Pathogen-Triggered Ethylene Signaling Mediates Systemic-Induced Susceptibility to Herbivory in *Arabidopsis*[W]

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Multicellular eukaryotic organisms are attacked by numerous parasites from diverse phyla, often simultaneously or sequentially. An outstanding question in these interactions is how hosts integrate signals induced by the attack of different parasites. We used a model system comprised of the plant host *Arabidopsis thaliana*, the hemibiotrophic bacterial phytopathogen *Pseudomonas syringae*, and herbivorous larvae of the moth *Trichoplusia ni* (cabbage looper) to characterize mechanisms involved in systemic-induced susceptibility (SIS) to *T. ni* herbivory caused by prior infection by virulent *P. syringae*. We uncovered a complex multilayered induction mechanism for SIS to herbivory. In this mechanism, antiherbivore defenses that depend on signaling via (1) the jasmonic acid–isoleucine conjugate (JA-Ile) and (2) other octadecanoids are suppressed by microbe-associated molecular pattern–triggered salicylic acid (SA) signaling and infection-triggered ethylene signaling, respectively. SIS to herbivory is, in turn, counteracted by a combination of the bacterial JA-Ile mimic coronatine and type III virulence-associated effectors. Our results show that SIS to herbivory involves more than antagonistic signaling between SA and JA-Ile and provide insight into the unexpectedly complex mechanisms behind a seemingly simple trade-off in plant defense against multiple enemies.

INTRODUCTION

In both natural and agricultural environments, plants are challenged by numerous pathogens and herbivores, making multiway interactions between plants, pathogens, and herbivorous insects the rule rather than the exception. Bacterial phytopathogens of the genus *Pseudomonas* can spread efficiently across landscapes via the water cycle or insect herbivores (Hirano and Upper, 2000; Morris et al., 2008; Stavrinides et al., 2009; Chung et al., 2013),

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and plants may therefore encounter them early in the growing season. Such early *Pseudomonas* infections affect subsequent plant–pathogen or plant–herbivore interactions, either compromising or aiding the response to a second attacker of similar or different identity (Cui et al., 2002, 2005; Fu and Dong, 2013). With only a few exceptions, most studies have focused on crosstalk that takes place locally in a pathogen-infected or partially consumed leaf, leaving questions about systemic crosstalk between defense response pathways largely unaddressed. However, systemic crosstalk is likely to be more important ecologically than local crosstalk because the entire plant rather than a single leaf is potentially involved in a change in susceptibility to attack. To study the underlying molecular mechanisms mediating the outcome of such complex interactions, we previously analyzed a three-way interaction system consisting of the model genetic plant *Arabidopsis thaliana*, the plant pathogen *Pseudomonas syringae*, and leaf-chewing caterpillars, *Trichoplusia ni* (Lepidoptera: Noctuidae) (Cui et al., 2002, 2005).

Plant defenses are regulated by a multilayered system that is shaped through coevolution of plants and their many enemies (Jones and Dangl, 2006). Plants recognize pathogens that have breached a first layer of constitutive barriers, such as the leaf cuticle, by the binding of conserved microbe-associated molecular pattern (MAMP) epitopes to pattern recognition receptors, which elicits pattern-triggered immunity (PTI). Examples of such epitopes are the synthetic peptides flg22 and elf26, which are synthetic 22- and 26-amino acid peptides derived from bacterial flagellin and elongation factor Tu, respectively (Zipfel et al., 2004, 2006). Pathogens

have evolved arrays of effector proteins to suppress PTI at various stages of the process (Lindeberg et al., 2012). Bacteria such as *P. syringae* inject these effectors into host cells via the type III secretion system. In a subsequent layer of the plant immune system, intracellular surveillance proteins of the nucleotide-binding site-leucine-rich repeat family directly recognize pathogen-encoded effectors or their effects on host factors to elicit effector-triggered immunity (ETI) (Jones and Dangl, 2006).

Downstream of pathogen recognition, the plant immune system is regulated by a hormonal signaling network that for simplicity can be considered to consist of four main signaling sectors that interact to provide specificity to the defense response: (1) salicylic acid (SA)-, (2) jasmonic acid (JA)-, (3) ethylene (ET)-, and (4) PHY-TOALEXIN DEFICIENT4 (PAD4)-mediated signaling (Tsuda et al., 2009). Dependent on the particular mode of attack and the activity of MAMPs, effectors, or other compounds that affect defense signaling, each pathogen generates a specific signal signature (de Vos et al., 2005). In the case of *P. syringae*, this signature is complex because all four main signaling sectors are induced, making the underlying mechanisms mediating crosstalk between them difficult to analyze (de Vos et al., 2005).

Defense against hemibiotrophic pathogens such as *P. syringae* is mediated primarily via SA signaling. SA production via the enzyme isochorismate synthase (ICS1; also referred to as SALICYLIC ACID INDUCTION DEFICIENT2 [SID2]) is stimulated by the lipase-like protein PAD4 (Wildermuth et al., 2001). Downstream SA responses are largely controlled by the transcriptional regulator NON-EXPRESSER OF PATHOGENESIS-RELATED GENES1 (NPR1) (Cao et al., 1994). SA-related signals also move systemically to immunize distal tissue in a process described as systemic acquired resistance (SAR) (Fu and Dong, 2013). Although SAR is generally triggered by effector recognition, it may also be induced by MAMPs (Mishina and Zeier, 2007).

Whereas the SA-signaling sector appears to be the main sector activated by pathogen attack, JA signaling is triggered by both herbivorous insects and necrotrophic fungal pathogens (Glazebrook, 2005). Even though JA is mainly active in an isoleucine-conjugated state (JA-Ile), other octadecanoid JA precursors and derivatives act as signaling molecules in their own right and have both overlapping and distinct roles (Stintzi et al., 2001; Taki et al., 2005; Bruce et al., 2008; Wang et al., 2008a). When triggered simultaneously, SA and JA-Ile signaling mostly counteract each other in a process referred to as SA/JA antagonism (Thaler et al., 2012). Some *P. syringae* strains exploit this antagonism by producing the JA-Ile–mimicking compound coronatine (COR), which weakens both local and systemic SA-mediated defenses (Zheng et al., 2012).

ET signaling functions in concert with JA-Ile or SA to activate particular branches of the defense network and repress others. When signaling in conjunction with JA-Ile, ET-mediated signaling results in the activation of ETHYLENE RESPONSE FACTOR family transcription factors, whereas JA-Ile signaling without the influence of ET leads to activation of MYC family transcription factors. The ETHYLENE RESPONSE FACTOR and MYC branches are mutually antagonistic, primarily activating antifungal and anti-herbivore defenses, respectively (Lorenzo et al., 2004; Fernández-Calvo et al., 2011; Verhage et al., 2011). ET can also have antagonistic effects on SA signaling. For example, ET blocks SA production by repressing transcription of *ICS1/SID2* (Chen et al.,

2009). Indeed, during a virulent *P. syringae* infection, at least two type III effectors, AvrPto and AvrPtoB, induce ET signaling in tomato (*Solanum lycopersicum*), which enhances susceptibility to the bacterial pathogen (Lund et al., 1998; Cohn and Martin, 2005). By contrast, during ETI, when plant immunity is triggered by recognition of pathogen-encoded effectors, the ET-signaling sector functions synergistically with the SA and other signaling sectors to robustly activate antimicrobial responses (Tsuda et al., 2009).

PAD4 is involved in regulating at least three important aspects of the plant immune signaling network. First, in association with its signaling partner ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), it regulates hormone-dependent and -independent signaling during PTI and ETI (Feys et al., 2001; Rustérucci et al., 2001; Rietz et al., 2011). Second, PAD4 stimulates SA production as part of a positive feedback loop that also includes ICS1/SID2 (Zhou et al., 1998; Jirage et al., 1999). Third, full production of ET after a plant is subjected to pathogen infection or other stresses relies on PAD4 (Heck et al., 2003; Mühlenbock et al., 2008). Regulation of these three processes is essential for the biosynthesis of sufficient antimicrobials during infection (Glazebrook et al., 1997).

In addition to the four main signaling sectors mediated by SA, JA-Ile, ET, and PAD4, the plant immune signaling network also receives inputs from other hormones, such as abscisic acid, gibberellic acid, brassinosteroid, cytokinin, and auxin, which have traditionally been studied in the context of plant growth and development and the response to abiotic stresses (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). Examples of *P. syringae* effectors that modify hormone signaling to suppress SA-mediated defenses are AvrPtoB, which modulates abscisic acid signaling (de Torres-Zabala et al., 2007), and AvrRpt2, which activates auxin signaling (Chen et al., 2007).

Here, we use the *Arabidopsis–P. syringae–T. ni* model system to investigate mechanisms behind systemic crosstalk in three-way plant pathogen–herbivore interactions. Previously, we found that infection of *Arabidopsis* lower rosette leaves with *P. syringae* pv *maculicola* (*Psm*) ES4326 triggers systemic-induced susceptibility (SIS) to herbivory by *T. ni* in the upper rosette leaves. Surprisingly, we found that SIS to herbivory is independent of SA signaling because SIS was still present in the SA-signaling mutant *npr1-1* and in SA-deficient transgenic *nahG* plants (Cui et al., 2002). In addition, we found that elicitation of SIS to herbivory was counteracted by COR produced by *P. syringae* (Cui et al., 2005) or by ETI triggered by *P. syringae* expressing the type III effectors AvrRpt2 or AvrB (Cui et al., 2002). Because SIS to herbivory is counteracted by COR and does not rely on SA signaling, we concluded that *P. syringae*–elicited susceptibility to *T. ni* occurs independently of SA/JA antagonism. This was a surprising conclusion since the limited number of previous studies in this area pointed at an important role for SA/JA antagonism in regulating the outcomes of crosstalk in three-way plant pathogen–herbivore interactions (Felton et al., 1999; Thaler et al., 2010).

In this study, we show that plant infection with COR-deficient *Psm* ES4326 *cfa6* leads to systemic transcriptional changes in the ET-, PAD4-, and EDS1-signaling sectors. Genetic analysis showed that ET signaling is both necessary and sufficient for pathogen-triggered SIS to herbivory. This is congruent with our previous results showing that SIS depends on PAD4 (Cui et al., 2002), as PAD4 is essential for boosting ET production after

pathogen infection and other stresses (Heck et al., 2003; Mühlenbock et al., 2008). We propose a multilayered induction mechanism underlying SIS to herbivory. In the first layer, MAMPs trigger SIS to herbivory dependent on SA/JA antagonism, which can be neutralized by COR and the action of certain type III bacterial effectors that stimulate signaling through the JA-Ile receptor complex containing CORONATINE INSENSITIVE1 (COI1) (He et al., 2004). In a subsequent layer, other type III effectors trigger SIS to herbivory by a mechanism that relies on ET and interferes with octadecanoid-dependent but not COI1-dependent defenses. Together, the results provide insight into the unexpectedly complex mechanisms behind a seemingly simple trade-off in plant defense against multiple attackers.

RESULTS

Pathogen-Triggered SIS to *T. ni* Requires PAD4 but not SA or JA Signaling

Even though their baseline resistance is greater than wild-type plants, pathogen-triggered SIS to *T. ni* herbivory is present in the *nahG* and *npr1-1* plants, suggesting that SIS is independent of SA signaling (Cui et al., 2002). To collect additional data to test this hypothesis more thoroughly, we tested the *sid2-2* mutant, which is deficient in SA biosynthesis. Since COR counteracts SIS to herbivory (Cui et al., 2005), we infiltrated *sid2-2* plants with COR-deficient *Psm* ES4326 *cfa6* to enhance the magnitude of the SIS phenotype. We found that infiltration of *Psm* ES4326 *cfa6* in the *sid2-2* mutant induced SIS to *T. ni* herbivory similar to wild-type plants (Figure 1A). By contrast, SIS was absent in a *pad4-1* mutant, confirming our earlier finding that SIS to herbivory relies on signaling through PAD4, but not SA (Figure 1A).

The fact that pathogen-triggered SIS to herbivory is independent of SA signaling suggests that it does not rely on SA/JA antagonism, which involves JA-Ile that is perceived via COI1. Indeed, although the *coi1-1* mutant was more susceptible to *T. ni* because JA-mediated defenses are severely compromised, we found that *Psm* ES4326 *cfa6* nevertheless triggered SIS to herbivory in the *coi1-1* mutant (Figure 1B). These data strongly suggest that pathogen-triggered SIS to herbivory is not a consequence of SA/JA antagonism, as the latter relies on functional SA and COI1-mediated JA signaling.

Psm ES4326 *cfa6* Induces Transcriptional Changes Associated with ET Signaling in Systemic Tissue

We performed whole-genome transcriptional profiling on systemic leaves of *Arabidopsis* plants infected with *Psm* ES4326 *cfa6* versus mock-inoculated plants to identify candidate genes that could underlie SIS to herbivory. The plants used in the expression profiling analysis exhibited SIS to herbivory when infiltrated with *Psm* ES4326 *cfa6* (similar to that shown for Columbia-0 [Col-0] in Figure 1A). We profiled the transcriptome at three time points representing early (3 h), intermediate (48 h), and late (96 h) stages of bacterial infection in our experimental setup. The 48-h time point has previously been identified as a stage where significant changes in gene expression and plant defense phenotype
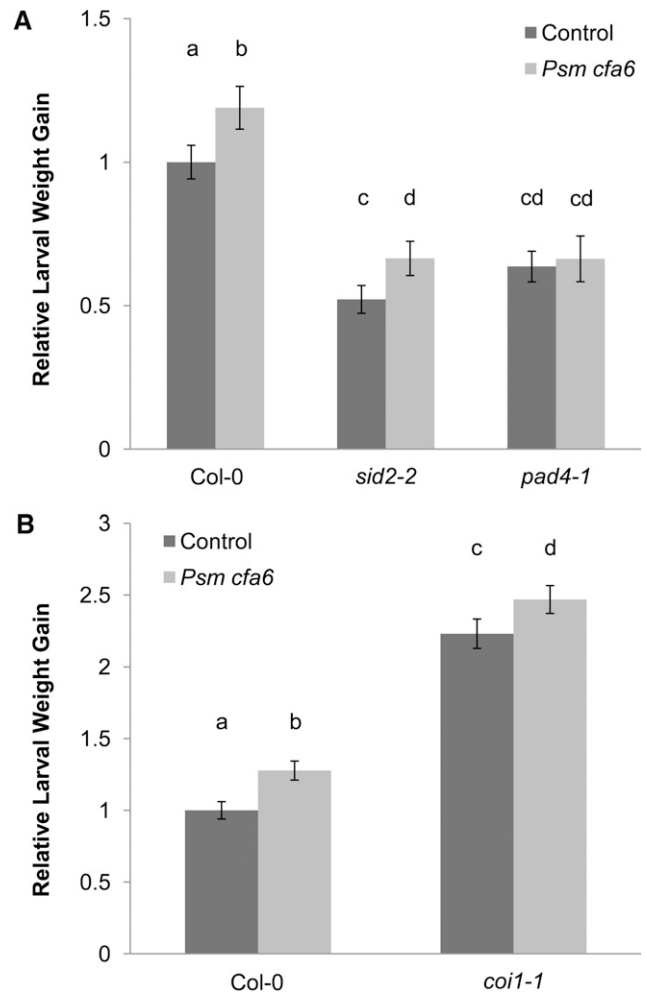

Figure 1. PAD4 Is Involved in Pathogen-Triggered SIS to Herbivory Independent of SA Production and the COI1 JA-Signaling Module.

Lower *Arabidopsis* rosette leaves were inoculated with a sterile 10 mM MgSO$_4$ control solution (dark-gray bars) or *Psm* ES4326 *cfa6* suspension (OD$_{600}$ = 0.2; light-gray bars). Four days later, the inoculated leaves were removed and newly hatched *T. ni* larvae were placed on the upper rosette leaves. Larval weight gain was measured after 7 d of feeding. For each experiment, weight gain data were normalized to the weight gain of larvae feeding on mock-inoculated wild-type (Col-0) plants. The bars represent the means (±SE) of relative weight gain from two independent experiments with n = 36 larvae per treatment per experiment.
(A) Relative larval weight gain in wild-type (Col-0), *sid2-2*, and *pad4-1* plants (ANOVA; treatment, $P < 0.05$; genotype, $P < 0.05$; treatment × genotype, $P < 0.05$).
(B) Relative larval weight gain in wild-type (Col-0) and *coi1-1* plants (ANOVA; treatment, $P < 0.05$; genotype, $P < 0.05$, treatment × genotype, not significant). Letters above the bars signify statistically significant differences among groups (Tukey, $P < 0.05$).

can be observed in systemic rosette leaves after infection of lower rosette leaves with *Psm* ES4326 (Mishina and Zeier, 2007).

Three independent biological RNA replicates per time point were individually hybridized to Affymetrix ATH1 GeneChips. For each probe set, signal intensities, mean expression fold change,

and P values were calculated after data normalization. Because changes in gene expression levels in the systemic rosette leaves were relatively modest following *Psm* ES4326 *cfa6* infiltration of lower rosette leaves (see Supplemental Table 1 online), we searched for differentially expressed genes without using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995) or a fold change cutoff. Lists of differentially expressed genes at the 3-, 48-, and 96-h time points are given in Supplemental Data Set 1 online. The maximum number of differentially expressed genes at $P < 0.05$ identified in this way in *Psm* ES4326 *cfa6*-infected plants compared with mock-inoculated controls was observed at the 48-h time point (see Supplemental Table 1 online), and we focused on this time point for further analysis. A primary objective of the transcriptional profiling was to determine whether any of the canonical plant hormonal and defensive signaling sectors could be involved in the elicitation of SIS to *T. ni* herbivory. To this end, we conducted a more detailed analysis of the data for the 48-h time point in which we only considered candidate genes involved in plant hormone biosynthesis or hormonal regulation of immune signaling based on The Arabidopsis Information Resource annotation (www.arabidopsis.org; see Supplemental Data Set 2 online). We found that many genes of interest (see below) were in the top 5% of those differentially regulated or were among the lowest 5% in terms of uncorrected P values (see Supplemental Data Set 2 online).

At the 48-h time point, several genes regulated by JA-Ile were repressed, including *PDF1.2b* and *CHI-B* (not listed in Supplemental Data Set 2 online as these are marker genes and not actively involved in hormonal regulation of immune signaling), and the DELLA protein-encoding *RGL3* (see Supplemental Table 2 online). The repressor of gibberellic acid–signaling RGL3 is necessary for full JA responses (Wild et al., 2012). By contrast, the ET precursor 1-aminocyclopropane-1-carboxylate (ACC) synthase-encoding gene *ACS2*, which mediates a rate-limiting step in ET production (Liu and Zhang, 2004) and whose transcriptional regulation correlates with enhanced pathogen-induced ET biosynthesis (Li et al., 2012), and *EOL2*, which encodes a negative regulator of ACS5, were induced 48 h after inoculation (see Supplemental Table 2 online). This could represent a switch from ACS5 to ACS2 as the main ACS involved in ET production, which has been previously observed after wounding (Tsuchisaka and Theologis, 2004). Two additional genes that were repressed at the 48-h time point were *NUDT7*, which encodes a hydrolase that negatively regulates SA-independent EDS1- and PAD4-mediated signaling (Bartsch et al., 2006), and the mitogen-activated protein kinase kinase kinase–encoding gene *MEKK1* (see Supplemental Table 2 online). MEKK1 is involved in the activation of MPK4, a negative regulator of EDS1 and PAD4 (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007).

The transcriptional profiling data showed that in plants that exhibited SIS to herbivory, very few differentially expressed genes could be identified in the systemic leaves, at least using Affymetrix GeneChip technology. Although the data were not sufficiently robust to correlate specific hormone signaling pathways with SIS, they suggested that ET, PAD4, and EDS1 signaling might be systemically affected following infection of lower rosette leaves with *Psm* ES4326 *cfa6*. Because (1) ET, PAD4, and EDS1 are associated with susceptibility to tissue-chewing insect herbivores (Kahl et al., 2000; Stotz et al., 2000; Winz and Baldwin,

2001; Cui et al., 2002), (2) ET production and transcription of PAD4 and EDS1 are induced by *P. syringae* and/or associated with leaf senescence in local, infected tissue (Bent et al., 1992; Magalhaes et al., 2000; Huang et al., 2005; Wang et al., 2008b; Mur et al., 2009), and (3) pathogen-triggered SIS to herbivory relies on PAD4 (Figure 1A), we hypothesized that SIS to herbivory may depend on ET signaling, and this prompted us to test a variety of ET-related signaling mutants for their ability to display the SIS to herbivory phenotype.

Pathogen-Triggered SIS to Herbivory Depends on ET Signaling

To test the hypothesis that pathogen-triggered SIS to herbivory depends on ET signaling, we phenotyped mutants with reduced and enhanced ET signaling. Indeed, although the *ein2-1* and *etr1-3* mutants, which are disrupted in ET signaling, were more resistant to *T. ni* herbivory than wild-type plants, we found no SIS to herbivory in these mutants (Figure 2A). Given that the *ein2-1* and *etr1-3* mutants were more resistant to *T. ni*, in agreement with the published data demonstrating that ET suppresses antiherbivore defense (Kahl et al., 2000; Stotz et al., 2000; Winz and Baldwin, 2001), we reasoned that the *eto1-1* mutant, which overproduces ET, would be more susceptible to *T. ni*, which was indeed the case (Figure 2A). Moreover, infiltration of *eto1-1* with *Psm* ES4326 *cfa6* did not induce systemic susceptibility, again showing that ET represses systemic antiherbivore defense in response to bacterial infection (Figure 2A). To confirm that ET signaling has systemic effects and can elicit susceptibility to *T. ni* in distal leaves, we treated three lower rosette leaves of wild-type or *ein2-1* mutant plants with the ET precursor ACC. As expected, ACC elicited SIS to herbivory in wild-type plants but not in the ET-insensitive *ein2-1* mutant (Figure 2B), showing that local induction of ET production, which has been observed previously after virulent *P. syringae* infection and associated senescence (Bent et al., 1992; Magalhaes et al., 2000; Huang et al., 2005; Mur et al., 2009), is sufficient to block antiherbivore defenses systemically.

ET Regulates Octadecanoid-Dependent Defenses Independently of COI1

SIS to herbivory does not rely on the core COI1 JA-signaling component (Figure 1). However, other octadecanoids (including JA unconjugated from Ile and other JA precursors or derivatives) also regulate antiherbivore defenses independently of COI1 (Stintzi et al., 2001; Schweizer et al., 2013). Production of JA and its precursor 12-oxo-phytodienoic acid (OPDA) is disrupted in the *aos* mutant, which is defective in the enzyme ALLENE OXIDE SYNTHASE (AOS; CYP74A) (Park et al., 2002). As expected, the *aos* mutant and the *coi1-1* mutant were highly susceptible to *T. ni* (Figure 3). However, in contrast with the *coi1-1* mutant (Figure 1B), *Psm* ES4326 *cfa6* did not induce further susceptibility in *aos* plants (Figure 3), suggesting that repression of the activity of JA precursors or derivatives other than JA-Ile contribute to SIS to *T. ni*.

ET signaling is required for SIS to *T. ni* and ET-signaling mutants are much more resistant to herbivory (Figure 2), presumably because they have a high level of JA-mediated signaling (Adie et al.,
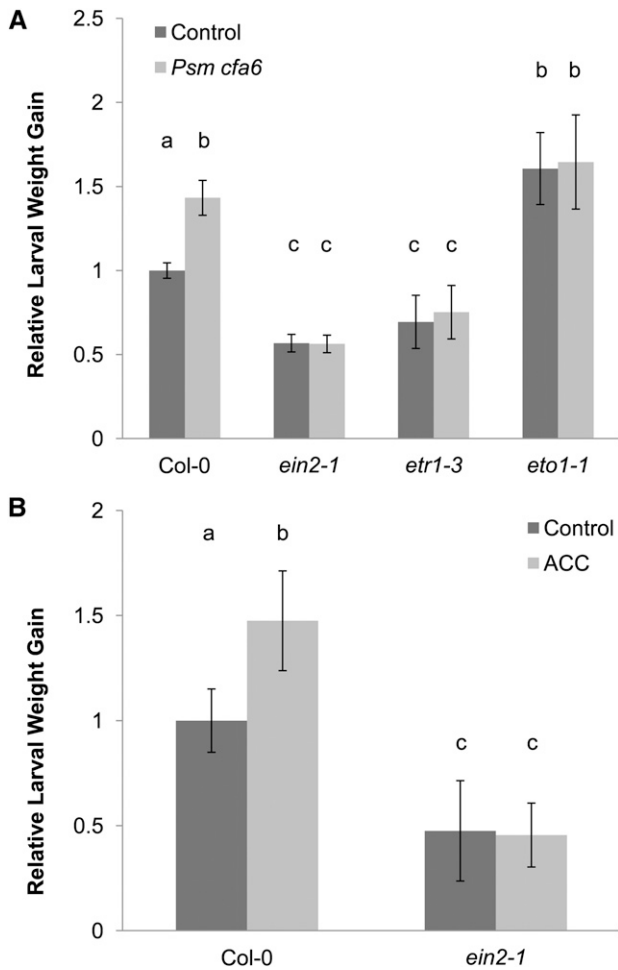
Figure 2. Pathogen-Triggered SIS to Herbivory Is Mediated via ET Signaling.

Lower *Arabidopsis* rosette leaves were inoculated with a control solution (dark-gray bars), *Psm* ES4326 *cfa6* suspension (OD_{600} = 0.2; light-gray bars; **[A]**), or a 100 μM solution of the ET precursor ACC (light-gray bars; **[B]**), respectively. Four days later, the inoculated or treated leaves were removed and newly hatched *T. ni* larvae were placed on the upper rosette leaves. Larval weight gain was measured after 7 d of feeding. For each experiment, weight gain data were normalized to the weight gain of larvae feeding on mock-inoculated **(A)** or control-treated **(B)** wild-type (Col-0) plants. The bars represent the means (±SE) of relative weight gain from two independent experiments with n = 36 larvae per treatment per experiment. **(A)** Relative larval weight gain in wild-type (Col-0), *ein2-1*, *etr1-3*, and *eto1-1* plants (ANOVA; treatment, P < 0.05; genotype, P < 0.05; treatment × genotype, P < 0.05). **(B)** Relative larval weight gain in wild-type (Col-0) and *ein2-1* plants (ANOVA; treatment, P < 0.05; genotype, P < 0.05; treatment × genotype, P < 0.05). Letters above the bars signify statistically significant differences among groups (Tukey, P < 0.05).

2007). If this interpretation is correct, we reasoned that the enhanced susceptibility of the *aos* mutant would be epistatic to the enhanced resistance of the *ein2-1* mutant. Indeed, disruption of *AOS* (as in the *aos* mutant allele) in an *ein2-1* background completely rescued the enhanced resistance to herbivory of the

ein2-1 mutant and even elevated susceptibility to *T. ni* herbivory to a level indistinguishable from that seen in the *aos* single mutant (Figure 3). Moreover, as in the *ein2-1* and *aos* single mutants, pathogen-triggered SIS to herbivory was absent in an *aos ein2-1* double mutant (Figure 3), strengthening the conclusion that ET is a negative regulator of octadecanoid-dependent, but COI1-independent, antiherbivore defense signaling.

MAMP-Triggered SIS to Herbivory Depends on SA/JA Antagonism, Not on ET Signaling

MAMPs have previously been shown to induce both ET production and SAR to subsequent infection with virulent *P. syringae* (Navarro et al., 2004; Mishina and Zeier, 2007). We hypothesized that MAMPs might induce an ET response strong enough to elicit SIS to herbivory. Because expression of the flagellin (flg22) receptor FLS2 depends on ET signaling (Boutrot et al., 2010; Mersmann et al., 2010), we primarily used treatment with the MAMP peptide elf26 in these experiments. Expression of the elf26 receptor EFR does not depend on ET signaling (Tintor et al., 2013). Instead of being infiltrated with bacteria, three lower rosette leaves were treated with 10 μM elf26 or flg22, and 4 d later *T. ni* larvae were added to systemic rosette leaves of the plants, after clipping off the infiltrated (local) leaves. Surprisingly, SIS to
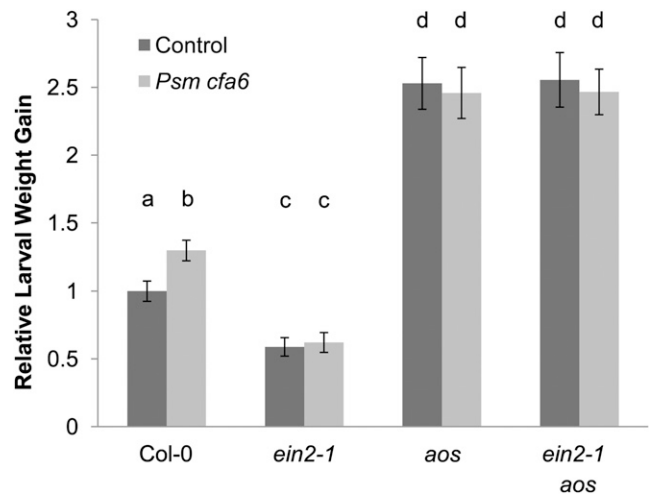

Figure 3. During Pathogen-Triggered SIS to Herbivory, ET Regulates Octadecanoid-Dependent, JA-Ile–Independent Defenses.

Lower *Arabidopsis* rosette leaves were inoculated with a sterile 10 mM $MgSO_4$ control solution (dark-gray bars) or *Psm* ES4326 *cfa6* suspension (OD_{600} = 0.2; light-gray bars). Four days later, the inoculated leaves were removed and newly hatched *T. ni* larvae were placed on the upper rosette leaves. Larval weight gain was measured after 7 d of feeding. For each experiment, weight gain data were normalized to the weight gain of larvae feeding on mock-inoculated wild-type (Col-0) plants. The bars represent the means (±SE) of relative weight gain from two independent experiments with n = 36 larvae per treatment per experiment. Relative larval weight gain in wild-type (Col-0), *ein2-1*, *aos*, and *ein2-1 aos* plants (ANOVA; treatment, P < 0.05; genotype, P < 0.05; treatment × genotype, P < 0.05). Letters above the bars signify statistically significant differences among groups (Tukey, P < 0.05).

herbivory was observed in *ein2-1* but not in *sid2-2* after treatment with elf26 (Figure 4A). Likewise, SIS to herbivory did not occur in *sid2-2* mutants after treatment with flg22 (see Supplemental Figure 1 online). In agreement with the dependency of FLS2 accumulation on functional ET signaling, SIS to herbivory did not occur in flg22-treated *ein2-1* mutants either (see Supplemental Figure 1 online).

The finding that MAMP-triggered SIS to herbivory (in contrast with pathogen-elicited SIS to herbivory) depends on SA but not ET signaling suggests that it may depend on SA/JA antagonism. SA/JA antagonism depends on functional COI1 and NPR1. When *aos*, *coi1-1*, and *npr1-1* mutants were treated with 10 μM elf26, MAMP-induced SIS to herbivory was absent in *aos*, *coi1-1*, and *npr1-1*, whereas it was present in wild-type plants (Figure 4B). Because pathogen-triggered SIS to herbivory does not rely on functional COI1 (Figure 1B) and NPR1 (Cui et al., 2002), these results indicate that pathogen-triggered SIS to herbivory and MAMP-triggered SIS to herbivory rely on different mechanisms. Pathogen-triggered SIS to herbivory relies on ET and octadecanoid signaling but is independent of COI1, SA signaling, and NPR1. By contrast, MAMP-triggered SIS to herbivory relies on SA/JA antagonism and depends on functional COI1 and NPR1.

Pathogen-Triggered SIS to Herbivory Is Mediated by Pathogen Effectors

Having established that pathogen-triggered SIS to herbivory is not mediated solely by MAMPs, we hypothesized that bacterial type III effectors might elicit SIS to herbivory. Type III effectors are possible candidates because they enhance pathogen virulence (if they or their actions are not recognized by a corresponding nucleotide-binding site-leucine-rich repeat resistance receptor) by interfering with PTI-mediated signaling, in some cases suppressing SA or stimulating ET signaling (Cohn and Martin, 2005; de Torres-Zabala et al., 2007; Jelenska et al., 2007). This hypothesis is consistent with our results showing that pathogen-triggered SIS to herbivory relies on ET but not on SA signaling. Moreover, the finding that hemibiotrophic pathogens activate ET signaling, which may enhance pathogen proliferation and disease symptoms, suggests some manipulation of the interaction by the pathogen, which could be achieved through the action of pathogen-encoded effectors (Lund et al., 1998; Magalhaes et al., 2000; Cohn and Martin, 2005; Huang et al., 2005; Chen et al., 2009; Mur et al., 2009; Wi et al., 2012).

To study the role of pathogen-encoded effectors in SIS to *T. ni* herbivory, we inoculated wild-type, *ein2-1*, and *npr1-1* plants with *P. syringae* strains that were unable to produce COR (*cfa6 cmaA* mutants) or unable to inject type III effectors into plant cells in addition to being COR deficient (*hrcC cfa6 cmaA* mutants) or with the isogenic wild-type *P. syringae* pv *tomato* (*Pto*) DC3000 parent. In these experiments, we used *Pto* DC3000 instead of *Psm* ES4326 so we could further test if pathogen-triggered SIS to herbivory is elicited by more than one virulent *P. syringae* strain. We found that infiltrating COR-deficient *Pto cfa6 cmaA* into wild-type plants elicits SIS to herbivory (see Supplemental Figure 2 online), similar to *Psm* ES4326 *cfa6* (Cui et al., 2005). Compared with the COR-deficient mutant *Pto cfa6 cmaA*, wild-type *Pto* DC3000 elicited lower levels of SIS in wild-type plants (see
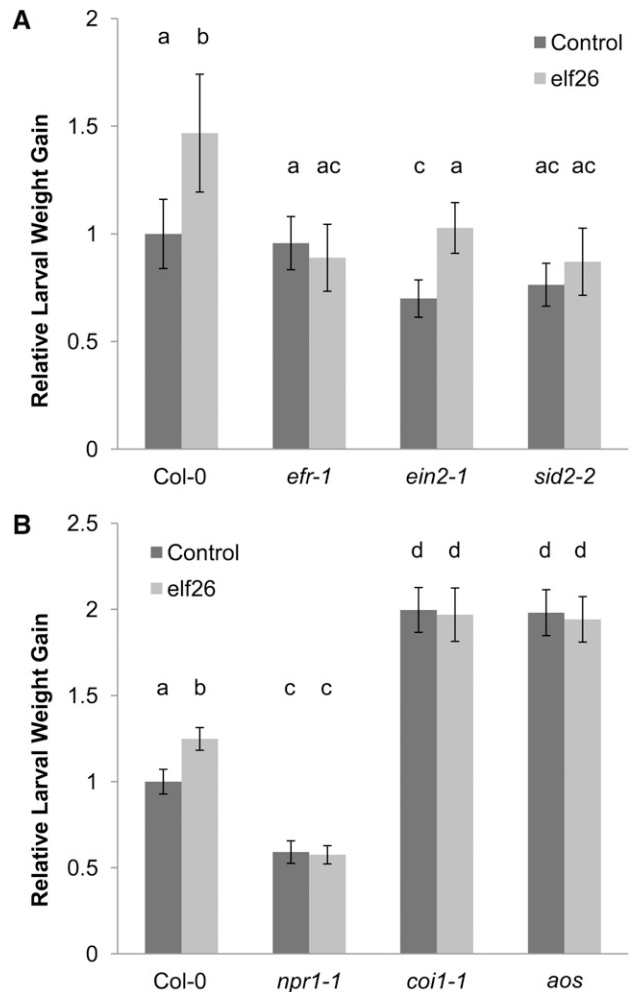

Figure 4. MAMP-Triggered SIS to Herbivory Depends on SA/JA Antagonism, not ET Signaling.

Lower *Arabidopsis* rosette leaves were inoculated with a control solution (dark-gray bars) or suspensions of 10 μM elf26 (light-gray bars). Four days later, the treated leaves were removed and newly hatched *T. ni* larvae were placed on the upper rosette leaves. Larval weight gain was measured after 7 d of feeding. For each experiment, weight gain data were normalized to the weight gain of larvae feeding on control-treated wild-type (Col-0) plants. The bars represent the means (±SE) of relative weight gain from two independent experiments with $n = 36$ larvae per treatment per experiment.
(A) Relative larval weight gain in wild-type (Col-0), *efr-1*, *ein2-1*, and *sid2-2* plants (ANOVA; treatment, $P < 0.05$; genotype, $P < 0.05$; treatment × genotype, $P < 0.05$). Letters above the bars signify statistically significant differences among groups (Tukey, $P < 0.05$).
(B) Relative larval weight gain in wild-type (Col-0), *npr1-1*, *coi1-1*, and *aos* plants (ANOVA; treatment, $P < 0.05$; genotype, $P < 0.05$; treatment × genotype, $P < 0.05$). Letters above the bars signify statistically significant differences among groups (Tukey, $P < 0.05$).

Supplemental Figure 2 online), again similar to our previous observations on plants infected with wild-type *Psm* ES4326 compared with *Psm* ES4326 *cfa6* (Cui et al., 2005) and consistent with the fact that COR induces JA signaling, which activates antiherbivore defenses (Cui et al., 2005).

By testing these *Pto* mutants on wild-type, *ein2-1*, and *npr1-1* *Arabidopsis*, we were able to separate the effects of MAMPs and effectors. COR-deficient *Pto cfa6 cmaA*, which is still able to employ type III effectors, elicited SIS in *npr1-1* but not *ein2-1* plants (see Supplemental Figure 2 online). By contrast, the *Pto hcC cfa6 cmaA* mutant, which also has a disrupted type III se-cretion system due to the *hrcC* null mutation, triggered slightly enhanced susceptibility in *ein2-1* but not in *npr1-1* plants (see Supplemental Figure 2 online). These results suggest that type III effectors suppress SA signaling, which interferes with COI1-dependent antiherbivore defenses via SA/JA antagonism, and trigger ET signaling, which interferes with COI1-independent antiherbivore defenses. In line with the result in wild-type plants, when COR was added to the interaction by infiltrating plants with wild-type *Pto* DC3000, SIS to herbivory was diminished in *npr1-1* plants and weak resistance to herbivory was induced in *ein2-1* plants (see Supplemental Figure 2 online).

In *ein2-1*, the weak additional resistance to *T. ni* on *Pto* DC3000–infiltrated plants and the slightly induced susceptibility to *T. ni* on *Pto hcC cfa6 cmaA* resulted in a significant difference in sus-ceptibility to herbivory between these two treatment groups (see Supplemental Figure 2 online), in line with results of previous experiments showing a resistance-enhancing role for COR (Cui et al., 2005) and a susceptibility-enhancing role for MAMPs (Figure 4). The result that *Pto hcC cfa6 cmaA* did not induce significant SIS on wild-type and *ein2-1* plants (see Supplemental Figure 2 online), but treatment of plants with the MAMP elf26 did (Figure 4), may be explained by the fact that this *P. syringae* mutant does not reach as high a titer as wild-type *Pto* DC3000 and the signaling it induces is simply not strong enough. Taken together, our data support a role for type III effectors suppressing MAMP-triggered SIS to herbivory by inhibiting SA signaling and simultaneously eliciting pathogen-triggered SIS to herbivory by suppressing COI1-independent antiherbivore defenses via ET signaling.

DISCUSSION

A Multilayered Induction Mechanism behind SIS to Herbivory

Using a combination of *Arabidopsis* and *P. syringae* mutants in sensitive *T. ni* feeding assays, we uncovered a multilayered mech-anism behind pathogen-triggered SIS to herbivory. Although not yet biochemically validated, our genetic studies lead to a new model for SIS to herbivory described in Figure 5. This model resembles the zigzag model illustrating the quantitative output of plant immune signaling in response to microbial attack (Jones and Dangl, 2006). In one layer, MAMP-triggered systemic SA signaling an-tagonizes COI1-dependent JA signaling via SA/JA antagonism that relies on NPR1, thereby weakening antiherbivore defenses and causing SIS to herbivory. This is neutralized in a second layer where SA signaling is suppressed by COR and some type III effectors that interfere with MAMP-triggered signaling. Additional-ly, enhanced ET signaling, also mediated by effectors, interferes with antiherbivore defenses that rely on octadecanoids produced via AOS but that function independent of COI1 (Stintzi et al., 2001; Schweizer et al., 2013), leading again to SIS to herbivory. When
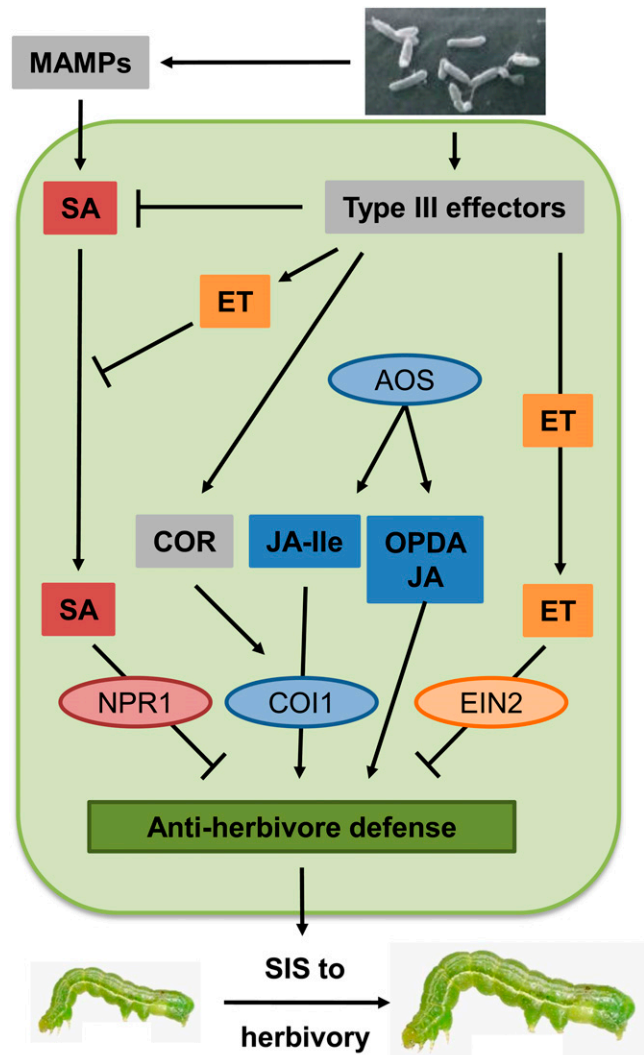

Figure 5. Simplified Model of Pathogen-Triggered SIS to Herbivory.

In the first layer, MAMP-triggered SA signaling (red) antagonizes COI1-dependent JA signaling (blue) via SA/JA antagonism that relies on NPR1, thereby weakening antiherbivore defenses and causing SIS to herbivory. This is neutralized in the next layer where SA signaling is suppressed by COR and some type III effectors that interfere with MAMP-triggered signaling. At the same time, enhanced ET signaling (orange), also me-diated by type III effectors, interferes with antiherbivore defenses that rely on octadecanoids produced via AOS, but that function independent of COI1, leading again to SIS to herbivory. When one of the type III ef-fectors elicits ETI, the induced susceptibility is nullified and effector-triggered signaling synergizes with COR to induce systemic resistance to herbivory. *P. syringae*–associated factors are indicated in gray squares, *Arabidopsis* hormones in colored squares, and *Arabidopsis* proteins in colored ellipses. (*T. ni* photo courtesy of Nestor Bautista Martínez.)

one of the type III effectors elicits ETI, the induced susceptibility is nullified and effector-triggered signaling synergizes with COR to induce systemic resistance to herbivory (Cui et al., 2002, 2005).

The dominant role of effector-mediated ET signaling over MAMP-triggered SA signaling in SIS to herbivory is consistent

with previous studies, which found that ET suppresses transcription of the SA biosynthesis enzyme ICS1 (SID2) (Chen et al., 2009) and overrides NPR1-mediated SA-JA antagonism (Leon-Reyes et al., 2009, 2010). The involvement of ET signaling in SIS to herbivory may also explain our earlier observation that variability exists in the strength of the SIS to herbivory phenotype dependent on environmental circumstances (Cui et al., 2005). ET levels respond to environmental cues such as light, water, and temperature. However, while ET signaling is both necessary and sufficient for pathogen-triggered SIS to herbivory, signaling by other hormones in addition to ET could potentially be involved in SIS. Type III effectors, such as HopI1, which actively perturbs SA biogenesis (Jelenska et al., 2007), AvrRpt2, which activates auxin signaling (Chen et al., 2007), and AvrPtoB, which modulates abscisic acid signaling (de Torres-Zabala et al., 2007), all interfere with SA-regulated pathogen defenses and could also influence antiherbivore defenses. The contributions of individual type III effectors and plant hormones other than ET should be investigated in future studies.

Virulent Hemibiotrophic Pathogens May Generally Induce ET Production

Our findings contribute to a growing body of literature showing that virulent hemibiotrophic pathogens actively suppress plant immunity by enhancing ET signaling. In tobacco (*Nicotiana tabacum*), both of the virulent hemibiotrophic pathogens *P. syringae* pv *tabaci* and *Phytophthora parasitica* var *nicotianae* induce ET production starting around 24 to 48 h and peaking at 72 h after inoculation (Huang et al., 2005; Wi et al., 2012). Despite the fact that we have not measured ET levels directly, we found evidence of an ET response in our transcriptional profiling data of the systemic response to *Psm* ES4326 *cfa6* in *Arabidopsis* as well, which corroborates previous observations of ET production induced by virulent *P. syringae* infection and/or associated tissue senescence in local, infected *Arabidopsis* leaves (Bent et al., 1992; Magalhaes et al., 2000; Mur et al., 2009).

By inducing ET signaling, virulent hemibiotrophic pathogens may subvert successful plant immune responses. ET-defective or -insensitive mutants of *Arabidopsis*, tobacco, and tomato show reduced pathogen proliferation and/or disease symptoms after infection (Bent et al., 1992; Lund et al., 1998; Wi et al., 2012). The hypothesis that hemibiotrophic pathogens actively induce ET signaling to enhance plant disease susceptibility is supported by the findings that ET production is induced by several pathogen effectors (Cohn and Martin, 2005) and that ET suppresses production of SA that is necessary for full immune signaling (Chen et al., 2009). From this perspective, SIS to herbivory may be a by-product of pathogens subverting the plant immune system to their own benefit.

Pathogen-Induced Susceptibility to Insects Could Have Epidemiological Consequences

Our results suggest that inducing susceptibility to insect herbivores could conceivably have epidemiological consequences and facilitate the dispersal of both insect and pathogen, especially when the insect can act as vector to transmit the pathogen to new hosts. Many examples of this phenomenon exist in the

plant virus literature (Mauck et al., 2012), including one provided by the begomovirus tomato yellow leaf curl China virus. This virus makes tomato more susceptible to the invasive silverleaf whitefly *Bemisia tabaci* B, which also transmits the virus, promoting the spread of both (Jiu et al., 2007). The suppression of JA-mediated signaling by the viral virulence factors C2 and βC1 likely contributes to the facilitation of the herbivore (Yang et al., 2008; Lozano-Durán et al., 2011).

Pseudomonas spp can be transported by leaf-chewing and phloem-feeding insects (Lilley et al., 1997; Hirano and Upper, 2000; Stavrinides et al., 2009; Chung et al., 2013), and *Pseudomonas* spp facilitate feeding by the chewing herbivore *Leptinotarsa decemlineata* or Colorado potato beetle (Chung et al., 2013). Interestingly, a positive association between endophytic *Pseudomonas* spp abundance and damage by specialist, dipteran herbivores from the genus *Scaptomyza* (Whiteman et al., 2011) has been observed in the wild mustard species *Cardamine cordifolia* in a relatively intact ecosystem (Parris T.H. Humphrey and Noah K. Whiteman, unpublished data). Moreover, the suppression of antiherbivore defenses in JA-signaling mutants of the wild tobacco *Nicotiana attenuata* led to increased vulnerability of plants to insect herbivores in field experiments and natural populations (Kessler et al., 2004; Kallenbach et al., 2012), as well as the attraction of herbivores that did not normally feed on these plants (Kessler et al., 2004). Future field studies with these and other pathosystems may shed further light on the role that SIS to herbivory might play in shaping plant–herbivore interactions in nature.

METHODS

Plant Lines and Growth Conditions

Seeds of *Arabidopsis thaliana* accession Col-0 (wild-type) plants and mutants (all Col-0 background) were cold-stratified in 0.1% agarose for 3 d at 4°C. Subsequently, plants were grown in 36-cell trays under a 12-h-day:12-h-night cycle on Fafard #2 soil mix at 23/20°C day/night receiving water twice per week until 5 weeks old. The *Arabidopsis* mutants used were as follows: *efr-1* (SALK_044334, At5g20480), *fls2-c* (SAIL_691C04, At5g46330), *sid2-2* (At1g74710), *pad4-1* (At3g52430), *ein2-1* (At5g03280), *etr1-3* (At1g66340, formerly *ein1*), *eto1-1* (At3g51770), *aos/cyp74a* (SALK_017756, At5g42650), *coi1-1* (At2g39940,), and an *aos ein2-1* double mutant. Homozygous *coi1-1* mutants were selected by spraying seedling offspring of selfing *coi1-1/COI1* heterozygotes with 1 mM methyl jasmonate in a solution containing 5% ethanol. After 7 d, heterozygotes and *COI1/COI1* homozygotes were removed. Wild-type control plants and other plants accompanying *coi1-1* mutants in the same experiments were treated with 5% ethanol in parallel.

Bacterial Strains and Cultivation

The COR-deficient bacterial strain *Pseudomonas syringae* pv *maculicola* ES4326 *cfa6*::Km^r (*Psm* ES4326 *cfa6*) carrying the empty vector pLAFR3 (Tet^r) has been described previously (Cui et al., 2005). *Pto* CUCPB5532 (*Pto hcC cfa6 cmaA*; originally created by Brian Kvitko, Cornell University, Ithaca, NY) and *Pto* DB29 (*Pto cfa6 cmaA*) are derived from wild-type *Pto* DC3000. *Pto* DB29 has been described previously (Millet et al., 2010). *P. syringae* bacterial strains were cultured on King's B media plates supplemented with appropriate antibiotics: 50 mg mL^{-1} rifampicin for *Pto* DC3000 and *Pto* CUCPB5532; 50 mg mL^{-1} streptomycin for *Psm* ES4326;

50 mg mL^{-1} kanamycin for *Pto* DB29, and *Psm* ES4326 *cfa6*. On the third day, single colonies were inoculated into King's B media liquid cultures containing the same antibiotics. Overnight cultures were grown in a shaking incubator at 28°C to OD$_{600}$ = 1.0. The tubes harboring the cultures were then spun down at 5000 rpm for 5 min. The King's B media was removed, and the bacterial cells were washed by resuspending in sterile 10 mM MgSO$_4$. This was repeated twice, and prior to inoculation in *Arabidopsis* leaves, bacteria were resuspended at a final concentration of OD$_{600}$ = 0.2.

Trichoplusia ni Weight Gain Assay

Trichoplusia ni eggs (Benzon Research) were incubated in a box containing a moist paper towel at 30°C for 2 d to synchronize hatching. One neonate caterpillar was transferred to each experimental plant using a fine paint brush. The plants with caterpillars were kept individually in Magenta GA-7 boxes (Sigma-Aldrich) with insect-proof mesh lids or in pots wrapped in mesh cloth (DC May), and caterpillars were weighed to the nearest 0.01 mg after 7 d of feeding on the plants using a micro-balance (Mettler-Toledo). The weight of the caterpillars reflects weight gain because the weight of the neonates, including relative differences between individuals at the start of the feeding trial, is negligible. Each feeding trial was performed at least twice independently with comparable results. All weight gain assays were run blind in that student volunteers did not know which plant accessions were fed to which caterpillars and had no prior expectation regarding experimental outcome.

T. ni SIS to Herbivory Weight Gain Assay

Three lower rosette leaves (true leaves three, four, and five) of 5-week-old plants were inoculated with a suspension of *Psm* ES4326 *cfa6* or *Pto* DC3000 (and mutants thereof) at a concentration OD$_{600}$ = 0.2 (or ~10^8 colony-forming units mL^{-1}; described in Cui et al., 2005). As a control, a solution of sterile 10 mM MgSO$_4$ was used. After 4 d, the leaves infected with bacteria were cut off and a neonate *T. ni* caterpillar was added to each plant. This ensured minimal direct contact between insect and pathogen, thereby allowing the interpretation that the observed phenotypes are plant-mediated through systemic leaves. The latter part of the assay is as described above for the *T. ni* weight gain assay.

T. ni Weight Gain Assay after Preinoculation with ACC, elf26, or flg22

The herbivore weight gain assay was executed as described above for the *T. ni* SIS to herbivory weight gain assay with the exception that instead of live bacteria, a solution of 100 μM ACC (Sigma-Aldrich) or solution of 10 μM flg22 or elf26 in sterile 10 mM MgSO$_4$ was used to pretreat plants (Navarro et al., 2004).

Statistical Analysis of Weight Gain Assays

All statistical analyses were performed using Minitab. Weight gain data from experiments on *Arabidopsis* mutants alongside wild-type plants subjected to different treatments were analyzed with analysis of variance (ANOVA) including plant line and treatment as factors.

Harvesting Leaf Tissue for the Microarray Experiment

Plants were grown and infiltrated as described above for the *T. ni* SIS to herbivory weight gain assay. For subsets of plants at the three time points (3, 48, and 96 h) after infection, leaves 6 and 7 were cut off at the petiole with scissors, flash-frozen in liquid nitrogen in paper envelopes, and stored at −80°C until RNA was extracted. Leaves 6 and 7 were stored separately; leaf 6 was used for gene expression profiling unless the yield of RNA had a concentration below 500 ng μL^{-1}: in that case, leaf 7 was used. Leaves from eight replicate plants were pooled to form each RNA sample.

RNA Extraction for the Microarray Experiments

For the microarray experiments, total RNA was extracted from ~200 mg of leaf tissue using the Qiagen RNeasy plant mini kit with the on-column DNase digestion (Qiagen). Pooled leaves from each treatment group were briefly ground in liquid nitrogen using a mortar and pestle. Two aliquots of ~100 mg of tissue were processed following the manufacturer's protocol. RNA yield and quality were assessed using fluorimetry in an Agilent bioanalyzer. For labeling and hybridization, standard Affymetrix protocols were used (Affymetrix). Five micrograms of RNA was taken to generate cDNA for each sample. After subsequent synthesis of complementary strand cDNA and purification, samples were transcribed into biotinylated cRNA, cut into fragments, and hybridized to Affymetrix ATH1 microarrays.

Microarray Experimental Design and Data Analysis

Eighteen Affymetrix ATH1 whole-genome arrays were divided evenly among a combination of the following factors: treatment group (inoculation with sterile 10 mM MgSO$_4$ as a mock control and *Psm* ES4326 *cfa6*); time point (3, 48, and 96 h after inoculation); and trial (three independent trials). Successful establishment of infection was confirmed visually by following the progress of disease symptoms on the inoculated leaves. Leaves inoculated with *Psm* ES4326 *cfa6* started showing symptoms after 2 to 3 d when the leaves began to turn yellow compared with mock-inoculated leaves. Furthermore, to ascertain that infection with *Psm* ES4326 *cfa6* was sufficient to cause the expected phenotype, a *T. ni* weight gain assay was conducted. The assays were performed as described above. Raw data for each experiment are available at NASCArrays (experiment reference: NASCARRAYS-705 "systemic response of *Arabidopsis* to *Pseudomonas syringae* pv *maculicola* ES4326 *cfa6*").

Data from the microarray experiment were analyzed using Bioconductor (Gentleman et al., 2004). Affymetrix (.cel) files were loaded into the program. Background correction, normalization, and expression summaries were performed using the robust multiarray average approach (Irizarry et al., 2003) and then combined into experiment definitions to compare the different time points. Linear modeling of microarray data and identification of differentially expressed genes were performed with the *limma* package (Smyth, 2005). P values were adjusted to control the FDR using the method of Benjamini and Hochberg (1995), which was performed with the statistical package implemented in R 2.10.1 (Ihaka and Gentleman, 1996). Because this proved to be too stringent to detect significant changes in gene expression levels in the systemic leaves following *Psm* ES4326 *cfa6* infiltration of lower leaves, no FDR method or fold change cutoffs were used to generate the lists of differentially expressed genes in Supplemental Data Set 1 online.

However, subsequently, a more detailed analysis of 750 candidate genes involved in plant hormone biosynthesis or hormonal regulation of immune signaling, which were selected based on The Arabidopsis Information Resource annotation (www.arabidopsis.org), was conducted (see Supplemental Data Set 2 online). Although applying the FDR method on the resulting group of genes revealed that none of the genes were below the new significance threshold, genes in the top 5% of those differentially regulated (based on absolute fold change) or among the lowest 5% in terms of P values without FDR correction were considered for generating hypotheses to test in the *T. ni* weight gain experiments.

Supplemental Data

The following materials are available in the online version of this article.

  Supplemental Figure 1. Flg22 Triggers SIS to Herbivory, which Requires SA and ET Signaling.

  Supplemental Figure 2. Bacterial Type III Effectors Mediate Pathogen-Triggered SIS to Herbivory via ET Signaling Suppressing NPR1-Mediated MAMP-Triggered SIS to Herbivory.

Supplemental Table 1. Number of Differentially Expressed Genes in Systemic Leaves of *Psm* ES4326 *cfa6-* versus 10 mM MgSO$_4$-Inoculated Plants.

Supplemental Table 2. Selected Differentially Expressed Genes in Systemic Leaves of *Psm* ES4326 *cfa6-* versus 10 mM MgSO$_4$-Inoculated Plants.

Supplemental Data Set 1. Genes Differentially Expressed in Systemic Leaves of *Psm* ES4326 *cfa6-* versus 10 mM MgSO$_4$-Inoculated Plants.

Supplemental Data Set 2. Response of Genes Involved in Hormone Biosynthesis and Hormonal Regulation of Immune Signaling in Systemic Leaves of *Psm* ES4326 *cfa6-* versus 10 mM MgSO$_4$-Inoculated Plants at 48 h after Inoculation.

AUTHOR CONTRIBUTIONS

S.C.G., N.K.W., A.K.B., J.C., and A.C.-J. performed experiments and data analysis. A.K.B., A.M.W., and J.A.R. designed and conducted microarray experiments. I.A.B., J.D.R., and G.-H.H. organized and carried out feeding trials. J.B. grew plants and provided advice on the project. S.C.G., N.K.W., F.M.A., and N.E.P. formulated the project, were responsible for the coordination and supervision of research, and wrote the article. F.M.A. and N.E.P. are joint senior authors. All authors contributed to and approved the final article.

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