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Characterization of a Salicylic Acid–Insensitive Mutant (*sai1*) of *Arabidopsis thaliana*, Identified in a Selective Screen Utilizing the SA-Inducible Expression of the *tms2* Gene

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Salicylic acid (SA) plays an important signaling role in the resistance of many plants to pathogen invasion. Increases in endogenous SA levels have been associated with the hypersensitive response as well as systemic acquired resistance (SAR). SA also induces the expression of a subset of the pathogenesis-related (PR) genes. However, relatively little is known about the events occurring subsequent to SA accumulation during a resistance response. In order to identify mutations in components of the SA signal transduction pathway, we have developed a genetic screen in *Arabidopsis thaliana* that utilizes the *Agrobacterium tumefaciens tms2* gene as a counter-selectable marker. SA-inducible expression of the *tms2* gene from the tobacco *PR-1a* promoter confers sensitivity to α-naphthalene acetamide (α-NAM), resulting in inhibition of root growth in germinating transgenic *Arabidopsis* seedlings. Mutants in which root growth is insensitive to α-NAM have been selected from this *PR-1a:tms2* transgenic line with the expectation that a subset will lack a regulatory component downstream of SA. The *sai1* mutant so identified expressed neither the *PR-1a:tms2* transgene nor the endogenous *Arabidopsis PR-1, PR-2,* and *PR-5* genes in response to SA. These genes also were not induced in *sai1* by 2,6-dichloroisonicotinic acid (INA) or benzothiadiazole (BTH), two chemical inducers of SAR. As expected of a mutation acting downstream of SA, *sai1* plants accumulate SA and its glucoside in response to infection with an avirulent pathogen and are more susceptible to this avirulent pathogen than the wild-type parent. *sai1* is allelic to *npr1*, a previously identified SA-noninducible mutation. The recessive nature of the noninducible *sai1* mutation suggests that the wild-type *SAI1* gene acts as a positive regulator in the SA signal transduction pathway.

Additional keyword: enhanced disease susceptibility.

In plants, disease resistance can be manifested as a hypersensitive response (HR) to the pathogen. The HR involves localized death of host cells at the site of ingress and restricted pathogen growth and spread (Matthews 1991). Within hours to a few days of the HR, the entire plant can develop a long-lasting, broad-spectrum resistance to future pathogen assault (systemic acquired resistance, SAR; Chester 1933; Ross 1961). Associated with the HR and SAR are the increased expression of a subset of pathogenesis-related (PR) genes, many of which possess antimicrobial activities and are good molecular markers for a resistance response (Alexander et al. 1993; Broglie et al. 1991; Klessig and Malamy 1994).

Salicylic acid (SA) is an important component of plant defense against disease. Exogenously applied SA induces expression of a subset of PR genes in tobacco (Antoniw and White 1980) and *Arabidopsis* (Uknes et al. 1992) and confers increased resistance to pathogen attack. Several studies of plants following infection have shown a strong correlation between increased levels of SA and both the expression of these SA-inducible PR genes and disease resistance (Dempsey et al. 1997; Malamy et al. 1990; Métraux et al. 1990; Uknes et al. 1993). Moreover, *Arabidopsis* mutants (*acd2*, *cpr1*, *lsd6*, *lsd7*) that contain a high level of SA constitutively express these PR genes and show enhanced resistance to pathogens (Bowling et al. 1994; Greenberg et al. 1994; Weymann et al. 1995). Furthermore, preventing the accumulation of SA in plants through ectopic expression of the bacterial *nahG* gene that encodes salicylate hydroxylase, an enzyme that converts SA to catechol, prevents induction of these PR genes by SA and increases susceptibility of these plants to both virulent and avirulent pathogens (Delaney et al. 1994; Gaffney et al. 1993; Lawton et al. 1995).

Very little is known about the events occurring subsequent to SA accumulation during the HR and SAR. SA and its biologically active analogs have been shown to reversibly bind to catalase and suppress the H_2O_2-degrading activity of catalase, both in vivo and in vitro (Chen et al. 1993; Conrath et al. 1995). In addition, the other major H_2O_2-scavenging enzyme, ascorbate peroxidase, is also inhibited by SA (Durner and Klessig 1995). It has been proposed that an altered redox state resulting from the inhibition of catalase and ascorbate peroxidase might be involved directly or indirectly in the activation of defense responses (Chen et al. 1993; Conrath et al. 1995; Dempsey and Klessig 1995; Durner and Klessig 1995). The involvement of catalase inhibition by SA and the resulting in-

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crease in H_2O_2 levels in a plant's resistance response is, however, in debate. H_2O_2 and H_2O_2-generating chemicals induce *PR-1* at much higher levels in wild-type tobacco than in NahG transgenic plants, where the SA signal is destroyed (Bi et al. 1995; Neuenschwander et al. 1995). In addition, elevated levels of H_2O_2 were not detected during development of SAR (Neuenschwander et al. 1995), and application of very high concentrations of H_2O_2 were found to stimulate SA accumulation (León et al. 1995; Neuenschwander et al. 1995; Summermatter et al. 1995). These results suggest that H_2O_2 functions upstream of SA rather than, or in addition to, acting downstream of SA. Hence, currently it is not very clear how the SA signal is perceived and propagated.

A genetic approach can be used to help address these questions by identifying mutations in components of the SA signal transduction pathway. To date, four mutants are known that block the transmission of the SA signal leading to the expression of the PR genes and disease resistance in *Arabidopsis*. The *npr1* mutant was initially identified in a screen for mutants that do not express the SA-inducible *BGL2:GUS* transgene (Cao et al. 1994). Subsequently, the *eds5* and *eds53* mutants that are allelic to *npr1* were obtained based on their enhanced susceptibility to *Pseudomonas syringae* pv. *maculicola* (Glazebrook et al. 1996). The *nim1* mutant was isolated in a screen for plants defective in 2,6-dichloroisonicotinic acid (INA)–induced enhanced resistance to pathogens (Delaney et al. 1995). In these four mutants the SA-inducible PR genes are not induced by SA or INA. How these four mutations affect the transmission of the SA signal is not known, although the recessive nature of these mutations suggests that the corresponding wild-type alleles are positive regulators of the SA signal transduction pathway. A fifth mutant, *ndr1-1,* which shows enhanced susceptibility to avirulent bacterial and fungal pathogens, could very well contain a lesion in the SA signal transduction pathway. However, the effect of SA application on the expression of PR genes and disease resistance was not evaluated in *ndr1-1;* hence, its role in the SA signal transduction pathway, if any, is still unclear (Century et al. 1995).

We have devised a genetic screen in *Arabidopsis thaliana* (L.) Heynh. to rapidly identify mutants defective in their response to SA. The *tms2* gene from *Agrobacterium tumefaci-*

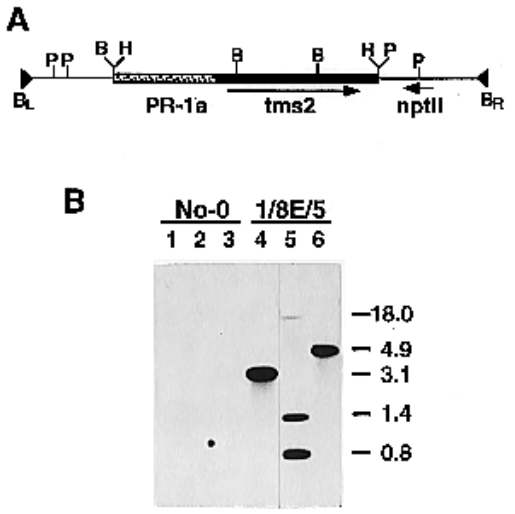

Fig. 1. A, Structure of the T-DNA containing the *PR-1a:tms2* transgene. The left (B_L) and right (B_R) borders of the T-DNA, the PR-1a promoter, the *tms2* DNA, and the neomycin phosphotransferase II (nptII) gene are indicated. Arrows indicate the direction of transcription. B, *Bgl*II; H, *Hin*dIII; P, *Pst*I. **B,** Southern blot analysis of total genomic DNA digested with *Hin*dIII (lanes 1 and 4), *Bgl*II (lanes 2 and 5), and *Pst*I (lanes 3 and 6) from nontransgenic parental No-0 (lanes 1 to 3) and the *PR-1a:tms2* transgenic line (1/8E/5; lanes 4 to 6) probed with the *tms2* DNA. The fragment sizes are indicated on the right in kilobases.

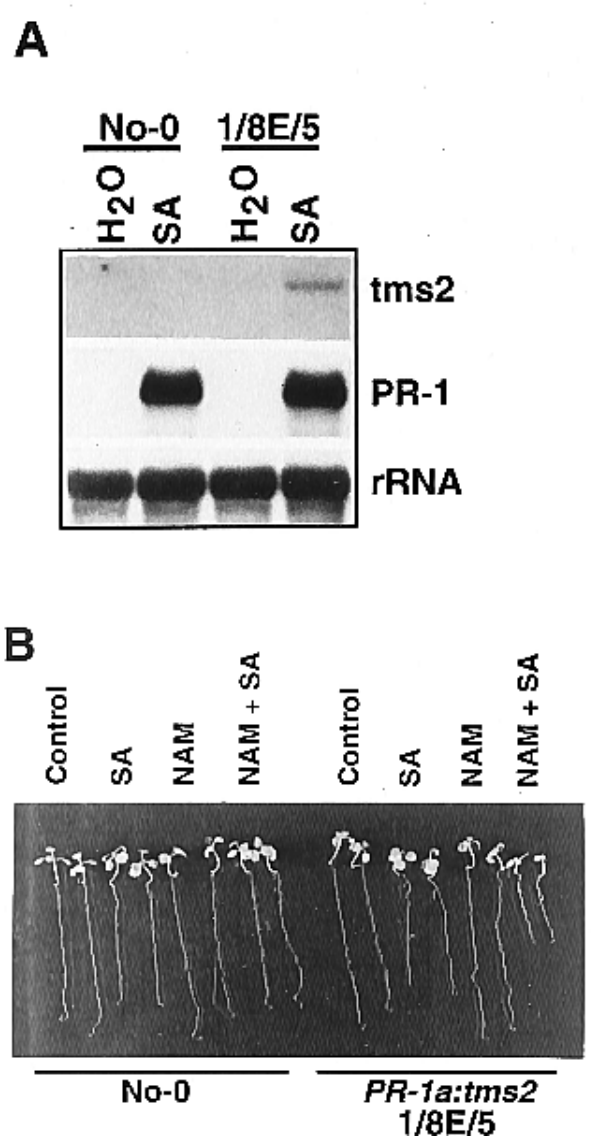

Fig. 2. A, Salicylic acid (SA)–inducible expression of the *PR-1a:tms2* transgene monitored by Northern (RNA) blot analysis. RNA was extracted from nontransgenic parental (No-0) and the *PR-1a:tms2* transgenic line 1/8E/5, 24 h after treatment with 500 µM SA (SA) or water (H_2O). The blot was sequentially probed for the transgene (tms2), the *Arabidopsis PR-1* gene (PR-1), and ribosomal RNA (rRNA) as an internal control for gel loading and transfer. **B,** Sensitivity of root growth to α-naphthalene acetamide (α-NAM) plus SA in the nontransgenic parental (No-0) and the *PR-1a:tms2* transgenic line (1/8E/5). Nine-day-old seedlings germinated on minimal agar (Control), or minimal agar containing 25 µM SA (SA), or 1 µM α-NAM (NAM), or 1 µM α-NAM plus 25 µM SA (NAM + SA) are shown. All minimal agar media contained $AgNO_3$ (25 µM) to prevent induction of the transgene by ethylene. Two representative seedlings are shown for each treatment.

ens (Klee et al. 1987), which has been previously utilized as a counter-selectable marker in *Arabidopsis* (Karlin-Neumann et al. 1991; Sundaresan et al. 1995), encodes an amidohydrolase that converts the biologically inactive α-naphthalene acetamide (α-NAM) to the biologically active auxin α-naphthalene acetic acid (α-NAA; Thomashow et al. 1984). Elevated levels of auxin are toxic to germinating seedlings and this causes a significant reduction in root growth (Boerjan et al. 1995; King et al. 1995). We have expressed the *tms2* gene in *Arabidopsis* from the SA-inducible tobacco *PR-1a* promoter. The SA-induced expression of this chimeric construct confers α-NAM sensitivity to the transgenic plants at concentrations that are nontoxic to nontransgenic plants. Mutants that do not express the transgene in response to SA can be rapidly identified among a population of wild-type plants on agar plates containing α-NAM plus SA, due to their long roots. We have successfully used this screen to identify a salicylic acid–insensitive (*sai1*) mutant that is allelic to *npr1* (Cao et al. 1994).

RESULTS

Construction of the *PR-1a:tms2* transgenic *Arabidopsis* line.

Expression of the *PR-1a* gene is SA inducible in tobacco. A chimeric *PR-1a:uidA*(GUS) gene that contains the tobacco *PR-1a* promoter sequences is SA inducible in tobacco. When stably integrated into *A. thaliana* this chimeric gene was SA inducible, suggesting the conservation of components involved in SA signaling between tobacco and *Arabidopsis* (J. Shah and D. F. Klessig, unpublished results). The *tms2* gene from *Agrobacterium tumefaciens* (Klee et al. 1987) was cloned downstream of this SA-inducible tobacco *PR-1a* gene promoter. The T-DNA containing the *PR-1a:tms2* transgene (Fig. 1A) was transformed by *Agrobacterium tumefaciens*–mediated root transformation (Márton and Browse 1991), into the *A. thaliana* ecotype Nössen (No-0). Kanamycin-resistant (kan^r) progeny from several primary transformants containing the chimeric *PR-1a:tms2* transgene were allowed to self-fertilize and set seeds. *Arabidopsis* lines homozygous for the transgene were identified in the T_4 progeny by their inability to segregate kanamycin-sensitive plants.

Southern blot analysis was performed on the T_4 progeny of homozygous kan^r lines to identify those with a single, intact copy of the T-DNA. The *PR-1a:tms2* transgenic line 1/8E/5 was shown to have an intact copy of the transgene since the *tms2* probe hybridized to fragments of expected sizes with genomic DNA from 1/8E/5 digested with various restriction enzymes (Fig. 1B, lanes 4 to 6). Additionally, it contains only a single copy of the transgene, as indicated by the *Bgl*II restriction pattern (Fig. 1B, lane 5). *Bgl*II cuts three times within the T-DNA; therefore, the *tms2* probe should hybridize to the 0.8- and 1.4-kb internal fragments and one border fragment of >4 kb containing flanking plant DNA for each insert (Fig. 1A). Besides the 0.8- and 1.4-kb fragments, the *tms2* probe hybridized to only one other fragment (≈18 kb), indicating that 1/8E/5 carries only one copy of the transgene. The presence of a single copy was also confirmed by digestion with *Eco*RV (data not shown). As expected, DNA from nontransgenic parental No-0 plants did not hybridize with the *tms2* probe (Fig. 1B, lanes 1 to 3).

The *PR-1a:tms2* transgene is induced by SA and confers α-NAM sensitivity on transgenic seedlings.

Expression of the *PR-1a:tms2* transgene was monitored by Northern (RNA) analysis (Fig. 2A). The basal level of transgene expression in the transgenic line 1/8E/5 was usually very low, although somewhat variable. Twenty-four hours after treatment with SA there was a marked increase in the steady state level of the *tms2* gene transcript. In contrast, RNA from nontransgenic parental No-0 plants did not hybridize with the *tms2* probe. To ensure that the SA treatment was effective, expression of the SA-inducible endogenous *Arabidopsis PR-1* gene was monitored. As expected, both the nontransgenic parental and 1/8E/5 transgenic plants showed comparably high steady state levels of the *PR-1* transcript after treatment with SA.

The effect of the *PR-1a:tms2* transgene expression on primary root elongation in the presence of α-NAM plus SA was analyzed next. We have observed that SA is phytotoxic to germinating *Arabidopsis* seedlings grown on agar medium at concentrations above 125 μM. At the concentration of SA used in our screen (25 μM), some inhibition of root elongation occurred in both the transgenic 1/8E/5 and nontransgenic parental plants (Fig. 2B; compare plants marked control versus SA). Under the conditions used, α-NAM (1 μM) alone showed no adverse effects on root growth of either plants. However, the combination of α-NAM (1 μM) plus SA (25 μM) inhibited primary root growth of the *PR-1a:tms2* transgenic line 1/8E/5 by 40 to 60% (compare plants marked NAM + SA with plants marked either SA or NAM). As anticipated with the nontransgenic parental No-0 plants, addition of α-NAM plus SA to the medium did not increase the level of root growth inhibition beyond that seen with SA alone. These results suggested that the *PR-1a:tms2*–based screen should be successful for the isolation of mutants in the SA signal transduction pathway. This conclusion was further supported by the finding that, among the progeny of several independent transgenic *PR-1a:tms2* lines, the level of transgene expression correlated with sensitivity to α-NAM plus SA (data not shown).

Isolation of the *sai1* mutant.

M_2 progeny of ethylmethyl sulfonate (EMS)–mutagenized M_1 seeds from the *PR-1a:tms2* transgenic line 1/8E/5 were screened on minimal agar plates containing α-NAM (1 μM) plus SA (25 μM) for seedlings whose primary root lengths re-

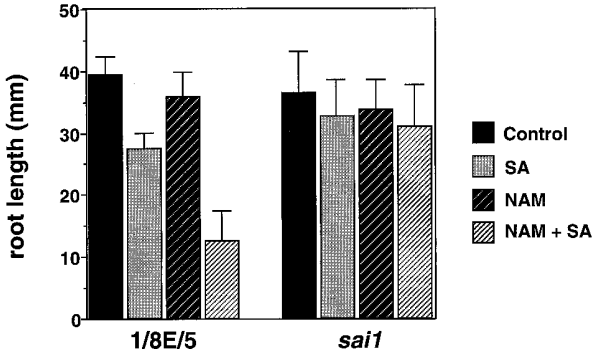

Fig. 3. Length of primary roots of 9-day-old wild-type transgenic (1/8E/5) and the *sai1* mutant seedlings grown on minimal agar medium containing the indicated chemicals (Fig. 2B caption). Root lengths were measured for seven to 12 seedlings per treatment.

sembled those of the nonmutagenized 1/8E/5 seedlings grown on medium containing only SA (25 μM). Among the various classes of mutants that could be identified by this screen (see below), two SA-insensitive (sai) mutants were isolated among the 20,000 M_2 seedlings screened. M_3 progeny of one of these mutants, *sai1*, were rescreened with the root length assay. In the secondary screen *sai1* and wild-type *PR-1a:tms2* transgenic seeds were germinated on plates containing α-NAM plus SA, α-NAM alone, SA alone, or neither chemical (control). Primary root lengths were measured on 9-day-old seedlings from each plate (Fig. 3). The *sai1* mutant repeatedly showed very little reduction in the length of the primary roots (only 4%) in the presence of α-NAM plus SA, compared with the 53% decrease observed in the root length of the wild-type transgenic line 1/8E/5 (compare SA versus NAM plus SA).

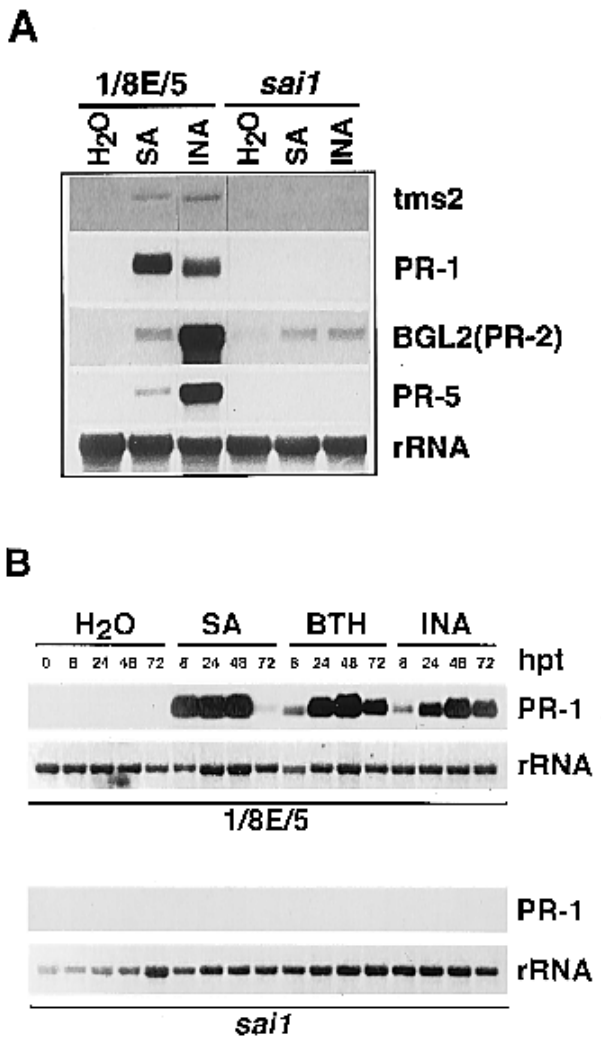

Fig. 4. Northern (RNA) blot analysis of RNA extracted from wild-type transgenic (1/8E/5) and the mutant *sai1* M_4 plants after chemical treatment. **A,** RNA was extracted from plants treated with salicylic acid (SA, 500 μM), 2,6-dichloroisonicotinic acid (INA) (100 μM) or H_2O, 24 h post treatment (hpt). **B,** RNA was extracted from plants before chemical treatment (0) or 8, 24, 48, and 72 hpt with SA (500 μM), benzothiadiazole (BTH) (100 μM), INA (100 μM), or H_2O. The blot in **A** was sequentially probed for the expression of the *tms2* transgene, the endogenous *Arabidopsis PR-1*, *BGL2* (*PR-2*), *PR-5*, and the rRNA genes while the blot in **B** was probed for the *Arabidopsis PR-1* and rRNA genes.

SA, INA, and BTH fail to induce the endogenous PR genes, as well as the *PR-1a:tms2* transgene, to high levels in *sai1* plants.

With the above screen, we expected to identify five major classes of mutants: (i) mutations in genes that are components of the SA signal transduction pathway; (ii) mutations affecting uptake and/or metabolism of SA; (iii) mutations in the promoter of the *PR-1a:tms2* transgene that would alter the SA responsiveness of the transgene; (iv) mutations in the open reading frame (ORF) of the *tms2* gene that would lower the amount of functional enzyme formed, but not affect the SA inducibility of the transgene; and (v) mutations that alter the auxin responsiveness of the plant. Only mutations in class i and class ii will reduce SA inducibility of the endogenous *Arabidopsis* PR genes in addition to that of the transgene. SA induction of the *PR-1*, *PR-2* (β-1,3-glucanase 2 [*BGL2*]), and *PR-5* genes, as well as that of the transgene, was monitored in three subsequent generations of *sai1*. SA responsiveness of all four genes was depressed in *sai1* (Fig. 4A). This lack of inducibility was most evident for the *PR-1* and *PR-5* genes. With the transgene and the *BGL2* gene, induction by SA in *sai1* plants was also poor, compared with that seen in wild-type transgenic plants; however, the basal expression levels and SA inducibility of these genes were more variable from experiment to experiment. Thus, the *sai1* mutation defines an important component necessary for the SA inducibility of all three *Arabidopsis* PR gene families.

INA and benzothiadiazole (BTH) are synthetic chemicals that simulate many of the biological properties of SA, including its ability to induce *PR-1* gene expression (Conrath et al. 1995; Görlach et al. 1996; Lawton et al. 1996; Métraux et al.,1991; Ward et al. 1991). The induction of the *PR-1* gene by INA or BTH does not require elevated levels of SA (Lawton et al. 1996; Malamy et al. 1996; Vernooij et al. 1995), suggesting that INA and BTH act either via a different signal transduction pathway or, alternatively, through the SA signal transduction pathway at the same step or at a step downstream of SA. INA did not induce either the transgene or the endogenous *PR-1*, *PR-2*, and *PR-5* genes to high levels in the *sai1* mutant plants (Fig. 4A). Similarly, BTH failed to induce the *PR-1* gene in the *sai1* mutant (Fig. 4B). These results argue that the *sai1* mutation is in a component common to the SA, INA, and BTH signal transduction pathways.

Pathogen infection induces accumulation of higher than wild-type levels of SA and SAG in *sai1*.

Since the *sai1* mutation appeared to disrupt the transmission of the SA signal leading to inducible expression of the *PR-1*, *PR-2*, and *PR-5* genes, we anticipated that the mutant should accumulate SA and its glucoside, SAG, upon infection with an avirulent pathogen. The levels of SA and SAG were monitored in wild-type and *sai1* plants, 24 h post infection (hpi) with *P. syringae* pv. *tomato* DC3000 (Pst), which contains the plasmid-borne *avrRpt2* avirulence gene. The *sai1* mutant is in the No-0 ecotype genetic background, which contains the resistance gene *RPS2* (Bent et al. 1994). Thus, wild-type No-0 is resistant to strains of Pst carrying the *avrRpt2* gene. In mock-infected wild-type and *sai1* plants, free SA levels were barely

detectable (<0.01 μg per gram of fresh weight [FW]) while the average SAG levels were 0.8 μg per gram of FW (Fig. 5). As expected, the *sai1* mutant plants accumulated high levels of SA and SAG upon infection with the avirulent pathogen. However, the levels of SA in *sai1* plants (15.3 ± 3.6 μg per gram of FW) were 12 times higher than in wild-type plants (1.3 ± 0.41 μg per gram of FW). SAG levels in pathogen-infected wild-type (19.2 ± 1.7 μg of FW) as well as *sai1* plants (27.3 ± 5.3 μg per gram of FW) increased 23- and 35-fold, respectively, over the corresponding mock-infected plants.

***sai1* shows enhanced susceptibility to pathogen.**

To test whether resistance in the *sai1* mutant is compromised due to this mutation in the SA signal transduction pathway, leaves of *sai1* and wild-type transgenic 1/8E/5 plants were infiltrated with Pst containing the *avrRpt2* gene. In the wild-type transgenic plants, the pathogen grew 100-fold, attaining maximal numbers by 72 hpi. This 100-fold increase of Pst containing the *avrRpt2* gene, in wild-type plants, is typical for *RPS2*-mediated resistance (Bent et al. 1994). In contrast, *sai1* plants supported 20 times more growth of the pathogen (Fig. 6A). Thus, as anticipated, *sai1* plants were more susceptible to this avirulent Pst.

The accumulation of *PR-1* gene transcript was also monitored in these plants after infection (Fig. 6B). In wild-type transgenic plants, *PR-1* gene induction was evident by 8 hpi (visible on longer exposures of the blot) and reached maximal levels by 16 hpi. In comparison, in the *sai1* mutant appearance of the *PR-1* transcript was delayed, being first detected at 16 hpi (visible on longer exposures of the blot). Moreover, the steady state levels of the *PR-1* transcript never attained the maximal levels seen in the wild-type transgenic plants, even after 72 hpi. Nonetheless, this activation of the *PR-1* gene in *sai1* plants upon infection with pathogen was surprising, as treatment with even high levels of SA (500 μM), INA (100 μM), or BTH (100 μM) failed to induce this gene in *sai1* plants even after 48 or 72 h post treatment (hpt) (Fig. 4B).

Genetic analysis of the *sai1* locus.

Plants homozygous for the *sai1* locus were backcrossed to the wild-type parent 1/8E/5. The *sai1* mutant phenotype was followed in the F_1 and F_2 progeny by the inability of SA to induce the accumulation of *PR-1* mRNA. All 18 F_1 plants accumulated high wild-type levels of *PR-1* transcript after treatment with SA (Table 1), suggesting that the *sai1* mutant allele is recessive to the wild-type allele. The *sai1* mutant

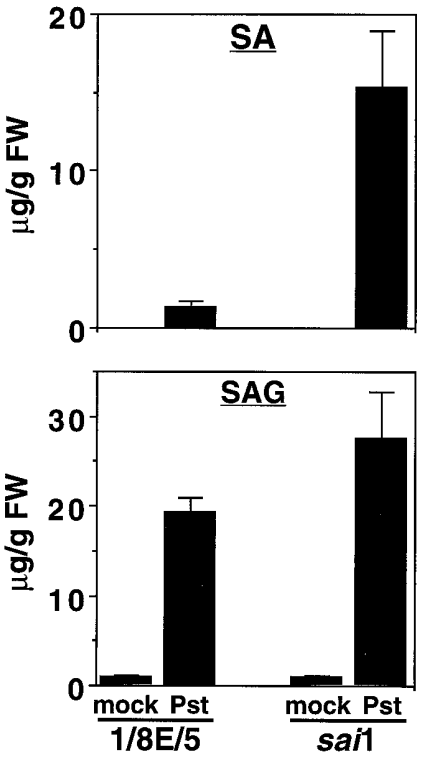

Fig. 5. Salicylic acid (SA) and SA with its glucoside (SAG) levels in wild-type transgenic (1/8E/5) and *sai1* plants 24 h after infection with *Pseudomonas syringae* pv. *tomato* DC3000 containing the *avrRpt2* gene (Pst; OD_{600} of 0.001) or mock-infected with 10 mM $MgCl_2$ (mock). The SA and SAG values presented as micrograms of fresh weight (FW) per gram are averages of four sets of samples per treatment.

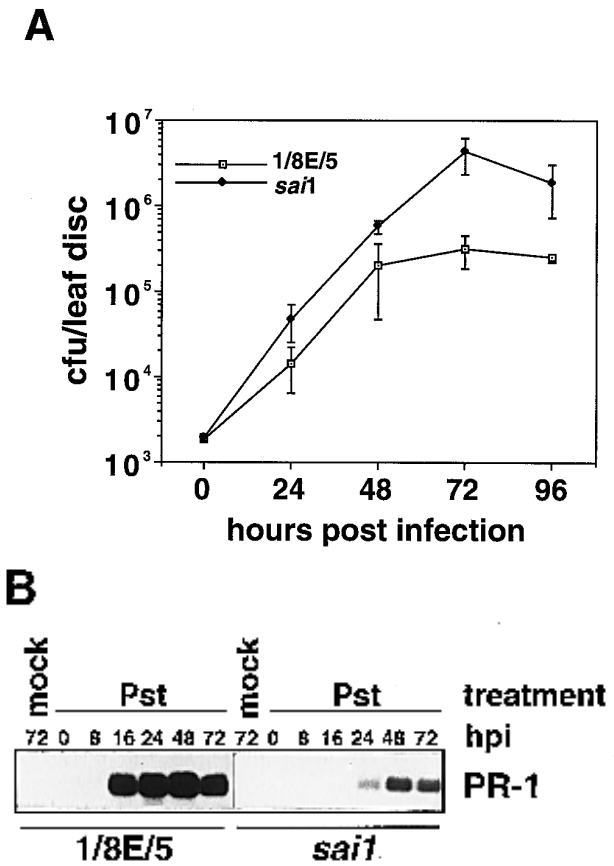

Fig. 6. A, Growth of *Pseudomonas syringae* pv. *tomato* DC3000 (Pst) carrying the *avrRpt2* gene in wild-type transgenic (1/8E/5) and *sai1* plants. The bacteria (OD_{600} of 0.001) in 10 mM $MgCl_2$ were injected in the abaxial surface of 12 leaves per time point. CFU of Pst per leaf disk (0.28 cm^2) were determined at various time points in hours post infection (hpi) as described in Materials and Methods. The values represent the average of four samples (each containing 3 leaf disks) ± standard deviation. **B,** Northern (RNA) blot analysis of RNA extracted at different time points from the same group of plants used in **A.** The blot was probed with the *Arabidopsis PR-1* gene. Equal loading of RNA was monitored by ethidium bromide staining.

phenotype segregated in a 3:1 (wild-type:mutant; $\chi^2 = 0.19$; $P > 0.5$) Mendelian ratio in the F_2 progeny (Table 1), thus confirming the recessive nature of the mutation and demonstrating that the mutant phenotype is due to a mutation at a single genetic locus.

The *sai1* and *npr1* mutations belong to the same complementation group.

The *npr1* mutation was identified by Cao et al. (1994) in a screen for mutants defective in the induction of an SA-inducible *BGL2:GUS* transgene. In addition, a subsequent screen for mutants showing enhanced disease susceptibility to strains of *P. syringae* pv. *maculicola* also identified alleles of *npr1* (Glazebrook et al. 1996). Similar to the case with *sai1*, neither SA nor INA can induce the expression of the endogenous *PR-1*, *PR-2*, and *PR-5* genes in *npr1* plants. Furthermore, the *npr1* mutant phenotype is due to a recessive mutation. The similar characteristics of *sai1* and *npr1* phenotypes suggested that these mutations might be in different genes affecting the same signal transduction pathway; alternatively, these mutations could be allelic. To distinguish between these possibilities, plants homozygous for the *sai1* mutant allele were crossed to plants homozygous for the *npr1* mutant allele (in the ecotype Columbia). The success of the cross was confirmed by performing CAPS analysis (Konieczny and Ausubel 1993) on the F_1 progeny (data not presented). All of the F_1 plants lacked induction of the endogenous *PR-1* gene by SA (Table 2), suggesting that *sai1* and *npr1* mutations are in the same complementation group. Additionally, *PR-1* mRNA was not induced in any of the 30 F_2 plants analyzed after treatment with SA (Table 2), again suggesting that *npr1* and *sai1* are allelic.

DISCUSSION

To date, only a few mutations have been obtained that prevent induction by SA (or INA) of plant defense responses. In part, this may reflect the absence of easily scorable phenotypes for such mutants. To isolate more such mutants, we have developed a genetic screen based on an SA-inducible tobacco *PR-1a* promoter-driven counter-selectable *tms2* gene. The *tms2* gene encodes an amidohydrolase that converts the biologically inactive α-NAM to the biologically active auxin α-NAA (Thomashow et al. 1984), which is toxic to *Arabidopsis* root growth. Thus, mutants that carry the *PR-1a:tms2* gene but are defective in their response to SA, such as the prototypic *sai1* mutant, can readily be obtained because growth of their roots shows little inhibition in the presence of α-NAM and SA (Figs. 2B and 3).

A variety of mutations could account for the inability of α-NAM plus SA to inhibit root elongation. These include, in addition to the desired mutations in components of the SA signaling pathway (class i), mutations that affect SA uptake and/or metabolism (class ii), mutations in the promoter or ORF of the *PR-1a:tms2* transgene (classes iii and iv, respectively), and mutations that alter auxin responsiveness (class v). The responsiveness of the endogenous *PR-1*, *PR-2*, and *PR-5* genes, as well as the chimeric transgene, to SA was altered in *sai1* (Fig. 4A), suggesting that the mutation was not in the transgene or in a gene affecting auxin responsiveness. The latter was confirmed by demonstrating that *sai1* seedling root growth was as sensitive to the auxin α-NAA as that of the wild-type transgenic plant carrying the *PR-1a:tms2* transgene (data not shown). The *sai1* mutant, besides being unable to express the *PR-1*, *PR-2*, and *PR-5* genes at high levels in response to SA, was also less sensitive to the phytotoxic effects of SA. Unlike the wild-type plants, growth of roots of *sai1* seedlings was only marginally inhibited by SA (Fig. 3; compare control versus SA). Additionally, the leaves of *sai1* plants showed little or no symptoms of leaf burning when sprayed with SA (1 mM), in contrast to wild-type plants that showed extensive leaf burning (data not shown). This reduced sensitivity of *sai1* to the phytotoxic effects of SA could be due to the poor uptake of SA in the mutant (class ii) or, alternatively, due to its insensitivity to SA because of a lesion in the SA signaling pathway (class i).

To help distinguish between class i and ii mutations, we utilized several chemical inducers of plant defense responses. INA mimics many of the functions of SA, including induction of the *PR-1* gene (Ward et al. 1991), enhancement of resistance (Métraux et al. 1991), and inhibition of catalase (Conrath et al. 1995) and ascorbate peroxidase activities (Durner and Klessig 1995). Since INA does not stimulate SA production and can induce PR genes and enhanced resistance in NahG transgenic plants that fail to accumulate SA (Malamy et al. 1996; Vernooij et al. 1995), it has been suggested that INA acts either downstream of SA or at the same step as SA (Conrath et al. 1995; Durner and Klessig 1995; Malamy et al.

Table 1. Genetic analysis of *sai1*

		SA inducibility of *PR-1*[b]		
Cross[a]	Generation	Tested	wt[c]	Mutant
1/8E/5 × 1/8E/5	F_1	16	16	0
sai1 × *sai1*	F_1	16	0	16
sai1 × 1/8E/5	F_1	18	18	0
sai1 × 1/8E/5	F_2	114	88	26[d]

[a] The plant receiving pollen is listed first for each cross.
[b] Twenty-one-day-old plants were sprayed, as well as subirrigated for 10 min, with 500 µM SA and RNA extracted 24 h later. The *sai1* mutant phenotype was followed by Northern (RNA) blot analysis as the inability of the mutant plant to express the endogenous *PR-1* gene in response to SA.
[c] Wild-type with respect to SA-inducible expression of *PR-1*.
[d] $\chi^2 = 0.19$; $0.7 > P > 0.5$.

Table 2. Complementation analysis between *sai1* and *npr1*

		Salicylic acid (SA) inducibility of *PR-1*[b]		
Cross[a]	Generation	Tested	wt[c]	Mutant
sai1 × Col	F_1	19	19	0
npr1 × *npr1*	F_1	16	0	16
sai1 × *npr1*	F_1	3	0	3
sai1 × *npr1*	F_2	30	0	30

[a] The *sai1* mutation is in the ecotype Nössen (No-0) while the *npr1* mutation is in the ecotype Columbia (Col). The plant receiving pollen is listed first for each cross.
[b] Twenty-one-day-old plants were sprayed, as well as subirrigated for 10 min with 500 µM SA, and RNA extracted 24 h later. The *sai1* and *npr1* mutant phenotypes were followed by Northern (RNA) blot analysis as the inability of the mutant plants to express the endogenous *PR-1* gene in response to SA.
[c] Wild-type with respect to SA-inducible expression of *PR-1*.

1996; Vernooij et al. 1995). In *sai1*, INA was unable to induce expression of endogenous *PR-1*, *PR-2*, and *PR-5* genes as well as the transgene (Fig. 4A). This result further confirms that SA and INA share a common signal transduction pathway, of which the wild-type *SAI1* gene is an important component. Similar to SA and INA, BTH, a commercially available activator of SAR (Görlach et al. 1996), also did not induce *PR-1* gene expression in *sai1* (Fig. 4B). The inability of both INA and BTH to induce the *PR-1* gene to high levels and the fact that *sai1* can accumulate high endogenous levels of SA and SAG (Fig. 5) rules out the possibility that the mutant phenotype of *sai1* plants is due to a defect in the uptake or metabolism of SA. Thus, it appears that the *sai1* mutation is in a bona fide component of the SA signal transduction pathway. Since the noninducible *sai1* mutant allele is recessive to its wild-type allele and is inherited as a single Mendelian locus, the wild-type *SAI1* gene is predicted to function as a positive regulator of the SA signal transduction pathway.

In contrast to the failure of exogenously applied SA, INA, or BTH to induce *PR-1* gene expression in *sai1* plants, infection with Pst containing the *avrRpt2* gene resulted in the accumulation of *PR-1* mRNA, although expression was delayed and lower than in infected wild-type plants. The lack of any induction of *PR-1* gene expression in *sai1* plants after application of SA, INA, or BTH argues that the low level of induction after infection is not the result of a leaky mutation but is due to the presence of a second pathway, independent of *SAI1*, for the induction of the *PR-1* gene. Similar results have also been reported by Glazebrook et al. (1996) and Delaney et al. (1995) with mutant alleles of *npr1* and *nim1*, respectively. In addition, the finding that transgenic *Arabidopsis*, which are unable to accumulate SA due to the expression of the *nahG* gene, accumulate *PR-1* transcript after infection with Pst containing the *avrRpt2* gene at levels higher than those seen in mock-infected leaves (Delaney et al. 1994; Lawton et al. 1995), provides further support for the existence of a second pathway for *PR-1* gene induction that probably is independent of SA.

The *sai1* mutant accumulated much higher levels of free SA than the wild-type plant upon infection with Pst containing the *avrRpt2* gene (Fig. 5). Two explanations for this result can be envisioned: the presence of feedback control, which regulates the maximal level of free SA that accumulates in the wild-type plant, or a mutation in *sai1* that negatively affects the conversion of SA to SAG. Since (i) SAG levels in *sai1* after infection were higher than those observed in wild-type plants (Fig. 5), and (ii) a defect in conversion of SA to SAG is inconsistent with the inability of SA to induce PR genes in *sai1* plants, as SA and not SAG is the active molecule (Hennig et al. 1993), it is likely that *sai1* disrupts a feedback loop that in wild-type plants negatively controls SA biosynthesis and accumulation. Disruption of this feedback loop permits uncontrolled SA biosynthesis and accumulation, as seen in *sai1*, after infection with pathogen. Precedence for negative feedback regulation of the biosynthesis of signaling molecules exists in *Arabidopsis* for the hormone ethylene. Application of Ag^+, which blocks ethylene action, leads to an increase in the biosynthesis and accumulation of ethylene. Additionally, the accumulation of higher-than-wild-type levels of ethylene in the leaves of the *Arabidopsis* ethylene-insensitive mutants *ein2* and *ein3* suggests that a block in ethylene action or response leads to an increase in the biosynthesis of ethylene (Guzmán and Ecker 1990).

Two other recessive mutations, *npr1* and *nim1*, that are nonresponsive to SA and INA have been isolated in *Arabidopsis* (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996). Like *sai1*, both of these mutants show increased susceptibility to virulent and avirulent pathogens, as well as lack of induction of the *PR-1*, *PR-2*, and *PR-5* genes by SA or INA. We tested allelism between *sai1* and *npr1* by analyzing F_1 and F_2 progeny of a cross between the two mutants for the SA inducibility of their *PR-1* genes. The *sai1* and *npr1* mutations did not complement each other, suggesting that they are allelic (Table 2). Whether *nim1* is allelic to *sai1*/*npr1* is currently not known. The inability of the *sai1*, *npr1*, and *nim1* mutants to express the *PR-1*, *PR-2*, and *PR-5* genes in response to chemical inducers of plant disease resistance (SA, INA, and BTH), combined with their decreased resistance to both virulent and avirulent pathogens, provides further evidence that SA is an important signal in disease resistance. Thus, these SA-insensitive mutants will serve as useful genetic tools to address important issues surrounding plant-pathogen interactions. For example, it has been shown that SA levels increase within and surrounding the spontaneous lesions formed in several lines of lesion mimic mutants (Greenberg et al. 1994; Weymann et al. 1995). Whether these increases in SA and the corresponding activation of the SA signal transduction pathway are involved in the programmed cell death that leads to lesion formation is currently not known. However, with the *sai1*/*npr1*/*nim1* class of mutants some of these questions can now be addressed. Since *sai1* is blocked in its ability to respond to SA, formation of spontaneous lesions in *sai1 lsd* double mutants would indicate that spontaneous lesion formation in these *lsd* mutants is not dependent on the *SAI1* gene and the events occurring downstream of *SAI1*. On the other hand, absence of spontaneous lesions in these *sai1 lsd* double mutants would argue strongly for the involvement of the SA signal transduction pathway in the formation of these spontaneous lesions in the *lsd* mutants.

MATERIALS AND METHODS

Growth conditions for plant and bacteria.

Arabidopsis plants were grown in soil at 22°C in growth chambers programmed for a 14-h light (7,000 to 9,000 lux) and 10-h dark cycle unless otherwise stated. *P. syringae* pv. *tomato* DC3000 carrying a plasmid-borne *avrRpt2* gene (Bent et al. 1994) was propagated at 30°C on King's B medium (King et al. 1954) containing rifampicin (100 µg/ml) and kanamycin (25 µg/ml).

Bacterial infection of plants.

For both the wild-type transgenic 1/8E/5 and *sai1*, 9-day-old seedlings in soil were transferred to a Conviron growth chamber (22°C, 75 to 80% relative humidity, 12-h light [13,000 to 14,000 lux] and 12-h dark cycle). Two weeks later, four leaves per plant were infiltrated with either 10 mM $MgCl_2$ (mock) or a suspension (OD_{600} of 0.001 in 10 mM $MgCl_2$) of *P. syringae* pv. *tomato* DC3000 carrying a plasmid-borne *avrRpt2* gene (Bent et al. 1994). Infiltration was performed with a 1-ml syringe (without a needle) on the abaxial side of the leaf. Leaf disks, 0.6 cm in diameter (0.28 cm^2),

from three Pst-infected leaves were ground in 1 ml of 10 mM $MgCl_2$ and appropriate dilutions were plated on King's B medium containing rifampicin (100 µg/ml) and kanamycin (25 µg/ml). Four such samples were taken per treatment per time point. Plates were incubated at 30°C for 2 days before counting the bacterial colonies. Mock-infected and Pst-infected leaves were also harvested at each time point for extraction of RNA.

DNA manipulation.

Standard cloning techniques for DNA manipulation as described in Sambrook et al. (1989) were used. The borders of all constructs were confirmed by dideoxy sequencing with Sequenase version 2 according to the manufacturer's protocol (USB, Cleveland, OH).

Construction of the *PR-1a:tms2* chimeric gene.

pJA3 contains a genomic clone of the tobacco *PR-1a* gene inserted in the *Hin*dIII site of pUC118 (J. Hennig, personal communication). A *Nco*I site overlaps the translation start site of the *PR-1a* gene in pJA3. The translation start site in pJA3 was deleted by digesting with *Nco*I followed by S1 nuclease and Klenow treatment. The resulting DNA was digested with *Sal*I to excise the *PR-1a* ORF, and the vector backbone was self-ligated to give plasmid JS116. pJS116 contains a 930-bp fragment of the *PR-1a* promoter from –933 to –3 upstream of the *PR-1a* translation start codon. A 2-kb *Bam*HI fragment, containing 15 bp of 5′ transcribed but untranslated sequences of the *Agrobacterium tumefaciens tms2* gene, its ORF, and transcription termination sequences, was excised from pMON544 (Klee et al. 1987) and cloned into the *Bam*HI site of pJS116, with the *tms2* gene 3′ to the *PR-1a* promoter, to give pJS120. The *PR-1a:tms2* chimeric construct was excised as a 3.1-kb *Hin*dIII fragment from pJS120 and cloned into the *Hin*dIII site of the binary vector pGA482 (An 1986). The resultant plasmid pJS125 was used to transform *A. thaliana*.

***Arabidopsis* transformation.**

The *PR-1a:tms2* chimeric construct in pJS125 was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating (Ditta et al. 1980). Roots of *A. thaliana* ecotype Nössen (No-0) were transformed essentially as described by Márton and Browse (1991). Rooting of well-developed kanamycin-resistant T_1 shoots was induced according to the procedure of Huang and Ma (1992) on rooting medium containing 2 mg of α-NAA per liter. Primary transformants (T_1) with well-developed roots were transferred to soil and allowed to set seeds.

Mutagenesis and selection of SA-insensitive mutants.

Twenty thousand seeds from T_5 plants of a *PR-1a:tms2* transformant, 1/8E/5, with a single T-DNA insertion were placed in 100 ml of 0.3% (vol/vol) solution of ethylmethyl sulfonate (EMS; Sigma, St. Louis, MO) at room temperature with gentle agitation. Twelve hours later the seeds (M_1) were washed with 15 changes of water over a period of 3 h. The M_1 seeds were sown in soil and allowed to self-fertilize. M_2 seeds were harvested as pools, each pool containing M_2 seeds derived from approximately 1,000 M_1 seeds. The M_2 seeds were imbibed in water for 30 min and then surface sterilized by a 5 min treatment with 70% ethanol plus 0.1% Triton X-100 followed by a 10-min treatment with 30% household bleach plus 0.1% Triton X-100. After four washes in sterile water the M_2 seeds were placed in a single row on agar plates of selection medium. The selection medium consisted of minimal agar (MS salts pH 5.9 [Gibco-BRL, Gaithersburg, MD], 0.7% Difco agar, 1.3 mM KH_2PO_4 and 25 µM $AgNO_3$) containing 25 µM sodium salicylate and 1 µM α-NAM. SA, α-NAM, and $AgNO_3$ were added to the medium after autoclaving. $AgNO_3$ was added to inhibit ethylene responsiveness of the transgene; ethylene can induce the *PR-1a* promoter. As controls, minimal agar media lacking either SA or α-NAM, or lacking both, were also used. Unmutagenized seeds from the ecotype Nössen and from the *PR-1a:tms2* transgenic line 1/8E/5 were also sowed on all four kinds of plates, as controls for each experiment. The plates were sealed with Scotch 3M filter tape and after 2 days of incubation at 4°C the plates were placed vertically in the growth room exposed to continuous light (5,000 to 6,000 lux). Nine days after transfer to the growth room, mutant seedlings with primary roots 30% or more longer than that of the unmutagenized parent were transferred to soil and allowed to set seeds. M_3 progeny of these putative mutants were tested for SA insensitivity in the root length assay as well as for the induction of the transgene and the endogenous *PR-1*, *BGL2*(*PR-2*), and *PR-5* genes by SA.

Treatment of plants with SA, INA, and BTH.

For treatment with SA (500 µM) and INA (100 µM), 21- to 25-day-old plants in soil were sprayed, as well as subirrigated for 10 min, with a solution of pH 6.9. Wherever possible, another set of control plants was similarly treated for 10 min with water. BTH (100 µM active ingredient) was applied, as above, as a water-dispersible formulation. Plants were transferred to growth chamber and leaf tissue were harvested at different times after treatment and quick frozen in liquid nitrogen. Leaf samples were stored at –80°C. For analysis of individual plants, two leaves were harvested before any chemical treatment and quick frozen in liquid nitrogen. This sample served as the untreated control. The plant was then treated with inducing chemicals as above and two leaves were harvested 24 h later.

Southern and Northern blot analyses.

Genomic DNA from *A. thaliana* plants was extracted according to the protocol of Das et al. (1990). Two micrograms of DNA digested with appropriate restriction enzyme was resolved on a 1% agarose gel, denatured, and then renatured as described by Sambrook et al. (1989). The DNA was transferred to Nytran Plus membrane (Schleicher and Schuell, Keene, NH) as recommended by the manufacturer. After UV-cross-linking, the blot was hybridized and processed according to the protocol of Church and Gilbert (1984) with a random-primed, labeled 2-kb *Bam*HI fragment of pMON544 (Klee et al. 1987) containing the *tms2* gene. Large-scale preparation of RNA from *Arabidopsis* was according to the protocol of Das et al. (1990). Small-scale extraction of RNA from one or two leaves was performed with the TRIzol reagent (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's protocol. Five micrograms of RNA was resolved on a 1.5% agarose gel containing 1.1% formaldehyde as described by Ausubel et al. (1987). Ethidium bromide was included in the loading buffer to visualize the RNA. The RNA

was transferred onto Nytran Plus membrane, UV-cross-linked, hybridized, and processed as for the Southern analysis. Probes specific for the *Arabidopsis PR-1*, *BGL2*(*PR-2*), and *PR-5* genes were random-primed, labeled, gel-purified cDNA clones. The *tms2* gene probe is the same as that used for Southern analysis. The rDNA probe was generated by random priming of plasmid DNA containing the rDNA.

Genetic analysis of *sai1*.

For all crosses, *sai1* plants in the ecotype Nössen were used as the recipient of pollen. Mature F_1 seeds were harvested from individual siliques, 21 to 23 days post pollination and germinated on MS medium containing 50 µg of kanamycin per ml. Nine-day-old seedlings were transferred to soil and treated with SA 2 to 3 weeks later. Crosses of *sai1* to the *npr1* mutant, in the ecotype Columbia, were confirmed by CAPS analysis (Konieczny and Ausubel 1993) of the F_1 progeny.

SA and SAG estimations.

SA and SAG were extracted and estimated from 0.25 to 0.5 g of fresh weight leaf tissue as described by Bowling et al. (1994).

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