Plastidial fatty acid levels regulate resistance gene-dependent defense signaling in *Arabidopsis*

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In *Arabidopsis*, resistance to Turnip Crinkle Virus (TCV) depends on the resistance (*R*) gene, *HRT*, and the recessive locus *rrt*. Resistance also depends on salicylic acid (SA), *EDS1*, and *PAD4*. Exogenous application of SA confers resistance in *RRT*-containing plants by increasing *HRT* transcript levels in a *PAD4*-dependent manner. Here we report that reduction of oleic acid (18:1) can also induce *HRT* gene expression and confer resistance to TCV. However, the 18:1-regulated pathway is independent of SA, *rrt*, *EDS1*, and *PAD4*. Reducing the levels of 18:1, via a mutation in the *SSI2*-encoded stearoyl-acyl carrier protein-desaturase, or by exogenous application of glycerol, increased transcript levels of *HRT* as well as several other *R* genes. Second-site mutations in the *ACT1*-encoded glycerol-3-phosphate acyltransferase or *GLY1*-encoded glycerol-3-phosphate dehydrogenase restored 18:1 levels in *HRT ssi2* plants and reestablished a dependence on *rrt*. Resistance to TCV and *HRT* gene expression in *HRT act1* plants was inducible by SA but not by glycerol, whereas that in *HRT pad4* plants was inducible by glycerol but not by SA. The low 18:1-mediated induction of *R* gene expression was also dependent on *ACT1* but independent of *EDS1*, *PAD4*, and *RAR1*. Intriguingly, TCV inoculation did not activate this 18:1-regulated pathway in *HRT* plants, but instead resulted in the induction of several genes that encode 18:1-synthesizing isozymes. These results suggest that the 18:1-regulated pathway may be specifically targeted during pathogen infection and that altering 18:1 levels may serve as a unique strategy for promoting disease resistance.

glycerol | salicylic acid | stearoyl-acyl carrier protein-desaturase | Turnip Crinkle Virus | *ssi2*

Plants respond to pathogen perception by triggering a cascade of responses. Perception involves strain-specific detection of a pathogen-encoded elicitor, through direct or indirect interaction, with the corresponding resistance (*R*) gene product. Such an interaction (also known as incompatible interaction) triggers one or more defense signaling pathways and is often associated with the induction of the hypersensitive response (HR) at the site of pathogen entry. HR is one of the first visible manifestations of the host-induced defense response and is thought to prevent development and movement of the pathogen by inducing deliberate death of the infected cells. R protein-mediated recognition of pathogen can also lead to the accumulation of various phytohormones including salicylic acid (SA), jasmonic acid, and/or ethylene, which in turn signal the activation of defense gene expression. Each hormone activates a specific pathway, and these act individually, synergistically, or antagonistically, depending on the pathogen involved, the ultimate effect of which confers disease resistance and prevents spread of the pathogen to uninoculated parts of the plant.

Several components of the SA-mediated pathway have been identified, mutations in which lead to enhanced susceptibility to various pathogens [supporting information (SI) Table 2] (1–3). Mutations in *eds1* (4), *eds5* (5), *pad4* (6), and *sid2* (7) lower or abolish the pathogen-induced increase in SA levels. The EDS1, EDS5, PAD4, and SID2 proteins participate in basal disease resistance to virulent pathogens as well as R protein-mediated resistance to avirulent pathogens. Defense signaling mediated via a majority of R proteins, which contain Toll–IL1-like domains at

their N termini, depends on EDS1. Conversely, the NDR1 protein is required for many R proteins that contain coiled-coil domains at their N termini. Besides EDS1 and NDR1, R protein-mediated signaling is also known to require the RAR1 and SGT1 proteins, which are implicated as possible regulators of protein ubiquitylation (8, 9). However, unlike EDS1 and NDR1, RAR1 and SGT1 proteins can mediate signaling via R proteins that contain either Toll–IL1-like or coiled-coil domains at their N termini.

Unlike most *R* genes encoding for coiled coil-containing proteins, *HRT*, which confers resistance to Turnip Crinkle Virus (TCV), depends on *EDS1*. *HRT*-mediated resistance is also dependent on *PAD4*, *EDS5*, and *SID2*, but is independent of *RAR1* and *SGT1* (10). In addition to *HRT*, resistance to TCV also requires the recessive locus *rrt* (11). However, this requirement for *rrt* can be overcome by increasing the levels of *HRT* transcript via exogenous application of SA (10). Although SA appears to be downstream of the initial recognition event, it cannot confer resistance in the absence of *HRT* and *PAD4* (10). Mobilizing *HRT* into susceptible mutant backgrounds carrying high SA, such as *ssi2* or *cpr5*, can constitutively increase expression of *HRT* and, thereby, resistance to TCV (10). The *ssi2* and *cpr5* plants contain mutations in genes encoding for stearoyl-acyl carrier protein-desaturase (S-ACP-DES) (12) and a transmembrane protein of unknown function (13, 14), respectively. Although molecular mechanisms responsible for constitutive defense in *cpr5* are not clear, characterization of *ssi2* and its suppressors has implicated low levels of oleic acid (18:1) in altering defense signaling (15–17). The diverse biology of *ssi2* and *cpr5* mutants suggests that high levels of endogenous SA may be responsible for the up-regulation of *HRT* in *HRT ssi2* and *HRT cpr5* plants (10–21).

In the present study we demonstrate the presence of an alternate, SA-, *rrt*-, *PAD4*-, and *EDS1*-independent pathway, which confers resistance to TCV by increasing *HRT* transcript levels. This pathway, controlled by the levels of oleic acid, also regulates expression of several other *R* genes, which encode structurally different R proteins. These data present insights into the regulation of plant defense pathways.

Results

The *ssi2* Mutation Up-Regulates *HRT* Gene Expression in an SA- and *PAD4*-Independent Manner.
To examine the role of high endogenous SA in the up-regulation of *HRT* gene expression, we mobilized the *sid2* mutation into *HRT ssi2* and *HRT cpr5* plants and obtained *HRT ssi2 sid2* and *HRT cpr5 sid2* plants. A mutation in *sid2* was expected
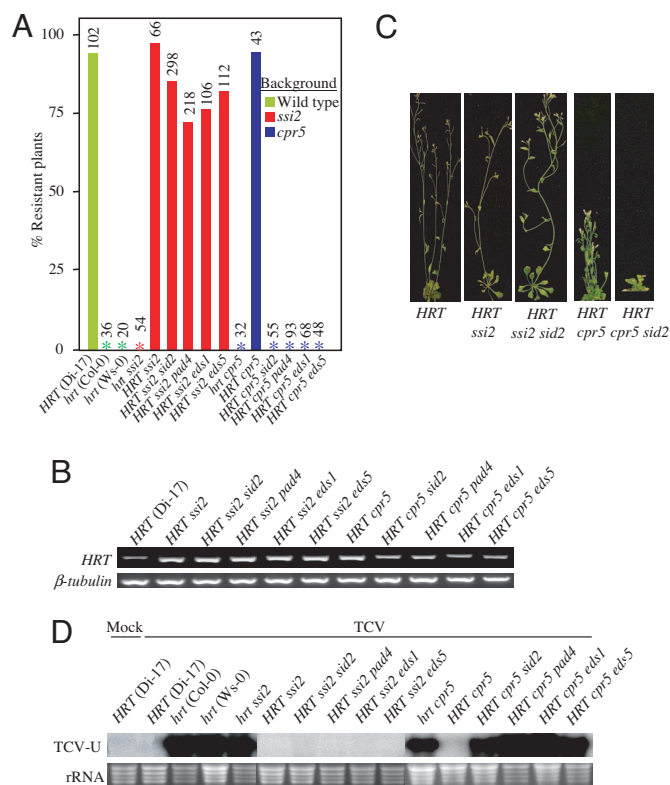
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Fig. 1. *ssi2*- and *cpr5*-mediated resistance signaling in various double mutant backgrounds. (*A*) Percentage of TCV-resistant plants in various genetic backgrounds. The number of plants tested is indicated above each bar. All plants were analyzed 3 weeks after inoculation. Asterisks indicate 100% susceptibility. (*B*) RT-PCR analyses showing *HRT* transcript levels in indicated genotypes. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. (*C*) Typical morphological phenotypes of TCV-inoculated plants; susceptible plants showed crinkling, stunted bolt development, and drooping of bolts. The resistant plants were morphologically similar to the mock-inoculated plants. The plants were photographed 2 weeks after inoculation. (*D*) Systemic spread of TCV to uninoculated tissue in TCV-inoculated plants. RNA was extracted from the uninoculated tissues at 18 days after inoculation and analyzed for the presence of the viral transcripts (TCV-U). Ethidium bromide staining of rRNA was used as a loading control.

to abolish the elevated levels of SA in these plants (7, 17) and, thereby, down-regulate *HRT* gene expression, resulting in susceptibility to TCV. As expected, *HRT cpr5 sid2* plants showed basal level expression of *HRT* and pronounced susceptibility (Fig. 1), confirming that high endogenous SA levels in *HRT cpr5* plants were likely responsible for elevated *HRT* gene expression and enhanced resistance. In contrast to these results, *HRT ssi2 sid2* plants continued to show high levels of *HRT* transcript (Fig. 1*B*). Consistent with the increased transcript levels of *HRT*, the *HRT ssi2 sid2* plants continued to show heightened resistance to TCV (Fig. 1 *A*, *C*, and *D*). This suggested that induction of *HRT* and enhanced TCV resistance in *HRT ssi2 sid2* plants were independent of SA. The *ssi2*-mediated induction of *HRT* was also independent of mutations in *eds1*, *pad4*, and *eds5*, indicating that, in *ssi2* plants, a factor(s) other than SA was responsible for the induced expression of *HRT*. However, as compared with *HRT ssi2* plants, the *HRT ssi2 sid2*, *HRT ssi2 eds5*, *HRT ssi2 eds1*, and *HRT ssi2 pad4* plants showed ≈15%, ≈18%, ≈20%, and ≈25% reductions in the number of resistant plants, respectively (Fig. 1*A*). These findings indicate that both SA-dependent and -independent signaling pathways triggered in *HRT ssi2* plants have an additive effect on resistance to TCV. In contrast to *HRT ssi2* plants, mutations in *pad4*, *eds1*, and *eds5*

abolished induced expression of *HRT* as well as resistance to TCV in *HRT cpr5* plants (Fig. 1).

HRT Promoter Responds to SA and Low 18:1 Conditions. Because altered defense signaling in the *ssi2* plants has been attributed to the reduced levels of 18:1 (15–17, 22), we checked the responsiveness of *HRT* promoter to SA and low 18:1 conditions. Histochemical assays for transient β-glucuronidase (GUS) activity in cells expressing the GUS protein under control of the *HRT* promoter were carried out. *Agrobacterium* cells transformed with an *HRT* promoter–GUS fusion vector were infiltrated into leaves from *sid2* or *ssi2 sid2* plants. Leaves from *sid2* plants displayed GUS activity only when pretreated with SA (Fig. 2*A*) or glycerol (data not shown). By comparison, GUS activity was detected constitutively in *ssi2 sid2* leaves. Taken together, these observations suggest that the *HRT* promoter responds to both SA and low 18:1 conditions.

Restoration of 18:1 Levels in HRT ssi2 Plants Compromises Resistance to TCV. We next asked whether restoration of 18:1 levels would down-regulate *HRT* gene expression to WT-like levels. We crossed Di-17 plants (resistant, *HRT*/*HRT rrt*/*rrt*) with *ssi2 act1* and *ssi2 gly1* plants (both susceptible, *hrt*/*hrt RRT*/*RRT*); mutations in *act1* and *gly1* restore WT phenotypes in *ssi2* plants by increasing the 18:1 content (15, 16) (SI Fig. 6*A*). Interestingly, *HRT ssi2 act1* and *HRT ssi2 gly1* plants showed basal level expression of *HRT* (Fig. 2*B*). Furthermore, resistance to TCV segregated with the recessive *rrt* allele; only 25% of *HRT ssi2 act1* or *HRT ssi2 gly1* plants showed resistance to TCV (Table 1 and Fig. 2 *C* and *D*). Fatty acid (FA) profiling revealed that, unlike *HRT ssi2* and *HRT ssi2 sid2* plants (17), the *HRT ssi2 act1* and *HRT ssi2 gly1* plants showed higher than or WT-like levels of 18:1, respectively (Fig. 2*B*). These data indicate that restoration of 18:1 levels in *HRT ssi2* plants was sufficient to restore the basal level expression of *HRT* as well as susceptibility to TCV. Control crosses between Di-17 and *act1* or *gly1* segregated normally for HR and resistance (Table 1), suggesting that these phenotypes were not influenced by mutations in *act1* or *gly1*.

To determine whether exogenous application of 18:1 in *HRT ssi2* plants had the same effect as the *act1* or *gly1* mutations, we infiltrated Di-17, Col-0 (susceptible, *hrt*/*hrt RRT*/*RRT*), and *HRT ssi2 sid2* plants with 18:1 (Fig. 2*E*). Infiltration of 18:1 did not appear to alter TCV replication in the inoculated leaves of WT plants; similar levels of TCV transcript were detected in water- and 18:1-treated Di-17 or Col-0 leaves. As expected, the susceptible Col-0 plants supported increased replication of the virus; TCV transcript levels in the inoculated leaves were severalfold higher than in the resistant Di-17 plants. In contrast, the levels of TCV transcript in the inoculated leaves of *HRT ssi2 sid2* plants were 2- to 3-fold lower than those in Di-17, suggesting that these plants repressed viral replication as compared with Di-17. Strikingly, infiltration of 18:1 into *HRT ssi2 sid2* leaves increased the amount of TCV transcript by ≈6- to 8-fold. However, TCV replication remained unaffected if 18:1 was infiltrated 24 h after TCV inoculation (Fig. 2*E*, last lane), indicating that 18:1 levels during the initial stages of pathogen perception were crucial for resistance signaling against TCV. Interestingly, although 18:1 injections increased TCV transcript at the localized site of inoculation, it did not allow systemic spread of the virus in *HRT ssi2 sid2* plants (data not shown). This could be because constitutive expression of *HRT* is likely to initiate downstream events leading to resistance, and a temporary surge in 18:1 levels at the localized site of infiltration may not be sufficient to promote systemic spread of the virus. Indeed, exogenous application only normalized 18:1 levels locally, at the site of infiltration, whereas the remaining untreated leaves continued to show low levels of 18:1 (SI Fig. 6*B* and SI Table 3). The above assumption is further supported by our results related to light-mediated resistance signaling to TCV (18), which showed that dependence on light can be overcome by initiating downstream signaling before dark treatment. Thus, exogenous application of SA

Fig. 2. *HRT* promoter assay and *HRT* transcript levels, 18:1 content, TCV resistance, and viral replication in leaves containing normal or higher oleate levels. (*A*) Transient GUS assay. Leaves were infiltrated with untransformed cells (-ve) or *Agrobacterium* transformed with HRT–GUS fusion construct. The *sid2* plants were treated with water (−) or SA (+) for 2 days before infiltration. Leaf discs (*sid2*) or whole leaves (*ssi2 sid2*) were processed for GUS histochemical staining as described before (45). (*B*) RT-PCR analyses showing basal-level expression of *HRT* in *HRT ssi2 act1* and *HRT ssi2 gly1* plants. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. The 18:1 levels are a mean of six independent replicates. (*C*) Systemic spread of TCV to uninoculated tissue (TCV-U) in TCV-inoculated plants. RNA was extracted from the uninoculated tissues at 18 days after inoculation and analyzed for the presence of the viral transcripts. Ethidium bromide staining of rRNA was used as a loading control. (*D*) Typical morpho-

logical phenotypes of mock- and TCV-inoculated *HRT ssi2 act1* and *HRT ssi2 gly1* plants. The susceptible plants showed crinkling, stunted bolt development, and drooping of bolts. Plants were photographed at 8 days after inoculation. (*E*) Effect of 18:1 infiltrations on viral replication in the inoculated leaf. Oleic acid (O, 1 mM) or water (W) was injected 24 h before or after (last two lanes) TCV inoculation, and the samples were harvested 72 h after inoculation. Ethidium bromide staining of rRNA was used as a loading control.
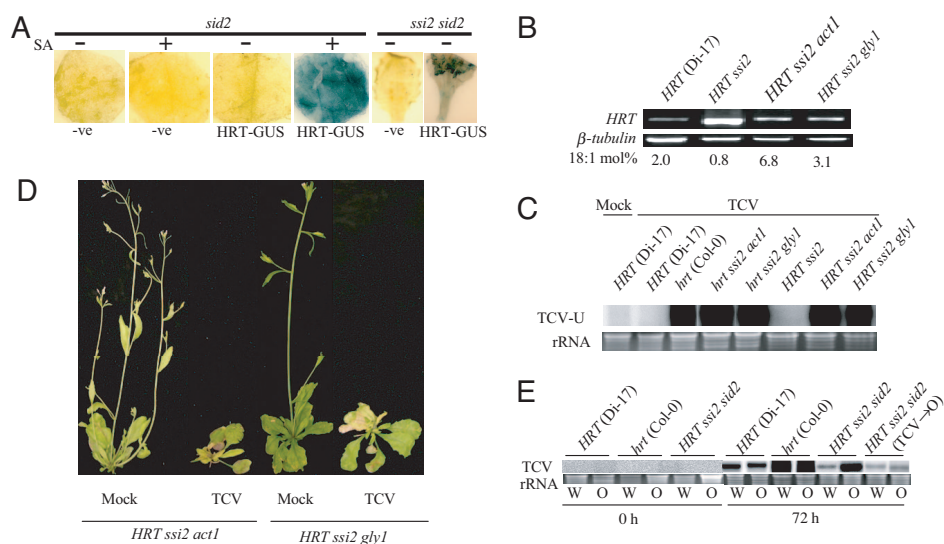
before dark treatment of Di-17 plants, or the presence of the *ssi2* or *cpr5* mutations in *HRT* plants, prevents dark-triggered susceptibility (18).

Exogenous Application of Glycerol Confers Resistance to TCV in an SA-Independent but *ACT1*-Dependent Manner. Additional evidence supporting a role for 18:1 in regulating *HRT* gene expression was obtained by glycerol treatment of several genotypes. Previously, we have shown that exogenous application of glycerol lowered 18:1 content in WT, *sid2*, and *pad4* backgrounds but not in *act1* plants (16, 17). This is because the *act1* plants are blocked in the step leading to the acylation of glycerol-3-phosphate with 18:1 (SI Fig. 6*A*). In addition, we earlier demonstrated that glycerol application induced high levels of SA and that this induction depended on the presence of a functional SID2 protein (17). As predicted, exogenous application of glycerol lowered 18:1 content in WT, *HRT sid2*, and *HRT pad4* plants, but not in *HRT act1* plants (Fig. 3*A*). Consequently, WT and *HRT pad4* plants were up-regulated in *PR-1* gene expression as opposed to *HRT act1* plants (Fig. 3*A*). *HRT sid2* plants

failed to induce *PR-1* gene expression in response to exogenous glycerol because of their inability to increase SA levels. Most importantly, glycerol-mediated reduction in 18:1 levels induced the levels of *HRT* transcript in WT, *HRT sid2*, and *HRT pad4*, but not in *HRT act1*, plants (Fig. 3*B*).

Because the up-regulation of *HRT* abolishes visible HR to TCV, we next monitored the effect of glycerol pretreatment on TCV-induced HR development in various genotypes. With the exception of *HRT act1* plants, all other genotypes, including *HRT* (Di-17), *HRT sid2*, and *HRT pad4* plants, did not develop visible HR if they were pretreated with glycerol (Fig. 3*C*; data not shown for Di-17). Thus, although the SA-mediated repression of HR to TCV depends on *PAD4* (10), 18:1-mediated repression of HR to TCV is independent of *PAD4*. The glycerol bioassay further confirmed that *HRT* expression and HR to TCV can be induced in an SA-independent manner by lowering the levels of 18:1 and that this effect was specific, because *act1* plants failed to show these responses upon glycerol application.

Consistent with the increase in *HRT* transcript levels, exogenous

Table 1. Epistatic analyses of F₂ populations generated by crossing Di-17 with various WT or mutant lines

Cross	Total no. of plants analyzed	Genotype	No. of plants obtained	HR	Resistant	Susceptible	χ^2	P value*
Di-17 × Col-0	136	*HRT/−*	93	+	18	75	1.58	0.21
Di-17 × Nössen	132	*HRT/−*	94	+	22	72	0.13	0.72
Di-17 × *ssi2 act1*	378	*HRT/− SSI2/− ACT1/−*	157	+	36	121	0.36	0.55
		HRT/− ssi2	66	ND	62	4	167.2	0.00†
		HRT/− act1	54	+	11	43	0.62	0.43
		HRT/− ssi2 act1	37	+	9	28	0.03	0.86
Di-17 × *ssi2 gly1-3*	301	*HRT/− SSI2/− GLY1/−*	108	+	26	82	0.05	0.82
		HRT/− ssi2	53	ND	51	2	143.4	0.00†
		HRT/− gly1	44	+	12	32	0.12	0.73
		HRT/− ssi2 gly1	31	+	6	25	0.53	0.47
Di-17 × *act1*	123	*HRT/− ACT1/−*	84	+	19	65	0.25	0.62
		HRT/− act1	12	+	3	9	0.00	1.00
Di-17 × *gly1-3*	126	*HRT/− GLY1/−*	71	+	16	55	0.23	0.63
		HRT/− gly1	22	+	8	14	1.51	0.22

The genotype at *HRT* and various mutant loci was determined by cleaved amplified polymorphic sequence analysis. ND, not determined; these plants show spontaneous HR.
*One degree of freedom.
†Statistically significant.

Fig. 3. *PR-1* and *R* gene expression levels, 18:1 levels, HR formation, and TCV resistance in plants treated with water (W), SA, or glycerol (G). (*A*) *PR-1* gene expression and 18:1 content in treated plants. The glycerol-treated *HRT ssi2* plants were harvested 24 h after treatment. Ethidium bromide staining of rRNA was used as a loading control. The 18:1 levels are a mean of six independent replicates. (*B*) RT-PCR analyses showing expression of *HRT* and *SSI4* genes in treated plants. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. (*C*) Visible HR formation in treated plants at 3 days after inoculation. (*D*) Percentage of TCV-resistant plants obtained after exogenous application of water, SA, or glycerol. Resistance was analyzed 3 weeks after inoculation. The number of plants tested is indicated above each bar. Asterisks indicate 100% susceptibility. (*E*) Systemic spread of TCV to uninoculated tissue in TCV-inoculated plants. RNA was extracted from the uninoculated tissues at 18 days after inoculation and

analyzed for the presence of the viral transcripts (TCV-U). Ethidium bromide staining of rRNA was used as a loading control. (*F*) Typical morphological phenotypes of TCV-inoculated plants. The susceptible plants showed crinkling, stunted bolt development, and drooping of bolts. Plants were photographed at 18 days after inoculation.
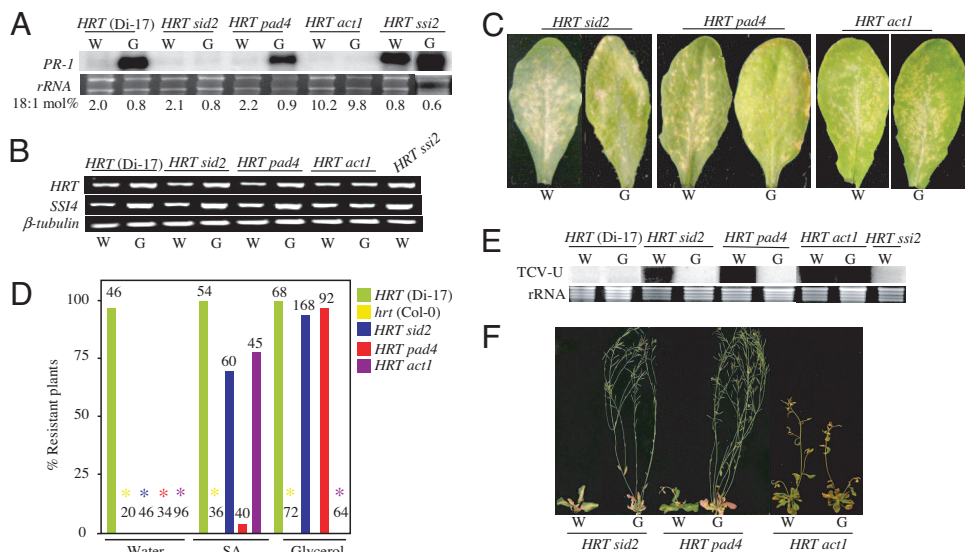
application of glycerol significantly enhanced resistance to TCV in *HRT pad4* and *HRT sid2* plants but not in *HRT act1* plants (Fig. 3 *B* and *D–F*). By contrast, SA pretreatment resulted in a substantial increase of TCV resistance in *HRT act1* plants but only a marginal increase in *HRT pad4* plants (Fig. 3*D*). SA or glycerol were unable to confer resistance in *hrt*-containing plants. These results suggest that SA- and glycerol-triggered pathways are mutually exclusive. This was further confirmed by generating *HRT cpr5 act1* and *HRT cpr5 ssi2 act1* plants; unlike *HRT ssi2 act1* plants, resistance in *HRT cpr5 act1* and *HRT cpr5 ssi2 act1* plants was comparable to that seen in *HRT cpr5* plants (data not shown). Taken together, these data indicate that there are at least two mechanisms leading to the induction of *HRT*, one dependent on SA and *PAD4* and the other responsive to reduced levels of 18:1. Furthermore, these data suggest that glycerol treatment is more effective at enhancing resistance; in comparison to SA, pretreatment with glycerol produced ≈30% more resistant *HRT sid2* plants (Fig. 3*D*).

Low 18:1 Conditions Up-Regulate Expression of Structurally Divergent *R* Genes. To examine whether the SA-independent and 18:1-modulated induction was specific for *HRT*, we analyzed the expression of several other *R* genes in the *ssi2*, *ssi2 sid2*, *HRT ssi2*, and *HRT ssi2 sid2* backgrounds (Figs. 3*B* and 4 *A* and *B*). A majority of the *R* genes analyzed were up-regulated in their expression levels in *ssi2 sid2* and *HRT ssi2 sid2* plants, indicating that low 18:1 levels induced *R* gene expression in an SA-independent manner. This was further confirmed upon exogenous application of glycerol on *sid2* plants, which induced the expression of several *R* genes (Fig. 4*C*). As predicted, *R* gene expression was restored to basal levels in the presence of the *act1* mutation, which normalizes 18:1 levels in *ssi2* plants (Fig. 4*B*). In contrast to *ssi2* plants, induction of *R* genes in the *cpr5* background was SA-dependent (Fig. 4*D*).

R protein-mediated signaling requires EDS1 and/or PAD4 functions, and mutations in these proteins have been shown to down-regulate *R* gene expression (20, 21, 23–26). Therefore, we next analyzed low 18:1-mediated induction of *R* genes in the presence of *eds1* or *pad4* mutations. Interestingly, both *ssi2 eds1* and *ssi2 pad4* plants showed elevated transcripts of all *R* genes tested (Fig. 4*E*). Glycerol treatment of *eds1* plants also elevated *R* gene transcript levels comparable to those induced in WT plants (Fig. 4*F*). Glycerol treatment of *rar1*, which encodes another essential regulator of *R*

gene-mediated signaling, also resulted in an increased accumulation of *R* gene transcripts. Taken together, these results suggest that 18:1-regulated expression of *R* genes does not depend on *EDS1*, *PAD4*, or *RAR1*.

To investigate whether increased accumulation of *R* gene transcripts in the *HRT ssi2 sid2* background also conferred SA-independent disease resistance to nonviral pathogens, we assayed *RPS2*-mediated resistance to the avirulent bacterial pathogen *Pseudomonas syringae* containing *AvrRPT2* (Fig. 4*G*). The *HRT sid2* plants showed severe susceptibility, consistent with the SA-dependent nature of *RPS2*-mediated resistance (27). By comparison, *HRT ssi2* and the WT ecotypes (Di-17, Col-0, or Nössen) showed resistance (Fig. 4*G*; data not shown for Nössen). Interestingly, although *HRT ssi2 sid2* plants were more susceptible than *HRT ssi2* or WT plants, these plants showed an ≈70-fold decrease in bacterial titer as compared with *HRT sid2* plants. These results indicate that SA-independent enhanced resistance, mediated by reduced 18:1 levels, was not specific to a certain group of pathogen and was conferred because of increased expression of the corresponding *R* gene. Because the SA-depleted *ssi2 nahG* plants have previously been shown to display enhanced resistance to virulent pathogens (28) and insects (29), the SA-independent induction of multiple *R* genes is also likely to contribute to generalized defense in these plants.

TCV Inoculation Induces Expression of Various S-ACP-DES Isoforms. Because a reduction in oleic acid levels induces defense signaling, we next asked whether resistance response to TCV triggers the 18:1-mediated pathway. FA profiling of mock- or TCV-inoculated plants did not show significant differences between the 18:1 levels, suggesting that the 18:1-derived pathway may not be activated during a resistance response to TCV (Fig. 5*A*). The *Arabidopsis* genome contains seven S-ACP-DES enzymes, and four of these are expressed in leaves and are capable of synthesizing 18:1 (22). To determine whether TCV inoculation of Di-17 plants altered the expression profile of any of these seven S-ACP-DES isoforms, we analyzed their transcript levels in mock- and TCV-inoculated plants. Inoculation of TCV increased the transcript levels of *SSI2*, *S-ACP-DES3*, and *S-ACP-DES5* genes (Fig. 5*B*). Because 18:1 levels in plants are under posttranscriptional and posttranslational controls (22), induction of various desaturases is unlikely to increase
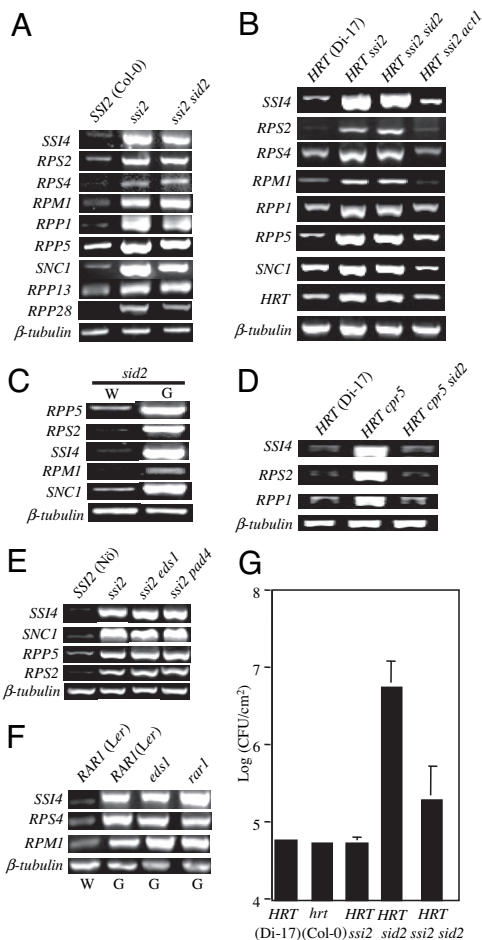
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Fig. 4. Oleic acid-modulated expression of *R* genes and resistance to bacterial pathogen. (*A*) RT-PCR analysis of various *R* genes in WT (*SSI2*), *ssi2*, and *ssi2 sid2* backgrounds. (*B*) RT-PCR analysis of various *R* genes in WT (*HRT*), *HRT ssi2*, *HRT ssi2 sid2*, and *HRT ssi2 act1* backgrounds. (*C*) RT-PCR analysis of various *R* genes in water (W)- or glycerol (G)-treated *sid2* plants. (*D*) RT-PCR analysis of various *R* genes in WT (*HRT*), *HRT cpr5*, and *HRT cpr5 sid2* backgrounds. (*E*) RT-PCR analysis of various *R* genes in WT (*SSI2*), *ssi2*, *ssi2 eds1*, and *ssi2 pad4* backgrounds. (*F*) RT-PCR analysis of various *R* genes in water (W)- or glycerol (G)-treated WT (*RAR1*), *eds1*, or *rar1* plants. The levels of β-tubulin were used as internal control to normalize the amount of cDNA template in experiments shown in *A*–*F*. (*G*) Growth of *P. syringae* on WT (*HRT* or *hrt*), *HRT ssi2*, *HRT sid2*, and *HRT ssi2 sid2* leaves. The Nössen ecotype was also tested and showed resistance similar to that seen in Di-17 and Col-0 plants (data not shown). Four leaf discs were harvested from infected leaves 3 days after inoculation and ground in 10 mM MgCl₂, and the bacterial numbers were titered. The bacterial numbers ± SD (*n* = 4) are presented as log of cfu per cm². The experiment was independently performed twice with similar results.

18:1 above WT levels, although it is likely to prevent a decline in the 18:1 content. Taken together, these data suggest that induction of several *S-ACP-DES* isoforms in TCV-inoculated Di-17 plants is likely to ensure normal 18:1 levels. Because SA-dependent and 18:1-regulated pathways have additive effects, it is possible that pathogens have developed mechanisms to shut off one or more defense pathway(s) to increase their chances of survival in the host. The ability of the bacterial effector proteins to suppress host immune responses further supports this notion (30).

Discussion

Oleic acid, a major monounsaturated FA in plants and animals, is known to influence mammalian immunity by participating in a variety of cellular processes. These include activation of signaling
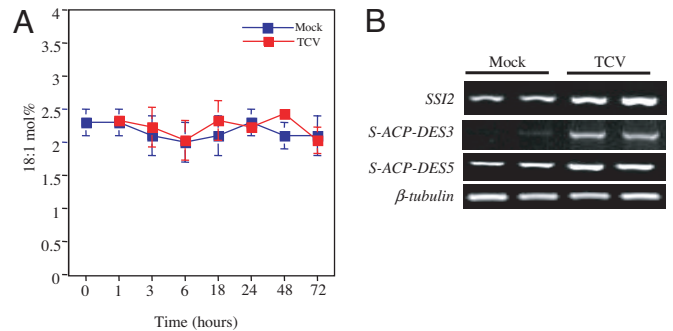

Fig. 5. Oleic acid and S-ACP-DES transcript levels after mock and TCV inoculation. (*A*) The inoculated leaves from Di-17 plants were sampled at 0–72 h after inoculation and processed for FA levels. The values are a mean of six independent replicates. The error bars represent SD. (*B*) *S-ACP-DES* transcript levels in mock- and TCV-inoculated plants 72 h after inoculation. Data from two independently extracted RNA samples are shown here. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. Transcript levels of *S-ACP-DES1*, *S-ACP-DES2*, *S-ACP-DES4*, and *S-ACP-DES6* either were not detected or showed no difference between mock- and TCV-inoculated plants.

cascades (31), regulation of protein activities (32), and transport of proteins across cellular compartments (33). The human α-lactalbumin, in its 18:1-conjugated form (HAMLET), induces apoptotic cell death specifically in tumor cells (34). The cancer protective properties of olive oil were also recently attributed to 18:1 (35).

In mammalian cells 18:1 biosynthesis is catalyzed by stearoyl-CoA desaturase (SCD), and altered expression of SCD is associated with abnormal physiology, as in diabetes, obesity, and neurological disorders (36). The soluble plant S-ACP-DES is functionally similar to SCD and catalyzes *de novo* synthesis of 18:1 in plastids. Our earlier work has shown that a mutation in the *SSI2*-encoded S-ACP-DES affects many cellular responses in the plant, and we have demonstrated that the altered defense-related phenotypes in *ssi2* plants are specifically associated with their low 18:1 levels (12, 15–17, 22). Previous research by Nandi *et al.* (37, 38) has implicated a reduction in hexadecatrienoic acid (16:3) to be responsible for restoration of *ssi2* phenotypes. However, several results contradict a role for 16:3. First, we have characterized several suppressor mutants in *ssi2* background, which restore all of the *ssi2*-trigrered phenotypes but contain normal levels of 16:3 (ref. 39 and unpublished data). Second, *ssi2* plants themselves show a slight reduction in 16:3 levels as compared with the WT plants (15–17). Third, age-dependent reappearance of *ssi2* phenotypes in *ssi2 gly1* plants does not alter 16:3 levels (ref. 16 and unpublished data). Fourth, mutations in *fad5*, *fad6*, *fad7*, *fad8*, and *dgd1* lower 16:3 levels but do not restore *ssi2* phenotypes (15, 17). Fifth, glycerol application converts WT plants into *ssi2* mimics but is not associated with any alteration of 16:3 levels (ref. 16 and unpublished data). Sixth, overexpression of *SSI2* (15) or *SSI2* isoform (22) complements *ssi2* phenotypes by increasing 18:1 levels but does not lower 16:3 content. Thus, it is possible that a reduction in 16:3 levels in various *ssi2* suppressors is merely coincidental because most mutations that influence 18:1 levels are likely to affect the prokaryotic pathway of glycerolipid biosynthesis.

Oleic acid is already known to both activate and inhibit protein activities in plants. Examples include the activation of phospholipase D activity in *Arabidopsis* (40) and inhibition of glucose-6-phosphate transporter activity in *Brassica* embryos (41). Thus, induction of *R* gene transcripts by low 18:1 conditions could be a result of activation/inactivation of molecules directly or indirectly involved in their regulation. Indeed, 18:1 is known to up-regulate a transcriptional repressor of the oncogene *HER2* (42) and induces genes required for neuronal differentiation by activating a tran-

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scription factor (43). An alternate possibility is that the altered ratio of saturated to unsaturated FAs is responsible for induction of *R* genes. In either case, the SSI2-catalyzed synthesis of 18:1 appears to influence defense responses by regulating *R* gene expression. Furthermore, this regulation is independent of *R* gene-signaling components *EDS1*, *PAD4*, and *RAR1*. It is intriguing that the 18:1-regulated pathway does not distinguish between *R* genes based on the structure of the encoded protein and influences transcription of *R* genes encoding either coiled-coil domain-carrying or Toll–IL1-like-domain-carrying proteins. We propose that this constitutive up-regulation of a large variety of *R* genes in *ssi2* plants is responsible for their altered defense-related phenotypes.

In conclusion, we report here an oleic acid-dependent signaling pathway that confers resistance in an SA-independent manner. Further molecular and biochemical characterization of 18:1-dependent regulation of *R* genes should provide exciting insights into the role of 18:1 in plant defense.

Methods

Plant Growth Conditions, Genetic Analysis, and Pathogen Inoculations. Plants were grown in MTPS 144 Conviron (Winnipeg, MB, Canada) walk-in-chambers at 22°C, 65% relative humidity, and 14-h photoperiod. Crosses were performed by pollinating flowers of Di-17 plants with pollen from Col-0, Nössen, *ssi2*, *cpr5*, *ssi2 act1*, *ssi2 gly1*, *act1*, and *gly1*. The double-mutant combinations in *ssi2* and *cpr5* backgrounds were created by crossing *HRT ssi2* or *HRT cpr5* plants with *eds1*, *sid2*, *eds5*, and *pad4*. The *HRT ssi2 act1 cpr5* plants were derived from a cross between *HRT ssi2 act1* and *HRT cpr5*. The *HRT cpr5 act1* plants were derived from a cross between *HRT cpr5* and *act1*. The *ssi2 eds1*, *ssi2 sid2*, and *ssi2 pad4* plants have been described earlier (17). All genotypes were initially screened in the F$_2$ generation and confirmed further in F$_3$ and F$_4$ generations. The genotypes at various loci were determined by conducting cleaved amplified polymorphic sequence analysis (CAPS) or derived CAPS (d-CAPS), as described before (10–12, 15–17). Inoculations with TCV and *P. syringae* were conducted as described before (10, 17).

Construction of *HRT* Promoter–GUS Fusions and GUS Assays. The *HRT* promoter was PCR-amplified from a clone obtained from the Di-17 genomic library (44) by using HindIII- and BamHI-linkered prim-

ers TGAGAAGCTTGTTGTTGCTGTCTCCTCTC and AA-GAGGATCCAACAACTAGTCGTCGAG. The PCR fragment was cloned upstream of the GUS ORF in pBI121. The HRT–GUS fusion construct was transformed into *Agrobacterium* strain MP90, and a culture grown overnight was used for agroinfiltrations as described before (45).

FA Analysis and Oleic Acid Infiltration. FA analysis was carried out by using gas chromatography as described previously (16). Oleic acid (Sigma–Aldrich, St. Louis, MO) was dissolved in water and infiltrated into leaves by using a needle-less syringe. To determine the 18:1 concentration required to elevate the leaf 18:1 content in *ssi2* plants to WT levels, we injected 50 μm to 10 mM into *HRT ssi2 sid2* leaves followed by FA profiling (SI Table 3). A concentration of 500 μm to 1 mM was considered optimal, with similar results being obtained upon infiltration of either of these concentrations.

SA and Glycerol Treatments. SA and glycerol treatments were carried out by spraying 500 μM and 50 mM solutions, respectively, prepared in sterile water. Control plants were treated with water. Plants were sampled or inoculated 2 days after SA treatment and 3 days after glycerol treatment, unless otherwise mentioned.

RNA Extraction, RT-PCR, and Northern Blot Analyses. RNA extraction, RT-PCR, and Northern blot analyses were conducted as described before (10, 22). For RT-PCR, the number of amplification cycles was reduced to 22–25 to evaluate and quantify any differences among transcript levels before they reached saturation. Primers used for PCR amplifications are described in SI Table 4.

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