Silverleaf Whitefly Induces Salicylic Acid Defenses and Suppresses Effectual Jasmonic Acid Defenses

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ABSTRACT

The basal defenses important in curtailing the development of the phloem-feeding silverleaf whitefly (Bemisia tabaci type B, SLWF) on Arabidopsis thaliana were investigated. Sentinel defense gene RNAs were monitored in SLWF-infested and control plants. SA-responsive gene transcripts accumulated locally (PR1, BGL2, PR5, SID2, EDS5, PAD4) and systemically (PR1, BGL2, PR5) during SLWF nymph feeding. In contrast, JA- and ET-dependent RNAs (PDF1.2, VSP, HEL, THI2.1, FAD3, ERS1, ERF1) were repressed or not modulated in SLWF-infested leaves. To test for a role of SA and JA pathways in basal defense, SLWF development on mutant and transgenic lines that constitutively activate or impair defense pathways was determined. By monitoring the percentage of SLWF nymphs in each instar, we show that mutants, which activate SA defenses (cim10) or impair JA-defenses (coi1), accelerated SLWF nymphal development. Reciprocally, mutants that activate JA defenses (cev1) or impair SA defenses (npr1, NahG) slowed SLWF nymphal development. Furthermore, when npr1 plants, which do not activate downstream SA defenses, were treated with MeJA, a dramatic delay in nymph development was observed. Collectively these results showed that SLWF-repressed, JA-regulated defenses were associated with basal defense to the SLWF.
Plants defend themselves from pathogens and herbivores using constitutive and induced resistance mechanisms (Karban, 1997; Thomma et al., 1998; Vorwerk et al., 2004). Emerging research has shown that plants utilize induced defense mechanisms that are dependent on the attacker and, in certain interactions, a subset of responses are species-specific (McDowell and Dangl, 2000; Walling, 2000; Kaloshian and Walling, 2005). The study of salicylic acid- and jasmonic acid/ethylene-dependent signaling pathways in defense against pathogens, pests and wounding have identified the key genes involved in defense gene regulation and have uncovered complex signaling networks and cross-talk between the pathways (Walling, 2000; Glazebrook, 2001; Rojo et al., 2003). In Arabidopsis thaliana, activation of the salicylic acid (SA) pathway has been shown to be important in both basal and resistance gene (R)-mediated biotrophic pathogen defense, while the jasmonic acid (JA)/ethylene (ET) pathway is activated in response to necrotrophic pathogens, feeding by tissue-damaging herbivores and wounding (Kessler and Baldwin, 2002; Glazebrook, 2005), although exceptions do exist (Thaler et al., 2004; Glazebrook, 2005; Musser et al 2002, 2005).

The SA-dependent signaling pathway regulates the expression of a wide array of defense-response genes including the PATHOGENESIS-RELATED PROTEIN (PR) genes. In addition, the SA-dependent pathway confers a broad-spectrum resistance known as systemic acquired resistance (SAR), which is a long-acting, induced resistance mechanism against a wide variety of invading pathogens (Ryals et al., 1996; Durrant and Dong, 2004). In contrast, the JA/ET pathway induces genes whose protein products have anti-microbial and anti-fungal activity, such as plant DEFENSIN (PDF1.2) and THIONIN (THI2.1), and accumulate in response to necrotrophic pathogens (Rojo et al., 2003). In addition, in caterpillar-infested tomato, JA signaling is important in the regulation of wound-response genes such as proteinase inhibitors, polyphenol oxidases, threonine deaminase, and arginase, which have roles in antagonizing caterpillar growth and development (Felton et al., 1989; Ryan, 2000; Chen et al., 2005). In addition, the JA-regulated VEGETATIVE STORAGE PROTEIN 1 (VSP1) has been shown to have anti-insect phosphatase activity against Drosophila (fruit fly), Diabrotica undecimpunctata howardi (corn root worm) and Callosobruchus maculatus (cow pea weevil) (Liu et al., 2005). Along with the aforementioned direct defenses, the JA/ET-pathway induces indirect defenses through the production and release of plant volatiles that attract both predators and parasitoids of the herbivore (Kessler and Baldwin, 2002).
Unlike chewing insects, less is known about molecular responses to insects from other feeding guilds. Phloem-feeding insects are intriguing due to their “stealthy” feeding mechanisms that cause little damage to the plant tissue as they establish direct access to amino acids and carbohydrates through the vascular tissue. To date, most studies of phloem-feeding insects have examined aphid interactions including *Myzus persicae* (green peach aphid) with tomato or Arabidopsis, *Myzus nicotianae* (tobacco aphid) with tobacco, *Macrosiphum euphorbiae* (potato aphid) with tomato, and *Schizaphis graminum* (greenbug aphid) with sorghum (Fidantsef et al., 1999; Moran and Thompson, 2001; Ellis et al., 2002; Martinez de Ilarduya et al., 2003; Voelckel, 2004; Zhu-Salzman et al., 2004; De Vos et al., 2005; Li et al., 2006; Thompson and Goggin, 2006). By monitoring the RNA levels of sentinel defense genes after aphid feeding, studies in Arabidopsis show that SA-regulated transcripts increase (Moran and Thompson, 2001; Moran et al., 2002; De Vos et al., 2005). Wound- and JA/ET-regulated genes are induced transiently or at lower levels during *M. persicae*-Arabidopsis and *M. euphorbiae*-tomato aphid feeding (Moran and Thompson, 2001; Martinez de Ilarduya et al., 2003).

Transcriptome analysis after aphid feeding on Arabidopsis further confirmed the trends observed by Moran and Thompson (Moran et al., 2002; De Vos et al., 2005). These changes in RNA levels suggest that responses to aphid feeding are more similar to “pathogen” defense responses than “chewing insect” defenses. While SA-regulated transcripts are induced, the role of SA in Arabidopsis’ basal defense to aphids remains controversial (Moran and Thompson, 2001; Mewis et al., 2005; Pegadaraju et al., 2005). In addition, recent experiments have shown mutations in *PAD4*, which is regulated by SA, increase susceptibility to *M. persicae*; *pad4* susceptibility is correlated with a delayed aphid-induced senescence (Pegadaraju et al., 2005). In contrast to *M. persicae*-Arabidopsis interactions, basal SA defenses decrease *M. euphorbiae* longevity in tomato and SA is important in *Mi1*-mediated resistance to potato aphids (Li et al., 2006).

Like aphids, the silverleaf whitefly (SLWF; *Bemisia tabaci* type B; *B. argentifolii*) is an obligate phloem-feeding pest. Although these animals share membership in the same feeding guild, aphid and whitefly feeding are not synonymous. Unlike aphids, which probe extensively and are more mobile in their feeding habits, whitefly nymphs feed continuously from the same location throughout their 28+-day nymphal development (Gill, 1990; Byrne and Bellows, 1991; Johnson, 1999; Freeman et al., 2001). The continuous and long-term interaction between whiteflies and their host results in an intimate relationship and possibly pronounced and distinct defense responses when
compared to aphids. In addition, whiteflies and aphids are likely to have different salivary components that may elicit different responses from their host (Walling, 2000).

The response of crop plants to SLWF feeding suggests that the JA/ET and novel defense pathways are induced (van de Ven et al., 2000; Walling, 2000). In tomato, JA/ET-regulated basic PR genes accumulate to higher levels than SA-regulated acidic PR gene transcripts (Puthoff et al., unpublished results). Genes identified through differential RNA display in response to SLWF feeding in tomato and squash, Whitefly Induced1 (Wfi1) and SILVERLEAF WHITEFLY INDUCED 1 (SLW1), respectively, have also been shown to be JA inducible (van de Ven et al., 2000; Walling, 2000). Novel pathways appear to contribute to SLWF defense in crop plants (van de Ven et al., 2000; Walling, 2000). For example, SLW3 transcripts do not accumulate in response to feeding by a closely related whitefly biotype (B. tabaci Type A) or after application of known defense-response chemicals (van de Ven et al., 2000). Although these studies demonstrate the complexity and dynamic interactions between crops and the SLWF, these plants lack the powerful genetic and genomic resources afforded by the plant model system Arabidopsis thaliana. Further studies that examine the role of both the JA and SA-defense pathways in Arabidopsis in response to SLWF are necessary to allow comparisons with aphid-induced responses.

The SLWF is a generalist and infests a wide variety of crop plants including members of the Brassicaceae. Infestations of Brassica oleracea in the field have been reported as high as 10 nymphs per cm² indicating that members of this family are natural hosts for this phloem-feeding pest (Liu, 2000). Therefore, it is timely to harness the genetic resources in the model plant Arabidopsis, a member of the Brassicaceae, to provide insights into the mechanisms that contribute to basal resistance to phloem-feeding whiteflies. Here, a foundation for Arabidopsis responses to SLWF feeding is provided by comparing RNA levels of well-characterized SA-, JA- and ET-defense genes. Here we show that SLWFs induced SA-signaling pathways and suppressed or did not alter expression of JA/ET-regulated genes. To test for the role of these defense pathways in basal resistance, five Arabidopsis SA and JA mutant/transgenic lines (npr1, cim10, coi1, cev1, and NahG) and wild-type Columbia were utilized to monitor SLWF nymphal development and sentinel SA- and JA-defense gene RNAs. These experiments and infestation studies with MeJA-treated npr1 plants demonstrated that basal defenses suppressed by SLWF-feeding were critical for constraining nymphal development.
RESULTS

Regulation of Known SA-, JA-, and ET-defense Transcripts in Response to SLWF Instar Feeding

To date, over 30 defense genes are aligned into complex SA-, JA- and ET-signaling cascades (Glazebrook, 2001; Devoto and Turner, 2003; Shah, 2003). Other defense genes have been identified, but their role or placement in defense signaling has yet to be determined. Transcriptome analysis of SLWF feeding in Arabidopsis thaliana ecotype Columbia has implicated that the SA-dependent pathway is induced, while the JA-dependent pathway shows no change or is repressed (Kempema et al. 2006). These transcript profile studies suggest that Arabidopsis perceives and responds to SLWF more like a pathogen than a tissue-damaging herbivore. Figure 1 summarizes the trends in known defense gene expression gleaned from Kempema et al. (2006).

The microarray data indicated that increases in SA-regulated defense gene RNAs are detected by 28 days after SLWF feeding (Kempema et al., 2006). To assess the timing of defense gene activation in response to SLWF nymph feeding, the levels of two sentinel defense gene RNAs were assessed at 0, 7, 14, 21 and 28 days after SLWF infestation (Fig. 2A). Transcripts for the SA-regulated PATHOGENESIS-RELATED PROTEIN 1 (PR1) gene were first detected at 14 days after infestation and increased to highest levels by 28 days. In contrast, the levels of the JA-regulated PLANT DEFENSIN PROTEIN 1.2 (PDF1.2) RNAs were not detected in control, non-infested or SLWF-infested leaves within the 28-day period.

To confirm the microarray data reported by Kempema et al (2006), RNAs control, non-infested and 21-day SLWF-infested plants from three independent biological experiments were used (Fig. 2B). Transcripts for the SA-regulated genes, PR1, PR5, and β-1,3-GLUCANASE 2 (BGL2; PR2) increased after 21 days of nymph feeding compared to non-infested control plants. The RNAs for genes important in events upstream of SA or for the synthesis of SA, such as SALICYLIC ACID INDUCTION DEFICIENT 2 (SID2), ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5), and PHYTOALEXIN DEFICIENT 4 (PAD4) were also elevated after nymphal feeding (Fig. 2B). These results indicated that, like biotrophic pathogens, the SA-defense pathway was activated. If similar to pathogen-plant interactions, this pathway could have a role in basal defense to SLWFs.
In contrast, RNAs encoded by genes known to be involved in JA biosynthesis, such as OMEGA-3 FATTY ACID DESATURASE (FAD3), or respond to JA, such as PDF1.2, decreased in infested leaves relative to control non-infested leaves (Figs. 1 and 2B). Unlike FAD3 and PDF1.2, THIONIN 2.1 (THI2.1) RNAs were not detected in non-infested controls or after SLWF nymph feeding (Figs. 1 and 2B). Two ethylene-responsive genes were also examined. ETHYLENE RESPONSE SENSOR1 (ERS1) RNA levels declined in SLWF-infested leaves relative to control leaves, while ETHYLENE RESPONSE FACTOR (ERF1) RNA levels were at similar levels in both infested and control leaves (Fig. 2B).

**Local and Systemic Induction of Defense Genes in Response to SLWF Infestation**

To evaluate if Arabidopsis mounts a systemic response to SLWF feeding, the change in SA and JA sentinel gene RNAs were examined both in local infested leaves and apical non-infested leaves (systemic) after a 21-day infestation. RT-PCR with genespecific primers showed that, unlike responses to aphids, the trends identified in SLWF-infested Arabidopsis leaves were also observed in apical, non-infested leaves. SA-regulated gene transcripts (PR1, PR5, and BGL2) accumulated both locally and systemically after nymph feeding (Fig. 3). JA-responsive RNAs (VSP1 and PDF1.2) were not present or were at lower levels in both local and systemic leaves. Collectively, the whole-plant response to SLWF infestation was distinctive from what has been observed with other phloem-feeders in Arabidopsis.

**Repression of JA Responses Enhances SLWF Development**

To assess the role of SA- and JA-signaling pathways in defense against SLWFs, lines with impaired SA (npr1 and NahG) and JA (coi1) signaling were examined (Cao et al., 1994; Feys et al., 1994; Lawton et al., 1995). This was complemented with lines that constitutively activated SA (cim10) and JA (cev1) defenses (Ellis and Turner, 2001; Maleck et al., 2002). Although cim10 is less well-characterized than some mutants that constitutively express SA defenses, it was chosen for these studies because it does not display a dwarf phenotype, nor does it produce the spontaneous lesions that are commonly observed in SA over-expression lines (Maleck et al., 2002).
Mutant and wild-type plants were infested with SLWFs (> 100 nymphs/plant) to assess impacts on nymphal development using a no-choice bioassay (Fig. 4). SLWF development was assayed by scoring the total number of insects at each developmental stage (1st, 2nd, 3rd or 4th instars) on each of the 8 replicate plants. The percent of insects that had reached advanced stages of development (4th instars) by day 24 was calculated and compared between all six lines using a Tukey Multiple Comparison Test (Fig. 4; Supplemental Fig. 1). In addition, to assess defense pathway activation during SLWF feeding, the changes in levels of marker genes PR1, BGL2, PDF1.2, and VSP1 RNAs were monitored in all lines. Most SA- and JA-defense mutants have not been utilized in long-term infestation or infection studies. Therefore, the examination of defense gene transcripts in these defense mutants provided further characterization of both SA- and JA-dependent gene expression at later times in plant development. Some defense genes are expressed at higher basal levels in older plants (Kus et al., 2002). Therefore it was important that the defense gene transcripts were monitored in the mutant non-infested plants and after challenge with SLWFs in order to interpret bioassay results.

At the time of infestation, PR1 RNAs were abundant in non-infested cim10 plants and at lower levels in WT plants, confirming the cim10 constitutive immunity phenotype (Supplemental Fig. 2). After 24 days of infestation, 76% of total insects that developed on cim10 plants were in their fourth instar (Fig. 4; Supplemental Fig. 1). This contrasted to the slower development of SLWF nymphs on WT plants, where approximately 45% of insects were 4th instars. These data indicated that SLWF development was significantly accelerated on the SA-over-expression mutant cim10. Similarly, insects on coi1 mutant plants, which do not perceive JA, showed accelerated development trends (65% 4th instars). After 24 days of infestation, the JA/ET-regulated transcripts PDF1.2 and VSP1 accumulated to lower levels in the cim10 and coi1 plants relative to infested WT plants (Fig. 5). In addition, SA-dependent transcripts (PR1 and BGL2) in cim10 and coi1 mutants were at similar or elevated levels relative to the WT plants. Collectively, these data suggested that either elevated SA and/or reduced JA responses compromise Arabidopsis’ basal resistance to the SLWF, as reflected by enhanced nymphal development.

This hypothesis was further supported by the development rates of SLWF nymphs on the SA mutant lines npr1 and NahG, which impair SA signaling and catabolize SA, respectively. The percent of 4th instars on npr1 and NahG plants was significantly different from WT plants. Only 18% and 16% of SLWF nymphs were in their 4th instar on
npr1 and NahG plants, respectively, when compared to WT plants (Fig. 4). In accordance with this finding, the percentage of SLWFs in their 2nd and 3rd instars rose. In these mutants, the SA-regulated RNAs PR1 and BGL2 accumulated to lower levels than in wild-type in both non-infested and infested leaves and, in a reciprocal fashion, JA-dependent PDF1.2 and VSP1 transcripts increased compared to WT (Fig. 5). These data indicated that by abolishing SA defenses and/or enhancing JA defenses in npr1 and NahG plants enhanced defenses active against SLWF nymphs, as reflected in significant delays in nymphal development, were displayed.

Similarly, on the JA-pathway over-expression mutant cev1 significantly fewer nymphs reached the 4th instar (13%) than on wild-type plants (Fig. 4). Consistent with the constitutive activation of JA defenses in cev1 plants (Ellis and Turner, 2001), the JA-dependent transcripts PDF1.2 and VSP1 accumulated to high levels in uninfested cev1 than WT leaves (Fig. 5). Despite elevated JA defenses, SLWF nymph feeding caused SA transcripts (PR1, BGL2) to accumulate in infested cev1 leaves; in fact, PR1 and BGL2 RNAs accumulated to similar levels in RNA blot analysis in the cev1 mutant and wild type plants (data not shown). PDF1.2 transcripts increased, while VSP1 transcripts decreased after SLWF infestation. Collectively, these data indicated that the SLWF nymphs provided signals that allowed for strong expression of SA-regulated genes and repression of VSP1 in the cev1 mutant. The fact that nymphs feeding on cev1 plants exhibited delayed development relative to WT plants and the SA-pathway gene RNAs accumulated in both cev1 and WT plants suggested that enhanced JA responses, and not SA defenses, were responsible for delaying the development of SLWF nymphs.

As cross-talk between JA and SA defense pathways is commonly associated with responses to biotic threats and displayed in defense mutant studies, it was important to further dissect the relative importance of the suppressed JA and induced SA defenses in SLWF basal resistance. npr1 plants uncouple the cross-talk between SA and JA signaling. For example, during Pseudomonas syringae pv. tomato infection, npr1 plants have reduced levels of SA and PR1 RNAs but JA signaling is preserved (Spoel et al., 2003). Therefore, comparisons of untreated and MeJA-treated npr1 plants should allow the role of JA-regulated defenses to be assessed. npr1 plants were treated with MeJA or served as controls. Unlike previous experiments (Fig. 4), these infestations were performed at 24°C, which significantly accelerated nymphal development (Fig. 6); the temperature dependence of whitefly nymph development is well established (Nava-Camberos et al., 2001). After 17 days of infestation at 24°C, over 74% of the SLWF
nymphs feeding on control npr1 plants were in their 4th instar. Smaller numbers of 1st, 2nd and 3rd instars were also noted in control plants (Fig. 6). In contrast, the MeJA-treated npr1 plants had no 4th-instar nymphs. Nymphs were primarily in their 1st (33%) and 2nd (65%) instars (Fig. 6). MeJA had a dramatic effect on SLWF nymphal development on npr1 plants clearly demonstrating the importance of JA-regulated defenses in basal resistance and curtailing SLWF nymphal development.

DISCUSSION

The SA and/or JA/ET-regulated defense pathways are important in basal and gene-for-gene resistance to pathogens and herbivores. After perception of a biotic threat, plants fine-tune the balance of defense pathways to orchestrate the “best” defense response to its intruder (Reymond and Farmer, 1998; Walling, 2000; Kunkel and Brooks, 2002). The cross-talk between the SA and JA pathways is thought to minimize expression of costly and ineffective defenses that divert C- and N- resources from plant vegetative growth, thereby avoiding compromises to plant vitality and reproduction. This view is supported by the facts that SA-induced defenses are important in the induced basal and gene-for-gene defenses against biotrophic pathogens (Glazebrook, 2005). Similarly, JA-induced defenses confer resistance to necrotrophic pathogens and insects.

Pests and pathogens have leveraged this molecular communication mechanism to enhance their success on host plants (Mudgett, 2005; Chisholm et al., 2006). While some pathogens evade host defenses by actively catabolizing antimicrobial compounds (Bouarab et al., 2002), there is a growing evidence that plant pathogens produce effectors that antagonize defense signaling networks (Hammond-Kosack and Parker, 2003; Kamoun, 2006). The complexity of the arms race between host and attacker is exemplified by Pseudomonas syringae, which uses an array of effectors to suppress expression of defense genes and secondary metabolites, suppress programmed cell death, avoid R gene-mediated resistance, suppress cell wall remodeling, and potentially alter gene expression programs and turnover of defense regulatory proteins (He et al., 2004; Cui et al., 2005; Mudgett, 2005; Chisholm et al., 2006; Janjusevic et al., 2006). By simultaneously evaluating SLWF nymph development on mutants from both SA and JA defense pathways and after exogenous MeJA-treatments, it appears that SLWFs should be added to the set of pathogens and pests that manipulate host-plant defense responses to their own advantage.
SLWF nymphs have an intimate and long-term interaction with their host plants. With the exception of the crawler, which emerges from the egg, SLWF nymphs are immobile and feed almost continuously for approximately 28 days under optimal Arabidopsis conditions. SLWF nymphs provided strong and reproducible signals that were perceived by Arabidopsis resulting in increases in SA-regulated defenses and suppression of JA-regulated defenses (Fig. 2). The accumulation of PR gene RNAs after SLWF feeding in Arabidopsis was SA- and NPR1-dependent, as transcripts did not accumulate to wild-type levels in NahG and npr1 plants (Fig. 5). SA-dependent defense gene RNAs accumulated both in local, infested leaves and systemically in non-infested apical leaves (Fig. 3). Previous studies in squash and tomato also show local and systemic induction of defense genes after SLWF feeding (van de Ven et al., 2000; Walling, 2000). In contrast, systemic activation of defenses was not observed in compatible M. persicae-Arabidopsis and M. euphorbiae-tomato interactions (Moran and Thompson, 2001; Martinez de Ilarduya et al., 2003). This suggests that SLWFs may provide more potent signals, more mobile signals, or larger quantities of signals (due to its prolonged feeding habits) to its host plant.

By using mutant and transgenic lines that alter SA and JA defenses, the branch of Arabidopsis defense signaling that antagonizes SLWF nymph development was identified. There was a strong correlation of SLWF success (as measured by the rate of nymphal development) with the absence of JA defenses and presence of SA defenses (Fig. 4). For example, SLWF nymph development was more rapid on cim10 and coi1 than WT plants (Fig. 4); coi1 and cim10 plants accumulated the SA-regulated PR1 and BGL2 RNAs and displayed reduced JA defenses (PDF1.2 and VSP1 RNAs) (Fig. 5). Reciprocally, cev1, NahG and npr1 mutants had an enhanced basal resistance to SLWFs; the delayed SLWF nymph development was correlated with enhanced JA-regulated defenses in these lines (Fig. 5). The fact that SA-dependent RNAs were abundant in cev1, cim10, and WT plants, but only cev1 displayed an increased basal resistance suggested that JA-dependent defenses, and not SA defenses, were responsible for the delays in nymph development observed on cev1 and cim10 plants (Figs. 4 and 5).

The importance of JA-regulated defenses in basal resistance to SLWFs was also supported by comparing SLWF development on untreated and MeJA-treated npr1 plants. npr1 mutants lack the ability to activate SA defenses (Spoel et al., 2003) and MeJA treatments accentuated the npr1 delay in SLWF nymph development relative to
the untreated *npr1* plants (Fig. 6). Collectively, these data and those reported above indicated that the suppressed JA-regulated defenses were important in slowing SLWF nymphal development. Furthermore, the highly induced SA defenses did not appear to significantly contribute to the basal resistance to SLWFs in Arabidopsis; although SA-dependent defenses may have a role in other aspects of the SLWF-Arabidopsis interaction, such as host choice, fecundity or longevity. These data contrasted to the preferential induction of SA defenses observed in biotrophic pathogen-plant interaction and the importance of SA defenses in both basal and R gene-mediated resistance (Glazebrook, 2005).

The data presented here support the idea that SLWFs enhance their success on Arabidopsis plants by failing to activate or suppressing the effectual JA-regulated defenses. It is possible that SLWFs evade activation of the JA pathway, since SLWFs cause little tissue damage (intracellular punctures) until they establish feeding sites at a minor veins of the phloem (Cohen et al., 1996; Walling, 2000). SLWFs could also prevent the activation of JA defenses by introducing inhibitors that directly or indirectly antagonize JA signaling pathway activation or action. Finally, SLWFs strongly activated SA defenses, even in *cev1* plants. Therefore, it is possible that SLWFs down-regulated the effectual JA defenses via SA cross-talk in WT plants. The SLWF effector(s) that induce SA defenses and/or suppress JA defenses are unknown, but are presumed to be salivary components synthesized by the whitefly or one of its endosymbionts (Walling, 2000). While whitefly saliva is not well characterized biochemically (Funk, 2001), the watery and sheath salivas of other hemipterans, such as aphids, are rich in potential defense signaling molecules including pectinases, complex carbohydrates, proteins, peroxidases, phospholipids, amylases, lipases and/or phosphatases (Miles, 1999; Walling, 2000).

Additional evidence for herbivore manipulation of plant defenses (the “decoy” hypothesis) to enhance insect performance is accumulating from studies with both tissue-damaging herbivores and phloem-feeding aphids (Zhu-Salzman, 2005; Thompson and Goggin, 2006). For example, *Helicoverpa zea* larvae egest saliva containing glucose oxidase (GOX) into their feeding sites to suppress the JA-regulated defenses that deter larval growth (Musser et al., 2002). While GOX uses glucose to produce hydrogen peroxide to activate SA defenses, such as *PR1* protein accumulation, an SA-independent mechanism is responsible for suppression the effectual JA-mediated defenses of tobacco, such as nicotine production (Musser et al., 2002; 2005).
Several studies from the molecular plant-aphid interaction literature also support the “decoy” hypothesis. It should be noted that changes in JA- or SA-defense gene RNA levels and aphid population dynamics on defense mutants have varied, presumably due to the differences in aphid-infestation experimental design (Moran and Thompson, 2001; Moran et al., 2002; De Vos et al., 2005; Mewis et al., 2005; Pegadaraju et al., 2005). In general, rises in PR RNAs have been noted and, like SLWFs, JA-defense gene RNAs are often suppressed or not highly induced after aphid feeding on Arabidopsis (Moran and Thompson, 2001; Ellis et al., 2002; Moran et al., 2002; De Vos et al., 2005). More variation is observed in defense mutant studies. The clear reciprocal phenotypes of SA- and JA-defense mutants, as was seen for SLWFs, have not been documented in the Arabidopsis-aphid literature. While several studies have shown that *M. persicae* population growth is slowed in *cev1*, *npr1* and *NahG* lines or after MeJA treatments (Moran and Thompson, 2001; Zhu-Salzman et al., 2004; Mewis et al., 2005), other studies showed neither NahG, *npr1*, nor *coi1* changed *M. persicae* population dynamics relative to WT plants (Moran and Thompson, 2001; Ellis et al., 2002; Mewis et al., 2005; Pegadaraju et al., 2005).

Given the variability in the aphid-plant interactions studies to date, the simultaneous analyses of five defense mutants were crucial in providing a comprehensive and reproducible picture establishing the importance of JA-regulated defenses in deterring SLWF nymphaal development. While the specific JA-dependent genes important in SLWF defense have yet to be identified, basal defense towards SLWF in Arabidopsis appeared to be antibiotic. Preliminary no-choice egg-deposition and choice bioassays show that SLWF exhibits no preference for any of the mutants altered in constitutive defenses including cell wall composition, secondary metabolites and trichome density (data not shown). Both generalist (*M. persicae*) and specialist (*Brevicoryne brassicae*) aphid interactions with Arabidopsis suggest that JA-dependent defenses have antibiotic effects on aphids (Mewis et al., 2005). However, in tomato, *Mi1.2*-mediated resistance towards SLWF feeding is antixenotic in that it acts to deter the whitefly from establishing a feeding site (Nombela et al., 2003). If SLWFs establish a feeding site on *Mi1.2* plants, nymphs develop similar to plants lacking *Mi1.2*.

If viewed in the broadest terms, the SLWF-Arabidopsis interactions bear a semblance to Arabidopsis interactions with fungal biotrophs like *Erysiphe spp* (Reuber et al., 1998, Kempema et al., 2006); both sets of organisms induce SA-dependent defenses. However, when basal resistance mechanisms are investigated, the SLWF and
fungal biotrophs are distinct. While SA defenses are essential for the basal and induced resistance mechanisms for control of fungal biotrophs, SA-induced defenses did not appear to contribute to the mechanisms that dictate basal resistance to SLWFs. Interestingly, Arabidopsis appeared to mount a completely ineffectual response to SLWF feeding as effective JA-dependent defenses were not induced in WT plants. Further experiments that examine the role of crosstalk, SLWF salivary components and downstream responses will allow identification of elicitor(s) and mechanism(s) for retarding nymphal development, which contributes to the basal resistance in Arabidopsis. The JA-mediated delays in SLWF nymph development could be used in the future to engineer resistance to SLWFs. Delays in insect development are considered important resistance mechanisms impacting insect population dynamics and providing a longer period of time for natural enemies, such as parasitoid wasps, to attack the insects (Pechan et al., 2000; Dicke and Hilker, 2003).

**METHODS**

*Plant Growth and Insect Maintenance*

*Arabidopsis thaliana* ecotype Columbia (wild-type, WT) plants used in the local and systemic defense gene transcript studies (Figs. 1-3) were grown and infested as described in Kempema et al. (2006). Plants used in the bioassays WT, *coronatine insensitive 1-1 (coi1-1), nonexpressor of PR1 (npr1-1), constitutive expression of vsp1 (cev1), constitutive immunity 10 (cim10)*, and transgenic NahG plants were grown in 4-inch pots in Sunshine Mix No. 1 soil (SunGro, Bellevue, WA) supplemented with fertilizer (Osmocote 14-14-14, Scott Horticulture Solutions). All plants were grown under fluorescent and incandescent lights (180 µE m⁻² s⁻¹) at 22°C under long-day (16-hr light: 8-hr dark) conditions for two weeks (with the exception of *cev1*) before infestation for 21 or 24 days under short-day (8-hr light: 16-hr dark) conditions.

Plant size (rosette diameter) influenced SLWF oviposition preference (Kempema, Zarate and Walling, unpublished results). At two weeks, all plants used in these studies had the same rosette diameter and number of leaves at the time of infestation and at the completion of the experiment. Due to its slower growth, *cev1* plants were planted seven days prior to other genotypes to allow an additional week of growth before infestation.
The rosette diameter and number of leaves on three-week-old cev1 plants was approximately the same as two-week-old plants from the other lines.

coi1 plants were identified from a F2 seed pool on ½ Murashige and Skoog medium (10 g L⁻¹ Suc and 0.8% [w/v] agar content) containing 30 µM methyl jasmonate (MeJA)/0.01% ethanol (EtOH) (Bedoukian Research, Danbury, CT). At seven days, homozygous coi1 seedlings were identified by elongated roots and normal above-ground organ morphology (Feys et al., 1994). The coi1 plants were transferred to pots containing soil.

cim10 mutants have WT stature, do not display necrotic lesions and constitutively over express SA and SA-regulated defense genes (Maleck et al., 2002). These features distinguish cim10 relative to other constitutive immunity mutant and made cim10 an excellent choice for SLWF infestations. The levels of PR1 RNAs in cim10 and WT plants were determined using RT-PCR and gene-specific primers in non-infested 2-week-old and 3-week-old plants to confirm the cim10 constitutive immunity phenotype prior to the time of SLWF infestation (Supplementary Fig. 2).

A virus-free silverleaf whitefly colony (B. tabaci type B; Bemisia argentifolii Bellows and Perring) was maintained on Brassica napus var ‘Florida Broad Leaf’ (W. Atlee Burpee & Co., Warminster, PA) grown at 27°C, 55% relative humidity under long-day (16-hr light: 8-hr dark) conditions in the Insectory and Quarantine Facility at the University of California, Riverside. Adults were collected by aspiration. B. napus plants were germinated under the same conditions in a growth chamber for four weeks before being transferred to the Insectory and Quarantine Facility.

**Whitefly Infestations**

Adult male and female whiteflies (totaling 30 to 100 depending on the experiment) were collected from SLWF-infested B. napus leaves by aspiration into 15-ml falcon tubes. A tube containing male and female SLWFs was placed upright in each pot. This number of whiteflies per plant resulted infestation levels similar to infestation levels experienced by Brassica plants in the field (Liu, 2000). Arabidopsis plants typically had > 100 feeding nymphs per plant. Nylon bags (5 by 10 inch) were placed around each pot and secured with a rubber band. Whiteflies were released by unscrewing the falcon tube. Control pots were bagged but not infested. After seven days, all adult flies were removed from the plants by aspiration and the pots re-bagged. For the time-course
experiment, rosette leaves were collected from infested and control plants at 0, 7, 14, 21 and 28 days. Three biological replicates of these experiments were performed. For most defense gene expression studies, rosette leaves were collected after 21 days, when 2\textsuperscript{nd} and 3\textsuperscript{rd} instars were observed on WT Columbia plants. In order to identify the non-infested apical leaves (systemic), leaves were examined under the microscope after 21 days. Leaves were considered “local” if nymphs or eggs were observed on a rosette leaf. Leaves were considered “systemic” if no nymphs or eggs were observed on the rosette leaf. Developmentally matched systemic and local leaves were collected from non-infested plants as controls.

In each no-choice bioassay experiment, eight replicate plants per line were grown and infested as described above. However, adults were removed 2 days after infestation. The number of whiteflies and their developmental stages (1\textsuperscript{st} through 4\textsuperscript{th} instars) were recorded after 24 days. Developmental progression was estimated by calculating the percentage of fourth instars (red-eye stage) on each plant (number of 4\textsuperscript{th} instars/total nymphs). The no-choice bioassays were repeated for a total of 24 replicate plants per line. To assure an unbiased reporting of insect numbers and developmental stages, infested plants were randomly assigned letters to conceal the genotype identity. Immediately after nymphs were counted, infested leaves were placed into liquid N\textsubscript{2} and stored at -80\textdegree C until used for RNA isolation.

**MeJA treatment of npr1 plants**

*npr1* plants were grown under long-day conditions for two weeks at 24\textdegree C. At this time, the growth chambers were changed to short-day conditions and plants were infested with SLWF and/or treated with MeJA. Ten replicate plants were treated with 25 µl per leaf of 100 µM MeJA/0.001% EtOH or 0.001% EtOH three hours prior to infestation and every three days after infestation. Solutions were added to the adaxial side of the leaves where whiteflies tend not to feed or deposit eggs. Plants were caged with nylon bags as previously described and were infested with 30 adult whiteflies. Adults were removed after 2 days of infestation. MeJA-treated and 0.001% EtOH-treated plants were placed in separate but comparable growth chambers (24\textdegree C) to control for volatiles. Because these no-choice experiments were performed at 24\textdegree C, the SLWF nymphs developed more rapidly; the temperature dependence of insect development and specifically SLWF development is well established (Nava-Camberos et al., 2001).
Therefore after 17 days, plants were scored for number of nymphs at each developmental stage as described for the developmental bioassays above. The experiment was replicated twice.

Data Analysis

Defense genes induced or repressed 1.5-fold by microarray analysis were identified by Kempema et al. (2006). Briefly, microarray data was background adjusted using Robust Multiarray Analysis (RMA) and differential analysis performed using Significant Analysis of Microarray (SAM). Data from the no-choice bioassay was analyzed using a one-way unstacked ANOVA and Tukey Multiple Comparison Test with Minitab software (Minitab, State College, PA). Data for the npr1 MeJA treatment experiment was analyzed using Student’s T-Test.

Reverse Transcription and Polymerase Chain Reactions (PCR)

Total RNA was extracted from rosette leaves using TRIzol reagent (Invitrogen, Carlsbad, CA). The quality of the RNA was checked on a 1% agarose denaturing gel (0.5% MOPS, 0.8% formaldehyde). Before the reverse transcriptase reaction, 1 µg of RNA was treated with TURBO DNase as indicated in the manufacturer’s instructions (Ambion, Austin, TX). Oligo dT₂₀ primer (0.5 µg) was added and RNA denatured for 5 minutes at 70°C. Reverse transcription was performed using ImpromII reverse transcriptase and RNasin ribonuclease inhibitor as indicated in the manufacturer’s instructions (Promega, Madison, WI).

Polymerase chain reactions (95°C 5 min, 95°C 35 sec, 55-64°C 35 sec, 72 °C 2 min (20 cycles), 72 °C 10 min final extension time) using ACTIN7 primers were used to check the cDNA synthesis and equalize cDNA amounts between reactions (25 mM MgCl₂, 8 µM forward primer, 8 µM reverse primer, 1 unit Taq polymerase, 8 mM dNTPs). ACTIN7 primers were designed to span an intron to verify that no genomic DNA contamination was amplified during reverse transcription (ACT7/2, At5g09810: forward 5' -CTCATGAAGATTCTCACTGAG-3', reverse 5'-ACAACAGATAGTTCAATTCCCA-3'; genomic 753 cDNA 652 bp). For ACTIN7, 20 PCR cycles were used. For JA/ET- and SA-regulated defense genes, transcripts were amplified using 27 and 25 cycles, respectively, and numbers of cycles are indicated in Figure legends. For the analysis of
PR1 RNAs in the non-infested leaves of cim10 and WT plants, 22 PCR cycles were used (Supplemental Fig. 2). The following primer sequences were designed: PDF1.2, At5g44420: forward 5'-TTCTCTTTGCTGCTTCGAC-3', reverse 5'-GTCATAAAGTTACTCATAGAGTGACAG-3' (258-bp product); THI2.1, At1g72260: forward 5'-TCGATTGCTAAGAGGAAAGTC-3', reverse 5'-GAGGTTTCTCTTTCCAGATTCTTTTC-3' (260-bp product); VSP1, At5g24780: forward 5'-TTTTACGCCAAAGGACTTGC-3', reverse 5'-GTCATAAAGTTACTCATAGAGTGACAG-3' (258-bp product); FAD3, At2g29980: forward 5'-TGACTCCAAGGACTTGC-3', reverse 5'-GTCATAAAGTTACTCATAGAGTGACAG-3' (258-bp product); ERS1, At2g40940: forward 5'-GACTGTTTTCCTCTTTCAACGCAA-3', reverse 5'-GAGGAATGTGCGTTGTGATG-3' (820-bp product); EDS5, At4g39030: forward 5'-TGAGGTAATCTTCAGAGGAGT-3', reverse 5'-TTTGAGCAACCAATCCAACA-3' (520-bp product); PAD4, At3g52430: forward 5'-TTGTCGATTCGAGACGAGTG-3', reverse 5'-TGGCTCGGCTAAGAGTTGAT-3' (1174-bp product). PCR products were fractionated on a 1% agarose, 0.5 X TBE gels. Gels were imaged using LabWorks (UVP Inc., Upland CA) and scanned using Adobe Photoshop 6.0 (San Jose, CA). There were two to three biological replications for all experiments (see above). RT and PCR reactions for all experimental replications were repeated twice.

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LEGENDS

Figure 1. Summary of Arabidopsis defense gene express patterns after SLWF 2nd and 3rd instar feeding. Genes involved in salicylic acid (SA) and jasmonic acid/ethylene (JA/ET) defense signaling and SA and JA biosynthesis pathways are shown as colored boxes. Green and red denote an increase or decrease in RNA levels, respectively (<1.5 fold). Blue indicates no change in expression. Several ethylene receptor genes have been identified including ETR1, ERS1, EIN4, ERS2, ETR2; only ETR1 and ERS1 are illustrated. These gene expression trends are based on microarray studies reported by Kempema et al (2006).

Figure 2. Defense gene transcript accumulation after infestation with SLWF nymphs. Total RNA was extracted from SLWF-infested or control non-infested rosette leaves. Infestations were performed at 22°C. cDNAs were synthesized and used in PCR reactions with gene-specific primers for defense genes involved in SA-dependent pathway (SID2, EDS5, PAD4, PR1, BGL2, PR5; 25 cycles) and JA/ET-dependent pathway (ERS1, ERF1, THI2.1, VSP1, PDF1.2, FAD3; 27 cycles). ACTIN7 was used as a control (20 cycles). A, RT-PCRs were performed on RNAs isolated from SLWF-infested and non-infested plant leaves collected at 0, 7, 14, 21 and 28 days. B, Leaves from 21-d infested (I) and control non-infested (C) plants were collected. RT-PCRs were performed on the RNAs used in the microarray experiments (Kempema et al, 2006) and RNAs from two additional infestation experiments (see Materials and Methods). A representative experiment is displayed.

Figure 3. Local and systemic accumulation of SA- and JA-defense gene RNAs. Infested leaves (local) and non-infested, apical leaves (systemic) from 21-d SLWF-infested plants (I) were collected. Control tissue (C) was collected from developmentally matched leaves on non-infested plants. PCR was performed on cDNA using gene-specific primers (25 cycles for SA-regulated genes and 27 cycles for JA-regulated genes) ACTIN7 was used as a control (20 cycles). Three biological replicate infestations at 22°C were performed. One representative experiment is shown.

Figure 4. SLWF nymph development on mutant, transgenic and WT plants. At 24 dpi, the numbers of total nymphs and nymphs in their 1st (grey), 2nd (dotted), 3rd (white), and
4th (black) instars were counted on wild-type Columbia (WT) and SA and JA signaling mutant/transgenic lines. The percent of insects in each instar was determined for wild-type Columbia (WT) and each SA and JA signaling mutant/transgenic lines. Defense signal mutants and lines are described within Results and include: SA- deficient (npr1-1, NahG), JA-deficient (coi1-1), SA over-expression (cim10), and JA over-expression (cev1) plant lines. Three biological replicate infestations were performed at 22oC and analyzed and a representative experiment is shown. The infestation level and biological variation within the replicate plants of each genotype can be found in Supplemental Fig. 1. Significance of variation in % 4th instars across genotypes was determined using the Tukey Multiple Comparison analysis at the 99.6% individual confidence level. The significance is indicated by: a, b, or c. On average infested plants had approximately 107 nymphs.

**Figure 5.** Local accumulation of defense gene RNAs. RNAs for sentinel SA (PR1, BGL2) and JA (VSP1, PDF1.2) defense-response genes were monitored by RT-PCR using gene-specific primers and 25 and 27 cycles, respectively. cDNAs were synthesized from RNAs from leaves from uninfested (C) mutant and control plants or 24-day infested leaves (I) from mutant and control plants. ACTIN7 was used as a control (20 cycles). Each infested plant had approximately 107 feeding nymphs. Three biological replicate infestations at 22oC were performed. One representative experiment is shown.

**Figure 6.** SLWF development on MeJA-treated or control npr1-1 plants. At 17 dpi, the numbers of nymphs in each instar were counted on npr1-1 plants treated with 100 µM MeJA/0.001% EtOH (diagonal bars) or 0.001% EtOH (black bars; control). The percent nymphs at each developmental stage (1st to 4th instar) was determined. Infestations were performed at 24oC, which accelerated SLWF nymph development. Each infested plant had approximately 225 feeding nymphs. Two biological replicate infestations were performed. One representative experiment is shown.

**Supplementary Figure 1.** Box plot of the percent fourth instar raw data. Minimum and maximum percentage of 4th instars are depicted by black dots, the box signifies the upper and lower quartiles, and the median is represented by a short black line within the box for each plant line. Infestation levels varied from 39 to 255 nymphs per plant; on
average, there were greater than 100 nymphs per plant. This experiment was repeated three times and the representative experiment from Figure 4 is shown to show the variability in individual plants in the SLWF no-choice developmental assay.

**Supplemental Figure 2.** PR1 RNA levels in 2- and 3-week-old cim10 plants. RNAs from leaves of two- and three-week-old cim10 and WT plants were isolated and PR1 and ACTIN7 RNAs were detected after RT-PCR using gene-specific primers and 22 or 20 cycles of PCR, respectively.
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|       | WT  | npr1-1 | NahG  | cim10 | coi1-1 | cev1  |
|-------|-----|--------|-------|-------|--------|-------|
|       | C   | I      | C     | I     | C      | I     | C     | I     | C     | I     |
| PR1   |     |        |       |       |        |       |       |       |       |       |
| BGL2  |     |        |       |       |        |       |       |       |       |       |
| PDF1.2|     |        |       |       |        |       |       |       |       |       |
| VSP1  |     |        |       |       |        |       |       |       |       |       |
| ACT2  |     |        |       |       |        |       |       |       |       |       |
