Positive and Negative Regulation of Salicylic Acid-Dependent Cell Death and Pathogen Resistance in Arabidopsis *lsd6* and *ssi1* Mutants

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Salicylic acid (SA) is a key defense molecule in higher plants that is required for resistance to diverse pathogens. A number of mutants of Arabidopsis with elevated resistance to pathogens and constitutive activation of defense-related genes and cell death have been shown to require SA for all of their phenotypes. These mutants potentially identify interesting regulatory genes that control diverse SA responses. When dominant mutations confer SA-dependent phenotypes, it is important to know the genetic basis of dominance in order to draw conclusions on the possible mechanisms of action of the genes identified. Here I characterize the basis of the dominant phenotypes conferred by the *ssi1* and *lsd6* mutations. I show that *ssi1* is haploinsufficient, while *lsd6* is a gain-of-function mutation. Thus, SA-dependent responses are under both negative and positive regulation.

Additional keywords: defense responses, *P. syringae*, triploid.

Salicylic acid (SA) is an important defense signal molecule in many plant species. It can act on its own to induce defense-related responses and disease resistance or with an additional signal(s) to induce or enhance cell death, the oxidative burst, phytoalexin accumulation, some defense-related genes, and cell growth (Shirasu et al. 1997; Thulke and Conrath 1998; Zhou et al. 1998; Rate et al. 1999). These observations have prompted several laboratories to identify and characterize mutants with altered SA-dependent responses. Two genes involved in transducing the SA signal include nonexpressor of PR genes 1 (*NPR1/NIMI*), whose product, an ankyrin repeat-containing protein, acts positively to activate defenses and disease resistance (Cao et al. 1997; Ryals et al. 1997) and suppressor of *npr1-1*, inducible 1 (*SNII*), whose product acts negatively to modulate SA responses (Li et al. 1999). Phytoalexin deficient 4 (*PAD4*) is a positive regulatory gene required for the induction of SA synthesis in response to *Pseudomonas syringae* pv. *maculicola* (Zhou et al. 1998). Enhanced disease susceptibility 5 (*eds5/sid1*) and salicylic acid induction deficient 2 (*sid2*) mutants identify additional genes required for SA biosynthesis and/or regulation (Nawrath and Metraux 1999). Additional mutants identifying genes with

possible SA-regulating functions include accelerated cell death 2 (*acd2*; Greenberg et al. 1994), lesions simulating disease (*lsd1-lsd5*, *lsd7* (Dietrich et al. 1994; Weymann et al. 1995)), constitutive expressor of PR genes (*cpr1*, *cpr5*, and *cpr6* (Bowling et al. 1994, 1997; Clarke et al. 1998)) and defense no death 1 (*dnd1*; Yu et al. 1998). It is unclear, however, whether these latter genes act directly or indirectly to regulate SA levels and/or SA-dependent responses, since many of them are pleiotropic and affect some SA-independent responses.

Three additional genes, *LSD6* (Weymann et al. 1995), suppressor of SA insensitivity (*SSII*; Shah et al. 1999), and *ACD6* (Rate et al. 1999) have been implicated in the regulation of SA-dependent responses. Mutations in all of these genes are dominant and result in phenotypes that are strictly SA dependent, including cell death, small stature, disease resistance, and defense-related gene expression. In wild-type plants, although SA is necessary, it is not sufficient to induce many of these phenotypes, including cell death and certain defense-related markers (Zhou et al. 1998; Rate et al. 1999). Because these phenotypes are SA dependent in wild-type plants, as they are in the mutants, pathogen attack might stimulate a second signal required for defense, a signal that is constitutive in *acd6-1*, *lsd6*, and *ssi1* mutants.

acd6-1 is a dominant gain-of-function allele and, therefore, may identify a positively acting regulator of the SA signaling pathway (Rate et al. 1999). Because *acd6-1* is only partially suppressed by *npr1-1*, *ACD6* may act both upstream of *NPR1* and in a second *NPR1*-independent pathway to activate SA-dependent responses (Rate et al. 1999). *ssi1* completely suppresses *npr1* and, therefore, *SSII* is proposed to act in an *NPR1*-independent manner (Shah et al. 1999). It is not known where *LSD6* acts relative to *NPR1* in activating defenses.

Understanding the genetic basis of the dominance of the *lsd6* and *ssi1* mutants would give insight into the possible functions of these genes and, therefore, is worthwhile to explore. Here I show that the dominant *ssi1*-conferred phenotypes are caused by haploinsufficiency (i.e., one wild-type copy is insufficient to rescue the defect caused by the *ssi1* mutation), implicating *SSII* as a negative regulator of SA-dependent functions. I also show that the *SSII* locus is distinct from *ACD6*, although the two loci are tightly linked. Finally, I demonstrate that *lsd6* is a dominant gain-of-function mutation that results in the activation of cell death and resistance to *P. syringae*. Together, these results suggest that the SA pathway is under both positive and negative regulation.

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RESULTS AND DISCUSSION

I used triploid analysis to determine the genetic basis of dominance in *lsd6* and *ssi* mutants. In general, the genetic basis of dominant phenotypes can be determined by comparing the behavior of triploid plants harboring one mutant allele of a gene and two wild-type alleles with heterozygous diploid plants harboring one mutant allele and one wild-type allele. If the mutant copy is a gain-of-function allele, the triploid and heterozygous diploid plants have the same phenotype. If, however, the mutant is dominant due to haploinsufficiency, then the triploid plants appear phenotypically wild type because the two wild-type alleles complement the mutant allele; the heterozygous diploids appear phenotypically mutant due to

an insufficient gene dose of the wild-type copy to complement the mutation.

To determine the basis of dominance in *lsd6* and *ssi* mutants, I created triploid plants with one dose of each mutant allele, respectively, and compared the phenotype with heterozygous mutant plants. I crossed each diploid homozygous mutant to an ecotype Columbia wild-type tetraploid line (CS3432) with a late flowering mutation (*co-1*) to create triploid plants with one copy of the respective mutation and two wild-type copies. In all cases, the tetraploid was the female in the cross and the success of the cross was verified by noting the flowering timing. *co-1* is recessive, and triploids bearing two copies of *co-1* and one copy of *Co-1* flowered 2 to 3 weeks earlier than the *co-1* tetraploid. As controls, I crossed

Fig. 1. Trypan blue staining for cell death and morphological analysis of *lsd6* and *ssi* leaves. Three-week-old leaves were stained with trypan blue to visualize dead cells. **A**, *lsd6* heterozygous leaf (Columbia $\times$ *lsd6*). **B**, *lsd6* triploid (Columbia tetraploid CS3432 $\times$ *lsd6*). **C**, *ssi* heterozygous leaf (Columbia $\times$ *ssi*). **D**, *ssi* triploid (Columbia tetraploid CS3432 $\times$ *ssi*). **E**, Columbia $\times$ Nossen (*ssi* parent ecotype). **F**, Nossen triploid (Columbia tetraploid CS3432 $\times$ Nossen). Columbia did not stain with trypan blue (data not shown). Bar is 1 mm. White arrowheads point to a type 1 lesion composed of clusters of dead cells. Asterisk marks a ring of dead cells. Black arrowhead points to a large patch of dead cells. Similar results were obtained in two separate experiments.

the tetraploid with the diploid parents of *lsd6* and *ssi1*, ecotypes Columbia and Nossen, respectively. For comparison with the triploid plants, I created heterozygous mutant diploids by crossing *lsd6* and *ssi1* with ecotype Columbia plants. Success of these crosses, where Columbia was pollinated, was monitored by the appearance of lesions on the F₁ leaves. As a control, I used the parent of *lsd6* (Columbia) or a cross of the parent of *ssi1* (ecotype Nossen) with Columbia. Because Nossen and Columbia have differently shaped leaves, it was possible to monitor the success of the cross by observing a new hybrid leaf shape in the Columbia/Nossen F₁ plants. I first verified the dominant phenotypes of *lsd6* and *ssi1* heterozygous plants, respectively, and then tested the phenotypes of the triploids harboring one copy of *lsd6* or *ssi1*, respectively.

Diploid heterozygous plants harboring *ssi1* or *lsd6* showed the distinctive morphologies previously reported (Weymann et al. 1995; Shah et al. 1999): *ssi1* heterozygotes had visible cell

death patches by week 3 after planting and showed mild leaf epinasty and yellowing late in development at week 4 (data not shown), while *lsd6* heterozygotes showed wavy leaves with cell death patches visible with a dissecting microscope by week 2 after planting (see below and data not shown). As expected, at week 3 after planting, I also saw spontaneous cell death patches visualized by trypan blue staining (Fig. 1A and C) on both the *ssi1* and *lsd6* heterozygotes. Wild-type control tissue showed no staining (Fig. 1E). I noticed predominantly

Fig. 2. Disease symptoms 2 days after inoculation with *Pseudomonas syringae* in *lsd6* and *ssi1* plants. Three-week-old plants were infected with *P. syringae* (PsmES4326) at OD₆₀₀ = 0.006. Left panels, diploid plants; right panels, triploid plants. Arrowheads point to infected leaves. Yellowing of leaves is diagnostic of disease caused by *P. syringae*. Inset shows a mature uninfected leaf (with lack of yellowing). Bar is 1 cm.

Fig. 3. Growth of *Pseudomonas syringae* in *lsd6* and *ssi1* diploid and triploid plants. Plants were inoculated as in Figure 2. Six leaves were sampled for each time point. Leaf punches were 6 mm in diameter. Bars represent standard errors. **A**, Diploid plants. Growth in *col/ssi1* was significantly different from *col/nos* on days 1, 2 and 3 (unpaired *t* test, *P* = 0.0169, 0.0008, 0.0030, respectively). Growth in *col/lsd6* was significantly different from *col* on days 1, 2 and 3 (unpaired *t* test, *P* = 0.0018, 0.001, 0.0241, respectively). **B**, Triploid plants. Growth in *col4x/ssi1* was not significantly different from *col4x/nos* on days 1, 2, and 3 (unpaired *t* test, *P* = 0.3772, 0.2432, 0.1389, respectively). Growth in *col4x/lsd6* was significantly different from *col4x/col* on days 1, 2, and 3 (unpaired *t* test, *P* = 0.0184, 0.0069, 0.0104, respectively). *col4x* is tetraploid Columbia CS3432; *col* is Columbia; *nos* is Nossen. This experiment was performed twice, with similar results.

two types of lesions on heterozygous *lsd6* leaves: type 1, in which dead cells occurred in clusters, and type 2, in which dead cells occurred in rings (Fig. 1A). These two types of lesions were also present in homozygous *lsd6* leaves (data not shown). This extends the previous observation of lesion morphology in *lsd6* plants (Weymann et al. 1995). In *ssi1* heterozygotes, I saw dead cells in large patches at leaf margins (Fig. 1C). A similar pattern of cell death was evident in the *ssi1* homozygous leaves although the cell death extended further toward the midvein than in the heterozygous plants (data not shown).

Both the *lsd6* and *ssi1* diploid heterozygous plants showed significantly greater resistance to the *P. syringae* pv. *maculicola* strain PsmES4326 than their respective parents in hand-inoculated plants. After 2 days, *ssi1* and *lsd6* heterozygous plants showed little if any disease symptoms (Fig. 2), while the respective controls of Columbia/Nossen and Columbia showed extensive loss of chlorophyll, visible as yellowed tissue, and water soaking (Fig. 2). I monitored the growth of PsmES4326 in the two mutants and their respective parents and found a significant decrease in the growth of this strain in both *lsd6* and *ssi1* heterozygous plants (Fig. 3A). *lsd6* heterozygotes showed a greater resistance to PsmES4326 than did *ssi1* heterozygotes (Fig. 3A). These results confirmed the dominance of these two mutants in the cell death and pathogen resistance assays and extended the disease resistance phenotype of *lsd6* to include a necrotrophic bacterial pathogen in addition to the previously determined resistance to the biotrophic fungal pathogen *Peronospora parasitica* (Weymann et al. 1995).

Triploid plants harboring one copy of *lsd6* resembled the *lsd6* heterozygotes in all the phenotypes examined (Table 1). These *lsd6* triploids showed wavy leaves (Fig. 2) and harbored both type 1 and type 2 lesions visible by trypan blue staining (Fig. 1B). No trypan blue staining was visible in the control triploids (data not shown). In addition, the *lsd6*-containing triploids were dramatically more resistant to *P. syringae* than the control triploids (Figs. 2 and 3B). Triploid wild-type plants were generally more resistant than the diploid wild-type plants (Rate et al. 1999); thus, comparisons should only be made between diploid or triploid lines, respectively. At the time of seed set, the triploid plants harboring *lsd6* were larger than the diploid heterozygous *lsd6* plants, as expected for the increase in ploidy (data not shown). These results indicate that *lsd6* is a dominant gain-of-function mutation.

In contrast to *lsd6*, *ssi1* did not confer a dominant phenotype in triploid plants harboring a single copy of *ssi1* (Table 1). *ssi1*-containing triploid plants and *SSI*⁺ triploid controls were indistinguishable based on their visible phenotypes (data

not shown and Figure 2), their lack of trypan blue staining (Fig. 1D and F) and their high disease susceptibility (Figs. 2 and 3B). Thus, the *ssi1*-containing triploid plants showed a different phenotype than the *ssi1*-containing heterozygous diploid plants due to the fact that the *ssi1* defect was complemented by the presence of two wild-type copies of *SSI* in the triploids. Since *ssi1* does not show any dominance in the triploid plants, it is likely that *ssi1* is either a loss-of-function or hypomorphic (reduced function) mutation.

Although *ssi1* maps very close to the *acd6-1* mutation and the two mutants share some similarities in their phenotypes (Rate et al. 1999; Shah et al. 1999), it is unlikely that these two mutations are allelic. This is because *ssi1* and *acd6* show dominance due to different mechanisms as well as different epistasis relationships with the *npr1* mutant. To demonstrate definitively that *acd6-1* and *ssi1* are distinct loci, I crossed the two mutants, with *acd6-1* as the female recipient, and found that the F₁ plants showed very reduced stature and many visible lesions and/or yellowed leaves, a phenotype typical of both homozygous *ssi1* or *acd6-1* plants. Both *ssi1* and *acd6-1* homozygous plants show very reduced stature relative to plants heterozygous for either mutation (Rate et al. 1999; Shah et al. 1999). Such *acd6-1* or *ssi1* heterozygous plants show only a modest reduction in size and show leaves with lesions (Rate et al. 1999; Shah et al. 1999). The F₂ population from the *acd6-1* cross with *ssi1* contained 304 plants with very reduced stature and many lesions and/or yellowed leaves. Three plants had an apparent *acd6-1* or *ssi1* heterozygous phenotype, consisting of slightly reduced stature and a modest number of lesions. The three apparent heterozygous plants likely resulted from crossover events between *ssi1* and *acd6-1*, indicating that these mutants are not allelic. The recombination frequency shows that *ssi1* and *acd6-1* are approximately 1 cM apart. In agreement with this genetic analysis, sequencing of the recently cloned *ACD6* gene from the *ssi1* mutant revealed no mutations in the *ACD6* gene (D. Rate and J. T. Greenberg, unpublished observations).

lsd6 and *ssi1* were previously shown to be dominant mutants whose phenotypes depend on SA. It is therefore reasonable to propose that their respective gene products function to modulate SA and/or SA-dependent processes. The genetic analysis presented here is summarized in Table 1 and suggests that *ssi1* diploid heterozygous plants showed a defense phenotype due to haploinsufficiency of *SSI*. Thus, *SSI* is a negative regulator of a number of SA-dependent processes. In this capacity, *SSI* could act directly or indirectly. If *SSI* acts directly, it could be a repressor of defense responses. In this

Table 1. Summary of genotypic and phenotypic traits in *ssi1* and *lsd6* mutants

Genotype ^a	Lesion phenotype	Disease resistance ^b	Leaf morphology	Stature ^c
<i>ssi/ssi</i>	Large patches throughout leaf	ND	Very curved (epinastic) leaves ^d	Very small
<i>ssi/SSI</i>	Large patches at leaf margins	Modest resistance	Curved (epinastic) leaves ^d	Intermediate
<i>ssi/SSI/SSI</i>	None	Susceptible	Normal	Normal
<i>lsd6/lsd6</i>	Discrete clusters and rings	ND	Very wavy leaves	Very small
<i>lsd6/LSD6</i>	Discrete clusters and rings	High resistance	Wavy leaves	Intermediate
<i>lsd6/LSD6/LSD6</i>	Discrete clusters and rings	High resistance	Wavy leaves	Intermediate

^a Heterozygous and triploid plants harbor wild-type alleles from the Columbia ecotype.

^b Disease was caused by *Pseudomonas syringae*. Resistance refers to a reduction of pathogen growth relative to control plants of the same ploidy. ND = not determined (plants were too small to infect).

^c Stature was scored relative to the wild-type of the same ploidy.

^d This phenotype was most obvious in mature rosettes at the time of bolting.

scenario, the haploinsufficiency could be due to limiting *SSII* product in *ssi1/SSII* heterozygous plants. Because *ssi1* plants completely suppress *npr1* mutants and *acd6-1* mutants require *npr1* for some of their phenotypes, *SSII* and *ACD6* may act at different steps to alter defense responses. If *SSII* acts indirectly, its partial absence in *ssi1/SSII* plants could cause the generation of a signal or cellular alteration that mimics a pathogen infection that leads to SA accumulation, cell death, and defense activation.

Because *lsd6* is a gain-of-function mutation, *LSD6* may function to positively regulate SA-dependent cell death and defenses involved in disease resistance. Although the phenotypes of *lsd6* and *acd6-1* are similar, it is not clear whether the *ACD6* and *LSD6* genes act at the same or different steps in defense signaling. In particular, it is unclear where *LSD6* acts in relationship to other regulators of SA signaling such as *NPR1*. An important caution in the interpretation of gain-of-function mutant phenotypes is the possibility of functions for the mutant allele that are not possessed by the wild-type gene. With this as a caveat, it is tempting to speculate that *LSD6* normally acts to positively activate SA-dependent defenses and cell death at a step in the pathway near where *ACD6* acts. The roles of *LSD6* and *ACD6* will be clarified by the identification and characterization of the wild-type genes and mutants with loss-of-function alleles of these genes, respectively.

MATERIALS AND METHODS

Growth of *Arabidopsis* and *P. syringae*, and pathogenicity assays.

Arabidopsis thaliana was grown under a 16-h light/8-h dark cycle in temperature-controlled growth rooms at 20°C, 50% relative humidity as described (Rate et al. 1999). *lsd6* seed was obtained from Novartis (Research Triangle Park, NC), *ssi1* seed was obtained from J. Shah (Kansas State University, Manhattan). Tetraploid seed of line CS3432 (Columbia, *co-1*, *gi-2*) was obtained from G. Copenhagen and D. Preuss (The University of Chicago, Chicago). Infections and growth curves were done as described previously with *P. syringae* pv. *maculicola* PsmES4326 obtained from F. M. Ausubel (Massachusetts General Hospital and Harvard University, Boston). Three to four of the youngest leaves on each plant were infected. After inoculation, plants were covered with a plastic dome to increase the relative humidity. For each genotype, at least six replicates were used for each time point. Leaf disks were 6 mm in diameter.

Visualization of cell death.

Fresh tissue was boiled in lactophenol containing trypan blue dye and cleared as described previously (Rate et al. 1999). Stained tissue was viewed on a petri dish with a Leica Wild M3Z binocular microscope (Leica, Rockleigh, NJ). Photographs were taken with Kodak 100 Elite Chrome slide film, scanned with a Microtek ScanMaker 4 with Adobe PhotoShop 4.0LE software (Adobe Systems, San Jose, CA), assembled into a composite and annotated with Canvas 5 (Deneba Software, Miami, FL).

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