

Role of Salicylic Acid and Fatty Acid Desaturation Pathways in *ssi2*-Mediated Signaling^{1[W]}

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Stearoyl-acyl carrier protein desaturase-mediated conversion of stearic acid to oleic acid (18:1) is the key step that regulates the levels of unsaturated fatty acids (FAs) in cells. Our previous work with the *Arabidopsis thaliana* *ssi2/fab2* mutant and its suppressors demonstrated that a balance between glycerol-3-phosphate (G3P) and 18:1 levels is critical for the regulation of salicylic acid (SA)- and jasmonic acid-mediated defense signaling in the plant. In this study, we have evaluated the role of various genes that have an impact on SA, resistance gene-mediated, or FA desaturation (FAD) pathways on *ssi2*-mediated signaling. We show that *ssi2*-triggered resistance is dependent on *EDS1*, *PAD4*, *EDS5*, *SID2*, and *FAD7 FAD8* genes. However, *ssi2*-triggered defects in the jasmonic acid pathway, morphology, and cell death phenotypes are independent of the *EDS1*, *EDS5*, *PAD4*, *NDR1*, *SID2*, *FAD3*, *FAD4*, *FAD5*, *DGD1*, *FAD7*, and *FAD7 FAD8* genes. Furthermore, the *act1*-mediated rescue of *ssi2* phenotypes is also independent of the *FAD2*, *FAD3*, *FAD4*, *FAD5*, *FAD7*, and *DGD1* genes. Since exogenous application of glycerol converts wild-type plants into *ssi2* mimics, we also studied the effect of exogenous application of glycerol on mutants impaired in resistance-gene signaling, SA, or *fad* pathways. Glycerol increased SA levels and induced pathogenesis-related gene expression in all but *sid2*, *nahG*, *fad7*, and *fad7 fad8* plants. Furthermore, glycerol-induced phenotypes in various mutant lines correlate with a concomitant reduction in 18:1 levels. Inability to convert glycerol into G3P due to a mutation in the *nho1*-encoded glycerol kinase renders plants tolerant to glycerol and unable to induce the SA-dependent pathway. A reduction in the NHO1-derived G3P pool also results in a partial age-dependent rescue of the *ssi2* morphological and cell death phenotypes in the *ssi2 nho1* plants. The glycerol-mediated induction of defense was not associated with any major changes in the lipid profile and/or levels of phosphatidic acid. Taken together, our results suggest that glycerol application and the *ssi2* mutation in various mutant backgrounds produce similar effects and that restoration of *ssi2* phenotypes is not associated with the further desaturation of 18:1 to linoleic or linolenic acids in plastidal or extraplastidal lipids.

Plants have evolved various defense mechanisms to resist infection by pathogens. Upon recognition, the host plant initiates one or more signal transduction pathways that activate various plant defenses and thereby avert pathogen colonization. In many cases, resistance is associated with increased expression of defense genes, including the pathogenesis-related (PR) genes and the accumulation of salicylic acid (SA) in the inoculated leaf. The SA signal transduction pathway

plays a pivotal role in plant defense signaling (for review, see Durrant and Dong, 2004). When SA accumulation is suppressed in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* by expression of the *nahG* transgene, which encodes the SA-degrading enzyme SA hydroxylase, susceptibility to both compatible and incompatible pathogens is enhanced and *PR* gene expression is suppressed (Gaffney et al., 1993; Delaney et al., 1994). Similarly, *Arabidopsis* mutants that are impaired in SA responsiveness, such as *npr1* (Cao et al., 1997; Ryals et al., 1997; Shah et al., 1997), or pathogen-induced SA accumulation, such as *eds1* (Falk et al., 1999), *eds5* (Nawrath et al., 2002), *sid2* (Wildermuth et al., 2001), and *pad4* (Jirage et al., 1999), exhibit enhanced susceptibility to pathogen infection and impaired *PR* gene expression.

In addition to the major phytohormone-mediated defense pathways, fatty acid (FA)-derived signaling has also started to emerge as one of the important defense pathways (Vijayan et al., 1998; Kachroo et al., 2001, 2003b, 2004; Weber, 2002; Li et al., 2003; Yaeno et al., 2004). Desaturation of stearic acid (18:0)-acyl carrier protein (ACP) to oleic acid (18:1)-ACP catalyzed by the *SSI2/FAB2*-encoded stearoyl-ACP desaturase (S-ACP-DES) is one of the key steps in the FA biosynthesis pathway that regulates levels of

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unsaturated FAs in the cell (see also Fig. 8). A mutation in *ssi2* confers stunted phenotype, constitutive *PR* gene expression, spontaneous lesion formation, and enhanced resistance to both bacterial and oomycete pathogens (Kachroo et al., 2001; Shah et al., 2001). By contrast, the *ssi2* plants are unable to induce jasmonic acid (JA)-responsive gene *PDF1.2* and show enhanced susceptibility to necrotrophic pathogen *Botrytis cinerea* (Kachroo et al., 2001, 2003b). The activity of the mutant S-ACP-DES enzyme was reduced 10-fold, resulting in elevation of 18:0 content in *ssi2* plants (Kachroo et al., 2001). However, an increase in 18:0 does not contribute to altered defense signaling because several *ssi2* suppressors show wild type-like signaling and yet accumulate high levels of 18:0 (Kachroo et al., 2003a).

A mutation in *ssi2* also results in reduction in 18:1 content. The altered morphology and defense phenotypes in the *ssi2* plants are restored by a loss-of-function mutation in the *ACT1*-encoded glycerol-3-P (G3P) acyltransferase, or in the *GLY1*-encoded G3P dehydrogenase (G3Pdh), both of which elevate 18:1 levels in the *ssi2* plants (Kachroo et al., 2003b, 2004). A mutation in *gly1* and *act1* results in reduced carbon flux through the prokaryotic pathway, which leads to a reduction in the hexadecatrienoic (16:3) acid levels (Kunst et al., 1988; Miquel et al., 1998). However, the *gly1* and *act1* plants continue to show normal growth characteristics, suggesting that increased flux through the eukaryotic pathway compensates for their defect. Because both 18:1 and G3P are required for the acyltransferase-catalyzed reaction, a reduction in either is likely to reduce the carbon flux through *ACT1*.

The levels of G3P and of 18:1 can also be modulated by exogenous application of glycerol. The glycerol treatment leads to an increase in the endogenous G3P levels, which results in quenching of 18:1. Since the *ACT1*-catalyzed step is rate limiting, the quenching of 18:1 is more drastic in glycerol-treated *ACT1*-overexpressing lines (Kachroo et al., 2004). A reduction in the 18:1 in wild-type plants confers phenotypes similar to that of the *ssi2* mutant.

In this study, we have attempted to establish a link between glycerol metabolism and *ssi2*-mediated defense signaling. We show that the glycerol-mediated decline in 18:1 levels occurs in mutants that are impaired in the SA signaling pathway or affected in various FAD steps. The defense phenotypes associated with glycerol application are dependent on the ability of plants to utilize glycerol or acylate the glycerol-derived G3P with 18:1. Consistent with this result, a mutation in *nho1* (*gli1*) renders plants tolerant to glycerol and unable to induce *PR-1* gene expression in response to glycerol. We also show that *ssi2*-triggered phenotypes were not influenced by mutations that impair the SA pathway or that alter the levels of 16:3 or trienoic acids (16:3 and linolenic [18:3]). Since mutations in various FADs did not affect any of the *ssi2* *act1* phenotypes, we conclude that complementation of the *ssi2* mutation in the *act1* background is not associated with the further conversion of 18:1 to

linoleic acid (18:2) or 18:3 in plastidal or extraplastidal lipids.

RESULTS

Glycerol-Induced SA Levels Are Dependent on *SID2*

Previously, we showed that exogenous application of glycerol on wild-type plants lowers 18:1 levels and results in the induction of *PR-1* gene expression (Kachroo et al., 2004). To determine the molecular components participating in this glycerol-mediated effect on the SA pathway, we tested the response of *sid2*, *npr1*, *eds1*, *pad4*, *ndr1*, and *eds5* mutants and *nahG* transgenic plants to exogenous application of glycerol (Table I). The glycerol- and water-sprayed plants were evaluated for cell death, SA/SA glucoside (SAG) levels, *PR* expression, pathogen resistance, and 18:1 levels. All genotypes showed cell death on their leaves, suggesting that glycerol-induced cell death was independent of mutations analyzed or the *nahG* transgene (Fig. 1A). Next, we determined the levels of SA and SAG in water- and glycerol-treated plants. Both Nössen (Nö) and Columbia-0 (Col-0) plants showed a 10- and 13-fold induction in SA levels and an 8- and 10-fold induction in SAG levels, respectively (Fig. 1B). The SA/SAG levels in glycerol-treated *eds1*, *eds5*, and *pad4* plants were higher compared to the water-treated plants, but significantly lower compared to the glycerol-treated wild-type plants. The *sid2* plants showed near-basal levels of SA/SAG, whereas *ndr1* and *npr1* plants accumulated higher than wild-type levels. These results indicate that SA levels generated upon glycerol treatment are partially dependent on *EDS1*, *EDS5*, and *PAD4*, and completely dependent on *SID2*. These results also suggest that *NDR1* and *NPR1* negatively regulate the glycerol-triggered increase in SA/SAG levels.

To determine whether a correlation exists between glycerol-induced SA levels and *PR* gene expression, we evaluated the expression of *PR-1* and *PR-2* genes in various genotypes treated with water or glycerol (Fig. 1C). Although basal or low levels of *PR-1* and *PR-2*, respectively, were seen in glycerol-treated *sid2* plants, all the other mutant lines showed induction of these transcripts upon glycerol treatment. Interestingly, the glycerol-treated *ndr1*, *eds5*, and *npr1* plants induced higher levels of the *PR-2* gene as compared to the glycerol-treated wild-type plants. Taken together, these data suggest that glycerol-induced *PR* gene expression is dependent on the presence of a certain threshold level of SA/SAG, and these levels are derived via a *SID2*-dependent pathway.

We further evaluated the effect of mutations impairing the SA pathway on glycerol-induced resistance to *Peronospora parasitica* biotype Emco5 (Fig. 1D). Under the conditions tested, the wild-type Col-0 plants showed developmental resistance to Emco5, revealing less than 20% susceptibility as compared to the mock-inoculated plants. By comparison, both Wassilewskija

Table 1. Mutant and transgenic lines used in this study and functional properties of the proteins encoded by their wild-type alleles

Mutant	Functional Properties	References
<i>fad2</i>	Endoplasmic reticulum-localized oleoyl-phosphatidylcholine desaturase, desaturates 18:1 to 18:2	Miquel and Browse (1992)
<i>fad3</i>	Endoplasmic reticulum-localized linoleoyl-phosphatidylcholine desaturase, desaturates 18:2 to 18:3	Browse et al. (1993)
<i>fad4</i>	Desaturates palmitate at sn-2 position of PG in plastids	Browse et al. (1985)
<i>fad5</i>	Desaturates palmitate at sn-2 position of MGDG in plastids	Kunst et al. (1989)
<i>fad6</i>	Plastid-localized oleoyl desaturase	Falcone et al. (1994)
<i>fad7</i>	Plastid-localized linoleate desaturase	Iba et al. (1993)
<i>fad8</i>	Plastid-localized linoleate desaturase	Gibson et al. (1994)
<i>nho1 (gli1)</i>	Encodes glycerolkinase, converts glycerol to G3P	Eastmond (2004); Kang et al. (2003)
<i>gly1</i>	Encodes G3Pdh, converts DHAP to G3P	Kachroo et al. (2004); Miquel (2003)
<i>act1</i>	Encodes G3P acyltransferase, plastid localized, acylates 18:1 on G3P backbone	Kunst et al. (1988)
<i>dgd1</i>	Plastid-localized galactosyltransferase; involved in synthesis of DGDG from MGDG	Dormann et al. (1995)
<i>eds1</i>	Encodes lipase-like protein, involved in <i>R</i> gene- and SA-mediated signaling	Falk et al. (1999)
<i>pad4</i>	Encodes lipase-like protein, involved in SA-mediated signaling	Jirage et al. (1999)
<i>eds5</i>	Encodes membrane-bound multidrug and toxin-extrusion protein transporter-like protein, involved in SA-mediated signaling	Nawrath et al. (2002)
<i>ndr1</i>	Encodes plasma membrane-localized protein required for nonrace-specific disease resistance, involved in <i>R</i> gene-mediated signaling	Century et al. (1997)
<i>sid2</i>	Encodes isochorismate synthase involved in biosynthesis of SA	Wildermuth et al. (2001)
<i>nahG</i>	Bacterial salicylate hydroxylase that converts SA to catabol	Yamamotoj et al. (1965)
<i>npr1</i>	Encodes a protein containing an ankyrin repeat domain, a key positive regulator of SA signaling and systemic acquired resistance	Cao et al. (1997); Ryals et al. (1997)

(Ws) and Nö showed marked susceptibility (Fig. 1D; Kachroo et al., 2004). The glycerol-treated Col-0 plants were as tolerant as the resistant ecotype *Landsberg erecta* (*Ler*) and glycerol treatment of Ws and Nö showed significant reduction in pathogen growth and sporulation (Fig. 1D; Kachroo et al., 2004). The water-treated *eds1* (derived from Ws ecotype), *eds5*, *pad4*, *sid2*, *ndr1*, *npr1*, and *nahG* plants showed marked susceptibility to Emco5. Glycerol treatment had little or no effect on the *nahG* plants. A partial enhancement in resistance was observed in glycerol-treated *pad4*, *eds5*, *ndr1*, and *sid2* plants. A more pronounced effect was observed in glycerol-treated *npr1* plants, which showed an approximately 60% reduction in the number of susceptible plants. The glycerol-treated *eds1* plants showed only a marginal, approximately 10% reduction in the number of susceptible plants. Taken together, these data suggest that glycerol-mediated resistance to Emco5 requires the functions of *EDS1*, *PAD4*, *EDS5*, *SID2*, and *NDR1*. Similar to Col-0 plants, only approximately 10% of water-treated *act1* plants displayed susceptibility. Consistent with the glycerol-insensitive phenotype of *act1* plants, glycerol treatment of *act1* did not enhance resistance to Emco5 (Fig. 1D).

To establish a correlation between the glycerol-induced phenotypes and 18:1 levels, we next determined the leaf 18:1 content 3 d after glycerol application. Both wild-type and plants impaired in the SA signaling pathway showed a drastic reduction in their 18:1 levels after glycerol application (Fig. 1E), and these were comparable to 18:1 levels in *ssi2* plants. By comparison, *act1* plants, which are unable to

acylate 18:1, did not show a decrease in their 18:1 levels.

SA Signaling Mutants Affect the SA Levels But Do Not Restore Altered Defense Signaling in *ssi2* Plants

Previously, we suggested that glycerol application on wild-type plants converts these into *ssi2* mimics by lowering their 18:1 levels (Kachroo et al., 2004). To determine whether the glycerol effect seen on single mutants defective in SA or resistance (*R*) gene signaling pathways was comparable to the presence of the *ssi2* mutation in these backgrounds, we constructed *ssi2* double-mutant plants in the *eds1*, *pad4*, *eds5*, *ndr1*, and *sid2* backgrounds. All the double-mutant plants showed hypersensitive response-like lesions on their leaves and, except *ssi2 ndr1*, all other double-mutant and *ssi2 nahG* plants were slightly bigger than the *ssi2* plants (Fig. 2, A and B). In comparison to *ssi2*, the *ssi2 eds1*, *ssi2 pad4*, *ssi2 eds5*, and *ssi2 ndr1* plants accumulated lower levels, whereas the *ssi2 sid2* plants showed basal levels, of SA and SAG (Fig. 2C). Thus *EDS1*, *PAD4*, *EDS5*, *NDR1*, and *SID2* appear to contribute to the SA levels in *ssi2* plants and the SA/SAG in *ssi2* plants is possibly derived via a *SID2*-dependent pathway. This possibility was further supported by the observation that exogenous application of glycerol was able to up-regulate the SA/SAG levels in *ssi2 gly1-3* plants, but not in *ssi2 sid2* and *ssi2 act1* plants (Fig. 2C). Consistent with the reduced levels of SA, *PR-1* gene expression decreased to basal levels in *ssi2 sid2* and *ssi2 nahG* plants (Fig. 2D). A moderate reduction in *PR-1* levels was also observed in *ssi2 npr1* and *ssi2*

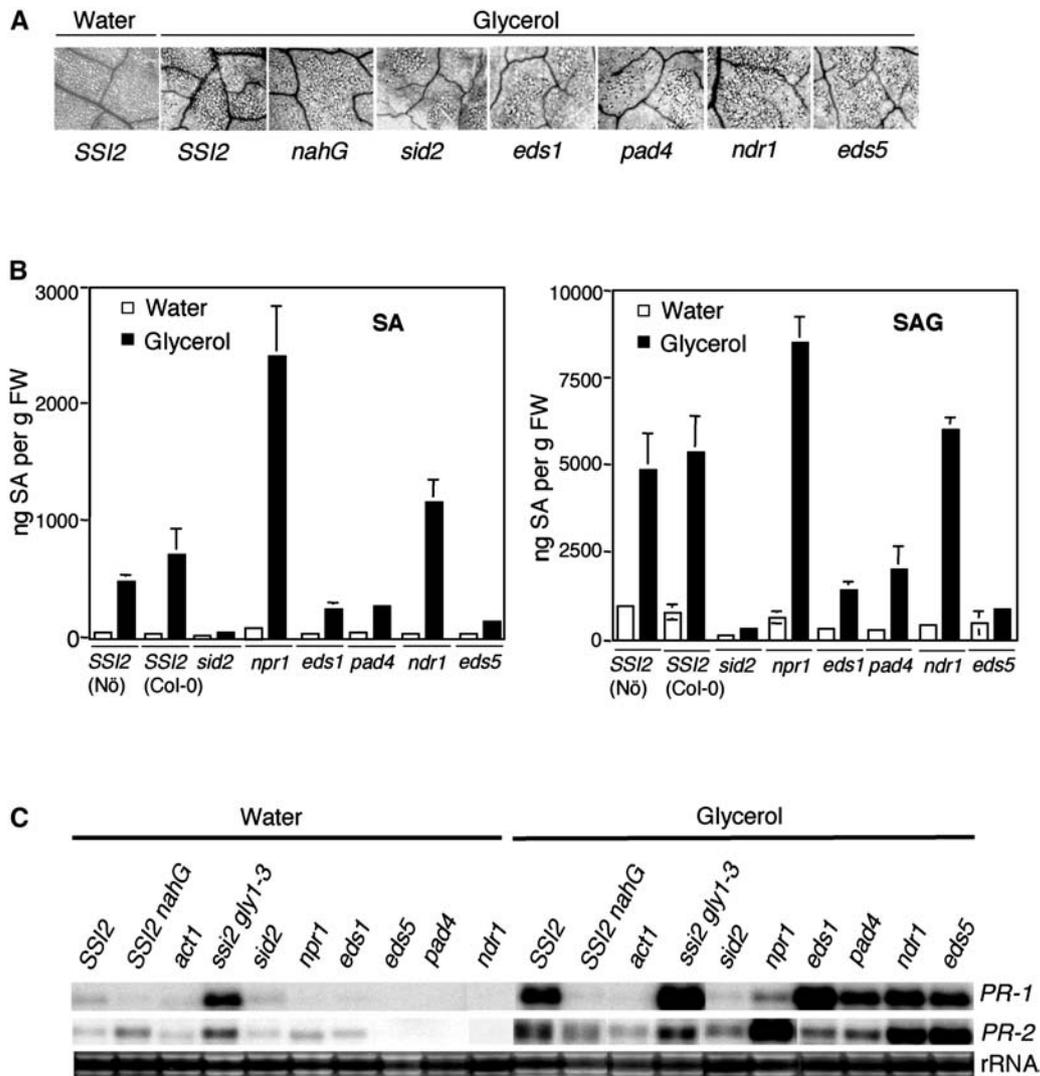
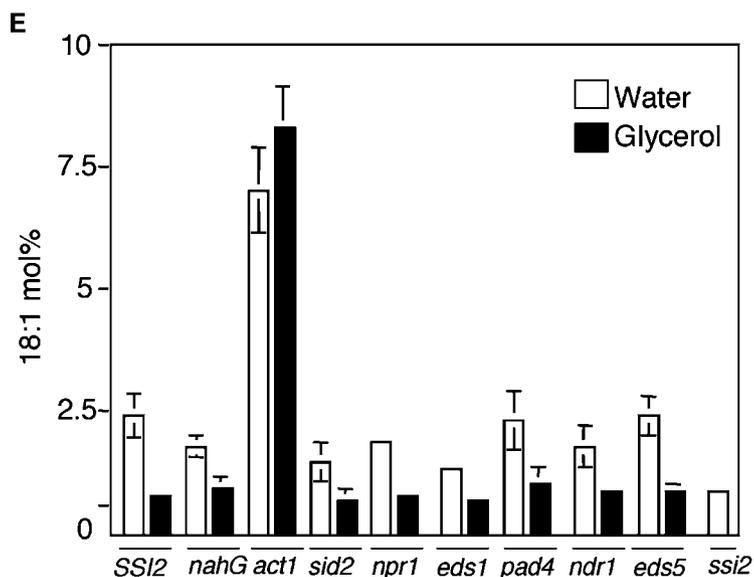
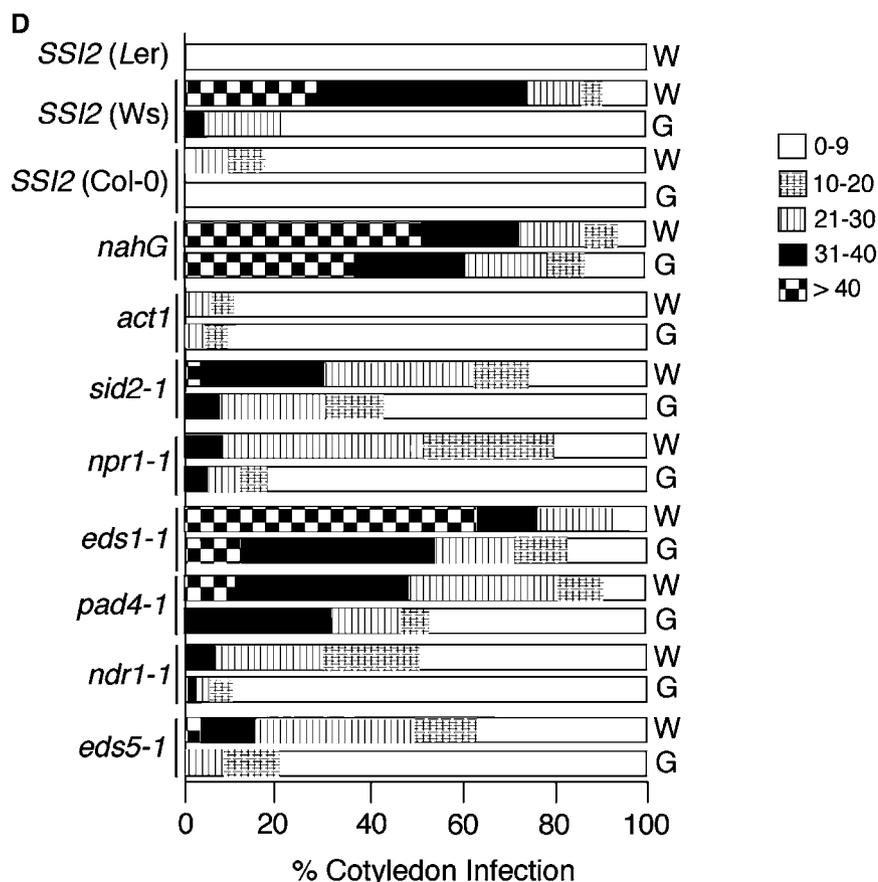


Figure 1. Glycerol-mediated effects on mutants impaired in SA or R gene signaling. A, Microscopy of trypan blue-stained leaves from indicated genotypes treated with water or 50 mM glycerol. *SSI2* indicates Col-0 ecotype. B, Endogenous SA and SAG levels in the leaves of indicated 4-week-old soil-grown plants treated with water or glycerol. The values are presented as the mean of three replicates. Error bars represent SD. C, Expression of the *PR-1* and *PR-2* genes in indicated genotypes. RNA gel-blot analysis was performed on 7 μ g of total RNA extracted from 4-week-old soil-grown plants treated with water or glycerol. *SSI2* indicates Col-0 ecotype. Ethidium bromide staining of rRNA was used as a loading control. D, Growth of *P. parasitica* biotype Emco5 on various plant genotypes listed at the left. The Ler and Ws ecotypes were used as the resistant and susceptible controls, respectively. The plants were treated with water (W) or glycerol (G) for 72 h prior to pathogen inoculation and approximately 60 to 75 cotyledons were scored for infection. The shade of each box indicates the severity of infection, based on the number of sporangiophores per cotyledon (see key at the right). Except *eds1-1* (Ws background) and *nahG* (Nö background), all other mutant lines were in Col-0 background. E, Glycerol-induced changes in the 18:1 levels in leaf tissue of 4-week-old plants. Plants were treated with glycerol or water, and samples taken 72 h post treatment were analyzed for FAs using gas chromatography (GC). *SSI2* indicates Col-0 ecotype. The values are presented as the mean of six to eight replicates. Error bars represent SD.

eds5 plants, whereas *ssi2 eds1*, *ssi2 pad4*, and *ssi2 ndr1* plants showed levels similar to those in *ssi2* plants. Interestingly, as seen in glycerol-treated single-mutant plants, the expression of *PR-2* was up-regulated by mutations in *npr1* and *eds5* and increased basal-level expression was also seen in *ssi2 sid2* and *ssi2 nahG* plants (Figs. 1C and 2D). These results indicate that high levels of *PR* expression in *ssi2* plants can be triggered by the presence of a certain threshold of

SA/SAG (Fig. 2D). To determine whether any correlation exists between SA levels/*PR* gene expression and pathogen resistance, we tested the response of *ssi2 pad4*, *ssi2 eds5*, *ssi2 sid2*, and *ssi2 nahG* plants to the virulent pathogen Emco5 (Fig. 2E). Strikingly, and similar to the resistance spectrum seen in glycerol-treated single mutants (Fig. 1D), a mutation in *pad4*, *eds5*, or *sid2* increased the susceptibility of *ssi2* plants to Emco5. Since *ssi2 eds1-2* plants were in the *RPP8* background

Figure 1. (Continued.)



(McDowell et al., 1998), which confers resistance to Emco5, we inoculated these and *ssi2 ndr1* plants with a virulent bacterial pathogen, *Pseudomonas syringae*. The *eds1-2* mutation compromised the enhanced bacterial resistance conferred by the *ssi2* mutation (Fig. 2F). By contrast, the *ssi2 ndr1* plants were as resistant as the *ssi2*

plants. These data suggest that *ssi2*-mediated resistance was dependent on *EDS1* and independent of *NDR1*. A higher degree of susceptibility seen in *ssi2 pad4* as compared to *ssi2 eds5* plants further suggests that *ssi2*-conferred resistance does not correlate with either the levels of SA or the expression of *PR-1*.

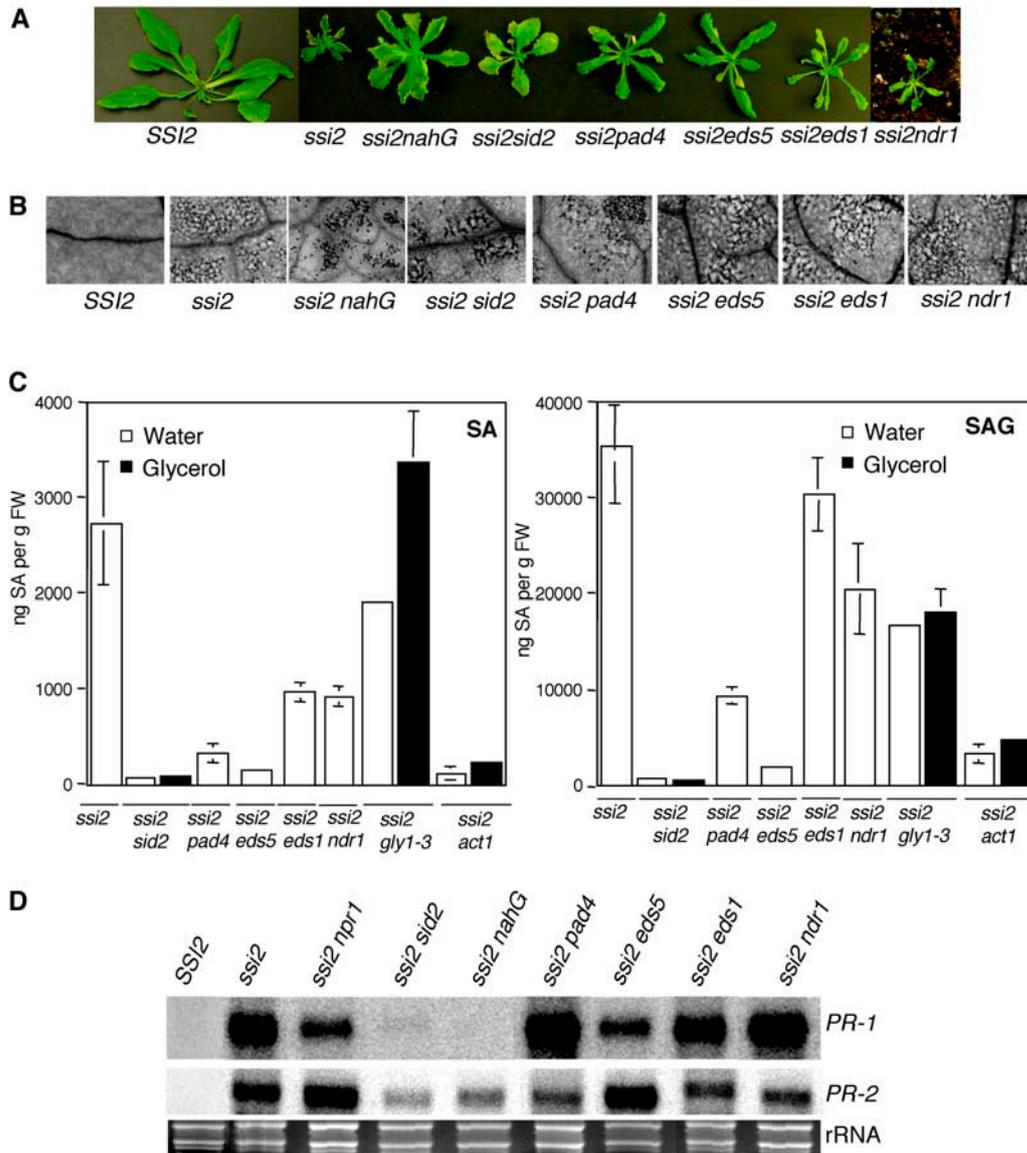


Figure 2. Morphological, molecular, and biochemical phenotypes of wild-type, *ssi2*, *ssi2 nahG*, *ssi2 sid2*, *ssi2 pad4*, *ssi2 eds1*, *ssi2 eds5*, and *ssi2 ndr1* plants. A, Comparison of the morphological phenotypes displayed by the wild-type (*SSI2*, Nö ecotype), *ssi2*, and various double-mutant plants in the *ssi2* background. B, Microscopy of trypan blue-stained leaves from wild-type (*SSI2*, Nö ecotype), *ssi2*, and various double-mutant plants in the *ssi2* background. C, Endogenous SA and SAG levels in the leaves of indicated 4-week-old soil-grown plants treated with water or glycerol. The values are presented as the mean of three replicates. Error bars represent sd. D, Expression of the *PR-1* and *PR-2* genes in indicated genotypes. RNA gel-blot analysis was performed on 7 μ g of total RNA extracted from 4-week-old soil-grown plants. *SSI2* indicates Nö ecotype. Ethidium bromide staining of rRNA was used as a loading control. E, Growth of *P. parasitica* biotype Emco5 on various plant genotypes listed at the left. The Ler and Nö ecotypes were used as the resistant and susceptible controls, respectively. The numbers against each box indicate cotyledons scored. The shade of each box indicates the severity of infection, based on the number of sporangiophores per cotyledon (see key at the right). F, Growth of *P. syringae* on *SSI2*, *ssi2*, *ssi2 eds1*, and *ssi2 ndr1*. Four leaf discs were harvested from infected leaves at 3 d postinoculation, ground in 10 mM $MgCl_2$, and the bacterial numbers tittered. The bacterial numbers \pm sd ($n = 4$) presented as colony forming units (CFU) per unit leaf area of 25 mm². The experiment was independently performed twice with similar results.

We then analyzed various *ssi2*-containing genotypes for their FA levels to determine whether a correlation exists between 18:1 levels and phenotypes exhibited by these plants (Table II). All double-mutant and *ssi2 nahG* plants showed increased levels of 18:0 and

a decrease in 18:1 content, which is consistent with the presence of the *ssi2* mutation in these plants. Taken together, these results suggest that a mutation in *eds1*, *pad4*, *eds5*, *ndr1*, or *sid2* does not restore 18:1 levels or normal signaling in *ssi2* plants.

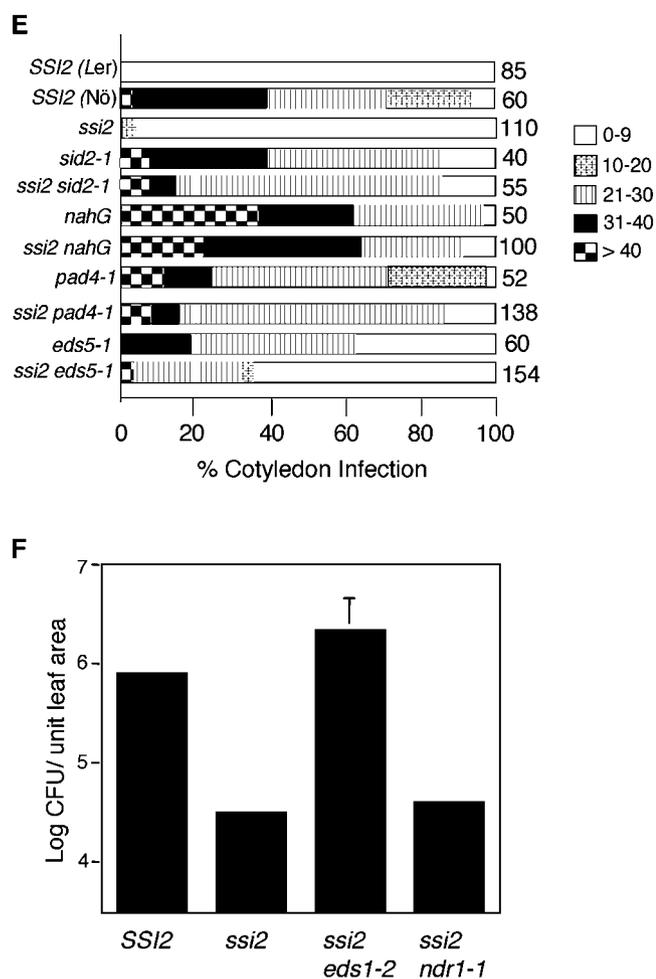


Figure 2. (Continued.)

Glycerol Application or Presence of the *ssi2* Mutation in *fad* Mutants Produce Similar Effects

To examine the role of the FA desaturation pathway in the induction of glycerol-mediated *ssi2*-like pheno-

types, we treated mutants affected in various steps of FAD with glycerol (Table I). The *fad2*, *fad3*, *fad4*, *fad5*, *fad6*, *fad7*, and *fad7 fad8* plants showed visible and microscopic cell death lesions on their leaves upon exposure to glycerol (Fig. 3A). The extent of lesion formation was maximal in *fad5* plants and minimal in *fad7* and *fad7 fad8* plants (Fig. 3A). The glycerol-induced morphological, microscopic, and *PR-1* phenotypes in *fad7* and *fad8* plants correlated with SA levels; upon glycerol treatment, *fad7* and *fad7 fad8* accumulated approximately 3-fold lower levels of SA as compared to wild-type plants (Fig. 3, A–C). The SA levels after glycerol treatment were highest in *fad5* plants followed by *fad3*, *fad2*, wild-type, *fad4*, and *fad6* plants. Although glycerol treatment induced higher expression of *PR-1* in *fad3* plants, their morphological phenotype was not as pronounced as that of *fad5* plants. FA profiling of water- and glycerol-treated *fad2*, *fad3*, *fad4*, *fad5*, and *fad6* plants showed a glycerol-triggered decline in 18:1 levels; however, this decline was not as drastic in *fad2* and *fad6* plants because they contain high levels of membranous 18:1 (Fig. 3D). As compared to *fad3*, *fad4*, and *fad5* plants, the glycerol-treated *fad7* and *fad7 fad8* plants showed a moderate reduction in 18:1 and these levels were higher than those in the *ssi2* plants. As expected, while the *ssi2 act1* plants did not show any drop in 18:1 levels, *ssi2 gly1-3* plants showed a significant decline. Taken together, these results imply a positive correlation between reduction in 18:1 levels and the appearance of glycerol-induced phenotypes in *fad* mutants.

To further test the hypothesis that the glycerol sensitivity of *fads* is related to *ssi2*-associated signaling, we generated *ssi2 fad3*, *ssi2 fad4*, *ssi2 fad5*, and *ssi2 fad7* double-mutant and *ssi2 fad7 fad8* triple-mutant plants. The morphological and biochemical characteristics of *ssi2 fad2* and *ssi2 fad6* plants have been described earlier (Kachroo et al., 2003b). The morphological phenotypes of *ssi2 fad3* and *ssi2 fad4* were similar to that of *ssi2* (Fig. 3E). Interestingly, *ssi2 fad5* plants

Table II. FA composition from leaf tissues of *SSI2*, *ssi2*, *eds1*, *ssi2 eds1*, *ndr1*, *ssi2 ndr1*, *eds5*, *ssi2 eds5*, *pad4*, *ssi2 pad4*, *sid2*, *ssi2 sid2*, *nahG*, and *ssi2 nahG* plants

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

Genotype	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	Morphology
<i>SSI2</i>	14.85 ± 1.41	4.10 ± 0.34	0.99 ± 0.33	16.30 ± 0.33	0.63 ± 0.08	2.31 ± 0.59	13.1 ± 1.55	47.72 ± 1.71	Wild type
<i>ssi2</i>	14.81 ± 2.10	2.86 ± 0.44	0.45 ± 0.18	9.0 ± 1.06	15.8 ± 2.82	0.6 ± 0.18	15.68 ± 1.72	40.8 ± 1.45	Stunted
<i>eds1</i>	13.73 ± 0.41	4.66 ± 0.12	0.55 ± 0.04	17.95 ± 0.84	0.70 ± 0.04	1.27 ± 0.13	10.41 ± 0.64	50.73 ± 0.96	Wild type-like
<i>ssi2 eds1</i>	14.77 ± 2.75	3.85 ± 0.54	0.45 ± 0.1	10.18 ± 1.29	16.9 ± 1.99	0.8 ± 0.15	13.8 ± 2.39	39.25 ± 3.53	<i>ssi2</i> -like
<i>ndr1</i>	14.6 ± 0.41	4.65 ± 0.12	1.01 ± 0.1	14.81 ± 0.67	0.54 ± 1.3	2.1 ± 0.48	13.87 ± 1.04	48.42 ± 0.98	Wild type-like
<i>ssi2 ndr1</i>	12.34 ± 0.33	2.0 ± 0.1	0.5 ± 0.1	9.84 ± 1.53	18.52 ± 1.3	0.70 ± 0.48	11.16 ± 1.04	45.30 ± 0.98	<i>ssi2</i> -like
<i>eds5</i>	14.05 ± 0.36	4.29 ± 0.37	1.17 ± 0.14	16.39 ± 0.74	0.60 ± 0.06	2.39 ± 0.39	14.72 ± 1.05	46.39 ± 0.79	Wild type-like
<i>ssi2 eds5</i>	16.68 ± 2.31	3.88 ± 0.96	0.28 ± 0.04	8.88 ± 1.86	14.84 ± 2.59	0.94 ± 0.20	12.66 ± 0.65	41.84 ± 5.00	<i>ssi2</i> -like
<i>pad4</i>	14.09 ± 0.88	4.09 ± 0.65	1.18 ± 0.22	15.91 ± 1.59	0.64 ± 0.11	2.27 ± 0.60	15.44 ± 2.29	46.38 ± 1.78	Wild type-like
<i>ssi2 pad4</i>	15.16 ± 0.48	3.05 ± 0.72	0.5 ± 0.1	8.62 ± 0.70	17.54 ± 2.36	0.88 ± 0.08	12.25 ± 0.73	42.00 ± 3.95	<i>ssi2</i> -like
<i>sid2</i>	13.00 ± 0.36	4.23 ± 0.35	0.84 ± 0.19	17.24 ± 0.92	0.63 ± 0.06	1.42 ± 0.39	12.91 ± 1.90	49.73 ± 1.62	Wild type-like
<i>ssi2 sid2</i>	16.68 ± 1.51	5.1 ± 0.75	0.18 ± 0.02	10.68 ± 2.08	17.58 ± 1.80	0.68 ± 0.21	8.68 ± 0.62	40.42 ± 1.19	<i>ssi2</i> -like
<i>nahG</i>	14.86 ± 0.90	4.18 ± 0.68	1.14 ± 0.51	16.06 ± 2.30	1.19 ± 0.50	1.75 ± 0.92	14.27 ± 3.29	46.55 ± 3.47	Wild type-like
<i>ssi2 nahG</i>	14.60 ± 2.53	3.32 ± 0.31	0.31 ± 0.08	10.0 ± 1.24	18.01 ± 1.07	0.9 ± 0.25	13.68 ± 0.91	39.18 ± 2.81	<i>ssi2</i> -like

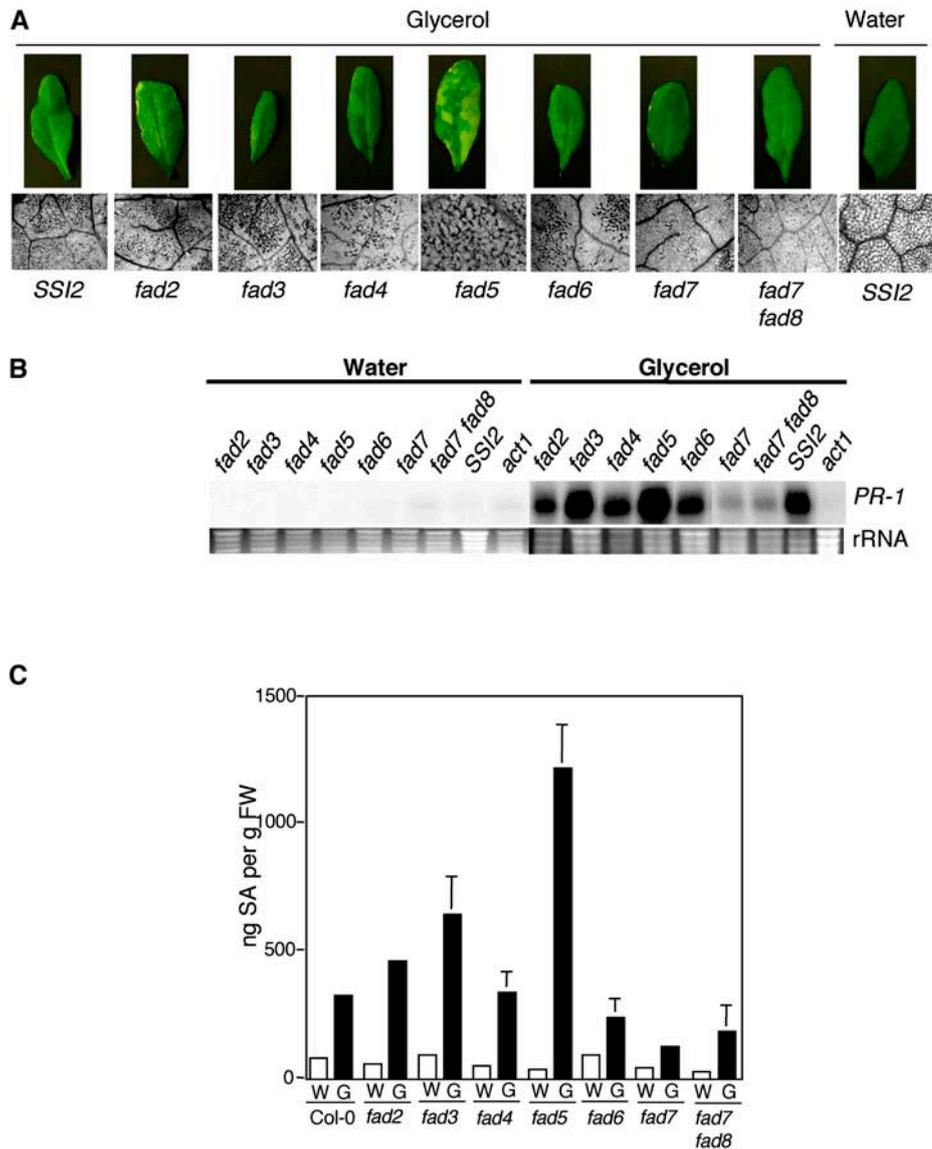


Figure 3. Glycerol-mediated effects on mutants impaired in various FAD steps and double-mutant analysis of *ssi2* in different *fad* backgrounds. **A**, Comparison of the morphological and cell death phenotypes displayed by the wild-type (*SSI2*, Col-0 ecotype), *fad2*, *fad3*, *fad4*, *fad5*, *fad6*, *fad7*, and *fad7 fad8* plants. The plants were treated with water or glycerol and photographed 3 d post treatment. **B**, Expression of the *PR-1* gene in water- and glycerol-treated *fads*, wild-type (*SSI2*, Col-0 ecotype), and *act1* plants. RNA gel-blot analysis was performed on 7 μ g of total RNA extracted from 4-week-old soil-grown plants. Ethidium bromide staining of rRNA was used as a loading control. **C**, Endogenous SA levels in the leaves of 4-week-old soil-grown wild-type (Col-0), *fad2*, *fad3*, *fad4*, *fad5*, *fad6*, *fad7*, and *fad7 fad8* plants treated with water (W) or glycerol (G). The values are presented as the mean of three replicates. Error bars represent sd. **D**, Glycerol-induced changes in the 18:1 levels in leaf tissue of 4-week-old plants. The *ssi2 gly1* and *ssi2 act1* plants were used as controls. Plants were treated with glycerol (G) or water (W), and samples taken 72 h post treatment were analyzed for FAs using GC. The values are presented as the mean of six to eight replicates. Error bars represent sd. **E**, Comparison of the morphological phenotypes displayed by the *ssi2* and various *ssi2 fad* double- and triple-mutant plants. **F**, Microscopy of trypan blue-stained leaves from *ssi2* and various *ssi2 fad* double- and triple-mutant plants. **G**, Expression of the *PR-1* and *PR-2* genes in wild-type (*SSI2*, Nö ecotype), *ssi2*, and various *ssi2 fad* double- and triple-mutant plants. RNA gel-blot analysis was performed on 7 μ g of total RNA extracted from 4-week-old soil-grown plants. Ethidium bromide staining of rRNA was used as a loading control. **H**, Endogenous SA and SAG levels in the leaves of 4-week-old soil-grown *SSI2* (Col-0), *ssi2*, *ssi2 fad7*, and *ssi2 fad7 fad8* plants. Values are presented as the mean of three replicates. Error bars represent sd. **I**, Growth of *P. syringae* on *SSI2*, *ssi2*, *fad5*, *ssi2 fad5*, *fad7*, *ssi2 fad7*, *fad7 fad8*, and *ssi2 fad7 fad8*. Four leaf discs were harvested from infected leaves at 3 d postinoculation, ground in 10 mM MgCl₂, and the bacterial numbers tittered. The bacterial numbers \pm sd ($n = 4$) presented as colony forming units (CFU) per unit leaf area of 25 mm². The experiment was independently performed twice with similar results. **J**, Expression of the *PDF1.2* gene in *SSI2*, *ssi2*, *ssi2 fad7*, and *ssi2 fad7 fad8* plants in response to 50 μ M JA. Samples were harvested 48 h post treatment and analyzed by RNA gel-blot analysis performed on 7 μ g of total RNA. Ethidium bromide staining of rRNA was used as a loading control.

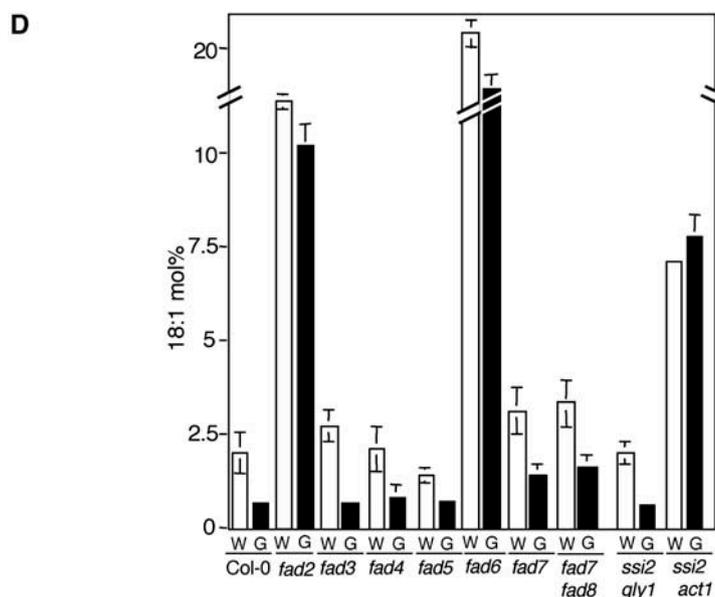
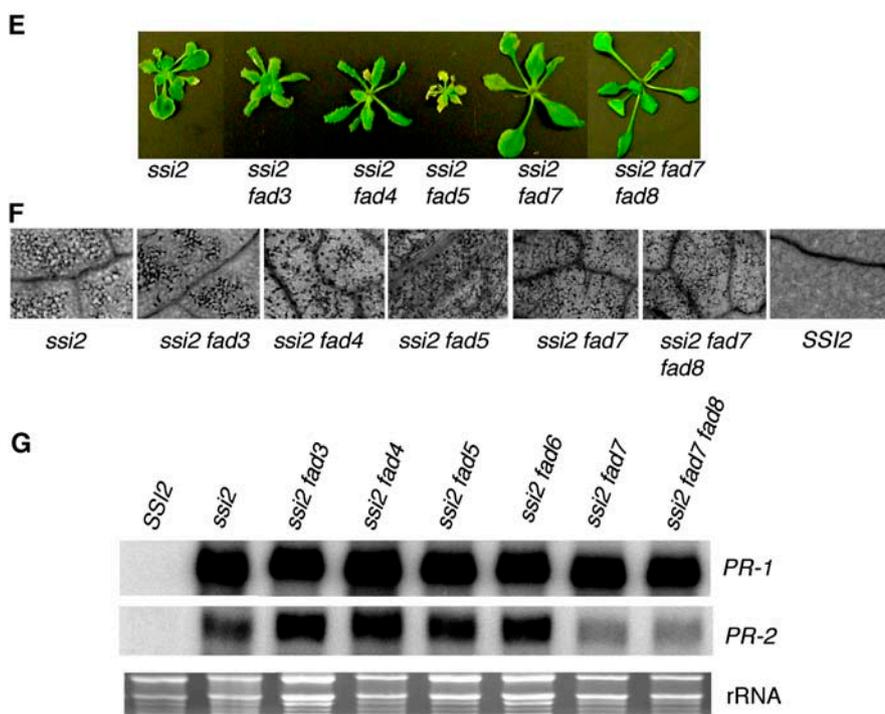


Figure 3. (Continued.)



showed a more stunted and chlorotic phenotype and *ssi2 fad7* plants were slightly less stunted than *ssi2* plants. The *ssi2 fad7* and *ssi2 fad7 fad8* plants displayed normal leaf morphology during initial stages of growth, but developed lesions on their leaves after 2 weeks of growth (Fig. 3F). The *ssi2 fad3*, *ssi2 fad4*, and *ssi2 fad5* plants all developed lesions and showed microscopic cell death. The morphological phenotype also correlated with expression of *PR-1* in these plants; all the *ssi2 fad* plants showed high-level expression of the *PR-1* gene (Fig. 3G). By contrast, the *ssi2*-triggered *PR-2* gene expression was drastically reduced in *ssi2*

fad7 and *ssi2 fad7 fad8* plants, but remained high in other *ssi2 fad* plants. Analysis of SA/SAG levels in *ssi2 fad7* and *ssi2 fad7 fad8* plants showed that these genotypes contained significantly lower levels of SA/SAG as compared to *ssi2* (Fig. 3H). However, the levels of SA, and particularly those of SAG in *ssi2 fad7* and *ssi2 fad7 fad8* plants, were higher than those in wild-type plants. This suggests that *fad7* and *fad8* mutations have a partial effect on the *ssi2*-triggered phenotypes. To determine whether any correlation exists between SA levels and pathogen resistance, we tested the response of *ssi2 fad7* and *ssi2 fad7 fad8* plants

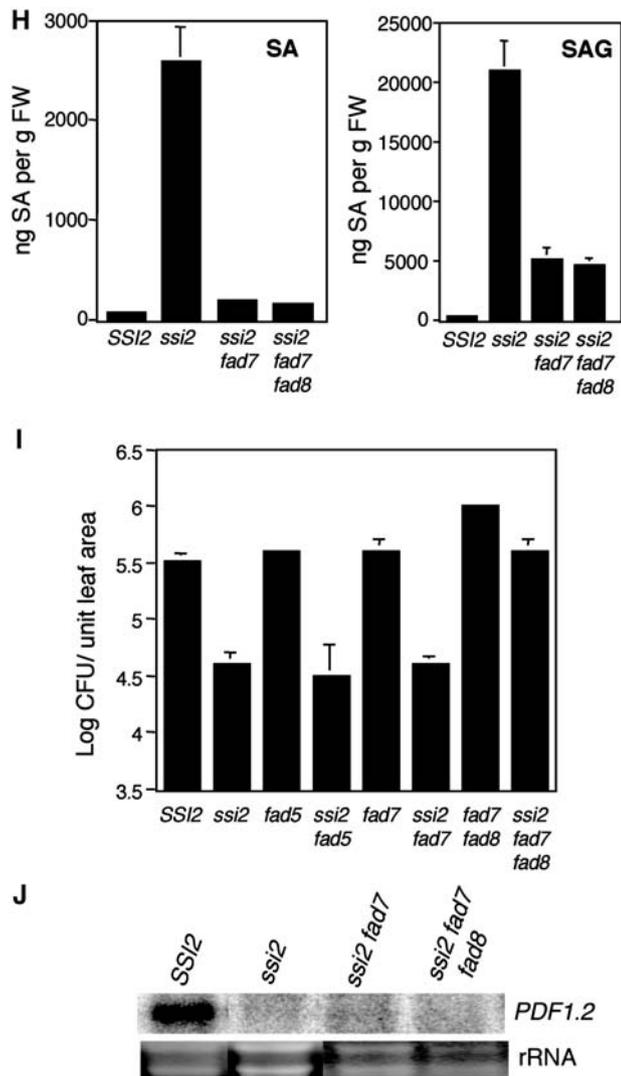


Figure 3. (Continued.)

to a virulent bacterial pathogen, *P. syringae*. Since *ssi2 fad5* plants showed more pronounced *ssi2*-like phenotypes, these served as an additional control for the experiment. The *fad7 fad8* mutations compromised the enhanced bacterial resistance conferred by the *ssi2* mutation, but the *fad5* and *fad7* mutations did not have any effect (Fig. 3I). A decrease in the SA/SAG levels in *ssi2 fad7* and *ssi2 fad7 fad8* plants did not alter their JA responsiveness and, like *ssi2*, these plants continued to show basal-level expression of *PDF1.2* upon exogenous application of JA (Fig. 3J). The *ssi2 fad3*, *ssi2 fad4*, and *ssi2 fad5* plants were also nonresponsive to JA (data not shown). FA profiling of *ssi2 fad3*, *ssi2 fad4*, and *ssi2 fad5* plants showed these contained 18:1 levels comparable to those seen in the *ssi2* plants (Table III). By comparison, the 18:1 levels in *ssi2 fad7* and *ssi2 fad7 fad8* plants were higher than those in *ssi2* and lower compared to wild-type plants. These results suggest that *ssi2* phenotypes are independent of *FAD3*, *FAD4*,

and *FAD5* genes and partially dependent on *FAD7* and *FAD7 FAD8* genes.

Phosphatidic Acid or Phosphatidic Acid-Derived Downstream Signaling Is Not Associated with the Glycerol- or *ssi2*-Triggered Phenotypes

The *act1* mutation restores all the *ssi2*-triggered defense phenotypes because it increases the 18:1 levels in these plants (Kachroo et al., 2003b). Since the *ACT1*-catalyzed reaction eventually leads to the biosynthesis of phosphatidic acid (PA), it is possible that the reduced levels of PA in *ssi2 act1* plants contribute to restoration of mutant phenotypes. This is plausible because PA levels are known to be induced during host-pathogen interactions (De Jong et al., 2004) and PA is likely to have a role in signaling (Zhang et al., 2003). To test the role of PA in *ssi2*-triggered defense phenotypes, we first generated a lipid profile of *ssi2* plants. The levels of PA in *ssi2* plants were similar to those in wild-type plants (Fig. 4A), suggesting that high levels of PA are not responsible for *ssi2* phenotypes. Since glycerol application to wild-type plants induces an *ssi2*-like phenotype, we measured PA levels in water- and glycerol-treated wild-type and *act1* plants. As expected, the PA levels in *act1* plants were approximately 2.8-fold lower than the wild-type plants, and these levels did not change significantly after glycerol application. By comparison, a marginal reduction in PA levels seen in glycerol-treated wild-type plants was not statistically significant. These results suggest that glycerol application has an impact on the *ACT1*-mediated acylation step without altering PA levels.

PA produced in the prokaryotic pathway is converted to diacylglycerol, which then serves as a precursor for the synthesis of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG; also see Fig. 8). To understand the role of these components in *ssi2*-triggered signaling, we generated *ssi2 dgd1* double-mutant plants, which are impaired in the synthesis of DGDG. The *ssi2 dgd1* double-mutant plants showed rosette leaf arrangement like that of *dgd1* plants, but much smaller, and showed visible and microscopic cell death lesions on their leaves (Fig. 4, B and C). The double-mutant plants contained low levels of 18:1 and accumulated high levels of *PR-1* (Table IV; Fig. 4D). These results were further corroborated by glycerol application to *dgd1* plants; as in wild-type plants, glycerol treatment of *dgd1* plants caused a decline in 18:1 levels and induced high-level expression of *PR-1* (Fig. 4E). Together, these results suggest that a mutation in *dgd1* is not sufficient to restore *ssi2* phenotypes. Furthermore, the *ssi2*-triggered phenotypes in *dgd1* plants were restored by the *act1* mutation, as judged by the lack of cell death lesions on *ssi2 act1 dgd1* leaves (data not shown). Other *ssi2*-like phenotypes could not be verified in the *ssi2 act1 dgd1* triple-mutant plants because they were severely

Table III. FA composition from leaf tissues of *SSI2*, *fad2*, *ssi2 fad2*, *fad3*, *ssi2 fad3*, *fad4*, *ssi2 fad4*, *fad5*, *ssi2 fad5*, *fad6*, *ssi2 fad6*, *fad7*, *ssi2 fad7*, *fad7 fad8*, and *ssi2 fad7 fad8* plants

All measurements were made on plants grown at 22°C and data are described as mol% ± SD calculated for a sample size of six. nd, Not detected.

Genotype	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	Morphology
<i>SSI2</i>	15.1 ± 0.47	4.5 ± 0.46	0.9 ± 0.36	16.02 ± 0.77	0.68 ± 0.13	2.9 ± 0.07	12.52 ± 2.36	47.38 ± 2.24	Wild type
<i>ssi2</i>	14.12 ± 1.10	3.01 ± 0.65	0.45 ± 0.10	9.4 ± 1.23	16.1 ± 2.22	0.7 ± 0.10	13.68 ± 2.12	42.54 ± 2.47	Stunted
<i>fad2</i>	13.7 ± 1.10	4.47 ± 0.42	1.58 ± 0.81	20.58 ± 0.72	0.42 ± 0.13	14.65 ± 2.47	4.18 ± 0.35	40.42 ± 1.43	Wild type-like
<i>ssi2 fad2</i>	17.08 ± 1.23	2.66 ± 0.49	0.38 ± 0.08	7.82 ± 1.52	10.83 ± 0.5	19.54 ± 2.02	3.84 ± 0.40	37.85 ± 1.82	<i>ssi2</i> -like
<i>fad3</i>	14.58 ± 0.53	4.22 ± 0.32	1.44 ± 0.27	16.4 ± 0.68	0.6 ± 0.12	3.42 ± 0.37	19.84 ± 1.73	39.5 ± 2.25	Wild type-like
<i>ssi2 fad3</i>	12.48 ± 1.80	3.66 ± 0.28	0.4 ± 0.1	10.82 ± 2.71	14.72 ± 2.1	1 ± 0.41	20.3 ± 4.89	36.62 ± 5.6	<i>ssi2</i> -like
<i>fad4</i>	17.88 ± 0.38	0.7 ± 0.07	1.18 ± 0.15	16.22 ± 0.31	0.6 ± 0	2.38 ± 0.35	13.00 ± 1.43	48.04 ± 1.59	Wild type-like
<i>ssi2 fad4</i>	21.02 ± 1.34	– nd	–	9.94 ± 0.39	9.82 ± 1.49	0.8 ± 0.1	10.78 ± 1.06	47.64 ± 2.01	<i>ssi2</i> -like
<i>fad5</i>	25.64 ± 0.63	4.04 ± 0.61	–	–	1.3 ± 0.1	2.5 ± 0.36	14.7 ± 1.94	52.18 ± 1.36	Wild type-like
<i>ssi2 fad5</i>	20.22 ± 1.05	1.88 ± 0.14	0.4 ± 0.1	–	21.66 ± 2.0	1.1 ± 0.22	12.44 ± 0.40	42.3 ± 1.67	<i>ssi2</i> -like
<i>fad6</i>	14.62 ± 0.38	17.25 ± 1.30	–	–	0.88 ± 0.1	22.72 ± 0.39	13.46 ± 0.97	31.07 ± 0.85	Wild type-like
<i>ssi2 fad6</i>	17.6 ± 0.62	8.3 ± 0.55	–	–	11.36 ± 0.05	11.58 ± 0.87	16.4 ± 0.92	34.76 ± 1.76	Larger than <i>ssi2</i>
<i>fad7</i>	13.42 ± 0.35	4.6 ± 0.45	10.9 ± 0.35	4.1 ± 0.64	0.84 ± 0.15	3.84 ± 0.21	31.2 ± 1.94	31.10 ± 1.50	Wild type-like
<i>ssi2 fad7</i>	19.22 ± 1.38	4.27 ± 0.55	11.2 ± 0.8	2.55 ± 0.5	15.47 ± 2.03	1.9 ± 0.42	24.85 ± 1.29	20.39 ± 1.40	Larger than <i>ssi2</i>
<i>fad7 fad8</i>	12.26 ± 0.31	5.36 ± 0.18	16.24 ± 0.78	–	0.52 ± 0.04	3.28 ± 0.63	53.92 ± 0.97	8.42 ± 1.22	Wild type-like
<i>ssi2 fad7 fad8</i>	14.93 ± 1.43	4.65 ± 0.50	14.81 ± 0.20	0.51 ± 0.5	14.93 ± 2.61	2.05 ± 0.25	35.9 ± 1.48	12.11 ± 1.74	Larger than <i>ssi2</i>

dwarfed (similar to *act1 dgd1* plants; Klaus et al., 2002) and did not produce any viable seeds.

Glycerol Application Lowers Total Lipid Content in Wild-Type Plants

To determine whether the glycerol-mediated induction of cell death and defense gene expression in wild-type plants is associated with an altered lipid profile, we measured the levels of individual lipids and the levels of total acyl carbons on each of these lipids. The lipid profiles were obtained for leaves of wild-type and *act1* plants treated with water or glycerol and compared with those of *ssi2* plants. Glycerol application resulted in a marginal decline in the levels of leaf MGDG and DGDG in wild-type plants and a marginal increase in the levels of phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). By comparison, *act1* plants did not show any significant alteration in the levels of MGDG, DGDG, PG, and PE (Fig. 5A). The levels of total acyl carbons and the total double bonds on each individual lipid did not alter significantly between glycerol- and water-treated samples (Supplemental Fig. 1). These results suggest that glycerol application does not significantly alter the levels of plastidal and/or extraplastidal lipids or the number of acyl carbon groups on the individual lipids.

Following up on the observation that the *ssi2* plants showed a significant reduction in total lipid content in

comparison to wild-type plants (Fig. 5B), we next determined total lipid content in glycerol- and water-treated wild-type plants. Interestingly, the wild-type plants showed a statistically significant decline in the total nanomoles of lipids per milligram dry weight (223 versus 164) upon glycerol application (Fig. 5B). Although the total lipid content of *act1* plants was significantly lower than wild-type plants, it was not further altered upon glycerol treatment. These results suggest that glycerol application possibly slows down the growth rate of the plants similar to that observed in the *ssi2* plants.

Inability to Metabolize Glycerol Abolishes the Glycerol-Induced Responses

To determine the specificity of the glycerol-mediated induction of defense responses, and to define other components of the glycerol-triggered defense pathway, we analyzed a mutant line, which is impaired in glycerol metabolism due to a mutation in the gene encoding glycerol kinase (GK; *NHO1/GLI1*; Kang et al., 2003; Eastmond, 2004). As seen in the *act1* plants, the *nho1* plants did not show any reduction in their 18:1 levels upon glycerol treatment (Fig. 6A). By comparison, wild-type, *ssi2 gly1-3*, and *ACT1*-overexpressing (*35S-ACT1*) plants showed a significant reduction in 18:1 (Fig. 6A). A glycerol-mediated decline in 18:1 levels also correlated with the amount of SA and SAG

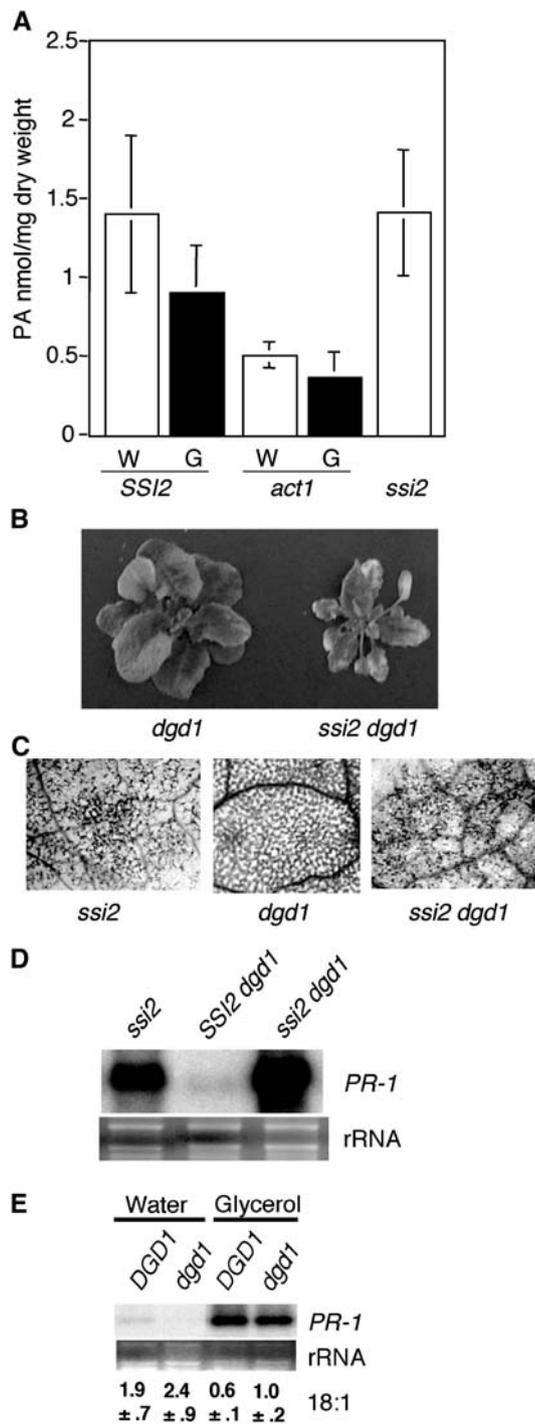


Figure 4. PA levels and morphological and molecular analyses of *ssi2 dgd1* plants. **A**, PA levels in *ssi2* and wild-type (Col-0) and *act1* plants treated with water (W) or glycerol (G). The values are presented as the mean of five replicates. Error bars represent sd. According to Student's *t* test, the difference in PA levels in water- and glycerol-treated samples was not significant ($P < 0.05$). **B**, Comparison of the morphological phenotypes displayed by the 4-week-old soil-grown *dgd1* and *ssi2 dgd1* plants. **C**, Microscopy of trypan blue-stained leaves from *ssi2*, *dgd1*, and *ssi2 dgd1* plants. **D**, Expression of the *PR-1* gene in *ssi2*, *dgd1*, and *ssi2 dgd1* plants. RNA gel-blot analysis was performed on 7 μ g of total RNA extracted from 4-week-old soil-grown plants. Ethidium bromide staining of rRNA was used as a loading control. **E**, Expression

accumulated in these plants and the levels of *PR-1* gene expression. Upon glycerol treatment, the highest levels of SA and *PR-1* gene expression were seen in 35S-*ACT1* plants, followed by wild-type plants (Fig. 6, B and C). The *nho1* plants accumulated near-basal levels of SA/SAG, similar to the *act1* and *ssi2 sid2* plants, and expressed basal levels of *PR-1* (Figs. 6, B and C, and 2C). These results further support the idea that the inability to metabolize glycerol confers tolerance to glycerol and abolishes glycerol-induced defense responses.

Because glycerol treatment has an impact on the *ACT1*-catalyzed step (Kachroo et al., 2004), it is very likely that the GK-generated G3P is channeled into the prokaryotic pathway of lipid biosynthesis. Our previous results show that a reduction in G3P levels leads to the accumulation of 18:1 and reversion of *ssi2* phenotypes in an age-dependent manner (Kachroo et al., 2004). To determine the relative contribution of GK toward the generation of G3P pools and its effect on *ssi2* phenotypes, we mobilized the *nho1* mutation in the *ssi2* background and analyzed double-mutant plants for various *ssi2*-like phenotypes. During the initial stages of growth, the *ssi2 nho1* double-mutant plants were slightly larger than *ssi2* plants and developed significantly fewer visible or microscopic cell death lesions on their leaves (Fig. 6, D and E). Although cell death lesions in *ssi2 nho1* plants did appear toward the later phase of growth (Fig. 6E), absence of cell death during the initial growth phase did not reduce or abolish constitutive expression of *PR-1* (Fig. 6F). Furthermore, the *PR-1* phenotype in *ssi2 nho1* plants correlated well with reduced levels of 18:1, similar to those seen in the *ssi2* plants (Table IV). These results suggest that conversion of exogenous glycerol to G3P is required for the glycerol-mediated induction of defense and that NHO1 may not be the major source of the plastidal G3P pool.

The *act1*-Mediated Rescue of *ssi2*-Triggered Phenotypes Does Not Involve Further Desaturation of 18:1

Previously, we reported that *ssi2 act1* plants show wild type-like morphological and molecular phenotypes (Kachroo et al., 2003b). It was also shown that high 18:1 levels in these plants restore their phenotypes. However, it could not be ruled out that the increased 18:1 was further converted to 18:2 and/or 18:3 in membrane lipids, and these were responsible for the phenotypic reversion of *ssi2* plants. Since SSI2 also acts on palmitic acid (16:0) substrate (Kachroo

of the *PR-1* gene and 18:1 levels in water- and glycerol-treated *DGD1* (Col-0 ecotype) and *dgd1* plants. RNA gel-blot analysis was performed on 7 μ g of total RNA extracted from 4-week-old soil-grown plants. Ethidium bromide staining of rRNA was used as a loading control. The 18:1 levels are described as mol% and the values shown are the average of six replicates (\pm sd).

Table IV. FA composition from leaf tissues of *SSI2*, *ssi2*, *nho1*, *ssi2 nho1*, *dgd1*, and *ssi2 dgd1* plants

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

Genotype	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	Morphology
<i>SSI2</i>	16.16 ± 0.8	4.24 ± 0.29	1.24 ± 0.08	16.44 ± 0.38	0.92 ± 0.32	2.58 ± 0.08	13.44 ± 0.77	44.98 ± 0.89	Wild type
<i>ssi2</i>	14.62 ± 2.13	3.12 ± 0.59	0.52 ± 0.18	10.55 ± 0.83	12.97 ± 2.71	0.95 ± 0.31	12.32 ± 2.23	44.95 ± 2.96	Stunted
<i>nho1</i>	15.58 ± 0.45	4.04 ± 0.23	1.06 ± 0.05	16.48 ± 0.25	0.76 ± 0.11	2.58 ± 0.38	13.06 ± 0.89	46.44 ± 1.40	Wild type-like
<i>ssi2 nho1</i>	17.1 ± 1.66	2.73 ± 0.6	0.18 ± 0.16	10.2 ± 1.1	13.8 ± 2.4	0.95 ± 0.1	9.78 ± 0.45	45.26 ± 2.59	<i>ssi2</i> -like
<i>dgd1</i>	18.37 ± 1.55	4.38 ± 0.6	0.48 ± 0.26	5.87 ± 2.37	1.82 ± 1.91	2.5 ± 0.3	12.5 ± 1.82	54.08 ± 3.59	Smaller than wt
<i>ssi2 dgd1</i>	18.83 ± 0.97	2.65 ± 0.25	0.25 ± 0.2	4.89 ± 1.24	12.66 ± 1.51	1.12 ± 0.21	11.26 ± 0.70	48.34 ± 2.59	<i>ssi2</i> -like

et al., 2001), it is also possible that 16:0-derived FAs may play a role in *ssi2 act1* plants. If palmitoleic acid (16:1), 18:2, or 18:3 were important, the *ssi2 act1* plants would require function of the FAD genes for their phenotypes. We therefore created triple-mutant plants in *fad2*, *fad3*, *fad4*, *fad5*, *fad6*, and *fad7* backgrounds and analyzed these for various *ssi2*-like phenotypes. The conversion of 18:1 to 18:2 in plastidal and extraplastidal lipids is catalyzed by *FAD6* and *FAD2*, respec-

tively. The conversion of 18:2 to 18:3 in plastidal and extraplastidal lipids is catalyzed by *FAD7*-, *FAD8*-, and *FAD3*-encoded desaturases, respectively (Ohlrogge and Browse, 1995). The *FAD4*- and *FAD5*-encoded plastidal desaturases catalyze the conversion of 16:0 to 16:1 on PG and MGDG, respectively (Ohlrogge and Browse, 1995). All of the triple-mutant plants showed *ssi2 act1*-like pale leaf morphology and absence of any visible or microscopic cell death (Fig. 7, A and B).

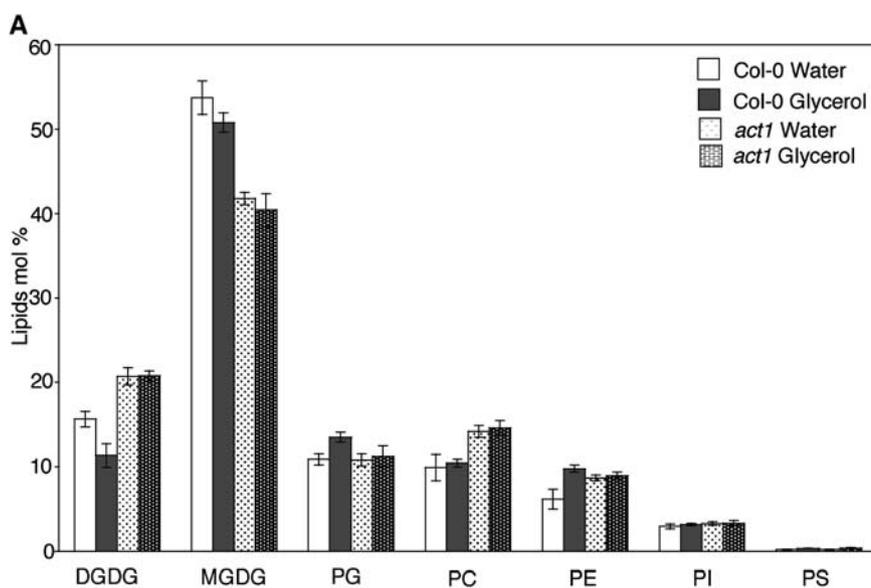
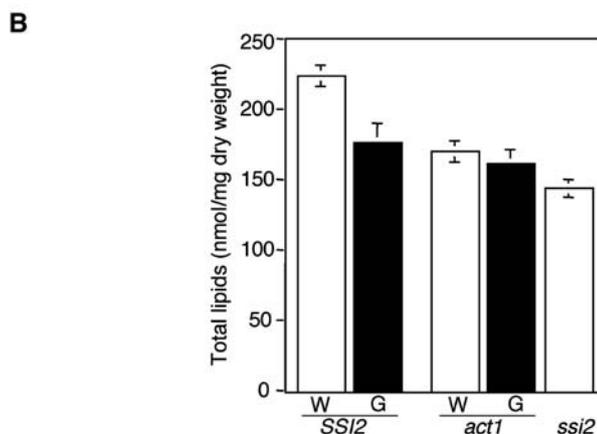


Figure 5. Lipid profile and total lipid content. A, Profile of total lipids extracted from Col-0 and *act1* plants treated with water or glycerol. The values are presented as the mean of five replicates. Error bars represent sd. PC, Phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine. B, Comparison of total lipid content in water- and glycerol-treated *SSI2* (Col-0) and *act1* plants with that of *ssi2*. The values are presented as the mean of five replicates. Error bars represent sd.



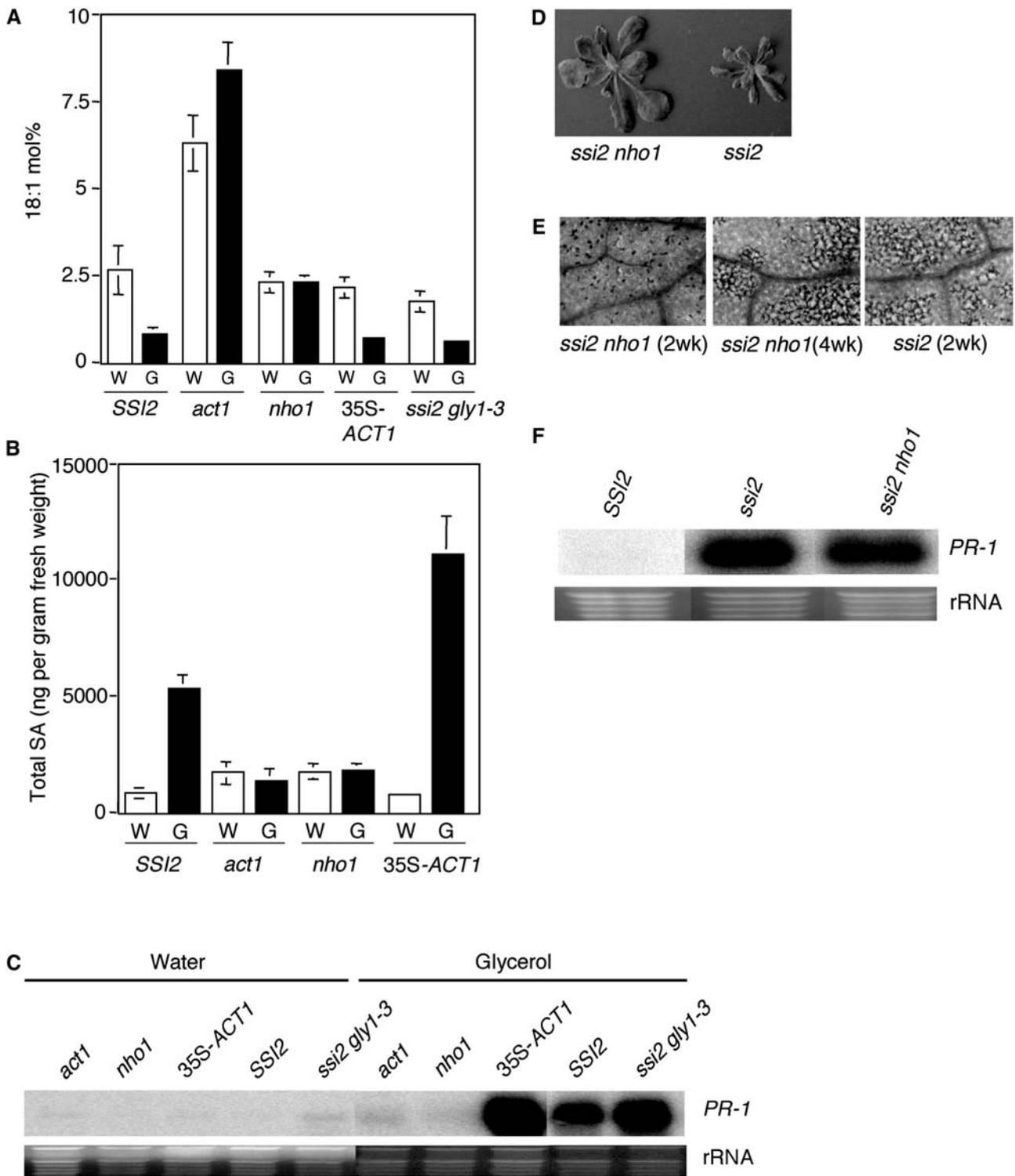


Figure 6. Comparison of glycerol-responsiveness in wild-type, *act1*, *nho1*, *ssi2 gly1-3*, and 35S-ACT1 plants and double-mutant analysis of *ssi2 nho1* plants. A, Glycerol-induced changes in the 18:1 levels in leaf tissue of 4-week-old plants. Plants were treated with glycerol (G) or water (W) and samples taken 72 h post treatment were analyzed for 18:1 content using GC. The values shown are an average of six independent replicates. Error bars represent sd. B, Endogenous SA levels in the leaves of 4-week-old soil-grown plants. SSI2 indicates Col-0 ecotype. The values presented are averages of three replicates. Error bars represent sd. C, Expression of the *PR-1* gene in water- and glycerol-treated plants. RNA gel-blot analysis was performed on 7 μ g of total RNA extracted 72 h after glycerol treatment. The *ssi2 gly1-3* plants used in this study were 3 weeks old. Ethidium bromide staining of rRNA was used as a loading control. D, Comparison of the morphological phenotypes displayed by the 16-d-old soil-grown *ssi2* and *ssi2 nho1* plants. E, Microscopy of trypan blue-stained leaves from *ssi2* and various *ssi2 nho1* plants. F, Expression of the *PR-1* gene in *ssi2* and *ssi2 nho1* plants. RNA gel-blot analysis was performed on 7 μ g of total RNA extracted from 16-d-old soil-grown plants. Ethidium bromide staining of rRNA was used as a loading control.

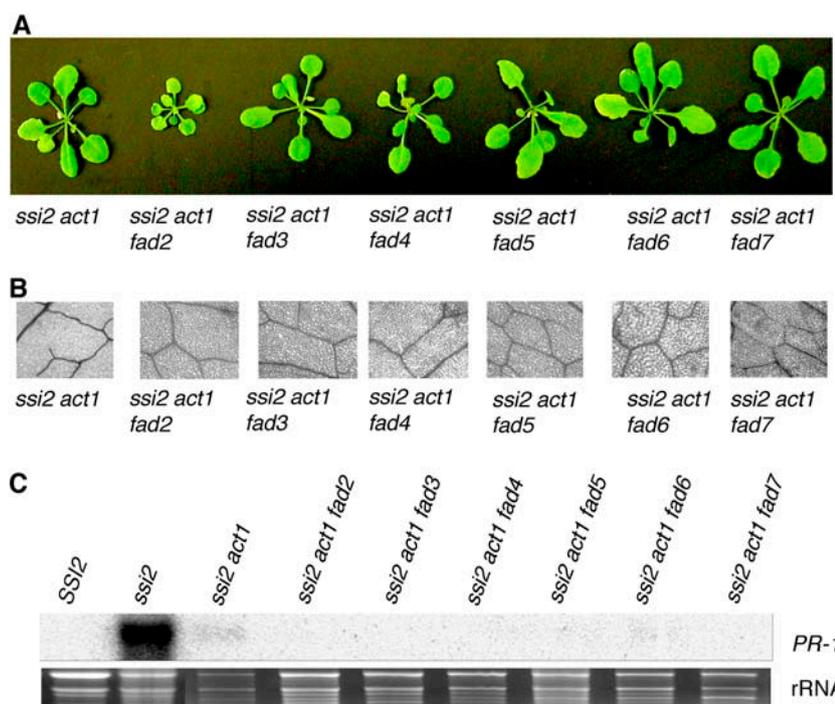


Figure 7. Morphological and molecular phenotypes of *ssi2 act1* and various *ssi2 act1 fad* triple-mutant plants. **A**, Comparison of the morphological phenotypes displayed by the 4-week-old soil-grown *ssi2 act1* and various *ssi2 act1 fad* triple-mutant plants. **B**, Microscopy of trypan blue-stained leaves from *ssi2 act1* and *ssi2 act1 fad* triple-mutant plants. **C**, Expression of the *PR-1* gene in wild-type, *ssi2*, *ssi2 act1*, and various *ssi2 act1 fad* triple-mutant plants. RNA gel-blot analysis was performed on 5 μ g of total RNA extracted from 4-week-old soil-grown plants. Ethidium bromide staining of rRNA was used as a loading control.

Except for *ssi2 act1 fad2* plants, which grew slowly, all the other triple-mutant plants had similar growth rates to *ssi2 act1* plants. Further, all triple-mutant plants showed basal-level expression of *PR-1* (Fig. 7C) and retained their JA responsiveness (data not shown). The FA profiling showed that *ssi2 act1* and all triple-mutant plants accumulated high levels of 18:1 in their leaves (Table V). The highest levels of 18:1 were found in the *ssi2 act1 fad2* plants, which could be because these plants are blocked in the utilization of 18:1 in both the prokaryotic and the eukaryotic pathways. Consistent with the presence of the *act1* mutation, both *ssi2 act1* and *ssi2 act1 fad* plants contained negligible amounts of 16:3. A reduction was also seen in 18:3 levels in *ssi2 act1 fad2*, *ssi2 act1 fad6*, and *ssi2 act1 fad7* plants. However, this did not have an impact on JA responsiveness in these plants, as both *fad2*, *fad6*, and *fad7* single-mutant plants and *ssi2 act1 fad2*, *ssi2 act1 fad6*, and *ssi2 act1 fad7* triple-mutant plants expressed high levels of *PDF1.2* in response to JA treatment (data not shown). Taken together, these results show that restoration of *ssi2* phenotypes by a mutation in *act1* is not affected by the inability of these plants to convert 18:1 to 18:2 or 18:3 in plastidic or extraplastidic lipids and 16:0 to 16:1 in plastidic lipids, and thus confirms that 18:1, not 18:2, 18:3, or 16:1, is responsible for this restoration.

DISCUSSION

Glycerol plays a major role in various metabolic processes, including its conversion to G3P, which serves as a building block for glycerolipid biosynthesis. In plants, G3P is synthesized via the GK-mediated phosphoryla-

tion of glycerol or via the G3Pdh-mediated reduction of dihydroxyacetone phosphate (DHAP; Kang et al., 2003; Eastmond, 2004; Kachroo et al., 2004). Both GK and G3Pdh have already been shown to participate in host-pathogen interactions (Kang et al., 2003; Kachroo et al., 2004; Nandi et al., 2004). Our previous observation that exogenous application of glycerol induces defense responses in plants further reinforces the importance of glycerol-mediated signaling. In this study, we attempted to delineate the components of the glycerol- and *ssi2*-mediated defense pathway and assess their impact on resistance to pathogens.

Exogenous glycerol application to wild-type plants results in the accumulation of SA and induction of *PR* genes, which suggests that the SA pathway is up-regulated in these plants. These findings are further supported by the observations that both the *sid2* mutation and the expression of the *nahG* transgene were able to abolish the glycerol-mediated increases in SA and *PR* expression (Fig. 1, B and C). However, both *sid2* and *nahG* plants showed glycerol-induced leaf cell death, thus disassociating SA accumulation from cell death. The defense phenotypes induced upon glycerol application to mutants impaired in *R* gene and SA signaling were similar to the phenotypes seen in *ssi2* double mutants containing the respective alteration in *R* gene or SA pathway. For example, *ssi2 sid2* and *ssi2 nahG* plants showed cell death and basal-level expression of the *PR-1* gene (Fig. 2, B and D). In addition, similar to glycerol-treated *npr1* and *eds5* plants, the *ssi2 npr1* and *ssi2 eds5* plants showed an increase in *PR-2* and a decrease in *PR-1* expression (Figs. 1C and 2D). Similarly, *ssi2 eds1*, *ssi2 pad4*, *ssi2 eds5*, *ssi2 ndr1*, *ssi2 sid2*, and *ssi2 nahG* plants as well as the glycerol-treated

Table V. FA composition from leaf tissues of *ssi2*, *act1*, *ssi2 act1*, *ssi2 act1 fad2*, *ssi2 act1 fad3*, *ssi2 act1 fad5*, *ssi2 act1 fad6*, and *ssi2 act1 fad7* plants

All measurements were made on plants grown at 22°C and data are described as mol% ± SD calculated for a sample size of six. nd, Not detected.

Genotype	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	Morphology
<i>ssi2</i>	14.81 ± 2.10	2.86 ± 0.44	0.45 ± 0.18	9.0 ± 1.06	15.8 ± 2.82	0.6 ± 0.18	15.68 ± 1.72	40.8 ± 1.45	Stunted
<i>act1</i>	12.5 ± 0.71	2.92 ± 0.32	0.2 ± 0.16	0.35 ± 0.12	1.15 ± 0.1	10.7 ± 1.71	14.58 ± 1.27	57.6 ± 2.72	Wild type-like
<i>ssi2 act1</i>	9.17 ± 1.30	1.62 ± 0.22	0.45 ± 0.12	– nd	9.5 ± 2.23	7.22 ± 2.2	16.52 ± 1.23	55.52 ± 3.12	Pale wild type-like
<i>fad2</i>	13.7 ± 1.10	4.47 ± 0.42	1.58 ± 0.81	20.58 ± 0.72	0.42 ± 0.13	14.65 ± 2.47	4.18 ± 0.35	40.42 ± 1.43	Wild type-like
<i>ssi2 fad2</i>	4.2 ± 0.23	1.2 ± 0.5	–	–	13.2 ± 1.36	40.9 ± 2.08	3.2 ± 0.55	37.3 ± 2.95	Pale wild type-like
<i>act1fad2</i>									
<i>fad3</i>	14.58 ± 0.53	4.22 ± 0.32	1.44 ± 0.27	16.4 ± 0.68	0.6 ± 0.12	3.42 ± 0.37	19.84 ± 1.73	39.5 ± 2.25	Wild type-like
<i>ssi2 fad3</i>	8.18 ± 0.82	2 ± 0.45	–	–	9.88 ± 1.20	3.32 ± 0.79	24.16 ± 3.6	52.46 ± 4.01	Pale wild type-like
<i>act1fad3</i>									
<i>fad4</i>	17.88 ± 0.38	0.7 ± 0.07	1.18 ± 0.15	16.22 ± 0.31	0.6 ± 0	2.38 ± 0.35	13.00 ± 1.43	48.04 ± 1.59	Wild type-like
<i>ssi2 fad4</i>	16.7 ± 0.36	–	–	–	6.46 ± 0.61	2.54 ± 0.1	22 ± 1.24	52.3 ± 1.23	Pale wild type-like
<i>act1fad4</i>									
<i>fad5</i>	25.64 ± 0.63	4.04 ± 0.61	–	–	1.3 ± 0.1	2.5 ± 0.36	14.7 ± 1.94	52.18 ± 1.36	Wild type-like
<i>ssi2 fad5</i>	8.16 ± 0.74	1.54 ± 0.25	–	–	13.24 ± 0.32	3.38 ± 1.06	17.76 ± 2.04	55.92 ± 1.97	Pale wild type-like
<i>act1fad5</i>									
<i>fad6</i>	14.62 ± 0.38	17.25 ± 1.30	–	–	0.88 ± 0.10	22.72 ± 0.39	13.46 ± 0.97	31.07 ± 0.85	Wild type-like
<i>ssi2 fad6</i>	13.4 ± 1.2	14.6 ± 0.7	–	–	11.5 ± 1.0	18.2 ± 1.2	13.7 ± 2.1	28.6 ± 2.2	Pale wild type-like
<i>act1fad6</i>									
<i>fad7</i>	13.42 ± 0.35	4.6 ± 0.45	10.9 ± 0.35	4.1 ± 0.64	0.84 ± 0.15	3.84 ± 0.21	31.2 ± 1.94	31.10 ± 1.50	Wild type-like
<i>ssi2 fad7</i>	11.67 ± 1.84	–	–	–	16.05 ± 0.44	7.02 ± 0.74	30.27 ± 5.4	34.99 ± 5.0	Pale wild type-like
<i>act1fad7</i>									

single mutants showed *ssi2*-like reduced levels of 18:1 (Fig. 1E; Table II). These observations argue that exogenous application of glycerol induces the same signaling pathways as the *ssi2* mutation.

A comprehensive analysis of the *ssi2* phenotypes in double-mutant backgrounds defective in SA or R gene signaling pathways showed that several of these mutations had an effect on the morphology of the plant. The *ssi2 eds1*, *ssi2 eds5*, *ssi2 pad4*, *ssi2 sid2*, and *ssi2 nahG* plants were larger than the *ssi2* plants. A larger morphology is likely due to a reduction in SA/SAG levels in these plants (Fig. 2, A and C). However, *ssi2 ndr1* plants also showed a reduction in SA/SAG levels, but these were morphologically similar to *ssi2* plants. This suggests that factors other than SA may also contribute to stunted morphology of *ssi2* plants. High levels of SA in *ssi2* plants induce expression of *EDS1* and *PAD4* genes and these levels are abolished by the *eds1* and *pad4* mutations (Chandra-Shekhara et al., 2004). Thus, a dependence of *ssi2*-triggered resistance on *EDS1* and *PAD4* is likely to be associated with increased expression of these in the *ssi2* background.

Besides the SA-signaling mutants, mutations in *fad7* and *fad7 fad8* were also able to significantly reduce SA/SAG levels in the *ssi2* plants (Fig. 3H). Although the *fad7* and *fad7 fad8* mutations down-regulated *ssi2*-triggered *PR-2* gene expression, these mutations were unable to completely restore SA- or JA-mediated defenses in *ssi2* plants (Fig. 3, G and J). One possible explanation for these observations would be that *fad7* and *fad7 fad8* mutations allow increased accumulation of 18:1 in *ssi2* plants, which partially restored the *ssi2*-triggered phenotypes. Since *fad7* and *fad8* mutations

block the step leading to the synthesis of 18:3 in plastidal membrane lipids, these mutations might cause a backup effect, resulting in the accumulation of an 18:1-ACP precursor. This idea is supported by the observation that 18:1 levels in *fad7* and *fad7 fad8* mutants were higher than in the wild-type plants. Similarly, the 18:1 levels in *ssi2 fad7* and *ssi2 fad7 fad8* plants were consistently higher compared to those of the *ssi2* plants (Table III). The *fad7* and *fad7 fad8* plants also showed a slower and less drastic decline in their 18:1 levels upon exogenous application of glycerol (Fig. 3D). Interestingly, the partial restoration of phenotypes displayed by *ssi2 fad7* and *ssi2 fad7 fad8* plants was similar to that seen in *ssi2 fad6* (Kachroo et al., 2003b). The *fad6* mutants have increased accumulation of 18:1 in membranous lipids; thus it is likely that this mutation causes a similar backup effect resulting in accumulation of free 18:1 or 18:1-ACP in the plastids. Another plausible explanation for glycerol tolerance of *fad7* and *fad7 fad8* plants is that levels of trienoic acids regulate SA levels and a reduction in these compromises the SA-mediated responses. This is supported by the observation that glycerol treatment or the presence of the *ssi2* mutation was unable to trigger accumulation of high levels of SA in the *fad7* or *fad7 fad8* plants (Fig. 3, C and H).

Epistatic analysis between *ssi2* and various *fad* mutations showed that, except *fad7 fad8*, these mutations are unable to restore altered defense signaling in the *ssi2* plants. The *fad7 fad8* mutation prevents the synthesis of trienoic FAs in the chloroplast resulting in compromised resistance to several avirulent *P. syringae* strains (Yaeno et al., 2004). Our study shows that *fad7*

slightly improves the morphological phenotype of *ssi2*, but does not alter the FA profile in leaf tissues or the *ssi2*-triggered defense phenotypes (Fig. 6; Table IV). This suggests that the NHO1-derived G3P may contribute only marginally to the plastidal G3P pool (Fig. 8, dashed line). However, these data do not correlate with the observation that exogenous application of glycerol can have an impact on the ACT1-mediated acylation step and lower 18:1 levels. A likely scenario explaining both results would be that exogenous application of glycerol generates an excess of G3P in the cytoplasm, which in turn increases the proportion of NHO1-derived G3P entering into plastids. An increase in plastidal G3P levels would in turn quench 18:1 levels and also lead to hypersensitivity toward glycerol in *ACT1*-overexpressing lines (Fig. 6). Since *NHO1* is vital for limiting the growth of nonhost isolates of *P. syringae* (Kang et al., 2003), it is likely that G3P levels and/or G3P-glycerol metabolism in *nho1* plants contribute to nonhost resistance, but do not affect defense responses to virulent pathogens. Presence of several isoforms of G3Pdh in the Arabidopsis genome (Wei et al., 2001; Shen et al., 2003) also suggests that there are several other enzymes contributing to G3P biosynthesis and that relative contributions of these may also influence defense responses.

In conclusion, we show that *ssi2*-triggered signaling requires components of both SA and FA pathways and is independent of the levels of 16:1, 16:3, 18:2, 18:3, and PA. A reduction in *ssi2*-triggered increase in SA levels by mutations in the *fad7 fad8* genes further establishes a link between SA and FA pathways. We also show that glycerol- and *ssi2*-mediated signaling pathways overlap and have presented a link between glycerol catabolism and SA signaling. However, it is not yet clear how a reduction in 18:1 levels in *ssi2* plants triggers the SA pathway and more work will be required to unravel the biochemical complexity of this novel signaling pathway.

MATERIALS AND METHODS

Plant Growth Conditions and Genetic Analysis

Plants were grown in the MTPS 144 Conviron walk-in chambers at 22°C, 65% relative humidity, and 14-h photoperiod. Mutations affecting the SA signal transduction pathway were transferred in the *ssi2* background by pollinating flowers of the *ssi2* plants with pollen from *npr1-5*, *eds1-2*, *pad4-1*, *eds5-1*, *ndr1-1*, or *sid2-1* plants. The *nahG* and *ssi2 nahG* transgenic plants used in this study have been described by Shah et al. (2001). Mutations affecting FAD or lipid biosynthesis were transferred in the *ssi2* background by pollinating flowers of the *ssi2* plants with pollen from *fad3-1*, *fad4-1*, *fad5-1*, *fad6-1*, *fad7-2*, and *fad7-1 fad8-1*. To mobilize various *fad* mutations in the *ssi2 act1* background, pollen from *fad2-1*, *fad3-1*, *fad4-1*, *fad5-1*, *fad6-1*, and *fad7-1* plants was used to pollinate the *ssi2 act1* plants. The *ssi2 dgd1* and *nho1* plants were obtained by pollinating flowers of the *ssi2* plant with pollen from *dgd1-1* and *nho1* plants, respectively. The *ssi2 dgd1 act1* plants were obtained by pollinating flowers of the *ssi2 dgd1* plant with pollen from *ssi2 act1* plants.

The double and triple mutants were identified by cleaved amplified polymorphic sequence (CAPS) or derived-CAPS analyses and by analyzing FA profiles. The genotypes at the *ssi2* and *act1* loci were determined (Kachroo et al., 2001, 2003b). To distinguish *FAD3* and *NHO1* wild-type and mutant

alleles, we sequenced the *fad3* and *nho1* genes and developed CAPS and derived-CAPS markers, respectively.

RNA Extraction and Northern Analyses

Small-scale extraction of RNA from one or two leaves was performed in the TRIzol reagent (Invitrogen) following the manufacturer's instructions. Northern-blot analysis and synthesis of random primed probes for *PR-1*, *PR-2*, and *PDF1.2* were carried out (Kachroo et al., 2001).

Trypan Blue Staining

Leaf samples were taken from 2- or 4-week-old plants grown on soil. Trypan blue staining was performed as described (Chandra-Shekara et al., 2006).

SA, FA, and Lipid Analyses

SA and SAG were extracted and measured from 0.2 to 0.3 g fresh weight of leaf tissue (Chandra-Shekara et al., 2004). FA analysis (Dahmer et al., 1989; He et al., 2002) and lipid extraction (Welti et al., 2002) were carried out as described previously. Lipid profiles and acyl group identification were carried out using an automated electrospray ionization-tandem mass spectrometry facility available at the Kansas Lipidomics Center.

Glycerol and JA Treatments

Glycerol treatments were carried out by spraying 50 mM solution of glycerol prepared in sterile water. All observations were made 3 d post treatment. JA treatments were carried out as described by Kachroo et al. (2001).

Pathogen Infection

Inoculations with *Peronospora parasitica* Emco5 (Kachroo et al., 2001) and *Pseudomonas syringae* were conducted as described previously (Shah et al., 2001).

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