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The *Arabidopsis* Gain-of-Function Mutant *ssi4* Requires *RAR1* and *SGT1b* Differentially for Defense Activation and Morphological Alterations

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A gain-of-function mutation in resistance (*R*) gene *SSI4* causes constitutive activation of defense responses, spontaneous necrotic lesion formation, enhanced resistance against virulent pathogens, and a severe dwarf phenotype. Genetic analysis revealed that *ssi4*-induced H_2O_2 accumulation and spontaneous cell death require *RAR1*, whereas *ssi4*-mediated stunting is dependent on *SGT1b*. By contrast, both *RAR1* and *SGT1b* are required in a genetically additive manner for *ssi4*-induced disease resistance, SA accumulation, and lesion formation after pathogen infection. These data point to cooperative yet distinct functions of RAR1 and SGT1b in responses conditioned by a deregulated nucleotide-binding leucine-rich repeat protein. We also found that *RAR1* and *SGT1b* together contribute to basal resistance because an *ssi4 rar1 sgt1b* triple mutant exhibited enhanced susceptibility to virulent pathogen infection compared with wild-type *SSI4* plants. All *ssi4*-induced phenotypes were suppressed when plants were grown at 22°C under high relative humidity. However, low temperature (16°C) triggered *ssi4*-mediated cell death via an *RAR1*-dependent pathway even in the presence of high humidity. Thus, multiple environmental factors impact on *ssi4* signaling, as has been observed for other constitutive defense mutants and *R* gene-triggered pathways.

Additional keywords: Arabidopsis, dwarfism

Nucleotide binding-leucine rich repeat (NB-LRR) proteins encoded by a major class of race-specific disease resistance (*R*) genes recognize their cognate avirulence (AVR) factors directly or indirectly and trigger a hypersensitive response (HR) and innate immunity (Jones and Dangl 2006). Based on their N-terminal sequences, NB-LRR proteins can be divided into two subgroups, one sharing homology to Drosophila Toll protein and mammalian interleukin-1 receptors (the TIR domain) and the other containing a coiled-coil (CC) motif. Of 149 *NB-LRR* genes identified in the *Arabidopsis* genome, 83 belong to the TIR-NB-LRR subgroup, 51 to the CC-NB-LRR subgroup, and the remainder either lack TIR or CC homology or contain additional domains (Meyers et al. 2003). By contrast, none of the 480 *NB-LRR* genes identified in the rice genome have a TIR structure (Zhou et al. 2004b).

Identification of the *ssi4* (*suppressor of salicylic acid insensitive*4) mutant that contains a gain-of-function mutation in a *TIR-NB-LRR*-type *R* gene provided an opportunity to study signal transduction and phenotypic changes caused by *R* gene activation (Shirano et al. 2002; Zhou et al. 2004a). The *ssi4* plants display many characteristics of constitutively active defense mutants, such as elevated expression of pathogenesis-related (*PR*) genes, broad-spectrum resistance to virulent pathogens, spontaneous necrotic lesion formation, and a severe dwarf morphology (Shirano et al. 2002). Epitasis analysis indicated that *ssi4*-induced *PR* gene expression and disease resistance is salicylic acid (SA) and *EDS1* dependent but *NPR1* and *NDR1* independent (Shirano et al. 2002). When grown under standard conditions, *ssi4* accumulates elevated H_2O_2 and SA levels prior to lesion formation and displays constitutive activation of the MAP kinases AtMPK6 and AtMPK3 (Zhou et al. 2004a). All *ssi4*-induced responses are suppressed by high relative humidity (HRH; 95%) (Zhou et al. 2004a), raising the possibility that a humidity-sensitive factor (HSF) functions at an early point in the *ssi4* signaling pathway.

To unravel further *ssi4* signaling processes leading to disease resistance and developmental defects, we assessed the involvement of two known plant defense regulators, *RAR1* and *SGT1*. *RAR1* encodes a small protein with two cysteine and histidine-rich, zinc-binding domains (CHORD I and II) that was first identified as a rate-limiting positive regulator of multiple *R* gene-triggered responses (Liu et al. 2002a; Muskett et al. 2002; Shirasu et al. 1999; Tornero et al. 2002). *SGT1* was characterized in yeast as a factor required for kinetochore assembly and SKP1/Cullin1/F-box (SCF) E3 ubiquitin ligase activity through interaction with SKP1 and the molecular chaperone HSP90 (Catlett and Kaplan 2006; Kitagawa et al. 1999). Accordingly, SGT1 has domains resembling the fold structures of two HSP90 co-chaperones Sti1/Hop and p23 (Garcia-Ranea et al. 2002; Muskett and Parker 2003). SGT1 proteins from yeast, humans, and plants are highly sequence related and the ability of plant or human SGT1 to complement cell-cycle defects in yeast *sgt1* mutants suggests that their bio-

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logical properties are conserved (Azevedo et al. 2002; Kitigawa et al. 1999). In *Arabidopsis*, two highly similar *SGT1* genes (*SGT1a* and *SGT1b*) are expressed that, together, are essential for early development (Azevedo et al. 2006). Although only *SGT1b* has been implicated genetically in plant *R* gene-triggered responses, overexpression of *SGT1a* complements an *sgt1b* null mutant, suggesting that both *Arabidopsis* SGT1 proteins are involved in signaling resistance (Azevedo et al. 2006).

The precise modes of action for RAR1 and SGT1 in plants are unclear. However, both RAR1 and SGT1 interact transiently with HSP90 (Hubert et al. 2003; Liu et al. 2004; Takahashi et al. 2003), suggesting that these proteins assist the assembly of functional R protein complexes. Consistent with this possibility, RAR1 is needed to stabilize the NB-LRR proteins MLA1 and MLA6 (barley) and RPM1 (*Arabidopsis*) in their preactivation states (Bieri et al. 2004; Holt et al. 2005; Hubert et al. 2003; Tornero et al. 2002) and SGT1 stabilizes Rx in tobacco (Azevedo et al. 2006). Genetic analyses established that certain *R* genes require either *SGT1b* or *RAR1* to signal resistance (Muskett and Parker 2003). For certain *R* genes, combined *rar1* and *sgt1b* mutations were genetically additive or antagonistic (Austin et al. 2002; Azevedo et al. 2002; Holt et al. 2005). These data imply that RAR1 and SGT1 have distinct but potentially interactive functions in resistance signaling that may depend on the accumulation and folding characteristics of particular NB-LRR proteins.

Plant RAR1 and SGT1 also were shown to interact with subunits of the COP9 signalosome, a multiprotein complex that regulates ubiquitin-proteasome-mediated degradation (Azevedo et al. 2002; Liu et al. 2002b). In addition, barley SGT1 interacts with SKP1 and CUL1, two core subunits of the SCF E3 ligase, another complex involved in protein degradation (Azevedo et al. 2002). Silencing *NbSKP1* or the *NbCOP9* signalosome in *Nicotiana benthamiana* compromised *N*-mediated resistance to *Tobacco mosaic virus* (TMV); therefore, ubiquitin-mediated protein degradation appears to play a role in plant defense, possibly by targeting a negative regulator for degradation (Liu et al. 2002b). Furthermore, the observation that SGT1 interacts with RAR1, SKP1, and CUL1 whereas RAR1 interacts only with SGT1 suggests that SGT1 functions independently of RAR1 via its interaction with the SCF E3 ligase (Azevedo et al. 2002). Indeed, *SGT1b* but not *RAR1* is required for the activities of the *Arabidopsis* SCF E3 ligases SCF^{-TIR1} and SCF^{-COI1} that regulate responses to the phytohormones auxin and jasmonic acid, respectively (Gray et al. 2003). Together, these data suggest that SGT1 works in conjunction with RAR1 to mediate R protein complex assembly or maturation but also suggest additional functions of SGT1 in regulating the ubiquitination machinery that may, for example, remove a negative regulator of R protein function or promote downstream signal transduction.

In this study, we investigated the roles of *RAR1* and *SGT1b* in *ssi4*-mediated disease resistance, cell death, and development. Our results reveal overlapping yet distinct functions of *RAR1* and *SGT1b* in signaling from a deregulated NB-LRR protein. This distinction is particularly evident in *ssi4*-induced developmental changes that require *SGT1b* but not *RAR1* and resemble those modulated by the phytohormone auxin.

RESULTS

SGT1b but not *RAR1* is necessary for the *ssi4*-conditioned dwarf phenotype.

To test whether *RAR1* and *SGT1b* are required for *ssi4*-mediated signaling, *ssi4 rar1* and *ssi4 sgt1b* double mutants were generated by crossing *ssi4* (Nö) with two null mutants, *rar1-10* (L*er*) and *sgt1b-1* (L*er*), and selecting genetic combinations from segregating F_2 populations using gene-specific polymerase chain reaction (PCR)-based markers (discussed below). Because the *erecta (er)* mutation in L*er* affects plant morphology, morphological characterization was performed in two subgroups of mutants that contained either the *ER* or *er* allele. When grown under standard conditions (22°C and 50 to 70% relative humidity [RH]), the phenotype of *ssi4 rar1 ER* plants, like that of *ssi4 ER*, was stunted. By contrast, *ssi4 sgt1b ER* double mutants were almost indistinguishable from *SSI4 ER* (Fig. 1A, B, and C). Measurement of the height of 4-week-old *ER*-containing double mutants revealed that *ssi4 rar1 ER* plants were as stunted as *ssi4 ER*, whereas *ssi4 sgt1b ER* were only slightly smaller than *SSI4 ER* (Fig. 1D). Consistent with their respective sizes, the length and width of the fifth leaf of *ssi4 rar1 ER* plants were as reduced as those of *ssi4 ER*, whereas the leaves on *ssi4 sgt1b ER* were nearly as large as those of *SSI4 ER* (Fig. 1D). In the *er* background, the size of *ssi4 sgt1b er* plants was indistinguishable from *rar1 er* or *sgt1b er*, whereas *ssi4 rar1 er* plants were stunted and the length and width of their leaves dramatically reduced compared with *rar1 er* or *sgt1b er* (Fig. 1C; data not shown). Thus, *sgt1b* substantially suppresses *ssi4*-induced stunting, whereas *rar1* does not. When *ssi4 rar1 ER* plants were grown at HRH, a condition that suppresses all *ssi4* (Nö) phenotypes (Zhou et al. 2004a), stunting was reversed (Fig. 1E), confirming that the dwarfism was due to the presence of *ssi4*.

RAR1 but not *SGT1b* is required for *ssi4*-induced H_2O_2 production and spontaneous cell death.

In addition to their small stature, *ssi4* plants developed spontaneous necrotic lesions on cotyledons and true leaves. Visual inspection of *ssi4 rar1* double mutants revealed that these plants did not form lesions although their leaves were chlorotic (Fig. 2A). By contrast, lesions were visible on fully expanded leaves of *ssi4 sgt1b* double mutants, although their appearance was delayed approximately 3 to 5 days compared with those on *ssi4* plants. Lesion formation triggered by *ssi4* was not affected by either *ER* or *er*. To determine whether spontaneous lesion formation was fully suppressed in *ssi4 rar1*, cell death (monitored by trypan blue staining) and H_2O_2 production (by 3,3′-diaminobenzidine [DAB] staining) were examined microscopically. No cell death was detected in the leaves of *rar1*, *sgt1b*, and *ssi4 rar1* mutants or the two wild type controls, *SSI4* and L*er*. By contrast, leaves of the *ssi4 sgt1b* double mutant and the *ssi4* single mutant exhibited similar levels of cell death and H_2O_2 accumulation once necrotic lesions became visible (Fig. 2B and C). Based on these results, we concluded that *RAR1* is required for *ssi4*-mediated cell death, whereas *SGT1b* is not.

In the course of these experiments, we found that low temperature (16°C) induces *ssi4*-mediated cell death, SA accumulation, and *PR* gene expression even when the plants are grown in HRH, which otherwise would suppress all *ssi4* phenotypes (Fig. 2D and E; data not shown) (Zhou et al. 2004a). Low temperature combined with HRH failed to induce cell death in wild-type or *ssi4 rar1* plants but did so in *ssi4 sgt1b* (Fig. 2D and E). This finding further supports the conclusion that *ssi4*-mediated cell death requires *RAR1* but not *SGT1b*. Analysis of 10 independent lines for each double mutant over four generations revealed that the differences between *ssi4 rar1* and *ssi4 sgt1b* phenotypes were robust and heritable.

The stunted phenotype of *ssi4 rar1* is largely due to a reduction in cell number.

The reduced stature of *ssi4* and *ssi4 rar1* plants (Fig. 1) could be caused by a reduction in cell number, cell size, or both. To exclude distortions due to spontaneous cell death, mi-

croscopic analysis was performed on wild-type (*SSI4 RAR1 ER*) and *ssi4 rar1 ER* plants that, unlike the *ssi4* single mutant, failed to form spontaneous lesions. Examination of the same square area of leaves from *ssi4 rar1 ER* and wild-type plants revealed that the epidermis of *ssi4 rar1 ER* double mutants contained slightly more cells that were smaller than those in the epidermis of wild-type leaves. In addition, the guard cells in *ssi4 rar1 ER* were approximately 30% smaller than those in the wild type (Fig. 3A). This decrease in cell size is unlikely to account for the large difference in leaf size between wild-type and *ssi4 rar1 ER* plants (Figs. 1 and 3A). Therefore, we investigated whether the number of cells in *ssi4 rar1 ER* leaves was reduced by examining cross-sections of the stem at comparable locations. Analysis of *ssi4 rar1 ER* stems revealed a dramatic reduction in cell number and a slight reduction in cell size compared with the wild type (Fig. 3B and C). Additionally, the stems of *ssi4 rar1 ER* plants contained seven vascular bundles, whereas those of the wild type contained eight, and the number of cells in the primary phloem and xylem was dramatically reduced (Fig. 3B and C). Analysis of pollen grains in wild-type versus *ssi4 rar1 ER* plants revealed that their size was comparable, but the number of pollen grains per anther was largely reduced (Fig. 3D; data not shown). Taken together, these results suggest that the dwarf phenotype of *ssi4 rar1 ER* plants, and by extension *ssi4* plants, results mainly from a reduction in cell number.

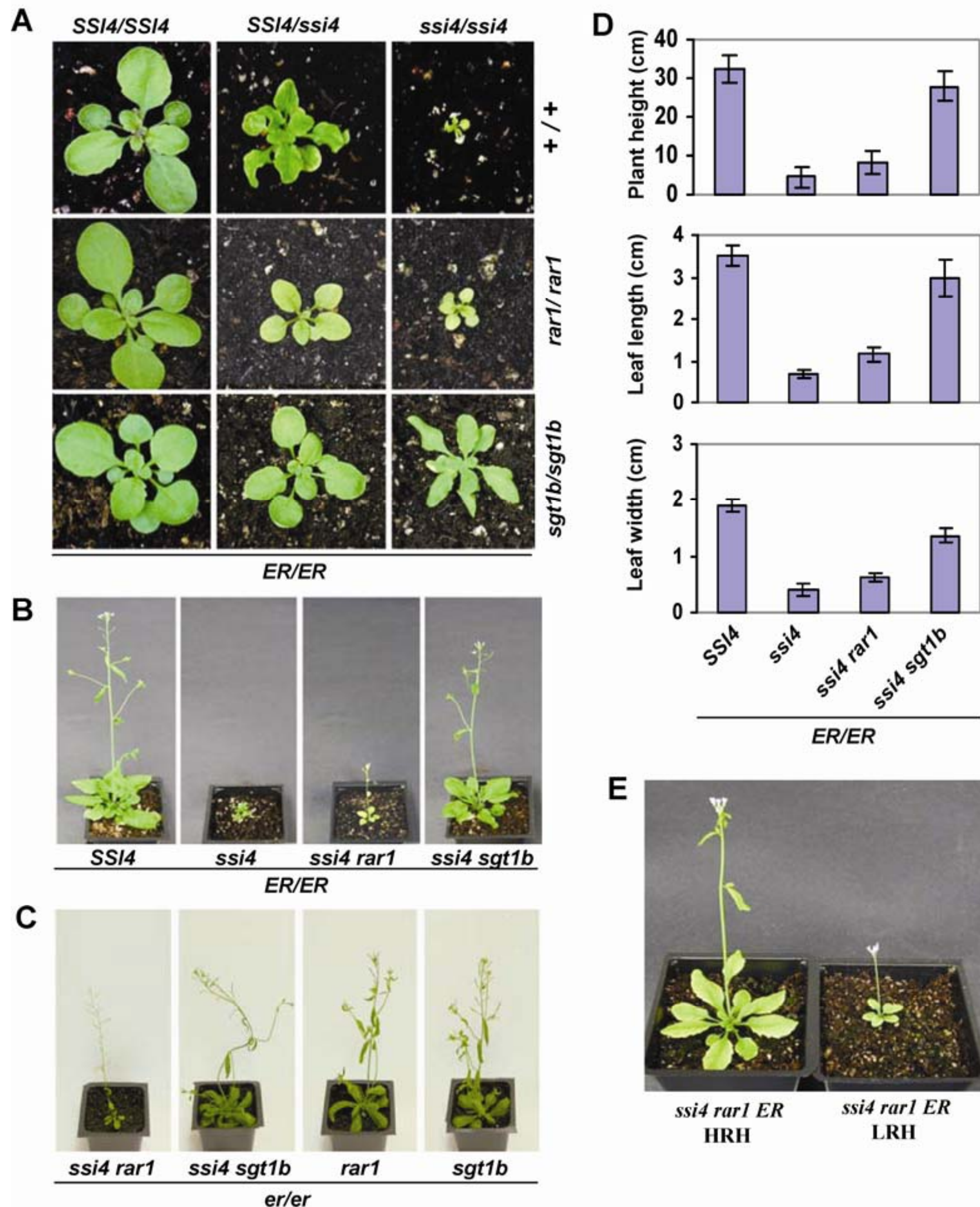


Fig. 1. Stunting induced by *ssi4* requires *SGT1b* but not *RAR1*. **A,** Photographs of representative 4-week-old plants showing the effect of *rar1* or *sgt1b* on the morphology of *ssi4* or *SSI4* plants containing the *ER* allele. The *ssi4 rar1 ER* plants were as stunted as *ssi4 ER* plants, although their leaves were lighter green, and *ssi4 sgt1b ER* plants were nearly the same in size as *SSI4 ER*, but retained wrinkled leaves. All plants were from F_2 populations. **B,** Representative *SSI4*, *ssi4, ssi4 rar1*, and *ssi4 sgt1b* plants in the *ER* background were from F_2 populations and photographed at 8 weeks of age. **C,** Representative *ssi4 rar1*, *ssi4 sgt1b, rar1*, and *sgt1b* plants in *er* background were from F_2 populations and photographed at 8 weeks of age. **D,** Quantitative measurement of the height of 12-week-old plants as well as the length and width of their fifth leaves at 4 weeks of age. The histogram represents the mean ± standard deviation of 20 plants for each genotype with the same genetic background as in B. **E,** Representative plants of *ssi4 rar1 ER* grown in high relative humidity (HRH) (95%) or moderate RH (60%), showing that HRH suppresses the dwarf phenotype of *ssi4 rar1 ER* but does not change leaf color.

The *ssi4 rar1 sgt1b* mutant displays hypersusceptibility to an oomycete pathogen.

We assessed whether *RAR1* and *SGT1b* play roles in *ssi4*-mediated resistance to *Hyaloperonospora parasitica* isolate EMCO5, a pathogen that is virulent on wild-type (Nö) plants but avirulent on *ssi4* (Nö) (Shirano et al. 2002). Because L*er* plants contain the *RPP8* gene which confers *RAR1*- and *SGT1b*-independent resistance to EMCO5 (Muskett and Parker 2003), we used a cleaved amplified polymorphic sequence (CAPS) marker for *RPP8* to select *ssi4 rar1* and *ssi4 sgt1b* double mutants that did not carry *RPP8*. Three *ssi4 rar1* and three *ssi4 sgt1b* lines without *RPP8* were inoculated with EMCO5. Microscopic analysis of 10-day-old and 4-week-old plants revealed that *ssi4 rar1* and *ssi4 sgt1b* double mutants supported similar levels of hyphal growth and sporangiophore development that were significantly greater than the levels observed on comparable *ssi4* single mutants but lower than those exhibited by *SSI4* plants (Fig. 4A and B). The 10-day-old and 4-week-old plants of the *ssi4 rar1* and *ssi4 sgt1b* double-mutant lines also developed similar levels of host cell death after infection, although this response was less severe than that of similarly inoculated *ssi4* single mutants (Fig. 4C). By contrast, no host cell death was detected in *SSI4* plants at 7 days after infection.

To assess whether the suppressive effect of *rar1* or *sgt1b* on *ssi4*-mediated resistance is additive, we generated *ssi4 rar1 sgt1b* triple mutant lines in an *RPP8*-deficient background by crossing susceptible *ssi4 rar1* and *ssi4 sgt1b* double mutants and genotyping the progeny using *rar1*- and *sgt1b*-specific PCR markers. The triple mutants were morphologically indistinguishable from *SSI4* plants. Both dwarfism and necrotic lesion phenotypes caused by *ssi4* were completely suppressed. Analysis of *ssi4 rar1 sgt1b* triple mutant lines in response to EMCO5 inoculation revealed that these were substantially more susceptible to the pathogen than either double-mutant line. The triple mutants supported more than twofold greater sporangiophore development than *SSI4* plants and failed to develop an HR (Fig. 4B and C). The extreme susceptibility of *ssi4 rar1 sgt1b* plants suggests that *RAR1* and *SGT1b* are needed not only for *ssi4*-mediated resistance but also for basal resistance to virulent pathogens.

***ssi4*-induced SA accumulation and defense gene expression require *RAR1* and *SGT1b*.**

Previously, it was demonstrated that *ssi4* accumulates elevated levels of the defense signaling hormone SA and constitutively expresses several classes of defense genes (Zhou et al. 2004a).

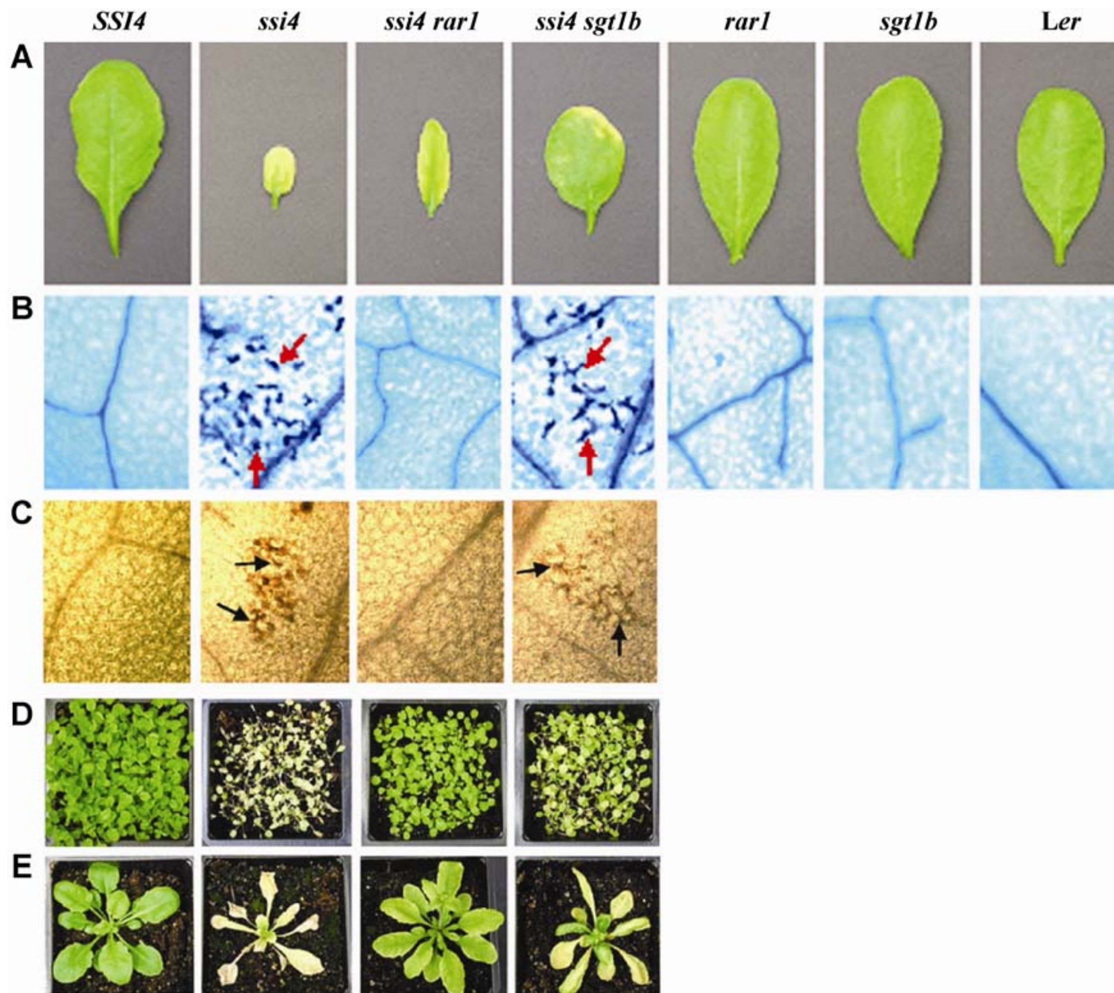


Fig. 2. H_2O_2 production and spontaneous cell death in *SSI4*, *ssi4*, and the *ssi4 rar1* and *ssi4 sgt1b* double mutants. **A,** The fifth leaf of 4-week-old plants grown under moderate relative humidity (RH) (60%) at 22°C was photographed. Note the necrotic lesions on leaves from *ssi4* and *ssi4 sgt1b* plants but not *ssi4 rar1*, although the latter had yellowish leaf color. All plants, except for L*er*, were F_2 population. **B,** Trypan blue staining of similar leaves as in A revealed mesophyll cell death in *ssi4* and *ssi4 sgt1b* plants, as indicated by arrows. **C,** 3,3′-Diaminobenzidine staining of similar leaves as in A revealed H_2O_2 accumulation in *ssi4* and *ssi4 sgt1b* plants, as indicated by the dark-brown regions noted by arrows. **D,** Ten-day-old plants grown in high relative humidity (HRH) (95%) conditions at 22°C were shifted to 16°C at HRH and photographed 5 days after the temperature shift. **E,** Six-week-old plants were shifted from 22°C at HRH to 16°C at HRH and photographed 5 days after the temperature shift.

To assess whether the *rar1* or *sgt1b* mutations affect these *ssi4*-induced responses, SA levels were monitored in *ssi4 rar1* and *ssi4 sgt1b* double mutants, an *ssi4 rar1 sgt1b* triple mutant, and parental lines. As expected, *ssi4* single mutants accumulated approximately 18-fold more SA and approximately 90-fold more SA glucoside (SAG) than wild-type plants, *SSI4* (Nö), or *SSI4* (L*er*) (Fig. 5A and B). SA and SAG levels were substantially reduced in *ssi4 sgt1b* double mutants, although they were still 5- and 15-fold greater than the levels in the wild type, respectively. Even lower levels of SA and SAG were observed in *ssi4 rar1* double mutants, although these also were elevated (approximately three- and sixfold, respectively) over wild-type plants. By contrast, SA and SAG levels in the *ssi4 rar1 sgt1b* triple mutant were similar to the wild type. Analysis of *rar1* and *sgt1b* single and double mutants in the *SSI4* background revealed no change in SA or SAG levels compared with wild-type L*er*.

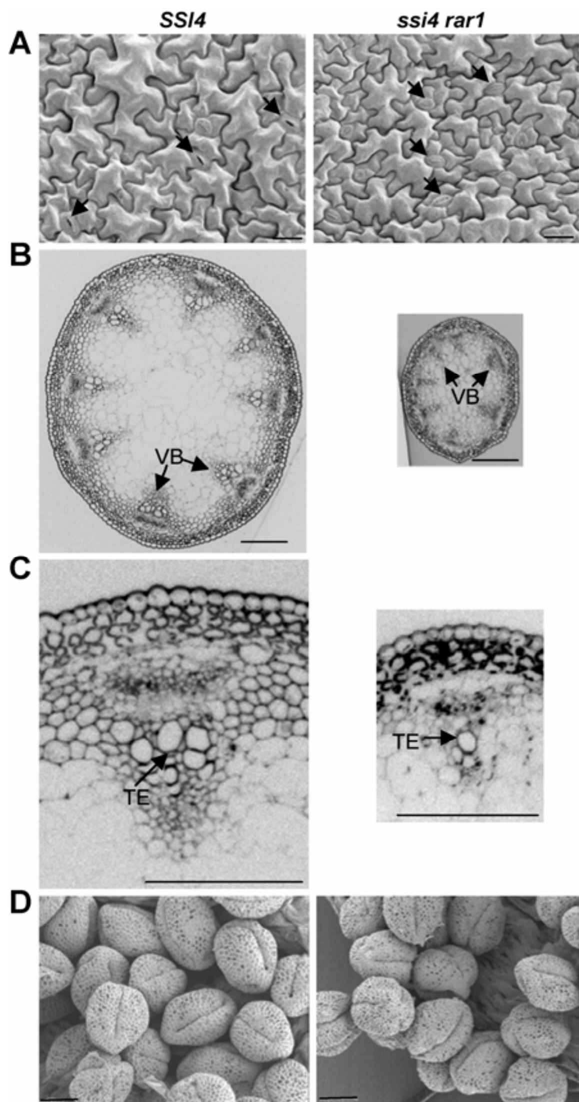
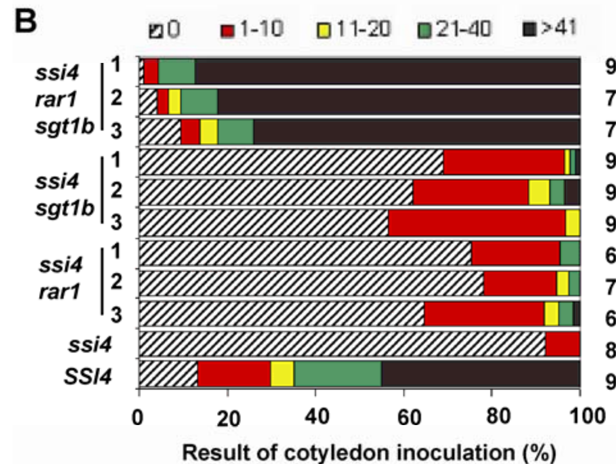


Fig. 3. Anatomical characteristics of cell morphology in different organs of wild-type and *ssi4 rar1 ER* plants. **A,** Scanning electron microscopic images of the epidermal cells of rosette leaves from 4-week-old *SSI4 RAR1 ER* and *ssi4 rar1 ER* plants. The bar represents 20 µm and the arrows point to stomata. **B,** Light microscopic images of a cross section of the stem at the second internode from the bottom of 8-week-old *SSI4 RAR1 ER* and *ssi4 rar1 ER* plants. The bar represents 100 µm and VB = vascular bundle. **C,** Close-up view of the stem cross sections showing epidermal cells and a vascular bundle. The bar represents 100 µm and TE = tracheary element. **D,** Scanning electron microscopic images of pollen grains from *SSI4 RAR1 ER* and *ssi4 rar1 ER* plants. The bar represents 10 µm.



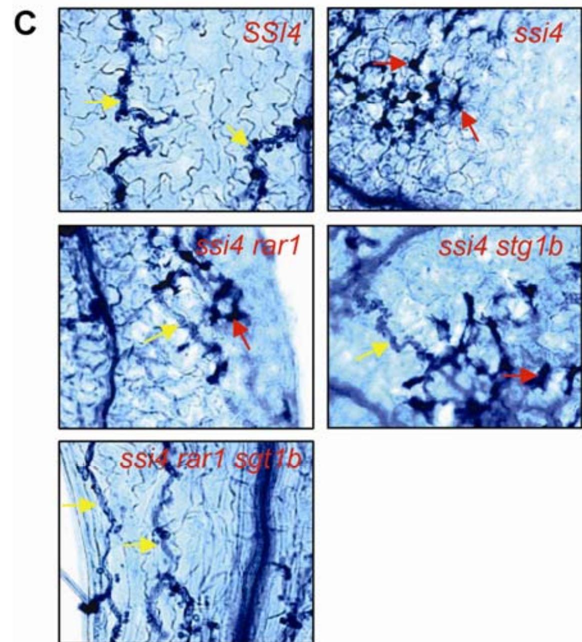


Fig. 4. *RAR1* and *SGT1b* function synergistically to mediate *ssi4*-induced resistance to *Hyaloperonospora parasitica.* **A,** Plants (4 weeks old) of various genotypes from F_3 lines were spray inoculated with *H. parasitica* EMCO5 (10^5 spores/ml). At 7 days postinoculation (dpi), representative leaves were photographed. **B,** Resistance to *H. parasitica* EMCO5 was quantitated in *SSI4* plants and in *ssi4* single, double, and triple mutants by counting the number of sporangiophores on cotyledons of 10-day-old seedlings collected at 7 dpi. The cotyledons were grouped into five categories based on the number of sporangiophores detected. The percentage of each category was calculated based on examination of approximately 60 to 100 cotyledons (the number examined is shown to the right of each bar). **C,** Trypan blue staining of infected cotyledons to reveal hyphal growth (as indicated by yellow arrows) and mesophyll cell death (as indicated by red arrows). Please note that the hyphae have normal morphology. However, in order to capture the hyphae and dead host cells in the same view, the hyphae or dead host cells are somewhat out of focus and, therefore, hyphae beads-on-a-string morphology is not apparent.

To investigate the correlation between SA accumulation and defense gene expression, *PR-1* transcript levels were monitored by RNA gel blot analysis in all genotypes used for SA measurements. The *ssi4*-induced *PR-1* expression was reduced substantially in the presence of *rar1* or *sgt1b*, although *PR-1* transcript levels in *ssi4 sgt1b* were higher than in wild-type plants (Fig. 5C). RNA gel blot analysis of additional defense genes, including *PR-2*, *PR-5*, *RPW8.1 RPW8.2*, *WRKY6, WRKY29*, and *EDS1,* similarly revealed that their *ssi4*-induced expression was reduced or fully suppressed by the loss of *RAR1* and/or *SGT1b* (Fig. 5D). Mutations in either *RAR1* or *SGT1b* substantially reduce *ssi4*-induced SA accumulation and defense gene expression; therefore, we concluded that both genes are required for full activation of these responses in the *ssi4* mutant.

DISCUSSION

Previous studies have demonstrated that *RAR1* and *SGT1b* are required for disease resistance mediated by various plant *R* genes in response to corresponding pathogen effectors (Austin et al. 2002; Azevedo et al. 2002; Holt et al. 2005; Liu et al. 2002a; Muskett et al. 2002; Shirasu et al. 1999; Tör et al. 2002; Tornero et al. 2002). Here, we show that *RAR1* and *SGT1b* are as important for defense activation and developmental alterations conditioned by a deregulated NB-LRR protein, ssi4. Thus, ssi4 (behaving as activated TIR-NB-LRR protein) cannot override many *sgt1b* and *rar1* defects that are associated with the regulation of properly constrained NB-LRR receptors. Although *SGT1b* and *RAR1* have cooperative functions in *ssi4*-induced pathogen resistance, they have distinct roles in conditioning developmental changes and triggering cell death, respectively (Figs. 2 and 3). These findings raise the question of whether RAR1 and SGT1 are needed only in the assembly of preexisting NB-LRR complexes or have broader functions during NB-LRR activation or downstream signaling. It is likely that a certain threshold of ssi4 protein needs to be attained to induce constitutive resistance, as is the case for authentically activated plant NB-LRR receptors (Azevedo et al. 2002; Bieri

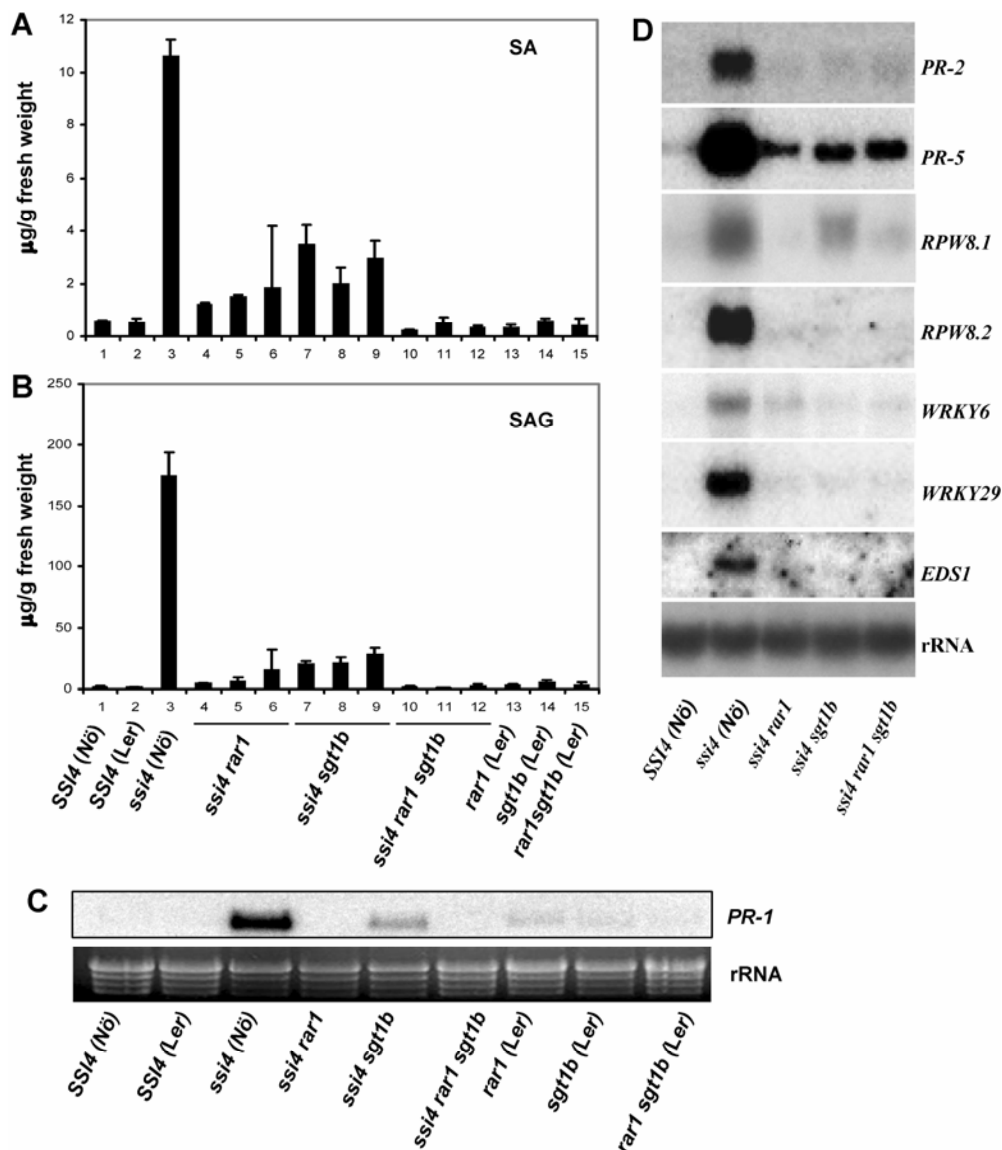


Fig. 5. Salicylic acid (SA) accumulation and defense gene expression in wild-type and *ssi4* single, double, and triple mutants. **A,** Free SA content in 4-week-old plants grown in moderate relative humidity (RH) (60%). The histogram represents the mean ± standard deviation (SD) of four independent samples. Three different lines were analyzed for the double and triple mutants. **B,** The SA glucoside (SAG) content in the same tissue as in A. The histogram represents the mean ± SD of four independent samples. **C,** Northern analysis of *PR-1* transcripts in wild-type and mutant lines grown at 60% RH; 10 µg of total RNA was loaded on each lane and ethidium bromide-stained rRNA was used as a control for loading. **D,** Northern analysis of transcripts for several defense genes in wild-type and mutant lines grown at 60% RH; 10 µg of total RNA was loaded on each lane and rRNA was used as a control for loading.

et al. 2004; Holt et al. 2005). The observation that *ssi4* is a semi-dominant mutation is consistent with a threshold model (Azevedo et al. 2006) and, therefore, both RAR1 and SGT1 may assist ssi4 protein assembly. High levels of *ssi4* transcripts detected in *ssi4* homozygous plants (Shirano et al. 2002) may explain why *ssi4*-mediated resistance is only partially dependent on *RAR1*. An alternative explanation is that RAR1 or SGT1 function after NB-LRR protein activation. Activities of RAR1 and SGT1b at different points of *ssi4*-conditioned signaling could account for the uncoupling of *rar1* and *sgt1b* phenotypes observed in this study. The differential recruitment of *SGT1b* in conditioning developmental alterations and *RAR1* in triggering cell death is more difficult to explain by quantitative differences in ssi4 protein accumulation in these mutants. In transient *N. benthamiana* expression assays, SGT1 was necessary to generate intramolecular folding intermediates of the pepper Bs2 NB-LRR protein that may be important for receptor activation (Leister et al. 2005). It also is notable that analyses of two mammalian NB-LRR receptors, Nod1 and Nod2, suggest a role of SGT1 in receptor signaling leading to the activation of downstream immune responses (da Silva Correia et al. 2007; Mayor et al. 2007).

Both *SGT1b* and *RAR1* were required for *ssi4*-mediated resistance to *H. parasitica* isolate EMCO5, as well as for pathogen-induced HR development, SA accumulation, and defense gene expression. Loss of *SGT1b* or *RAR1* in the *ssi4* background diminished all these responses (with the exception of defense gene expression) to a level that was intermediate between that observed in *ssi4* single mutants and *ssi4 sgt1b rar1* triple-mutant plants. Thus, RAR1 and SGT1b appear to have distinct activities that work cooperatively to activate most *ssi4*-induced defense responses. Genetic analyses in *Arabidopsis* and barley suggested that *RAR1* and *SGT1* also work cooperatively to signal *RPP5*-mediated resistance to *H. parasitica* isolate Noco2 and *Mla6*-mediated resistance to powdery mildew (Austin et al. 2002; Azevedo et al. 2002). In addition to mediating *R* gene-triggered defenses, our results show that *RAR1* and *SGT1b* are required for basal resistance to a virulent *H. parasitica* (Fig. 4). In contrast to wild-type *SSI4* plants that are susceptible to EMCO5 and display high levels of sporangiophores on approximately 45% of their cotyledons, *ssi4 rar1 sgt1b* triple mutants were hypersusceptible, supporting high-level sporulation on approximately 80% of their cotyledons (Fig. 4). Previous analyses of *rar1 sgt1* double mutants in *Arabidopsis* did not detect enhanced susceptibility over that associated with loss of the corresponding *R* gene (Austin et al. 2002; Azevedo et al. 2002; Holt et al. 2005). However, hypersusceptibility to a virulent bacterial pathogen was observed in *rar1* single-mutant *Arabidopsis* (Holt et al. 2005). Also, silencing of *SGT1* in *N. benthamiana* led to increased replication of TMV and *Potato virus X* over that detected in plants silenced for either *N* or *Rx*, respectively, and abolished nonhost resistance to certain bacterial pathogens (Peart et al. 2002). Those findings, combined with our results, implicate RAR1 or SGT1 in the regulation of basal, race-specific, and nonhost resistance consistent with these responses having at least some signaling mechanisms in common. Alternatively, instability of NB-LRR proteins in *rar1* or *sgt1b* mutants as described earlier (Azevedo et al. 2002; Bieri et al. 2004; Holt et al. 2005) may account for the various forms of enhanced susceptibility in these mutants if different NB-LRR proteins are involved in basal, race-specific, or nonhost resistance.

Although both SGT1 and RAR1 are required for *R* gene-mediated and basal resistance, they appear to work separately to condition other *ssi4*-induced morphological alterations. Importantly, *ssi4*-induced stunting requires SGT1b but not RAR1. Analysis of stem cross-sections and pollen grains from *ssi4 rar1* plants suggested that the stunted phenotype is largely due to a reduction in cell number. The reduced vasculature in these plants is reminiscent of the vascular defects displayed by auxin-deficient tobacco expressing a *35S-iaaL* construct, which encodes an indoleacetic acid (IAA)-lysine synthetase that converts IAA to IAA-lysine (Romano et al. 1991). SGT1b previously was shown to be required for SCF^{TIR1}, a ubiquitin protein ligase that functions as a key regulator of the auxin response in *Arabidopsis* (Gray et al. 2003). Thus, SGT1b may mediate *ssi4*-induced stunting by altering auxin signaling.

By contrast, *ssi4*-induced spontaneous cell death was abolished in the absence of *RAR1* but not *SGT1b*. However, spontaneous lesion formation was delayed by several days in *ssi4 sgt1b* mutants compared with *ssi4* plants, suggesting that *SGT1b* influences the timing of cell death. Other studies revealed activities of RAR1 or SGT1 in *R* gene-mediated HR development after pathogen infection (Austin et al. 2002; Freialdenhoven et al. 1994; Holt et al. 2005; Muskett et al. 2002; Peart et al. 2002; Shirasu et al. 1999; Tornero et al. 2002). Because spontaneous cell death shares many characteristics with pathogen-induced HR (Dietrich et al. 1994), both phenomena are thought to be activated via the same signaling pathway. The discovery that *RAR1* is indispensable for *ssi4*-induced spontaneous cell death but a quantitative factor in pathogen-induced HR development could be explained by the fact that they are different processes or, more likely, that they are influenced by the extent of accumulation and competence of the NB-LRR protein involved.

Constitutive activation of defense responses frequently causes two closely associated phenotypes: spontaneous cell death leading to necrotic lesion formation and inhibition of growth resulting in a dwarfism. These have been described in several *Arabidopsis* mutants, such as *cpr22*, *ssi2*, *cpn1/bon1*, and others (Hua et al. 2001; Jambunathan et al. 2001; Kachroo et al. 2001; Yoshioka et al. 2001). The generation of a large quantity of reactive oxygen species (ROS), know as oxidative burst, is an early event in *R* gene-mediated defense signaling which was believed to trigger necrotic lesion formation and a dwarf phenotype. Consistent with this, we found that growth of *ssi4* under HRH conditions abrogated not only H_2O_2 accumulation but also lesion formation and dwarfism (Zhou et al. 2004a). One explanation is that the toxic effect of ROS causes cell death and growth inhibition. However, such a scenario recently has been ruled out in the *flu* mutant by the identification of a suppressor, *executer1* (Wagner et al. 2004), which reverses *flu*-mediated cell death and dwarfism but did not suppress singlet oxygen production. This result suggests that *flu* triggers cell death and dwarfism by activation of an intrinsic signaling pathway involving *EXECUTER1* rather than via the direct damaging effect of ROS. Because both dwarfism and cell death in *flu* are suppressed by *executer1*, researchers were unable to separate the two phenotypes. In this study, we separated *ssi4*-mediated cell death from its dwarf phenotype by introducing *rar1* or *sgt1b* into the *ssi4* background. Analyses of *ssi4 rar1* and *ssi4 sgt1b* revealed that *ssi4* triggers necrotic lesion formation and dwarfism through two distinct signaling pathways or branches, which require *Rar1* and *Sgt1b*, respectively. The *ssi4*-mediated signaling via *SGT1b* leads to dwarfism, whereas signaling via *RAR1* triggers lesion formation.

H_2O_2 accumulation prior to lesion formation in *ssi4 sgt1b* suggests that H_2O_2 may be a signaling component for *ssi4*-triggered cell death. This is consistent with suppression of both H_2O_2 accumulation and cell death in *ssi4 rar1*. In contrast, retention of the dwarf phenotype in *ssi4 rar1* argues that H_2O_2 accumulation is not involved in triggering dwarfism. However, it is possible that a small, not readily detectable, increase in H_2O_2 in *ssi4 rar1* leads to dwarfism.

Previous analysis revealed that all *ssi4* phenotypes are completely suppressed by high-humidity conditions, and an HSF was proposed to regulate an early step in the *ssi4* pathway (Zhou et al. 2004a). Here, we demonstrate that *ssi4*-induced spontaneous cell death, SA accumulation, and *PR* gene expression are triggered by low temperature (LT) even in plants grown under HRH conditions. LT-induced spontaneous cell death required *RAR1*, although the presence of *SGT1b* influenced the timing. Thus, LT appears to activate *ssi4*-induced spontaneous cell death via the same pathway as in plants grown at normal temperatures, possibly by restoring HSF activity. High humidity suppresses constitutive resistance activated by other mutations, as well as *R* gene-induced defenses in *Arabidopsis* and tomato, consistent with the notion that these pathways all are regulated by one or more HSFs (Hammond-Kosack et al. 1996; Jambunathan et al. 2001; May et al. 1996; Weymann et al. 1995; Xiao et al. 2003; Yoshioka et al. 2001). Strikingly, high-humidity suppression of the *cpn1/bon1* induced spontaneous lesion formation; enhanced disease resistance, constitutive *PR* gene expression, and stunting; and was overridden by LT (Jambunathan and McNellis 2003; Yang and Hua 2004). CPN1/BON1 is a negative regulator of SNC1, a homolog of the TIR-NB-LRR R protein RPP5 (Yang and Hua 2004; Zhang et al. 2003). Thus, the constitutive activation of defense responses in *cpn1/bon1* plants, like that in *ssi4* plants, is caused by deregulated activity of an R protein. HRH conditions also suppress resistance and spontaneous lesion formation conferred by overexpression of the powdery mildew resistance genes *RPW8.1* and *RPW8.2* (Xiao et al. 2003). These genes encode small, basic proteins with a putative N-terminal transmembrane domain and a CC domain but no NB or LRR. Although the effect of LT on the *RPW8.1* and *RPW8.2* overexpression phenotype is not known, transcripts for these genes accumulate to high levels in the *ssi4* mutant in a humidity-sensitive manner (Zhou et al. 2004a). Establishing that the ssi4, SNC1, RPW8.1, and RPW8.2 signaling pathways are dependent on SA and EDS1 and that their corresponding *R* genes are positively regulated by SA via a feedback loop (Li et al. 2001; Shirano et al. 2002; Xiao et al. 2003; Yang and Hua 2004) suggests that a common mechanism underlies environmental control of these defense pathways.

MATERIALS AND METHODS

Plant materials and growth conditions.

Arabidopsis rar1-10 and *sgt1b-1* mutants (L*er* background) were described previously (Austin et al. 2002, Muskett et al. 2002). To generate *ssi4 rar1* and *ssi4 sgt1b* double mutants, *ssi4* (Nössen [Nö] background) was crossed reciprocally with *rar1-10* and *sgt1b-1*; *ssi4* also was crossed with L*er* as a control. F_1 plants in all crosses showed a phenotype similar to heterozygous *SSI4/ssi4* plants. F_2 progeny were genotyped using CAPS markers specifically designed for *ssi4*, *rar1-10*, or *sgt1b* (Austin et al. 2002; Muskett et al. 2002; Shirano et al. 2002). To screen for the loss of *RPP8* in *ssi4 rar1, ssi4 sgt1b*, and *ssi4 rar1 sgt1b* plants, an approximately 375-bp fragment was amplified using the forward primer 5′-ACGAACATATAACCT GATGATTCAC-3′ and the reverse primer 5′-CTTAGATTAGC TCATGATCTTATAC-3′ and digested with *Fnu*4HI. This generated either a 375-bp undigested fragment in the amplified product from L*er RPP8* or 300- and 75-bp fragments in the amplified product from Nö plants. Homozygous *ssi4 rar1* and *ssi4 sgt1b* double mutants were identified from the F_2 populations and further divided into two subgroups containing the *ER* or *er* allele based on characteristic morphology alterations. Over 10 independent individual lines of each double mutant, *ssi4 rar1 ER and ssi4 sgt1b ER*, were screened for the absence of *RPP8*, and three lines of each double mutant without *RPP8* were selected for disease resistance analysis. To generate *ssi4 rar1 sgt1b* triple-mutant lines, *ssi4 rar1 rpp8* and *ssi4 sgt1b rpp8* plants were crossed and CAPS markers were used to screen for the homozygous triple mutant. Characterization was performed using F_4 homozygous mutant lines. All plants were grown in growth chambers set for 100 μmol $m^{-2} \cdot s^{-1}$ light intensity, light and dark periods of 14 and 10 h, respectively, 22°C, and moderate RH of 60%, except for the humidity shift experiments, where HRH was set at 95%, and for the temperature shift experiments, where low temperature was set at 16°C.

Plant anatomical analysis.

For scanning electron microscopy, 2-by-3-mm pieces were taken from the fully expanded fifth leaf of 4-week-old plants and immediately fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 6.8. Entire flowers were collected just before opening and fixed in the same solution. After an overnight incubation in the fixative at 4°C, the specimens were rinsed in cold buffer (0.1 M sodium cacodylate buffer, pH 6.8) 3 times for 10 min each, then transferred to 2% osmium tetraoxide and kept at 4°C overnight. Cold water was used to rinse the specimens three times for 10 min each. After dehydration in an ethanol series (10, 30, 50, 70 plus 2% uranyl acetate, 90, and 100%), specimens were dried using Balzers CPD 030 critical point dryer (Balzers Union, Furstentum, Liechtenstein). The dried specimens were mounted on an aluminum stub with double-sided tape. Approximately 45 nm of gold/palladium was coated onto the specimens in Balzers scd 050 sputter coater (Balzers Union, Liechtenstein). Selected specimens were examined and photographed using a Hitachi S4500 Scanning Electron Microscope (Hitachi High Technologies America Inc., Pleasanton, CA, U.S.A.).

For light microscopy, the same procedures were followed as described by Zhou and associates (2000). The sections were stained with Azure B.

DAB and trypan blue staining.

To monitor H_2O_2 accumulation in situ, DAB (Sigma-Aldrich, St. Louis) staining was performed according to Thordal-Christensen and associates (1997), with modifications. The fifth leaf was detached from 4-week-old plants and put in an Eppendorf tube containing 1 ml of DAB solution with the leaf petiole being submerged. DAB solution was taken up by the leaf through transpiration. After 6 h of treatment, the leaf was cleared by boiling in ethanol/lactic acid/glycerol (4:1:1) for 5 min. For microscopy and photography, the cleared leaf segments were mounted on glass slides in 30% glycerol. Representative images were taken using a digital camera (FIH033947; Olympus, Goleta, CA, U.S.A.) adapted to the microscope (Axioskop; Zeiss, Oberkochen, Germany). To visualize cell death and fungal growth in mesophyll tissue, whole leaf samples were collected from pathogen-infected plants at 7 days postinoculation, stained with trypan blue solution (10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, and 10 mg of trypan blue dissolved in 10 ml of distilled water) by boiling for approximately 1 min, and then cleared in chloral hydrate (2.5 g of chloral hydrate dissolved in 1 ml of distilled water). They were mounted in chloral hydrate and examined under a light microscope as described above.

RNA isolation and Northern blot analysis.

Total RNA was isolated from *Arabidopsis* leaf tissues using TRIzol reagent (Life Technologies, Rockville, MD, U.S.A.) according to manufacturer's instructions. Total RNA (10 μg from each sample) was resolved in 1.5% agarose gels containing 0.6% formaldehyde and ethidium bromide at 0.075 μg/ml.

RNA was transferred onto Hybond-NX membrane (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) following the manufacturer's instructions. RNA blots were hybridized with ^{32}P-dCTP-labeled gene-specific probes following the procedures described by Shirano and associates (2002). The hybridization probes were prepared as describe by Zhou and associates (2004a) and Shirano and associates (2002).

SA measurement.

SA and its glucoside were extracted from 0.2 to 0.5 g of leaf tissue and their levels were quantified by high-performance liquid chromatography as previously described (Bowling et al. 1994).

Pathogen inoculation.

Inoculation with *H. parasitica* biotype EMCO5 was performed as described by Zhou and associates (2004a), with the following modifications. Inoculations were performed on 10-day-old seedlings or 4-week-old plants grown at moderate RH (60%). Seedlings were sprayed with a freshly prepared suspension (10^5 spores/ml) of conidiospores suspended in water. The infected cotyledons were collected 7 days postinoculation and stained with trypan blue. The number of sporangiophores per cotyledon was counted on 80 infected cotyledons for each treatment. The infection phenotype of 4-week-old plants was scored visually and representative leaves were photographed.

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