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Plastidial Fatty Acid Signaling Modulates Salicylic Acid– and Jasmonic Acid–Mediated Defense Pathways in the Arabidopsis *ssi2* Mutant

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A mutation in the Arabidopsis gene *ssi2*/*fab2*, which encodes stearoyl–acyl carrier protein desaturase (S-ACP-DES), results in the reduction of oleic acid (18:1) levels in the mutant plants and also leads to the constitutive activation of NPR1-dependent and -independent defense responses. By contrast, *ssi2* plants are compromised in the induction of the jasmonic acid (JA)–responsive gene *PDF1.2* and in resistance to the necrotrophic pathogen *Botrytis cinerea*. Although S-ACP-DES catalyzes the initial desaturation step required for JA biosynthesis, a mutation in *ssi2* does not alter the levels of the JA precursor linolenic acid (18:3), the perception of JA or ethylene, or the induced endogenous levels of JA. This finding led us to postulate that the S-ACP-DES–derived fatty acid (FA) 18:1 or its derivative is required for the activation of certain JA-mediated responses and the repression of the salicylic acid (SA) signaling pathway. Here, we report that alteration of the prokaryotic FA signaling pathway in plastids, leading to increased levels of 18:1, is required for the rescue of *ssi2*-triggered phenotypes. 18:1 levels in *ssi2* plants were increased by performing epistatic analyses between *ssi2* and several mutants in FA pathways that cause an increase in the levels of 18:1 in specific compartments of the cell. A loss-of-function mutation in the soluble chloroplastic enzyme glycerol-3-phosphate acyltransferase (*ACT1*) completely reverses SA- and JA-mediated phenotypes in *ssi2*. In contrast to the *act1* mutation, a loss-of-function mutation in the endoplasmic reticulum–localized ω6 oleate desaturase (*FAD2*) does not alter SA- or JA-related phenotypes of *ssi2*. However, a mutation in the plastidial membrane–localized ω6 desaturase (*FAD6*) mediates a partial rescue of *ssi2*-mediated phenotypes. Although *ssi2 fad6* plants are rescued in their morphological phenotypes, including larger size, absence of visible lesions, and straight leaves, these plants continue to exhibit microscopic cell death and express the *PR-1* gene constitutively. In addition, these plants are unable to induce the expression of *PDF1.2* in response to the exogenous application of JA. Because the *act1* mutation rescues all of these phenotypes in *ssi2 fad6 act1* triple-mutant plants, *act1*-mediated reversion may be mediated largely by an increase in the free 18:1 content within the chloroplasts. The reversion of JA responsiveness in *ssi2 act1* plants is abolished in the *ssi2 act1 coi1* triple-mutant background, suggesting that both JA- and *act1*-generated signals are required for the expression of the JA-inducible *PDF1.2* gene. Our conclusion that FA signaling in plastids plays an essential role in the regulation of SSI2-mediated defense signaling is further substantiated by the fact that overexpression of the N-terminal–deleted SSI2, which lacks the putative plastid-localizing transit peptide, is unable to rescue *ssi2*-triggered phenotypes, as opposed to overexpression of the full-length protein.

INTRODUCTION

Plants resist pathogen infection by inducing a defense response that is targeted specifically to combat invasion by the pathogen (Keen, 1990; Van der Hoorn et al., 2002). In many cases, the induction of these responses is accompanied by localized cell death at the site of pathogen entry, which often is able to restrict the spread of pathogen to cells within and immediately surrounding the lesions. This phenomenon, known as the hypersensitive response, is one of the earliest visible manifestations of induced defense response and resembles programmed cell death in animals (Flor, 1971; Greenberg et al., 1994; Dangl et al., 1996; Hammond-Kosack and Jones, 1996; Jabs et al., 1996; Gray, 2002). Concurrent with hypersensitive response development, defense reactions are triggered locally and in parts distant from the site of primary infection. This phenomenon, known as systemic acquired resistance, is one of the most studied induced defense responses and is accompanied by a local and systemic increase in endogenous salicylic acid (SA) and a concomitant upregulation of a large set of defense genes, including genes that encode pathogenesis-related (PR) proteins (Ward et al., 1991; Gaffney et al., 1993; Uknes et al., 1993; Dong, 2001).

A substantial body of evidence has demonstrated that SA plays an important signaling role in the activation of *PR* gene

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expression and resistance to certain pathogens (Delaney et al., 1994; Dempsey et al., 1999; Feys and Parker, 2000; Dong, 2001; Thomma et al., 2001; Kunkel and Brooks, 2002; Shah, 2003). In addition to SA, jasmonic acid (JA) and ethylene are two other important signal molecules in plant defense against pathogens. The role of JA in defense signaling was proven conclusively by creating the *fad3 fad7 fad8* triple mutant, which is unable to accumulate JA and highly susceptible to infection by *Pythium* spp (Vijayan et al., 1998). Similarly, the JA-insensitive *coi1* and *jar1* mutants show enhanced susceptibility to fungal pathogens (Penninckx et al., 1996; Staswick et al., 1998; Thomma et al., 1998; Kachroo et al., 2001) and suppress the expression of the JA-inducible defense genes *PDF1.2* and *THI2.1* (Epple et al., 1995; Penninckx et al., 1998; Kachroo et al., 2001). A defect in ethylene signaling also impairs the expression of *PDF1.2* and renders plants susceptible to infection by *Alternaria brassicicola* (Thomma et al., 1999).

Several lines of evidence suggest the existence of an intricate signaling network involving SA, JA, and ethylene, which leads to fine tuning of defense responses. Although SA and JA activate distinct signaling pathways, there is a growing body of literature that shows that these pathways do not function entirely independently. Rather, they are involved in a complex signaling network that influences the magnitude or amplitude of various signals derived from these pathways (Kunkel and Brooks, 2002). One of the major roles postulated for JA is its antagonistic action on SA-dependent signaling pathways (Creelman and Mullet, 1997; Seo et al., 1997). Similarly, various pharmacological and genetic experiments have shown that SA is a potent suppressor of the JA signaling pathway (Pena-Cortes et al., 1993; Doares et al., 1995; Harms et al., 1995; Niki et al., 1998; Gupta et al., 2000; Spoel et al., 2003).

The mechanism of crosstalk between SA and JA signaling pathways in the plant defense response remains to be elucidated. Results from several laboratories suggest that NPR1, a positive regulator of systemic acquired resistance, also may play a role in crosstalk and modulate the expression of the JA-inducible gene *PDF1.2* (Shah et al., 1999; Devadas et al., 2002; Spoel et al., 2003). Recently, Spoel et al. (2003) showed that the NPR1-interacting protein TGA2 also can bind to a TGACG motif in the *PDF1.2* promoter. Although the TGACG motif does not appear to be required for NPR1-dependent crosstalk, these results indicate that SA- and JA-mediated pathways are likely to share common components that may have a regulatory role.

Although NPR1 is a key transducer of SA signal (Cao et al., 1997; Ryals et al., 1997), several NPR1-independent pathways also have been identified that regulate *PR* gene expression and/or resistance to certain bacterial and fungal pathogens (Bowling et al., 1997; Clarke et al., 1998; Rate et al., 1999; Shah et al., 1999, 2001; Kachroo et al., 2000; Shirano et al., 2002). The *ssi2* mutant represents one such component of the NPR1-independent signaling pathway. The recessive *ssi2* mutation confers constitutive *PR* gene expression, spontaneous lesion formation, and enhanced resistance to both bacteria and oomycete pathogens (Shah et al., 2001). By contrast, a subset of defense responses regulated by the JA signaling pathway, including expression of the defensin gene *PDF1.2* and resistance to *Botrytis cinerea*, are impaired in *ssi2* plants (Kachroo et al., 2001). *SSI2* encodes a stearoyl–acyl carrier protein desaturase (S-ACP-DES), which is an archetype member of a family of soluble fatty acid (FA) desaturases; these enzymes play an important role in regulating the overall level of desaturated FAs in the cell. The activity of the mutant S-ACP-DES enzyme was reduced 10-fold, resulting in an increase of the stearic acid (18:0) content in *ssi2* plants (Kachroo et al., 2001). Because reduced S-ACP-DES activity leads to the induction of certain defense responses and the inhibition of others, we proposed that a FA-derived signal modulates crosstalk between different defense signaling pathways.

In plants, FAs have started to emerge as important molecules that participate in diverse biological processes (Lee et al., 1997; Ryu and Wang, 1998; Shanklin and Cahoon, 1998; Piffanelli and Murphy, 1999; Kachroo et al., 2001, 2003; Laxalt and Munnik, 2002; Maldonado et al., 2002; Weber, 2002; Li et al., 2003). Interestingly, FA signaling in plants and animals show several intriguing parallels. For example, both JA and its structural equivalent in animals, prostaglandins, are derived from similar enzymatic reactions starting with the hydrolysis of polyunsaturated FAs (Straus and Glass, 2001). Furthermore, the stearoyl desaturase–catalyzed enzymatic step that leads to the synthesis of JA and prostaglandin precursors, linolenic acid and arachidonic acid, respectively, also is conserved between plants and animals (Shanklin and Somerville, 1991). In animals, altered stearoyl-CoA desaturase activity has been implicated in the regulation of cell growth, differentiation, and signal transduction (Kates et al., 1984; Gyorfy et al., 1997, Kim et al., 1999; Kumar et al., 1999). Additionally, altered activity of this enzyme is correlated with apoptosis (de Vries et al., 1997) and neoplasia (Li et al., 1994); these phenomena are similar to the cell death and altered defense signaling phenotypes associated with the *ssi2* mutation. Thus, although the mechanism(s) by which a mutation in S-ACP-DES affects defense response activation in plants is unclear, the discovery that a defect in FA desaturation can modulate defense responses opens novel approaches in the study of pathways that lead to disease resistance. Here, we show that a loss-of-function mutation in the chloroplastic enzymes glycerol-3-phosphate acyltransferase and ω6 desaturase can reverse various *ssi2*-triggered phenotypes. We also show that alteration of the prokaryotic FA signaling pathway in plastids, leading to increased levels of 18:1, can rescue *ssi2*-triggered phenotypes. Our analysis suggests that FA signaling plays an important role in modulating the defense response against pathogens.

RESULTS

ssi2 Plants Are Not Altered in the Perception or Biosynthesis of JA

SSI2-encoded S-ACP-DES preferentially desaturates stearic acid (18:0) between carbons 9 and 10 to yield 18:1. Compared with wild-type S-ACP-DES, the activity of the mutant protein is reduced 10-fold, which causes an increase in 18:0 levels and a reduction in 18:1 levels in *ssi2* plants (Table 1). The inability of the *ssi2* mutant plants to induce *PDF1.2* expression upon JA treatment could be attributable to any of the following causes:

Table 1. Fatty Acid Composition of Total Leaf Lipids from *SSI2*, *ssi2*, *act1*, *fad2*, *fad6*, *ssi2 act1*, *ssi2 fad6*, and *ssi2 fad2* Plants

Genotype	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
SSI2	16.6 ± 0.9	3.1 ± 0.7	0.8 ± 0.1	14.5 ± 0.5	0.8 ± 0.2	2.0 ± 1.0	15.4 ± 0.6	46.8 ± 2.0
ssi2	13.3 ± 1.0	2.7 ± 0.3	0.7 ± 0.0	10.1 ± 0.5	14.3 ± 1.0	0.7 ± 0.2	14.7 ± 0.4	43.5 ± 2.4
act1	13.1 ± 0.3	2.5 ± 0.2	0.5 ± 0.1	1.5 ± 0.2	0.7 ± 0.0	5.8 ± 0.5	15.8 ± 1.0	60.1 ± 4.2
ssi2 act1	13.1 ± 0.9	1.9 ± 0.3	—	1.7 ± 0.2	7.4 ± 0.5	11.1 ± 1.3	14.7 ± 0.8	50.1 ± 2.1
fad6	12.9 ± 0.6	16.4 ± 0.5	0.8 ± 0.1	0.1 ± 0.0	0.9 ± 0.3	25.0 ± 1.3	15.0 ± 1.6	28.9 ± 1.5
ssi2 fad6	13.8 ± 0.8	13.1 ± 0.4	0.5 ± 0.0	—	9.8 ± 1.2	17.3 ± 1.3	17.4 ± 1.1	28.1 ± 2.1
fad2	16.0 ± 1.0	3.2 ± 0.5	0.6 ± 0.0	14.1 ± 0.9	0.7 ± 0.1	12.1 ± 1.1	5.5 ± 0.8	47.8 ± 2.3
ssi2 fad2	14.2 ± 1.1	2.7 ± 0.4	0.7 ± 0.1	8.2 ± 1.1	8.6 ± 0.8	15.9 ± 1.3	5.5 ± 0.5	44.2 ± 1.1

All measurements were made on plants grown at 22°C, and data are described as mol% ± SE calculated for a sample size of six.

(1) *ssi2* plants are insensitive to JA and therefore unable to respond to exogenous treatment; (2) *ssi2* plants are impaired in the JA biosynthetic pathway and therefore unable to accumulate endogenous JA; or (3) *ssi2* plants lack a JA-coactivating signal, which is derived from S-ACP-DES. To assess these possibilities, we first determined the ability of *ssi2* plants to perceive JA by conducting a root-growth bioassay (Staswick et al., 1998). Approximately 30 to 40 seeds each of *SSI2*, *jar1-1*, and *ssi2* were grown on plates containing either 25 μM JA or an equal proportion of alcohol and allowed to grow vertically. Mean root length was measured 14 days later. As expected, JA inhibited root growth of the wild-type plants, whereas root growth in the JA-insensitive mutant *jar1-1* remained largely unaffected (Figure 1A). *ssi2* plants behaved like the wild-type plants and showed inhibition of root growth on JA-containing medium. These results suggest that *ssi2* plants perceive JA in a manner similar to that of wild-type plants.

To assess if the inability of *ssi2* plants to induce the expression of *PDF1.2* was associated with a defect in JA biosynthesis, we determined the levels of JA in wild-type and *ssi2* plants after pathogen infection and wounding. Maximum levels of JA induction in wild-type plants were seen at 1 h after wounding or 96 h after spraying of *A. brassicicola* spores (Figure 1B). At these time points, the induced levels of JA in *ssi2* were comparable to or higher than the levels in wild-type plants. These results suggest that *ssi2* plants are not defective in the pathway leading to JA biosynthesis. Because *ssi2* plants do not appear to be impaired in the perception or biosynthesis of JA, our results strongly support the third possibility mentioned above, that the lack of JA responses in *ssi2* may be caused by the absence or reduced levels of a JA-coactivating signal.

ssi2 Seedlings Are Not Affected in Their Response to Ethylene

Because expression of the *PDF1.2* gene requires the concomitant activation of both JA and ethylene signal transduction pathways (Penninckx et al., 1998), it also is possible that *ssi2* plants do not induce *PDF1.2* expression because they carry a defect in the ethylene signaling pathway. To test this possibility, we compared the seedling responses of *ssi2*, wild-type, and ethylene-insensitive *etr1* plants to the ethylene precursor aminocyclopropane carboxylic acid (ACC) (Guzman and Ecker, 1990; Smalle et al., 1997). Arabidopsis plants grown under continuous light on water-agar plates containing ACC exhibit enhanced elongation of the hypocotyl (Smalle et al., 1997). Compared with *etr1*, both *ssi2* and wild-type seedlings showed elongated hypocotyls on plates containing ACC as opposed to seedlings grown on plates without ACC (Figure 2A). In addition, both *ssi2* and wild-type seedlings showed a drastic inhibition of root growth on ACC plates as opposed to only a partial inhibition in *etr1* plants (Figure 2B). These data suggest that *ssi2* plants are not impaired in their response to ethylene.

A Mutation in the *act1* Gene Causes Reversion of the *ssi2* Phenotypes

Because *ssi2* plants contain highly reduced levels of 18:1 compared with 18:2 or 18:3, we proposed that the JA-coactivating signal might be derived from 18:1 or that the balance between 18:0 and 18:1 may be critical for normal signaling. This notion is supported by our previous work, in which we showed that the administration of 18:1 rescues the JA responsiveness of *ssi2* plants (Kachroo et al., 2001). However, because a pharmacological approach is fairly limiting for determining the effects of increased levels of 18:1 on SA and JA signal transduction pathways, we chose a genetic approach to determine if increasing 18:1 levels in *ssi2* plants can restore the normal signaling of SA and JA pathways.

The Arabidopsis genes *FAD2* (Miquel and Browse, 1992) and *ACT1* (Kunst et al., 1989) encode enzymes that are localized in extrachloroplast or chloroplast, respectively, and mutations in these genes result in the upregulation of 18:1 levels in the cell. By contrast, the *FAE1* gene product is expressed specifically in seeds, and a mutation in this gene causes an increase in 18:1 levels in the seeds (James et al., 1995). To determine if mutations in these genes can upregulate the levels of 18:1 in *ssi2* and thereby complement defective crosstalk, we crossed *ssi2* with *fad2-1*, *act1-1*, and *fae1-1* mutants and screened the F2 populations for double-mutant plants. Interestingly, F2 analysis of the *ssi2* × *act1* cross revealed that a certain proportion of these plants, corresponding to recessive double mutants, had nearly wild-type-like leaf morphology but were slightly pale compared with either parent (Figure 3A). By contrast, the *ssi2 fad2* (Figure 3A) and *ssi2 fae1* (data not shown) plants showed *ssi2*-like phenotypes. Because *ssi2 act1* plants contained the

ssi2 mutation in the homozygous state, we concluded that the presence of the *act1* mutant allele had resulted in the reversion of *ssi2*-conferred phenotypes. To confirm the genotype at the *act1* locus, we sequenced the *act1* gene and developed a cleaved amplified polymorphic sequence (CAPS) marker, which allowed us to score for the mutant allele. As expected, analysis of the double-mutant plants with the ACT1-CAPS marker showed that these contained the *act1* mutation in the homozygous state. Unlike *ssi2* plants, *ssi2 act1* double-mutant plants did not show spontaneous cell death or constitutively increased levels of *PR-1* gene expression (Figures 3B and 3C). To determine if these double mutant plants had regained sensitivity to the exogenous application of JA, we treated *SSI2* (wild-type), *ssi2*, *act1*, and *ssi2 act1* plants with JA or methyl jasmonate. As expected, *ssi2* showed no induction of *PDF1.2*. By contrast, high levels of *PDF1.2* expression were induced in wild-type, *act1*, and *ssi2 act1* plants (Figure 3D). These results indicate that the *act1* mutation was sufficient to revert *ssi2* plants to wild-type-like plants.

The *act1* Mutation Restores the Altered Pathogen Response of *ssi2* Plants

To determine if the *act1* mutation also restored wild-type-like resistance to necrotrophic pathogens in *ssi2* plants, we inoculated *ssi2*, *act1*, *ssi2 act1*, and wild-type plants with *B. cinerea* and assayed necrosis at 8 days after inoculation. Interestingly, *ssi2 act1* plants were as resistant to infection by *B. cinerea* as wild-type plants (Figure 4A). By contrast, *ssi2* plants either in the Nössen background or derived from a cross between Columbia (Col-0) and *ssi2* were highly susceptible to *B. cinerea*, and >80% of these plants showed necrosis accompanied by extensive proliferation and sporulation of the fungal mycelia (Figure 4A).

In contrast to a susceptible response toward *B. cinerea*, *ssi2* plants show heightened resistance to biotrophic pathogens, and this resistance is partially compromised in a NahG background, which abolishes the accumulation of SA (Shah et al., 2001). To determine the effect of the *act1* mutation on *ssi2*-conferred resistance, *ssi2*, *act1*, *ssi2 act1*, and wild-type plants were challenged with a virulent biotype of *Peronospora parasitica*. As anticipated, *ssi2* plants and a resistant ecotype of Arabidopsis supported 5% or no growth of the pathogen, respectively, and *act1*, like its parental ecotype Col-0, showed partial susceptibility to this pathogen. By contrast, *ssi2 act1* plants supported profuse growth of the pathogen, similar to that observed in the susceptible wild-type plants (Figure 4B).

The *act1* Mutation Restores Wild-Type-Like SA Levels in *ssi2* Plants and Does Not Impair SA Responsiveness

To test if reversion of the enhanced resistance phenotype conferred by the *ssi2* mutation also influences the accumulation of increased levels of SA, we determined the levels of SA and SA glucoside (SAG) in wild-type, *ssi2*, *act1*, and *ssi2 act1* plants (Figure 4C). The *ssi2 act1* plants showed a dramatic decrease in the levels of SA/SAG compared with the *ssi2* plants, suggesting that the *act1* mutation suppresses all of the SA-related phenotypes of the *ssi2* mutant. Interestingly, suppression of SA levels alone is not sufficient to abolish any of the *ssi2* phenotypes, because *ssi2 nahG* plants continue to remain stunted, show spontaneous cell death, express *PR* genes, and show partial resistance to bacterial and oomycete pathogens (Shah et al., 2001).

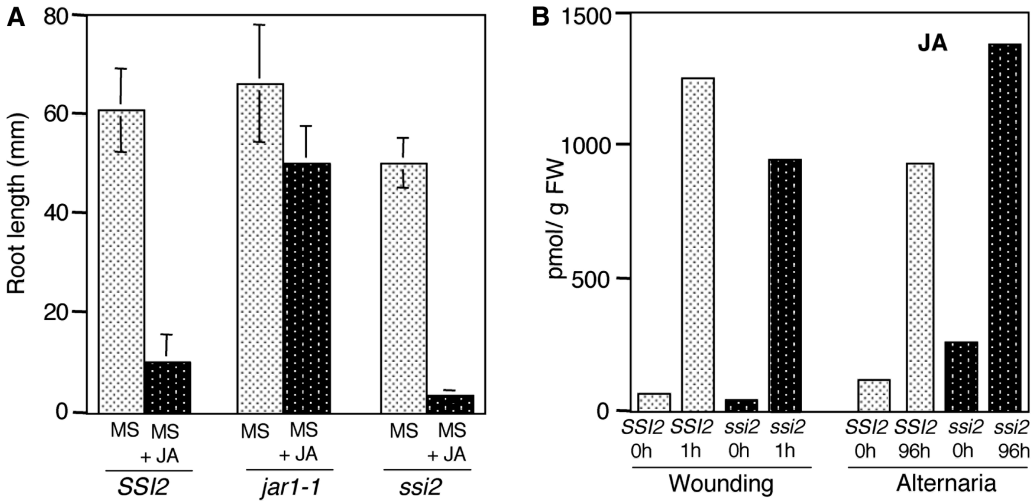

Figure 1. Root Growth Bioassay and JA Levels in the Leaves of *SSI2* (Wild-Type) and *ssi2* Plants after Wounding or Pathogen Infection.

(A) Approximately 30 to 40 seeds each of *SSI2*, *jar1-1*, and *ssi2* plants were sown on Murashige and Skoog (1962) (MS) plates with or without 25 μM JA and allowed to grow vertically for 14 days. Mean root length (mm ± SD) was determined for 30 seeds from each line.

(B) Leaves were wounded with a razor blade or inoculated by spraying with *A. brassicicola* spores at a concentration of 10^6 spores/mL. Maximum JA levels were observed at 1 h after wounding and 96 h after inoculation and are shown as averages of two to three replicates. FW, fresh weight.

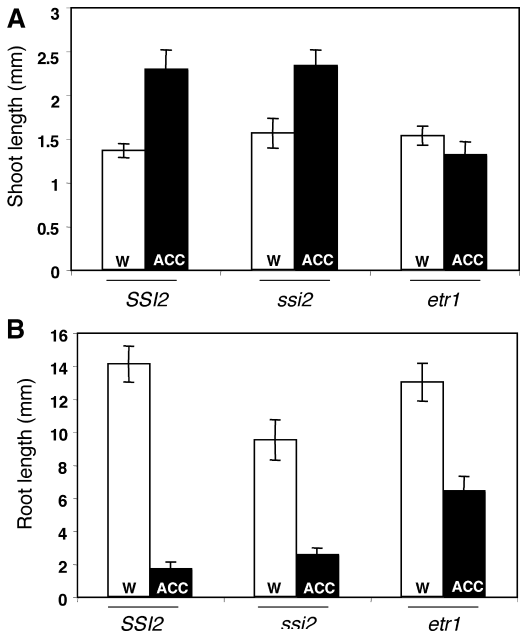

Figure 2. Effect of ACC Treatments on Seedlings of *SSI2*, *ssi2*, and *etr1* Plants.

(A) Comparison of hypocotyl lengths of *SSI2*, *ssi2*, and *etr1* seedlings grown on water-agar plates (w) or water-agar plates containing 50 μM ACC (ACC). Approximately 30 to 40 seeds each of *SSI2*, *ssi2*, and *etr1* were grown on water-agar plates with or without ACC, and the plates were incubated at 22°C in continuous light. Hypocotyl lengths were measured after 10 days of growth.
(B) Comparison of root lengths of 10-day-old seedlings from the same plants used in **(A)**.

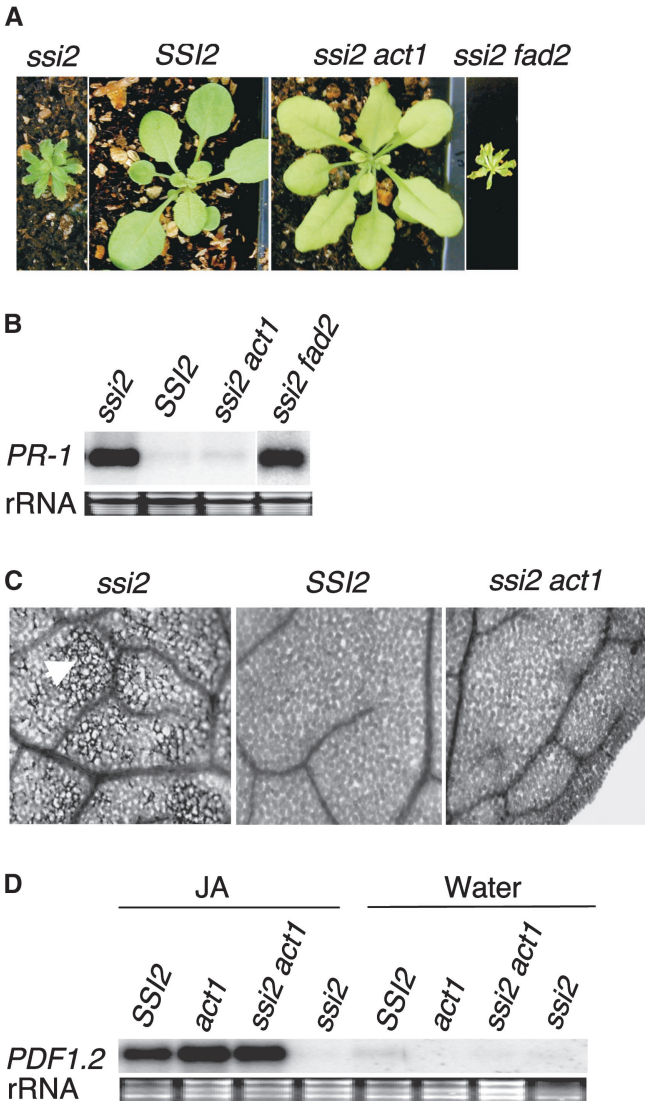

Figure 3. Morphological and Molecular Phenotypes of *SSI2*, *ssi2*, *ssi2 act1*, and *ssi2 fad2* Plants.

(A) Comparison of the morphological phenotypes displayed by *SSI2*, *ssi2*, and *ssi2 act1* plants. Plants were grown on soil and photographed after 3 (*SSI2*) or 4 (*ssi2*, *ssi2 act1*, and *ssi2 fad2*) weeks.
(B) Expression of the *PR-1* gene in *SSI2*, *ssi2*, *ssi2 act1*, and *ssi2 fad2* plants. RNA gel blot analysis was performed on 5 μg of total RNA extracted from 4-week-old soil-grown plants. Ethidium bromide staining of rRNA was used as a loading control.
(C) Microscopy of trypan blue–stained leaves from *SSI2*, *ssi2*, and *ssi2 act1* plants. Leaves from *ssi2* plants contain intensely stained areas of dead cells (arrowhead), whereas *ssi2 act1* leaves show a wild-type-like phenotype.
(D) Expression of the *PDF1.2* gene in *SSI2*, *act1*, *ssi2 act1*, and *ssi2* plants in response to 50 μM JA or water treatment. Samples were harvested at 48 h after treatment and examined by RNA gel blot analysis performed on 5 μg of total RNA. Ethidium bromide staining of rRNA was used as a loading control.

To determine if the *act1* mutation restores various *ssi2* phenotypes by impairing SA signaling, we sprayed wild-type, *act1*, and *ssi2 act1* plants with water or benzo(1,2,3)thiadiazole-7-carbothioic acid and analyzed these for the induction of *PR-1* gene expression. Both *act1* and *ssi2 act1* plants showed high levels of *PR-1* expression, which was comparable to the induced levels of *PR-1* seen in the wild-type plants (Figure 4D). These results suggest that *ssi2 act1* plants are SA responsive and that the *act1* mutation does not impair SA signaling. Therefore, *act1*-mediated suppression of the SA signaling pathway in *ssi2 act1* plants may involve the generation of a signal that acts as a repressor and shuts off the SA signaling pathway.

The *act1* Mutation Causes the Upregulation of 18:1 Levels in *ssi2* Plants

ACT1 encodes glycerol-3-phosphate acyltransferase, which functions in the first step of the prokaryotic pathway of glycerolipid biosynthesis (Kunst et al., 1988) (Figure 5). The FAs 16:0 and 18:1 synthesized de novo in the chloroplasts either enter into the "prokaryotic pathway" in the chloroplast envelope or are exported to the endoplasmic reticulum as CoA thioesters, where they are incorporated into lipids via the "eukaryotic path-

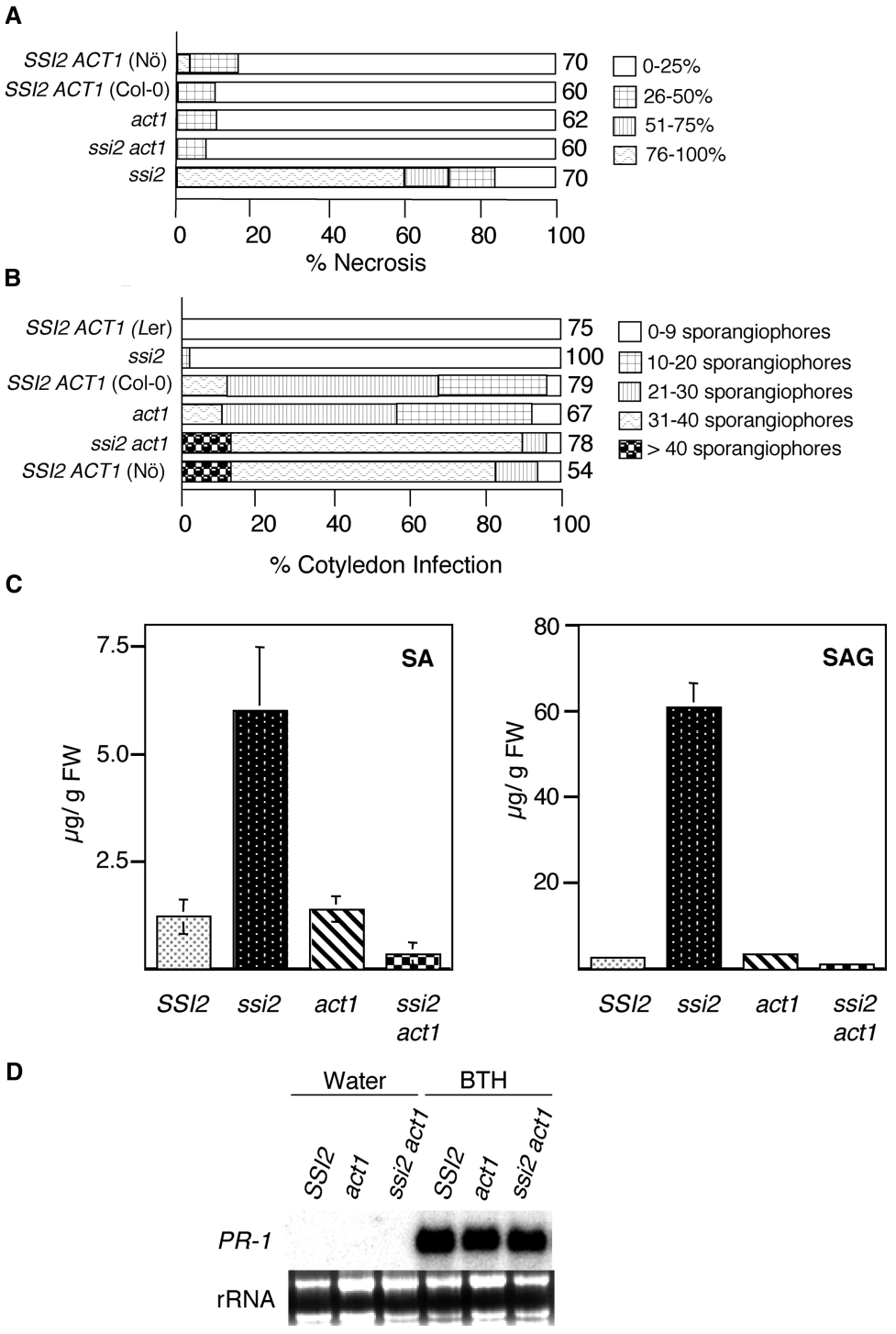

Figure 4. Resistance Response, Levels of SA and SA Glucoside, and SA Responsiveness.

(A) Comparison of necrosis caused by *B. cinerea* in *SSI2* (Col-0 and Nössen [Nö] ecotypes), *ssi2*, *ssi2 act1*, and *act1* genetic backgrounds. Inoculations were performed on leaves of 4-week-old plants pricked with a needle at two to three well-spaced spots per leaf. The shading of each box indicates the severity of necrosis, and the numbers at right of the sample boxes indicate the number of leaves assayed.

(B) Growth of *P. parasitica* ecotype Emco5. Cotyledons of 7-day-old seedlings from the various plant genotypes listed at left were inoculated by spraying conidiospores at a concentration of 10^6 spores/mL. There was no difference in the size of cotyledons between wild-type and mutant plants at the time of infection. Pathogen growth was assessed by counting the number of sporangiophores per cotyledon at 8 days after inoculation. The shading of each box indicates the severity of infection, based on the number of sporangiophores per cotyledon (see key at right). Numbers at right of the sample boxes indicate the number of cotyledons assayed. Ler, Landsberg *erecta*.

(C) Endogenous SA and SA glucoside levels in leaves of 4-week-old soil-grown *SSI2*, *ssi2*, *act1*, and *ssi2 act1* plants. The values presented are averages of three replicates. FW, fresh weight.

(D) SA responsiveness of *act1* and *ssi2 act1* plants. Wild-type, *act1*, and *ssi2 act1* plants were treated with water or 100 μM benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH) and analyzed for *PR-1* gene expression after 48 h. Ethidium bromide staining of rRNA was used as a loading control.

way." A mutation in *act1* effectively converts a 16:3 plant such as Arabidopsis to an 18:3 plant, resulting in the accumulation of 18:1-ACP and the redirection of C-16 chains into the eukaryotic pathway (Kunst et al., 1988, 1989). Therefore, *act1* mutant plants are deficient in their levels of 16:3 FA. To determine how *act1* influences the FA content of *ssi2* plants, levels of the various FAs were determined in leaf tissue of *ssi2*, *act1*, *ssi2 act1*, and wild-type plants (Table 1). As expected, the FA profile of *ssi2 act1* plants overlapped with the profiles from single-mutant plants; *ssi2 act1* plants contained lower levels of 16:3, similar to *act1* plants, and high levels of 18:0, similar to *ssi2* plants. However, the 18:1 content in *ssi2 act1* plants was ~15-fold higher compared with that in *ssi2* plants, whereas the levels of all other FAs in general remained similar to those of control plants. These data suggest that the restoration of phenotypes in *ssi2 act1* plants is likely to be mediated by increased levels of 18:1 in these plants. Because the *act1*-mediated increase in 18:1-ACP levels was initiated within chloroplasts, this result also suggests that reversion of the *ssi2* phenotype may be linked to chloroplastic 18:1 content. This finding is supported by the fact that *ssi2 fad2* plants accumulated an ~17-fold increase in 18:1 content but continued to show *ssi2*-like phenotypes (Table 1).

Partial Reversion of *ssi2* Phenotypes by a Mutation in Plastidial ω6 Oleate Desaturase

The 18:1 present in the chloroplasts can be found as either free 18:1-ACP or 18:1 bound to membrane lipids. To investigate the importance of free versus membrane-bound chloroplastic 18:1 in rescuing *ssi2*-related phenotypes, we crossed *ssi2* to the *fad6* mutant (Falcone et al., 1994). The *fad6* mutant is defective in the activity of chloroplastic ω6 desaturase, which converts 16:1 and 18:1 to 16:2 and 18:2, respectively, in chloroplastic membrane lipids. Therefore, a mutation in *fad6* results in increased 16:1 and 18:1 levels in the membrane lipids. To isolate double-mutant plants, we sequenced the *fad6* gene and identified the mutant base that leads to a loss of function of *fad6*-encoded desaturase. A CAPS marker was developed for this mutant base, and double-mutant plants were identified based on CAPS primer analysis and subsequently confirmed by FA profiling. Analysis of the double-mutant plants revealed that they were much larger than *ssi2* plants but smaller than wild-type plants (Figure 6A). Like *ssi2 act1* plants, *ssi2 fad6* plants showed no visible lesions on their leaves, although their leaves were not pale like those of *ssi2 act1* plants. Trypan blue staining of *ssi2 fad6* plants revealed that these plants showed microscopic cell death, although the dead cells were less dense than those in *ssi2* plants (Figure 6B). Surprisingly, even though 90% of the randomly selected *ssi2 fad6* leaves showed prominent cell death, 10% or fewer showed a marked reduction in the cell-death phenotype (Figure 6B, marked as I and II). This was observed particularly in one of the four F4 *ssi2 fad6* homozygous lines and may result from the fact that *ssi2* or *fad6* segregates for another mutant loci that has a suppressive effect on cell death. Nonetheless, both types of leaves were analyzed for various *ssi2*-like phenotypes. As expected, the expression levels of *PR-1* correlated well with the extent of dead cells present in the *ssi2 fad6* leaves; leaves showing less density of dead cells showed reduced accumulation of *PR-1* transcript compared with leaves showing prominent cell death (Figure 6C). RNA gel blot analysis of RNA extracted from one individual plant in which a few leaves showed no cell death revealed that *ssi2 fad6* plants expressed high levels of *PR-1* and that these levels were similar to those found in *ssi2* plants (Figure 6B, marked as III). These results suggest that the *fad6* mutation was unable to suppress cell death and *PR-1* gene expression in *ssi2 fad6* plants.

To determine if a partial rescue of the morphological phenotype in *ssi2 fad6* plants also includes the rescue of JA responsiveness, we treated *ssi2 fad6* and control plants with JA and analyzed them for the expression of *PDF1.2*. As expected wild-type, *fad6*, and *ssi2 act1* plants induced high levels of *PDF1.2* transcript, whereas *ssi2* plants showed basal-level expression of this gene (Figure 6D). The *ssi2 fad6* leaves displaying prominent cell death showed basal-level expression of *PDF1.2*, similar to that seen in *ssi2* plants. The *ssi2 fad6* leaves, which contained reduced numbers of dead cells, showed a slightly higher expression of *PDF1.2*, but these levels were appreciably lower than those seen in wild-type, *fad6*, or *ssi2 act1* plants. These

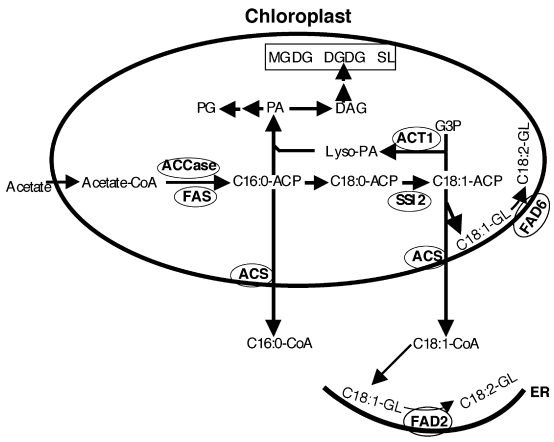

Figure 5. Condensed Scheme for Lipid Biosynthesis in the Chloroplasts of Arabidopsis Leaves.

De novo FA biosynthesis from acetyl-CoA occurs exclusively in the plastids of all cells (represented by the oval). Acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS) complex are key enzymes involved in the biosynthesis of C16:0 FA. Upon elongation to C18:0, this FA undergoes desaturation to C18:1. This step is catalyzed by the *SSI2*-encoded S-ACP-DES. The product of this reaction (C18:1-ACP) either enters the prokaryotic pathway of lipid biosynthesis through the acylation of glycerol-3-phosphate (G3P) or is exported out of the plastids as a CoA thioester to enter the eukaryotic pathway. The acylation of G3P is catalyzed by an *ACT1*-encoded G3P acyltransferase. Desaturation of the C18:1 present on membrane glycerolipids (GL) is catalyzed by *FAD2*- or *FAD6*-encoded ω6 desaturases that are present on the endoplasmic reticulum (ER) or the plastid envelop, respectively. Esterification of the CoA group is mediated by acyl-CoA synthetase (ACS). Symbols for various components are as follows: ACP, acyl carrier protein; C, carbon; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; Lyso-PA, 1-acyl-G3P; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PG, phosphatidylglycerol; SL, sulfolipid.

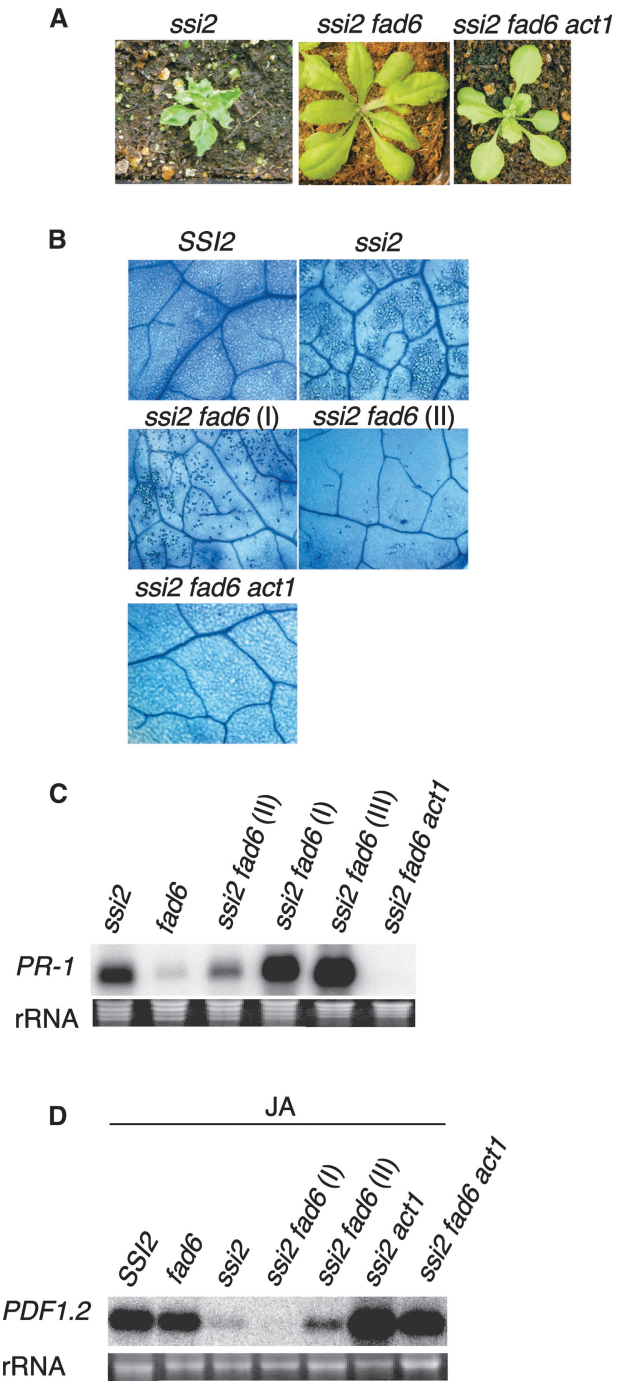

Figure 6. Morphological and Molecular Phenotypes of *ssi2*, *ssi2 fad6*, and *ssi2 fad6 act1* Plants.

(A) Comparison of the morphological phenotypes displayed by the *ssi2*, *ssi2 fad6*, and *ssi2 fad6 act1* plants. Plants were grown on soil and photographed after 4 weeks.

(B) Microscopy of trypan blue–stained leaves from *SSI2*, *ssi2*, *ssi2 fad6*, and *ssi2 fad6 act1* plants. More than 90% of the *ssi2 fad6* leaf samples showed prominent cell death (shown as I), whereas the remaining samples showed a marked reduction in the cell-death phenotype (shown as II).

(C) Expression of *PR-1* in *ssi2*, *fad6*, *ssi2 fad6*, and *ssi2 fad6 act1* plants. For *ssi2 fad6* plants, *PR-1* gene expression was analyzed for three different sets of leaves. The lanes marked I and II represent RNA extracted from *ssi2 fad6* leaves that showed high and low numbers of dead cells (shown in **[B]**), respectively. The lane marked III represents RNA extracted from leaves of one individual *ssi2 fad6* plant in which a few leaves showed no cell death. RNA gel blot analysis was performed on 5 μg of total RNA extracted from 4-week-old soil-grown plants. Ethidium bromide staining of rRNA was used as a loading control.

(D) Expression of *PDF1.2* in *SSI2*, *fad6*, *ssi2 fad6*, and *ssi2 fad6 act1* plants in response to 50 μM JA. Lanes marked I and II represent RNA extracted from *ssi2 fad6* plants that showed high and low numbers of dead cells, respectively. Samples were harvested at 48 h after treatment and examined by RNA gel blot analysis performed on 5 μg of total RNA. Ethidium bromide staining of rRNA was used as a loading control.

results suggest that a partial rescue of phenotypes by the *fad6* mutation was insufficient to rescue JA responsiveness in *ssi2* plants.

We next determined the FA profiles of *fad6* and *ssi2 fad6* plants and found that both *fad6* and *ssi2 fad6* plants contained reduced levels of 16:3 and 18:3 and increased levels of 16:1 and 18:1, compared with wild-type plants (Table 1). Compared with *ssi2* plants, *ssi2 fad6* plants accumulated ~24-fold greater levels of 18:1; these levels were ~10-fold higher than those in *ssi2 act1* plants. This finding suggests that an increase in the 18:1 content of chloroplastic membrane lipids is not sufficient to completely revert the altered defense signaling in *ssi2* plants.

The *act1* Mutation Mediates a Complete Rescue of *ssi2 fad6* Plants

A partial recovery of *ssi2* phenotypes by the *fad6* mutation suggests that a simple increase in membranous 18:1 levels may not be enough to restore defective signaling in *ssi2* plants. On the other hand, a complete reversal of *ssi2* phenotypes by the *act1* mutation suggests that the *act1* mutation may generate free 18:1 in addition to channelizing 18:1 into chloroplastic membrane lipids. If these assumptions were correct, then we would expect the *act1* mutation to completely restore all *ssi2*-related phenotypes in *ssi2 fad6* plants. We generated *ssi2 fad6 act1* triple-mutant plants by crossing *ssi2 act1* with *fad6* and identified triple mutants plants by CAPS and derived CAPS analysis of *act1*, *fad6*, and *ssi2*. The triple-mutant plants were morphologically similar to *ssi2 act1* plants and showed no visible or microscopic cell death in their leaves (Figures 6A and 6B). These plants showed basal levels of *PR-1* gene expression (Figure 6C) and high levels of the JA-inducible *PDF1.2* gene (Figure 6D). Together, these results suggest that the *act1* mutation is epistatic to the *fad6* mutation and can completely suppress the *ssi2*-related phenotypes in *ssi2 fad6* plants.

Induction of *PDF1.2* in *ssi2 act1* Plants Requires a Functional JA Pathway

Because the JA-induced expression of *PDF1.2* requires the *COI1* gene (Xie et al., 1998), we further assessed if the rescue

of JA responsiveness in *ssi2 act1* plants requires a fully functional JA pathway. To obtain triple-mutant plants, *ssi2 act1* plants were crossed with *coi1* plants and the F2 population was scored for plants homozygous for the *ssi2* and *act1* loci. Because tight linkage between the *ssi2* and *coi1* loci did not allow us to obtain *ssi2 act1* plants that were homozygous for the *coi1* locus, we isolated *ssi2 act1* homozygous plants that were heterozygous at the *coi1* locus. Three such plants were isolated after screening ~460 F2 plants, and these were selfed to isolate *ssi2 act1 coi1* triple-mutant plants. Genotypes at the *coi1*, *act1*, and *ssi2* loci were determined by CAPS or derived CAPS marker analysis. The triple-mutant plants were morphologically similar to *ssi2 act1* plants. Single-, double-, and triple-mutant plants between *ssi2*, *act1*, and *coi1* were sprayed with water or JA, and the RNA isolated from these plants was analyzed for the expression of *PDF1.2* after 48 h of treatment (Figure 7). As observed previously, *ssi2* plants failed to induce any expression of *PDF1.2*, as opposed to the high levels seen in *ssi2 act1* and *ssi2 act1 COI1/−* plants. By contrast, *ssi2 act1 coi1* plants behaved like *coi1* and *ssi2* single-mutant plants and did not allow any JA-mediated induction of *PDF1.2*. These results suggest that *COI1* is required for the induction of *PDF1.2* in *ssi2 act1* plants and that the *act1*-mediated rescue of JA responsiveness in *ssi2* plants involves a step upstream of the *COI1* gene in the JA pathway. However, it is likely that the *act1* mutation regenerates a putative signal that in combination with JA is required for the induction of *PDF1.2*. In such a situation, blocking either the step leading to the generation of this putative signal or the JA signaling pathway will block the expression of the *PDF1.2* gene. This notion is supported by our observation that exogenous application of both JA and 18:1 are required for the rescue of *PDF1.2* expression in *ssi2* plants (Kachroo et al., 2001).

SSI2 Lacking the N-Terminal Transit Peptide Does Not Complement the *ssi2* Mutation

SSI2-encoded S-ACP-DES is a soluble chloroplastic desaturase that shows ~83 to 93% identity with similar enzymes from other plant species (Lightner et al., 1994; Ohlrogge and Browse, 1995) (data not shown). Phylogenetic analysis of various S-ACP-DES revealed that SSI2 shares a high level of amino acid identity with S-ACP-DES from castor bean. Based on the aligned amino acid sequence and the presence of conserved domains between the castor bean and the SSI2-encoded S-ACP-DES, the predicted N-terminal transit peptide was removed from SSI2. Previously, we showed that SSI2 protein with an N-terminal deletion (SSI2 ΔN-t) had 10-fold higher activity compared with the mutant ssi2 protein (Kachroo et al., 2001). The SSI2 ΔN-t also showed the expected 18:16 substrate preference and Δ^9 regiospecificity, suggesting that the N-terminal truncation had not caused any alteration in the predicted enzymatic activity of SSI2. Because removal of the N terminus from SSI2 was likely to abolish its targeting to the chloroplasts, we predicted that removal of the signal peptide would prevent its ability to complement the *ssi2* mutant. We first overexpressed the full-length *SSI2* cDNA in *ssi2* plants and scored these plants for various *ssi2* phenotypes. Transgenic plants overexpressing *SSI2* (35S:SSI2) were similar to wild-type plants in morphology (Figure 8A) and showed the absence of spontaneous cell death (data not shown) and *PR-1* gene expression (Figure 8B). By contrast, transgenic plants overexpressing *SSI2* ΔN-t (35S:SSI2 ΔN-t) were similar to *ssi2* plants in morphology and showed high levels of *PR-1* expression (Figures 8A and 8B). Both groups of transgenic plants showed comparable levels of the *SSI2* transcript, and as predicted, the size of the *SSI2* ΔN-t transcript was slightly lower than that of the endogenous transcript (Figure 8B). These results suggest that targeting of SSI2 to the chloroplasts is important for the rescue of *ssi2*-associated phenotypes.

To ascertain if the ability to complement *ssi2* phenotypes was associated with an increase in 18:1 levels, we conducted FA profiling of 35S:SSI2 and 35S:SSI2 ΔN-t plants (Table 2). Surprisingly, even though 35S:SSI2 plants showed complementation of all *ssi2* phenotypes, they still continued to accumulate higher levels of 18:0. However, the levels of 18:0 in 35S; SSI2 plants were appreciably lower than those of 35S:SSI2 ΔN-t plants or *ssi2*-like F2 segregants from the 35S:SSI2 T2 population. The high levels of 18:0 in 35S:SSI2 may be the result of transcriptional or translational regulation of S-ACP-DES genes, which are under negative feedback control (Bené et al., 2001), or of low levels of S-ACP-DES protein transcribed during the initial stages of growth. Alternatively, it may represent the 18:0 levels channeled outside the chloroplasts (Lightner et al., 1994). As expected, 35S:SSI2 plants showed a fourfold increase in the levels of 18:1 compared with *ssi2* and 35S:SSI2 ΔN-t plants. 35S:SSI2 plants resembled wild-type plants in their levels of all other FAs, including 16:3, which consistently showed lower levels in *ssi2* plants. Together, these results reinforce the role of 18:1 in the restoration of altered defense signaling in *ssi2* plants.

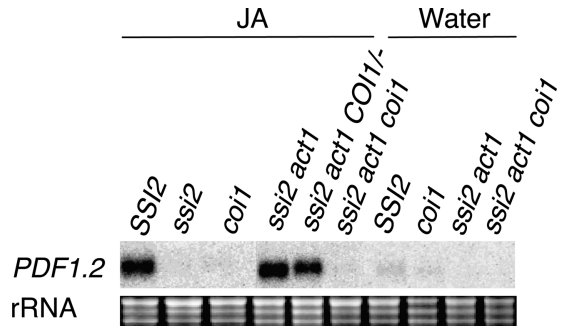

Figure 7. JA Responsiveness in *ssi2 act1 coi1* Triple-Mutant Plants.

Expression of *PDF1.2* in *SSI2*, *ssi2*, *coi1*, *ssi2 act1*, *ssi2 act1 COI1/−*, and *ssi2 act1 coi1* plants in response to 50 μM JA or water treatment. Samples were harvested at 48 h after treatment and examined by RNA gel blot analysis performed on 5 μg of total RNA. Ethidium bromide staining of rRNA was used as a loading control.

DISCUSSION

Here, we have attempted to reveal an as yet unexplored role of FAs as signal molecules in the regulation of various plant defense signaling pathways. The *ssi2* plants accumulate high lev-

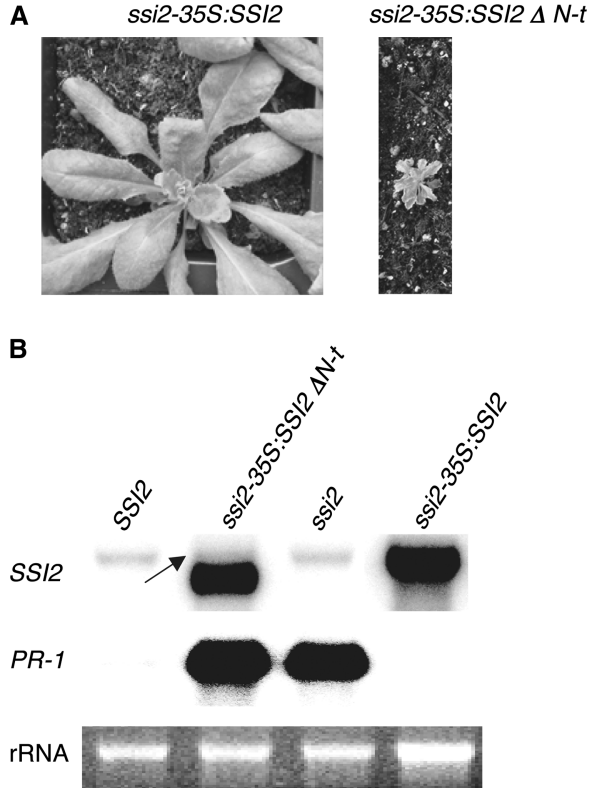

Figure 8. Complementation of *ssi2* Plants with Full-Length and N-Terminal Truncated *SSI2* cDNAs.

(A) Morphological phenotypes displayed by the transgenic plants overexpressing *SSI2* or *SSI2* ΔN-t cDNA. Plants were grown on soil and photographed after 4 weeks.

(B) Expression of the *SSI2* and *PR-1* genes in transgenic plants overexpressing *SSI2* or *SSI2* ΔN-t cDNA. The band marked by the arrow represents endogenous *SSI2* transcript. RNA gel blot analysis was performed on 5 μg of total RNA extracted from 4-week-old soil-grown plants. Ethidium bromide staining of rRNA was used as a loading control.

els of 18:0 FA, which could have a direct or indirect effect on these plants and cause stress-induced constitutive SA signaling. However, high levels of 18:0 are unlikely to induce SA signaling, because *ssi2 act1* plants continue to accumulate high levels of 18:0 but have normal SA levels and *PR* gene expression. Moreover, we isolated several mutants in the *ssi2* background that are restored in *ssi2*-triggered altered defense signaling but continue to accumulate high levels of 18:0 (Kachroo et al., 2003). In addition, we also studied *shs1*, a previously characterized mutant in the *fab2* background, that shows nearly wild-type levels of 18:0 but continues to show spontaneous cell death and high levels of *PR* gene expression (Lightner et al., 1997; Kachroo et al., 2003). These results suggest that activation of the SA pathway in the *ssi2* mutant most likely is caused by the absence of SSI2-mediated repression.

There are two possible ways that a mutation in *act1* can rescue various *ssi2*-mediated phenotypes. First, because *act1* suppresses SA/SAG levels in *ssi2*, it is likely that the *act1* mutation renders plants defective in the SA signaling pathway, thereby shutting off constitutive SA signaling conferred by the *ssi2* mutation. However, removal of SA by expressing the NahG transgene in *ssi2* plants does not abolish any of its phenotypes (Shah et al., 2001). Furthermore, *act1* plants are able to respond to the exogenous application of SA and are not altered in their resistance response to virulent or avirulent pathogens (Figures 4B and 4D). These results suggest that *ACT1* may not participate in the SA signaling pathway and that reducing SA levels is not sufficient to cure the defect in JA-mediated responses in *ssi2* plants. The second and most plausible explanation is that the *act1* mutation acts indirectly by increasing the levels of 18:1 FA, and this increase leads to the restoration of *ssi2*-mediated signaling. This notion is supported by the observations that the injection of 18:1 rescues the responsiveness of *ssi2* to JA (Kachroo et al., 2001) and that *ssi2 act1* plants accumulate ~15-fold higher levels of 18:1 compared with *ssi2* plants, whereas levels of other FAs remain similar to those in control plants.

The mechanism underlying the wild-type-like reversion of *ssi2* by a mutation in *act1* is likely to involve FA signaling and to be mediated by 18:1 or its derivative. However, it also is possible that the signaling between various defense pathways is regulated by the ratios of saturated versus unsaturated FAs. This notion is supported by the observation that unsaturated FAs can stimulate or inhibit the activities of various regulatory enzymes (Klumpp et al., 1998; Baudouin et al., 1999). Furthermore, in animal systems, the ratio of saturated to unsaturated FAs is thought to control many cellular functions, including cell growth, differentiation, and apoptosis (Kates et al., 1984; Ntambi, 1995). Therefore it is likely that the *act1* mutation mediates its effect by increasing the proportion of unsaturated 18:1 and balancing the ratio between 18:0 and 18:1. Interestingly, the ratios of 18:0 to 18:1 were more similar in wild-type and *ssi2 act1* plants than in *ssi2* plants, in which 18:0/18:1 ratios were 10-fold or more higher.

It is interesting that *ssi2* plants continue to make low levels of 18:1 even though they are defective in the step leading to the formation of 18:1 (Figure 5). Similarly, *fab2*, which contains a null mutation in the *SSI2* gene, also accumulates low levels of 18:1 (Lightner et al., 1994), indicating that trace amounts of 18:1 in *ssi2* plants are not contributed by the residual activity of the mutant enzyme. This finding indicates that in addition to *SSI2*, several other isozymes of S-ACP-DES contribute to the generation of 18:1, although SSI2 appears to be the major enzyme regulating 18:1 levels in the chloroplast. Chloroplasts have been shown to harbor signaling components for both the SA and JA pathways, and a close proximity of various intermediates from these pathways would not only favor crosstalk but provide tighter control over their regulation (Schmid and Amrhein, 1995; Verberne et al., 2000; Weber, 2002). This also would explain why other mutations, including *fad2*, which upregulates 18:1 levels in the extrachloroplast membranes, are unable to complement a defect in *ssi2* plants.

One important difference between the cellular locations of ACT1 and FAD2 is that although the former is a soluble chloroplastic enzyme, the later is a membrane-bound extrachloroplastic enzyme. These membrane-bound desaturases increase

Table 2. Fatty Acid Composition of Total Leaf Lipids from *SSI2*, *ssi2*, and Transgenic Plants Overexpressing *SSI2* or the N-Terminal Truncated Form of *SSI2*

Genotype	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
SSI2	16.6 ± 0.9	3.1 ± 0.7	0.8 ± 0.1	14.5 ± 0.5	0.8 ± 0.2	2.0 ± 1.0	15.4 ± 0.6	46.8 ± 2.0
ssi2	13.3 ± 1.0	2.7 ± 0.3	0.7 ± 0.0	10.1 ± 0.5	14.3 ± 1.0	0.7 ± 0.2	14.7 ± 0.4	43.5 ± 2.4
35S-SSI2 F2 wild-type-like	11.5 ± 1.0	3.0 ± 0.7	0.9 ± 0.1	14.8 ± 1.1	7.2 ± 0.8	4.4 ± 0.3	13.6 ± 1.0	44.6 ± 2.2
35S-SSI2 F2 *ssi2*-like	14.2 ± 1.4	2.6 ± 0.3	0.8 ± 0.1	10.4 ± 0.9	16.8 ± 1.3	0.5 ± 0.0	12.3 ± 0.9	42.4 ± 2.1
35S-SSI2 ΔN-t	12.6 ± 0.9	2.3 ± 0.7	0.7 ± 0.2	9.3 ± 1.1	14.0 ± 1..5	0.5 ± 0.1	20.6 ± 1.3	40.0 ± 1.8

All measurements were made on plants grown at 22°C, and data are described as mol% ± SE calculated for a sample size of six.

the unsaturated levels of membrane lipids and are less likely to cause an increase in the free FA pool in the cell. By contrast, a mutation in *act1* is more likely to increase the free 18:1-ACP levels in the cell (Figure 5). This leads to the possibility that the reversion of phenotypes in *ssi2 act1* plants may be attributable to the free chloroplastic 18:1-ACP pool rather than to the increased 18:1 content in the chloroplastic membrane lipids. However, the partial reversion of the morphological phenotype seen in *ssi2 fad6* plants suggests that increases in both the free pool of 18:1-ACP and the 18:1 content of chloroplastic membrane lipids may be required for the restoration of *ssi2* phenotypes. These results also suggest that *act1* may generate certain factor(s) in addition to channelizing 18:1 into chloroplastic membrane lipids. This appears to be the case, because the *act1* mutation is epistatic to the *fad6* mutation and restores all of the *ssi2*-triggered phenotypes in *ssi2 fad6* plants. Because SSI2 must be targeted to the chloroplast to be able to complement the *ssi2* phenotypes (Figure 8), signaling within chloroplasts appears to account for the altered defense signaling in *ssi2*. Thus, it may be reasonable to assume that free 18:1 levels within the chloroplasts are able to balance the high levels of 18:0 generated as a result of the *ssi2* mutation. Alternatively, the free 18:1 generated within the chloroplasts may generate a signal that participates in the modulation of defense signaling between the SA and JA pathways.

Several studies in both plants and animals have suggested a role for 18:1 in various cellular processes. In parsley cells, 18:1 levels have been shown to be upregulated in a biphasic manner at 2 h after treatment with an elicitor from *Phytophthora sojae* (Kirsch et al., 1997). By contrast, the levels of 18:2 declined and the levels of 18:3 increased steadily and doubled at 12 h after treatment. These findings indicate that the pathway from 18:1 to 18:3 is not linear and that 18:1 could play other roles besides serving as a precursor for 18:2. Studies in mammalian systems have shown that 18:1 can induce the translocation of protein kinase C from the cytosol toward the membranes via a direct or indirect process (Diaz-Guerra et al., 1991). Interestingly, in a recent study by Spoel et al. (2003), nucleocytoplasmic localization of NPR1 was suggested to regulate the crosstalk between the SA and JA pathways. Therefore, it is conceivable that 18:1 levels in plants regulate crosstalk by inducing the translocation of regulators of defense gene expression.

In addition to increasing the levels of 18:1 in *ssi2* plants, the *act1* and *fad6* mutations also decrease 16:3 levels in the double-mutant plants. Thus, it is possible that the lower levels of 16:3 may play a role in restoring various phenotypes in *ssi2 act1* and *ssi2 fad6* plants. It is equally probable that the reduction in 16:3 levels is merely coincidental, because both *act1* and *fad6* affect the prokaryotic pathway of glycerolipid biosynthesis. Several results support this view. First, we have isolated several suppressor mutants in the *ssi2* background that restore all of the *ssi2*-triggered phenotypes but contain normal levels of 16:3 (Kachroo et al., 2003). Second, *ssi2* plants themselves show slight reductions in 16:3 levels compared with wild-type plants. Third, 35S:SSI2 transgenic plants show increased levels of 16:3 similar to wild-type plants. Fourth, although both *ssi2 act1* and *ssi2 fad6* plants have reduced 16:3 levels, only *ssi2 act1* plants are completely rescued for all the *ssi2*-triggered phenotypes. Fifth, a mutation in *fad5*, which also decreased the levels of 16:3, did not restore wild-type-like phenotypes in *ssi2* plants (A. Kachroo and P. Kachroo, unpublished results).

Our results also show that in addition to JA and ethylene, expression of the *PDF1.2* gene is dependent on normal levels of FAs. The lack of induction of *PDF1.2* in *ssi2* plants does not appear to involve the JA or ethylene pathway, because *ssi2* plants are responsive to both of these hormones (Figures 1 and 2). Furthermore, the restoration of JA responsiveness in *ssi2 act1* plants can be abolished by the *coi1* mutation, which indicates that functional FA and JA signaling pathways are required for the expression of *PDF1.2*.

In conclusion, our results clearly demonstrate the importance of FAs in modulating signaling between SA- and JA-dependent defense pathways. Further analysis of the FA signaling pathway should provide useful insights into the complex networking of these pathways and their regulation.

METHODS

Plant Growth Conditions and Genetic Analysis

Plants were grown in MTPS 144 Conviron walk-in-chambers (Winnipeg, Manitoba, Canada) at 22°C with 65% RH and 14-h photoperiods. Crosses were performed by pollinating flowers of *ssi2* or wild-type Nössen plants with pollen from *fad2*, *act1*, *fad6*, or *fae1* plants. The *ssi2 act1 coi1* triple-mutant plants were obtained by pollinating flowers from *coi1* with pollen from *ssi2 act1*. The *ssi2 fad6 act1* triple-mutant plants were obtained by pollinating *ssi2 act1* flowers with pollen from *fad6*.

The genotype at the *ssi2* locus was determined by derived cleaved amplified polymorphic sequence (CAPS) analysis (Kachroo et al., 2001). The genotype at the *act1-1* locus was determined by identifying the

mutant base, amplifying a 0.5-kb region flanking the mutant base, and digesting the amplified product with BsmFI. Primers used to amplify *act1*-specific CAPS were 5′-GCCATCAAGTGTTCATCTACT-3′ and 5′-GGAAGTCATACAAGGTTGCTA-3′. The genotype at the *coi1* locus was determined as described previously (Xie et al., 1998). The genotype at the *fad6* locus was identified by CAPS analysis. Sequence analysis of the *fad6-1* gene revealed a mutation from G to A at bp 478 in *fad6* cDNA, which abolishes the restriction enzyme site for AlwNI in the *fad6* amplified DNA. Primers used for FAD6-CAPS were 5′-GGATACACTTCC-CAAAGAGGTG-3′ and 5′-AGTTCACCCAGTGAGCTATGG-3′. The genotype at the *fad2* locus was determined by sequence analysis. The *fad2-1* mutation was determined by sequence analysis of the mutant gene and found to contain A instead of G at 310 bp in the cDNA. The genotype at the *fae1* locus was determined indirectly by scoring for plants that segregated like ecotype Columbia for markers on the lower arm of chromosome 4.

RNA Extraction and RNA Gel Blot Analysis

Small-scale extraction of RNA from one or two leaves was performed with the TRIzol reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. RNA gel blot analysis and the synthesis of randomly primed probes for *PR-1* and *PDF1.2* were performed as described (Kachroo et al., 2001).

Generation of Transgenic Plants Overexpressing *SSI2* and *SSI2* ΔN-t cDNAs

The putative transit peptide region of SSI2 was predicted by aligning it with the protein sequence from castor bean S-ACP-DES. *SSI2* ΔN-t cDNA was amplified such that it lacked 34 amino acids of the putative signal peptide and the 35th amino acid was converted to Met. Both full-length and N-terminal truncated cDNAs were amplified as NcoI-XbaI–linked PCR products and cloned downstream of the double 35S promoter in pRTL2.GUS (β-glucuronidase) vector. For transformation of *Arabidopsis thaliana*, the fragment containing promoter, *SSI2* cDNA, and terminator was removed from the pRTL2-SSI2 or pRTL2-SSI2 ΔN-t vectors and cloned into the HindIII site of the binary vectors pBAR1 and pVK18. Transgenic seeds containing pBAR1-derived vector were selected on soil sprayed with BASTA, and transgenic seeds containing pVK18 were selected on Murashige and Skoog (1962) medium containing hygromycin.

Trypan Blue Staining

Leaf samples were taken from 2-week-old plants grown on soil. Trypan blue staining was performed as described previously (Bowling et al., 1997).

Salicylic Acid, Jasmonic Acid, and Fatty Acid Analysis

Salicylic acid and salicylic acid glucoside were extracted and measured from 0.4 g (fresh weight) of leaf tissue as described previously (Bowling et al., 1994). Jasmonic acid and fatty acid analysis were performed as described previously (Dahmer et al., 1989; He et al., 2002). Endogenous levels of jasmonic acid were induced by spray inoculating *Alternaria brassicicola* spores at a concentration of 10^6 spores/mL or by wounding leaf tissue with a razor blade.

Pathogen Infection

Infection with *Peronospora parasitica* Emco5 was performed by spraying or applying a single drop of asexual inoculum suspension as described previously (Kachroo et al., 2001). Infection with *Botrytis cinerea* was performed by spraying the spores at a concentration of 10^7/mL on plants that were wounded by needle pricks. Plants were covered with a transparent plastic dome and kept in a plexiglass container to create maximum humidity conditions. Necrosis and fungal growth were scored at 8 to 10 days after inoculation. Necrosis was quantified by estimating the percentage of leaf tissue showing decay and was confirmed microscopically by determining fungal spread and the extent of sporulation.

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 Pradeep Kachroo, pk62@uky.edu.

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