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Arabidopsis *sfd* Mutants Affect Plastidic Lipid Composition and Suppress Dwarfing, Cell Death, and the Enhanced Disease Resistance Phenotypes Resulting from the Deficiency of a Fatty Acid Desaturase

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A loss-of-function mutation in the Arabidopsis *SSI2*/*FAB2* gene, which encodes a plastidic stearoyl–acyl-carrier protein desaturase, has pleiotropic effects. The *ssi2* mutant plant is dwarf, spontaneously develops lesions containing dead cells, accumulates increased salicylic acid (SA) levels, and constitutively expresses SA-mediated, NPR1-dependent and -independent defense responses. In parallel, jasmonic acid–regulated signaling is compromised in the *ssi2* mutant. In an effort to discern the involvement of lipids in the *ssi2*-conferred developmental and defense phenotypes, we identified suppressors of fatty acid (stearoyl) desaturase deficiency (*sfd*) mutants. The *sfd1*, *sfd2*, and *sfd4* mutant alleles suppress the *ssi2*-conferred dwarfing and lesion development, the NPR1-independent expression of the *PATHOGENESIS-RELATED1* (*PR1*) gene, and resistance to *Pseudomonas syringae* pv *maculicola*. The *sfd1* and *sfd4* mutant alleles also depress *ssi2*-conferred *PR1* expression in *NPR1*-containing *sfd1 ssi2* and *sfd4 ssi2* plants. By contrast, the *sfd2 ssi2* plant retains the *ssi2*-conferred high-level expression of *PR1*. In parallel with the loss of *ssi2*-conferred constitutive SA signaling, the ability of jasmonic acid to activate *PDF1.2* expression is reinstated in the *sfd1 ssi2 npr1* plant. *sfd4* is a mutation in the *FAD6* gene that encodes a plastidic ω6-desaturase that is involved in the synthesis of polyunsaturated fatty acid–containing lipids. Because the levels of plastid complex lipid species containing hexadecatrienoic acid are depressed in all of the *sfd ssi2 npr1* plants, we propose that these lipids are involved in the manifestation of the *ssi2*-conferred phenotypes.

INTRODUCTION

Lipids are important structural components of biological membranes. In addition, lipid-derived second messengers participate in signal transduction mechanisms to influence plant growth, development, and responses to environmental cues (Munnik et al., 1998; Somerville et al., 2000; Laxalt and Munnik, 2002; Weber, 2002). Many of these signaling molecules are derived from fatty acids (Weber, 2002). De novo fatty acid synthesis in higher plants occurs solely in the plastids (Ohlrogge et al., 1991), resulting in the synthesis of palmitic acid (16:0)–, stearic acid (18:0)–, and oleic acid (18:1)–acyl-carrier protein (ACP) (McKeon and Stumpf, 1982; Somerville et al., 2000). These are either converted to the plastidic lipids phosphatidylglycerol (PG), monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol by the prokaryotic pathway localized in the plastid inner envelope or exported as CoA thioesters to the cytoplasm, where they enter the eukaryotic lipid biosynthesis pathway, which primarily synthesizes phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (Somerville et al., 2000; Wallis and Browse, 2002). Some PC species, or fragments thereof, are returned to the plastid, where an acyl-glycerol component is incorporated into complex plastid lipids (Browse et al., 1986; Mongrand et al., 2000). Differences in the enzymes present in the two compartments result in differences in the positions at which 16- and 18-C acyl species are esterified to glycerol, the extent of 16- and 18-C acyl desaturation, and the lipid head group classes formed in the cytoplasm and plastid. The desaturation of 16:0 and 18:1 occurs on 16-C and 18-C fatty acids incorporated into complex lipids. In Arabidopsis, the desaturation of 16:0 to hexadecatrienoic acid (16:3) occurs primarily on plastid-localized complex lipids. Hence, 16:3-containing lipids are found predominantly in the plastid lipid, MGDG (Wallis and Browse, 2002).

Polyunsaturated fatty acids (PUFAs) released from membrane lipids are precursors for the synthesis of oxylipins, a large family of oxidized lipids. For example, jasmonic acid (JA) and its methyl ester (MeJA) are synthesized from linolenic acid (18:3) via the octadecanoid pathway. In addition, a parallel hexadecanoid pathway in Arabidopsis synthesizes JA-related compounds from 16:3 (Weber, 2002). Oxylipins are important signaling molecules in plant development and defense against

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wounding, insect feeding, and pathogen infection (Reymond and Farmer, 1998; Weber, 2002). For example, JA is required for pollen development, and several Arabidopsis mutants blocked in JA synthesis or signaling are male sterile as a result of improper pollen development (Feys et al., 1994; McConn and Browse, 1996; Stinzi and Browse, 2000). In addition, JA biosynthesis and signaling mutants exhibit increased susceptibility to fungal pathogens and insects (Penninckx et al., 1996; Staswick et al., 1998; Vijayan et al., 1998). By contrast, JA application activates the expression of the antifungal protein defensin gene *PDF1.2* (Penninckx et al., 1998). Moreover, high-level expression of *PDF1.2* correlates with increased resistance to the necrotrophic fungus *Botrytis cinerea* (Berrocal-Lobo et al., 2002).

Salicylic acid (SA) is another important signaling molecule in plants, influencing plant development and defense responses (Shah and Klessig, 1999). The involvement of SA in plant defense has been studied extensively. SA levels are increased in plants resisting pathogen attack, and these increases correlate with the activation of *PATHOGENESIS-RELATED* (*PR*) gene expression and resistance (Durner et al., 1997; Shah and Klessig, 1999). In addition, the application of SA activates *PR* gene expression and disease resistance. Several PR proteins have proven antimicrobial activities, and their expression serves as a molecular marker for the activation of defense responses (Klessig and Malamy, 1994; Hammond-Kosack and Jones, 1996; Hunt and Ryals., 1996; Ryals et al., 1996). Blocking SA biosynthesis in the Arabidopsis *eds5* (*sid1*) and *sid2* (*eds16*) mutants depresses *PR* expression in pathogen-infected plants and enhances susceptibility to pathogens (Nawrath and Metraux, 1999; Dewdney et al., 2000). Likewise, preventing SA accumulation by expressing the SA-degrading salicylate hydroxylase, which is encoded by the bacterial *nahG* gene, suppresses the expression of *PR* genes and confers enhanced susceptibility to pathogens (Gaffney et al., 1993; Delaney et al., 1994). The *NPR1* gene is an important regulator of the SA-dependent expression of *PR* genes and resistance in Arabidopsis. The loss of NPR1 activity affects the SA-inducible expression of *PR* genes and resistance in *npr1*/*nim1* mutants (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). However, SA signaling also occurs via NPR1-independent mechanism(s) in addition to the NPR1-dependent pathway (Shah et al., 1999, 2001; Clarke et al., 2000).

The *ssi* (*suppressor of SA insensitivity*) mutants were identified in a screen for mutants that restore SA signaling in the *npr1* genetic background (Shah et al., 1999, 2001; Shirano et al., 2002). SA signaling through the NPR1-dependent and -independent pathways is constitutively active in the *ssi2* mutant (Shah et al., 2001). *PR* genes are expressed at increased levels, and resistance to *Peronospora parasitica* is enhanced in *ssi2* and *ssi2 npr1* plants (Shah et al., 2001). In addition, enhanced resistance to an avirulent strain of *Pseudomonas syringae* pv *tomato* is observed in the *ssi2* mutant. The *ssi2*-conferred *PR* expression and resistance phenotypes are associated with the accumulation of increased levels of SA. Moreover, increased SA levels are required for the *ssi2*-conferred *PR* expression and enhanced disease resistance; *PR1* expression and resistance to *P. parasitica* and *P. syringae* are depressed in the *ssi2 nahG* plant (Shah et al., 2001). Crosstalk between SA and JA signaling pathways is important for the fine-tuning of plant defense responses. Both synergistic and antagonistic interactions between SA and JA signaling have been reported (Reymond and Farmer, 1998; Kunkel and Brooks, 2002). Similarly, in the *ssi2* mutant, the constitutive activation of SA-dependent defense responses is paralleled by the inability of JA to activate *PDF1.2* expression and enhanced susceptibility to *B. cinerea* (Kachroo et al., 2001). In addition to altering defense responses, *ssi2* and the allelic *fab2* mutant are dwarf and develop lesions that contain dead cells (Lightner et al., 1994a, 1994b; Shah et al., 2001). Figure 1 summarizes the impact of *ssi2* on Arabidopsis defense signaling.

The *ssi2* phenotypes are caused by a deficiency in a plastidic stearoyl-ACP desaturase activity (Kachroo et al., 2001), sug-

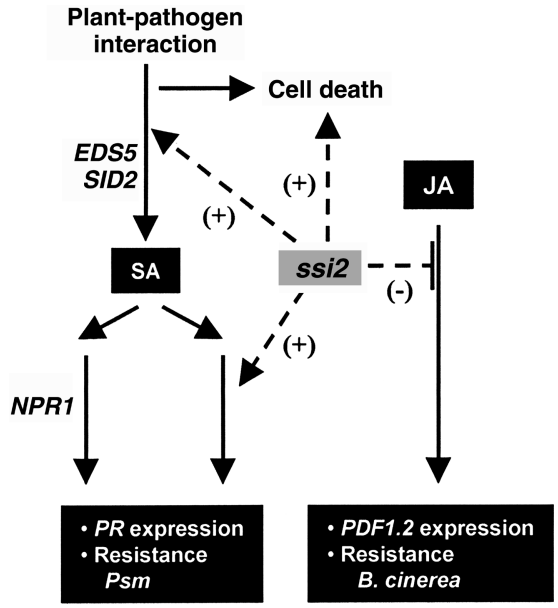

Figure 1. Impact of the *ssi2* Mutant on Defense Signaling in Arabidopsis.

SA and JA are important signaling molecules in plant defense. JA signaling is required for the pathogen-activated expression of *PDF1.2* and resistance to the necrotrophic pathogen *B. cinerea*. SA signaling is required for the pathogen-activated expression of the *PR* genes and resistance to *Psm*. The *EDS5* and *SID2* genes are required for SA synthesis; the loss-of-function *eds5* and *sid2* mutations block SA synthesis. SA signaling is activated via both NPR1-dependent and -independent mechanisms. In addition to SA, an unknown pathogen-activated factor is required for signaling through the NPR1-independent pathway. The loss of the SSI2-encoded stearoyl-ACP desaturase activity in the *ssi2* mutant has pleiotropic effects on plant defense responses. The *ssi2* mutant allele promotes (+) the spontaneous development of lesions containing dead cells, the accumulation of increased SA levels, and the constitutive expression of NPR1-dependent and -independent defense mechanisms, which confer high-level expression of *PR* genes and enhanced resistance to *Psm*. By contrast, the *ssi2* mutant interferes with (−) the ability of JA/MeJA to activate *PDF1.2* expression and exhibits enhanced susceptibility to *B. cinerea*. 18:1 application restores the JA-inducible expression of *PDF1.2* in the *ssi2* mutant plant (Kachroo et al., 2001), suggesting a role for an 18:1-derived factor, which is limiting in the *ssi2* mutant plant, in promoting JA signaling.

gesting the involvement of lipids and lipid signaling in plant development and in modulating SA- and JA-dependent defense mechanisms. The *FAB2* (*SSI2*)-encoded stearoyl-ACP desaturase preferentially desaturates 18:0-ACP to yield 18:1-ACP (Shanklin and Somerville, 1991; Shanklin and Cahoon, 1998). Compared with those in wild-type plants, 18:0 levels were increased and 18:1 levels were depressed in the *ssi2* mutant. Moreover, coapplication of 18:1 restored JA-activated *PDF1.2* expression in the *ssi2* mutant, suggesting that 18:1 deficiency, rather than the increased 18:0 level, was responsible for the inability of the *ssi2* mutant to express *PDF1.2* (Kachroo et al., 2001). However, the role of altered lipid composition in other *ssi2*-conferred phenotypes was unclear.

To further elucidate the role of lipids in the *ssi2*-conferred growth and developmental phenotypes and defense responses, we conducted a suppressor screen in the *ssi2 npr1* genetic background for mutants that lack *ssi2*-conferred dwarfing and *PR1* expression. The *sfd1*, *sfd2*, and *sfd4* mutant alleles suppress the *ssi2*-conferred dwarfing and lesion development, the NPR1-independent expression of *PR1*, and resistance to *Pseudomonas syringae* pv *maculicola* (*Psm*). In addition, *sfd1* overcomes the *ssi2*-conferred block on MeJA activation of *PDF1.2* expression. *sfd4* contains a mutation in the 16:1/18:1 ω6 desaturase gene *FAD6*. Moreover, levels of the PUFA 16:3 were depressed in all *sfd ssi2 npr1* plants, suggesting a role for PUFA-derived signals in promoting the *ssi2*-conferred plant developmental and defense response phenotypes.

RESULTS

sfd Mutant Alleles Suppress the *ssi2*-Conferred Dwarfing and Lesion Development and the NPR1-Independent Expression of the *PR1* Gene

To isolate suppressors of the stearoyl-ACP desaturase–deficient *ssi2* mutation, seeds from *ssi2 npr1* plants were mutagenized with ethyl methanesulfonate as described previously (Shah et al., 1997). Four-week-old M2 progeny of these ethyl methanesulfonate–mutagenized M1 seeds were screened for large plants and for the loss of *ssi2*-conferred constitutive *PR1* expression. Seven *sfd* mutants were identified among the 4000 M2 plants screened. These *sfd* mutants define four complementation groups: *sfd1* (two alleles: *sfd1-1* and *sfd1-2*), *sfd2* (three alleles: *sfd2-1*, *sfd2-2*, and *sfd2-3*), *sfd3*, and *sfd4*. Mutants in the *sfd1*, *sfd2*, and *sfd4* complementation groups were characterized further. As shown in Figures 2A and 3A, mutants in the *sfd1*, *sfd2*, and *sfd4* complementation groups ameliorate the *ssi2*-conferred dwarf phenotype and depress the *ssi2*-conferred NPR1-independent expression of the *PR1* gene. The suppressive effect of these *sfd* alleles on the *ssi2*-conferred *BGL2* expression was less pronounced.

Compared with the wild type and *npr1*, leaves of all *sfd ssi2 npr1* plants were lighter green. Leaves of the *ssi2* and *ssi2 npr1* mutant plants develop macroscopic lesions containing dead cells (Shah et al., 2001). However, leaves of all *sfd1 ssi2 npr1* and *sfd2 ssi2 npr1* plants and the *sfd4 ssi2 npr1* plant do not exhibit macroscopic lesions. We examined trypan blue–stained leaves from the *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants to determine whether they had microscopic lesions containing dead cells. Trypan blue stains dead cells dark blue. As shown in Figure 2B, unlike the *ssi2 npr1* plant, but like the *npr1* control, leaves of the *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants lacked intensely stained dead cells.

sfd1 and *sfd4* Suppress the *ssi2*-Conferred SA Accumulation

Previously, we had shown that the *ssi2*-conferred accumulation of increased SA is not required for cell death and dwarfing (Shah et al., 2001). Dwarfing, cell death, and SA accumulation may be activated independently by *ssi2*. Alternatively, dwarfing and/or the activation of cell death in the *ssi2* mutant may stimulate SA accumulation. Therefore, we measured the total SA plus SA-glucoside levels in leaves of *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants. As shown in Table 1, the total SA content of *sfd1-1 ssi2 npr1* and *sfd4 ssi2 npr1* plants was comparable to the total SA level in wild-type and *npr1* plants and 25- to 50-fold lower than the total SA level in the *ssi2 npr1* plant. By contrast, the total SA content in the *sfd2-1 ssi2 npr1* plant was ~13-fold higher than the total SA level in wild-type and *npr1* plants and one-quarter of that in the *ssi2 npr1* plant.

To determine if the loss of *ssi2*-conferred NPR1-independent *PR1* expression in *sfd1-1 ssi2 npr1* is attributable to the lack of SA accumulation, we examined *PR1* expression in leaves of the SA-treated *sfd1-1 ssi2 npr1* plant. SA-treated wild-type, *ssi2*, and *ssi2 npr1* plants served as positive controls for monitoring the efficacy of SA application in this experiment, and the *npr1* mutant served as a negative control. As shown in Figure 3B, SA was ineffective at restoring *ssi2*-conferred *PR1* expression in the *sfd1-1 ssi2 npr1* plant. Likewise, SA application was ineffective at restoring *PR1* expression in the *sfd2-1 ssi2 npr1* (Figure 3B) and *sfd4 ssi2 npr1* plants (data not shown). By contrast, the SA-treated wild-type and *ssi2* control plants expressed high levels of *PR1*. Similar results were obtained with the application of benzothiadiazole, a functional analog of SA (data not shown). Hence, another *ssi2*-modulated factor(s), in addition to SA, is required for the NPR1-independent *PR1* expression observed in the *ssi2* mutant.

sfd1, *sfd2*, and *sfd4* Suppress the *ssi2*-Conferred NPR1-Independent Resistance to *Psm*

We had shown previously that the activation of SA signaling in the *ssi2 npr1* plant confers enhanced resistance to *P. syringae* pv *tomato* DC3000 containing the avirulence gene *avrRpt2* and the oomycete *Peronospora parasitica* Emco5 (Shah et al., 2001). Similarly, as shown in Figure 4A, compared with wild-type and *npr1* plants, the *ssi2 npr1* double mutant plant exhibited enhanced resistance to *Psm*. Bacterial numbers at 3 days after inoculation in the *ssi2 npr1* plant were 20-fold lower than those in the *npr1* plant. Therefore, we tested if the *sfd1*, *sfd2*, and *sfd4* mutant alleles could suppress the *ssi2*-conferred NPR1-independent resistance to *Psm*. Bacterial numbers were monitored in *sfd1-1 ssi2 npr1*, *sfd1-2 ssi2 npr1*, *sfd2-1 ssi2 npr1*, *sfd2-2 ssi2 npr1*, *sfd2-3 ssi2 npr1*, and *sfd4 ssi2 npr1* plants. As shown in Figure 4A, bacterial numbers in *sfd1-1 ssi2*

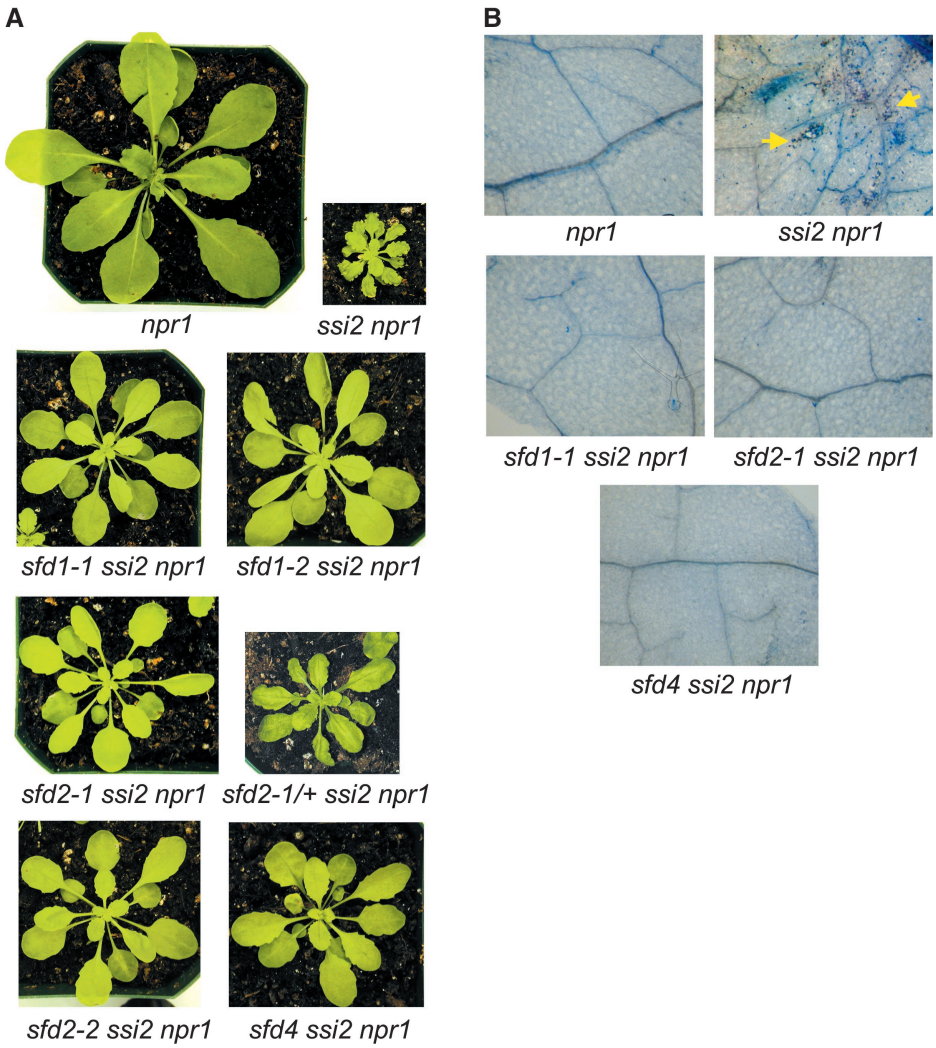

Figure 2. Comparison of Morphological and Cell Death Phenotypes of the *sfd ssi2 npr1* Mutants.

(A) Comparison of the morphology of 4-week-old *npr1*, *ssi2 npr1*, *sfd1-1 ssi2 npr1*, *sfd1-2 ssi2 npr1*, *sfd2-1 ssi2 npr1*, *sfd2-1*/+ *ssi2 npr1*, *sfd2-2 ssi2 npr1*, and *sfd4 ssi2 npr1* plants. The *sfd2-1*/+ *ssi2 npr1* plant is heterozygous for the *sfd2-1* mutant allele. All plants were photographed from the same distance.

(B) Light microscopy of trypan blue–stained leaves from *npr1*, *ssi2 npr1*, *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants. The arrows mark areas containing intensely stained dead cells in *ssi2 npr1*. All photographs were taken at the same magnification.

npr1, *sfd1-2 ssi2 npr1*, *sfd2-1 ssi2 npr1*, *sfd2-2 ssi2 npr1*, *sfd2-3 ssi2 npr1*, and *sfd4 ssi2 npr1* plants at 3 days after inoculation were 8- to 10-fold higher than those in the *ssi2 npr1* plant.

sfd2 Does Not Suppress the *ssi2*-Conferred NPR1-Dependent Expression of *PR1*

The *ssi2* mutant activates SA signaling through the NPR1-dependent pathway in addition to via the NPR1-independent mechanism (Shah et al., 2001). Therefore, we tested if the *sfd1-1* and *sfd2-1* mutant alleles could suppress *ssi2*-conferred *PR1* expression in the wild-type *NPR1*-containing *sfd1-1 ssi2* and *sfd2-1 ssi2* plants. As shown in Figure 3C, the *sfd1-1* allele effectively suppressed *ssi2*-conferred constitutive *PR1* expression in the *sfd1-1 ssi2* plant. By contrast, the *sfd2-1* allele was not very effective at blocking *ssi2*-conferred *PR1* expression in the *sfd2-1 ssi2* double mutant (Figure 3A). Compared with the *PR1* transcript levels in the *ssi2* mutant plant, only a slight reduction in the accumulation of *PR1* transcript, probably as a result of the suppression of the NPR1-independent pathway, was observed in the *sfd2-1 ssi2* plant, suggesting that *sfd2* specifically affects NPR1-independent signaling.

sfd1 Bypasses the *ssi2*-Conferred Block on the JA-Activated Expression of *PDF1.2*

The loss of SSI2 activity in the *ssi2* mutant prevents the JA-activated expression of the *PDF1.2* gene (Kachroo et al., 2001).

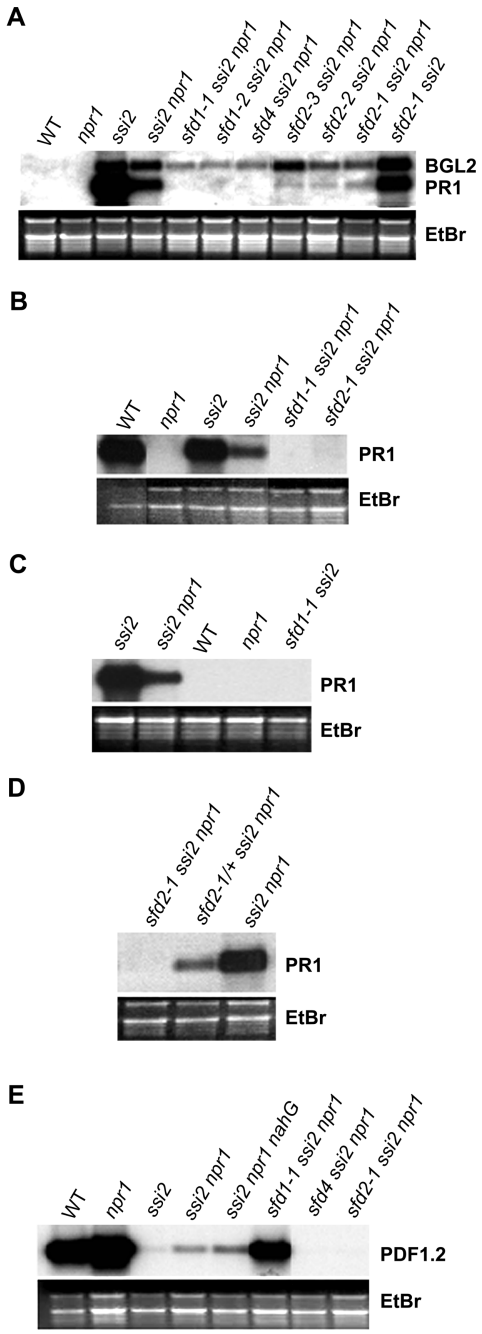

Figure 3. Defense Gene Expression in the *sfd* Mutants.

(A) Comparison of *PR1* and *BGL2* expression in leaves of 4-week-old soil-grown wild-type (WT), *npr1*, *ssi2*, *ssi2 npr1*, *sfd1-1 ssi2 npr1*, *sfd1-2 ssi2 npr1*, *sfd4 ssi2 npr1*, *sfd2-3 ssi2 npr1*, *sfd2-2 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd2-1 ssi2* plants.

(B) Comparison of *PR1* expression in leaves of 4-week-old wild-type, *npr1*, *ssi2*, *ssi2 npr1*, *sfd1-1 ssi2 npr1*, and *sfd2-1 ssi2 npr1* plants 48 h after treatment with 500 μM SA.

(C) Comparison of *PR1* expression in leaves of 4-week-old soil-grown *ssi2*, *ssi2 npr1*, wild-type, *npr1*, and *sfd1-1 ssi2* plants.

(D) Comparison of *PR1* expression in *sfd2-1 ssi2 npr1*, *sfd2-1/+ ssi2 npr1*, and *ssi2 npr1* plants.

(E) Comparison of *PDF1.2* expression in leaves of 4-week-old wild-type, *npr1*, *ssi2*, *ssi2 npr1*, *ssi2 npr1 nahG*, *sfd1-1 ssi2 npr1*, *sfd4 ssi2 npr1*, and *sfd2-1 ssi2 npr1* plants 48 h after treatment with 5 μM MeJA dissolved in 0.1% ethanol.

All RNAs were resolved on denaturing gels, transferred to Nytran Plus membranes (Schleicher & Schuell), and probed for the indicated genes. Gel loading was monitored by photographing the ethidium bromide–stained gel (EtBr) before transferring the RNA to a Nytran Plus membrane.

Kloek et al. (2001) have shown that JA signaling antagonizes SA signaling in plant defense, including the expression of *PR1* and resistance to *P. syringae*. The lack of *ssi2*-conferred *PR1* expression in the *sfd1 ssi2 npr1*, *sfd2 ssi2 npr1*, and *sfd4 ssi2 npr1* plants could result from the restoration of JA signaling. Therefore, we examined the expression of the *PDF1.2* gene in MeJA-treated leaves of the *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants. MeJA-treated leaves of wild-type and *npr1* plants served as positive controls for this experiment, and *ssi2*, *ssi2 npr1*, and *ssi2 npr1 nahG* plants served as negative controls. As shown previously (Kachroo et al., 2001), MeJA application conferred high-level expression of the *PDF1.2* gene in leaves of wild-type and *npr1* plants but not in *ssi2*, *ssi2 npr1*, and *ssi2 npr1 nahG* plants (Figure 3E). The presence of the *sfd1-1* allele restored *PDF1.2* expression in MeJA-treated leaves of the *sfd1-1 ssi2 npr1* plant (Figure 3E). However, MeJA was unable to activate *PDF1.2* expression in the leaves of *sfd2-1 ssi2 npr1* and *sfd4 ssi2 npr1*. As expected, control plants treated with ethanol, which was used to dilute the MeJA, did not activate the expression of *PDF1.2* (data not shown).

ssi2-Conferred Susceptibility to *B. cinerea* Is Unaffected by *sfd1*, *sfd2*, and *sfd4*

An increased susceptibility to *B. cinerea* parallels the inability of the *ssi2* mutant to express the *PDF1.2* gene in response to JA application (Kachroo et al., 2001). Therefore, we tested whether the restoration of JA-inducible *PDF1.2* expression in the *sfd1-1 ssi2 npr1* plant is associated with the reestablishment of resistance to *B. cinerea*. As shown in Figure 4B, compared with the wild-type plant, the *sfd1-1 ssi2 npr1* plant retained the *ssi2*-conferred enhanced susceptibility to infection with *B. cinerea*. The relative infection rating of *sfd1-1 ssi2 npr1* was comparable to those of *ssi2 npr1* and *ssi2* plants. Similarly, the *sfd1-2 ssi2 npr1* plant retained the *ssi2*-conferred enhanced susceptibility to *B. cinerea*. The relative infection ratings for *sfd2-1 ssi2 npr1*, *sfd2-2 ssi2 npr1*, and *sfd4 ssi2 npr1* plants were comparable to that of the *ssi2 npr1* plant as well.

sfd1, *sfd2*, and *sfd4* Modify the Lipid Profile of the *ssi2* Mutant

It was hypothesized previously that decreased levels of 18:1 contribute directly or indirectly to the *ssi2* phenotypes (Kachroo et al., 2001). Alternatively, high levels of 18:0 in membrane lipids of the *ssi2* mutant might activate one or more *ssi2*-con-

Table 1. Total SA Content in *sfd* Plants

Genotype	Total SA ± SD (μg/g FW)
Wild type	0.5 ± 0.2
npr1	0.5 ± 0.1
ssi2 npr1	24.0 ± 1.8
sfd1-1 ssi2 npr1	0.5 ± 0.1
sfd2-1 ssi2 npr1	6.6 ± 1.3
sfd4 ssi2 npr1	0.9 ± 0.3

Total SA values for each line are averages of five samples ± SD. All values are corrected based on a 73% recovery. FW, fresh weight.

ferred phenotypes. The *sfd*-mediated suppression of the *ssi2*-conferred phenotypes could result from restoration or other alterations in the lipid composition of the mutant plants. Therefore, we compared the fatty acid composition of polar lipids from *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants with those of wild-type, *npr1*, and *ssi2 npr1* plants. As shown previously (Kachroo et al., 2001), the mol % of 18:0 in *ssi2 npr1* was sixfold higher and that of 18:1 was one-third of the level in the *npr1* plant (Table 2). The mol % of 18:0 in *sfd1-1 ssi2 npr1* was comparable to that in *ssi2 npr1*, whereas the mol % of 18:0 in the *sfd2-1 ssi2 npr1* and *sfd4 ssi2 npr1* plants were slightly lower than that in *ssi2 npr1*. However, these levels still were fivefold higher than the mol % of 18:0 in the *npr1* plant. Hence, it seems unlikely that increased 18:0 mol % is responsible for the *ssi2* phenotypes.

By contrast, the mol % of 18:1 in *sfd1-1 ssi2 npr1* was 2.5-fold higher and that in *sfd2-1 ssi2 npr1* was 2-fold higher than that in the *ssi2 npr1* plant. The mol % of 18:1 in the *sfd4 ssi2 npr1* plant was ~18-fold higher than in the *ssi2 npr1* plant and 6-fold higher than that in *npr1*. This increase in the mol % of 18:1 in *sfd4 ssi2 npr1* was paralleled by a corresponding reduction in the mol % of 18:3. One striking and consistent alteration in the fatty acid compositions of all of the *sfd ssi2 npr1* plants was the decrease in the mol % of hexadecatrienoic acid (16:3). 16:3 mol % in the *sfd1-1 ssi2 npr1* mutant was 40% lower than that in the *ssi2 npr1* plant, whereas 16:3 mol % in *sfd2-1 ssi2 npr1* and *sfd4 ssi2 npr1* were 82 and 90% lower, respectively, than that in *ssi2 npr1*. The decreased mol % of 16:3 in *sfd4 ssi2 npr1* was paralleled by an increase in the mol % of 16:1.

The lipid compositions were examined in more detail by comparing the molecular species of the membrane lipids in the leaves of *ssi2*, *ssi2 npr1*, *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants. Wild-type and *npr1* plants served as controls for this experiment. Compared with the wild-type (214 nmol diacyl species/mg dry weight) and the *npr1* mutant (213 nmol diacyl species/mg dry weight), the *ssi2* and *ssi2 npr1* plants contained lower amounts of lipid (147 and 155 nmol diacyl species/mg dry weight, respectively). As shown in Figure 5A, the lower lipid levels in *ssi2* and *ssi2 npr1* were attributable to a lower content of the lipids that are primarily plastid synthesized and localized; PG and MGDG species decreased by ~40%, whereas DGDG levels decreased by ~50%. The total lipid content was increased somewhat in the *sfd1-1 ssi2 npr1* (191 nmol diacyl species/mg dry weight) and *sfd2-1 ssi2 npr1* (166 nmol diacyl species/mg dry weight) plants, compared with

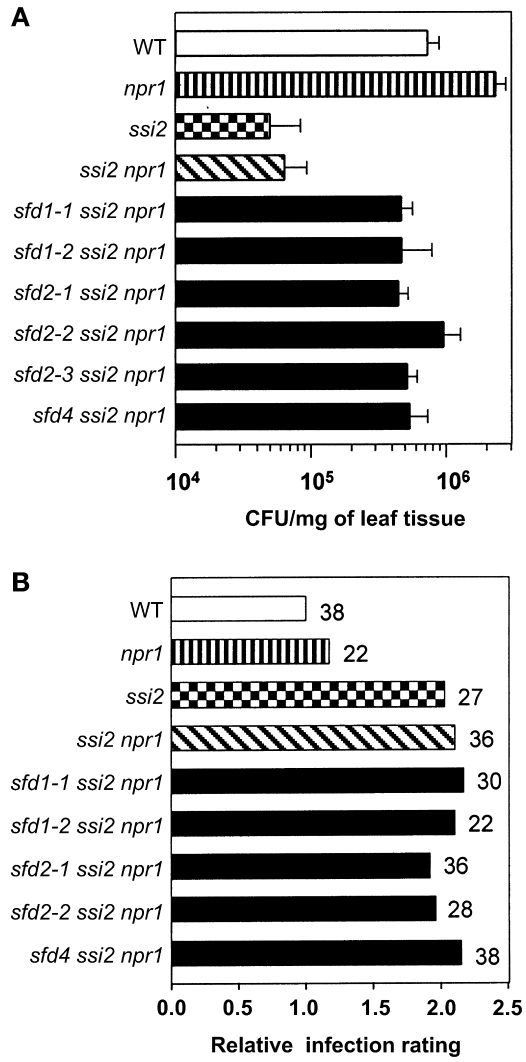

Figure 4. Growth of Pathogen in the *sfd* Mutants.

(A) Bacterial numbers in the *sfd* mutants. *Psm* (OD_{600} = 0.0002) was infiltrated into the abaxial surfaces of leaves of wild-type (WT), *npr1*, *ssi2*, *ssi2 npr1*, *sfd1-1 ssi2 npr1*, *sfd1-2 ssi2 npr1*, *sfd2-1 ssi2 npr1*, *sfd2-2 ssi2 npr1*, *sfd2-3 ssi2 npr1*, and *sfd4 ssi2 npr1* plants with a needleless syringe. Leaf discs were harvested from the inoculated leaves at 3 days after inoculation, weighed, and ground in 10 mM $MgCl_2$, and the bacterial numbers were titered. Each sample contained five leaf discs. The bacterial numbers, presented as colony-forming units (CFU) per mg of leaf tissue, represent the average of five samples ± SD.

(B) *B. cinerea* disease ratings in the *sfd* mutants. Leaves of 4-week-old soil-grown wild-type, *npr1*, *ssi2*, *ssi2 npr1*, *sfd1-1 ssi2 npr1*, *sfd1-2 ssi2 npr1*, *sfd2-1 ssi2 npr1*, *sfd2-2 ssi2 npr1*, and *sfd4 ssi2 npr1* plants were inoculated with spores of *B. cinerea*. Four days later, plants were scored for the extent of spreading necrosis. Leaves from each line were grouped based on the extent of necrosis. A four-step grading system was used. Leaves with <25%, 25 to 50%, 50 to 75%, and 75 to 100% of leaf area exhibiting necrosis were given scores of 0.25, 0.5, 0.75, and 1, respectively. The leaves in each category were counted, multiplied by the score, and divided by the total number of leaves inoculated (given next to each bar) to give an infection rating for each line. The "relative infection rating" for each line was calculated as the ratio of the infection rating for the line to the infection rating of the wild type.

Table 2. Fatty Acyl Profile of Polar Lipids in *sfd* Plants

Genotype	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
Wild type	15.7 ± 0.5	4.3 ± 0.1	0.8 ± 0.1	15.5 ± 0.7	1.4 ± 0.1	2.5 ± 0.4	14.1 ± 0.5	45.3 ± 0.6
npr1	18.4 ± 1.4	5.6 ± 0.7	1.0 ± 0.2	14.0 ± 1.2	1.9 ± 0.5	2.7 ± 0.5	14.5 ± 0.6	41.1 ± 1.8
ssi2 npr1	13.9 ± 0.7	3.4 ± 0.4	0.6 ± 0.1	12.4 ± 1.0	12.6 ± 0.4	1.0 ± 0.1	15.5 ± 0.4	40.4 ± 0.7
sfd1-1 ssi2 npr1	13.6 + 2.6	5.8 ± 0.7	0.3 ± 0.1	7.3 ± 0.8	13.2 ± 1.9	2.4 ± 0.6	16.3 ± 0.6	40.3 ± 3.5
sfd2-1 ssi2 npr1	20.0 ± 2.1	4.9 ± 0.5	0.2 ± 0.1	2.2 ± 0.2	10.0 ± 0.6	2.1 ± 0.3	15.2 ± 0.5	45.2 ± 2.7
sfd4 ssi2 npr1	16.0 ± 1.4	15.9 ± 0.6	0.3 ± 0.2	1.3 ± 1.6	9.6 ± 0.8	17.7 ± 2.8	14.6 ± 0.4	24.3 ± 1.2
sfd2-1 ssi2	19.3 ± 1.6	4.0 ± 0.4	0.3 ± 0.1	2.7 ± 0.3	8.4 ± 1.7	2.0 ± 0.4	14.4 ± 0.5	48.5 ± 1.6

All values are given as mol % of fatty acids. The value for each line is the average of five samples ± SD. Values for 14:0 are not listed.

155 nmol diacyl species/mg dry weight for *ssi2 npr1*. This increase was primarily the result of an increase in the content of 36:6-MGDG (18:3-18:3 acyl combination) in *sfd1-1 ssi2 npr1* and *sfd2-1 ssi2 npr1* (Figure 5A). Total lipid in the *sfd4 ssi2 npr1* mutant plant (153 nmol diacyl species/mg dry weight) was not increased. The large reduction in 16:3 levels in *sfd2-1 ssi2 npr1* and *sfd4 ssi2 npr1* were reflected in a >80% reduction in the level of 34:6-MGDG (16:3-18:3 acyl combination) (Figure 5A). 34:6 levels in *sfd1-1 ssi2 npr1* were only 17% lower than those in *ssi2 npr1*. However, the ratio of 34:6-MGDG to 36:6-MGDG species was low in each of the *sfd* mutants, with ratios of 0.8 in *sfd1-1 ssi2 npr1*, 0.2 in *sfd2-1 ssi2 npr1*, and 0.3 in *sfd4 ssi2 npr1*, compared with 2.6 in the wild type and 2.3 in *ssi2 npr1*.

In addition, there were considerable differences in the molecular species within the phospholipid classes of *ssi2* and *ssi2 npr1* plants compared with those in wild-type and *npr1* plants. For example, *ssi2* and *ssi2 npr1* plants contained higher proportions of PC, PE, and PI containing either a 36:2 or 36:3 acyl composition (Figure 5B). Analysis of the acyl fragments of these 36:2 and 36:3 molecular species in *ssi2 npr1* plants indicated that they represent primarily the 18:0-18:2 and 18:0-18:3 combinations, respectively. The levels of 34:2-PC, 34:2-PE, and 34:2-PI were lower in the *ssi2* and *ssi2 npr1* plants than in wild-type or *npr1* plants, whereas 34:3-PC, 34:3-PE, and 34:3-PI were similar or slightly higher in *ssi2* and *ssi2 npr1* (Figure 5B). Acyl fragment analysis showed that 34:2 species in *npr1* and *ssi2 npr1* represented mainly a combination of 18:2 and 16:0, whereas 34:3 species were mainly a 16:3-18:0 combination, but lower amounts of 16:1-18:2 and 18:0-16:3 combinations were detected. Interestingly, the trace amounts of 18:0-16:3 in phospholipids were higher in *ssi2 npr1* plants than in *npr1* plants. For example, in *npr1* plants, 16:3-18:0 represents 0.3% of the 34:3-PE species, whereas in *ssi2 npr1*, 16:3-18:0 represents 1.4% of the 34:3-PE species. Levels of the 34:2-, 36:2-, and 36:3 PC, PE, and PI species in *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* were comparable to the levels of these species in *ssi2 npr1* (Figure 5B). However, compared with the levels in wild-type, *npr1*, and *ssi2 npr1* plants, the levels of 34:3-PC, 34:3-PE, and 34:3-PI species were lower in the *sfd1-1 ssi2 npr1* and *sfd4 ssi2 npr1* plants.

Genetic Characterization of the *sfd1* and *sfd2* Mutants

The genetic basis of the *sfd1* and *sfd2* phenotypes was characterized in detail. F1 plants resulting from a cross between *sfd1-1 ssi2 npr1* and the *ssi2 npr1* parent exhibited the typical small-plant phenotype associated with the *ssi2* mutant, suggesting that *sfd1-1* is recessive to the wild-type *SFD1* allele (Table 3). These F1 plants also developed lesions containing dead cells and expressed the *PR1* gene at high levels (data not shown). The F2 progeny of these F1 plants segregated wild-type and *ssi2*-like plants in a nearly 1:3 ratio. Moreover, only the *ssi2*-like dwarf segregants expressed *PR1*, confirming that *sfd1-1* is a recessive mutation at a single genetic locus. Likewise, the *sfd1-2* phenotype also was confirmed to be a monogenic recessive trait (data not shown). The F1 plants generated by crossing the *sfd1-1 ssi2 npr1* plant with the *sfd1-2 ssi2 npr1* plant were large, devoid of lesions, and lacked *PR1* expression. Moreover, none of the 72 F2 progeny derived from these F1 plants exhibited the dwarf and lesion-bearing phenotypes associated with *ssi2*, confirming that *sfd1-1* and *sfd1-2* are allelic (Table 3).

F1 plants (*sfd2-1/+ ssi2 npr1*) generated by crossing the *sfd2-1 ssi2 npr1* plant with a *ssi2 npr1* plant were intermediate in size compared with the parents (Figure 2A). In addition, the level of *PR1* transcript in these *sfd2-1/+ ssi2 npr1* plants was intermediate to levels of *PR1* transcript in the *ssi2 npr1* and homozygous *sfd2-1 ssi2 npr1* plants (Figure 3D), suggesting that *sfd2-1* is semidominant. Likewise, in contrast to the *sfd2-1 ssi2 npr1* plant, the *sfd2-1*–mediated suppression of *ssi2*-conferred resistance to *Psm* was less pronounced in the *sfd2-1/+ ssi2 npr1* plant (A. Nandi and J. Shah, unpublished data). The semidominance of *sfd2-1* was confirmed in the F2 progeny, which segregated large, intermediate, and dwarf plants in a nearly 1:2:1 ratio (Table 3). Negligible levels of *PR1* transcript were observed in the large F2 progeny plants, whereas moderate and high levels of constitutive *PR1* expression were observed in the intermediate and dwarf plants, respectively (data not shown). F3 progeny of the intermediate F2 plants segregated large, intermediate, and dwarf plants, confirming that these F2 plants were heterozygous at the *sfd2* locus. Similarly, the *sfd2-2* and *sfd2-3* alleles also exhibited a semidominant phenotype (data not shown). Crosses between *sfd2-1 ssi2 npr1* and *sfd2-2 ssi2 npr1*, *sfd2-1 ssi2 npr1* and *sfd2-3 ssi2 npr1*, and *sfd2-2 ssi2 npr1* and *sfd2-3 ssi2 npr1* suggested that *sfd2-1*, *sfd2-2*, and *sfd2-3* are allelic. All F1 plants from these crosses retained the large *sfd2* phenotype. Furthermore, none of them segregated dwarf *ssi2*-like F2 progeny plants. Further support for allelism among *sfd2-1*, *sfd2-2*, and *sfd2-3* is provided by the identical lipid profiles of these mutants (A. Nandi, C. Buseman, M. Li, R. Welti, and J. Shah, unpublished data).

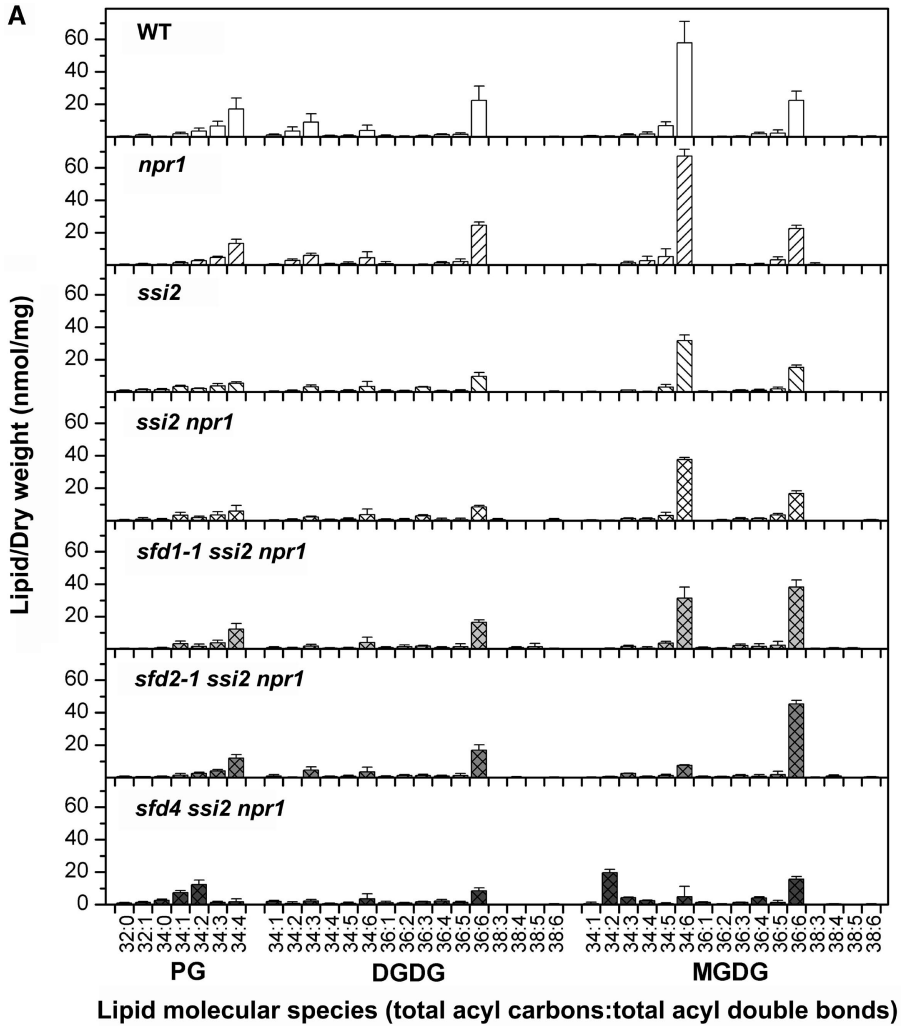

Figure 5. Lipid Profiles of the *sfd* Mutants.

(A) Electrospray ionization–tandem mass spectrometry-generated profiles of the plastidic glycerolipids PG, DGDG, and MGDG in wild-type (WT), *npr1*, *ssi2*, *ssi2 npr1*, *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants.

(B) Electrospray ionization–tandem mass spectrometry-generated profiles of the extraplastidic glycerolipids PC, PE, and PI in wild-type (WT), *npr1*, *ssi2*, *ssi2 npr1*, *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants.

In our attempts to outcross *sfd1* away from the *ssi2* mutant allele, we discovered that *sfd1* was linked to the *ssi2* locus on chromosome 2. As shown in Table 3, of the 146 F2 progeny of an F1 plant obtained from a cross between *sfd1-2 ssi2 npr1* and a wild-type plant, only two morphologically *ssi2*-like segregants were observed. Mapping populations composed of large and lesionless (*sfd1*/*sfd1*) F2 progeny of F1 plants derived from a cross between a *sfd1-1 ssi2 npr1* plant in ecotype Nössen and the *fab2* (allelic with *ssi2*) mutant in ecotype Columbia confirmed that *sfd1* maps in the vicinity of *SSI2*. Likewise, mapping populations generated by crossing the *sfd1-2 ssi2 npr1* plant with the *fab2* mutant also localized *sfd1-2* on chromosome 2, near *SSI2*. Cleaved amplified polymorphic sequence (Konieczny and Ausubel, 1993) and simple sequence length polymorphism (Bell and Ecker, 1994) analyses performed on 29 *sfd1-1*/*sfd1-1* and 26 *sfd1-2*/*sfd1-2* F2 progeny plants mapped the *sfd1* locus between the SSLP markers F27D4 and the *SSI2* gene, 20 centimorgan (cM) from F27D4 and 3 cM from *SSI2*. The size of the mapping population was increased subsequently to 301 F2 *sfd1*/*sfd1* plants to map *sfd1* between the SSLP marker nga168 (2.1 cM; 13 recombinant chromosomes) and the *SSI2* gene (2.6 cM; 16 recombinant chromosomes).

Mapping populations generated by crossing *sfd2-1 ssi2 npr1* with *fab2* were used to map *sfd2*. Analysis of 20 large *sfd2-1*/*sfd2-1* F2 progeny plants mapped the *sfd2* locus to a 27-cM interval on chromosome 3, between the cleaved amplified polymorphic sequence marker T6H20 and the SSLP nga6, 20 cM from T6H20 and 7 cM from nga6.

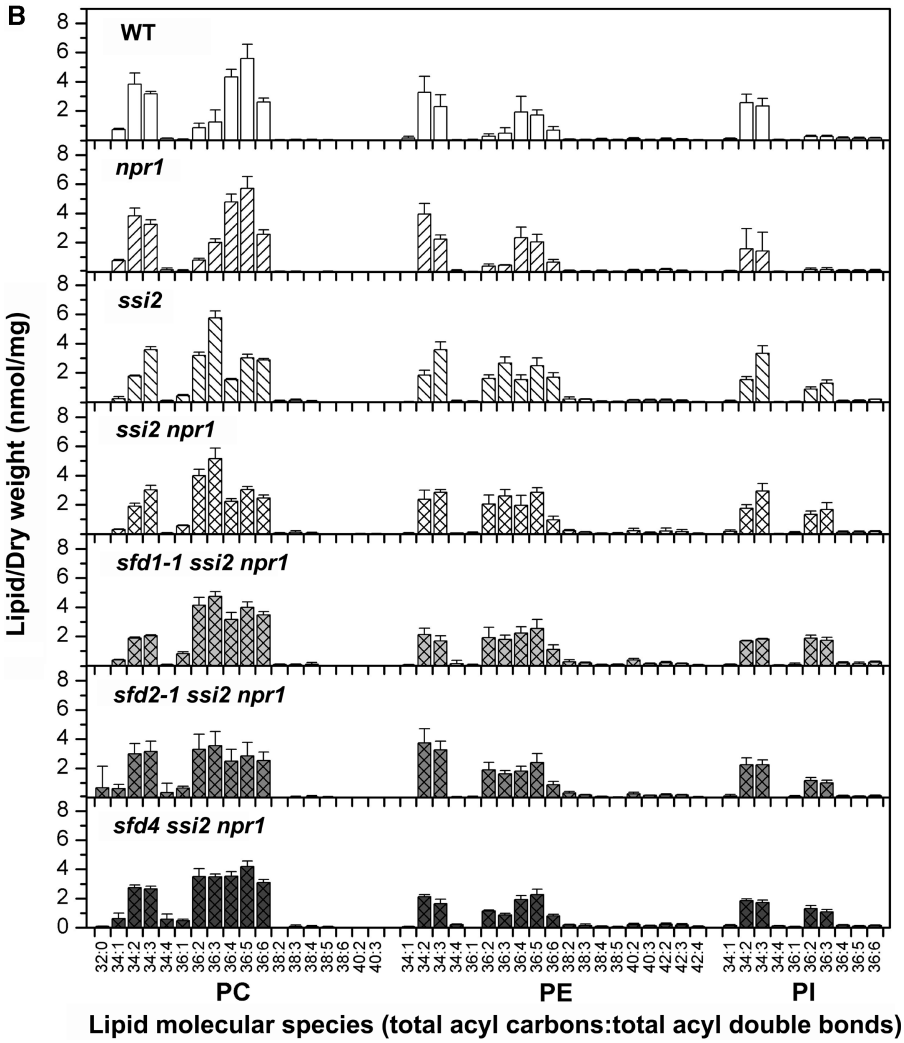

Figure 5. (continued).

sfd4 Contains a Mutation in the *FAD6* Gene

The fatty acid composition of polar lipids in *sfd4 ssi2 npr1* showed a decrease in content of the trienoic acids 16:3 and 18:3 and a corresponding increase in the concentrations of 16:1 and 18:1 (Table 2), suggesting that it is blocked in the desaturation of 16:1 and 18:1. This is similar to the defect in the *fad6* mutant, which is deficient in a plastidic 16:1/18:1 ω6 desaturase activity (Browse et al., 1989). Therefore, we sequenced the *FAD6* locus from wild-type Nössen and *sfd4 ssi2 npr1* plants. Indeed, the *sfd4 ssi2 npr1* plant contained a single nucleotide change (C→T transition) in the *FAD6* gene (Falcone et al., 1994), which is expected to result in a Ser-133→Phe-133 change in the corresponding protein. Ser-133 is highly conserved among FAD6 homologs from various plant and cyanobacterial species (Figure 6A). This C→T transition mutation in the *sfd4 ssi2 npr1* plant resulted in an EcoRV restriction polymorphism (Figure 6B), thus confirming the presence of a mutation in the *FAD6* gene. To confirm that *FAD6* is required for the manifestation of the *ssi2*-conferred development and defense phenotypes, we generated the *ssi2 fad6* double mutant. As shown in Figures 6D and 6E, the *fad6* mutant allele suppressed the *ssi2*-conferred dwarfing and spontaneous cell death phenotypes. In addition, the *ssi2*-conferred constitutive *PR1* expression also was suppressed by the *fad6* allele (Figure 6C). These effects of the *fad6* allele on the *ssi2* phenotype were recessive to the wild-type *FAD6* allele, and the *ssi2 fad6*/+ plant retained all of the *ssi2*-conferred phenotypes.

DISCUSSION

The *sfd1*, *sfd2*, and *sfd4* mutations suppress key phenotypes of the *ssi2* mutation, including dwarfing, lesion development, the NPR1-independent expression of the *PR1* gene, and resistance to *Psm*. The high mol % of 18:0 in leaves of *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants imply that increased 18:0 levels are not sufficient to cause the *ssi2* phenotypes. The

Table 3. Genetic Analysis of the *sfd1* and *sfd2* Mutants

Cross	F1 Phenotype[a]	F2 Progeny							
		Large	Intermediate	Dwarf	Mendelian Ratio	Hypothesis	χ^2	P[b]	Fit?/Remarks
sfd1-1 ssi2 npr1 × *ssi2 npr1*	Dwarf	11	0	32	1:2.9	Monogenic recessive	0.05	$0.9 > P > 0.5$	Yes
sfd1-1 ssi2 npr1 × *sfd1-2 ssi2 npr1*	Large	72	0	0					Allelic
sfd1-2 ssi2 npr1 × *SFD1 SSI2 NPR1*	Large	144	0	2					Linked to *ssi2*
sfd2-1 ssi2 npr1 × *ssi2 npr1*	Intermediate	9	17	10	1:1.9:1.1	Monogenic semidominant	0.17	$P > 0.9$	Yes

[a] Plants were scored for the presence/absence of the dwarf phenotype associated with *ssi2*.
[b] Degrees of freedom used to calculate probability: *sfd1-1 ssi2 npr1* × *ssi2 npr1* ($df = 1$); *sfd2-1 ssi2 npr1* × *ssi2 npr1* ($df = 2$).

increases in mol % of 18:1 in the leaves of the *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants could account for the suppression of some *ssi2*-conferred phenotypes. However, pharmacological experiments argue against this increase in 18:1 mol % suppressing the *ssi2*-conferred *PR1* expression. To the contrary, the application of 18:1 free fatty acid to leaves of the wild-type plant activates *PR1* expression (A. Nandi and J. Shah, unpublished data). It is likely that other changes in the *sfd* plants contribute to the suppression phenotype.

A striking change in the fatty acid composition of each of the *sfd* mutants, but especially *sfd2-1 ssi2 npr1* and *sfd4 ssi2 npr1* plants, was the decreased content of 16:3 (Table 2). This decrease in 16:3 content was reflected by a reduction in the content of the major plastid-localized lipid species, 34:6-MGDG, in the *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants (Figure 5A). 16:3 is the precursor for the synthesis of C-16 fatty acid–derived oxylipin compounds (e.g., dinor-12-oxo-phytodienoic acid), which has been postulated to fine-tune defense signaling in plants (Weber et al., 1997; Stinzi et al., 2001). In addition, dinor-12-oxo-phytodienoic acid may modulate crosstalk between C-16 and C-18 fatty acid–derived oxylipins (Stinzi et al., 2001). A 16:3-derived molecule(s) that is depressed in the *sfd* mutants may contribute to some of the *ssi2*-conferred phenotypes. Indeed, compared with the *npr1* plant, increased numbers of species with 16:3-18:0 acyl combinations were found in the phospholipids of the *ssi2 npr1* plant. 16:3 usually is not found at more than trace levels outside of the plastids. This incorporation of 16:3 into new molecular species and potentially into nonplastidic compartment(s) in the *ssi2 npr1* plant, coupled with the decrease in 16:3-containing complex lipid levels in all of the *sfd* mutants, suggests the potential importance of a complex lipid(s) containing 16:3, and perhaps a signal derived from this species, in the *ssi2*-conferred phenotypes.

Barring the *ssi2*-conferred increased 18:0 levels, the fatty acid composition and lipid species profile of *sfd1-1 ssi2 npr1* are strikingly similar to those in the *gly1* mutant (Miquel et al., 1998). *gly1* and *sfd1* map to the same region on chromosome II, in the vicinity of *SSI2*, and may contain mutations in the same gene. Glycerol application restores the lipid composition of the *gly1* mutant, suggesting that the defect in *gly1* is caused by the limited availability of glycerol-3-phosphate for complex lipid biosynthesis in the plastids (Miquel et al., 1998). Similarly, glycerol application restores *ssi2*-conferred *PR1* expression in the *sfd1-1 ssi2 npr1* and *sfd1-2 ssi2 npr1* plants (A. Nandi and J. Shah, unpublished data), further suggesting that *gly1* and *sfd1* contain mutations in the same gene.

The *sfd4* mutation is predicted to alter the highly conserved Ser-133 to Phe-133 in the FAD6 protein. *FAD6* encodes a 16:1/18:1 ω6 desaturase that catalyzes the desaturation of plastid-localized 16:1 and 18:1 to 16:2 and 18:2, respectively (Browse et al., 1989; Falcone et al., 1994). In wild-type plants, 16:2 and 18:2 can be converted further to 16:3 and 18:3, respectively, by other desaturases. Like the *fad6* mutant, *sfd4 ssi2 npr1* contains higher levels of the monounsaturated 16:1 and 18:1 fatty acids at the expense of the trienoic fatty acids 16:3 and 18:3, the two predominant PUFAs in Arabidopsis (Wallis and Browse, 2002). Mutations in the *FAD5* gene also affect the synthesis of 16:3 (Kunst et al., 1989). However, unlike the *fad6* mutation, mutations in *fad5* do not affect 18:3 levels. *FAD5* encodes a plastidic palmitoyl desaturase (Mekhedov et al., 2000). Compared with *ssi2 npr1* and *npr1*, 16:3 levels are very low in leaves of the *sfd2 ssi2 npr1* plant, and no decrease in the level of 18:3 was observed (Table 2). These data suggest that SFD2, like FAD5, might affect the synthesis or accumulation of 16:3. However, *sfd2* does not map to the *FAD5* locus; thus, it identifies a novel gene that affects 16:3 biosynthesis or accumulation. Therefore, our results suggest an important role for 16:3, or complex lipids containing it, in the manifestation of one or more *ssi2*-conferred phenotypes.

SA Signaling in the *sfd* Mutants

The activation of JA signaling can antagonize SA signaling in Arabidopsis (Kloek et al., 2001). It is possible that the *ssi2*-conferred *PR1* expression is the result of the loss of this inhibitory effect of JA. Indeed, the loss of *ssi2*-conferred *PR1* expression in *sfd1 ssi2 npr1* plants correlates with the restoration of JA-activated *PDF1.2* expression. However, JA responses are not restored in the *sfd2 ssi2 npr1* and *sfd4 ssi2 npr1* mutants, yet *ssi2*-conferred NPR1-independent *PR1* expression is sup-

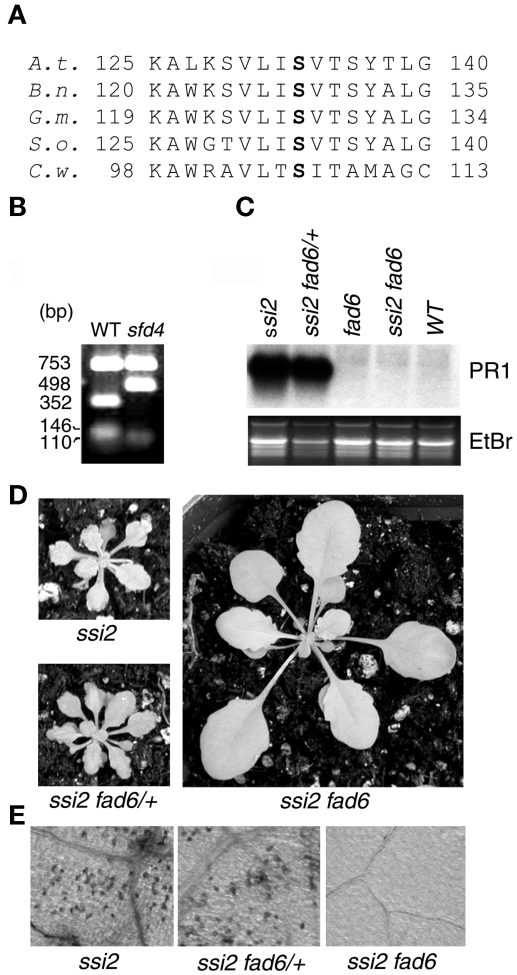

Figure 6. *sfd4* Contains a Mutation in the *FAD6* Gene.

(A) Alignment of amino acid sequences in a conserved region of plastidic ω6 desaturases from *Arabidopsis thaliana* (*A.t.*), rape (*Brassica napus*; *B.n.*), soybean (*Glycine max*; *G.m.*), spinach (*Spinacia oleracea*; *S.o.*), and the green alga *Chlamydomonas* sp W80 (*C.w.*). The Ser-133 that is mutated to Phe-133 in *sfd4* is shown in boldface. Numbers at left and right indicate amino acid positions.

(B) EcoRV restriction polymorphism generated by a C→T mutation at the *FAD6* locus in the *sfd4 ssi2 npr1* plant. PCR-amplified products from the wild type (WT) and *sfd4 ssi2 npr1* were digested with EcoRV and resolved on an agarose gel. The size of each band in base pairs is listed at left.

(C) Comparison of *PR1* expression in the leaves of 4-week-old soil-grown *ssi2* plants that are homozygous for the wild-type *FAD6* allele (*ssi2*), heterozygous for the *fad6-1* mutant allele (*ssi2 fad6*/+), and homozygous for the *fad6-1* mutant allele (*ssi2 fad6*). All RNAs were resolved on denaturing gels, transferred to Nytran Plus membranes, and probed for the indicated genes. Gel loading was monitored by photographing the ethidium bromide–stained gel (EtBr) before transferring the RNA to a Nytran Plus membrane.

(D) Morphological phenotype of 4-week-old soil-grown *ssi2* plants that are homozygous for the wild-type *FAD6* allele (*ssi2*), heterozygous for the *fad6-1* mutant allele (*ssi2 fad6*/+), and homozygous for the *fad6-1* mutant allele (*ssi2 fad6*). All plants were photographed from the same distance.

pressed in these plants, arguing against JA signaling per se affecting *PR1* expression in the *ssi2* mutant. However, we cannot exclude the possibility that the *sfd2* and *sfd4* mutants affect a step after the point of this crosstalk between the JA and SA pathways.

The simultaneous absence of *ssi2*-conferred cell death, dwarfism, SA and *PR1* transcript accumulation, and enhanced resistance to *Psm* in the *sfd1 ssi2 npr1* and *sfd4 ssi2 npr1* plants suggests that SFD1 and SFD4 target a step common to the expression of these *ssi2* phenotypes (Figure 7). The *sfd1-1* and *sfd4*/*fad6* mutants blocked the *ssi2*-conferred *PR1* expression equally well in wild-type *NPR1* and *npr1* mutant backgrounds (Figures 3C and 6C). The lack of an increased SA level could account for the absence of *ssi2*-conferred *PR1* expression in the *sfd1-1 ssi2* and *sfd4*/*fad6 ssi2* plants. However, unlike the *sfd1 ssi2 npr1* and *sfd4 ssi2 npr1* plants, the *sfd2-1 ssi2 npr1* plant accumulates increased SA levels. Furthermore, in contrast to the *sfd2-1 ssi2 npr1* plant, *PR1* was expressed constitutively in *sfd2-1 ssi2*, which contains the wild-type *NPR1* gene. Thus, *SFD2* is required only for the *ssi2*-conferred cell death, dwarfing, and for the functioning of the NPR1-independent defense pathway. The fatty acid composition in *sfd2-1 ssi2* was comparable to that in *sfd2-1 ssi2 npr1* (Table 2). Hence, the inability of *sfd2-1* to suppress the NPR1-dependent *PR1* expression is not caused by differences in lipid composition between the *sfd2-1 ssi2* and *sfd2-1 ssi2 npr1* plants. Instead, the high SA content may suffice to activate *PR1* expression through the NPR1-regulated pathway in the *sfd2-1 ssi2* plant.

The inability of applied SA to restore *PR1* expression in the *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants (Figure 3B and data not shown) suggests the requirement for an additional factor, in addition to SA, for the activation of NPR1-independent *PR1* expression. A 16:3-derived product and/or regulated activity could be this second factor required for the activation of SA signaling. Lipid signaling was shown by Anderson et al. (1998) to be an important factor in modulating the SA-activated expression of the *PR1* gene in tobacco. Alternatively, this second factor could be made available by the cell death and/or dwarfing that occurs in the *ssi2* mutant.

JA Signaling in *sfd* Mutants

Lack of an 18:1-derived signal/factor was suggested to be responsible for the inability of JA to activate *PDF1.2* expression in the *ssi2* mutant (Kachroo et al., 2001). The restoration of wild-type levels of 18:1 (Table 2) could account for the establishment of JA-activated *PDF1.2* expression in the *sfd1-1 ssi2 npr1* plant (Figure 3E). However, comparable levels of 18:1 in the *sfd2-1 ssi2 npr1* plant and the eightfold higher levels of 18:1 in *sfd4 ssi2 npr1* were not sufficient to restore JA-activated

(E) Light microscopy of trypan blue–stained leaves of 4-week-old soil-grown *ssi2* plants that are homozygous for the wild-type *FAD6* allele (*ssi2*), heterozygous for the *fad6-1* mutant allele (*ssi2 fad6*/+), and homozygous for the *fad6-1* mutant allele (*ssi2 fad6*). All photographs were taken at the same magnification.

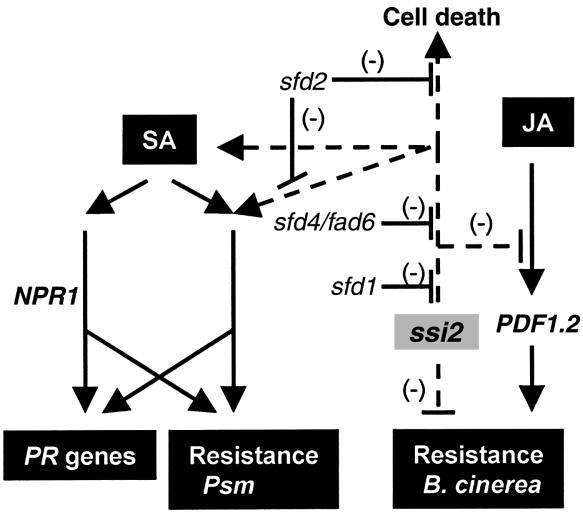

Figure 7. Working Model of the Interplay of *ssi2*, *sfd1*, *sfd2*, and *sfd4* in Defense Signaling in Arabidopsis.

This model is a refinement of Figure 1. The *sfd1*, *sfd2*, and *sfd4* mutant alleles suppress (−) the *ssi2*-conferred dwarfing, spontaneous development of lesions containing dead cells, NPR1-independent expression of *PR1*, and enhanced resistance to *Psm*. In addition, *sfd1* and *sfd4* also suppress the *ssi2*-conferred accumulation of high SA levels. However, SA application is ineffective in restoring *PR1* expression in *sfd1 ssi2 npr1*, *sfd2 ssi2 npr1*, and *sfd4 ssi2 npr1* plants, implicating the involvement of another *ssi2*-contributed factor in the activation of the NPR1-independent pathway leading to the expression of *PR1* and enhanced resistance to *Psm*. The absence of SA accumulation in the *ssi2 nahG* and *ssi2 eds5* plants does not ameliorate the *ssi2*-conferred cell death phenotype, suggesting that high levels of SA do not have a causal role in the cell death phenotype. The *sfd2 ssi2 npr1* plants accumulate increased SA levels despite the lack of spontaneous cell death, suggesting that cell death is not the primary factor that promotes SA accumulation in the *ssi2* mutant. Hence, *ssi2*-conferred cell death and SA accumulation are shown to be independent of each other. The *sfd1* mutant alleles restore JA-inducible *PDF1.2* expression in *sfd1 ssi2 npr1* plants. *sfd1* is shown to impinge on a step that is common to the activation of cell death, SA accumulation, the activation of the NPR1-independent defense pathway, and the repression of JA signaling in the *ssi2* mutant. However, because *sfd1* does not restore resistance to *B. cinerea*, despite restoring *PDF1.2* expression in the *sfd1 ssi2 npr1* plants, an additional *ssi2*-modulated mechanism is shown to suppress (−) the defense against *B. cinerea*. *sfd4* does not restore JA-inducible *PDF1.2* expression in *sfd4 ssi2 npr1*. Hence, *sfd4* is shown to suppress (−) a step that is common to the activation of cell death and dwarfing, SA accumulation, and the activation of the NPR1-independent defense pathway. *sfd2* is shown to interfere with (−) a step common to the activation of *ssi2*-conferred cell death and the activation of NPR1-independent signaling.

PDF1.2 expression, suggesting that factors in addition to 18:1 are involved. Because JA is ineffective at activating *PDF1.2* expression in SA-deficient *ssi2 nahG* plants (Figure 3E) (Kachroo et al., 2001) and in *sfd4 ssi2 npr1* (Figure 3E), it is highly unlikely that the increased SA levels in *ssi2 npr1* and *sfd2-1 ssi2 npr1* suppress JA-activated *PDF1.2* expression. The restoration of JA-activated *PDF1.2* expression in the *sfd1-1 ssi2 npr1* plant suggests that an SFD1-dependent signal may be an inhibitor of *PDF1.2* expression in the *ssi2* plant (Figure 7). In wild-type plants, the 18:1-derived signal may antagonize this inhibitory activity, allowing JA to activate *PDF1.2* expression.

JA application activates *PDF1.2* expression and confers protection against the necrotrophic pathogen *B. cinerea* (Penninckx et al., 1996, 1998; Thomma et al., 1999). In addition, enhancing *PDF1.2* expression by the overexpression of *ETHYLENE RESPONSE FACTOR1*, a regulator of *PDF1.2* expression in Arabidopsis, enhances resistance to *B. cinerea* (Berrocal-Lobo et al., 2002). An increased susceptibility to *B. cinerea* parallels the inability of the *ssi2* mutant to express the *PDF1.2* gene in response to JA application (Kachroo et al., 2001). However, the restoration of MeJA-activated *PDF1.2* expression in the *sfd1-1 ssi2 npr1* plant (Figure 3E) was not sufficient to restore resistance to *B. cinerea* (Figure 4B). However, we cannot exclude the possibility that the *sfd1-1 ssi2 npr1* plant, although responsive to MeJA, may not be very sensitive to it or to other factors that activate *PDF1.2*. For example, although exogenously applied MeJA activated *PDF1.2* expression in *sfd1-1 ssi2 npr1* plants, the *PDF1.2* gene transcript accumulated to lower levels than in the MeJA-treated control *npr1* plant (Figure 3E). In addition, application of 1-aminocyclopropane-1-carboxylic acid was ineffective at activating *PDF1.2* expression in *sfd1-1 ssi2 npr1* plants (A. Nandi and J. Shah, unpublished data).

Govrin and Levine (2000) have shown that hypersensitive response–associated cell death in Arabidopsis enhances susceptibility to *B. cinerea*. It is likely that cell death in *ssi2* predisposes the mutant plant to infection by *B. cinerea*. However, the prevalence of the *ssi2*-conferred enhanced susceptibility to *B. cinerea* in the *sfd1-1 ssi2 npr1*, *sfd2-1 ssi2 npr1*, and *sfd4 ssi2 npr1* plants (Figure 4B), despite the absence of *ssi2*-conferred cell death (Figure 2B), argues against cell death being an important factor in the susceptibility of the *ssi2* mutant to *B. cinerea*. The loss of *ssi2*-activated high-level SA accumulation in the *sfd1-1 ssi2 npr1* and *sfd4 ssi2 npr1* plants also excludes any involvement of SA in the susceptibility of the *ssi2* mutant to *B. cinerea*.

In conclusion, we show here that the mere increase in 18:0 level resulting from the deficiency of a plastidic fatty acid desaturase activity is not sufficient to cause the development defects and the altered defense phenotypes observed in the *ssi2* mutant. Instead, the signals/factors derived from the PUFA 16:3 may be involved in the activation of one or more of the *ssi2*-conferred phenotypes. Although the exact identity of these signaling molecules and the mechanism by which they exert their effects on plant development and defense responses are unclear, the genetic dissection of these signaling mechanisms in concert with lipid compositional analysis in mutants should facilitate our understanding of how plants generate and decipher complex information involving lipids.

METHODS

Cultivation of Plants and Pathogens

Arabidopsis thaliana seeds were germinated at 22°C in a tissue culture chamber exposed to a 14-h-light (80 $\mu E \cdot m^{-2} \cdot s^{-1}$)/10-h-dark cycle on

Murashige and Skoog (1962) (Sigma, St. Louis, MO) agar supplemented with 1% sucrose. Nine-day-old seedlings were transferred to soil and grown at 22°C in growth chambers programmed for a 14-h-light (90 $\mu E \cdot m^{-2} \cdot s^{-1}$)/10-h-dark cycle.

Pseudomonas syringae pv *maculicola* (*Psm*) 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 to infect plants. *Botrytis cinerea* IMI169558 was cultivated on maltose medium at 22°C in a growth chamber programmed for a 14-h-light (80 $\mu E \cdot m^{-2} \cdot s^{-1}$)/10-h-dark cycle. Spores were harvested and infections performed as described previously (Nandi et al., 2003).

Infection of Plants with Bacterial and Fungal Pathogens

Infections with *Psm* ES4326 were performed on 4-week-old soil-grown plants as described previously (Nandi et al., 2003). For every set of infections, 25 (five replications of five leaves in each sample) leaf discs (0.15 cm^2) were harvested at 3 days after infection and placed in preweighed tubes. After the weight of each sample was determined, bacterial counts were determined as described previously (Shah et al., 1997). Bacterial counts were expressed as colony-forming units per milligram of leaf tissue.

B. cinerea infections were performed on 4-week-old soil-grown plants as described previously (Nandi et al., 2003). Three leaves per plant were pricked with a needle. Infection was initiated by placing a 10-μL drop of freshly harvested spore suspension (5×10^5 spores/mL) on each needle prick. The inoculum was allowed to air dry. Thereafter, plants were covered with a transparent plastic dome and cultivated at 22°C in a growth chamber programmed for a 14-h-light (90 $\mu E \cdot m^{-2} \cdot s^{-1}$)/10-h-dark cycle. The number of leaves showing varied levels of necrosis were scored at 4 days after inoculation.

Chemical Treatment of Plants

For methyl jasmonate treatment, leaves from 4-week-old plants, excised at the base of the petioles, were floated on 5 mL of a methyl jasmonate solution (5 μM in 0.1% ethanol; Bedoukian Research, Danbury, CT) on tissue culture plates. As the control, leaves from the same plants were floated simultaneously on 5 mL of 0.1% ethanol solution. Leaves were harvested 48 h later, quick frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

Salicylic acid (SA; 500 μM) treatment of 4-week-old soil-grown plants was performed as described previously (Shah et al., 1997). As controls, plants were treated similarly with water. Leaves were harvested 48 h after treatment and quick-frozen in liquid nitrogen. Leaf samples were stored at −80°C until RNA extraction.

RNA Extraction and RNA Gel Blot Analyses

Leaf tissue was ground under liquid nitrogen, and RNA was extracted using acid guanidinium thiocyanate-phenol-chloroform as described by Chomczynski and Sacchi (1987). RNA gel blot analysis and the synthesis of random primed probes for *PR1*, *BGL2*, and *PDF1.2* were performed as described previously (Shah et al., 1997, 1999).

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 the *npr1-5* mutant alleles were performed as described previously (Shah et al., 1999). A cleaved amplified polymorphic sequence (CAPS) marker was used to distinguish between the *ssi2-1* and *SSI2* alleles (Kachroo et al., 2001). For studies with *sfd4*, the primers FAD6-F1 (5′-GTCGCTTCTTCTGCATTTTC-3′) and FAD6-R1 (5′-TGATGACTCAAACTCCTCTG-3′) were used for PCR. Thirty cycles of PCR were performed. Each cycle consisted of incubation at 95°C for 45 s followed by 56°C for 30 s and 72°C for 2 min. The PCR products were digested with EcoRV to distinguish the *sfd4* mutant allele from the wild-type *SFD4* allele. PCR amplification of the other CAPS and simple sequence length polymorphism markers used in this study were performed as described at http://www.arabidopsis.org.

Histochemistry and Microscopy

Leaves from 4-week-old soil-grown plants were used for trypan blue staining of dead cells. Samples were processed and analyzed as described by Rate et al. (1999).

SA Quantitation

Total SA (SA plus SA-glucoside) in 0.25 to 0.5 g (fresh weight) of leaf tissue was extracted in methanol. The methanol extracts were dried and resuspended in 1.25 mL of 100 mM sodium acetate buffer, pH 5.5, containing 20 units of β-glucosidase (EC 3.2.1.21; almond). After 1.5 h of incubation at 37°C, extracts were acidified to pH 1.0 with 10% (w/v) trichloroacetic acid and subjected to SA extraction and quantification by spectrofluorescence HPLC as described previously (Enyedi, 1999).

Fatty Acid and Lipid Profiling

For fatty acid analysis, leaves from two to three plants were cut and immediately transferred to 3 mL of isopropanol containing 0.01% butylated hydroxytoluene at 75°C. After 15 min, 1.5 mL of chloroform plus 0.6 mL of water were added. The tubes were shaken for 1 h, followed by removal of the extract. The leaves were reextracted five times with chloroform:methanol (2:1) containing 0.01% butylated hydroxytoluene. During each extraction, the tubes were agitated for 30 min. The extracted leaf tissue was heated overnight at 105°C and weighed. The weight of the dried and extracted tissue was the "dry weight" of the sample. Dry weights ranged from 14 to 78 mg. The combined extracts were washed once with 1 mL of 1 M KCl and once with 2 mL of water. The solvent was evaporated under nitrogen, and the lipid extract was dissolved in 1 mL of chloroform.

For analysis of the fatty acyl species in complex lipids, 200 μL of the sample was applied to a silicic acid (0.3 g) column in chloroform. Neutral lipids were eluted with 10 mL of chloroform, and complex lipids were eluted with 10 mL of methanol. The solvent was evaporated from the methanol fraction, and fatty acid methyl esters were formed from 4% of each sample in duplicate by derivatization in 1.5 M methanolic HCl. After heating at 78°C for 90 min, 2 volumes of water was added to the reaction mixture. Each mixture was extracted twice with pentane and dried with sodium sulfate. The solvent was evaporated, and each sample was dissolved in carbon disulfide and analyzed by gas-liquid chromatography with a Supelco 30-meter Omegawax 250 capillary column (Sigma-Aldrich) at 155°C. The temperature of the injector was 220°C, and the flame ionization detector was also at 220°C. The fatty acyl composition for each sample was an average of the two analyses. The fatty acyl composition of each Arabidopsis line is the average ± SD of five samples.

An automated electrospray ionization–tandem mass spectrometry approach was used to profile lipid composition in Arabidopsis leaves. Sample preparation, processing, data acquisition and analysis, and acyl group identification were as described previously (Welti et al., 2002).

Mutagenesis and Selection of the *sfd* Mutants

Four thousand *ssi2 npr1-5* seeds (ecotype Nössen) were mutagenized with 0.3% ethyl methanesulfonate (Sigma-Aldrich) as described previ-

ously (Shah et al., 1997). M2 seeds were harvested as pools; each pool contained M2 seeds derived from ~150 ethyl methanesulfonate–mutagenized M1 seeds. The M2 seeds were germinated in soil, in parallel with seeds of *ssi2 npr1* and wild-type plants. Large plants were identified, and RNA was extracted from the leaves. RNA was analyzed by RNA gel blot analysis for *PR1* expression. RNA extracted from wild-type and *ssi2 npr1* plants served as negative and positive controls, respectively. Individual plants were allowed to set seeds. *sfd* phenotypes were confirmed in M3 progeny.

Genetic Analysis

Backcrosses were performed by pollinating flowers of the *sfd* mutant lines with pollen from the *ssi2 npr1* parental line (Shah et al., 2001). This line contains the *npr1-5* allele (Shah et al., 1997, 1999). A backcrossed homozygous line was used for all other genetic analyses. To generate the *sfd2-1 ssi2* line, flowers of a *sfd2-1 ssi2 npr1* plant were crossed with pollen from a wild-type plant. The success of the cross was confirmed by CAPS analysis of F1 plants for heterozygosity at the *npr1* and *ssi2* loci. The genotype of the F1 generation is *sfd2-1/+ ssi2/+ npr1/+*. Large plants segregating in the F2 generation were screened using PCR to identify plants that were homozygous for the *ssi2* mutant allele. Because of their large stature, these plants were presumed to be homozygous for the *sfd2-1* allele. These plants then were tested by PCR to identify those that were homozygous for the wild-type NPR1 allele. The F2 lines that did not segregate any dwarf plants were confirmed to be *sfd2-1 ssi2*.

The *sfd1-1 ssi2* line was generated from a cross involving the *sfd1-1 ssi2 npr1* and *ssi2* plants. The genotype of these dwarf and lesion-bearing F1 plants is *sfd1-1+ ssi2/ssi2 npr1/+*. Large plants segregating in the F2 generation were analyzed using PCR for plants that are homozygous for the *NPR1* allele. F3 seeds from these *sfd1-1 ssi2* plants were used in experiments.

Mapping populations for *sfd1* were generated by crossing the *sfd1-1 ssi2 npr1* plant in ecotype Nössen with the *fab2* mutant in ecotype Columbia. The resulting F1 plants were dwarf and expressed the *PR1* gene at increased levels. The large F2 progeny plants were used to map *sfd1-1*. Similarly, F2 progeny plants from a cross between *sfd1-2 ssi2 npr1* and *fab2* were used to map *sfd1-2*. F2 progeny from a cross of a *sfd2-1 ssi2 npr1* plant (ecotype Nössen) and the *fab2* mutant (ecotype Columbia) were used to map *sfd2-1*. F2 progeny homozygous for *sfd2-1*, identified as large plants, were used for mapping.

A cross between the *ssi2* and *fad6-1* (ecotype Columbia) plants was used to generate the *ssi2 fad6* double mutant. F2 progeny plants from this cross were analyzed for the presence of the *ssi2* mutant allele by PCR (Kachroo et al., 2001). One-quarter of the plants that were homozygous for the *ssi2* mutant allele lacked the *ssi2*-conferred dwarf phenotype, suggesting recessive epistasis, with *fad6-1* epistatic to *ssi2*. The presence of the *fad6* or wild-type *FAD6* allele was monitored by PCR for the AGa and AthDET1 markers, which flank the *FAD6* locus and exhibit polymorphism between ecotypes Columbia and Nössen. All of the *ssi2/ssi2* F2 plants that did not exhibit the *ssi2*-conferred dwarf phenotype were homozygous for the Columbia pattern at these two markers, confirming that they were homozygous for the *fad6-1* mutant allele.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Jyoti Shah, shah@ksu.edu.

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