Arabidopsis ssi2*-Conferred Susceptibility to *Botrytis cinerea* Is Dependent on *EDS5* and *PAD4

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Loss of a stearyl-ACP desaturase activity in the *Arabidopsis thaliana ssi2* mutant confers susceptibility to the necrotroph, *Botrytis cinerea*. In contrast, the *ssi2* mutant exhibits enhanced resistance to *Pseudomonas syringae*, *Peronospora parasitica*, and *Cucumber mosaic virus*. The altered basal resistance to these pathogens in the *ssi2* mutant plant is accompanied by the constitutive accumulation of elevated salicylic acid (SA) level and expression of the pathogenesis-related 1 (*PR1*) gene, the inability of jasmonic acid (JA) to activate expression of the defensin gene, *PDF1.2*, and the spontaneous death of cells. Here, we show that presence of the *eds5* and *pad4* mutant alleles compromises the *ssi2*-conferred resistance to *Pseudomonas syringae* pv. *maculicola*. In contrast, resistance to *B. cinerea* was restored in the *ssi2 eds5* and *ssi2 pad4* double-mutant plants. However, resistance to *B. cinerea* was not accompanied by the restoration of JA responsiveness in the *ssi2 eds5* and *ssi2 pad4* plants. The *ssi2 eds5* and *ssi2 pad4* plants retain the *ssi2*-conferred spontaneous cell death phenotype, suggesting that cell death is not a major factor that predisposes the *ssi2* mutant to infection by *B. cinerea*. Furthermore, the high SA content of the *ssi2 pad4* plant, combined with our previous observation that the SA-deficient *ssi2 nahG* plant succumbs to infection by *B. cinerea*, suggests that elevated SA level does not have a causal role in the *ssi2*-conferred susceptibility to *B. cinerea*. Our results suggest that interaction between an *SSI2*-dependent factor or factors and an *EDS5*- and *PAD4*-dependent mechanism or mechanisms modulates defense to *B. cinerea*.

Additional keywords: plant defense.

Plants have evolved multiple mechanisms to defend themselves against pathogens. Salicylic acid (SA) is an important signaling molecule in plant defense (Durner et al. 1997; Feys and Parker 2000; Shah 2003; Shah and Klessig 1999). Stimulation of SA signaling activates expression of the pathogenesis-related (*PR*) genes, some of which have antimicrobial activities, and enhances resistance to several bacterial and oomycete

pathogens (Dempsey et al. 1999; Durner et al. 1997; Shah and Klessig 1999). SA-dependent defense responses are compromised in the *Arabidopsis npr1* mutant plant (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997) and in transgenic *NahG* plants (Delaney et al. 1994; Gaffney et al. 1993), resulting in a heightened susceptibility to bacterial and oomycete pathogens. The *nahG* gene encodes a salicylate hydroxylase that converts SA to catechol. Similarly, presence of the *sid2* and *eds5* mutant alleles, which depress SA synthesis in *Arabidopsis*, compromise the pathogen-induced expression of the *PR1* gene and enhance susceptibility to bacterial and oomycete pathogens (Nawrath and Métraux, 1999; Wildermuth et al. 2001). *SID2* is an isochorismate synthase that is involved in the biosynthesis of SA (Wildermuth et al. 2001). *EDS5* exhibits homology to multidrug and toxin extrusion (MATE) transporter proteins from animals (Nawrath et al. 2002). It has been suggested that *EDS5* transports precursors for SA biosynthesis (Nawrath et al. 2002). The accumulation of SA and the activation of SA-dependent defense responses, which are required for basal resistance to virulent bacterial and oomycete pathogens, also are compromised in the *Arabidopsis pad4* mutant (Jirage et al. 1999, 2001; Zhou et al. 1998). However, *pad4* does not affect SA accumulation in response to some avirulent pathogens (Zhou et al. 1998), suggesting that *PAD4* is not required for SA accumulation in all the cases of plant–pathogen interaction. In contrast to the *eds5* and *pad4* mutants, the *ssi1*, *ssi2*, *ssi4*, *cpr1*, and *cpr5* mutants, which constitutively accumulate high levels of SA, exhibit enhanced resistance to virulent strains of *Pseudomonas syringae* and *Peronospora parasitica* (Bowling et al. 1994, 1997; Shah et al. 1999, 2001; Shirano et al. 2002).

Jasmonic acid (JA) and ethylene are two other important signal molecules in plant defense. In *Arabidopsis*, JA- and ethylene-dependent mechanisms modulate resistance to the necrotrophic pathogen *Botrytis cinerea*. JA insensitivity conferred by the *Arabidopsis coi1* mutant and ethylene insensitivity conferred by the *ein2* mutant depress resistance to *B. cinerea* (Thomma et al. 1998, 1999). Exposure to *B. cinerea* enhances expression of the *Arabidopsis PDF1.2* gene, which encodes the antifungal protein defensin (Penninckx et al. 1998; Thomma et al. 1998, 1999). Constitutive expression of *PDF1.2* is accompanied by enhanced resistance to *B. cinerea* in transgenic *Arabidopsis* plants overexpressing the transcription factor *ERF1* (Berrocal-Lobo et al. 2002). Moreover, JA and ethylene treatment activate *PDF1.2* expression, and pathogen induction of *PDF1.2* expression requires JA and ethylene signaling (Penninckx et al. 1998). A complex network of cooperative and antagonistic interactions between the SA and JA or ethylene signaling pathways fine tune defense responses and

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*The e-Xtra logo stands for “electronic extra” and indicates the HTML abstract available on-line contains a supplemental figure showing the effect of the *eds5* and *pad4* mutant alleles on *ssi2*-conferred susceptibility to *B. cinerea*.

determine the spectrum and magnitude of expression of different defense responses (Kunkel and Brooks 2002; Pieterse et al. 2001; Thomma et al. 2001). For example, JA and SA are required for the NPR1-independent defense mechanism to *Pseudomonas syringae* and *Peronospora parasitica* in *Arabidopsis* (Clarke et al. 2000; Nandi et al. 2003a). In contrast, JA signaling antagonizes a SA- and NPR1-dependent mechanism that limits growth of *Pseudomonas syringae* (Kloek et al. 2001). Conversely, SA application depresses JA synthesis (Peña-Cortés et al. 1993) and the JA-inducible expression of *PDF1.2* (Spoel et al. 2003). Furthermore, the SA-conferred repression of JA-activated *PDF1.2* expression requires NPR1 (Spoel et al. 2003), and constitutive *PDF1.2* expression that is prevalent in the *Arabidopsis ssi1* and *cpr6* mutants is enhanced in the absence of a functional NPR1 (Clarke et al. 2000; Shah et al. 1999).

SA- and JA- or ethylene-regulated defense mechanisms are altered in the *Arabidopsis ssi2* mutant (Kachroo et al. 2001). *SSI2* is involved in lipid biosynthesis. It encodes a stearyl-

ACP desaturase, which converts 18:0-acyl carrier protein (ACP) to 18:1-ACP (Kachroo et al. 2001). The *ssi2* mutant plants contain high levels of SA, constitutively express the *PR* genes, and exhibit heightened resistance to *P. syringae* pv. *maculicola*, *Peronospora parasitica*, and *Cucumber mosaic virus* (CMV) (Kachroo et al. 2001, 2003a,b, 2004; Nandi et al. 2003b; Sekine et al. 2004; Shah et al. 2001). In contrast, presence of the *ssi2* mutant allele confers enhanced susceptibility to *B. cinerea* (Kachroo et al. 2001, 2003a; Nandi et al. 2003b). Furthermore, JA- and pathogen-induced expression of *PDF1.2* is blocked in the *ssi2* plants (Kachroo et al. 2001). Experiments with the *ssi2 nahG* plant suggest that the high level of SA does not have a causal role in the inability of *ssi2* to express *PDF1.2* and its enhanced susceptibility to *B. cinerea* (Kachroo et al. 2001; Shah et al. 2001).

In addition to altering defense responses, the *ssi2* plant is dwarfed and spontaneously develops lesions containing dead cells (Shah et al. 2001). Necrotrophs obtain nutrients from dead or dying cells. The propensity for cell death may predispose the *ssi2* mutant to infection by *B. cinerea*. Gorvin and Levine (2000) showed that activation of the hypersensitive response (HR), which is characterized by cell death, was associated with enhanced susceptibility of *Arabidopsis* to *B. cinerea*. Conversely, overexpression of animal antiapoptotic genes in tobacco and tomato enhances resistance to necrotrophs (Dickman et al. 2001; Lincoln et al. 2002). Here we show that the *ssi2*-conferred cell death is not sufficient to account for its enhanced susceptibility to *B. cinerea*. Furthermore, our previous studies with *ssi2 nahG* plant and experiments described in this study indicate that, although the high level of SA present in leaves of the *ssi2* mutant plant is associated with its heightened resistance to *P. syringae*, this high level of SA does not have a causal role in the susceptibility to *B. cinerea*. Our results suggest that the interaction between an SSI2-dependent or -modulated factor and an EDS5- and PAD4-dependent negative regulatory mechanism modulates defense to *B. cinerea*.

RESULTS

EDS5 is required for *ssi2*-conferred resistance to *P. syringae* pv. *maculicola*.

Previously, we showed that presence of the *nahG* transgene in the *ssi2 nahG* plant compromises *ssi2*-conferred enhanced resistance to *P. syringae* (Shah et al. 2001). However, the *ssi2*-conferred dwarf and cell death phenotypes, the inability to express *PDF1.2*, and the enhanced susceptibility to *B. cinerea* were retained in the *ssi2 nahG* plant (Kachroo et al. 2001; Shah et al. 2001). However, recent studies indicate that the *nahG*-encoded salicylate hydroxylase modifies some defense reactions, independent of its effect on SA (Heck et al. 2003; Van Wees and Glazebrook 2003). Therefore, we further tested the involvement of SA in *ssi2*-conferred phenotypes by studying the impact of the *eds5* and *pad4* mutant alleles on *ssi2*-conferred phenotypes. We compared resistance to *P. syringae* pv. *maculicola* between *ssi2* and the double mutants *ssi2 eds5* and *ssi2 pad4* (Fig. 1A and B). Bacterial numbers were monitored at 3 days post inoculation (dpi). As previously shown by us, the bacterial titer in leaves of the *ssi2* mutant was 35-fold lower than in leaves of the wild-type ecotype Nössen (wt-No) and Columbia (wt-Col) plants. In contrast, bacterial titer in leaves of the *ssi2 eds5* double-mutant plant was comparable to that observed in the wild-type plants and threefold lower than those observed in the *eds5* single-mutant plant (Fig. 1A). This loss of the *ssi2*-conferred resistance in the *ssi2 eds5* plant was paralleled by almost complete loss of *ssi2*-conferred constitutive *PR1* gene expression (Fig. 2). In contrast to the robust effect of the *eds5* allele on the *ssi2*-conferred *PR1* expression and re-

Fig. 1. *Arabidopsis thaliana ssi2*-conferred resistance to *Pseudomonas syringae* pv. *maculicola* in *eds5*- and *pad4*-containing plants. **A**, Comparison of *P. syringae* pv. *maculicola* titer in the leaves of wild-type ecotype Nössen (wt No) and Columbia (wt Col) plants, and the *ssi2*, *eds5*, and *ssi2 eds5* mutant plants. F₃ progeny of the *ssi2 eds5* F₂ plant no. 29 were used in this experiment. **B**, Comparison of *P. syringae* pv. *maculicola* titer in the leaves of wt No and wt Col plants, and the *ssi2*, *pad4*, and *ssi2 pad4* mutant plants. **A** and **B**, *P. syringae* pv. *maculicola* ES4326 (optical density at 600 nm = 0.0005 in 10 mM MgCl₂) was infiltrated into the abaxial surface of leaves with a syringe. In all, 20 leaf discs (five replications of four discs in each tube) were harvested from infected leaves at 3 days post inoculation; they then were weighed and ground in 10 mM MgCl₂, and the bacterial numbers titered. The bacterial numbers ± SD are presented as CFU per milligram of leaf tissue.

sistance to *P. syringae* pv. *maculicola*, the *pad4* mutant allele had an intermediate effect on the *ssi2*-conferred resistance to *P. syringae* pv. *maculicola*. Bacterial titer in the *ssi2 pad4* double mutant was 15-fold higher than in the *ssi2* mutant and 20-fold lower than in the *pad4* mutant (Fig. 1B). Likewise, the *ssi2 pad4* double mutant constitutively expressed the *PR1* gene, albeit at levels lower than that observed in the *ssi2* mutant (Fig. 2).

EDS5 and PAD4 are required for the *ssi2*-conferred susceptibility to *B. cinerea*.

We compared the susceptibility to *B. cinerea* between the *ssi2* mutant and the *ssi2 eds5* double mutant. The wild-type (wt-No and wt-Col) and the *eds5* single mutant, which served as controls, were resistant to *B. cinerea* (Fig. 3). Furthermore, none of the pathogen-inoculated leaves from these plants exhibit fungal sporulation (data not shown). In contrast, as previously shown by us (Kachroo et al. 2001; Nandi et al. 2003b), the *ssi2* mutant was highly susceptible to the fungus. Further-

more, >60% of pathogen-inoculated leaves of the *ssi2* plant exhibited profuse sporulation. Compared with the *ssi2* mutant, disease was limited in the *ssi2 eds5* mutants (Fig. 3). Moreover, compared with the *ssi2* mutant, fungal sporulation was observed in <5% of *B. cinerea*-inoculated leaves of the *ssi2 eds5* plant. The *ssi2* and *eds5-1* mutant alleles which were used in this study are in the ecotype Nössen and Columbia background, respectively. To control for any contribution of interaccession differences between the Nössen and Columbia ecotypes on the results with the *ssi2 eds5* double-mutant plants, we studied growth of *B. cinerea* in the F₃ progeny derived from four F₂ *ssi2 eds5* double-mutant plants. All four *ssi2 eds5* lines exhibited resistance to *B. cinerea*, suggesting that interaccession differences do not have a major contribution to the resistance observed in the *ssi2 eds5* double mutant. Compared with the *ssi2* mutant, disease also was limited in the *ssi2 pad4* double mutant (Fig. 3). Similarly, a high level of resistance to *B. cinerea* also was observed in multiple *ssi2 eds5 npr1* and *ssi2 pad4 npr1* lines (A. Nandi and J. Shah, unpublished).

EDS5 is required for *ssi2*-conferred SA accumulation.

To determine whether the effects of the *eds5* and *pad4* mutant alleles on the *ssi2*-conferred defense-responses are associated with a reduction in *ssi2*-conferred SA accumulation, we compared the total SA (SA plus SA-glucoside) content in the leaves of *ssi2*, *ssi2 eds5*, and *ssi2 pad4* mutant plants. Compared with the wild-type (wt-No) plant, the *ssi2* mutant contained >50-fold higher levels of total SA (Fig. 4A and B). In contrast, total SA content in the *ssi2 eds5* plant was only two- and threefold higher than the total SA content in the wild-type and *eds5* mutant plant, respectively, and approximately 25-fold lower than in the *ssi2* mutant (Fig. 4A). Unlike the *eds5* mutant allele, the *pad4* mutant allele had a weak effect on the *ssi2*-conferred SA accumulation; the total SA content in leaves of the *ssi2 pad4* mutant was only 2.5-fold lower than in leaves

Fig. 2. Effect of the *eds5* and *pad4* mutant alleles on *ssi2*-conferred *PR1* expression. RNA was extracted from leaves of 4-week-old, soil-grown, wild-type ecotype Nössen (wt No), *eds5*, *pad4*, *ssi2*, *ssi2 eds5*, and *ssi2 pad4* plants. F₃ progeny of the *ssi2 eds5* F₂ plant no. 29 were used in this experiment. The blot was probed for accumulation of the *PR1* gene transcript. Gel loading was monitored by photographing the ethidium bromide-stained gel (EtBr) before transferring the RNA to a Nytran Plus membrane.

Fig. 3. Effect of the *eds5* and *pad4* mutant alleles on *ssi2*-conferred susceptibility to *Botrytis cinerea*. Leaves of 4-week-old, soil-grown, wild-type ecotype Nössen (wt No) and Columbia (wt Col) plants and the *ssi2*, *eds5*, *ssi2 eds5*, *pad4*, and *ssi2 pad4* mutant plants were inoculated with freshly harvested spores of *B. cinerea*. F₃ progeny of the *ssi2 eds5* F₂ plant nos. 29, 39, 45, and 120 were used in this experiment. For each line, 20 to 30 leaves were inoculated. Seven days later, each inoculated leaf was scored for the extent of spreading necrosis. The number of inoculated leaves exhibiting necrosis covering <25, 25 to 50, 50 to 75, and >75% of leaf area were determined for each line. The X-axis shows the percentage of inoculated leaves in each category.

of the *ssi2* mutant plant. However, these levels were approximately 20-fold higher than in leaves of the wild-type plant.

EDS5 and PAD4 are not required for *ssi2*-conferred cell death.

The spontaneous cell death that is prevalent in the *ssi2* mutant may predispose it to *B. cinerea* and simultaneously confer resistance to *P. syringae*. To determine whether the *eds5*- and *pad4*-conferred restoration of resistance to *B. cinerea* and depressed resistance to *P. syringae* in the *ssi2 eds5* and *ssi2 pad4* double-mutant plants is associated with the amelioration of the

ssi2-conferred cell death, we examined trypan blue-stained leaves from the *ssi2*, *ssi2 eds5*, and *ssi2 pad4* mutant plants for the presence of dead cells. Trypan blue stains dead cells intense blue. Cell death was not observed in the leaves of the wild-type (wt-No) or the *eds5* and *pad4* single mutants, which served as controls (Fig. 5A). In contrast, the *ssi2*-conferred cell death was retained in the leaves of the *ssi2 eds5* and *ssi2 pad4* double mutants. The *ssi2 eds5* and *ssi2 pad4* double mutants were slightly larger than the *ssi2* mutant, even though they were dwarfed compared with the wild-type plant (Fig. 5B).

Resistance to *B. cinerea* is not accompanied by the restoration of JA responsiveness in the *ssi2 eds5* and *ssi2 pad4* double mutants.

To determine whether, compared with the *ssi2* mutant, restoration of resistance to *B. cinerea* and susceptibility to *P. syringae* pv. *maculicola* in the *ssi2 eds5* and *ssi2 pad4* mutants are accompanied by restoration of JA signaling, we compared methyl-JA (MeJA) induction of *PDF1.2* expression in the *ssi2* plant versus the *ssi2 eds5* and *ssi2 pad4* plants. MeJA was a very effective inducer of *PDF1.2* expression in the wild-type (wt-No and wt-Col), *eds5* and *pad4* plants (Fig. 6A). In contrast, MeJA was ineffective in enhancing *PDF1.2* expression in the *ssi2*, *ssi2 eds5*, and *ssi2 pad4* mutants. Furthermore, although the *PDF1.2* transcript accumulated to very high levels in *B. cinerea*-inoculated leaves of wild-type (wt-No), *eds5*, and *pad4* plants, inoculation with *B. cinerea* did not enhance accumulation of the *PDF1.2* transcript in the *ssi2*, *ssi2 eds5*, and *ssi2 pad4* plants (Fig. 6B). However, compared with the wild-type, *eds5*, *pad4*, and *ssi2* mutants, we repeatedly observed constitutive expression of *PDF1.2* in the *ssi2 eds5* and *ssi2 pad4* double mutants (Fig. 6A and B). Similarly, constitutive expression of *PDF1.2* also was observed in multiple *ssi2 eds5 npr1* and *ssi2 pad4 npr1* lines (A. Nandi and J. Shah, unpublished).

DISCUSSION

Our observation that the *eds5* mutant allele abolishes *ssi2*-conferred high-level accumulation of SA and resistance to *P. syringae* pv. *maculicola* confirms our prior proposition (Shah et al. 2001) that elevated SA levels are required for the enhanced resistance to this pathogen in the *ssi2* mutant. In contrast, several lines of evidence suggest that hyperactivation of SA signaling and high SA content in the *ssi2* mutant do not have a causal role in its enhanced susceptibility to *B. cinerea*. First, like *ssi2*, the *ssi2 pad4* double mutant contained very high levels of SA and constitutively expressed the *PR1* gene (Figs. 2 and 4). However, compared with the *ssi2* mutant, the *ssi2 pad4* double mutant was resistant to *B. cinerea*. Second, we previously showed that the absence of the *ssi2*-conferred SA accumulation in the *ssi2 nahG* plant was not accompanied by restoration of resistance to *B. cinerea* (Kachroo et al. 2001; Shah et al. 2001). Last, *ssi2*-conferred susceptibility was retained in *sfd1 ssi2 npr1* and *sfd4 ssi2 npr1* plants, despite the lack of *ssi2*-conferred SA accumulation in these plants (Nandi et al. 2003b). The application of benzothiadiazole, a functional analog of SA, enhances resistance to *B. cinerea* (Zimmerli et al. 2001), suggesting that SA signaling promotes resistance to *B. cinerea* instead of antagonizing it.

Restoration of resistance to *B. cinerea* in the *ssi2 eds5* and *ssi2 pad4* plants (Fig. 3), despite the retention of the *ssi2*-conferred cell death (Fig. 5A), suggests that widespread cell death in the *ssi2* mutant plant is not sufficient to confer enhanced susceptibility to *B. cinerea*. This conclusion also is supported by our studies of the *sfd* mutants, *sfd1*, *sfd2*, and *sfd4*, which were identified as suppressors of *ssi2* (Nandi et al. 2003b). The *sfd1 ssi2 npr1*, *sfd2 ssi2 npr1*, and *sfd4 ssi2 npr1* plants retain

Fig. 4. Effect of the *eds5* and *pad4* mutant alleles on *ssi2*-conferred salicylic acid (SA) accumulation. **A**, Total SA (SA plus SA-glucoside) content in the leaves of the wild-type ecotype Nössen (wt-No), *ssi2*, *eds5*, and *ssi2 eds5* plants. F₃ progeny of the *ssi2 eds5* F₂ plant no. 29 were used in this experiment. **B**, Total SA content in the leaves of the wt No, *ssi2*, and *ssi2 pad4* plants. **A** and **B**, Total SA was extracted from leaves of 4-week-old, soil-grown plants. The total SA values presented as micrograms of SA per gram of fresh weight (FW) are averages of three sets of samples per line.

Fig. 5. *Arabidopsis thaliana* *ssi2*-conferred cell death and morphology in *eds5*- and *pad4*-containing plants. **A**, Comparison of the extent of cell death in leaves of the wild-type ecotype Nössen (wt-No), *ssi2*, *eds5*, *pad4*, *ssi2 eds5*, and *ssi2 pad4* plants. Leaves of 4-week old, soil-grown plants were stained with trypan blue and photographed under a dissection microscope. The black arrows point to areas containing intensely stained dead cells. **B**, Comparison of morphology of 4-week old, soil-grown wild-type ecotype Nössen, *ssi2*, *ssi2 eds5*, and *ssi2 pad4* plants. **A** and **B**, F_3 progeny of the *ssi2 eds5* F_2 plant no. 29 were used. All photographs are at the same magnification.

the *ssi2*-conferred susceptibility to *B. cinerea* despite lacking the *ssi2*-conferred cell death phenotype. In addition, factors that contribute toward the dwarfing of the *ssi2* mutant are not involved in susceptibility to *B. cinerea*, because the *ssi2 eds5* and *ssi2 pad4* double mutants retain the *ssi2*-conferred dwarf phenotype, although to a lesser degree (Fig. 5B). Moreover, the *sfd1 ssi2 npr1*, *sfd2 ssi2 npr1*, and *sfd4 ssi2 npr1* plants lack the *ssi2*-conferred dwarf status yet retain enhanced susceptibility to *B. cinerea* (Nandi et al. 2003b).

Berrocal-Lobo and associates (2002) showed that the enhanced resistance to *B. cinerea* observed in transgenic *Arabidopsis* plants, which constitutively express the transcription factor ERF1, is accompanied by constitutive expression of

Fig. 6. *PDF1.2* expression in wild-type and mutant plants. **A**, *PDF1.2* expression in leaves from wild-type ecotypes Nössen (wt-No) and Columbia (wt-Col) plants and *eds5*, *pad4*, *ssi2*, *ssi2 eds5*, and *ssi2 pad4* mutant plants 36 h after floatation on 0.1% ethanol (control) or 5 μ M methyl-jasmonic acid (MeJA) in 0.01% ethanol. **B**, *PDF1.2* expression in leaves from wt-No and *eds5*, *pad4*, *ssi2*, *ssi2 eds5*, and *ssi2 pad4* mutant plants 48 h after inoculation with *B. cinerea*. **A** and **B**, F_3 progeny of the *ssi2 eds5* F_2 plant no. 29 and 4-week-old soil-grown plants were used. The blots were probed for accumulation of the *PDF1.2* gene transcript. Gel loading was monitored by photographing the ethidium bromide stained gel (EtBr) before transferring the RNA to a Nytran Plus membrane.

PDF1.2. Similarly, the restoration of resistance to *B. cinerea* in the *ssi2 eds5* and *ssi2 pad4* double mutants is accompanied by the constitutive expression of *PDF1.2* (Fig. 6A and B). It is probable that, to counter the *ssi2*-dependent or modulated susceptibility factors, a higher basal level of *PDF1.2* expression or factors coregulated with it are required for conferring resistance to *B. cinerea* in the *ssi2 eds5* and *ssi2 pad4* plants. However, this needs to be experimentally tested.

Previously, we showed that the inability of the *ssi2* mutant to express *PDF1.2* and its hypersusceptibility to *B. cinerea* are not due to a defect in JA synthesis (Kachroo et al. 2003a). In this study, we have shown that the presence of the *eds5* and *pad4* alleles does not overcome the JA insensitivity conferred by the *ssi2* allele; MeJA was unable to activate *PDF1.2* expression in the *ssi2 eds5* and *ssi2 pad4* double mutants (Fig. 6A). Furthermore, like the *ssi2* mutant, *B. cinerea* inoculation was ineffective in enhancing *PDF1.2* expression in the *ssi2 eds5* and *ssi2 pad4* mutants. However, the *ssi2 eds5* and *ssi2 pad4* plants constitutively express the *PDF1.2* gene (Fig. 6A and B). Similarly, the *eds5* and *pad4* mutant alleles also enhance the basal level of *PDF1.2* expression in *ssi1 eds5* and *ssi1 pad4* plants (A. Nandi and J. Shah, unpublished). Other studies similarly have noted an inhibitory effect of the wild-type *EDS5* and *PAD4* alleles on the expression of *PDF1.2* (Clarke et al. 2000; Ferrari et al. 2003; Jirage et al. 2001). Our data suggests that this effect of *EDS5* and *PAD4* on *PDF1.2* expression in *ssi2*-containing plants probably is due to factors other than SA. A similar effect of *EDS5* on the *ssi2*-conferred resistance to CMV has been described (Sekine et al. 2004). The *EDS5* protein is predicted to participate in trafficking of organic molecules across membranes (Nawrath et al. 2002). Perhaps, *EDS5* is involved in the trafficking of a molecule that modulates *PDF1.2* expression and basal resistance to *B. cinerea*. *EDS5* and *PAD4* expression are induced in leaves inoculated with bacterial pathogens (Jirage et al. 1999; Nawrath et al. 2002). This increase in *EDS5* and *PAD4* expression may further contribute to repression of defense responses to *B. cinerea* in leaves inoculated with avirulent bacterial pathogens. In part, this may account for the increased susceptibility to *B. cinerea* of *Arabidopsis* plants undergoing the HR (Gorvin and Levine 2000).

Previously, we had suggested that the *ssi2* mutant lacks a factor, other than JA, that is required for resistance to *B. cinerea* (Kachroo et al. 2001, 2003a). *EDS5* and *PAD4* may negatively affect the generation or activity of this factor. However, the fact that the *eds5* and *pad4* mutant alleles bypass the requirement of *SSI2* for resistance to *B. cinerea* argues against this possibility. Alternatively, the *SSI2*-derived or -modulated factor or factors may negatively affect the repressive effect of the *EDS5*- and *PAD4*-modulated mechanisms on basal resistance to *B. cinerea*. In support of this possibility, in several experiments we have observed the *eds5* mutant to be more resistant to *B. cinerea* than the wild-type plant. However, the available evidence does not rule out the possibility that two parallel pathways, one requiring *SSI2* and the other dependent on *EDS5* and *PAD4*, modulate basal resistance to *B. cinerea*. Although both the *eds5* and *pad4* mutant alleles have a qualitatively similar effect on *ssi2*-conferred defense response, we cannot rule out the possibility that the impact of the *eds5* and *pad4* mutant alleles on *ssi2*-conferred phenotypes are exerted via different mechanisms. Indeed, several differences between the *eds5* and *pad4* mutant alleles on plant defense responses have been reported. For example, unlike the *eds5* allele, the *pad4* allele compromises camalexin biosynthesis (Zhou et al. 1998; Nawrath and Métraux 1999) and resistance to aphids (V. Pegadaraju and J. Shah, unpublished). Furthermore, microarray studies confirm that, in response to pathogen inoculation, the

eds5 and *pad4* mutants display some differences in gene expression profiles (Glazebrook et al. 2003).

The *Arabidopsis* *SSI2* protein is involved in lipid metabolism. Similarly, the *PAD4* protein exhibits homology to lipases and esterases (Jirage et al. 1999). Recently, several other studies also have argued that lipids play an important role in plant defense to pathogens (Kachroo et al. 2003a, 2004; Kumar and Klessig 2003; Maldonado et al. 2002; Nandi et al. 2003b, 2004). The availability of *Arabidopsis* mutants defective in lipid biosynthesis and metabolism combined with the recent development of lipidomics technology (Welti et al. 2002) should aid in further characterizing the role of lipids in plant defense.

MATERIALS AND METHODS

Growth conditions for plant and pathogens.

Arabidopsis seed were germinated at 22°C in a tissue culture chamber exposed to a cycle of 14 h of light (80 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 10 h of darkness on Murashige and Skoog agar (Sigma-Aldrich, St. Louis) supplemented with 1% sucrose. Nine-day-old seedlings were transferred to soil and grown at 22°C in growth chambers programmed for a cycle of 14 h of light (90 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 10 h of darkness.

P. syringae pv. *maculicola* ES4326 was propagated at 28°C on King's B medium (King et al. 1954) containing streptomycin (100 $\mu\text{g/ml}$). *B. cinerea* IMI169558 was cultivated on maltose media at 22°C in a growth chamber programmed for a cycle of 14 h of light (80 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 10 h of darkness. To induce sporulation, three 1-cm² maltose agar pieces containing *B. cinerea* mycelia were placed on potato dextrose agar and 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 cheesecloth and spores counted on a hemacytometer slide.

Pathogen inoculations.

Infections with *P. syringae* pv. *maculicola* ES4326 were performed on 4-week-old soil-grown plants. An overnight *P. syringae* pv. *maculicola* ES4326 culture was harvested and resuspended in 10 mM MgCl_2 to an optical density at 600 nm of 0.0005. 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 20 (five replications of four discs in each tube) leaf discs (0.15 cm²) were harvested at 3 dpi and placed in preweighed tubes. After the weight of each sample was determined, the samples were processed for bacterial counts as described earlier (Nandi et al. 2003a; Shah et al. 1997). Bacterial counts were expressed as CFU per milligram of fresh weight of leaf tissue.

Inoculation with *B. cinerea* spores was done on 4-week-old soil-grown plants. Three leaves per plant were pricked with a needle at well-spaced spots. Infection was initiated by placing a drop of freshly harvested spore suspension (5×10^5 spores/ml) on the needle prick. To account for the size differences, each leaf of the wild-type ecotype Nössen and Columbia and the *eds5* and *pad4* mutant plants were pricked at three spots and spotted with 10 μl of inoculum, and each leaf of the *ssi2 eds5* and *ssi2 pad4* plants and that of the *ssi2* plant were pricked at two and one spot, respectively, and spotted with 5 μl of inoculum. The inoculum was allowed to air dry. The plants were covered with a transparent plastic dome and cultivated at 22°C in a growth chamber programmed for a cycle of 14 h of light (90 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 10 h of darkness. Plants were uncovered after 2 days. Disease symptom and fungal sporulation were scored 7 dpi.

Construction of double-mutant lines.

The *eds5* and *pad4* mutants are in the ecotype Columbia while *ssi2* is in the ecotype Nössen. To generate the *ssi2 eds5* and *ssi2 pad4* lines, pollen from an *ssi2 npr1* plant was used to pollinate flowers from *eds5-1* and *pad4-1* plants, respectively. The success of the cross was confirmed by cleaved amplified polymorphic sequence (CAPS) analysis of F₁ plants for heterozygosity at the *NPR1* locus (Shah et al. 1999). Segregation of the *npr1-5*, *eds5-1*, *pad4-1*, and *ssi2* mutant alleles in the F₂ progeny was monitored with polymerase chain reaction (PCR)-based markers. F₃ generation plants were used for all studies.

DNA extraction and 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* allele and between the wild-type *SSI2* and *ssi2* mutant allele was performed as described previously (Nandi et al. 2003b; Shah et al. 1999). The simple sequence length polymorphism (SSLP) marker nga1107 (Nawrath and Métraux 1999) which is 2 cM from *EDS5* and polymorphic between Columbia and Nössen ecotypes was used to follow the *eds5-1* and *EDS5* alleles. Subsequently, a derived-CAPS (dCAPS) marker that distinguishes between the *EDS5* and *eds5-1* alleles was used to confirm the genotype at the *EDS5* locus. A CAPS also was used to distinguish between the *pad4-1* and *PAD4* alleles. CAPS and dCAPS information for the above alleles are available on request.

Histochemistry and microscopy.

Leaves from 4-week-old soil-grown plants were stained with trypan blue to visualize dead cells. Samples were processed and analyzed as described by Rate and associates (1999).

SA and SAG estimations.

Total SA was extracted and estimated from 0.25 to 0.5 g of fresh weight leaf tissue obtained from 4-week-old plants as described by Bowling and associates (1994).

Chemical treatment of plants.

Leaves from 4-week-old plants were excised at the base of the petiole and placed in a tissue culture dish, which contained 5 ml of a 5- μ M MeJA (Bedoukian Research, Danbury, CT, U.S.A.) solution prepared in 0.1% ethanol. The tissue culture dishes were placed at 22°C in a tissue culture chamber exposed to a cycle of 14 h of light (80 μ E m⁻² s⁻¹) and 10 h of darkness. Leaves were harvested 36 h after treatment and quick-frozen in liquid nitrogen before being processed for extracting RNA.

RNA extraction and RNA blot analyses.

Leaf tissue was ground under liquid nitrogen and RNA extracted using acid guanidinium thiocyanate-phenol-chloroform as described by Chomczynski and Sacchi (1987). Northern blot analysis and synthesis of random primed probes for *PR-1* and *PDF1.2* were synthesized as described earlier (Shah et al. 1997, 1999).

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