Enhanced Resistance to *Cucumber mosaic virus* in the *Arabidopsis thaliana* *ssi2* Mutant Is Mediated via an SA-Independent Mechanism

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The *Arabidopsis thaliana* *SSI2* gene encodes a plastid-localized stearoyl-ACP desaturase. The recessive *ssi2* mutant allele confers constitutive accumulation of the pathogenesis-related-1 (*PR-1*) gene transcript and salicylic acid (SA), and enhanced resistance to bacterial and oomycete pathogens. In addition, the *ssi2* mutant is a dwarf and spontaneously develops lesions containing dead cells. Here, we show that the *ssi2* mutant also confers enhanced resistance to *Cucumber mosaic virus* (CMV). Compared with the wild-type plant, viral multiplication and systemic spread were diminished in the *ssi2* mutant plant. However, unlike the *ssi2*-conferred resistance to bacterial and oomycete pathogens, the *ssi2*-conferred enhanced resistance to CMV was retained in the SA-deficient *ssi2 nahG* plant. In addition, SA application was not effective in limiting CMV multiplication and systemic spread in the CMV-susceptible wild-type plant. The *acd1*, *acd2*, and *cpr5* mutants which, like the *ssi2* mutant, accumulate elevated SA levels, constitutively express the *PR-1* gene, spontaneously develop lesions containing dead cells, and are dwarfs, are, however, fully susceptible to CMV. Our results suggest that dwarfing, cell death, and constitutive activation of SA signaling are not important for the *ssi2*-conferred enhanced resistance to CMV. However, the *sfd1* and *sfd4* mutations, which affect lipid metabolism, suppress the *ssi2*-conferred enhanced resistance to CMV, thus implicating a lipid or lipids in the *ssi2*-conferred resistance to CMV. Interestingly, the *ssi2*-conferred resistance to CMV was compromised in the *ssi2 eds5* plant, suggesting the involvement of an SA-independent, EDS5-dependent mechanism in the *ssi2*-conferred resistance to CMV.

Additional keywords: plant defense, signaling pathway.

Plant defense responses are regulated by various signaling mechanisms (Dong 1998; Glazebrook 2002; Kunkel and Brooks 2002). Salicylic acid (SA), ethylene, and jasmonic acid (JA) signaling have a critical role in plant defense response (Alvarez 2000; Durner et al. 1997; Feys and Parker 2000; Kunkel and Brooks 2002; Shah and Klessig 1999). SA application induces the expression of genes for the defense-associated pathogenesis-related (PR) proteins, and enhances resistance to several pathogens (Dempsey et al. 1999; Durner et al. 1997; Shah and Klessig 1999). Some of these PR pro-

teins exhibit antimicrobial activities (Hunt and Ryals 1996). In addition, SA is also involved in the activation of systemic acquired resistance (SAR), which confers resistance to a broad spectrum of pathogens (Dempsey et al. 1999; Ryals et al. 1996; Shah and Klessig 1999). Preventing SA accumulation by expressing a salicylate hydroxylase, which converts SA to catechol in the transgenic NahG tobacco and *Arabidopsis thaliana* plants, depresses the pathogen-stimulated expression of the PR genes, confers enhanced susceptibility to pathogens, and prevents the activation of SAR (Delaney et al. 1994; Gaffney et al. 1993). Similarly, loss-of-function mutations in the *Arabidopsis* *EDS5* gene block SA biosynthesis and confer enhanced susceptibility to bacterial and oomycete pathogens (Nawrath et al. 1999). It has been suggested that EDS5, which exhibits homology to multidrug and toxin extrusion (MATE) transporter proteins from animals, transports precursors for SA biosynthesis (Nawrath et al. 2002). In contrast to the SA-deficient mutants, the *Arabidopsis* *acd1*, *acd2*, *cpr5*, *ssi1*, and *ssi2* mutants accumulate elevated SA levels and constitutively express the PR genes (Bowling et al. 1997; Greenberg and Ausubel 1993; Mach et al. 2001; Shah et al. 1999, 2001). Furthermore, the *acd1*, *acd2*, *cpr5*, *ssi1*, and *ssi2* mutations confer enhanced resistance to some pathogens. These mutations have pleiotropic effects; the *acd1*, *acd2*, *cpr5*, *ssi1*, and *ssi2* mutants are dwarfs and spontaneously develop lesions containing dead cells. The elevated levels of SA that accumulate in some of these mutants are required for their enhanced resistance to pathogens. For example, the inability to accumulate elevated levels of SA due to expression of the *nahG* transgene in the *ssi1 nahG* plants suppressed the *ssi1*-conferred disease resistance (Nandi et al. 2003a; Shah et al. 1999). Similarly, the *ssi2*-conferred expression of the *PR-1* gene and enhanced resistance to bacterial and oomycete pathogens was significantly compromised in the *ssi2 nahG* plant (Shah et al. 2001).

A. thaliana has proven to be an excellent model plant for studying host–virus interaction. Turnip crinkle virus (TCV) (Dempsey et al. 1997; Simon et al. 1992), *Cucumber mosaic virus* (CMV) (Takahashi et al. 1994), *Beet curly top virus* (Lee et al. 1994), *Cauliflower mosaic virus* (Balázs and Lebeurier 1981; Leisner et al. 1993; Melcher 1989), *Oilseed rape mosaic virus* (Aguilar et al. 1996; Martín et al. 1997), the Cg strain of *Tobacco mosaic virus* (TMV) (Ishikawa et al. 1991), *Tobacco ring spot virus* (Lee et al. 1996), *Tomato spotted wilt virus* (German et al. 1995), *Turnip mosaic virus* (Nicolas and Laliberté 1992), and *Turnip vein clearing tobamovirus* (Lartey et al. 1997) can infect *Arabidopsis* spp. and spread systemi-

cally through the plant. However, *Arabidopsis* ecotypes resistant to some of these viruses have been reported. For example, although many *Arabidopsis* ecotypes are susceptible to TCV, the dominant *HRT* allele in the ecotype Dijön confers resistance to TCV (Dempsey et al. 1997; Kachroo et al. 2000). Similarly, in contrast to the *Arabidopsis* ecotypes Columbia (Col) and Nössen (Nö), which are susceptible to the yellow strain of CMV (CMV-Y) and permit systemic spread of the virus, the ecotype C24 is resistant to CMV-Y (Takahashi et al. 1994, 2001). The dominant *RCY1* allele, which encodes a coiled-coil, nucleotide-binding site and leucine-rich repeat domain containing protein, confers a gene-for-gene type resistance to CMV-Y in C24 (Takahashi et al. 2002). C24 responds to CMV-Y exposure with a hypersensitive response (HR) and restricts the virus to the inoculated leaf. Previously, we have shown that SA and ethylene signaling, and an unknown mechanism or mechanisms, are required for the full extent of *RCY1*-conferred resistance to CMV-Y (Takahashi et al. 2002). In contrast, the *HRT*-mediated gene-for-gene resistance to TCV in the *Arabidopsis* ecotype Dijön is dependent on SA, but independent of ethylene (Kachroo et al. 2000). In tobacco, the *N* gene-mediated gene-for-gene resistance to TMV requires SA. However, Chivasa and associates (1997) have proposed that, in tobacco, a distinct branch of the SA signaling pathway specific to viruses is associated with resistance to TMV. Therefore, our understanding about the signaling pathways for the resistance to virus, including SA-mediated signaling, is limited primarily.

Several studies have uncovered an important role for lipids in plant defense mechanisms (Shah 2003; Weber 2002). For example, mutations in the *Arabidopsis* *PAD4* and *EDS1* genes that encode proteins with homology to the active sites of lipases esterases compromise the activation of SA signaling and exhibit enhanced susceptibility to some pathogens (Falk et al. 1999; Jirage et al. 1999). Lipids also have a role in the activation of SAR. In tobacco, the SA-binding protein 2 (SABP2) is a lipase esterase that is required for the *N* gene-mediated resistance to TMV and the subsequent activation of SAR (Kumar and Klessig 2003). In *Arabidopsis* spp., mutations in the *SFD1* and *DIR1* genes, which encode a dihydroxyacetone phosphate reductase that is involved in lipid biosynthesis and a putative lipid transfer protein, respectively, compromise the activation of SAR (Maldonado et al. 2002; Nandi et al. 2004). The *sfd1* mutant allele was identified in a screen for suppressors of the *ssi2* mutant (Nandi et al. 2003b). Similarly, the *sfd4* mutation in a gene encoding a $\omega 6$ fatty acid desaturase also suppressed the *ssi2*-conferred phenotypes (Nandi et al. 2003b). The *SSI2* gene encodes a plastid localized stearyl ACP desaturase, which catalyzes the desaturation of stearic acid to oleic acid (Kachroo et al. 2001) and alters membrane lipid composition (Nandi et al. 2003b). In addition to constitutively

expressing SA-dependent defense responses and conferring enhanced resistance to bacterial and oomycete pathogens, the *ssi2* mutant allele also affects cross talk between signaling pathways in plant defense. JA and ethylene are two potent inducers of expression of the *Arabidopsis* *PDF1.2* gene (Penninckx et al. 1998). However, JA- and ethylene-activated *PDF1.2* expression was compromised in the *ssi2* mutant plant (Kachroo et al. 2001). In addition, compared with *Alternaria brassicicola*-inoculated leaves of the wild-type plant, *PDF1.2* expression was compromised in the pathogen-challenged leaves of the *ssi2* mutant plant (Kachroo et al. 2001). Furthermore, the *ssi2* mutant exhibits enhanced susceptibility to the fungal pathogen *Botrytis cinerea* (Kachroo et al. 2001; Nandi et al. 2003b).

In this study, we show that the *ssi2* mutant also enhances basal resistance to two different isolates of CMV. In addition, systemic spread of CMV is depressed in the *ssi2* mutant. However, unlike the *ssi2*-conferred enhanced resistance to bacterial and oomycete pathogens, the high SA content of the *ssi2* mutant is not required for the *ssi2*-conferred resistance to CMV. The *EDS5*, *SFD1*, and *SFD4* genes are required for the *ssi2*-conferred enhanced resistance to CMV. Our results suggest the involvement of a lipid or lipids in the *ssi2*-conferred enhanced resistance to CMV.

RESULTS

The *ssi2* mutant confers enhanced resistance to CMV.

We previously had shown that the *RCY1*-conferred gene-for-gene resistance to CMV in the *Arabidopsis thaliana* ecotype C24 is partially dependent on ethylene and SA signaling (Takahashi et al. 2002). Therefore, we tested whether the constitutive expression of ethylene signaling in the *ctr1* mutant and SA signaling in the *acd1*, *acd2*, *cpr5*, *ssi1*, and *ssi2* mutants were sufficient to restrict the systemic spread of CMV-Y in these *RCY1*-deficient plants. The *ctr1*, *acd1*, *acd2*, and *cpr5* mutants are in the ecotype Col and the *ssi1* and *ssi2* mutants are in the ecotype Nö backgrounds; therefore, wild-type plants of ecotypes Col and Nö were included as CMV-susceptible controls. Like the wild-type Col and Nö plants, systemic spread of the virus was observed in >90% of the *ctr1*, *acd1*, *acd2*, and *cpr5* and 79% of the *ssi1* mutant plants (Tables 1 and 2; Figs. 1B and 2). Interestingly, only <50% of the CMV-Y-inoculated *ssi2* mutant plants exhibited systemic spread of the virus (Figs. 1B and 2; Tables 1 and 2). Similarly, in contrast to the wild-type and *ctr1* mutant plants, spread of the Indonesian isolate of CMV (CMV-B2) also was restricted in the *ssi2* mutant plant (Table 2). These results indicate that the constitutive expression of ethylene or SA signaling is not sufficient to enhance resistance to CMV. The *acd1*, *acd2*, *cpr5*, and *ssi1* mutants spontaneously develop lesions containing dead

Table 1. Systemic spread of *Cucumber mosaic virus*-yellow strain (CMV-Y) in the *Arabidopsis* *ssi1*, *ssi2*, *cpr5*, *acd1*, and *acd2* mutants

Plant ^b	Number of plants ^a			CMV-Y susceptible (%)
	Total inoculated	Susceptible	Resistant	
Wild-type (Nö)	23	22	1	96
<i>ssi2</i>	31	13	18	42
<i>ssi1</i>	47	37	10	79
Wild-type (Col)	10	10	0	100
<i>cpr5</i>	26	25	1	96
<i>acd1</i>	21	20	1	95
<i>acd2</i>	25	24	1	96

^a Distribution of virus coat protein in the virus-inoculated plants was detected by the tissue printing method. The number of plants in which CMV-Y had spread to the noninoculated leaves (susceptible) or was restricted to the inoculated leaves (resistant) was determined. CMV-Y at 100 µg/ml was used as the inoculum.

^b Two-week-old *Arabidopsis thaliana* plants were used. The *ssi1* and *ssi2* mutants are in the ecotype Nössen (Nö) background and *cpr5*, *acd1*, and *acd2* are in the ecotype Columbia (Col) background.

cells (Bowling et al. 1997; Greenberg and Ausubel 1993; Mach et al. 2001; Shah et al. 1999, 2001). In addition, these and the *ctr1* mutant (Fig. 1A) are dwarfs. Hence, the above results also argue against any role for heightened cell death and dwarfing in restricting the spread of CMV.

We further tested whether viral multiplication also was suppressed in the CMV-Y-inoculated leaves of the *ssi2* mutant. The accumulation of viral coat protein was used as an indicator of viral multiplication. An enzyme-linked immunosorbent

assay (ELISA) was used to quantify the amount of viral coat protein present in the virus-inoculated leaves of the wild-type CMV-Y-susceptible plant and the *ssi2* mutant plant at varied time points after inoculation with CMV-Y. CMV-Y-inoculated leaves of the *ssi2* mutant plant accumulated <30% of the amount of coat protein present in the virus-inoculated leaves of the wild-type plants (Fig. 3). In contrast, coat protein levels in the CMV-Y-inoculated leaves of the *ctr1*, *acd1*, *acd2*, *cpr5*, and *ssi1* mutants were comparable to the levels in the wild-

Table 2. Systemic spread of *Cucumber mosaic virus* (CMV) strains Y and B2 in the *Arabidopsis ssi2* and *ctr1* mutants

Plant ^b	Virus ^c	Number of plants ^a			CMV-Y-susceptible (%)
		Total inoculated	Susceptible	Resistant	
Wild type (Col)	CMV-Y	50	50	0	100
Wild type (Nö)	CMV-Y	67	67	0	100
<i>ctr1</i>	CMV-Y	189	179	10	95
<i>ssi2</i>	CMV-Y	150	69	81	46
Wild type (Col)	CMV-B2	50	50	0	100
Wild type (Nö)	CMV-B2	43	42	1	98
<i>ctr1</i>	CMV-B2	76	69	7	91
<i>ssi2</i>	CMV-B2	80	51	29	64

^a Distribution of virus coat protein in the virus-inoculated plants was detected by the tissue printing method. The number of plants in which the virus had spread to the noninoculated leaves (susceptible) or was restricted to the inoculated leaves (resistant) was determined.

^b Two-week-old *Arabidopsis thaliana* plants were used. The *ssi2* mutant is in the ecotype Nössen (Nö) background and the *ctr1* mutant is in the ecotype Columbia (Col) background.

^c CMV-Y and CMV-B2 at 100 µg/ml were used as the inoculum.

Fig. 1. Comparison of morphology of the *ssi2* and *ctr1* mutant plants and systemic disease symptoms after exposure to *Cucumber mosaic virus*-yellow strain (CMV-Y). **A**, Three-week-old wild-type ecotype Nössen (Nö) and the *ssi2* and *ctr1* mutant plants were photographed from the same distance. **B**, Comparison of the systemic spread of disease in CMV-Y-inoculated *ctr1* and *ssi2* mutant plants. Two lower leaves of 2-week-old *ctr1* plants were inoculated with CMV-Y. In parallel, 0.01 M potassium phosphate buffer (pH 8.0)-inoculated (Mock-inoculated) *ctr1* and *ssi2* plants served as negative controls. Plants were photographed 7 days later.

type plant (Fig. 3). These results indicate that the *ssi2*-conferred enhanced resistance to CMV results from the suppression of viral multiplication in the virus-inoculated leaves and the inhibition of long-distance movement of the virus.

The *ssi2*-conferred enhanced resistance to CMV is independent of SA accumulation.

Previously, we have shown that the elevated level of SA that accumulates in the *ssi2* mutant is in part responsible for the *ssi2*-conferred enhanced resistance to bacterial and oomycete pathogens (Shah et al. 2001). Similarly, the high SA content of the *ssi2* mutant may have a causal role in the *ssi2*-conferred enhanced resistance to CMV. We previously have shown that SA has a role in the *RCY1*-conferred gene-for-gene resistance to CMV-Y (Takahashi et al. 2002). Furthermore, SA application enhances the resistance of tobacco plants to CMV (Naylor et al. 1998). Therefore, we compared viral multiplication and spread in CMV-inoculated *ssi2*, and the SA-deficient *ssi2 nahG* (Shah et al. 2001) and *ssi2 eds5* (A. Nandi and J. Shah, *unpublished*) plants. The wild-type Nö and Col plants provided the CMV-susceptible control. Northern analysis confirmed the expression of the *nahG* transgene in the *ssi2 nahG* plant (Fig. 4B). Furthermore, as previously shown by us, the presence of the *nahG* transgene in the *ssi2 nahG* plant depressed the *ssi2*-conferred constitutive expression of *PR-1* (Fig. 4A). Similarly, the *ssi2*-conferred constitutive expression of *PR-1* was depressed in the

ssi2 eds5 mutant plant (Fig. 4A). In contrast to the wild-type and the *nahG* transgenic plant, but comparable to the *ssi2* mutant plant, viral multiplication was depressed in the leaves of the CMV-Y-inoculated *ssi2 nahG* plant (Fig. 5). Viral coat protein levels in the CMV-Y-inoculated leaves of the *ssi2 nahG* plant were comparable to the coat protein levels in the virus-inoculated leaves of the *ssi2* mutant plant. Furthermore, in approximately 60% of the *ssi2* and *ssi2 nahG* plants inoculated with CMV-Y, the virus was restricted to the inoculated leaves (Fig. 6; Table 3). In comparison, the virus spread systemically in all the control wild-type and *nahG* transgenic plants. In contrast to the *ssi2* and *ssi2 nahG* plants, viral multiplication in the CMV-Y-inoculated leaves of the *ssi2 eds5* double-mutant plant was comparable to viral multiplication in the *eds5* single mutant and the wild-type Col and Nö plants (Fig. 5). Furthermore, viral spread was observed in >80% of the CMV-Y-inoculated *ssi2 eds5* double-mutant plants (Fig. 6; Table 3). Although the *ssi2* mutant is in the ecotype Nö background, the *eds5-1* allele used in the above experiment is in the ecotype Col background. Hence, the *ssi2 eds5* double-mutant plant has a hybrid genetic background. To control for any contribution of genetic differences between the Col and Nö ecotypes on the results with the *ssi2 eds5* double-mutant plants, we studied the systemic spread of CMV-Y in the F₃ progeny derived from three F₂ *ssi2 eds5* and two F₂ *SSI2 EDS5* (wild type), *SSI2 eds5*, and *ssi2 EDS5* plants (Table 3; Figs. 5 and 6). Loss of the *ssi2*-conferred re-

Fig. 2. Systemic spread of *Cucumber mosaic virus-yellow* strain (CMV-Y) in *Arabidopsis* mutant plants. Two lower leaves of the wild-type ecotype Columbia (Col) and Nönnen (Nö) plants and the *acd1*, *acd2*, *cpr5*, *ctr1*, *ssi1*, and *ssi2* mutant plants were inoculated with CMV-Y. Seven days later, the presence of viral coat protein was detected by tissue printing method: press-blotting all the plants on a filter paper and probing the filter with an anti-CMV-Y coat protein probe. The blue color indicates presence of the viral coat protein.

sistance to CMV-Y cosegregated with *eds5-1*; compared with the *ssi2* single mutant lines 11 and 31, systemic spread of virus was observed in >80% of plants of the *ssi2 eds5* lines 1, 30, and 45. In all, 100% of the control wild-type lines 9 and 32 and the *eds5* single-mutant lines 70 and 121 exhibited systemic spread of virus. The contrasting effects of the *nahG* transgene and the *eds5* mutant allele on the *ssi2*-conferred resistance to CMV-Y in the *ssi2 nahG* and *ssi2 eds5* plants, respectively, suggest that the *ssi2*-conferred enhanced resistance to CMV is mediated by an SA-independent but largely *EDS5*-dependent mechanism, which is independent of the effect of *EDS5* on SA biosynthesis. The inability of applied SA to limit viral multiplication and systemic spread (Table 4) in the wild-type Nö plant provides further support to this hypothesis.

The *sfd* mutant alleles suppress the *ssi2*-conferred resistance to CMV.

The suppressor of fatty acid desaturase deficiency (*sfd*) mutants *sfd1* and *sfd4* suppress the *ssi2*-conferred enhanced resistance to *Pseudomonas syringae* (Nandi et al. 2003b). To determine if the *sfd1* and *sfd4* mutant alleles also suppress the *ssi2*-conferred enhanced resistance to CMV, the *ssi2 sfd1-1* and *ssi2 sfd4-1* mutant plants were inoculated with CMV-Y and systemic spread of virus in these plants was compared with viral spread in the wild-type and *ssi2* mutant plants. The *ssi2*-conferred restriction of systemic spread of CMV-Y was suppressed in the *ssi2 sfd1-1* and *ssi2 sfd4-1* mutant plants (Fig. 5; Table 5). These results suggest the involvement of lip-

ids in the *ssi2*-conferred resistance to CMV. We previously have shown that the *sfd1* and *sfd4* mutations affect lipid metabolism and that the *ssi2*-conferred enhanced resistance to bacterial pathogens is associated with changes in membrane lipid composition (Nandi et al. 2003b).

DISCUSSION

We previously have shown that the loss of a stearyl-ACP desaturase activity in the *Arabidopsis ssi2* mutant confers enhanced resistance to the bacterial pathogen *P. syringae* and the oomycete pathogen *Peronospora parasitica* (Kachroo et al. 2001; Nandi et al. 2003b; Shah et al. 2001). Here we show that the *ssi2* mutant also confers enhanced resistance to two different strains of CMV, thus providing further evidence suggesting the activation of broad-spectrum disease resistance mechanisms in the *ssi2* mutant. However, the signaling mechanism or mechanisms associated with the *ssi2*-conferred resistance to CMV are partly distinct from the signaling mechanisms involved in the *ssi2*-conferred resistance to bacterial and oomycete pathogens. Although an elevated SA level is required for the full extent of *ssi2*-conferred resistance to *Pseudomonas syringae* and *Peronospora parasitica* (Shah et al. 2001), which was compromised in the SA-deficient *ssi2 nahG* plant, an elevated SA level is not important for the *ssi2*-conferred resistance to CMV. The *ssi2*-conferred enhanced resistance to CMV was comparable between the *ssi2* mutant and the SA-deficient *ssi2 nahG* plant (Figs. 5A and 6; Table 3). In addition, SA application was ineffective in restricting CMV multiplication and systemic spread in the CMV-susceptible wild-type plants (Table 4). This is unlike the previously documented ability of SA and SA analogs to

Fig. 3. Virus multiplication in the *Cucumber mosaic virus*-yellow strain (CMV-Y)-inoculated leaves of *Arabidopsis* mutants. **A**, Time course of virus multiplication in CMV-Y-inoculated leaves of the wild-type ecotype Columbia (Col) (filled black circle with solid black line) and the *cpr5* (filled black square with solid black line), *acd1* (filled gray triangle with solid gray line), *acd2* (filled gray circle with solid gray line), and *ctr1* (open circle with broken black line) mutant plants, all of which are in the Columbia background. **B**, Time course of virus multiplication in CMV-Y-inoculated leaves of the wild-type ecotype Nösssen (Nö) (filled black circle with solid black line) and the *ssi1* (solid gray square with solid gray line) and *ssi2* (open black circle with broken black line) mutant plants, which are in the Nösssen background. In **A** and **B**, CMV-Y coat protein accumulation was measured by enzyme-linked immunosorbent assay and expressed as absorbance at 405 nm (A_{405}) per 0.025 mg per milliliter of total protein. The experiments in **A** and **B** were conducted simultaneously.

Fig. 4. Accumulation of the *PR-1* and *nahG* transcripts in *Arabidopsis* mutant plants. **A**, Accumulation of the *PR-1* gene transcript in salicylic acid (lane 1)- and Me-jasmonic acid (lane 2)-treated wild-type ecotype Nösssen (Nö), untreated Nö (lane 3), and Columbia (lane 4) plants; the *ssi2* (lane 5), *eds5* (lane 6), and *nahG* (lane 8) single mutant plants; and the *ssi2 eds5* (lane 7) and *ssi2 nahG* (lane 9) double-mutant plants was analyzed by northern hybridization. **B**, Accumulation of the *nahG* transgene transcript in the wild-type ecotype Nö plant (lane 1) and the *ssi2* (lane 2), *nahG* (lane 3), and *ssi2 nahG* (lane 4) mutant plants was analyzed by Northern hybridization. In both **A** and **B**, the gel was stained with ethidium bromide to monitor equal loading of RNA. The rRNA band is shown.

enhance basal resistance to *Pseudomonas syringae* and *Peronospora parasitica* in wild-type *Arabidopsis* spp. (Cao et al. 1997; Delaney et al. 1995). In support of the noninvolvement of elevated an SA level in the *ssi2*-conferred resistance to CMV, we show that the comparably high level of SA that accumulates in the *ssi1* mutant (Shah et al. 1999, 2001) does not result in enhanced resistance to CMV (Fig. 2; Table 1). Similarly, presence of elevated SA levels in the *acd1*, *acd2*, and

cpr5 mutants (Bowling et al. 1997; Greenberg and Ausubel 1993; Mach et al. 2001) does not confer enhanced resistance to CMV (Fig. 2; Table 1). The CMV susceptibility of the *acd1*, *acd2*, *cpr5*, and *ssi1* mutant plants, which also exhibit dwarfing and spontaneously develop lesions containing dead cells, also argues against dwarfing and cell death being important factors in the *ssi2*-conferred enhanced resistance to CMV.

The *EDS5* gene is predicted to encode a member of the MATE family of transporter protein (Nawrath et al. 2002). Based on the inability of the *eds5* mutant plant to synthesize SA in response to abiotic and biotic stresses, it was proposed that the EDS5 protein is involved in the transport of phenolic precursors of SA. As expected, the *eds5-1* mutant allele does block the *ssi2*-conferred SA accumulation and *PR-1* gene expression in the *ssi2 eds5* double-mutant plant and compromises the *ssi2*-conferred resistance to *Pseudomonas syringae* (A. Nandi and J. Shah, unpublished). In addition, as shown in Figures 5 and 6 and Table 3, the *eds5-1* mutant allele also compromised the *ssi2*-conferred resistance to CMV in the *ssi2 eds5* double-mutant plant. However, resistance to CMV was not compromised in the SA-deficient *ssi2 nahG* plant (Figs. 5 and 6; Table 3); therefore, it is unlikely that the *eds5*-conferred block in SA accumulation is responsible for the suppression of the *ssi2*-conferred resistance to CMV. Rather, an EDS5-modulated, SA-independent mechanism is involved in the *ssi2*-conferred resistance to CMV. Similarly, we have observed the involvement of EDS5 in an SA-independent mechanism that modulates defense responses to *Botrytis cinerea* (A. Nandi and J. Shah, unpublished). The MATE family proteins are involved in the transport of different kinds of organic molecules (Diener et al. 2001). Likewise, EDS5 may be involved in the transport of another molecule or molecules that modulates a defense mechanism or mechanisms targeting CMV.

The *RCY1*-conferred resistance to CMV in the *Arabidopsis* ecotype C24 is associated with the HR (Takahashi et al. 1994, 2001). The *ssi2* mutant spontaneously develops HR-like lesions that contain dead cells. However, our study indicates that activation of cell death is not sufficient to restrict CMV multiplication and systemic spread in plants lacking the *RCY1* gene; the *acd1*, *acd2*, *cpr5*, and *ssi1* mutants, which spontaneously develop lesions that contain dead cells, do not exhibit enhanced resistance to CMV. In addition, the *ssi2 eds5* plant, in which the *ssi2*-conferred resistance to CMV is

Fig. 5. Accumulation of viral coat protein in the *Cucumber mosaic virus-yellow* strain (CMV-Y)-inoculated leaves of *Arabidopsis* mutants. **A**, Time course of viral multiplication in the CMV-Y-inoculated leaves of the wild-type ecotype Nö (Nö) plant (filled black circle with solid black line) and the *ssi2* (filled gray circle with solid gray line), *nahG* (open black circle with solid black line), and *ssi2 nahG* (solid black square with broken black line) mutant plants. **B**, Time course of viral multiplication in the CMV-Y-inoculated leaves of F₃ progeny of the wild-type *SSI2 EDS5* F₂ line 9 (filled black circle with solid black line), the *ssi2 EDS5* F₂ line 11 (filled black square with broken black line), the *ssi2 eds5* F₂ line 45 (open gray circle with broken gray line), and the *SSI2 eds5* F₂ line 36 (open black circle with solid black line), which were obtained from a cross between the *ssi2* and *eds5* single-mutant plants. In **A** and **B**, CMV-Y coat protein accumulation was measured by enzyme-linked immunosorbent assay and expressed as absorbance at 405 nm (A_{405}) per 0.025 mg per milliliters of total protein. The experiments in **A** and **B** were conducted simultaneously.

Table 3. Systemic spread of *Cucumber mosaic virus-yellow* strain (CMV-Y) in the *Arabidopsis ssi2 nahG* and *ssi2 eds5* mutant plants

Plant ^b	Number of plants ^a			CMV-Y susceptible (%)
	Total inoculated	Susceptible	Resistant	
Wild type (Nö)	23	23	0	100
<i>ssi2</i>	47	19	28	40
<i>nahG</i>	20	20	0	100
<i>ssi2 nahG</i>	100	42	58	42
Wild type (Col)	14	14	0	100
<i>eds5</i>	33	33	0	100
<i>SSI2 EDS5</i> # 9	5	5	0	100
<i>SSI2 EDS5</i> # 32	5	5	0	100
<i>SSI2 eds5</i> # 70	14	14	0	100
<i>SSI2 eds5</i> # 121	5	5	0	100
<i>ssi2 EDS5</i> # 11	8	4	4	50
<i>ssi2 EDS5</i> # 31	6	2	4	33
<i>ssi2 eds5</i> # 1	117	103	14	88
<i>ssi2 eds5</i> # 30	41	33	8	80
<i>ssi2 eds5</i> # 45	14	14	0	100

^a Distribution of virus coat protein in the virus-inoculated plants was detected by the tissue printing method. The number of plants in which the virus had spread to the noninoculated leaves (susceptible) or was restricted to the inoculated leaves (resistant) was determined. CMV-Y at 100 µg/ml was used as the inoculum.

^b Two-week-old *Arabidopsis thaliana* plants were used. The *ssi2*, *nahG*, and *ssi2 nahG* plants are in the ecotype Nö (Nö) background and the *eds5* mutant is in the ecotype Columbia (Col) background.

significantly compromised, retains the *ssi2*-conferred cell death phenotype, strongly arguing against cell death having a direct role in the *ssi2*-conferred resistance to CMV. Likewise, the prevalence of the dwarf phenotype in the *ssi2 eds5*, *ssi1*, *acd1*, *acd2*, *cpr5*, and *ctr1* mutant plants, all of which are susceptible to CMV, argues against *ssi2*-conferred dwarfing influencing resistance to CMV.

SSI2 encodes a plastid-localized stearyl-ACP desaturase, which catalyzes the conversion of stearic acid (18:0) to oleic acid (18:1) (Kachroo et al. 2001). The *ssi2* mutation alters membrane lipid composition (Nandi et al. 2003b). It is very likely that alterations in the composition of one or more lipids may have a causal role in the *ssi2* phenotypes. Indeed, studies with the *sfd* mutants, which suppress the *ssi2*-conferred enhanced disease resistance, dwarfing, and cell death

and alter membrane lipid composition, provide support to the involvement of lipids in the *ssi2*-conferred broad-spectrum disease resistance, including resistance to CMV. Changes in fatty acid composition have been observed during the activation of HR in TMV-inoculated *Nicotiana tabacum* cv. Xanthi nc (Ruzicska et al. 1983). However, the importance of these alterations in the development of HR and resistance to virus is not known. Likewise, the lipid or lipid-derived molecule in the *ssi2* mutant involved in modulating resistance to CMV and other pathogens remains to be identified. The availability of mutants in lipid metabolism and the recent development of an electrospray ionization tandem mass spectrometry (ESI-MS/MS) technology to profile changes in membrane lipid compositions (Walti et al. 2002) will provide a better understanding of the role of lipids in resistance to pathogens.

Table 4. Systemic spread of *Cucumber mosaic virus*-yellow strain (CMV-Y) in salicylic acid (SA)-treated plants

Plant ^b	SA	Number of plants ^a			CMV-Y susceptible (%)
		Total inoculated	Susceptible	Resistant	
Wild type (Nössen)	+	25	24	1	96
Wild type (Nössen)	–	25	23	2	92
<i>ssi2</i>	–	30	12	18	40

^a Distribution of virus coat protein in the virus-inoculated plants was detected by the tissue printing method. The number of plants in which the virus had spread to the noninoculated leaves (susceptible) or was restricted to the inoculated leaves (resistant) was determined. CMV-Y at 100 µg/ml was used as the inoculum.

^b Two-week-old *Arabidopsis thaliana* plants were used. Plants were treated with 0.5 mM SA (+) or H₂O (–) before inoculation with CMV-Y.

Table 5. Systemic spread of *Cucumber mosaic virus*-yellow strain (CMV-Y) in the *ssi2 sfd* plants

Plant ^b	Number of plants ^a			CMV-Y susceptible (%)
	Total inoculated	Susceptible	Resistant	
Wild type (Nö)	20	20	0	100
<i>ssi2</i>	25	9	16	36
<i>ssi2 sfd1-1</i>	25	23	2	92
<i>ssi2 sfd4-1</i>	28	28	0	100

^a Distribution of virus coat protein in the virus-inoculated plants was detected by the tissue printing method. The number of plants in which the virus had spread to the noninoculated leaves (susceptible) or was restricted to the inoculated leaves (resistant) was determined. CMV-Y at 100 µg/ml was used as the inoculum.

^b Two-week-old *Arabidopsis thaliana* plants were used. All plants are in the ecotype Nössen (Nö) background.

Fig. 6. Systemic spread of virus in *Cucumber mosaic virus*-yellow strain (CMV-Y)-inoculated *Arabidopsis* mutants. Two lower leaves of the *nahG*, *ssi2 nahG*, and *ssi2 sfd1-1* mutant plants and the F₃ progeny of the wild-type *SSI2 EDS5* F₂ line 9, the *ssi2 EDS5* F₂ line 11, and the *ssi2 eds5* F₂ line 1 were inoculated with CMV-Y. Seven days later, the presence of viral coat protein was detected by tissue printing method: press-blotting all the plants on filter papers and probing the filters with an anti-CMV-Y coat protein probe. The blue color indicates presence of the viral coat protein.

We previously had shown that, in addition to SA, an ethylene-dependent mechanism or mechanisms and another unknown mechanism or mechanisms are required to activate the full extent of *RCY1*-conferred resistance to CMV-Y (Takahashi et al. 2002). *HRT* in the *Arabidopsis* ecotype Dijön is an *R* gene required for gene-for-gene resistance to TCV (Cooley et al. 2000; Dempsey et al. 1997). Although *HRT* in the ecotype Dijön is the ortholog of *RCY1* in the ecotype C24, the *HRT*-conferred HR and resistance to TCV is dependent on an SA-dependent signaling mechanism and does not require ethylene signaling (Kachroo et al. 2000). In addition, unlike as was shown here for CMV, the *ssi2* mutant does not exhibit enhanced resistance to TCV (Kachroo et al. 2000). These different requirements between *Arabidopsis* resistance to CMV and TCV strongly suggest that resistance to CMV and TCV require distinct signaling pathways.

MATERIALS AND METHODS

Virus and plant.

Two strains of CMV, yellow strain CMV-Y (Tomaru and Hidaka 1960) and an Indonesian isolate of CMV-B2 (Suastika et al. 1995), were propagated on tobacco (*N. tabacum* cv. Xanthi nc) and the viral particles purified as previously described (Takahashi and Ehara 1993). *A. thaliana* was grown at 25°C under continuous illumination (8,000 lux) on a mixture of vermiculite and perlite (1:1 mixture). Plants were irrigated with nutrient medium every 3 days. The *Arabidopsis* mutants *ssi1*, *ssi2*, *nahG*, *ssi2 sfd1-1*, *ssi2 sfd4-1*, and *ssi2 nahG*, which are in the ecotype Nö background (Nandi et al. 2003b; Shah et al. 1999, 2001), and the *acd1-20* (Greenberg and Ausubel 1993), *acd2-2* (Mach et al. 2001), *cpr5-2* (Bowling et al. 1997), *eds5-1* (Nawrath et al. 1999), and *ctrl-1* (Kieber et al. 1993) mutant alleles, which are in the ecotype Col background, were used in this study. To generate the *ssi2 eds5* double-mutant plant, an *eds5-1* mutant plant was crossed with the *ssi2* mutant plant. The resulting F₁ plants were allowed to self. F₂ progeny which were homozygous for the *ssi2* and *eds5-1* allele were identified. The presence of the *ssi2* allele was followed by a dCAPS (Kachroo et al. 2001) whereas presence of the *eds5-1* and *EDS5* alleles were followed by the closely linked sequence length polymorphism marker nga1107 (Nawrath and Métraux 1999) which is 2 centimorgans from the *EDS5* locus and polymorphic between the Col and Nö ecotypes. F₃ progeny derived from three independent F₂ plants of genotype *ssi2 eds5* and two independent F₂ plants of the genotypes *SSI2 eds5-1* and *ssi2 EDS5* were used for this study.

Virus inoculation and detection.

Virus inoculations were performed on 2-week-old plants as previously described (Takahashi et al. 1994). To quantify the amount of virus in CMV-inoculated leaves, CMV coat protein was detected immunologically by the modified tissue-printing method (Takahashi et al. 2002). To quantify the multiplication of virus in CMV-inoculated leaves, the amount of the coat protein in the inoculated plants was measured by ELISA (Koenig 1981). A polyclonal anti-CMV coat protein antibody was used. Six independent CMV-Y-infected plants of each genotype were homogenized in 50× volume of 0.01 M potassium phosphate buffer (pH 8.0). The protein concentration in the homogenate was determined with the Bradford reagent (Bradford 1976). The homogenate was adjusted to 0.025 mg/ml of total protein with 0.01 M potassium phosphate buffer, and then used for ELISA. The amount of coat protein was expressed as an 'arbitrary unit,' which is the absorbance at 405 nm per 0.025 mg/ml of protein.

Chemical treatment of plants.

Two-week-old *A. thaliana* ecotype Col plants were subirrigated with a solution of 0.5 mM SA in distilled water. At 24 h post treatment, leaf tissue was harvested and processed as described below to extract total RNA and analyzed by northern blots for expression of the *PR-1* gene. To analyze the activation of resistance to CMV by exogenously applied SA, plants were irrigated with a solution of 0.5 mM SA. Two days later, leaves of these SA-treated and control water-treated plants were rub-inoculated with CMV-Y at 100 µg/ml. Systemic spread of the virus was monitored 7 days after viral inoculation by the modified tissue-printing method as previously described (Takahashi et al. 2002).

Detection of defense-related gene expression.

Total RNA was isolated from the leaves of an *A. thaliana* plant by the acid guanidium-phenol-chloroform method (Chomczynski and Sacchi 1987). In all, 15 µg of total RNA from each sample was loaded on a 1.5% agarose gel. Northern hybridization was performed according to standard protocols (Sambrook and Russell 2001). A cDNA clone containing a 450-bp region of the *PR-1* gene spanning nucleotides 50 to 500 and a genomic DNA fragment of the *nahG* gene spanning the open reading frame were used to study expression of the *PR-1* gene and the *nahG* transgene, respectively. ³²P-labeled probes were prepared using the Megaprime DNA labeling systems (Amersham-Biosciences, Piscataway, NJ, U.S.A.).

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