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Hemipteran and dipteran pests: Effectors and plant host immune regulators

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Invited Expert Review

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Abstract Hemipteran and dipteran insects have behavioral, cellular and chemical strategies for evading or coping with the host plant defenses making these insects particularly destructive pests worldwide. A critical component of a host plant's defense to herbivory is innate immunity. Here we review the status of our understanding of the receptors that contribute to perception of hemipteran and dipteran pests and highlight the gaps in our knowledge in these early events in immune signaling. We also highlight recent advances in identification of the effectors that activate pattern-triggered immunity and those involved in effector-triggered immunity.

Keywords: Aphids; effectors; endosymbionts; Hessian fly; planthoppers; resistance genes
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INTRODUCTION

Plants are sessile and cannot escape the continuous attack by pests and pathogens. Therefore, they have acquired a multilayered form of defense, including innate immunity, to protect themselves from these foreign invaders. Plant perception of pathogens and consequent triggering of immune responses is mediated by two classes of immune receptors distinct in their cellular localization: Plasma membrane or intracellular. The plasma membrane-localized receptors are pattern-recognition receptors (PRRs) with variable extracellular domains, a transmembrane domain with or without intracellular kinase domain. Pathogen perception by PRRs induces pattern-triggered immunity (PTI), the core of immune responses (Zipfel 2014). PRR-dependent PTI is also activated by host-derived damage-associated molecular patterns (DAMPs) released by pathogen or pest attack (Heil and Land 2014). The intracellular receptors, mostly with nucleotide-binding leucine-rich repeat (NLR) domains, directly or indirectly recognize pathogen virulence effectors delivered inside the plant cell inducing effector-triggered immunity (ETI) (Dodds and Rathjen 2010). Insect pests, belonging to the order Hemiptera, which includes aphids, whiteflies and planthoppers, appear to be recognized by similar classes of immune receptors (Smith and Clement 2012; Kaloshian and Walling 2015). In addition to hemiptera, indirect evidence suggests the presence of such recognition receptors against gall-forming dipteran herbivores (Stuart 2015). This review focuses on plant immunity against hemipteran and dipteran insects (Figure 1) and the counter defenses employed by these pests to overcome host immunity.

PLASMA MEMBRANE-LOCALIZED IMMUNE RECEPTORS AND ASSOCIATES

Pattern-recognition receptors perceive microbe-associated molecular patterns (MAMPs), which are molecules with highly conserved motifs. The PRRs, FLS2 (FLAGELLIN SENSING 2) and EFR (EF-TU RECEPTOR), are well characterized and recognize the microbial MAMPs flagellin and elongation factor Tu (EF-Tu), respectively (Newman et al. 2013). As a trans-membrane leucine-rich repeat receptor kinase (LRR-RK), FLS2 recognizes flagellin by binding to a conserved set of 22 amino acids (flg22) (Chinchilla et al. 2007). First characterized in *Arabidopsis* (*Arabidopsis thaliana*), FLS2 homologs are found in numerous plant genomes and functional orthologs have been characterized in several crops including rice (*Oryza sativa*), tomato (*Solanum lycopersicum*) and grapevine (*Vitis vinifera*) (Robatzek et al. 2007; Takai et al. 2008; Trda et al. 2014). Unlike the wide distribution FLS2 in monocots and eudicots, EFR is found only within the Brassicaceae. Like FLS2, EFR is a membrane-localized LRR-RK and recognizes the N-acetylated

Figure 1. From left to right, Hessian fly (*Mayetiola destructor*), whitefly (*Bemicia tabaci* B, Middle-east Asia Minor 1 (MEAM1)), potato aphid (*Macrosiphum euphorbiae*), and brown planthopper (*Nilaparvata lugens*)

elf18 peptide, which spans the first 18 amino acids of EF-Tu (Kunze et al. 2004; Zipfel et al. 2006). While EFR is not conserved, recent data suggest there may be other mechanisms for recognizing EF-Tu that are deployed by other plants. For example, a distinct 50-amino acid epitope of EF-Tu (EFa50) is recognized by rice via a yet uncharacterized receptor suggesting that EF-Tu recognition has evolved independently in different plant species (Furukawa et al. 2014).

Cell surface-localized receptors also confer resistance to insects. Recently, the rice *Bph3* locus conferring broad-spectrum resistance to different biotypes of brown planthopper (*Nilaparvata lugens*) and to white-backed planthopper (*Sogatella furcifera*) was cloned using mapped-based cloning approach (Liu et al. 2015). The *Bph3* resistance is durable and has been effective in the field for over 30 years (Fujita et al. 2013). This locus comprises a cluster of three genes encoding lectin RKs, namely *Os*LecRK1, *Os*LecRK2 and *Os*LecRK3. *Os*LecRK1-3 contain an N-terminal extracellular B-lectin domain, a transmembrane domain and a C-terminal cytosolic Ser/Thr kinase domain (Figure 2). Similar to plant kinases associated with recognition of conserved molecular patterns, the kinase domains of the *Os*LecRKs lack the conserved arginine-aspartate (RD) motif and, therefore, belong to the non-RD kinases (Dardick et al. 2012). *Os*LecRK2 and *Os*LecRK3 are most closely related sharing 89% amino acid identity, while OsLecRK1 is more diverged with 67%–68% amino acid identity with *Os*LecRK2 and *Os*LecRK3.

The three *Os*LecRKs belong to the G-type of LecRK class, which have an extracellular lectin domain resembling the *Galanthus nivalis* agglutinin (GNA) (Vaid et al. 2012). To date, the G-type lectin-binding domain has not been shown to bind to a sugar ligand. *Os*LeKRK1-3 also contain a PAN/APPLE-like (plasminogen-apple-nematode motif) domain. This motif is present in both G-type LecRKs, as well as plant S-domain (SD)-RLKs that are secreted glycoproteins involved in self-incompatibility (Nasrallah 1997). Although the role of G-type LecRKs in self-incompatibility is well documented, their role in plant immunity is only starting to emerge (Lannoo and Van Damme 2014). For example, the rice Pi-d2 is a membrane-localized RLK with G-type B-lectin domain and confers race-specific resistance to the blast fungus *Magnaporthe grisea* (Chen et al. 2006).

There are four classes of plant plasma membrane-localized LecRKs: G-type, C-type (calcium-dependent), L-type (legume-like), and LysM-type (lysine motif). In both *Arabidopsis* and rice, the G-type and L-type LecRKs are the most abundant. There are 35 G-type and 45 L-type LecRKs in *Arabidopsis* and in rice, these protein families are expanded to 100 G-type and 72 L-type LecRKs (Vaid et al. 2012). Several L-type LecRKs have been implicated in plant resistance to pathogens in *Arabidopsis* and *Nicotiana benthamiana* (Bouwmeester et al. 2011; Singh et al. 2012; Huang et al. 2014). Recently, DORN1 (AtLecRKI.9), an *Arabidopsis* L-type LecRK, was shown to be the likely receptor for extracellular ATP (eATP), as *dorn1* mutants are insensitive to ATP (Choi et al. 2014). eATP is emerging as an important danger signal that heralds a breach in cellular integrity and may function in triggering

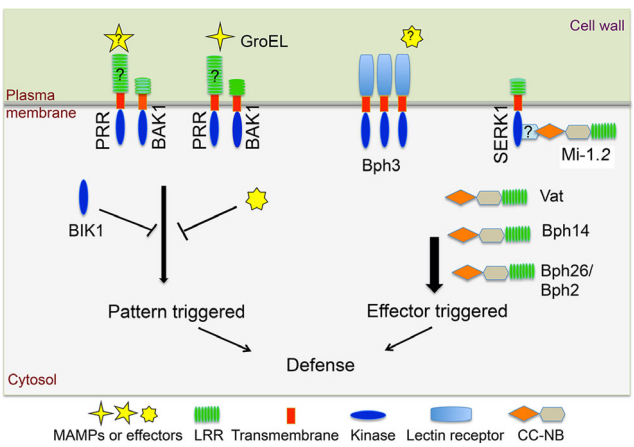

Figure 2. A model for plant resistance to hemipteran insects
Unknown, aphid-associated molecular pattern(s) and the GroEL of the aphid endosymbiont *Buchnera aphidicola* are recognized by presumed plasma membrane-localized pattern recognition receptors (PRR). The plasma membrane-localized receptor-like kinase (RLK) BAK1 is required for both recognition events. The cytosolic RLK BIK1 acts as negative regulator of aphid defense. The rice *Bph3* locus consists of three plasma membrane-localized lectin receptor kinases, OsLecRK1, OsLecRK2 and OsLecRK3, and confers broad-spectrum resistance to brown and white-backed planthoppers. The tomato coiled-coil nucleotide-binding leucine-rich repeat receptor (CC-NLR) Mi-1.2 confers broad-spectrum resistance to potato aphids, whiteflies, psyllids, and root-knot nematodes. For aphid resistance, Mi-1.2 requires the plasma membrane-localized RLK SERK1. The rice CC-NLRs Bph14 and Bph26/Bph2 confer resistance to brown planthoppers and the melon CC-NLR Vat confers resistance to the cotton aphid.

defenses against pathogen and pest attacks (Cao et al. 2014). In addition, a L-type LecRK from *Nicotiana attenuata* (LecRK1) is negative regulator of wound-induced defenses against the chewing herbivore *Manduca sexta* (Gilardoni et al. 2011).

Like most phloem-feeding aphids and whiteflies, the brown and white-backed planthoppers use their slender mouthparts (stylets) to weave between cells, puncture mesophyll cells, and to ultimately establish feeding sites on sieve elements. While the rice planthoppers cause plasmolysis of punctured mesophyll cells and can cause hopper burn when feeding at high densities, they do not cause the extensive cellular damage associated with caterpillar feeding (Sogawa 1982). Therefore, it is currently unclear if the rice G-type LecRKs are associated with the damage associated with cellular plasmolysis of punctured cells, eATP signaling, or if *Os*Leck1-3 are involved in direct recognition of planthoppers. Since *Bph3*/*OsLeck1-3* confers broad-spectrum planthopper resistance, they may recognize a conserved planthopper-associated molecular pattern. Lectin receptors often form homo- or hetero-dimers that are essential for the activation of downstream intracellular signaling (Lannoo and Van Damme 2014). Heterodimers are frequently formed with Lec receptor proteins (RP) lacking a cytosolic kinase domain or RKs having a non-functional kinase domain. Considering that all three *Os*LecRK1-3 appear to have functional kinase domains, it is possible that they are the PRRs for planthopper-associated molecular pattern(s). However, in the absence of a ligand, the conclusive role for the *OsLecRK1-3*s as receptors or co-receptors cannot be made.

An atypical characteristic of *OsLecRK1-3* is their additive function in planthopper resistance. *OsLecRK2* and OsLecRK3 confer 50% of the resistance conferred by the *Bph3* locus, while *OsLecRK1* alone confers 25% of the resistance (Liu et al. 2015). This suggests that each of these Lec-RKs recognize a planthopper-associated molecular pattern or DAMP or the combination of these three proteins allow recognition of additional molecular patterns.

A common partner of both *At*FLS2 and *At*EFR is BAK1/SERK3 (BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE3/). BAK1 is a LRR-RK that is a co-receptor for flg22 recognition and is required for both flg22- and elf18-induced PTI (Heese et al. 2007; Sun et al. 2013). Although it is unclear if BAK1 is a co-receptor for other MAMPs, BAK1 is required for PTI by a number of MAMPs/DAMPs (Zipfel 2014). Recent information indicates that the *bak1-5* mutant, which has no pleiotropic effects on brassinosteriod signaling or cell death (Roux et al. 2011), displays enhanced susceptibility to the *Arabidopsis* non-adapted pest the pea aphid (*Acyrthosiphon pisum*) but not to the adapted pest the green peach aphid (*Myzus persicae*) indicating that green peach aphids are able to circumvent the *BAK1*-dependent immune responses (Prince et al. 2014) (Figure 2). Moreover, aphid whole-body extracts triggered accumulation of reactive oxygen species, callose deposition and induction of defense genes indicating PTI activation (Prince et al. 2014). In addition, when infiltrated into *Arabidopsis* leaves, aphid extracts induced resistance to aphids (Prince et al. 2014). Similarly, *Arabidopsis* treatments with green peach aphid saliva resulted in defense gene activation and reduction in aphid population growth suggesting enhanced aphid resistance (De Vos and Jander 2009). Aphid-derived small proteinaceous molecule(s) of 3- to 10-kDa triggered both PTI activation and enhanced resistance (De Vos and Jander 2009; Prince et al. 2014). It is not clear whether these responses are triggered by the same molecule as the identities of the active constituent in the aphid extracts or saliva have not yet been revealed.

To activate PTI by microbial pathogens, several PRRs also require the cytoplasmic RLK BIK1 (BOTRYTIS-INDUCED KINASE 1) (Lu et al. 2010; Zhang et al. 2010). BIK1 positively regulates PTI to microbial pathogens (Veronese et al. 2006; Lu et al. 2010; Zhang et al. 2010; Laluk et al. 2011). In contrast, it negatively regulates resistance to aphids, since the *bik1* mutants exhibit reduced green peach aphid fecundity (Figure 2) (Lei et al. 2014). Together, these data suggest that the same proteins contribute to both aphid- and microbial-induced PTI; however, it appears that the molecular mechanisms that determine these early events in aphid perception and signaling may be distinct.

INTRACELLULAR-LOCALIZED IMMUNE RECEPTORS

A number of loci conferring resistance to aphids, planthoppers and gall midges have been genetically identified and mapped (Smith and Clement 2012; Yasala et al. 2012; Fujita et al. 2013; Kamphuis et al. 2013; Harris et al. 2015). While only a small number of these resistance (*R*) genes have been cloned and functionally tested for their roles in resistance, several *R* loci span genes with NLR domains (Zhang 2007; Kamphuis et al. 2013; Sama et al. 2014; Divya et al. 2015). To date, *R* genes to phloem-feeding hemipteran insects have been isolated from rice, tomato and melon. In addition to the *Bph3* locus (see above), two rice genes, *Bph14* and *Bph26* (also known as *Bph2*), conferring resistance to brown planthoppers have been cloned (Du et al. 2009; Tamura et al. 2014; Liu et al. 2015). Moreover, two aphid resistance genes, the tomato (*Solanum lycopersicum*) *Mi-1.2* gene and the melon (*Cucumis melo*) *Vat* gene conferring resistance to the potato aphid (*Macrosiphum euphorbiae*) and the cotton aphid (*Aphis gossypii*), respectively, were cloned and characterized (Rossi et al. 1998; Vos et al. 1998; Dogimont et al. 2014). All four loci encode proteins with coiled-coil (CC)-NLR domains and belong to the largest class of disease R proteins (Figure 2) (Rossi et al. 1998; Vos et al. 1998; Du et al. 2009; Rafiqi et al. 2009; Dogimont et al. 2014; Tamura et al. 2014). All four CC-NLRs lack organellar localization signals and therefore are presumed to be confined to the cytosol.

Among the cloned insect NLRs, a unique characteristic of *Mi-1.2* is its broad-spectrum pest resistance. Besides resistance to potato aphids, *Mi-1.2* confers resistance to whiteflies (*B. tabaci* B (MEAM1) and Q (MED)), psyllids (*Bactericerca cockerelli*), and three species of root-knot nematodes (*Meloidogyne arenaria*, *M. incognita* and *M. javanica*) (Roberts and Thomason 1986; Milligan et al. 1998; Rossi et al. 1998; Vos et al. 1998; Nombela et al. 2000, 2001, 2003; Casteel et al. 2006). After two decades of deployment in commercial tomato cultivars, the effectiveness of Mi-1.2 in the field has been compromised; isolates of both root-knot nematodes and potato aphid populations overcoming *Mi-1.2*-

mediated resistance have been reported (Kaloshian et al. 1996; Goggin et al. 2001).

Besides aphid resistance, the *Vat* gene is also associated with resistance to viruses transmitted by the cotton aphid in a non-persistent manner (Lecoq et al. 1979; Lecoq et al. 1980). The virus resistance does not appear to be due to the altered ability of the cotton aphid to deliver the virus to *Vat* plants, nor ability to acquire viruses from virus-infected *Vat* plants. In addition, resistance to the virus does not seem to involve direct or indirect recognition by *Vat* but rather is a consequence of *Vat*'s impact on down-stream broad-spectrum defense responses that impact virus infection (Dogimont et al. 2014).

DOWNSTREAM SIGNALING PARTNERS IN HERBIVORE RESISTANCE

Nucleotide-binding leucine-rich repeat-mediated resistance to microbial pathogens often requires additional signaling partners including the chaperone Hsp90 (Heat Shock Protein 90) and the co-chaperones Rar1 (Required for MLA12 resistance 1) and Sgt1 (Suppressor of the G2 allele of SKP1). To date, there is relatively little know about the signaling components acting downstream of hemipteran R proteins, as this has only been investigated for tomato's Mi-1.2-mediated resistance to the potato aphid. Using virus-induced gene silencing (VIGS), Hsp90 and Sgt1, but not Rar1, was demonstrated to have a role in *Mi-1.2*-mediated resistance to aphids (Bhattarai et al. 2007). Consistent with the roles of these chaperones/co-chaperones in the regulation of other NLR proteins (Kadota et al. 2010), tomato's Hsp90 and Sgt1 may enhance Mi-1.2 stability, facilitate the folding of Mi-1.2 to stabilize active and inactive conformational states of this NLR, or enable the assembly and stability of a Mi-1.2 multi-protein signaling complex, as recent studies suggest that Mi-1.2 is present in a plasma membrane-associated protein complex (Figure 2) (Peng and Kaloshian, unpublished results). In plant-microbe interactions, Sgt1 is also known to negatively regulate NLR-mediated resistance by enabling R protein degradation via the proteasome (Liu et al. 2002). Therefore, it is possible that the tomato Sgt1 may stimulate Mi-1.2 turnover to avoid hyper-accumulation of this immune receptor, which could have negative consequences to plant fitness. Alternatively, after Mi-1.2 release from its plasma-membrane complex, Sgt1 may be critical for degrading effector-activated Mi-1.2 to initiate defense signaling. The role of both Hsp90 and Sgt1 in Mi-1.2-mediated resistance suggests that these chaperones may have more global roles in NLR immune complex regulation including resistance to hemipteran pests.

In addition to Hsp90 and Sgt1, the receptor-like kinase *Sl*-SERK1, mitogen-activated protein kinase cascades, and three WRKY transcription factors (WRKY70, WRKY72a and WRKY72b) are important for mediating *Mi-1.2* resistance to aphids (Bhattarai et al. 2010; Mantelin et al. 2011; Atamian et al. 2012). Using VIGS in tomato, it was shown that the plasma membrane-localized SERK1 is required for resistance to the potato aphid. Silencing of *Sl*SERK1 in *Mi-1.2* plants compromised aphid resistance and *SlWRKY72* expression. In contrast, silencing *Sl*SERK1 did not influence aphid performance on aphid-susceptible tomato lines indicating that it is not an essential virulence target.

Unlike aphid resistance, *SlSERK1* is not required for Mi-1.2-mediated nematode resistance. However, the down-stream transcription factors, *WRKY70*, *WRKY72a* and *WRKY72b*, are required for *Mi-1.2*-mediated resistance to nematodes suggesting some signaling elements are deployed in both the leaf and root resistance to aphids and nematodes, respectively (Bhattarai et al. 2010; Atamian et al. 2012). Finally, *Rme1 (Required for resistance to Meloidogyne)*, which was originally identified as being required for *Mi-1.2*-mediated resistance to the root-knot nematode *Meloidogyne javanica*, is required for resistance against aphids and whiteflies (Martinez de Ilarduya et al. 2001, 2004). While the identity of *Rme1* has yet to be discovered, it is clear that mutation of *Rme1* compromises the resistance to both hemiptera and nematodes and for this it could be the target for different effectors from these pests.

Far less is known about the signaling events required for *Mi-1.2*-mediated resistance to whiteflies and psyllids. While *Rme1* is required for whitefly resistance, the role of *Hsp90*, *Sgt1*, *SERK1*, and the WRKY transcription factors in whitefly or psyllid resistance has yet to be tested. Since aphid resistance is phloem-mediated, whitefly resistance is apoplastic localized, and psyllid resistance is associated with volatiles, branching of Mi-1.2-triggered immune responses may be revealed (Kaloshian et al. 1997; Jiang et al. 2001; Casteel et al. 2006).

HEMIPTERAN-ASSOCIATED BENEFICIAL MICROBES SIGNAL INSECT ATTACK

Hempiteran insects transmit viruses, mycoplasmas and pathogenic bacteria to the plants they probe or feed on (Weintraub and Beanland 2006; Grafton-Cardwell et al. 2013; Sugio et al. 2015; Whitfield et al. 2015). In addition to these pathogenic microbes, hempiteran insects harbor obligate and facultative symbiotic microbes (Hansen and Moran 2014; Douglas 2015). A growing body of evidence indicates that these hemiptera-associated microbes are directly involved in modulating host defenses (Chaudhary et al. 2014; Elzinga et al. 2014; Kaloshian and Walling 2015).

While feeding, aphids secrete watery and gelling salivas into the plant and excrete honeydew, which is mainly composed of sugars and amino acids (Auclair 1963). Besides aphid-derived proteins, aphid saliva and honeydew contain proteins from its primary bacterial endosymbiont, *Buchnera aphidicola* (Sabri et al. 2013; Chaudhary et al. 2014; Vandermoten et al. 2014). Aphid honeydew also contains proteins originating from the insect gut microflora (Sabri et al. 2013). *Buchnera* is an obligate mutualist endosymbiotic γ-Protobacterium essential for aphid reproduction and survival (Wilson et al. 2010). These microbial proteins could act as aphid-associated signals that activate plant immune responses.

The chaperonin GroEL is the most abundant endosymbiont protein constituting 10% of the total *Buchnera* proteins (Baumann et al. 1996). *Buchnera* GroEL has been identified in aphid saliva and honeydew (Sabri et al. 2013; Chaudhary et al. 2014; Vandermoten et al. 2014). Interestingly, when expressed *in planta* or infiltrated into plant leaves, the *Buchnera* GroEL

induces PTI defense responses in both tomato and *Arabidopsis* (Chaudhary et al. 2014). These defense responses include oxidative burst, induction of defense marker genes and callose deposition suggesting that GroEL is a MAMP (Chaudhary et al. 2014). Consistent with its role as a molecular pattern, heat treatment of GroEL did not affect its PTI elicitor activity. In addition, perception of GroEL in *Arabidopsis* requires BAK1 suggesting the presence of a yet unidentified cell-surface receptor that functions with BAK1 in GroEL recognition (Figure 2). Unlike chewing insect-associated microbes, that activate ineffective plant defenses to suppress effective defense against their host insect (Chung et al. 2013), GroEL-induced PTI interferes with aphid colonization. Expressing GroEL in transgenic *Arabidopsis* or delivery of GroEL into tomato plants, using bacterial type-three secretion system, reduces aphid reproduction (Chaudhary et al. 2014; Elzinga et al. 2014). Recognition of *Buchnera* GroEL is similar to the recognition of bacterial flagella or EF-Tu and this recognition could be reminiscent of bacterial recognition by plants. Indeed, GroEL-expressing plants exhibit enhanced resistance to pathogenic and non-pathogenic bacteria similar to flg22 and elf18 peptide treatments (Chaudhary and Kaloshian, unpublished results). Since the aphid-*Buchnera* mutualism is obligate, it is intriguing to speculate that the plant immune system is exploiting this strict mutual dependency to recognize the aphid intruder.

Contrary to the role of the *Buchnera* GroEL in activating effective defenses against the aphid, a secondary endosymbiont of whiteflies *Hamiltonella defensa* exploits the antagonistic relationship between salicylic acid (SA) and jasmonic acid (JA) defense hormones to suppress effective plant defenses and thereby benefit its insect host (Su et al. 2015). This interaction is similar to the chewing insect oral cavity-associated bacteria that are known to utilize the SA/JA antagonist relationship for the benefit of its insect host (Chung et al. 2013). An important distinction between *Buchnera* and *H. defensa* association with their insect hosts exists. Unlike *Buchnera*, *H. defensa* is a facultative endosymbiont of whiteflies and strains of whiteflies lacking *H. defensa* exist. Whiteflies harboring *H. defensa* have greater longevity and fecundity. While the nature of the *H. defensa*-associated signal is unknown, it appears to be a small non-proteinaceous molecule in the whitefly saliva (Su et al. 2015).

HEMIPTERAN AND DIPTERAN EFFECTORS

Effectors

While microbial pathogen effectors have been extensively studied, the study of effectors in hempiteran and dipteran herbivores has only recently attracted attention. Most of the work on hemiptera and diptera herbivores has been carried out with aphids and the Hessian fly *Mayetiola destructor* (Elzinga and Jander 2013; Rodriguez and Bos 2013; Harris et al. 2015; Stuart 2015). These herbivore effectors are delivered into plant tissues during feeding. To feed, aphids use stylets, a hypodermal needle-like structure, to reach their feeding site the phloem, while Hessian flies use tiny mandibles to attack leaf epidermal cells (Harris et al. 2015). Another distinction between aphids and Hessian flies is that while all stages of aphids feed, several stages of the Hessian fly, including some nymphal stages and adults, do not feed.

Biochemical studies have shown that aphid saliva is a complex mixture of biomolecules with potential roles in overcoming plant immune responses (Miles 1999; Will et al. 2013). Recent advances in genomics technology and molecular approaches have allowed identification and direct investigation of the role of aphid effectors in interactions with their hosts. Transcriptome analysis of the pea aphid salivary glands identified the first aphid effector, C002 (Mutti et al. 2006). *C002* encodes an unknown protein, is secreted into the plant tissues, and reducing *C002* expression by RNAi results in shortened aphid lifespan and reduced host colonization (Mutti et al. 2006; Mutti et al. 2008; Pitino et al. 2011). A pea aphid effector Armet has enriched expression in the salivary glands, is secreted into host phloem sap, and is important for aphid survival and host colonization (Wang et al. 2015a). In Drosophila, Armet has two distinct roles, intracellularly as a component of the unfolded protein response in the endoplasmic reticulum and as extracellular neutrophic factor (Palgi et al. 2009, 2012). Although the exact role of Armit in aphids is not well understood, reduced expression of *Armet* in pea aphids by dsRNA injection enhances aphid salivation and reduces sap feeding resulting in shortened life span (Wang et al. 2015a). In addition, treating *N. benthamiana* with recombinant pea aphid Armit induces plant defense genes suggesting Armet is recognized by the plant immune system (Wang et al. 2015a).

Salivary gland transcriptomes of the green peach aphid and potato aphid identified additional components of the aphid secretome (Ramsey et al. 2007; Bos et al. 2010; Atamian et al. 2013). When tested for function in plant-aphid interactions, several of these proteins elicited plant defenses, while others suppressed host plant defenses (Bos et al. 2010; Atamian et al. 2013; Pitino and Hogenhout 2013; Elzinga et al. 2014; Rodriguez et al. 2014).

Proteome analysis of the pea aphid salivary glands and saliva, collected *in vitro* in artificial diets, from a number of aphid species identified a large number of secreted proteins and demonstrating the diverse constituents of aphid saliva (Harmel et al. 2008; Carolan et al. 2009, 2011; Cooper et al. 2010, 2011; Nicholson et al. 2012; Rao et al. 2013; Chaudhary et al. 2014, 2015; Nicholson and Puterka 2014; Vandermoten et al. 2014). A few key findings from these salivary protein studies have been revealed. First, aphid species differ in their ability to salivate in artificial diets. Second, the composition of the saliva varies among aphids with different host ranges. Third, salivary proteins have a plethora of functions. Fourth, many salivary proteins do not have canonical secretion signals. Finally, aphid saliva contains proteins from its primary endosymbionts. The identification of *Buchnera* proteins in the aphid saliva and the demonstration of a direct role for these microbes in plant-aphid interactions further highlights the role of endosymbionts in plant-herbivore interactions.

A recent comprehensive analysis of salivary proteome of the potato aphid was performed (Chaudhary et al. 2014, 2015). Collecting saliva from a large number of potato aphids, 105 salivary proteins were identified. Comparison of the potato aphid secretome with the available secretomes from the pea aphid, the grain aphids, vetch aphid, and green peach aphid indicates that only a fraction of the potato aphid salivary

proteins have been reported from other aphid species (Chaudhary et al. 2015). Of the salivary proteins with functions inferred by protein similarity, only a few correspond to effectors that are deployed by microbial plant pathogens (Carolan et al. 2011; Chaudhary et al. 2015). In addition, many of the potato aphid salivary proteins are annotated as unknowns. Collectively, this indicates that novel effectors are used in plant-aphid interactions and suggests the existence of novel mechanisms of aphid salivary protein action *in planta*.

Hemiptera secrete two types of saliva. The watery saliva is the liquid saliva secreted at the surface of plant tissues and during stylet penetration, as well as during feeding. The second type of saliva is the sheath saliva, which is secreted during stylet penetration of the apoplast and polymerizes forming a protective sheath that envelops the stylets. The sheath saliva remains in the plant tissue after the stylets retract and provides a history of the movement of stylets within their host plant (Miles 1999; Tjallingii 2006). The aphid sheath protein (structural sheath protein, SHP) is present in the saliva of most aphids and shares no sequence similarity with proteins of other organisms (Will et al 2012; Chaudhary et al 2015). Recently, the role of SHP was demonstrated using two RNAi strategies. *SHP* was silenced by dsRNA injection directly into pea aphids. In addition, grain aphids (*Sitobion avenae*) were allowed to feed on transgenic barley expressing a *SHP* dsRNA to silence the grain aphid *SHP*. Both strategies resulted in aphids with deformed sheaths that affected their ability to feed and ultimately reduced aphid fecundity (Abdellatef et al. 2015; Will and Vilcinskas 2015). On transgenic barley plants, aphids also displayed early mortality, delayed maturation, smaller bodies, and a higher percentage of adults with wings. Interestingly, the morphological and physiological characteristics of delayed maturation and wing formation were observed over seven aphid generations when these insects were transferred to and maintained on wild-type plants. These data indicate a potent transgenerational effect on aphid development and suggests *SHP*-silencing strategies could be used for the control of aphid pests (Abdellatef et al. 2015). However, there may be considerable trade-offs that will need to be seriously evaluated prior to deploying a SHP-based control strategy. While delayed aphid development may enhance the efficacy of biocontrol strategies, the increased production of winged adults is likely to promote aphid dispersal and thereby encourage colonization of and virus transmission to neighboring host plants.

Several proteins with similarities to angiotensin-converting enzymes (ACEs) have been identified in pea aphid saliva (Carolan et al. 2009). ACEs are zinc-metallopeptidases with the ability to remove C-terminal dipeptides (Isaac and Shirras 2013). Two of these, *ACE1* and *ACE2*, were shown to have redundant effector functions as simultaneous reduction in their expression by dsRNA injection results in increased aphid mortality (Wang et al. 2015b). Surprisingly, the reduced expression of *ACE1* and *ACE2* enhances both the ability of the aphid to reach the phloem and to ingest phloem sap (Wang et al. 2015b). The mechanism of ACE1 and ACE2 action is currently unknown. As peptidases, these proteins may hydrolyze phloem-localized proteins or peptides that are critical for mounting an effective defense response; in the absence of ACE1 and ACE2 activity, the aphid encounters enhanced defenses, which reduces aphid viability. Alternatively, since *ACE1* and *ACE2* are also expressed in aphid tissues other than the salivary glands (Wang et al. 2015b), the increased mortality could be due to inhibition of essential metallopeptidase activities in these tissues.

Similarly, interrogation of the Hessian fly genome and salivary gland transcriptome identified a large repertoire of salivary-secreted proteins as putative Hessian fly effectors (Chen et al. 2004; Chen et al. 2010; Stuart 2015). These effectors comprise over 7% of the Hessian fly genes indicating the importance of secreted effectors for the biology of this insect (Zhao et al. 2015). Similar to aphids, a large portion of these putative effectors do not have homologs in other organisms. Among these putative effectors are the Secreted Salivary Gland Proteins (SSGPs). With 426 members, the *SSGP-71s* form the largest arthropod gene family identified to date and *SSGP-71* genes are dispersed throughout the Hessian fly genome. Although the majority of the *SSGP-71* genes do not have orthologs in other arthropods, a subset of these proteins do (Zhao et al. 2015). SSGP-71 proteins have a LRR domain and many SSGP-71s also contain a cyclin-like F-box domain. Many F-box proteins are critical components in E3 ligase complexes that mediate protein turnover by the 26S proteasome. Interestingly, some bacterial plant pathogen effectors have both F-box and LRR domains (Hicks and Galan 2010; Mukhtar et al. 2011; Kobayashi et al. 2014; Stuart 2015). These microbial effectors act as mimics of plant F-box LRRs. Therefore, it is likely that the Hessian fly SSGP-71 effectors with F-box and LRR motifs will also function as plant F-box mimics and influence the turnover plant proteins critical for host plant immunity via the 26S proteasome. Consistent with this proposal is the fact that using a yeast-two-hybrid screen, the Hessian fly SSGP-71-142 was shown to interact with the wheat SKP6, a component of the SKP-Cullin-F-box E3 ubiquitin ligase complex (Zhao et al. 2015).

Effectors recognized in R-mediated resistance

In spite of the recent insights into the complex repertoire of aphid salivary proteins, no aphid effectors recognized by R proteins have been identified at this time. This is mainly due to genetic intractability of this group of herbivores. While a brown leafhopper effector locus, *vBph1*, which triggers *Bph1*-mediated ETI has been mapped, the only cloned insect effectors are from the genetically amenable Hessian fly (Kobayashi et al. 2014; Stuart 2015).

Using genetic crosses, the first Hessian fly virulence locus (*vH13, virulence to Hessian fly 13*) was identified using a map-based cloning strategy (Rider et al. 2002; Lobo et al. 2006; Aggarwal et al. 2009). Hessian flies with *vH13* are able to overcome resistance conferred by the *R* gene *H13* of wheat (*Triticum* spp.). The *vH13* locus contains two genes encoding short, secreted proteins. Using RNAi and gene expression analyses, the identity of *vH13* was revealed (Aggarwal et al. 2014). *vH13* RNAs are detected in the salivary glands of *H13*-avirulent Hessian fly larvae but not in *H13*-virulent flies. Interestingly, vH13 has no homologs in databases. RNAi knockdown of *vH13*, allowed some *H13*-avirulent Hessian flies to escape the *H13*-mediated resistance in wheat.

Recent completion of the Hessian fly genome has enabled identification of two additional dipteran effectors, *vH6* and *vH9*, that are able to overcome resistance mediated by wheat

H6 and *H9* R genes, respectively. Using several Hessian fly field populations and structured mapping populations of virulent and non-virulent insects, as well as gene expression analyses, the Hessian fly *vH6* and *vH9* avirulence gene loci were identified (Zhao et al. 2015). *vH6* and *vH9* encode SSGP-71-like proteins. In avirulent *vH6* Hessian flies, a *SSGP-71* gene (Mdes009086-RA) is not expressed. While in avirulent *vH9* flies, two candidate *SSGP-71* genes, both lacking F-box domains, were the candidate H9 effectors. Alleles for the *Mdes015365-RA* gene and avirulence were strictly associated in several mapping populations, making this gene the likely H9 effector. These data suggest that the *SSGP-71* family may play a critical role in the evolution of Hessian fly avirulent biotypes. Given the large size of the *SSGP-71* gene family, it is not surprising that *R* gene deployment against Hessian flies in wheat can be overcome quickly by newly emerging virulent populations. In the absence of a cognate R protein, the recognition mechanism of these Hessian fly effectors remains to be investigated.

EMERGING OPPORTUNITIES IN INSECT RESISTANCE

Pattern-triggered immunity is an effective form of defense that limits pathogen and pest attack. Adapted pathogens and pests have acquired the ability to attenuate PTI through the secretion of effector molecules, suppressing defense and, thus, enabling infection. This outcome, where the pathogen or pest is virulent and the host is susceptible, is also known as effector-triggered susceptibility (ETS) (Chisholm et al. 2006). Therefore, virulence effector targets could be key to understanding how a pathogen causes disease and pests are able to colonize and feed on their host plants. The targets of these virulence effectors are susceptibility factors required by biotrophic pathogens and pests to complete their life cycles (de Almeida Engler et al. 2005). Other effectors target immune-signaling regulators to suppress defense (Dodds and Rathjen 2010). One of the earliest examples of susceptibility factors was derived from the interaction of the necrotrophic fungus *Cochliobolus victoriae* and *Arabidopsis* (Lorang et al. 2007; Lorang et al. 2012). *C. victoriae* produces the effector, victorin toxin. Victorin inhibits the activity of the defense-associated thioredoxin (TRX-hr) and in the absence of LOV-1 (a NBS-LRR protein), defenses are compromised but disease symptoms (e.g., extensive necrosis) do not develop. Whereas in the presence of LOV-1, disease susceptibility is seen. The victorin-bound TRX-hr activates LOV-1 to stimulate a resistance response including cell death, which *C. vitoriae* exploits and disease symptoms develop.

Only a few susceptibility targets have been characterized for herbivore pests. A wheat *HSP* gene (*Mayetiola destructor susceptibility gene-1; Mds-1*) plays an important role in susceptibility to the Hessian fly (Liu et al. 2013). Consistent with its beneficial role in Hessian fly survival, *Mds-1* RNAs are abundant in leaf sheaths of susceptible Hessian fly-infested wheat plants and are at low levels in infested resistant plants. In addition, when *Mds-1* is silenced transiently by VIGS or transgenically by RNAi, these plants are more resistant to Hessian flies indicating requirement of *Mds-1* for Hessian fly survival. Resistant wheat plants ectopically expressing MDS-1 displayed susceptibility, further confirming the role of *Mds-1* in Hessian fly susceptibility. Although it is not clear whether a Hessian fly effector directly targets MDS-1 or if another mechanism is used to confer *Mds-1*-dependent susceptibility, it is clear that MDS-1 suppresses plant defense response and activates nutrient metabolic pathway genes. Although *Mds-1* is activated by heat stress, *Mds1*-dependent susceptibility is independent of heat stress. Interestingly, *Mds-1* is also required for wheat's susceptibility to powdery mildew (*Blumeria graminis* f. sp. *tritici*) (Liu et al. 2013). Together these findings suggest that MDS-1 may be a susceptibility hub targeted by a number of pests and pathogens. In spite of this unique role of *Mds-1* in pathogen and pest susceptibility, it is unclear whether *Mds-1* can be used to develop broad-spectrum resistance in wheat, as plants silenced for this gene display agronomically undesirable traits such as partial sterility, smaller grain weight, reduced plant height, and low seed germination rates. A future challenge will be to determine if transgenic manipulation of MDS-1 levels can eliminate the undesirable effects while reserving the desirable effects.

Evidence for susceptibility factors have also been garnered from wheat-Russian wheat aphid (*Diuraphis noxia*) interactions. In this system, the aphid-induced 1,3:1,4-β-glucanase is implicated as a susceptibility factor. When this β-glucanase is silenced by VIGS in susceptible wheat, reduced aphid reproduction, less infestation-associated chlorosis and increased plant biomass is observed (Anderson et al. 2014). It is possible that a Russian wheat aphid effector targets this 1,3:1,4-β-glucanase. At the present time, the mechanism of β-glucanase-enhanced susceptibility has not yet been explored.

CONCLUSIONS

During the past decade, rapid technological advances for the discovery and interrogation of plant and insect genomes, transcriptomes, and proteomes have been made (Mochida and Shinozaki 2011; Chen et al. 2013; Yonekura-Sakakibara et al. 2013; Walling and Kaloshian 2015). These developments have provided momentum for exploration of signals delivered by genetically intractable organisms (such as herbivores) that stimulate or suppress plant immunity. Combined with the efficacy of VIGS and RNAi strategies to silence plant and/or insect genes, the importance of these signals and the key plant-signaling components are getting revealed. These discoveries have advanced our knowledge of the genetic basis of plant immune responses to hemipteran and dipteran pests. Among these critical breakthroughs are the identification of a large number of putative hemipteran effectors and the discovery of dipteran effectors triggering *R*-mediated resistance (Kaloshian and Walling 2015; Stuart 2015). While the number of *R* genes against herbivores has now risen to five, new planthopper *R* loci have brought to light the diversity of herbivore immune receptors, which now includes NB-LRRs and lectin-RKs.

In spite of these advances, major gaps in our understanding of herbivore-plant interactions remain. The PRRs that bind hemipteran and dipteran molecular signatures have yet to be identified. Moreover, none of the cognate effectors of the cloned *R* genes have been discovered, and while three

effectors critical in ETI have been identified from Hessian flies, the corresponding *R* genes have yet to be cloned. Furthermore, no plant targets of hemipteran or dipteran effectors have yet been identified. The rapidly growing number of putative effectors encode unknown proteins suggesting that herbivore-associated effectors are likely to be diverse in structure, function and perhaps target protein identity, as these elicitors are derived from either the insect or its resident microbes. In addition, the emergence of a wealth of herbivore and associated microbe genomes, transcriptomes and proteomes will accelerate the discovery of putative effectors (Walling and Kaloshian 2015). Finally, while RNAi-based technologies are currently used for testing gene function in insect, their success is often gene or insect specific. Therefore, the new emerging genetic technologies, such as CRISPR, which allow site-directed mutagenesis, gene deletions and gene replacement, holds great promise for the future manipulation of both insect and plant genomes. These emerging genetic technologies will be invaluable to rapidly test the roles of insect-derived effectors and plant target protein functions in the modulation of plant immunity that occurs in response to hemipteran and dipteran attack. Novel technological approaches promise great expectations in deciphering herbivore-pest interactions in the next decade.

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