Ethylene and Jasmonic Acid Signaling Affect the NPR1-Independent Expression of Defense Genes Without Impacting Resistance to *Pseudomonas syringae* and *Peronospora parasitica* in the *Arabidopsis ssi1* Mutant

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Salicylic acid (SA), ethylene, and jasmonic acid (JA) are important signaling molecules in plant defense to biotic stress. An intricate signaling network involving SA, ethylene, and JA fine tunes plant defense responses. SA-dependent defense responses in *Arabidopsis thaliana* are mediated through NPR1-dependent and -independent mechanisms. We have previously shown that activation of an NPR1-independent defense mechanism confers enhanced disease resistance and constitutive expression of the pathogenesis-related (*PR*) genes in the *Arabidopsis ssi1* mutant. In addition, the *ssi1* mutant constitutively expresses the defensin gene *PDF1.2*. Moreover, SA is required for the *ssi1*-conferred constitutive expression of *PDF1.2* in addition to *PR* genes. Hence, the *ssi1* mutant appears to target a step common to SA- and ethylene- or JA-regulated defense pathways. In the present study, we show that, in addition to SA, ethylene and JA signaling also are required for the *ssi1*-conferred constitutive expression of *PDF1.2* and the NPR1-independent expression of *PR-1*. Furthermore, the ethylene-insensitive *ein2* and JA-insensitive *jar1* mutants enhance susceptibility of *ssi1* plants to the necrotrophic fungus *Botrytis cinerea*. However, defects in either the ethylene- or JA-signaling pathways do not compromise *ssi1*-conferred resistance to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* and the oomycete pathogen *Peronospora parasitica*. Interestingly, *ssi1* exhibits a marginal increase in the levels of ethylene and JA, suggesting that low endogenous levels of these phytohormones are sufficient to activate expression of defense genes. Taken together, our results indicate that although cross talk in *ssi1* renders expression of ethylene- or JA-responsive defense genes sensitive to SA and vice versa, it does not affect downstream signaling leading to resistance.

Additional keywords: cell death.

Salicylic acid (SA) is a key signal molecule in the activation of plant defense responses (Dempsey et al. 1999; Durner et al. 1997; Ryals et al. 1996). The endogenous level of SA and its conjugates are elevated in various plants resisting disease, and this increase correlates with the increased expression of the pathogenesis-related (*PR*) genes (Dempsey et al. 1999; Durner et al. 1997), some of which encode proteins with antimicrobial activities (Hunt and Ryals 1996; Klessig and Malamy 1994). Moreover, exogenous application of SA induces expression of the *PR* genes and disease resistance (Antoniw and White 1980; Uknes et al. 1992; Ward et al. 1991). Preventing accumulation of SA in transgenic tobacco and *Arabidopsis* plants expressing the bacterial *nahG* gene, which encodes the SA-degrading enzyme salicylate hydroxylase, compromises *PR* gene expression and resistance (Delaney et al. 1994; Gaffney et al. 1993).

The *Arabidopsis NPR1/NIM1* gene is a key component of the SA signal transduction pathway. Loss of NPR1 activity compromises pathogen resistance in the *npr1/nim1* mutants (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). The inability of the allelic *npr1* and *nim1* mutants to resist pathogen attack is due to their failure to respond to SA. SA and its functional analog benzothiadiazole (BTH) are unable to induce expression of the *PR* genes and SAR in these mutants. However, SA signaling also occurs via one or more NPR1-independent pathways. For example, in contrast to the poor expression in pathogen-infected NahG plants (Delaney et al. 1994), the *PR-1* gene is expressed at elevated levels in the leaves of pathogen-infected *npr1* plant (Delaney et al. 1995; Glazebrook et al. 1996; Kachroo et al. 2000; Shah et al. 1997). Furthermore, the SA-deficient NahG plants are 10- to 50-fold more susceptible to pathogens than the *npr1* mutant (Delaney et al. 1994, 1995; Shah et al. 2001). Likewise, studies with the *ssi1* (Shah et al. 1999), *ssi2* (Shah et al. 2001), *cpr5* (Bowling et al. 1997), *cpr6* (Clarke et al. 1998), and *acd6* (Rate et al. 1999) mutants have also implicated the involvement of an NPR1-independent pathway in mediating SA responses. These mutants constitutively express the *PR-1* gene and enhanced disease resistance independently of NPR1.

Jasmonic acid (JA) and ethylene are two other important signal molecules in plant defense against pathogens. Endogenous levels of JA and ethylene evolution are elevated in *Alternaria brassicicola*-inoculated *Arabidopsis* (Penninckx, et al. 1998).

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In addition, JA application induces expression of the *Thi2-1* and *PDF1.2* genes, which encode the antimicrobial proteins thionin and defensin, respectively (Epple et al. 1995; Penninckx et al. 1998; Vignutelli et al. 1998). Moreover, the *Arabidopsis fad3-2 fad7-2 fad8* triple mutant, which is unable to accumulate JA, is susceptible to infection by *Pythium* spp. (Vijayan et al. 1998). Similarly, the JA-insensitive *coi1* and *jar1* and the ethylene-insensitive *etr1* mutants show enhanced susceptibility to some fungal pathogens (Penninckx et al. 1996; Staswick et al. 1998; Thomma et al. 1998) and suppress expression of *PDF1.2* and *Thi2-1* (Epple et al. 1995; Penninckx et al. 1998). In addition, the *etr1*, *ein2*, and *jar1* mutant plants are incapable of developing induced systemic resistance (ISR), which is activated by root-colonizing strains of *Pseudomonas fluorescens* (Pieterse et al. 1998).

Several lines of evidence point to the existence of an intricate signaling network involving SA, JA, and ethylene in plant defense, leading to fine tuning of defense responses. Synergistic and antagonistic interactions between these signaling pathways in plant defense have been observed. For example, coapplication of methyl JA (MeJA) and SA superinduces tobacco *PR-1* expression (Xu et al. 1994). Likewise, ethylene potentiates SA-mediated *PR-1* expression (Lawton et al. 1995). By contrast, SA-activated expression of some *PR* genes is antagonized by JA (Niki et al. 1998). In addition, ethylene and JA biosynthesis is inhibited by salicylates in apple and tomato, respectively (Shah and Klessig 1999). Furthermore, NPR1 negatively regulates expression of the ethylene plus JA-responsive *PDF1.2* gene (Clarke et al. 1998; Shah et al. 1999). By contrast, the *NPR1* gene is required for ethylene- and JA-dependent activation of ISR in addition to the activation of SA-dependent defense responses (Pieterse et al. 1998). The mechanism of this cross talk between SA, ethylene, and JA signaling pathways and its significance to disease resistance is unclear.

We have previously shown that the *Arabidopsis ssi1* mutant targets a step common to SA and ethylene or JA signaling in plant defense response (Shah et al. 1999). The *ssi1* mutant allele confers high level expression of ethylene- and JA-dependent defense responses in addition to SA-dependent defense

responses (Shah et al. 1999). For example, expression of the SA-regulated *PR-1*, *BGL2* (*PR-2*), and *PR-5*, as well as the ethylene/JA regulated *PDF1.2* genes, were elevated in *ssi1*. Moreover, in parallel with *PR* expression, constitutive *PDF1.2* expression is rendered SA-dependent in the *ssi1* plant. The increased expressions of these defense-related genes are associated with enhanced resistance to *Pseudomonas syringae*.

Fig. 1. *PR-1* and *PDF1.2* expression in methyl jasmonic acid (MeJA)- and benzothiadiazole (BTH)-treated *ssi1 npr1 nahG* plants. Comparison of *PR-1* and *PDF1.2* expression in leaves of 4-week-old soil-grown plants of the indicated genotypes injected with MeJA (50 μ M solution in 0.1% ethanol) or sprayed plus subirrigated with BTH (100 μ M active ingredient in water). Untreated *ssi1 npr1* plants served as a positive control. RNA was extracted from leaves harvested 48 h after treatment. Gel loading was monitored by photographing the ethidium bromide-stained gel (EtBr) before transferring the RNA to a Nytran Plus membrane. The blot was sequentially probed for the indicated genes.

Fig. 2. Ethylene and jasmonic acid (JA) signaling requirements in *ssi1*-conferred defense gene expression. **A**, Benzothiadiazole (BTH)-induced expression of *PR-1* and *PDF1.2* in water- (W), α -aminoisobutyrate- (A), and silver thiosulphate (S)-treated wild-type, *npr1*, *npr1 nahG*, and *ssi1 npr1 nahG* plants. Four fully expanded leaves per plant were injected with either water, α -aminoisobutyrate (2 mM in water), or silver thiosulphate (4 mM in water) prior to spraying the plants with BTH (100 μ M active ingredient in water). RNA was extracted from injected leaves, 48 h posttreatment. **B**, Comparison of *PR-1*, *BGL2*, and *PDF1.2* expression between *ssi1*/+ *npr1*, *ssi1*/+ *npr1 ein2*, and *ssi1*/+ *npr1 jar1* plants. The wild-type Nöessen (Nö) and Columbia (Col), *npr1*, *npr1 ein2*, and *npr1 jar1* plants served as negative controls. Plants were homozygous for all other wild-type and mutant alleles. **C**, Comparison of *PDF1.2* expression between *ssi1*/+ and the *ssi1*/+ *etr1*-1/+ and *ssi1*/+ *coi1* double mutants. RNA was extracted from leaves of 4-week-old soil-grown plants. Gel loading was monitored by photographing the ethidium bromide-stained gel (EtBr) before transferring the RNA to a Nytran Plus membrane. The blot was sequentially probed for the indicated genes.

Fig. 3. Effect of ethylene- and jasmonic acid (JA)-insensitive mutants and *nahG* on *ssl1*-conferred, NPR1-independent resistance to *Pseudomonas syringae* pv. *maculicola*. **A**, Comparison of *Pseudomonas syringae* pv. *maculicola* cell numbers in *ssl1/+ npr1* and *ssl1/+ npr1 ein2* plants. Wild-type plants of ecotype Nössen (Nö) and Columbia (Col) and the *npr1* and *npr1 ein2* mutant plants served as controls. *Pseudomonas syringae* pv. *maculicola* ES4326 ($OD_{600nm} = 0.0005$ in 10 mM $MgCl_2$) was infiltrated into the abaxial surface of leaves with a syringe. **B**, Comparison of *Pseudomonas syringae* pv. *maculicola* cell numbers in *ssl1/+ npr1* and *ssl1/+ npr1 jar1* plants. Wild-type Nö and Col plants and the *npr1* and *npr1 jar1* mutant plants served as controls. *Pseudomonas syringae* pv. *maculicola* ES4326 ($OD_{600nm} = 0.0005$ in 10 mM $MgCl_2$) was infiltrated into the abaxial surface of leaves with a syringe. **C**, Comparison of *Pseudomonas syringae* pv. *maculicola* cell numbers in *ssl1/+ npr1* and *ssl1/+ npr1 nahG* plants. The wild-type and *npr1 nahG* plants served as controls. *Pseudomonas syringae* pv. *maculicola* ES4326 ($OD_{600nm} = 0.0002$ in 10 mM $MgCl_2$) was infiltrated into the abaxial surface of leaves with a syringe. Four leaf discs were harvested from infected leaves at 3 days postinoculation; they were then weighed and ground in 10 mM $MgCl_2$, and the bacterial numbers were titrated. The bacterial numbers $\pm$ SD, presented as CFU per mg of leaf tissue, represent the average of five samples. The above experiments were independently performed twice with similar results.

Interestingly, *ssil*-conferred constitutive expression of *PR* genes, and resistance to *P. syringae* is comparable in wild-type and *npr1* mutant backgrounds, implying the activation of a SA-dependent, NPR1-independent defense mechanism in *ssil*. In addition, *ssil* plants are stunted and spontaneously develop hypersensitive response (HR)-like lesions.

Here, we report that ethylene and JA signaling are required for NPR1-independent expression of *PR-1* in *ssil* plants. In addition, loss of ethylene and JA signaling suppresses *PDF1.2* expression and enhances susceptibility of *ssil* to the necrotrophic fungus *Botrytis cinerea*. The ethylene- or JA-dependence of *ssil*-conferred *PR-1* and *PDF1.2* expression is associated with small increases in synthesis of ethylene and accumulation of JA, suggesting that defense gene expression in *ssil* is rendered responsive to the low endogenous levels of these signal molecules. Interestingly, activation of ethylene and JA defense signaling does not have any obvious role in the elevated resistance to *Pseudomonas syringae* and *Peronospora parasitica* prevalent in *ssil* plants, suggesting that enhanced resistance and defense gene expression can be uncoupled.

RESULTS

Ethylene and JA signaling are required for *ssil*-conferred *PDF1.2* expression.

We have previously shown that constitutive *PDF1.2* expression conferred by the semidominant *ssil* mutant allele is SA-dependent; it is abolished in the SA-deficient *ssil npr1 nahG* plants but can be restored by treatment with BTH (Shah et al. 1999). This is unlike the situation in wild-type plants where *PDF1.2* expression is ethylene/JA inducible, but not SA inducible. To test whether *PDF1.2* expression in *ssil* was still responsive to jasmonates, we treated the *ssil npr1 nahG* plant with MeJA and, as a control, with BTH. Untreated *ssil npr1 nahG* plants and BTH-treated wild-type, *npr1* and *npr1 nahG* plants, which lack the mutant *ssil* allele, served as negative controls for monitoring *PDF1.2* expression. MeJA and BTH

application were very effective activators of *PDF1.2* expression in *ssil npr1 nahG* (Fig. 1). By contrast, only MeJA was capable of inducing *PDF1.2* expression in plants lacking the *ssil* mutant allele. However, unlike BTH, MeJA was unable to induce *PR-1* expression in the *ssil npr1 nahG* plants, indicating that MeJA application cannot bypass the requirement of SA for *PR-1* expression in *ssil* plants. Likewise, 1-aminocyclopropane-1-carboxylate (ACC), a precursor of ethylene, was capable of activating *PDF1.2* expression but not *PR-1* expression in the *ssil npr1 nahG* plant (data not shown). Thus, the SA- or BTH-activated step in *ssil* occurs before activation of ethylene or JA signaling.

To determine if ethylene signaling is required for the *ssil*-conferred *PDF1.2* expression, we tested *PDF1.2* expression in BTH-treated *ssil npr1 nahG* plants, simultaneously injected with the ethylene biosynthesis and signaling inhibitors, α -aminobutyrate (α -AIB) and silver thiosulphate, respectively. Leaves of water-injected, BTH-treated *ssil npr1 nahG* served as a positive control and water-, α -AIB-, and silver thiosulfate-injected, BTH-treated wild-type, *npr1*, and *npr1 nahG* plants served as negative controls. While BTH induced a high level expression of *PDF1.2* in leaves of water-injected *ssil npr1 nahG* plants, it was ineffective in activating *PDF1.2* expression in α -AIB- and silver thiosulfate-injected *ssil npr1 nahG* plants (Fig. 2A), suggesting that ethylene synthesis and signaling are required for SA- and BTH-induced *PDF1.2* expression in *ssil*.

To further evaluate the requirement of known components of ethylene and JA signal transduction in *ssil*-conferred constitutive *PDF1.2* expression, we crossed the *ssil npr1* double mutant with the ethylene-insensitive *ein2-1* (Guzman and Ecker 1990) and the JA-insensitive *jar1-1* (Staswick et al. 1992) mutants. We have previously shown that *ssil/+* heterozygous plants express higher levels of *PDF1.2* than homozygous *ssil* plants (Shah et al. 1999). Moreover, in comparison to *ssil/+*, the *ssil/+ npr1* double mutant accumulated higher levels of the *PDF1.2* transcript (Shah et al. 1999). Hence, constitutive

Fig. 4. Effect of ethylene- and jasmonic acid (JA)-insensitive mutants and *nahG* on *ssil*-conferred, NPR1-independent resistance to *Peronospora parasitica*. Comparison of growth of *P. parasitica* biotype Emco5 on *ssil*, *ssil npr1*, *ssil npr1 nahG*, *ssil npr1 ein2*, and *ssil npr1 jar1* plants. The wild-type, *npr1*, *npr1 nahG*, *npr1 ein2*, and *npr1 jar1* plants served as controls. Cotyledons of 7-day-old plants were infected with spores of *P. parasitica*. The number of sporangiophores on each cotyledon were counted 8 days postinoculation. For each line, the percentage of cotyledons exhibiting 0 to 10, 11 to 20, 21 to 30, 31 to 40, and ≥ 41 sporangiophores are shown. Numbers to the right of each bar denote number of inoculated cotyledons.

PDF1.2 expression was assessed in the *ssi1/+ npr1 ein2* and *ssi1/+ npr1 jar1* triple mutant plants. The *ein2* mutant effectively suppressed *ssi1*-conferred constitutive *PDF1.2* expression (Fig. 2B). Moreover, lack of *PDF1.2* expression in the *ssi1/+ etr1/+* plant confirmed the requirement of ethylene signaling for *ssi1*-conferred constitutive *PDF1.2* expression (Fig. 2C). By contrast, *jar1* was ineffective in suppressing *ssi1*-conferred constitutive *PDF1.2* expression (Fig. 2B). To further explore the involvement of JA signaling in *ssi1*-conferred *PDF1.2* expression, we analyzed *PDF1.2* expression in *ssi1/+ coi1* plants. The JA-insensitive *coi1-1* allele suppressed *ssi1*-conferred *PDF1.2* expression (Fig. 2C), implying that *COIL*, but not *JAR1*, is required for *PDF1.2* expression in *ssi1*.

Ethylene and JA signaling are required for *ssi1*-conferred NPR1-independent high level expression of *PR-1*.

Our experiments with α -AIB (Fig. 2A) suggested that ethylene biosynthesis, in addition to SA or BTH, is required for the *ssi1*-conferred NPR1-independent expression of *PR-1*. Blocking ethylene synthesis by coapplication of α -AIB prevented a high expression level of the *PR-1* gene in BTH-treated *ssi1 npr1 nahG* plants. Surprisingly, silver thiosulfate application by itself activated *PR-1* expression in a SA-independent manner in *Arabidopsis*. Ethylene and JA previously have been shown to potentiate *PR* expression in other systems (Lawton et al. 1995; Xu et al. 1994). Hence, we further evaluated the involvement of ethylene and JA signaling in *ssi1*-conferred, NPR1-independent expression of the *PR* genes by studying the impact of the *ein2* and *jar1* mutants on *ssi1*-conferred expression of the *PR-1* and *BGL2* genes. Constitutive accumulation of the *PR-1* and *BGL2* transcripts was compared among the *ssi1/+ npr1 ein2*, *ssi1/+ npr1 jar1*, and *ssi1/+ npr1* plants. Both *ein2* and *jar1* reduced *ssi1*-conferred *PR-1* transcript accumulation (Fig. 2B), confirming the involvement of ethylene and JA signaling, in addition to SA signaling, in the *ssi1*-con-

ferred, NPR1-independent accumulation of *PR-1* transcript. However, neither *ein2* nor *jar1* were effective in suppressing *ssi1*-conferred constitutive *BGL2* expression.

ssi1-conferred, NPR1-independent resistance to *Pseudomonas syringae* and *P. parasitica* is SA-dependent but independent of ethylene and JA.

Since, the *ein2* and *jar1* mutant alleles compromised *ssi1*-conferred, NPR1-independent *PR-1* transcript accumulation, we evaluated the effect of the *ein2* and *jar1* mutations, and hence ethylene and JA signaling, on the *ssi1*-conferred enhanced resistance to virulent strains of the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* and the oomycete pathogen *P. parasitica* biotype Emco5. Due to the very small size of plants homozygous for the *ssi1* mutant allele, *Pseudomonas syringae* pv. *maculicola* infections were carried out on the relatively larger sized *ssi1/+* heterozygous plants. The *npr1-5* allele was included to discount any contribution of the NPR1 pathway in the *ssi1*-conferred enhanced resistance. Since the *ein2* and *jar1* mutants are in the Columbia ecotype (Guzman and Ecker 1990; Staswick et al. 1992) while *ssi1* and *npr1-5* are in the Nöessen (Nö) ecotype (Shah et al. 1997, 1999), wild-type Columbia (Col) plants were also included as controls. As expected, in comparison with the wild-type Nö plant, the lack of NPR1 activity was associated with higher bacterial numbers in the *npr1* mutant. However, as previously shown by us (Shah et al. 1999), the *ssi1* mutant allele restored resistance in the *ssi1/+ npr1* plant. Interestingly, while the *npr1 ein2* double mutant is more susceptible than the wild-type Nö and Col plants, the *ssi1/+ npr1 ein2* plant was highly resistant to *Pseudomonas syringae* pv. *maculicola* (Fig. 3A). Bacterial numbers in *ssi1/+ npr1 ein2* were 200-fold fewer than in the *npr1 ein2* double mutant, implying that ethylene signaling is not important for *ssi1*-conferred enhanced resistance to *Pseudomonas syringae* pv. *maculicola*. Similarly, bacterial

Fig. 5. Growth of *Botrytis cinerea* on *ssi1* plants. Four leaves of four-week-old soil-grown wild-type, *ein2*, *ssi1/+*, *ssi1 npr1 nahG*, *ssi1/+ ein2*, and *ssi1/+ jar1* plants were inoculated with freshly harvested spores of *B. cinerea*. Plants were photographed 7 days postinfection from the same distance and at the same magnification. The yellow arrows and the white circles mark the primary inoculated leaves and the noninoculated but infected leaves, respectively.

numbers in the *ssil/+ npr1 jar1* plants were 30-fold fewer than in the *npr1 jar1* double mutant (Fig. 3B), suggesting that JA signaling is not important for *ssil*-conferred enhanced resistance to *Pseudomonas syringae* pv. *maculicola*. By contrast, as expected, preventing SA accumulation in the *ssil npr1 nahG* plants severely compromised the *ssil*-conferred enhanced resistance to *Pseudomonas syringae* pv. *maculicola* (Fig. 3C).

In addition to *Pseudomonas syringae* pv. *maculicola*, we also used the oomycete pathogen *P. parasitica* biotype Emco5 to assess the role of ethylene and JA signaling pathways in *ssil*-conferred, NPR1-independent resistance to downy mildew. *P. parasitica* biotype Emco5 is virulent on wild-type Nössen and Columbia plants. While the wild-type, *npr1*, *npr1 nahG*, *npr1 ein2*, and *npr1 jar1* plants, all of which lack the *ssil* mutant allele, support profuse growth and sporulation of *P. parasitica*, the *ssil* and *ssil npr1* plants show high level resistance to *P. parasitica* (Fig. 4). By contrast, preventing SA accumulation in the *ssil npr1 nahG* plant abolished *ssil*-conferred resistance to *P. parasitica*. Similar to our observations with *Pseudomonas syringae* pv. *maculicola* (Fig. 3), the *ein2* and *jar1* alleles did not compromise *ssil*-conferred resistance to *P. parasitica* in the *ssil npr1 ein2* and *ssil npr1 jar1* plants. Hence, the *ssil*-conferred, enhanced resistance to *Pseudomonas syringae* pv. *maculicola* and *P. parasitica* are dependent on SA but independent of JA and ethylene signaling.

***ein2* and *jar1* enhance susceptibility of *ssil* to *B. cinerea*.**

Exogenously applied ethylene and JA confer protection against the necrotrophic pathogen *B. cinerea* (Thomma et al. 1999). Likewise, overexpression of *ETHYLENE RESPONSE FACTOR1* (*ERF1*), a regulator of *PDF1.2* expression in *Arabidopsis*, enhances resistance to *B. cinerea* (Berrocal-Lobo et al. 2002). Hence, we tested the influence of suppressing ethylene and JA signaling due to the *ein2* and *jar1* mutations, respectively, on growth of *B. cinerea* in *ssil* plants. In comparison to *ssil/+*, the *ssil/+ ein2* and *ssil/+ jar1* plants exhibited increased susceptibility to *B. cinerea* (Fig. 5, Table 1). While none of the *ssil/+* plants exhibited spread of fungal infection to uninoculated leaves, systemic spread of fungus was observed in 54% and 20% of *ssil/+ ein2* and *ssil/+ jar1* plants, respectively. In addition, the *ssil/+ jar1* plants in particular exhibited profuse sporulation on a majority of inoculated leaves as early as 3 days postinoculation (dpi). Interestingly, compared with wild type, the *ssil/+* plants exhibit increased susceptibility to *B. cinerea*; spreading necrosis was observed in 88% of inoculated *ssil/+* leaves compared with 19% of inoculated wild-type leaves. By comparison, 86% and 92% of *B. cinerea*-inoculated *ssil/+ ein2* and *ssil/+ jar1* leaves, respectively, exhibited spreading necrosis.

We have previously shown that the *nahG* transgene suppresses all known *ssil*-conferred phenotypes in the *ssil npr1 nahG* plants (Shah et al. 1999). To determine if the increased susceptibility to *B. cinerea* is indeed associated with expression of *ssil*-activated responses, we tested susceptibility of *ssil npr1 nahG* plants to infection by *B. cinerea*. *ssil*-conferred enhanced susceptibility to *B. cinerea* was suppressed in *ssil npr1 nahG* plants (Fig. 5, Table 1); only 25% of inoculated *ssil npr1 nahG* leaves exhibited spreading necrosis.

Ethylene and JA signaling are not required for *ssil*-conferred stunted growth and development of spontaneous lesions.

In addition to the defense-associated phenotypes, *ssil* plants are stunted and spontaneously develop HR-like lesions (Shah et al. 1999). High levels of SA are required for the development of spontaneous lesions and stunted appearance of *ssil* (Shah et al. 1999). Hence, we evaluated the involvement of

ethylene and JA signaling in these *ssil*-conferred phenotypes. The *ein2-1* and *jar1-1* mutant alleles did not suppress the *ssil*-conferred stunted plant phenotype (Fig. 6A). Except for the *ssil npr1 jar1* triple mutant, which was marginally larger than the *ssil npr1* plant, no obvious increases in plant size were observed for the other double and triple mutant lines. Moreover, trypan blue staining of leaves showed that the *ein2* and *jar1* mutant alleles did not alleviate the *ssil*-conferred spontaneous cell death phenotype in *ssil/+ npr1 ein2* and *ssil/+ npr1 jar1* plants (Fig. 6B). Similar results were observed with *ssil/+ ein2* and *ssil/+ jar1* double mutants (data not shown). Likewise, the *etr1-1* and *coi1-1* mutant alleles did not suppress the *ssil*-conferred spontaneous cell death and small plant phenotypes (data not shown). Hence, we can conclude that the induced ethylene and JA signaling does not have a causal role in the stunted plant and lesion mimic phenotypes of *ssil*.

***ssil* exhibits small increases in basal levels of ethylene and JA.**

The elevated expression of *PDF1.2* and NPR1-independent expression of *PR-1* in *ssil* could be due to increased accumulation of ethylene, JA, or both in the *ssil* mutant or, alternatively, could be due to increased sensitivity of *ssil* to ethylene, JA, or both. Therefore, we compared ethylene production and JA accumulation in *ssil* and wild-type plants. Since, *PDF1.2* expression was previously observed to be higher in plants heterozygous for *ssil* compared with homozygous *ssil* plants, ethylene and JA measurements were taken in plants heterozygous for *ssil* (*ssil/+*). Basal levels of ethylene synthesis and JA accumulation in *ssil/+* were 1.3-fold and twofold higher than in wild type, respectively (Table 2). By contrast, mock-inoculated, *A. brassicicola*-infected wild-type plants showed a 34-fold increase in JA accumulation (data not shown). However, *ssil* seedlings do not show a constitutive triple response, suggesting that ethylene levels are not physiologically high in germinating, etiolated *ssil* seedlings (data not shown).

Growth of *ssil* seedlings is not hypersensitive to ethylene and JA.

We next tested whether growth of *ssil* seedlings is more sensitive than that of wild-type seedlings to ethylene. One of the characteristics of ethylene-induced triple response is inhibition of hypocotyl elongation in etiolated seedlings (Guzman and Ecker 1990). ACC is a precursor of ethylene that is converted into ethylene, resulting in triple response in etiolated seedlings (Guzman and Ecker 1990). We therefore compared the effect of varying concentrations of ACC on hypocotyl elongation in wild-type and *ssil* plants. As controls, we also measured hypocotyl elongation in the ethylene-insensitive *etr1* and *ein2* mutants (Guzman and Ecker 1990). Hypocotyl growth of *etr1* and *ein2* was not inhibited by 10 μ M of ACC (Fig. 7A). By contrast, hypocotyl elongation in the wild type and *ssil* mutant

Table 1. Growth of *Botrytis cinerea* on *ssil* plants

Genotype ^a	No. inoculated leaves	% Exhibiting spreading necrosis ^b	% Exhibiting systemic spread ^c
Wild type	36	19	0
<i>ein2</i>	47	81	0
<i>ssil/+</i>	60	88	0
<i>ssil/+ ein2</i>	74	86	54
<i>ssil/+ jar1</i>	66	92	20
<i>ssil npr1 nahG</i>	36	25	0

^a The *ein2-1* and *jar1-1* alleles were used in this experiment.

^b Percentage of inoculated leaves with greater than 50% of area exhibiting necrosis.

^c Percentage of inoculated plants exhibiting spreading necrosis on systemic noninoculated leaves.

was markedly inhibited in the presence of 1 μ M ACC. Wild-type and *ssi1* seedlings were equally sensitive to ACC at all concentrations tested.

Root elongation in germinating *Arabidopsis* seedlings is sensitive to presence of JA in the growth medium (Staswick et al. 1992). To test whether germinating *ssi1* seedlings are more sensitive to JA than are wild-type plants, we compared the effect of varying concentrations of MeJA on root elongation in germinating wild-type and *ssi1* seedlings. Root elongation in the wild-type and *ssi1* plants were equally sensitive to MeJA (Fig. 7B). By comparison, roots of the JA-insensitive *jar1* mutant (Staswick et al. 1992) were less sensitive to MeJA. Hence, growth of *ssi1* does not exhibit a general increase in sensitivity to ethylene or JA.

DISCUSSION

We have previously shown that the semidominant *ssi1* allele conferred NPR1-independent expression of *PR* genes and en-

hanced resistance to an avirulent strain of *Pseudomonas syringae* pv. *tomato* (Shah et al. 1999). In addition, the *ssi1* mutant constitutively expressed *PDF1.2*. Interestingly, constitutive expression of *PDF1.2*, in addition to expression of the *PR* genes in *ssi1*, was SA dependent, implying that *ssi1* or a *ssi1*-derived signal targets a step common to SA and ethylene and JA signaling. This is unlike several other mutants exhibiting constitutive *PDF1.2* expression, in which SA is not essential for *PDF1.2* expression (Bowling et al. 1997; Clarke et al. 1998).

To understand the role of the ethylene and JA signaling pathways in the *ssi1* mutant, we evaluated the requirement of ethylene and JA and the corresponding signal transduction pathways in the *ssi1*-conferred phenotypes. These studies show that a functional ethylene signal transduction pathway is required for *ssi1*-conferred *PDF1.2* expression; the *ein2-1* and *etr1-1* mutant alleles, which confer ethylene insensitivity, compromised *ssi1*-conferred constitutive *PDF1.2* expression (Fig. 2B and C). In addition, α -AIB and aminovinylglycine, inhibitors of ethylene biosynthesis, and silver thiosulfate, an inhibitor of ethylene

Fig. 6. Comparison of morphological and cell death phenotypes of double and triple mutant lines. **A**, Comparison of morphological phenotype of *ssi1*, *ssi1 ein2*, *ssi1 jar1*, *ssi1 npr1*, *ssi1 npr1 ein2*, and *ssi1 npr1 jar1* lines. Four-week-old soil-grown plants were photographed. The *ssi1*, *ssi1 ein2*, *ssi1 jar1*, *ssi1 npr1*, *ssi1 npr1 ein2*, and *ssi1 npr1 jar1* plants are at a 4 $\times$ higher magnification than the wild-type, *jar1*, and *ein2* plants that served as controls. All plants are homozygous for the indicated wild-type and mutant alleles. **B**, Comparison of spontaneous lesions in *ssi1/+ npr1*, *ssi1/+ npr1 ein2*, and *ssi1/+ npr1 jar1* plants. Leaves of 4-week-old soil-grown plants were stained with trypan blue and were photographed under a dissection microscope. The white arrows point to areas containing intensely stained dead cells. The *npr1*, *npr1 ein2*, and *npr1 jar1* plants served as negative controls.

action, blocked *PDF1.2* expression in BTH-treated *ssi1 npr1 nahG* plants (Fig. 2A and data not shown), further confirming the requirement of ethylene signal transduction in *ssi1*-conferred *PDF1.2* expression. However, both ACC and MeJA were capable of activating *PDF1.2* expression in the SA-deficient *ssi1 npr1 nahG* plant, suggesting that SA and BTH activate a step before ethylene and JA action in *ssi1*.

Our experiments with the *ssi1/+ npr1 jar1* plant suggested that JA signaling might not be involved in *ssi1*-conferred *PDF1.2* expression (Fig. 2B). However, lack of *PDF1.2* expression in the *ssi1/+ coil* plant suggested that JA signaling is essential for *ssi1*-conferred constitutive *PDF1.2* expression (Fig. 2C). Leakiness of the *jar1-1* allele could explain the above results. Alternatively, *ssi1*-generated signal activates the JA signal transduction pathway resulting in *PDF1.2* expression at a point after JAR1 action, but before COI1 action. Similarly, *jar1-1* is unable to efficiently suppress *cpr5*- and *cpr6*-conferred *PDF1.2* expression in *cpr5 npr1 jar1* and *cpr6 npr1 jar1* plants (Clarke et al. 2000). However, the effect of *coil* on *cpr5*- and *cpr6*-conferred *PDF1.2* expression is not known.

Basal levels of ethylene production and JA accumulation in *ssi1/+* are 1.3- and twofold higher, respectively, than in the wild-type plants (Table 2). By comparison, *A. brassicicola* infection results in a >30-fold increase in JA accumulation in wild-type plants. Likewise, by comparison with wild-type plants, JA levels in leaves of the *acd2* mutant, which, like *ssi1*, constitutively expresses *PDF1.2*, were ninefold and threefold higher in necrotic and asymptomatic leaves, respectively (Pieterse et al. 1996). Whether the small increases in basal ethylene and JA levels have a causal role in the *ssi1*-conferred *PDF1.2* and *PR-1* expression is presently unclear. However, it is likely that *ssi1* may be more sensitive than the wild-type plants to small increases in ethylene and JA levels. This is similar to the situation in ISR, activation of which requires ethylene and JA signaling but is not associated with increases in ethylene and JA accumulation (Pieterse et al. 2000). Potentiation has been proposed to account for induction of defense responses by otherwise noninductive conditions, for example during ISR (Conrath et al. 2002). Analogously, *ssi1*-activated SA accumulation, cell death, or some other factor might effectively increase sensitivity of *ssi1* tissues to suboptimal levels of ethylene and JA, leading to expression of *PDF1.2*. This would also explain the absence of *ssi1*-conferred constitutive *PDF1.2* expression in *ssi1 npr1 nahG* plants and the ability of BTH application to restore *PDF1.2* expression in *ssi1 npr1 nahG* plants (Fig. 1). Potentiation is predicted to be an effective mechanism only at suboptimal levels of the primary activation signal. This would explain why SA is not required for *PDF1.2* expression in *ssi1 npr1 nahG* plants treated with high levels of MeJA (Fig. 1). However, *ssi1* plants do not show a constitutive triple response (data not shown), nor is growth of *ssi1* seedlings hypersensitive to low levels of ethylene and JA (Fig. 7A and B). Thus, *ssi1* plants do not show a general increase in sensitivity and activity of ethylene and JA signaling pathways. Rather, a subset of responses in *ssi1* might be primed for increased sensitivity to suboptimal levels of ethylene and JA. The fact that *ssi1* seedlings are resistant to *P. parasitica* indicates that the *ssi1* mutant allele does exert its effect at the seedling stage. However, we cannot discount the possibility that *ssi1* exerts other responses only at later stages of development and not at the early seedling stage, thus explaining the lack of ethylene and JA hypersensitivity in *ssi1* seedlings.

The *ssi1*-conferred, NPR1-independent *PR-1* transcript accumulation is also partly dependent on ethylene and JA signaling. The ethylene biosynthesis inhibitor α -AIB prevented high level expression of *PR-1* in BTH-treated *ssi1 npr1 nahG* plants (Fig. 2A). In addition, the *ein2-1* and *jar1-1* mutant alleles reduced

accumulation of *PR-1* transcript in the *ssi1/+ npr1 ein2* and *ssi1/+ npr1 jar1* plants (Fig. 2B). However, this suppression of *PR-1* expression in *ssi1/+ npr1 ein2* and *ssi1/+ npr1 jar1* plants was not as severe as in SA-deficient *ssi1 npr1 nahG* (Fig. 1) (Shah et al. 1999). By contrast, the *ssi1*-conferred, NPR1-independent accumulation of *BGL2* transcript is independent of ethylene and JA signaling; the *ein2-1* and *jar1-1* alleles did not compromise *BGL2* expression in *ssi1/+ npr1 ein2* and *ssi1/+ npr1 jar1* plants (Fig. 2B). Potentiation of SA-dependent *PR-1* expression by ethylene and JA could account for the NPR1-independent *PR-1* expression in *ssi1* plants. Ethylene signaling has been demonstrated to potentiate SA-mediated *PR-1* expression in *Arabidopsis* (Lawton et al. 1995). Furthermore, coapplication of MeJA plus SA has been shown to superinduce tobacco *PR-1* expression (Xu et al. 1994). Like *ssi1*, the NPR1-independent accumulation of *PR-1* gene transcript in the *cpr5* and *cpr6* mutants is dependent on ethylene and JA signaling (Clarke et al. 2000).

Fig. 7. Ethylene and jasmonic acid (JA) sensitivity of *ssi1* seedlings. **A**, Comparison of hypocotyl lengths of 5-day-old etiolated wild-type, *ssi1*, and ethylene-insensitive *ein2-1* and *etr1-1* seedlings on Murashige and Skoog (MS) agar plates containing increasing concentrations of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC). **B**, Comparison of root lengths of 9-day-old, light-grown wild-type, *ssi1*, and JA-insensitive *jar1-1* seedlings on MS agar plates containing increasing concentrations of the methyl jasmonic acid (MeJA).

Interestingly, despite suppressing *ssil*-conferred NPR1-independent *PR-1* expression, *ein2* and *jar1* were unable to repress *ssil*-conferred resistance to the virulent pathogen *Pseudomonas syringae* pv. *maculicola* and *P. parasitica* (Figs. 3A and B, 4). It is likely that the residual expression of one or more *PR* genes in *ssil/+ npr1 ein2* and *ssil/+ npr1 jar1* plants is sufficient for restricting pathogen growth. By contrast, severe reduction of *ssil*-conferred defense gene expression in the SA-deficient *ssil npr1 nahG* plant is accompanied by loss of *ssil*-conferred resistance to *Pseudomonas syringae* pv. *maculicola* and *P. parasitica* (Figs. 3C, 4). The scenario is different in the *cpr5* and *cpr6* mutants (Clarke et al. 2000). Loss of NPR1-independent *PR-1* expression in *cpr5 npr1 ein2*, *cpr5 npr1 jar1*, *cpr6 npr1 ein2*, and *cpr6 npr1 jar1* correlated with loss of *cpr5*- and *cpr6*-conferred resistance to *Pseudomonas syringae* pv. *maculicola* and *P. parasitica*. Our observation that *ein2-1* does not compromise resistance to *Pseudomonas syringae* pv. *maculicola* and *P. parasitica* despite abolishing *ssil*-conferred constitutive *PDF1.2* expression in the *ssil/+ npr1 ein2* plant suggests that *PDF1.2* expression does not correlate with resistance to *Pseudomonas syringae* pv. *maculicola* and *P. parasitica* in *ssil* plants. Thus, *ssil*-conferred resistance to *Pseudomonas syringae* pv. *maculicola* and *P. parasitica* is due to activation of an NPR1-independent resistance mechanism that does not evoke ethylene and JA signaling. However, we cannot rule out the possibility that differences between the *npr1* mutant alleles and the *P. parasitica* biotypes used in this study and by Clarke and associates (2000) and the different ecotype backgrounds of the *ssil* and the *cpr* mutants could partly account for the observed differences between *ssil* and the *cpr* mutants. In addition, we cannot discount the possibility that, while defense gene expression in *ssil* requires ethylene and JA signaling in addition to SA, resistance to *Pseudomonas* and *Peronospora* isolates in *ssil* is activated after the point of action of EIN2 and JAR1. Whether cell death has a causal role in activation of defense gene expression and resistance to *Pseudomonas* and *Peronospora* isolates in the *ssil* mutant is still unclear.

Ethylene and JA signaling have important roles in resistance of *Arabidopsis* to the necrotrophic fungal pathogen *A. brassicicola* and *B. cinerea*. Ethylene insensitivity conferred by *ein2* and JA insensitivity conferred by *coi1* and *jar1* enhance susceptibility to *B. cinerea* (Kachroo et al. 2001; Thomma et al. 1998, 1999). By contrast, ethylene and JA application increases resistance against *B. cinerea* (Thomma et al. 1999). In addition, overexpression of *ERF1*, a regulator of ethylene responses in *Arabidopsis*, confers enhanced resistance to necrotrophic fungi (Berrocal-Lobo et al. 2002). In agreement with the role of ethylene and JA in resistance to *B. cinerea*, *ssil/+ ein2* and *ssil/+ jar1* plants exhibit enhanced susceptibility to *B. cinerea* compared with *ssil/+* (Fig. 5, Table 1). Interestingly however, despite the constitutive expression of the ethylene- and JA-dependent *PDF1.2* gene, the *ssil/+* plant exhibited increased susceptibility to *B. cinerea* (Fig. 5, Table 1), suggesting that constitutive expression of *PDF1.2* is not sufficient for resistance to *B. cinerea*.

Table 2. Ethylene evolution and jasmonic acid (JA) accumulation in wild type and *ssil*

	Wild type	<i>ssil/+</i>
Ethylene ^a	2.59 ± 0.04	3.43 ± 0.35
JA ^b	60.3 ± 14.4	118.0 ± 4.9

^a Ethylene levels ± standard deviation (SD) are given as nanoliters of ethylene produced per gram of fresh weight tissue per hour

^b JA levels ± SD are given as pmole JA accumulated per gram of fresh weight tissue

HR-associated cell death in *Arabidopsis* has been previously shown to facilitate infection by *B. cinerea* (Gorvin and Levine 2000). It is likely that spontaneous cell death in *ssil* predisposes it to infection by *B. cinerea*. However, the *ssil npr1 eds5* plant is highly resistant to *B. cinerea* despite exhibiting extensive cell death (J. Shah and A. Nandi, unpublished data), suggesting that cell death by itself is not a sufficient factor in *ssil* for conferring susceptibility to *B. cinerea*. Since resistance to *B. cinerea* is restored in the *ssil npr1 nahG* plants, elevated SA levels may have a role in susceptibility to *B. cinerea*. However, since *nahG* suppresses all *ssil*-conferred phenotypes, it is equally likely that this effect of SA on *ssil*-conferred susceptibility to *B. cinerea* is an indirect consequence of SA being required for other *ssil*-conferred phenotypes. We have previously shown that a comparable high level of SA in the *ssil2* mutant is not associated with increased susceptibility to *B. cinerea* (Kachroo et al. 2001).

In conclusion, we provide evidence that resistance to bacterial and oomycete pathogens conferred by activation of an SA-dependent, NPR1-independent mechanism in the *ssil* mutant does not evoke ethylene and JA signaling and can be uncoupled from *PR-1* and *PDF1.2* expression. In addition, our work further highlights the complexity of plant defense mechanisms and the networking in plant defense signaling. We have shown that interaction between the SA and ethylene or JA pathways in *ssil* can be altered with only small increases in levels of ethylene and JA.

MATERIALS AND METHODS

Growth conditions for plants and pathogens.

Arabidopsis seeds were germinated at 22°C in a tissue-culture chamber exposed to a 14 h light (80 µE/m/s) and 10 h dark cycle on Murashige-Skoog (MS) (Sigma, St. Louis) agar supplemented with 1% sucrose. Nine-day-old seedlings were transferred to soil and were grown at 22°C in growth chambers programmed for a 14 h light (90 µE/m/s) and 10 h dark cycle.

Pseudomonas syringae pv. *maculicola* ES4326 was propagated at 28°C on King's B medium (King et al. 1954) containing streptomycin (100 µg/ml). An overnight culture was used for infecting plants. *P. parasitica* biotype Emco5 was cultivated on the susceptible ecotype Nössen, as described by Dangl and associates (1992). *Botrytis cinerea* IMI169558 was cultivated on maltose media at 22°C in a growth chamber programmed for a 14 h light (80 µE/m/s) and 10 h dark cycle. To induce sporulation, three 1-cm² maltose agar pieces containing *B. cinerea* mycelia were placed on potato dextrose agar and were incubated at 22°C under continuous light for 7 to 10 days. Spores were harvested by flooding the plate with potato dextrose broth and then gently stroking the surface with a sterile paintbrush. The spore suspension was filtered through cheese-cloth, and spores were counted on a haemocytometer slide.

ACC and JA sensitivity test.

MS agar supplemented with 1% sucrose, and different concentrations of the ethylene precursor ACC (Sigma) were used for measuring sensitivity to ethylene. ACC is converted into ethylene in planta. Surface-sterilized seeds were sown on the above medium and, after a 2-day cold (4°C) treatment, the seeds were allowed to germinate in the dark at 22°C. Seven days later, seedlings were examined for presence of a triple response, and the lengths of hypocotyls were measured on 10 to 15 randomly picked seedlings for each plant type.

MS agar supplemented with 1% sucrose and different concentrations of MeJA (Bedoukian Research, Danbury, CT, U.S.A.) were used for measuring sensitivity of roots to MeJA. Surface-sterilized seeds were sown in a straight line on the sur-

face of the above medium and, after a 2-day cold (4°C) treatment, the plates were incubated vertically in a tissue-culture chamber exposed to a 14 h light (80 µE/m/s) and 10 h dark cycle. Seven days later, the length of the primary root was measured on 10 to 15 randomly picked seedlings for each plant type.

Pathogen infection of plants.

Infections with *Pseudomonas syringae* pv. *maculicola* ES4326 were performed on 4-week-old soil-grown plants. An overnight *Pseudomonas* strain ES4326 culture was harvested and resuspended in 10 mM MgCl₂ to an optical density specified for each experiment. The bacterial suspension was infiltrated into the abaxial surface of leaves with the help of a needleless 1-ml syringe. For every set of infections, a total of 25 (five replications of five leaves in each sample) leaf disks (0.15 cm²) were harvested at 3 days postinfection and were placed in preweighed tubes. After the weight of each sample was determined, the samples were processed for bacterial counts and RNA extraction as described earlier (Shah et al. 1997). Bacterial counts were expressed as CFU per milligram of fresh weight of leaf tissue.

Inoculation with the virulent *P. parasitica* biotype Emco5 was done on 7-day-old soil-grown plants. Spore suspensions of *P. parasitica* were prepared as described (Dangl et al. 1992). Plants were sprayed with a freshly prepared suspension of conidiospores in water (10⁶ spores per ml). Inoculated plants were kept covered with a clear plastic dome to maintain high humidity throughout the course of the experiment, and pathogen growth was evaluated under a dissecting microscope 8 dpi by counting the number of sporangiophores per cotyledon.

Inoculation with *B. cinerea* spores was done on 4-week-old soil-grown plants. Four leaves per plant were pricked with a needle at three well-spaced spots per leaf. Infection was initiated by placing a 10 µl drop of freshly harvested spore suspension (5 × 10⁵ spores per ml) on each needle prick. The inoculum was allowed to air dry. The plants were covered with a transparent plastic dome and were cultivated at 22°C in a growth chamber programmed for a 14 h light (90 µE/m/s) and 10 h dark cycle. Plants were uncovered after two days. Fungal growth was scored 7 dpi.

Chemical treatment of plants.

Four-week-old plants were either injected with a needleless syringe through the abaxial surface with solutions of MeJA (50 µM in 0.1% ethanol), α-AIB (2 mM in water) (Sigma), and silver thiosulphate (4 mM in water) or sprayed till run-off and subirrigated with a BTH formulation (100 µM active ingredient) in water, as previously described (Shah et al. 1997). As controls, plants were similarly treated with water. Leaves were harvested 48 h after treatment and were quick-frozen in liquid nitrogen. Leaf samples were stored at -80°C.

RNA extraction and Northern analyses.

Extraction of RNA was performed in the TRIzol reagent (GIBCO-BRL, Gaithersburg, MD, U.S.A.) following the manufacturer's instructions. Northern blot analysis and synthesis of random primed probes for *PR-1*, *BGL2*, and *PDF1.2* were synthesized as described earlier (Shah et al. 1997, 1999).

DNA extraction and

polymerase chain reaction (PCR) analyses.

Arabidopsis genomic DNA from leaf tissue was isolated by the method of Konieczny and Ausubel (1993). PCR analyses to distinguish between the wild-type *NPR1* and *npr1-5* mutant alleles were performed as described previously (Shah et al. 1999). A dCAPS (derived cleaved amplified polymorphic se-

quence) was used to distinguish between the *etr1-1* and wild-type *ETR1* alleles (Kachroo et al. 2000). Likewise, a CAPS marker polymorphic for the *COI1* and *coi1-1* alleles (Feys et al. 1994) was used to identify *coi1-1/coi1-1* plants. To distinguish between the *EIN2* and *ein2-1* alleles, two primers (5'-GTGCGGTTGGAAAGCAGGGTAAAGGAT-3' and 5'-CTC-CAGTTAAGACTAAGTT-3') were used to amplify a 175-bp fragment flanking the *ein2-1* mutation. After denaturation at 95°C for 5 min, 30 cycles of amplification were performed. Each amplification cycle was 95°C for 30 s, followed by 55°C for 30 s, followed by 72°C for 45 s. The wild-type amplicon lacks an *Afl*III restriction site, whereas the *ein2-1* mutation generates an *Afl*III restriction site causing two fragments of 24 bp and 151 bp, which are resolved on 3% agarose gel. The *ssil* mutation maps to the lower arm of chromosome IV (Shah et al. 1999). A PCR-based polymorphic marker tightly linked to *ssil* (approximately 0.4 cM) (P. Kachroo, J. Shah, and D. F. Klessig, unpublished data) that can distinguish between the Columbia and Nössen ecotypes was used to confirm the presence of *ssil* in various crosses.

Histochemistry and microscopy.

Leaves from 4-week-old soil-grown plants were used for trypan blue staining for dead cells. Trypan blue staining on *P. parasitica*-infected leaves was carried out on samples harvested 8 dpi. Samples were processed and analyzed as described by Rate and associates (1999).

SA/SA-glucoside (SAG), JA, and ethylene estimations.

SA and SAG were extracted and estimated from 0.25 to 0.5 g of fresh weight leaf tissue, as described by Bowling and associates (1994). Ethylene concentrations were measured by gas chromatography. Four-week-old plants were gently uprooted from soil, were placed in closed airtight rubber-cap containers (25 ml), and were incubated in a growth chamber. After 2 h of incubation, 1 ml of air was removed from each container with a 1-ml syringe and was analyzed by gas chromatography. The amount of ethylene was calculated from the peak area by comparing against the standard curve obtained by using pure ethylene. JA was extracted from leaf tissue and was assayed as described previously (Wang et al. 1999).

Construction of double and triple mutant lines.

Construction of the *ssil*, *ssil npr1*, and *ssil npr1 nahG* lines has been previously described (Shah et al. 1999). For all other genetic analyses, progeny from a backcrossed line homozygous for the *ssil* and *npr1-5* alleles was used. To generate *ssil ein2*, *ssil/+ ein2*, *ssil npr1 ein2*, and *ssil/+ npr1 ein2* lines, pollen from a *ssil npr1* plant was used to pollinate flowers from an *ein2-1* plant. The success of the cross was confirmed by CAPS analysis of F₁ plants for heterozygosity at the *NPR1* locus (Shah et al. 1999). Segregation of the *ssil* mutant allele was monitored in the F₂ progeny by the presence of the lesioned phenotype and by Northern blot for constitutive *PR-1* gene expression. Presence of the *ssil* locus from ecotype Nössen was further confirmed by a tightly linked polymorphic CAPS marker. CAPS analysis was performed, as previously described (Shah et al. 1999), on DNA from these phenotypically *ssil* plants to identify plants homozygous for the wild-type *NPR1* or the *npr1-5* mutant allele. F₃ progeny of these *ssil/+ NPR1* and *ssil/+ npr1* plants were screened for ethylene sensitivity, as described above. F₂ segregants that gave 100% ethylene-insensitive F₃ progeny were assumed to be homozygous for the *ein2-1* mutant allele. The genotype at the *ein2-1* locus was confirmed by PCR. Amongst these F₃ plants, those that were very small were presumed to be homozygous for *ssil*. This was confirmed by PCR for the *ssil* locus and in the F₄ progeny, which

were all small plants. To generate *ssil jarl*, *ssil/+ jarl*, *ssil npr1 jarl*, *ssil/+ npr1 jarl*, *ssil/+ coil*, and *ssil/+ etr1/+* plants, a strategy similar to that described above for *ssil ein2* plants was used to follow the *ssil* locus. Insensitivity of *jarl-1* roots to MeJA was used to identify plants homozygous for *jarl-1* mutant allele, while PCR-based polymorphisms between wild-type *COI1* and *coil-1* and wild-type *ETR1* and *etr1-1* were used to identify *coil-1/coil-1* and *etr1-1/+* segregants.

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