ROS damage and mitigation arising from quenching failures associated with end-product inhibition of photosynthesis. Several studies have suggested that tolerant plants appear to counteract deleterious effects of ROS accumulation and, consequently, PCD in response to phloem-feeding insects through up-regulation of detoxification mechanisms (Heng-Moss et al., 2004; Franzen et al., 2007; Gutsche et al., 2009; Smith et al., 2010; Ramm et al., 2013, 2015; Sytykiewicz et al., 2014). Sytykiewicz et al. (2014) described a significant increase of superoxide anion radicals (O_2) in maize seedlings infested with *Rhopalosiphum padi* (Bird cherry-oat aphid). Accordingly, aphid infestation also resulted in a significant increase in transcript abundances of genes encoding GSTs in the resistant maize plants, relative to the susceptible variety, suggesting a potential role of GST in limiting the adverse effects of oxidative stress within the resistant maize (Sytykiewicz et al., 2014). GSTs are central to redox balance in plant cells, and have been implicated in resistance to exogenous stress (Perez and Brown, 2014).

Transcriptional profiling in tolerant and susceptible buffalograsses suggests that a chinch bug tolerant genotype may be physiologically better prepared for chinch bug attack than susceptible plants as a result of relatively high basal levels of *POX* and *POX-1* (peroxidases), *CAT* (catalase), and *GRAS* [a gibberellic acid insensitive (GAI), repressor of GAI and scarecrow] transcripts (Ramm et al., 2013). Ramm et al. (2015) further noted that prior to chinch bug feeding the tolerant buffalograss had significantly higher expression of seven POXs, including five *GPXs*, relative to the susceptible buffalograss. Collectively, this suggest that constitutively elevated levels of ROS scavenging enzymes in tolerant plants may confer the ability to more readily detoxify ROS induced by chinch bug injury without suffering the negative consequences of high cellular levels of ROS. In wheat, transcriptional profiling also revealed that a resistant line, which was better able to tolerate *D. noxia* injury, had elevated levels of transcripts related to ROS metabolism, including *POX* and *GST*, whereas the susceptible line generally showed an increase in *AUX* related transcripts (Smith et al., 2010).

Taken together, these studies suggest that plant tolerance to hemipterans involves reprogramming of plant physiology and requires some degree of interaction particularly between primary metabolism, photosynthesis and plant defense responses. In cabbage (*Brassica oleracea*), radish (*Raphanus sativus*) and *Arabidopsis* seedlings infested with the green peach aphid (*Myzus persicae*), there was a differential regulation of nitrogen metabolism in aphid-infested plants relative to uninfested plants. Infestation led to greater enrichment of ^{15}N in the infested plants, primarily as a result of changes in host N-metabolism. These changes were attributed to increased nitrate reductase activities along with changes in nitrate flux, resulting in greater incorporation of ^{15}N. When coupled to selective removal of ^{14}N by aphids, the net result was increasing levels of ^{15}N in infested plants (Wilson et al., 2011). These data provide more clues into how aphids could modulate plant primary processes, and how tolerant plants might have evolved compensatory mechanisms impacting plant primary metabolism.

Key aspects of cellular changes occurring in a tolerant phenotype are summarized in **Figure 2**. Perception of hemipteran pests appears to occur within a short time frame <1 h, with some changes observed at an even shorter interval (Santamaria et al., 2013; Tzin et al., 2015). These changes appear to be triggered by a number of cell wall-anchored proteins, including receptors, kinases and RBOHs (Maffei et al., 2007; Louis and Shah, 2013; Hettenhausen et al., 2015; Foyer et al., 2016). Reaction cascades impacted by these proteins include changes in intercellular calcium content and production of superoxide and related ROS. Some of these events are likely part of the innate immunity of plants to pests and/or pathogens (Foyer et al., 2016). Piercing-sucking insects subsequently trigger more specific responses, because the removal of phloem and xylem contents disturbs both the water and nutrient balance in the plant, and effectively modulates chloroplast functions.

Chloroplasts are critical gatekeepers of leaf health, and altering chloroplast physiology has a significant effect on transcription through retrograde signaling and via shifts in the levels of metabolites such as starch, sugars (trehalose), and JA among others (Singh et al., 2011; Schwarzlander et al., 2012; De Clercq

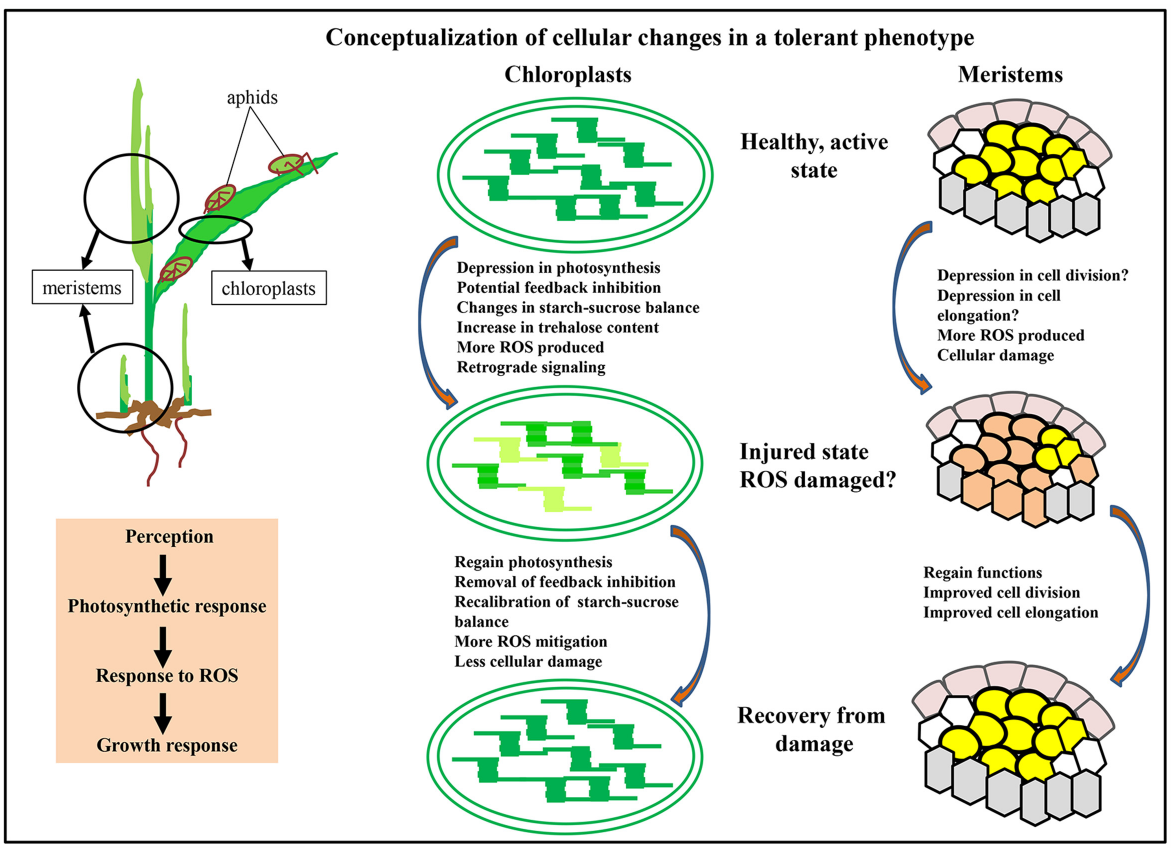

FIGURE 2 | Conceptualization of cellular changes in a tolerant phenotype. Initial aphid probing of leaves, followed by continued feeding leads to multiple plant responses. Initial perception of the pest is accompanied by a photosynthetic response in the chloroplasts, and mitigation of ROS that is likely to involve a number of cellular compartments. A consequence of these physiological changes is a repression of growth of meristems. As physiological processes return to normal, growth is reinitiated. Within chloroplasts, these changes are represented as change from dark green to light green to denote loss of functions, and from light green to dark green to indicate recovery of functions. Similarly, in the apical meristems, orange colored cells indicate a stressed state and the other colors indicate a healthy state.

et al., 2013). Thus, negative changes in chloroplast metabolism have a larger effect on other leaf functions, including increased production of ROS and overall slower rates of C and N assimilation. In a tolerant phenotype, mechanisms need to be present to minimize damage from increased ROS, support a defense response, while balancing leaf functions to compensate for nutrient removed by pests. Diverting energy to defense can be expected to impact growth, either by depressing growth of existing meristems and/or by reducing the formation of new meristems. In addition, ROS could damage meristematic cells in the shoots or roots directly as well (**Figure 2**). How these different processes influence each other at a biochemical level is largely unexplored, and deep transcriptional sequencing of multiple tissues in aphid-tolerant plants is absent. Recovery of leaf, meristem and other plant growth functions are the hallmark of a "tolerant phenotype" in the face of manageable pest populations. Recovery of growth requires overcoming the cellular reprogramming caused by hemipteran pests discussed earlier. However, it is not entirely clear if there is successive or simultaneous rebalancing of chloroplast functions, ROS mitigation and increased delivery of nutrients to sinks to promote growth. It is plausible that integration of these processes might be involved in attenuation of the defense response, maintaining higher levels of ROS mitigating systems, compensation of photosynthates lost due to insect herbivory and renewed growth of the meristems (**Figure 2**). These features appear to be consistent with much of the literature discussed above.

Plant Tolerance to Other Insect Pests and Pathogens

Interestingly, a recent study on comparing the molecular mechanisms of plant responses to phloem-feeding insects indicate unique and different signaling networks being activated following attack by the generalist and specialist aphids (Foyer et al., 2015). Predictably, plant responses to chewing insect herbivores differ significantly from those to piercing-sucking insects such as hemipterans (Zhou et al., 2015). Chewing insects feed primarily by the defoliation and consumption of plant tissues such as leaves, stems, flowers, and/or roots. Accordingly, while plant tolerance to sucking insects is primarily associated with molecular mechanisms such as ROS-detoxification and changes in photosynthetic activity, tolerance mechanisms in response to chewing herbivory are more frequently described by over-compensation via the production of new tissues, changes in plant architecture, and the allocation of resources to less vulnerable locations (Trumble et al., 1993; Strauss and Agrawal, 1999; Stowe et al., 2000; Tiffin, 2000; Zhou et al., 2015; Krimmel and Pearse, 2016).

The most extensive research on plant tolerance to chewing insects has focused on the plant's ability to compensate for loss of tissue or damage by producing more organs and increasing growth rates. Compensation-mediated tolerance to the cinnabar moth (*Tyria jacobaeae*) is due to the induced production of new capitula on regrowth shoots in ragwort (Islam and Crawley, 1983). The growth response of plant height and number of stems following defoliation by beetles (*Altica subplicata*) demonstrated genetic variation in tolerance to herbivory in *Salix cordata* (Shen and Bach, 1997). Examples of tolerance to chewing insects have been reported in the constitutive or basal differences in plant architecture. In the wild maize relative *Zea diploperennis*, a greater number of pre-existing tillers and leaves allowed for greater developmental plasticity in response to a stem boring caterpillar, *Diatraea grandiosella* (Rosenthal and Welter, 1995). Further evidence suggests that reallocation of resources upon insect attack may be a key mechanism in tolerance. Upon attack by *Manduca sexta* (tobacco hornworm) on *Nicotiana attenuata* (tobacco), carbon and sugar were allocated to the roots, a less-vulnerable location (Schwachtje et al., 2006). In another study, the application of *M. sexta* regurgitant on defoliated tomato accelerated leaf regrowth via responses similar to resource appropriation (Korpita et al., 2014). This reallocation of resources such as carbon has also been observed in response to root herbivory by western corn rootworm (*Diabrotica virgifera virgifera*). Upon below-ground herbivory, maize plants allocated more carbon to above-ground foliage, thickening stem tissues and increasing crown-root growth as a means of compensation (Robert et al., 2014). Tolerance can also be influenced on a multi-trophic level. Milkweed (*Asclepias*) symbiosis with arbuscular mycorrhizal fungi (AMF) enhances tolerance to herbivory though changes in nutrient status, allocation patters, and growth rate (Tao et al., 2016).

Instances of tolerance have also been observed in a plant's response to pathogens (Newton, 2016). One of the earliest cellular responses following pathogen infection is an oxidative burst of ROS as a part of the plant's hypersensitive response (Hammond-Kosack and Jones, 1996; Grant and Loake, 2000; Grün et al., 2006). Upon pathogen infection, activity levels of ROS scavenging enzymes PX and CAT are suppressed (Klessig et al., 2000). This suppression of ROS scavenging and accumulation of ROS in response to the pathogen is central to PCD of infected cells, leading to pathogen resistance (Mittler et al., 1999). With tolerance to pathogens, however, the role of cell death, and potentially ROS detoxification differs. A common trend in pathogen tolerance is actually the reduction or suppression of cell death, rather than the upregulation seen in pathogen resistance. Sublethal levels of H_2O_2 as a signaling molecule have been shown to induce the expression of defense genes that lead to an enhanced pathogen tolerance (Chamnongpol et al., 1998). Mach et al. (2001) found that the reduction of chlorophyll catabolism reduced cell death without affecting *Pseudomonas syringae* growth in *Arabidopsis*. Other instances of pathogen tolerance were found in ET -insensitive, -deficient, and SA-deficient tomato and *Arabidopsis* plants. Plants unable to produce ET and SA had attenuated cell death and chlorophyll loss, resulting in reduced symptoms without affecting pathogen replication (Bent et al., 1992; O'Donnell et al., 2001). This suppression of PCD in pathogen-tolerant plants is similar to that seen in several hemipteran-tolerant plants as mentioned previously. Taken together, these studies indicate that plants often compensate for damage caused by herbivory or pathogen infection by increasing chlorophyll concentrations, increasing

nutrient uptake, delaying senescence, and increasing the size or number of tissues such as leaves (Paige and Whitham, 1987; Rosenthal and Welter, 1995; Marquis, 1996; Strauss and Agrawal, 1999).

QUANTIFYING TOLERANCE

While tolerance has gained attention as a viable way of managing insect pests, traditional phenomic screening for hemipteran tolerance in plants is often time consuming and labor intensive. Currently, several different phenotyping approaches are used to quantify plant defense against piercing-sucking insects such as aphids. These approaches include insect population assays, use of EPG technique to measure feeding behavior, hand-held spectrophotometry (SPAD meter) to measure chlorophyll content in leaves, ELISAs to measure virus transmission, and plant metabolite assays (McLean and Kinsey, 1964; Tjallingii, 1988; Deol et al., 1997; Girma et al., 1998; Walker, 2000; Chan et al., 2010; Chen et al., 2012; Ménard et al., 2013). Most basic screening methods for tolerance include approximating insect population size and estimating plant damage by visual estimations to compare to a known susceptible genotype or cultivar. As insect populations are comparable to those seen on susceptible plant varieties, populations can become extremely high and visual estimations of damage can limit precision and result in bias.

High-Throughput Phenotyping (HTP)

Because of the tedious nature of these methods, newer techniques of phenotype screening have allowed plants to be measured for specific defensive/tolerant traits. HTP systems quantify a number of traits within plant populations through automated image collection and analysis, effectively streamlining the search process, and contributing further to plant phenomics.

HTP is gaining momentum due to its non-destructive sampling methods, rapid screening of a large number of plants, and automation of data analysis. Current HTP systems utilize image capture to quantify numerous plant traits, including insect-related symptoms. Kloth et al. (2015) proposed an automated video tracking of aphid feeding behavior as a means of phenotyping resistance in plants. Through this method, they were able to successfully screen a large number of *Arabidopsis* genotypes for resistance to the green peach aphid, *M. persicae*. As a means to measure tolerance, visual cameras can measure plant growth, architecture, and, chlorosis, and necrosis, all of which can be negatively affected by insect infestation. Fluorescence cameras can also be used to measure chlorophyll fluorescence, which can be indicative of the plant's photosynthetic activity (Buschmann and Lichtenthaler, 1998), which, as mentioned above, may be indicative of tolerance mechanisms occurring in response to hemipteran attack. HTP can have numerous applications in the measure of insect damage and plant resistance to insects as reviewed by Goggin et al. (2015). Ultimately the use of HTP systems could reduce the amount of labor and screening time put to identify plants that are tolerant to hemipterans.

RETHINKING PLANT TOLERANCE

An alternate scenario to explore is whether tolerance is really a manifestation of "less susceptibility" (i.e., a broad–based genetic response to intermittent pest pressure), rather than a resistance-mechanism *per se*. An outcome of this hypothesis is that finding hemipteran-tolerant plants might be easier in less domesticated or undomesticated wild species (Koch et al., 2014). It is known that finding tolerant genotypes in established crops is time consuming and requires extensive screening to identify tolerant genotypes. As examples, approximately 150 genotypes had to be screened to find a chinch bug tolerant buffalograss (Heng-Moss et al., 2002; Gulsen et al., 2010) and a soybean-aphid tolerant soybean (Pierson et al., 2010). These data suggest that selecting for yield or other agronomically desired traits, especially over a sustained period, may select against tolerant genotypes present in breeding nurseries (Mitchell et al., 2016). Another limiting factor in understanding tolerance has been the lack of genetically closely related lines that have a tolerant versus a susceptible response. Most frequently, these comparisons have used either a susceptible or resistant (antixenosis or antibiotic) plant of unrelated genetics to compare against a tolerant genotype, making head-to-head comparisons somewhat more challenging. To our knowledge, two tolerant plants of different genetics or tolerant and susceptible plants have not been crossed to evaluate their progeny for the tolerance phenotype. However, the fact that tolerance to hemipteran pests is present in most plant species specifically evaluated for this response indicates that continued research to find the molecular mechanisms underlying tolerance will be fruitful.

Based on research reported so far, it is possible to envisage at least two different routes to tolerance, one where tolerance is induced, and the other where tolerance is constitutive (**Figure 3**). In the case of induced-tolerance, infestation elicits a strong response across a spectrum of plant cells and pathways (**Figure 3A**; light orange colored cells). This strong initial response subsequently reprograms metabolism to counter the negative impact of herbivory, such as, by recalibrating cellular redox, photosynthesis and nutrient acquisition.

In a plant with a constitutive tolerant response, several biochemical/physiological aspects of tolerance can be expected to be present, for example: structural fortification (**Figure 3B**; blue line over the epidermis), increased transcript/protein and antioxidant abundances for ROS mitigation, and possibly greater photosynthetic capacity (**Figure 3B**; represented as gray colored cells). Under insect pressure, there is a more modulated response, potentially resulting in a shorter duration in suppressed growth (**Figure 3B**).

Graphically, plant responses can be envisaged as shown in **Figure 3C**. In resistant plants [R], there may or may not be a biochemical response to infestation, but this response if present is short lived, because inherent antibiosis and/or antixenosis significantly limit length of insect herbivory. If tolerance is constitutive [Tc], the response to infestation is more nuanced, with a subsequent faster recovery of growth processes as compared to plants with induced-tolerance [Ti].

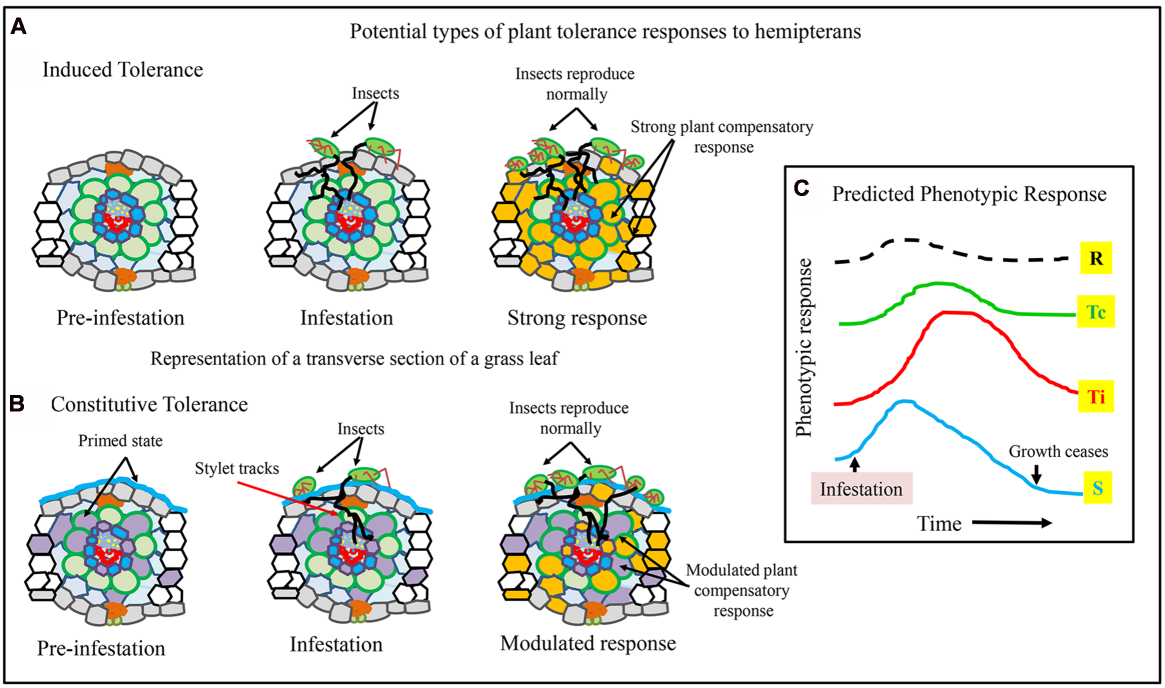

FIGURE 3 | Potential differences between an induced and constitutive plant tolerance response to hemipterans. A cartoon of a transverse section of monocot leaf with or without aphids is shown. In both types of predicted tolerance, there is a pre-infestation state, an infested state, followed by a sustained plant response to herbivory. **(A)** Induced tolerance, where there is a strong response to infestation and herbivory. **(B)** Constitutive tolerance, where plants have higher levels of protective mechanisms, and have a more attenuated response to hemipteran pests. **(C)** Plausible plant responses (arbitrary) to infestation over time. In resistant plants R (black dashed line), there is a minimal and short response due to underlying resistance mechanisms. In plants with constitutive tolerance Tc (green line), the response is present, but is more modulated and growth presumably begins sooner. For plants with induced tolerance Ti (red line), the responses to herbivory are more pronounced and last for a longer duration before reverting to conditions that permit plant growth. For susceptible plants S (blue line), there is a strong initial response to herbivory, but this response is not sustainable and the plant dies from accumulated insect damage.

In Ti plants, the initial response to hemipteran herbivory is rapid and strong, which are sustained for a longer period of time before plant growth is resumed. For susceptible plants [S], defensive mechanisms are initiated in response to herbivory, but they are unable to maintain these responses, and eventually succumb due to increasing tissue damage (**Figure 3C**; arrow, growth ceases). Identifying underlying mechanisms of tolerance will provide meaningful insight into our understanding of plant-insect interactions and have utility for breeding plants with more durable pest resistance.

CONCLUDING REMARKS

Continued focus on the contributions of specific mechanisms underlying plant tolerance to hemipterans will be critical for the development of tolerant germplasm. Additionally, the role of phytohormones in the expression of tolerance to hemipterans presents an appealing avenue of future research. Phytohormones are not only instrumental in regulating plant development, but they are also significantly involved in mediating plant responses to abiotic and biotic stresses. JA and SA in particular have been implicated in defense against pathogens and herbivores alike, however, the life-styles of the stressors determines which pathways are activated. Piercing-sucking insects such as hemipterans are homologous to biotrophic pathogens in the sense that they feed on the plant's nutrients without killing host cells. It is generally assumed that SA is a crucial signaling molecule required for the plant defense response against biotrophic pathogens and sucking insect pests: in contrast, JA is associated more in the defense against necrotrophic pathogens and chewing insects (Delaney et al., 1994; Dempsey et al., 1999; Ozawa et al., 2000; Glazebrook, 2005).

However, to date, few studies have investigated the role of phytohormones in plant tolerance to insects. Marimuthu and Smith (2012) reported that a tolerant barley line had significantly greater constitutive expression of JA-, ET- and auxin-indole acetic acid (IAA) pathway genes when challenged with *D. noxia*, and this heightened constitutive expression may help to attenuate stress associated with *D. noxia* feeding immediately after attack, through adjustments in stomatal opening and root growth. Correspondingly, upregulation of transcripts related to abscisic acid and ET signaling pathways have also been reported in *D. noxia*-resistant wheat plants suggesting their importance in *D. noxia* tolerance (Boyko et al., 2006; Smith et al., 2010). However, further work is needed to elucidate the mechanism by which these phytohormones may help condition tolerance to herbivory.

Plant tolerance to insect herbivory is a compelling category of resistance, consistent with integrated pest management strategies (Smith, 2005; Stout, 2013). Because tolerance does not interfere with the insect pests' physiology or behavior, selection for virulent insect populations and the threat of emerging biotypes is presumed to be limited (Smith, 2005). Moreover, it may also help promote the effects of beneficial arthropods in agricultural settings (Smith, 2005). While tolerance has received increasing attention, its deployment has been limited to date, due in part to the lack of information regarding the complex mechanisms involved (Mitchell et al., 2016). Another concern is the uncertainty of plant and insect interactions in response to climate change, which could require multiple strategies, including breeding for tolerance to maintain adequate crop yields in the future (Mitchell et al., 2016). As discussed earlier, recent studies are providing growing evidence for the role of photosynthetic compensation and ROS scavenging in tolerant plants. Additionally, the involvement of plant hormones in effecting a tolerant phenotype is captivating, yet mechanistically unexplored.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported in part by grants from the USDA-National Institute of Food and Agriculture grant number 2011-67009-30096 (GS and THM), by the USDA-ARS CRIS project 5440-21000-030-00D, and University of Nebraska-Lincoln start-up research funds and a faculty seed grant to JL. The U.S. Department of Agriculture, Agricultural Research Service, is an equal opportunity/affirmative action employer and all agency services are available without discrimination. Mention of commercial products and organizations in this manuscript is solely to provide specific information. It does not constitute endorsement by USDA-ARS over other products and organizations not mentioned.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 15 December 2016
doi: 10.3389/fpls.2016.01840

Functional Evaluation of Proteins in Watery and Gel Saliva of Aphids

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Gel and watery saliva are regarded as key players in aphid–plant interactions. The salivary composition seems to be influenced by the variable environment encountered by the stylet tip. Milieu sensing has been postulated to provide information needed for proper stylet navigation and for the required switches between gel and watery saliva secretion during stylet progress. Both the chemical and physical factors involved in sensing of the stylet's environment are discussed. To investigate the salivary proteome, proteins were collected from dissected gland extracts or artificial diets in a range of studies. We discuss the advantages and disadvantages of either collection method. Several proteins were identified by functional assays or by use of proteomic tools, while most of their functions still remain unknown. These studies disclosed the presence of at least two proteins carrying numerous sulfhydryl groups that may act as the structural backbone of the salivary sheath. Furthermore, cell-wall degrading proteins such a pectinases, pectin methylesterases, polygalacturonases, and cellulases as well as diverse Ca^{2+}-binding proteins (e.g., regucalcin, ARMET proteins) were detected. Suppression of the plant defense may be a common goal of salivary proteins. Salivary proteases are likely involved in the breakdown of sieve-element proteins to invalidate plant defense or to increase the availability of organic N compounds. Salivary polyphenoloxidases, peroxidases and oxidoreductases were suggested to detoxify, e.g., plant phenols. During the last years, an increasing number of salivary proteins have been categorized under the term 'effector'. Effectors may act in the suppression (C002 or MIF cytokine) or the induction (e.g., Mp10 or Mp 42) of plant defense, respectively. A remarkable component of watery saliva seems the protein GroEL that originates from *Buchnera aphidicola*, the obligate symbiont of aphids and probably reflects an excretory product that induces plant defense responses. Furthermore, chitin fragments in the saliva may trigger defense reactions (e.g., callose deposition). The functions of identified proteins and protein classes are discussed with regard to physical and chemical characteristics of apoplasmic and symplasmic plant compartments.

Keywords: aphids, gel saliva, hemipterans, proteome, salivary proteins, watery saliva

OPEN ACCESS

Edited by:
Linda Walling,
University of California, Riverside, USA

Reviewed by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *15 June 2016*
Accepted: *22 November 2016*
Published: *15 December 2016*

Citation:
van Bel AJE and Will T (2016) Functional Evaluation of Proteins in Watery and Gel Saliva of Aphids. Front. Plant Sci. 7:1840. doi: 10.3389/fpls.2016.01840

INTRODUCTION

Evidence is accumulating that host-plant proteins and salivary proteins of aphids play a major role in the "battle" between them. Aphid saliva contains proteins aimed to pave the way for the aphid stylet and to undermine plant defense and resistance (Tjallingii, 2006). Conversely, a high number of plant proteins are encountered along the stylet pathway. A part of the plant proteins is associated

with defense responses, while others are involved in, e.g., metabolic and regulatory processes. Although some cortex-expressed proteins are able to deter or combat aphids, the majority of proteins of high relevance for plant defense against aphids may occur in the sieve-tube sap in view of the much longer stylet residence times there (Tjallingii, 2006; Will et al., 2013). Up to thousands of proteins have been detected in sieve-tube exudates of, e.g., grasses (Aki et al., 2008), *Arabidopsis thaliana* (Batailler et al., 2012) and cucurbits (Lin et al., 2009; Dinant and Lucas, 2013). Unproven as yet, some of them may function as deterrents, while several others are involved in immediate plant defense on the protein level (Will et al., 2013). Again other proteins with a high impact on aphid–plant interaction may be integral part of local and long-distance signaling pathways/cascades (e.g., van Bel et al., 2011, 2014).

Harmel et al. (2008) detected more than 200 different polypeptides in the saliva of the green peach aphid *Myzus persicae*, of which only nine proteins were identified having a known function in other insects (*Aedes aegyptii* and *Aphis mellifera*). Others were related to expressed sequence tags (EST) of *A. pisum* and *M. persicae*. Later, Carolan et al. (2011) identified 925 proteins by mass spectrometry in salivary gland extracts of the pea aphid *A. pisum*. Over 300 proteins, most of them with an unknown function, were reported to possess secretory signals. The latter property was regarded as an essential characteristic of proteins belonging to the salivary gland secretome (Carolan et al., 2011). Recently, Atamian et al. (2013) studied the protein composition of salivary gland extracts of the potato aphid *Macrosiphum euphorbiae*. They allocated 125 of the 460 detected proteins to the secretome due to the presence of a signal peptide in accordance with the previous definition (Carolan et al., 2011).

However, the numbers of salivary proteins should be treated with care, because proteins without a secretory sequence may also be part of the salivary secretome (Chaudhary et al., 2015). Secretory or signal peptides located at the N-terminus of proteins mediate their transfer to specific regions inside the cell or their secretion out of the cell. Signal peptide sequences are predicted by software tools such as SignalP, which selected proteins in salivary glands having an N-terminal signal peptide (used by Carolan et al., 2011; Atamian et al., 2013), while TargetP is employed for prediction of the subcellular location of eukaryotic proteins. In eukaryotes, proteins with or without a secretory peptide end can be secreted in an unconventional way (Nickel and Rabouille, 2009), which might explain the presence of proteins without a secretory signal in aphid saliva (Chaudhary et al., 2015).

Like in related hemipteran families (Sogawa, 1965; Ramm et al., 2015), aphids produce salivary proteins in two pairs of glands at either side of the aphid head (Ponsen, 1972). Secretory proteins are released into the salivary channel and then secreted via their mouth parts into the plant tissues. The larger (principal) and smaller (accessory) pair of glands probably produce two mixtures of saliva. Gel saliva is predominantly secreted into the apoplasm during stylet movement along the cell walls. Watery saliva is mainly secreted intracellularly during penetration of different plant cell types (Tjallingii, 2006), but is also reported to be secreted into the apoplasm (Moreno et al., 2011). The principal glands may mainly produce gel saliva as indicated by immuno-histochemical labeling of a 154 kDa protein, while watery saliva is hypothesized to be produced for the major part by the smaller accessory glands (Cherqui and Tjallingii, 2000). As for the protein production, a specialization seems to exist between the cells of the principal glands (Pan et al., 2015). While some proteins like C002 (Mutti et al., 2008) or ACYP100646 (Pan et al., 2015) are only produced in the principal glands, other proteins like ACYPI39568 are expressed in both primary and accessory glands (Guo et al., 2014). The secretory activity of the glands is possibly regulated by the environmental conditions around the stylet tip (Will et al., 2012), perceived by receptors located in the precibarium (Wensler and Filshie, 1969).

On its way to the sieve tube, the stylet follows an apoplasmic path (Tjallingii, 2006; Hewer et al., 2011). In the apoplasmic space or cell wall, continuously secreted drops of gel saliva are pierced successively by the stylet to form a tubular corridor after hardening (Will et al., 2012) that envelops, protects and guides the stylet. Cells along the pathway are regularly probed by the stylet (Tjallingii and Hogen Esch, 1993). During stylet penetration of, e.g., mesophyll cells, watery saliva is secreted into the intracellular space, followed by ingestion of some cell sap (Tjallingii, 2006). There are indications that aphids navigate to the sieve tubes following a "cell rejection mode" (Hewer et al., 2011). As long as the cells do not contain a threshold level of sucrose and do not have a pH of approximately 7.5 (the alkaline conditions in the sieve-tube sap; Hafke et al., 2005), the aphids "reject" such cells after cell-sap probing. After penetration of vascular cells having the pH-value and sucrose concentration incentive to feeding, the stylet progress halts (Hewer et al., 2010, 2011). It was speculated that the directional orientation of the stylet is enabled by an inborne autopilot, guiding the stylet in radial direction (Hewer et al., 2011).

Once the stylet has reached the sieve tubes, watery saliva is secreted for a period of 40–60 s (Tjallingii and Hogen Esch, 1993). Aphids then start ingestion of sieve-tube sap that is interrupted by regular intervals of saliva secretion (Tjallingii, 2006). Saliva is not delivered into the plant any more but mixes with ingested sieve-tube sap in the common duct at the tip of the stylet (Tjallingii, 2006). Given the specificity of aphid-plant interactions – many aphids are monophagous, while some are poly- or oligophagous – several intriguing questions regarding the protein composition of aphid saliva emerge. Does protein composition of watery saliva differ between aphid species? Can the protein composition of watery saliva be adapted when general feeders like *M. persicae* switch the host plant species? Is it possible to separate gel and watery saliva and, if so, is there a clear distinction in protein composition between the two saliva types? And as the most prominent question here, which are the functions of salivary proteins identified thus far and how do they interfere with plant actions? These and associated questions are addressed here.

SECRETION OF GEL AND WATERY SALIVA

Sensing the Stylet Environment for Stylet Navigation

Observations by Hewer et al. (2010, 2011) and Will et al. (2012) point to the pH and the carbohydrate species as cues for stylet navigation to the sieve tubes and secretion of a saliva mixture that is adapted to the needs for stylet progress, orientation or feeding in dependence of the location of the stylet tip. Moreover, aphids appear to be able to perceive the presence of amino acids in artificial diets (Cherqui and Tjallingii, 2000). Because the stylet tip exclusively contains mechanoreceptors (Powell et al., 2006), aphids are likely capable of sensing the chemical stylet environment by chemosensillae in the precibarium (Wensler and Filshie, 1969; Backus and McLean, 1985), which requires frequent ingestion of cellular probes.

Together with other prameters, pH sensing would enable aphids to assess the stylet location. Through the clear distinction between cytosolic (pH 7.5; e.g., Felle and Bertl, 1986; Plieth et al., 1997; Bethmann et al., 1998; Felle, 2001; Hafke et al., 2001) and vacuolar pH (pH 5.5; e.g., Foyer et al., 1982; Nishimura, 1982; Weigel and Weis, 1984; Guern et al., 1986; Mathieu et al., 1989), aphids are able to identify the cell type punctured. Given the mechanical forces required to drive the stylet through the cell wall, it is expected that the stylet tip will instantly cross the thin cytosolic layer of parenchyma cells during penetration (Petterson et al., 2007) and reach the vacuole, which makes up almost the entire cell volume. Upon sensing the acidic vacuolar pH, aphids will retract the stylet and continue their search for a source of nutrition, until a sieve tube (pH 7.5, Hafke et al., 2005) is identified (for a simple model, see Hewer et al., 2011). The standard pH of 7.3 to 7.5 in sieve elements (Hafke et al., 2005) is due to the lack of vacuoles.

One of the crucial, albeit disputable, claims in this "rejection" hypothesis is that the stylet becomes inserted into the vacuole of parenchyma cells. It has been argued that aphid-transmitted viruses must be released from the stylet into the cytoplasm of parenchyma cells (Martin et al., 1997; Marchetti et al., 2009) to enable virus multiplication (Martelli and Castellano, 1971; Shalla et al., 1980) and the stylet tip must therefore reside in the cytoplasm during the entire cell-probing period (Powell et al., 2006). This view excludes stylet piercing of the tonoplast. However, pictures of stylet tips inside the vacuole (Hewer et al., 2011), traces of irreversible damage of intracellular membranes (Spiller et al., 1985; Hewer et al., 2011), and the presence of viruses inside the vacuole (Wan et al., 2015) corroborate the view that the vacuolar pH is sensed by aphids and thus seem to support the "rejection" hypothesis.

As a second cue for stylet orientation, the sucrose concentration has been proposed (Hewer et al., 2010, 2011). Aphids are able to discriminate between sugar species and sugar quantities (Mittler and Dadd, 1964; Auclair, 1969; Hewer et al., 2010). Many aphids show a clear preference for sucrose at high concentrations (Hewer et al., 2010), which is the common transport sugar in the majority of higher plants (Zimmermann and Ziegler, 1975) and mostly occurs in high concentrations in sieve-tube sap [e.g., barley 1080 mM (Lohaus et al., 1995), plantain 645 mM, celery 389 mM (Nadwodnik and Lohaus, 2008), and spinach 830 mM (Gerhardt et al., 1987; Lohaus et al., 1995)]. In contrast, vacuoles of parenchymatous cells contain concentrations of sucrose varying between 0.9 mM in plantain, 45 mM in celery, 64 mM in peach (Nadwodnik and Lohaus, 2008), and 40 mM in spinach (Gerhardt et al., 1987). Together with the low pH, a low sucrose concentration may be an incentive to retract the stylet and move on to the next cell. Like pH values, sucrose concentrations may be monitored by chemosensory cells in the precibarium (cf. Backus and McLean, 1985).

It has become obvious from tests with artificial diets that amino acids are indispensable substances for aphid feeding (Turner, 1971; Wille and Hartmann, 2008; Will, 2016a). The question arises, whether specific amino acids – as sucrose presumably does – act simultaneously as nutrients and as indicators for stylet orientation. The latter issue has not been investigated yet to the best of our knowledge.

In addition to chemical cues such as pH, sucrose and – perhaps – amino acids, the fourth sensory element in orientation may be turgor pressure, as aphids are able to perceive changes in turgor pressure in sieve tubes as demonstrated by an artificial feeding system (Will et al., 2008), and thus seem to sense turgor differences between the successive cells along the stylet pathway. All in all, the attractiveness of the "rejection" concept is that it provides a universal model for aphid orientation to the sieve tubes that is not dependent on species-specific traits.

Sensing the Stylet Environment for Appropriate Saliva Secretion

When aphids are feeding from an artificial diet, a salivary sheath is formed that is attached to a Parafilm cover at the side facing the diet (e.g., Cherqui and Tjallingii, 2000; Cooper et al., 2010). The pearl necklace structure of gel saliva secreted *in vitro* suggests rhythmic pulses of saliva secretion (McLean and Kinsey, 1965; Will et al., 2012; Morgan et al., 2013). According to a long-lasting concept (McLean and Kinsey, 1965), the salivary sheath was formed, because gel drops were inflated from the inside by watery saliva, quickly solidified and then were pierced by the stylet to be followed by the next drop of gel saliva (McLean and Kinsey, 1965; Miles, 1972). Inflation, however, would lead to round cavities that do not form a tight-fit tube around the stylet. The discrete and straight tubular structure, visible by confocal laser scanning microscopy (Will et al., 2012), infers that the gel saliva hardens after that the stylet has pierced the gel saliva at the front side before release of the next drop without intervention of watery saliva. Occasional side branches of the gel-saliva puffs *in vitro* (Will et al., 2012) and regular side-branches of gel saliva tracts in plant tissues (Hewer et al., 2011) are indicative of an auto-programmed process switching between stylet movement, gel saliva secretion, and regular cell probing along the stylet pathway (Tjallingii, 2006).

The question arises, whether the predicted chemical cues (pH, carbohydrate species) are not only responsible for stylet navigation, but also control the secretion of different types

of saliva (gel saliva in the apoplasmic, watery saliva in the symplasmic compartments). Saliva was collected in artificial diets mimicking apoplasmic and symplasmic solutions. Gel saliva depositions and diet fluids were collected at pH 5 (mimicking apoplasmic pH conditions) and pH 7 (mimicking neutral to weakly alkaline sieve-tube conditions). To study the protein composition of salivary sheaths a protocol was developed by Will et al. (2012). After solubilisation of salivary sheaths attached to Parafilm by breaking up the structural framework of the sheaths in several steps, the free proteins were separated by 1D SDS-PAGE (Will et al., 2012). At pH 5, hardly any proteins occurred in the diet fluid, whereas the Parafilm-adhesive gel saliva showed a multitude of protein bands in 1D SDS-PAGE gels (Will et al., 2012). By contrast, the diet fluid contained a rich diversity of protein bands at pH 7, whereas the number of proteins increased in gel saliva depositions (Will et al., 2012). pH 5 mimics the acidic conditions inside the apoplasmic compartment (Cosgrove, 2005), where secretion of gel saliva is needed to facilitate stylet progress (Miles, 1999). Furthermore, low pH may be optimal for the activity of enzymes in gel saliva as it is for other enzymes being active in the cell-wall compartment such as cell-wall invertase (Hothorn et al., 2010). In conclusion, the stylet-tip milieu elicits the secretion of watery saliva only under diet conditions that mimic the composition of sieve-tube sap.

Separation of Watery and Gel Saliva Proteins

The capacity of aphids to discriminate between the diets paves the way for an almost unequivocal separation and assessment of gel and watery saliva proteins (Will et al., 2012). The risk of cross-contamination (watery saliva proteins enclosed in the sheath structure and proteins leaking from the sheath structure leaking into the diet) is high under sieve-tube conditions in the diet. Under conditions mimicking the apoplasmic environment, the amount of proteins in the diet fluid (containing watery-saliva proteins) was so low, that the gel saliva depositions are virtually devoid of watery-saliva proteins and, hence, the protein bands obtained from sheath solubilisation primarily represent gel-saliva proteins. Thus, the composition of watery saliva may be disclosed by subtraction procedures facilitated by previous identification of gel-saliva proteins. However, it should be stressed that some proteins may be part of both types of saliva as discussed above.

A Side-Step: Physical Components of Aphid–Plant Interaction and Putative Consequences for the Salivary Proteome

Little attention has been paid to the physical components of interaction between aphids and plants (Will and van Bel, 2006) and the inherent consequences for protein composition of the saliva types. The stylet penetration site is usually located at the wall junction of two epidermal cells and the labium is anchored to the plant surface by a salivary flange made of gel saliva (e.g., Will et al., 2013). The wall junction probably offers the weakest spot in the cell-wall barrier and provides a strategic spatial location for stylet penetration. The stylet mainly moves along the primary cell wall (Tjallingii and Hogen Esch, 1993) that has a jelly and loosely woven structure. It represents the softest part of the cell walls with the lowest mechanical resistance. It is possible that stylet movement leverages the rigid cell-wall sandwich bordering either side of the primary wall. Because the stylet proceeds along the cell walls, aphids must generate a considerable force to thrust the stylet forward through the tortuous path inside the walls, where the fragile mouth parts could be damaged. Cell-wall degrading enzymes could be useful to reduce the wall resistance, but this view is under dispute (Cherqui and Tjallingii, 2000).

To create the strength, needed for stylet propulsion, the movement of the four subunits of the stylet (two maxillary and two mandibulary mouth parts) must be well coordinated. The maxillary and mandibulary pair at one side move together forward alternating with the other pair. Stabilization and support of the mouth parts during movement must be an important function of the salivary sheath. In conclusion, stylet movement requires a mixture of proteins that softens and digests the walls, lubricates the pathway to decrease the mechanical resistance, and forms a corridor through which the fragile stylet finds its way along the brisk cell wall angles and that stabilizes the movement of the mouthparts.

That the sheath does not possess the necklace structure *in planta* as obtained *in vitro*, where shots of gel saliva assume a spherical shape, may be due to physical constraints imposed by the cell walls around the sheath. Hardening of the salivary sheath is ascribed to oxidation of sulfhydryl groups (Miles, 1965; Tjallingii, 2006). Under ambient *in vitro* oxygen concentrations (∼20%; Hewer et al., 2011), "the pearls of the necklace" are discretely roundish having a rimmed texture (Will et al., 2012). Under reduced oxygen conditions, in contrast, the spheres were less delimited and the texture smoother, while the single drops are eliminated in the presence of DTT that impedes cross-bridging of sulfhydryl groups (Will et al., 2012). Due to the low oxygen level in the plant cortex (∼7%; van Dongen et al., 2003), sheath polymerization is anticipated to be less rapid than *in vitro* and initial bulging of the gel saliva drops will vanish during hardening. Moreover, the turgescence of the plant cells enveloping the sheath may compress the gel saliva during solidification. Due to retarded hardening under low-oxygen conditions, the sheath takes the shape of the mold presented by the cell walls.

Turgor of plant cells may also provide auxiliary information on cell identity. High sugar concentrations and high turgor are mostly linked, so that sieve elements stand out by a high turgor pressure. Moreover, pressure sensing might be relevant for initiating the secretion of saliva. Recognition of the atmospheric pressure or even negative pressure inside the apoplasm may trigger the secretion of gel saliva. Alternatively, secretion of gel saliva may result from sensing mechanical resistance as experienced during piercing of the Parafilm cover on diet solutions (Will et al., 2007; Carolan et al., 2009; Cooper et al., 2010; Chaudhary et al., 2015). The latter event (Cherqui and Tjallingii, 2000; Cooper et al., 2010; Will et al., 2012; Morgan et al., 2013) may mimic the resistance experienced during cell-wall penetration.

Cell punctures along the stylet pathway (Tjallingii, 2006) may cause local waves of electrical depolarization, which propagate to

the nearby sieve tubes (van Bel et al., 2014) and prepare them to aphid attack. Plasmodesmata would provide a symplasmic lateral pathway (Kempers et al., 1998) for propagation of the depolarization wave, in which voltage-activated Ca^{2+}-permeable channels are involved (van Bel and Ehlers, 2005; van Bel et al., 2014). Cell punctures made by stylets may be quickly repaired by plasma membranes in analogy to their reaction toward microelectrode impalement. Immediately after insertion of microelectrodes into cells or vacuoles, the pierced membranes seal off around the electrode tip and completely shut off, shortly after the electrode is retracted (e.g., Bates et al., 1983). Since stylet tips and conventional microelectrode tips have approx. the same diameter (1 micron, Will and van Bel, 2006), plasma-membrane wounds inflicted by punctures will probably close shortly after stylet retraction. According to this concept, secretion of gel saliva to seal off the puncture (Tjallingii, 2006) is therefore needed to prevent bulging of the turgescent protoplast associated with undesired physiological consequences rather than sealing the protoplast. Moreover, the chemical incompatibility of the hydrophilic gel-saliva and the lipophilic membrane material renders their fusion to seal the plasma membrane highly unlikely (Will and van Bel, 2006).

Stylet puncture in sieve elements has a number of physical consequences. First of all, free Ca^{2+} ions present in the cell walls will readily invade the sieve-element lumen via the puncture (Will and van Bel, 2006). Furthermore, the inevitable turgor drop linked with stylet penetration will activate mechano-sensitive Ca^{2+}-permeable channels (Demidchik and Maathuis, 2007) causing Ca^{2+} influx into the sieve element (Furch et al., 2009; van Bel et al., 2014). In plant/aphid systems, where Ca^{2+} levels are instantly reduced by Ca^{2+} scavenging salivary proteins (Will and van Bel, 2006), the consequences of stylet penetration could be limited. In plant/aphid interactions with low Ca^{2+}-binding capacities, however, we speculate that the temporary Ca^{2+} upsurge in sieve elements is sufficient to activate voltage-activated Ca^{2+}-permeable channels (McAinsh and Pittman, 2009). The subsequent cascade of ion movements would be responsible for a strong local depolarization and the initiation of an electrical potential wave along successive sieve elements (e.g., van Bel et al., 2014, and references therein; Hedrich et al., 2016). Consequently, stylet impalement into a sieve element may trigger the propagation of an electric potential wave resulting in a series of reactions in cells adjacent to the sieve tube (van Bel et al., 2014). All above types of Ca^{2+} influx will collectively boost Ca^{2+} concentration in sieve-element mictoplasm (Furch et al., 2009; Hafke et al., 2009), which will in turn evoke gating of Ca^{2+}-activated Ca^{2+} channels (CICR channels; Muir and Sanders, 1996) giving rise to massive Ca^{2+} release from the sieve-element endoplasmic reticulum. In conclusion, diverse ways of Ca^{2+} influx, brought about by physical events owing to stylet penetration, potentiate the elevation of Ca^{2+} level in sieve elements leading to cascades of local and remote events. Given the involvement of enhanced Ca^{2+} in a range of plant defense responses (Will and van Bel, 2006; van Bel et al., 2014) Ca^{2+}-binding proteins in aphid saliva would be helpful to suppress plant defense.

SPECIES-SPECIFICITY OF SALIVARY PROTEINS AND POTENTIAL ADAPTATION TO HOST PLANTS

Protein Profiles of Watery Saliva of Various Aphid Species Reared on One Host Plant Species

To our knowledge, the very first comparative study that included more than one aphid species feeding the same host plant species was conducted by Madhusudhan and Miles (1998). They detected differences and overlaps between the salivary protein bands in SDS-PAGE profiles of the pea aphid (*A. pisum*) and the spotted alfalfa aphid (*Therioaphis trifolii maculata*), both feeding on *Medicago sativa*. This observation coincided with distinct differences and overlaps (e.g., pectin methylesterase and endopolygalacturonase) in enzyme activities between saliva probes from the two species. Similarly, the SDS-PAGE band patterns of salivary proteins from *A. pisum*, *M. viciae*, and *A. fabae*, all reared on *Vicia faba*, disclosed a wide diversity between species-specific protein profiles (Will et al., 2009). Experiments similar to those of Will et al. (2009) using *A. pisum*, *M. viciae*, and *M. persicae* reared on *V. faba* produced almost identical, but less discrete protein bands (Vandermoten et al., 2014). Although protein identification was not executed, some proteins seem to be identical based on their molecular weight, while others appear to differ between aphid species.

A more sophisticated approach using mass spectrometry for protein identification (Rao et al., 2013) demonstrated that the salivary proteomes of *A. pisum* and the cereal aphid species *Sitobion avenae* and *Metopolophium dirhodum* showed several overlaps [e.g., trehalase and GMC (glucose-methanol-choline) oxidoreductase]. The results indicate that some elements of the salivary proteome have universal functions, while others may be adapted to the host-plant species. It raises the question if aphids are able to adapt their salivary composition to the host-plant species.

Are Salivary Proteins Involved in the Adaptation to Host-Plant Species?

Biotypes of *A. pisum* have been reported to be adapted to diverse legume species due to minor variations in the genetic background of aphids (Via, 1991). Biotype variation may be regarded as long-term adaptation to different host-plant species, which was demonstrated by a genomic approach including different biotypes of the polyphagous aphid species *A. pisum* (Peccoud et al., 2009). They identified 11 different biotypes feeding on different legume species, showing significant adaptations of the aphid genome. In a genome-wide study on pea aphid, Jaquiéry et al. (2012) identified regions enclosing salivary protein genes and olfactory receptor genes that are likely involved in host-plant adaptation. The authors point out that the genetic markers used only cover a small percentage of the aphid genome (Jaquiéry et al., 2012).

Adaptation to host plants is of paramount importance in host alternation, an obligatory seasonal shifting between aphid

species and host plants of distant genetic relationship. The gene expression patterns of the mealy aphid *Hyalopteris personikus* feeding on *Phragmites australis* in summer and several members of the Rosaceae in winter showed enormous seasonal variations (Cui et al., 2016). In summer, several secretory proteins, attributed to watery saliva, were highly expressed, while a salivary sheath protein was highly expressed in winter. All in all, aphids seem to be able to adapt their salivary proteome to the host plant.

REMARKS CONCERNING COLLECTION AND SEPARATION PROCEDURES OF SALIVARY PROTEINS

Proteins Obtained from Extracts of Salivary Glands or by Collection of Secreted Saliva: Advantages and Disadvantages

Protein composition of aphid saliva has been assessed by analysis of either the proteome of salivary gland extracts or stylet exudates. Analysis of the salivary proteome of gland extracts has the presumptive advantage that the samples are hardly prone to oxidation, degradation by proteases, or bacterial breakdown, all risks when using artificial diets. Furthermore, Carolan et al. (2011) identified a higher number of proteins in salivary gland extracts than in the proteome collected with artificial diets. Salivary glands extracts may be better suited to detect low-abundance proteins, as extracts often have higher protein concentrations and do not need to be concentrated prior to analysis by SDS-PAGE or MS/MS. In addition, extracts enable the detection of peptides of molecular weights lower than 3–10 kDa. These are the molecular cut-off sizes for ultrafiltration, commonly used for concentrating saliva-diet mixtures (Will et al., 2007; Carolan et al., 2009; Cooper et al., 2011; Chaudhary et al., 2015).

For the above reasons, the number of proteins in gland extracts is anticipated to be higher than in diet fluids. This difference may also illustrate a severe drawback of the method: proteins identified in gland extracts may profoundly deviate from the actual and functional salivary proteome. As an additional disadvantage of extract sampling, excision of intact salivary glands is demanding and one needs at least 300 glands to enable proteomic analysis (Rao et al., 2013).

An unmistakable advantage of saliva collection by using artificial diets is the unequivocal identification of proteins engaged in plant-aphid interaction. A further advantage of using artificial diets is that diets can be manipulated to provide preferential conditions for the secretion of either gel or watery saliva (Will et al., 2012). As noted above, however, proteins secreted in artificial diets may be subject to degradation due to long incubation times of 16–48 h needed for saliva collection (e.g., Will et al., 2007; Harmel et al., 2008; Carolan et al., 2009; Chaudhary et al., 2015). Furthermore, the procedures are laborious: pooled diets containing saliva of 1000s of aphids have to be concentrated by ultrafiltration to reach quantities needed for reliable protein analysis (Will et al., 2007; Carolan et al., 2009; Chaudhary et al., 2015). A major drawback of using diets may be the presence of traces of host-plant sieve-element proteins in the collected saliva (Chaudhary et al., 2015). As a final important issue, the composition of the saliva secreted into diet fluids in the absence of various host-plant cues may deviate from that secreted into plants (Guo et al., 2014; Wang et al., 2015b). Surprisingly, it has been demonstrated (Morgan et al., 2013) that gel saliva can also be collected *in aera*, thus without the intervention of artificial diets. This opens perspectives for a more amenable mode of collection and separation of gel saliva.

Salivary Proteomics and Functional Assessment of Salivary Proteins

As soon as aphids invade a plant, a cross-fire of attacks and counterattacks bursts out. In the present paper, only the "weapons" from the aphid-side are discussed. While the number of salivary proteins that has been identified continuously increases, the most exhaustive list of hemipteran salivary proteins thus far (Sharma et al., 2014) includes more than 60 salivary proteins identified in diverse hemipterans. We will focus here on the few classes of aphid salivary proteins, of which the functional relevance has been identified.

One should bear in mind that almost none of the proteomic studies aimed or succeeded thus far to discriminate between gel and watery saliva. Almost all diet studies thus far were executed using diets at pH 7, a value at which gel and watery saliva are mixed (Will et al., 2012). Nevertheless, separation of the two types seems to be an absolute prerequisite for meaningful functional assessment, the more as the saliva types may have overlapping protein compositions as a further issue of complication (Will et al., 2012). Separately collected extracts of primary and accessory glands may give a rough impression of the degree of distinction between gel and watery saliva proteins. In contrast to diet studies, extract contents do not guarantee the presence of these proteins in the saliva as argued above. Hence, the following discussions suffer from uncertainties regarding the origin of the salivary proteins and the location of secretion inside the plant. Attribution to either gel or watery saliva follows an apparent logic rather than one based on solid experimental evidence.

FUNCTIONAL ASPECTS OF SALIVARY PROTEINS

Predicted Gel Saliva Components

The major component of the salivary sheath is most likely a 154-kDa protein (ACYP1009881-PA; Carolan et al., 2009, 2011) having a high content of cysteine residues. This protein, termed "sheath protein", SHP (Carolan et al., 2011), was first detected in the saliva of *A. pisum* (Carolan et al., 2009), and later in the saliva of *S. avenae* and *M. dirhodum* (Rao et al., 2013). Oxidation of the SHP sulfhydryl groups would lead to the formation of disulfide bridges (Miles, 1965; Tjallingii, 2006), catalyzed by disulfide isomerases, several of which were found in the salivary gland secretome of *A. pisum* (Carolan et al., 2011). Linked SHPs

form the backbone of the salivary sheath. This scenario was supported by a diminished degree of polymerization, exemplified by a disorganized sheath structure, in the presence of the reducing compound dithiothreitol that breaks disulphide bonds (Will et al., 2012).

Inhibition of sheath formation by silencing of *shp* expression in *A. pisum* by means of injection of double-stranded RNA demonstrated that SHP is an important structural sheath protein (Will and Vilcinskas, 2015). Reduced sheath hardening weakened the capability of aphids to withdraw nutrition from sieve elements (Will and Vilcinskas, 2015). *S. avenae* individuals feeding on barley plants containing a similar dsRNA sequence suffered from decreased reproduction and reduced survival rates in comparison to aphids on control plants (Abdellatef et al., 2015). A necessity of salivary sheath proteins for the interaction between sucking insects and plants was also demonstrated for the brown plant hopper on rice plants (Huang et al., 2015).

Recently a second *A. pisum* cysteine-rich protein carrying 14 cysteine domains has been discovered (Guo et al., 2014). This zinc-dependent protein (ACYP139568), found enriched in extracts of the salivary glands of *A. pisum*, may constitute another part of the sheath backbone. The expression was enhanced when the aphids were feeding on plants instead of on artificial diets (Guo et al., 2014), which is a phenomenon; reported more often in transcriptome studies (Wang et al., 2015b).

It is to be expected that gel saliva contains wall-softening and -degrading enzymes facilitate stylet progress (see section: A side step: physical components of aphid–plant interaction and putative consequences for the salivary proteome). Therefore, it is no surprise that several cell-wall degrading enzymes were found among salivary proteins, although only a few have been attributed to gel saliva with certainty. Among the potential cell-wall degrading enzymes are pectinases (Ma et al., 1990; Cherqui and Tjallingii, 2000), pectin methylesterases (Ma et al., 1990; Madhusudhan and Miles, 1998), and polygalacturonases (Ma et al., 1990; Madhusudhan and Miles, 1998). Cellulase-(like) activity was detected in aphid saliva (Adams and Drew, 1965) and in aphid homogenates (Campbell and Dreyer, 1985). Several cellulase transcripts were recently detected in *M. persicae* and *Myzus cerasi*, but were absent in *R. padi* (Thorpe et al., 2016) which raises questions regarding their function.

Thus far, none of the effects of cell-wall degrading enzymes has been demonstrated unequivocally. Miles (1999) had even doubts on the effectiveness of the wall-degrading enzymes in general, given the presumptively low rates of catalytic activity and the rapid stylet progress so that the residence times of the stylet tip are short. In this context, it is worthwhile to note that cell-wall degradation products (e.g., cellodextrins and polygalacturonides) act as pathogen-induced molecular patterns evoking plant defense responses (Aziz et al., 2007; Will and van Bel, 2008). Polygalacturonides elicit an increase in cytosolic Ca^{2+} (e.g., Messiaen et al., 1993) which might act as a second messenger in plant defense (Maffei et al., 2007; War et al., 2012).

As a last note, cell walls contain a wealth of phenolic substances (Nicholson and Hammerschmidt, 1992), several of which interfere with aphid infestation by mechanical hindrance, while others are aphid deterrents. It is anticipated that gel saliva contains detoxifying proteins such as phenoloxidases to combat deterring and poisonous phenolics in cell walls and protoplasmic compartments (Miles and Oertli, 1993). Further information on saliva-mediated detoxification of phenolics is given in the Section "Detoxifying Proteins."

Predicted Watery Saliva Proteins

Ca^{2+}-Binding Proteins

The targets of aphids are the sieve elements, nutrient-rich cells in plants. As explained above, puncturing the sieve tubes implies that the sieve elements are damaged by sudden impalement of the stylet. Damage of sieve elements provokes a dual sieve-plate occlusion mechanism in dicotyledons that can extend over considerable distances from the site of wounding inside a plant (Furch et al., 2007, 2010; van Bel et al., 2014). An immediate sealing by protein plugs as a first response is followed by a slower deposition of callose along the sieve pores (Furch et al., 2007, 2008, 2009). Both reactions are Ca^{2+}-dependent and respond to an increase of Ca^{2+} in the sieve-element lumen (Knoblauch et al., 2001, 2003, 2012; Furch et al., 2007, 2009; Hafke et al., 2009). Ca^{2+} increase may arise from influx of cell-wall Ca^{2+} via the imperfectly sealed wound inflicted by the stylet (Will and van Bel, 2006) or via Ca^{2+} channels in the sieve-element plasma membrane or in the sieve-element ER-membranes (Buchen et al., 1983; Sjolund and Shih, 1983; Furch et al., 2009; Hafke et al., 2009). Ca^{2+} influx further away from the site of wounding is triggered by electro-potential waves propagating along the sieve elements (Hafke et al., 2009; van Bel et al., 2014). Sieve-element depolarisations spread into adjacent cells (Rhodes et al., 1996; van Bel et al., 2014), which might explain the occlusion of neighboring sieve tubes during feeding of the generalist aphid species *Macrosiphon euphorbiae* and *M. persicae* (Medina-Ortega and Walker, 2015).

Sieve-tube occlusion by protein plugs was observed in legume sieve tubes that contain forisomes, giant spindle-like protein complexes (e.g., Lawton, 1978; van Bel and van Rijen, 1994; Knoblauch and van Bel, 1998). Forisomes, which seem to be composed of distinctly demarcated subunits (Schwan et al., 2009; Müller et al., 2014), expand in response to wounding and fully occlude the sieve tubes (Knoblauch et al., 2001, 2012). Their expansion is reversible (Knoblauch et al., 2001): forisomes re-condense after some time (30 min to a few hours) provided that the sieve elements are not severely damaged (Furch et al., 2007, 2008, 2009). Forisome expansion is induced by Ca^{2+} influx into the sieve elements (Knoblauch et al., 2001, 2003). It occurs, if the Ca^{2+} level in the sieve elements that is usually extremely low (50 nM; Furch et al., 2009), surpasses a certain threshold, most likely at membrane-located Ca^{2+} hotspots in sieve elements (Hafke et al., 2009). These hotspots arise, since Ca^{2+} ions do not quickly diffuse away from the Ca^{2+} channel apertures and tend to accumulate at sites where Ca^{2+} channels are aggregated. Ca^{2+} hotspots have strong local impact. Ca^{2+} hotspots visualized in transfer cells, for instance, co-localized with cell-wall apposition giving rise to cell wall protuberances (Zhang et al., 2015). The reversibility of the forisome dispersion may depend on the activity of Ca^{2+} pumps in sieve-element membranes as observed in other cell types (Kudla et al., 2010; Huda et al., 2013).

Forisomes are conglomerates of several types of SEO-(sieve-element occlusion) proteins (Noll et al., 2007; Pélissier et al., 2008; Tuteja et al., 2010; Rüping et al., 2010; Ernst et al., 2012; Zielonka et al., 2014; Müller et al., 2014). Remarkably, no known Ca^{2+}-binding motifs were found so far in legume SEOs (Tuteja et al., 2010; Srivastava et al., 2016), but the pea forisome protein PsSEO-F1 showed a cleat-cut Ca^{2+}-binding capability (Srivastava et al., 2016) so that identification of Ca^{2+} binding motifs in SEO protein structures may be a matter of time.

Other members of the SEO-protein family occur in a non-crystalline filamentous form in plant families other than legumes (Rüping et al., 2010; Anstead et al., 2012). Whether these SEO proteins present as heterodimers (Anstead et al., 2012; Jekat et al., 2013) bind Ca^{2+} ions and occlude the sieve plates in an aggregated state is a matter of debate. That mass flow is not blocked by dense aggregates of SEO-proteins near the sieve plates of intact *A. thaliana* plants was taken as evidence that filamentous SEO proteins lack occlusion properties (Froelich et al., 2011). However, this conclusion seems over-hasty, because the occluding capacities of SEO proteins only come to light, when the sieve tubes are damaged. Similar experiments with damaged *A. thaliana* (Jekat et al., 2013) and tobacco (Ernst et al., 2012) plants indicate SEO-mediated sieve-plate occlusion. Moreover, aphid feeding, recorded by using the electrical penetration graph technique, showed an instantaneous interruption in response to remote burning in a range of plant species. This reaction suggests that protein-mediated sieve-tube occlusion also occurs in plant species lacking forisomes (Will et al., 2009).

Not every protein clogging event in sieve tubes is Ca^{2+} dependent. In cucurbits, phloem protein 1 (PP1) and phloem protein 2 (PP2) aggregate to insoluble polymeric plugs in response to oxidation leading to sieve-pore occlusion in cut sieve tubes (Kleinig, 1975; Alosi et al., 1988; Golecki et al., 1998). Within a couple of minutes, interaction between PP1 and PP2 seals the cut surface of cucurbit plants by exudate gelling (Clark et al., 1997; Zimmermann et al., 2013). A similar coagulation of phloem exudate was observed shortly after excision of stylets from aphids feeding on *V. faba* (Fisher and Frame, 1984). It is hard to conceive that this gelling is due to dispersing forisomes, since their number is likely to be extremely low in the exudate (none to a few). Hence, the phenomenon infers oxygen-sensitive protein gelling in broadbean sieve-tube sap as well (Arsanto, 1986).

Oxygen-stimulated linkage of PP1 and PP2 may be corroborated by Ca^{2+}-binding sites. Cucurbit sieve-tubes occlude several centimeters away from a site of burning (Furch et al., 2010). The distinct decrease in soluble PP1 and PP2-dimers in sieve elements there (Furch et al., 2010) is supportive of PP1–PP2 cross-linking. Latter may depend on a longitudinal electric potential wave evoked by burning (Furch et al., 2010). The electric potential wave induces a rise in Ca^{2+} concentrations along the sieve tubes (Furch et al., 2009). PP1 is a candidate for Ca^{2+}-mediated sieve-element occlusion (Furch et al., 2010) in view of its potential Ca^{2+}-binding sites (McEuen et al., 1981; Arsanto, 1986). PP2 is abundantly present in the sieve-tubes of *A. thaliana* (Batailler et al., 2012), but interaction with environmental factors remains unclear. Simultaneously, the Ca^{2+} wave may enhance the level of reactive oxygen species in sieve elements (Görlach et al., 2015; Evans et al., 2016). As yet it is difficult to discriminate between the potential cues of PP1–PP2 cross-linking there. Is either the rise in Ca^{2+} or ROS or is it a collaborative action?

Obviously, sieve-tube occlusion endangers food ingestion by aphids, because their nutrients are transported by mass flow through the sieve tubes. Sequestration of Ca^{2+} ions would prevent sieve-plate occlusion and preserve mass flow. Based upon this idea, it has been postulated that aphid saliva contains Ca^{2+}-binding proteins (Will and van Bel, 2006). Ca^{2+}-binding properties of aphid saliva were substantiated by experiments using the *in vitro* Ca^{2+} reactivity of forisomes from *V. faba* (Knoblauch et al., 2001, 2012; Will et al., 2007). Concentrated aphid saliva, as well as EDTA, showed similar condensation effects on isolated dispersed forisomes (Will et al., 2007). It has been argued that these *in vitro* experiments using salivary probes and forisomes (Will et al., 2007) lead to an enormous overestimation of the Ca^{2+}-binding capacity of saliva proteins. It is difficult to rebut this critical point using quantitative arguments. The actual saliva concentration is probably much lower in sieve tubes, punctured by one or a few aphids. On the other hand, Ca^{2+} influx brought about by a single stylet puncture is much lower than the amount of Ca^{2+} ions administered in the *in vitro* forisome experiments (Will et al., 2007). Moreover, Ca^{2+} can be readily sequestered, since saliva is being secreted near the site of puncture.

Separation by SDS-PAGE and selective staining and radio-labeling disclosed the presence of several Ca^{2+}-binding proteins in the saliva of *M. viciae* (Will et al., 2007). Later proteomic analysis of saliva from *A. pisum* showed the presence of regucalcin, a Ca^{2+}-binding protein involved in Ca^{2+} homeostasis (Carolan et al., 2009). Regucalcin sequesters Ca^{2+} and activates Ca^{2+} pumps (Yamaguchi, 2005). This 43-kDa protein belongs to the SMP-30 superfamily and coincides in molecular size with a 43-kDa Ca^{2+}-binding protein identified by one-dimensional SDS-PAGE in the saliva of *M. viciae* (Will et al., 2007). Regucalcin was not found in the saliva of cereal aphids (Rao et al., 2013), which once again illustrates the diversity of saliva between the aphid species.

The second Ca^{2+}-binding protein, detected in *A. pisum* saliva, was an ARMET protein (Wang et al., 2015a) that was identified by proteomic analysis (Carolan et al., 2011). ARMET proteins are associated with the unfolded protein response (UPR) in ER stacks (Hampton, 2000; Apostolou et al., 2008). It was argued that salivary protein concentrations in sieve-tube sap are generally low, so that Ca^{2+} sequestration by salivary ARMET should be highly localized (Wang et al., 2015a). As sieve-element wounding is likely correlated with Ca^{2+} release from ER compartments via CICR-channels (Hafke et al., 2009; Evans et al., 2016), interference with Ca^{2+} trafficking at the sieve-element ER-membranes (Hafke et al., 2009) may be a feasible option for ARMET functioning.

Recently, a third potential mode of lowering the Ca^{2+} concentration in sieve elements by salivary proteins became apparent (Sinha et al., 2016). Ca^{2+} release from the sieve-element ER stacks (Buchen et al., 1983; Sjolund and Shih, 1983;

Furch et al., 2009; Hafke et al., 2009) likely contributes to increased mictoplasmic Ca^{2+} levels in response to aphid attack. Mastoparan treatments of staminal hairs of *Setcreasea purpurea* demonstrated that IP_3-activated Ca^{2+} channels appear to be relevant for Ca^{2+} efflux from the ER compartments (Tucker and Boss, 1996), where the Ca^{2+} concentration is at least 10,000 times higher (Montero et al., 1995) than in the sieve-element lumen (Furch et al., 2009). As a speculation, an interference of salivary proteins with the phosphoinositide metabolism, would lead to a reduced IP_3 production, and thus would suppress the release of Ca^{2+} ions into the sieve-element lumen. *D. noxia* biotype-2 aphids that overexpress proteins inhibiting the key enzyme phospopholipase C, relevant for IP_3 formation, are virulent on aphid-resistant wheat plants (Sinha et al., 2016). However, it should be noted that, in contrast to overwhelming evidence for IP_3 involvement in Ca^{2+} release in animal cells, the presence of cytosolic IP_3 in plants has not been convincingly demonstrated as yet (Kudla et al., 2010).

The presence of Ca^{2+}-binding proteins is not limited to aphids. An EF-hand motif – a helix-loop-helix structural domain in a large family of Ca^{2+}-binding proteins – was found in a salivary protein of the green rice leafhopper *Nephotettix cinticeps* (Hattori et al., 2012), so that Ca^{2+}-binding properties of saliva may be universal among plant sucking Hemiptera.

Ca^{2+} sequestration by salivary proteins with the objective to suppress sieve-tube occlusion may be wide-spread among aphid species. The sudden change in feeding behavior in response to remote heat shocks in diverse plant-aphid combinations (Will et al., 2009) hints at intensified saliva secretion to counteract imminent sieve-element occlusion. As a speculation, binding of Ca^{2+} by salivary proteins can be regarded as an adaptation of specialized feeders like *A. pisum* to those plants that highly rely on Ca^{2+}-based occlusion. Penetration of *A. pisum* stylets did not trigger sieve-tube occlusion in *V. faba* (Walker and Medina-Ortega, 2012). This lack of reaction has been ascribed to the high specialization of pea aphids to legumes (Medina-Ortega and Walker, 2015). The saliva would sequester Ca^{2+} to such an extent that condensed forisomes are not sufficiently challenged (Will, 2016b). The latter conclusion was drawn, since feeding of generalists like *M. persicae* and *M. euphorbiae* indeed triggered forisome-mediated occlusion (Medina-Ortega and Walker, 2015). It is no surprise that Ca^{2+}-channel blockers favored feeding of *M. persicae* by preventing sieve-element occlusion (Medina-Ortega and Walker, 2015), since the Ca^{2+}-channel blocker gadolinium prevented forisome dispersion in sieve-element protoplasts (Hafke et al., 2007).

Indirect Suppression of Callose Production by Salivary Proteins?

The second slower type of sieve-tube occlusion by callose partly overlaps in time with protein occlusion (Furch et al., 2007) and is executed by β-1,3 glucan depositions around the sieve plate pores, termed "callose plugs" (van Bel, 2005). These "plugs" reside unlike protein plugs in the extracellular space and are, in fact, callose collars apposed against the cell-wall areas bordering the plasma membrane corridor that crosses the sieve pores (Evert, 1990; Ehlers et al., 2000). As result of callose deposition, the mictoplasmic corridors between sieve elements become "strangled" and phloem transport stops. It should be noted that callose is not always deposited in the extracellular space. Intracellular callose plugs of plasmodesmata are delivered by multivesicular bodies as an early step in the hypersensitive response (An et al., 2006). Furthermore, callose plugs inside plasmodesmal corridors are involved in dormancy processes (Rinne et al., 2001).

Since sieve pores are evolutionary and ontogenic "descendants" of plasmodesmata (Evert, 1977, 1990), one may expect that fundamental traits such as callose homeostasis have strong similarities between both structures and that plasmodesmal physiology might tell us much about sieve-pore physiology. Unlike the continuous callose collars in the sieve pores, plasmodesmata have a ring of callose around both neck regions (sphincters) of the cytoplasmic corridors that connect neighboring cells (Radford et al., 1998). The permanence of callose rings around the sphincters of standard plasmodesmata is a matter of debate (Levy and Epel, 2009). In sieve elements, callose depositions – necessary for the formation of sieve pores and PPUs (Evert, 1977, 1990; Barratt et al., 2011) – permanently surround the mictoplasmic corridors (through the sieve pores) between sieve elements after maturation (Ehlers et al., 2000). Plasmodesmal fluxes are inversely correlated with the degree of callose deposition (e.g., Zavaliev et al., 2011; Tilsner et al., 2016), which suggests a strong resemblance with sieve-pore functionality.

According to recent reviews (Zavaliev et al., 2011; De Storme and Geelen, 2014; Kumar et al., 2015; Tilsner et al., 2016), callose deposition results from an equilibirum imposed by the activities of plasma transmembrane β-1,3 glucan synthases or callose synthases (GSLs) and plasma-membrane anchored extracellular β-1,3 glucanases or glucan endo-1,3-hydrolases (BGs).

Callose synthases present a multigene family of large (200–220 kDa) plasma-membrane spanning proteins with both the N- and C-terminus residing in the cytoplasm (Farrokhi et al., 2006; Brownfield et al., 2007). They are typically clustered in two transmembrane regions connected by an extensive cytoplasmic loop, including an UDP-glucose catalytic site and a glysosyltransferase domain (see for reviews: De Storme and Geelen, 2014; Tilsner et al., 2016). Together with the N-terminal region, the hydrophilic loop acts as a site for interaction with regulatory molecules due to several glycosylation and phosphorylation domains (Verma and Hong, 2001). Callose synthases are probably incorporated into complex protein structures (CalS holoenzyme complexes; Amor et al., 1995; Hong et al., 2001), that include several proteins participating in the polymerization of the substrate UDP-glucose and the delivery of glucan chains into the apoplasmic space. Three genes were found to be directly associated with plasmodesmal callose deposition. Most likely, *GSL 8* is responsible for callose deposition in plasmodesmata in a wide variety of tissues (Guseman et al., 2010), *GSL7* is involved in sieve-pore shaping during sieve-element development (Barratt et al., 2011; Xie et al., 2011), and *GSL12* has a major role in adjusting the functional diameter of plasmodesmata (Vaten et al., 2011). Their distribution over the

cell types seems to indicate that all three genes are engaged in callose homeostasis in sieve elements.

Glucanases are the functional counterpart of callose synthases. In *Arabidopsis*, the glucanase family comprises 50 representatives of β-1,3-glucan-degrading enzymes (Doxey et al., 2007). Thus far, three of them were found to be associated with plasmodesmal regions and were characterized: one in leaves (Zavaliev et al., 2013) and two in roots (Benitez-Alfonso et al., 2013). They are likely anchored by a C-terminal glycophosphatidylinositol (Gaudioso-Pedraza and Benitez-Alfonso, 2014) to the outer leaflet of the plasma membrane and thus face the inner side of the cell wall. β-1,3-glucanases are produced in the Golgi system and secreted into the apoplasmic space via exocytosis (De Storme and Geelen, 2014). In conclusion, it is of paramount importance to note that the regulation of callose deposition is located in entirely different cell compartments. Callose synthases operate in the cytosol, glucanases in the extracellular space.

Pioneer experiments of Erwee and Goodwin (1983) demonstrated that the plasmodesmal diameter is under the control of Ca^{2+} ions. A rise in Ca^{2+} concentration, probably from the ER stores (Tucker, 1988; Tucker and Boss, 1996), coincides with enhanced callose deposition (Tucker and Boss, 1996; Holdaway-Clarke et al., 2000) and, hence, increased obstruction of symplasmic transports (Sager and Lee, 2014). As a result, abrupt changes in intercellular Ca^{2+} concentration have an immediate effect on intercellular communication. The molecular mechanisms that control this crucial Ca^{2+} effect on callose synthesis has yet to be thoroughly investigated (Zavaliev et al., 2011; De Storme and Geelen, 2014; Kumar et al., 2015; Tilsner et al., 2016).

Similar Ca^{2+}-controlled mechanisms are supposed to regulate mass flow through sieve tubes (Kauss, 1987). The uneven deployment of Ca^{2+} channels along sieve elements renders credence for a strong relationship between Ca^{2+} level and callose deposition (Furch et al., 2009). The modified plasmodesmata of sieve elements (sieve pores and pore-plasmodesm units or PPUs, the symplasmic connections with the companion cells) possess constitutive callose linings (Evert, 1990; Ehlers et al., 2000). Sieve pores and PPUs perfectly co-localize with Ca^{2+} hotspots (Furch et al., 2009; Hafke et al., 2009; see section Ca^{2+}-binding proteins for a hotspot definition). Aggregates of Ca^{2+} channels along the sieve-element plasma membrane allow changes in Ca^{2+} level focused at sites, where they are physiologically relevant for immediate action such as callose deposition and protein dispersion (Hafke et al., 2009). Such a phenomenon is by no means unique. In developing transfer cells, cytosolic Ca^{2+} plumes co-localize with the deposition of cell-wall protrusions, which may consist in part of callose to provide a plastic matrix for embedment of stiffer cell-wall components (Zhang et al., 2015).

In disturbed, yet undamaged sieve elements, supplementary callose depositions at sieve pores and PPUs disappear within a couple of hours after disturbance (Furch et al., 2007, 2010). The quick build-up and slower breakdown of callose in sieve elements raises questions regarding the control mechanisms. The basic level of callose deposition may be balanced by the counteraction of callose synthases and glucanases (Zavaliev et al., 2011; De Storme and Geelen, 2014; Kumar et al., 2015).

The rapidity of callose build-up in response to wounding (seconds to minutes) suggests a regulation on the protein level. Ca^{2+} released into the sieve element mictoplasm may readily bind to the CalS complex (Hong et al., 2001) with the putative participation of ATP and calmodulin (Levy and Epel, 2009) under favorable redox conditions (see Zavaliev et al., 2011). As a speculation, a rapid rise in intracellular Ca^{2+} will boost the activity of the callose synthases to an extent that cannot be counteracted by glucanase activity and additional callose will be deposited. If however, Ca^{2+} is removed from the local cytosol to the ground level as will happen in undamaged sieve elements (Furch et al., 2007), glucanases have the chance to reduce the callose layer to the original thickness in sieve elements.

It is possible that callose synthases and glucanases are upregulated at the transcript level during peaks of callose turnover. It should be realized, however that the situation in enucleate sieve elements differs radically from that in nucleate cells. Gene expression of callose synthases and glucanases must take place in cells adjacent to the sieve elements, most probably the companion cells, from which the glucanases are released into the apoplasmic space by exocytosis (De Storme and Geelen, 2014).

In conclusion, the previous considerations infer altogether at least two potential modes of callose suppression by aphid saliva. As callose sealing is considered to be a general, but quantitatively species-specific (Saheed et al., 2009) defense mechanism against aphids and other sucking insects (Hao et al., 2008), Ca^{2+} sequestration by salivary proteins may enable the aphids to prevent callose deposition by suppression of Ca^{2+}-stimulated callose-synthase activity. When thick callose depositions are found in sieve tubes as a response to aphid attack, Ca^{2+} sequestration has likely been insufficient to prevent callose synthesis (Saheed et al., 2009). Ca^{2+}-binding proteins may also disturb the equilibrium between callose build-up and degradation so that stimulation of glucanase action is conceivable. Breakdown of callose depositions by salivary glucanases is unlikey, since they would be introduced into the luminal compartment of sieve elements, out of reach of the callose located in the cell-walls. It is much more logical; therefore, that callose breakdown is facilitated by upregulation of plant glucanases (Van der Westhuizen et al., 1998) probably making use of salivary effectors. In keeping with this idea, infestation with *R. padi* induced a transcript abundance of three β-1,3-glucanases in 15 barley breeding lines (Mehrabi et al., 2016). In Mp55-expressing *Arabidopsis* plants, callose deposition in response to aphid infestation is reduced (Elzinga et al., 2014), but the molecular action of Mp55 is yet to be identified.

Proteases

The presence of proteases in the alimentary tract of aphids is an established fact (Rahbé et al., 1995; Cristofoletti et al., 2003, 2006; Pyati et al., 2011). It was suspected for a long time that aphid saliva as well contains several proteases, although the first functional assays were not successful (Cherqui and Tjallingii, 2000). Some proteases were recently identified using proteomics on aphid saliva and salivary gland extracts of *A. pisum* (Carolan et al., 2009, 2011). In mammalian systems, the M1

zinc metalloprotease and M2 metalloproteases (angiotensin-converting enzymes, ACEs; Wang et al., 2015b) bind zinc at their catalytic domain (Lausten et al., 2001; Kim et al., 2003), while Ca^{2+} regulates their catalytic activity (Goto et al., 2007). ACEs remove dipeptides from short oligopeptides (Ehlers and Riordan, 1989) which contrasts the action of M1 metalloproteases cleaving the terminal amino acids from proteins (Itturioz et al., 2001; Naqvi et al., 2005). It is not excluded that salivary proteases are species-specific in aphids: the metalloproteases detected in *A. pisum* (Carolan et al., 2009, 2011) were not found in the saliva of *S. avenae* and *M. dirhodum* (Rao et al., 2013). Like Ca^{2+}-binding proteins, metalloproteases were also found in the saliva of other insect groups such as the phytophagous thrips *Frankliniella occidentalis* (Stafford-Banks et al., 2014) and the blood-feeding tick *Ixodes scapularis* (Francischetti et al., 2003; Decrem et al., 2008).

The putative proteolytic activity of saliva was verified by two functional assays (Furch et al., 2015). Albumin was degraded by salivary probes of *A. pisum* in the presence or absence of EDTA. Rapid albumin breakdown, in particular in the absence of EDTA, indicated the involvement of metalloproteases in protein degradation (Furch et al., 2015). Mixing salivary probes of *A. pisum* and *M. euphorbiae*, respectively, with sieve-tube exudate of *Cucurbita maxima* demonstrated that PP1 and PP2 in the protein-rich exudate were degraded with time (Furch et al., 2015). This is supportive of protease-mediated breakdown of sieve-element proteins by salivary proteases. PP1, the protein specialized in occlusion, was more rapidly broken down than PP2, which appeared to be recalcitrant to breakdown by proteases in the alimentary tract (Rahbé et al., 1995). In conclusion, proteases may act as an auxiliary tool to remove proteinaceous occlusions.

A third functional aspect of protease activity pertains to aphid nutrition (Carolan et al., 2009; Furch et al., 2015). As sieve-tube sap is poor in several essential amino acids (Gündüz and Douglas, 2009), aphids are mostly short of these indispensable compounds. The deficiency is compensated by endosymbiotic bacteria that transform non-essential into essential amino acids (Baumann et al., 1995). Amino acids from degraded proteins in sieve tubes could enhance the aphid's diet quality. The breakdown strategy may be more successful in dicots than in monocots given the higher protein contents in sieve-tube sap of dicots (2–100 mg ml^{-1}: Richardson et al., 1982; Schobert et al., 1998; Zimmermann et al., 2013) as compared to monocots (0.1–1.0 mg ml^{-1}: Fisher et al., 1992; Schobert et al., 1998; Gaupels et al., 2008).

It is unclear, if protein breakdown by salivary proteases merely has a non-selective character as indicated by *in vitro* breakdown of proteins by salivary proteases (Furch et al., 2015). In view of the presence of so many other vital proteins in the saliva, however, additional selective protein-breakdown machinery seems logical. Selective protease activity is usually associated with ubiquitin tagging accompanied by several accessory enzymes (van der Hoorn, 2008) and the final transfer of the ubiquitin-protein complex to the proteasome. The question arises, whether ubiquitin as a first indicator of selective protein degradation occurs in sieve elements or aphid saliva. After the first detection of ubiquitin in *Ricinus communis* sieve-tube exudate (Schobert et al., 1995), proteomics disclosed the presence of at least 116 components involved in proteasome-associated protein degradation in the sieve-tube sap of cucurbits (Lin et al., 2009) This indication for a complete proteolytic system in sieve elements was consistent with the presence of ubiquination compounds in the sieve-tube sap of rice (Aki et al., 2008) and rapeseed (Giavalisco et al., 2006) and the likeliness of proteasomes in sieve elements (Ingvardsen and Veierskov, 2001). In contrast, native ubiquitin nor proteasomal components have not been found in aphid saliva thus far (Chaudhary et al., 2015). Should selective protein degradation be executed by salivary proteases, they likely make use of the sieve-element breakdown machinery.

Detoxifying Proteins

The presence of polyphenoloxidases (Peng and Miles, 1988; Miles and Oertli, 1993; Madhusudhan and Miles, 1998; Urbanska et al., 1998; Cherqui and Tjallingii, 2000; Harmel et al., 2008; Cooper et al., 2011; Chaudhary et al., 2015) and peroxidases in saliva (Miles and Peng, 1989; Cherqui and Tjallingii, 2000; Chaudhary et al., 2015) is interpreted as a reductive weapon against plant phenols and reactive oxygen species (Leszczynski et al., 1985; Moloi and van der Westhuizen, 2006). A similar function has been attributed to oxidoreductases (Miles and Oertli, 1993) and GMC-oxidoreductases (Carolan et al., 2009; Nicholson et al., 2012; Rao et al., 2013). Given the differences in phenol localization (epidermis, cortex, mesophyll, sieve tubes) and phenol quantities between plant species, some of the oxidases may be most effective against toxic substances along the stylet pathway and others against phenols or other toxic substances residing inside the sieve tubes. Peroxidases may also play a role in the breakdown of hydrogen peroxide, a booster of Ca^{2+}-channel gating (Lecourieux et al., 2006), and hence may reduce the occlusion of sieve tubes. The same may be true for GMC-oxidoreductases, which possess ROS (reactive oxygen species)-scavenging properties. However, the low pH optima of these enzymes raise some reserve as for their efficiency in view of the alkaline sieve-tube milieu (Will, 2016b). The ROS-scavengers possibly interact with the native ROS-scavenging system in sieve tubes (Walz et al., 2002).

Evidence has emerged that the redox status impacts on callose synthesis. In *Arabidopsis* mutants defective in the thioredoxin-m3 gene (*TRX-m3*), GFP diffusion out of the sieve elements was reduced in comparison with wild-type plants. These mutants accumulated ROS and contained higher levels of callose in root tips (Benitez-Alfonso et al., 2009). Overexpression of TRX-m3, by contrast, resulted in enhanced intercellular transport compared to wild-type plants. Moreover, reduced macromolecular trafficking in wild-type plants treated with oxidants (Benitez-Alfonso et al., 2009) and in *Arabidopsis* mutants defective in the production of glutathione (Cairns et al., 2006) inferred that oxidants stimulate callose production. Consequently, oxidoreductases and other ROS scavengers in aphid saliva may limit the cellular damage caused by ROS (e.g., lipids and proteins), but also impair callose production by withdrawal of oxidants. This conclusion may be premature as in *Arabidopsis ise1* mutants in which the ROS level

exceeds that in wild-type plants, intercellular transport is stimulated (Stonebloom et al., 2009). It seems beyond doubt that redox homeostasis regulates intercellular trafficking probably by interaction with callose metabolism. It is unclear, whether ROS directly act upon callose deposition or if Ca^{2+} channels are gated by ROS as demonstrated for hydrogen peroxide (Lecourieux et al., 2006).

Salivary dehydrogenases and glucose oxidases possibly interfere with plant defense systems regulated by jasmonic acid (JA) and salicylic acid (SA) during aphid infestation (Louis and Shah, 2013). The suppressed JA-controlled defense responses of *A. thaliana* to *Brevicoryne brassicae* infestation (Kusnierczyk et al., 2011) could be due to a diminished production of JA (Takemoto et al., 2013) conferred by the above-mentioned enzymes. It is unclear, if the aphid-imposed modulation of genes engaged in SA-synthesis (Zhu-Salzman et al., 2004) depends on the cross-talk with the JA pathway (Pieterse et al., 2012) or on so-called salivary effectors.

Chaudhary et al. (2015) detected several other defense modulators in the saliva of *M. euphorbia*, mainly interfering with regulatory enzymes of the oxidative burst, a hallmark in plant immunity. In addition, lipase-like proteins in the same secretome may function as virulence factors to promote aphid colonization (Chaudhary et al., 2015).

Effector Proteins of Unknown Function

In contrast to the protein classes discussed above, the majority of salivary proteins may not interfere in a direct disruptive manner with events in cell walls or inside sieve elements. They probably interact in an unknown fashion with the protein network involved in plant defense. Effectors act in a broad spectrum of pathogens and are generally defined as molecules that alter function and/or structure of host cells (Hogenhout et al., 2009). This definition includes a group of proteins that effect on aphid fecundity and behavior. Their effect on suppression or elicitation of plant defense, respectively, are inferred from the rates of aphid colonization or fecundity (Bos et al., 2010; Pitino et al., 2011; Atamian et al., 2013; Elzinga and Jander, 2013; Pitino and Hogenhout, 2013; Rodriguez and Bos, 2013; Elzinga et al., 2014; Rodriguez et al., 2014; Chaudhary et al., 2015).

As a first salivary protein, C002 was termed "effector". C002 discovered in the saliva of *A. pisum* and is required for continuous aphid feeding (Mutti et al., 2008). When C002 is transiently overexpressed in plants that were subsequently infested by *M. persicae*, aphid fecundity is increased (Bos et al., 2010). In reverse, suppression of C002 expression by RNA interference is lethal in *A. pisum* (Mutti et al., 2006) and causes reduced fecundity in *M. persicae* (Pitino et al., 2011). Interestingly, C002 is only required for feeding on plants, not on diets (Mutti et al., 2008), which implies a role in compatibilty. C002 may have significance for the host range of an aphid species. The ability of *M. persicae* to feed on a broad range of host plants may be due to the presence of a repeated 7-amino acid motif in C002 of *M. persicae* that is absent in C002 of *A. pisum* (Pitino and Hogenhout, 2013).

Aphid colonization is also promoted by two further salivary proteins detected in *M. persicae* (Pitino and Hogenhout, 2013). These effector proteins [PIntO1, (Mp1) and PintO2 (Mp2)] are acting in a plant species-specific manner (Pitino and Hogenhout, 2013). The effector Mp55 also suppresses plant defense. Mp55-expressing *A. thaliana* plants were more attractive to aphids in choice assays, whereas Mp55 silencing in aphids led to reduced reproduction rates (Elzinga et al., 2014).

Likewise, saliva of *M. euphorbiae* contains two proteins, Me10 and Me23 that promote aphid colonization, as they seem to suppress plant defense when expressed in *Nicotiana benthamiana* (Atamian et al., 2013). Me23 possesses a conserved glutathione peroxidase domain and might interfere with plant defense responses, while the function of Me10 is unknown (Chaudhary et al., 2015). They are both phosphorylated, but the molecular significance of these phosphorylation sites is elusive (Chaudhary et al., 2015). When expressed in tomato, only Me10 increased aphid fecundity underlining once again the species-specificity of effectors (Atamian et al., 2013), the more as expression of the Me10-homologue Mp58 resulted in a decreased fecundity in *Nicotiana tabacum* and *Arabidopsis* (Elzinga et al., 2014). As for the function of these proteins, the fundamental question remains as whether the increased fecundity of aphids is due to the suppression of plant defense or to a promoted efficiency of aphid feeding.

By contrast, some salivary proteins such as Mp10 and Mp42 from *M. persicae* suppress aphid reproductive performance and appear to elicit plant defense reactions (Bos et al., 2010). *In planta* overexpression of Mp10 and Mp42 in *N. benthamiana* reduced aphid feeding (Bos et al., 2010). Further work revealed that Mp10 and Mp42 are engaged in elicitation of plant defense in distinct ways at different subcellular locations. Transient overexpression activated JA and SA signaling pathways, while Mp42 did not (Rodriguez and Bos, 2013; Rodriguez et al., 2014).

Recently, a macrophage migration inhibitory factor (MIF) was identified in the saliva of *A. pisum* using 2D-DIGE-MALDI-TOF/MS (Vandermoten et al., 2014; Naessens et al., 2015) that sheds some light on effector action. In mammals MIFs are pro-inflammatory cytokins that regulate immune responses (Calandra and Roger, 2003). Of five members of a MIF multigene family in *A. pisum* (Dubreuil et al., 2014), just one (*ApMIF1*) is present in the saliva (Naessens et al., 2015; Reymond and Calandra, 2015). This protein was postulated to suppress the immune response of plants by inhibiting the expression of defense-related genes (Naessens et al., 2015).

Endosymbiont-Derived Proteins

Beside proteins that possess a secretory signal sequence and were classified as salivary components, a number of proteins that have been identified in salivary glands are lacking such a sequence (Carolan et al., 2011). It has been a matter of dispute, if such proteins belong to the salivary proteome. Recently, eleven proteins from *Buchnera aphidicola* proteins were detected in the saliva of *M. euphorbia*, four of which were attributed to gel saliva only (Chaudhary et al., 2014). One of the identified proteins was the chaperone GroEL, a heat shock protein engaged in protein folding, which is abundant in *B. aphidicola* (Baumann et al., 1996). Several GroEL expression studies in plants demonstrated that GroEL is recognized intra- and

extracellularly and functions as a microbe associated molecular pattern (MAMP) triggering an oxidative burst and the expression of a number of immunity marker genes (Chaudhary et al., 2014). This suggests that GroEL has to be regarded as a biologically relevant contaminant that elicits plant defense. Aphids reacted to increased GroEL expression by diminished fecundity (Chaudhary et al., 2014; Elzinga et al., 2014). The investigators interpreted the plant defense response as being targeted to *B. aphidicola*, which impacts negatively on the reproduction of the aphids (Chaudhary et al., 2014). Removal of *B. aphidicola* activity indeed led to a delayed aphid development and a considerable decrease of reproduction rates (Sasaki et al., 1991).

It is of major importance to investigate the route followed by GroEL into the salivary glands. Such studies may give a clue, as if proteins without a secretory sequence are produced by the gland cells or are imported into the glands after being produced by other organs.

Impact of Chitin Fragments in Aphid Saliva on Plant Defense Responses?

Although chitin does not belong to the salivary secretome, aphid saliva may contain chitin fragments. They may be rubbed away by mechanical stress during stylet movement or are liberated by plant chitinases. In the presence of chitin, the lysin domain-containing glycophosphatidylinositol-anchored protein 2 (LYM2) which is an *Arabidopsis* homologue of a chitin receptor-like protein (Kaku et al., 2006), becomes involved in the reduction of plasmodesmal traffic (Faulkner et al., 2013). As a speculation, chitin loss from the stylet surface may impact on the cellular response to stylet penetration by callose deposition, mediated by the plasmodesma-located LYM2-protein. Thus, chitin fragments may act as pathogen-associated molecular patterns that strengthen the plant-defense response. It should be noted, however, that CERK1 (CHITIN ELECITOR RECEPTOR KINASE1) does not seem to be engaged in chitin recognition (Prince et al., 2014).

CONCLUDING REMARKS

Unequivocal separation of gel and watery saliva is an absolute prerequisite for the functional assessment of salivary proteins, but suffers from cross-contamination in dietary solutions up till now. Analysis of proteins collected in specific diets (e.g., pH 5 or 7) or *in aera* will enable a distinct separation of the saliva types. It should be taken into account that salivary protein patterns obtained with artificial diets and plants often differ.

Protein profiles of watery saliva exhibit large interspecific variations. The evolutionary challenges imposed by the highly variable symplasmic conditions between plant species appear to have had a strong impact on the nature of the salivary proteins. The protein profiles of watery saliva indicate that there is a wealth of possibilities for interaction between host plant and aphid species.

The protein composition of gel saliva is anticipated to be more conservative in view of the lower interspecific variability of the cell-wall milieu.

Studies point to an adaptation of the salivary protein composition to the plant host, but more research is required, i.e., on the time-dependence and on the transgenerational nature of the adaptation.

The proven and putative functions of (putative) salivary proteins are in keeping with the stylet itinerary and the proposed mode of orientation. Several functional groups of salivary proteins have been distinguished or postulated thus far:

(1) Proteins that provide the structural backbone for the salivary sheath.
(2) Proteins that degrade cell-wall carbohydrates and by doing so facilitate stylet movement and give rise to the production of pathogen-induced molecular patterns.
(3) Proteins that function in prevention or degradation of sieve-plate occlusion (by proteins and callose) by sequestration of Ca^{2+} ions, in Ca^{2+} homeostasis, and in triggering several signaling cascades under the control of Ca^{2+} ions.
(4) Proteins engaged in proteolysis, which provide supplementary supply of organic N-compounds to the aphid diet, in the degradation of protein plugs on sieve plates, or in the sabotage of protein-mediated plant defense mechanisms.
(5) Proteins that regulate ROS levels, which in turn are associated with local signaling cascades or are engaged in processes that trigger long-distance signals and distant signaling cascades.
(6) Proteins involved in detoxification of a variety of poisonous compounds such as phenols.
(7) Proteins denominated simply "effectors" with an unknown involvement in host-plant defense responses, but with a clear impact on aphid fitness, e.g., fecundity. Some have phosphorylation traits, others suppress immune responses of host plants.
(8) Other salivary components such as endosymbiont-derived proteins interfere with the protein-mediated interaction between aphids and host plants.

With the aid of their vast arsenal of salivary proteins, aphids trigger and suppress plant defense in parallel. If and how activation and suppression of plant defense go hand in hand and if aphids benefit from a local induction of plant defense, remains to be investigated. Identification of salivary proteins of interest by use of proteomics is the first step. The search for their functional and, hence, biological relevance is the next essential step.

AUTHOR CONTRIBUTIONS

The authors made equal intellectual contributions to the work and approved it for publication.

ACKNOWLEDGMENTS

We express our gratitude to Kathrin Steckbauer Ph.D., Angela Hewer Ph.D., Sara Ruoss Ph.D., and Alexander Becker MSc for fruitful discussions and we are grateful for the constructive comments of the reviewers. They all contributed to upgrading of this review.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

METHODS
published: 09 August 2016
doi: 10.3389/fpls.2016.01171

Optimization of Agroinfiltration in *Pisum sativum* Provides a New Tool for Studying the Salivary Protein Functions in the Pea Aphid Complex

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OPEN ACCESS

Edited by:
Jyoti Shah,
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Reviewed by:
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Utrecht University, Netherlands
Karam B. Singh,
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *29 April 2016*
Accepted: *20 July 2016*
Published: *09 August 2016*

Citation:
Guy E, Boulain H, Aigu Y, Le Pennec C, Chawki K, Morlière S, Schädel K, Kunert G, Simon J-C and Sugio A (2016) Optimization of Agroinfiltration in Pisum sativum Provides a New Tool for Studying the Salivary Protein Functions in the Pea Aphid Complex. Front. Plant Sci. 7:1171. doi: 10.3389/fpls.2016.01171

Aphids are piercing-sucking insect pests and feed on phloem sap. During feeding, aphids inject a battery of salivary proteins into host plant. Some of these proteins function like effectors of microbial pathogens and influence the outcome of plant–aphid interactions. The pea aphid (*Acyrthosiphon pisum*) is the model aphid and encompasses multiple biotypes each specialized to one or a few legume species, providing an opportunity to investigate the underlying mechanisms of the compatibility between plants and aphid biotypes. We aim to identify the aphid factors that determine the compatibility with host plants, hence involved in the host plant specialization process, and hypothesize that salivary proteins are one of those factors. *Agrobacterium*-mediated transient gene expression is a powerful tool to perform functional analyses of effector (salivary) proteins in plants. However, the tool was not established for the legume species that *A. pisum* feeds on. Thus, we decided to optimize the method for legume plants to facilitate the functional analyses of *A. pisum* salivary proteins. We screened a range of cultivars of pea (*Pisum sativum*) and alfalfa (*Medicago sativa*). None of the *M. sativa* cultivars was suitable for agroinfiltration under the tested conditions; however, we established a protocol for efficient transient gene expression in two cultivars of *P. sativum*, ZP1109 and ZP1130, using *A. tumefaciens* AGL-1 strain and the pEAQ-HT-DEST1 vector. We confirmed that the genes are expressed from 3 to 10 days post-infiltration and that aphid lines of the pea adapted biotype fed and reproduced on these two cultivars while lines of alfalfa and clover biotypes did not. Thus, the pea biotype recognizes these two cultivars as typical pea plants. By using a combination of ZP1109 and an *A. pisum* line, we defined an agroinfiltration procedure to examine the effect of *in planta* expression of selected salivary proteins on *A. pisum* fitness and demonstrated that transient expression of one candidate salivary gene increased the fecundity of the aphids. This result confirms that the agroinfiltration can be used to perform functional analyses of salivary proteins in *P. sativum* and consequently to study the molecular mechanisms underlying host specialization in the pea aphid complex.

Keywords: pea aphid, *Acyrthosiphon pisum*, Leguminosae, agroinfiltration, salivary proteins, biotypes, host specialization, effector

INTRODUCTION

Herbivorous insects present a high level of species diversity and a large majority of them is specialized to feed on certain host plant species. Specialization to different host plants also occurs within single insect species and leads to the existence of distinguishable "host races" or "biotypes" (Dres and Mallet, 2002). The mechanisms of host plant adaptation in herbivorous insects are poorly understood, although these could explain a large part of insect species richness (Simon et al., 2015). Therefore, insect species displaying an array of races or biotypes provide interesting opportunities to study the process of host plant specialization due to the possibility to compare genomes and feeding strategies between closely related races or biotypes.

The pea aphid, *Acyrthosiphon pisum* Harris, is the first aphid to be genome sequenced and owing to its long history of research, it is the model of aphids and sap-feeding insects (hemipterans; International Aphid Genomics Consortium, 2010). In addition, *A. pisum* encompasses a range of biotypes each specialized to one or a few closely related legume species but cannot survive or reproduce well on non-host legume plants. So far, 15 biotypes are described (Peccoud et al., 2015), of which alfalfa, clover and pea biotypes are the ones most studied in host specialization (Hawthorne and Via, 2001; Ferrari et al., 2008; Peccoud et al., 2009; Jaquiery et al., 2012; Via et al., 2012). In addition to show strong differences in performances on host and non-host plants, these biotypes are genetically distinct and can be distinguished by using microsatellite markers (Ferrari et al., 2008; Peccoud et al., 2009). Interestingly, all the *A. pisum* biotypes studied so far feed well on *Vicia faba*, which is considered as a universal host plant for pea aphids (Ferrari et al., 2008; Peccoud et al., 2009). Many of these *A. pisum* biotypes can be crossed with other biotypes (Peccoud et al., 2014), and QTL analyses have been used to identify aphid factors that determine the compatibility with the host plants (Hawthorne and Via, 2001; Via et al., 2012; Kanvil et al., 2015).

Aphids feed on plant phloem sap using a specialized mouthpart called stylet. During feeding, aphids may transmit plant pathogenic viruses, inject toxic saliva and remove nutrients from host plants. Hence, aphids are considered among the most serious crop pests. Recent studies gradually revealed that there are intricate molecular interactions between the proteins secreted with aphid saliva and host plant proteins (Elzinga and Jander, 2013; Rodriguez and Bos, 2013; Kaloshian and Walling, 2016). In some cases, salivary proteins trigger plant defense responses (De Vos and Jander, 2009; Chaudhary et al., 2014; Elzinga et al., 2014), in others, they suppress plant defense reactions and promote aphid proliferation (Will et al., 2007; Bos et al., 2010; Atamian et al., 2013; Elzinga et al., 2014; Naessens et al., 2015). Hence, aphid salivary proteins are considered to be analogous to effectors of plant pathogens, and their functions have been examined using similar techniques, such as silencing of salivary genes or *in planta* expression of salivary proteins (Elzinga and Jander, 2013; Rodriguez and Bos, 2013). The first characterized aphid salivary gene was an *A. pisum* gene named C002, which is strongly expressed in salivary glands and was detected in plants infested by the aphids. Silencing of *A. pisum C002* (*ApC002*) was achieved by injection of siRNA in aphids. It prevented aphids from feeding on *V. faba,* while aphid feeding on artificial diet was unaffected (Mutti et al., 2006, 2008). In line with these studies, transient or stable expression of *Myzus persicae* orthologue of ApC002, MpC002, in *Nicotiana benthamiana* and *Arabidopsis thaliana,* respectively, increased the fecundity of *M. persicae* feeding on these plants, indicating the conserved role of C002 as an effector required for aphid feeding on host plants (Bos et al., 2010; Pitino and Hogenhout, 2013; Elzinga et al., 2014).

Since then, several *A. pisum* salivary proteins required for aphid full performance have been identified and characterized mostly by using gene silencing induced by siRNA injection to aphids (Guo et al., 2014; Pan et al., 2015; Wang et al., 2015a,b) while several salivary proteins from other aphids, such as *M. persicae* have been identified using transient or stable *in planta* expression of salivary genes (Bos et al., 2010; Pitino and Hogenhout, 2013; Elzinga et al., 2014). However, since the *A. pisum* genome is extensively duplicated and more than 2000 gene families show massive expansion compared to published insect genomes (Rispe et al., 2008; International Aphid Genomics Consortium, 2010; Jaubert-Possamai et al., 2010), it is often difficult to select a siRNA or dsRNA fragment that specifically targets the gene of interest for silencing. In some cases, co-silencing of multiple gene family members need to be examined to determine whether the phenotype observed is due to the silencing of single gene or multiple genes. Furthermore, there is a possibility that gene silencing does not show a strong phenotypic effect on plant–aphid interactions if genes with redundant functions exist or if gene silencing is too transient.

On the other hand, *in planta* expression of saliva gene allows simple characterization of single gene in plant-aphid interactions. While the construction and multiplication of transgenic plants require several months to years of preparation before testing, *Agrobacterium* mediated transient gene expression (agroinfiltration) can be achieved in a few days; therefore, it is a commonly used technique to identify and characterize effector functions. However, the efficiency of agroinfiltration is highly variable and often depends on the compatibility between the *Agrobacterium tumefaciens* strain and the plant species or cultivar used (Wroblewski et al., 2005). The technique has been developed in *N. benthamiana* using a disarmed strain where the virulence factors encoded by the Ti plasmid were deleted (Goodin et al., 2008). Then, the technique was optimized for different plants such as potato (Bhaskar et al., 2009), lettuce (Chen et al., 2016), grapevine (Santos-Rosa et al., 2008), *Medicago truncatula* (Picard et al., 2013) and recently in soybeans (King et al., 2015). However, the technique is not established in the legume plants, which are hosts for *A. pisum*.

As mentioned earlier, *A. pisum* encompasses multiple biotypes which cannot survive on the plants they are not specialized to. We study the commonest and most studied pea aphid biotypes to identify the factors that determine the compatibility between the aphid and legume species as such factors are likely be involved in the host plant specialization process of the aphids. Based on

our recent genome analysis of three aphid biotypes respectively specialized on clover, alfalfa and pea, we hypothesized that salivary proteins are one of the factors that are involved in the host plant specialization process in *A. pisum* (Jaquiery et al., 2012). Hence, we envisaged to identify salivary proteins with biotype specific polymorphisms and to characterize their effects on specific plant–aphid interactions. Some salivary proteins from non-adapted biotypes may induce resistance responses in non-host plants while some salivary proteins from adapted biotypes may suppress specific plant defense reactions and allow non-adapted aphids to feed on non-host plants.

Here, as the first step to reach the objectives and to facilitate identification and functional characterization of *A. pisum* salivary proteins, we undertook optimization of agroinfiltration in *Medicago sativa* (alfalfa) and *Pisum sativum* (pea). We focused on these two plants because (1) significant amount of studies have been done on the aphid biotypes that feed on these plants (Hawthorne and Via, 2001; Jaquiery et al., 2012; Via et al., 2012), (2) these two biotypes show clear-cut performance difference on these two plants (Peccoud et al., 2009), and (3) seeds of various cultivars are easily available in our research center.

MATERIALS AND METHODS

Aphids, Bacteria Strains, Plasmids and Growth Conditions

Aphid lineages, and bacterial strains and plasmids used in this study are listed in Supplementary Tables S1 and S2, respectively. All aphid lineages were reared in a growth chamber at 18°C with a 16 h day/8 h night photoperiod on the broad bean, *Vicia faba* (Castel), at low density to avoid the production of winged individuals. *Escherichia coli* and *A. tumefaciens* strains were grown on Luria-Bertani medium at 37°C and 30°C, respectively. For solid media, agar was added at a final concentration of 1.5% (w/v). Antibiotics were used at the following concentrations: for all bacteria, 50 μg/ml kanamycin; for *A. tumefaciens*, 50 μg/ml rifampicin; for *E. coli*, 10 μg/ml gentamycin.

Plants and Growth Conditions

Pisum sativum (Supplementary Table S3) and *Medicago sativa* plants were grown in a growth chamber at 18°C with a 16 h day/8 h night photoperiod for 2 and 3 weeks, respectively.

Measurements of Aphid Performances on Pea Cultivars

Life traits of five aphid lineages from pea (Ar_Po_28, Ar_Po_58), alfalfa (L9Ms14) and clover (YR2, T8005) biotypes (Supplementary Table S1) were measured on *P. sativum* cultivars ZP1130 and ZP1109 (Supplementary Table S3). Adult aphids were installed on both pea cultivars and removed 24 h later, giving them enough time to produce 10 larvae that were left on the plants (day 1). Survival rate of the 10 larvae was measured at day 9 (when they reach adulthood), three surviving adult aphids were then reinstalled on the plants and biomass (the cumulated weight of the three adults and their offspring) of the aphid population was weighted at day 17. The biomass is a good proxy of the number of nymphs produced by adult aphids and reflects well their overall fitness (Peccoud et al., 2009). Five replicates for each aphid lineage on the two tested plants were performed.

Construction of Plasmids

All primers used in this study are listed in Supplementary Table S4. The genes encoding eGFP and the β-glucuronidase with a plant derived intron (GUSi; Vancanneyt et al., 1990) were amplified using GFP-Fw/GFP-Rv primers and GUS-Fw/GUS-Rv primers, respectively, and were added complete *attB1* and *attB2* sequences by the second PCR with attB1 and attB2 primers. In order to clone aphid salivary genes, cDNAs produced from aphid head total RNA were used to enrich transcripts encoding salivary genes. Adult aphids feeding on *V. faba* were flash frozen in liquid nitrogen, and decapitated with a scalpel between the first and second pairs of legs. Head RNA was extracted from 10 to 20 individuals using the RNeasy plant mini kit (Qiagen). cDNA synthesis was performed with poly-T primers using the AMV reverse transcriptase system (Promega) according to the manufacturers' instructions. ACYPI009919 (Ap25) and ACYPI008617 (ApC002) open reading frames encoding mature proteins were amplified from the cDNA of the Ar_Po_58 line (pea biotype) with Phusion DNA polymerase (ThermoFisher Scientific) using AP25-Fw/AP25-Rv and APC002-Fw/APC002-Rv primers (Supplementary Table S4), respectively. *attB1* and *attB2* sites were added with a second PCR using attB1 and attB2 primers. All amplicons, eGFP, GUSi and two salivary genes, were recombined by BP reaction into pDONR207 (Invitrogen) using BP clonase II (Invitrogen) and produced entry vectors (Table S2). Entry vectors were recombined by LR reaction using LR clonase II (Invitrogen) into pEAQ-HT-DEST1 expression vector (Supplementary Table S2; Sainsbury et al., 2009). Expression vectors were transformed in electro-competent *A. tumefaciens* cells (Supplementary Table S2).

Infiltration of *Agrobacterium*

Agrobacterium tumefaciens-mediated transient expression was performed as described (Rivas et al., 2002). Freshly cultured cells were resuspended in induction buffer [10 mM $MgCl_2$, 10 mM Mes (2-(*N*-morpholino) ethanesulfonic acid), pH 5.6, and 150 μM acetosyringone] to an $O.D._{600}$ (optical density at 600 nm) of 0.5. Cells were syringe infiltrated into leaves of 2 week-old *P. sativum* (Supplementary Table S1) and 3 week-old *M. sativa* plants.

GUS Staining

Plant leaves infiltrated with *Agrobacterium* were detached 3 days post infiltration (dpi) and GUS activity was visualized as described (Jefferson, 1987). Briefly, leaves were vacuum infiltrated with GUS staining solution (61 mM Na_2HPO_4, 39 mM $NaHPO_4$, 0.1% triton X-100, 10 mM EDTA, 0.3% H_2O_2 and 1.5 mM 5-bromo, 4-chloro, 3-indolyl glucuronide (X-Glc, Biosynth), pH 7.0) and incubated overnight at 37°C. Then chlorophyll discoloration was performed with successive washes with ethanol at 37°C.

Protein Extraction and Western-Blot Analyses

Three leaf disks per leaf were sampled using a cork borer (area = 0.79 cm^2) at 0, 7, and 10 days post-infiltration for GFP protein detection. Leaf disks were flash frozen in liquid nitrogen and stored at −80°C. Proteins were extracted in 120 µl extraction buffer (50 mM tris pH 7.5, 1 µM Dithiothreitol, glycerol 10%, 1 mM PMSF (Phenylmethylsulfonyl fluoride), 0.05% triton X-100). Extracts from pea plants were prepared as described (Canonne et al., 2011) and supernatants were resuspended in 5X loading buffer (0.5 M Tris pH 6.8, SDS 10%, glycerol 50 and 0.001% bromophenol blue). Fifteen microliters of samples were separated by SDS-PAGE (12% polyacrylamide) and transferred on PVDF (Polyvinylidene fluoride) membranes, (Merck Millipore) as described (Witte et al., 2004) with following modifications: PVDF membranes were soaked in methanol before and after transfer, and then washed in water. Methanol in transfer buffer was replaced by ethanol. The rabbit anti-GFP antibody (Biorad) and secondary antibodies (polyclonal goat anti-rabbit antibody peroxidase conjugated; Sigma–Aldrich) were both used at 1:10000. Detection was performed by chemiluminescence using Clarity Western ECL Substrate (Biorad) and CL-XPosure^{TM} Film (Lifetechnologies) according to manufacturer's instructions. Coomassie stains were performed with 0.2% Coomassie Brilliant Blue R250 (Sigma) in 50:40:10 water, methanol, acetic acid.

Aphid Performance Test on Agroinfiltrated Leaves

One young leaf of the *P. sativum* ZP1109 cultivar was syringe-infiltrated with *A. tumefaciens* AGL-1 strain harboring expression vectors. Three days later (at 3 dpi), 6 new-born aphids (1 day-old) born on *V. faba* were installed on *P. sativum* agroinfiltrated leaves in custom-built clip cages (area = 2.54 cm^2). When aphids were 8 days-old (10 dpi), clip cages were opened and the number of surviving aphids was recorded to estimate the survival rate. From the survivors, one average sized aphid was selected and transferred to a new *P. sativum* leaf that was infiltrated with the same construct of *Agrobacterium* 3 days before the transfer. Clip cages were opened when aphids were 12 and 15 days-old to assess the fecundity by counting the number of nymphs produced by each aphid. The nymphs were removed after each counting to avoid overcrowding of the cages. In one experiment, 10 replicates per gene were performed and the same experiment was repeated twice, producing 20 replicates. All the experiments were conducted at 20°C, 16 h day/8 h night photoperiod.

Statistical Analyses

All statistical analyses were conducted in R version 3.1.2 (R Core Team, 2014). Data were checked for approximate normal distribution by graphical visualizing of residuals. The effects of the different factors (pea cultivar, aphid lineage, expressed gene) were tested and the simplest model explaining the data was used. Analyses of survival rates (**Figures 2A** and **3A**) and fecundity counts (**Figure 3B**) were performed by classical linear regressions using generalized linear models (GLM) with binomial and Poisson distributions, respectively. Both tests were followed by multiple comparisons of means by the Tukey contrast method implemented in the package "multcomp" (Hothorn et al., 2008). The influence of pea cultivars and aphid lineage on aphid biomass (**Figure 2B**) was analyzed by a two-way ANOVA. Tukey's *post hoc* multiple comparisons of means from the R package "agricolae" (De Mendiburu, 2014) were used to reveal differences between groups.

RESULTS

Screening of *P. sativum* and *M. sativa* Cultivars for Agroinfiltration

Combinations of *A. tumefaciens* and various *M. sativa* and *P. sativum* cultivars were tested using the β-glucuronidase containing a plant derived intron (GUSi) as a reporter gene (Vancanneyt et al., 1990). Green fluorescence protein (GFP) could not be used as a reporter due to strong auto fluorescence induced in the leaf surface by the infiltration. Initially, we tested two plant expression vectors pGWB402Ω (Nakagawa et al., 2007) and pEAQ-HT-DEST1 (Sainsbury et al., 2009) in some pea cultivars, but the difference in expression levels between the two vectors was not very clear or slightly better when pEAQ-HT-DEST1 was used. Therefore, we used pEAQ-HT-DEST1 for the rest of screening. Also, our initial test showed that a bacterial suspension with an $O.D._{600}$ less than 0.3 resulted in a weak transgene expression and more than 0.7 triggered leaf chlorosis a few days after infiltration. Therefore, for the rest of the screening, agroinfiltrations were performed using syringe infiltration method and a bacterial suspension with an $O.D._{600}$ = 0.5. Seventeen *P. sativum* (Supplementary Table S3) and five *M. sativa* cultivars were selected based on geographic origin and phylogenetic groups in order to screen a large genetic diversity. Each cultivar was infiltrated with three *Agrobacterium* strains [C58C1, GV3101 and AGL-1 (Supplementary Table S2)] each harboring pEAQ-HT-DEST1-GUSi to identify the combination of plant and bacterium genotypes that produce high amount of GUS proteins. Leaves were analyzed histochemically for GUS activity at 3 dpi. At least three independent experiments were performed for each combination and results are summarized in **Table 1**. None of the *M. sativa* cultivars was suitable for *Agrobacterium*-mediated transient expression in leaves as no GUS staining could be observed in these plants under the tested conditions. High differences between pea cultivars were observed. Most of the pea cultivars had no or weak intensities of GUS staining. Of the three *Agrobacterium* strains used in this study, AGL-1 induced the highest expression of GUS, and C58C1 was the lowest inducer. Two pea genotypes, ZP1130 and ZP1109, inoculated with AGL-1 showed most intense coloration during GUS staining (**Figure 1A**). GUS staining could be observed at 3 dpi for both cultivars, ZP1130 and ZP1109. To confirm protein expression in these two cultivars, transient expression of eGFP and detection by western-blot was performed (we could not visualize GFP fluorescence due to autofluorescence induced by wounding). eGFP protein was detected at 7 and 10 dpi for both ZP1109 and

TABLE 1 | Results of screening of *P. sativum and M. sativa* cultivars for agroinfiltration.

	C58C1[b]	GV301	AGL-1
Pisum sativum[a]			
AP3783	N	I	W
AP3830	N	N	N
WP1018	W	W	W
ZP690	N	N	N
ZP748	N	N	N
ZP750	N	W	W
ZP793	N	W	W
ZP747	N	N	W
ZP1109	N	I	I
ZP1124	N	N	W
ZP1130	N	W	S
ZP3495	N	W	N
ZP3508	N	N	N
ZP3514	N	N	N
ZP3535	N	N	N
ZP3570	N	N	N
ZP3664	N	W	W
Medicago sativum			
Comète	nd	nd	N
Harpe	nd	nd	N
Lux Timbale	nd	nd	N
Lux Galaxie	nd	nd	N
Cannelle	nd	nd	N

[a]*pea or alfalfa cultivars used in this study.* [b]*A. tumefaciens strains used in this study. N, no coloration; W, weak; I, intermediate coloration; S, strong coloration (**Figure 1A**), nd, not determined.*

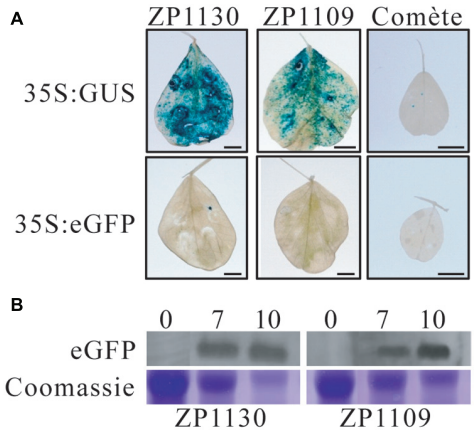

FIGURE 1 | Optimization of agroinfiltration in legume plants. (A) GUS expression in *P. sativum* and *M. sativa*. Leaves of ZP1130 and ZP1109 cultivars of *P. sativum* and comète cultivar of *M. sativa* were syringe-infiltrated with *A. tumefaciens* AGL-1 carrying the pEAQ-HT-DEST1 plasmid encoding the gene for β-glucuronidase with an intron (GUSi), under the control of the CaMV 35S promoter (35S:GUSi). GUS staining was performed 3 days post infiltration. Results are representatives of four independent experiments. *A. tumefaciens* carrying the pEAQ-HT-DEST1 plasmid encoding the gene for enhanced GFP, under the control of the CaMV 35S promoter (35S:eGFP) was used as control. Scale bars represent 0.5 cm. **(B)** Total protein extracts of leaves from ZP1130 and ZP1109 cultivars expressing eGFP using *Agrobacterium*-mediated transformation were separated by 12% SDS-PAGE and analyzed by immunoblot using the anti-GFP antibody. Samples were harvested at 0, 7 and 10 days post infiltration. Coomassie stained portions of the gel (Rubisco) are shown to compare sample loading between lanes.

ZP1130 (**Figure 1B**). During this study, yellowing of the leaves starting at 9–10 dpi for ZP1130 and at 12–13 dpi for ZP1109 was observed. This leaf yellowing was probably due to AGL-1 infection as the yellowing was observed in the leaves infiltrated with *Agrobacterium* with empty vector control, and no yellowing was observed in buffer infiltrated leaves (data not shown). Taken together, we identified two pea cultivars, ZP1130 and ZP1109, and the *A. tumefaciens* strain AGL-1 as the combinations that are suitable for transient gene expression, and we presumed that 3–8 dpi for ZP1130 and 3–10 dpi for ZP1109 are the timing to examine the effect of transgene expression in the plant or plant–aphid interactions.

Pea Cultivars ZP1130 and ZP1109 Are Hosts Only for the *A. pisum* Pea Biotype

Survival rate and biomass of the five *A. pisum* lineages belonging to three biotypes (pea, alfalfa and clover; Supplementary Table S1) were assessed on the ZP1130 and ZP1109 pea cultivars we identified as suitable for agroinfiltration (**Figure 2**). Analysis revealed that the two plant cultivars did not influence the survival rate and produced aphid biomass [$\chi^2 = 0.14$, $P = 0.243$; $F_{(5,44)} = 129.7$, $P = 0.261$; for survival and biomass, respectively], but pea aphid lineages differed significantly in their survival rates ($\chi^2 = 19.04$, $P < 0.001$) and biomass production [$F_{(4,45)} = 128.9$, $P < 0.001$]. The pea adapted lineages Ar_Po_28 and Ar_Po_58 showed a higher survival rate on the pea cultivars at day 9 compared to L9Ms14 (alfalfa biotype), YR2 and T8005 (clover biotype). The difference in survival was very pronounced between pea and alfalfa specialized lineages, and intermediate for lineages of the clover biotype (**Figure 2A**). On both ZP1130 and ZP1109 cultivars, only the lineages of the pea biotype (Ar_Po_28 and Ar_Po_58) produced a substantial biomass. Although Ar_Po_28 had a significantly higher biomass than Ar_Po_58, both lineages performed well on the tested cultivars that they seem to recognize as favorable hosts. By contrast, alfalfa and clover adapted lineages hardly reproduced on the pea cultivars that seem to be non-host plants in these interactions (**Figure 2B**). Thus, the ZP1130 and ZP1109 cultivars are selective hosts for *A. pisum* biotypes, allowing to assess host and non-host interactions using agroinfiltration experiments.

Transient Expression of AP25 in ZP1109 Increased *A. pisum* Fecundity

Next, we expressed two salivary genes in ZP1109 by agroinfiltration using strain AGL-1 and examined their effects on *A. pisum* feeding on the infiltration site. We chose Ar_Po_58 as a test aphid line as it belongs to the pea biotype and harbors no secondary symbiont, which may interfere with plant–aphid interactions. Mature proteins encoding ACYPI008617 (ApC002) and ACYPI009919, which we named Ap25, were

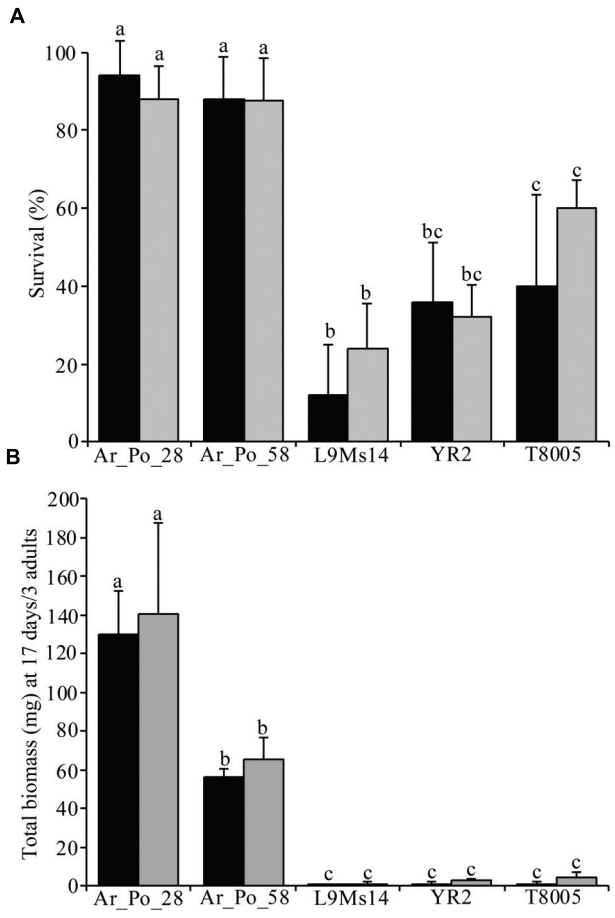

FIGURE 2 | ZP1109 and ZP1130 allow only *A. pisum* pea biotype reproduction. Survival **(A)** and biomass **(B)** of five aphid lineages are measured on the pea cultivars ZP1109 (black bars) and ZP1130 (gray bars). Bars show the average of survival or biomass and standard deviation for five replicates per conditions. Statistical differences between groups are indicated by different letters. a, b, c; indicate groups determined by multiple comparisons tests after GLM and ANOVA analyses for survival and biomass data, respectively.

transiently expressed using pEAQ-HT-DEST1 vector. The genes were expressed by CaMV 35S promoter, which is known to be ubiquitously and constitutively activated in various plant tissues including epidermal, mesophyll and phloem tissues (Stockhaus et al., 1989). In the process of establishing phloem feeding, *A. pisum* punctures various tissues and salivates (Schwarzkopf et al., 2013). When the aphid attempts to feed on non-host legume plant, it punctures epidermal and mesophyll cells but cannot establish phloem feeding: therefore, the factors that determine the compatibility between *A. pisum* and host plants are present in those tissues (Schwarzkopf et al., 2013). Based on these informations, we thought it is important to express salivary proteins ubiquitously to fully assess their functions in plants and used 35S promoter for transient expression. 35S promoter has been successfully used in other studies on aphid salivary proteins (Bos et al., 2010; Naessens et al., 2015). *ApC002* was chosen because it is one of the most studied salivary proteins and is shown to be essential for *A. pisum* to feed on the universal host plant (*V. faba*; Ferrari et al., 2008; Peccoud et al., 2009). *Ap25* was selected because the gene presents the same features as that of *ApC002*: the gene was identified in salivary glands by transcriptomic analyses (Carolan et al., 2011), is specifically expressed in salivary glands (Akiko Sugio et al., unpublished data), and encodes a signal peptide and a small (13.9 kDa) mature protein with no predicted function. Although many genes are duplicated in *A. pisum* genome, *Ap25*, like *ApC002*, is single copy in *A. pisum* and its orthologues exist only in the Aphididae family (Hélène Boulain et al., unpublished data).

In this study, transient protein expression was observed from 3 (detected by GUS activity) to 10 days (detected by western blot) at 20°C after infiltration of *Agrobacterium*. *A. pisum* starts to reproduce around 9th day after birth, reaches its peak of reproduction around 5 days later, and slows down but continues to reproduce until its death at an age of approximately 30 days (Tsuchida et al., 2004). By supplying newly infiltrated leaves, we extended the duration of the experiment to characterize the effect of transgene expression on aphid fecundity. Leaves of ZP1109 were infiltrated with AGL-1 harboring expression plasmids of eGFP, ApC002 or Ap25. Three days after the infiltration, six new-born aphids of the pea adapted clone Ar_Po_58 were clip caged on the infiltrated leaves. When the aphids were 8 days-old (at 10 dpi) the cages were opened to count the number of survivors. One aphid was transferred to a new 3-day-post-infiltrated leaf. Production of nymphs of the caged adult was measured when the aphid was 12 and 15-day old corresponding to the peak of reproduction of adults. Survival rate and total number of nymphs of the aphids are shown in **Figure 3**. There was no difference in the survival rate of the aphids that were fed on the leaves expressing the three tested genes ($\chi^2 = 0.01$, $P = 0.96$). Production of nymphs of Ar_Po_58 feeding on ApC002 expressing leaves was same as that of aphids feeding on eGFP expressing leaves, while the aphids produced approximately 12% more offspring on Ap25 expressing leaves than on eGFP expressing leaves (20 biological replicates, $\chi^2 = 18.75$, $P < 0.001$). The results indicate that Ap25 plays a role in promoting *A. pisum* feeding on *P. sativum*.

DISCUSSION

Here, we screened cultivars of *P. sativum* and *M. sativa* using GUS activity as a reporter and identified two *P. sativum* cultivars, ZP1130 and 1109, that are amenable to *Agrobacterium* mediated transient gene expression. We noted that *A. tumefaciens* strain AGL-1 was the most efficient strain among the three strains tested. This can be explained by the presence of extra virulent factors in this strain (Jin et al., 1987). We also noted that a few days upon infiltration with high concentration of *A. tumefaciens* ($O.D._{600} > 0.7$), chlorosis appeared and was restricted to the agroinfiltrated area. Pruss et al. (2008) also observed that fully virulent and disarmed *A. tumefaciens* strains also triggered chlorosis restricted to the infiltrated area in tobacco plants.

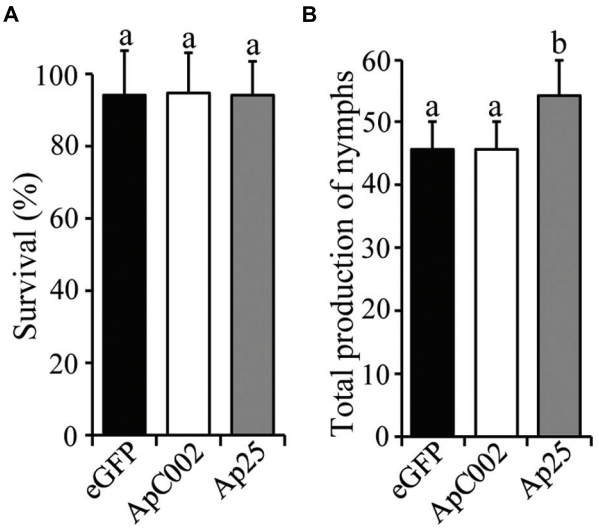

FIGURE 3 | Transient expression of Ap25 promotes reproduction of *A. pisum* on ZP1109. Leaves of ZP1109 cultivar of *P. sativum* were syringe-infiltrated with *A. tumefaciens* AGL-1 carrying the pEAQ-HT-DEST1 plasmid encoding the genes for *ACYPI008617* (*ApC002*; white bar) and *ACYPI009919* (*Ap25*; gray bar), under the control of the CaMV 35S promoter. *A. tumefaciens* carrying the pEAQ-HT-DEST1 plasmid encoding the gene for enhanced GFP (eGFP; black bar), under the control of the CaMV 35S promoter was used as control. **(A)** Survival rate of Ar_Po_58 line is not affected by the transient expression of the salivary genes *ApC002* and *Ap25*. At 3 days post infiltration, six new-born aphids were clip caged on agroinfiltrated leaves and counted when aphids were adults (8 days-old) to check the survival rate. Bars show the average percentage of survivors plus the standard deviation of 20 biological replicates. After the survival test, one aphid per plant was kept and placed on a new 3-day-post-infiltrated leaf to check the fecundity. **(B)** The transient expression of different genes influenced aphid nymph production. Bars show the average number of nymphs produced plus the standard deviation of 20 biological replicates. Different letters indicate significant differences between groups.

Although the mechanisms underlying this chlorosis have not been well understood, it could be due to a defense response to the *A. tumefaciens* involving the chloroplasts (Pruss et al., 2008).

Two tested *A. pisum* lines belonging to the pea biotype reproduced well on these two cultivars, while members of the alfalfa and clover biotypes could not survive and reproduce well on them. This indicates that these two cultivars serve as host plants of the pea biotype only and can be used to characterize candidate aphid salivary genes that may determine the compatibility of *A. pisum* biotypes with *P. sativum*. Interestingly, we found differences in aphid performances, as measured by survival and biomass, between the two *P. sativum* adapted lines on both pea cultivars. In particular, biomass production by Ar_Po_28 was about twice more than that of Ar_Po_58. Since the two lines differ in both genotype and symbiont composition (Ar_Po_28 harbors *Rickettsia* and *Serratia* secondary symbionts while Ar_Po_58 is free of any secondary symbiont, Supplementary Table S1), it is difficult to tell which factor (aphid genome or symbiont status), alone or in interaction, accounts for these differences in performances.

Although we optimized agroinfiltration in *P. sativum* to study the host specialization mechanisms in *A. pisum*, the system can be used to study the functions of *P. sativum* genes or effectors of other pea parasites. *P. sativum* is an important legume crop used in arable rotations for the production of nutritious food for both humans and animals. Various projects to identify genes involved in *P. sativum* biotic and abiotic stress resistances are ongoing (Lejeune-Henaut et al., 2008; Hamon et al., 2013; Desgroux et al., 2016) and whole-genome sequencing of *P. sativum* is underway (Alves-Carvalho et al., 2015). Therefore, the *P. sativum* research community is in need of various tools to analyze the genes of agronomical interest that will be identified in near future. Though *P. sativum* is reported to be stably transformed (Svabova et al., 2005), it remains to be a time consuming and difficult task. Recent application of virus vectors in *P. sativum* provides a new tool to express transgene in pea plant relatively quickly, but it is still time consuming (in a few weeks; Meziadi et al., 2016) and the agroinfiltration method described here provides another way to express transgenes in a few days. By using various Gateway™ compatible vectors available for agroinfiltration (Karimi et al., 2002; Nakagawa et al., 2007), fusion proteins or dsRNA will be easily produced in *P. sativum* leaves. Furthermore, coexpression of a few proteins may be realized by infiltration of *A. tumefaciens* with different expression constructs.

We transiently expressed ApC002 and Ap25 in *P. sativum* leaves and examined the survival and fecundity of an *A. pisum* line of the pea adapted biotype. The aphids grew well in the clip cages fixed on the agroinfiltrated leaves and produced offspring. Since ApC002 is required for *A. pisum* feeding on *V. faba* plant, which is a universal plant of all *A. pisum* biotypes (Ferrari et al., 2008; Peccoud et al., 2009), and *in planta* (*Arabidopsis* and *N. benthamiana*) expression of MpC002 increases the fecundity of *M. persicae* feeding on the plants, we expected that ApC002 expression in *P. sativum* leaves would also increase the fecundity of the aphids. However, the survival and fecundity of the aphids fed on ApC002 expressing plants were at the same level as that of the aphids feeding on eGFP expressing plants. As C002 is one of the abundantly expressed salivary genes in *A. pisum* (Mutti et al., 2006), the aphids may produce enough of this protein and may not benefit significantly from extra production of ApC002 in *P. sativum* leaves. On the other hand, expression of Ap25 in *P. sativum* leaves increased the fecundity of the aphids. *Ap25* is an Aphididae specific gene which encodes a small protein with a signal peptide. As the protein does not show homology with known proteins, the function of Ap25 is unknown. It is possible that the protein interferes with plant defense reactions triggered by aphid feeding and facilitates nutrient acquisition from the pea plant. Carolan et al. (2011) identified more than 300 salivary genes in *A. pisum* and more than half of the identified genes encode proteins with unknown function (Carolan et al., 2011). The agroinfiltration method described here provides a mean to examine the functions of those salivary proteins in relatively short time and also allows us to investigate whether those genes are determinants of compatibility between *P. sativum* and *A. pisum* biotypes. As the second step of this study, we envisage to express salivary proteins with biotype specific sequences in the pea leaves and examine how they affect the performance

of different pea biotypes installed on the leaves. Further, the agroinfiltration technique can be combined with aphid gene silencing to investigate whether a gene expressed in leaves can complement the silenced gene function (Naessens et al., 2015). Studies on plant–insect interactions at a molecular level are less advanced compared to plant-microbe interaction studies partly because it is not yet possible to transform insect herbivores. The tools to manipulate host plants, like the method described here, can provide alternative ways to examine plant–insect interactions at a molecular level and will be able to contribute to advance the field.

AUTHOR CONTRIBUTIONS

EG, HB, YA, CLP, KC, SM, J-CS, and AS designed the experiments. EG, HB, YA, CLP, KC, SM, KS, J-CS and AS conducted the experiments. EG, HB, GK, J-CS and AS edited the manuscript. GK, J-CS and AS provided funding for the project.

ACKNOWLEDGMENTS

Authors acknowledge to Gaëtan Denis and Jean-François Le Gallic for experimental support, Drs Marie-Laure Pilet-Nayel and Alain Baranger and their team for providing pea seeds, Anne Moussart for providing alfalfa seeds, Dr. George P. Lomonossoff for providing pEAQ vectors, Prof. Tsuyoshi Nakagawa for providing pGWB vectors and Mathew Smoker for *Agrobacterium* strains. This project was supported by the French Agence Nationale de la Recherche grant (13-JSV7-0012-01-Bugspit) to AS and by Procope German-French collaboration program funded by the German Academic Exchange Service (DAAD) and Campus France to GK (57049694) and J-CS (30753SE). Ph.D. grant to HB is funded by INRA SPE and Région Bretagne.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01171

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

CORRECTION
published: 10 January 2017
doi: 10.3389/fpls.2016.02046

Corrigendum: Optimization of Agroinfiltration in *Pisum sativum* Provides a New Tool for Studying the Salivary Protein Functions in the Pea Aphid Complex

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Keywords: pea aphid, *Acyrtosiphon pisum*, Leguminosae, agroinfiltration, salivary proteins, biotypes, host specialization, effector

A corrigendum on

Optimization of Agroinfiltration in *Pisum sativum* Provides a New Tool for Studying the Salivary Protein Functions in the Pea Aphid Complex
by Guy, E., Boulain, H., Aigu, Y., Le Pennec, C., Chawki, K., Morlière, S., et al. (2016). Front. Plant Sci. 7:1171. doi: 10.3389/fpls.2016.01171

Corrigendum on Supplemental Table 2.

In the original article, description of helper plasmids in C58C1 (pGV2260) and GV3101 (pMP90) were missing. The correct information of these two strains appears below. The authors apologize for the missing information. This error does not change the scientific conclusions of the article in any way.

Bacteria	Features	References or sources
BACTERIA		
Agrobacterium tumefaciens C58C1	Rif^r, harbors pGV2260 (pTiB6S3ΔT-DNA)	Deblaere et al., 1985
Agrobacterium tumefaciens GV3101	Rif^r, harbors pMP90 (pTiC58ΔT-DNA)	Koncz and Schell, 1986

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *19 December 2016*
Accepted: *21 December 2016*
Published: *10 January 2017*

Citation:
Guy E, Boulain H, Aigu Y, Le Pennec C, Chawki K, Morlière S, Schädel K, Kunert G, Simon J-C and Sugio A (2017) Corrigendum: Optimization of Agroinfiltration in Pisum sativum Provides a New Tool for Studying the Salivary Protein Functions in the Pea Aphid Complex. Front. Plant Sci. 7:2046. doi: 10.3389/fpls.2016.02046

frontiers in Plant Science

ORIGINAL RESEARCH
published: 03 August 2016
doi: 10.3389/fpls.2016.01142

The Potato Aphid Salivary Effector Me47 Is a Glutathione-*S*-Transferase Involved in Modifying Plant Responses to Aphid Infestation

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Polyphagous aphid pests cause considerable economic damage to crop plants, primarily through the depletion of photoassimilates and transfer of viruses. The potato aphid (*Macrosiphum euphorbiae*) is a notable pest of solanaceous crops, however, the molecular mechanisms that underpin the ability to colonize these hosts are unknown. It has recently been demonstrated that like other aphid species, *M. euphorbiae* injects a battery of salivary proteins into host plants during feeding. It is speculated that these proteins function in a manner analagous to secreted effectors from phytopathogenic bacteria, fungi and oomycetes. Here, we describe a novel aphid effector (Me47) which was identified from the potato aphid salivary secretome as a putative glutathione-*S*-transferase (GST). Expression of Me47 in *Nicotiana benthamiana* enhanced reproductive performance of green peach aphid (*Myzus persicae*). Similarly, delivery of Me47 into leaves of tomato (*Solanum lycopersicum)* by *Pseudomonas* spp. enhanced potato aphid fecundity. In contrast, delivery of Me47 into *Arabidopsis thaliana* reduced GPA reproductive performance, indicating that Me47 impacts the outcome of plant–aphid interactions differently depending on the host species. Delivery of Me47 by the non-pathogenic *Pseudomonas fluorescens* revealed that Me47 protein or activity triggers defense gene transcriptional upregulation in tomato but not *Arabidopsis*. Recombinant Me47 was purified and demonstrated to have GST activity against two specific isothiocyanates (ITCs), compounds implicated in herbivore defense. Whilst GSTs have previously been associated with development of aphid resistance to synthetic insecticides, the findings described here highlight a novel function as both an elicitor and suppressor of plant defense when delivered into host tissues.

Keywords: effector, potato aphid, glutathione-*S*-transferase, GST, secretome

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *25 April 2016*
Accepted: *18 July 2016*
Published: *03 August 2016*

Citation:
Kettles GJ and Kaloshian I (2016) The Potato Aphid Salivary Effector Me47 Is a Glutathione-S-Transferase Involved in Modifying Plant Responses to Aphid Infestation. Front. Plant Sci. 7:1142. doi: 10.3389/fpls.2016.01142

INTRODUCTION

Aphids are a large family of hemipteran insects that feed from the vasculature tissue of plants. They feed by inserting their flexible hypodermal needle-like mouthpart or stylets into plant tissue and navigate mostly between cells until they puncture the phloem tissue and feed from the sugar-rich sap (Tjallingii and Esch, 1993; Tjallingii, 2006). Both during initial probing and feeding, aphids secrete watery saliva from their stylets (Tjallingii, 2006). It is known that salivary secretions

from aphids play important roles in the establishment and maintenance of successful feeding sites (Will et al., 2007). For example, phloem-plugging in fava bean is dependent on the expansion of forisomes in sieve elements. This process can be inhibited by application of aphid salivary extracts (Will et al., 2007). Saliva from numerous aphid species is known to contain a complex mix of proteins (Harmel et al., 2008; Carolan et al., 2009, 2011; Cooper et al., 2011; Nicholson et al., 2012; Rao et al., 2013; Nicholson and Puterka, 2014; Vandermoten et al., 2014; Chaudhary et al., 2015). It is speculated that salivary proteins act in ways similar to protein effectors from plant microbial pathogens. That is, to inhibit or suppress the activation of host immune processes and enable successful colonization. Whilst the salivary protein complement of several aphid species is now known, functional characterization of individual proteins has extended to just a handful of examples (Jaouannet et al., 2014; Kaloshian and Walling, 2016). The pea aphid *Acyrthosiphon pisum* protein C002 is injected into fava bean during feeding and is required for effective feeding behavior (Mutti et al., 2008). Two proteins (Mp10, Mp42) were identified from the green peach aphid *Myzus persicae* that reduced aphid performance when transiently expressed in *Nicotiana benthamiana* by *Agrobacterium tumefaciens* (Bos et al., 2010). Mp10 induces chlorosis/cell death in *N. benthamiana* suggesting direct recognition of this salivary protein through mechanisms that are distinct from Mp42 (Bos et al., 2010; Rodriguez et al., 2014). Further effectors from *M. persicae* (Mp1, Mp2, Mp55, Mp56, Mp57, and Mp58) have been reported that have various impact on aphid fecundity when either transiently or stably expressed in hosts (Pitino and Hogenhout, 2013; Elzinga et al., 2014). However, the molecular functions of these proteins are unknown. Two effectors (Me10, Me23) have to date been identified from the potato aphid *Macrosiphum euphorbiae* (Atamian et al., 2013). Me10 and Me23 both increase aphid performance when delivered by the bacterium *Pseudomonas syringae* type three secretion system (T3SS) into *N. benthamiana*; however, only Me10 had a similar effect when introduced into tomato (*Solanum lycopersicum*) leaves using the same delivery method (Atamian et al., 2013). As for other aphid effectors, the specific performance-enhancing activities of Me10 and Me23 are unknown.

The mechanisms by which plants defend themselves against aphid attack are wide-ranging. Preformed physical defenses include barriers such as trichomes, waxy cuticles and oily secretions to discourage aphid settling. There are also inducible changes that occur following the onset of aphid feeding. These include transcriptional modifications, generation of reactive oxygen species (ROS), callose deposition and the production of toxic phytoalexins (Moran et al., 2002; Martinez de Ilarduya et al., 2003; De Vos et al., 2005; Kusnierczyk et al., 2008; Louis et al., 2010; Kettles et al., 2013). The perception of microbial plant attackers, lately shown for aphids as well, has been conceptualized in a multi-layered model of plant defense (Jones and Dangl, 2006; Kaloshian and Walling, 2016). In the first instance, immune recognition of conserved Pathogen-Associated Molecular Patterns (PAMPs) results in PAMP-triggered immunity (PTI) which in most cases is enough to prevent infection or colonization. Only if the pathogen or pest has means to overcome PTI and suppress these inducible changes, typically through the action of proteinaceous effectors or other metabolites, can disease or colonization be achieved.

In order to overcome powerful host defenses, aphids must evolve ways of either suppressing the activation of plant immune processes or detoxifying the resulting chemical assault mounted by the host. Glutathione-*S*-transferases (GSTs) are a class of detoxification enzyme found throughout the eukaryotic kingdom that catalyzes conjugation of reduced glutathione (GSH) to both natural and synthetic xenobiotics (Li et al., 2007). Specifically for insect pests of plants, they are often grouped with classes of other detoxifying enzymes such as cytochrome P450s and carboxy/cholinesterases (Li et al., 2007; Ramsey et al., 2010) and have been linked to the development of resistance against chemical insecticides (Vontas et al., 2001, 2002). In addition to their role in insecticide resistance, GSTs are assumed to protect insects from xenobiotics encountered in nature. Aphid GSTs are induced when feeding on resistant plants (Bansal et al., 2014) or when fed on toxins in artificial diet (Francis et al., 2005). It has been speculated that diversity of GSTs may contribute to host-range of aphids due to capacity to metabolize a greater variety of host toxins (Ramsey et al., 2010). Study of GSTs in insect pests has largely focussed on those present in gut tissue and their interaction with compounds ingested during feeding. The role of GSTs deployed out on or into plant tissues and their interaction with host immune systems is unexplored.

Recent bioinformatic and proteomic analyses of the *M. euphorbiae* salivary secretome (Atamian et al., 2013; Chaudhary et al., 2014, 2015) revealed the presence of a single putative GST in aphid saliva. In this investigation, we describe the functional characterization of this candidate effector which we have named Me47. The impact of Me47 expression on performance of two aphid species across three different hosts was examined. Additionally, we find an inverse correlation between Me47-dependent activation of defense responses and aphid performance. Finally, we present evidence of substrate specificity of Me47 which helps explain the role of this GST in plant–aphid interactions.

MATERIALS AND METHODS

Phylogenetic Analysis and Secretion Signal Prediction

Glutathione sequences from *A. pisum* (Ramsey et al., 2010) were obtained from AphidBase 2.1 (INRA). *M. persicae* GST sequences were recovered from Myzus DB (INRA) by low stringency ($E < 0.1$) Blastp analysis of both *M. persciae* clone O and clone G006 genomes using Me47 sequence as query. Me47 coding sequence was aligned to GST sequences from *A. pisum* and *M. persicae* (both clones G006 and O; Supplementary Table S2) using ClustalW and displayed using a Neighbor-Joining tree with 100 bootstrap replicates using Geneious software (Biomatters). *M. persicae* protein identifiers are presented as MpG006 or MpO to indicate clonal origin.

The predicted amino acid sequences of the GSTs were subjected to *de novo* signal peptide prediction analysis using SignalP 4.1 and TargetP 1.1 programs (Emanuelsson et al., 2000; Petersen et al., 2011). For SignalP a Hidden Markov model scores higher than 0.45 was used. For TargetP predictions were determined by predefined set of cutoffs that yielded specificity >0.95 on the test sets.

Me47 Cloning and Bacterial Transformation

Me47 coding sequence lacking the secretion signal was amplified from 100 ng of potato aphid cDNA using primers attB1 Me47-F and attB2 Me47-R (Supplementary Table S1) and high-fidelity Phusion polymerase (New England Biolabs) with the following thermocycle (30 s at 95°C, 30 s at 55°C, 30 s at 72°C × 30 cycles). The attB-flanked Me47 PCR product was recombined into pDONRzeo using BP clonase (Invitrogen) following the manufacturer's instructions. Me47 was sequence verified by Sanger sequencing before subsequent shuttling into the destination vectors pEARLEYGATE100 for *in planta* Agroexpression (Earley et al., 2006), pVSP_*Ps*SPdes for bacterial delivery in tomato and *Arabidopsis* (*Arabidopsis thaliana*; Rentel et al., 2008) and pDEST17 (Invitrogen) for recombinant protein expression using LR clonase (Invitrogen). For initial cloning, electrocompetent DH5α cells were used for all transformations and *Agrobacterium* strain GV3101 was used for Agroexpression following standard procedures

Plant Materials and Aphid Colonies

Tomato cultivars (cv.) UC82B and Moneymaker, *N. benthamiana,* tobacco (*Nicotiana tabacum)* NC-95, mustard India, and *Arabidopsis* Col-0 were used. Seeds were planted directly into autoclaved soil or transplanted after seeding into soil. Plants were maintained in growth rooms at 22–24°C with 16 h day length and 200 μmol m^{-2} s^{-1} light intensity. Solanaceous plants were weekly fertilized with MiracleGro (18-18-21; Stern's MiracleGro Products).

Colonies of the parthenogenetic *M. euphorbiae* were reared on tomato cv. UC82B, while *M. persicae* was reared on tobacco NC-95 or mustard plants. The colonies were maintained in insect cages in a pesticide-free greenhouse at 22–26°C supplemented with light for 16 h day length. One-day old age synchronized *M. euphorbiae* adults were produced as described by Bhattarai et al. (2007).

Aphid Performance Assays

To assess *M. persicae* performance on *N. benthamiana*, *Agrobacterium* carrying either pEARLEYGATE100-GFP or pEARLEYGATE100-Me47 were grown in LB supplemented with appropriate antibiotics for 36 h at 28°C. Cells were washed thrice and resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 100 μM acetosyringone, pH 5.6) to an OD_{600} = 0.3. Bacteria were infiltrated into fully expanded leaves using a needleless syringe. After 24 h, four adult *M. persicae* were applied to infiltrated leaves within clip cages and left to produce a population of age-synchronized nymphs (day 0). After 48 h, all adults and excess nymphs were removed leaving five nymphs on each leaf (day 2). Nymphs were allowed to feed for two further days before being transferred to a second set of plants which had been similarly infiltrated 24 h previously (day 4). Aphids were allowed to feed from the second set of leaves for 4 days, before transfer to a final set of plants infiltrated 24 h previously (day 8). Experiments were terminated on day 12. This method allowed nymphs to mature to adulthood whilst being continuously exposed to high levels of transgene expression. Aphids typically began production of the next generation of nymphs on day 8. Aphid counts were made daily on days 8–12 and nymphs were continuously removed, such that each count represented fecundity over a 24 h period. Counts from all days were pooled for analysis. The experiment was conducted three times with similar results. Comparison of aphid fecundity on GFP-expressing and Me47-expressing leaves was assessed by two-tailed *t*-test.

To assess *M. euphorbiae* performance on tomato, GUS or Me47 was delivered by either semi-virulent *P. syringae* pv. *tomato* (*Pst*) DC3000 ΔAvrPto ΔAvrPtoB or non-pathogenic *P. fluorescens* (*Pfo*) EtHAn engineered with a T3SS (Thomas et al., 2009). In both systems, bacteria were cultured on Kings B plates with appropriate antibiotics for 36 h at 30°C. Cells were washed from plates in 10 mM $MgCl_2$ and resuspended to a density of 1×10^3 CFU/mL in infiltration buffer (1 mM $MgCl_2$, 0.02% Silwet L-77) in 2.5 L total volume. Whole plants were upturned and submerged in infiltration buffer, placed in a vacuum chamber and infiltrated for 2 min at 20 inHg. Plants were immediately transferred to a growth cabinet and allowed to recover overnight. At 24 h post infection (hpi), 10 mature age-synchronized adult *M. euphorbiae* were applied to the leaves of each plant with a fine paintbrush. Counts of both the surviving adults and newly born nymphs were made daily for 5 days and all nymphs were removed each day such that each count represented fecundity over a 24 h period. The counts from all days were pooled for analysis and each experiment was conducted three times with similar results. Comparison of aphid fecundity on GUS-expressing and Me47-expressing leaves was assessed by two-tailed *t*-test.

For *Arabidopsis* performance assays, *Pfo* EtHAn strain was prepared as for the tomato assay except that leaves were individually syringe-infiltrated rather than whole-plant submersion infiltration. At 24 hpi, single age-synchronized adult *M. euphorbiae* were applied to the center of each rosette and the whole plant caged. Counts of newly born nymphs were made daily for 5 days as described for the tomato assay. The experiment was conducted three times (**Figure 2B**; Supplementary Figure S3B) and results were analyzed as for the tomato assay.

Induction of Plant PAMP Responses in Tomato and *Arabidopsis*

High-dose *Pfo* inoculum was prepared following the method described above, with the exception that bacteria were infiltrated at OD = 0.01 (~1×10^6 CFU/mL; Nguyen et al., 2010) compared to the lower dose used for aphid performance assays. Following infiltration, plants were returned to growth conditions until sample harvest at 6 hpi. Experiments were conducted twice

with three biological replicates per experiment. Expression data from both experiments were combined and analyzed together. Comparisons of expression levels of defense-related genes in GUS-expressing and Me47-expressing leaves were made using a two-tailed *t*-test.

qRT-PCR

Leaf tissues from *Pfo*-infiltrated tomato or *Arabidopsis* plants were harvested at 6 hpi and snap frozen. Samples were ground in collection tubes using pellet pestles (Sigma) and total RNA extracted using Trizol (Invitrogen) as per the manufacturer's instructions. The 260/280 ratios of all samples were checked using a Nanodrop spectrophotometer and all were between 1.8 and 2.1 μg of total RNA was treated with DNaseI (NEB) and samples were subsequently tested for gDNA contamination by PCR amplification using either *UBI3* (tomato) or *PEX4* (*Arabidopsis*) primer pairs. First strand cDNA synthesis was performed using the SuperScript III kit (Invitrogen). cDNA was diluted 1:10 with dH_2O prior to qRT-PCR and 1 μl of this dilution was used per reaction.

Duplicate reactions for each sample/primer-pair combination were conducted using clear 96-well PCR plates (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad). Reactions were carried out using an iCycler real-time PCR system (Bio-Rad) using the following thermocycle (5 min at 95°C followed by 30 s at 95°C, 30 s at 58°C, 30 s at 72°C × 40 cycles). Relative expression values for defense-related genes were calculated using the formula $2^{-\Delta Ct}$ (Pfaffl, 2001) relative to the *TIP41* reference gene (tomato) or *PEX4* (*Arabidopsis*). Expression values were rescaled for presentation such that the buffer treatment is equal to 1.

Protein Purification and Western Blot Analysis

Escherichia coli ArcticExpress cells (Agilent) carrying the pDEST17-Me47 construct were grown in LB media at 37°C to an OD_{600} of 0.8. Recombinant Me47 production was induced by addition of 1 mM IPTG followed by incubation at 12°C for 16 h. Cells were recovered by centrifugation, resuspended in chilled lysis buffer (300 mM NaCl, 50 mM sodium phosphate, pH7.2) and lysed using sonication (6 × 30 s pulses). The soluble protein fraction was collected and incubated with Ni-NTA agarose beads (Qiagen) for 1 h at 4°C with gentle agitation. Non-specifically bound proteins were removed with four washes of lysis buffer containing 25 mM imidazole. His-tagged Me47 protein was eluted with two washes of lysis buffer containing 250 mM imidazole. Aliquots were taken at all stages of the purification process and protein content assessed by Bradford assay. Twenty micrograms of all samples were separated by SDS-PAGE using a 12% acrylamide gel. To confirm the identity of purified His-tagged Me47, protein was transferred to nitrocellulose membrane and probed with HisProbe-HRP conjugate antibody (Santa Cruz Biotechnology) in PBST with 2% milk powder. Signal was detected by Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and imaged using X-ray film.

Glutathione Depletion Assay

Fractions of N-terminal His-tagged Me47 protein were pooled and imidazole removed by buffer exchange using PBS (pH 6.5) and PD10 buffer exchange columns (GE Healthcare). N-terminal His-tagged GroEL was prepared using the same method. To assess activity against ITC substrates, 2 μg of each protein treatment (equine liver GST, His-Me47, His-GroEL) were incubated in the presence of 50 μM glutathione and 200 μM of three ITCs (AITC, BITC, and PEITC) at pH 7.0 for 20 min at room temperature. Buffer-only control reactions with no protein treatment were also included. The concentration of free glutathione remaining in each reaction was assessed using the Glutathione Assay Kit (Sigma) following the manufacturer's instructions.

ROS Burst Assay

GFP, Me47, and Mp10 (Bos et al., 2010) were expressed in *N. benthamiana* leaf tissue following Agroinfiltration with GV3101 containing pEARLEYGATE100-GFP, pEARLEYGATE100-Me47, or pEARLEYGATE100-Mp10 as described above. At 2 dpi, 2 mm × 2 mm leaf squares from the Agroinfiltrated leaves were cut using a razor blade and soaked overnight in dH_2O. Leaf squares were subsequently exposed to flg22 (100 nM) in a luminol-based assay (Chaudhary et al., 2014) and luminosity was recorded using a Mithras LB 940 Multimode Reader luminometer (Berthold Technologies) for 25 min. For assays to test elicitor activity of Me47, naive *N. benthamiana* leaf disks were prepared as described above before exposure to reaction cocktail containing flg22 (100 nM), Me47 (1.5 μM) or PBS as negative control.

RESULTS

Identification and Phylogenetic Analysis of Me47

The identification of the proteinaceous components of *M. euphorbiae* saliva and the correlation with salivary gland EST data has been described previously (Atamian et al., 2013; Chaudhary et al., 2014, 2015). This analysis revealed the presence of a single protein (contig Me_WB05003; Me47), encoding 261 amino acids, with predicted GST activity based on homology to known enzymes of this type. To characterize Me47 and to perform phylogenetic analysis, using BLASTp at low stringency ($E < 0.1$), we identified the GST homologs from the two aphid species with publically available genome sequences. These are the legume specialist *A. pisum* and the generalist *M. persicae* with genome sequences for two distinct clones (The International Aphid Genomics Consortium [TIAGC], 2010; Myzus DB). These analyses identified 17 GSTs (AphidBase 2.1; Ramsey et al., 2010) from *A. psium* and nine GSTs from each of the *M. persicae* clones O and G006 (Myzus DB). We identified several alternate spliced forms of some of these GSTs and only one representative of these was included in further analysis. Phylogenetic analysis of Me47 coding sequence relative to the GST predicted proteins identified from *A. pisum* and *M. persicae* revealed that Me47

is more similar to GSTs from *A. pisum* (**Figure 1A**). *A. pisum* has three different classes of GST and the closest homolog to Me47 is the delta-class GST ACYPI006899 which encodes a 241 amino acid protein (Chelvanayagam et al., 2001; Ramsey et al., 2010). Direct comparison between Me47 and ACYPI006899 showed 62% identity at the amino acid level (**Figure 1B**) with conservative or semi-conservative substitutions at 2/6 positions of the GSH-binding site (G site; **Figure 1B** black asterisks) and 2/9 positions of the substrate-binding pocket (H site; **Figure 1B** red asterisks).

Since Me47 peptides were detected in the *M. euphorbiae* saliva (Chaudhary et al., 2014, 2015), we investigated the presence of a secretion signal peptide cleavage site in the predicted Me47 protein. Using SignalP, the presence of a 28 amino acid secretion signal was identified in Me47 confirming secretion of this GST in aphid saliva (Petersen et al., 2011). Curiously, ACYPI006899 does not contain a secretion signal cleavage site predicted by SignalP. Indeed, of the 36 aphid GSTs in this analysis, only three (Me47, ACYPI009586 and MpO_000127080.4) contain putative canonical secretion signal cleavage sites. Secretion of proteins could also be predicted by TargetP in the absence of a secretion signal peptide cleavage site (Emanuelsson et al., 2000). Using TargetP with the remaining *M. persicae* and *A. pisum* GSTs, predicted secretion for two additional *A. pisum* GSTs (ACYPI006899 and ACYPI006691) including the Me47 homolog (ACYPI006899) was identified. Taken together this information indicates that aphid GSTs have evolved different mechanisms for secretion and that their secretion into either extracellular spaces or saliva is relatively uncommon.

Me47 Modifies Aphid Performance in Multiple Fecundity Systems

To examine the role of Me47 during aphid colonization, we used Agrobacterium-mediated transient expression to express Me47 in leaf tissue of *N. benthamiana*. As *M. euphorbiae* does not reproduce successfully on *N. benthamiana*, we assessed the fecundity of *M. persicae,* which is able to feed on this host, over a 12-day period in an assay similar to experiments conducted previously (Bos et al., 2010; Atamian et al., 2013). In these experiments, we found that *M. persicae* fecundity was significantly increased on Me47-expressing leaves compared to GFP-expressing control leaves (**Figure 2A**, $P < 0.001$, Supplementary Figure S3A). This indicates that Me47 may function as a suppressor of plant immunity to enhance aphid colonization of tobacco.

To assess the role of Me47 in wider plant–aphid interactions, we transformed the semi-virulent bacterial strain *Pst* DC3000 Δ*AvrPto*/Δ*AvrPtoB* with the construct pVSP_*Ps*SPdes Me47 (pVSP-Me47). Whole tomato plants were vacuum-infiltrated with this semi-virulent inoculum. Using this method, the aphid protein of interest is delivered into tomato leaf cells via the bacterial T3SS, allowing performance of *M. euphorbiae* to be assessed on its natural host (Atamian et al., 2013). In these trials, *M. euphorbiae* fecundity was significantly increased on plants infected with *Pst* DC3000 Δ*AvrPto*/Δ*AvrPtoB* (pVSP-Me47) relative to those infected with the *Pst* DC3000 Δ*AvrPto*/Δ*AvrPtoB* (pVSP-GUS) control (**Figure 2C**, $p < 0.01$, Supplementary Figure S3C). This indicates that Me47 can function as a pathogenicity determinant in at least two host plant species with impact on two distinct aphid pests. However, as *Pst* DC3000 Δ*AvrPto*/Δ*AvrPtoB* possesses its own effector complement and is semi-virulent to tomato, we chose to assess the effect of Me47 delivery in the absence of other pathogen effectors. For this experiment, we used the non-pathogenic *Pfo* EtHAn strain (Thomas et al., 2009), which has been engineered to express the T3SS. This strain was transformed with the same constructs used in experiments described for *Pst* DC3000 Δ*AvrPto*/Δ*AvrPtoB*. In fecundity assays, *M. euphorbiae* performed significantly better on tomato infected with *Pfo* EtHAn (pVSP-Me47) compared to a *Pfo* EtHAn (pVSP-GUS) control (**Figure 2D**, $p < 0.01$, Supplementary Figure S3D). This confirmed our previous result in tomato, and indicates that the choice of *Pseudomonas* species for delivery of Me47 has minimal impact on the role of this protein in modifying *M. euphorbiae* fecundity on tomato. Finally, we used *Pfo* EtHAn with the same constructs to assess *M. persicae* fecundity on *Arabidopsis*. Interestingly in these experiments, *M. persicae* fecundity was significantly reduced on plants infected with *Pfo* EtHAn (pVSP-Me47) relative to the *Pfo* EtHAn (pVSP-GUS) control (**Figure 2B**, $p < 0.001$, Supplementary Figure S3B). This indicates that in specific host–aphid interactions, Me47 can have a host-dependent deleterious impact on aphid fecundity.

Me47 Induces PAMP-Responsive Genes in Tomato But Not in *Arabidopsis*

Our data indicated that in some experimental systems, Me47 made a positive contribution to aphid fecundity (**Figures 2A,C,D**) but in others the impact was negative (**Figure 2B**). As a non-pathogen, *Pfo* has been shown to induce PTI-related defense genes following infiltration into leaves of several plant species (Nguyen et al., 2010). We therefore made use of the *Pfo* EtHAn strain to assess ability of Me47 to suppress the PTI responses induced by this non-pathogenic bacterium. In these experiments, tomato plants were challenged with *Pfo* EtHAn delivering either GUS or Me47. The tomato defense genes *Lrr22* and *Pti5* have previously been shown to be inducible at 6 hpi following *Pfo* treatment (Nguyen et al., 2010). In our experiments, *Lrr22* and *Pti5* were only slightly induced by *Pfo* EtHAn (pVSP-GUS) but this increase was not statistically significant relative to the buffer control (**Figures 3A,B**). However, as the initial study used tomato cultivar Rio Grande-prf3, and the cultivar used in experiments described here is Moneymaker, it is possible that there is temporal variation in defense gene induction between tomato cultivars.

As we previously showed that Me47 can enhance aphid performance on tomato (**Figure 2D**), we suspected that Me47 might further suppress expression of these two defense genes. To our surprise, we found that delivery of Me47 by *Pfo* EtHAn enhanced the induction of both *Lrr22* and *Pti5* when transcript abundance was assessed at 6 hpi (**Figures 3A,B**, $p < 0.05$). For the PAMP-inducible gene *Gras2*, Nguyen et al. (2010) reported no induction at 6 hpi following *Pfo* infection.

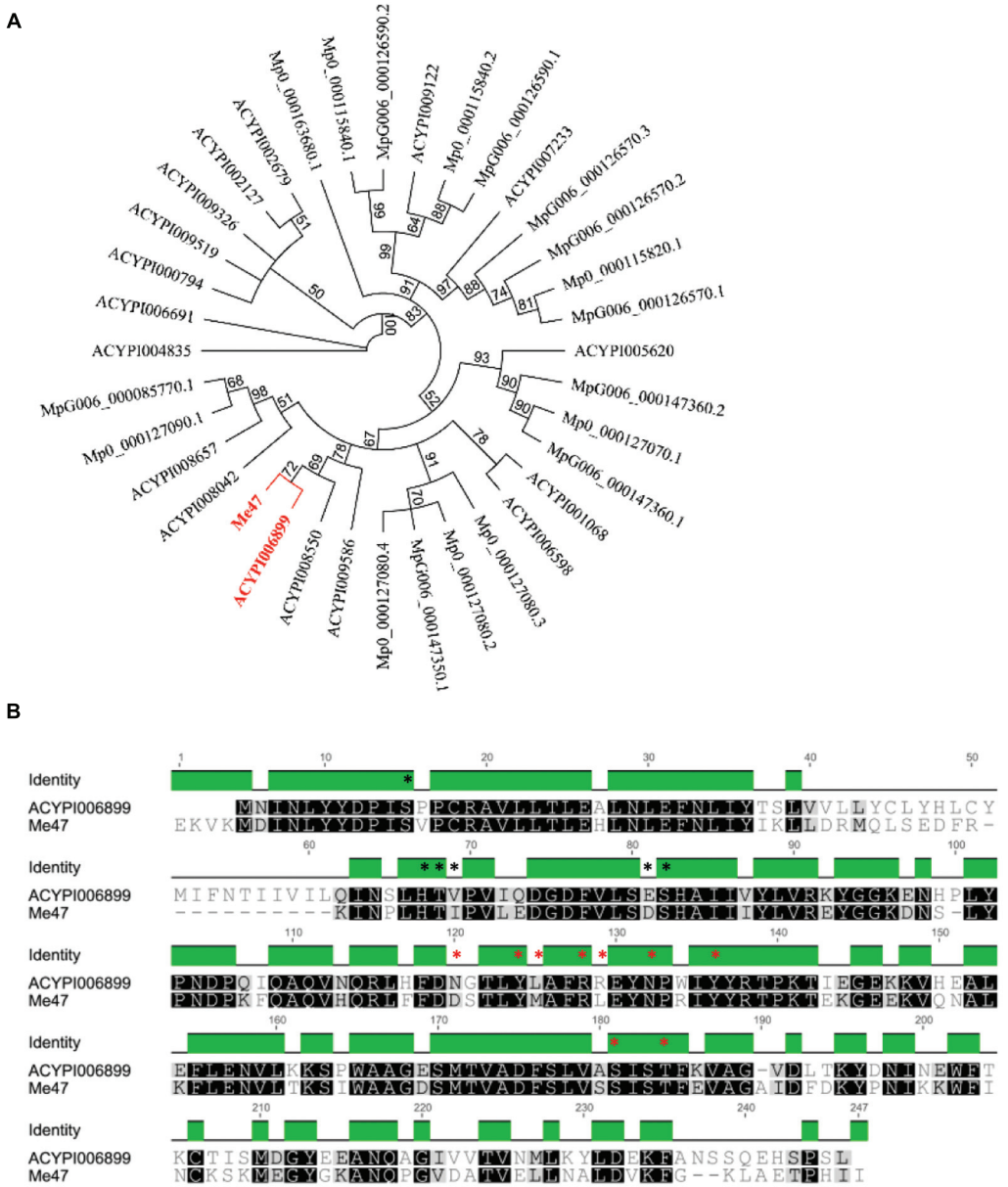
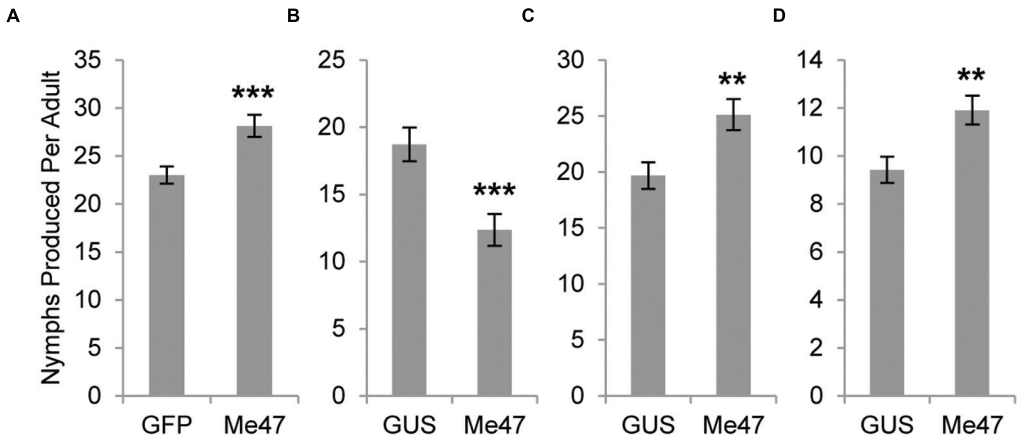


FIGURE 1 | Phylogenetic analysis of Me47 relative to *A. pisum* and *M. persicae* GST sequences. (A) Predicted Me47 amino acid sequences were aligned with *M. persicae* (both clones G006 and O) and *A. pisum* GSTs using ClustalW and presented as a phylogenetic tree using the Neighbor-joining method (Geneious, 100 bootstrap replicates). Me47 and the closest *A. pisum* ortholog (ACYPI006899) are highlighted in red. **(B)** Alignment of Me47 with ACYPI006899. Residues forming the GSH binding site (G site; black) and substrate binding pocket (H site; red) are marked with asterisks.

Similarly, we found no change in expression between the buffer and *Pfo* EtHAn (pVSP-GUS) treatments (**Figure 3C**). However, *Gras2* was significantly induced following *Pfo* EtHAn (pVSP-Me47) treatment (**Figure 3C**, $p < 0.05$). In this experiment, we also analyzed the expression of the *PR1a* reporter gene as it is frequently observed to be inducible both during pathogen infection and by PAMP treatment. Similar to the other genes tested, *PR1a* was highly induced following *Pfo* EtHAn (pVSP-Me47) treatment relative to both *Pfo* EtHAn (pVSP-GUS) and the buffer control (**Figure 3D**, $p < 0.05$). Together, this defense gene expression dataset illustrates the surprising observation that delivery of Me47 into tomato leaves enhances the expression of PAMP-responsive genes during bacterial challenge.

To assess whether a similar phenomenon is present in another host used in our aphid bioassay, we conducted a parallel experiment to monitor defense gene induction in *Arabidopsis* following delivery of Me47 by *Pfo* EtHAn (**Figures 4A–D**). Unlike tomato, a specific defense marker assay for *Pfo* infection has not been developed for *Arabidopsis*. However, numerous



Host	*N.benthamiana*	*A. thaliana*	*S. lycopersicum*	*S. lycopersicum*
Aphid	*M. persicae*	*M. persicae*	*M. euphorbiae*	*M. euphorbiae*
Delivery	*A. tumefaciens*	*P. fluorescens*	*P. syringae*	*P. fluorescens*

FIGURE 2 | Me47 changes aphid reproductive performance. (A) *M. persicae* fecundity assessed on *N. benthamiana* transiently expressing either GFP or Me47 by Agroinfiltration. **(B)** *M. persicae* fecundity assessed on *A. thaliana* infected with either *Pfo* EtHAn (pVSP-GUS) or *Pfo* EtHAn (pVSP-Me47). **(C)** *M. euphorbiae* fecundity assessed on tomato cv. Moneymaker infected with either *Pst* DC3000 Δ*AvrPto*/Δ*AvrPtoB* (pVSP-GUS) or *Pst* DC3000 Δ*AvrPto*/Δ*AvrPtoB* (pVSP-Me47). **(D)** *M. euphorbiae* fecundity assessed on tomato cv. Moneymaker infected with either *Pfo* EtHAn (pVSP-GUS) or *Pfo* EtHAn (pVSP-Me47). $^{**}P < 0.01$ and $^{***}P < 0.001$ as determined by two-tailed *t*-test. Data from single experiments presented and data from additional experiments are shown in Supplementary Figure S3.

studies have used *Arabidopsis* for dissection of aphid-relevant defense pathways (De Vos et al., 2005; Couldridge et al., 2007; Kusnierczyk et al., 2008; Kettles et al., 2013). The camalexin biosynthetic gene *PAD3* is known to be involved in resistance to numerous pathogens in addition to aphids. We therefore hypothesized it would be a good choice for assessing defense activation in plants challenged with *Pfo*. Whilst *Pfo* infection indeed caused a significant increase in *PAD3* expression (**Figure 4A**), there was no difference in expression levels between the *Pfo* EtHAn (pVSP-GUS) and *Pfo* EtHAn (pVSP-Me47) treatments at 6 hpi (**Figure 4A**). *CYP81F2* has been reported to be involved in the production of indolic glucosinolates that have activity against some pathogens and also aphids (Bednarek et al., 2009; Pfalz et al., 2009). Again, we found that expression of this gene was highly responsive to *Pfo* treatment at 6 hpi irrespective of the expressed construct (**Figure 4B**). *PDF1.2* is routinely used as a defense marker of specific relevance to the jasmonic acid (JA)/ethylene signaling pathways, whilst *PR1* has long been known to be highly responsive to many pathogens/pests and as a marker for salicylic acid (SA)-related defense signaling. Neither of these genes showed statistically significant responses either to *Pfo* treatment or delivery of Me47 relative to GUS at 6 hpi (**Figures 4C,D**). Together, we found no evidence of enhanced defense marker gene expression in *Arabidopsis* leaves treated with *Pfo* EtHAn (pVSP-Me47) relative to *Pfo* EtHAn (pVSP-GUS) at 6 hpi, thus illustrating differential responses of tomato and *Arabidopsis* to the Me47 effector protein.

Me47 Is a Glutathione-*S*-Transferase with Activity against Isothiocyanates

To confirm that Me47 is a functional GST, recombinant N-terminal His-tagged Me47 (His-Me47) was expressed and purified from bacterial cell lysates (Supplementary Figure S1) for use in a GST activity assay utilizing the broad-spectrum GST substrate 1-Chloro-2,4-dinitrobenzene (CDNB). Surprisingly, purified His-Me47 showed no ability to conjugate glutathione to CDNB when compared to commercially available GST preparations (data not shown). Nonetheless, we developed a glutathione depletion assay based on the method of Wadleigh and Yu (1988) to assess activity of Me47 against a selection of isothiocyanates (ITCs). These volatile defense compounds are specific to members of Brassicaceae, have toxic activity against insects and are known substrates for both insect and human GSTs (Wadleigh and Yu, 1988; Kolm et al., 1995). The bacterial chaperonin GroEL, also expressed and purified with an N-terminal His-tag (His-GroEL), was used as a negative control. In this assay, His-Me47 depleted the free glutathione in the presence of benzyl isothiocyanate (BITC; **Figure 5A**) and phenylethyl isothiocyanate (PEITC; **Figure 5B**) to a level comparable to the commercially prepared equine GST (eqGST) positive control. As expected, His-GroEL did not have any glutathione-depleting activity in the presence of either BITC or PEITC similar to the buffer-only control. In contrast, His-Me47 was unable to utilize allyl isothiocyanate (AITC) as substrate and the free glutathione level remained consistent with the buffer and His-GroEL protein controls (**Figure 5C**). These data illustrate

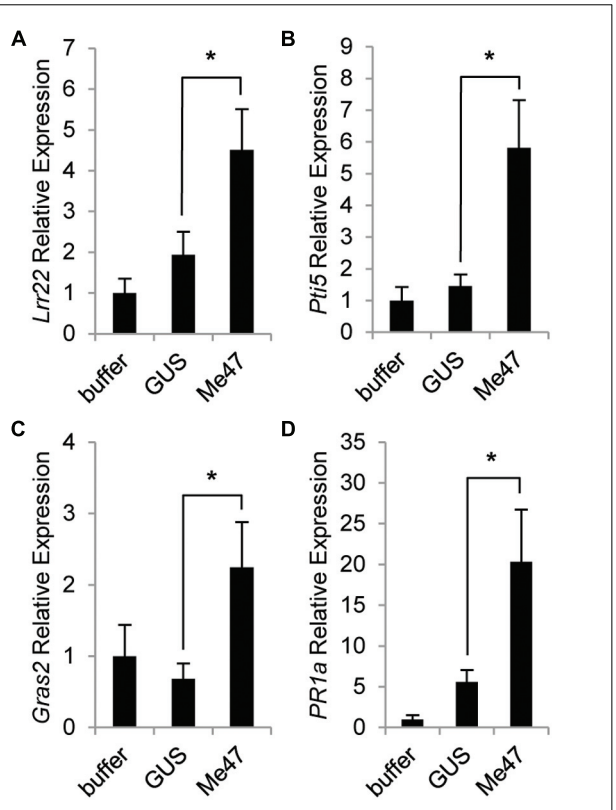


FIGURE 3 | Me47 induces defense genes in tomato. Tomato cv. Moneymaker was infiltrated with buffer-only, *Pfo* EtHAn (pVSP-GUS) or *Pfo* EtHAn (pVSP-Me47) and leaves harvested at 6 hpi. Expression analysis of genes involved in PTI (*Lrr22*, *Pti5*, *Gras2*) **(A–C)** and salicylic acid (SA)-dependent defense (*PR1a*) **(D)** conducted by qRT-PCR. $*P < 0.05$ as determined by two-tailed *t*-test. Bars represent means and standard error across six biological replicates from two independent experiments. Buffer treatment rescaled to 1 for presentation.

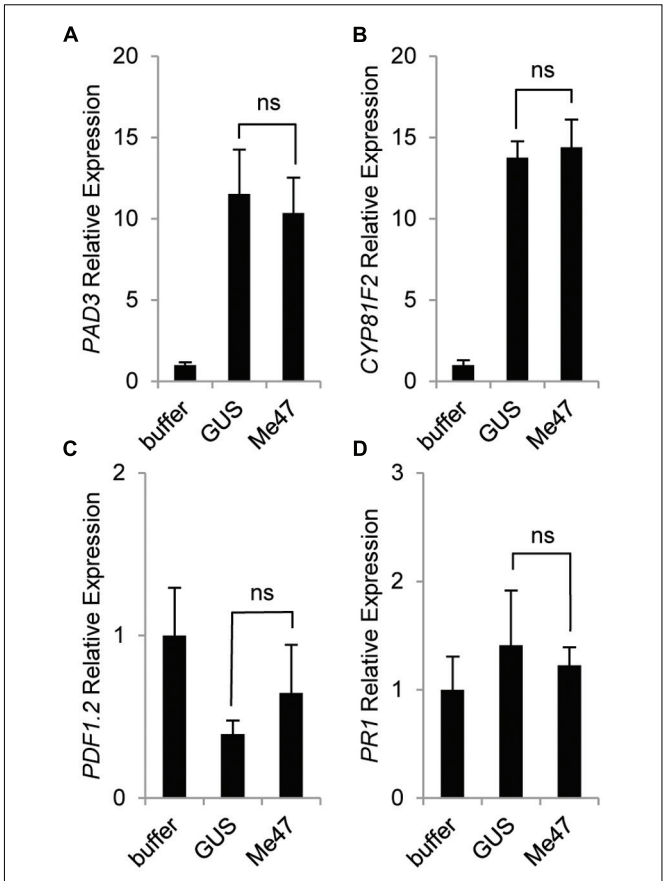


FIGURE 4 | Me47 has no effect on defense gene induction in *Arabidopsis*. *Arabidopsis* Col-0 was treated as described in **Figure 3** and leaves harvested at 6 hpi. Expression analysis of genes involved in **(A)** camalexin (*PAD3*), **(B)** indole glucosinolate (*CYP81F2*), **(C)** Jasmonic acid/ethylene (JA/ET; *PDF1.2*) and **(D)** SA pathway (*PR1*) done by qRT-PCR. Bars represent means and standard error across six biological replicates from two independent experiments. Buffer treatment rescaled to 1 for presentation. Differences between GUS and Me47 treatments were not significant (ns).

that Me47 is a functional GST with ability to utilize known plant defense compounds as substrates.

Me47 Does Not Interfere with the PAMP-Induced ROS Burst in *N. benthamiana*

The *M. persicae* effector Mp10 was previously demonstrated to suppress the flg22-induced ROS burst when transiently expressed in *N. benthamiana* (Bos et al., 2010). We conducted a similar experiment to test whether Me47 might have similar properties. In contrast to Mp10, Me47 had no suppressive effect on the flg22-induced ROS burst relative to leaf tissue expressing a GFP control transgene (Supplementary Figure S2A). Given that Me47 was found to induce PAMP-responsive defense genes when delivered into tomato leaves (**Figure 3**), we then tested whether Me47 might trigger a ROS burst in *N. benthamiana* using the same recombinant Me47 protein as used for the glutathione depletion assay (**Figure 5**, Supplementary Figure S2). Me47 protein was unable to induce ROS production above the PBS control levels (Supplementary Figure S2B) indicating that it is not an elicitor of ROS burst in *N. benthamiana*.

DISCUSSION

To date, only a handful of aphid effectors have been reported and for all the specific function or activity is unknown. For *M. euphorbiae*, effectors Me10 and Me23 are the only examples known to have impact on aphid fecundity when expressed *in planta* (Atamian et al., 2013). Agroexpression of both Me10 and Me23 increased *M. persicae* fecundity on *N. benthamiana*, whilst only Me10 increased potato aphid fecundity on tomato when delivered through bacterial T3SS (Atamian et al., 2013). In studies on *M. persicae* effectors, Mp10 and Mp42 were found to reduce aphid fecundity on *N. benthamiana* (Bos et al., 2010). Additional *M. persicae* effectors, Mp55-58, were described by Elzinga et al. (2014) that have either beneficial or deleterious impact on aphid success across *N. benthamiana*, *N. tabacum* or *Arabidopsis*. In

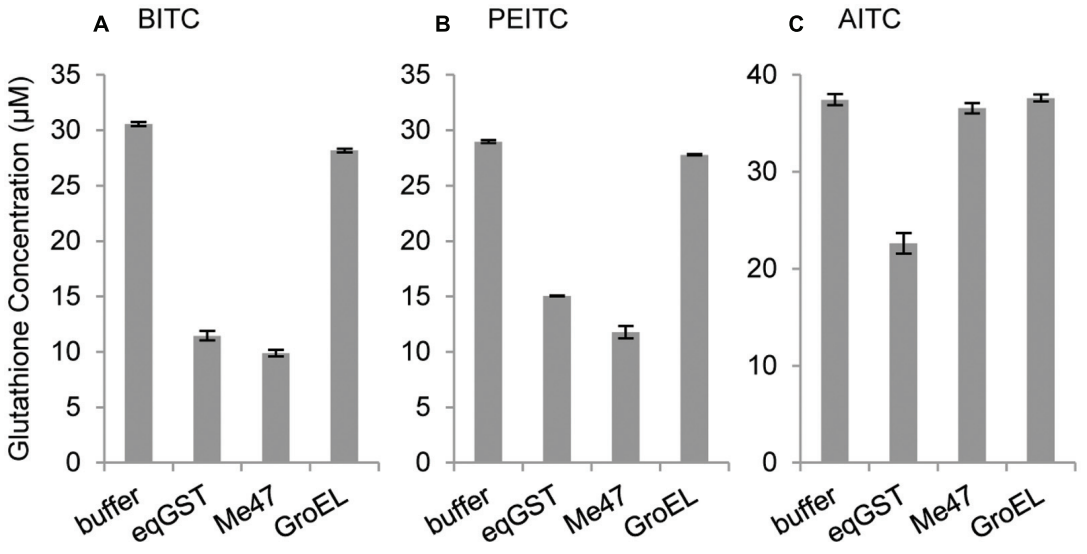


FIGURE 5 | Me47 can utilize selected isothiocyanates as substrates. Glutathione depletion assay analysis of substrates **(A)** benzyl isothiocyanate (BITC), **(B)** phenylethyl isothiocyanate (PEITC), and **(C)** allyl isothiocyanate (AITC), incubated with buffer control, commercial equine GST (eqGST; Sigma), recombinant N-terminal His-tagged Me47 (Me47) and non-enzymatic recombinant N-terminal His-tagged GroEL (GroEL).

each case, the change in aphid performance was consistent across the host species assayed. This indicates that well-conserved defense mechanisms may be subject to manipulation by these effectors.

Previously it has been also demonstrated that some aphid effectors have host-specific activity (Bos et al., 2010; Pitino and Hogenhout, 2013). For example, *M. persicae* effectors Mp1 and Mp2 enhanced *M. persicae* performance when stably expressed in *Arabidopsis*. In contrast, transient expression of the *A. pisum* orthologs of these genes had no effect on *M. persicae* performance. Me47 is the first aphid effector observed to have both beneficial and detrimental impact on aphid colonies that is host-dependent. In tomato, Me47 improved *M. euphorbiae* reproductive success (**Figures 2C,D**) yet Me47 protein or activity was recognized and induced the expression of multiple defense genes (**Figure 3**). Remarkably, Me47 decreased *M. persicae* fecundity on *Arabidopsis* (**Figure 2B**), yet there was no immune recognition of Me47 protein or activity, at an early effector delivery time point (6 hpi), as indicated by expression levels of several genes previously linked to aphid defense (**Figure 4**). These observations are surprising, as it is expected that an increase in aphid fecundity (as on tomato) would align with some degree of immune suppression and not immune activation. Furthermore, a decrease in aphid fecundity (as on *Arabidopsis*) might be expected to accompany a degree of immune stimulation. Together, these observations suggest that the direct recognition of either Me47 or its activity does not underpin the likelihood of successful aphid colonization. Additionally, the immunogenicity of Me47 may be suppressed in natural aphid infestations by the action of other, as yet unidentified, effector proteins present in the salivary milieu.

It has been speculated that aphids might actively trigger host defenses that have little efficacy against this class of plant attacker (Walling, 2008). For example, it is known that the JA and SA signaling pathways can act antagonistically where induction of one leads to suppression of the other. Aphid infestations primarily elicit SA-dependent defenses (De Vos et al., 2005; Kettles et al., 2013), yet other studies have reported JA-mediated defense to be more effective (Ellis et al., 2002; Zhu-Salzman et al., 2004). It is possible that the defense pathways triggered by Me47 delivery in tomato have little impact on aphid colonization, but might supress more effective defense responses not included as part of this investigation. Indeed, *PR1a* is frequently used as a marker gene for SA-dependent defense responses and was highly induced by Me47 in tomato (**Figure 3D**) but not in *Arabidopsis* (**Figure 4D**) consistent with this hypothesis.

Me47 delivery in tomato induces defense-related genes as determined by qRT-PCR (**Figure 3**). However, the elicitor activity of Me47 remains to be determined. From limited available data, it appears many endogenous *Arabidopsis* GSTs have non-specific subcellular localization and are present in the cytosol (Dixon et al., 2009), although a limited number are nuclear- or peroxisome-localized. We were unable to precisely localize Me47 in plant cells as transient expression of yellow fluorescent protein (YFP)-tagged Me47 in *N. benthamiana* revealed localization to both the cytosol and nucleus similar to YFP control (Data not shown). Since the Me47-YFP size is 57 kDa the protein could defuse through the nuclear pore. Nevertheless, it is unlikely that Me47 is present in plant organelles in the absence of endogenous GSTs. One possibility is that it is not Me47 that is recognized but the metabolomic products of its activity. Me47 substrate specificity may be different from endogenous GSTs, such that Me47-catalyzed reaction products are hallmarks of a foreign GST. Aberrant GST activity might therefore be open to recognition and stimulate immunity in a manner analogous to the perception of PAMPs during the PTI phase of host–microbe interactions.

A plethora of secondary plant metabolites exists and is speculated the primary function of many is for defense against herbivory. To overcome these defenses, insects have evolved large and diverse classes of detoxification enzymes, including GSTs, to negate the potentially lethal effects of toxic phytochemicals (Li et al., 2007; Ramsey et al., 2010). In aphids, GSTs have been linked to detoxification of glucosinolates (Francis et al., 2005) and nicotine (Ramsey et al., 2014) and the cereal hydroxamic acid 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA; Mukanganyama et al., 2003). A single GST from the cotton bollworm (*Helicoverpa armigera*) was also found to detoxify the plant JA pathway precursor 12-oxophytodienoic acid (*cis*-OPDA; Dabrowska et al., 2009). However, the focus of insect GSTs has been almost exclusively on their role in the gut (for phytochemical detoxification) or in the cuticle/body (for insecticide detoxification; Vontas et al., 2001, 2002). To our knowledge, no prior study has described the role of a single GST, from any insect, out of the producing organism and in direct mediation of host–pest interactions. It is perhaps not surprising that such a mechanism has evolved in insects, as longer exposure time of toxic phytochemicals to detoxification enzymes likely reduces the concentration of toxin ingested and exposed to cells in the gut. Whilst this function for Me47 is therefore novel, it is unlikely to be the only example of such a phenomenon. Indeed, a catalytically active GST, expressed in salivary glands of wheat-infesting Hessian fly (*Mayetiola destructor*; Yoshiyama and Shukle, 2004) suggest that additional examples will exist in other groups of plant pests.

In our characterization of Me47, we found that Me47 substrate specificity did not include CDNB, a model substrate found to be metabolized by total GST preparations from *M. persicae* (Francis et al., 2005). The activity spectrum of total GST extracts from *M. euphorbiae* have not been described, but it is possible that other GSTs aside from Me47 show activity against this model substrate. In this study, we identified two ITCs (BITC, PEITC) as Me47 substrates (**Figure 5**). Me47 did not metabolize AITC, however, suggesting some degree of enzymatic specificity within this class of defensive metabolite. ITCs are defense compounds associated with insect resistance and are specific for cruciferous plants. During aphid infestations of plants belonging to this family, such as *Arabidopsis*, the function of Me47 is therefore clear. However, natural substrates of Me47 from the other hosts used in this study, tobacco and tomato, remain to be identified. It is therefore not yet possible to assess whether Me47 is a highly promiscuous, broad-spectrum GST or moderately promiscuous in its activity against plant defense compounds of relevance to natural *M. euphorbiae* infestations. Our initial data regarding metabolism of ITCs, coupled with the inability of Me47 to metabolize the model substrate CDNB would suggest the latter, but this requires further biochemical investigation.

AUTHOR CONTRIBUTIONS

GK and IK conceived and planned the experiments. GK performed the experiments. GK and IK analyzed the data. GK wrote the manuscript with help from IK.

FUNDING

This work was funded by grant (2010-65106-20675) to IK from the United States Department of Agriculture, National Institute of Food and Agriculture.

ACKNOWLEDGMENT

The authors would like to thank Hagop Atamian and Ritu Chaudhary for helpful discussions during the course of this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01142

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 30 August 2016
doi: 10.3389/fpls.2016.01241

Small RNA Regulators of Plant-Hemipteran Interactions: Micromanagers with Versatile Roles

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Non-coding small RNAs (sRNAs) in plants have important roles in regulating biological processes, including development, reproduction, and stress responses. Recent research indicates significant roles for sRNA-mediated gene silencing during plant-hemipteran interactions that involve all three of these biological processes. Plant responses to hemipteran feeding are determined by changes in the host transcriptome that appear to be fine-tuned by sRNAs. The role of sRNA in plant defense responses is complex. Different forms of sRNAs, with specific modes of action, regulate changes in the host transcriptome primarily through post-transcriptional gene silencing and occasionally through translational repression. Plant genetic resistance against hemipterans provides a model to explore the regulatory roles of sRNAs in plant defense. Aphid-induced sRNA expression in resistance genotypes delivers a new paradigm in understanding the regulation of *R* gene-mediated resistance in host plants. Unique sRNA profiles, including changes in sRNA biogenesis and expression can also provide insights into susceptibility to insect herbivores. Activation of phytohormone-mediated defense responses against insect herbivory is another hallmark of this interaction, and recent studies have shown that regulation of phytohormone signaling is under the control of sRNAs. Hemipterans feeding on resistant plants also show changes in insect sRNA profiles, possibly influencing insect development and reproduction. Changes in insect traits such as fecundity, host range, and resistance to insecticides are impacted by sRNAs and can directly contribute to the success of certain insect biotypes. In addition to causing direct damage to the host plant, hemipteran insects are often vectors of viral pathogens. Insect anti-viral RNAi machinery is activated to limit virus accumulation, suggesting a role in insect immunity. Virus-derived long sRNAs strongly resemble insect piRNAs, leading to the speculation that the piRNA pathway is induced in response to viral infection. Evidence for robust insect RNAi machinery in several hemipteran species is of immense interest and is being actively pursued as a possible tool for insect control. RNAi-induced gene silencing following uptake of exogenous dsRNA was successfully demonstrated in several hemipterans and the presence of *sid-1 like* genes support the concept of a systemic response in some species.

Keywords: sRNAs, hemiptera, resistance, RNAi, viral immunity

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *30 May 2016*
Accepted: *04 August 2016*
Published: *30 August 2016*

Citation:
Sattar S and Thompson GA (2016) Small RNA Regulators of Plant-Hemipteran Interactions: Micromanagers with Versatile Roles. Front. Plant Sci. 7:1241. doi: 10.3389/fpls.2016.01241

INTRODUCTION

Small RNAs (sRNAs) are essential regulators of eukaryotic gene expression and function. These 20–30 nucleotide (nt) regulatory elements (Aravin et al., 2003), common to both plants and animals, control endogenous gene expression in response to external stimuli and protect the host from invasive viruses. Plants respond to changing environmental conditions by altering their transcriptome, which is actively modulated by sRNAs. Altered expression of sRNA and their gene targets, in response to abiotic and biotic stress have firmly established the importance of these regulatory elements. During biotic stress, plants identify the pathogen associated molecular patterns (PAMPs), which initiates a downstream signaling cascade leading to PAMP-triggered immunity (PTI). Pests and pathogens have simultaneously evolved effector proteins to halt PTI and launch effector-triggered susceptibility (ETS). Plants have co-evolved to acquire resistance (R) proteins that recognize these effectors, resulting in a secondary immune response called effector-triggered immunity (ETI) (Pieterse et al., 2009). Global sRNA profiling for specific pest or pathogen interactions have provided useful information regarding the sRNAs involved in immunity and the altered expression of genes, and sRNAs have become the molecular signatures of specific PTI or ETI events. Such molecular markers have been reported for several pathogens, including markers for bacterial, fungal, and viral infections in different plant species (Navarro et al., 2006; Jagadeeswaran et al., 2009; Li et al., 2010; Campo et al., 2013; Feng et al., 2013; Pablo Peláez and Sanchez, 2013). Similar events have been reported during insect herbivory, where several sRNA-regulated defense responses have been identified during herbivory by nematodes and chewing insects (Pandey et al., 2008; Li et al., 2012). Plants infested by phloem-feeding insects belonging to the order hemiptera appear to elicit significantly different responses than chewing insects and might be more closely aligned with responses to biotrophic pathogens. Unlike the chewing pests, sucking insects do not cause massive mechanical wounding to the plant tissue during herbivory. The specialized mouthparts of hemipterans, called stylets, penetrate the cortical tissues to reach the vascular tissues, causing minimal mechanical damage, and evading many of the specialized host defense responses to wounding. However, plants respond to phloem-feeding insects by activating a suite of specific defense responses that are also regulated by sRNAs. This review will primarily focus on the sRNAs involved in plant-hemipteran interactions and will emphasize the role of both plant and insect derived sRNAs in susceptible and resistant host interactions to inform strategies using sRNAs as tools for pest management in agriculture.

sRNAs in Plants

Plants have two major classes of small endogenous RNAs, microRNA (miRNA) and small interfering RNA (siRNA) that are distinguished by their structure and biogenesis. MicroRNAs are derived from single-stranded long primary transcripts (pri-miRNA) that are primarily processed by Dicer-like-1 (DCL1) to a double-stranded hairpin structure called pre-miRNA (Jones-Rhoades et al., 2006; Voinnet, 2009). The pre-miRNA is further processed into the miRNA/miRNA∗ duplex, which is then methylated by Hua Enhancer 1(HEN1) and loaded into the Argonaute-1 (AGO1)-containing RNA induced silencing effector complex (RISC) (Zhu, 2008; Chen, 2009). Mature miRNA guides RISC to the target mRNA resulting in cleavage and post-transcriptional regulation of the target gene (Mallory and Vaucheret, 2010). In Arabidopsis, miRNAs have also been shown to inhibit the translation of target mRNAs (Li S. et al., 2013). In contrast, siRNAs are derived from double-stranded RNA (dsRNA) precursors that are processed by DCL3 or DCL4 and then loaded in AGO1, AGO7, AGO4, and other AGO complexes (Jones-Rhoades et al., 2006). Other notable characteristics differentiate these two classes of sRNAs. MicroRNAs typically originate from intergenic regions and target unrelated gene loci. In contrast, siRNAs target either the gene from which they are derived or closely related genes. Furthermore, miRNAs are often conserved across closely related species, whereas endogenous siRNAs are highly divergent (Jones-Rhoades et al., 2006).

Small interfering RNAs can be further classified into heterochromatic siRNAs, secondary siRNAs, and NAT-siRNAs (Vaucheret, 2006; Axtell, 2013). Heterochromatic siRNAs are usually 23–24 nt in length and originate from the repetitive and intergenic regions in the chromosome. They are processed by DCL3 and recruit AGO4 as part of the RNAi-induced transcriptional silencing complex and take part in silencing chromatin (Jones-Rhoades et al., 2006; Axtell, 2013). Secondary siRNAs are generated as a "secondary effect" of miRNA-mediated target cleavage. Sometimes the miRNA-mediated cleaved target is used by RNA-dependent RNA polymerase (RDR) to produce secondary siRNAs (Allen et al., 2005; Manavella et al., 2012). This can either give rise to a phased set of siRNAs or trans-acting siRNAs (tasiRNAs) that have the ability to target genes that are different from their loci of origin. Natural-antisense transcript siRNAs (NAT-siRNAs) are generated from dsRNA precursors as a result of hybridization of independently transcribed complementary RNA strands (Borsani et al., 2005; Vaucheret, 2006; Axtell, 2013). These can be further distinguished as *cis*-NAT-siRNA generated from precursors that are transcribed from overlapping regions of the same gene but in opposite polarity, and *trans*-NAT-siRNA whose dsRNA precursors are transcribed from non-overlapping regions, but are complementarity to each other (Borsani et al., 2005; Vaucheret, 2006). There are other classes of siRNAs such as repeat-associated siRNAs (rasiRNA) that have been studied in detail in the maize genome (Barber et al., 2012) and are essential for transcriptional gene silencing and maintaining DNA methylation (Chan et al., 2004; Onodera et al., 2005; Chellappan et al., 2010). The two most recent additions to the repertoire of plant sRNAs are the 21-nt epigenetically activated small interfering RNAs (easiRNA) and siRNAs independent of DCLs (sidRNAs) (Creasey et al., 2014; Ye et al., 2016).

Of all the sRNAs, the miRNAs are the best characterized with well-defined roles in plant development, metabolism, reproduction, defense, and stress biology (Katiyar-Agarwal and Jin, 2010; Sunkar, 2010; Khraiwesh et al., 2012). MicroRNAs can also be classified into two categories: the lineage specific miRNAs found in a single species or across closely related species and the

long miRNAs of 23–24 nt in length that are functionally similar to heterochromatic siRNAs (Axtell, 2013).

sRNAs in Insects

Insect sRNAs can be classified into three classes: miRNAs, endogenous-siRNAs (endo-siRNAs), and piwi-interacting RNAs (piRNAs) (Golden et al., 2008). The classification is based on their distinct characteristics, biogenesis, and association with AGO proteins (Kim et al., 2009). Like their plant counterparts, insect miRNAs are well characterized; however, the biogenesis of insect miRNA involves the enzymatic action of two RNase III proteins, Drosha and Dicer. The pri-miRNA hairpin-structure originates from the intergenic region by the polymerase activity of RNA polII and is processed within the nucleus into ∼70-nt pre-miRNA by Drosha. The pre-miRNA hairpin lacking perfect complementarity is exported in to the cytoplasm by Exportin-5 where it is processed by Dicer-1 into the miRNA/miRNA∗ duplex (Lucas and Raikhel, 2013). The 21-nt endo-siRNAs in insects and mammals are produced in an RNA-dependent RNA polymerase (RdRP) independent manner, requiring a Dicer-2-dependent process (Kim et al., 2009). Endo-siRNAs primarily originate from perfect or near complementary regions of transposon transcripts, intergenic repetitive elements, or endo-siRNA cluster loci (Tomari and Zamore, 2005). Piwi-interacting RNAs also originate from intergenic repetitive elements, including retro-transposons, but do not require Dicer for processing. Piwi-interacting RNAs were originally reported from Drosophila germ cells (Lin and Spradling, 1997). Other than their distinct biogenesis, these three classes of sRNA can be distinguished by their size; miRNAs are typically 22 nt, endo-siRNAs are 21 nt, and piRNAs are 24–30 nt (Golden et al., 2008).

Another important characteristic distinguishing the three species of insect sRNAs is their association with distinct members of the Argonaute family. In Drosophila, endo-siRNAs typically use the effector protein Ago-2, an association that is considered to be a distinguishing feature for this class of sRNAs (Golden et al., 2008). Ago-1 acts as the effector protein for miRNAs and in association with GW182 protein, forms the miRISC complex in Drosophila (Tomari et al., 2007; Carthew and Sontheimer, 2009). As their name indicates, piRNAs interact with Piwi proteins. Piwi-interacting RNAs are primarily involved in silencing selfish genetic elements and contribute to germ line stability (Aravin et al., 2007; Hartig et al., 2007). The final distinguishing mark between these three classes is the presence or absence of a 2′-o-methyl modification at the 3′end; siRNAs and piRNAs are modified, whereas miRNAs lack this modification and are therefore susceptible to perioxidate oxidation and beta-elimination (Golden et al., 2008).

sRNAs in Regulating Plant Interactions with Insect Pests and Pathogens

Plants have developed various defensive strategies to disarm attacks by different insect pests and pathogens. In the last decade, an active role for sRNAs during these plant biotic interactions has been increasingly recognized. Plant-derived sRNAs participate in PTI as well as ETI as defense mechanisms against insect pests and pathogens. However, virulence and host immunity can be affected by pathogen-derived sRNAs that function as effector molecules to overcome the plant immune response (Weiberg et al., 2014). Evidence for the role of miRNAs in PTI was provided by Navarro et al. (2006) when they demonstrated that overexpressing miR393 in Arabidopsis provided enhanced resistance to the bacterial pathogen *Psuedomonas syringae*. Arabidopsis miR393 decreased the steady-state levels of mRNAs encoding auxin receptors transport inhibitor response 1 (TIR1) and auxin signaling F-box 2 and 3 (AFB2, and AFB3), disrupting auxin signaling. As a consequence, auxin-mediated suppression of salicylic acid (SA) is inhibited, impacting plant defense through accumulation of SA and activation of SA signaling. Additionally, in miR393 overexpressing plants, the secondary metabolic pathway is re-directed away from camelaxin toward glucosinolates. The combined effects of enhanced SA signaling and increased levels of glucosinolates contributed to *P. syringae* resistance (Robert-Seilaniantz et al., 2011). Other miRNAs that impact auxin signaling also have been implicated in regulating bacterial pathogenesis. Altered expression of miR160 and miR167 during bacterial infection was linked to differential regulation of the auxin signaling pathway by targeting members of the auxin-response factor (ARF) family of transcription factors (Fahlgren et al., 2007). Plant-derived miRNAs have been implicated in other biotic interactions involving fungi (Lu et al., 2007) and viruses (He et al., 2008). A diverse set of miRNAs was reported to be affected by powdery mildew infection in wheat (Xin et al., 2010). Similarly, Gonzalez-Ibeas et al. (2011) identified a large number of conserved miRNA families in the melon sRNA transcriptome analyzed from watermelon mosaic virus (WMV) and melon necrotic spot virus (MNSV) susceptible (Tendril) and resistant (T-111, and TGR-1551) cultivars. Wheat miR408 negatively regulates plantacyanin TaCLP1, which is responsible for enhanced susceptibility to wheat stripe rust fungus (Feng et al., 2013). Evidence for miRNA-mediated PTI in basal defense against rice blast fungus, has been reported for rice miR169a, miR172a, and miR398b (Li Y. et al., 2014). An exhaustive list of the miRNA families that are involved in bacterial and fungal pathogenesis in several plant species is documented in recent reviews by Weiberg et al. (2014) and Huang et al. (2016).

Specific, and perhaps unique, roles for plant sRNAs have been identified during nematode infection and insect herbivory. Altering global sRNA biogenesis in *dcl* and *rdr* mutants of Arabidopsis showed reduced susceptibility to nematodes (Hewezi et al., 2008), whereas silencing *rdr1* in *Nicotiana attenuata* increased the susceptibility of the plant to herbivory by chewing pests (Pandey et al., 2008). The *rdr1*-silenced *Nicotiana* plants had attenuated expression of jasmonic acid (JA) and ethylene (ET) biosynthetic genes as well as reduced accumulation of JA indicate that sRNAs negatively impact host-defense signaling in response to *Manduca sexta* feeding (Pandey et al., 2008). Additionally, Rasmann et al. (2012) have shown that Arabidopsis mutants deficient in sRNA biogenesis do not inherit the trans-generational priming of jasmonic acid (JA)-dependent defense response against chewing herbivores. Chewing insect herbivory results in significant wound damage to the plant tissues, and several conserved and novel miRNA

families a large number of loci generating phased siRNA and tasi-RNA were identified in tobacco in response to herbivory (Tang et al., 2012).

Biotic stress induced by insect pests and pathogens can trigger *R* gene mediated defense responses in plants. Evidence for sRNA regulation of *R* genes in several plant species has increased our understanding of the molecular switch that controls *R* gene mediated responses in plants. During normal plant growth, *R*-gene expression could trigger autoimmunity redirecting the plant metabolism from growth to defense. In the Solanaceae, miRNAs and secondary siRNA have conserved roles in regulating NBS-LRR receptors and innate immunity (Li et al., 2012). For example, NBS-LRR resistance gene mRNAs are specifically targeted by miR482/2118 in tomato and other members of the Solanaceae (Shivaprasad et al., 2012). Similar results were observed in Medicago and soybean where three 22 nt miRNAs (miR1507, miR2118, and miR21090) generated phased-siRNAs that regulate NBS-LRR genes (Zhai et al., 2011).

HOST sRNA PATHWAY COMPONENTS AND INDUCED RESPONSES AGAINST HEMIPTERAN HERBIVORY

Phloem-feeding insects belonging to the order hemiptera have adopted a unique feeding niche that exploits the sugar-rich plant phloem sap as a primary food source. Phloem sap is under high turgor pressure that is maintained by low osmotic potentials within transport phloem sieve elements (Taiz and Zeiger, 2010). This sugar-rich environment also contains proteins, peptides, and a high ratio of non-essential: essential amino acids. Phloem-feeding hemipterans have co-evolved to exploit this challenging diet by acquiring several unique adaptions. Phloem feeders have specialized mouth parts, called stylets, which mechanically and enzymatically penetrate cortical cell layers to tap into the sieve element. The high turgor pressure in the punctured sieve element allows sustained passive feeding from the phloem. The osmotic challenges presented by the ingested phloem sap are managed by gut sucrose-transglucosidases that transform excess sugar into long-chain oligosaccharides that is expelled as honeydew (Douglas, 2006). Another unique adaptation is the vertical transfer of symbiotic microorganisms within the gut tissues, providing the insects with essential amino acids that are nutritionally unavailable from the phloem sap diet (Baumann et al., 1997; Douglas, 2006).

Plants are well equipped to protect themselves from phloem feeders. The phloem sap not only provides food, but also has the ability to provide defense against these hemipteran pests (Hagel et al., 2011). The phloem tissue contains secondary metabolites and other defensive compounds that can deter phloem feeders and microbial pathogens. Glucosinolates are sulfur-rich compounds confined in the vacuole of specialized S-cells located in the periphery of phloem tissue of brassicas. During tissue damage, myrosinases, and thioglucosidases present in the M-cells of the phloem parenchyma mix with these glucosinolates to produce toxic isothiocyanates, nitriles, or thiocyanates (Hagel et al., 2011). However, phloem feeders most often evade these defenses by careful stylet insertion during feeding (Tjallingii and Hogen Esch, 1993). Structural phloem proteins also contribute to defense through physical interactions within sieve elements that possibly impact hemipteran feeding. This phenomenon has been best characterized in members of Fabaceae, where spindle-shaped forisomes regulate sieve element occlusion by expanding to spherical structures at sieve plates that occlude the sieve element (Knoblauch et al., 2001; Knoblauch and Peters, 2004; Tuteja et al., 2010). The reversible crystalline to amorphous structural change is determined by calcium flux within sieve elements. Perception of a stress signal activates calcium influx into the phloem sap, resulting in sieve element occlusion. Interestingly, it appears that aphids have salivary calcium chelators that could prevent forisome structural transitions by scavenging calcium within the phloem sap (Will et al., 2007). Emerging evidence suggests that proteases in aphid saliva degrade the very abundant phloem protein 1 (PP1), suppressing a putative phloem defense and providing an additional nitrogen source for the aphids (Furch et al., 2015).

Defense responses against phloem feeders are almost certainly not limited to vascular tissues. While stylet probing is primarily intercellular through the middle lamella of cortical cell walls, intracellular stylet penetration of cells of the cortical tissues is common. This is clearly illustrated by the large number of hemipteran-transmitted viruses that are not phloem-limited and unequivocally confirmed by countless EPG analyses. One weakness in understanding defenses against phloem-feeding insects at the molecular level has been an overall lack of high resolution localization data. Many studies have shown that hemipteran herbivory induces global transcriptional reprogramming in plant tissues that shifts primary metabolism to secondary metabolism and defense (Giordanengo et al., 2010). Defense pathways and related phytohormone-mediated responses are strongly induced in response to hemipteran feeding (Moran and Thompson, 2001; Smith and Boyko, 2007; Morkunas et al., 2011). During the last decade, studies have revealed that sRNAs serve as important modulators of plant stress responses in response to phloem-feeding insects (Greyling, 2012; Sattar et al., 2012b; Barah et al., 2013; Kettles et al., 2013; Xia et al., 2015) (**Table 1**). Important milestones in our understanding of sRNA function in basal immunity against hemipteran insects have been made in Arabidopsis; however, parallel investigations in non-model systems are revealing the role of sRNAs in host plant resistance. Both approaches are contributing to the future development of integrated pest management strategies.

Identifying sRNA Co-expression Networks and Biogenesis Pathway Components during Arabidopsis-Hemipteran Interactions

Comparative analyses of the transcriptional changes in Arabidopsis in response to the microbial pathogen *P. syringae* or cabbage aphid (*Brevicoryne brassicae*) revealed commonalities between the two biotic stress signals, as well

TABLE 1 | sRNA profiling studies in host plants in response to aphid infestations.

Host Plant[a]	Insect[b]	Interaction	Duration	Analysis	sRNAs Identified	References
Arabidopsis	Cabbage aphid	Susceptible	72 h	miRNA:mRNA co-expression network analysis	Not applicable	Barah et al., 2013
Arabidopsis	Green peach aphid	Susceptible	14 days	sRNA pathway mutant analysis	Not applicable	Kettles et al., 2013
Melon	Cotton-melon aphid	Resistant and susceptible	2–12 h	sRNA sequencing, qRT-PCR	23 conserved miRNA families, 5 novel miRNAs	Sattar et al., 2012a
Chrysanthemum	Chrysanthemum aphid	Resistant	0–48 h	sRNA sequencing	303 conserved miRNAs, 234 novel miRNAs	Xia et al., 2015
Wheat	Russian wheat aphid	Resistant	12–24 h	Subtractive sRNA cloning, qRT-PCR	86 putative miRNAs	Greyling, 2012

[a]*Melon (Cucumis melo); Chysanthemum (Chysanthemum morifolium); Wheat (Triticum aestivum).*
[b]*Cabbage aphid (Brevicoryne brassicae); Green peach aphid (Myzus persicae); Cotton-melon aphid (Aphis gossypii); Chysanthemum aphid (Macrosiphoniella sanbourni); Russian wheat aphid (Duiraphis noxia).*

as aphid-specific responses (Barah et al., 2013). Pathways regulating defense responses, signaling, and metabolic processes were common to both *P. syringae* and the cabbage aphid. Integration of the two data sets by *in silico* analysis of data generated through microarray studies with publicly available gene expression and miRNA datasets for Arabidopsis described a theoretical co-expression network of mRNAs and their cognate miRNAs. The aphid-response network consisted of 82 transcripts, including mRNAs encoding 42 transcription factors and 21 conserved targets for Arabidopsis miRNAs. Further analysis identified 17 miRNA families as regulators of differentially expressed transcripts in response to aphid infestations. Some of these miRNA target transcripts belonged to WRKY and bZIP transcription factor families that have well established functions in biotic stress, reflecting some level of conservation among the different stress responses. The co-expression network also revealed that aphid-specific transcripts were connected to more than one miRNA node, indicating that transcripts are under the regulation of more than one member of a miRNA family or multiple miRNAs belonging to different miRNA families. Additional network complexity was displayed when a single member of a miRNA family was shown to target two different transcripts. While informative, this *in silico* mRNA:miRNA network analysis lacked supporting experimental evidence for miRNA regulation during aphid infestation.

The availability of Arabidopsis mutants for sRNA and defense related pathways provided tools to assess the effects of sRNAs on green peach aphid (*Myzus persicae*) fecundity (Kettles et al., 2013). The reproduction of aphids feeding on *RDR* mutants (*rdr1*, *rdr2*, *rdr6*) did not show any differences between these and Col-0 control plants, indicating that interruption of the siRNA pathway had minimal effect on green peach aphid performance in Arabidopsis. Interestingly, *DCL* mutants had differential responses: *dcl1* mutants showed greater resistance toward aphids, but *dcl2*, *dcl3,* and *dcl4* had no effect on aphid fecundity. Double mutants for *dcl2/3* and *dcl2/4* and triple mutant *dcl2/3/4* also showed no significant change in aphid fecundity. *AGO* mutant *ago1-25* showed significantly reduced aphid fecundity; however, *ago2*, *ago4*, or *ago7* mutants did not impact aphid performance. Taken together, these data indicate that impaired miRNA processing by specific members of *DCL* and *AGO* multigene families negatively affects reproduction of green peach aphid. This was further confirmed by reduced aphid performance on *hen1*, *hst* (*hasty*), and *se (serrate)* mutants that also were defective in miRNA processing. Since all the miRNA-processing pathway mutants had a dwarf phenotype, an Arabidopsis line exhibiting a similar phenotype (PDLP1a:GFP overexpression line) was used as a control. It was confirmed that the reduced fecundity was not a result of dwarfism but due to the compromised miRNA processing.

Further analysis of the miRNA-processing mutants revealed that *PAD3* (a marker for camalexin biosynthesis) and *CYP81F2* (member of indolic glucosinolate pathway) (Pfalz et al., 2009) were highly induced at 12 h post aphid infestation in the *dcl1* mutants. HPLC and mass spectrometry analysis confirmed enhanced camalexin content in *dcl1* plants in response to aphid herbivory and it was shown that aphids raised on these mutants ingested camalexin during phloem feeding. Artificial diet assays supplemented with camalexin substantiated the negative impact of this metabolite on aphid fecundity; however, no toxicity was reported for adult aphids. Aphid fecundity assays on *pad3* and *cyp81f* mutants validated the role of camalexin in aphid performance. The impaired miRNA processing pathway also affected phytohormone-mediated defense signaling (Kettles et al., 2013). *LOX2* expression in *dcl1* mutants in response to aphid herbivory was enhanced, whereas, aphid fecundity on *coi1, jar1*, and *35S:LOX2* mutants, defective in JA signaling did not significantly differ from control plants. ET-responsive *HEL* transcript was also induced in response to aphid feeding in *dcl1* plants. Fecundity assays on ET-insensitive *etr-1* or *ein2-5* mutants revealed that aphid reproduction was greater on *ein2* mutant plants, whereas, aphid reproduction on *etr1* mutant plants was not significantly different from control plants. In contrast, previous studies have shown that aphid saliva-induced plant defenses in Arabidopsis did not involve EIN2 and ET signaling (De Vos and Jander, 2009). Thus, EIN2 appears to have some role in green peach aphid resistance that can be seen in either *dcl1* mutants or in the presence of the bacterial effector harpin protein (Liu et al., 2011; Kettles et al., 2013).

sRNA-Mediated Resistance Against Hemipteran Insect Pests in Non-model Host Plants

Changes in the miRNA profile in response to aphid herbivory have been reported in the ornamental species *Chysanthemum morifolium* showing resistance to chrysanthemum aphid (*Macrosiphoniella sanbourni*) infestations (Xia et al., 2015). Three sRNA libraries were generated from no treatment control plants, plants receiving mock punctures, and aphid-infested plants, respectively. Eighty miRNAs were differentially regulated when comparing the control and aphid-infested libraries; among these 39 miRNAs showed increased expression and 41 miRNAs were down-regulated during aphid herbivory. Comparisons between mock punctures (wounding) and aphid infestation libraries revealed 79 differentially regulated miRNAs, with 39 miRNAs up-regulated and 40 miRNAs down-regulated. Novel miRNAs were also identified from these libraries. Further analysis revealed 24 conserved miRNAs and 37 novel miRNAs were specific to aphid infestations, while of 52 conserved and 9 novel miRNAs were associated with mock puncture (wounding) treatment. In the absence of chrysanthemum genome, the transcriptome was used for *in silico* miRNA target prediction; however, several of the *in silico*-predicted targets could not be verified by experimental methods due to poor coverage of the transcriptome. Because of the lack of validated miRNA targets for chrysanthemum, specific roles for miRNAs in aphid-induced plant defense signaling in the resistant cultivar could not be further explored.

Resistance toward Russian wheat aphid (RWA, *Diuraphis noxia*) is due to the presence of *Dn* genes. Eleven *Dn* genes have been reported from cereals, including *Dn1-9*, *Dnx*, and *Dny* (Botha et al., 2005). The wheat cultivar TugelaDN contains the *Dn1 R*-gene that confers resistance against RWA biotype 1 (Jankielsohn, 2011). Matsioloko and Botha (2003) observed significant transcriptional changes in response to RWA infestation in the resistant TugelaDN wheat. Genes related to the defense response including receptor and signaling pathway were reported to be differentially regulated within 1–2 h of RWA feeding (Gill et al., 2004; Botha et al., 2005). Subtractive sRNA libraries were constructed from RWA-infested susceptible (Tugela) and resistant (TugelaDN) wheat leaf tissues collected at 12, 18, and 24 h post feeding. The *Dn*-resistance specific sRNAs included 86 putative miRNAs with targets predicted by *in silico* methods (Greyling, 2012). Q-PCR analysis for three selected miRNAs (TaDn-miR65, TaDn-miR15, and TaDn-miR104) showed enhanced expression of these miRNAs in the resistant cultivar in response to aphid feeding in time-course study. Putative targets were predicted for these miRNAs: *β-1, 3 glucanase,* and *cytochrome-P450* targeted by TaDn-miR15 and *WRKY13* and *MYB* targeted by TaDn-65. This demonstrated the potential role for TaDn-miRNAs in aphid resistance.

R gene-mediated resistance conferred by the *Vat* (virus aphid transmission) gene against cotton-melon aphids (*Aphis gossypii*) and cotton-melon aphid-transmitted viruses is well documented in melon (*Cucumis melo*) (Kennedy et al., 1978; Dogimont et al., 2014). Resistance to cotton-melon aphids is exhibited as antixenosis (non-preference), antibiosis (delayed growth and development and reduced reproduction), and host plant tolerance (Bohn et al., 1972). The melon miRNA expression profile was determined using sRNAseq combined with comparative analysis of miRNA expression patterns in response to aphid herbivory during resistant and susceptible interactions (Sattar et al., 2012b). Libraries generated from leaf tissues of Vat^+ aphid-resistant melon plants with and without aphids compared the sRNA expression at initial stages of the interaction to distinguish between the molecular cues that are associated with early (2, 4, and 6 h) and late (8, 10, and 12 h) stages that corresponded with pre- and post-sustained phloem ingestion, respectively (Klingler et al., 2001). In total, 23 families of conserved plant miRNAs were identified from the three libraries. Next generation sequence profiling, qPCR, and sRNA blot data revealed that members of 18 conserved miRNA families preferentially accumulated during the early stages of aphid herbivory in the resistant interaction. Twenty-two conserved miRNAs were down-regulated, whereas only one was up-regulated in the early response to aphid infestations. Eight miRNAs were up-regulated during the late stages of aphid herbivory in the Vat^- susceptible melon. Five miRNA families showed statistically significant down-regulation during early stages and two during the late stages of aphid infestation in the susceptible interaction. Overall, the resistant interaction showed enhanced miRNA expression, whereas the susceptible interaction showed down-regulation of miRNAs. The opposing trends in these nearly-isogenic lines could be due to differences in miRNA transcription or biogenesis. Eighteen cucurbit-specific miRNAs were also identified, five of which were melon-specific, while the remaining 13 sequences were identified from both melon and pumpkin. The expression profiles of all five novel melon-specific miRNAs in Vat^+ resistant melon line did not change significantly during early and late stages of aphid herbivory, but in the Vat^- susceptible line three were significantly down-regulated during early stages of aphid infestation.

Melon miRNA targets were empirically identified by degradome sequencing and further verified by 5′RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) (Sattar et al., 2012b, 2016). Degradome sequencing identified 70 miRNA: mRNA target pairs for the 23 conserved miRNA families that included 28 novel target pairs not found in other plant species. Interestingly, 11 miRNA target gene transcripts encode proteins with established roles in regulating phytohormone (auxin, JA, ET, ABA, and GA) biosynthesis and signaling pathways. A detailed analysis of the miRNA:mRNA interactome revealed six miRNA:mRNA target pairs that impact auxin perception and signal transduction. The auxin-miRNA interactome provided evidence for a series of redundant mechanisms resulting in auxin insensitivity that appears to be a component of *Vat*-mediated resistance (Sattar et al., 2016). Aphid feeding on Vat^+ resistant melon tissues results in miR393-mediated loss of *TIR-1* and *AFB2* auxin receptors. Loss of auxin receptors prevents the formation of $SCF^{receptor}$-ubiquitin ligase complex and degradation of AUX/IAA proteins via the complex. AUX/IAA proteins negatively regulate auxin signaling by inactivating a class of ARF that are transcriptional activators

of auxin-induced genes. Simultaneously, miR167 targets ARF activators (*ARF6* and *ARF8*) as a redundant mechanism contributing to auxin insensitivity in the resistant Vat^{+} tissue (Sattar et al., 2016). Reduced expression of auxin downstream signaling genes after 12 h of aphid infestation in resistant plants provides indirect evidence for the proposed auxin insensitivity model. Experimental evidence directly linking the inactivation of the auxin receptor with a reduction in aphid fecundity was obtained by treating susceptible melon leaf tissues with a chemical inhibitor (PEO-IAA) of the TIR-1 auxin receptor.

Additional components of the auxin-miRNA interactome in *Vat*-mediated resistance have conserved roles in auxin homeostasis. MicroRNA miR160 targets transcriptional repressor ARF17 that in turn regulates the expression of the gene encoding the *GH3* auxin-conjugating enzyme. MicroRNAs miR164 and miR319 are involved in auxin feedback loops through *NAC* and *TCP* transcription factor genes, respectively, and miR390 mediates miRNA cleavage that generate secondary tasiRNA that target *ARF2* and *ARF3*.

INSECT-DERIVED SRNAS AND THEIR ROLE IN HERBIVORY

The advent of new sequencing technologies has made it possible for sRNA profiling in hemipteran insect species that have either extensive or limited genomic information. Experimental and *in silico* sRNA profiling studies have been reported for the following phloem feeding insects: pea aphid (*Acyrthosiphon pisum*), cotton-melon aphid (*A. gossypii*), whitefly (*Bemisia tabaci*), brown planthopper (*Nilaparvata lugens*), small brown planthopper (*Laodelphax striatellus*), and white-backed planthopper (*Sogatella furcifera*) (**Table 2**). Small RNA profiling was reported from the xylem sap feeder glassy-winged sharpshooter (*Homalodisca vitripennis*) and both xylem and phloem feeders Asian citrus psyllid (*Diaphorina citri*) and large milkweed bug (*Oncopeltus fasciatus*) (**Table 2**). To date, sRNA studies in hemipteran species have primarily focused on identifying sRNA sequences and categorizing those sequences as miRNA, piRNAs, or virus-derived siRNAs (viRNAs). Other studies have identified sRNA biogenesis pathways and sRNAs that are specific to developmental stages, growth, reproduction, or insect immunity. These reports are beginning to provide evidence for sRNA regulation of important biological processes in hemipteran insects and an understanding of insect-host plant and vector-pathogen relationships.

Identification of sRNA Pathways in Hemipteran Insects

The pea aphid has become the model hemipteran species due to an international collaborative effort to obtain the fully sequenced and annotated genome, which has opened avenues for fundamental studies to be conducted in this species. MicroRNA sequences as well as genes involved in siRNA and miRNA biogenesis from pea aphid were initially predicted by *in silico* probing of the genome sequence (Jaubert-Possamai et al., 2010; Legeai et al., 2010; Kozomara and Griffiths-Jones, 2011). Phylogenetic analysis revealed duplicated miRNA biogenesis genes in the pea aphid (two *Ago-1*, two *Dcr-1*, and four *Pasha* gene copies) that retain their functionality (Jaubert-Possamai et al., 2010). These duplication events occurred at different time periods with the *Dcr-1* duplication being a recent event, while *Ago-1* occurred as an ancestral event in the subfamily Aphidinae. The *Ago-1* and *Dcr-1* duplicated genes were differentially expressed in four different reproductive morphs of the pea aphid (Ortiz-Rivas et al., 2012). Duplication events were also reported for genes from the pea aphid piRNA pathway (Lu et al., 2011).

Aphids have unusually high phenotypic plasticity and can switch from sexual to asexual reproduction (Miura et al., 2003), which presents a unique system to investigate the role of duplication events in the piRNA biogenesis pathway during asexual and sexual reproduction. Expression of the duplicated *Piwi* and *Ago* genes was tissue specific in certain reproductive morphs (Lu et al., 2011). During embryogenesis, *Api-Piwi2*, *Api-Piwi6*, and *Api-Ago-3a* were expressed in germ cells, whereas duplicated copies *Api-Piwi5*, *Api-Piwi3,* and *Api-Ago3b* were localized in somatic cells. Semi-qPCR detected differential expression for *Api-Piwi* and *Api-Ago3* genes in the different reproductive morphs. *Ago-3b* was most abundant in the sexuparae female morph, whereas *Ago-3a* was abundantly expressed in all of the female morphs. Both the *Ago-3* duplicates were expressed at very low levels in the sexual males, indicating *Ago-3* was not involved in male sexual reproduction. Expression studies of *Api-Piwi* genes in the different reproductive morphs revealed germ line-specific *Api-Piwi2* and somatic cell-specific *Api-Piwi3* were abundant in all the female reproductive morphs. Interestingly another somatic cell-specific *Api-Piwi5* was strongly expressed in the sexual males. *Api-Piwi6* was strongly expressed in the germline cells of the female oviparae. These data indicate additional functions for *Piwi* genes during both sexual and asexual phases of aphid reproduction.

Several genes belonging to the different sRNA pathways were identified from the soybean aphid (*Aphis glycines*) (Bansal and Michel, 2013). Single copies of *Dcr2, R2d2*, *Ago2,* and *Sid-1* were identified in soybean aphid. Expression analysis of the sRNA pathways genes at different developmental stages showed *Dcr2, R2d2*, and *Ago2* were highest during the first and second instar stage. However, *Sid-1* was uniformly expressed throughout all the developmental stages in the soybean aphid. Tissue-specific qPCR analysis detected the presence of *Dcr2, R2d2*, *Ago2*, and *Sid-1* in the epidermis, gut, and fat body of the insect. Because *Sid-1* is essential for systemic response of RNAi in both *Apis mellifera* and *Caenorhabditis elegans* (Winston et al., 2002; Aronstein et al., 2006), its presence throughout all the developmental stages opens up the possibility of designing effective RNAi-mediated control of the soybean aphid.

Small RNA pathways also have been evaluated and characterized in brown planthoppers (Zha et al., 2011; Xu et al., 2013). Brown planthopper *Sid-1* and *Aub* genes encoding proteins involved in the RNAi pathway were identified, as were members of the *Ago* and *Dcr* families (Zha et al., 2011). Genome and transcriptome sequence analyses revealed one *Drosha*, three *Dcr* genes, and one ortholog each of the RNA-binding protein *R2D2*, *Loquacious* (*Loqs*), and *Pasha* (Xu et al., 2013).

TABLE 2 | Hemipteran sRNAs identified.

Hemipteran pest[a]	Experimental design	Analysis	sRNAs identified	References
Whitefly	Comparative analysis of sRNA profile Q and B biotype raised on susceptible host cotton	sRNA sequencing	170 conserved miRNAs and 15 novel candidates	Guo et al., 2014
	miRNA profiles for viruliferous and nonviruliferous whiteflies on tomato	sRNA sequencing, qPCR	112 and 136 conserved miRNAs from nonviruliferous and viruliferous whiteflies	Wang et al., 2016
Glassy- winged sharpshooter	miRNA profiling of insects raised on basil, cotton and cowpea	sRNA sequencing	345 conserved and 14 novel miRNAs	Nandety et al., 2015
Pea aphid	*In silico* prediction of miRNAs from genome sequence	Solexa sequencing	149 miRNAs including 55 conserved and 94 new miRNAs	Legeai et al., 2010
	miRNA and siRNA pathway identification	Annotation of the miRNA and siRNA pathway genes and expression profiling of these genes	Not applicable	Jaubert-Possamai et al., 2010
	Evolutionary analysis of the miRNA machinery	Phylogenetic analysis of *ago-1* and *dcl-1*	Not applicable	Ortiz-Rivas et al., 2012
Cotton- melon aphid	Comparative analysis of insects feeding on susceptible and resistant melons	sRNA sequencing	81 conserved miRNAs, 12 aphid-specific miRNAs, 9 novel miRNA candidates	Sattar et al., 2012a
	Analysis of ESTs	*In silico*	16 potential miRNAs	Rebijith et al., 2014
Brown planthopper	Prediction of novel miRNA	*In silico*	9 novel miRNAs	Asokan et al., 2013
	Comparative analysis of sRNA from the insect developmental stages	sRNA sequencing	452, 430, and 381 conserved miRNAs from adult male, adult female and female nymph libraries	Chen et al., 2012
	Genome-wide screening for siRNA, miRNA pathway		Not applicable	Xu et al., 2013
	Analysis of fecundity-related miRNAs	Dual-luciferase assay, miRNA injection	38 potential miRNAs regulating 9 fecundity-related genes	Fu et al., 2015
	Identification of miRNAs regulating molting	sRNA sequencing, miRNA injections, qRT-PCR	miR-8-5p and miR-2a-3p regulate chitin synthesis	Chen et al., 2013
	Analysis of sRNA biogenesis gene *dcl-1*	Cloning and sequencing of *dcl1*, qRT-PCR of *dcl* in different tissues	Not applicable	Zhang et al., 2013
Small brown planthopper	RBSDV infection	sRNA seq	59 conserved miRNA, 20 novel miRNAs	Li et al., 2015
	HiPV-derived sRNAs	sRNAseq	Virus derived RNAs are 21–22 nt	Li J. et al., 2014
Asian citrus psyllid	Prediction of virulence-regulatory miRNAs and phylogenetic analysis of miRNA clades	*In silico*	10 major clades	Khalfallah et al., 2015
Large milk-weed bug	Prediction of miRNAs	*In silico*	96 candidate mature miRNAs	Ellango et al., 2016
White-backed plant hopper	Small RNA libraries from viruliferous and non-viruliferous insects	sRNA sequencing	106 conserved miRNAs, 276 novel miRNAs	Chang et al., 2016

[a]*Whitefly (Bemicia tabaci); Glassy-winged sharpshooter (Homalodisca vitripennis); Pea aphid (Acyrthosiphon pisum); Cotton-melon aphid (Aphis gossypii); Brown planthopper (Nilaparvata lugens); Small brown planthopper (Laodelphax striatellus); Asian citrus psyllid (Diaphorina citri); Large milkweed bug (Oncopeltus fasciatus); White-backed planthopper (Sogatella furcifera).*

Three members of the *Ago* family (*Ago-1*, *Ago-2*, and *Ago-3*), were also identified, indicating the presence of siRNA, miRNA, and piRNA pathways in the brown planthopper (Xu et al., 2013). The brown planthopper sRNA pathway genes were cloned, sequenced, and their functionality confirmed by gene knockdown assays using dsRNA microinjections. The brown planthopper nymphs with *Sid-1* knockdown lost systemic RNAi for other targets, confirming the conserved role for *Sid-1* in this insect. Third-instar brown planthopper nymphs with silenced *Dcr-1* and *Ago-1* showed lethal defects, and the few that survived could not complete metamorphosis nor were able to stretch their wings (Xu et al., 2013). These experiments suggest that

miRNA pathways impact insect development and ecdysis. Zhang et al. (2013) observed a similar effect for *Dcr-1* down-regulation in brown planthopper adult females. Microinjection of *Dcr1* into adult females caused significant loss of *Dcr-1* transcripts in both whole body and ovaries. Furthermore, the oocytes of the adults with *Dcr-1* knockdown were poorly developed with abnormal follicular development. As a result the number of eggs produced by *Dcr-1*-silenced brown planthopper females where significantly less than those in the control group. Also, the expression of several ubiquitously found conserved miRNAs (*bantam, miR-7, miR-8,* and *miR-9*) decreased significantly in ds*Dcr1*-treated brown planthopper adult females 3 days following microinjection.

Zhou et al. (2016) demonstrated the differential expression of *Ago-1* and *Ago-2* in small brown planthoppers under different stress conditions. Although both *Ago* genes are expressed during all developmental stages of the insect, reduced expression of both *Ago-1* and *Ago-2* was reported in second-instar small brown planthopper nymphs in response to rice black-streaked dwarf virus. Both high and low temperature extremes negatively affected *Ago-1* expression; however, *Ago-2* expression was markedly reduced only in response to low temperature stress. Changing host plants initially caused reduced expression of both the *Ago* genes, but the expression of *Ago* genes recovered to their normal state after a 7-day period on the new host, indicating that *Ago* genes have important roles host specificity as well as stress responses. Other important genes from the RNAi pathway such as *Eri-1* and *Sid-1* were also identified from the small brown planthoppers.

sRNAs Regulating Insect Development, Growth, and Reproduction

Hemipterans are paurometabolous insects with three life stages (egg, nymph, and adult) that undergo gradual metamorphosis (Bybee et al., 2015). For example, aphid nymphs molt 6–8 times and then metamorphose into an adult. Reproduction in hemipterans can be sexual or asexual. Some hemipterans, such as aphids, are economically important agricultural pests with prolific reproductive ability. When favorable conditions exist, aphids reproduce asexually, giving birth to live females rather than laying eggs. As days shorten and become cooler, aphids produce winged males and females that can mate and reproduce sexually to overwinter as eggs on perennial host plants (Ogawa and Miura, 2014). Female aphids begin reproducing parthenogenetically 7–10 days after birth. The reduced pre-reproductive period is possible because of "telescoping of generations" where aphids complete much of their development, including their reproductive system before they are born (Dixon, 1998). Aphid growth and development are reliable indicators of insect performance on host plants because they correlate with fecundity and are directly impacted by environmental factors (Awmack and Leather, 2007).

Insect growth, development, reproductive potential, and interactions with plant hosts can be influenced by sRNAs (Asgari, 2013; Lucas and Raikhel, 2013). In Drosophila, miRNAs have been identified as regulators of reproductive biology, including differentiation and maintenance of germlines within the ovaries (Park et al., 2007). Genome-wide association studies have identified several Drosophila miRNAs as well as epigenetic modifications associated with sexual reproduction and ageing (Zhou G. et al., 2014; Zhou S. et al., 2014). The potential role sRNAs on pea aphid reproduction and life cycle was first suggested by Ortiz-Rivas et al. (2012) when they reported differential expression of *Ago-1* and *Dcr-1* genes in the asexual and sexual reproductive morphs. As the aphid lifecycle transitions from asexual to sexual reproduction, the sexupara females parthenogenetically produce sexual morphs and the females carrying eggs mate with the male. PCR-based expression assays confirmed *Ago-1a* and *Dcr-1b* overexpression in sexupara females. The *Ago-1a* was down-regulated in sexual female morphs, whereas *Ago-1b* was down-regulated in asexual females reproducing parthenogenetically, and *Dcr-1b* was not expressed in the sexual males. These observations indicate specific functions for the duplicated gene copies of *Ago-1* and *Dcr-1* during the reproductive transition in pea aphid (Ortiz-Rivas et al., 2012).

Differential expression of sRNAs across different reproductive morphs was also observed in other hemipteran insects. Comparative analyses of the sRNA libraries from different developmental stages of the brown planthopper were conducted to identify sRNAs associated with insect growth and development (Chen et al., 2012). A bimodal distribution pattern of sRNAs were observed for the three libraries: 21–22 nt sRNAs were predominant in adult males; 26–27 nt sRNAs were abundant in adult females; and an almost equal distribution of 22-nt and 28-nt sRNAs in the last instar of female nymphs. Analysis of a subset of the conserved miRNAs revealed that miR30d was specific to female adults and nymphs, whereas miR-144* and miR-20d were exclusively expressed in female nymphs. Certain miRNAs (miR-1, miR-184, miR-278, and miR-34) were highly expressed in adult males. The conserved miRNAs bantam and miR-10 were ubiquitously present in all three reproductive morphs. Novel miRNAs identified from brown planthoppers also showed differential expression within the reproduction morphs. MicroRNA bph-m0032 was exclusively expressed in female adults, whereas bph-m0045 was only found in female nymphs, and two novel miRNAs bph-m0057 and bph-m0041 were found in both male and female adults.

Additional studies of sRNAs in the brown planthopper identified two conserved miRNAs miR-8-5p and miR-2a-3p that modulate the chitin biosynthetic pathway membrane-bound trehalase (*Tre-2*) and phosphoacetylglucosamine mutase (*PAGM*), respectively (Chen et al., 2013). Both miR-8-5p and miR-2a-3p were highly expressed in nymphs and both female and male adults. During molting, miR-8-5p and miR-2a-3p and their respective target genes *Tre-2* and *PAGM* showed anti-correlated expression patterns with the enhanced expression of both miRNAs and down-regulation of the respective targets on the last day of 3rd, 4th, and 5th instars. The differential expression of miR-8-5p and miR-2a-3p and their respective targets between the first day of a new instar and last day of previous instar suggests a strong correlation to changes induced by the steroid hormone 20-hydroxyecdysone (20E) during the molting process.

Co-transfection of miR-8-5p and miR-2a-3p along with the respective targets fused to a luciferase reporter gene in the human embryonic kidney cell line HEK293T and Drosophila derived S2 cell lines showed decreased expression in dual luciferase assays. Microinjection experiments with synthetic dsRNA copies of endogenous miRNAs (miRNA mimic) in the 5th instar confirmed the dual luciferase assay results and showed reduced expression of the target proteins. Nymphs feeding on an artificial diet containing the miR-8-5p mimic experienced starvation-related mortality, while those fed a diet containing the miR-2a-3p mimic showed severe molting defects. Diet assays with miRNA inhibitors had no adverse effect on brown planthopper nymphs. Chitin content in these nymphs was significantly reduced in those fed with miRNA mimics, whereas the nymphs from the inhibitor assay had enhanced chitin content as compared to the control group. Furthermore, experimental evidence showed that both miR-8-5p and miR-2a-3p were negatively regulated by ecdysone-inducible gene *BR-C* by 20E signaling during brown planthopper molting. This study directly links miRNAs to chitin biosynthesis during insect development that is regulated by the steroid hormone 20E.

Insect fecundity is an important trait to predict population growth rates on host plants and forecast their performance under field conditions (Awmack and Leather, 2007). Fecundity also serves as a reliable measure of the plant host-insect interaction and is especially valuable when screening plant genotypes for resistance. Reduced fecundity is a hallmark of *Vat*-mediated resistance in melon to the cotton-melon aphid (Klingler et al., 1998). In addition to reduced fecundity, aphids on resistant plants have an extended pre-reproductive period and shortened reproductive and post-reproductive periods resulting in fewer progeny. The overall life span of an individual aphid is reduced and after the final molt, aphids feeding on resistant plants are smaller in size than those feeding on the susceptible melon plants (Kennedy and Kishaba, 1977; Klingler et al., 1998). Comparative analysis of sRNA libraries from aphids feeding on Vat^{+} and Vat^{-} plants for 48 h showed a differential bimodal size distribution pattern for sRNAs in the two libraries with the Vat^{+} library over-represented by longer 26–27 nt sequences (Sattar et al., 2012a). Approximately half of these longer sRNA sequences mapped to transposable elements. In insects, a vast majority of the sRNA sequences that arise from the transposable elements are endogenous piRNAs involved in maintaining genome integrity (Biryukova and Ye, 2015). A search of *Buchnera aphidicola* homology revealed 4.6% of the 26–27 nt sequences in the Vat^{+} library were of bacterial origin. Although there is no direct experimental evidence implicating the role of endosymbiont-derived sRNAs in aphid reproduction during Vat^{+} interactions, previous studies in other aphid species have confirmed that the endosymbiont *B. aphidicola* is required for successful reproduction (Srivastava and Auclair, 1976; Douglas, 1992; Dunbar et al., 2007; Shigenobu and Wilson, 2011). A detailed discussion of endosymbiont-derived sRNAs by Hansen and coworkers is presented in this focus issue. In addition to the longer sequences, a total of 81 miRNAs belonging to 56 miRNA families were identified from cotton-melon aphid libraries (Sattar et al., 2012a). While putative target genes have been predicted by *in silico* methods the role that these miRNAs play in aphid reproduction and their relationship to host plant resistance remains to be determined.

Reduced fecundity was observed for soybean aphids, feeding on bean pod mottle virus (BPMV)-infected host plants (Cassone et al., 2014). BPMV is not vectored by soybean aphids, yet the presence of the virus showed a negative impact on aphid fecundity. Although RNAseq analysis of the aphids did not reveal the presence of transcripts associated with viral immunity, sRNA biogenesis genes belonging to the siRNA, miRNA, and piRNA biogenesis pathways were down-regulated in aphids feeding on BPMV-infected host plants, indicating a defense response. However, viral replication for BPMV was not observed in the soybean aphid and Cassone et al. (2014) speculate that the loss of fecundity may be a result of aphids investing more in "survival rather than reproduction" due to limited resources available in virus-infected plants.

MicroRNAs regulating fecundity were identified in adult brown planthoppers (Fu et al., 2015). MicroRNA-binding regions in the 3′-UTR of fecundity-associated genes detected *in silico* led to the identification of 38 miRNAs targeting nine fecundity genes. Among these 38 putative miRNAs, miR-4868b showed perfect complementarity to the 3′UTR region of the *glutamine synthetase* (*GS*) gene. The miR-4868b:*GS* target pair was confirmed using the dual-luciferase assay reporter assay for the *GS* target in S2 cell lines. Microinjecting newly emerged adult female planthoppers with the miR-4868b mimic reduced GS protein levels within 48 h; however, the accumulation of GS mRNA did not change, indicating miR-4868b regulated the expression of GS protein by translational repression. GS protein also accumulated following treatment with a miR-4868b binding inhibitor. The number of offspring in the miR-4868b-mimic treatment decreased by 32% compared with the control group, illustrating the effect of reduced GS protein on fecundity. Ovaries isolated from adult females 2 days after the miR-4868b mimic treatment showed delayed development, fewer ovarioles, and fewer developed eggs per ovary. Earlier studies using RNAi-mediated knockdown of GS protein in brown planthopper have resulted in severe defects in ovary development and egg deposition (Zhai et al., 2013). Taken together they confirm miR-4868b plays a role in brown planthopper reproduction via regulation of GS. Vitellogenin (*Vg*) was also reduced by the miR-4868b mimic treatment. However, negative effects of microinjecting the miR-4868b mimic on *Vg* expression and ovarian development were transient with no significant differences between the treatment and control groups 6–7 days post-microinjection. The link between *GS* and *Vg* in brown planthopper reproduction is not fully understood, but may be through the glutamine-activated TOR signaling pathway. Several studies have shown TOR signaling pathway plays a role in insect fecundity by regulating *Vg* accumulation and ovary development (Patel et al., 2007; Zhai et al., 2015).

Hemipteran sRNAs in Response to Virus Infection

Hemipteran insects, especially members of the Aphididae, are common vectors of plant viruses and play significant roles in

viral epidemiology. Viruses transmitted by aphids outnumber those transmitted by whiteflies, leafhoppers, and planthoppers combined (Nault, 1997). The majority of aphids transmit "stylet borne" viruses in a non-persistent manner, where a very brief stylet penetration of less than a minute is required for viral acquisition and inoculation of the host plant (Katis et al., 2006). Some aphids, however, transmit viruses in a semi-persistent manner where longer periods are required for acquisition and inoculation of viral particles. Persistent transmission requires a latent period between viral acquisition and viral inoculation allowing the virus to propagate or only circulate within the aphid during the course of its lifetime (Katis et al., 2006).

Antiviral immunity in both plants and insects is mediated by RNA interference (RNAi) (Ding and Vionnet, 2007; Obbard et al., 2009). Virus-derived siRNAs accumulate during viral infection in plants and insects cleaving the viral dsRNA into short fragments causing silencing of the viral genes in a systemic manner (Ding and Vionnet, 2007; Wieczorek and Obrępalska-Stęplowska, 2015). Concurrently, viruses evolved a counter mechanism for viRNA-mediated silencing by producing viral suppressors of silencing (VSR). VSR proteins interfere with RNA silencing by specifically targeting components of the RNA-silencing pathway (Ding, 2010). Members of the RNA silencing (*Dcr-2* and *R2D2*) and piRNA biogenesis pathways have been implicated in insect viral immunity (Zambon et al., 2006; Vodovar and Saleh, 2012). Long viral-derived sRNAs similar to endogenous piRNAs have been reported upon viral infection in Drosophila ovarian somatic sheet cells, although it could not be confirmed if they originated from the piRNA biogenesis pathway (Wu et al., 2010). Understanding the role hemipteran sRNAs play in viral immunity could enable new approaches in preventing the systemic spread of plant viruses.

Researchers have investigated sRNA pathways in several hemipteran species as a response to virus acquisition and infection in host plants (Li et al., 2013a; Sekhar Nandety et al., 2013; Li J. et al., 2014; Li et al., 2015; Chang et al., 2016; Wang et al., 2016). Comparing sRNA sequences from small brown planthoppers infected with rice black-streaked dwarf virus (RBSDV) and rice stripe virus (RSV) revealed the greatest accumulation of viRNAs during RBSDV infection (Li et al., 2013a). RBSDV induced viRNAs were predominantly 21–22 nt in length originating in equal proportions from the sense and antisense strands. Hotspots for viRNA initiation were restricted to the 5′ or 3′ terminal regions of viral genome. Double infection of RBSDV and RSV induced more viRNA from the RBSDV RNA segments. In addition to the RBSDV- and RSV-derived virRNAs, Himetobi P virus (HiPV)-derived viRNAs were identified in the sRNA libraries (Li J. et al., 2014). Subsequently, HiPV infection was confirmed in the insect host. Analysis of all virus-infected and uninfected samples revealed greater accumulation of HiPV-derived RNA in the RSV library than in the RBSD or the double-infection library, suggesting that HiPV abundance is determined by the RSV infection. Although viral infection in insects typically produces *dcr-2* derived 21–23 nt viRNAs, HiPV-derived viRNAs showed a wide range of size distribution from 18 to 30 nt. Majority of the 21–22 nt viRNAs were generated from the antisense strand, whereas the longer viRNAs came from the sense strand. While initially thought to be piRNAs, these long sequences lacked the characteristic piRNA peak at 27–28 nt and uracil bias at the 5′-terminal end. The authors concluded that these long RNAs were likely derived from the sense strand from the viral genome by an unknown sRNA biogenesis pathway.

Differentially expressed miRNAs in response to the virus infection were identified in RBSDV-infected small brown planthoppers (Li et al., 2015). Nine up-regulated and 12 down-regulated conserved miRNAs were identified from the RBSD-infected library. Several miRNAs (miR-2765-5p, miR-87-3p, and miR-1-3p) were induced, while others were repressed (miR-750-3p, miR-727-5p, miR-124-3p, and miR-133-3p) in the insect host. Twenty novel miRNA candidates were also identified in this interaction. Target identification for these miRNAs was hampered by the lack of small brown planthopper genome sequence data. In the future, validated miRNA targets will provide a better understanding of the physiological significance of miRNAs in RBSD infection of small brown planthoppers.

Analysis of sRNA libraries prepared from white-backed planthoppers (*S. furcifera*) infected with southern rice black-streaked dwarf virus (SRBSDV) identified eight up-regulated miRNAs and four down-regulated miRNAs, among which two, miR-14 and miR-2798, are conserved miRNAs and the remaining 10 are unique to the insect (Chang et al., 2016). MicroRNAs miR-14 and the novel miR-n98a target genes involved in viral immunity. The highly expressed miR-14 targets transcripts encoding the patched (Ptc) protein, a positive regulator of hedgehog signaling. The hedgehog signaling pathway has been implicated in host interactions with dengue virus in *Aedes aegypti* (Chauhan et al., 2012). SFU-20.387 mRNA, encoding a Rab-5 interacting protein with a well-established role in Hepatitis C virus genome replication in mammals is the putative target for *S. furcifera* miR-n98a (Stone et al., 2007). Based on these observations, it was speculated that miR-14 and miR-n98a are involved in SRBSDV virus infection and immunity (Chang et al., 2016).

Homologs of sRNA biogenesis genes *ago-1* and *dcr-1* have been identified from whiteflies infected with begomovirus (Wang et al., 2016). sRNA profiling from viruliferous and non-viruliferous whiteflies carrying tomato yellow leaf curl China virus (TYLCCNV) showed an abundance of larger 29–30 nt sRNAs in the non-viruliferous library, whereas the viruliferous library was enriched in smaller 21–22 nt sRNA sequences. The whitefly miRNA profile was also analyzed in response to virus infection. Among the 52 miRNAs that were differentially expressed in the nonviruliferous and viruliferous libraries, 26 were specific to the viruliferous library. The expression of these miRNAs was confirmed by qPCR: miR-bantam, miR-1, miR-2b, and miR-124 were significantly up-regulated and miR-307, miR-317, and miR-993a were down-regulated in the viruliferous library. In addition to conserved miRNAs, seven novel miRNAs were identified from both the libraries. *In silico* predicted target genes of the differentially expressed miRNAs primarily belonged to three main GO categories: biological processes, cellular processes, and molecular function.

The glassy-winged sharpshooter is a xylem-feeding leafhopper that is an important pest on a wide range of plants including

citrus, grapes, and almonds and vectors *Xylella fastidiosa*, the causal agent of Pierce's disease of grapevines and citrus-variegated chlorosis disease. Sekhar Nandety et al. (2013) identified viRNAs in glassy- winged sharp shooters infected with either *Homalodisca coagulata virus-1* (HoCV-1) or *H. vitripennis reovirus* (HoVRV) and mapped the viRNAs to the viral genomes. Most of the viRNA sequences for HoCV-1 were derived from the positive strand, whereas HoVRV sequences were evenly distributed across the genome. In contrast to HoCV-1 viRNAs, several hotspots were identified for HoVRV on both 5′ and 3′ ends of the viral segments. The distinct mapping patterns for viRNAs from two taxonomically different viruses in the same insect vector raises the possibility of unique anti-viral immunity targets for each virus.

The combined effect of two taxonomically different viruses on viral immunity in an insect host was documented in soybean aphid (Vijayendran, 2014). A novel viral pathogen, *A. glycines virus* (AGV) was identified from the transcriptome sequencing of the soybean aphid. AGV infection was ubiquitously present in several clonal populations of soybean aphids collected from different geographical locations. The enhanced transfer rate of AGV to different insect hosts is possibly due to its ability to evade the RNAi-mediated anti-viral host defense. AGV is susceptible to RNAi-mediated anti-viral immunity in the host, but only in the presence of another viral pathogen *Aphid lethal paralysis virus* (ALPV). This was clearly demonstrated by a reduction of viRNAs produced from the AGV genome as compared during AGV infection alone. In contrast, a large number of viRNAs were produced in response to the double infection by AGV and ALPV, and the majority of these viRNAs were mapped to the ALPV genome.

sRNAS AS A TOOL IN AGRICULTURE FOR HEMIPTERAN PEST CONTROL

Insects and the microbial pathogens they vector are major causes of economic losses in production agriculture. Developing species-specific and environmentally benign approaches are important considerations when designing pest management strategies. RNA interference (RNAi) technology appears to be a promising candidate for such an approach. During RNAi, dsRNA is cleaved by Dicer to generate 21–24 nt siRNAs. The siRNAs separate into guide and passenger strands; the guide strand is introduced into the RISC and the passenger strand is degraded (Agrawal et al., 2003; Meister, 2013). The discovery of RNAi machinery in economically important hemipteran pests, including pea aphids, soybean aphids, whiteflies, brown planthoppers, and small brown planthoppers provides a robust rationale to pursue RNAi-based pest management strategies for hemipterans (Jaubert-Possamai et al., 2010; Ortiz-Rivas et al., 2012; Bansal and Michel, 2013; Xu et al., 2013; Wang et al., 2016; Zhou et al., 2016). RNAi protocols for hemipterans typically introduce dsRNA into the insect by one of several experimental methods: microinjection where dsRNAs are directly injected into the body of the insect; feeding dsRNAs in artificial diets or *in planta*; direct topical application by spraying or soaking insects in dsRNA solutions; or incorporating dsRNAs into nanoparticles (Scott et al., 2013). The mode of introducing dsRNA into the insect and the tissue in which the target gene is expressed are important criteria to obtain successful gene silencing in hemipteran insects.

Direct Delivery of dsRNA or siRNA via Injections in Hemiptera

Microinjection has been successfully used to deliver RNAi in several insect species belonging to lepidoptera, coleoptera, diptera, as well as hemiptera (Yu et al., 2013). RNAi-mediated silencing of *Hox, wg*, and *decapentaplegic (dpp)* in large milkweed bug (*O. fasciatus*) (Angelini and Kaufman, 2005) and salivary gland gene *Coo2*, gut-specific *cath-L* genes, and *calreticulin* in pea aphid (Mutti et al., 2006; Jaubert-Possamai et al., 2007) employed microinjection to deliver the dsRNA. Microinjecting brown planthoppers with dsRNA against *calreticulin, cathepsin-B,* and nicotinic acetylcholine receptors (nAChRs) β2 subunit *Nlβ2* resulted in ∼50% silencing effect; however, high insect mortality is often reported especially in smaller insects as a result of wounding during microinjection (Liu et al., 2010; Li et al., 2013b).

Oral Delivery of dsRNA in Hemiptera

Oral delivery through diet is a less invasive method for introducing dsRNA into hemipteran insects (Scott et al., 2013). Unlike microinjection, oral delivery of dsRNA through feeding sachets does not result in wounding-induced mortality and can be a useful tool when working with smaller insects. However, it is difficult to quantitate the dsRNA dose ingested by the insects to produce the silencing effect and thus, higher dosages are often required for oral delivery. In the absence of systemic RNAi machinery, the success of oral delivery may be limited to gut-specific target genes. Pea aphids feeding on an artificial diet supplemented with dsRNA against *aquaporin* showed 50% silencing of aquaporin transcript (Shakesby et al., 2009), whereas lethal effects were obtained in response to orally administered dsRNAs against gut *vATPase* (Whyard et al., 2009). Such effects could be species specific, as in the brown planthopper where orally-delivered *vATPase* dsRNA resulted in only ∼50% silencing of vATPase subunit E (Li et al., 2011), whereas dsRNA against *trehalose phosphate synthase (TPS)* showed a marked reduction in *TPS* activity in the fat body, ovary, and midgut (Chen et al., 2010). Diet-delivered dsRNA-mediated silencing of *sugar transporter gene 6 (Nlst6)* showed reduced *Nlst6* expression in the midgut with a negative effect on brown planthopper growth and fecundity (Ge et al., 2015). In whitefly, diet-delivered dsRNA against *glutathione S-transferase (GST)* showed significant decreases in mRNA levels that correlated with mortality in the insects (Asokan et al., 2015).

Chaitanya et al. (2016) studied effect of gene silencing using the sachet diet method to deliver dsRNA to cotton-melon aphid. Aphids fed on sachet diets containing dsRNA specific to sodium channel (*AgSCN*) or ultraspiracle genes (*AgUSP*) resulted in high levels of mortality that corresponded to decreased transcript levels for both genes. Oral delivery of dsRNA to silence cotton-melon aphid *juvenile hormone binding*

protein (JHBP) and *vacuolar ATPase subunit H (V-ATPase-H)* showed a 10–73% reduction in mRNA for both JHBP and *V-ATPase-H* and mortality in the range of 10–63% for both treatments (Rebijith et al., 2016). Comparative analysis of microinjection and oral delivery of dsRNA targeting the *cathepsin-L* gene in pea aphid demonstrated efficacy for each method that was tissue or organ specific (Sapountzis et al., 2014). Microinjection was most successful for gene knockdown in the head and carcass that induced altered morphology. In contrast, diet delivery showed enhanced silencing effect in the gut and gut-specific epithelial cells, possibly due to weak systemic spread of the RNAi signal. A similar study in potato/tomato psyllid (*Bactericera cockerelli*) compared the efficacy of microinjection and oral feeding (Wuriyanghan et al., 2011). Double-stranded RNAs introduced into the psyllids were experimentally shown to be processed into 21-nt siRNAs. Although microinjections were effective for dsRNA-mediated silencing for *actin*, mortality rates due to wounding were also higher. Sachet feeding of dsRNA or siRNA targeting *actin, ATPase*, *hsp70,* and *CLIC* showed tissue-specific gene knockdown of actin in the gut tissue, whereas silencing of the other genes was achieved in the whole insect (Wuriyanghan et al., 2011). The gut-specific knockdown of actin could be due to the lack of a systemic RNAi response for potato/tomato psyllid *actin* transcript.

In Planta Delivery of dsRNA

Expressing dsRNA within the host plant, either transiently or through stable integration, allows the effects of specific gene targeting on insect performance to be evaluated in the most relevant environment. The effect of *Rack1* and *Coo2* gene silencing on green peach aphid performance and fecundity was evaluated in *Nicotiana benthamiana* and Arabidopsis plant tissues (Pitino et al., 2011). Aphid gut-specific *Rack1* and salivary gland-specific *Coo2* transcripts were down-regulated in aphids feeding on *N. benthmiana* leaves transiently expressing ds*Rack1* and ds*Coo2*. Transient expression experiments reduced aphid fecundity by 25%, whereas, ds*Rack1* and ds*Coo2* transgenic plants showed a 50–60% decrease in mRNA levels with a 20% reduction in aphid fecundity. Neither method negatively affected aphid survival. These results deviated from the earlier microinjection studies in pea aphid where ds*Coo2* was lethal (Mutti et al., 2006).

Guo et al. (2014) compared two distinct approaches to gene silencing by developing *N. benthamiana* transgenic lines carrying intron-spliced hairpin RNA (hpRNA)-expressing plant vectors for *acetylcholinesterase 2 (MpAChE2)*, *vATPase,* and *tubulin folding cofactor D (TBCD)* or artificial miRNAs (amiRNAs) targeting two different sites in the Mp-*AChE2*. Transgenic tobacco plants expressing Mp-*vATPase* and Mp-*TBCD* hpRNAs showed enhanced resistance toward green peach aphids with ∼30% reduction in fecundity. Aphids feeding on transgenic plants expressing Mp-*AChE2* amiRs showed significantly more silencing of Mp-*AChE2* as compared to those feeding on hpRNA-expressing plant vectors for Mp-*AChE2.* Also the transgenics expressing Mp-*AChE2* amiRs showed improved insect resistance. The improved efficacy of Mp-*AChE2* amiRs over the hpRNA, could be due to the stability and the specificity of the amiRNAs compared to hpRNAs, which could be a better strategy for implementing RNAi *in planta*.

RNAi silencing of three gut-specific brown planthopper genes, hexose transporter gene *NlHT1*, carboxypeptidase gene *Nlcar*, and the trypsin-like serine protease gene *Nltry* in transgenic rice plants expressing dsRNA constructs failed to generate phenotypic changes in the insect (Zha et al., 2011). Third instar brown planthopper nymphs feeding on transgenic rice plants reduced the *NlHT1* and *Nlcar* transcript levels by about half in the midgut. However, such a significant reduction in the expression of target mRNA did not induce lethal phenotype, possibly due to either multiple copies of the target gene or limited changes at the protein level. In contrast, RNAi silencing of the abnormal wing disc (*Awd*) gene in Asian citrus psyllid had phenotypic effects (Hajeri et al., 2014). Pysillds feeding on citrus trees infected with recombinant Citrus tristeza virus (CTV) expressing *Awd*-silencing constructs had malformed wings and increased adult mortality. Gene expression analysis detected significant reduction in *Awd* transcripts in psyllids feeding on CTV-Awd infected citrus plants. The successful application of RNAi for Asian citrus psyllid control could significantly impact Huanglongbing (HLB) disease caused by the psyllid-vectored bacterial pathogen *Candidatus Liberibacter asiaticus* (CLas) (Hajeri et al., 2014).

Hemipteran insects readily develop resistance to pesticides, which could be overcome by targeting pesticide resistance genes using RNAi. The *carboxylesterase (CbE E4)* gene in grain aphids (*Sitobian avenae*) is responsible for developing resistance to a wide range of chemical pesticides that are routinely applied in agricultural fields (Xu et al., 2014). Grain aphids feeding on stable transgenic wheat plants expressing *CbE E4* dsRNA showed a 30–60% decrease in the *CbE E4* mRNA levels and reduced aphid numbers. Decreasing *CbE E4* gene expression could delay the development of resistance in this insect pest extending the utility of chemical management tools.

A novel method for *in planta* delivery of RNAi was tested for whiteflies by Luan et al. (2013). In separate experiments, uptake of dsRNA through the cut end of a tomato leaflet was accomplished by dipping petioles into solutions containing dsRNAs targeting whitefly genes *Cyp315a1* and *Cyp18a1*, involved in ecdysone 20E synthesis and degradation, respectively, or ecdysone response genes *EcR* and *E75*. In each of these treatments, silencing of these genes did not impact the survival and fecundity of the adult whiteflies. The exception was *EcR*-silenced adults, which laid fewer eggs. In all treatments, nymphs showed delayed development and poor survival rates (Luan et al., 2013).

Proof of principle for RNAi application in hemipteran insect control is demonstrated in these studies. However, successful deployment of RNAi technologies depends on the mode of delivery, effective dose, and target gene selection. *In planta* and spray delivery RNAi has potential for field applications, whereas microinjections and artificial diets are primarily limited to laboratory studies. As the cost of production of RNAi products become more economical, sprays, direct delivery of dsRNAs through plant cuttings or rooted seedlings, injecting trees and

drip irrigation becomes more feasible (Hunter et al., 2012; Luan et al., 2013; Camargo et al., 2015).

CROSS-KINGDOM TRANSFER OF sRNAS

The ability to target insect genes by expressing dsRNAs in host plants provides compelling evidence for the cross-kingdom transfer of sRNAs; however, the role of endogenous plant-derived sRNAs directly impacting hemipteran insects has not been demonstrated. Phloem sap contains mobile sRNAs that are likely consumed by phloem-feeding hemipterans. Indeed, conserved plant miRNAs have been identified in phloem sap isolated by aphid stylectomy (Varkonyi-Gasic et al., 2010) and detected in aphid sRNA libraries (Sattar et al., 2012a). Direct evidence that sRNAs are readily consumed during normal feeding was demonstrated by aphids feeding on an artificial diet containing radio-labeled 24 nt dsRNA, which was detected in whole aphid tissues and in the honeydew excretia (Sattar et al., 2012a). However, the functional consequences for these dietary derived plant-sRNAs on the insect herbivore remains to be clarified (Cottrill and Chan, 2014; Witwer and Hirschi, 2014).

CONCLUDING REMARKS

Recent studies have recognized that sRNAs are important regulatory components of plant-hemipteran interactions. Within host plants, transcriptional changes in response to this unique form of insect herbivory are beginning to be correlated with concurrent changes in sRNA profiles. Co-expression networks and mRNA:sRNA interactomes are being assembled that are providing additional and sometimes unexpected information on the regulation of plant responses to insect herbivory. It is becoming increasingly clear that sRNAs are responsible for fine-tuning responses in a wide variety of plant-hemipteran interactions; however, unifying concepts for sRNA-mediated regulation across systems have yet to fully emerge. Understanding specific roles of sRNAs in host plant resistance along with advanced knowledge about the different components of the sRNA biosynthesis pathways can inform new pest control strategies for agricultural applications. Insects have co-evolved strategies to suppress plant immunity. Understanding these strategies, along with the contribution of insect sRNAs in regulating insect fitness and fecundity, provides additional insights that could allow sRNAs to be utilized in pest control. Insect anti-viral viRNAs that offer immunity against viral pathogens provide a new paradigm in understanding the complex plant-insect-virus interactions. The accumulation of viRNAs in response to virus acquisition leads to silencing of the viral genes, contributing to the vitality of the insect vector and its ability to infect new host plants. Emerging technologies based on our increasing knowledge of the role of sRNAs in regulating different aspect of plant-hemipteran interactions will greatly aid in developing next-generation alternatives to chemical pesticides. Ongoing work to identify and deliver effective RNAi approaches for hemipterans is paving the way for the rational design of target-specific pesticides that can complement current IPM techniques in the field.

AUTHOR CONTRIBUTIONS

SS has done the literature search and SS and GT have written this manuscript.

ACKNOWLEDGMENTS

This work was made possible by funds made available to SS from the Jeanne and Charles Rider endowment for support of research on the biotechnology of food crops awarded by College of Agricultural Sciences, Penn State University.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 November 2016
doi: 10.3389/fpls.2016.01759

Small RNAs from *Bemisia tabaci* Are Transferred to *Solanum lycopersicum* Phloem during Feeding

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The phloem-feeding whitefly *Bemisia tabaci* is a serious pest to a broad range of host plants, including many economically important crops such as tomato. These insects serve as a vector for various devastating plant viruses. It is known that whiteflies are capable of manipulating host-defense responses, potentially mediated by effector molecules in the whitefly saliva. We hypothesized that, beside putative effector proteins, small RNAs (sRNA) are delivered by *B. tabaci* into the phloem, where they may play a role in manipulating host plant defenses. There is already evidence to suggest that sRNAs can mediate the host-pathogen dialogue. It has been shown that *Botrytis cinerea*, the causal agent of gray mold disease, takes advantage of the plant sRNA machinery to selectively silence host genes involved in defense signaling. Here we identified sRNAs originating from *B. tabaci* in the phloem of tomato plants on which they are feeding. sRNAs were isolated and sequenced from tomato phloem of whitefly-infested and control plants as well as from the nymphs themselves, control leaflets, and from the infested leaflets. Using stem-loop RT-PCR, three whitefly sRNAs have been verified to be present in whitefly-infested leaflets that were also present in the whitefly-infested phloem sample. Our results show that whitefly sRNAs are indeed present in tomato tissues upon feeding, and they appear to be mobile in the phloem. Their role in the host-insect interaction can now be investigated.

Keywords: small-RNA, whitefly, RNAseq, phloem, tomato

OPEN ACCESS

Edited by:
Linda Walling,
University of California, Riverside, USA

Reviewed by:
Hailing Jin,
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *30 May 2016*
Accepted: *08 November 2016*
Published: *24 November 2016*

Citation:
van Kleeff PJM, Galland M, Schuurink RC and Bleeker PM (2016) Small RNAs from Bemisia tabaci Are Transferred to Solanum lycopersicum Phloem during Feeding. Front. Plant Sci. 7:1759. doi: 10.3389/fpls.2016.01759

INTRODUCTION

Bemisia tabaci (Hemiptera), commonly known as whitefly, is a polyphagous insect that is a threat for many crops across the globe. These insects can reduce crop yield in a number of ways; (1) through transmission of yield-limiting plant viruses (Navas-Castillo et al., 2011); (2) via honeydew excrement, which results in growth of sooty molds leading to a reduction of photosynthesis (Walling, 2008) or the release of the glycoside of salicylic acid (VanDoorn et al., 2015) or; (3) ingestion of phloem sap thereby depleting plants of photosynthetic compounds (Buntin et al., 1993).

Plants can defend themselves against herbivores and pathogens in various ways e.g., via physical barriers, volatile or non-volatile compounds, and through induction of defense responses controlled by various phytohormones (Walling, 2008; Kant et al., 2015). Trichomes can act both as physical barriers and as metabolite production facilities. Trichomes on the leaves will hinder small herbivores in their movement and finding suitable feeding places (Simmons and Gurr, 2005). In

addition, trichomes can produce specialized metabolites such as repellent volatiles or exudates that can be toxic or that trap herbivores (Simmons and Gurr, 2005; Walling, 2008; Bleeker et al., 2009). The phytohormones involved in herbivore-defense responses are predominantly jasmonic acid (JA) and salicylic acid (SA). SA can antagonize the JA-mediated signaling responses (Koornneef and Pieterse, 2008). Adult whiteflies feeding on tomato induce the SA-response thereby suppressing the JA-response (Shi et al., 2014). During the feeding of whitefly nymphs on Arabidopsis, transcript levels of SA-induced genes became higher while JA-related transcript levels decreased (Kempema et al., 2007; Zarate et al., 2007).

After hatching from the egg, whitefly nymphs are mobile and will select the site where they will feed and develop into an adult while being immobile. Feeding is initiated by insertion of a specialized mouthpiece (stylet) through the leaf surface toward the phloem sieve elements in a mostly intercellular fashion (Pollard, 1955; Jiang et al., 1999; Jiang and Walker, 2003). This insertion is facilitated by the excretion of gel-like saliva, in a similar way as an aphid, and other stylet- and phloem-feeding insects (Jiang et al., 1999; Moreno et al., 2011). After the stylet enters the sieve element, watery saliva is excreted and ingestion of phloem sap starts (Jiang et al., 1999; Jiang and Walker, 2003). Plants try to close the opening made by the stylet by depositing callose and proteins (Kempema et al., 2007) and phloem-feeding insects try to counteract this (Will et al., 2007).

There is evidence that herbivore saliva contains factors that can manipulate plant defenses (Will et al., 2013; Sharma et al., 2014; Su et al., 2015; Peng et al., 2016; Villarroel et al., 2016). For hemipterans most knowledge stems from work with aphids: several salivary proteins (effectors) have been identified that affect aphid reproductive rate (Bos et al., 2010; Pitino and Hogenhout, 2013). The aphid salivary proteins C002, Mp1, and Mp2 increase fecundity, while Mp10 and Mp42 reduce aphid fecundity (Bos et al., 2010; Pitino and Hogenhout, 2013). The production of effector proteins by aphids seems to be analogous to that of plant pathogens to establish disease. Such plant pathogens can interfere with the defense response of their host by secreting effectors that interact with host proteins and modulate these to their benefit.

Besides effector-protein interactions, small non-coding RNAs (sRNAs) between 21 and 24 nucleotides long (nts) have been shown to mediate interactions between hosts and pathogens (Knip et al., 2014; Baulcombe, 2015). Regarding plants, one of the best-studied examples is the *Botrytis cinerea* infection of Arabidopsis and tomato (Weiberg et al., 2013). After fungal infection, 73 sRNAs from Botrytis were found in infected leaves (Weiberg et al., 2013). These Botrytis sRNAs take advantage of the plant's own silencing machinery to mediate their action (i.e., targeting ARGONAUTE 1). Another example comes from the green peach aphid (*Myzus persicae*) that displays reduced fecundity on Arabidopsis mutants affected in their miRNA biogenesis pathway (i.e., Dicer-like1 *dcl1* and Argonaute1 *ago1*; Kettles et al., 2013). These results indicate that sRNA pathways are not only involved in plant resistance against a phloem-feeding insect, but also suggest that aphids produce sRNAs that can influence plant-defense responses (Kettles et al., 2013).

Here we show that whiteflies transfer sRNAs to the host plant they are feeding from. To detect whitefly-specific sRNAs within the phloem of tomato plants small RNA sequencing was utilized. These sRNAs are detected in isolated phloem sap indicating they are mobile. The presence of three whitefly sRNAs in tomato was confirmed by means of stem-loop RT-PCR. Our findings are, to our knowledge, the first confirmation of the transfer of insect sRNA to phloem.

METHODS AND MATERIALS

Whitefly Rearing and Tomato Infestation

Whiteflies (*B. tabaci* biotype B) were reared in a climatised chamber (Snijders, Tilburg; 28°C, 16 h light 150 μE m^{-2} s^{-1}, RH 75%) as previously described (Bleeker et al., 2011), on a diet of cucumber plants (*Cucumis sativus*, Ventura, RijkZwaan). Two weeks after sowing, 5 tomato plants (*Solanum lycopersicum*, cultivar Moneymaker) were placed in a netted insect dome (60 × 60 × 90 cm) and infested with ±200 adult whiteflies (greenhouse 22–25°C, 16/8 h photoperiod at 500 μE m^{-2} s^{-1}). Aiming for a consistent treatment with the different instar stages present, whiteflies (±100) were added 3 times per week until week 4 after sowing. In week 6 after sowing the samples for small RNA sequencing were collected.

Phloem, Nymph, and Leaf Collection for sRNA-seq

For phloem collection plants were kept in the greenhouse under standard greenhouse conditions (22–25°C, 16/8 h photoperiod at 500 μE m^{-2} s^{-1}). Phloem sap was collected from control and whitefly-infested leaflets using the "EDTA" method (King and Zeevaart, 1974) during the light-period (see also Figure S1 and Tetyuk et al., 2013). Adult whiteflies were removed from treated leaflets by aspiration. Leaflets with a high density of nymphs were excised and the petioles were carefully submerged in phloem collection buffer (5 mM EDTA, 5 mM phosphate buffer pH 6.8). The petioles of 3–6 leaflets were then cut once more while submerged in buffer and placed in a 2-ml Eppendorf vial containing phloem-collection buffer to bleed for 30 min under high humidity. After this the leaflets were transferred to collection tubes with fresh phloem-collection buffer supplemented with protease inhibitor (1 Complete Protease Inhibitor Tablet (Roche) 100 ml^{-1} water) and phloem samples were collected for 6 h under high humidity before being snap frozen in liquid nitrogen. Nymphs (1st, 2nd, and 3rd instar) were collected from a total of 4 infested leaflet using an insect pin, pooled, and transferred to 100% acetone. For the infested sample (LW) in addition to nymphs, eggs were removed as accurate as possible as well, after which the leaflet samples were separately snap frozen in liquid nitrogen ($n = 4$). Untreated control leaflets (LC) were harvested in exactly the same way and at the same time point ($n = 4$). For an additional control, leaflets with the eggs remaining were included (LE, $n = 4$). For this adult whiteflies were placed on leaflets for 24 h after which the adults were removed.

Total and Small RNA Isolation

Total RNA from phloem samples was isolated using concentrated TRIzol reagent (Life Technologies). The leaf samples (for each treatment four replicates pooled) and one nymph sample were ground in liquid nitrogen. Total RNA was isolated using the E.Z.N.A.® MicroElute RNA Clean Up Kit (Omega Bio-Tek). Briefly, TRIzol Reagent (Life Technologies) and chloroform was added according to the manufacturer's instructions. After centrifugation, the RNA-containing aqueous phase was collected, mixed with 1.5 volume of 100% ethanol and applied to a MicroElute spin column (Omega Bio-Tek). The column was washed according to the manufacturers's instructions: once with RWT buffer (Qiagen), once with RPE washing buffer (Qiagen) and finally with 80% ethanol. The RNA concentration was measured on a NanoDrop ND-2000 (Thermo Scientific) and RNA integrity was examined using the 2200 TapeStation System with Agilent RNA ScreenTapes (Agilent Technologies).

Total RNA was spiked with ERCCs spike-in mix 1 (Life Technologies) as well as a synthetic spike-in set for Size Range Quality Control (SRQC) together with an External Reference for Data Normalization (ERDN; Locati et al., 2015). Both phloem samples from the control and whitefly-infested plants were not spiked. The total RNA was divided in a large and a small fraction. The large RNA fraction was bound to a mirVana™ spin column (mirVana™ miRNA Isolation Kit, Life Technologies) according to the manufacturer's instructions. Small RNAs (<200 nts) were purified from the flow-through by adding ethanol to a final concentration of 65% (v/v) and bound to an E.Z.N.A.® MicroElute spin column. The column was washed once with RWT buffer, once with RPE buffer and once with 80% ethanol (Qiagen). The concentration and integrity of small RNA was examined as described above.

Next-Generation Sequencing

Bar-coded small RNA libraries of the 6 different samples were generated according to the manufacturer's protocols using the Ion Total RNA-Seq Kit v2 and the Ion Xpress™ RNA-Seq bar-coding kit (Life Technologies). The size distribution and yield of the bar-coded libraries were assessed using the 2200 TapeStation System with Agilent D1K ScreenTapes (Agilent Technologies). Sequencing templates were prepared on the Ion Chef™ System using the Ion PI Hi-Q Chef Kit (Life Technologies). Sequencing was performed on an Ion Proton™ System using Ion PI v3 chips (Life Technologies) according to the manufacturer's instructions.

Bioinformatic Analyses

Bioinformatic analyses were done using the Snakemake workflow management tool (Köster and Rahmann, 2012) to generate bioinformatic pipelines. Software used was Bowtie2 v2.1.0 (Langmead and Salzberg, 2012), Samtools v1.2 (Li et al., 2009), Python v3.3.3, Python package Pandas 0.14.1, and Biopython 1.64 (Cock et al., 2009), STAR v2.4.0 (Dobin et al., 2013), R v3.2.1 (R Core Team, 2016). All sequences <18 and >40 nts were removed. Contaminating sequences were removed by alignment to plant virus databases (Adams and Antoniw, 2006), other types of RNA (rRNA, tRNA, snoRNA, degraded messenger RNA, mitochondrial RNA) using the RFAM 12.0 database for tomatoes (excluding microRNA; Nawrocki et al., 2015) and the publically available tomato transcriptome [ITAG2.3, solgenomics.net Tomato Genome Consortium, 2012]. sRNAs were normalized for comparisons and expressed as RPKM (Reads Per Kilobase per Million mapped reads; Table S1).

The online psRNATarget tool (Dai and Zhao, 2011) was used to retrieve mRNA targets of selected miRNAs (using the *Solanum lycopersicum* ITAG2.4 cDNA reference) with default parameters: a seed region length of 20 nts to score complementarity between target and miRNA and a target accessibility (maximum energy required to open the mRNA secondary structure around the target site) of 25.

Stem-Loop RT-PCR of Small RNAs

Stem-loop RT-PCR of small RNAs was conducted to confirm findings of the sRNA-seq. For this analyses total RNA was isolated from four biological replicates of leaflet samples using the method described above and primers for the specific small RNAs were designed (Varkonyi-Gasic et al., 2007; Kramer, 2011; Table S2). A total of 100 ng RNA per sample was used for a reverse transcriptase reaction (RevertAid H Minus reverse Transcriptase, Thermo fisher) in a total volume of 20 μL with small RNA specific RT-primers (Table S2). A pulse reverse-transcriptase reaction (RT) was used (Varkonyi-Gasic et al., 2007). For the stem-loop RT-PCRs, 2 μL of RT reaction was used as template in a total volume of 50 μL for 35 cycles with an annealing temperature of 58°C. PCR products were cloned with CloneJET PCR cloning kit (Thermo scientific) and sequenced to validate amplicon specificity.

RESULTS

Small RNA Sequencing: Detection of Small RNAs in Phloem and Leaves

Phloem samples were collected from uninfested tomato leaflets (Phloem control, PC) and compared to phloem samples from tomato leaflets infested with whitefly nymphs and eggs (Phloem whitefly, PW, **Figure 1**). Whitefly nymphs (WN) were separately collected from leaflets of the same plants used to obtain phloem sap exudates (**Figure 1**). After sRNA sequencing we obtained 31,231,948 sRNAs in the WN sample and 5,370,176 and 7,321,768 sRNAs in the PC and PW samples respectively (see also Table S1). To determine which sRNAs originated from whiteflies and were present in phloem, a bioinformatic pipeline was designed (**Figure 1**). For this analysis, sequences shorter than 18 nucleotides (nts) and longer than 40 nts were removed. Next, we removed sequences that aligned to plant viruses (Adams and Antoniw, 2006), other types of RNA (rRNA, tRNA, snoRNA, degraded messenger RNA, mitochondrial RNA) using the RFAM 12.0 database for tomatoes (excluding microRNA; Nawrocki et al., 2015) and the publically available tomato transcriptome (ITAG2.3, Tomato Genome Consortium, 2012). To eliminate sequences

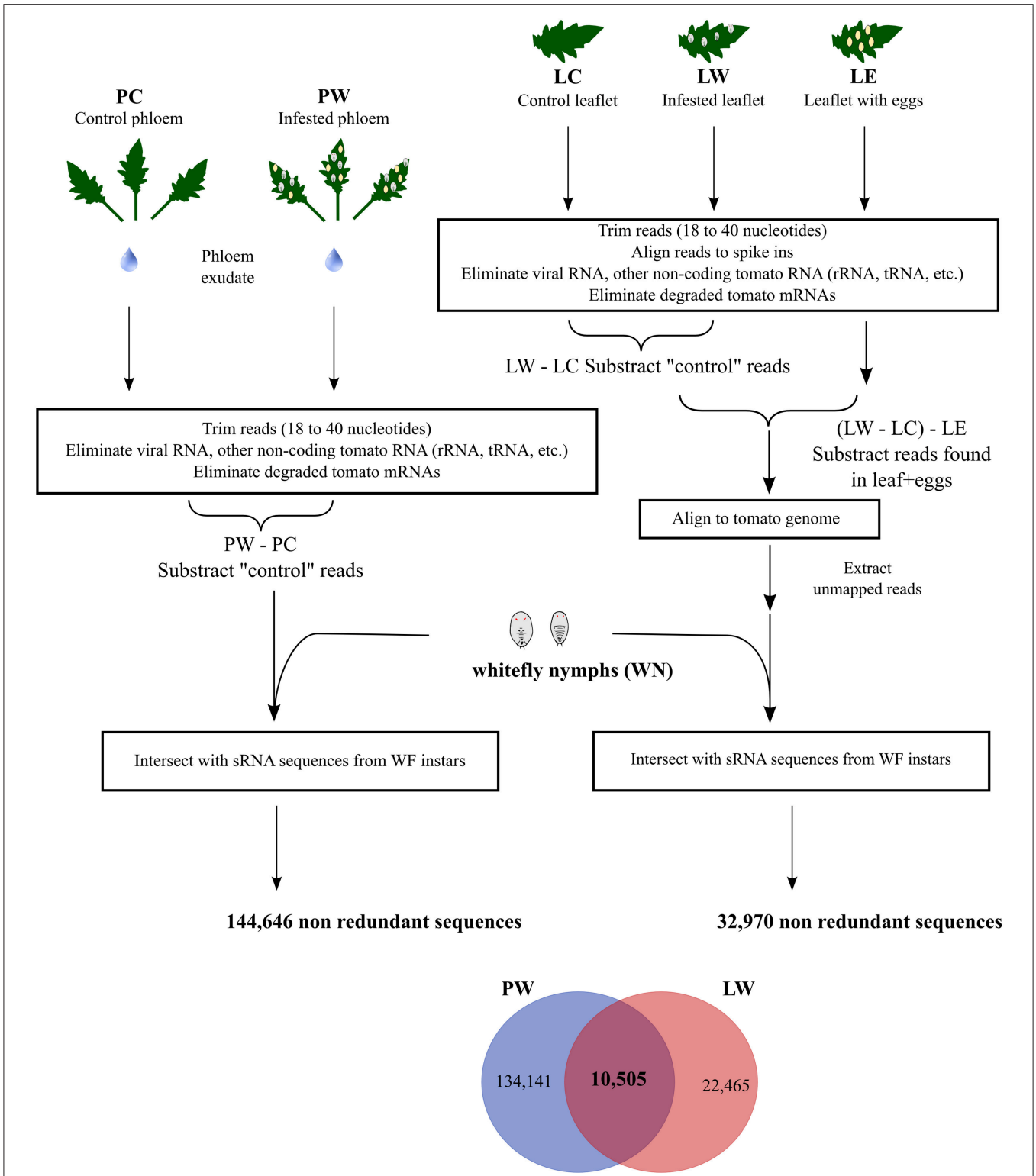

FIGURE 1 | Bioinformatic pipeline. Small RNAs (sRNAs) were isolated from tomato-phloem exudates from either control leaflets (PC) or leaflets infested with whitefly nymphs (PW), and from control tomato leaflets (LC), whitefly nymphs (WN), leaflets first infested with whiteflies but with adults and nymphs subsequently removed (LW) and tomato leaflets with only eggs (LE). A bioinformatic workflow (see main text for details) was implemented yielding 10,505 non-redundant small RNAs present in feeding whitefly nymphs plus identified in both infested tomato phloem and leaflets.

present in PW but unrelated to whitefly infestation, PC sRNAs were excluded from the PW sRNAs (PW–PC, **Figure 1**). Of these PW-PC sRNAs a final 144,646 non-redundant sequences overlapped with WN sequence (**Figure 1**, Table S1) and were regarded as putatively transferred from the insect into the phloem.

In order to further validate whitefly-specific sRNAs present in tomato, we additionally sequenced sRNAs isolated from whole tomato leaflets (**Figure 1**, Table S1). We obtained 36,793,380 sRNAs from uninfested tomato leaflets (Leaf Control, LC), 33,780,469 sRNAs from infested leaflets after removal of nymphs (Leaf whitefly, LW) and 32,730,583 sRNAs from leaflets with only eggs (Leaf Eggs, LE). LC sRNAs were subtracted from the LW sRNAs (LW–LC, **Figure 1**). Next, to correct for any whitefly-specific egg sRNA that could have been left on the leaf surface of LW, the LE sequences were removed (**Figure 1**). The remaining whitefly nymph sRNAs were subsequently aligned against the tomato genome and the unmapped sequences were aligned with the tomato-fed WN sequences to find nymph-specific sRNAs. By doing so, we ended up with 32,970 non-redundant sequences (**Figure 1**). Finally, we searched (qualitatively) for sRNAs that would be both present amongst the 144,464 sRNAs coming from PW sample and the 32,970 sRNAs coming from infested LW sample, and found in WN nymph sample. This resulted in 10,505 non-redundant (**Figure 1**, Table S1) putative whitefly sRNAs found in both phloem exudate and leaflets containing phloem of whitefly-infested plants.

Length Distribution

The sRNA-length distributions from the different libraries ranging from 18 to 40 nts are shown in **Figure 2**. The sRNA length distributions of the three leaf samples (LC, LW, and LE) were very comparable with an expected major peak at 24 nts (35–40% of all sequences) and a minor peak at 21 nts (**Figure 2**). Compared to tomato leaf samples, both phloem samples exhibited a slightly different length distribution with a peak at 23–24 nts (∼18–25% of all sequences). However, the sRNA length distribution of the whitefly nymphs (WN) was distinctly different from the other samples with two major peaks; one at 22 nts (∼12% of all sequences) and one at 29–30 nts (∼15–20% of all sequences; **Figure 2**). The 29–30 nt sequences, apparent in the nymph sample, appeared to be a well-defined peak in the phloem sample from the nymph-infested leaflet (PW), compared to the control phloem (PC).

Next, the sRNAs from whitefly-infested samples LW, WN, and PW were compared to 185 whitefly miRNA sequences found in two different biotypes of *B. tabaci* (Guo et al., 2013). All previously described whitefly miRNAs had a length comprised between 20 and 24 nts (Guo et al., 2013). Accordingly, 150 sRNAs of our WN sample could be exactly matched to the whitefly miRNAs published earlier (Table S3) and had a length predominantly centered around 22 nts, a feature characteristic of insect miRNAs (**Figure 2**, insets). The most abundant miRNAs found in the WN sample were miR-276a, miR-317 and miR-14 that appear to be conserved as well in other insects e.g., *Bombyx mori, Apis mellifera, Drosophila melanogaster* (Yin et al., 2016; Table S3).

Confirmation of Small RNAs in Leaf Samples

A sensitive stem-loop RT-PCR (Varkonyi-Gasic et al., 2007) was used to confirm the presence or absence of whitefly sRNAs in four biological replicates of whitefly-infested tomato leaflets (LW), non-infested leaflets (LC), leaflets with eggs (LE), and the *B. tabaci* nymph sample (WN). To verify there was no sRNA from whitefly nymph contaminating the LW sRNAs other than those transferred by the whitefly, a 29-nt sRNA (# 29691) was amplified as this sRNA proved particularly abundant in the WN sample. **Figure 3A** shows that sRNA #29691 was indeed specific to the nymph sample and was absent in the LW, LC, or LE samples, indicating that there is no whitefly nymph contamination in the LW sample after the infestation and that nymphs had been successfully removed. Sample quality was further checked using a known *B. tabaci* specific sRNA (Bta_miR2A; Guo et al., 2013) and a known tomato miR172 that is conserved among land plants (Taylor et al., 2014). Accordingly, the plant-specific miR172 was detected in all leaf samples (**Figure 3B**) while the whitefly Bta_miR2A was detected in the nymph samples of *B. tabaci* reared on tomato and in the leaf samples containing eggs (LW and LE; **Figure 3C**).

Whitefly Small RNAs Transferred to Tomato

sRNA sequencing of the phloem samples identified the presence of mobile whitefly-originating sRNAs. We selected three whitefly candidate sRNAs from the final list (**Table 1**) for validation using stem-loop PCR on leaflets. The criteria for selecting these specific candidates from the sRNAseq data were (1) a length between 23 and 24 nt (**Figure 2**), (2) present among highest counts in nymphs, (3) presence in whitefly-infested leaflets (LW), in phloem from leaflets infested with whiteflies (PW) and in the *B. tabaci* nymph (WN) sample, while absent in the control leaflet (LC), absent in leaflets with only eggs (LE), and absent in the phloem control sample, and finally (4) preferably matching an insect-like or an unknown small RNA in the miRBase. From the three selected sRNAs, sRNA #13120, and #18833 were annotated as insect miR305 and miR1175-3p, respectively, using the miRBase (Kozomara and Griffiths-Jones, 2014). sRNAs #13120 (**Figure 4A**) and #18833 (**Figure 4B**) were present in nymph and were found back in three out of four LW samples while being absent in all LC and LE samples. sRNA #3182 did not provide a match in the miRBase but could be amplified in nymph and all infested leaf samples, however it was found in one out of four control samples. Overall, whitefly sRNAs could be detected within the leaflet samples (**Figure 4C**) on which nymph feeding took place. Since two out of three candidate small RNAs were found exclusively in the infested samples PW and LW, we conclude that whiteflies transfer small RNAs to the phloem, which then have the potential to move.

Prediction of Whitefly sRNAs Targets in Tomato

To get insight into putative roles of these three whitefly sRNAs**,** the online psRNAtarget tool was used (Dai and Zhao,

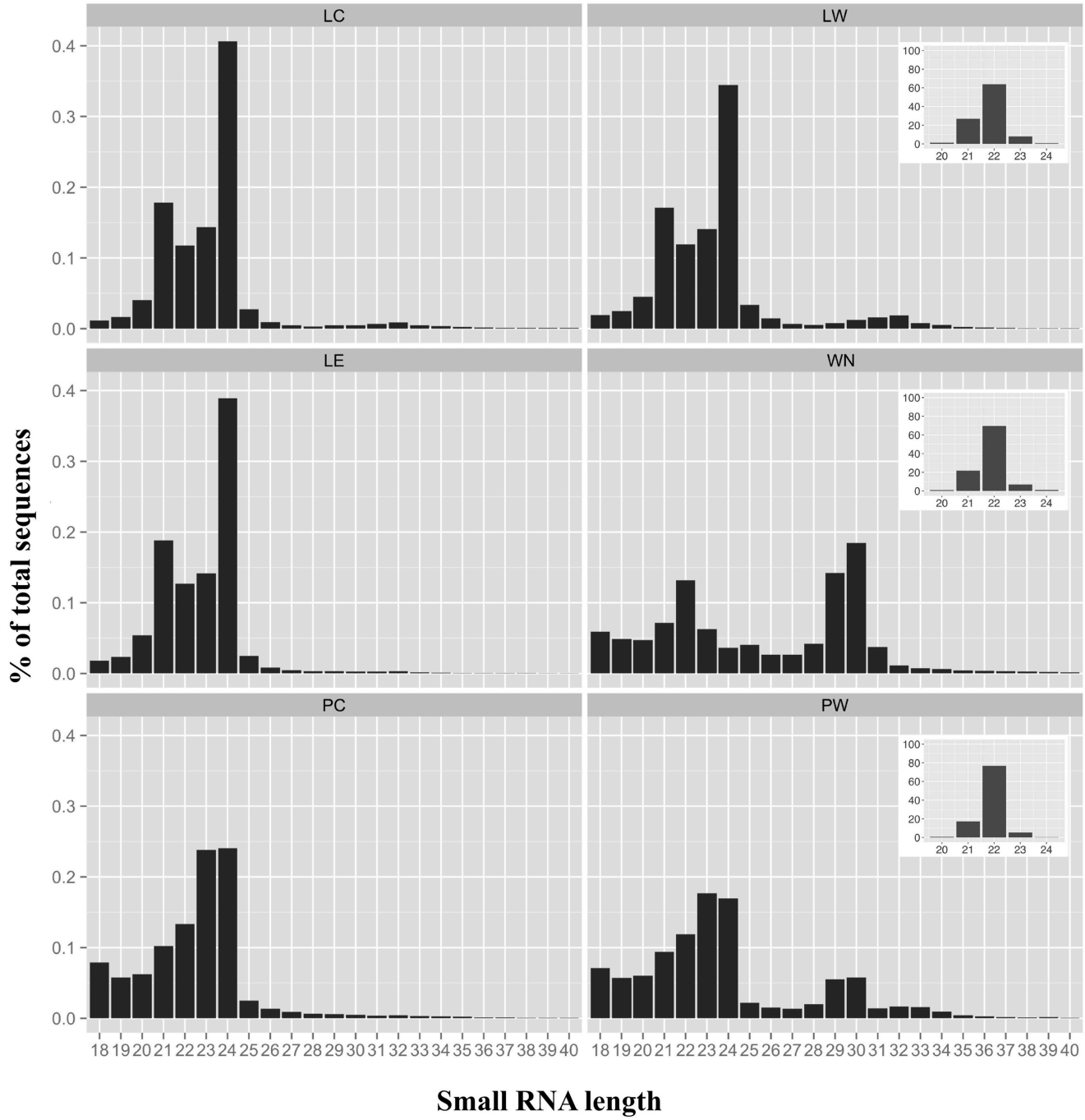

FIGURE 2 | Size distribution of sRNAs. Size distributions and percentages of small RNAs (sRNAs) between 18 and 40 nucleotides are indicated for each sample. LC, control leaflets (not infested with whitefly nymphs); LW, leaflets first infested with whiteflies but with adults and nymphs subsequently removed; LE, leaflets with whitefly eggs; WN, whitefly nymphs; PC, phloem control (not infested); PW, phloem infested with whitefly nymphs. Insets for LW, WN, and PW represent the size distributions and percentages of the sRNAs matching previously described whitefly miRNAs (Guo et al., 2013).

2011) to predict putative tomato mRNA targets. By doing so, putative targets for the sRNAs #13120 and #18833 were found. sRNA #13120 is predicted to target four different exocyst complex proteins of which two are expressed in tomato leaves (**Table 2**). Another interesting putative targets of sRNA #18833 are three closely related xylanase inhibitors (**Table 2**). They are moderately similar (between 56 and 58% identity at the amino acid level) to a previously described tomato xyloglucan-specific endoglucanase inhibitor (Qin et al., 2003).

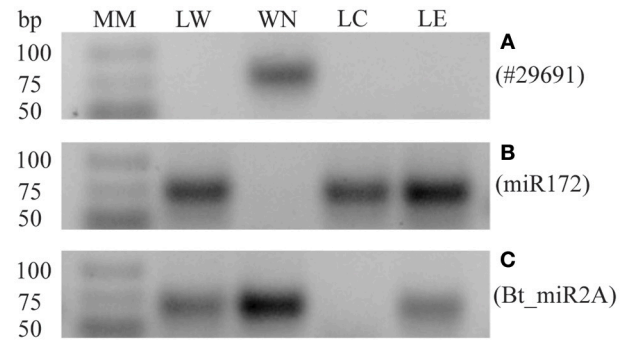

FIGURE 3 | Quality check of leaflet samples by stem-loop RT-PCRs. (A) Expression of nymph-specific sRNA #29691 in: leaflet first infested with whiteflies but with adults and nymphs subsequently removed (LW), whitefly nymphs (WN), control leaflet (LC), or leaflet with eggs (LE), showing a specific band in WN only. **(B)** Validation of the expression of tomato specific sRNA Sly_miR172 in the different samples. Expression is detected in LW, LC, and LE, but not in the WN sample. **(C)** Expression of the known whitefly-specific miRNA Bta_miR2A is also detected in LE. Similar results were obtained in four biological replicates. Molecular Mass (MM), GeneRuler Ultra Low Range DNA ladder (Thermo Scientific).

DISCUSSION

Cross-Kingdom Interactions Mediated by sRNAs

Besides ingesting plant sap, phloem-feeding insects such as aphids manipulate plant defenses by secreting protein effectors that can improve host colonization and reproductive fitness (Louis and Shah, 2013). However, the precise molecular mode of action of aphid effectors remains elusive. Along with an array of notorious plant-viruses (Rosen et al., 2015), whiteflies theoretically could also transfer effector proteins into the phloem while feeding. Here we show that *B. tabaci* also appears to transfer sRNAs into the tomato phloem. Transfer of sRNAs from whiteflies could putatively be involved in transcriptional or post-transcriptional gene silencing inside the host and form an additional way for the insect to manipulate host defenses. There is increasing experimental evidence that sRNAs can mediate cross-kingdom interactions between plant and microorganisms (Knip et al., 2014), with perhaps the most convincing examples being those of Botrytis-Arabidopsis and Botrytis-tomato (Weiberg et al., 2013). One of the major difficulties in the field is to establish with certainty that a specific sRNA has been transferred by the "invader organism" into the host rather than being produced by the attacked host. Genome availability of *B. cinerea*, tomato and *Arabidopsis thaliana* was a major advantage that led to the discovery that sRNA can promote fungal pathogenicity (Weiberg et al., 2013). We did not have a draft or complete *B. tabaci* genome sequence to our disposal and this work therefore relied on a bioinformatic pipeline to search for whitefly sRNAs present in the phloem of whitefly-infested tomato (**Figure 1**). To further verify the sequences found in the tomato phloem, the sRNAs present only in the PW phloem were compared to the sRNAs of the whitefly nymph (WN) sample. Additionally, they were cross-referenced to sRNAs from whitefly-infested leaflets (LW) after elimination of non-infected leaves (LC) and leaflet with eggs (LE), in case not all eggs were removed from the LW leaflet surface. sRNAs were subsequently aligned to the tomato genome to remove tomato sRNAs especially from repetitive regions (**Figure 1**), leaving us with potentially 10,505 whitefly-specific sRNAs present in plant tissue. The final LW sample (nymph sRNAs in LW–LC–LE) contains less phloem than phloem exudate itself (nymph sRNAs in PW–PC) as the starting material contains many other tomato cell types that have been consequently filtered out. The remaining 22,465 sequences that do not match the phloem sequences can contain e.g., whitefly sRNAs from phloem companion cells. The phloem exudate samples are more concentrated and thus contain more (134,141) putative whitefly sRNAs than the leaflet samples.

A great diversity of sRNAs has been found in the phloem sap of several species including pumpkin, cucumber, lupin, and Arabidopsis, in the absence of major pathogen infection or pest infestation (Yoo et al., 2004). These phloem sRNAs typically had a length between 18 and 25 nts with a major peak at 23 nts (Yoo et al., 2004), which is consistent with the sRNA length distribution found in our non-infested control samples (LC and PC, **Figure 2**). In leaves, a major peak at 24 nts is very common (see, Itaya et al., 2008 for an example in tomato), which has long been associated with transcriptional gene silencing especially of repetitive sequences e.g., transposons (Borges and Martienssen, 2015). In both phloem and leaflet samples, we found conserved miRNAs such as miR156 and miR172 known to act in concert to regulate flowering time (Spanudakis and Jackson, 2014) and miR159 previously identified in cucurbit phloem (Yoo et al., 2004). Finding such miRNAs among the most abundant phloem sRNAs is consistent with previous studies (Yoo et al., 2004; Rodriguez-Medina et al., 2011; Bhogale et al., 2014). We also observed Solanaceae-specific miR482 and miR6022 among the most abundant miRNAs in the non-infested phloem PC and LC sRNAs (Table S4). The collection of phloem was performed after an initial "bleed" period of 30 min to limit sample contamination by other types of cellular content. Nymphs were feeding on the leaflet at 2–3 cm distance from the petiole phloem collection site indicating mobile whitefly sRNAs in the plant phloem. It was estimated that contamination of phloem exudates by companion cell breakage composed around 2% of the exudate (Atkins et al., 2011). Thus, it is likely that the identified sRNAs in the phloem originate from the sieve elements although some contamination from neighboring cells cannot be completely ruled out.

The presence of whitefly nymphs on tomato leaflets caused 29–30 nts sRNAs to appear in the phloem of infested plants (**Figure 2**). These longer sRNAs were indeed also observed in the whitefly nymphs (**Figure 2**). A similar sRNA length distribution has previously been found in adult whiteflies (Guo et al., 2013; Wang et al., 2016) and similar sized sRNAs (between 26 and 30 nts) have been reported for other insects e.g., cotton-melon aphid (*Aphis gossypii*) or the brown planthopper (*Nilaparvata lugens*; Chen et al., 2012; Sattar et al., 2012). These longer sRNAs are assumed to be Piwi-interacting RNAs (piRNAs) and are known to be a large class of non-coding RNAs in animals, specifically linked to genome stability in germ-line

TABLE 1 | List of selected putative whitefly sRNA candidates with normalized counts (RPKM).

Id	sRNA sequence (5′–3′)	Counts	Length	Best miRBase21 homolog
–	ACCGGCGGCGCGGUGAGGCACC	44	22	Unknown
–	CACCGGCGGCGCGGUGAGGCACC	47	23	Unknown
–	CACCGGAAGGAUUGACAGAUU	66	21	*Acyrthosiphon pisum* miR-263b
–	UGAGAUUCAACUCCUCCAUCUUAU	1574	24	*Bombyx mori* miR-1175
–	AGCAGAGUGGCGCAGUGGAAGC	386	22	*Monodelphis domestica* miR-885
3182	**UAGUAGCUAACGACGAUUCCUUU**	**957**	**23**	**NA**
–	UAAGGCACGCGGUGAAUGCCAUU	1105	23	*Panagrellus redivivus* miR-124
–	UGGUAACUCCACACCACCGUUGGC	1713	24	*Acyrthosiphon pisum* miR-2765
–	GCGGGUGUCGGCGGCCGUG	52	19	*Pongo pygmaeus* miR-118
–	UGAGAUCAUCGUGAAAGCUGAUA	543	23	*Apis mellifera* bantam stem-loop
–	CAAGCUCGUUGAAGUAUACCCAU	531	23	*Petromyzon marinus* miR-133a
–	UAAGUACUCCGUGCCGCAGGA	899	21	*Daphnia pulex* miR-252a
–	UCAGGCGGGCAAUCGCCGGG	157	20	*Ectocarpus siliculosus* miR3453
–	UCGCGGGUGUCGGCGGCCGUGAGC	31	24	*Pongo pygmaeus* miR-1181
–	GGCGGCAAUCGCCGGGGCCCU	9	21	*Mus musculus* miR-3104
–	UGGACGGAGAACUGAUAAGGGCU	553	23	*Drosophila melanogaster* miR-184
–	AUACAGGGGAGUAAGGGUUUGU	316	22	*Monodelphis domestica* miR-7398j
18833	**UGAGAUUCAACUCCUCCAUCUUA**	**1166**	**23**	***Bombyx mori* miR-1175**
–	GAAGGCCCUACAACGCGGACCCC	1557	23	*Equus caballus* miR-1905a
–	UAUCACAGCCAUUUUGACGUGCCU	1037	24	*Drosophila melanogaster* miR-13b-1
13120	**AUUGUACUUCAUCAGGUGCUCUGU**	**1275**	**24**	***Drosophila melanogaster* miR-305**
–	UUAAAAAGUGAUUUCACCACGG	750	22	*Ornithorhynchus anatinus* miR-1334

The sRNAs indicated in bold were detected with stem-loop RT-PCR.

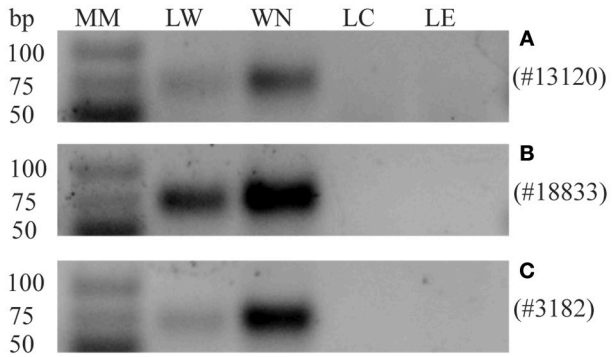

FIGURE 4 | Detection of candidate transferred whitefly sRNAs in the nymph and leaflet samples by stem-loop RT-PCRs. Expression of sRNAs in: leaflet first infested with whiteflies but with adults and nymphs subsequently removed (LW), whitefly nymphs (WN), control leaflet (LC) or leaflet with eggs (LE). **(A)** sRNA #13120, a specific band is detected in LW and WN but not in LC and LE. **(B)** #18833, a specific band is detected in LW and WN but not in LC and LE. Similar results for #13120 and #18833 were obtained in four biological replicates. **(C)** #3182, a specific band is detected in LW and WN but not in LC and LE in three out of four replicates. One biological replicate showed also a band in LC and LE. Molecular Mass (MM), GeneRuler Ultra Low Range DNA ladder (Thermo Scientific).

cells and silence transposons (Vagin et al., 2006). In Drosophila (*D. melanogaster*), for example, piRNAs are produced in a Dicer-independent manner from transposon-rich genomic clusters and specifically silence transposon expression in the germline (Iwasaki et al., 2015). Sattar et al. (2012) found that sRNAs with a length between 26 and 27 were overrepresented in the cotton-melon aphid, *A. gossyppii*, when infesting melon plants containing the *Vat* aphid resistance gene. Similar to *B. tabaci*, there is no annotated genome for *A. gossypii* but in this case the authors could make use of an *A. pisum* transposon database to show that around 50% of these 26–27 sRNAs actually derived from transposons. Another ∼5% matched from the primary endosymbionts in aphids, *Buchnera aphodicola* (Sattar et al., 2012). In this study, we cannot completely rule out that the 29–30 nts sRNA originate from tomato. Nevertheless, when trying to align the five most abundant 29 or 30 nts sRNA to the tomato Heinz genome sequence (**Figure 1**), no full-length alignments were found. Assuming these particular sRNAs are indeed piRNAs involved in insect germline development, it remains elusive as to if and how the enrichment in the phloem of whitefly-infested leaves (**Figure 2**) is biologically relevant.

Insect Salivary Small RNAs Transferred into Host

Our bioinformatic pipeline identified whitefly sRNAs in tomato phloem of leaflets where nymphs were feeding. These sRNAs most likely found their way into the phloem via the whitefly saliva. During feeding whiteflies salivate into the phloem after which they ingests phloem sap (Figure S1; Pollard, 1955; Jiang et al., 1999; Jiang and Walker, 2003). Since whitefly nymphs are immobile and feed for long periods of time, one can expect to find components of whitefly saliva in the phloem. Salivary

TABLE 2 | List of predicted mRNA targets from the selected whitefly small RNA found in tomato tissues.

id	sRNA sequence (5′–3′)	Length	mRNA target	Annotation
13120	AUUGUACUUCAUCAGGUGCUCUGU	24	Solyc09g075400.2.1	Putative DNA-ligase
13120	AUUGUACUUCAUCAGGUGCUCUGU	24	Solyc10g019140.1.1	Exocyst complex component protein
13120	AUUGUACUUCAUCAGGUGCUCUGU	24	Solyc10g019110.1.1	Exocyst complex component protein
13120	AUUGUACUUCAUCAGGUGCUCUGU	24	Solyc11g050710.1.1	Exocyst complex component protein
13120	AUUGUACUUCAUCAGGUGCUCUGU	24	Solyc03g095410.2.1	Exocyst complex component protein
13120	AUUGUACUUCAUCAGGUGCUCUGU	24	Solyc02g085940.2.1	Unknown protein
13120	AUUGUACUUCAUCAGGUGCUCUGU	24	Solyc08g062170.1.1	Unknown protein
13120	AUUGUACUUCAUCAGGUGCUCUGU	24	Solyc01g009030.2.1	ATP synthase regulation protein
13120	AUUGUACUUCAUCAGGUGCUCUGU	24	Solyc01g111270.2.1	Armadillo protein
18833	UGAGAUUCAACUCCUCCAUCUUA	23	Solyc01g079980.2.1	Xylanase inhibitor
18833	UGAGAUUCAACUCCUCCAUCUUA	23	Solyc01g079960.2.1	Xylanase inhibitor
18833	UGAGAUUCAACUCCUCCAUCUUA	23	Solyc03g082470.2.1	Leucine-rich repeat receptor-like protein kinase
18833	UGAGAUUCAACUCCUCCAUCUUA	23	Solyc02g084980.2.1	Galactinol synthase
18833	UGAGAUUCAACUCCUCCAUCUUA	23	Solyc11g008350.1.1	Kinesin-like protein
18833	UGAGAUUCAACUCCUCCAUCUUA	23	Solyc01g079970.2.1	Xylanase inhibitor
18833	UGAGAUUCAACUCCUCCAUCUUA	23	Solyc02g037490.1.1	Acyl-activating enzyme
18833	UGAGAUUCAACUCCUCCAUCUUA	23	Solyc00g005160.1.1	Retrovirus-related Pol polyprotein from TNT transposon
3182	UAGUAGCUAACGACGAUUCCUUU	23	Solyc02g085990.1.1	Unknown protein

glands of phloem feeding insects like aphids and whitefly have been subjected to RNA sequencing and proteomics to obtain insight in the transcriptome and proteome (Carolan et al., 2011; Su et al., 2012; Rao et al., 2013). Also, aphid saliva has been collected and used for proteomics studies (Rao et al., 2013; Chaudhary et al., 2015). In the current study, phloem from whitefly-infested tomato was used, as obtaining salivary glands from adult whiteflies, though feasible (Ghanim et al., 2001; Su et al., 2012), proved too challenging in nymphs. In addition, the saliva composition of insects is not necessarily the same as the composition of the salivary gland, which includes cell membranes and ducts (Rao et al., 2013). Moreover, in order to collect sufficient saliva, it requires the culturing of large amounts of adult whiteflies for a prolonged period on an artificial diet (Su et al., 2015; VanDoorn et al., 2015), which was technically not feasible. Also, it has been reported that the composition of insect saliva differs when feeding on artificial diet and on different plant species (Habibi et al., 2001; Cooper et al., 2010). Finally, as nymphs are immobile while feeding for long periods of time it might increase the chances of actually identifying whitefly sRNAs in phloem.

To our knowledge, miRNAs have so far only been identified in the saliva of mosquito (*Aedes aegypti*; Maharaj et al., 2015). Interestingly, miRNAs closely related (one nucleotide difference) to our #13120 (Bta_miR305-pGtoU) and #18833 (Bta_miR1175-3p+A) were also found in the saliva of *A. aegypti,* particularly after sucrose feeding (Maharaj et al., 2015). Whether saliva secreted miRNAs are conserved among fluid-feeding insects remains to be seen. Since sRNAs of prokaryotes are generally bigger than 100 nt (Gottesman and Storz, 2011) and our cut-off for analysis was <40 nt, it is unlikely that the miRNAs presented here originate from symbionts present in the whitefly. Nevertheless, we aligned the 10,505 sRNAs to the genome of *Rickettsia* sp. Strain MEAM1 (Genbank AJWD00000000.2), and found no matches.

A possible source of contamination for the sRNA sequencing of leaflet samples could be part of nymphs still attached to the leaflet. However, in **Figure 3A** it was shown that nymph tissue was removed from leaflets or at least below the level of detection. The bands visible in the LW samples (**Figures 4A–C**) are therefore very unlikely to originate from nymph tissue still present on the leaflets. The plant specific sRNA miR172 was only found in leaf samples and not in the whitefly nymphs (**Figure 3B**) though this could have been possible since sRNAs have been found previously ingested by aphids (Sattar et al., 2012). Similarly, Bta_miR2A was detected only in the nymph and whitefly-infested samples (**Figure 3C**), showing that the miR2A of the eggs on leaflet (LE) samples was even detectable by stem-loop RT-PCR.

The three sRNAs investigated here were very likely transferred from whitefly into tomato. All three candidates were identified in whitefly-infested material and in the nymphs themselves. sRNA #13120 (Bta_miR305-pGtoU) (**Figure 4A**) and #18833 (Bta_miR1175-3p+A) (**Figure 4B**) have been detected in LW samples but not in the LC or LE sample. Family members of two of these candidates have been previously identified in whitefly (Guo et al., 2013). For the third candidate (#3182), no similarity was found with previously identified whitefly sRNAs or with other sequences in the miRBase. This sRNA has been detected in all of the replicates of whitefly-infested leaf samples and is very abundant in the nymph sample but could be detected once out of 4 in control leaflets. Despite the fact that it cannot be completely ruled out, it is highly unlikely that #3182 derives from tomato. Sly-miR172, a very abundant tomato miRNAs present in our phloem sample could not be detected in our nymph sample while #3182 was found in the insect in a relative high level.

Small RNA as Effectors?

Pathogens and insects are known to transfer proteins into host plant cells in order to suppress host immunity (Dangl et al., 2013; Will et al., 2013; Su et al., 2015). In fact, it has been postulated that phloem-feeding insects employ a suit of proteins that are passed from the saliva into the phloem during feeding which could act as effector proteins that suppress plant defenses. Besides proteins, non-protein salivary factors can act as an effector (Su et al., 2015). Here we describe, for the first time, the transfer of putative salivary non-coding sRNAs from whitefly and postulate that they might target tomato host proteins. Small RNAs could facilitate the interaction between organisms by improving the attackers chance of survival (Weiberg et al., 2013; Knip et al., 2014) or improve fecundity (Sattar et al., 2012). In plants, the high base complementarity between the sRNA and the target mRNA has been successfully used to predict post-transcriptional regulations by sRNAs (Ding et al., 2012). Further validation and characterization of these mRNA targets is currently under investigation.

AUTHOR CONTRIBUTIONS

PvK and MG contributed equally to this paper and shared first authors. MG analyzed the RNAseq data and PvK did the wet-lab experiments. PB conceived the project and supervised MG. RS supervised PvK and contributed to discussions. All four authors wrote and carefully read and approved the final manuscript.

FUNDING

This work was supported by funding from the European Union (H2020, DURETO project #655656) to MG and the NWO VIDI grant 12988 awarded to PB.

ACKNOWLEDGMENTS

The authors would like to thank Suzanne Hoogstrate for careful reading of the manuscript. We are grateful to Selina van Leeuwen, Wim Ensink, and Marina van Olst, Wim de Leeuw, and Han Rauwerda of the "RNA Biology and Applied Bioinformatics' group (SILS, University of Amsterdam) for sRNAs sequencing and bioinformatics support and thank Ludek Tikovsky, Harold Lemereis, and Fernando Inchauste Callahuara for taking care of the tomato plants. This work was supported by funding from the European Union (H2020, DURETO project #655656) and by the Dutch Technology Foundation STW, part of the Netherlands Organization for Scientific Research (NWO, VIDI project #12988).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01759/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer HJ and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Plant Science

REVIEW
published: 04 August 2016
doi: 10.3389/fpls.2016.01164

Novel Insights into Insect-Microbe Interactions—Role of Epigenomics and Small RNAs

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It has become increasingly clear that microbes form close associations with the vast majority of animal species, especially insects. In fact, an array of diverse microbes is known to form shared metabolic pathways with their insect hosts. A growing area of research in insect-microbe interactions, notably for hemipteran insects and their mutualistic symbionts, is to elucidate the regulation of this inter-domain metabolism. This review examines two new emerging mechanisms of gene regulation and their importance in host-microbe interactions. Specifically, we highlight how the incipient areas of research on regulatory "dark matter" such as epigenomics and small RNAs, can play a pivotal role in the evolution of both insect and microbe gene regulation. We then propose specific models of how these dynamic forms of gene regulation can influence insect-symbiont-plant interactions. Future studies in this area of research will give us a systematic understanding of how these symbiotic microbes and animals reciprocally respond to and regulate their shared metabolic processes.

Keywords: symbiosis, epigenomics, DNA methylation, small RNAs, insect-plant interactions

OPEN ACCESS

Edited by:
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University of California, Riverside, USA

Reviewed by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *26 April 2016*
Accepted: *20 July 2016*
Published: *04 August 2016*

Citation:
Kim D, Thairu MW and Hansen AK (2016) Novel Insights into Insect-Microbe Interactions—Role of Epigenomics and Small RNAs. Front. Plant Sci. 7:1164. doi: 10.3389/fpls.2016.01164

INTRODUCTION

Microbial associates that interact with insects can produce a wide array of metabolic products that complement the metabolic needs of their herbivorous hosts (Hansen and Moran, 2014). Consequently, microbes that form persistent but noninvasive associations with their hosts have the potential to provide their hosts with useful novel gene products in a short evolutionary timespan. How these symbiotic microbes and animals reciprocally respond to and regulate shared metabolic processes is a nascent but emerging area of research.

Animals, including insects, can biosynthesize some but not all of the amino acids that are required for building proteins. Food sources that are deficient in those essential amino acids (EAA), such as plant sap, present a nutritional challenge to consumers. Most insect herbivores that feed on a phloem or xylem sap diet are able to feed on this essential amino acid-deficient niche because they harbor one or more nutritional symbionts (Hansen and Moran, 2014). One model system that has been productive for teasing apart the regulatory mechanisms of shared animal-microbe metabolic processes is the pea aphid, *Acyrthosiphon pisum,* a sap-feeding insect in the order Hemiptera, and the bacterium *Buchnera aphidicola*, the mutualistic endosymbiont found in most aphids. Aphid and *Buchnera* physiologies are integrated for the production of amino acids and this occurs within specialized aphid cells called bacteriocytes. Specifically, *Buchnera* relies on the aphid for the biosynthesis of aphid-encoded non-essential amino acid pathways, and the aphid relies on *Buchnera* for the biosynthesis of *Buchnera*-encoded essential amino acid pathways.

Previous work on this system supports the prevailing hypothesis that this integrated metabolism is regulated primarily by the aphid host via aphid-encoded transporters (Price et al., 2014) and aphid genes that complement *Buchnera*'s EAA pathways (Hansen and Moran, 2011; Poliakov et al., 2011). Moreover, an aphid-encoded protein of bacterial origin can be transported into *Buchnera* cells and therefore a cross-domain protein translocation system exists for this intimate symbiosis (Nakabachi et al., 2005, 2014).

Gene expression of aphid bacteriocytes has been characterized at the transcriptome and proteome level (Hansen and Moran, 2011; Poliakov et al., 2011). As expected pathways involved in the amino acid metabolism are especially enriched in bacteriocytes compared to other aphid body cells (Hansen and Moran, 2011; Poliakov et al., 2011). However, the regulatory factors that lead to the development of these tissues and their signature expression profiles are not well-understood. In other animal systems, the primary regulatory factors that determine a eukaryotic cell's fate and its potential reprograming include histones, DNA methylation, noncoding RNAs, and transcription factors (Peter and Davidson, 2015). Work by Braendle et al. (2003) did identify three transcription factors that are expressed in temporal order, Dll, En, and Ubx or Abd-A, during bacteriocyte development in aphid embryos. The timing and expression of this subset of transcription factors is unique compared to any other cell type in insect embryos (Braendle et al., 2003). Currently it is unclear how these transcription factors may regulate metabolic processes or if other unknown co-factors are involved during embryonic stages or later during maternal bacteriocyte development. Moreover, it is unknown if chemical marks on histones and/or DNA (i.e., epigenetic mechanisms) (Hansen and Moran, 2014) are important in the regulation and metabolic reprogramming of these symbiotic cells, especially in response to environmental signals such as host plant nutrients or secondary plant compounds. Therefore, further understanding of how different subsets of host genes turn on and off in bacteriocyte development in response to environmental stimuli is required in order to fully understand how these intimate symbioses evolved, how they are maintained, and how they may ultimately influence host-plant-interactions.

Although evidence from the aphid and *Buchnera* model system suggests that the host exerts the majority of the regulatory control on this symbiosis, the role of the symbiont in gene regulation is not well-defined (Hansen and Moran, 2014). Given that *Buchnera* has lost the majority of the canonical mechanisms for gene regulation its capacity to be a participant in gene regulation has long seemed compromised (Shigenobu et al., 2000). Nevertheless, it was recently suggested that *Buchnera* regulates its own protein expression via putative post-transcriptional mechanisms (Hansen and Degnan, 2014). This recent study presents somewhat of a paradigm shift in understanding the regulation of these intracellular host-microbe mutualisms. It is now clear that symbionts with reduced genomes can potentially regulate their genomes through post-transcriptional processes, such as through regulatory small RNAs (sRNAs; Hansen and Degnan, 2014). Consequently, the potential role of microbe-mediated gene regulation cannot be ignored in these shared insect-microbe regulatory networks.

Recent advancements in the field of molecular biology and genome sequencing technologies allow for the first time the ability to predict how epigenetic mechanisms and regulatory sRNAs may impact the regulation and evolution of animal and microbial genomes. This review draws upon these emerging fields of gene regulation to investigate the potential role of epigenetics and sRNAs in *both* insect and microbe-mediated regulation of shared metabolisms. By understanding the dynamics and the intersection of these regulatory mechanisms, we can begin to disentangle the evolution of these shared herbivore-microbe metabolisms. Ultimately, if we can identify key molecular mechanisms that are responsible for regulating shared amino-acid metabolisms, which are widespread in symbiont-enabled herbivory, we can determine if the evolution of these mechanisms impacts insect-plant interactions.

For the first part of this review we will discuss previous literature on epigenetic mechanisms in eukaryotic gene regulation, and how epigenetics are potentially involved in herbivore-microbe interactions. For the second part of this review we will discuss previous literature on regulatory RNAs and how they are potentially involved in the regulation of these insect-microbe interactions.

GENE REGULATION VIA EPIGENOMIC MECHANISMS

Under Darwinian natural selection random genetic mutations in the DNA molecule are inherited from parent to offspring, and increase in frequency within a population if a given mutation contributes to differential reproductive success (Dobzhansky, 1937). Alternative mechanisms of adaptation, such as Lamarkian inheritance, where an individual can pass down acquired traits that are obtained during its lifetime, have been hotly debated (Pilpel and Rechavi, 2015). Evolutionary theory that involves different variations of Lamarkian inheritance has been resurrected multiple times through history (Burkhardt, 2013). One controversial variation of Lamarkian inheritance, neo-Lamarkism (Skinner, 2015), has been proposed to explain epigenetic inheritance (Jablonka and Lamb, 2015) and CRISPR-*cas* immunity in Bacteria and Archaea (Koonin and Wolf, 2009), because these acquired traits are not random but are induced by the environment in a predictable fashion and are inherited through generations.

Epigenetic marks referred to here as chemical marks on DNA and histones are responsible for tissue-specific gene expression in eukaryotes and thus can lead to a change in an organism's phenotype (Gama-Sosa et al., 1983). If the environment induces epigenetic marks in a repeatable way and these marks are inherited across generations then there is potential for epigenetics to play important roles in organismal adaptation in natural populations (Gadjev, 2015), which can then affect the organism's interaction with other organisms. Therefore, we propose that epigenetic mechanisms may be important for the evolution of both insect-plant and insect-microbe interactions.

Patterns of DNA Methylation in Different Phyla

One type of epigenetic mark that is widespread and generally occurs across all domains of life is DNA methylation. DNA methylation involves the enzymatic addition of a methyl group to individual nucleotide bases of DNA in chromosomes by DNA methyltransferases (DMNTs). Now more than ever, DNA methylation has become more tractable to study due to the recent advancement in sequencing technology and bioinformatics. In turn the field of epigenomics is more accessible to researchers for both model and non-model organisms. As such the number of research articles on DNA methylation has been steadily increasing (Romanoski et al., 2015). For example, according to the Web of Science (2016), the number of epigenetic research articles has doubled every 5 years since 1971, reaching just over 110,000 articles between 2011 and 2015.

The role and patterns of DNA methylation vary widely among the three domains of life (Jeltsch, 2010). Several independent losses of DNA methylation have occurred in various eukaryotic taxa, suggesting that the role of DNA methylation may not be essential for all eukaryotic species (Field et al., 2004; Wion and Casadesús, 2006). For example, in the model systems *Caenorhabditis elegans* (roundworm) and *Drosophila melanogaster* (fruit fly) DNA methylation is not functional, because of lineage specific losses of DMNTs (Goll and Bestor, 2005). Nevertheless, DMNTs are present and DNA methylation is prevalent and functional in a wide diversity of other eukaryotic taxa including plants, vertebrates, and invertebrates (Jeltsch, 2010).

In vertebrates, including humans, cytosine methylation is widespread in the genome, specifically at cytosine-phosphate-guanine (CpG) dinucleotide sites (Bird, 1986). However, CpG-rich regions called CpG islands, which are typically 300- to 3000- base pairs long and are located primarily in the promoter regions of vertebrate genes, are largely un-methylated (Bird, 1986). Methylation of even a single CpG site in a promoter region can significantly inhibit transcription of the downstream genes (Robertson et al., 1995). Such transcriptional inhibition or gene silencing is an important role for DNA methylation as it also helps maintain the integrity of the genome by silencing transposable elements (Zamudio et al., 2015). DNA methylation also has a well-established role in imprinting, such as mammalian X-chromosome inactivation (Augui et al., 2011; Balaton et al., 2015), and differential expression of parental-specific alleles (Reik et al., 1987; Li et al., 1993; Razin and Cedar, 1994). Furthermore, DNA methylation in gene-body regions (e.g., un-translated regions, exons, and introns) can also affect the activity of genes in vertebrate genomes. For example, in human cell lines the inhibition of DNA methylation in gene-body regions resulted in the alternative splicing of exons (Maunakea et al., 2013).

In invertebrates, cytosine methylation also plays an important role in gene regulation. Epigenomic research in invertebrates initially lagged behind, because the two main invertebrate model species in genetics, *C. elegans* and *D. melanogaster*, do not have active copies of DNMTs (detailed above). Nevertheless, DNA methylation has been observed in a diversity of other invertebrate species. For example, in the Pacific oyster *Crassostrea gigas*, different levels of CpG methylation have been observed that correlate with gene functions (Gavery and Roberts, 2010). In the Chinese white shrimp, *Fenneropenaeus chinensis,* tissue-specific DNA methylation was observed (He et al., 2015). Furthermore, DNA methylation has been reported in many insect species of various orders including Diptera, Hemiptera, Hymenoptera, Lepidoptera, Coleoptera, Odonata, and Orthoptera (Field et al., 2004; Richards et al., 2008; Walsh et al., 2010; Xiang et al., 2010; Zhang J. et al., 2015; Zhang M. et al., 2015). In general, methylation levels of invertebrate CpG sites are relatively low, ranging from 0.36–20% (Regev et al., 1998), compared to mammalian systems where 60–90% of all CpG dinucleotides are subject to methylation (Suzuki and Bird, 2008).

Methylome studies across different invertebrate taxa have revealed that DNA methylation is often confined to genic regions (promoters, exons, and introns) of the genome, whereas intergenic regions remain largely unmethylated (Suzuki and Bird, 2008). In hymenopteran genomes, such as parasitoid wasps, ants, and bees, low levels of DNA methylation occur within transposable elements (TEs) compared to vertebrate genomes. These results suggest that DNA methylation has no or very little association with the repression of TEs as shown in vertebrates (Yan et al., 2015). DNA methylation within invertebrate genes has been associated with gene activation and alternative splicing. For example, loss of DNA methylation from multiple CpG sites within the insecticide-detoxifying esterase gene E4 of the green peach aphid *Myzus persicae* was associated with a reduction of transcription of the esterase gene E4, and thus increased sensitivity to pesticides (Field et al., 1989; Field, 2000). Also, several studies have proposed that DNA methylation is associated with alternative splicing of mRNA transcripts, which leads to behavioral regulation and caste specificity in eusocial insects including bees (Foret et al., 2012; Li-Byarlay et al., 2013), ants (Bonasio et al., 2012), and termites (Terrapon et al., 2014).

Insect DNA Methylation and Adaptation to Variable Environments

Mounting evidence from the handful of non-model animal systems that have been studied suggests that environmental cues can trigger the reprogramming of cells through DNA methylation, resulting in the regulation of adaptive traits (Kucharski et al., 2008; Moczek and Snell-Rood, 2008; Alvarado et al., 2015; **Table 1**). As such, differential methylation patterns have the potential to produce an adaptive regulatory response to current environmental conditions. Environmental signals such as diet, stress, and anxiety have been shown to alter DNA methylation patterns during an organism's lifetime (Weaver et al., 2004; Jankard and Herman, 2008; Schwenk et al., 2013). For example, in the honey bee nutritional cues from royal jelly regulate queen determination via epigenetic mechanisms. Specifically, the gene, *dynactin p62* is differentially methylated in queens compared to workers, and it is hypothesized to be a key gene in regulating different developmental pathways (Kucharski et al., 2008). In this study, when DNA methyltransferase 3 (*Dnmt3*) is silenced in larvae that feed on protein-rich royal

TABLE 1 | DNA methylation in various insects and its associated phenotypic effects.

Species	Common name	Phenotype	References
Acyrthosiphon pisum	Pea aphid	Color morph differentiation	Dombrovsky et al., 2009
Aedes aegypti	Mosquito	Wolbachia infection and gene transcription	Ye et al., 2013
Apis mellifera	Honeybee	Caste determination	Elango et al., 2009; Foret et al., 2012; Herb et al., 2012; Patalano et al., 2012
Apis mellifera	Honeybee	Learning and memory processing	Lockett et al., 2010; Biergans et al., 2012
Bombus terrestris	Bumblebee	Reproductive caste formation	Kankanamge and Eranthi, 2015
Bombyx mori	Silkworm	Immune response against bacterial infection	Xiang et al., 2010; Zhang Q. L. et al., 2015
Componotus floridanus	Florida carpenter ant	Caste determination	Bonasio et al., 2012
Coptotermes formosanus	Subterranean termite	Gene regulation	Glastad et al., 2012
Lucusta migratoria	Migratory locust	Alternative migratory phenotypes	Robinson et al., 2015
Medauroidea extradentata	Stick insect	Gene regulation	Krauss et al., 2009
Myzus persicae	Peach-potato aphid	Upregulation of insecticide detoxifying esterases	Field et al., 1989; Hick et al., 1996; Field and Blackman, 2003
Nasonia vitripennis	Jewel wasp	Photoperiodic response on diapause	Werren et al., 2010; Park et al., 2011; Pegoraro et al., 2016
Nasonia vitripennis	Jewel wasp	Embryo development	Zwier et al., 2012
Nilaparvata lugens	Brown planthopper	Female fecundity	Zhang J. et al., 2015
Onthophagus sp.	Horned Beetle	Nutritional plasticity	Snell-Rood et al., 2012
Pogonomyrmex barbatus	Red harvester ant	Caste determination	Smith et al., 2012
Reticulitermes flavipes	Subterranean termite	Gene regulation	Glastad et al., 2012
Schizaphis graminum	Greenbug aphid	Upregulation of insecticide detoxifying esterases	Ono et al., 1999
Sogatella furcifera	Rice planthopper	Sexual dimorphism	Zhang M. et al., 2015
Sogatella furcifera	Rice planthopper	Wing dimorphism	Zhou et al., 2013
Zootermopsis nevadensis	Dampwood termite	Caste differentiation	Terrapon et al., 2014

jelly these larvae develop into fertile queens with fully developed ovaries (Kucharski et al., 2008). This result indicates that a nutritional signal can alter epigenetic patterns resulting in caste determination. In another honeybee study, social stimuli of bees were highly correlated with changes in DNA methylation patterns between worker and nurse bees of the same age (Lockett et al., 2012). One particular CpG site in the gene Protein kinase C-binding protein 1 (*PKCbp1*) with variable levels of methylation between worker and nurse bees was strongly correlated with the alternative splicing of its gene product. The direct consequences of this alternative splicing however are unclear (Lockett et al., 2012). DNA methylation also plays a role in caste determination of another social hymenopteran, the carpenter ant *Camponotus floridanus*, by modifying ant body size, a key trait associated with the division of labor (Alvarado et al., 2015).

In the parasitoid wasp *Nasonia vitripennis* changes in photoperiod are hypothesized to induce genome-wide DNA methylation changes (Pegoraro et al., 2016). When day length is decreased female *N. vitripennis* wasps induce developmental arrest of their progeny (diapause). This photoperiodic response allows the larvae to survive throughout winter. Knock-down of either DNMT1a or DNMT3 in *N. vitripennis* parents disrupted the photoperiod-induced developmental arrest of their larvae. Although the exact mechanisms are yet to be elucidated, these results suggest that environmentally induced diapause in *N. vitripennis* are linked to DNA methylation.

In addition to hymenopterans, methylation contributes to adaptive regulatory responses to environmental conditions in aphids. In the green peach aphid, *M. persicae*, individuals resistant to an organophosphate pesticide encode a differentially methylated esterase gene that confers the resistant phenotype (Field et al., 1989, 1996; Hick et al., 1996). Biotypes of the Russian wheat aphid, *Diuraphis noxia*, that differ in virulence toward their host plant display differential methylation patterns for key genes that are expressed in their salivary glands, suggesting that methylation may play an important role in this insect's ability to feed on different host plant cultivars (Gong et al., 2012). In *A. pisum*, it has been shown that extreme temperatures may result in variation in DNA methylation patterns, which are correlated to different color phenotypes within genetic clones (Dombrovsky et al., 2009). This study revealed that the intensity of methylation in CpG-islands within aphid cuticular genes varied dramatically between three different *A. pisum* color morphs (white, pink, and green). Furthermore, the authors identified correlations between CpG island methylation and growth rate, morph development, and pigmentation of the aphid population by pharmacologically inhibiting the DNA methyltransferases (Dombrovsky et al., 2009). In sum, DNA methylation may help drive rapid and precise gene regulation to variable environmental conditions.

Role of DNA Methylation in Symbioses

In the sections above we detailed several examples of how environmental cues can induce specific DNA methylation patterns in insects, which can result in adaptive gene expression profiles. Although microbial associations are ubiquitous in many insect systems, as of yet, there has not been extensive research on how microbes affect insect host epigenomics. Nevertheless, we predict that epigenomics may play major roles broadly

in insect-plant and insect-microbe ecology and evolution. For example, insect microbial associations have facilitated numerous host plant niche expansions, and the diversification of insect lineages (Hansen and Moran, 2014). Moreover, insect symbionts can contribute to a variety of extended insect host phenotypes, which include: defense against viral pathogens, fungal pathogens, and parasitoids (Kaltenpoth et al., 2005; Oliver et al., 2005; Scarborough et al., 2005; Scott et al., 2008; Teixeira et al., 2008; Vorburger et al., 2009), conferring thermal tolerance (Dunbar et al., 2007; Brumin et al., 2011), facilitating food digestion (Brownlie et al., 2009; Salem et al., 2012), and manipulating sexual reproduction (Stouthamer and Werren, 1993). Currently, we are still in the discovery phase in identifying specific genetic mechanisms that facilitate these important microbe-induced, insect-extended phenotypes.

To the best of our knowledge the only studies that demonstrate an effect of microbes on insect epigenomics are of *Wolbachia* and mosquitos. *Wolbachia* is an intracellular bacterial symbiont that is both vertically and horizontally inherited in numerous insect species and commonly enhances its transmission through reproductive manipulations (Stouthamer and Werren, 1993). Nevertheless, mosquitos like *Drosophila,* do not have functional DNA methylation, because they do not encode DMNT1 and DMNT3 (Holt et al., 2002; Nene et al., 2007). However, they do encode the methyltransferase, DMNT2, which has substrate specificity for tRNAs (Goll et al., 2006), contributes to antiviral defense in *Drosophila* (Durdevic et al., 2013), and is involved in random genome methylation patterns (Kunert et al., 2003). In one study, when the pathogenic strain of *Wolbachia* (*w*MelPop) infects the mosquito, *Aedes aegypti*, the mosquito is hypomethylated because *Wolbachia* suppresses DNMT2 (Zhang G. et al., 2013). When DMNT2 is overexpressed in mosquito cell lines *Wolbachia* replication is inhibited, suggesting that suppression of DNMT2 is beneficial for *Wolbachia* survival. Conversely, in *A. aegypti* DNMT2 is induced by the Dengue virus, and this induction promotes Dengue virus replication. *In vivo* this antagonistic interaction ultimately results in *Wolbachia* suppressing the Dengue virus via DNMT2 suppression (Zhang G. et al., 2013). In another study, the *Wolbachia* strain *w*MelPop results in both the methylation and de-methylation of *A. aegypti's* genome (Ye et al., 2013). For the most part these changes in methylation primarily appeared to be random (Ye et al., 2013). In this study, the direct effect of differential methylation on transcription in *w*MelPop-infected compared to uninfected mosquitos remains unclear.

Given the paucity of evidence of symbionts affecting invertebrate host epigenetics and studies of insect hosts with functional DNA methylation systems investigating the effect of symbionts on vertebrate hosts may provide insights into possible ways symbionts may impact DNA methylation in insect genomes. In general, DNA methylation in vertebrate studies of gut-associated microbes has revealed that host immune responses and microbially derived metabolites affect host DNA methylation. For example, in mice Takahashi et al. (2011) found that the methylation level of the Toll-like receptor 4 gene in intestinal epithelial cells is significantly lower in germ-free mice compared to conventional mice. Moreover, results from this study suggests that this epigenetic modification is elicited by and important for the maintenance of commensal microbes in the gut. In another study, when the human pathogen *Helicobacer pylori* infects the human gut DNA methylation increases in the promoter regions of the human genes filamin C and thrombomodulin. This results in the silencing of these genes and a concomitant increase in the risk of gastric cancer (Nakajima et al., 2009). In another human microbiome study, an increase in abundance of two members of the human oral microbiome that belong to Enterobacteriaceae and Tenericutes is associated with the hypermethylation of the promoter regions of the human host gene *MDR1*. Hypermethylation of *MDR1* can result in head and neck squamous cell carcinoma (Bebek et al., 2012). In another study on host pathogens, pathogenic viruses including human adenovirus, hepatitis B virus and HIV are known to increase genome-wide levels of methylation of their host by up-regulating DNMT1 (Fang et al., 2001; Burgers et al., 2007; Jung et al., 2007).

In addition to pathogenic and non-pathogenic gut microbes mediating human immune responses through DNA methylation, microbe-derived metabolites can also influence DNA methylation in humans and ultimately impact expressed phenotypes. For example, nutritional uptake in early postnatal humans modifies the infant's gut microbiome, which in turn affects the epigenetic patterns of the individual (Mischke and Plosch, 2013). This study proposes that changes in the composition of the gut microbiome results in altered profiles of microbe-produced metabolites such as folate and short-chain fatty acids. The same study proposed that an increase in such metabolites may influence the DNA methylation patterns of adjacent intestinal cells, which in turn results in the predisposition to obesity.

Microbial symbionts and pathogens of humans and some insects have been demonstrated to alter patterns of DNA methylation. As such microbial symbionts have the capacity to (radically) alter host phenotypes. We hypothesize that this ability is widespread in insect symbionts, particularly among co-evolved insect symbionts. These intimate partners may influence methylation of their insect hosts with functional DNA methylation systems by modulating their host's immune responses to microbes. For example, attenuating immune responses so as to permit their intracellular persistence. In addition, these symbionts can encode novel biosynthetic pathways, which may contribute microbially derived metabolites, such as folate; folate is a key source of the one carbon group used to methylate DNA (**Table 2**). Moreover, in co-evolved insect symbioses tissue- specific, DNA-methylation patterns in specialized insect cells that harbor obligate symbionts may facilitate the development and regulation of this long-term symbiotic relationship. Nevertheless, our understanding of the development and regulation of these symbiotic cells in insects is still nascent (Braendle et al., 2003; Hosokawa et al., 2016). Therefore, by investigating if and how epigenetic modifications affect the regulation of insect-microbe interactions, we will gain a better understanding of key biological mechanisms in symbiosis and evolution in general.

TABLE 2 | Presence and absence of pathways from obligate symbionts of insects with fully sequenced genomes.

Insect host	Microbe symbiont	Folate biosynthesis	Methionine metabolism	Sulfur metabolism	Genome size (Mbp)
Coptotermes formosanus	*Azobacteroides pseudotrichonymphae*	Complete	Partial	Partial	1.22
Graphocephala atropunctata	*Baumannia cicadellinicola BGSS*	Complete	Absent	Complete	0.64
Camponotus chromaiodes	*Blochmannia chromaiodes*	Complete	Complete	Complete	0.79
Camponotus floridanus	*Blochmannia floridanus*	Complete	Complete	Complete	0.71
Camponotus pennsylvanicus	*Blochmannia pennsylvanicus*	Complete	Complete	Complete	0.79
Camponotus vafer	*Blochmannia vafer*	Complete	Complete	Complete	0.72
Heteropsylla texana	*Carsonella ruddii HT*	Absent	Absent	Absent	0.16
Heteropsylla cubana	*Carsonella ruddii HC*	Absent	Absent	Absent	0.17
Ctenarytaina spatulata	*Carsonella ruddii CS*	Absent	Absent	Absent	0.16
Ctenarytaina eucalypti	*Carsonella ruddii CE*	Absent	Absent	Absent	0.16
Pachypsylla venusta	*Carsonella ruddii PV*	Absent	Absent	Absent	0.16
Pachypsylla celtidis	*Carsonella ruddii PC*	Absent	Absent	Absent	0.16
Bemisia tabaci	*Portiera aleyrodidarum*	Absent	Absent	Absent	0.35
Acyrthosiphon pisum	*Buchnera aphidicola APS*	Partial	Absent	Complete	0.66
Acyrthosiphon kondoi	*Buchnera aphidicola AK*	Partial	Absent	Complete	0.65
Uroleucon ambrosiae	*Buchnera aphidicola UA*	Partial	Absent	Complete	0.63
Schizaphis graminum	*Buchnera aphidicola Sg*	Partial	Absent	Complete	0.64
Baizongia pistaciae	*Buchnera aphidicola Bp*	Partial	Absent	Absent	0.62
Cinara tujafilina	*Buchnera aphidicola Ct*	Absent	Absent	Absent	0.44
Aspidiotus nerii	*Uzinura diaspidicola*	Partial	Absent	Absent	0.26
Planococcus citri	*Tremblaya princeps*	Absent	Absent	Absent	0.14
Planococcus citri	*Moranella endobia*	Absent	Absent	Absent	0.54
Homalodisca vitripennis	*Sulcia muelleri Hc*	Absent	Absent	Absent	0.15
Diceroprocta semicincta	*Sulcia muelleri DSEM*	Absent	Absent	Absent	0.28
Clastoptera arizonana	*Sulcia muelleri CARI*	Absent	Absent	Absent	0.28
Megacopta punctatissima	*Ishikawaella capsulata*	Complete	Complete	Partial	0.75
Diceroprocta semicincta	*Hodgkinia cicadicola*	Absent	Partial	Absent	1.11
Graphocephala atropunctata	*Baumannia cicadellinicola BGSS*	Complete	Absent	Partial	0.76
Macrosteles quadrilineatus	*Nasuia deltocephalinicola*	Absent	Partial	Absent	0.11
Pediculus humanus	*Riesia pediculicola*	Complete	Absent	Absent	0.58
Blattella germanica	*Blattabacterium* sp. *Bge*	Complete	Partial	Partial	0.64
Mastotermes darwiniensis	*Blattabacterium* sp. *MADAR*	Complete	Absent	Partial	0.59
Cryptocercus punctulatus	*Blattabacterium* sp. *Cpu*	Complete	Absent	Absent	0.61
Blaberus giganteus	*Blattabacterium* sp. *BGIGA*	Complete	Partial	Absent	0.63
Panesthia angustipennis spadica	*Blattabacterium* sp. *BPAA*	Complete	Partial	Absent	0.63
Diaphorina citri	*Profftella armatura*	Absent	Absent	Partial	0.46
Glossina morsitans morsitans	*Wigglesworthia glossinidia*	Complete	Absent	Absent	0.72

"Complete" denotes that all of the enzymes for a particular pathway are encoded in the genome. "Partial" denotes that only some enzymes for a particular pathway are encoded in the genome. "Absent" denotes that no orthologs of the enzymes of a particular pathway are present in the genome. Information for gene presence and absence is based on GenBank, KEGG, and BioCyc data.

The selected pathways produce important microbially derived metabolites that may influence insect host DNA methylation patterns.

GENE REGULATION VIA SMALL RNAs

In recent years, there has been a rapid increase in the identification of non-coding, regulatory RNAs in Bacteria, Archaea, and Eukaryotes (Babski et al., 2014). With the improvement of sequencing technology it has been found that the non-coding RNAs expressed within in a cell include more than just the rRNAs, tRNAs, small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs). What has become clear is that these non-coding RNAs of varying sizes are important in a myriad of biological functions, which includes gene regulation. One large class of non-coding RNAs is regulatory small RNAs (sRNAs). sRNAs can be categorized depending on their function, structure, conservation among taxa, and/or size range (Kim et al., 2009; Waters and Storz, 2009). In general, the term sRNAs encompasses a large diversity of expressed RNAs that have various size cut offs depending on what domain you are studying (Babski et al., 2014). This review will broadly define sRNAs as regulatory elements that

are transcribed from coding and/or non-coding regions, which have both perfect and/or imperfect base-pairing interactions with their target RNA, and are <300 nt in length.

Bacterial sRNAs

In general, sRNAs fall into two categories: *cis*-encoded and *trans*-encoded sRNAs. Within bacteria, *trans*-encoded sRNAs are encoded at genomic locations that are distant from their target mRNA(s), as such they often have partial complementarity with their targets. *Trans*-encoded sRNAs are generally dependent on the RNA chaperone Hfq (Storz et al., 2011). Hfq proteins protect sRNAs from ribonuclease degradation and facilitate intermolecular contacts between sRNAs and their mRNA targets in the Enterobacteriaceae (reviewed in Vogel and Luisi, 2011; Sauer, 2014). Despite its important role, Hfq cannot be detected in some bacterial phyla including the Chlamydiae, Spirochaetae, Actinobacteria, Deinococcus-Thermus, Cyanobacteria, Chlorobi, and Bacteroidetes (Sun, 2002). In the Firmicutes, a gram-positive bacterial phylum, some species encode Hfq, however, unlike the Enterobacteriaceae, it does not play a major role in gene regulation (reviewed in Bouloc and Repoila, 2016). There is evidence that some of these gram-positive bacteria have analogous proteins that carry out similar roles to Hfq (reviewed in Durand et al., 2015). In many insect endosymbionts belonging to gram-negative bacteria, the homolog of Hfq has been lost (Sun, 2002). This suggests *trans*-encoded sRNAs are not expressed, and/or if they are expressed, other stabilizing mechanisms are employed (Hansen and Degnan, 2014).

Currently, *trans*-encoded sRNAs are the most extensively characterized sRNAs in bacteria. This is because of the historical difficulties that arose when trying to identify *cis*-encoded sRNAs (Georg and Hess, 2011). Improvements in sequencing technologies and methodologies, and bioinformatics prediction programs have led to an increase in the identification of candidate *cis*-encoded sRNAs, however validating the functional role of many of these accumulating candidates still lags behind (Barquist and Vogel, 2015). *Cis*-encoded sRNAs can be transcribed in the 5′ and/or 3′ regions of a coding sequence, within the coding sequence, or within the non-coding regions, and have perfect complementarity with their target RNAs. Many *cis*-encoded sRNAs in free-living bacteria have been observed to have a variety of roles, such as expression within mobile genetic elements like plasmids, transposons and phage, aiding in mRNA stability and degradation, and/or translational inhibition and attenuation (Wagner and Simons, 1994; Brantl, 2007). In addition, *cis*-encoded sRNAs have been identified to inhibit the synthesis of toxic proteins (Fozo et al., 2008).

Riboswitches are another common bacterial sRNA. Riboswitches are characterized by having complex folded domains—"the riboswitch," and they encompass a non-coding portion of the mRNA that binds to various metabolites (Nahvi et al., 2002; Winkler et al., 2002; Lai, 2003; Winkler and Breaker, 2003). In the presence of specific metabolites, riboswitches undergo a conformational change which then influences transcription, translation, or other processes related to protein production (Mandal and Breaker, 2004). Early surveys of riboswitches found that within many gram-positive bacteria such as the Firmicutes, riboswitches tended to modulate transcriptional attenuation, whereas in gram-negative bacteria such as the Proteobacteria, many of the riboswitches identified regulated translational attenuation (Nudler and Mironov, 2004; Barrick and Breaker, 2007). However, as riboswitches are described in more species, the regulation of the riboswitch seems to be more closely associated to ligand/type of riboswitch (Ray and Chakdar, 2015).

Eukaryotic sRNAs

In general, eukaryotic sRNAs can be divided into two families: micro RNAs (miRNAs) and small-interfering RNAs (siRNAs). Within eukaryotes, the RNAi pathway is responsible for the regulation of a diversity of endogenous genes via miRNAs, and for protecting the organism from invasive genetic material, such as viruses and transposable elements via siRNAs (Hannon, 2002). These sRNAs act as template RNA that bind to the RNA-induced silencing complex (RISC), and degrade complementary RNA sequences (Hannon, 2002). This pathway is conserved across most eukaryotes, however it has been lost in some fungal lineages and, notably, in the model system, *Saccharomyces cervisiae* (Billmyre et al., 2013).

miRNAs are ∼21–24 nucleotides in length and are transcribed from genes or within introns and subsequently form hairpin structures that are cleaved to result in the final mature miRNA (Ha and Kim, 2014). In animals, miRNAs share partial sequence complementarity to multiple target mRNAs, much like bacterial *trans*-encoded sRNAs. Plant miRNAs on the other hand have high sequence complementarity (Jones-Rhoades et al., 2006). Among plants and animals, miRNAs mediate post-transcriptional gene regulation in various shared aspects of physiology and development (Flynt and Lai, 2008; de Lima et al., 2012; Vidigal and Ventura, 2015).

Unlike miRNAs, siRNAs are ∼21 nt long and have endogenous or exogenous origins such as transposons and viruses. Furthermore, they are formed from double-stranded RNAs (Carthew and Sontheimer, 2009; Piatek and Werner, 2014). Also unlike miRNAs, siRNAs generally share perfect complementarity with their targets. Animal siRNAs are important in regulating transposons, heterochromatic sequences, intergenic regions, long RNA transcripts with extensive structure, and mRNAs (Ghildiyal and Zamore, 2009). It has been found that many expressed endo-siRNAs have vital roles in animal development (reviewed in Ghildiyal and Zamore, 2009). Within plants, there are three types of endo-siRNAs: *cis*-acting siRNAs (casiRNAs), *trans*-acting siRNAs (tasiRNAs), and natural antisense transcript-derived siRNAs (natsiRNAs; reviewed in Ghildiyal and Zamore, 2009; Axtell, 2013; Pattanayak et al., 2013). In general these endo-siRNAs are important in methylation (Mette et al., 2000; Llave et al., 2002; Tran et al., 2005), development (Allen and Howell, 2010), modulation of the plant's responses to stress (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Borsani et al., 2005; Fujii et al., 2005) and plant immunity (Katiyar-Agarwal et al., 2006).

In both plants and animals, miRNAs and siRNAs share biosynthesis pathways that include RNA endonucleases

Argonaute proteins and proteins Drosha and Dicer (Carthew and Sontheimer, 2009; Piatek and Werner, 2014). Though the genes involved in the biosynthesis of these sRNAs may share a common ancestor (Shabalina and Koonin, 2008), plant and animal sRNAs differ in biogenesis, function, and subsequent evolutionary trajectories (Shabalina and Koonin, 2008; Axtell et al., 2011; Mukherjee et al., 2013).

Evolution of Small RNAs in Reduced Genomes

Intracellular symbionts, pathogens, and organelles are subject to repeated genetic bottlenecks, which contribute to a suite of associated genome-wide changes including genome-size reduction. This evolutionary constraint also results in biases in nucleotide composition (increased %A+T), elevated mutation rates and the fixation of deleterious and neutral mutations due to genetic drift, ultimately resulting in gene inactivation and loss (Moran et al., 2009; Moran and Bennett, 2014). Moreover, selection becomes less effective in maintaining the mechanisms of gene regulation in reduced genomes compared to free-living relatives with larger genomes and access to more diverse environments (Lambert and Moran, 1998; Hansen and Moran, 2012; Hansen and Degnan, 2014). Thus, over evolutionary time, genome sizes tend to shrink dramatically, losing numerous functional capabilities and becoming structurally rearranged compared to free-living relatives (Moran and Bennett, 2014).

Despite these dramatic changes, reduced genomes still display strong purifying selection for key genes that are essential for the host-microbe relationship, including core housekeeping genes (Moran et al., 2009; Williams and Wernegreen, 2011). Among these are genes encoding biologically active non-coding RNAs, such as tRNAs and rRNAs that display higher %G+C composition relative to coding sequences, and accumulate compensatory base substitutions to maintain important secondary structural regions (e.g., stems; Lambert and Moran, 1998; Hansen and Moran, 2012). The increased genome wide %A+T composition in reduced genomes results in numerous polyA and polyT homopolymers. Such homopolymers are readily subject to insertions or deletions (indels) that can disrupt promoters or result in non-functional proteins because of frame shifts (Dunbar et al., 2007). These indels can initiate the processes of genome erosion (Moran et al., 2009) but in several instances transcriptional slippage can occur at these polyA/T tracts, restoring the reading frame of the mRNA and yielding functional proteins (Tamas et al., 2008; Wernegreen et al., 2010). Importantly, transcriptional slippage has been: (i) observed in symbionts with reduced genomes, (ii) has been maintained over evolutionary time, and (iii) functionally has been shown to rescue the activity of important genes for the symbiont's lifestyle (Tamas et al., 2008; Wernegreen et al., 2010). Collectively these patterns indicate that even though these reduced genomes experience severe genetic drift, purifying selection is strong enough to maintain and/or co-opt compensatory mechanisms to maintain fundamental processes for the persistence of microbe lineages.

Thus, we hypothesize that bacterial symbiont genomes subject to radical gene loss and structural rearrangements over evolutionary time can compensate for the loss of *essential* regulatory machinery by maintaining and/or co-opting compensatory mechanisms, such as sRNAs to aid in the regulation of vital symbiotic genes and core housekeeping processes, as suggested in Hansen and Degnan (2014).

sRNAs in Bacteria and Eukaryotes have been associated with rapid diversification and adaptation of lineages to local environments (Horler and Vanderpool, 2009; Yu et al., 2010; Ames and Lovell, 2011; He et al., 2011; Raghavan et al., 2012). It has been suggested that sRNAs can rapidly evolve from non-adaptive transcripts (Raghavan et al., 2012). Non-adaptive transcripts, i.e., transcriptional noise, can result when RNA polymerase accidentally recognizes non-promoter sequences as promoter sequences, because promoters contain low information content (Mendoza-Vargas et al., 2009; Raghavan et al., 2012). In reduced genomes that experience severe genetic drift, it is unclear how novel regulatory sRNAs could evolve. However, given that purifying selection has been identified in these reduced genomes for both coding and non-coding functional RNA sequences (Lambert and Moran, 1998; Moran et al., 2009; Williams and Wernegreen, 2011; Hansen and Moran, 2012), we hypothesize that purifying selection is strong enough to maintain and/ or co-opt emergent regulatory sRNAs that are essential for the symbiont's gene regulation. The failure to maintain such essential sRNAs would be lethal for the microbe and in turn its host, if other host and/or microbe factors failed to compensate.

One example of how reduced genomes compensate to maintain essential gene expression processes comes from previous research on *Buchnera* from several diverse aphid species (Hansen and Moran, 2012). In this study, the authors revealed that *Buchnera* tRNA genes in four divergent taxa are often shorter in length than their homologs in *E. coli*, *Buchnera*'s distant free-living relative. The difference in length is typically three nucleotides, and mostly reflects the loss of the encoded 3′ CCA sequence in the *Buchnera* tRNA genes. At the 3′ end of tRNAs, the nucleotide sequence CCA is required for amino acid activation and must either be encoded in the tRNA gene or added during tRNA maturation by the CCA-adding enzyme (*cca*). In *Buchnera*'s close relatives, such as *E. coli*, *Vibrio* spp., and *Pseudomonas* spp., 3′ CCA is encoded in all tRNA genes that are present in *Buchnera*; most likely to maintain efficient translation. Nevertheless, only half of *Buchnera* tRNA genes encode 3′ CCA (Hansen and Moran, 2012). Importantly, using directional RNAseq, Hansen and Moran (2012) found that mature RNA transcripts of these genes possess a CCA at the 3′ end, implying CCA-addition and thus activation of amino acids. The role of the CCA-adding enzyme in *E. coli* and other organisms that already encode CCA in their tRNA gene sequences is to monitor the stability of tRNAs by tagging unstable tRNAs with an additional CCA for degradation (Wilusz et al., 2011; Kuhn et al., 2015). In turn, this study revealed that *Buchnera* is compensating for the loss of CCA in the tRNA gene sequence by co-opting the CCA-adding enzyme. If *Buchnera* did not co-opt this regulatory machinery for this additional role, which is not observed in *Buchnera*'s closest free-living relatives, then the symbiont would

not be able to translate proteins. Moreover, the authors observed numerous compensatory base substitutions in tRNA stems and high %GC in tRNA genes compared to coding sequences that help maintain tRNA secondary structure and function. In sum, these results provide evidence that purifying selection is strong enough in reduced genomes to (1) co-opt existing machinery to compensate for core regulatory processes and (2) maintain existing machinery to conserve essential regulatory functions.

sRNAs in Organelle Genomes

Organelles represent the most dramatic example of tiny genomes that express regulatory sRNAs. Within eukaryotes there are two general types of organelles that have a unique evolutionary history with a microbial origin, such as mitochondria and plastids (Smith and Keeling, 2015). Mitochondria and plastids arose from bacteria that were once endosymbionts in early host eukaryotic cells (reviewed in Katz, 2012). In lineages that harbor both types of organelles, acquisition of plastids occurred after the early mitochondrial-eukaryotic lineage diversification (Gould et al., 2008). Mitochondria are believed to have evolved from a single endosymbiotic event involving an Alphaproteobacterium most likely from a Rickettsiales ancestor (Williams et al., 2007). Plastids on the other hand have a more complicated evolutionary history. All plastids evolved from the primary endosymbiosis of a cyanobacterium; however, across lineages there have also been secondary and tertiary endosymbiotic events (Gould et al., 2008). Currently, there is a growing body of research focused on organelle-associated sRNAs and their regulation (**Table 3**). Overall there is an emerging trend that gene regulation within the organelle is controlled by both host nuclear-encoded sRNAs and organelle-encoded sRNAs.

In the early 2000's it was thought that few if any sRNAs were expressed within organelle genomes (e.g., Lung et al., 2006). However, the identification of sRNAs has increased rapidly over the last decade for uncultivable organelles and microbes, because of lower sequencing costs and advances in sequencing technology (Hotto et al., 2011; Wang L. et al., 2011; Ro et al., 2013; Wu et al., 2015). There are two types of sRNAs identified within organelles: nuclear-encoded sRNAs, that are eukaryotic-like in origin and organelle-encoded sRNAs, that are bacterial-like in origin. Currently, the method by which nuclear-encoded sRNAs are transported into the organelle is unclear. However, there is evidence to suggest that tRNAs and 5S rRNAs can be imported into mammalian mitochondria and indirect evidence that tRNAs also can be imported into plastids (Schneider, 2011). In turn, similar pathways may be used for sRNA uptake into organelles. It is important to note that not only are nuclear-encoded sRNAs present within organelles, but argonaute (specifically, Ago2), the nuclear-encoded protein of the RISC complex, is also present within mitochondria (Bian et al., 2010; Bandiera et al., 2011). The presence of Ago2 and both nuclear-encoded and organelle-encoded sRNA within organelles supports the hypothesis that there is a complex interplay of gene regulation between the host and the organelle.

In mammals, besides providing energy for cellular functioning, mitochondria are also important in apoptosis (Lee et al., 2004; Suen et al., 2008), calcium concentration regulation (O'Rourke, 2004; Chen et al., 2005), and reactive oxygen species production (Chen et al., 2003). As such, in mammals, mitochondrial dysfunction has been linked to various diseases (Au et al., 2005; Baloyannis, 2006; Lemieux et al., 2010; Ritov et al., 2010; Gong et al., 2011). Some mitochondrial disease phenotypes are associated with the dysregulation of mitochodrial sRNAs (mitomiRs; **Table 3**). In general, there is less work reported in plant organelle sRNAs (Budak and Akpinar, 2015). Wang L. et al. (2011) is one of the few studies that show that chloroplast-encoded sRNAs can respond to environmental changes in the Chinese cabbage (*Brassica rapa* ssp. *Chinensis*). From these studies, we can see a potential trend emerging that organelle sRNAs expressed either from the organelle genome or the nuclear genome of their hosts are important for the regulation of these small, eroded genomes.

sRNAs in Reduced Bacterial Genomes

Overall, organelles, especially animal mitochondria, share many genomic characteristics to endosymbionts: organelles have reduced genomes when compared to free living relatives (Green, 2011; Gray, 2012), are generally low in GC content (McCutcheon and Moran, 2012; Smith, 2012), and their operons are highly fragmented (Barbrook et al., 2010; Brinza et al., 2010; Hansen and Degnan, 2014). Unlike organelles, most intracellular symbionts and pathogens with reduced genomes have not transferred regulatory genes to their host's chromosomes and still have autonomous housekeeping functions (Bennett and Moran, 2013).

Within bacterial genomes, operon structure, regulatory proteins, and sigma factors govern gene regulation. Organisms that possess reduced genomes, such as intracellular pathogens and mutualistic symbionts have lost many of these hallmark regulatory features (e.g., *Mycoplasma* sp. and *Buchnera*; Dandekar et al., 2000; Shigenobu et al., 2000). Within the human pathogen, *Mycoplasma pneumoniae*, it has been revealed that ∼13% of coding genes have a corresponding antisense RNA (Güell et al., 2009). The function of a few of these antisense sRNA candidates has been determined and have been shown to down-regulate their target gene (Güell et al., 2009). Two of these sRNAs, NEW87 and NEW8, potentially have roles in metabolism and DNA repair and replication, respectively (Güell et al., 2009). Several of these antisense sRNAs are conserved in the closely related species *Mycoplasma genitalium*, however, the functionality of these sRNA transcripts including NEW87 and NEW8 have not yet been validated (Lluch-Senar et al., 2007). There is evidence that suggests that many of the transcripts that are found within reduced bacterial genomes are simply the by-product of transcriptional noise (Raghavan et al., 2012; Llorens-Rico et al., 2016), highlighting the importance of validating the function of identified sRNAs.

Another type of intracellular parasite, *Rickettsia,* is an intracellular alphaproteobacterial endosymbiont that is found widely within arthropod lineages. The most widely studied *Rickettsia* species cause vertebrate diseases such as Typhus and Rocky Mountain spotted fever (Weinert et al., 2009). These pathogenic species also spend at least part of their lifecycles in arthropod vectors. A recent study observed sRNA expression within 13 *Rickettsia* species and identified 1785 novel sRNAs

TABLE 3 | Summary of studies isolating mitochondrial/plastid-encoded sRNAs and nuclear-encoded sRNAs found within organelles.

Study	Organism	Organelle	sRNAs identified
ORGANELLE-ENCODED sRNAs			
Lung et al., 2006	*Nicotiana tabacum*; Leaves	Plastid	*Ntc-1, Ntc-2, Ntc-3, Ntc-4, Ntc-5, Ntc-6, Ntc-7, Ntc-8, Ntc-9, Ntc-10, Ntc-11, and Ntc-12*
	Mouse liver and kidney	Mitochondria	*Mt-1, Mt-2, Mt-3, Mt-4, Mt-5, and Mt-6*
Mercer et al., 2011	Human 143B cells	Mitochondria	31 (26 mapping to tRNAs)
Smalheiser et al., 2011	Mouse hippocampus	Mitochondria	18 (9 mapping to a tRNA)
Sripada et al., 2012	HEK293 and HeLa	Mitochondria	miR-4461, miR-4463, miR-4484, miR-4485, and 7 punitive miRNA
Ro et al., 2013	Human	Mitochondria	2540 miRNAs
	Mouse	Mitochondria	1499 miRNAs
Zhou et al., 2014	*Laodephax striatellus*	Mitochondria	3977 [mRNAs (1546), tRNAs (308), and rRNAs (2091)]
Wu et al., 2015	*Silene noctiflora*	Mitochondria	9 miRNAS
NUCLEAR-ENCODED sRNAs ISOLATED WITH MITOCHONDRIA			
Kren et al., 2009	Rat liver	Mitochondria	*mirR-130a/b, mirR-140, mirR-320, mirR-494, mirR-671, mirR-202, mirR-763, mirR-198, mirR-765, mirR-705, mirR-709, mirR-721, and mirR-761*
Bian et al., 2010	mouse liver	Mitochondria	Top 20 expressed: *miR-122, miR-805, miR-690, miR-494, miR-705, miR-721, miR-720, miR-188-5p, miR-101, miR-let-7f, miR-711, miR-432, miR-181b, miR-361-5, miR-680, miR-181d, miR-29c, miR-29a, and miR-762*
Barrey et al., 2011	Human skeletal muscle myoblasts	Mitochondria	*miR-720, miR-133b, miR-1974, miR- 24, miR-133a, miR-125a-5p, miR-1979, miR-103, miR-125b, miR-103, miR-221, miR-23a, miR-let-7b, miR-423-3p, miR-106a, miR-23b, miR-92a, miR-193b, and miR-365*
Bandiera et al., 2011	HeLa cells	Mitochondria	*miR-1973, miR-1275, miR-494, miR-513-a-5p, miR-1246, miR-328-5p, miR-1908, miR-1972, miR-1977, miR-638, miR-1974, miR-1978, and miR-1201*
Sripada et al., 2012	HEK293 and HeLa	Mitochondria	209 nuclear coded with punitive and 230 references in miRBase

KNOWN FUNCTIONS OF NUCLEAR SRNAS ON MITOCHONDRIAL BIOLOGY AND METABOLISM		
Study	**Organelle process**	**sRNA**
Aoi et al., 2010; Li et al., 2010; Wang J.-X., et al., 2011; Wang et al., 2012; Yamamoto et al., 2012; Kang et al., 2013; Long et al., 2013; Zhang Y. et al., 2013; Li J. et al., 2014; Li X. et al., 2014; Tak et al., 2014	Mitochondrial fission and biogenesis	*miR-696, miR-30, miR-499, miR-484,* miR-494, *miR-761, miR-106b, miR-140, miR-196, and miR-27a/b*
Zhu et al., 2009; Zhang et al., 2010; Frankel et al., 2011; Xiao et al., 2011	Mitophagy	*miR-101, miR-204, miR-30a, and miR-21*
Aschrafi et al., 2008; Gao et al., 2009; Fang et al., 2012; Jiang et al., 2012; Latronico and Condorelli, 2012; Sun et al., 2012; Bienertova-Vasku et al., 2013; Das et al., 2014; Tomasetti et al., 2014	Mitochondrial metabolism	*miR-155, miR-143, miR-326, miR-124, miR-137, miR-340, miR- 183, miR-743a, miR-181c, miR-210, miR-338, miR-14, miR-15b, miR-16, miR-195, miR-424 miR-338, and miR-23a/b*

Nuclear-encoded sRNAs have been found to affect various aspects of organelle biology. Included are examples of the sRNAs that regulate genes affecting mitochondrial biogenesis and fusion, mitophagy, and metabolic functions.

(Schroeder et al., 2015). The number of sRNAs identified within each species varied from 15–191 and there was no correlation with genome size and number of sRNAs identified (Schroeder et al., 2015).

Wolbachia, another intracellular alphaproteobacterium, which is closely related to *Rickettsia*, is found within many insect taxa and other invertebrates (Hilgenboecker et al., 2008). It has varied effects on its host, such as reproductive manipulation (Werren et al., 2008), inhibition of vector-bone pathogen transmission (Moreira et al., 2009; Bian et al., 2013), and as a symbiont providing vitamins to its host (Hosokawa et al., 2010). When *Wolbachia* parasitically infects mosquito (*A. aegypti*) cell lines, it has been found to express sRNAs that potentially increase bacterial fitness by upregulating host genes that help facilitate its own replication (Mayoral et al., 2014a). It has been found that *Wolbachia* infection of *A. aegypti* results in differential expression of host miRNAs compared to uninfected control mosquitos (Hussain et al., 2011; Mayoral et al., 2014b). Hussain et al. (2011) identified that the host sRNA, aae-miR-2940, which upregulates a metalloprotease, is necessary for successful *Wolbachia* infection.

Until recently, there was little evidence that gene regulation occurred within unculturable mutualistic endosymbionts. It has been hypothesized that symbionts like *Buchnera,* display minimal gene regulation because they live in a stable intracellular environment (Shigenobu et al., 2000). *Buchnera* microarray experiments have provided limited evidence that this endosymbiont is able to regulate the transcription of genes underlying essential amino-acid (EEA) pathways in response to aphid nutritional demand (Moran et al., 2003, 2005; Reymond

TABLE 4 | Outstanding questions in regard to the relative importance of regulatory mechanisms that are key to shared herbivore-microbe metabolisms.

EPIGENETIC REGULATORY MECHANISMS
Can co-evolved insect symbionts modulate their host's immune responses toward them by influencing their host's methylation patterns in symbiotic host cells?
Can insect symbionts modulate their host's DNA-methylation patterns by producing folate and/or methy-groups for their host?
Is DNA inside of specialized insect host cells that harbor symbionts (e.g., bacteriocytes) differentially methylated compared to DNA in other body tissues? If so, is this linked to gene expression patterns inside of these specialized cells?
When insect herbivores feed on different host plants do different DNA methylation profiles result in specialized insect symbiont cells? If so, are these inherited and thus can they dynamically influence host plant adaptation?
Are methylation patterns conserved in specialized insect symbiont cells for insect orthologs that are hypothesized to play a conserved core role in the shared insect-microbe metabolism?
Can environmental stimuli, such as host plant nutritional quality, trigger a regulated metabolic response via DNA methylation to compensate for deficient nutrients?
sRNA REGULATORY MECHANISMS
Are post-transcriptional gene expression processes widespread in obligate symbionts of sap-feeding insects?
In addition to *Buchnera*, are conserved sRNAs expressed in other obligate symbiont lineages of sap-feeding insects?
What are the functions of conserved sRNAs in obligate symbionts of sap-feeding insects? Are they important in the regulation of essential amino acid pathways and if so can they respond to aphid nutrient demand?
How do sRNAs evolve in reduced bacterial genomes?

et al., 2006; Viñuelas et al., 2011). In turn, *Buchnera*'s gene regulation at the RNA level is generally assumed to be non-existent (Hansen and Moran, 2014). However, a recent study showed that *Buchnera* exhibits differential expression of 80 proteins between two different *Buchnera* life-stages, including proteins that are involved in essential amino acid biosynthesis with no concomitant changes in mRNA expression suggesting that post-transcriptional regulation was occurring (Hansen and Degnan, 2014). To support this hypothesis, Hansen and Degnan (2014), also found that across highly divergent *Buchnera* lineages, there is widespread conservation of *cis*- and/or *trans*-encoded sRNAs. They also revealed that of the 80 differentially expressed proteins identified between the two *Buchnera* life stages, 86% have evidence of possible *cis*-acting regulatory sRNAs (Hansen and Degnan, 2014). Many of the differentially expressed proteins in EEA pathways also had *cis* sRNAs identified within their transcriptional unit or flanking them (Hansen and Degnan, 2014). These observations led the authors to hypothesize that these patterns in differential protein expression may result from post-transcriptional regulation, such as conserved sRNAs (Hansen and Degnan, 2014). Based on this study's results, we predict that these novel sRNAs potentially have important regulatory roles, especially for *Buchnera's* EAA pathways.

In summary, there is growing evidence that both organelles and bacteria with highly reduced genomes, have maintained key sRNAs to aid in the regulation of genes that are vital to their core biological functioning. Organelles have not only evolved mechanisms to uptake vital eukaryotic nuclear encoded sRNAs but also the necessary machinery (Ago2) to process these sRNAs. There is evidence that shows that bacteria can produce sRNAs that target eukaryotic genes, suggesting that these sRNAs either mimic eukaryotic sRNAs or can be co-opted into the RNAi pathway. This type of trans-kingdom communication via sRNAs has also been observed in fungal plant pathogens (Weiberg et al., 2013) and the malarial parasite (LaMonte et al., 2012). There is also evidence demonstrating that organelles have maintained sRNAs within their highly eroded bacterial genome. Within endosymbionts, there is evidence that sRNAs are important for regulating functions important in maintaining the symbiosis with their host. Overall, these examples of sRNA utilization supports the hypothesis that within organisms that have undergone radical gene loss, sRNAs may aid in the regulation of vital symbiotic genes and core housekeeping processes.

FUTURE DIRECTIONS AND CONCLUSIONS

Recent studies on epigenomic and small-RNA regulation in eukaryotes and bacteria have challenged historical preconceptions of how these genomes are regulated and evolve. The implications of these new studies on the understanding of the regulation of shared animal-microbe metabolic processes warrant further investigation in future studies. Such studies are critical because of the importance these regulatory mechanisms have in the evolution and maintenance of these widespread herbivore symbioses. To this end, we conclude this review by proposing future directions that will aid in teasing apart the regulatory underpinnings and dynamics of shared metabolic processes that are ubiquitous between insect herbivores and their microbial symbionts.

Epigenomics in Insect-Plant Interactions

Elucidating the mechanisms and patterns of DNA methylation and its inheritance is important for understanding the molecular biology, ecology, and the evolution of non-model animals. For example, methylation patterns are linked not only to numerous human diseases (Robertson, 2001; Richardson, 2003; Lund et al., 2004; Stenvinkel et al., 2007; Mastroeni et al., 2010; Benton et al., 2015) but also to insect and mammalian behavior (Kucharski et al., 2008; Dias and Ressler, 2014; Alvarado et al., 2015), and pesticide resistance in insects (Field et al., 1989, 1996; Hick et al., 1996; **Table 1**). Some of these epigenetic-associated phenotypes are inherited from parent to offspring (Dias and Ressler, 2014), which influences current views of the mechanisms of evolution, particularly the central dogma (Szyf, 2014). CpG

DNA methylation is present in divergent eukaryotic taxa such as fungi, animals, and plants (Wang et al., 2006; Feng et al., 2010; Zemach et al., 2010), and therefore its effects on gene regulation and organismal ecology and evolution are of broad interest to science in general.

Aphids are an attractive and tractable model for studying epigenetic regulation of shared herbivore-microbe metabolisms. Aphids display functional DNA methylation (Walsh et al., 2010) in contrast to many classical model organisms (e.g., *D. melanogaster, S. cervisiae*, *C. elegans*; Goll and Bestor, 2005; Feng et al., 2010). Moreover, *A. pisum* can be reared in culture, maintained asexually or sexually, have short generation times, have co-evolved microbial symbionts, and exhibit a wide range of host plant interactions. Thus, aphids are ideally suited for characterizing novel aspects of epigenetic regulation. Results produced from this research can thus be important for understanding insect nutrition specifically, as well as the ecology and evolution of insect-host plant interactions and symbiosis more generally. In addition, many obligate symbionts in insect systems produce folate and/or other methyl- groups, which may influence insect host DNA-methylation patterns (**Table 2**). In turn, more work is needed on the epigenetics of animal-microbe interactions, especially for non-classical model insect systems. Currently, many of the outstanding questions on this topic (**Table 4**) can now be addressed using cutting-edge molecular genetics, genomics, and bioinformatics tools.

Small RNAs in Insect-Plant Interactions

Insect taxa in several of the most diverse insect orders are known to harbor obligate symbionts with reduced genomes. Most of these symbionts have been characterized in blood and sap-feeding insects; the smallest known bacterial genome is a symbiont from a sap-feeding insect (Bennett and Moran, 2013). Similar to organelles, such as mitochondria, these obligate symbiont genomes are greatly reduced due to genetic drift, primarily encode genes essential for their host, are unculturable, and have lost most genes for transcriptional regulation. Nevertheless, unlike organelles these symbionts encode core housekeeping genes, and in general none of its genes were transferred to the host's chromosome (Bennett and Moran, 2013). To this end obligate symbionts like the model *Buchnera* are still autonomous bacterial cells, however some of these minimal genomes can regulate gene expression at the post-transcriptional level (Hansen and Degnan, 2014). Potentially in these small genomes the loss of canonical regulatory proteins has resulted in the evolution of compensatory regulatory mechanisms, such as small regulatory RNAs (Hansen and Degnan, 2014). In sum, if these genomes rely primarily on small RNAs for gene regulation, instead of proteins, this could be a prime example of how genomes revert to the "RNA world" for gene regulation. More functional and comparative genomic studies using novel manipulative techniques on unculturable microbes are required to further understand the putative regulatory role of sRNAs in symbionts with reduced genomes. Ultimately, these future studies will determine the relative importance of these microbial regulatory mechanisms in these intimate symbioses. Outstanding questions on this topic are presented in **Table 4** and can now be addressed using classical microbiology techniques, cutting-edge genomics, and bioinformatics tools.

AUTHOR CONTRIBUTIONS

DK, MT, and AH all substantially wrote and edited the manuscript.

ACKNOWLEDGMENTS

We would like to thank Patrick Degnan, Amanda Adams, Katie Frye, and two reviewers for their helpful comments and suggestions on earlier drafts and the University of Illinois at Urbana-Champaign for funding.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 09 August 2016
doi: 10.3389/fpls.2016.01163

Vector-Borne Bacterial Plant Pathogens: Interactions with Hemipteran Insects and Plants

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Hemipteran insects are devastating pests of crops due to their wide host range, rapid reproduction, and ability to transmit numerous plant-infecting pathogens as vectors. While the field of plant–virus–vector interactions has flourished in recent years, plant–bacteria–vector interactions remain poorly understood. Leafhoppers and psyllids are by far the most important vectors of bacterial pathogens, yet there are still significant gaps in our understanding of their feeding behavior, salivary secretions, and plant responses as compared to important viral vectors, such as whiteflies and aphids. Even with an incomplete understanding of plant–bacteria–vector interactions, some common themes have emerged: (1) all known vector-borne bacteria share the ability to propagate in the plant and insect host; (2) particular hemipteran families appear to be incapable of transmitting vector-borne bacteria; (3) all known vector-borne bacteria have highly reduced genomes and coding capacity, resulting in host-dependence; and (4) vector-borne bacteria encode proteins that are essential for colonization of specific hosts, though only a few types of proteins have been investigated. Here, we review the current knowledge on important vector-borne bacterial pathogens, including *Xylella fastidiosa*, *Spiroplasma* spp., *Liberibacter* spp., and '*Candidatus* Phytoplasma spp.'. We then highlight recent approaches used in the study of vector-borne bacteria. Finally, we discuss the application of this knowledge for control and future directions that will need to be addressed in the field of vector–plant–bacteria interactions.

Keywords: vector-borne bacteria, vascular bacteria, phloem, xylem, plant–insect interactions, plant–microbe interactions, leafhoppers, psyllids

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *14 May 2016*
Accepted: *20 July 2016*
Published: *09 August 2016*

Citation:
Perilla-Henao LM and Casteel CL (2016) Vector-Borne Bacterial Plant Pathogens: Interactions with Hemipteran Insects and Plants. Front. Plant Sci. 7:1163. doi: 10.3389/fpls.2016.01163

INTRODUCTION

The plant vascular system is a rich source of nutrients and represents a transport pathway for colonizers. It consists of phloem and xylem tissues, two different host environments for plant pathogens. Phloem tissue consists of companion cells, providing metabolic and regulatory components to the phloem sap, and sieve elements, forming a long distance transport system throughout the plant (Lucas, 2006; Will et al., 2013). Because this specialized transport system offers access to a rich source of carbohydrates, proteins, and amino acids, numerous viral and bacterial microbes colonize the phloem specifically (Bove and Garnier, 2003; Lough and Lucas, 2006). In contrast, the xylem vessels mainly transport water and contain lower nutrient levels in comparison to the phloem (Bae et al., 2015). Despite the low nutrient content of xylem, plant pathogens have also been identified that can colonize the xylem (Purcell and Hopkins, 1996; Bae et al., 2015).

In addition to viral and bacterial microorganisms, macro-organisms also rely on the plant vascular system for their primary nutrient source. These include hemipteran pests, such as whiteflies, aphids, psyllids, and leafhoppers. Specialized mouthparts, known as stylets, allow hemipterans to penetrate the plant's epidermal tissues and reach their preferred tissue. Some hemipterans feed from the mesophyll and vascular system, while others only probe the mesophyll and feed exclusively from the phloem or xylem. As a result of this specialized feeding, hemipterans interact with microbes colonizing the plant vascular system and can serve as vectors. A vector is the specific organism that transmits a pathogen (Purcell, 1982) and hemipteran insects are by far the most important vectors of plant-infecting pathogens (Nault and Ammar, 1989; Orlovskis et al., 2015).

While the interactions between plant–pathogenic viruses and their hemipteran vectors have been studied in depth, far less is known about the interactions between plant–infecting bacteria and their hemipteran vectors (Ng and Falk, 2006; Hogenhout et al., 2008a; Walling, 2008; Ammar et al., 2011; Blanc et al., 2011, 2014; Gray et al., 2014; Gilbertson et al., 2015; Whitfield et al., 2015). In recent decades, vector-borne bacteria have caused some of the most devastating plant diseases in perennial and annual crops. For example, in North America '*Candidatus* Liberibacter asiaticus,' the causative agent of citrus greening, has rapidly spread across several regions of the world. Citrus greening continues to cost growers over $4 billion each year and has resulted in the loss of 1000s of jobs (Gottwald, 2010). Here, we review the current mechanistic knowledge of interactions shared among vector-borne bacteria, hemipteran vectors, and host plants. As most vector-borne bacteria cannot be cultured and are difficult to study in the lab, we then highlight current approaches used to study these tri-partite systems. Finally, we discuss application of recent knowledge for control and propose future directions for research on vector-borne bacteria and their hemipteran vectors.

REDEFINING THE RELATIONSHIPS VECTOR-BORNE BACTERIA SHARE WITH HEMIPTERAN INSECTS

Early studies of plant pathogens used microscopy, serological testing, and host inoculation to determine the etiological agents of diseases. While insect transmission of plant viruses was first described in 1920, insect transmission of plant bacteria was not reported until 1967 (Purcell, 1982). Because of the historic precedence of research on vector-borne viruses, concepts and terminology from virus research were applied to the study of vector-borne bacteria. In spite of this methodological connection, the actual similarities between viruses and bacteria as vector-transmitted plant pathogens may be quite limited. Here, we briefly define the common terminology found in the literature for describing pathogen–vector interactions and highlight terms that are useful for vector-borne bacteria specifically.

Persistence: Non-persistent, Semi-persistent, or Persistent

The transmission process of vector-borne viruses is categorized by two features: (1) the time period required by the vector for acquisition of the virus and inoculation of the virus, and (2) the retention time of viral particles in the vector (Ng and Falk, 2006). Based on these features, virus-vector relationships can be categorized as non-persistent, semi-persistent, or persistent. For non-persistent viruses, transmission can occur within minutes of acquiring the viral particles (virions) and particles are retained in the stylet or in the alimentary canal of the insect (Ng and Falk, 2006; Uzest et al., 2007; Whitfield et al., 2015). Viral particles can be lost quickly in this transmission mode and multiple encounters with infected plants are required for vectors to remain viruliferous (Ng and Falk, 2006). Semi-persistent retention of virions can last for days and retention sites are found in the alimentary canal or gut lumen of the insect for the majority of these viruses (Chen et al., 2011; Ng and Zhou, 2015). For semi-persistent relationships, feeding for hours to days is required to acquire the virus and if acquisition occurs during vector immature stages, infectivity is lost after each molt. Finally for persistent associations, vectors remain infective until death after a single encounter with an infected plant. Long feeding periods (hours to days) are required for acquisition of persistent viruses by vectors.

Persistence of vector-borne bacteria varies according to plant-tissue specialization. *Xylella fastidiosa*, the only known vector-borne xylem specialist, has a semi-persistent association with its vectors (**Table 1**). Dozens of crops and native plants are hosts for *X. fastidiosa* and a diverse array of vectors transmits the pathogen compared to other species of vector-borne bacteria (Redak et al., 2004). The ability to utilize diverse plant and vector species may be due to *X. fastidiosa*'s semi-persistent relationship with insects. For example, semi-persistent bacteria may be more easily acquired and transmitted by vectors to diverse host species during pre-feeding and host finding behavior. In contrast, all known phloem-limited bacteria appear to establish persistent associations with their respective vectors (**Table 1**). Persistence of phloem specialists may be due to the intracellular relationship they share with plant and insect hosts. However, conclusion about tissue trophisms may be premature, as only one vector-borne xylem specialists is known so far.

Location: Circulative or Non-circulative

The interactions plant viruses share with their insect vectors can either be "non-circulative" or "circulative." In non-circulative interactions, the virus does not enter the insect body as part of the transmission process and the virus particles are retained in the stylet or the foregut region (Ng and Zhou, 2015). Viruses that are transmitted in a circulative manner in contrast, pass beyond the foregut into the insect intestine and enter the body as part of the transmission process. Circulative viruses can be retained for the life of the insect vector (Gray et al., 2014). For vector-borne bacteria both non-circulative and circulative relationships exist among pathogen–vector interactions (**Table 1**); and like persistence, relationships correlate

TABLE 1 | Vector-borne phloem limited plant pathogenic bacteria.

Class Family	Pathogen	Genome size (Mb)	Plant tissue tropism	Plant host	Vectors	Location/ Insect organs	Reference
Gammaproteobacteria *Xanthomonadaceae*	*Xylella fastidiosa*	2.7	Xylem **(a)**	Wide host range	*Homalodisca vitripennis, Graphocephala atropunctata* (+ Others)	Non-Circulative/ Cybarium, foregut	Backus and Morgan, 2011
Mollicutes *Spiroplasmataceae*	*Spiroplasma citri*	1.8	Phloem **(a)**	Citrus	*Circulifer tenellus*	Circulative/	Fletcher et al., 1998
	Spiroplasma kunkelii			Corn	*Dalbulus maidis*	Hemolymph bacteriocyte, salivary glands	
Mollicutes *Acholeplasmataceae*	"*Candidatus* Phytoplasma spp."	0.8	Phloem **(b)**	Wide host range: Asteraceae horticulture crops	*Macrosteles quadrilineatus* (+ Others)	Circulative/ Hemolymph bacteriocyte, salivary glands	Beanland et al., 2000
Alphaproteobacteria *Rhizobiaceae*	"*Candidatus* Liberibacter spp."	1.2	Phloem **(b)**	Citrus Solanaceae Apiaceae	*Diaphorina citri*, *Bactericera cockerelli, Bactericera trigonica, Trioza apicalis*	Circulative/ Hemolymph bacteriocyte, salivary glands	Ammar et al., 2011

*The bacterial group is routinely culturable **(a)** or non-culturable **(b)**.*

with plant-tissue specialization of the pathogen. For example, the xylem colonizer, *X. fastidiosa,* is non-circulative, while all known phloem colonizers interact in a circulative manner with vectors (**Table 1**).

Differences in pathogen location within vectors may be explained by ancestral origins (Nadarasah and Stavrinides, 2011). In one scenario, bacteria pre-adapted to plant environments may have evolved to use insects as alternative hosts. Alternatively, insect pathogens or symbionts, pre-adapted to thrive in hemipterans, may have found an additional niche in plants (Nadarasah and Stavrinides, 2011). *X. fastidiosa* is most closely related to the genus *Xanthomonas* (**Table 1**). Members of *Xanthomonas* are exclusively plant-associated and commonly plant pathogens. Inability to cross insect membranes may be due to the fact that *X. fastidiosa* has evolved to be restricted to dead cells of the plant (xylem). The ability to cross plant cellular membranes may have been lost from its genetic arsenal over time. Liberibacters also are related to plant pathogens as a member of the family Rhizobiaceae, yet liberibacters have circulative relationships with insect vectors. A more striking phylogenetic observation for liberibacters is that many members of the Rhizobiaceae, have intracellular associations with hosts as pathogens and symbionts (insect and plant hosts; genera *Bradyrhizobium*, *Bartonella*, *Brucella,* and *Afipia*; Jagoueix et al., 1994). This trend may explain the origin of the circulative associations of liberibacters with their vectors. As microbiome projects for hemipterans expand, the relationship among these bacteria and the traits responsible for interactions inside the insect will likely be revealed.

Replication: Propagative or Non-propagative

Circulative viral pathogens can either circulate through the insect vector's body without reproducing, in which case they are described as "non-propagative," or they can circulate and multiply within the insect vector, in which case they are described as "propagative." In the latter case, the vector serves as an alternative host for the plant pathogen (Nadarasah and Stavrinides, 2011). Typically, the vector acquires the plant pathogen by feeding on infected plants. Once inside the insect body, the virus crosses intestinal barriers, internal organs, and visceral muscles, and can be found throughout the hemolymph (Hogenhout et al., 2008a; Orlovskis et al., 2015). From the hemolymph the virus must spread to the salivary glands before the vector can subsequently transmit the pathogen to a new plant host. Only a few families of vector-borne plant viruses have propagative relationships with vectors. These families include *Rhabdoviridae*, *Reoviridae,* and *Bunyaviridae* (Hogenhout et al., 2008a; Ammar et al., 2009; Whitfield et al., 2015).

All described vector-borne bacteria utilize their insect vectors as alternative hosts, and are thus considered propagative (Bove and Garnier, 2003; Orlovskis et al., 2015). Vector-borne bacteria can propagate extracellularly (between host cells) or intracellularly (within host cells; **Table 1**). For example, the xylem colonizer, *X. fastidiosa,* propagates extracellularly within the vector and is non-circulative. This is in contrast to all vector-borne viruses, which can only be propagative and circulative, as they are all parasites of the cellular replication machinery. It is assumed all phloem colonizers propagate intracellularly within their vectors as they are found in diverse tissues and hemolymph (**Table 1**). However, detailed intracellular propagation of bacteria is not easily studied and the current knowledge may reflect methodological limitations for evaluating bacterial replication in different insect organs and cavities. Specific mechanisms mediating insect recognition, attachment, and multiplication in organs are also not yet clear, and seem to be unique for each bacteria–vector interaction.

HEMIPTERANS AND THEIR ROLE AS VECTORS OF BACTERIAL PLANT PATHOGENS

The ability to serve as a viral and/or bacterial vector appears to vary across hemipteran lineages (**Figure 1**; Supplementary Table S1). Vector-borne bacteria most commonly rely on members of the suborder Auchenorrhyncha for transmission, including leafhoppers (Membracoidea), froghoppers/spittlebugs (Cercopoidea), and planthoppers (Fulgoroidea; **Figure 1**) (Bove and Garnier, 2003). However, several psyllids (Psylloidea) from the subgroup Sternorrhyncha are also important vectors of bacterial plant pathogens (**Figure 1**; Supplementary Table S1). In these groups, transmission has been demonstrated for mesophyll, xylem, and phloem-feeding hemipterans (**Figure 1**; Supplementary Table S1). The efficiency of pathogen transmission, however, depends on the specific insect–plant interaction and on pathogen biology.

Vectors of bacterial plant pathogens and vectors of viral plant pathogens have been reported in multiple superfamilies of the Euhemiptera lineage (Auchenorrhyncha, Coleorrhyncha, and Heteroptera; **Figure 1**). Surprisingly, only a few individual species within these superfamilies have been reported to serve as efficient vectors for both bacterial and viral plant pathogens (Weintraub and Beanland, 2006). One such example is the Beet Leafhopper (*Circulifer tenellus*, Baker), which is a vector for the *Beet curly top virus* ([BCTV], *Geminiviridae*), as well as two different bacterial pathogens ("*Candidatus* Phytoplasma trifolii" and *Spiroplasma citri*; Weintraub and Beanland, 2006).

The suborder Sternorrhyncha contains psyllids, aphids (Aphidoidea), whiteflies (Aleyrodoidea), and scales (Coccoidea), though the latter three are more closely related to one another phylogenetically (Gullan and Cranston, 2014). This is interesting because aphids, whiteflies, and scales have only been reported as vectors of viruses, while psyllids have only been reported as vectors of bacteria (**Figure 1**; Supplementary Table S1). In fact, aphids and whiteflies are the most important vectors of plant viruses, transmitting 46% of all described plant-infecting viruses (Hogenhout et al., 2008a; Gilbertson et al., 2015). Recent work on the Asian citrus psyllid's (*Diaphorina citri*) viral metagenome found viral sequences from diverse groups of animal viruses but no sequences related to any known viral plant pathogens were reported (Nouri et al., 2015). These results provide further evidence that psyllids may lack the ability to transmit plant viruses, however, additional work in this area is needed.

Our literature review suggests that some hemipteran groups are capable of transmitting bacterial pathogens while other groups are not (**Figure 1**). Despite extensive research into various vector systems, the mechanisms that mediate vector specificity remain largely unknown for all but a few vector-borne phytopathogens (Uzest et al., 2007; Chen et al., 2011; Blanc et al., 2014). Variations in vector specificity among lineages suggest physical, physiological, or temporal constraints on vector-pathogen relationships. Vectors that transmit viruses and bacteria may have fewer constraints, or constraints that are easier for the pathogens to overcome. Differences in insect physiology, immunity, or feeding behavior among groups may mediate some aspects of vector specificity. However, conclusions on differences in insect biology are difficult to make at this point, as the basic biology of many hemipterans remains poorly understood and complete genomes are available for only a few vectors of plant pathogens (Leshkowitz et al., 2006; Ramsey et al., 2007; Legeai et al., 2010; Chen et al., 2015; Upadhyay et al., 2015). Variations in vector specificity may also depend on location and timing of vectors and pathogens. Geographic factors, environmental conditions, and agricultural economics are all dynamic forces that may limit host distribution, insect populations, and plant-pathogen-vector associations. Alternatively, these observations may be the result of a lack of information on the full extent of vector-borne pathogens and their hemipteran vectors, as the current inventory is still likely underrepresented (Malmstrom et al., 2011).

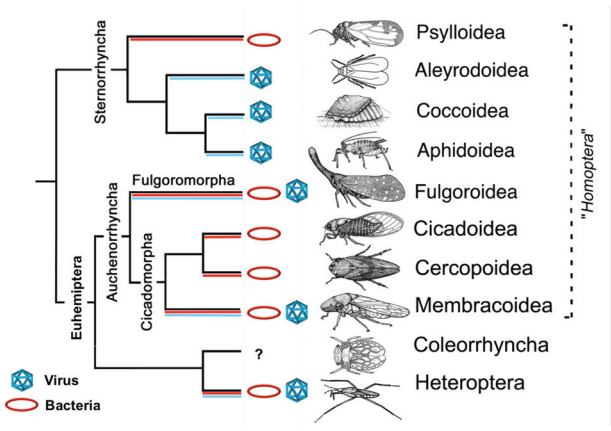

FIGURE 1 | Hemiptera taxa: reported vectors and groups of plant pathogens. Specific plant pathogens are listed in Supplementary Table S1. Branches where species have been reported as transmitting virus are labeled in blue, while those transmitting bacteria are labeled in red. Figure modified with permission from Gullan and Cranston (2014).

VECTOR-BORNE BACTERIA: DUAL HOST INTERACTIONS

All known vector-borne bacteria share certain biological features, including plant vascular tissue specialization, propagative relationships with vectors, and complete dependence on their hosts. Host dependence is likely a result of genome degradation, where essential biosynthetic pathways from the bacterial ancestor have been lost where the same resources can be obtained from the host environment (Nadarasah and Stavrinides, 2011). The xylem colonizer, *X. fastidiosa,* has the largest genome of the group (**Table 1**). This may be due to the fact that xylem represent an inferior nutrient sources as compared to the phloem. Therefore, more essential biosynthetic pathways in the genome may be required for the bacteria to survive in the nutrient limited xylem. Despite these similarities, the bacterial groups that depend on

hemipteran vectors for transmission occur in several different phyla and orders (**Table 1**). Accordingly, many differences exist, including diverse mechanisms for promoting host colonization and dispersal (Orlovskis et al., 2015).

Xylem-Limited Vector-Borne Bacteria

The only known xylem-limited bacterial pathogen that is also transmitted by hemipteran vectors is *X. fastidiosa. X. fastidiosa* (class Gammaproteobacteria) has a very wide host range, colonizing and causing disease in grapes (Pierce's disease), citrus (citrus variegated chlorosis), olives (leaf scorch), almonds (leaf scorch), and several other plant species (Chatterjee et al., 2008a; Saponari et al., 2014; Almeida and Nuney, 2015). Because of its economic importance, *X. fastidiosa* was the first bacterial plant pathogen genome to be completely sequenced (Simpson et al., 2000). The genome of *X. fastidiosa* is ∼2.7 Mb, half the size of its closest relatives (Xanthomonas group; **Table 1**). *X. fastidiosa* is transmitted in a non-circulative manner by a diverse set of xylem-feeding hemipterans including members from the superfamilies Membracoidea, Cercopoidea, and Cicadoidea (PaiãO et al., 1996; Purcell and Hopkins, 1996; Saponari et al., 2014). Experimental evidence suggests that a wide range of additional xylem feeding hemipterans are potential vectors, however, efficiency of transmission may vary depending on the vector species (Redak et al., 2004). The planthoppers *Homalodisca vitripennis* and *Graphocephala atropunctata* are the two most well studied vectors of *X. fastidiosa*. Knowledge of these two vectors has been used to model the relationship *X. fastidiosa* share with vectors in general (Chatterjee et al., 2008b; Backus and Morgan, 2011; Rapicavoli et al., 2015).

Numerous research tools have been developed to study *X. fastidiosa,* including *in vitro* culture techniques, transformation, and bacterial strain mutants (Killiny et al., 2012; Purcell, 2013; Webster et al., 2014; Rapicavoli et al., 2015). These tools have facilitated the dissection of many genetic components involved in pathogenicity. *X. fastidiosa* colonize and propagate extracellularly in the plant and insect (Chatterjee et al., 2008a), with some overlapping mechanisms. *X. fastidiosa* uses a cell-to-cell signaling sensor (RpfC), which acts as a negative regulator. This signaling system is mediated by diffusible signaling factors (DSF) in order to modulate different aspects of behavior in a population dependent manner (i.e., quorum sensing). DSF is secreted into the extracellular environment, activating motility, biofilm formation, and virulence mechanisms when a threshold concentration is reached outside the cell (Chatterjee et al., 2008a). Aspects of *X. fastidiosa* colonization that are dependent on quorum sensing include the production of toxins, extracellular polysaccharides (EPS), adhesins, and hemaglutinins (**Table 2**) (Chatterjee et al., 2008b; Nascimento et al., 2016). Inside the insect, the bacteria do not invade the epithelial gut, hemolymph, or salivary glands, and are retained in the alimentary canal. Transmission can occur within minutes of acquisition (Killiny et al., 2012). Recently it was determined that lipopolysaccharide (LPS), the outermost layer of sugar polymers surrounding Gram-negative bacteria, is critical for attachment to the vector and subsequent transmission (**Table 2**) (Rapicavoli et al., 2015).

In plants, *X. fastidiosa* relies on bacterial multiplication, attachment, and dispersion into neighboring vessels to colonize the xylem (Chatterjee et al., 2008b; Nascimento et al., 2016). Phytotoxicity during early stages of infection is associated with the lipase/esterase effector LesA, a type II secreted enzyme produced abundantly in culture (Nascimento et al., 2016). By degrading plant cell walls, nutrients are acquired and the bacteria are able to disperse throughout the plant (Purcell, 2013; Fatima and Senthil-Kumar, 2015). Degradation of vascular plant tissue requires the combined action of multiple enzymes, such as β-1,4 endoglucanases, xylanases, xylosidases, and polygalacturonases (Purcell, 2013; Fatima and Senthil-Kumar, 2015). *X. fastidiosa* mutants impaired in polygalacturonases enzyme (PglA) production, lack pathogenicity, and systemic movement in the plant (**Table 2**) (Roper et al., 2007). Given the reduced nutritional content of the xylem and the extracellular location of *X. fastidiosa*, many differences may exist for pathogenicity strategies among xylem and phloem colonizers.

Phloem-Limited Vector-Borne Bacteria

Diverse phylogenetic groups converge in phloem specialization and hemipteran transmission and it is hypothesized that those traits have been acquired independently multiple times over the course of bacteria evolution (Orlovskis et al., 2015). The majority of phytoplasmas (class Mollicutes) and liberibacters (class Alphaproteobacteria) are vector-borne phytopathogens (Bressan, 2014; Fagen et al., 2014a). For other groups, such as spiroplasmas (class Mollicutes), only some species are phytopathogens. Despite this diversity, all known phloem-limited vector-borne bacteria appear to colonize both the insect vector and the plant host intracellularly (Orlovskis et al., 2015). The bacteria cross the gut barrier and circulate in the vector body, eventually reaching the hemolymph, and salivary glands (**Table 1**) (Gasparich, 2010). The journey to the salivary glands requires a latent period, ranging from days to months, before transmission can occur (Thebaud et al., 2009). Here, we will discuss three examples of phylogenetic groups containing vector-borne bacteria: spiroplasmas, phytoplasmas, and liberibacters.

Spiroplasmas

Spiroplasma spp. have a distinctive helical morphology and use pili–like structures to move in a corkscrew-like motion (Ammar et al., 2004). They are classified as Mollicutes as they lack a cell wall. Spiroplasmas share diverse relationships with plant and insect hosts spanning pathogenic, commensal, and mutualistic interactions (Ammar et al., 2004). Most are associated with diverse insect orders, such as Hymenoptera, Coleoptera, Diptera, Lepidoptera, and Hemiptera (Gasparich, 2010). However, there are three phytopathogenic spiroplasmas that are also transmitted by leafhoppers (Cicadellidae): *Spiroplasma citri, S. kunkelii*, and *S. phoeniceum* (**Table 1**) (Gasparich, 2010). *S. citri*, the causal agent of citrus stubborn, was the first vector-borne bacteria to be cultured. It was first discovered in 1970 and culture methods were developed shortly after this (Bove and Garnier, 2003). Cultivation of spiroplasmas is not trivial, as it requires

TABLE 2 | Reported gene product (or structure) associated with host interaction for vector-borne bacteria.

Vector-borne bacteria	Gene product	Descriptions	Mechanisms	Phenotype	Reference
Xylella fastidiosa	RpfC	Signaling sensor	Negative regulator for DSF	Mutants show hyper attachment phenotype in xylem vessels and cybarium of insect vector	Chatterjee et al., 2008b
	FimA, FimF	Type I fimbrial adhesins	Facilitates cell–cell aggregation	–	Chatterjee et al., 2008b
	HxfA, HxfB	Hemaglutinins	Facilitates cell–cell aggregation and cell–surface interactions	HxfA mutants slightly reduced attachment	Chatterjee et al., 2008b
	PglA	Polygalacturonase	–	Mutants lack pathogenicity and systemic movement in plants	Roper et al., 2007
	β-1,4 endoglucanases Xylanases Xylosidases	Cell degrading enzymes	Degradation of plant cell wall components	–	Chatterjee et al., 2008b
	LesA	Lipase/esterase Type II toxin	Phytotoxicity	Phytotoxicity in early plant infection	Nascimento et al., 2016
	O-antigen in LPS	*O*-Lipopolysaccharide		Mutants lack full pathogenicity	Rapicavoli et al., 2015
Spiroplasma kunkelii	–	Pili, extracellular structure	Attachment to insects	–	Ammar et al., 2004
Spiroplasma citri	P58	Membrane protein	Attachment to insects	–	Ye et al., 1997
	SARP1	Membrane protein	Attachment to insects	–	Berg et al., 2001
	Spiralin	Membrane protein	Attachment to insects	Mutants have reduced transmission by insects	Gasparich, 2010
	P32	Membrane protein encoded in plasmid (pSci6)	Attachment to insects	Mutants have reduced attachment to insects	Berho et al., 2006
"*Candidatus* Phytoplasma spp."	SAP11	Sec-exported, NLS signal	Block JA biosynthesis in plants		Sugio et al., 2011
	Amp	Sec-exported, Transmembrane domain	Interacts with insect proteins	Increase vector fecundity	Rashidi et al., 2015
	SAP54/PHYL	Sec-exported	Interaction floral transcription factors	Floral abnormalities as phyllody	MacLean et al., 2014
		NLS signal	Degrades MADS-box proteins		Maejima et al., 2014
	TENGU	Sec-exported	Inhibits auxin-related pathway	Dwarf plants	Minato et al., 2014
	P38	Adhesin domain	Interacts with insect proteins	–	Neriya et al., 2014
	HflB	Protease	Virulence factor	–	Seemüller et al., 2013
	VmpA	Membrane protein	Interaction with insects	–	Renaudin et al., 2015
"*Candidatus* Liberibacter asiaticus"	LasAI	Autotransporter	Unknown	–	Hao et al., 2013
	SC2_gp095	Glutathione peroxidase	Detoxify ROS	–	Jain et al., 2015

complex media enriched with cholesterol and fatty acids. Tools and information derived for spiroplasmas culture methods have served as references for attempts to culture phytoplasmas and liberibacter.

After acquisition, spiroplasmas adhere to receptors in the lumen of the insect midgut, where endocytosis occurs (Fletcher et al., 1998; Gasparich, 2010). Intracellular vesicular transport mediates migration to the hemolymph and exocytosis (Fletcher et al., 1998). Once inside the hemolymph, the bacteria are transported throughout the insect body, eventually reaching the salivary glands after additional intracellular crossings (Fletcher et al., 1998). Currently, the specific insect receptors/factors

mediating the journey inside the vector remain unknown for spiroplasmas. However, several potential proteins required for insect attachment have been identified using *S. citri* mutants impaired in insect transmission and with *S. citri* strains that have lost insect attachment properties after multiple *in vitro* cultivations (Ye et al., 1997; Fletcher et al., 1998; Berho et al., 2006; Mutaqin et al., 2011) (**Table 2**).

One of the first approaches developed to study bacterial protein–insect interactions was the use of leafhopper (*C. tenellus*) monolayer cell culture assays with spiroplasmas. In this technique, researchers exposed insect cells (CT1) *in vitro* to *S. citri*. After exposure, electron microscopy (Wayadande and Fletcher, 1998) or immunofluorescence assays (Labroussaa et al., 2010) were used to evaluate bacterial phenotypes. Numerous candidate attachment proteins have been identified in this way, including P58, SARP, and the plasmid-borne protein P32 (**Table 2**) (Wayadande and Fletcher, 1998; Berg et al., 2001). Another very abundant membrane protein of *S. citri* that has been implicated in transmission is spiralin. *S. citri* mutants compromised in spiralin production exhibit reduced transmission by the vector, *Circulifer haematoceps* (**Table 2**) (Gasparich, 2010). This suggests that spiralin may mediate pathogen interactions within the insect vector, though specific mechanisms remain unknown.

Phytoplasmas

Phytoplasmas are another category of the Mollicutes that depend on insect vectors for transmission (**Table 1**), but unlike *Spiroplasma*, they have pleomorphic shapes and are very difficult to culture (Contaldo et al., 2012). Phytoplasmas are a diverse monophyletic group, with more than 30 "*Candidatus* Phytoplasma" species described and 100s of subgroups (Hogenhout et al., 2008b). As a taxon they have a wide host range, infecting more than 800 different plant species (Hogenhout et al., 2008b), but individual strains have highly restricted insect and plant hosts. Collectively, more than 1000 plant diseases are caused by phytoplasmas that are transmitted by leafhoppers and, to a lesser extent, a few other hemipterans (Mitchel, 2004; Weintraub and Beanland, 2006).

Phytoplasmas have the smallest genomes of all described phytopathogenic bacteria, averaging ∼0.7 Mb with a low G+C content, high number of repetitive regions, and interesting variability in genome features across the taxon (**Table 2**) (Kube et al., 2012, 2014). At least six types of ATP-binding cassette (ABC) transporters are conserved in the evaluated genomes. ABC transporters shuttle metabolites across bacterial membranes, and are predicted to allow nutrient and metabolite uptake from the host. Other common features include a superoxide dismutase enzyme (SOD), possibly used to counteract reactive oxygen species produced by hosts, and a protease (HflB), which is a virulence factor for '*Candidatus* Phytoplasma mali (Wang et al., 2014). Recently a conserved Mollicutes adhesion motif (MAM) was identified in the Onion Yellow Phytoplasma genome. This candidate protein (P38) interacts with crude insect extracts and weakly with plants extracts (**Table 2**) (Neriya et al., 2014), however, specific host targets are unknown.

Phytoplasmas also encode translocase *SecA*, part of the Type II secretion system for bacteria. This secretion system allows the delivery of functionally distinct proteins with a characteristic signal peptide at the *n*-terminal to the bacterial membrane. Because phytoplasmas have a single membrane, after the signal peptide is cleaved the proteins are released into the host environment (secreted). Secreted phytoplasma proteins can alter host functions and act as effectors (Bai et al., 2009). A single phytoplasma genome can encodes over 50 secreted proteins (SAP's), however, the function of each one during host colonization and propagation is only known for a few (Bai et al., 2009). SAP effectors often alter host function by manipulating plant hormone homeostasis. For example, the effector TENGU inhibits auxin-related pathways leading to a dwarf plant phenotype and floral sterility (**Table 2**) (Minato et al., 2014). Further, *Arabidopsis* transgenic lines expressing SAP11, produce less jasmonic acid (JA) compared to controls (**Table 2**) (Sugio et al., 2014). This leads to abnormal vegetative growth and increased fecundity for leafhopper vectors on infected plants (Lu et al., 2014b). SAP effectors can also modulate pathogenicity through changes in development. SAP54/PHYL interacts with floral transcription factors and promotes degradation of the MADS-box proteins. MADS-box proteins are critical for floral meristem development and plants expressing SAP54/PHYL flower abnormally (**Table 1**) (MacLean et al., 2014; Maejima et al., 2014).

A second group of proteins delivered by the Sec-secretion system are the immunodominant membrane proteins (IMPs), which remain anchored and decorate the external membrane of phytoplasmas. IMPs are unique for phytoplasmas and are categorized into three subgroups depending on whether the n- or c- terminal side of the protein is exposed extracellularly (Amp, IdpA, or Imp; Kakizawa et al., 2006). When a monoclonal anti-AMP from "*Candidatus* Phytoplasma asteris" Chrysanthemums Yellows strain (CPY) was fed to the leafhopper vector, internalization of the phytoplasma and transmission efficiency was reduced. These results imply that anti-Amp impedes attachment of the bacteria in the vector gut (**Table 2**) (Rashidi et al., 2015).

Liberibacter

The genus *Liberibacter* spp. contains six species of phloem-limited bacteria (Haapalainen, 2014): "*Ca.* Liberibacter africanus," "*Ca.* Liberibacter americanus," "*Ca.* Liberibacter asiaticus," "*Ca.* Liberibacter solanacearum," "*Ca.* Liberibacter europaeus," and *Liberibacter crescens*. "*Ca.* Liberibacter africanus," "*Ca.* Liberibacter americanus," and "*Ca.* Liberibacter asiaticus" are associated with citrus greening disease, also referred as Huanglongbing (HLB) in different regions around the globe (Gottwald, 2010). "*Ca.* Liberibacter solanacearum" (="*Ca.* Liberibacter psyllaurous") is phytopathogenic to members of the Apiaceae and Solanaceae plant families. These four species all depend on psyllid vectors for transmission and as alternative hosts (Fagen et al., 2014b; Haapalainen, 2014). "*Ca.* Liberibacter europaeus" has also been associated with psyllids, but its role as a plant pathogen has not been demonstrated. To date, only *Liberibacter crescens* has been cultured *in vitro*, but

it is not considered phytopathogenic and it is not vector-borne (Fagen et al., 2014a). *L. crescens* was first isolated as a bacterial endophyte from papaya and has not been re-isolated in nature. Non-psyllid hemipterans may also be able to pick up the bacteria during feeding as bacterial DNA has been found in mealybugs (Pitino et al., 2014), however, liberibacter transmission by other hemipterans is currently not clear.

Liberibacters have a small genome of ∼1.2 Mb. Comparative genomics have shown a similar gene organization across the genus and evidence of horizontal gene transfers as prophages integrated into the genomes (**Table 1**) (Thompson et al., 2015). Similar to phytoplasmas, liberibacters lack biosynthesis genes for amino acids, sugars, and nitrogenated bases, which imply they obtain those metabolic products from their host (Thompson et al., 2015). Accordingly, many ABC transporters are encoded in liberibacter genomes (Lin et al., 2011; Mafra et al., 2013; Yan et al., 2013). Active importation of nutrients from phloem and insect vectors may lead to nutrient imbalances, partially explaining the foliar symptoms observed in liberibacter-infected plants (Rashed et al., 2013).

Potential pathogenicity mechanisms of liberibacters have recently been suggested based on comparative bioinformatics with other phloem-limited bacteria. Liberibacters encode the basic proteins for Sec-dependent translocation, similar to phytoplasmas (Lin et al., 2011; Yan et al., 2013). However, as liberibacters have two membranes of different composition in contrast to phytoplasmas, it is not known whether putative liberibacter Sec-transported proteins cross the outer membrane and interact with the plant or insect host. Recently, two unusual autotransporters were identified in the liberibacter genome (*LasAI* and *LasAII*) and these may serve as an alternative secretion system to the Sec-system (**Table 2**) (Hao et al., 2013). Evidence suggests that plant transcripts and metabolites related to salicylic acid (SA) production are altered during '*Candidatus* Liberibacter solanacerum' infection (Casteel et al., 2012; Chin et al., 2014). SA is an important signaling molecule involved in plant defense to pathogens and insects (Glazebrook, 2005; Walling, 2009; Erb et al., 2012). Recently, a NahG-like salicylate hydroxylase gene was found in the liberibacter genome. NahG is predicted to cleave salicylates derived from SA (Lin et al., 2013) and may be used to modify the plant defense system. Although comparative bioinformatics has revealed many potential proteins used by liberibacter to alter plant and vector metabolism and vector–plants interactions, exact mechanisms for host colonization and transmission remain largely unknown.

APPROACHES TO STUDY VECTOR-BORNE BACTERIA

The current understanding of pathogenicity mechanisms in vector-borne bacteria is largely influenced by the ability to culture those bacteria. To date only *X. fastidiosa* and *Spiroplasma* spp. have been cultured *in vitro* and both require very specific conditions (Dourado et al., 2015; Renaudin et al., 2015). Because of this limitation, much of the biology and mechanisms of host colonization for phytoplasmas and liberibacters are still poorly understood (Bove and Garnier, 2003). Another challenge of working with phloem-limited vector-borne bacteria in particular is the non-homogenous distribution in the phloem tissue. This makes choosing sampling locations difficult and can result in false negatives during detection. Further, symptoms vary significantly across plant hosts and do not necessarily correlate with pathogen titer. Despite these difficulties, approaches combining genomics, bioinformatics, transcriptomics, and genetic manipulation have contributed to recent advances in the understanding of how these bacterial pathogens colonize their host environments.

Whole Genome Sequencing and Bioinformatics of Vector-Borne Bacteria

The complete genome sequences for many strains of vector-borne bacteria have recently become available (**Table 1**). This has allowed scientists to study bacterial gene function within these systems without the need to culture the organism. Bioinformatics can be used to compare genome sequences with the annotated genomes of close relatives or analyze sequences using server-based algorithms to assign predicted functions to each coding region (Rutherford et al., 2000). Amino acid sequences can be further explored to identify conserved patterns and domains. In this way, proteins with low average similarity can be assigned to a predicted function (Yu et al., 2010; Caccia et al., 2013; Cortazar et al., 2015). Finally, functions of unknown proteins can even be predicted using dedicated algorithms that identify patterns associated with signal peptides, localization, cleavage sites, phosphorylation, and transmembrane domains (Yu et al., 2010).

A limitation of these various bioinformatics approaches is that all programs are trained using cultured organisms. For unculturable bacteria, many unique sequences with no homologs in cultured species exist, making comparisons and inferences difficult (Kube et al., 2008). Despite these limitations, bioinformatics have been used successfully to study gene function for many phytoplasma effectors. Bai et al. (2009) identified 56 Secreted Aster Yellows Proteins (SAPs) in the genome of '*Candidatus* Phytoplasma asteris' strain Aster Yellows Witches'-broom (AY-WB). In this study, they utilized a pipeline to predict prokaryotic signal peptides recognized by Sec-translocases (SignalP v. 3.0) and then predicted transmembrane domains within this list to predict secretion (TMHMM v. 2.0; Bai et al., 2009). Finally, the list of 56 predicted effectors was examined for eukaryotic nuclear localization signals (predictNLS and pSORT) to select SAPs targeting plant nuclei for further investigation (Bai et al., 2009; Lu et al., 2014b).

Transcriptomics of Vector-Borne Bacteria in Their Hosts

After potential pathogenicity factors are identified, functional validation is required. For unculturable bacteria, transcription and translation of targets can only be evaluated within their hosts (plant or insect). This means RNA and protein isolations must be done from infected host tissue. By some estimates, only 0.1% of total RNA extracted from infected herbaceous

hosts represents the phytoplasma RNA. Others report only 0.02% of the mRNA from the woody host was associated with the phytoplasma genome (Abba et al., 2014). However, high throughput sequencing technologies have expanded the possibilities for studying pathogens inside their hosts. Now RNAseq can be used to quantify the complete RNA population in a sample (Westermann et al., 2012). This technique has advantages over microarrays and qRT-PCR because it affords higher sensitivity for monitoring gene expression levels, independence from examining only known sequences, and wider detection ranges (Westermann et al., 2012). However, for vector-borne bacterial pathogens, RNAseq approaches have thus far had low levels of success.

RNAseq has been used to examine "*Candidatus* Phytoplasma mali" transcription in tobacco (*Nicotiana occidentalis*; Siewert et al., 2014). Prior to preparation for sequencing, total RNA was treated with a plant ribosomal depletion kit to enrich the samples for bacterial RNA. Only 0.003% of the total reads (17,046,418 reads averaging 115 b) were mapped against the protein coding regions of the predicted "*Candidatus* Phytoplasma mali" genome. Mapped reads corresponded to 132 genes out of the 497 predicted genes. In another RNAseq study, RNA was enriched for bacterial transcripts using a ribosomal depletion kit to remove plant cytoplasmic, mitochondrial, and chloroplast ribosomal RNA (Abba et al., 2014). Despite the enrichment and relatively deep sequencing, only 0.01% of the total reads (125,813,174 and 129,412,231, for each library) were mapped to the draft genome of the phytoplasma flavescence dore (Abba et al., 2014). In two slightly more successful studies, total RNA from psyllid vectors was used to detect transcripts from "*Ca.* Liberibacter solanacearum" (Ibanez et al., 2014; Yao et al., 2016). However, only 0.3% of the total (70,869,948) reads were mapped to the bacteria genome after ribosomal depletion (Ibanez et al., 2014). Transcriptomics offer a unique opportunity to overcome the many difficulties posed by these difficult pathosystems, but as evident in the above examples, many technical challenges remain.

Genetic Manipulation of Vector-Borne Bacterial Phytopathogens

The first approach that permitted gene function discovery for vector-borne bacterial plant pathogens was the use of transposon mutagenesis with spiroplasmas in the early 1990s (Fletcher et al., 1998; Mutaqin et al., 2011). In this approach, a transposon with a selective marker was integrated randomly into the chromosome of *S. citri,* and recombinant colonies were selected in media with antibiotics. When transformed colonies were tested in the host, the transposon was retained for a few days without antibiotic pressure. This technique was used to determine that disruption of a solute binding protein (gene sc76) reduced transmission in the leafhopper vector (Boutareaud et al., 2004). Since this first study, numerous research groups have generated collections of *S. citri* mutants using this technique (Foissac et al., 1997; Boutareaud et al., 2004; Mutaqin et al., 2011). Currently, *X. fastidiosa* and *Spiroplasma* spp. are the only vascular plant pathogens transmitted by hemipterans for which genetic transformation protocols and mutant libraries are currently available.

Genetic manipulation using surrogate culturable bacteria and heterologous gene expression in plants has been used to test gene function for other vector-borne bacteria (Jain et al., 2015; Renaudin et al., 2015). In a study using the flavescence dore phytoplasma, the surface protein, variable membrane protein A (VmpA*)*, was expressed under the control of the *S. citri tuf* promoter in a recombinant *S. citri* (**Table 2**) (Renaudin et al., 2015). The *tuf* promoter was chosen because the *tuf* gene is expressed at high levels in most bacteria (Kim et al., 2009). In this system the leafhopper, *Euscelidius variegatus,* serves as a vector for both the phytoplasma and *S. citri*. Thus gain of function studies could be conducted with the recombinant *S. citri* in both hosts. In the case of phytopathogenic "*Candidatus* Liberibacter asiaticus," a peroxidase protein (SC2_gp095) has been expressed in the cultivable *L. crescens* as a surrogate (**Table 2**). However, biological inferences from this system may be restricted by the lack of host infection of *L. crescens* after culturing.

An alternative method for studying gene function is to overexpress bacterial candidate proteins in the plant host. Model plants such as *Arabidopsis thaliana* and *Nicotiana* spp. are routinely used to evaluate gene function for plant pathogens. Once the candidate gene is selected, the coding sequence is cloned into a suitable expression vector and transgenic plants can be generated. Several authors have utilized plant heterologous expression systems to investigate the function of phytoplasma SAPs (MacLean et al., 2011; Lu et al., 2014a; Yang et al., 2015). In these studies transgenic *A. thaliana* expressing individual phytoplasma SAPs were screened for symptom development and plant abnormalities (MacLean et al., 2011; Lu et al., 2014a; Yang et al., 2015). After a relevant phenotype was identified, plant gene expression changes and plant proteins interacting with the phytoplasma proteins were examined (**Table 2**). A limitation of this approach is that only profound disturbances caused by a single bacterial gene can be identified. In addition, model plants may not serve as natural hosts for all vectored-borne bacteria and relevance of findings may be limited to an artificial system.

APPLICATION OF KNOWLEDGE FOR 'NEXT GENERATION' CONTROL STRATEGIES

Controlling vector-borne pathogens is difficult. Chemical control of insect vectors is the most widely used method, but in most cases insecticidal applications are not sufficient to contain the spread of these pathogens and associated diseases. Furthermore, insect resistance and environmental regulations have limited the viability of long-term application of insecticides. Host plant resistance has been successful for several high value crops (Bisognin et al., 2008; Riaz et al., 2008), including grapevine tolerance to Pierce's disease. In these plants, *X. fastidiosa* infection occurs, but titer remains low in the plant (Riaz et al., 2008). Due to the long time periods required to identify resistance and produce new varieties, this method may not always be a practical choice for the more aggressive and devastating outbreaks. Overall, as research on vector-borne bacteria continues to

flourish, a focus on the 'next generation' of control strategies is needed.

One recent approach to block transmission of vector-borne bacteria used chemicals intended to saturate the pathogen-binding site in the insect or on the bacteria surface, so the insect picks up fewer pathogen cells (Killiny et al., 2012). In this study, vectors were fed artificial diet supplemented with *X. fastidiosa* cells and different potential transmission-blocking chemicals. Multiple lectins, carbohydrates, and antibodies were evaluated for potential transmission blocking characteristics. After feeding on the diet-bacteria mixture, insects were transferred to healthy plants to determine transmission efficiency with and without the different chemicals (Killiny et al., 2012). Diets containing certain lectins (wheat germ agglutinin and concanavalin A), *N*-acetyl glucosamine carbohydrates, and certain antibodies reduced the transmission efficiency under greenhouse conditions. The authors suggest that lectins probably compete with the bacteria for the binding sites inside the insect, while carbohydrate saturate *X. fastidiosa* adhesions on the cell surface (Killiny et al., 2012). The interference approach has also been explored in phytoplasmas using antibodies against the extracellular membrane protein Amp, with some success in the lab (Rashidi et al., 2015). Recently, phage-display libraries have been used to evaluate antibodies and protein–protein interactions inside the insect vector. In this approach, each phage contains a known peptide and the binding capacity of the peptide to an extracellular bacterial epitope is evaluated (Huang et al., 2012). The exact mechanisms mediating the ability of specific chemicals to block transmission is still unknown, and it is not clear how this technology could be used in large-scale application. How, for example, might a natural population of insects be exposed to the transmission-blocking chemical?

Some of the most extensive research efforts on 'next generation' control technologies for vector-borne bacteria have focused on the use of nucleic acids in gene drive systems (Sinkins and Gould, 2006), and with RNA interference strategies (Gordon and Waterhouse, 2007; Nandety et al., 2015). The first concept was explored initially in the field of medicine, and is based on the concept of 'selfish DNA'. Selfish DNA is a naturally occurring phenomenon where certain genetic elements, such as transposable elements and others, spread in the genome of an organism and in the population by making additional copies of themselves. It has been suggested that selfish genetic elements could be used for control as a gene drive system that carries additional genes with anti-pathogen effects (Sinkins and Gould, 2006; Gantz and Jasinskiene, 2015). Populations of insects transformed with transposable elements or with a transgenic *Wolbachia* strain could be released into the environment, permitting the gene drive system and 'gene of interest' to spread in the population and block plant pathogen associations (Sinkins and Gould, 2006). Obvious concerns with this method are public acceptance of transgenic organisms, non-target impacts, and the costs of implantation.

Nucleic acids can also be utilized as a control method by inducing RNA interference (RNAi). RNAi has already been successfully exploited in plants to control viruses in commercial production (Tricoll et al., 1995; Gonsalves, 2006; Fuchs and Gonsalves, 2007; Scorza et al., 2013) and successful control of bacteria has been demonstrated (Escobar et al., 2001). RNAi can also be used to control insect species, altering insect reproduction, physiology, or survival (Gordon and Waterhouse, 2007; Wuriyanghan et al., 2011; Nandety et al., 2015). Direct injection, bait feeding, or transgenic host plants can be used to induce RNAi in insects. As direct injection is not practical for large scale control, and bait feeding is not effective in field studies for hemipteran insects, transgenic plants are the best options for using RNAi to control vectors of bacterial pathogens. While there is much excitement about the use of RNAi as an alternative control strategy (Gordon and Waterhouse, 2007; Donald et al., 2012; Li et al., 2013; Yu et al., 2016), additional research on delivery, safety, and non-target effects needs to be explored. Despite these unknowns, RNAi studies still represent an excellent attempt at next-generation control for these important plant pathogens.

CONCLUSION AND FUTURE DIRECTIONS

Devastating outbreaks of citrus greening disease, Pierce's disease, and zebra chip disease in recent years have contributed to a rapid growth in the literature on bacterial plant pathogens and their hemipteran vectors (Haapalainen, 2014; Almeida and Nuney, 2015; Orlovskis et al., 2015). Whereas most plant-infecting viruses depend on hemipterans for transmission, most plant-infecting bacteria do not. The small subset of known bacteria that are vector-borne are able to propagate in both the plant host and the insect vector, organisms from diverse phylogenetic kingdoms (**Table 1**). This is in contrast to the non-propagative relationships most vector-borne plant viruses share with hemipteran vectors. The ability to transition between divergent hosts is remarkable considering that most vector-borne bacteria have highly reduced genomes compared their free-living ancestors, yet, we still do not understand the mechanisms which make this sort of transitioning possible. Variation in vector-pathogen specificity also exists across hemipteran groups (**Figure 1**; Supplementary Table S1), suggesting there are still unknown constraints on these relationships. Clear differences occur between the relationships that phloem and xylem colonizers share with insect vectors (**Table 1**). However, conclusions based on tissue tropisms should be made with caution, as only one xylem-limited vector-borne species has been identified so far.

The analysis of the genetic mechanisms mediating interactions between vector-borne bacteria and their hosts has focused largely on membrane-bound proteins and Sec-dependent peptides in gram-positive bacteria (**Table 2**). For gram-negative bacteria, the primary focus has been on toxins, enzymes, and aggregation factors (**Table 2**). Clearly, the role of membrane-associated proteins and extracellular structures represents the first target for investigating physical recognition inside the vector and initiation of host processes. However, the methodological bias toward these functional categories may limit our understanding of other important mechanisms mediating interactions with hosts. For example, in '*Candidatus* Phytoplasma mali' 32% of

the genome has no homology to any other sequences, and for '*Candidatus* Phytoplasma asteris' strain Onion Yellow's almost 50% of the genome is classified as unknown (Kube et al., 2008). Considering the highly reduced genomes and host-dependence of these bacteria, genes without an assigned function likely still play a significant role in the biology of the organism and will need to be investigated. The continued expansion of "omics" and other next-generation technologies in molecular biology will likely shed new light on the role of unknown coding sequences in host colonization, pathogenesis, and how host specificity may have evolved independently in different bacterial lineages.

Despite these advances, research on vector-borne pathogens is still in its infancy. Some of the most significant gaps in our understanding concern interactions with insect vectors. In particular, our understanding of leafhopper and psyllid feeding behavior, immunity, and plant responses to these insects needs to be improved. Genetic resources for these important vectors also need to be expanded. Promisingly, the genomes for the psyllid *Diaphorina citri*[1] and at least one planthopper have been sequenced (Noda et al., 2008) and several other genome projects for important vectors of bacterial pathogens are underway (Evans et al., 2013; Poelchau et al., 2015). However, accessibility and quality control of insect genomic data remains an ongoing concern for the entomological community. In response to this, several projects attempting to address these issues have been initiated (Legeai et al., 2010; Poelchau et al., 2015; Yin et al., 2016), though at the time of publication most of these online resources remain works-in-progress. This area of research is likely to progress rapidly in the coming years. While climate change and the global food economy will continue to drive emergence of additional vector-borne bacterial pathosystems, the advent of genome editing, single-cell–omics, and interference RNA techniques will contribute to the identification of vector-borne bacterial phytopathogens and advances in our knowledge.

[1] http://www.ncbi.nlm.nih.gov/genome/?term=txid121845[orgn]

AUTHOR CONTRIBUTIONS

CC conceived the project. CC and LP-H wrote the article.

FUNDING

This work was supported in part by a grant from the NIFA (award 2013-2013-03265) to CC and University of California start-up funds to CC; and by a Fulbright-COLCIENCIAS (Colombian Administrative Department of Science, Technology and Innovation) fellowship to LP-H.

ACKNOWLEDGMENT

We thank Aurelie Bak and MacKenzie Patton for helpful discussion and comments on earlier versions of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01163

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 16 March 2017
doi: 10.3389/fpls.2017.00355

The Involvement of Heat Shock Proteins in the Establishment of *Tomato Yellow Leaf Curl Virus* Infection

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Tomato yellow leaf curl virus (TYLCV), a begomovirus, induces protein aggregation in infected tomatoes and in its whitefly vector *Bemisia tabaci*. The interactions between TYLCV and HSP70 and HSP90 in plants and vectors are necessity for virus infection to proceed. In infected host cells, HSP70 and HSP90 are redistributed from a soluble to an aggregated state. These aggregates contain, together with viral DNA/proteins and virions, HSPs and components of the protein quality control system such as ubiquitin, 26S proteasome subunits, and the autophagy protein ATG8. TYLCV CP can form complexes with HSPs in tomato and whitefly. Nonetheless, HSP70 and HSP90 play different roles in the viral cell cycle in the plant host. In the infected host cell, HSP70, but not HSP90, participates in the translocation of CP from the cytoplasm into the nucleus. Viral amounts decrease when HSP70 is inhibited, but increase when HSP90 is downregulated. In the whitefly vector, HSP70 impairs the circulative transmission of TYLCV; its inhibition increases transmission. Hence, the efficiency of virus acquisition by whiteflies depends on the functionality of both plant chaperones and their cross-talk with other protein mechanisms controlling virus-induced aggregation.

Keywords: begomovirus, heat shock proteins, protein quality control, tomato, whitefly

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *09 December 2016*
Accepted: *01 March 2017*
Published: *16 March 2017*

Citation:
Gorovits R and Czosnek H (2017) The Involvement of Heat Shock Proteins in the Establishment of Tomato Yellow Leaf Curl Virus Infection. Front. Plant Sci. 8:355. doi: 10.3389/fpls.2017.00355

INTRODUCTION

Plants often grow in unfavorable environments such as poor soils, heat and drought, and have to cope with pathogens such as viruses, fungi, bacteria, and with sucking and chewing insects. Plants have adapted to these conditions and their genome contain genes conferring tolerance to various stresses, which are tapped by breeders to develop varieties adjusted to these environments. Plants have also developed strategies to cope with diseases transmitted by pathogens. They use either pre-formed structures and chemicals to stop spread and repel invaders or they respond to infection by inducing an immune-like response. Both type of responses sense and react to the pathogen by sending signals to other cells of the plant, leading to transcriptional reprogramming, and biosynthesis of compounds that limits pathogen spread (Jones and Dangl, 2006). Immunity comes in different forms, from PAMP/MAMP-triggered immunity to *R*-gene mediated immunity; often these processes are happening simultaneously. The HR does not always occur in *R* gene responses for pathogens or insects. However, the signaling cascades and downstream gene expression does occur in all interactions in host and non-host organisms. HR acts largely inside the cell by using

proteins encoded by *R* genes that cause an apoptotic hypersensitive response, or/and by activating resistance and defense genes. Plant may also respond to infection in one part of the plant enhancing the defense response in other parts (Hail and Bostock, 2002; Fu and Dong, 2013). Against viruses, plants frequently mobilize RNAi-mediated gene silencing mechanisms to suppress the expression of viral genes (Mandadi and Scholthof, 2013).

Tomato yellow leaf curl virus (TYLCV) is a begomovirus (genus *Begomovirus*, family *Geminiviridae*) transmitted by the whitefly *Bemisia tabaci* (*Hemiptera: Aleyrodidae*). It is one of the viruses causing the most damages to tomato crops (Czosnek, 2007; Scholthof et al., 2011). The TYLCV complex comprises many species and isolates discernable by their DNA sequence (Czosnek, 2008). Begomoviruses belong to the Geminiviridae family characterized by a 22 nm × 38 nm geminate virion containing one circular ssDNA genome of 2,700–2,800 nucleotides in length. The encapsidated TYLCV genome strand comprises two genes, V1 and V2; the complementary-sense strand (synthesized during the viral DNA replication) comprises four genes, C1 to C4 (Navot et al., 1991). V1 encodes the coat protein (CP), which is indispensable for cell-to-cell and long-distance movement, and transmission by whiteflies. All the other genes are also multifunctional and their activity is aimed at ensuring virus replication and spread, and at counteracting plant defenses (summarized by Díaz-Pendón et al., 2010).

The path of TYLCV (and begomoviruses in general) in the infected plant and in the whitefly vector is known in its broad features. Virions are inoculated into the phloem by viruliferous whiteflies during feeding and transported to the phloem-associated cells. The viral single-stranded DNA (ssDNA) genome is then freed from the capsid. Replication is initiated when host DNA polymerases synthesize the virus genome complementary strand, creating a double-stranded DNA (dsDNA) form of the viral genome. The proteins encoded by the complementary strand are expressed, especially the replication-associated protein (Rep encoded by the C1 gene), initiating the rolling-circle replication mechanism. The CP is expressed and the nascent viral genomic ssDNA is packaged into virions. The viral particles propagate cell-to-cell and long-distance via the phloem (see details in Hanley-Bowdoin et al., 2013). TYLCV-infected susceptible tomato plants are stunted, leaves are curled and swelled, and yields are reduced.

Bemisia tabaci acquires TYLCV with their stylets while feeding on infected plants. Then virions reach the esophagus and the midgut, which they cross into the haemolymph on their way to the salivary glands. Secretory cells mediate the transmission of begomoviral particles to plants together with saliva (Ghanim et al., 2001; Wei et al., 2014). TYLCV can express viral genes and replicate in the insect vector (Pakkianathan et al., 2015; Wang et al., 2016).

Tomato yellow leaf curl virus is associated with modifications of the expression patterns of many genes, as well as changes in the protein and metabolite contents of both host plant and insect vector. All these changes are thought to facilitate host invasion, virus genome replication and expression, and to resist host defenses. In this article, we summarize our knowledge on the association of TYLCV with tomato host and virus vector chaperone systems, a critical step that ensures a successful infection.

TYLCV INTERACTIONS WITH PLANT HOST AND INSECT VECTOR HEAT SHOCK AND QUALITY CONTROL PROTEINS

TYLCV Infection Leads to Changes in the Transcriptome, Proteome, and Metabolome of the Tomato Host Plant and of the Whitefly Vector

Transcriptome analyzes of tomato infection (using subtraction cDNA libraries and microarrays) revealed that TYLCV induces significant changes in the 1st days after inoculation, changes that exacerbate as infection progresses (Eybishtz et al., 2009; Chen et al., 2013; Sade et al., 2013; Miozzi et al., 2014). These responses include the activation of genes involved in general stress-response, hormone biosynthesis, signal transduction, RNA regulation and processing, induction of the ubiquitination pathway and initiation of autophagy. TYLCV-susceptible plants emitted high levels of reactive oxygen species (ROS), pathogenesis-related (PR), and wound-induced proteins. Sources of carbon and nitrogen were highly affected (Moshe et al., 2012). Tomato infection with TYLCV was accompanied with significant changes in the abundance of various classes of metabolites such as amino acids and polyamines, phenolic and indolic metabolites, indicating a tightly coordinated reprogramming of phenylpropanoid, tryptophan/nicotinate, urea/polyamine, and salicylic acid biosynthesis pathways leading to the production of defense compounds (Moshe et al., 2012; Sade et al., 2015).

Tomato infestation with non-viruliferous whiteflies induced a decrease in the amounts of MAPKs, heat shock proteins (HSPs), as well as increased activities of the *PR* genes, β-1,3-glucanase, and peroxidase. These effects were exacerbated when the insects carried TYLCV (Gorovits and Czosnek, 2007; Gorovits et al., 2007). In another study, it was shown that PR genes are expressed when *B. tabaci* and the greenhouse whitefly *Trialeurodes vaporariorum* are feeding on tomato plants (Puthoff et al., 2010). Transcriptome analyses of different plants (e.g., *Arabidopsis*, Kempema et al., 2007; tomato, Musser et al., 2014; cotton, Li et al., 2016) upon infestation by non-viruliferous whiteflies showed a specific expression of genes associated with photosynthesis, senescence, secondary metabolism, and stress.

The interactions of geminiviruses with their insect host also induced changes in signaling and defense pathways. The long-term presence of TYLCV in the whitefly host (sometimes for the remaining lifespan) has deleterious effects on the longevity and fertility of the insect (Rubinstein and Czosnek, 1997; Pan et al., 2013). In the recent few years, high-through put sequencing

has allowed studying the transcriptome of different species (previously referred as biotypes) of adult whiteflies from various locations, males and females, and their developmental stages (Leshkowitz et al., 2006; Wang et al., 2011, 2012, 2013; Seal et al., 2012). In addition to whole whiteflies, the transcriptome of several organs involved in begomovirus transmission such as the primary salivary gland (Su et al., 2012) and the gut (Ye et al., 2014) has been analyzed. Genes differentially expressed upon TYLCV (or *Tomato yellow leaf curl China virus*, TYLCCNV) acquisition and retention were identified by several methods, including subtractive hybridization (Li et al., 2011), microarrays (Götz et al., 2012) and transcriptome sequencing (RNA-Seq) (Luan et al., 2011). Results showed that more than 1,500 genes were differentially regulated. Among these were genes involved in the activation of the immune responses and of the autophagy pathway, as well as genes encoding HSPs. Several studies aimed at investigating the response of *B. tabaci* to plant defenses have shown that the insect is able to detoxify induced secondary metabolites (Alon et al., 2012; Elbaz et al., 2012).

Interactions of TYLCV and Other Viruses-Encoded Proteins with Host Proteins

Tomato yellow leaf curl virus, with only six genes (eight genes in begomovirus with bipartite genomes), needs to replicate, spread and counter host defenses (Hanley-Bowdoin et al., 2004, 2013). For instance, since begomoviruses do not encode their own replicase, they use the Rep protein (encoded by the C1 gene) to interact with the host DNA replication and cell cycle machineries. For example, the *Tomato golden mosaic virus* (TGMV) Rep cooperates with a retinoblastoma-like protein to promote the replication of the TGMV DNA (Arguello-Astorga et al., 2004), while the Rep of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) recruits a complex of proliferating cell nuclear antigen (PCNA) and plant DNA polymerase to the viral origin of replication (Castillo et al., 2003). TYLCV V2 is a Suppressor of Gene Silencing (Zrachya et al., 2007), which interacts with the host proteins SGS3 and CYP1 (Glick et al., 2008; Bar-Ziv et al., 2012). TYLCSV C2 interacts with the COP9 subunit of the signalosome (CSN), a complex involved in the regulation of the ubiquitination, preventing tagging the virus for destruction (Lozano-Durân et al., 2011). TYLCV C4 protein interacts with tomato plant defense proteins (Kim et al., 2016).

The ability of viruses to hijack cellular processes stipulates that the infected cell protects the structural and functional complexity of the virus proteins. Many viruses depend on host chaperones/heat stress proteins (HSPs) for folding, protein quality control (PQC) and maintenance of proteostasis (Mayer, 2005; Nagy and Pogany, 2012). HSPs affect virus expression, replication, and assembly and counter the plant responses to infection (Nagy et al., 2011). HSPs are involved in the assembly of the large virus-induced protein aggregates (coined viral factories, VFs), sheltering the virus, promoting their activity and their multiplication (the characteristics of VFs in mammalian cells have been reviewed by Wileman, 2006, 2007; Livingston et al., 2009; Netherton and Wileman, 2011).

HSP70 and HSP90 are the most frequent chaperons utilized by viruses. HSP90 promotes *Bamboo mosaic virus* replication by interacting with the virus replicase (Huang et al., 2012). Similarly, HSP70 and HSP90 form a 480-kDa multicomponent complex with the *Red clover necrotic mosaic virus* replicase and interact with p27, a viral-encoded component of the replicase complex on the endoplasmic reticulum membrane (Mine et al., 2012). The association of HSP70/HSC70 and HSP90 involves interactions with the HSP90 co-chaperone, SGT1 (for Suppressor of G2 allele of skp1) (Noel et al., 2007). In plants (and animals), SGT1 is essential to the function of many NLR (nucleotide-binding leucine-rich repeat receptor) proteins that induce plant defenses (Liu et al., 2004). SGT1 enhances *Potato virus X* multiplication, while *SGT1* silencing led to an increased accumulation of *Plantago asiatic mosaic virus* in *Nicotiana benthamiana* (Komatsu et al., 2010; Ye et al., 2012). Indeed, SGT1 is involved in PQC by associating with the ubiquitin and 26S proteasome protein degradation complexes (Muskett and Parker, 2003) and by interacting with two COP9 signalosome components (Azevedo et al., 2002; Liu et al., 2002).

The HSP70 family is actively participating in the biology of geminiviruses (Gorovits et al., 2013a). During the live cycle of the bipartite begomovirus *Abutilon mosaic geminivirus* (AbMV), the chloroplast cpHSC70-1 proteins binds to the virus movement protein (MP). *In planta*, cpHSC70-1/MP complexes were visualized at the cell periphery and within chloroplasts, suggesting that AbMV utilizes cpHSC70-1 to move intra- and inter-cellularly (Krenz et al., 2010, 2012). Silencing cpHSC70-1 inhibited AbMV movement, but not replication.

Heat shock proteins are also associated with the circulative transmission of begomoviruses in their whitefly vector. Microarray-based analyses of the *B. tabaci* transcriptome in response to the ingestion and retention of the monopartite TYLCV and the bipartite *Squash leaf curl virus* (SLCV) indicated that the insect *Hsp70* transcription is induced upon virus infection. Immuno-capture PCR (IC-PCR) and virus-overlay protein-binding confirmed the interaction of TYLCV and SLCV CP with HSP70 in *B. tabaci* (Götz et al., 2012). In the digestive tract, TYLCV and HSP70 co-localized exclusively in the insect filter chamber and cecae. Whiteflies membrane-fed with anti-HSP70 antibodies had enhanced capacities to transmit TYLCV, indicating that HSP70 limits virus transmission, possibly moderating some of the potential long-term harmful effects of the virus on the whitefly.

Cytosolic HSP70 isoforms were shown to be required at distinct steps of the life cycle of Dengue virus (DENV, genus *Flavivirus*, family *Flaviviridae*), a mosquito-borne virus causing a life-threatening disease in human (Bhatt et al., 2013). DENV generates a web derived from the ER (Welsch et al., 2009), where replication takes place. These processes are highly dependent on the proper folding of viral proteins and its control by cellular chaperones. Moreover, it was shown for the *Japanese encephalitis virus* (a *Flavivirus* related to DENV) that HSP70 protects proteins from degradation (Ye et al., 2013). HSP70 is involved in DENV entry, RNA replication, and virion biogenesis. Nine distinct DNAJ cofactors (also known as HSP40) are necessary for proper

HSP70 function: DnaJB11 promote viral RNA synthesis, while DnaJB6 in concert with the CP promotes assembly of viral particles (Taguwa et al., 2015).

TYLCV Infection Is Characterized by the Induction of Aggregates of Increasing Size, Reminiscent of Animal Viral Factories

It has been known for several decades that Azure-A stains aggregates/inclusion bodies that could be visualized with the light microscope in the phloem-associated cells of leaves of begomovirus-infected (including TYLCV) susceptible plants (Christie et al., 1986). The role of these aggregates in the process of geminivirus propagation and in the host immune response was intriguing.

Ultracentrifugation of native proteins in linear 10–50% sucrose gradients allowed to separate proteins aggregates according to size, from soluble and small (top fractions) to large bottom fractions, via mid-size (Gorovits et al., 2013b). Using *in situ* immuno-detection, cell fractionation and separation of proteins by ultracentrifugation, it was shown that TYLCV CP is localized in aggregates of increasing size as infection progresses (**Figure 1**). These aggregates occur first in the cytoplasm then in the nuclei of phloem-associated cells (Gorovits et al., 2013b). The large CP aggregates, which can be compared with VFs in animal cells (Wileman, 2006, 2007), is a major feature of a successful TYLCV infection. The role of small/mid-size aggregates in sheltering TYLCV components and protecting them from host degradation has been shown in plant and in insect cells (Gorovits et al., 2016). In both hosts, the proteolytic activities in the small/mid-size aggregates were low. At the beginning of plant and vector infection TYLCV proteins were found in mid-size aggregates. Altogether, aggregation may have a dual role: (1) from the virus point of view: protecting the virus from host proteases, and concentrating enzymes and other factors necessary for its replication, (2) from the host plant point of view: sequestrating virus components, isolate and neutralize its proteins, preventing virus expression and prepare viral components for destruction.

Co-localization of TYLCV CP and Host HSPs

HSP70 presents opposite behaviors in whitefly and in tomato in the presence of TYLCV. Microarray-based analyses showed that the insect *Hsp70* was upregulated as TYLCV is ingested (Götz et al., 2012). In plants, TYLCV does not induce the expression of *Hsp70*. On the contrary, increasing amounts of viral DNA and CP were accompanied by decreasing amounts of plant HSP60, HSP70, and HSP90 (Gorovits and Czosnek, 2007; Gorovits et al., 2007; Moshe et al., 2012).

HSP70 and viral CP in tomatoes and whiteflies was investigated *in situ* using fluorescently labeled antibodies. In leaves, HSP70 and CP aggregates of increasing size were found first in the cytoplasm then in the nucleus (**Figure 2**). At the late infection stages (49 dpi), the large aggregates contained both proteins (Gorovits et al., 2013a). Co-immunoprecipitation (Co-IP) assay revealed the ability of CP to interact with tomato and *B. tabaci* HSP70 (**Figure 3**), pointing on the development of potential complexes between TYLCV CP and HSP70 of both viral hosts.

Fluorescence microscopy detected co-localized TYLCV CP with HSP90 in leaves of infected tomato, first in cytoplasmic, then in large nuclear aggregates (**Figure 2**). Prior to infection, HSP90 was not found in large aggregates (Moshe et al., 2016). Co-IP showed that plant HSP90 and CP interacted in protein extracts from the nucleus, but not from the cytoplasm. Therefore, HSP70 and HSP90 formed complexes with TYLCV CP in large nuclear aggregates operating as VFs, but not in cytoplasmic aggregates. Interestingly chaperones such as HSP60 and the glucose related protein 78 (GRP78 or BiP) were not found in CP-containing large nuclear aggregates.

In whiteflies, fluorescent *in situ* hybridization and immuno-histology showed that TYLCV CP and HSP70 co-localized in the midgut epithelial cells. IC-PCR, protein Co-IP (**Figure 3**), and virus-overlay protein-binding assays pointed not only on possible co-localization, but also on CP-HSP70 interaction (Götz et al., 2012; Ghanim and Czosnek, 2016). The HSP90 localization and its relation with CP in whiteflies is currently being examined.

Decrease of HSP70 and HSP90 Affects Differently the Accumulation of TYLCV in Plants and Insects

In plant cells, the expression of *Hsp70* can be reduced by quercetin, a bioflavonoid that inhibits *Hsp70* transcription (Wang et al., 2009). Quercetin-treated infected tomato leaves contained reduced amounts of virus, decreased CP quantities in large nuclear aggregates and increased CP levels in cytoplasmic mid-size aggregates (Gorovits et al., 2013a). Taken as a whole, we propose that HSP70 plays an important role in the nuclear CP transportation and in TYLCV replication.

The involvement of HSP90 in TYLCV infection was studied using the benzoquinone antibiotic geldanamycin (GDA), which inhibits the activity of HSP90. Tomato leaves were treated with GDA and the location of TYLCV CP was examined. In contrast to HSP70, HSP90 did not affect the nuclear localization of CP and, therefore, is not required for the translocation of CP to the nucleus. However, silencing of *Hsp90* and *SGT1* led to enhanced accumulation of TYLCV CP as infection develops (Moshe et al., 2016). This increase in virus amounts could be connected with the HSP90 activity in the cell protein degradation machinery. Indeed, HSP90 has a key role in the function of the 26S proteasome. Proteins destined for degradation are attached to ubiquitin (Ub) and this complex is then degraded by the 26S proteasome, the major proteolytic system of eukaryotic cells (Voges et al., 1999; Smalle and Vierstra, 2004; Sadanandom et al., 2012). In plants, loss of function of the 26S proteasome leads to cell death (CD) and to the release of ROS (Kim et al., 2003). GDA-induced HSP90 inhibition causes the disruption of the 26S proteasome and the loss of its protease activity (Nishizawa-Yokoi et al., 2010). HSP90 inactivation also leads to a decrease in the

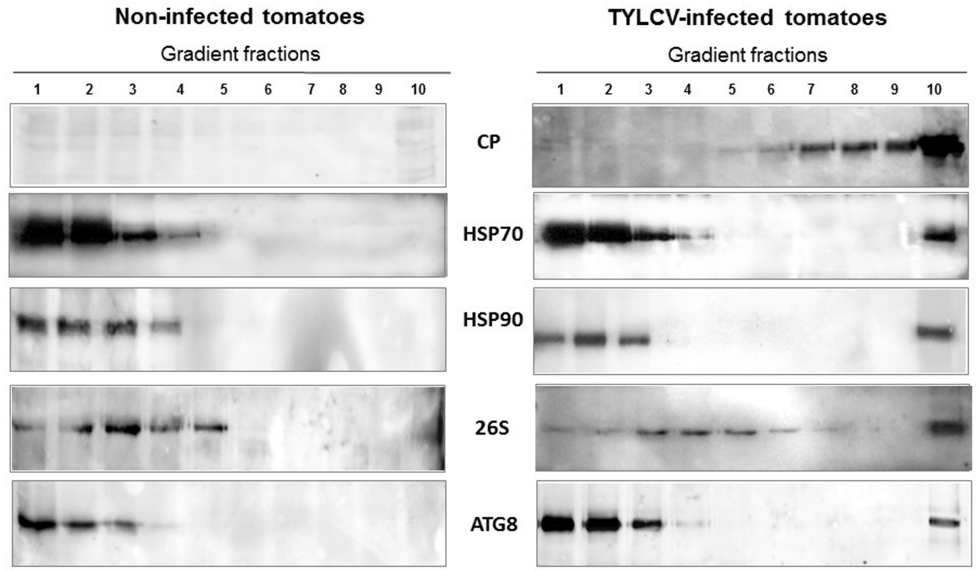

FIGURE 1 | *Tomato yellow leaf curl virus* (TYLCV) induces the aggregation of coat protein (CP) as well as of tomato HSP70 and HSP90 in infected tomato leaves. Distribution of TYLCV CP and plant HSP70 and HSP90 after sedimentation of leaf native proteins on linear 10–50% sucrose gradients (according to Gorovits et al., 2013b). Leaf homogenates were prepared from infected tomato plants at 28 dpi; non-infected plants of the same age were similarly processed. Gradients were divided into 10 fractions, 1 (top – contained soluble proteins) to 10 (bottom – contains large protein aggregates), and aliquots were subjected to SDS-PAGE, followed by western blot immunodetection with antibodies against TYLCV CP, and plant HSP70 and HSP90.

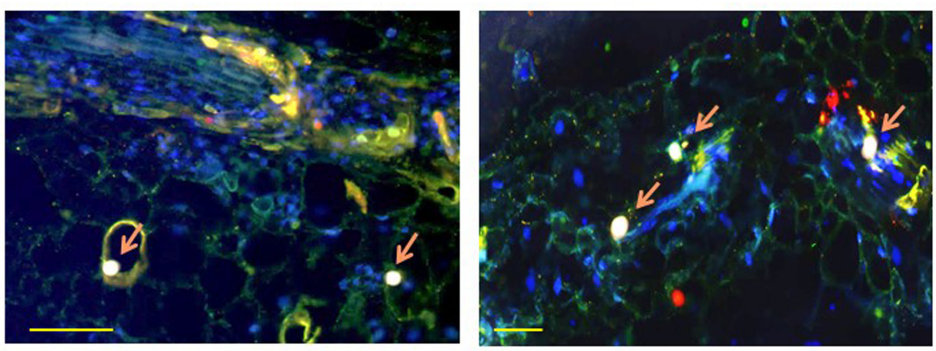

FIGURE 2 | Co-localization of TYLCV CP with tomato HSP70 (left) and with tomato HSP90 (right) in infected leaf at 49 dpi, as observed with a confocal microscope. Cross-section through the leaf blade. CP appears as red, cellular HSP70 or HSP90 as green, nuclei as blue; CP co-localizing with HSP70 or HSP90 in nuclei as pink (pink arrow). Bar is 100 nm. The left photograph is reproduced, with permission, from Gorovits et al. (2013a).

degradation of the TYLCV protein V2 by the 26S proteasome (Moshe et al., 2016). Loss of function of HSP70 and HSP90 had contrary effects on virus levels. As inhibition of *Hsp70* transcription by quercetin impaired the propagation of TYLCV, probably by slowing down the nuclear transport of the viral CP nuclear, GDA-treatment and *Hsp90* silencing inactivated the UPS, accompanied by increased viral CP and DNA levels.

Involvement of Whitefly Chaperones in the Circulative Transmission of TYLCV

Whiteflies contain two types of chaperones: those synthesized by the insect cells and those produced by their endosymbiotic bacteria, housed in cells named bacteriocytes (Baumann, 2005). The cellular chaperones belong to the HSP family found in other eukaryotic cells (e.g., HSP23, HSP70, and HSP90). The expression of the *Hsp* genes is upregulated in response to abiotic (pesticide applications, heat, and UV radiation) and biotic stresses (viruses, bacteria, fungi, and insect natural enemies) (Zhao and Jones, 2012). The endosymbiotic GroEL chaperones are barrel-shaped structure consisting of two superimposed rings of seven subunits each. The co-chaperonin GroES is necessary for GroEL activity (Hayer-Hartl et al., 2016). GroEL and GroES are structurally and functionally nearly identical to the eukaryotic proteins HSP60 and HSP10, respectively.

The different whitefly *B. tabaci* species (De Barro et al., 2011) harbor the obligatory primary endosymbiont *Portiera*

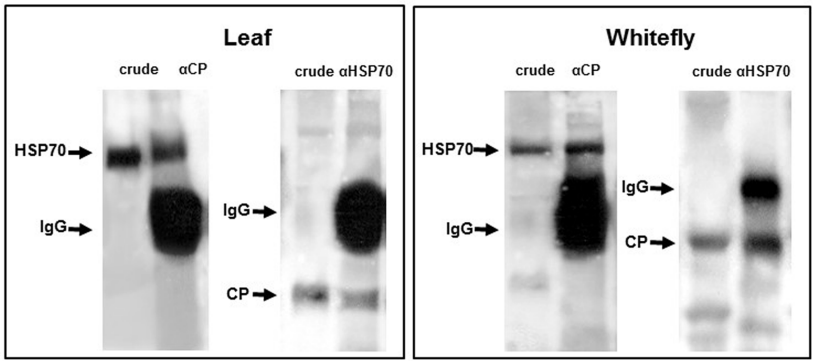

FIGURE 3 | Co-immunoprecipitations of cellular HSP70 with viral CP and *vice versa* in tomato leaves and *Bemisia tabaci*. Co-immunoprecipitation of CP with anti-TYLCV CP specific antibody and HSP70 with anti-HSP70 specific antibody in leaf and whitefly protein extracts. The direct immunodetection was designed as "crude."

aleyrodidarum, together with some of the facultative secondary endosymbionts such as *Arsenophonus*, *Cardinium*, *Fritschea*, *Hamiltonella*, *Rickettsia,* and *Wolbachia* (Chiel et al., 2007). The role of an endosymbiotic GroEL protein in the circulative transmission of a plant virus by its insect vector was first demonstrated for aphid-transmitted luteoviruses (van den Heuvel et al., 1994). Later it was found that a GroEL produced by *B. tabaci* B (also known as MEAM1) *Hamiltonella* ensured the transmission of TYLCV to tomato plants by protecting the virus from destruction in the hemolymph (Morin et al., 1999). GroELs from other secondary endosymbionts, whether in B or Q (also referred as MED) whiteflies do not contribute to substantial level of TYLCV transmission (Gottlieb et al., 2010).

Other *B. tabaci* cellular chaperones may play a role in begomovirus transmission. A 16-kDa protein belonging to the HSP20 family interacted with TYLCSV CP in a protein-protein binding assay (Ohnesorge and Bejarano, 2009). Microarray studies indicated that the expression of an *Hsp70* gene was modulated upon TYLCV acquisition. TYLCV and HSP70 interacted in *in vitro* tests. TYLCV (and the bipartite begomovirus *Watermelon chlorotic stunt virus*, WmCSV) co-immuno-localized with HSP70 within epithelial cells of the whitefly midgut. Feeding whiteflies with an anti-HSP70 antibody was associated with an increase in the efficiency of virus transmission to plants, suggesting that HSP70 may help restrain virus translocation from the gut into the hemolymph (Götz et al., 2012). Interestingly HSP70 in *B. tabaci* behaves inversely than in tomato plants, where downregulation of *Hsp70* led to a decrease in TYLCV amounts (Gorovits et al., 2013a).

Conversion of Cellular Chaperones from Soluble into Insoluble State upon TYLVCV Infection Is Part of the Cellular Protein Quality Control and Virus Degradation Process

Western blot analysis of native proteins from non-infected and TYLCV-infected tomato leaves, separated by ultracentrifugation in 10–50% sucrose gradients, showed that HSP70 and HSP90 were found in aggregates only in infected tissues (**Figure 1**). The large aggregates also contained the other components of the plant PQC system, such as the autophagy-related protein 8 (ATG8) (Gorovits et al., 2016), ubiquitin, and the regulatory subunit of the 26S proteasome (Gorovits et al., 2014) (**Figure 1**). Since ATG8 is needed for the formation of autophagosomes and is a key element of autophagy (Shpilka et al., 2011), autophagy may be a major mechanism induced by plants to cope with TYLCV infection. Similarly, when viruliferous whiteflies native proteins were separated in sucrose gradients, the fractions containing large aggregates included also the 26S proteasome; ATG8 was not immuno-detected, perhaps because the antibody did not recognize the insect protein (**Figure 4**).

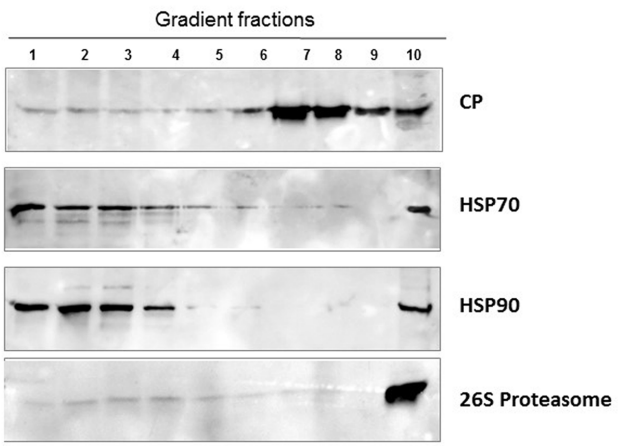

FIGURE 4 | Aggregates of TYLCV CP and whitefly HPS70 and HSP90, 26S proteasome. Native proteins of viruliferous whiteflies (after about a week feeding on infected tomato) were separated by ultracentrifugation of 10–50% sucrose gradients. The gradients were resolved in 10 fractions. Aliquots were subjected to SDS-PAGE, followed by western blot immunodetection with antibodies against TYLCV CP, HSP70 and HSP90 and 26S proteasome. As in tomato, TYLCV induced the formation of large HSP70/90 aggregates; the 26S proteasome was also in large aggregates.

In vitro tests showed that large aggregates exhibited proteolytic activities that could digest all the six proteins encoded by the TYLCV genome. Moreover, incubation of detached tomato leaves with the 26S proteasome inhibitor MG132, with the autophagy inhibitor wortmannin and with the autophagy inducer rapamycin (Yang et al., 2013) caused changes in the TYLCV CP and V2 aggregation patterns, pointing on the involvement of these degradation mechanisms in TYLCV infection. The amounts of the six TYLCV proteins changed upon MG132 or wortmannin treatment, indicating that 26S proteasome and autophagy are involved in the degradation of begomoviruses (Gorovits et al., 2016). The presence of PQC elements, including crucial chaperones, indicated that these aggregates could be similar to animal VFs (Netherton and Wileman, 2011).

It was recently shown that TYLCV activated the autophagy pathway in the *B. tabaci* B (Wang et al., 2016). Upon feeding on infected tomatoes, there was a steady increase in the amounts of viral DNA and CP during the first 48 h followed by a decrease. Virus depletion was correlated with the activation of autophagy, as the levels of ATG8-II greatly increased and the amounts of autophagosomes in the whitefly midgut was enhanced. The activation of whitefly autophagy inhibited virus transmission, whereas the inhibition of autophagy promoted virus transmission. Hence, *B. tabaci* uses autophagy to curb TYLCV amounts in the insect, possibly to restrain putative negative effects of the virus on the whitefly life cycle (Wang et al., 2016).

DISCUSSION

HSPs and PQC Elements Govern the Size of TYLCV-Containing Protein Aggregates, Which Serve to Protect or to Destroy TYLCV

Like the other begomoviruses, TYLCV is dependent on the host cell machinery for survival. During infection, begomoviruses remodel the host cells by interacting with cellular proteins. The limited set of viral proteins requires their multi-functionality and interaction with the host chaperones, such as HSP70 and HSP90. Protein aggregation is a major process in which viruses and viral proteins ensure their survival and replication in the infected cell. Based on their behavior in infected plants and viruliferous whiteflies, we suggest that movement, localization and degradation of the key chaperones (HSP70 and HSP90), together with PQC such as ubiquitin, 26S proteasome and autophagy proteins play a major role in TYLCV aggregation and consequently, in virus mobilization.

Tomato yellow leaf curl virus could induce a mechanism to sequester virus-induced misfolded or modified cellular proteins in aggregates to prevent the triggering of innate antiviral responses, inhibit the induction of cell death and prevent an activation of HSFs and their substrates, which suppress virus successful multiplication. Indeed, TYLCV-infected tomatoes are characterized by low levels of cell death; moreover, TYLCV is able to alleviate cell death, induced by the other stresses such as heat (Anfoka et al., 2016).

Tomato yellow leaf curl virus in tomato does not induce HSP70/HSP90 expression, but causes their shift from soluble proteins into aggregates (**Figure 1**). During the development of plant infection, HSP70 and HSP90 re-localize in an aggregated state, from the cytoplasm to the nucleus in the cells associated with the vascular system. The other PQC elements such as ubiquitin, 26S proteasome subunits, autophagy protein ATG8 are present in the large nuclear VFs together with TYLCV proteins (mainly CP), viral DNA, DNA-protein complexes and infectious virions.

Intracellular homeostasis depends on PQC, the constant degradation and re-synthesis of proteins. At one end, chaperons modulate protein folding and repair. At the other end, HSPs are involved in proteasome and autophagy removal of dysfunctional proteins. These systems may influence each other (Dokladny et al., 2013, 2015). Invading viruses are considered by cells under attack as entities to be destroyed or sequestered, while viruses have evolved mechanisms to subvert proteolysis, such as the autophagic response (reviewed by Chiramel et al., 2013). Apart from playing a major role in antiviral defense, autophagy can also enhance viral replication (Shoji-Kawata and Levine, 2009). Inhibitory effect of autophagy genes was demonstrated on *Tobacco mosaic virus* (TMV) replication in *N. benthamiana* plants (Liu et al., 2005). In *B. tabaci* B, TYLCV triggers the insect autophagy antiviral program, promoting the formation of autophagosomes and curbing TYLCV infection (Wang et al., 2016).

HSP70 co-localized with TYLCV CP in aggregates not only in tomato plants, but also in the TYLCV *B. tabaci* vector. Whiteflies are well adapted to high temperatures. When temperatures rise from 25 to 40°C, the expression of their *Hsp70* and *Hsp90* genes is upregulated, improving the fitness of the insect (Mahadav et al., 2009; Díaz et al., 2015). In contrast, the presence of TYLCV impairs the fitness of *B. tabaci* raised at high temperatures.

The completion of a successful infection by the begomovirus TYLCV depends on its interaction with cellular chaperones, among them HSP70 and HSP90, at all stages of the virus life cycle: in the tomato host, during acquisition by its whitefly vector and in the insect. The outcome of which was the formation of protein aggregates of different sizes, which could simultaneously serve to protect and destroy TYLCV, depending on the recruitment of PQC components.

Do Changes in the Amounts of Plant HSPs, and Thereby in the Size of TYLCV-Containing Aggregates, Modulate the Acquisition of TYLCV by *B. tabaci*?

Inhibitors of HSP70 and HSP90 are able to change the size and the pattern of protein aggregation in infected tomato, together with their content in viral DNA and CP (**Figure 5**). In tomato leaves with quercetin-inactivated HSP70, CP shifted from large to mid-size aggregates, accompanied by a significant decrease of the

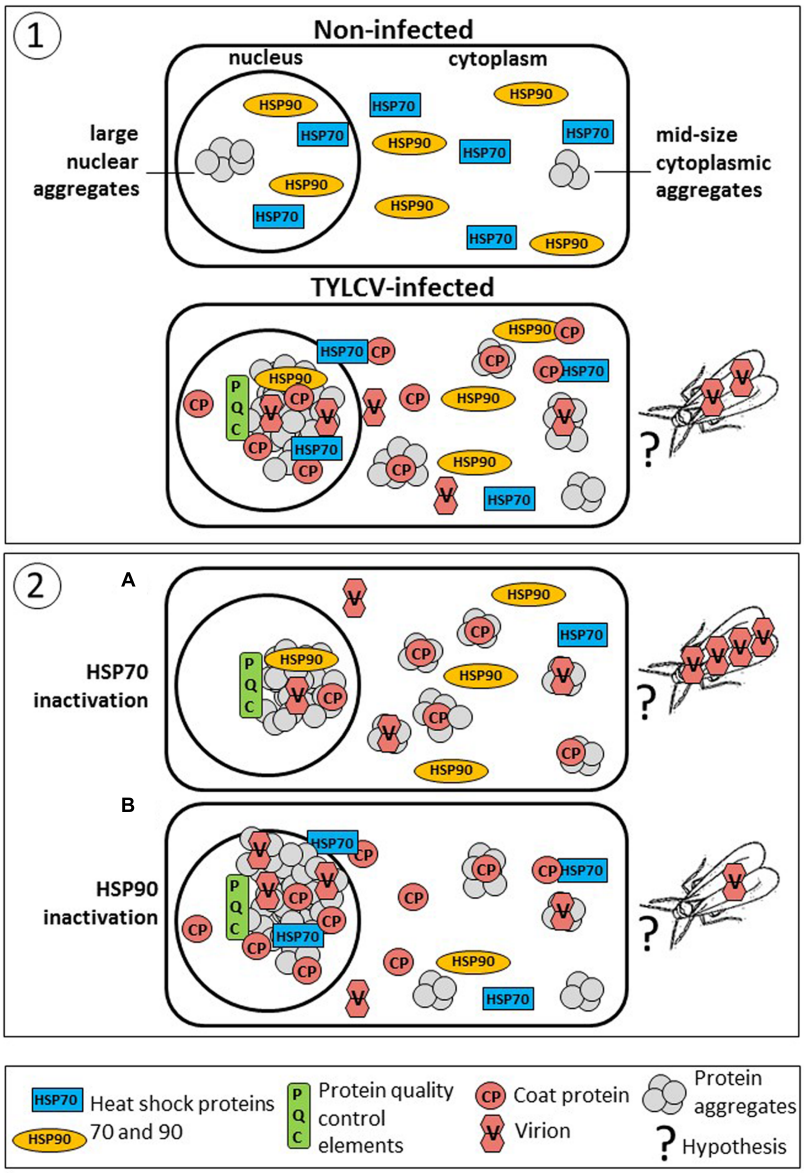

FIGURE 5 | Diagram summarizing the association of TYLCV with tomato host chaperons and how it may influence efficacy of virus acquisition by the whitefly vector. Panel (1): TYLCV causes changes in the amounts of plant chaperons and their intra-cellular location. Non-infected cells contain pool of free nuclear and cytoplasmic HSP70 and HSP90. Upon TYLCV infection there is a decline in chaperons amounts (Gorovits et al., 2007; Moshe et al., 2012). At the same time, infection leads to massive protein aggregation in the nucleus and cytoplasm. Large nuclear aggregates contain viral components (mainly, CP) together with virions (Gorovits et al., 2013b), and cellular proteins, including HSP70 (Gorovits et al., 2013b) and HSP90 (Moshe et al., 2016), as well as the other PQC elements, as in viral factories in mammalian cells. Mid-size cytoplasmic aggregates contain CP, but not HSP70 and HSP90. Whiteflies may acquire free virions or virions detached from aggregates that moved to the phloem. In the insect, TYLCV CP is localized with HSP70 in aggregates present in the filter chamber (Götz et al., 2012). Panel (2): The amounts and activities of chaperons influence the amounts of viral particles and their degree of aggregation in such a way that they may modulate TYLCV acquisition by whiteflies. (A) Inactivation of HSP70 leads to a decline in virus amounts, especially in nucleus, but results in increased abundance of CP and DNA (virions) in mid-size cytoplasmic aggregates (Gorovits et al., 2013a). Hence, it is likely that there are more virions in the phloem, and acquisition of increased virus amounts by whiteflies. (B) Inactivation of HSP90 leads to a significant increase in the total virus amounts especially in nuclear aggregates, but to decrease of virus in cytoplasmic aggregates (Moshe et al., 2016). Therefore, we hypothesize that since most virus is trapped in nuclear aggregates, less virus may reach the phloem, and therefore less virus is acquired by whiteflies.

viral DNA amounts present in untreated plants (Gorovits et al., 2013a). On the contrary, in leaves with GDA-inactivated HSP90, CP shifted from mid-size to large aggregates that contained higher amounts of viral CP and DNA (Moshe et al., 2016). Large aggregates are confined to the nucleus, while midsized aggregates are present mostly in the cytoplasm. Whiteflies acquire virus from the phloem of infected plants. Therefore, virions need to move from the phloem-associate cells to the plant vascular system.

Although it was shown that nuclear large aggregates contain infectious particles (Gorovits et al., 2013b), it is doubtful that large aggregates-containing virions translocate as such to the phloem. Therefore it is likely that the viral particles acquired by whiteflies originate from cytoplasmic free virus or/and from virus bound to mid-size aggregates. It is possible that virions move in an out from the nucleus. In, to find a shelter to avoid destruction and to serve as template for replication, and out, to provide free virions and virions attached to mid-size aggregates that will serve as inoculate in whitefly-mediated transmission. If this is the case, it might be possible to impair the ability of whiteflies to acquire begomoviruses by increasing the relative amounts of large aggregates with HSP90 inhibitors and by applied various abiotic stresses causing the formation of these TYLCV-containing structures (**Figure 5**).

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENT

This research was supported by a grant from the Israel Science Foundation, Award 1037/13.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 15 November 2016
doi: 10.3389/fpls.2016.01702

Implication of the Whitefly *Bemisia tabaci* Cyclophilin B Protein in the Transmission of *Tomato yellow leaf curl virus*

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Tomato yellow leaf curl virus (TYLCV) is a single-stranded (ssDNA) begomoviruses that causes severe damage to tomato and several other crops worldwide. TYLCV is exclusively transmitted by the sweetpotato whitefly, *Bemisia tabaci* in a persistent circulative and propagative manner. Previous studies have shown that the transmission, retention, and circulation of TYLCV in its vector involves interaction with insect and endosymbiont proteins, which aid in the transmission of the virus, or have a protective role in response to the presence of the virus in the insect body. However, only a low number of such proteins have been identified. Here, the role of *B. tabaci* Cyclophilin B (CypB) in the transmission of TYLCV protein was investigated. Cyclophilins are a large family of cellular prolyl isomerases that have many molecular roles including facilitating protein-protein interactions in the cell. One cyclophilin protein has been implicated in aphid-luteovirus interactions. We demonstrate that the expression of *CypB* from *B. tabaci* is altered upon TYLCV acquisition and retention. Further experiments used immunocapture-PCR and co-immunolocalization and demonstrated a specific interaction and colocalization between CypB and TYLCV in the the midgut, eggs, and salivary glands. Membrane feeding of anti-CypB antibodies and TYLCV-infected plants showed a decrease in TYLCV transmission, suggesting a critical role that CypB plays in TYLCV transmission. Further experiments, which used membrane feeding with the CypB inhibitor Cyclosporin A showed decrease in CypB-TYLCV colocalization in the midgut and virus transmission. Altogether, our results indicate that CypB plays an important role in TYLCV transmission by *B. tabaci*.

Keywords: TYLCV, *Bemisia tabaci*, Cyclophilin B, immunostaining, virus transmission

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *26 May 2016*
Accepted: *28 October 2016*
Published: *15 November 2016*

Citation:
Kanakala S and Ghanim M (2016) Implication of the Whitefly Bemisia tabaci Cyclophilin B Protein in the Transmission of Tomato yellow leaf curl virus. Front. Plant Sci. 7:1702. doi: 10.3389/fpls.2016.01702

INTRODUCTION

Begomoviruses are a group of icosahedral single-stranded DNA (ssDNA) viruses exclusively transmitted by the whitefly *Bemisia tabaci* in a persistent, circulative manner. Among the whitefly-transmitted viruses, 90% belong to the genera *Begomovirus* (Jones, 2003), which includes approximately 288 species[1], and have emerged as the most threatening group of plant viruses globally during the past two decades, as reported from dicotyledonous host-causing diseases of economic importance (Brown and Czosnek, 2002). Of all tomato begomoviruses,

[1]http://www.ictvonline.org/virustaxonomy.asp

Tomato yellow leaf curl virus (TYLCV) is the most threatening to tomato production worldwide (Czosnek, 2007). TYLCV is exclusively transmitted by *B. tabaci*, and many parameters for the virus acquisition, transmission, and retention have been extensively documented (Rubinstein and Czosnek, 1997; Brown and Czosnek, 2002). The 29-kDa virus coat protein (CP) is exclusively required for transmission and interaction with *B. tabaci* tissues and proteins (Briddon et al., 1990; Noris et al., 1998). TYLCV virions are acquired with the phloem sap, move along the food canal and foregut to reach the midgut, translocate across the gut epithelia into the hemolymph, then enter the primary salivary glands via endocytosis, from which they are expelled into the host plant with salivary secretions (Ghanim, 2014; Gray et al., 2014).

During this process, TYLCV is hypothesized to interact with insect proteins that influence and facilitate the virus transmission (Ghanim et al., 2001; Ghanim, 2014). Recent studies have investigated the importance of TYLCV CP in virus transmission and demonstrated its interaction with a member of the small heat-shock protein family (BtHSP16), which was identified using a yeast-two hybrid system screen against *Tomato yellow leaf curl Sardinia virus* (TYLCSV) CP (Ohnesorge and Bejarano, 2009). Another study has identified another heat-shock protein, HSP70, which interacts with the TYLCV CP *in vivo* and *in vitro*. Membrane feeding with anti-HSP70 antibodies resulted in an increase in TYLCV transmission. This result suggested that under normal conditions HSP70 restricts virus activity and transmission, thereby protecting the insect from deleterious effects of TYLCV (Gotz et al., 2012). Interactions with these proteins may be necessary for refolding of the virion particle and facilitating their circulation and translocation across membrane barriers especially the midgut-hemolymph and the hemolymph-salivary glands.

The identification of additional whitefly proteins that interact with and mediate virus transmission is critical for understanding viral strategies that aid in their highly successful transmission by whiteflies. The search for such proteins in many cases is based on results described in other circulative virus-vector systems in which other proteins facilitating virus transmission have been identified. One such example was demonstrated in recent studies in which genetic and proteomic analyses suggested that virus-binding aphid proteins belonging to the peptidyl-prolyl isomerases proteins (PPIases or Cyps) were specifically inherited and conserved in different aphid vector genotypes (Tamborindeguy et al., 2013). This suggested that they play a major role in regulating *Cereal yellow dwarf virus-RPV* (CYDV-RPV) transmission (Yang et al., 2008). Tamborindeguy et al. (2013) showed that both CypA and B interact with CYDV-RPV, and these interactions may be important but not sufficient to mediate virus transport from the hindgut lumen into the hemocoel.

Like aphids, in whiteflies, expressed *Cyp* genes were detected in EST libraries (Saripalli, 2008), and those were amplified from TYLCV non-viruliferous *B. tabaci* B adults. Members of the PPIase protein family (e.g., cyclophilins, FKBPs, and parvulins) are enzymes found in both eukaryotes and prokaryotes, participate in cell signaling, gene transcription and assist folding and localization of proteins, respectively (Hanes, 2015). More recently, Cyps were shown to facilitate dissociation of the human Papillomavirus Type 16 L1 and L2 capsid proteins from L2/DNA complexes following virus entry (Bienkowska-Haba et al., 2012), while CypA was shown to bind *Tomato bushy stunt tombusvirus* and inhibit tombusvirus replicase assembly (Kovalev and Nagy, 2013). Furthermore, Zhou et al. (2016) showed that *Cyp* genes contribute to the development and virulence of *Beauveria bassiana*, an entomopathogenic fungus. In the case of the whitefly-begomovirus system, interactions with these proteins may be necessary for refolding of virion particle, facilitating interactions with other whitefly proteins, and aiding the virus in crossing membrane barriers in the whitefly. In whiteflies, nothing is known about the function of Cyp proteins and whether they play a role in virus-whitefly interactions. In the current study we used an arsenal of biological and molecular methods to demonstrate that CypB, a member of this protein family plays an important role in *B. tabaci*-TYLCV interactions.

MATERIALS AND METHODS

Maintenance of Whiteflies and Plants

Population of the *B. tabaci* B infected with the secondary symbionts *Rickettsia* and *Hamiltonella*, rearing conditions and establishment of iso-female strains of *Rickettsia*-free (R−) and *Rickettsia*-infected (R+) strains, were conducted as previously described (Brumin et al., 2012). Briefly, the populations were reared on cotton seedlings (*Gossypium hirsutum* L. cv. Acala) maintained inside insect-proof cages and growth rooms under standard conditions of 25 ± 2°C, 60% relative humidity and a 14 h light/10 h dark photoperiod. The purity of the *B. tabaci* B population was confirmed by PCR with Bem 23 primers (**Table 1**). Healthy cotton plants were added once a month, while older plants were cut 2 days before, to make sure all the whiteflies move to the newly added plants. Both R− and R+ populations were handled in alternate days to avoid cross contamination. Unless otherwise indicated, all experiments in this work were conducted with the R+ strain.

Tomato (*Solanum lycopersicum* cv. Avigail) and cotton plants were grown in potting mix in 1.5-L pots under artificial light and maintained inside insect-proof cages in the greenhouse under controlled conditions as detailed above.

TYLCV Source and Insect-Mediated Transmission

Partial tandem repeat (PTR) construct of an Israeli isolate of TYLCV DNA A (Genbank Accession number X15656) described previously in Navot et al. (1991) was used. PTR construct was transformed to the *Agrobacterium tumefaciens* strain C58 by direct transformation. A 2-day-old *A. tumefaciens* culture carrying the construct was plated on LB broth with rifampicin (30 mg/ml) and kanamycin (50 mg/ml), and incubated at 28°C for 24 h (180 rpm), the *Agrobacterium* cells were collected by centrifugation for 10 min at 3,000 rpm and resuspended to an OD600 of 0.6–0.9 in suspension solution (MS medium supplemented with 10 mM MES and 200 mM

TABLE 1 | Primers used in this study.

Gene/Probe	Name	Primer sequence (5′→3′)	Expected product size (bp)	Reference
CypB	CypBF	ATGAAGAACCCGAAAGTTCA	615	This study
	CypBR	TTATTCGGTGGCATCAGCTT		
CypBs	CypBsF	ATGAAGAACCCGAAAGTTCA	216	This study
	CypBsR	GAAATTCTCGACAGTCTTCG		
CypDs	CypDsF	ATGGAGCTCCGCAATGATGT	155	This study
	CypDsR	GTGCCGTTGTGGTTTGTGAA		
CypGs	CypGsF	AGATGTACCGCAGCCCAAAT	188	This study
	CypGsR	GGGTCCGATACCTCAGGACT		
Bemisia tabaci β-Actin	Fβ-Actin	TCTTCCAGCCATCCTTCTTG	81	Sinisterra et al., 2005
	Rβ-Actin	CGGTGATTTCCTTCTGCATT		
B. tabaci B	Bem 23F	CGGAGCTTGCGCCTTAGTC	200	De Barro et al., 2003
	Bem 23R	CGGCTTTATCATAGCTCTCGT		
TYLCV CP	V61	ATACTTGGACACCTAATGGC	412	Ghanim et al., 1998
	C473	AGTCACGGGCCCTTACA		

acetosyringone) and incubated at room temperature for 2 h before agroinfiltration. The tomato plants were observed for leaf curl symptoms and subjected to PCR after 3 weeks using TYLCV-specific primers V1 and C473 (Ghanim et al., 1998). PCR positive plants were maintained under insect-free cages for further studies. Symptomatic leaves were used for immunocapture-PCR (IC-PCR) and virus acquisition and transmission experiments.

DNA and RNA Extraction from *B. tabaci*

Genomic DNA was isolated from single *B. tabaci* B adults using the CTAB (cetyltrimethylammonium bromide) method (Shahjahan et al., 1995). Briefly, single whitefly was homogenized in 30 μl of extraction buffer (10 mM Tris-HCl, 1.4 M NaCl, 2 mM EDTA, 2% CTAB, and 0.2% β-mercaptoethanol) and incubated at 65°C for 15 min, followed by incubation at 95°C for 10 min. Samples were centrifuged for 55 min at 16,300 × *g*, and an equal volume of phenol – chloroform – isoamyl alcohol (25:24:1) was added. The aqueous phase was transferred to clean tube, equal volumes of chloroform were added, followed by another centrifugation. The upper phase was collected, and nucleic acids were precipitated using 0.2 volumes of 5 M sodium chloride (NaCl) and one volume of isopropanol. The samples were incubated at 4°C overnight for DNA precipitation. The samples were then centrifuged at 14,000 × *g* for 30 min at 4°C. The pellet was washed with 70% ice-cold ethanol, air-dried and dissolved in 40 μl of double-distilled water.

For total RNA extractions, each replicate out of the three, which consisted about forty 6- to 7-day-old adults, was homogenized in 300 μl of Tri-reagent (Sigma), and 1 μl of polyacrylamide carrier (GenElute LPA; Sigma) was added and the tube vortexed for 10 s and incubated for 5 min at room temperature. Sixty microliters of chloroform were added, followed by vigorous mixing and centrifugation at 12,000 × *g* for 10 min at 4°C. The aqueous phase was transferred to a clean tube, gently mixed with 0.7 volumes of ice-cold isopropanol, and incubated overnight at −20°C for RNA precipitation. The samples were centrifuged at 14,000 × *g* for 30 min at 4°C. The supernatant was removed and pellet was washed with 75 % ice-cold ethanol. The air-dried pellet was dissolved in 40 μl of double-distilled water. DNA and RNA purity and yield were analyzed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

Plant DNA Extraction

Total DNA was extracted from 100 mg leaves of tomato plants showing typical leaf curl symptoms. The young leaf tissues were ground in liquid nitrogen using pestle and mortar and the homogenate was transferred to modified extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl; 20 mM EDTA, 2% CTAB, and 2% β-mercaptoethanol). The homogenized sap was transferred into microfuge tubes and incubated at 65°C for >30 min and mixed by inversion 3–5 times. Equal volume of chloroform:isoamyl alcohol (24:1) was added into each tube and tubes were mixed gently by inversion for 10 min followed by centrifugation at 13,000 rpm for 10 min at 4°C. The aqueous phase was transferred to a sterile microfuge tube. To the aqueous phase, 0.8 volume of isopropanol was added and mixed gently to precipitate the nucleic acids. The DNA was separated by centrifugation at 13,000 rpm for 10 min at 4°C, followed by washes with 80 and 70% ethanol, separately. The pellet was air-dried, and dissolved in 50 μl of double-distilled H_2O. The DNA was further purified by RNase A treatment and stored at −20°C for further use.

Preparation of cDNA from RNA and Whitefly Midguts

RNA (100 ng) was used as a template for cDNA synthesis in 25-μl reaction mixtures by using Verso cDNA kit (Thermo scientific, Fermentas). Each of the three biological replicates used RNAs from 10 dissected midguts for cDNA synthesis. Dissected midguts were washed using PBST (1X PBS with 0.05% [vol/vol] Tween 20) twice and dissolved in 20 μl of RNase-free double-distilled water. The samples were then incubated at 95°C for 10 min to disrupt the cells and ∼4 μl of the cells (not purified RNA) were used for cDNA synthesis. For midgut

cDNA synthesis, Maxima kit with dsDNase (Thermo Scientific) was used.

Gene Amplification, Sequence, and Phylogenetic Analysis

PCR primers for amplifications were designed using sequences obtained from *B. tabaci* transcriptome sequence data generated through several projects performed in our lab, and sequences available in GenBank, and were confirmed with the new assembled whitefly genome (Drs. Zhangjun Fei and Wenbo Chen, personal communication). PCR amplifications were performed using *CypB*, *CypB*s, *CypD*s, and *CypG*s forward and reverse primers (**Table 1**) with predicted sizes of 615, 216, 155, and 188 bp, respectively. The PCR mixture consisted of 50 ng of cDNA, 1 mM of the forward and reverse primer, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 2 units of Taq DNA polymerase (Hylabs, Rehovot, Israel), and the final volume was made up to 20 μl with sterile distilled water. PCR cycling conditions were as follows: 94°C for 3 min, 30 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 30 s, and final extension at 72°C for 5 min. The PCR product was resolved in 1% agarose gel, and 1-kb DNA ladder (Thermo Scientific) was used as a molecular weight marker. PCR products were extracted from the gel and sent for purification and sequencing (Hylabs, Rehovot, Israel).

Sequence and Phylogenetic Analysis

Bemisia tabaci B *Cyp* nucleotide and amino acid sequences were initially analyzed by using the BLASTN and BLASTP algorithm at NCBI website[2] and the Expert Protein Analysis System[3]. The 3D structure of CypB was predicted using SWISSMODEL[4]. Sequences were aligned with closely related arthropod Cyp sequences obtained from the Genbank sequences database using Bioedit program[5]. The predicted amino acid sequence of the protein with CypBs from *Melanoplus sanguinipes*, *Zootermopsis nevadensis*, *Cyphomyrmex costatus*, and *Tribolium castaneum* showed identity of 69, 68, 67, and 66%, respectively. A phylogenetic tree was generated using a neighbor-joining algorithm bootstrapped with 1000 replicates in MEGA6 (Tamura et al., 2013) to evaluate the statistical robustness of the internal tree branches.

qRT-PCR Analysis

To measure the expression levels of the *CypB*, *CypD*, and *CypG* genes, a quantitative RT-PCR (qRT-PCR) approach was used. The primer pairs used for the amplifications are listed in **Table 1**. Amplifications were performed using Absolute Blue qPCR SYBR green Rox mix (Thermo Scientific) and 5 pmol of each primer. To ensure the validity of the data, the expression of each gene was tested in triplicate in each of three biologically independent experiments. The cycling conditions were as follows: 15 min of activation at 95°C and 40 cycles of 15 s at 95°C, 30 s at 58°C (actin), 54°C (*CypB*, *CypD*, and *CypG*), and 30 s at 72°C. The channel source was 470 nm, with a detector at 510 nm. A Rotor-Gene 6000 machine (Corbett Robotics Pty. Ltd., Brisbane, QLD, Australia) and the accompanying software were used for qPCR data normalization and quantification. Average expression of actin cDNA, which was not regulated after virus acquisition experiments, was used as a reference as stated above, and the quantification was done using the relative expression method. For each run, standards were loaded onto the same plate to obtain the appropriate standard curve.

Immunocapture-PCR

Interaction between CypB and TYLCV CP was tested by IC-PCR assay. The wells of microtiter plates were coated with coating buffer (0.05 M sodium carbonate pH 9.5) including a polyclonal antibody against cyclophilin (Cyp) IgG1g/ml (Sigma–Aldrich catalog # SAB4200201) whose specificity was confirmed using western blot analysis in which an expected band of ∼20 KDa was obtained (data not shown). Anti-TYLCV CP antibody (a gift from Prof. Henryk Czosnek) was also used. The plated were coated with both antibodies for 4 h at room temperature. About 30 viruliferous whitefly adults or 100 mg leaf tissue were crushed in one volume of extraction buffer, 50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 2% polyvinylpyrrolidone, and 0.05% Tween 20. The extract was clarified at 13,000 × *g* for 15 min at 4°C. The clarified extract was treated with DNase I (1 U/l) for 30 min at 37°C to remove the viral replicative DNA to ensure that only encapsidated ssDNA served as the template (Kanakala et al., 2013). The plates were washed with 1X PBS three times to remove unbound antibody, and 200 μl aliquots of DNase treated plant and whitefly extract were added into the wells in three replicates. The plate was incubated overnight at 4°C to allow maximum capturing of the particles. The overnight-incubated plates were washed with 1X PBS to remove all unbound material. The bound particles were released by adding 50 μl/well of extraction buffer with 1% (v/v) Triton X-100 and the suspension was stored at 4°C until further use. PCR amplification of the 5 μl of viral DNA from the virions bound to the CypB protein, which was itself bound to the antibody-coated tubes, was performed with the TYLCV-specific primers V1 and C473 (Ghanim et al., 1998).

Immunostaining of TYLCV and CypB in *B. tabaci* B Midguts, Salivary Glands, and Eggs

Whitefly midguts, salivary glands and eggs were dissected on glass microscopic slides, and fixed with 4% paraformaldehyde in 1X PBS buffer (10 mM Tris HCl, 150 mM sodium chloride, pH 7.5) for 30 min at room temperature, followed by 200 μl of 0.1% triton-X100 for 0.5–1 h at room temperature. After washing with PBST (1X PBS with 0.05% [vol/vol] Tween 20) for three times, the specimens were blocked with 200 μl of blocking buffer (PBST+ 1% Bovine Serum Albumin, BSA) for 1–2 h at room temperature. Then TYLCV CP (Gorovits et al., 2013) primary antibody diluted in 1:100 in blocking buffer was incubated with the samples at 4°C overnight. After washing with PBST, samples were incubated for 1–2 h at 25°C with a Cy3-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch, USA) diluted to 1:200.

[2]http://www.ncbi.nlm.nih.gov/blast

[3]http://www.expasy.org/

[4]http://swissmodel.expasy.org/

[5]http://www.mbio.ncsu.edu/BioEdit

The samples were washed three times and blocked for 2 h. Then anti-CypB primary antibody (Sigma–Aldrich SAB4200201) diluted in 1:100 in blocking buffer was incubated with the samples overnight at 4°C. After washing with PBST, samples were incubated for 1–2 h at 25°C with a Cy2-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch, USA) diluted 1:200. After washing three times with PBST, the nuclei were stained with DAPI in PBST (Thermo Scientific DAPI, Pierce Protein Research Product), at 1 μg/ml for 20 min at 25°C, covered with a cover-slip, sealed with nail polish and viewed under aIX81 Olympus FluoView500 confocal laser-scanning microscope; TYLCV CP and CypB were detected as red and green fluorescent signals, respectively. For each treatment, 15–20 whitefly midguts, salivary glands, and eggs were viewed. Controls consisted of performing the same experiments but not including first antibodies, and also switching the order of using the primary antibodies to ensure specificity in binding, and to ensure that both secondary antibodies saturate the primary ones in each step, because both primary antibodies were raised in rabbit. Another control consisted performing the protocol without using the anti-CypB antibody as detailed in the results.

Transmission of TYLCV after Feeding on CypB Antibodies and Cyclosporin A

To assess the implication of CypB in the transmission of TYLCV, *B. tabaci* adults, 1 week after emergence, were fed on antibodies against CypB (2 μg/μl) or the Cyp inhibitor Cyclosporin A (CsA) (2 μg/μl; Sigma–Aldrich C1832). The antibody or the inhibitor were mixed with 25% sucrose solution and confined between two layers of stretched parafilm M (Bemis, Neenah, WI, USA) on a glass scintillation vial in which the adult whiteflies

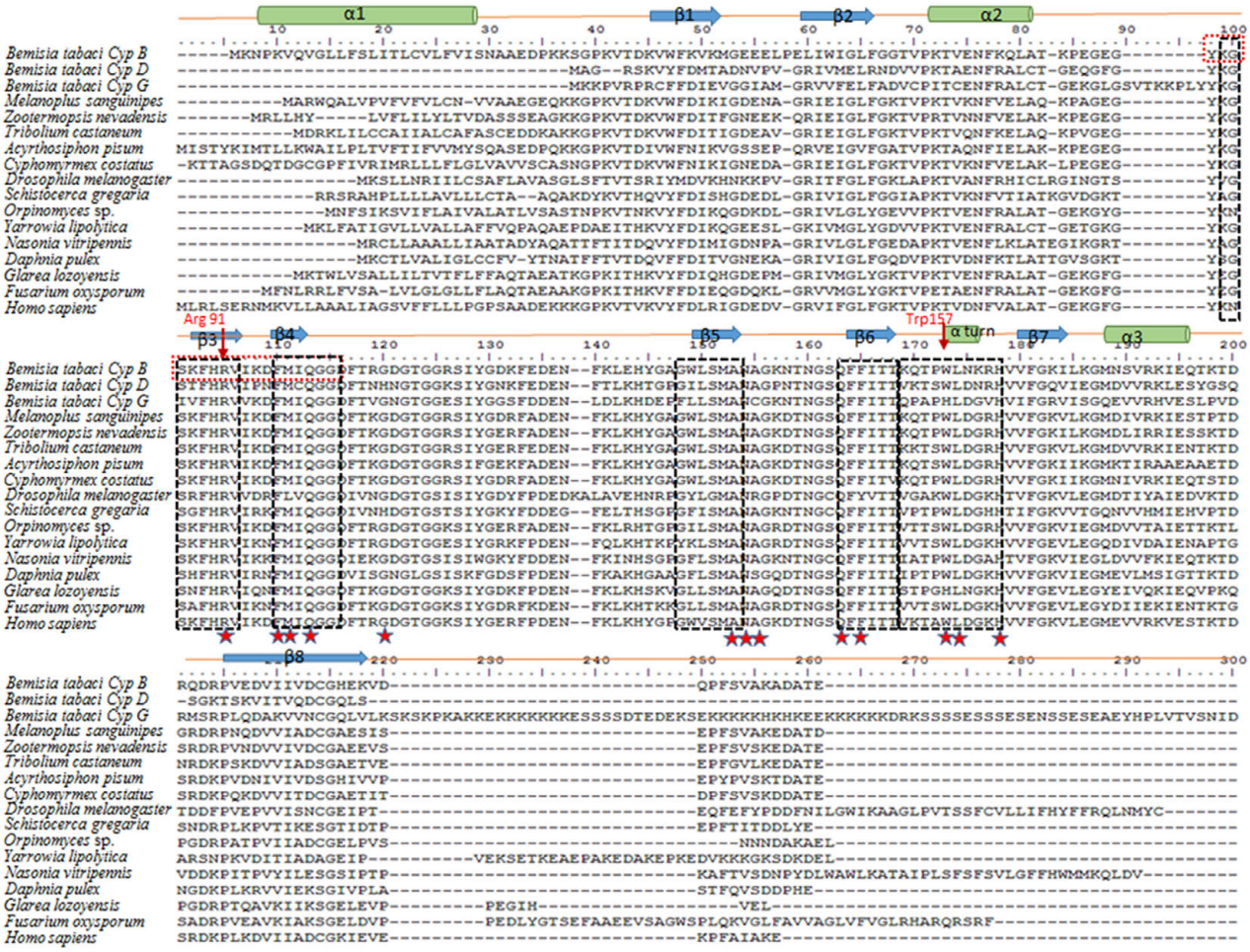

FIGURE 1 | Multiple alignments of *Bemisia tabaci* CypB, CypD, and CypG amino acid sequences with other arthropod, fungal, and human CypB amino acids sequences. 'β 1–8' indicates β-strand; 'α 1–3' indicates α-helix; The CypB inhibitor cyclosporin A (CsA)-binding domains (four β-strands) are indicated on the top of sequences and are boxed. The 13 well-conserved residues that constitute the CsA-binding site are marked with star symbol. The signature of peptidyl-prolyl-*cis-trans* isomerase (YKGSKFHRVIKDFMIQGG) is represented in red colour box. Accession numbers are: *Melanoplus sanguinipes* ALX00032, *Zootermopsis nevadensis* KDR13508, *Tribolium castaneum* XP 971028, *Acyrthosiphon pisum* NP 001156707, *Cyphomyrmex costatus* KYN04146, *Drosophila melanogaster* NP 476656, *Schistocerca gregaria* AEV89763, *Orpinomyces sp.* Q01490, *Yarrowia lipolytica* XP 504296, *Nasonia vitripennis* XP 001602615, *Daphnia pulex* EFX68543, *Glarea lozoyensis* EHK99826, *Fusarium oxysporum* EXL62949, *Homo sapiens* P23284.

were caged for feeding on this solution for 24 h. The insects were then transferred to TYLCV-infected tomato plants for a 48-h acquisition access period and subsequently single whiteflies were transferred to tomato plants in their three-leaf stage for 7 days of inoculation access period. The single whiteflies were confined to the plants in leaf-clip cages. Whiteflies fed on 25% sucrose solution for 24 h and TYLCV-infected plants for 48 h served as a control. Tomato plants were grown in a potting mix in 1.5-L pots under artificial light and maintained inside insect-free greenhouse under controlled temperature as detailed above. The whiteflies that were incubated with the plants were tested for TYLCV acquisition. The plants were monitored for the development of disease symptoms after 28 days post inoculation. DNA was extracted from symptomatic and non-symptomatic tomato plants and subjected to PCR for detecting TYLCV with specific CP primers V61 and V473 (Ghanim et al., 1998). The experiments were triplicated with 20 plants for each replicate.

Statistical Analyses

The significance of the differences between means in all comparisons performed on data from the qRT-PCR and virus transmission experiments was determined by replicating the experiments at least three times and using the Tukey–Kramer honest significance difference (HSD) test ($\alpha = 0.05$). JMP7 (SAS institute) was used for all statistical analysis. In all of the figures, asterisks indicate significant differences and the significance $p < 0.05$.

RESULTS

Sequence Analysis of *Cyp* Genes of *B. tabaci* B

Cyp EST sequences were obtained from several deep-sequencing projects with *B. tabaci* generated in our lab with the Illumina technology (not published), and were confirmed using the Nr or Swiss-Prot databases, and were finally confirmed with gene sequences from the whitefly genome project. Initially, *CypB* was amplified from whiteflies using corresponding primer pairs (**Table 1**), which yielded a single band of 615 bp. The complete gene sequence of *CypB*, *CypD*, and *CypG* cDNAs were recovered from the available sequences obtained in our sequencing projects. The complete sequence of *CypB* encodes a polypeptide of 216 amino acids with a predicted molecular weight of 24.15 kDa and an isoelectric point of 8.076. Alignment

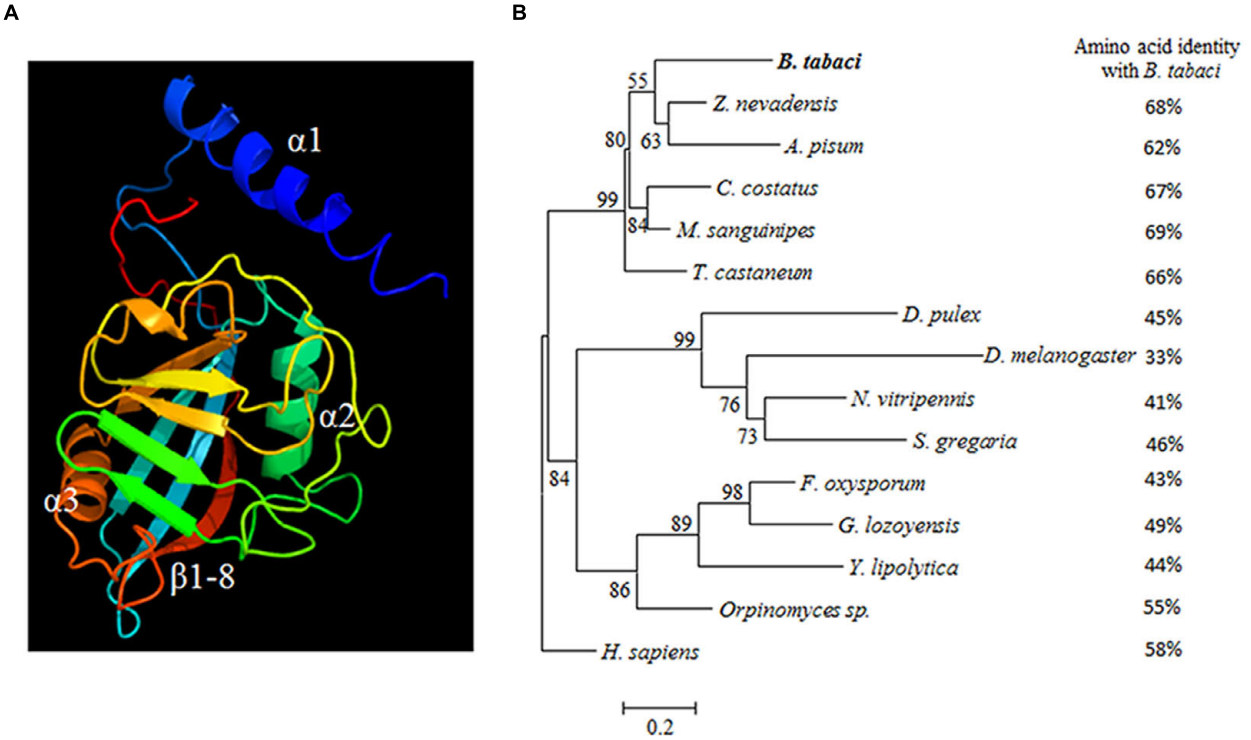

FIGURE 2 | (A) The 3-D structure of *B. tabaci*-CypB. The β-strands and α-helices are shown. **(B)** Phylogenetic tree of *B. tabaci*-CypB and other arthropods, fungal species and *H. sapiens*, were constructed using MEGA 6 with maximum likelihood method. Numbers next to the branches indicated bootstrap value of each internal branch in the phylogenetic tree nodes from 1000 replicates. Cyclophilin B sequences include *Zootermopsis nevadensis (*KDR13508); *Acyrthosiphon pisum* (NP 001156707); *Cyphomyrmex costatus* (KYN04146)*; Melanoplus sanguinipes (*ALX00032); *Tribolium castaneum* (XP 971028); *Daphnia pulex* (EFX68543); *Drosophila melanogaster* (NP 476656); *Nasonia vitripennis* (XP 001602615); *Schistocerca gregaria* (AEV89763); *Fusarium oxysporum* (EXL62949); *Glarea lozoyensis* (EHK99826); *Yarrowia lipolytica* (XP 504296); *Orpinomyces* sp. (Q01490), and *Homo sapiens* (P23284). Amino acid sequence identity with other arthropods and fungal species and *H. sapiens* are given for easy comparison.

of the deduced amino acid sequences of the three *Cyp* genes revealed strong identities with those of other *CypB*s genes of other arthropods obtained from Genebank (**Figure 1**). The nucleotide and deduced amino acid sequence of the *B. tabaci CypB*, *CypD*, and *CypG* have been deposited in GenBank under the accession number**s** KX268377, KX268378, and KX268379, respectively.

Further analysis on the Cyp protein sequence from *B. tabaci,* and amino acid sequences from five arthropods and four fungal species revealed the presence of a conserved domain of the protein. This single domain of Cyp PPIase is located between the amino acids 84–101 (YKGSKFHRVIKDFMIQGG) in CypB and was found to be highly conserved in *B. tabaci* and few other arthropods as shown in the deduced polypeptide of *B. tabaci*-CypB protein (**Figure 1**). The conserved amino acids residues (Y84, R91, and F96) are necessary for the function of the CypB PPIase activity. The 13-highly conserved CsA-binding residues (named R91, F96, M97, Q99, G108, A137, N138, A139, Q147, F149, W157, L158, and H162) were also found in *B. tabaci* CypB. In *B. tabaci* CypB, fold architecture resembled structures from other organisms from higher animals (*Bos taurus*) to unicellular parasites (plasmodium), and consisted of eight antiparallel β sheets and three α-helices that pack against the sheets. Among them four β-strands (K88-I93, F96-I98, W133-A137, F148-T151) and a loop (Q154-H162) that forms CsA-binding pocket (**Figure 1**). In addition, a short α-helical turn containing the active site residue Trp157 found in the β6–β7 loop region is also present (**Figure 2A**). To further validate the molecular evolution on the multiple alignments of CypBs, a phylogenetic tree was constructed based on the amino acid sequences of the protein sequences using maximum likelihood method (**Figure 2B**). According to the phylogenetic analysis, *B. tabaci* firstly clustered with CypB of *Z. nevadensis and Acyrthosiphon pisum* and then formed a sister group with CypBs from other arthropods. Subsequently, they clustered with CypBs from fungal species.

Expression Analysis of *Cyp* Genes in *B. tabaci* B

A previous report has suggested that cyclophilins play a role in aphid-luteovirus interactions. It was thus decided to measure the expression of *Cyp* genes from *B. tabaci* B after TYLCV acquisition. The expression of all three *Cyp* genes from *B. tabaci,*

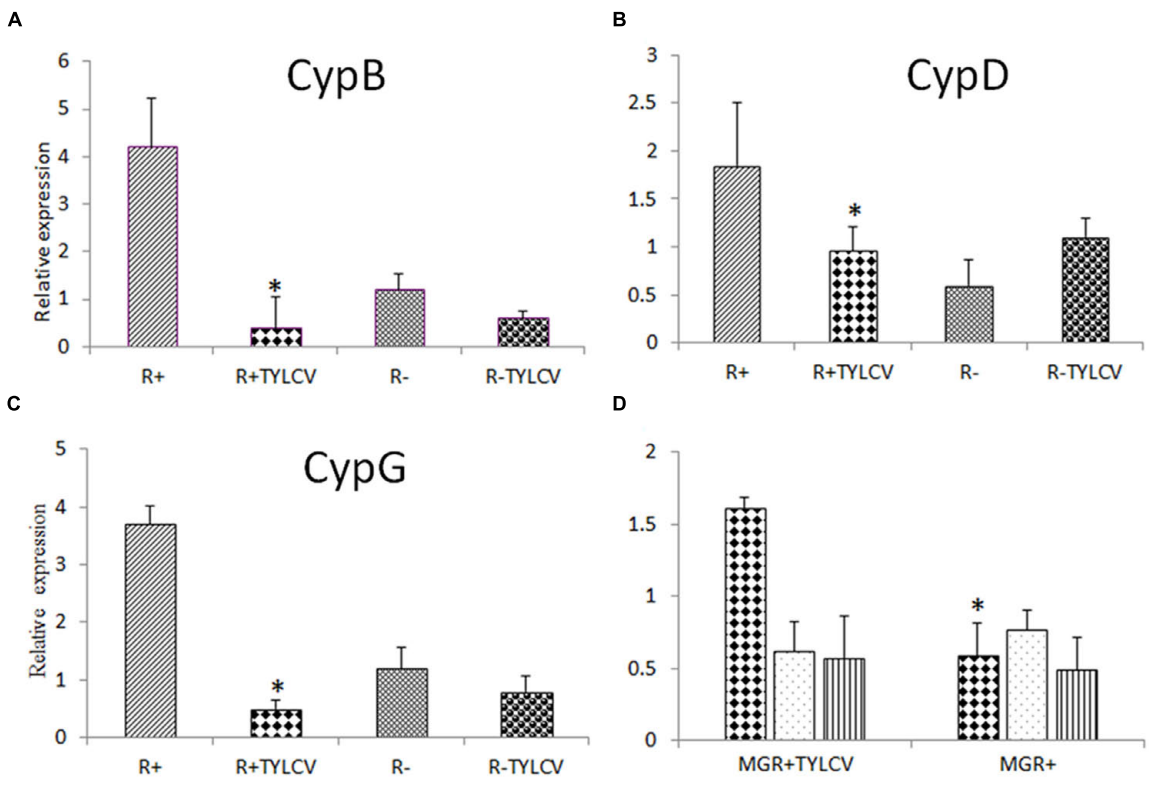

FIGURE 3 | Relative expression of cyclophilins in viruliferous and non-viruliferous whiteflies (NVBt) measured using quantitative RT-PCR (qRT-PCR), compared to the expression levels of the housekeeping gene β-actin gene. (A–C) Show relative expression of CypB, CypD, and CypG in viruliferous and NVBt, respectively, in *Rickettsia*-infected (R+) and uninfected (R−) whiteflies. Whiteflies were given acquisition access period for 10 h on *Tomato yellow leaf curl virus* (TYLCV)-infected plants, or healthy plants as control. **(D)** Relative expression of CypB (triangle columns), CypD (dotted columns), and CypG (vertical lines columns) in *B. tabaci* dissected midguts from viruliferous (MGR+TYLCV) and non-viruliferous (MGR+) *Rickettsia*-infected whitefly B adults after a 10 h acquisition access period on TYLCV-infected and healthy tomato (HT), respectively. Data shown are the means ± standard errors of the means of data from three independent experiments. Asterisk indicate statistically significant differences with $P < 0.05$ between viruliferous and non-viruliferous insects.

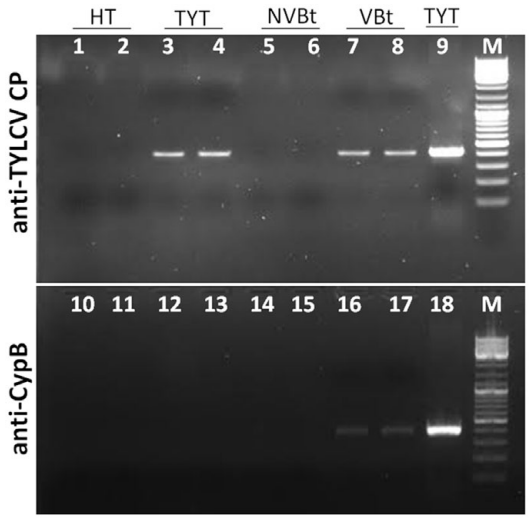

FIGURE 4 | Immunocapture-PCR assay to detect CypB-TYLCV CP interaction in viruliferous whiteflies (VBt). Lanes 1–8 and 10–17 represents microtiter plates coated with an anti-TYLCV CP and anti-CypB specific antibodies, respectively, and a PCR was performed on the caught virus-CypB complexes. Lanes 1, 2, 10, and 11 are leaf extract from HT, lanes 3, 4, 12, and 13 are leaf extract from TYLCV-infected tomato (TYT). Lanes 5, 6, 14, and 15 are extracts of NVBt, and lanes 7, 8, 16, and 17 are extracts of VBt. Lane 9 and 18 are genomic extracted from TYT (PCR positive control). TYLCV V61-C473 (**Table 1**) primers were used to amplify the virus bind to the anti-TYLCV CP and anti-CypB antibody.

CypB, CypD, and *CypG*, was measured using a quantitative realtime PCR approach, after TYLCV acquisition. All three genes were down regulated after virus acquisition in adults infected with the secondary symbiont *Rickettsia* (R+) (**Figures 3A–C**). However, the expression of the three genes was different in adults lacking the secondary endosymbiont *Rickettsia* (R−) (**Figures 3A–C**). The expression of the three *Cyp* genes was measured in dissected midguts from viruliferous and non-viruliferous R+ adults. Only *CypB* showed significantly higher expression in viruliferous midguts compared to non-viruliferous ones. Based on this result it was decided to further investigate the role of this specific protein in *B. tabaci*-TYLCV interactions (**Figure 3D**).

Interaction between TYLCV CP and CypB

Following the high expression of *CypB* in response to TYLCV in viruliferous midguts, we tested whether this response involves direct interaction between TYLCV and CypB protein. Two methods were used to test possible interaction. First, an IC-PCR assay was used and the controls included TYLCV-infected tomato leave extracts which were applied to microtiter well-plates coated with anti-CypB antibody (**Figure 4**). Other controls included extracts from non-viruliferous whiteflies and from TYLCV-uninfected plants which were applied to plates coated with anti-TYLCV CP antibody (**Figure 4**). The PCR results obtained with these controls were all negative. Extracts from viruliferous whiteflies applied to plates coated with anti-CypB antibody (**Figure 4**), or extracts from TYLCV-infected tomato applied to plates coated with anti-TYLCV CP antibody (**Figure 4**), provided positive PCR results, suggesting a specific interaction between CypB and TYLCV, very likely via the formation of CypB and TYLCV complexes. Second, co-immunolocalization of TYLCV (CP) and CypB using specific polyclonal antibodies, in the insect midguts, salivary glands, and eggs. As seen in **Figure 5**, colocalization of both CypB and TYLCV was observed as yellow patches in viruliferous whitefly midgut epithelial cells (**Figure 5**), and in primary salivary glands and oocytes (**Figure 6**), while the controls demonstrated the specificity of the procedure and the primary antibodies used (**Figure 5**).

Feeding Whiteflies with Anti-CypB Antibody or with the Cyp Inhibitor Cyclosporin A Decrease TYLCV Transmission

Transmission experiments were performed with whiteflies fed with anti-CypB antibody to further investigate the involvement of CypB in TYLCV transmission. *B. tabaci* B adults were first fed with artificial medium containing CypB antibody for 24 h and subsequently fed on TYLCV-infected tomato plants for 24 h. In the control experiments, adult whiteflies were fed with 25% sucrose, followed by feeding on infected plants. In the antibody-feeding experiment, 6 out of 20 plants in the first experiment and 5 out of 20 plants in the second and third experiments became infected, while 13 plants out of 20 in the first and second experiments, and 10 plants out of 20 in the third experiment became infected after feeding with 25% sucrose (control). Altogether, 17% transmission rates, on average, were obtained after feeding with anti-CypB antibody, compared to 60% transmission when the whiteflies were fed with 25% sucrose solution (**Figure 7**).

In another set of transmission experiments, adult *B. tabaci* B whiteflies were fed the Cyp inhibitor CsA to further investigate the involvement of Cyp in TYLCV transmission. The whiteflies were fed with CsA for 24 h, or with 25% sucrose as a control, and the insects from both groups were caged with TYLCV-infected plants for a 48 h acquisition access period. Interestingly the transmission rates dramatically decreased, with 3 out of 20 plants in the first and second experiments and 4 out of 20 plants in the third experiment became infected, when the insects were fed with Cyp inhibitor, and altogether, 27% transmission rates, on average, were obtained, compared to 60% when the whiteflies were fed with 25% sucrose solution (**Figure 7**).

The following experiment was conducted for demonstrating the internalization of the anti-CypB antibody by whiteflies and testing the effect of the CypB inhibitor on colocalization of CypB and/or TYLCV and their interaction. Midguts were dissected from whiteflies fed on the first antibody against CypB and the CypB inhibitor CsA, and then were fed goat-anti rabbit Cy2 secondary antibody. Typical CypB-TYLC colocalization was obtained when viruliferous whiteflies were fed only sucrose (**Figures 8A–C**), while atypical signal of TYLCV and no signal of CypB were observed in the midguts dissected from whiteflies fed with the Cyp inhibitor CsA (**Figure 8**).

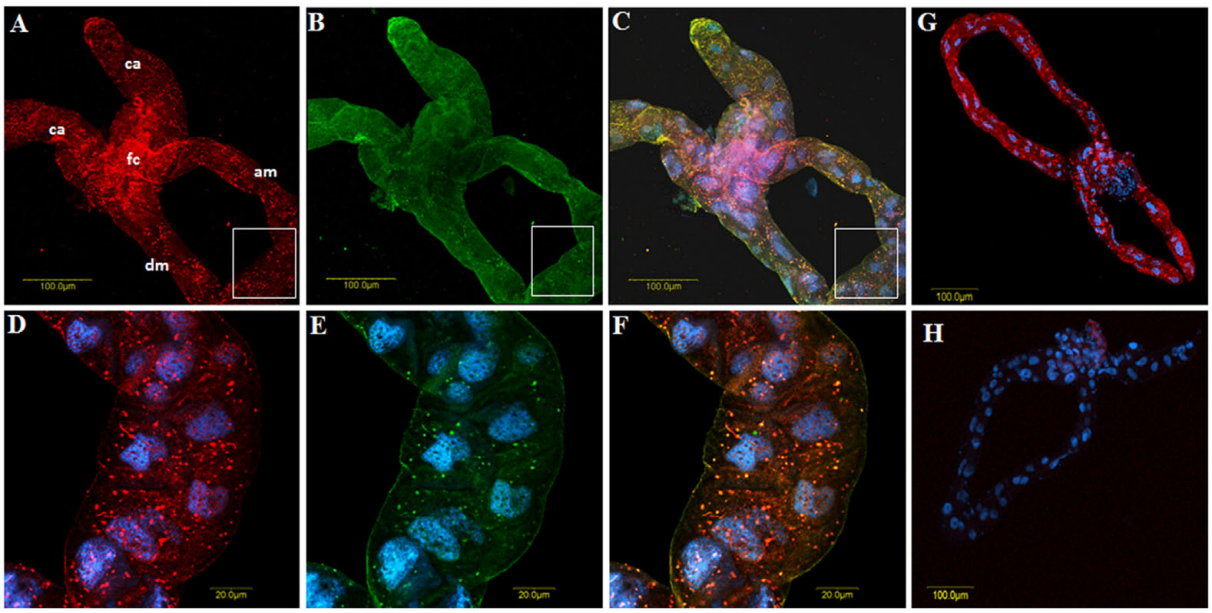

FIGURE 5 | Co-immunolocalization of TYLCV with first antibody against the virus CP and detected with secondary antibody conjugated to Cy3 (A, red), and CypB reacted with polyclonal anti-CypB first antibody and detected with secondary antibody conjugated to Cy2 anti-rabbit secondary antibody (**B**, green) in *B. tabaci* midguts dissected from viruliferous adults. **(C)** Shows the overlay of **(A** and **B)** and the yellow spots show the colocalization. **(D–F)** Are zoom in of the portions shown in the insets that appear in **(A–C)**, respectively. **(G)** Is a control gut in which the whole co-immunolocalization procedure was performed without adding the anti-CypB primary antibody, and **(H)** is a control gut in which only secondary antibodies were used. ca, cecae; fc, filter chamber; am, ascending midgut; dm, descending midgut.

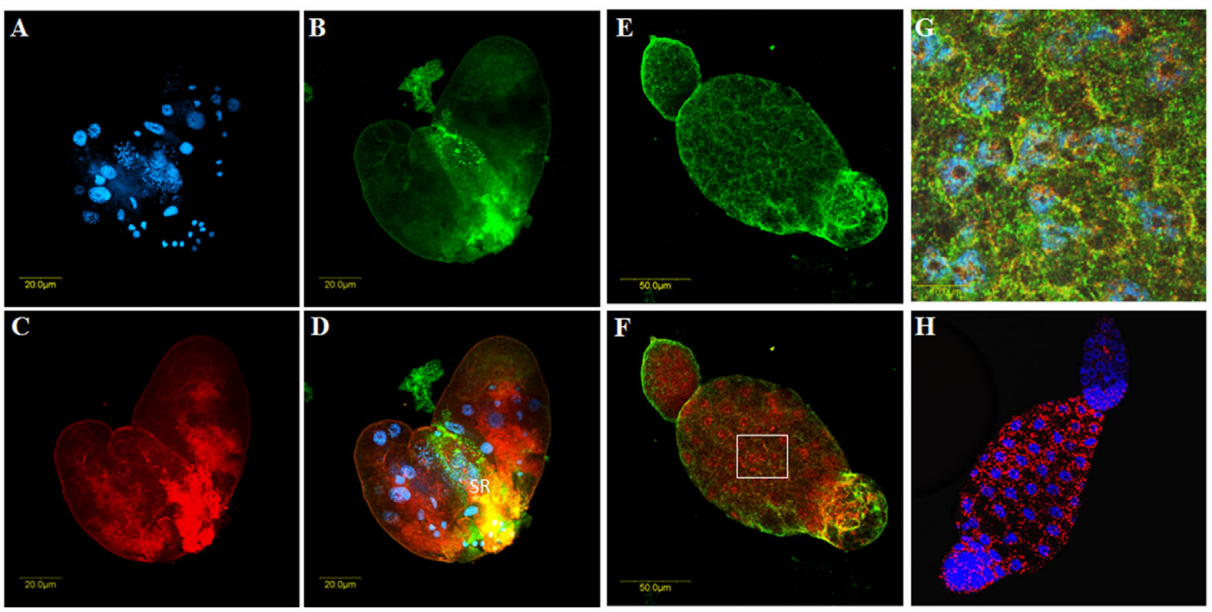

FIGURE 6 | Co-immunolocalization of TYLCV with first antibody against TYLCV CP of the virus, and detected with a secondary antibody conjugated to Cy3 (red), and first antibody against CypB detected with a secondary antibody conjugated to Cy2 anti-rabbit secondary antibody (green) in *B. tabaci* primary salivary glands (A–D) and in oocytes **(E–H)** dissected from viruliferous adults. **(A)** Shows one gland stained with DAPI for nuclei, **(B)** shows CypB immunolocalization, **(C)** shows TYLCV immunolocalization, and **(D)** is overlay of **(A–C)** showing the co-immunolocalization of CypB and TYLCV in the yellow portions near the secretory region of the primary salivary gland (SR). **(E)** shows one oocyte in which CypB is immunolocalized, **(F)** shows co-immunolocalization of TYLCV with CypB in the same oocyte, **(G)** is zoom in of the inset in **(F)** and **(H)** is a control oocyte in which the whole co-immunolocalization procedure was performed without adding the anti-CypB primary antibody. SR, secretory region.

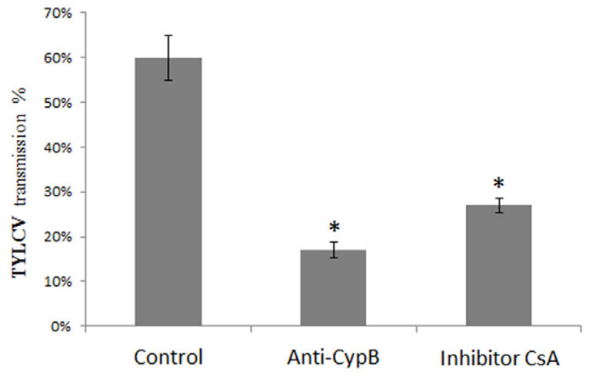

FIGURE 7 | Decrease in TYLCV transmission rates after feeding of adult whiteflies on artificial medium containing 25% sucrose (control), artificial medium containing anti-CypB antibodies (anti-CypB) and the cyclophilin inhibitor CsA, for 24 h, followed by feeding on the TYT plants for 48 h, before performing transmission experiments of one insect/plant as detailed in the text. Data shown are the means and standard errors from three independent experiments. Asterisks indicate statistically significant differences with $P < 0.05$ between transmission experiments compared to the control.

DISCUSSION

Successful infection cycle for whitefly-transmitted begomoviruses depends on the efficiency of acquisition, transmission, and retention of the virus in the whitefly vector (Rosen et al., 2015). Although these interactions have been studied for many years, very little is known about the molecular mechanisms that govern the transmission of this group of viruses by their hemipteran vectors. Several insect and endosymbiont proteins have been implicated in the recognition and translocation of TYLCV virions in the vector (Gottlieb et al., 2010; Gotz et al., 2012). TYLCV CP was shown to be the only and essential viral protein involved in virus transmission (Hofer et al., 1997). Two additional proteins were shown to interact with TYLCV CP. First, GroEL protein from the secondary symbiont *Hamiltonella* of *B. tabaci* B, which has been shown to be required for protecting virions from rapid proteolysis in the insect hemolymph (Gottlieb et al., 2010), thus ensuring safe and efficient transmission of the virus. Second, Heat-Shock Protein 70 HSP70 whose expression was shown to be induced upon virus retention and restricts virus transmission (Gotz et al., 2012).

Recently, peptidyl-prolyl isomerases proteins (PPIases or Cyps) have been implicated in B/CYDV circulation and transmission by aphids (Tamborindeguy et al., 2013), and were hypothesized to play a role in chaperoning these viruses to various membrane bound vesicles (Yang et al., 2008). Cyps are important in the proper folding of proteins and as modulators for human virus replication (Frausto et al., 2013). Furthermore, Cyps were shown to interact with the capsid protein of the human immunodeficiency virus type 1 (HIV-1; Schaller et al.,

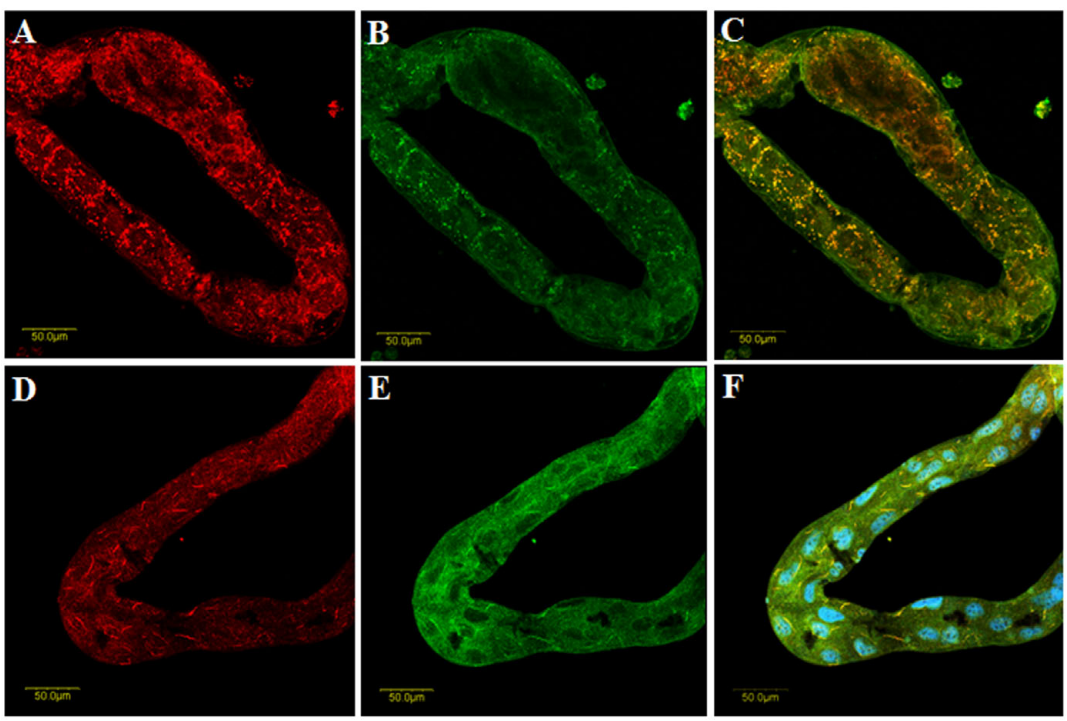

FIGURE 8 | Colocalization of TYLCV (A, red) and CypB (**B**, green) in midguts dissected from VBt fed on 25% sucrose. **(C)** Shows the yellow spots where CypB and TYLCV co-localize. **(D)** Shows immunolocalization of TYLCV after feeding with the CypB inhibitor CsA and **(E)** Shows immunolocalization of CypB after feeding with the CsA inhibitor. **(F)** Is an overlay of **(D** and **E)**. Blue is DAPI staining of the nuclei.

2011) and influenza A virus M1 protein (Liu et al., 2009) and were shown to play a key role in the viral replication cycle. In this study, we investigated whether Cyps from *B. tabaci* B have a role in begomovirus transmission. We used molecular, biological and microscopy approaches to show that *CypB* gene expression is altered upon TYLCV acquisition and retention by *B. tabaci,* and close proximity and possible physical interaction with the virus were verified. First, we have analyzed the expression of various Cyp genes in viruliferous and non-viruliferous whiteflies using a qRT-PCR approach (**Figures 3A–C**). Interestingly, the three *Cyp* genes tested in this study, *CypB*, *Cyp*D, and *Cyp*G, showed generally down regulation in their expression in R+ viruliferous whole insects, however, their expression was not similar and fluctuated in the R− strain. When the expression of Cyps was measured in midguts, only the expression of *CypB* was significantly induced by twofold in viruliferous R+ whiteflies, compared to the uninfected insects. Our results also show that infection with *Rickettsia* results in lower expression of *Cyp* genes after TYLCV acquisition. These results indicate that the role of Cyp proteins might involve multitrophic interactions between insect proteins, endosymbionts, and the virus CP, especially that when the expression of the three *Cyp* genes was measured in R+ and R− insects, without TYLCV acquisition, their expression was always significantly higher in the R+ insects (**Figures 3A–C**). These results support a previous raised hypothesis which suggested that *Rickettsia* plays an important role in TYLCV-*B. tabaci* interactions (Kliot et al., 2014). Further research will be required to verify the role of *Rickettsia* in altering expression of *Cyp* genes before and post TYLCV acquisition.

The expression results of *CypB* in the midgut were different from whole body results (**Figure 3D**), and combined with previous observations that the majority of TYLCV particles are translocated from the filter chamber in the midgut to the hemolymph (Ghanim et al., 2009; Skaljac and Ghanim, 2010; Gotz et al., 2012), suggest that Cyp might play an important role in TYLCV-*B. tabaci* interactions in the gut. Such roles include transport and translocation to the hemolymph. If indeed this is the role of Cyp, it was hypothesized that direct interaction between CypB and TYLCV CP might occur in the insect, specifically in the gut and the salivary glands, major sites along the transmission pathway. To demonstrate such interaction, we performed IC-PCR (**Figure 4**) and co-immunostaining (**Figure 5**). Both methods clearly demonstrated that CypB and TYLCV are likely to interact *in vivo*. The colocalization of CypB and TYLCV in the filter chamber, ceacae and the midgut further support this hypothesis and show close proximity of the proteins in these organs. The filter chamber is likely be the site where most of these interactions with the virus occur, suggesting that Cyp might play an important role in translocating the virus to the hemolymph. These colocalization results and the possible shuttling of the virus by Cyp in the filter chamber is supported by the increase in CypB concentration when measured by qRT-PCR in the midgut. Further subcellular localization experiments may confirm these colocalization results and the role of CypB protein in TYLCV-*B. tabaci* interactions.

Our results further confirmed CypB colocalization with TYLCV in the primary salivary glands of viruliferous insects (**Figure 6**). Gottlieb et al. (2010) demonstrated that *Hamiltonella*-GroEL protein plays a crucial role in safeguarding the virus in the hostile environment of the hemolymph, while proteins that aid in virus translocation from the midgut to the hemolymph and from the hemolymph to the salivary glands have not been described. CypB protein is a candidate that could have a major role in these two organs with regard to TYLCV transmission. Our results also show that CypB-TYLCV colocalization was observed in eggs from viruliferous whiteflies (**Figure 6**). A previous study has suggested that TYLCV is transovarially transmitted (Ghanim et al., 1998) through the reproductive system, and the interaction with CypB can be an important factor that determines the efficiency of TYLCV transmission from one generation to another through the reproductive system.

To further test whether the observed interaction are instrumental in influencing the transmission of the virus to test plants, we conducted virus transmission experiments after feeding adult whiteflies with anti-CypB antibodies and the CypB inhibitor CsA. The results showed that TYLCV transmission was reduced by 43% and 33 after feeding with anti-CypB and CsA, respectively (**Figure 7**). Those results are supported by the colocalization results in the midgut, in which decrease in the virus and CypB levels was observed, and their shape turned abnormal when fed with CsA. CsA is a general inhibitor of cyclophilins and it is possible that other cyclophilins in the insect were also affected following the feeding. However, since our results from other experiments indicate that CypB is possibly the protein involved in *B. tabaci*-TYLCV interactions, it is likely that the effect of CsA of virus transmission and localization is related to CypB. In aphids, two CypB proteins, S28 and S29, were identified in populations of *Schizaphis graminum* and those populations showed differences in their ability to transmit CYDV-RPV, and were related to the circulative transmission of luteoviruses, suggesting that those proteins play similar roles in whiteflies and aphids. The reduction in the virus and CypB levels, and their abnormal shape after feeding with the inhibitor suggests that CypB acts also to stabilize the virus in the gut, and might be aiding the virus to avoid or combat the insect immune responses. Recently, it was demonstrated through structural analyses that CycA stabilizes the HIV-1 capsid and is recruited to facilitate HIV-1 infection (Liu et al., 2016), suggesting that CypB might have a similar role with TYLCV in the whitefly.

CONCLUSION

We demonstrated in the present study an important role of *B. tabaci* B CypB protein in TYLCV circulative transmission. The results further suggest that this interaction might not only be relevant to the virus transmission to plants but also for the virus transovarial transmission between generations. Further studies in this system will focus on functional analysis of the role of CypB in the interaction with TYLCV including *CypB* gene silencing in whiteflies, the exact role of the protein in the virus translocation in the different insect organs, and whether this CypB-TYLCV

interaction is important for mediating the virus interaction with other insect proteins during the transmission process.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MG and SK. Performed the experiments: SK and MG. Analyzed the data: SK and MG. Contributed materials/analysis tools: MG. Wrote the paper: SK and MG.

FUNDING

Funded by Israel Science Foundation (ISF) grant 1127-13 to MG.

ACKNOWLEDGMENTS

SK was supported with a Postdoctoral Fellowship # 2014–2016 from ARO. The authors thank Prof. Henryk Czosnek and Prof. Rena Gorovits for providing the TYLCV CP antibody.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 27 April 2016
doi: 10.3389/fpls.2016.00552

Water Stress Modulates Soybean Aphid Performance, Feeding Behavior, and Virus Transmission in Soybean

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Little is known about how water stress including drought and flooding modifies the ability of plants to resist simultaneous attack by insect feeding and transmission of insect-vectored pathogen. We analyzed insect population growth, feeding behaviors, virus transmission, and plant amino acid profiles and defense gene expression to characterize mechanisms underlying the interaction between water stress, soybean aphid and aphid-transmitted, *Soybean mosaic virus*, on soybean plants. Population growth of non-viruliferous aphids was reduced under drought stress and saturation, likely because the aphids spent less time feeding from the sieve element on these plants compared to well-watered plants. Water stress did not impact population growth of viruliferous aphids. However, virus incidence and transmission rate was lowest under drought stress and highest under saturated conditions since viruliferous aphids took the greatest amount time to puncture cells and transmit the virus under saturated conditions and lowest time under drought stress. Petiole exudates from drought-stressed plants had the highest level of total free amino acids including asparagine and valine that are critical for aphid performance. Aphids did not benefit from improved phloem sap quality as indicated by their lower densities on drought-stressed plants. Saturation, on the other hand, resulted in low amino acid content compared to all of the other treatments. Drought and saturation had significant and opposing effects on expression of marker genes involved in abscisic acid (ABA) signaling. Drought alone significantly increased expression of ABA marker genes, which likely led to suppression of salicylic acid (SA)- and jasmonic acid (JA)-related genes. In contrast, ABA marker genes were down-regulated under saturation, while expression of SA- and JA-related genes was up-regulated. We propose that the apparent antagonism between ABA and SA/JA signaling pathways contributed to an increase in aphid densities under drought and their decrease under saturation. Taken together, our findings suggests that plant responses to water stress is complex involving changes in phloem amino acid composition and signaling pathways, which can impact aphid populations and virus transmission.

Keywords: drought, flooding, soybean aphid, soybean mosaic virus, amino acids, abscisic acid, salicylic acid, jasmonic acid

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *11 December 2015*
Accepted: *11 April 2016*
Published: *27 April 2016*

Citation:
Nachappa P, Culkin CT, Saya PM II, Han J and Nalam VJ (2016) Water Stress Modulates Soybean Aphid Performance, Feeding Behavior, and Virus Transmission in Soybean. Front. Plant Sci. 7:552. doi: 10.3389/fpls.2016.00552

INTRODUCTION

Water stress including drought and flooding is the most important factor affecting the outcome of plant- herbivore and plant–pathogen interactions (Rosenzweig et al., 2001). There is a wealth of information related to performance of different aphid species on drought-stressed plants (Huberty and Denno, 2004). For example, studies have reported positive (Khan et al., 2010; Mewis et al., 2012), negative (McVean and Dixon, 2001; Hale et al., 2003), and neutral (Salas and Corcuera, 1991; Pons and Tatchell, 1995) effects of drought on aphid performance. Drought stress can have equally complex consequences on plant–pathogen interactions. Most studies report reduced disease resistance in plants under drought stress, but there is considerable variation in the outcomes (Mauch-Mani and Mauch, 2005; Fujita et al., 2006; Asselbergh et al., 2008). For instance, drought stress increased the development of Pierce's disease symptoms caused by the bacterial pathogen, *Xylella fastidiosa* in grapevine (Thorne et al., 2006). Conversely, drought-stressed plants were shown to be resistant to certain pathogens. In tomato, for example drought reduced gray mold infection caused by *Botrytis cinerea* by 50% and also suppressed spread of the powdery mildew fungus, *Oidium neolycopersici* (Achuo et al., 2006). In contrast, there is limited information on the influence of flooding or saturation on plant–herbivore and plant–pathogen interactions. Studies have found that populations of the generalist aphid, *Myzus persicae* was reduced under saturated conditions, whereas the specialist aphid *Brevicoryne brassicae* was less affected by saturation (Khan et al., 2010; Mewis et al., 2012). Flooding has shown to benefit epidemics and prevalence of several fungal pathogens in corn, soybean, alfalfa, and wheat (Rosenzweig et al., 2001).

To date most studies have focused on understanding the direct effects of drought stress on plants and herbivores, but it is also important to understand indirect effects of drought on species interactions. There is accumulating information on the impact of drought stress on insect-transmitted pathogens. Krugner and Backus (2014) showed that drought stress reduced the frequency of probes by the glassy winged sharpshooter, *Homalodisca vitripennis* that are critical for transmission and spread of *Xylella fastidiosa*, bacterial pathogen of Pierce's diseases in grapes. In contrast, drought stress enhanced the plant-to-plant movement of bird cherry-oat aphids, *Rhopalosiphum padi* thereby increasing the proportion of plants infected with *Barley yellow dwarf virus* (BYDV) (Smyrnioudis et al., 2000). More recently, Davis et al. (2015b) showed that *R. padi* feeding on drought-stressed BYDV-infected plants had greater population growth rate compared to non-infected water stressed plants suggesting that virus infection helps aphids perform better on suboptimal plants. In addition, the authors found that BYDV infection in wheat increased total phytohormone concentration specifically that of SA in a time-dependent manner, which may play a role in plant resistance to drought (Davis et al., 2015a).

Plant responses during drought stress are mainly regulated by the stress hormone, ABA, which results in the activation of transcription factors and downstream functional genes that re-establish homeostasis in the plant (Finkelstein et al., 2002; Ramanjulu and Bartels, 2002; Asselbergh et al., 2008; Urano et al., 2009; Harb et al., 2010; Bostock et al., 2014). In addition to its function in abiotic stress, ABA also impacts plant resistance to pathogens and herbivores (Asselbergh et al., 2008; Studham and MacIntosh, 2013; Guo et al., 2016). There is evidence for both antagonistic and synergistic interactions between ABA and hormonal pathways that regulate plant defenses. ABA can suppress SA-mediated defenses (Mohr and Cahill, 2003, 2007; Thaler and Bostock, 2004; Asselbergh et al., 2008) and plant susceptibility to pathogens can increase following exogenous applications of ABA (McDonald and Cahill, 1999; Asselbergh et al., 2007, 2008). In certain instances, exogenous application of ABA can have the opposite effect on SA-mediated defenses resulting in increased resistance to pathogens (Ton and Mauch-Mani, 2004; Wiese et al., 2004; Melotto et al., 2006). The antagonism of SA-mediated defenses by ABA may be explained in part by the positive effect of ABA on JA biosynthesis (Adie et al., 2007). In addition, changes in proteins in ethylene (ET) and JA signaling were observed in maize leaves during drought (Bonhomme et al., 2012). There is also evidence that aphid feeding increases ABA levels in several crop species including, barley (Casaretto et al., 2004), eggplant, squash (El-Khawas and El-Khawas, 2008), and soybeans (Studham and MacIntosh, 2013). In soybeans, it has been hypothesized that soybean aphids induce ABA expression as a decoy strategy to suppress SA- and JA-mediated defense signaling (Studham and MacIntosh, 2013). Taken together, these results point toward a complex role of phytohormones in plant–pathogen and plant–herbivore interactions under drought stress.

Besides changes to plant signaling pathways, drought stress alters nutritional quality of the phloem (Huberty and Denno, 2004). Plants produce nitrogen-related osmoprotectants to counter the low osmotic pressure that occurs in response to drought stress resulting in increased nitrogen content in phloem sap (Brodbeck and Strong, 1987). Increased levels of proteins and amino acids have also been reported in leaf tissue during drought stress which may minimize water loss (Garg et al., 2001; Johnson et al., 2011). Analysis of aphid feeding behavior indicates that drought stress increases mesophyll/phloem resistance (Guo et al., 2016) plausibly due to change in phloem sap viscosity due to altered sugar and solute concentrations increasing the difficulty for aphids to acquire nutrients (Hsiao, 1973; Garg et al., 2001). Changes in the water potential of the host plant due to water stress can also impact the aphids' ability to consume xylem sap which allows aphids to deal with the high sugar concentration and osmotic pressure of the phloem sap (Pompon et al., 2010, 2011; Guo et al., 2016). Therefore, an integrative approach evaluating changes in gene expression and analyzing changes in host plant quality is essential to develop a better understanding of the impacts of water stress on plant–aphid interactions.

In the present study, we sought to investigate factors that influence performance of soybean aphids (*Aphis glycines* L.) and aphid-transmitted *Soybean mosaic virus* (SMV) on water-stressed soybean (*Glycine max* L.) plants. We adopted a broad approach by investigating plant nutritional quality and defense signaling as possible mechanisms underlining the interaction between water stress, herbivory and virus transmission. Our experimental setup comprised of three water stress regimes: drought, well-watered and saturated and two levels of aphid infestation: viruliferous (SMV-infected) and non-viruliferous (uninfected). We analyzed soybean aphid population growth, feeding behavior using electrical penetration graph (EPG) technique, and monitored virus infection and transmission. Further, we measured total and individual amino acid profiles and gene expression related to major plant signaling pathways in plants subjected to water stress, insect feeding and virus infection.

MATERIALS AND METHODS

Plant Growth Conditions

Soybean variety Asgrow® AG3432 (Monsanto, St. Louis, MO, USA) was grown in Mastermix® 830 soilless media (Mastermix, Quakertown, PA, USA). All plants were maintained at 60–70% relative humidity, temperature of 24 ± 1°C and a photoperiod of 16:8 (L:D) hours (h) at a photosynthetically active radiation (PAR) of 460 μmol/m^2/sec in an environmental chamber. Plants were watered three times per week ad libitum and received Miracle Gro® (Scott's Company, Marysville, OH, USA) solution as per label instructions once per week.

Virus Source

Soybean mosaic virus-infected seeds were provided by Dr. Glen L. Hartman, Laboratory for Soybean Disease Research at the University of Illinois, Urbana–Champaign. SMV was maintained through both mechanical inoculation and aphid transmission (Hunst and Tolin, 1982; Hill et al., 2001). Young leaves from SMV-infected plants were ground in 0.01 M phosphate buffer (pH 7.1). Virus inoculum was rub-inoculated using a cotton-tipped applicator to carborundum-dusted leaves. At least 2–3 almost fully expanded leaves were inoculated per plant. After 5 min leaves were then gently rinsed with water to remove excess carborundum. SMV infection was monitored and confirmed through presence of symptoms and RT-PCR analysis using primers listed in **Table 1**.

Insect Source

Soybean aphids were originally collected from a soybean field at the Pinney Purdue Agricultural Center (PPAC), Watanah, Indiana. In the laboratory, the aphid colony was maintained on AG3432 at temperature of 24 ± 1°C and a photoperiod of 16:8 (L:D) h in 30 × 30 × 76 cm insect cage (BioQuip, Rancho Dominguez, CA, USA). In order to obtain viruliferous aphids, adult non-viruliferous aphids from the lab colony were exposed to SMV-infected plant for 30 min (Clark and Perry, 2002).

Water-stress Treatments

To determine the level of water stress to be applied, a modified water stress procedure was used (Porcel and Ruiz-Lozano, 2004).

TABLE 1 | Quantitative reverse transcription -PCR (qRT-PCR) primer pair sequences and corresponding PCR efficiencies.

Gene	Locus/description	Primer sequences	PCR efficiency	Amplicon length (bp)	Reference
Internal Control					
FBOX	Glyma.12g051100/F-box only protein	AGATAGGGAAATTGTGCAGGT CTAATGGCAATTGCAGCTCTC	2.05	93	Le et al., 2012
SA marker genes					
PR1	Glyma.15g06790/Pathogenesis related protein 1	GCAGCTAGCAAGCTACCACT CACGCCACAACGTTCAAGA	2.26	196	Li et al., 2008
PAL2	Glyma.20g32135.1/Phenylalanine ammonia lyase	TCAGAAGCAAATGCTGCCAAC CTCTAGCATGCGCTTGACCT	1.88	144	This paper
JA marker genes					
JAR1	Glyma.16g03010.1/Jasmonic acid-amido synthetase	ACACCAAGATTCTCCTAGCTGC AGGATCCGTCCTCCCATTCA	1.75	208	This paper
AOS	Glyma.17g36530/Allene oxide synthase	TCCTCAACCAAACAACGCTCT GCGGGACTTGAAGAACTCGT	1.98	210	Studham and MacIntosh, 2013
ABA marker genes					
RD20A	Glyma03g41030/Responsive to desiccation 20	GTGGCACATGACTGAAGGAA ATCTTTCCAGCAGCACCTCT	1.98	195	Neves-Borges et al., 2012
SCOF1	Glyma.17g35430/Soybean zinc finger protein	GAGGTAAGGCCCATGAGTGC CGAAAAATCCGGAAAGGCCG	1.86	224	Studham and MacIntosh, 2013
Virus marker					
SMV413-CP	GU015011/ Soybean mosaic virus coat protein	TTCCAATGGTTGAAGGAAG CTTGCCCTGTTTGGTGTTTT	1.93	456	This paper

Briefly, 500 g of Mastermix 830 soilless media (Mastermix, Quakertown, PA, USA) was weighed, and fully saturated with water in 6″ pots (Hummert International, Earth City, MO, USA). Saturated media was weighed and a Waterscout® SM100 soil moisture probe (Spectrum Technologies, Aurora, IL, USA) was used to determine percent volumetric water content (VWC). The saturated media was allowed to air dry until all moisture was lost. VWC and weight of water lost were monitored daily. A calibration curve based on average VWC and corresponding mass of water was computed based on which the volume of water to be used to maintain each water stress treatment was determined (Supplementary Figure S1). Upon reaching V1 or first trifoliate leaf stage, plants were exposed to three water-stress treatments: drought-stress conditions (25% field capacity or FC corresponding to 7.6% VWC), well-watered conditions (75% FC corresponding to 17.9% VWC), and water-saturated or flooding condition (100% FC corresponding to 24.8% VWC). The plants were maintained at such conditions for 3 days prior to the start of the experiment and for 7 days during the duration of the experiment. In order to maintain the water stress conditions, soil water content of the soilless media was measured daily (early evening) and re-watered to restore the soil water content to required levels. During the 24 h period between measurement and re-watering, the water content only decreased by about 2–6% VWC in each of the treatments. Leaf water potential measurements were not performed because the protocol used has been previously shown to reduce water potential in drought stressed plants (Porcel and Ruiz-Lozano, 2004). With respect to the saturation treatment, previous studies have found that flooding or soil water saturation does not impact leaf water potential (Oosterhuis et al., 1990).

Experimental Design and Structure

The experimental design was a 3 × 3 factorial with three water stress treatment levels (drought, well-watered, and saturated) and three aphid infestations (uninfested plants, plants infested with non-viruliferous aphids, and plants infested with viruliferous aphids). For all treatments, 20 adult aphids were transferred using a camel-hair brush onto the adaxial surface of the first true leaves (V1 growth stage).

To assess quality of water-stressed plants as a food resource, aphid fecundity was measured daily by counting the number of nymphs and adults until day 7. At the end of the experiment, fresh weight and dry weight of the plants were obtained in order to compute water content and biomass in plants. Each treatment was replicated 3–6 times and the experiment was repeated three times (biological replicates).

Absolute Quantification of SMV

In order to accurately determine virus level, the SMV-coat protein gene was quantified from infected leaf tissue using SMV413-CP primers whose sequence are listed in **Table 1**. The PCR program consisted of 95°C for 5 min denaturation stage followed by 40 cycles of 95°C for 1 min, 55°C for 30 s, 72°C 30 s elongation, and a final elongation step of 5 min at 72°C. The PCR product was cloned following manufacturer's protocol (pCR8/GW/TOPO® vector, Thermo Scientific, Pittsburgh, PA, USA) and sequenced. The nucleotide sequences were 100% identical to the target sequences deposited in GenBank (Accession: GU015011). To quantify SMV level in a given leaf tissue, a standard curve was prepared using the aforementioned plasmid containing the SMV-coat protein target region at a known concentration of 187.8 ng/μL. The mass of the plasmid containing insert was estimated from the size of the plasmid 3,273 base pairs and the average molecular mass of a base pair in DNA 1.096×10^{-21} g, resulting in the mass of one copy of the plasmid being equal to 3.59×10^{-18} g. The initial concentration of the plasmid standard was adjusted in water to be 1.0×10^{10} copies/μL. To obtain a standard curve, 10-fold serial dilutions (ranging from 1×10^{7}copies/μL to 1×10^{1}copies/μL) from the initial concentration of plasmid. Quantitative polymerase chain reaction (qPCR) was then performed on plant tissue samples. Reactions for serial dilutions were performed in triplicates.

Electrical Penetration Graph

Aphid feeding behavior was analyzed using the electrical penetration graph technique (EPG) on a GIGA 8 complete system (EPG Systems, Wageningen, Netherlands) (Tjallingii and Esch, 1993). Adult soybean aphids were starved for 1 h prior to wiring. After wiring of aphids was completed, eight plants, two per water stress treatment were placed into a Faraday cage. Treatments were both tested and analyzed blindly. The wired plant electrodes were then placed into the soil, and insect probes adjusted to that the aphids could rest on the underside of the first trifoliate leaf allowing for contact between the plant and insect. Aphids were then allowed to feed for 8 h, while the aphid feeding behavior was recorded. This experiment was repeated until sufficient biological replications were obtained. Each feeding experiment was analyzed to determine the amount of time spent in each of the four main phases: pathway phase (PP), non-probing phase (NP), sieve element phase (SEP), and xylem phase (XP). Other parameters that were recorded include time to 1st probe, time to first potential drop (PD) and the number of PDs all of which provide an indication of aphid health/condition and also virus acquisition and transmission (Martin et al., 1997). EPG results were analyzed using Stylet+ software (EPG Systems, Wageningen, Netherlands).

Petiole Exudate Collection

In a separate experiment, plants were grown in pots with a 12″ diameter (Myers Industries Marysville, OH, USA) until the V1 growth stage. Plants were then subjected to moisture stress as described above and phloem exudates were extracted as per Nalam et al. (2012). In order to prevent bacterial contamination of the petiole exudates, trifoliates were cut and immersed in 50% ethanol, and then immediately moved to 0.05% bleach solution for no more than 2–3 s in to achieve surface sterilization of the leaf and cut surfaces. Trifoliates were then transferred to 1 mM EDTA solution (pH 8.0) until all of the trifoliates were collected from the treatment groups. Next, 1 cm of the stem was cut off, and three trifoliates were immediately placed into wells containing 4 mL of 1 mM EDTA. After all the trifoliates were transferred to fresh EDTA buffer, they were placed in an aquarium with a clear

lid lined with moistened paper towels for 24 h. Petiole exudates from three wells were then pooled per sample resulting in nine trifoliates per pooled sample. Samples were then filtered through 0.2 μm pore size filters and lyophilized. After lyophilization, samples were eluted in 750 μL of 1 mM EDTA solution and used in artificial feeding assays and nutrient analysis (described below).

Artificial Feeding Assay

An artificial diet previously tested for optimum soybean aphid performance (Diet C, Wille and Hartman, 2008), was used for all artificial feeding assays. An artificial feeding chamber consisted of 55 mm petri dishes (VWR, Radnor, PA, USA) with parafilm (Bemis, Neenah, WI, USA) stretched across the top to act as feeding sachets. Each sachet contained a total volume of 750 μL, which included the artificial diet with or without 25 μL petiole exudates or buffer used to collect the petiole exudates. Ten 3rd instar non-viruliferous aphid nymphs were placed in each feeding chamber and allowed to develop until adulthood. Total number of nymphs and adults were counted at the end of the experiment. We did not test viruliferous aphids because SMV is not a phloem-limited virus and thereby not likely to affect aphid biology in artificial feeding assays.

Amino Acid Analysis

Petiole exudates from each of the water-stress treatments were sent to the Donald Danforth Plant Science Center (St. Louis, MO, USA) for amino acid analysis. Samples were tested for amino acids using the AccQTag derivitization method (Waters Corporation, Milford, MA, USA). Samples were run in triplicate on an Acuity UPLC® System for 9.5 min and essential and non-essential amino acids were detected. Results from amino acid analysis were standardized to the average leaf mass for each treatment.

RNA Extraction, cDNA Synthesis and Reverse Transcriptase-Quantitative PCR (RT-qPCR)

For all experiments, 100 mg leaf tissue was harvested from each of the nine treatments (3 water stress × 3 aphid infestations), flash frozen, and stored at −80°C for further processing. Plant RNA was extracted using the Trizol® (Invitrogen, Grand Island, NY, USA) method, checked for purity and quantity using a Nanodrop ND 100 (Thermo Scientific, Pittsburgh, PA, USA). RNA was then treated with Turbo DNase® (Invitrogen, Grand Island, NY, USA) in order to remove DNA contamination. Complete removal of DNA was verified by PCR using DNase treated RNA as template for amplification with the internal control *FBOX* gene (Le et al., 2012). Two micrograms of RNA was used as a template for cDNA synthesis using the Verso® cDNA synthesis kit (Thermo Scientific, Pittsburgh, PA, USA).

RT-qPCR was performed using SYBR Green® (Biorad, Berkeley, CA, USA) on a CFX Connect® (Biorad, Berkeley, CA, USA) thermocycler. The cycling conditions used were: 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, and 60°C for 30 s. PCR efficiencies (E) of target and internal control genes were determined using the LinRegPCR software (Ruijter et al., 2009) and are shown in **Table 1**. Reactions for all samples were performed in duplicate and three biological replicates and a negative and positive control were used in each run. Fold change was determined by normalizing transcript levels of the genes of interest to the internal control gene (*FBOX*), followed by normalization to expression of the respective gene in a plant that was not subjected to water stress or aphid infestation using the following formula, $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Fold changes were $\log_2$ transformed in order to normalize data. Log_2 (fold change) data is presented and also used for all statistical analysis.

Statistical Analysis

All response variables conformed to assumptions of ANOVA and no transformations were performed with the exception of gene expression fold change. To determine the relationship between water content of plants and soil VWC a simple linear regression analysis was performed and 95% confidence intervals were calculated. Regression analysis was performed in Sigma Plot Version 12.5 (Systat Software® San Jose, CA, USA). To determine if water-stress treatments affected aphid population growth rate, a two-way ANOVA was conducted with water stress (drought, well-watered, saturated) aphid infestations (non-viruliferous and viruliferous) and their interactions as main effects. Fold change of plant defense genes was also analyzed using two-way ANOVA with the same fixed and interaction effects. To determine the effect of petiole exudates (from water-stressed plants) on aphid populations in artificial feeding assays, a one-way ANOVA was performed. A one-way ANOVA was also conducted to determine the effect of water stress on virus level (log copies) and amino acid levels. For EPG analysis, the mean time spent by the aphids in various activities was analyzed using non-parametric Kruskal–Wallis test. Parameters that showed a significance level close to 5% were further analyzed using a separate pair-wise comparison (Mann–Whitney *U*-test, $\alpha = 0.05$). All data was analyzed using Minitab Version 17 (Minitab® State College, PA, USA).

RESULTS

Effect of Water Stress on Plant Growth and Water Content

Water content was lowest in plants under drought and highest in plants under saturation ($P < 0.001$, **Figure 1A**). At the end of the experiment, there was a strong positive relationship between soil and plant water content indicating that water-stress treatments were consistent throughout experiments (R^2: 0.92 uninfested plants, 0.82 non-viruliferous aphid-infested plants, 0.84 viruliferous aphid-infested plants). Plant dry weight measured at the end of the experiment also showed the same pattern ($P < 0.001$) except there was no difference in dry weight between well-watered and saturated plants (**Figure 1B**). The expression of a drought-stress marker, *RD20A* (Neves-Borges et al., 2012), 3 days after the commencement of water stress, treatments showed a significant increase in expression under drought stress as compared to well-watered and saturated plants

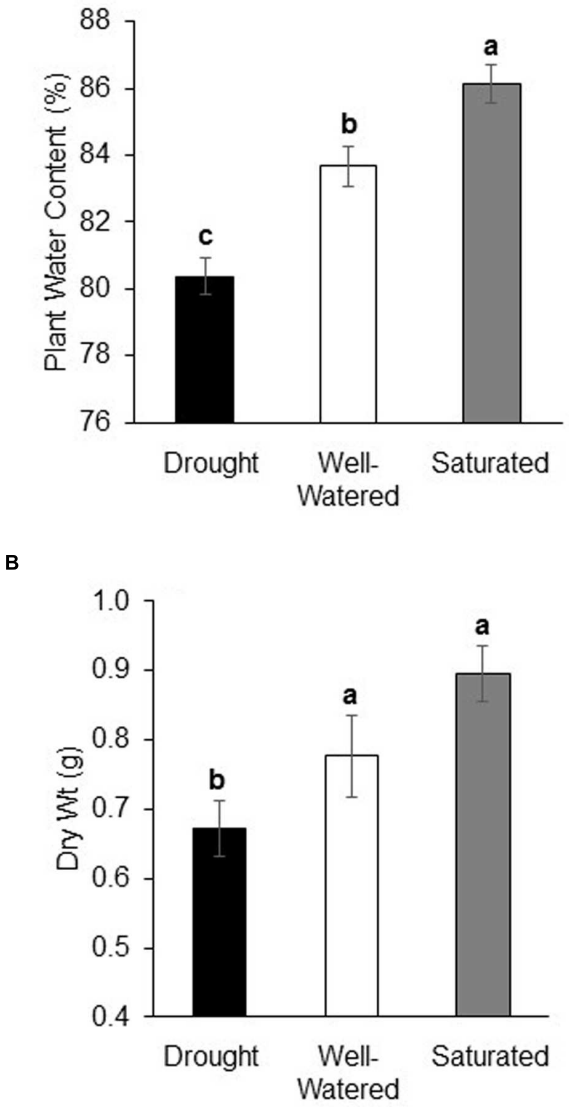

FIGURE 1 | Drought stress reduces plant water content and dry weight. (A) Water content (%) and **(B)** dry weight (g) of soybean plants subjected to drought and saturation treatments for a 10 days period. Soybean plants grown under well-watered conditions serve as control. Each bar represents the mean ± SE of $n = 3$–6 plants per experiment or biological replicate. Each experiment was repeated three times. Different letters indicate significant difference between treatments (Tukey's HSD $P < 0.001$).

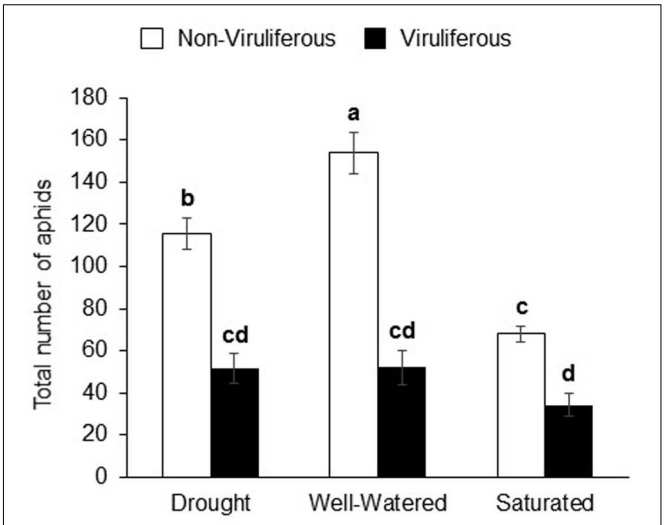

FIGURE 2 | Water stress and virus infection has a significant impact on aphid populations on soybean plants. The total number of aphids (Adults + nymphs) on soybean plants 7 days post-infestation. Each bar represents the mean ± SE of $n = 3$–6 plants per experiment or biological replicate. Each experiment was repeated three times. Different letters indicate significant difference between treatments (Tukey's HSD $P < 0.001$).

(Supplementary Figure S2). Feeding by either non-viruliferous or viruliferous aphids did not affect plant water content or dry weight in response to the water-stress treatments (Data not shown).

Effect of Water Stress on Aphid Populations

Water-stress treatments had significant and strong effects on aphid populations. The interaction effect (water stress × aphid infection levels) and main effects were significant for aphid populations (Supplementary Table S1). Non-viruliferous aphid populations were highest on well-watered plants and lowest on saturated plants (**Figure 2**). On the other hand, there was no significant difference in viruliferous aphid populations on any of the water-stress treatments (**Figure 2**). However, populations of viruliferous aphids were significantly lower than non-viruliferous aphids under all treatments.

Effect of Water Stress on SMV Infection and Transmission

Water-stress treatments also significantly impacted virus infection levels and aphid's ability to transmit SMV. Although there was no significant difference in viruliferous aphid populations under the different water-stress treatments (**Figure 2**), virus levels as measured by average number of SMV-coat protein molecules per 100 mg of leaf tissue differed between treatments. SMV infection was highest in saturated plants and lowest in drought-stressed plants ($P < 0.001$; **Figure 3**). Transmission rate (calculated as the proportion of soybean plants testing positive for the virus) showed similar patterns in that rates were lowest under drought stress and highest under saturated conditions, 50 and 77%, respectively, compared to well-watered plants where the rate was 60%

Aphid Feeding Behavior on Water Stressed Plants

Among the feeding behaviors recorded, a significant impact of water stress was observed on the amount of time spent

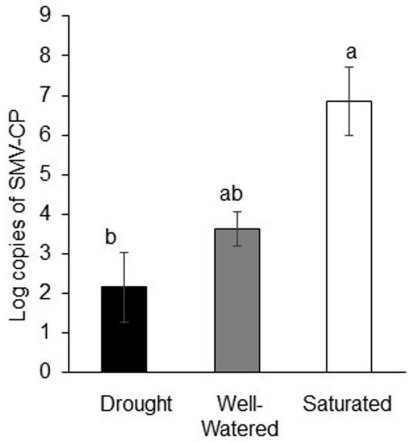

FIGURE 3 | Drought reduces virus infection but saturation enhances virus infection. Log copies of SMV-coat protein in soybean plants subjected to water stress and feeding by viruliferous aphids. Each bar represents the average of $n = 3$–6 plants per experiment or biological replicate. Each experiment was repeated three times. Different letters indicate significant difference between treatments (Tukey's HSD $P < 0.001$).

by non-viruliferous aphids in the sieve-element phase, SEP ($P = 0.02$; **Figure 4A**). Non-viruliferous aphids spent significantly less time in the SEP on saturated plants compared to drought and well-watered plants (**Figure 4A**). There were significant differences in the time spent by viruliferous aphids in both SEP and non-probing phase, NP ($P = 0.02$ and $P = 0.05$, respectively; **Figure 4B**). Viruliferous aphids spent lesser time in SEP on both drought-stressed and saturated plants compared to well-watered plants (**Figure 4B**). Viruliferous aphids spent more time in NP on plants under drought and saturation treatments compared to on plants that were well-watered plants (**Figure 4B**). Water-stress treatments did not affect aphid hydration status (Supplementary Figure S3).

Irrespective of the water-stress treatment, non-viruliferous aphids showed a significantly greater number of (PDs; i.e., when the stylet tip punctures a cell) compared to viruliferous aphids (**Table 2**). Additionally, non-viruliferous aphids took significantly less time to 1st PD under all conditions. In terms of behaviors critical for virus transmission, viruliferous aphids took least amount time for 1st PD under saturated conditions followed by well-watered and drought stress treatment (**Table 2**).

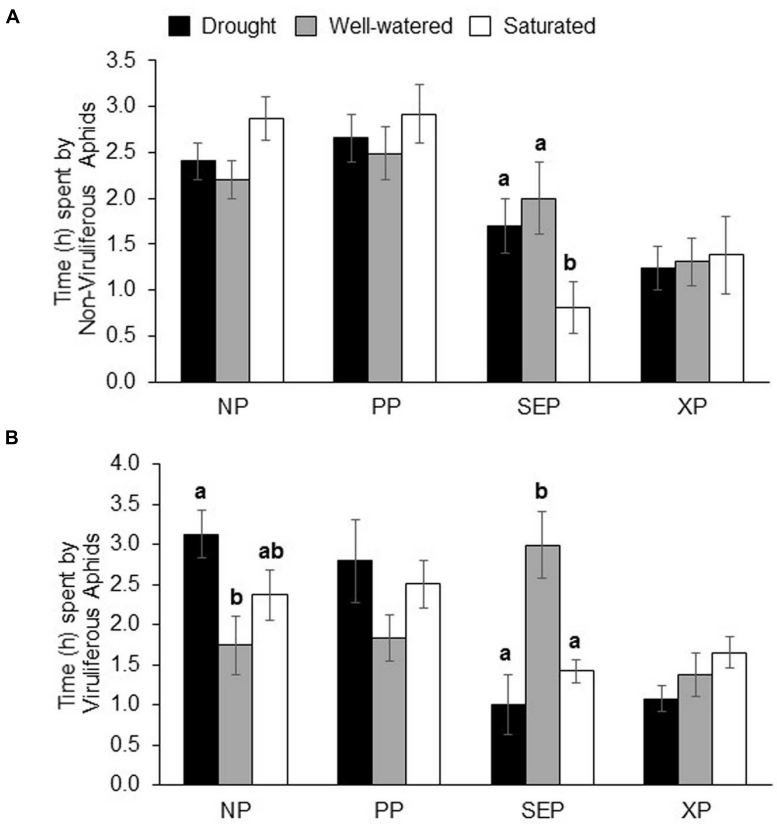

FIGURE 4 | Electrical penetration graph analysis of aphid behavior on water-stressed plants. Time (in h) spent by **(A)** non-viruliferous and **(B)** viruliferous aphids on drought, well-watered and saturated soybean plants over an 8 h of recording time. Each values represents the mean from 12 to 19 replications. The time spent by aphids on various activities (NP, non-probing phase; PP, pathway phase; SEP, Sieve element Phase; XP, Xylem Phase) was analyzed by the non-parametric Kruskal–Wallis test ($P < 0.05$). For parameters that showed a significance level of $P \leq 0.05$ a separate pairwise comparison using the Mann–Whitney U-test ($\alpha = 0.05$) was performed. Each bar represent the mean ± SE. Different letters indicate significant difference between treatments.

TABLE 2 | Probing behavior of non-viruliferous and viruliferous aphids on drought-stressed, well-watered and water-saturated plants.

Parameter		Drought		Well-Watered		Saturated		
	n	Non-viruliferous *19*	Viruliferous *16*	Non-viruliferous *16*	Viruliferous *14*	Non-viruliferous *13*	Viruliferous *12*	*P*-value
Number of PD	(#)	81.4 ± 5.3 a	45.6 ± 3.5 b	78.8 ± 6.9 a	51.1 ± 5.8 bc	70.9 ± 6.9 ac	59.0 ± 6.0 bc	0.001
Time to 1st potential drop (PD)	(min)	0.7 ± 0.1 a	5.0 ± 0.2 b	0.8 ± 0.2 a	3.0 ± 1.0 b	0.8 ± 0.3 a	1.4 ± 0.5 a	<0.0001
Time to 1st probe	(min)	42.5 ± 7.9 a	91.8 ± 14.4 b	47.7 ± 10.4 a	111.4 ± 20.8 b	36.6 ± 10.9 a	109.7 ± 15.6 b	<0.0001
Aphids with phloem phase	(#), (%)	17, 89.5	12, 75	16,100	14, 100	9, 69.2	12, 100	
Aphids with xylem phase	(#), (%)	17, 89.5	16, 100	14, 87.5	14,100	10,76.9	11, 91.7	

Total durations (min), numbers (#), or percentage of total time (%). Means followed by different letters within lines are significantly different.

Analysis of Petiole Exudates from Water Stressed Plants on Aphid Populations

A total of 18 amino acids were detected including both essential and non-essential amino acids (**Table 3**). There were significant differences in total amino acid content in vascular sap enriched petiole-exudates from water-stressed plants ($P < 0.001$). Petiole-exudates from drought-stressed plants had greater total amino acid content, but not significantly different from well-watered plants. Petiole-exudates from saturated plants had the lowest free amino acid content (**Table 3**). There were significant differences in eight amino acids due to water stress including four essential amino acids: isoleucine, leucine, threonine, and valine and four non-essential amino acids: asparagine, glutamic acid, proline (marginally significant), and tyrosine (**Table 3**). All amino acids were higher in petiole-exudates from drought-stressed plants compared to well-watered and saturated plants with the exception of tyrosine (**Table 3**).

Artificial feeding assays performed using petiole-exudates from soybean plants exposed to the various water-stress treatments indicated that aphid populations were highest on artificial diet and diet plus buffer, which served as the positive controls ($P < 0.0001$; **Figure 5**). Among the water-stress treatments, soybean aphid populations were highest in response to petiole-exudates from well-watered plants, which was not significantly different from the positive controls (**Figure 5**). The lowest aphid populations were observed in response to petiole-exudates collected from saturated plants which was not significantly different from drought stressed plants. Overall, non-viruliferous aphid numbers in artificial feeding assays

TABLE 3 | Concentrations of amino acid in petiole exudates of soybean plants subjected to different water-stress treatments.

Amino Acids	*P-values*	Drought	Well-Watered	Saturated
Alanine	0.302	182.74	119.92	17.81
Arginine	0.569	167.16	114.30	21.52
Asparagine	**0.05**	**23322.58 a**	**13481.85 ab**	**6316.14 b**
Aspartic Acid	0.376	2621.01	2262.47	1660.27
Glutamine	0.085	5735.13	5425.74	1788.18
Glycine	0.513	745.11	744.96	156.32
Glutamic Acid	**0.01**	**1229.81 a**	**536.74 b**	**507.06 b**
Isoleucine	**<0.001**	**528.75 a**	**214.88 b**	**27.83 c**
Leucine	**0.015**	**304.09 a**	**138.77 ab**	**48.64 b**
Lysine	0.467	218.89	277.51	29.95
Methionine	0.086	37.75	16.99	6.52
Phenylalanine	0.214	293.58	236.13	54.27
Proline	0.065	409.66	94.78	10.51
Serine	0.155	1490.07	2298.38	237.59
Threonine	**0.007**	**832.11 a**	**317.36 b**	**197.26 b**
Tryptophan	0.298	51.67	66.17	20.91
Tyrosine	**0.04**	**124.04 ab**	**159.50 a**	**25.23 b**
Valine	**0.005**	**555.48 a**	**261.68 ab**	**88.76 b**
Total	**<0.001**	**43445.36 a**	**37500.14 ab**	**12347 b**

Values are expressed as pmol/gm fresh weight. Values represent mean of n = 5 plants or replicates. Means followed by different letters indicate significant difference between treatments. Treatments with significant differences are in bold.

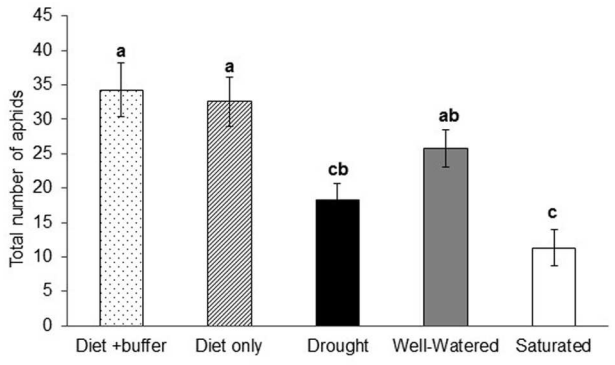

FIGURE 5 | Petiole exudates from water stressed plants alters aphid populations. Total number of aphids reared on artificial diet supplemented with buffer or petiole exudates collected from drought, well-watered and saturated plants in artificial feeding assays. Each bar represents the mean ± SE of $n = 8$ artificial feeding assays or replicates. Different letters indicate significant difference between treatments (Tukey's HSD $P < 0.001$).

showed the same pattern as aphid populations on whole plants (**Figure 2**).

Gene Expression Analysis

There was significant interaction between water stress and aphid infestation on ABA marker genes, *RD20A* but not *SCOF1* (Supplementary Table S2). *RD20A* has been previously shown to be highly induced by drought stress, and indeed we found a significant increase in *RD20A* expression in response to drought stress (**Figure 6A**). In contrast, saturation resulted in the suppression of *RD20A* expression (**Figure 6A**). Feeding by non-viruliferous aphids increased *RD20A* expression under all treatments (**Figure 6A**). There was a moderate increase in *SCOF1* expression under drought stress compared to well-watered and saturated conditions albeit not statistically significant. Similar to *RD20A*, non-viruliferous aphid feeding up-regulated *SCOF1* expression under drought stress (**Figure 6B**). The SA pathway marker, *PR1* was affected by the interaction between water stress and aphid infestation but not *PAL2*, a gene involved in SA biosynthesis (Supplementary Table S2). *PR1* expression was down-regulated in uninfested drought-stressed plants and significantly up-regulated in saturated plants. In addition, *PR1* expression was induced in response to both non-viruliferous and viruliferous aphid feeding in all treatments (**Figure 6C**). *PAL2* expression was reduced under drought stress, moreover, expression was down-regulated in response to feeding by viruliferous aphids (**Figure 6D**). Expression of the JA marker, *JAR1* was also affected by the interaction between water stress and aphid infestation, but only water stress had a significant main effect (Supplementary Table S2). *JAR1* expression was suppressed in drought-stressed plants and in well-watered plants whereas, expression was significantly up-regulated in saturated plants (**Figure 6E**). The expression of *AOS*, involved in JA biosynthesis, was not affected by water stress (Supplementary Table S2). However, the pattern of expression was similar to *JAR1*. In general, feeding by either non-viruliferous or viruliferous aphids significantly up-regulated *AOS* expression as compared to the uninfested control (**Figure 6F**).

DISCUSSION

We investigated plant responses to simultaneous exposure to abiotic (drought and saturation) and biotic stresses (insect feeding and virus transmission) in the model crop species, soybean at two levels: organismal (measured as fecundity, feeding behavior, and virus transmission), and sub-organismal (measured as free amino acid profiles and defense gene expression). Our results show that drought and saturation have different consequences for plant resistance to aphids and aphid-transmitted SMV. We hypothesize that these outcomes are a result of changes in amino acid content and interactions between the phytohormones ABA, SA, and JA.

Availability of water whether excess or deficit is critical for plant growth and maintenance, which are important determinants for plant resistance against insect herbivores and pathogens. In the current study, soybean plants were subjected to drought and saturation as per Porcel and Ruiz-Lozano (2004). Similar methods of water-stress treatments have been used in other studies that have attempted to elucidate the impact of drought stress on phloem-feeding insects (Mewis et al., 2005, 2012; Khan et al., 2010; Guo et al., 2016). The water stress regime implemented in the current study correlated well with plant water content at the end of the experiment; water content was lowest in plants under drought and highest in plants under saturation. Moreover, there was a strong positive relationship between soil and plant water content indicating that water-stress treatments were consistent throughout experiments. The increase in plant water content in saturated plants may suggest that they did not experience significant stress as a result of the treatment. A previous study did not find any impact of saturation on plant water content at day 7 but found that the total N content of the plants was significantly reduced (Bacanamwo and Purcell, 1999). So, it is possible that although a change in plant water content was not observed, the treatment significantly affected plant metabolism. Daily variation in VWC was minimized by maintaining the plants in an environmental chamber that was maintained at a constant temperature and humidity. Furthermore, daily monitoring of the VWC revealed that over a 24 h between re-watering, the VWC reduced by only 2–6% depending on the treatment with the highest fluctuation observed under saturated conditions. The effectiveness of drought treatment was further confirmed by the strong induction of *RD20A*, a known soybean drought-stress marker in drought stressed plants after 3 days of treatment. Taken together, these results confirm the reliability of water-stress treatments imposed in the current study.

Water stress especially drought in host plants is known to impact aphid performance (Huberty and Denno, 2004). We show that populations of non-viruliferous aphids were significantly reduced when plants were saturated or drought-stressed compared to well-watered conditions. This effect was reflected in aphid feeding behaviors as determined by EPG

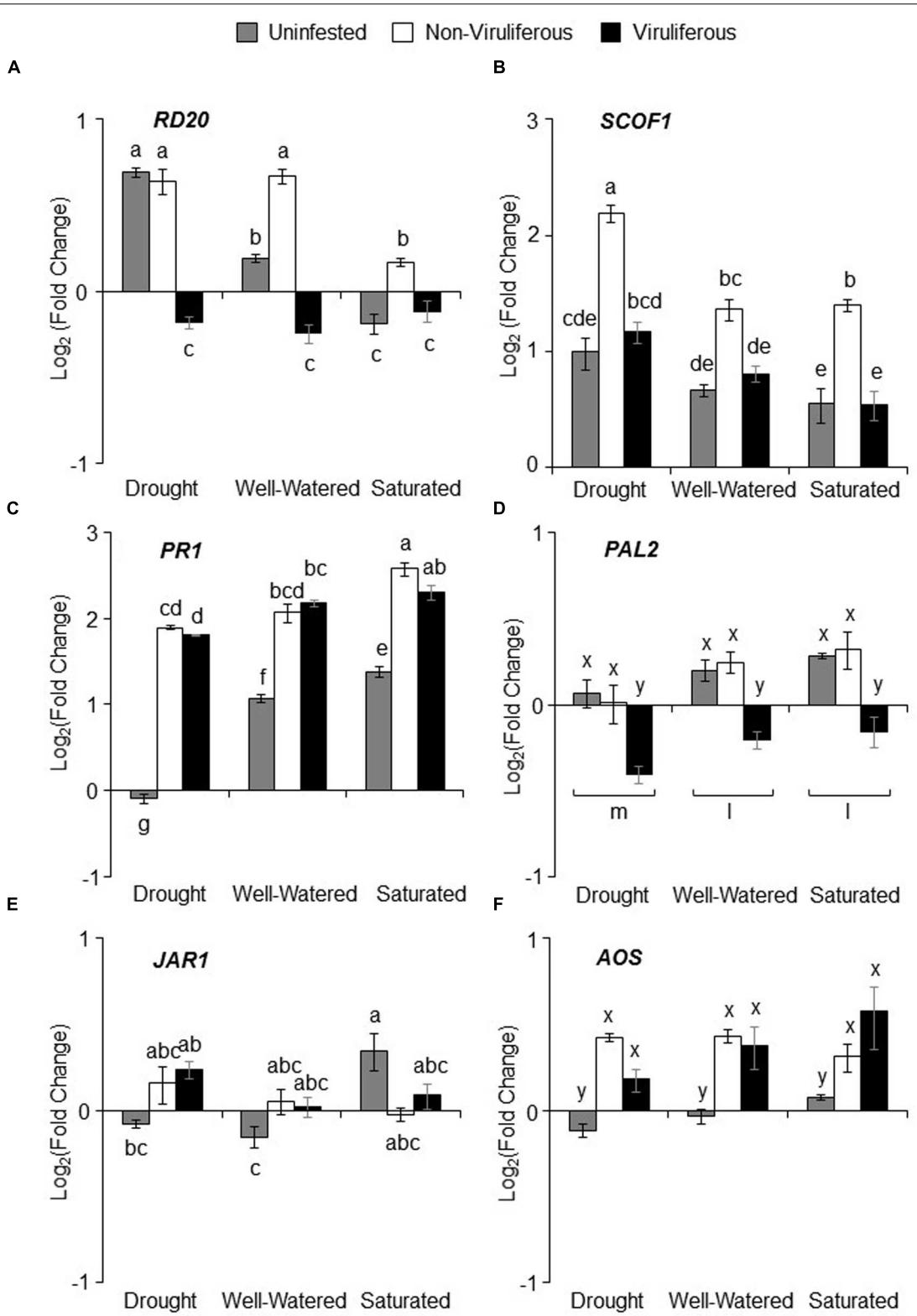

FIGURE 6 | Water stress and aphid feeding affects plant defense signaling genes. Log_2 (fold change) with respect to uninfested unstressed control of the following genes **(A)** *RD20A* (ABA/Drought marker), **(B)** *SCOF1* (ABA marker), **(C)** *PR1* (SA marker), **(D)** *PAL2* (SA biosynthesis), **(E)** *JAR1* (JA marker) and **(F)** *AOS* (JA biosynthesis). Relative gene expression and fold change was calculated using the comparative $2^{-\Delta\Delta C_T}$ method with *FBOX* as endogenous control. Values are shown as mean of Log_2 (fold change) ± SE. Each bar represents the average Cq values derived from of $n = 3$–6 plants pooled together from three independent experiments. Different letters indicate significant difference between treatments (Tukey's HSD $P < 0.001$).

analysis. We found that non-viruliferous aphids spent the least amount of time feeding from the sieve element (SEP) on saturated plants, which likely affected growth of aphid populations. In contrast, aphids tended to spend the greatest amount of time consuming sap from the phloem of well-watered plants and their populations grew significantly faster. Previously, it has been reported that drought stress resulted in increased populations of a generalist aphid, *M. persicae*, on broccoli or *Arabidopsis* plants, whereas saturation negatively impacted population growth (Khan et al., 2010; Mewis et al., 2012). In contrast, drought and saturation had no impact on the specialist, *Brevicoryne brassicae*. The authors found that drought and saturation increased secondary metabolite levels, which the specialist, *B. brassicae* were better able to tolerate compared to generalist, *M. persicae*. In the current study, however, populations of the specialist, soybean aphid, were reduced under drought stress, and saturation. There could be several reasons for the variation in outcomes from one system to another including plant hosts used (Hale et al., 2003), insect species, severity and type of stress (Huberty and Denno, 2004; Mody et al., 2009), and even experimental design (Koricheva et al., 1998). To summarize, soybean aphid performance was best on well-watered soybean plants owing to longer undisturbed feeding from the sieve element compared to saturated and drought-stressed plants where feeding from SEP was reduced.

Virus infection has been shown to improve drought tolerance in a variety of crop species (Xu et al., 2008). For instance, it was recently demonstrated that BYDV-infected wheat plants had increased growth, seed set, and germination compared to non-infected plants under drought stress (Davis et al., 2015b). In addition, fecundity of the aphid vector, *R. padi* increased by 47% when fed on BYDV-infected drought-stressed plants whereas fecundity increased by only 23% from feeding on BYDV-infected well-watered plants. Unlike the abovementioned study where plants were first inoculated with the virus, we first subjected plants to water stress and then exposed them to viruliferous aphids. By introducing aphids after application of water treatments we were not only able to monitor aphid performance but also virus transmission on water-stressed plants. Viruliferous aphid population was significantly reduced compared to non-viruliferous aphids irrespective of the water treatment. Our findings corroborate a previous report that showed that SMV infection negatively affects population growth of soybean aphids on soybeans (Donaldson and Gratton, 2007). Yet, we found that drought-stressed plants harbored lowest SMV infection and had the lowest transmission rate whereas saturated plants had highest level of infection and transmission rate. It has been previously shown that the time taken to first intracellular puncture or PD and the number of PD are important for efficiency of virus transmission in case of non-persistently transmitted plant viruses (Martin et al., 1997). Virus infection and transmission rate observed in the current study correlated well with aphid feeding behaviors on the respective plants. Viruliferous aphids took a longer time to first PD on drought-stressed plants and shortest time on saturated plants. Moreover, the number of PD was lowest on drought-stress plants and highest on saturated plants albeit not statistically significant. This response correlated well with the lower SMV level observed in plants under drought stress and highest under saturation. This is in contrast to a previous report that showed that drought stress increased aphid movement resulting in increased BYDV transmission (Smyrnioudis et al., 2000). The outcomes of the interaction between water stress and insect-transmitted disease is dependent on several abiotic and biotic factors (Bartels and Sunkar, 2005; Davis et al., 2015b). It is possible that mode of virus transmission, persistent (pathogen propagates within the vector) such as BYDV or non-persistent (pathogen does not propagates within the vector) such as SMV influences the outcomes. In non-persistent or stylet-borne virus, virions are attached to the distal tip of the stylet of the insect and when the insect feeds on a healthy plant, it inoculates the plant with the virus. In this case, transmission efficiency is greatest when vectors briefly puncture plant cells and decreases with longer feeding. In contrast, transmission efficiency increases with longer feeding duration in case of persistently transmitted viruses (Purcell and Almeida, 2005). It is plausible that enhanced host plant traits and vector performance is critical for transmission of persistent viruses compared to non-persistent viruses.

Water stress can modify nutritional quality of the phloem sap which has significant repercussions for aphids (Huberty and Denno, 2004). We therefore collected vascular sap-enriched petiole exudates from soybean plants exposed to the various water-stress treatments for artificial feeding assays. The collection of petiole exudates in the current study was modified from the protocol developed by King and Zeevaart (1974), which makes use of a chelating agent EDTA to enhance exudation from the cut petioles. Although criticized for the use EDTA that can hinder the identification of free amino acids this method has been used in several studies (Urquhart and Joy, 1981; Douglas, 1993; Karley et al., 2002; Khan et al., 2010; Mewis et al., 2012; Nalam et al., 2012; Guo et al., 2013; Zhang et al., 2015). We believe that the low concertation of EDTA (1 mM) used in the above-mentioned studies and in the current study is not detrimental and allows for adequate detection of free amino acids in petiole exudates. In artificial feeding assays, populations of non-viruliferous aphids were reduced on diet supplemented with petiole-exudates from saturated and drought-stressed plants mirroring results observed in whole plants assays. It is hypothesized that drought stress increases amino acid concentration in the phloem sap, but loss of turgor pressure can limit accessibility of phloem sap to aphids thereby reducing population growth (Huberty and Denno, 2004). We found a tendency for greater total free amino acid content in drought-stressed plants and also reduction in feeding duration. Conversely, decrease in amino acid content in saturated plants may have resulted in decrease in aphid populations. Besides changes in amino acid content, drought stress has been shown to enhance sugar content in the phloem which can have positive (Khan et al., 2010; Mewis et al., 2012) or negative (Douglas, 2006) impact on aphid performance. It is plausible that in addition to changes in amino acid composition, water-stress treatments could have caused alterations in the levels of other compounds which have not been evaluated in the study. Future research may be aimed at investigating such compounds using proteomic and metabolomic approaches. In the current study, however, aphids

performed poorly on drought-stressed plants which suggests that other changes may outweigh any benefit to the aphid from increased sugar content on drought-stressed plants.

The differential effects of water-stress treatments on growth of aphid populations may be also explained by differences in amino acid profiles. Asparagine and valine are critical for soybean aphid development and fecundity. Soybean aphids reared on diets low in asparagine and valine had longer development times, lower fecundity, and significantly fewer mature into adults (Wille and Hartman, 2008). Conversely, *M persicae* reared on a diet supplemented with asparagine and glutamate displayed enhanced growth (Karley et al., 2002). Moreover, tyrosine, alanine, leucine, and glutamic acid accounted for 43% of variations in the intrinsic rate of increase in populations of the *M. persicae* and *B. brassicae* (Cole, 1997). Proline, one of the markers for drought stress acts as an osmoprotectant in plant cells against water stress. However, we did not observe a significant change in proline concentration under any of the water-stress treatments. The accumulation of proline in soybean plants has been shown to be dependent on the growth stage of the plant and also on the level of drought tolerance (Silvente et al., 2012). In soybean plants, proline accumulation is mainly induced when drought occurs during the flowering and also in genotypes that are less tolerant to drought (Silvente et al., 2012). Furthermore, proline does not effect on aphid population growth rates (Douglas et al., 2001), so it is unlikely to have impacted soybean aphid numbers. To summarize, drought-induced enhancement of total amino acid content in the petiole-exudates did not benefit soybean aphid performance which may, in part, be due to the increase in specific amino acids that were detrimental to soybean aphid growth and development.

Water stress can alter plant's constitutive and induced defenses against both insect pests and pathogens (Mauch-Mani and Mauch, 2005; Fujita et al., 2006; Asselbergh et al., 2008; Ramegowda and Senthil-Kumar, 2015). We analyzed marker genes associated with various phytohormone signaling pathways in order to elucidate the impact of water stress, insect herbivory and virus transmission on plant responses. It is well-documented that the phytohormone, ABA is critical in plant response to drought, and, however, our knowledge regarding its functions in response to insects and pathogens is limited (Erb et al., 2012; Pieterse et al., 2012; Biere and Bennett, 2013). The induction of ABA marker genes, *RD20A* and *SCOF1* we observed in uninfested plants under drought stress highlight the importance of ABA. Studies have shown that ABA levels rapidly increased until 7 days and then start to plateau in response to drought stress (Vaseva et al., 2010). In contrast, both ABA-marker genes showed reduced expression under saturated conditions. Flooding can cause reduction in ABA levels due to downregulation by ethylene (Bailey-Serres and Voesenek, 2008). Interestingly, feeding by non-viruliferous aphids significantly increased ABA-marker gene expression under drought stress and well-watered conditions. Recently, it was shown that drought induced the accumulation of transcripts associated with ABA leading to suppression of SA-dependent defenses in *Medicago truncatula* plants that are susceptible to the pea aphid, *Acrythosiphon pisum* (Guo et al., 2016). With respect to soybean and soybean aphid defense response, our findings are in agreement with Studham and MacIntosh (2013) who showed that ABA levels significantly increased in response to soybean aphid feeding at day 7 in a susceptible cultivar. The cultivar, AG3432, used in our study is also a susceptible cultivar suggesting that our results support the hypothesis that a decoy strategy is initiated by aphids to suppress both SA- and JA-mediated defenses.

Salicylic acid signaling pathway is critical for plant resistance against aphids, but JA can also be involved (Goggin, 2007). There is mounting evidence that ABA antagonizes SA through various mechanisms including suppression of SA-inducible defense transcripts (Asselbergh et al., 2008). The suppression of SA-dependent transcripts in drought stressed plants indicates a potential antagonistic interaction between ABA and SA signaling. A corresponding decrease in non-viruliferous aphid numbers observed on saturated plants and increase in numbers in drought-stressed plants further highlights the antagonism between ABA and SA signaling. The effect of ABA on JA on the other hand is more complex with antagonistic and synergistic effects reported (Asselbergh et al., 2008). Similar to the pattern for SA-related genes, the expression of JA marker genes were lowest under drought stress and highest under saturation, which suggests that ABA had a negative impact on JA signaling as well. Taken together, our results suggest that antagonism of ABA on SA and JA is a key element in the interaction between water stress and aphid herbivory.

Plant responses to virus attack is mainly mediated via the SA pathway (Glazebrook, 2005; Koornneef and Pieterse, 2008), hence ABA antagonism of SA can affect plant resistance against virus. For instance, in tobacco plants, infection with the *Tobacco mosaic virus* resulted in an increase in ABA concentration which down–regulated β-1,3- glucanase resulting in increased resistance (Whenham et al., 1986). In the current study, *PR1* expression was highest in response to viruliferous aphid feeding in saturated plants where ABA levels were the lowest. These plants harbored highest amount of virus and transmission rates, suggesting that SA is not critical in virus resistance and there could be other phytohormones involved. We did not, however, find evidence for changes in JA-related genes due to viruliferous aphid feeding under water stress. Transcriptomic analysis of soybean leaf tissue with SMV infection showed that expression levels of many of the transcripts encoding phytohormones were either down-regulated or not affected during early stage of infection (day 7), but upregulated at late stages (day 14 and 21) indicating that plant immune response is not activated until later which may be critical for SMV to establish its systemic infection. (Babu et al., 2008). Hence, future studies may be aimed at analyzing the impact of water stress on virus infection over time.

CONCLUSION

This is among the first studies to investigate the effect of drought and saturation on insect herbivory and virus transmission, and the first to undertake a comprehensive analysis of the role of nutrition and defense signaling in plant responses to simultaneous attack by abiotic and biotic stresses. We report that

drought and saturation had different consequences for soybean aphids and virus infection and transmission on soybean. Drought and saturation reduced non-viruliferous aphid populations, but had no impact on viruliferous aphids. Nevertheless, virus level and transmission rate was highest in saturated plants and lowest in drought-stressed plants. We were able to show that variation in aphid populations and virus levels correlated with aphid feeding on the corresponding plants. For example, non-viruliferous aphids spent reduced amount of time in SEP on saturated and drought-stressed plants compared to well-watered plants, which presumably resulted in lower populations on these plants. Our findings suggests that plant responses to water stress is complex involving changes in nutrient quality and signaling pathways, which can impact aphid populations and virus transmission. The drought-mediated increase in free amino acid content did not benefit non-viruliferous aphids whereas, a reduction in amino acid content in saturated plants negatively impacted aphid populations. It is possible that quality rather than quantity of specific amino acids had a greater impact on aphid populations. In drought-stressed plants, there was an increase in ABA-related gene expression and decrease in the expression of SA- and JA-related genes compared to saturated plants where the ABA-related gene expression was reduced. These changes in gene expression may in part explain the higher aphid densities on drought-stressed plants compared to saturated plants. Further experimentation including phytohormone analysis and utilizing mutants of the plant defense signaling pathways would be useful to explore this result. Future experiments such as transcriptomic, proteomic and metabolomics approaches may also shed light on specific changes in genes, proteins and metabolites underlying the interaction between water stress, insect herbivory and virus infection.

AUTHOR CONTRIBUTIONS

PN and VN conceived and designed the experiments. CC, PSII, JH, and VN performed the experiments. VN and PN analyzed data. PN contributed reagents, consumables, and use of equipment. VN and PN wrote the manuscript.

FUNDING

This project was supported by a Graduate Research Fellowship to CC by the Graduate Office and Office of Research, Engagement, Sponsored Programs at Indiana University-Purdue University Fort Wayne, and startup funds provided to PN.

ACKNOWLEDGMENTS

We would like to thank Dr. Jyoti Shah for the invitation to contribute to this special issue. We would like to acknowledge Dr. Ada Szczepaniec for helpful comments on an earlier version of the manuscript. We are grateful to Drs., Houston Hobbs and Glen Hartman for kindly providing SMV-infected seeds and to Dr. Christian Krupke for soybean seeds.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00552

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 09 November 2016
doi: 10.3389/fpls.2016.01680

The Contrasting Effects of Elevated CO_2 on TYLCV Infection of Tomato Genotypes with and without the Resistance Gene, *Mi-1.2*

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Elevated atmospheric CO_2 typically enhances photosynthesis of C3 plants and alters primary and secondary metabolites in plant tissue. By modifying the defensive signaling pathways in host plants, elevated CO_2 could potentially affect the interactions between plants, viruses, and insects that vector viruses. *R* gene-mediated resistance in plants represents an efficient and highly specific defense against pathogens and herbivorous insects. The current study determined the effect of elevated CO_2 on tomato plants with and without the nematode resistance gene *Mi-1.2*, which also confers resistance to some sap-sucking insects including whitefly, *Bemisia tabaci*. Furthermore, the subsequent effects of elevated CO_2 on the performance of the vector whiteflies and the severity of *Tomato yellow leaf curl virus* (TYLCV) were also determined. The results showed that elevated CO_2 increased the biomass, plant height, and photosynthetic rate of both the Moneymaker and the *Mi-1.2* genotype. Elevated CO_2 decreased TYLCV disease incidence and severity for Moneymaker plants but had the opposite effect on *Mi-1.2* plants whether the plants were agroinoculated or inoculated via *B. tabaci* feeding. Elevated CO_2 increased the salicylic acid (SA)-dependent signaling pathway on Moneymaker plants but decreased the SA-signaling pathway on *Mi-1.2* plants when infected by TYLCV. Elevated CO_2 did not significantly affect *B. tabaci* fitness or the ability of viruliferous *B. tabaci* to transmit virus regardless of plant genotype. The results indicate that elevated CO_2 increases the resistance of Moneymaker plants but decreases the resistance of *Mi-1.2* plants against TYLCV, whether the plants are agroinoculated or inoculated by the vector. Our results suggest that plant genotypes containing the R gene *Mi-1.2* will be more vulnerable to TYLCV and perhaps to other plant viruses under elevated CO_2 conditions.

Keywords: elevated CO_2, resistance, *Mi-1.2* gene, tomato, TYLCV, whitefly

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *27 May 2016*
Accepted: *25 October 2016*
Published: *09 November 2016*

Citation:
Guo H, Huang L, Sun Y, Guo H and Ge F (2016) The Contrasting Effects of Elevated CO_2 on TYLCV Infection of Tomato Genotypes with and without the Resistance Gene, Mi-1.2. Front. Plant Sci. 7:1680. doi: 10.3389/fpls.2016.01680

INTRODUCTION

The atmospheric CO_2 concentration, which has risen from 280 to 400 ppm since the industrial revolution, now exceeds any level in the past 65,000 years and is predicted to reach 540–900 ppm by the end of this century (IPCC, 2013). Increases in atmospheric CO_2 alter photosynthetic rates, carbohydrate accumulation, transpiration, and other aspects of plant physiology

(Ainsworth and Long, 2005; Ainsworth et al., 2008). These effects can lead to changes in the primary and secondary metabolites in plant tissue, and may therefore affect interactions between plants and pathogens, between plants and insects, and between plants, viruses, and virus vectors (Chakraborty and Datta, 2003).

The effect of elevated CO_2 on the incidence and severity of diseases caused by plant pathogens differs among pathogens. Free-air CO_2 enrichment (FACE) studies have indicated that elevated CO_2 increases plant susceptibility to certain fungal species (Kobayashi et al., 2006; Melloy et al., 2010) but reduces susceptibility to certain bacterial pathogens and some fungal species (Jwa and Walling, 2001; Zhang et al., 2015). These results were largely explained by the cross-talk between jasmonic acid (JA)- and salicylic acid (SA)-signaling pathways, which are vital for plant resistance against different types of pathogens (Eastburn et al., 2011; Zhang et al., 2015). Elevated CO_2 increased plant resistance against *Potato virus Y* in tobacco and *Tomato yellow leaf curl virus* (TYLCV) in tomato (Matros et al., 2006; Huang et al., 2012). In the field, these plant viruses are transmitted by insect vectors, most of which are sap-sucking insects (i.e., aphids and whiteflies) whose performance could be affected by elevated CO_2 (Sun et al., 2013; Wang et al., 2014). Some aphid species exhibit increased fecundity, abundance, and survival under elevated CO_2 (Pritchard et al., 2007; Robinson et al., 2012). In contrast, elevated CO_2 reduced whitefly abundance at 1000 ppm but had no effect at 700 ppm (Butler et al., 1986; Tripp et al., 1992; Wang et al., 2014). It is unclear whether the effects of elevated CO_2 on the performance of insect vectors could in turn alter virus transmission to plants.

The interactions between insect vectors and plant viruses are often assumed to be mediated by plant defenses (Belliure et al., 2005; Colvin et al., 2006; Stout et al., 2006). A growing number of studies have reported that virus infection can decrease the resistance of host plants against insect vectors. Infection of tobacco plants by Tomato Yellow Leaf Curl China Virus (TYLCCNV) suppresses JA-dependent defenses and terpenoid synthesis, thereby favoring the performance of the whitefly vector, *Bemisia tabaci*, on virus-infected plants (Zhang et al., 2012; Luan et al., 2013). Viruliferous *B. tabaci* fed more than non-viruliferous *B. tabaci* and spent more time salivating into sieve tube elements, thereby enhancing virus infection and spread (Liu et al., 2013).

Tomato yellow leaf curl virus, which severely damages tomato crops in many tropical and subtropical regions worldwide (Czosnek and Laterrot, 1997; Zhang et al., 2009), is mainly transmitted by the whitefly *B. tabaci* in a persistent-circulative manner (Hogenhout et al., 2008). *B. tabaci* and TYLCV have a mutualistic relationship involving their shared host plants (McKenzie, 2002; Colvin et al., 2006; Jiu et al., 2007). Thus, the interaction between *B. tabaci* and the host plant is a key determinant of TYLCV transmission and infection.

In tomato, a well-studied *R* gene, *Mi-1.2*, encodes a protein with a nucleotide-binding domain and a leucine-rich repeat region (Milligan et al., 1998). Tomato plants with *Mi-1.2* are resistant to three species of root-knot nematodes (*Meloidogyne arenaria*, *M. incognita*, and *M. javanica*) and sap-sucking insects such as whiteflies, aphids and pysllids. This gene reduces nematode or insect reproduction and abundance (Kaloshian et al., 1995; Milligan et al., 1998 ; Vos et al., 1998; Nombela et al., 2003; Casteel et al., 2006). Given that the *Mi-1.2* gene confers a moderate level of resistance to whiteflies, we suspect that the *Mi-1.2* gene might also affect TYLCV acquisition and transmission by its vectors.

In host plants not infected with virus, SA-signaling defenses reduce the feeding efficiency of viruliferous *B. tabaci*, which may subsequently affect TYLCV transmission and infection of plants (Shi et al., 2013). TYLCV infection alone can induce SA-dependent defenses, which increases the defense against subsequent feeding by *B. tabaci* (Huang et al., 2012; Shi et al., 2013). Moreover, the SA-signaling pathway is involved in *R* gene *Mi-1.2*-mediated resistance (Li et al., 2006). The transcript levels of *PR1* in the resistant *Mi-1.2* plants accumulated faster and at higher amounts than in the susceptible *mi-1.2* plants after aphid infestation (Martinez de Ilarduya et al., 2003). Thus, the regulation of the SA-signaling pathway appears to be crucial for plant resistance against both virus and vector. In tomato and other crops, the SA-signaling pathway can be modified by the environment (Huang et al., 2012; Sun et al., 2013), suggesting that environmental change could affect phytohormone SA-induced defenses in *Mi-1.2* contained plants, which may affect the severity of TYLCV and the fitness of vector *B. tabaci*.

In the current study, we assessed the effects of elevated CO_2 on the tritrophic interactions among tomato, *B. tabaci*, and TYLCV. Two tomato cultivars were used: whitefly resistant cultivar Motelle (*Mi-1.2*) plants and its near-isogenic susceptible cultivar Moneymaker. We tested two hypotheses: (1) the *Mi-1.2* genotype of tomato would reduce TYLCV transmission and severity due to the higher resistance ability, which may indirectly suppress *B. tabaci* fitness; and (2) elevated CO_2 would enhance plant resistance against TYLCV and *B. tabaci* by up-regulating the SA- signaling pathway.

MATERIALS AND METHODS

Open-Top Field Chambers and CO_2 Levels and Plants

The experiment was carried out in eight open-top field chambers (OTCs). Four of the OTCs were continuously maintained at the current ambient level of CO_2 (about 400 ppm), and four were continuously maintained at an elevated level of CO_2 (about 750 ppm, the predicted level by the end of this century) (IPCC, 2013). Details of the automatic control system for CO_2 concentrations and OTCs are provided in Chen et al. (2005). Air temperature was measured three times daily (8:00, 14:00, and 18:00) throughout the experiment and did not differ significantly between the two sets of OTCs during the experiment.

Two near-isogenic tomato (*Solanum lycopersicum*) lines, the susceptible cultivar Moneymaker and the resistant cultivar Motelle (*Mi-1.2*), were used in our experiments. Motelle carries a 650-kb segment of *S. peruvianum* DNA that harbors the *Mi-1.2* gene, which makes it genetically distinct from Moneymaker (Milligan et al., 1998). These lines were selected for study due to whitefly resistance (Nombela et al., 2000). Seeds of

Moneymaker and *Mi-1.2* (Motelle) plants were obtained from the National Engineer and Research Center for Vegetable, Academy of Agricultural and Forestry Sciences, Beijing, China. One week after germination, when the cotyledons were beginning to expand, the seedlings were transplanted singly into plastic pots (25 cm × 21 cm × 22 cm) containing sterilized loamy field soil (organic carbon 75 g/kg; available N 500 mg/kg; available P 200 mg/kg; available K 300 mg/kg). The pots were placed in ventilated insect-proof cages in octagonal OTCs until they grew to the 3- to 4-leaf stage. Pot placement was re-randomized within each OTC once each week. No chemical fertilizers and insecticides were used. Water was added to each pot every 2 days. Five groups of plants were used for the experiments described in the following sections (Supplementary Figure S1).

Plant Growth Traits and Photosynthesis as Affected by Plant Genotype and CO_2 Level (Group 1)

Six undamaged 8-week-old plants of each genotype in each OTC (=24 plants per treatment and 96 plants in total) were selected for measurement of photosynthetic rate and plant growth traits. The net photosynthetic rate was determined according to Guo et al. (2012) with some modification. The net photosynthetic rate of each plant was measured with a Li-Cor 6400 gas exchange system (Li-Cor Inc., Lincoln, NE, USA). The fourth mature leaf from the base of the stem was selected for measurement. All measurements were done between 9:00 and 12:00 am. The CO_2 concentration of the incoming air was adjusted to 400 μmol mol^{-1} CO_2 or 750 μmol mol^{-1}. Relative humidity corresponded to ambient conditions. Before gas exchange was measured, photosynthetic active radiation for the leaf in the measuring cuvette was increased in steps to 1200 μmol m^{-2} s^{-1}. When the CO_2 assimilation rate was stable for at least 2 min, measurements were recorded. After that, the plants were harvested for measurement of biomass, stem diameter, and height.

TYLCV Incidence and Disease Index as Affected by Plant Genotype, CO_2 Level, and Agroinoculation vs. Whitefly Virus Inoculation (Group 2)

The plant–virus interactions could be affected by both plant physiology and vector transmission ability, thus, in current study, we determined the effects of elevated CO_2 on the disease incidence and index of TYLCV by either agroinoculation or transmitted by whitefly. For agroinoculation of TYLCV, 25 8-week-old plants of each genotype in each OTC (25 plants × 4 OTC × 2 genotypes × 2 CO_2 levels and 400 plants in total) were selected and agroinoculated as described previously (Huang et al., 2012). The TYLCV infection of tomato plants was achieved using *Agrobacterium tumefaciens*-mediated infectious inoculation (Zhang et al., 2009; Al Abdallat et al., 2010), and the infectious 2 clone (pBINPLUS-SH2-1.4A) of TYLCV- Israel [China: Shangai2] was constructed into *A. tumefaciens* strain EHA105 as described previously (Zhang et al., 2009). The infectious clone of TYLCV was provided by Professor Xueping Zhou (State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, China). The culture of TYLCV clone was grown in LB culture medium with kanamycin (50 μg/ml) and rifampicin (50 μg/ml) at 28°C (250 rpm) for 24 h (OD_{600} = 1.5). The bacteria culture was centrifuged for 10 min at 2500 *g* and resuspended with 50 ml buffer (10 mM $MgC1_2$, 10 mM 2-(N-morpholino) ethanesulfonic acid, 200 μM acetosyringone) after which 0.2 ml of the culture was injected three times into the phloem (about 1 mm in depth) of the tomato stem at the three to four leaf stage to achieve inoculation; a sterile syringe (1 ml) with a beveled needle (0.5 mm × 20 mm) was used for injection. Inoculated plants were grown in ventilated cages in the OTCs. The incidence of TYLCV infection (percentage of plants with disease symptoms) and the disease index were determined 6 weeks after agroinoculation. Disease index values were determined as follows (Curvers et al., 2010):

$$(DI) = \Sigma Ni \times Ri/(N \times Rh) \times 100$$

where Ni represents the number of plants in disease symptom ranking i, Ri represents the disease symptom rank (i = 0–4), *N* represents the total number of plants investigated, and Rh represents the highest disease symptom rank. Disease symptoms were ranked mainly according to Friedmann et al. (1998): 0 = no visible symptoms: inoculated plants show the same growth and development as non-inoculated plants; 1 = very slight yellowing of apical leaf margins; 2 = some yellowing and minor curling of leaf ends; 3 = widespread leaf yellowing, curling, and cupping, with some reduction in size, but plants continue to develop; 4 = severe plant stunting and yellowing, and pronounced cupping and curling of leaves; plants stop growing.

Bemisia tabaci of the B biotype (Middle East Asia Minor 1, aka MEAM 1), which were kindly provided by Professor Youjun Zhang of the Institute of Vegetable and Flower, Chinese Academy of Agricultural Sciences, were reared on cabbage (non-host of TYLCV) grown in insect-proof wooden cages as previously described (Jiu et al., 2007). Viruliferous whiteflies were caged on the TYLCV-infected tomato plants in a separate greenhouse. Whiteflies from the viruliferous colony were confirmed to be infected with TYLCV prior to infestation by PCR analysis (Zhang et al., 2009). For transmission of TYLCV to tomato plants by *B. tabaci*, 60 8-week-old plants of each genotype in each OTC were randomly selected, and each of 20 plants was infested by 5, 15, or 25 viruliferous *B. tabaci* for 48 h (20 plants × 4 OTC × 2 genotypes × 2 CO_2 levels × 3 whiteflies densities and 960 plants in total). The virus incidence and disease index of the tomato plants were determined 6 weeks after *B. tabaci* infestation.

The Abundance and Fecundity of *B. tabaci* as Affected by Plant Genotype, CO_2 Level, and TYLCV Infection (Group 3)

To determine the effect of *TYLCV* infection on *B. tabaci* numbers and fecundity on tomato, 16 5-week-old plants of each genotype in each OTC were randomly selected. Eight plants were agroinoculated with TYLCV, and the other eight were not. Three

weeks later, we checked the TYLCV copy numbers of the new emerged leaf by qPCR and confirmed that they are all successfully infected by TYLCV. Then, 4 8-week plants from each tomato genotype and TYLCV treatment per OTC (4 plants × 4 OTC × 2 genotypes × 2 CO_2 levels × 2 TYLCV treatment and 128 plants in total) were selected. Five newly emerged females and five newly emerged males were released onto each plant; each plant was kept in a separate whitefly proof, ventilated cage (120 mesh). After 28 days, the numbers of each developmental stage of *B. tabaci* were determined for each of the four replicates in each OTC.

To determine the effect of *TYLCV* infection of tomato on *B. tabaci* fecundity, 4 8-week plants from each tomato genotype and TYLCV treatment per OTC (4 plants × 4 OTC × 2 genotypes × 2 CO_2 levels × 2 TYLCV treatment and 128 plants in total) were randomly selected, one mated females were introduced into each plant with a whitefly proof, ventilated cage. The females were then transferred daily to fresh leaves until they died, and the number of eggs deposited by each female was determined.

Acquisition and Transmission of TYLCV by *B. tabaci* as Affected by Plant Genotype and CO_2 Level (Group 4)

Forty-eight 4-week-old tomato plants were agroinoculated with the virus. Once the plants exhibited obvious symptoms 4 weeks later and were confirmed as TYLCV infected by detecting the TYLCV copies with RT-PCR in the systemic leaves according to Zhang et al. (2009), we started to inoculate whiteflies. To determine the effects of plant genotype and CO_2 level on transmission of TYLCV by *B. tabaci*, 100 adult whiteflies were caged on the second true leaf (numbered from the apex down) of each TYLCV-infected tomato plants to obtain enough viruliferous whiteflies. After a 48-h acquisition access period, 20 viruliferous whiteflies were then caged on the second true leaf of each of four 5-week-old tomato plants (at the four-leaf stage) at three time points of each genotype in each OTC (4 plants × 4 OTC × 2 genotypes × 2 CO_2 levels × 3 three time points and 192 plants in total) (Rubinstein and Czosnek, 1997). The whiteflies were removed after 8, 24, and 48 h inoculation access period. Infection was assessed 4 weeks later based on the appearance of TYLCV symptoms and on the number of copies of TYLCV in the leaf tissue, which was determined according to Zhang et al. (2009).

To determine the effects of plant genotype and CO_2 level on the acquisition of TYLCV by *B. tabaci*, four six-leaf stage virus-infected tomato plants (9-week-old) of each genotype at each time point in each OTC were selected (4 plants × 4 OTC × 2 genotypes × 2 CO_2 levels × 3 three time points and 192 plants in total); the plants had been agroinoculated about 4 weeks earlier. Before releasing whiteflies, we confirmed as TYLCV infected by detecting the TYLCV copies with PCR in the systemic leaves according to Zhang et al. (2009). Fifty adult *B. tabaci* were caged on the second true leaf (numbered from the apex down). After acquisition access periods of 2, 8, and 24 h, ten *B. tabaci* were removed from each cage, and the TYLCV copy number in each group of ten *B. tabaci* was determined.

Quantification of Phytohormone Content, Defensive Enzyme Activity, and Defensive Gene Expression (Group 5)

For measurement of the contents of the phytohormones JA and SA and the activities of the defensive enzymes phenylalanine ammonia lyase (PAL) and lipoxygenase (LOX) in tomato plants as affected by TYLCV and CO_2 level, four 5-week-old plants of each genotype in each OTC were agroinoculated with TYLCV; another four plants of each genotype in each OTC were not inoculated and served as controls. Four weeks later, 500 mg of leaves were collected from each plant. The leaf samples were immediately stored in liquid N until analyzed.

For measurement of expression of JA- and SA-dependent defense genes, 16 5-week-old plants of each genotype in each OTC were agroinoculated with TYLCV, and another 16 plants of each genotype in each OTC were not inoculated and served as controls. After 0, 2, 8, and 24 h, the leaves of four plants (±inoculation) of each genotype in each OTC were harvested. The leaf samples were immediately stored in liquid N until analyzed.

Measurement of Phytohormone Content and Defensive Enzyme Activity

The contents of endogenous JA and SA in the plant leaves were measured as described by Sun et al. (2013). The activities of PAL and LOX were measured according to Guo et al. (2012).

Real-Time Quantitative PCR of Defensive Gene Expression

For real-time quantitative PCR, each treatment sample had four technical replicates for each of the biological replications. The RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was used to isolate total RNAs from tomato leaves (0.05 g from samples stored at −70°C), and about 2 μg quantities of the RNAs were used to generate the cDNAs with the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). The mRNA amounts of four target genes were quantified by real-time quantitative PCR: proteinase inhibitor (*PI-1*), lipoxygenase (*LOX2*), phenylalanine ammonia lyase (*PAL5*), and pathogenesis-related protein (*PR1a*). Specific primers for each gene were designed from the tomato EST sequences using PRIMER5 software (Supplementary Table S1). The PCR reactions were performed in a 20-μL total reaction volume including 10 μL of 2x SYBRs Premix EX TaqTM (Qiagen) master mix, 5 mM of each gene-specific primer, and 1 μL of cDNA template. PCR reactions were carried out on an Mx 3000P detection system (Stratagene, USA) as follows: 5 min at 95°C; then 40 cycles of 10 s at 95°C and 20 s at 62°C; and finally one cycle of 30 s at 95°C, 30 s at 55°C, and 30 s at 95°C. A standard curve was derived from the serial dilutions to quantify the copy numbers of target mRNAs. The relative level of each target gene was standardized by comparing the copy numbers of target mRNAs with the copy number of β-*actin* (*Actin7*) (the housekeeping gene; Zhai et al., 2013), which remains constant under different treatment conditions. The β-actin mRNAs of the control were examined in every plate of PCR to eliminate systematic error.

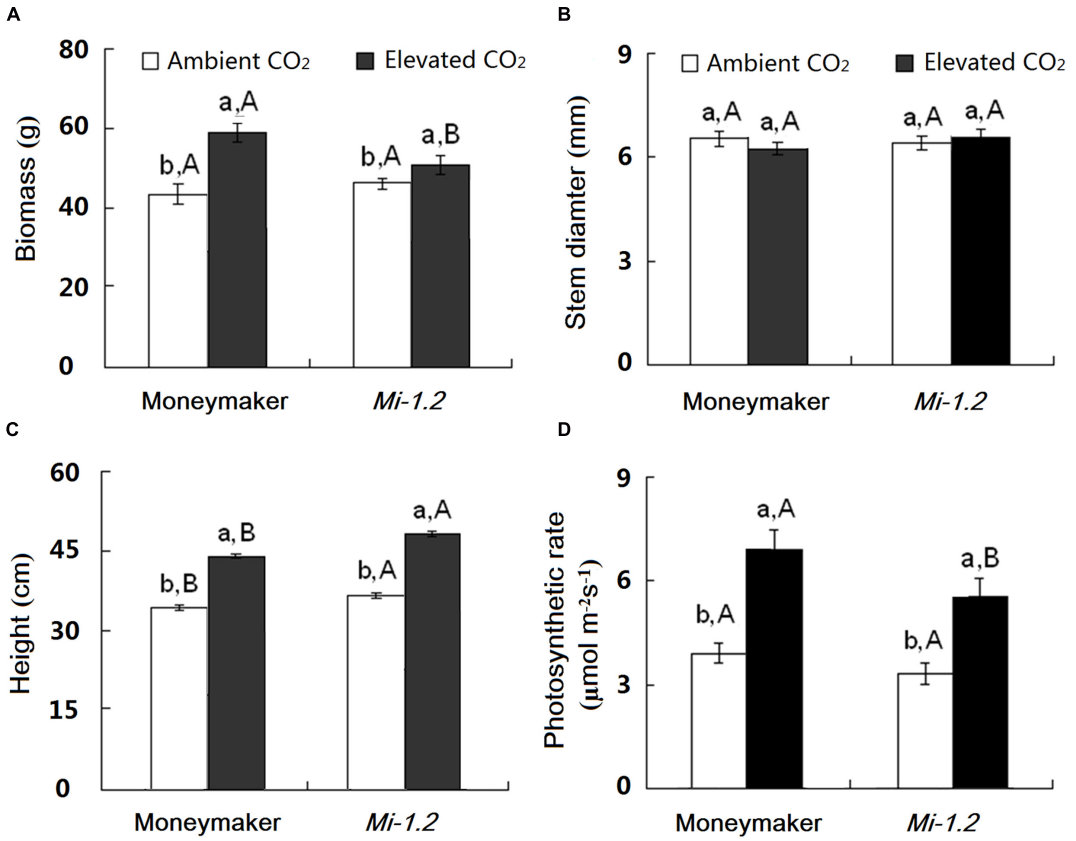

FIGURE 1 | Growth traits of two tomato genotypes (Moneymaker and *Mi-1.2*) grown under ambient CO_2 and elevated CO_2. (A) Biomass, **(B)** Stem diameter, **(C)** Height, and **(D)** Photosynthetic rate. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same genotype. Different uppercase letters indicate significant differences between genotypes within the same CO_2 treatment.

Statistical Analyses

All data were checked for normality and equality of residual error variances and were appropriately transformed (log or square-root) if needed to satisfy the assumptions of analysis of variance. A split-split plot design was used to analyze the univariate responses of the phytohormone contents, enzyme activities, and gene expression in plants (ANOVA, PASW Statistics 18.0, SPSS Inc., Chicago, IL, USA). In the following ANOVA model, CO_2 and block (a pair of OTCs with ambient and elevated CO_2) were the main effects, tomato genotype was the subplot effect, and TYLCV infection level was the sub-subplot effect:

$$X_{ijklm} = \mu + C_i + B(C)_{j(i)} + G_k + CG_{ik} + GB(C)_{kj(i)} + H_l + CH_{il} + HB(C)_{lj(i)} + GHB(C)_{klj(i)} + e_{m(ijkl)}$$

where C is the CO_2 treatment ($i = 2$), B is the block ($j = 4$), G is the tomato genotype ($k = 2$), and H is the virus infection treatment ($l = 2$). $e_{m(ijkl)}$ represents the error because of the smaller scale differences between samples and variability within blocks (ANOVA, SAS Institute). Effects were considered significant if $P < 0.05$. Because the effect of block and the interactive effects of block and other factors were not significant ($P > 0.45$), the effect of block and its interaction with other factors are not presented to simplify the presentation. Tukey's multiple range tests were used to separate means when ANOVAs were significant. For analysis of the plant growth traits (biomass, stem diameter, plant height, and photosynthetic rate), TYLCV incidence and index, and the ability of *B. tabaci* to acquire and transmit TYLCV under two CO_2 levels, a split-plot design was also applied, with CO_2 and block as the main effects and tomato genotype as the subplot effect.

RESULTS

Plant Growth Traits and Photosynthesis as Affected by Plant Genotype and CO_2 Level (Group 1)

Under ambient CO_2, growth and photosynthesis did not significantly differ between Moneymaker and *Mi-1.2* plants except for the height (**Figure 1**; Supplementary Table S2). Elevated CO_2 increased biomass by 38.2%, height by 28.6%, and photosynthetic rate by 75.1% for Moneymaker plants, and increased biomass by 15.5%, height by 33.3%, and photosynthetic

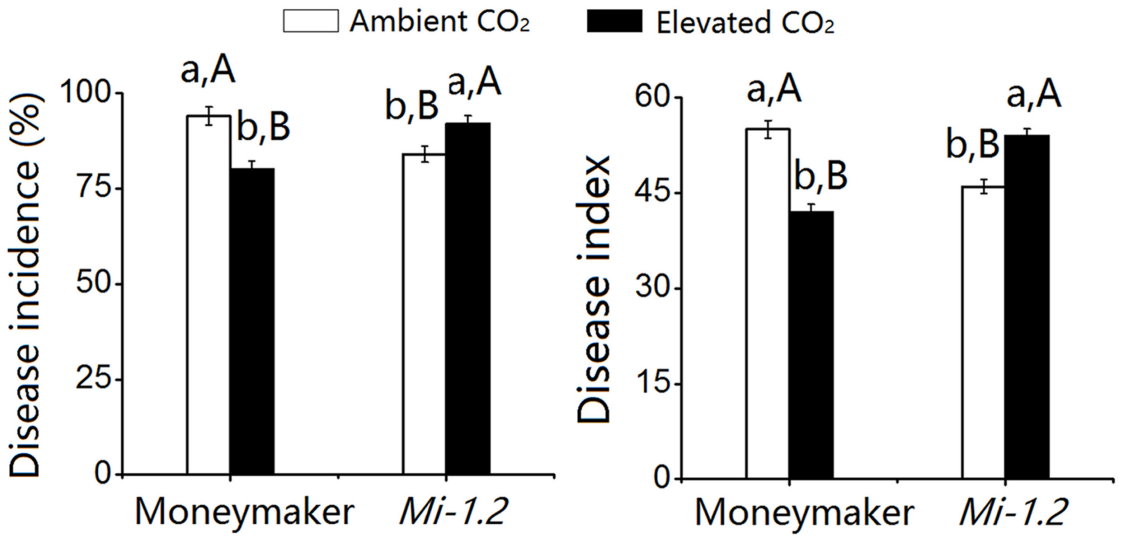

FIGURE 2 | *Tomato yellow leaf curl virus* (TYLCV) disease incidence and index values in two tomato genotypes (Moneymaker and *Mi-1.2*) that were agroinoculated with the virus and then grown under ambient CO_2 and elevated CO_2. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same genotype. Different uppercase letters indicate significant differences between genotypes within the same CO_2 treatment. Means were compared with Tukey's multiple range test at $P < 0.05$.

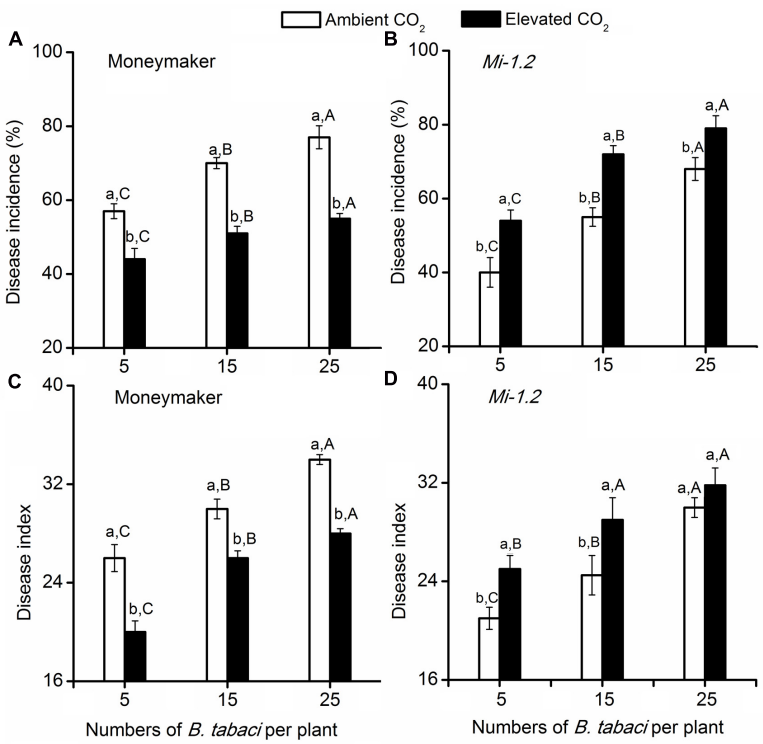

FIGURE 3 | *Tomato yellow leaf curl virus* disease incidence and index values in two tomato genotypes (Moneymaker and *Mi-1.2*) that were infested with different numbers of viruliferous *Bemisia tabaci* and grown under ambient CO_2 and elevated CO_2. (A) Disease incidence of Moneymaker, **(B)** Disease incidence of *Mi-1.2*, **(C)** Disease index of Moneymaker, and **(D)** Disease index of *Mi-1.2*. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same *B. tabaci* density. Different uppercase letters indicate significant differences among *B. tabaci* densities within the same CO_2 treatment.

rate by 62.3 % for *Mi-1.2* plants. *Mi-1.2* plants had a lower biomass, a lower photosynthetic rate, and a greater height than Moneymaker plants under elevated CO_2 (**Figures 1A,C,D**).

TYLCV Incidence and Disease Index as Affected by Plant Genotype, CO_2 Level, and Agroinoculation vs. Whitefly Virus Inoculation (Group 2)

For the plants that were agroinoculated with TYLCV, elevated CO_2 significantly decreased TYLCV disease incidence and index values for Moneymaker plants but increased those values for *Mi-1.2* plants (**Figure 2**; Supplementary Table S3). For plants that were inoculated with TYLCV by *B. tabaci*, TYLCV incidence and index values increased as the number of *B. tabaci* added increased (**Figure 3**; Supplementary Table S4). Elevated CO_2 decreased the TYLCV incidence and disease index values for Moneymaker plants but increased those values for *Mi-1.2* plants when infested by the same number of viruliferous *B. tabaci* (**Figure 3**).

Abundance and Fecundity of *B. tabaci* as Affected by Plant Genotype, CO_2 Level, and TYLCV Infection (Group 3)

Elevated CO_2 did not significantly affect the abundance or fecundity of *B. tabaci* on either healthy or virus-infected plants regardless of plant genotype (**Figure 4**; Supplementary Table S5). Fecundity was lower on healthy *Mi-1.2* plants than on healthy Moneymaker plants under ambient CO_2. Under elevated

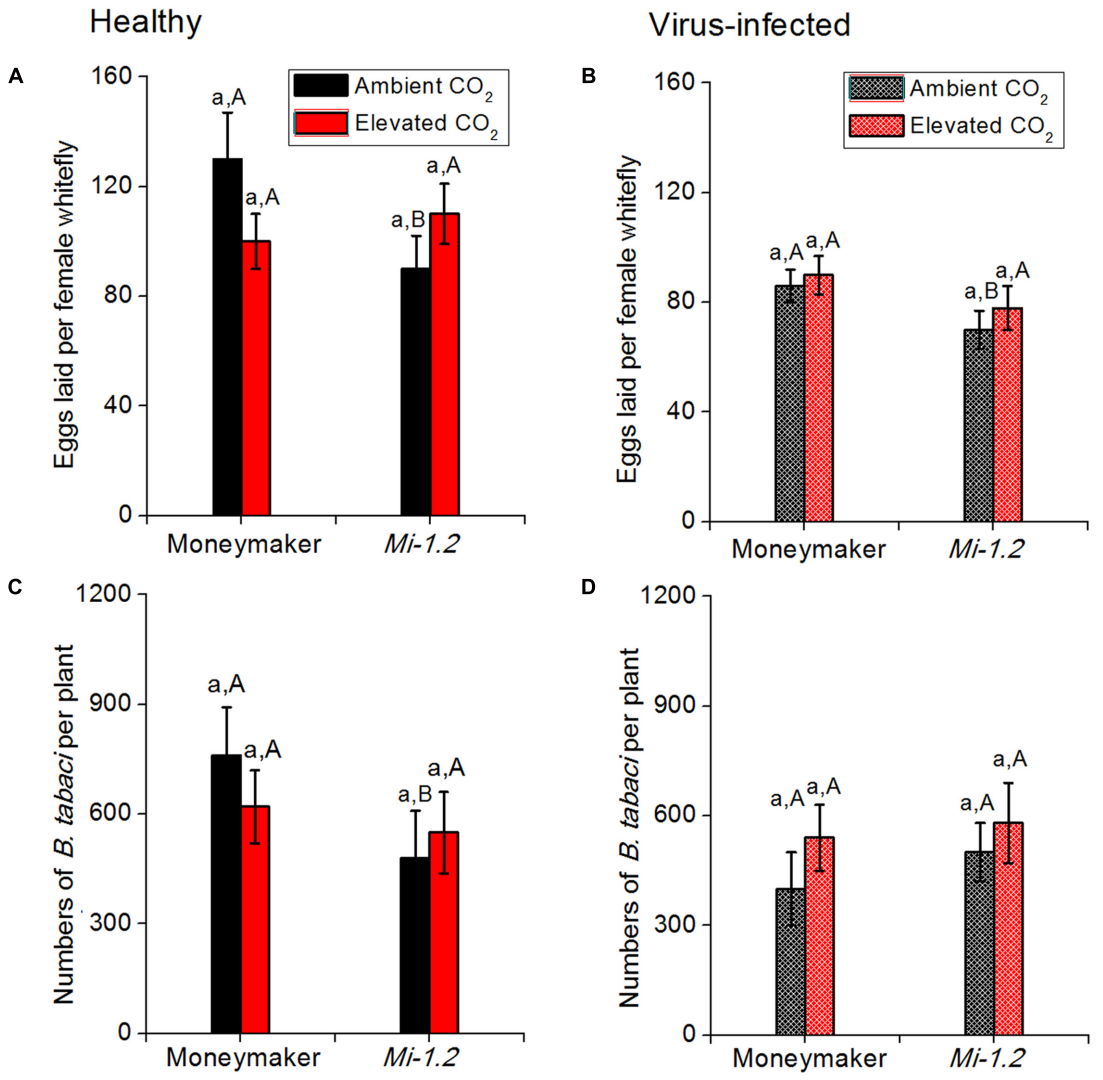

FIGURE 4 | Fecundity and abundance of *B. tabaci* on tomato plants (Moneymaker and *Mi-1.2*) that were agroinoculated or not infected with TYLCV and grown under ambient CO_2 or elevated CO_2. (A,B) Fecundity of *B. tabaci* on healthy and virus-infected plants; **(C,D)** Abundance of *B. tabaci* on healthy and virus-infected plants. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same genotype. Different uppercase letters indicate significant differences in *B. tabaci* numbers within the same CO_2 treatment.

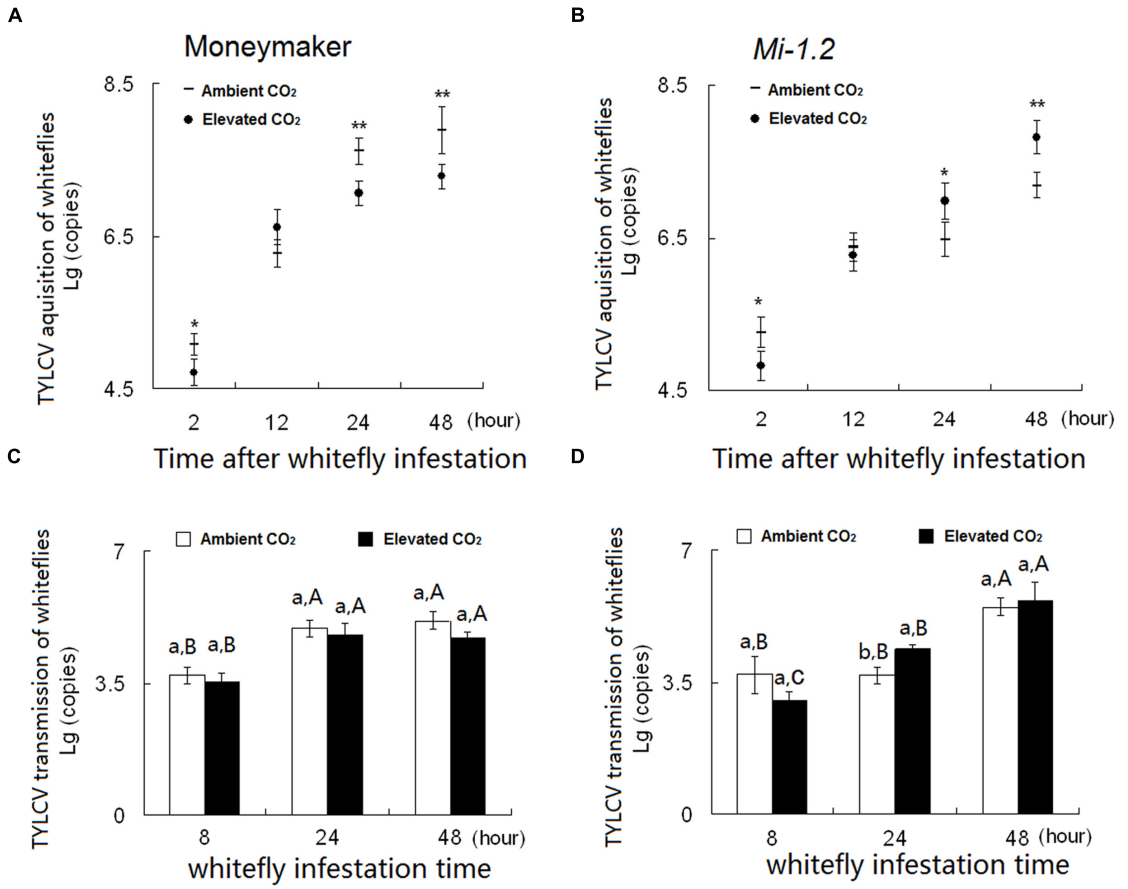

FIGURE 5 | Number of TYLCV-DNA copies acquired per *B. tabaci* when feeding on (A) Moneymaker plants and on **(B)** *Mi-1.2* plants. Number of TYLCV-DNA copies per gram of tissue in **(C)** Moneymaker plants and **(D)** *Mi-1.2* plants infested with viruliferous *B. tabaci*. Within **(A)** and **(B)**, * and ** indicate a significant difference in copy number between ambient and elevated CO_2 at the same time point at $P < 0.05$ and 0.01, respectively. Within **(C)** and **(D)**, different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 at the same time point, and different uppercase letters indicate significant differences within the same CO_2 treatment at $P < 0.05$. In all cases, means were compared with Tukey's multiple range test.

CO_2, in contrast, neither *B. tabaci* fecundity nor abundance significantly differed between the two plant genotypes. *B. tabaci* abundance and fecundity were lower on TYLCV-infected plants than on healthy plants regardless of CO_2 level or plant genotype (**Figure 4**).

Acquisition and Transmission of TYLCV by *B. tabaci* as Affected by Plant Genotype and CO_2 Level (Group 4)

After whiteflies had fed on the TYLCV-infected plants for 2, 24, or 48 h, the number of TYLCV-DNA copies per *B. tabaci* was significantly lower under elevated CO_2 than under ambient CO_2 in the case of Moneymaker plants but the opposite was true in the case of *Mi-1.2* plants (**Figures 5A,B**). Under ambient CO_2, *B. tabaci* contained fewer TYLCV-DNA copies when reared on TYLCV-infected *Mi-1.2* plants than on TYLCV-infected Moneymaker plants (**Figures 5A,B**). Under elevated CO_2, *B. tabaci* contained a higher number of TYLCV-DNA copies when reared on TYLCV-infected *Mi-1.2* plants than on TYLCV-infected Moneymaker plants (**Figures 5A,B**; Supplementary Table S6), which is consistent with the TYLCV disease incidence and index of both genotypes before whitefly acquired TYLCV from plants (Supplementary Figure S2).

After viruliferous *B. tabaci* had fed on plants for 24 h, numbers of TYLCV-DNA copies in Moneymaker plants were unaffected by CO_2 level but were higher in *Mi-1.2* plants under elevated CO_2 than under ambient CO_2 (**Figures 5C,D**). After a 48 h transmission access period, *Mi-1.2* plants contained fewer TYLCV-DNA copies than Moneymaker plants under ambient CO_2 but contained higher numbers of TYLCV-DNA copies under elevated CO_2 (**Figures 5C,D**).

SA and JA Content and Defensive Enzyme Activity

In Moneymaker plants that were not infected by TYLCV, elevated CO_2 increased SA content and PAL activity but decreased JA content and LOX activity (**Figure 6**; Supplementary Table S7).

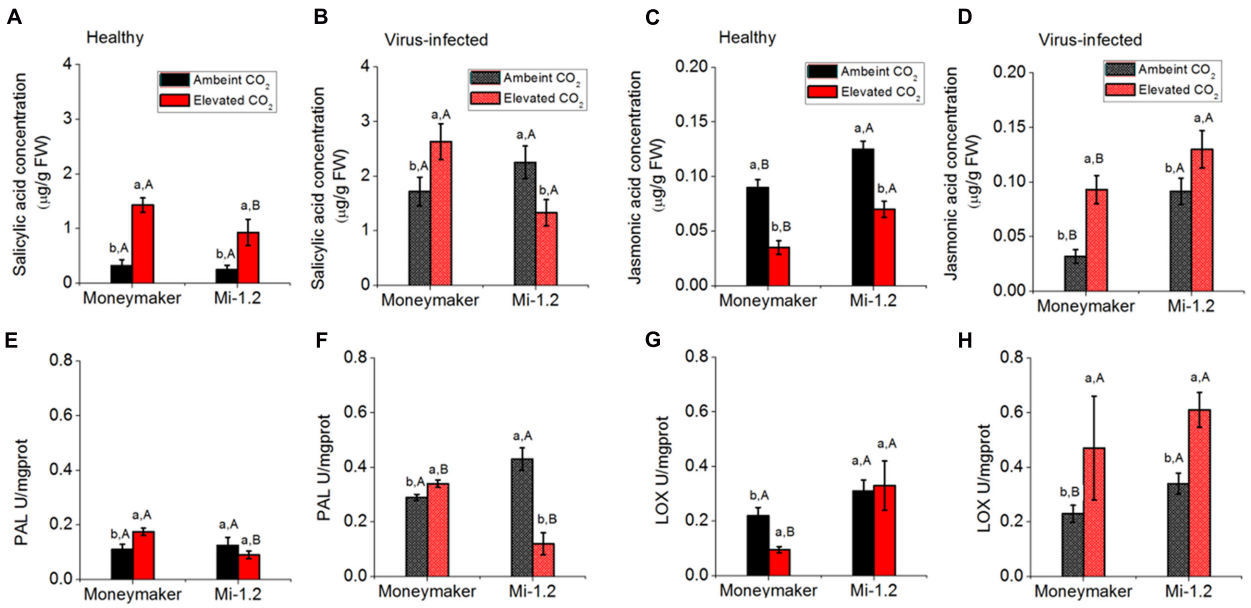

FIGURE 6 | Contents of phytohormones and activities of enzymes involved in the JA and SA signaling pathways of two tomato genotypes grown under ambient CO_2 and elevated CO_2 with and without TYLCV infection. (A,B) SA concentration in healthy and virus-infected plants; **(C,D)** JA concentration in healthy and virus-infected plants; **(E,F)** PAL activity in healthy and virus-infected plants; and **(G,H)** LOX activity in healthy and virus-infected plants. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same genotype. Different uppercase letters indicate significant differences in *B. tabaci* numbers within the same CO_2 treatment.

Elevated CO_2 increased SA content and decreased JA content of *Mi-1.2* plants (**Figures 6A,B**). After agroinoculation of TYLCV infection for 48 h, elevated CO_2 increased the SA and JA contents and PAL and LOX activities of Moneymaker plants. In contrast, elevated CO_2 decreased SA and PAL activity but increased JA content and LOX activity of *Mi-1.2* plants (**Figure 6**). Under ambient CO_2, SA content and PAL activity were lower in infected Moneymaker plants than in infected *Mi-1.2* plants. Under elevated CO_2, however, SA content and PAL activity were lower in the *Mi-1.2* plants than in Moneymaker plants regardless of TYLCV infection.

Expression of Genes Involved in the SA- and JA-Signaling Pathways

From 8 to 48 h post-infection with TYLCV artificially, elevated CO_2 increased the expression of genes encoding *PAL5* and *PR1a* involved in the SA-signaling pathway of Moneymaker plants but decreased their expression in *Mi-1.2* plants (**Figures 7A,B**; Supplementary Table S8). The expression of genes encoding *LOX2* and *PI1-1* in the JA-signaling pathway, however, was not greatly affected by elevated CO_2 (**Figures 7C,D**; Supplementary Table S8). TYLCV infection tended to up-regulate the expression of genes encoding *PAL5* and *PR1a* but to down-regulate the expression of *LOX2* and *PI1-1* regardless of plant genotype (**Figure 7**). Compared with Moneymaker plants, *Mi-1.2* plants had a higher expression of genes encoding *PAL5* and *PR1a* under ambient CO_2 but the reverse was true under elevated CO_2 (**Figure 7**). The expression pattern of genes involved in the SA- signaling pathway across the treatments suggested that the SA-signaling pathway is an important part of plant response to TYLCV infection.

DISCUSSION

The *Mi-1.2* gene in tomato mediates resistance to insect vectors by triggering an array of defense responses that could in turn affect virus infection (Tameling et al., 2002). Resistance against nematodes conferred by the *Mi-1.2* gene can be reduced by elevated temperature and other environmental variables (Holtzman, 1965). In the current study, we determined the effects of elevated CO_2 on *Mi-1.2* gene-mediated resistance against TYLCV and its vector, *B. tabaci*. Inconsistent with our hypotheses that elevated CO_2 would increase the resistance of plants to TYLCV in both genotype, we discovered that the effects of elevated CO_2 on TYLCV infection differed between Moneymaker and *Mi-1.2* plants. Under elevated CO_2, the responses of the SA-signaling pathway differed between the plant genotypes, which suggested that the SA-signaling pathway may help explain the differences in plant responses to TYLCV under elevated CO_2.

Elevated CO_2 is expected to affect plant–virus interactions by altering both plant physiology and vector transmission ability (Malmström and Field, 1997; Rúa et al., 2013). In the present study, we found that elevated CO_2 decreased the severity of disease caused by TYLCV on agroinoculated, Moneymaker plants, which is consistent with previous studies (Matros et al., 2006; Huang et al., 2012). The *Mi-1.2* plants, which were previously reported to be resistant to *B. tabaci* (Nombela et al.,

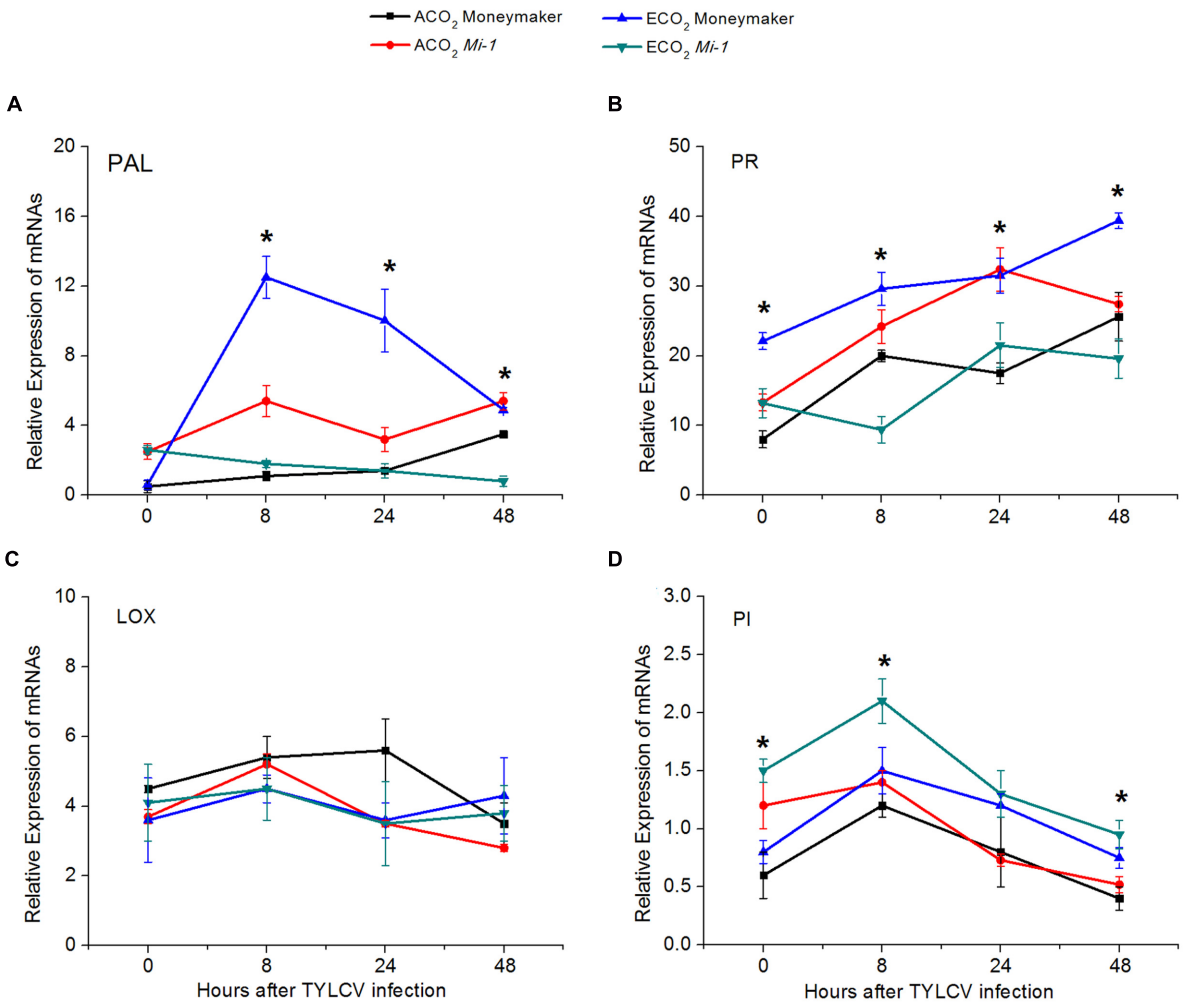

FIGURE 7 | Expression of key genes in the JA- and SA-signaling pathways of two tomato genotypes that were grown under ambient CO_2 and elevated CO_2 and that were infected with TYLCV for 0 to 48 h. (A) Phenylalanine ammonia lyase (PAL); **(B)** Pathogenesis-related protein 1 (PR); **(C)** Lipoxygenase 2 (LOX); and **(D)** Proteinase inhibitor (PI). Significant differences among different treatments in the same time point at $P < 0.05$ are indicated by an asterisk.

2003), were also resistant to TYLCV, i.e., they were less diseased than the Moneymaker plants under ambient CO_2. Under elevated CO_2, however, the *Mi-1.2* plants had a higher disease index and severity values than wild-type plants whether they were agroinoculated with the virus or inoculated by *B. tabaci*. This result indicated that elevated CO_2 tends to increase the resistance of Moneymaker plants but decrease the resistance of *Mi-1.2* plants against TYLCV.

In plant–virus interactions, the SA-signaling pathway is thought to provide efficient resistance against plant viruses. For example, exogenous application of SA reduces the levels of *Tobacco mosaic virus* and *Potato virus X* coat proteins in infected *Nicotiana benthamiana* leaves (Lee et al., 2011). In *N. tabacum* and *Arabidopsis*, the activation of the SA-signaling pathway inhibits the systemic movement of Cucumber Mosaic Virus (Alazem and Lin, 2015). Our results showed that tomato plants rapidly up-regulated the activity of enzymes and the expression of genes involved in the SA-signaling pathway to defend against TYLCV infection regardless of plant genotype under ambient CO_2. The SA-signaling pathway was also found to be involved in *Mi*-mediated resistance in plants when against nematodes and aphids (Branch et al., 2004; Li et al., 2006). In the current study, *Mi-1.2* plants had a higher SA content and greater SA signaling-related enzyme activity and gene expression than Moneymaker plants under ambient CO_2 when infected by TYLCV, which suggests that *Mi-1.2* plants have greater resistance against TYLCV infection than Moneymaker plants. Interestingly, we found that elevated CO_2 increased SA-signaling-related enzyme activity and gene expression in virus-infected Moneymaker plants but had the opposite effect in virus-infected *Mi-1.2* plants. To our knowledge, this is the first report that the effects of elevated CO_2 on the SA-signaling pathway differ greatly between plant genotypes differing in *R* gene-mediated resistance when those genotypes are infected by a plant virus.

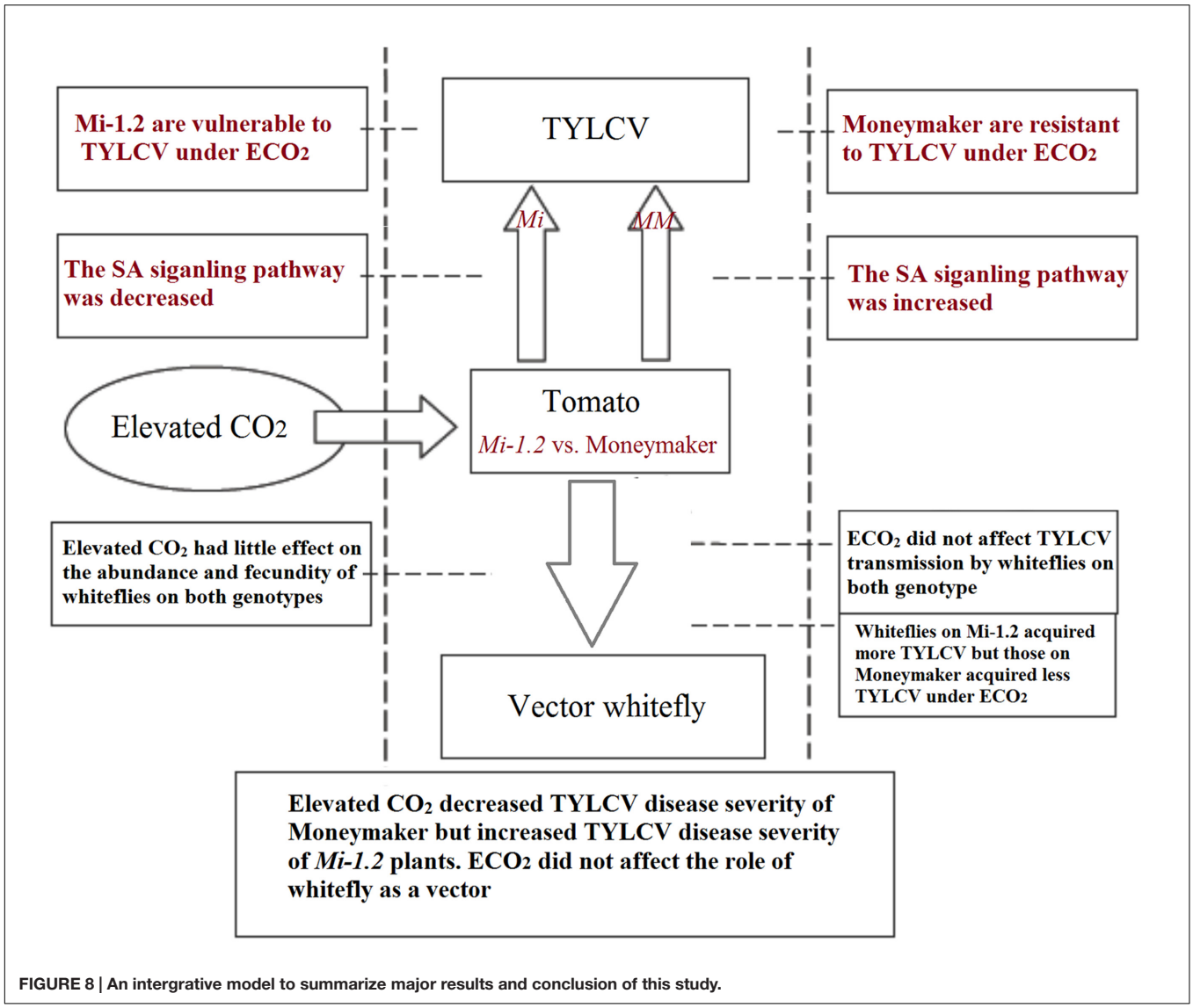

FIGURE 8 | An intergrative model to summarize major results and conclusion of this study.

Under natural conditions, TYLCV is mainly transmitted by whiteflies in a persistent-circulative, non-propagative manner (Hogenhout et al., 2008). Previous research has demonstrated that vector-borne viruses can modify vector behavior and fitness and thereby enhance virus spread by altering the host plant traits. For example, the virus could increase the nutritional quality of infected host plants, decrease the resistance of infected host plants, or increase the attractiveness of infected plants to their vectors (Jiménez-Martínez et al., 2004; Luan et al., 2013; Trębicki et al., 2016). Infection by TYLCCNV, for example, suppresses JA-induced defenses in tomato plants, which increases the feeding and the fitness of the whitefly vector, which in turn enhances the transmission of the virus (Zhang et al., 2012). In current study, we did not observe a positive effect of TYLCV infection on *B. tabaci* performance, even though TYLCV infection suppressed JA content and the expression level of *PI* in both tomato genotypes.

Most of the insects that vector plant viruses, like aphids, whiteflies, and planthoppers, have piercing-sucking mouthparts. The piercing-sucking insects could directly suppress plant efficient defense and subsequently increase the virus transmission (Zarate et al., 2007; Walling, 2008). The fitness of sap-sucking insects could be easily affected by abiotic environment. As reviewed by Sun et al. (2016), elevated CO_2 tends to increase the feeding efficiency of some aphids by decreasing JA-mediated resistance and by increasing nutrition content of host plants. As an exception, elevated CO_2 decreased the feeding efficiency of *Myzus persicae* on bell pepper. Thus, the decreased performance of *M. persicae* led to a twofold decrease in virus transmission under elevated CO_2 (Dáder et al., 2016). The current study showed that, regardless of plant genotype, elevated CO_2 had little effect on the abundance and fecundity of *B. tabaci*. As a result, elevated CO_2 did not affect TYLCV transmission by viruliferous *B. tabaci* regardless of plant genotype. The levels of TYLCV acquired by *B. tabaci* were positively correlated with the levels

of virus in the plants (Lapidot et al., 2001). Thus, during the virus acquisition process, elevated CO_2 decreased the numbers of TYLCV-DNA copies in *B. tabaci* feeding on Moneymaker plants but increased the numbers in *B. tabaci* feeding on *Mi-1.2* plants (**Figure 5**).

Plants have evolved sophisticated mechanisms to perceive biotic stress caused by herbivorous insects and virus pathogens (Dangl and Jones, 2001). Although tomato plants with *Mi-1.2* are resistant to sap-sucking vector whiteflies, aphids and pysllids and root-knot nematodes, the mechanisms are distinct. For instance, once infested by *B. tabaci*, the increased resistance of *Mi-1.2* prolonged the pathway stage prior to establishment of feeding site (Jiang et al., 2001). With respect to aphids, they feed for shorter periods on *Mi-1.2* plants, apparently perishing due to dehydration or starvation (Kaloshian et al., 2000). In contrast, psyllids exhibited a host selection preference and higher survival for the susceptible variety Moneymaker relative to the resistant *Mi-1.2* plants (Casteel et al., 2006). These may suggest that the effect of *Mi*-conferred resistance on different feeding stage of vector insects could further affect their virus transmission ability. In current study, although the TYLCV severity in *Mi-1.2* genotype was lower than Moneymaker, the mechanisms of defense may differ between the virus and its vector. For whiteflies, the *Mi-1.2* gene of tomato can directly recognize the elicitor and up-regulate Sgt1 (suppressor of G-two allele of Skp1) and Hsp90 (heat shock protein 90) to induce hypersensitive response (HR)-mediated effector-triggered immunity (ETI) if the same signaling mechanisms are used by *Mi-1.2* in response to aphids and whiteflies (Bhattarai et al., 2007). In contrast, the defense of *Mi-1.2* plants against TYLCV involves the up-regulation of SA-mediated resistance.

With respect to insect vectors, elevated CO_2 may accelerate the breakdown of *R* gene-mediated resistance in *Rubus idaeus* when that plant is attacked by the aphid *Amphorophora idaei* (Martin and Johnson, 2011). In contrast, we did not find any significant effect of elevated CO_2 on the resistance of *Mi-1.2* plants against *B. tabaci* whether the insect was feeding on virus-infected or healthy plants. With respect to the plant virus, elevated CO_2 decreased the SA-signaling pathway of *Mi-1.2* plants and therefore decreased the resistance against TYLCV. The different response of *B. tabaci* and TYLCV to elevated CO_2 on *Mi-1.2* plants suggests that the resistance mechanism in plants that contain *R* genes differs for pathogens vs. herbivorous insects and that those mechanisms may be respond differently to changes in the environment.

In summary, this study showed that the effects of elevated CO_2 on TYLCV transmission and infection differed greatly between tomato genotypes with and without the *R* gene *Mi-1.2*, i.e., elevated CO_2 decreased TYLCV disease severity of Moneymaker plants but increased TYLCV disease severity of *Mi-1.2* plants. The genotype-specific responses were closely related to the expression pattern of the SA-signaling pathway (**Figure 8**). Elevated CO_2 did not affect the role of *B. tabaci* as a vector. The results indicate that *Mi-1.2* plants are more vulnerable than Moneymaker plants to TYLCV and may suffer greater virus damage if atmospheric CO_2 levels continue to increase. The outcomes of this study have important implications for agricultural pest control and for transgenic breeding of resistant plants under future elevated CO_2 conditions.

AUTHOR CONTRIBUTIONS

HG contribute to data analysis and article writing. LH design and do the experiment. YS wrote and revised this article. HG performed the technical work. FG conceived the project.

ACKNOWLEDGMENTS

This project was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (no.XDB11050400) and the National Nature Science Fund of China (nos.31500332 and 31221091).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01680/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 07 September 2016
doi: 10.3389/fmicb.2016.01314

Aboveground Whitefly Infestation-Mediated Reshaping of the Root Microbiota

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Plants respond to various types of herbivore and pathogen attack using well-developed defensive machinery designed for self-protection. Infestation from phloem-sucking insects such as whitefly and aphid on plant leaves was previously shown to influence both the saprophytic and pathogenic bacterial community in the plant rhizosphere. However, the modulation of the root microbial community by plants following *insect* infestation has been largely unexplored. Only limited studies of culture-dependent bacterial diversity caused by whitefly and aphid have been conducted. In this study, to obtain a complete picture of the belowground microbiome community, we performed high-speed and high-throughput next-generation sequencing. We sampled the rhizosphere soils of pepper seedlings at 0, 1, and 2 weeks after whitefly infestation versus the water control. We amplified a partial 16S ribosomal RNA gene (V1–V3 region) by polymerase chain reaction with specific primers. Our analysis revealed that whitefly infestation reshaped the overall microbiota structure compared to that of the control rhizosphere, even after 1 week of infestation. Examination of the relative abundance distributions of microbes demonstrated that whitefly infestation shifted the proteobacterial groups at week 2. Intriguingly, the population of Pseudomonadales of the class Gammaproteobacteria significantly increased after 2 weeks of whitefly infestation, and the fluorescent *Pseudomonas* spp. recruited to the rhizosphere were confirmed to exhibit insect-killing capacity. Additionally, three taxa, including *Caulobacteraceae*, *Enterobacteriaceae*, and *Flavobacteriaceae*, and three genera, including *Achromobacter*, *Janthinobacterium*, and *Stenotrophomonas*, were the most abundant bacterial groups in the whitefly infested plant rhizosphere. Our results indicate that whitefly infestation leads to the recruitment of specific groups of rhizosphere bacteria by the plant, which confer beneficial traits to the host plant. This study provides a new framework for investigating how aboveground insect feeding modulates the belowground microbiome.

Keywords: bacterial community, rhizosphere, pyrosequencing, whitefly infestation, pepper, PGPR, *Pseudomonas*, microbiota

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Edited by:
Jyoti Shah,
University of North Texas, USA

Reviewed by:
Harsh Bais,
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Punya Nachappa,
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Microbiology

Received: *29 April 2016*
Accepted: *09 August 2016*
Published: *07 September 2016*

Citation:
Kong HG, Kim BK, Song GC, Lee S and Ryu C-M (2016) Aboveground Whitefly Infestation-Mediated Reshaping of the Root Microbiota. Front. Microbiol. 7:1314. doi: 10.3389/fmicb.2016.01314

INTRODUCTION

Insects and plants have been interacting and co-evolving over the past 0.4 billion years. Under natural conditions, insects have several beneficial effects on plants, including protection from herbivores and help with pollination, while the plants provide a habitat and food for the insects (Panda and Khush, 1995). However, herbivore infestation can in some cases lead to the death of the plant. To protect themselves from insect infestation,

plants have developed genetic and chemical defense mechanisms such as indirect defense via insect-derived plant volatiles (Birkett et al., 2003) and the production of toxic metabolites (Baldwin, 2001; Howe and Jander, 2008). At the same time, these organisms have established elaborate and varied relationships with microbes such as bacteria (Sugio et al., 2015). A growing body of studies on insect-plant-microbe interactions has broadened our knowledge of plant-derived modulation of microbe diversity to help plants survive under attack from insect pests (Pangesti et al., 2013).

Plant-insect-microbe interactions can be classified into two categories: microbial mediation of plant-insect interactions and insect mediation of plant-microbe interactions (Pineda et al., 2010, 2013; Biere and Bennett, 2013; Fu and Dong, 2013; Pangesti et al., 2013; Lazebnik et al., 2014; Sugio et al., 2015). Microbes influence plant-insect interactions by suppressing or enhancing infestation of the plant by herbivores. In this type of interaction, root colonization by the beneficial rhizobacterium *Azospirillum brasilense* provides insect resistance to corn plants and elicits the suppression of infestation by corn rootworm (*Diabrotica speciosa*) by increasing the emissions of *(E)-B*-caryophyllene in corn roots (Santos et al., 2014). Similarly, the presence of the plant growth-promoting rhizobacterium (PGPR) *Bacillus subtilis* leads to retarded development of whitefly (*Bemisia tabaci*) in tomato plants (Valenzuela-Soto et al., 2010). By contrast, the root application of certain soil bacteria enhances herbivore infestation by modulating plant immune signaling (Groen et al., 2013; Lazebnik et al., 2014). Pre-inoculation of *Pseudomonas fluorescens* WCS417r on the tomato root system increases the survivability of the nymph stages of whitefly (*B. tabaci*) by reducing the efficiency of defense responses related to the jasmonic acid (JA)-pathway (Shavit et al., 2013). In addition, the prior infection of *Pseudomonas syringae* on *Arabidopsis* leaves reduces plant resistance to cabbage looper (*Trichoplusia ni*) by enhancing ethylene signaling, thereby antagonizing salicylic acid (SA) signaling, which confers plant immunity to the target insect (Groen et al., 2013). Herbivores also modulate microbial behavior and community structure through regulating plant physiology and defense systems (Gehring and Bennett, 2009; Lakshmanan et al., 2012; Tack and Dicke, 2013). The belowground herbivorous insect *Agriotes lineatus* L. negatively affects the composition of fungal communities in the ragwort (*Jacobaea vulgaris*) rhizosphere (Kostenko et al., 2012). More specifically, infestation by the belowground insect wireworm (*Agriotes lineatus* L.) leads to the accumulation of the major plant defense compounds pyrrolizidine alkaloids in ragwort plants and reduces the levels of the pathogenic fungus *Fusarium oxysporum* in roots (Bezemer et al., 2013). By contrast, feeding by western corn rootworm larvae (*Diabrotica virgifera virgifera*) increases the density of the bacterial and fungal communities in maize (*Zea mays* L.) roots. Of all the members of the bacterial community whose populations increase in the rhizosphere due to insect infestation, the greatest increase occurs in *Acinetobacter calcoaceticus* (Dematheis et al., 2012). Even though recent studies have broadened our knowledge of plant-insect-microbe interactions, the effects of aboveground insect infestation on changes in commensal microbial communities were unknown until 2011.

In 2011, new information was obtained about how plants orchestrate resistance against the soil-borne pathogen *Ralstonia solanacearum* when whitefly (*Bemisia tabaci* Genn.) feeds on the leaf tissue of pepper (Yang et al., 2011). More intriguingly, whitefly infestation increases the populations of Gram-positive bacteria in the root zone known as the rhizosphere. These bacteria have beneficial effects on plants (Kloepper et al., 2004). Gram-positive *Bacillus* spp. act as a biological trigger to elicit plant systemic defense against subsequent whitefly infestation under field conditions (Murphy et al., 2000). Similarly, aphids, which like whitefly are sap-sucking insects, alter the population densities of *B. subtilis* GB03, as well as the Gram-negative bacterium *P. fluorescens* Pf-5, in the pepper rhizosphere (Lee et al., 2012). However, studies of insect-mediated changes in the populations of root-associated bacteria are limited due to their use of culture-dependent methodology. Analyses of variations in bacterial density due to whitefly or aphid infestation have traditionally been based on culture-dependent methods, but the diverse results obtained using molecular techniques suggest that reliance on culture-based approaches has led to an underestimation of bacterial diversity in the rhizosphere, which hampers estimation of the microbial diversity of plant rhizosphere microbiomes (Torsvik et al., 2002). To elucidate the functions of the altered bacterial populations, more sophisticated methods are needed to measure bacterial diversity.

Recently, the microbial diversity in the rhizosphere was investigated by a culture-independent method based on amplified rRNA sequences from environmental samples (Smalla et al., 2001; Kirk et al., 2005; Inceoglu et al., 2013). Pyrosequencing technologies are culture-independent methods based on the principle of sequencing by synthesis, enabling the systematic culture-independent investigation of the plant rhizosphere microbiome (Chaparro et al., 2014; Bulgarelli et al., 2015; van der Voort et al., 2016). Such techniques can reveal the profiles of complex microbial taxonomic structures and specific bacterial communities in various plants such as rice, maize, oat, and wheat (Uroz et al., 2010; Knief et al., 2012; Turner et al., 2013). The rhizosphere soil, a narrow zone surrounding plant roots, contains dense populations of microbes (Hartmann et al., 2008; Mendes et al., 2011). The rhizosphere provides nutrients to the microbial community and influences bacterial activity and diversity, while the bacterial community in the rhizosphere is influenced by plant species, root exudates, plant age, and fungal diseases (McSpadden Gardener and Weller, 2001; Kowalchuk et al., 2002; Haichar et al., 2008; Mendes et al., 2011; Berendsen et al., 2012; Lundberg et al., 2012). A recent study demonstrated that the *Arabidopsis thaliana* rhizosphere contained different bacterial communities from those of bulk soil, as revealed by pyrosequencing (Lundberg et al., 2012; Inceoglu et al., 2013; Bulgarelli et al., 2015). The populations of Comamonadaceae, Flavobacteriaceae, Rhizobiaceae, Actinobacteria, and Proteobacteria were enriched in the *A. thaliana* rhizosphere, which was influenced by plant genotype, plant growth, and soil type (Lundberg et al., 2012; Bulgarelli et al., 2015). Several studies based on culture-dependent and -independent procedures show that great

bacterial diversity exists in the rhizosphere (Bulgarelli et al., 2012, 2015; Lundberg et al., 2012; Chaparro et al., 2014). However, the rhizosphere bacterial communities of insect-infested plants are poorly understood. In this preliminary study, we performed next-generation sequencing (NGS) using the 454-pyrosequencing platform to evaluate the structure of the rhizosphere microbiome in the pepper plant rhizosphere in response to leaf infestation with whitefly. Collectively, the results of this study broaden our understanding of the role of the microbiome in insect–plant relations and the induction of systemic resistance, as well as the ecological value of the microbiome under natural conditions. The goal of this study was to provide new evidence that whitefly, a sucking insect that affects pepper, increases the populations of specific bacterial groups in the plant rhizosphere. Furthermore, we evaluated whether enriched *Pseudomonas* spp. have direct effects on insect herbivores (**Figure 1**). Investigating the effects of whitefly infestation on bacterial communities in the rhizosphere is important for understanding insect-plant-microbe interactions and their role in conferring beneficial traits to the host plant.

MATERIALS AND METHODS

Plant Growth Conditions

Pepper (*Capsicum annuum* L. cv. Bukwang) was used as a model system in this study as described previously (Yang et al., 2011). *C. annuum* seeds were surface-sterilized with 6% sodium hypochlorite, washed four times with sterile distilled water (SWD), and germinated at 25–28°C for 3 days on 1/2 Murashige and Skoog medium supplemented with 0.6% (w/v) agar and 1.5% (w/v) sucrose. The seedlings were transplanted to natural soil collected from a pepper field located in Cheongwon-gun, Chungcheongbuk-do, South Korea (conducted in Cheongwon-gun, Chungcheongbuk-do, South Korea, 36° 35′ 32.27″ North, 127° 30′ 34.75″ East) in the KRIBB greenhouse facility, Daejeon, South Korea and grown at 25 ± 2°C for 2 weeks under controlled conditions in a growth chamber (12 h/12 h day/night cycle, c. 7000 L × light intensity). Each pepper plant was placed into an acrylic plastic cylinder (diameter = 15 cm, height = 50 cm, and thickness = 3 mm), and the top of the cylinder was covered with a nylon stocking as described previously (Yang et al., 2011; Kim et al., 2016).

Whitefly Treatment

Whitefly (*B. tabaci*) were grown and maintained in the KRIBB greenhouse facility of Daejeon, South Korea in 2008–2010 as described previously (Yang et al., 2011; Park and Ryu, 2014). To investigate the effects of whitefly on the belowground bacterial microbiota in pepper plants, 2-week-old pepper plants were exposed to whitefly for 1 or 2 weeks (Whitefly at Week 1, WW1 and Whitefly at Week 2, WW2). The plants were exposed to an average of 18 ± 3.3 adult-stage whiteflies per pepper leaf (**Figure 1**). Control plants were grown without whitefly infestation at weeks 0, 1, and 2 (Control at Week 0, CW0, Control at Week 1, CW1, and Control at Week 2, CW2).

Sampling and Amplification of the 16S rRNA Gene

To investigate the influence of whitefly infestation on belowground bacterial communities, 1 g of soil was sampled from the rhizospheres of whitefly-infested and control pepper plants at 1 and 2 weeks after treatment with whitefly, respectively (**Figure 1**). Plants grown in a growth chamber were removed from acrylic plastic cylinders. The roots from each sample were gently shaken to remove loosely attached soil, and tightly associated soil was separated from the roots by vigorous shaking in SDW for 30 min. The separated soil solution was centrifuged at 8,000 rpm for 10 min to collect rhizosphere soil containing microbiomes. The rhizosphere soil samples were stored at −80°C until use for microbial community analysis. Soil bacterial genomic DNA was extracted using a PowerSoil DNA kit (Mo Bio Laboratories, Solana Beach, CA, USA). Amplification of 16S rRNA and DNA sequencing were performed by OmicsPia, Co.

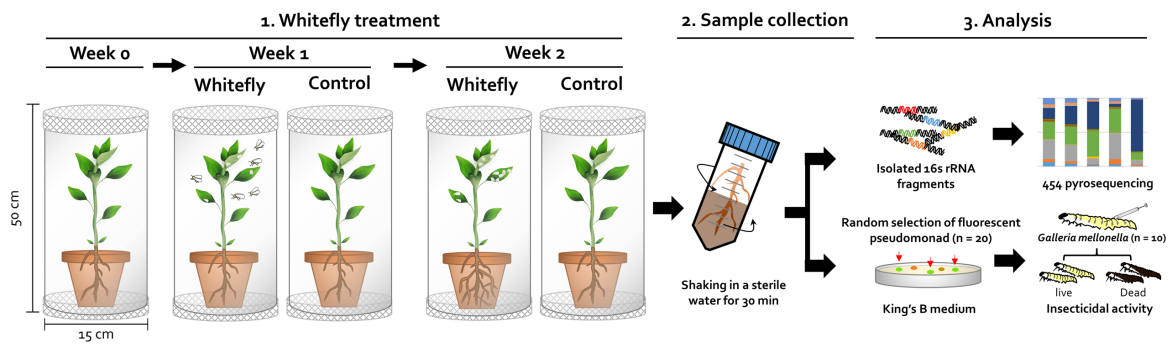

FIGURE 1 | Overview of workflow for investigating the microbial community in whitefly-infested pepper plants. (1) *Whitefly treatment*: two-weeks-old pepper seedlings were treated with an average of 18 whitefly adults for 2 weeks in a plastic cylinder. (2) *Sample collection*: the root system was collected at 0, 1, and 2 week after whitefly infestation. The bacteria were then separated by shaking in SDW for 30 min. (3) *Analysis*: to investigate bacterial diversity, PCR-based 454 pyrosequencing (culture independent techniques) was employed after extraction of 16S rRNA from rhizosphere bacteria. To assess the insecticidal capacity of randomly selected pseudomonads, a killing assay with a model insect *Galleria mellonella* was conducted with 2 μL of bacterial suspension (OD_{600} = 1.0). *G. mellonella* mortality was measured at 24 h after inoculation at 30°C.

Ltd (Daejeon, South Korea) according to the manufacturer's instructions.

The 16S rRNA genes were amplified with universal primers (27F-GAGTTTGATCMTGGCTCAG and 518R-WTTACCGCGGCTGCTGG), which were used to amplify the V1–V3 regions of the bacterial 16S rRNA genes. To enable the separation of samples, specific barcode sequences were fused to the 5′ ends of the universal primers.

The 16S rRNA genes were amplified in a 50 μL (total volume) reaction mixture containing 1 μL of 100 ng/μL template DNA, 5 μL of 10X Ex Taq buffer, each deoxynucleoside triphosphate at a concentration of 2.5 μM, each primer of 20 nM, and 1.25 units of EX-Taq DNA polymerase (Takara Suzo, Co. Ltd, Tokyo, Japan). The polymerase chain reaction (PCR) was performed with a PCR thermocycler (Bio-Rad, Germany) under the following conditions: an initial denaturation step of 95°C for 5 min; 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; and a final extension step at 72°C for 7 min. The amplified PCR products were sequenced using a GS-FLX Titanium Pyrosequencer (454 Life Sciences, Branford, CT, USA) at OmicsPia, Co. Ltd.

Pyrosequencing Analysis of Using the Mothur Pipeline

Amplicon reads of the partial 16S ribosomal RNA genes (V1–V3 regions) generated by the 454 GS FLX Titanium platform were initially trimmed for quality using the Pyrotrimmer program v1.1 (Oh et al., 2012). Bacterial 16S rRNA sequence data from the microbiota in the rhizosphere of pepper plants were processed through the mothur pipeline (Schloss et al., 2009). Reads were sorted into each sample based on their unique barcodes and were error-corrected using the PyroNoise algorithm. Chimeric sequences were filtered out using the UCHIME algorithm after the nearest alignment space termination based on the SILVA database (DeSantis et al., 2006; Edgar et al., 2011). High-quality controlled reads were taxonomically assigned using RDP classifier with a 0.8 confidence threshold. The reads were also used to determine diversity indices and unique sequences and to evaluate the abundance of observed operational taxonomic units (OTUs), which were clustered at 3% dissimilarity in each sample (Wang et al., 2007). Using these OTUs, construction of distance matrix and clustering were conducted using the mothur pipeline. Alpha diversity was estimated using various diversity and richness indices , such as the Shannon index, and Inverse Simpson index, abundance-based coverage estimators (ACEs), and Chao1 (a non-parametric richness estimator), which were calculated using mothur analyses (Schloss et al., 2009). For beta diversity analysis, principal coordinates analysis (PCoA) was conducted using the Bray–Curtis metric. The Bray–Curtis algorithm was used to calculate the distance between samples (Beals, 1984). PCoA was conducted using the Bray–Curtis metric. RDP LibCompare was used to estimate the probability of differences in the abundance of some observed phylogenetic taxa between samples. The pyrosequencing experiment at CW1 (Control at Week 1), CW2 (Control at Week 2), WW1 (Whitefly at Week 1), and WW2 (Whitefly at Week 2) was repeated at least twice.

Quantification of Rhizosphere Fluorescent Pseudomonads

The population of bacteria on the roots was measured at 0, 1, and 2 weeks after whitefly exposure as described previously (Yang et al., 2011). In brief, whitefly-infested pepper roots were incubated in 30 mL of SWD for 30 min in a shaking incubator at 30°C. The population of root-colonizing *Pseudomonas* spp. was determined by plating on King's B-agar medium (KB; 10 g proteose peptone No. 3, 1.5 g K_2HPO_4, 1.5 g $MgSO_4{\cdot}7H_2O$, 10 mL glycerol, 20 g agar, and 1 L distilled water) (King et al., 1954). The pseudomonad population was calculated based on the number of fluorescent colonies under UV light irradiation (UVP, Inc., Upland, CA, USA) at 365 nm. The experiment was conducted using a completely randomized design with 10 replications. Twenty fluorescent colonies per treatment were randomly selected for further evaluation of insecticidal activity. The experiment was repeated at least twice with 10 biological replications.

Galleria mellonella Killing Assay

Insecticidal activity analysis was performed with *Galleria mellonella* as described previously (Chung et al., 2016). Ten randomly chosen *G. mellonella* caterpillars were used for each selected bacterium in an experiment. Prior to inoculation, 20 of pre-selected pseudomonads per treatment as described above were adjusted to an optical density OD_{600} of 1.0 with phosphate-buffered saline (PBS). A 2 μl bacterial suspension was injected into the body cavity of each *G. mellonella* caterpillar using 10-μL Hamilton syringe (25-gauge, Hamilton, Co., Reno, NV, USA). After Injection, *G. mellonella* caterpillars were incubated in a growth chamber at 30°C to assess the number of dead caterpillars at 24 h after inoculation (**Figure 1**). The experiment was repeated at least twice with 20 biological replications.

Statistical Analysis

Analysis of pyrosequencing data was performed with the R program (R Core Team, 2014) with the additional multcomp packages (Hothorn et al., 2008). Statistical analyses of experimental datasets were performed using commercial statistical software (JMP v5.0, SAS Institute, Inc., Cary, NC, USA). Significant effects of treatment were determined based on the magnitude of the *F*-value ($P = 0.05$). When a significant *F*-test was obtained, separation of means was accomplished using Fisher's protected least significant difference (LSD) at $P = 0.05$.

RESULTS

Plant Rhizosphere Bacterial Community Is Affected by Whitefly

To profile the belowground bacterial community, we amplified 16S rRNA genes in the rhizosphere using 12 pepper plants,

including five control plants and seven plants whose leaves were infested with whitefly. A total of 341,009 reads were sorted by the Protrimmer program. After a de-replication step, 284,945 unique reads were obtained. After removing chimeric and chloroplastic sequences, 196,554 sequences were obtained for all samples.

After the reads were clustered into OTUs, those with sequence similarity >97% were discarded from the analysis, resulting in 23,596 OTUs (**Table 1**). A total of OTUs were obtained for the whitefly infested pepper rhizosphere, and microbial diversity analysis was performed based on species diversity and evenness index. The bacterial diversity of samples was estimated by the Shannon and Inverse Simpson metrics. Bacterial richness in whitefly-infested samples at week 2 (WW2) appeared to be significantly lower that of the control plant samples (CW0 and CW1, respectively; one-way ANOVA, $P < 0.05$). Chao1 and ACE metric, which are used for richness analysis, revealed similar patterns in the control and whitefly infested pepper rhizosphere (one-way ANOVA, $P < 0.08$; **Table 2**).

Among the total OTUs, 41 were exclusively detected in the control and 47 were exclusively detected in the whitefly infested plant rhizosphere. A total of 124 OTUs were shared with two other groups (**Figure 2A**). Furthermore, as observed in the Venn diagrams (**Figure 2B**), the samples at 2 weeks after whitefly infestation contained the highest number of endemic OTUs (41 OTUs).

TABLE 2 | Summary of the relationships between major taxa and genera.

	Taxa (significance value > 0.01)	Genus level
CW1 > WW1	Caulobacteraceae (*Brevundimonas, Asticcacaulis, and Phenylobacterium*)	*Massilia*
CW2 > WW2	Cytophagaceae (*Cytophaga, Flectobacillus, and Dyadobacter*)	-
	Oxalobacteraceae (*Massilia, Undibacterium, Naxibacter, and Herbaspirillum*)	-
	Xanthomonadaceae (*Rhodanobacter, Stenotrophomonas, Thermomonas, and Rudaea*)	-
	Paenibacillaceae (*Paenibacillus and Cohnella*)	-
CW1 < WW1	Microbacteriaceae (*Microbacterium and Leifsonia*)	*Ralstonia*
	Mycobacteriaceae (*Mycobacterium*)	*Sphingobium*
	Flavobacteriaceae (*Chryseobacterium*)	*Variovorax*
CW2 < WW2	Caulobacteraceae (*Brevundimonas*)	*Achromobacter*
	Enterobacteriaceae (*Escherichia/Shigella*)	*Janthinobacterium*
	Flavobacteriaceae (*Elizabethkingia*)	*Stenotrophomonas*

The bacterial taxa and genus represent the different major populations at the different treatments. CW1 = Control at Week 1, CW2 = Control at Week 2, WW1 = Whitefly at Week 1, WW2 = Whitefly at Week 2.

Structure of the Bacterial Community

We detected differentially abundant bacterial communities in the control versus whitefly infested plant rhizosphere. Bacterial community structure analysis at the class level showed that, in all samples, alpha-, beta-, and gammaproteobacteria were the major bacterial communities. However, the relative abundance of gammaproteobacteria was highest at 2 weeks after whitefly infestation (WW2; 76 ± 11%), whereas the abundance of alpha- (7 ± 4%) and betaproteobacteria (11 ± 4%) decreased (**Figure 3A**). At the order level, the abundance of the *Pseudomonadales* population (72 ± 12%) was higher at 2 weeks after whitefly infestation (WW2). By contrast, the populations of *Xanthomonadales* (13%), *Burkholderiales* (25%), and *Sphingomonadales* (5%) were larger in the control at the beginning of analysis (CW1; **Figure 3B**).

Principal coordinates analysis based on the Bray–Curtis dissimilarity index revealed clear differences between the two groups at the genus level. The first two axes as PCoA explained 64.7 and 15.8% of the variation, respectively. In the whitefly infested samples, we observed closer clustering, and the distances between sampling times of the two groups were variable (**Figure 4**). The abundances of *Brevundimonas*, *Asticcacaulis*, and *Phenylobacterium* of the family Caulobacteraceae were higher among abundant OTUs in the control at 1 week after infestation (CW1), whereas the abundances of Microbacteriaceae (genus *Microbacterium* and *Leifsonia*), Mycobacteriaceae (genus *Mycobacterium*), and Flavobacteriaceae (genus *Chryseobacterium*) were higher in whitefly infested plants at 1 week after infestation (WW1). The abundance of *Rhodanobacter*, *Stenotrophomonas*, *Thermomonas*, and *Rudaea* of the family Xanthomonadaceae increased among abundant OTUs in the control at 2 weeks after infestation (CW2), whereas the abundances of *Escherichia/Shigella* of the family

TABLE 1 | Total number of reads, observed operational taxonomic units (OTUs), Good's coverage, diversity index (Shannon's and Inverse-Simpson index), and richness (Chao1 and ACE) for each sample measured based on a 3% dissimilarity cutoff.

Treatments	Reads	Observed OTUs	Good's coverage	Shannon's Index	Inverse-Simpson	Chao1	ACE
CW0	7886^{b}	2914^{a}	0.72^{b}	6.7^{a}	178.07^{a}	10713^{a}	22809^{a}
CW1	$10798^{b} \pm 3347$	$2763^{a} \pm 196$	$0.78^{b} \pm 0.09$	$5.69^{a} \pm 1.20$	$139.39^{b} \pm 184.01$	$8818^{a} \pm 3235$	$18179^{a} \pm 7026$
CW2	$13863^{b} \pm 3122$	$2182^{b} \pm 294$	$0.875^{ab} \pm 0.05$	$4.02^{a} \pm 0.96$	$10.19^{d} \pm 6.66$	$5092^{ab} \pm 1557$	$11023^{ab} \pm 3940$
WW1	$7549^{b} \pm 132$	$1954^{b} \pm 50$	$0.80^{b} \pm 0.0$	$5.27^{ab} \pm 0.02$	$20.19^{c} \pm 1.05$	$7573^{a} \pm 285$	$16213^{a} \pm 126$
WW2	$24847^{a} \pm 2793$	$1376^{b} \pm 289$	$0.954^{a} \pm 0.02$	$2.04^{ab} \pm 0.47$	$2.36^{d} \pm 0.54$	$2360^{b} \pm 689$	$4657^{b} \pm 1514$

Richness represents the number of observed unique operational taxonomic units (OTUs), which was estimated by the estimator Chao1 and the abundance-based coverage estimator (ACE). Diversity is indicated by the Shannon and Inverse Simpson indexes. Evenness is measured as the ratio of Shannon index to the number of observed OTUs. All data were expressed as the mean ± SEM. Statistical significance was calculated using a one-way ANOVA.
Different letters such as a–d indicate significant difference based on LSD (P = 0.05).

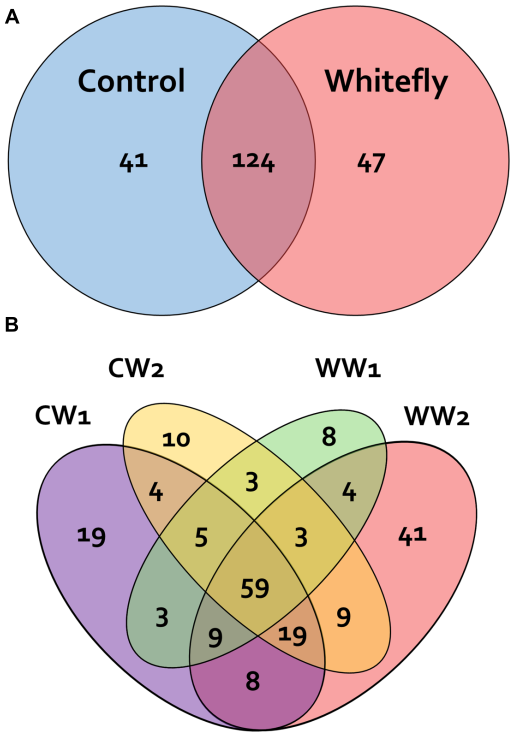

FIGURE 2 | Venn diagrams representing the number of unique and shared operational taxonomic units (OTUs 97% sequence similarity). Diagrams comparing pyrosequencing results from the control and whitefly-infested pepper plant rhizosphere **(A)** and after different durations of whitefly infestation and the control **(B)**. OTUs are defined at 97% sequence similarity. The relative abundance of shared OTUs across all samples is shown in parentheses.

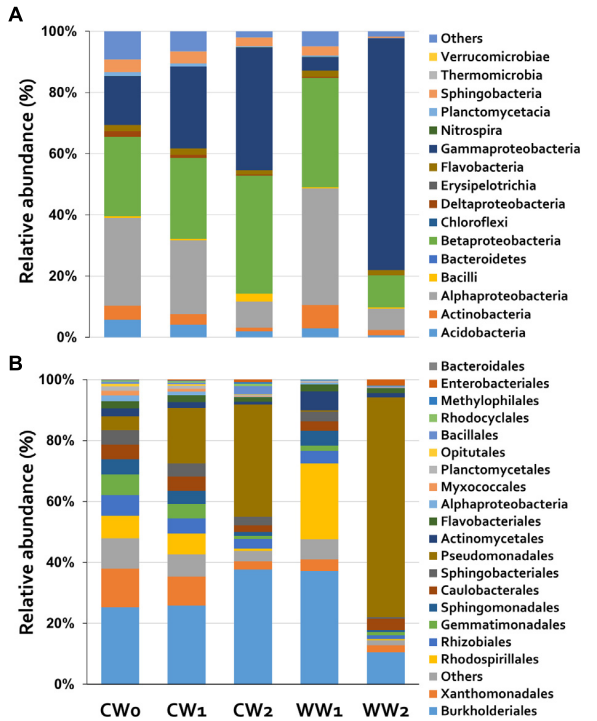

FIGURE 3 | Relative abundance (%) of rhizosphere bacteria. The composition of the bacterial community in the rhizospheres of three control treatments (CW: control) and two whitefly-infested treatments (WW: Whitefly treatments). Numbers indicate exposure time to whitefly (CW0: time zero; CW1 and WW1: 1 week; CW2 and WW2: 2 weeks). The distribution of the different bacterial phyla is based on data obtained by 454 sequencing. Distribution of classes with relative abundance (>0.3% dissimilarity; **A**) and orders **(B)** in control and whitefly infestation samples.

Enterobacteriaceae and *Elizabethkingia* from Flavobacteriaceae were higher in whitefly infested plants at 2 weeks after infestation (WW2; **Figure 4**).

Analysis of Fluorescent Pseudomonad Abundance and Insecticidal Effect

Assessment of the effects of whitefly infestation on the plant rhizosphere, specifically *Pseudomonas* spp. diversity against insect infestation, using a culture-based method on King's B medium showed that whitefly-infested plants had significantly ($P < 0.05$, $n = 10$) higher fluorescent pseudomonad populations at 2 weeks than 1 week and the control plants, whereas the control plants at 1 and 2 weeks were similar (**Figure 5A**). To investigate the effects of *Pseudomonas* spp. on insect killing, we randomly selected 20 fluorescent colonies to assess insecticidal activity for each time period. To determine whether such differences in pathogenicity to selected fluorescence *Pseudomonas* spp. occurred between the whitefly-infested samples and the control, we inoculated *G. mellonella* caterpillars with these *Pseudomonas* spp. As shown in **Figure 5B**, *G. mellonella* mortality was significantly higher in caterpillars inoculated with *Pseudomonas* spp. isolates from pepper root after whitefly application than those of the control ($P < 0.05$; **Figure 5B**).

DISCUSSION

The recent analysis of plant-associated microbiomes represents a new horizon in botanical and agricultural research (Mendes et al., 2013). Previous studies examining the role of microbes in insect-plant-microbe tritrophic interactions were limited, as few utilized culture-independent 16S rRNA amplicon sequencing technology, also referred to as NGS. In the current study, we demonstrated that whitefly (*B. tabaci* Genn.) infestation of pepper plants modulates the rhizosphere bacterial community, leading to the enrichment of Pseudomonadales of the class Gammaproteobacteria, as determined using a NGS platform, 454 pyrosequencing, and a culture-based method. The results of pyrosequencing indicate that the bacterial diversity and evenness in the plant rhizosphere were influenced by whitefly infestation rather than by the sampling times of the plant rhizosphere (**Table 1**; **Figure 3**). However, in a study of *Arabidopsis*, the bacterial diversity and evenness in the microbiomes in the rhizosphere were found to be unrelated to plant developmental time point (Lundberg et al., 2012; Chaparro et al., 2014). This finding indicates that the rhizosphere sampling time does not have much of an effect on bacterial diversity. However, in the current study, the bacterial communities in the rhizospheres

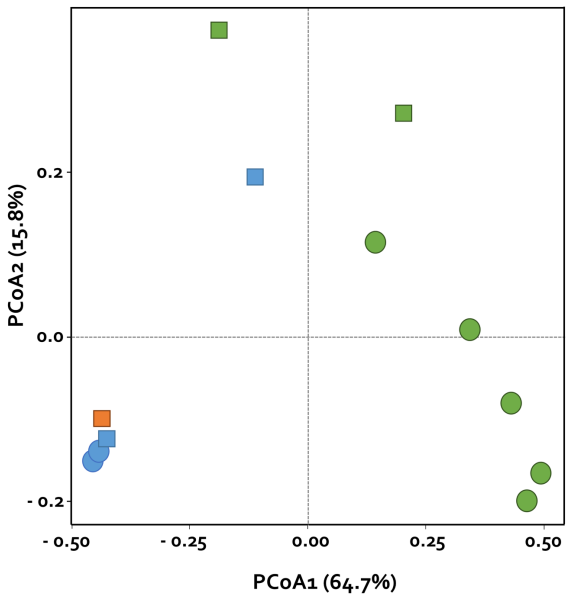

FIGURE 4 | Principal coordinates analysis (PCoA) based on Bray–Curtis distance matrix of bacterial community compositional structure in pepper plants. Plant rhizosphere without (squares) and with (circles) whitefly infestation. Samples were taken at two different time points after whitefly infestation and are represented as follows: orange indicates time zero, blue indicates 1 week, and green indicates 2 weeks after whitefly infestation. PCoA1 (64.7%) and PCoA2 (15.8%) are the first and second principal coordinates, respectively.

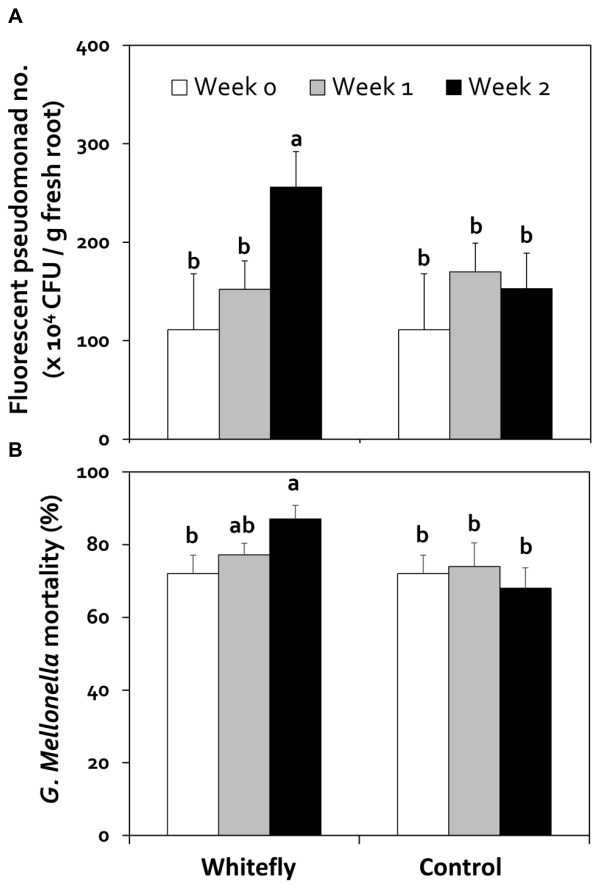

FIGURE 5 | Effects of whitefly infestation on fluorescent pseudomonad populations in the pepper rhizosphere and insecticidal activity. (A) Fluorescent pseudomonad community in the rhizosphere. The population of pseudomonads was quantified by plating on King's B medium at 0, 1, and 2 weeks after whitefly infestation ($n = 10$). The number of colony-forming units (CFUs) of fluorescent pseudomonads was determined under UV light and expressed per gram of root fresh weight. **(B)** Evaluation of *Galleria mellonella* mortality by *Pseudomonas* spp. ($n = 20$). *G. mellonella* mortality was evaluated for 24 h after injection of 2 μL *Pseudomonas* spp. suspension. *G. mellonella* caterpillars ($n = 10$) were incubated in a growth chamber at 30°C after Injection to assess the number of dead caterpillars. Values are mean ± SEM at $^*P < 0.05$ according to the LSD test.

of whitefly infested plants exhibited slight differences in OTUs, diversity, and evenness at 2 weeks of whitefly infestation (WW2) compared to 1 week (WW1; **Table 1**). These results indicate that specific bacterial populations were recruited to the rhizosphere due to whitefly infestation.

An intersection of OTUs in each sample, when grouped by treatment or sampling time, was observed for 28.9% of the OTUs, which shared 97% sequence similarity and were shared between the whitefly infested plant and control plant rhizospheres (**Figure 2**). The shared OTUs represent essential microbial communities in the plant rhizosphere, whereas the endemic OTUs in WW2 might be helpful for the whitefly infested plants. PCoA also indicated that each sample was clustered according to whitefly infestation and sampling time (**Figure 4**). The results indicate that specific bacterial populations were affected in the changing bacterial community. A previous study indicated that the level of a specific bacterial population, i.e., Gram-positive bacteria, increased in the whitefly infested pepper rhizosphere compared to the control (Yang et al., 2011). However, the current study demonstrates that whitefly feeding on pepper leaves led to a significant increase in Gram-negative bacteria (**Figure 3**). These different results might be attributed to the different techniques used: in the previous study, bacterial colonies on artificial media were measured, while, in the present study, we detected the number of OTUs based on the presence of 16S rRNA in the rhizosphere. Taken together, our results more comprehensively reflect the bacterial community.

Based on comprehensive analysis of the essential or endemic OTUs, we estimated the relative abundances of members of the bacterial community. Our results show that the population of Pseudomonadales of the class Gammaproteobacteria significantly increased after 2 weeks of whitefly infestation, as revealed through both culture-dependent and -independent methods (**Figure 3**). We propose three possible scenarios to explain these results: (1) plants secrete root exudates specifically to attract *Pseudomonas* spp. following whitefly infestation. This idea is supported by our current and previous finding that the variation in rhizosphere microbes between WW1 and WW2 may be influenced by the altered secretion of root exudates and the expression of plant signaling genes (**Figure 5A**).

Previously, we found that whitefly infestation induces four transporter genes, including the genes encoding ATP-binding cassette (ABC) transporter, peptide transporter, zinc transporter, and phosphate transporter, as well as two auxin-responsive genes, which can increase the root biomass and help recruit microbes in whitefly infested plants (Yang et al., 2011; Park and Ryu, 2014). In addition, recent genome sequencing of *Pseudomonas* spp. of diverse origins revealed that they contain insecticidal gene clusters such as *Fit, TccC*, and *Mcf* (Kupferschmied et al., 2013; Bruto et al., 2014; Flury et al., 2016). A study of natural variation across *Pseudomonas* spp. and field application of specific strains of *Pseudomonas* spp. demonstrated their insect-killing capacity. The *Pseudomonas* spp. are also distributed in both the phyllosphere and rhizosphere, indicating that plants indirectly protect themselves against subsequent whitefly infestation. (2) A recent study demonstrated that plant phenolic compounds such as anthocyanin and salicylic acid (SA) are major secreted products of plants when aphids attack their leaves (Park and Ryu, 2014; Song et al., 2015). Previously 6 μg/mL SA secretion by whitefly infestation was shown to be effective against soil microbiota (Song et al., 2015). Interestingly, most *Pseudomonas* spp. are resistant to SA, while other Gram-negative bacteria are sensitive, leading to the elimination of the SA-sensitive bacterial population (Ramos, 2004). (3) Finally, the accumulated SA in the rhizosphere leads to an increase in the remaining SA-resistant population, such as *Pseudomonas* spp. In addition, researchers have long investigated the beneficial effects of Pseudomonadales, typically including *Pseudomonas* spp. The *Pseudomonas* spp. include a large number of species that provide benefits for plants, such as plant growth promotion and biocontrol (Raaijmakers et al., 1995; McSpadden Gardener and Weller, 2001). Similarly, the populations of Burkholderiales of the class Betaproteobacteria and Rhodospirillales of the class Alphaproteobacteria significantly increased at 1 week of infestation (**Figure 3B**). Overall, these results indicate that rhizosphere microbiota react rapidly to whitefly infestation, leading to the dominance of different bacterial taxa over time. The reason that whitefly mediated changes in plant physiology lead to changes in the rhizosphere microbiome is still largely unknown. One possible explanation is that the recruitment of *Pseudomonas* spp. helps protect plants against possible subsequent attack from soil-borne insect pests. Many species of insect pests complete their life cycles from the larval stage in the soil to aboveground infestation.

A more detailed classification of the bacterial community at the genus level revealed that the populations of *Achromobacter*, *Janthinobacterium*, and *Stenotrophomonas* were altered with whitefly infestation, suggesting that whitefly infested plants specifically select microbes (**Table 2**). *Achromobacter* promotes the growth of oilseed-rape (*Brassica napus*), wheat (*Triticum aestivum*), and *Brassica juncea* by improving the absorption of nitrogen, producing indole acetic acid (IAA), and functioning in phosphate solubilization (Bertrand et al., 2000; Jha and Kumar, 2009; Ma et al., 2009). These findings are also in agreement with the previous observation (Park and Ryu, 2014) that plant auxin-related genes are upregulated at 1 week after whitefly infestation (Park and Ryu, 2014). Moreover, *Stenotrophomonas* strains can produce IAA *in vitro*, which influences root development (Suckstorff and Berg, 2003), and indole-dependent priming increases the levels of plant stress hormones such as jasmonate–isoleucine conjugate and abscisic acid (Erb et al., 2015). Under whitefly infestation, these hormones may elicit systemic resistance against bacterial pathogens and abiotic stress (Yang et al., 2011; Park et al., 2015; Song et al., 2015). Therefore, our results indicate that whitefly infestation enriches the population sizes of specific bacteria, including IAA-producing bacteria, which play an important role in plant growth both directly and indirectly, by priming plants for defense responses.

CONCLUSION

This is the first report demonstrating the transition of belowground microbial communities elicited by aboveground insect herbivores. Many studies using various ecological systems demonstrate that insect infestation aboveground systemically affects plant defense mechanisms. The effects of insect infestation on plant rhizosphere microbes have only recently begun to be understood. Moreover, the interactions of insect-plant-microbes remain poorly understood. Revealing the composition of the microbiome community in the whitefly infested plant rhizosphere and unraveling the underlying mechanisms will increase our understanding of the effects of insects and plants on the rhizosphere environment. Out of all communities of the microbiome, members of the Gammaproteobacterial group, including *Pseudomonas* spp. containing the insecticidal capacity, are the major enriched communities that respond to whitefly feeding. Moreover, the NGS technique and culture-base procedure employed in this study shed light on the novel insect-plant-microbe tritrophic interaction, thus representing a promising development. A more detailed study of the role of the recruited *Pseudomonas* spp. and other enriched bacterial genera in the rhizosphere of pepper plants infested by phloem-sucking insects should be performed in the near future. In addition, the ecological meaning behind the current results must also be determined to apply this information to pest management programs.

AUTHOR CONTRIBUTIONS

HGK: performed both *in vitro* screening of pseudomonad and insecticidal activity, analyzed the microbiome data, and wrote the paper. BKK: performed the bioinformatics analysis. GCS and SL: DNA isolation from plant rhizosphere and performed the experiments. CMR: Supervise the design of experiments and the processes. HGK, BKK, GCS, SL, and CMR read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

This research was financially supported by the Woo Jang-Choon program (PJ010939012016) of the Rural Development Administration, the Industrial Source Technology Development Program of the Ministry of Knowledge Economy (10044909) of Korea, and the KRIBB initiative program, Republic of Korea.

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