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The Arabidopsis *ssi1* Mutation Restores Pathogenesis-Related Gene Expression in *npr1* Plants and Renders Defensin Gene Expression Salicylic Acid Dependent

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The Arabidopsis *NPR1* gene was previously shown to be required for the salicylic acid (SA)– and benzothiadiazole (BTH)-induced expression of pathogenesis-related (*PR*) genes and systemic acquired resistance. The dominant *ssi1* (for suppressor of SA insensitivity) mutation characterized in this study defines a new component of the SA signal transduction pathway that bypasses the requirement of *NPR1* for expression of the *PR* genes and disease resistance. The *ssi1* mutation caused *PR* (*PR-1*, *BGL2* [*PR-2*], and *PR-5*) genes to be constitutively expressed and restored resistance to an avirulent strain of *Pseudomonas syringae* pv *tomato* in *npr1-5* (previously called *sai1*) mutant plants. In addition, *ssi1* plants were small, spontaneously developed hypersensitive response–like lesions, accumulated elevated levels of SA, and constitutively expressed the antimicrobial defensin gene *PDF1.2.* The phenotypes of the *ssi1* mutant are SA dependent. When SA accumulation was prevented in *ssi1 npr1-5* plants by expressing the SA-degrading salicylate hydroxylase (*nahG*) gene, all of the phenotypes associated with the *ssi1* mutation were suppressed. However, lesion formation and expression of the *PR* genes were restored in these plants by the application of BTH. Interestingly, expression of *PDF1.2*, which previously has been shown to be SA independent but jasmonic acid and ethylene dependent, was also suppressed in *ssi1 npr1-5* plants by the *nahG* gene. Furthermore, exogenous application of BTH restored *PDF1.2* expression in these plants. Our results suggest that SSI1 may function as a switch modulating cross-talk between the SA- and jasmonic acid/ethylene–mediated defense signal transduction pathways.

INTRODUCTION

In plants, the outcome of an interaction with a pathogen is governed by multiple factors, including the genotypes of the plant and pathogen as well as the complex exchange of signals between the host and the intruder (Yang et al., 1997). Over years of coevolution with pathogens, plants have evolved complex mechanisms to defend themselves against disease. Whereas some defense responses are constitutive, others are induced upon pathogen attack. One such induced response is systemic acquired resistance (SAR), which is triggered by prior exposure to pathogens that cause cell death (Ross 1961; Kuc, 1982; Ryals et al., 1996). SAR is long lasting and provides the plant with protection against a broad spectrum of pathogens (Dempsey and Klessig, 1995; Hunt and Ryals, 1996). A more rapid defense response that precedes SAR is the hypersensitive response (HR), which occurs at sites of pathogen entry and is characterized by programmed host cell death and restriction of pathogen growth and spread (Matthews, 1991; Hammond-Kosack and Jones, 1996). Increased expression of a subset of the pathogenesis-related (*PR*) genes, many of which encode proteins possessing antimicrobial activities, is tightly correlated with the development of the HR and SAR. Hence, the induction of these *PR* genes serves as a good molecular marker for a resistance response (Klessig and Malamy, 1994; Hunt and Ryals, 1996).

Considerable effort has been directed toward identifying signaling molecules responsible for activating the HR and SAR. Salicylic acid (SA) has emerged as a key signaling component in the manifestation of these phenomena (Hammond-Kosack and Jones, 1996; Ryals et al., 1996; Durner et al., 1997). In both tobacco and Arabidopsis, exogenous SA induces the expression of *PR* (*PR-1*, *PR-2*, and *PR-5*) genes (Antoniw and White, 1980; Ward et al., 1991; Uknes et al., 1992) and resistance (White, 1979; Uknes et al., 1993). Several studies have shown a good correlation between increases in the endogenous levels of SA and its conjugates in infected plants and both the expression of *PR* genes and the development of disease resistance (Malamy et al., 1990; Métraux et al., 1990; Uknes et al., 1993; Summermatter et

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al., 1995; Dempsey et al., 1997). Furthermore, when SA accumulation is prevented in tobacco and Arabidopsis plants because of the constitutive expression of an *nahG* transgene, which encodes the SA-degrading enzyme salicylate hydroxylase, *PR* gene expression and resistance to several pathogens is compromised (Gaffney et al., 1993; Delaney et al., 1994). Likewise, preventing SA synthesis by specifically inhibiting the activity of phenylalanine ammonia–lyase (PAL), the first enzyme in the SA biosynthetic pathway, makes otherwise resistant Arabidopsis plants susceptible to *Peronospora parasitica* (Mauch-Mani and Slusarenko, 1996). The ability of two synthetic functional analogs of SA, 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) (Vernooij et al., 1995; Görlach et al., 1996; Du and Klessig, 1997; Wendehenne et al., 1998), to restore resistance in SA-depleted NahG tobacco (Friedrich et al., 1996) and Arabidopsis (Lawton et al., 1996) plants further confirms the importance of SA in disease resistance.

To better understand the mechanisms of disease resistance and SAR, several Arabidopsis mutants with altered resistance responses have been identified. They can be broadly classified into two groups. The first group contains mutants that exhibit constitutive SAR, such as *acd2* (for accelerated cell death; Greenberg et al., 1994), *lsd* (for lesion-simulating disease; Dietrich et al., 1994; Weymann et al., 1995), *cpr* (for constitutive expresser of *PR* genes; Bowling et al., 1994, 1997; Clarke et al., 1998), and *cep* (for constitutive expression of *PR* genes; Klessig et al., 1996). These mutants constitutively accumulate high levels of SA and express the *PR* genes. They also show enhanced resistance to pathogens. The second group comprises the *eds* (for enhanced disease susceptibility; Glazebrook et al., 1996; Parker et al., 1996; Rogers and Ausubel, 1997), *ndr1* (for non-race-specific disease resistance; Century et al., 1995), and the allelic *npr1* (for nonexpresser of *PR* genes; Cao et al., 1994; Glazebrook et al., 1996), *nim1* (for noninducible immunity; Delaney et al., 1995), and *sai1* (for salicylic acid–insensitive; Shah et al., 1997) mutants, all of which exhibit compromised resistance to pathogens. The enhanced disease susceptibility phenotype of the allelic *npr1*, *nim1*, and *sai1* (renamed *npr1-5*) mutants is due to their inability to respond to SA. These mutants are incapable of expressing the *PR* (*PR-1*, *BGL2* [*PR-2*], and *PR-5*) genes or developing SAR in response to SA, INA, and BTH.

The recessive nature of most of the *NPR1* mutant alleles strongly suggests that NPR1 is a positive regulator of the SA signal transduction pathway. The *NPR1* gene was recently cloned (Cao et al., 1997; Ryals et al., 1997) and shown to encode a protein containing ankyrin repeat motifs. These ankyrin repeats appear to be important for NPR1 function because they contain missense mutations in three of the *npr1* mutants (*npr1-1*, *nim1-2*, and *npr1-5*; Cao et al., 1997; Ryals et al., 1997; H. Cao, J. Shah, D.F. Klessig, and X. Dong, unpublished results). In animals, ankyrin repeats have been implicated in mediating protein–protein interactions, such as those between 53BP2 and the tumor suppressor p53 (Gorina and Pavletich, 1996) and between IκBα and NF-κB (Krappmann et al., 1996). Interestingly, it has been suggested that *NPR1* is the plant homolog of IκBα (Ryals et al., 1997), which inhibits the activity of mammalian immune and inflammatory responses by binding to NF-κB. By analogy, NPR1 may regulate the SA-mediated activation of plant defense responses by interacting with another protein(s).

Expression of the *PR* genes can be induced in response to bacterial and fungal pathogens independently of *NPR1*. In *npr1-2*, *npr1-3* (Glazebrook et al., 1996), and *npr1-5* (Shah et al., 1997) plants, expression of the *PR-1*, *BGL2*, and *PR-5* genes was induced upon infection with a bacterial pathogen. Likewise, infection of *nim1-1* mutant plants with *P. parasitica* also induced expression of the *PR-1* gene (Delaney et al., 1995). Although the induction of *PR-1* expression in these mutants was delayed and never reached the maximal levels seen in pathogen-infected wild-type plants (Glazebrook et al., 1996; Shah et al., 1997), the *BGL2* and *PR-5* genes were induced with kinetics and magnitude similar to those observed in pathogen-infected wild-type plants (Glazebrook et al., 1996).

Even though SA is required for resistance to various pathogens in several plant species, some defense responses appear to be activated by an SA-independent pathway(s). For example, systemic resistance to *Fusarium oxysporum* as well as *Pseudomonas syringae* pv *tomato* can be induced in Arabidopsis, in the absence of SA accumulation and SA-mediated *PR* gene expression, by initially inoculating the roots with *P. fluorescens* (Pieterse et al., 1996). *Cf-2* and *Cf-9* gene–mediated resistance to *Cladosporium fulvum* species in tomato also seem to be SA independent because fungal growth is restricted equally well in wild-type and *nahG* transgenic plants (Hammond-Kosack and Jones, 1996). Likewise, the systemic induction in Arabidopsis of cysteine-rich antimicrobial peptides called defensins after *Alternaria brassicicola* infection appears to be mediated by an SA-independent pathway (Penninckx et al., 1996). Neither the presence of the *nahG* transgene nor mutations in *NPR1* adversely affected the *A. brassicicola*–mediated induction of the defensin *PDF1.2* gene. Systemic induction of *PDF1.2* has further been shown to be dependent on jasmonic acid (JA) and ethylene signaling.

Both JA and ethylene have been implicated as important signals during plant defense responses (Yang et al., 1997). Several lines of evidence suggest that there may be cross-talk between the JA, ethylene, and SA signaling pathways. Ethylene has been shown to potentiate the SA-mediated induction of *PR-1* in Arabidopsis (Lawton et al., 1995). Likewise, simultaneous application of methyl jasmonate and SA superinduces the expression of the SA-inducible *PR-1* gene in tobacco (Xu et al., 1994). The functional SA analog INA has also been shown to elevate JA levels and stimulate the expression of JA-responsive genes in rice (Schweizer et al., 1997) and the thionin gene in barley (Wasternack et al., 1994). Very recently, NPR1 has been shown to be required for the activation of ethylene- and JA-mediated systemic resistance induced by *P. fluorescens* (Pieterse et al., 1998).

Antagonistic effects between these signaling pathways have also been reported. For example, JA biosynthesis and signaling are inhibited by SA and its derivative acetyl SA (aspirin) in tomato (Peña-Cortés et al., 1993; Doares et al., 1995), and ethylene biosynthesis is inhibited by SA in apple (Pennazio et al., 1985; Leslie and Romani, 1988).

To identify other components of the SA signal transduction pathway, we set up a mutant screen for Arabidopsis to identify genetic suppressors of the *npr1-5* mutation. The *npr1-5*–conferred phenotype is due to a C-to-T transition mutation that causes a proline-to-serine change at amino acid 342 in one of the ankyrin repeats of NPR1 (H. Cao, J. Shah, D.F. Klessig, and X. Dong, unpublished results). Here, we report the identification and characterization of the dominant suppressor of SA insensitivity (*ssi1*) mutation, which confers constitutive expression of the *PR* genes and restores disease resistance in plants homozygous for the *npr1-5* mutant allele. Furthermore, *ssi1* plants spontaneously develop HR-like lesions and constitutively express the defensin *PDF1.2* gene. All of these *ssi1*-conferred phenotypes, including the expression of *PDF1.2*, are dependent on the ability of the mutant to accumulate high levels of SA. BTH application restores all of the *ssi1*-conferred phenotypes in SA-depleted *ssi1 npr1-5 nahG* plants, including the expression of the *PDF1.2* gene, suggesting that the SSI1 protein may be involved in one of the key steps regulating signaling through the SA-dependent and the JA/ethylene–dependent defense pathways.

RESULTS

The *ssi1* Mutation Causes *PR* Gene Expression and Spontaneous Development of HR-like Lesions in *npr1-5* Plants

To isolate suppressors of the SA-insensitive *npr1-5* mutation, seeds from *npr1-5* plants were mutagenized with ethyl methanesulfonate (EMS), as previously described (Shah et al., 1997). Three- to 4-week-old M_2 progeny of these EMS-mutagenized M_1 seeds were screened by RNA gel blot analysis for mutants that constitutively accumulated elevated levels of the *PR-1* gene transcript. Seven *ssi* mutants of *npr1-5* were identified among the 2400 M_2 plants screened. The *ssi1* mutant was further characterized. As shown in Figure 1A, unlike the wild-type (*SSI1 NPR1*) and the parental *npr1-5* plants (*SSI1 npr1-5*), the *ssi1 npr1-5* double mutants constitutively accumulated elevated levels of the *PR-1*, *BGL2*, and *PR-5* transcripts. In addition, the *ssi1 npr1-5* double mutants were smaller than was the parental *npr1-5* plant (*SSI1 npr1-5*; Figure 2A), and they developed macroscopic lesions on their leaves (Figure 2B). Trypan blue staining showed a heavy concentration of intensely stained dead cells in these necrotic areas (Figure 2C). Similar patterns and intensity of staining were not observed in wild-type or *npr1-5* leaves (data not shown). In addition to cell death and *PR* gene expression, the accumulation of autofluorescent material at lesion sites is associated with the HR. UV microscopy showed that the necrotic areas on the leaves of *ssi1 npr1-5* plants were associated with enhanced levels of autofluorescence (Figure 2D). This result indicates that the spontaneous lesions that develop on the *ssi1* mutant plants are HR-like.

Genetic Analysis of *ssi1*

M_3 progeny from the *ssi1 npr1-5* double mutant segregated in a ratio of three plants constitutively expressing *PR-1* (PR^+) to one plant lacking constitutive *PR-1* expression (PR^-), suggesting that the *ssi1* mutant allele is dominant over the wild-type allele. To confirm the dominant nature of the *ssi1* mutant allele, we backcrossed the *ssi1 npr1-5* double mutant to the parental *npr1-5* mutant (wild type for *SSI1*), which is in the ecotype Nössen background. The resulting F_1 and F_2 progeny were then monitored for constitutive *PR-1* gene expression. All of the F_1 plants constitutively expressed the *PR-1* gene, and this *ssi1*-conferred phenotype segregated in a 3 PR^+:1 PR^- (160 PR^+ plants to 61 PR^- plants; $\chi^2 = 0.79$; $0.5 > P > 0.3$) Mendelian ratio in the F_2 progeny. This indicates that *ssi1* is a dominant mutation at a single genetic locus. The spontaneous lesion formation and reduced size phenotypes cosegregated with constitutive *PR-1* expression, suggesting that they are due to a dominant mutation in either the *SSI1* gene or a gene(s) tightly linked to the *SSI1* locus. Approximately one-third of the plants exhibiting constitutive *PR-1* expression and lesions were very small in size (*ssi1 npr1-5*; Figure 2A) compared with the wild-type *SSI1* plants. The other two-thirds of the constitutive *PR-1*–expressing, lesion-bearing plants were intermediate in size (*ssi1*[het] *npr1-5*), suggesting that the reduced size phenotype of the *ssi1* mutant is dependent on the dose of the mutant *ssi1* allele. Analyses of F_3 progeny confirmed that the very small F_2 plants and the intermediate-sized F_2 plants were homozygous and heterozygous for the *ssi1* mutant allele, respectively.

A second site mutation within the *npr1-5* allele could potentially suppress the *npr1-5*–conferred phenotype. If so, this intragenic suppressor mutation should cosegregate with the *npr1-5* allele. To determine whether the *ssi1* mutation is an intragenic suppressor of *npr1-5*, the *ssi1 npr1-5* double mutant was crossed to wild-type (*SSI1 NPR1*) plants of ecotype Nössen, and F_2 progeny were analyzed for constitutive *PR-1* expression. This *ssi1*-conferred phenotype segregated in a 3 PR^+:1 PR^- ratio (33 PR^+ plants to 11 PR^- plants). Spontaneous lesion formation cosegregated with the PR^+ phenotype in these plants. Using codominant cleaved amplified polymorphic sequence (CAPS) analysis, we determined the genotype of these 33 phenotypically *ssi1* plants at the *NPR1* locus. The mutant *npr1-5* allele can be detected by the absence of an NlaIV restriction site that is present in

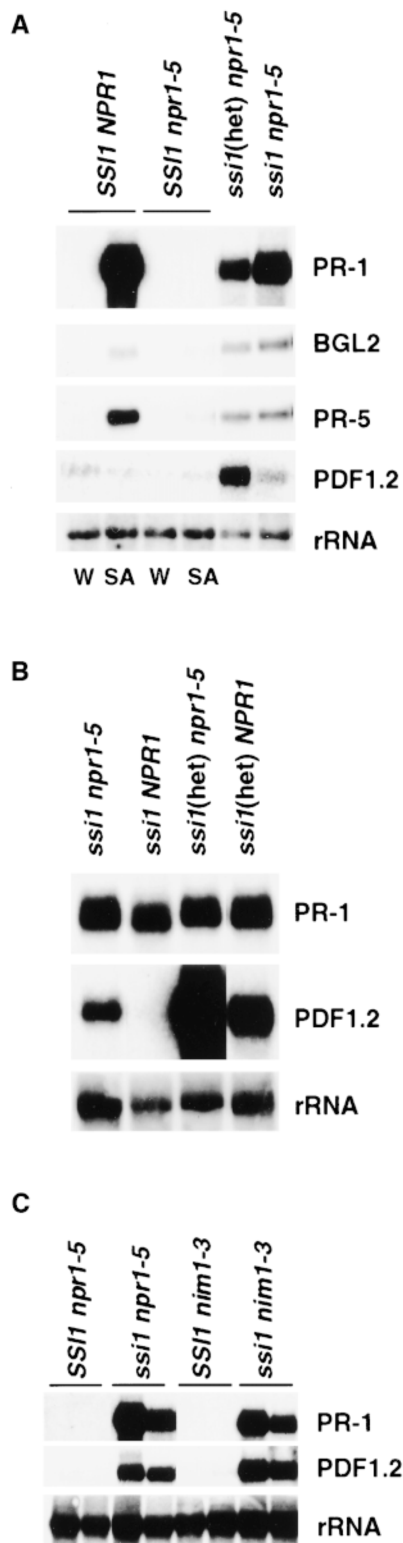

Figure 1. *PR* and *PDF1.2* Expression in *ssi1*.

(A) Expression of *PR-1*, *BGL2*, *PR-5*, and *PDF1.2* genes in water-treated or SA-treated wild-type (*SSI1 NPR1*) and *npr1-5* (*SSI1 npr1-5*) plants and in untreated heterozygous (*ssi1*[het] *npr1-5*) and homozygous (*ssi1 npr1-5*) *ssi1* plants. The heterozygous as well as homozygous *ssi1* plants analyzed were all homozygous for the *npr1-5* mutant allele. RNA was extracted from leaves of untreated *ssi1*(het) *npr1-5* and *ssi1 npr1-5* plants and, as controls, from water (W)- or SA-treated (SA) *SSI1 NPR1* and *SSI1 npr1-5* plants 24 hr after treatment.

(B) A comparison of the expression of *PR-1* and *PDF1.2* genes in *ssi1 npr1-5* double mutants and *ssi1 NPR1* plants homozygous (*ssi1 npr1-5* and *ssi1 NPR1*) or heterozygous (*ssi1*[het] *npr1-5* and *ssi1*[het] *NPR1*) for the *ssi1* mutant allele.

(C) *PR-1* and *PDF1.2* expression in *npr1-5* and *nim1-3* mutants homozygous for the wild-type *SSI1* (*SSI1 npr1-5* and *SSI1 nim1-3*) or the *ssi1* mutant allele (*ssi1 npr1-5* and *ssi1 nim1-3*). Two plants of each genotype were investigated.

The blots were sequentially probed for the indicated genes and rRNA as an internal control for gel loading and transfer. Plants were grown in soil and were 3 weeks old when sampled.

the wild-type *NPR1* allele. One-quarter of the F_2 plants with the *ssi1* phenotype (eight of 33) were homozygous for the *NPR1* wild-type allele. Analysis of *PR-1* expression in the F_3 progeny of these *ssi1 NPR1* plants (Figure 1B) confirmed that the *ssi1*-conferred phenotype is unlinked to the *npr1-5* allele and therefore is not an intragenic suppressor of *npr1-5.* Furthermore, the *ssi1*-conferred phenotype is not dependent on the *npr1-5* mutation.

To determine whether the *ssi1*-conferred phenotype requires NPR1, we analyzed the *ssi1*-conferred phenotype in the *nim1-3* (allelic with *npr1*) mutant background. The *nim1-3* allele contains a single base pair deletion causing a frameshift at amino acid 172, thus encoding a truncated protein lacking the C-terminal two-thirds of NPR1 (Ryals et al., 1997). *nim1-3* plants are insensitive to SA and are defective in activating SAR. The *ssi1 npr1-5* double mutant was crossed with a *SSI1 nim1-3* plant. F_2 plants homozygous for the *nim1-3* allele were identified using CAPS analysis, and the expression of the *PR-1* gene was analyzed in these plants. Figure 1C shows the expression of *PR-1* in two of these F_2 segregants. Three-fourths of the plants homozygous for the *nim1-3* allele constitutively expressed the *PR-1* gene and spontaneously developed lesions, thus strongly arguing that the *ssi1* mutant phenotypes do not require NPR1.

The *ssi1* Mutant Constitutively Accumulates High Levels of SA and SAG

Several studies have demonstrated the presence of an SA-dependent potentiation and feedback amplification loop in the expression of defense genes and the development of HR-like lesions (Weymann et al., 1995; Fauth et al., 1996;

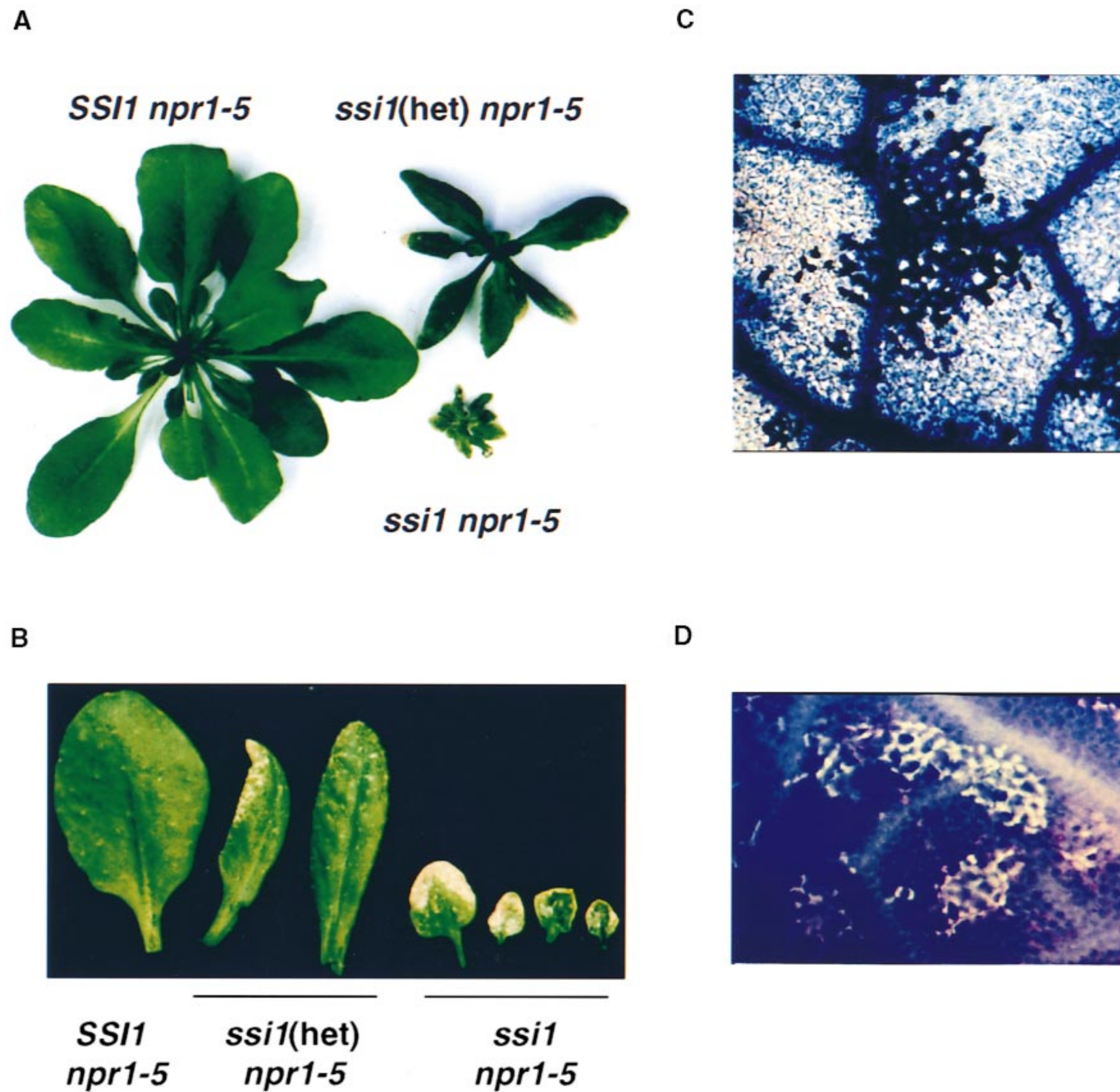

Figure 2. Morphological Phenotypes of *ssi1*.

(A) A comparison of the dominant small-size phenotype of *ssi1 npr1-5* double mutants heterozygous (*ssi1*[het] *npr1-5*) or homozygous (*ssi1 npr1-5*) for the *ssi1* mutant allele with an *npr1-5* mutant (*SSI1 npr1-5*) plant.
(B) A comparison of leaves from *SSI1 npr1-5* double mutants, heterozygous (*ssi1*[het] *npr1-5*) or homozygous (*ssi1 npr1-5*) for the *ssi1* mutant allele, showing chlorosis and spontaneous lesions with a leaf from an *npr1-5* mutant (*SSI1 npr1-5*) plant.
(C) Microscopy of a trypan blue–stained leaf containing lesions from an *ssi1 npr1-5* plant showing an intensely stained area of dead cells.
(D) UV microscopy of a leaf from an *ssi1 npr1-5* plant showing increased autofluorescence, above background, at the site of a lesion.
All plants were grown in soil and photographed when 3 weeks old.

Mur et al., 1996; Shirasu et al., 1997; Thulke and Conrath, 1998). Therefore, we analyzed the endogenous levels of SA and its glucoside (SAG) in *npr1-5* plants that were either homozygous or heterozygous for the *ssi1* mutant allele. As shown in Figure 3, SA (7.3 $\pm$ 0.4 μg per gram fresh weight of tissue) and SAG (80.0 $\pm$ 4.2 μg per gram fresh weight of tissue) levels in plants homozygous for the *ssi1* mutant allele (*ssi1 npr1-5*) were $\sim$20- and 200-fold higher, respectively, than in the parental *npr1-5* plants (*SSI1 npr1-5*). In comparison, plants heterozygous for the *ssi1* mutant allele (*ssi1*[het]

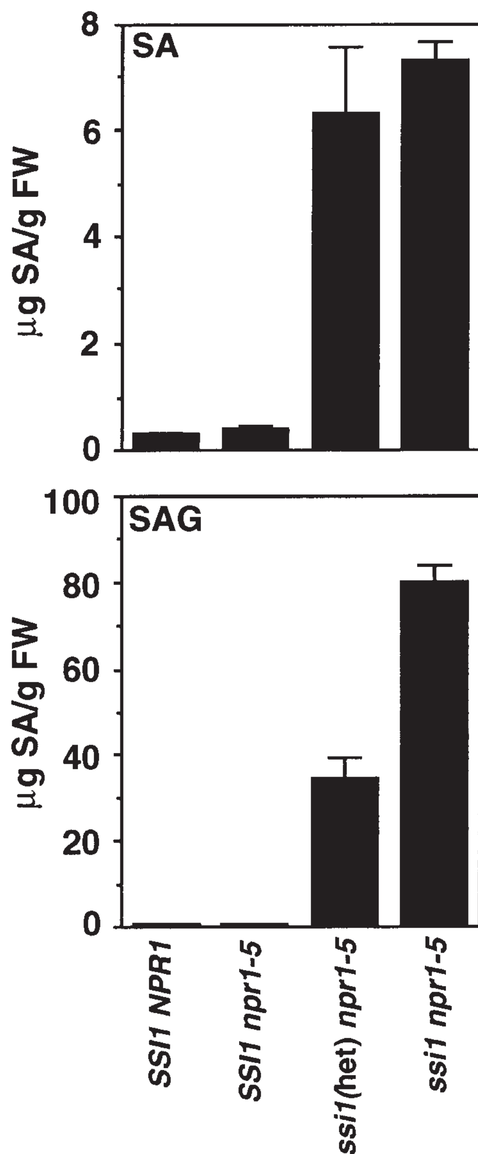

Figure 3. Comparison of SA and SAG Levels in the Wild Type (*SSI1 NPR1*), the *npr1-5* Mutant (*SSI1 npr1-5*), and *ssi1 npr1-5* Double Mutants Heterozygous (*ssi1*[het] *npr1-5*) or Homozygous (*ssi1 npr1-5*) for the *ssi1* Mutant Allele.

Leaves from 3-week-old soil-grown plants were harvested, extracted, and analyzed by HPLC, as described by Bowling et al. (1994). The SA and SAG values ±SD, presented as micrograms of SA per gram fresh weight (FW) of tissue, are averages of three to five sets of samples per line.

npr1-5) accumulated slightly less SA (6.3 ± 1.3 µg per gram fresh weight of tissue) and approximately twofold less SAG (34.4 ± 4.9 µg per gram fresh weight of tissue) than did the homozygous *ssi1* plants.

Expression of an *nahG* Transgene Suppresses Constitutive *PR* Gene Expression and Spontaneous Lesion Formation in *ssi1* Plants

To determine whether high endogenous levels of SA and SAG are required for the mutant phenotypes exhibited by the *ssi1* plants, *ssi1 npr1-5* double mutants were crossed with NahG (ecotype Nössen) plants, which are unable to accumulate elevated levels of SA. The resulting F_1 plants (*ssi1* × *nahG*) did not constitutively express the *PR-1*, *BGL2*, or *PR-5* genes (Figure 4). Furthermore, they were morphologically similar to wild-type plants and did not develop lesions or possess the reduced-size phenotype associated with the dominant *ssi1* mutant allele. These results were further confirmed in the F_2 progeny (data not shown). Thus, the elevated levels of endogenous SA detected in the *ssi1* mutant appear to be required for all of the *ssi1*-conferred phenotypes.

BTH Restores *PR* Gene Expression and Spontaneous Lesion Formation in SA-Depleted *ssi1 npr1-5 nahG* Plants

BTH, a functional analog of SA (Görlach et al., 1996; Lawton et al., 1996; Du and Klessig, 1997; Wendehenne et al., 1998), induces *PR* gene expression and disease resistance in both wild-type and *nahG*-expressing transgenic Arabidopsis plants (Lawton et al., 1996). However, BTH requires a functional SA signaling pathway because it is unable to induce *PR* expression in *npr1-5* plants (Shah et al., 1997). Because the *ssi1* mutation restores SA-mediated expression of the *PR* genes in *npr1-5* plants, we tested whether it could also restore BTH-induced *PR* gene expression in *npr1-5* plants. *ssi1 npr1-5 nahG* plants (homozygous for the *npr1-5* allele) were used for this experiment because they fail to accumulate elevated levels of SA and do not possess any of the phenotypes associated with the *ssi1* mutation. BTH treatment was found to restore lesion formation in the existing and newly emerging leaves of these plants (Figures 5A and 5B). It also induced to high levels the expression of *PR-1* (Figure 6), *BGL2*, and *PR-5* (data not shown). In comparison, BTH was unable to induce high levels of *PR* gene expression or lesion formation in *npr1-5 nahG* plants homozygous for the wild-type *SSI1* allele (*SSI1 npr1-5 nahG*). Trypan blue staining confirmed the absence of lesions in these plants after BTH treatment (Figure 5B). These results argue that the mere accumulation of high levels of SA may not necessarily cause the *ssi1*-conferred phenotypes. Rather, the *SSI1* gene

functions as a component of the SA signal transduction pathway, and the *ssi1* mutation bypasses the requirement of NPR1 function for expression of the *PR* genes.

ssi1 Restores Resistance to an Avirulent Bacterial Pathogen in *npr1-5* Plants

Because the *ssi1* mutation restores SA- and BTH-inducible *PR* gene expression in *npr1-5* plants, we tested whether it would also restore disease resistance. Wild-type Nössen plants contain the resistance gene *RPS2* (Bent et al., 1994) and are resistant to *P. s. tomato* DC3000 carrying the *avrRpt2* avirulence gene. In contrast, *npr1-5* plants show enhanced susceptibility to this pathogen (Shah et al., 1997). Because plants homozygous for the *ssi1* mutant allele are very small and difficult to infiltrate with *P. s. tomato*, we chose to infect plants heterozygous for the *ssi1* mutant allele. As shown in Figure 7A, the growth of *P. s. tomato* was 14-fold lower in *ssi1*(het) *npr1-5* double mutants than in *SSI1 npr1-5* plants at 3 days postinfiltration (DPI). The presence of the wild-type *NPR1* gene led to an additional twofold decrease in bacterial growth in *ssi1*(het) *NPR1* plants as compared with *ssi1*(het) *npr1-5* double mutant plants.

The accumulation of *PR-1* gene transcript was also monitored in these plants after *P. s. tomato* infection (Figure 7B). Unlike the wild-type (*SSI1 NPR1*) and the *SSI1 npr1-5* plants, the *PR-1* gene was constitutively expressed in the uninfected *ssi1*(het) *npr1-5* and *ssi1*(het) *NPR1* plants. Furthermore, as in the wild-type plants, *PR-1* expression increased after *P. s. tomato* infection and reached maximal levels by 1 DPI in the *ssi1*(het) *npr1-5* and *ssi1*(het) *NPR1* plants. In comparison, as previously demonstrated in *SSI1 npr1-5* plants (Shah et al., 1997), *PR-1* expression was delayed and never attained the maximal levels seen in the *P. s. tomato*–infected wild-type or the *ssi1* plants.

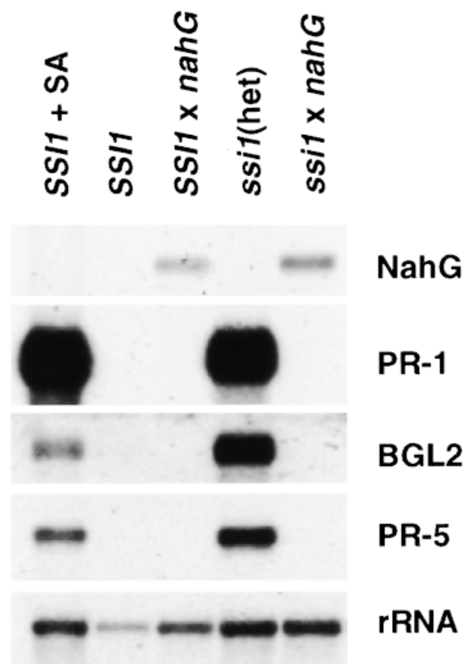

Figure 4. Expression of *PR* Genes in *ssi1* × *nahG* F_1 Plants.

RNA was extracted from *SSI1* × *nahG* and *ssi1* × *nahG* F_1 plants and a plant heterozygous for the *ssi1* mutant allele (*ssi1*[het]). These F_1 plants contain a wild-type *NPR1* allele derived from the wild-type or NahG parents. As a control, RNA was also extracted from an untreated wild-type plant (*SSI1*) and a wild-type plant 24 hr after SA (500 μM) treatment (*SSI1* + SA). All plants were grown in soil and sampled when 3 weeks old. The blot was sequentially probed for the Arabidopsis *PR-1*, *BGL2*, and *PR-5* gene transcripts and the *nahG* transgene transcript (NahG); rRNA was used as an internal control for gel loading and transfer.

Constitutive Defensin Gene Expression in the *ssi1* Mutant Is SA Dependent

Expression of the *PDF1.2* gene, which encodes defensin, an antifungal peptide, has previously been shown to be independent of both SA and NPR1 (Penninckx et al., 1996). This gene is constitutively expressed in an SA- and NPR1-independent manner in the Arabidopsis *cpr5* and *cpr6-1* mutants that, like *ssi1*, constitutively express the *PR* genes and exhibit SAR (Bowling et al., 1997; Clarke et al., 1998). Therefore, we examined the accumulation of *PDF1.2* transcripts in *ssi1* plants. As shown in Figures 1A and 1B, plants homozygous for both the *ssi1* and *npr1-5* alleles (*ssi1 npr1-5*) constitutively accumulated elevated levels of *PDF1.2* transcripts. Similarly, plants homozygous for the *ssi1* and *nim1-3* alleles also constitutively expressed the *PDF1.2* gene (Figure 1C). Interestingly, the expression of *PDF1.2*, unlike that of the *PR* genes, was observed to be higher in plants heterozygous for the *ssi1* allele (*ssi1*[het] *npr1-5*) compared with those homozygous for the *ssi1* allele (*ssi1 npr1-5*; Figure 1A). Constitutive *PDF1.2* expression was also repeatedly observed to be higher in *ssi1* plants homozygous for the *npr1-5* mutant allele compared with *ssi1* plants homozygous for the wild-type *NPR1* allele (Figures 1B and 7B). Unlike expression of the *PR-1* gene, *PDF1.2* expression was not induced by *P. s. tomato* infection in either the wild-type or the *ssi1* plants (Figure 7B). Instead, steady state levels of the *PDF1.2* transcript in the *ssi1* mutants transiently decreased 1 and 2 DPI, returning to the basal levels seen in uninfected plants by 3 DPI. In contrast, in two of four experiments, infection with *P. s. tomato* was found to induce *PDF1.2* expression in the *SSI1 npr1-5* mutant.

To determine whether the constitutive expression of *PDF1.2* observed in *ssi1* plants was associated with elevated SA levels, we analyzed its expression in *ssi1 npr1-5 nahG* plants. Expression of the *nahG* transgene was observed

A

B

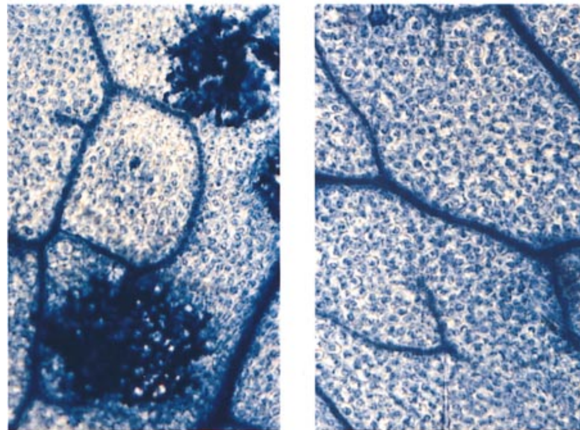

Figure 5. Comparison of Lesion Formation in Untreated and BTH-Treated *ssi1 npr1-5 nahG* and *SSI1 npr1-5 nahG* Plants.

(A) Leaves from an untreated and BTH-treated *ssi1 npr1-5 nahG* plant showing absence of macroscopic lesions in untreated plants and restoration of lesions when BTH was applied.

(B) Trypan blue–stained, BTH-treated leaves of an *ssi1 npr1-5 nahG* plant showing intensely stained areas of dead cells. As a control, leaves from a BTH-treated *SSI1 npr1-5 nahG* plant are shown; they do not have areas of intensely stained dead cells.

Three-week-old plants were treated with 100 μM BTH, and leaf tissue from untreated and BTH-treated plants was analyzed 6 days later.

to suppress *PDF1.2* expression. BTH treatment of these *nahG*-expressing *ssi1 npr1-5* mutants, however, restored *PDF1.2* expression (Figure 6). In contrast, BTH treatment did not induce *PDF1.2* expression in *nahG* plants homozygous for the wild-type *SSI1* and mutant *npr1-5* alleles (*SSI1 npr1-5 nahG*).

ssi1 Maps to Chromosome 4 and Defines a Novel Gene

To determine the map position of the *SSI1* locus, we crossed *ssi1* plants (ecotype Nössen) with wild-type plants from ecotype Columbia. As expected, the F_2 progeny segregated in a 3 PR^+:1 PR^- ratio (88 PR^+ plants to 28 PR^- plants) when scored for constitutive *PR-1* expression. The genotype at the *SSI1* locus was determined for these 116 F_2 plants by monitoring constitutive *PR-1* expression in the F_3 families. CAPS (Konieczny and Ausubel, 1993) and simple sequence length polymorphism (SSLP; Bell and Ecker, 1994) marker analyses were subsequently performed on 24 PR^+ (homozygous *ssi1*) F_2 plants. Based on these preliminary analyses, *ssi1* was mapped to chromosome 4 in the 28-centimorgan (cM) interval between the SSLP marker *nga8* and the CAPS marker *SC5.*

Because the *ssi1* mutant allele is dominant and homozygous *ssi1* plants are small and poor seed producers, the map position of the *SSI1* locus was further determined using 182 phenotypically wild-type (PR^-) F_2 plants. Based on this analysis, the *SSI1* gene was mapped within a 16.1-cM interval, 9.8 cM from the SSLP marker *AthDET1* and 6.3 cM from the CAPS marker *SC5.* The *CPR1*, *LSD1*, *ACD2*, and *CEP1* genes, which have recessive mutations and confer constitutive expression of *PR* genes, also map to chromosome 4 (Bowling et al., 1994; Dietrich et al., 1994; Greenberg et al., 1994; Klessig et al., 1996) but distal to *ssi1. CPR1* is the closest to *SSI1*; however, the *cpr1* mutation is recessive, and unlike the dominant *ssi1* mutation, *cpr1* plants do not constitutively express the *PDF1.2* gene or spontaneously develop lesions. From these results, we conclude that *cpr1* and *ssi1* are most likely mutations in two distinct genes.

DISCUSSION

To identify components of the SA-mediated defense signaling pathway, we isolated and characterized a suppressor of the *npr1-5* mutant designated *ssi1.* The dominant *ssi1* mutation suppresses all of the known phenotypes of the *npr1-5* mutant, including lack of SA-mediated expression of the *PR-1*, *BGL2*, and *PR-5* genes and reduced disease resistance. In addition, *ssi1 npr1-5* plants constitutively express these *PR* genes and the JA- and ethylene-responsive *PDF1.2* gene, spontaneously develop HR-like lesions, are smaller in size than either the wild-type or parental *npr1-5* plants, and accumulate elevated levels of SA and SAG.

The *ssi1*-conferred phenotypes appear to be dependent on high levels of SA because they are suppressed in SA-depleted *ssi1 npr1-5* plants expressing the *nahG* gene. Moreover, the ability of BTH to restore the phenotype conferred by *ssi1* in these plants suggests that SSI1 is a component of the SA-mediated signaling pathway leading to defense responses. Like the dominant *ssi1* mutant, the recessive *cpr5* (Bowling et al., 1997) and the dominant *cpr6-1* (Clarke et al., 1998) mutants, which map to chromosomes 5 and 1, respectively, also constitutively express the *PR* and *PDF1.2* genes, accumulate elevated levels of SA, show enhanced resistance to bacterial pathogens, and are small in size. In addition, like *ssi1*, *cpr5* plants spontaneously develop HR-like lesions. The constitutive expression of *PR* genes in *ssi1*, *cpr5*, and *cpr6-1* is dependent on SA. However, whereas *PR* gene expression in *cpr5* requires NPR1 function, it is independent of NPR1 in the *ssi1* and *cpr6-1* mutants. Moreover, in contrast to the ability of *ssi1* to restore resistance against bacterial pathogens in *npr1-5* plants, resistance to a bacterial pathogen in *cpr5* and *cpr6-1* was dependent on NPR1 function. Finally, unlike *ssi1*, constitutive *PDF1.2* expression in *cpr5* and *cpr6-1* is independent of SA.

The NPR1 protein has previously been shown to be required for SA-mediated expression of *PR* genes and the establishment of SAR in Arabidopsis (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). These studies have demonstrated that NPR1 functions downstream of SA. Because *ssi1* plants accumulate elevated levels of SA and require SA for constitutive *PR* expression, it is possible that SSI1 functions upstream of NPR1 in the SA-signaling pathway. This scenario would require *npr1-5* to be a leaky mutation. However, the *ssi1* mutation can confer constitutive *PR* expression in the *nim1-3* (allelic with *npr1*) mutant background (Figure 1C), which potentially expresses a protein lacking the C-terminal two-thirds of NPR1 (Ryals et al., 1997). This confirms that the *ssi1*-conferred phenotype does not depend on NPR1 function; hence, SSI1 does not function upstream of NPR1.

If SSI1 is not upstream of NPR1, where does it function in the defense signaling pathway? NPR1 contains ankyrin repeats, which are involved in protein–protein interactions in animals. These repeats appear to be important for NPR1 function because three mutant alleles of the *NPR1* gene contain missense mutations in the ankyrin repeat region (Cao et al., 1997; Ryals et al., 1997; H. Cao, J. Shah, D.F. Klessig, and X. Dong, unpublished results). Thus, it is possible that SSI1 physically interacts with NPR1 to propagate the SA signal. The mutation in *npr1-5* could adversely affect this interaction, thereby preventing the SA signal from being transmitted further. In the *ssi1* mutant, a compensatory mutation in the SSI1 protein could allow partial interaction between the mutant SSI1 and NPR1 proteins. This would then restore the SA signaling pathway leading to expression of the *PR* genes and disease resistance. However, the inability of the *nim1-3* mutant to suppress the *ssi1*-mediated constitutive *PR* expression phenotype strongly argues against SSI1 functioning as an NPR1-interacting protein.

A more plausible hypothesis is that SSI1 might either directly or indirectly influence the SA signaling pathway downstream of NPR1 (Figure 8A). SSI1 could function either downstream of NPR1 or produce a signal that, in conjunction with an NPR1-derived signal, activates *PR* gene expression and disease resistance. A mutation in the SSI1 protein might allow the requirement for the NPR1-derived signal to be bypassed while still requiring SA. SA either would be required to activate the SSI1 protein or may be required in any one of the downstream steps leading to expression of the *PR* genes and disease resistance. The recent observation that *PR* gene expression is not constitutively activated in Arabidopsis overexpressing NPR1 (Cao et al., 1998) can also

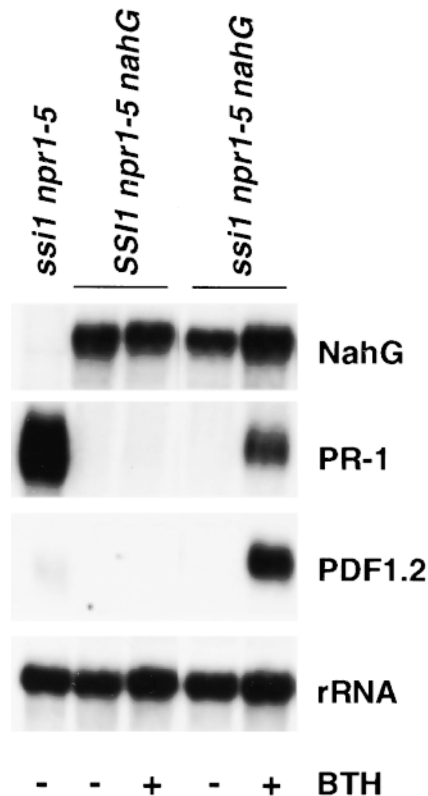

Figure 6. Expression of *PR-1* and *PDF1.2* Genes in BTH-Treated *ssi1 npr1-5 nahG* Plants.

RNA was extracted 6 days after BTH treatment (+; 100 μM) from leaves of 3-week-old *ssi1 npr1-5 nahG* and *SSI1 npr1-5 nahG* plants. As controls, RNA was also extracted from untreated (−) *ssi1 npr1-5 nahG*, *SSI1 npr1-5 nahG*, and *ssi1 npr1-5* plants. The blot was sequentially probed for the Arabidopsis *PR-1* and *PDF1.2* gene transcripts, the *nahG* transgene transcript (NahG), and rRNA as an internal control for gel loading and transfer.

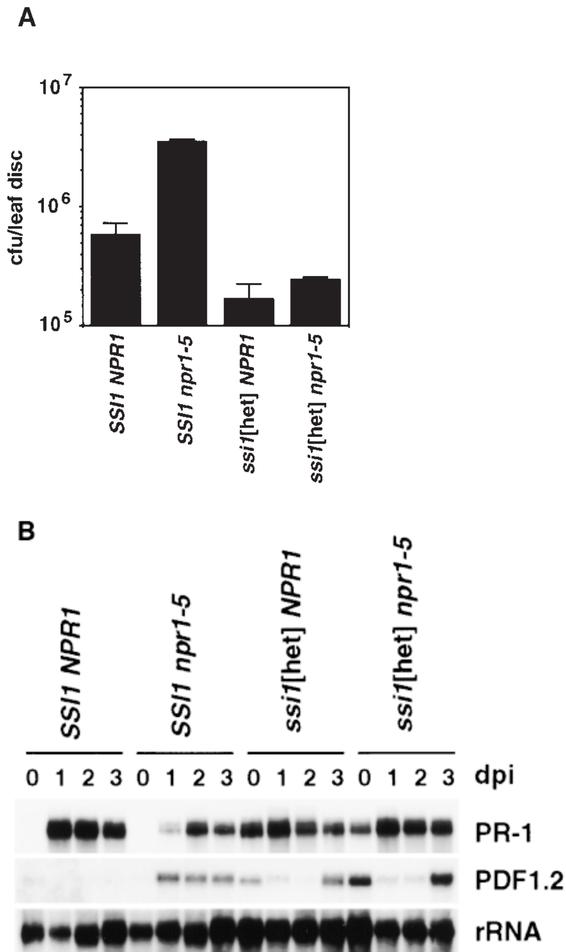

Figure 7. Growth of *P. s. tomato* DC3000 Containing the *avrRpt2* Avirulence Gene and *PR-1* and *PDF1.2* Expression in Wild-Type (*SSI1 NPR1*), *npr1-5* Mutant (*SSI1 npr1-5*), *ssi1* Mutant (*ssi1*[het] *NPR1*), and *ssi1 npr1-5* Double Mutant (*ssi1*[het] *npr1-5*) Plants.

(A) *P. s. tomato* DC3000 containing the *avrRpt2* avirulence gene ($OD_{600\ nm}$ = 0.001 in 10 mM $MgCl_2$) was infiltrated into the abaxial surface of leaves with a syringe. Four leaf discs were harvested 3 DPI from the *P. s. tomato*–infected leaves and ground in 10 mM $MgCl_2$, and bacterial numbers were titered. The bacterial numbers ±SD, presented as colony-forming units (cfu) per leaf disc (0.2 cm^2), are averages of three samples. The *ssi1 NPR1* (*ssi1*[het] *NPR1*) and *ssi1 npr1-5* double mutant (*ssi1*[het] *npr1-5*) plants were heterozygous for the *ssi1* mutant allele.

(B) Expression of the *PR-1* and *PDF1.2* genes in *P. s. tomato*–infected wild-type (*SSI1 NPR1*), *npr1-5* mutant (*SSI1 npr1-5*), *ssi1* mutant (*ssi1*[het] *NPR1*), and *ssi1 npr1-5* double mutant (*ssi1*[het] *npr1-5*) plants. *P. s. tomato*–infected leaf samples were taken at the indicated times, and RNA was extracted. The blot was sequentially probed for Arabidopsis *PR-1* and *PDF1.2*, and rRNA was used as an internal control for gel loading and transfer.

be explained by this hypothesis. Although these plants overexpress NPR1, they lack the SSI1-activated signal, which is also required for *PR* gene induction. Alternatively, SSI1 might function completely independently of NPR1 and might be a component of an SA-dependent but NPR1-independent pathway (Figure 8B) involved in the expression of *PR* genes and resistance. Previous studies of various *npr1* mutants have suggested the existence of such an NPR1-independent resistance pathway (Glazebrook et al., 1996; Shah et al., 1997). Expression of *PR* genes was activated in *npr1* plants upon pathogen infection. Because SA by itself cannot activate *PR* expression in *SSI1 npr1* plants, this scenario would require SA plus another pathogen-induced signal for the activation of SSI1-dependent *PR* gene expression. A mutation in the SSI1 protein could bypass the requirement for this unknown pathogen-induced signal, although SA would still be required.

Irrespective of where SSI1 acts in the SA signal transduction pathway, based on the dominance of the *ssi1* mutant allele over the wild-type SSI1 allele, the *ssi1*-conferred phenotype could be due either to a gain-of-function mutation in a positive regulator of the SA signal transduction pathway or, alternatively, to a dominant loss-of-function mutation in a negative regulator. The available data do not allow us to classify SSI1 as an activator or a repressor of the SA signaling pathway.

Even though SSI1 does not function upstream of NPR1, the *ssi1* mutant accumulates elevated levels of SA. In addition, SA is required to activate the *ssi1*-conferred phenotype in the SA-insensitive *npr1-5* background. This implies that SSI1-activated components of the defense pathway might subsequently regulate SA accumulation through a feedback loop. A feedback or SA-dependent potentiation loop has previously been identified in the pathways leading to several defense responses including cell death and the expression of PAL (Weymann et al., 1995; Fauth et al., 1996; Mur et al., 1996; Shirasu et al., 1997; Thulke and Conrath, 1998). PAL activity is required for SA biosynthesis in Arabidopsis (Mauch-Mani and Slusarenko, 1996), and induction of *PAL* expression by pathogen infection or elicitor treatment is potentiated by SA (Shirasu et al., 1997; Thulke and Conrath, 1998). In the *ssi1* mutant, the pathogen-inducible *PAL1* gene is constitutively expressed at elevated levels (J. Shah and D.F. Klessig, unpublished data). An SSI1-activated defense component(s) in the *ssi1* plant could activate expression of the *PAL1* gene, which in turn could lead to the increased production of SA. SA in turn would then activate SSI1-dependent signaling leading to expression of *PR* genes and disease resistance.

ssi1 plants spontaneously develop HR-like lesions. Lesion formation in *ssi1* is dependent on the accumulation of elevated levels of SA. However, cell death in *ssi1* plants is not a direct result of toxicity due to the enormously high levels of SA that accumulate in the mutant but rather is a result of the activation of an SSI1-dependent cell death pathway. This is evident from the fact that BTH application at concentrations

that are known not to induce cell death in wild-type *SSI1* plants activates cell death in SA-depleted *ssi1 npr1-5 nahG* plants but not in *SSI1 npr1-5 nahG* plants (Figures 5A and 5B).

HR-like cell death in several cases has been shown to be mediated through reactive oxygen species (ROS), and SA has been shown to potentiate pathogen-induced as well as ROS-induced cell death (Levine et al., 1994; Dangl et al., 1996). In the *lsd1* mutant, cell death was shown to be dependent on the accumulation of elevated levels of superoxide, and SA or its functional analog INA could induce cell death in *lsd1* under nonpermissive conditions (Dangl et al., 1996; Jabs et al., 1996). Whether activation of cell death in *ssi1* is a result of the production of ROS needs to be investigated. Although *PR* and *PDF1.2* gene expression and cell death in the *ssi1* mutant are tightly correlated, a cause and effect relationship between cell death and *PR* and *PDF1.2* expression is at present unclear. However, in several other cases, *PR* gene expression, SA accumulation, and resistance can occur independently of cell death. The Arabidopsis *cpr1* (Bowling et al., 1994), *cpr6-1* (Clarke et al., 1998), and *dnd1* (Yu et al., 1998) mutants constitutively accumulate elevated levels of SA and express *PR* genes without any evident spontaneous cell death. *cpr6-1* plants, like the *ssi1* mutant plants, also constitutively express the *PDF1.2* gene. Furthermore, the *dnd1* mutant also demonstrates gene-for-gene disease resistance in the absence of HR-associated cell death (Yu et al., 1998).

A. brassicicola–induced expression of the *PDF1.2* gene in Arabidopsis has previously been shown to occur independent of both SA and NPR1 (Penninckx et al., 1996). However, based on our analyses of *ssi1 npr1-5 nahG* plants, SA is required for constitutive *PDF1.2* expression in *ssi1* mutant plants. Possibly, SA is required in these plants to activate the mutant SSI1 protein or a downstream component and thus *PDF1.2* expression. Supporting this hypothesis, BTH treatment restores high levels of *PDF1.2* expression in *ssi1*

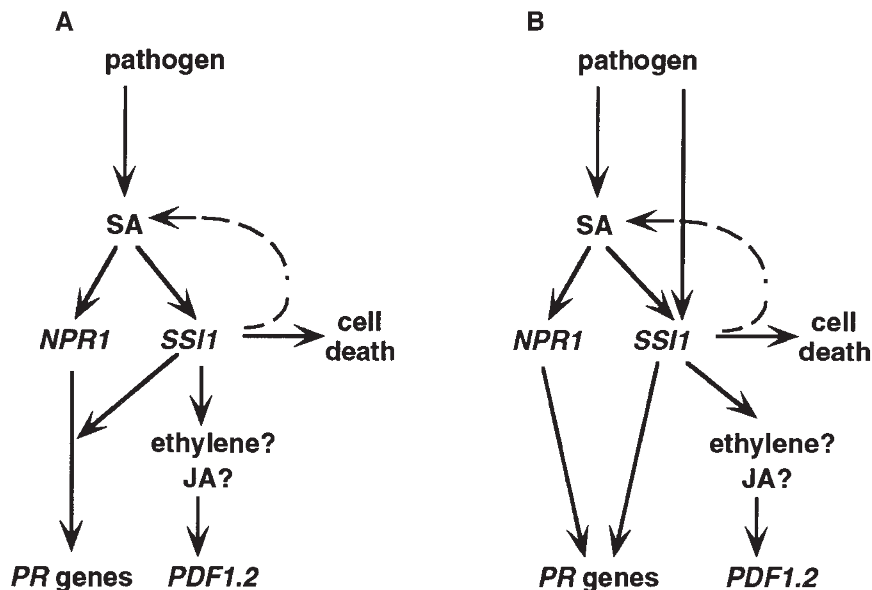

Figure 8. Possible Relationships among SA and the *NPR1*, *SSI1*, *PR*, and *PDF1.2* Genes.

(A) *SSI1* or an *SSI1*-generated signal enters the SA signaling pathway downstream of *NPR1*. The mutant SSI1 protein bypasses the requirement of the NPR1-generated signal for activation of the signaling pathway.

(B) *SSI1* activates expression of the *PR* genes and resistance via an SA-dependent but NPR1-independent pathway. A second pathogen-activated signal is required for activation of *SSI1*, in addition to SA, to account for the inability of exogenous SA to induce expression of *PR* genes in *npr1* mutant plants. The mutant SSI1 protein bypasses the requirement for this pathogen-derived signal, although it still requires SA.

In both **(A)** and **(B)**, *SSI1* is shown to regulate the accumulation of SA via an autoregulatory loop (dashed arrows). SSI1-mediated cell death and SA accumulation have been shown to be independent of each other in light of the accumulating genetic evidence that cell death may not be required for SA accumulation. Cell death is also shown to be independent of the *NPR1* gene because the HR develops normally in the *npr1* mutant when infected with avirulent bacterial pathogens (Cao et al., 1994; J. Shah and D.F. Klessig, unpublished results). Pathogen-induced expression of *PDF1.2* is dependent on JA and ethylene signaling. At the present time, it is not known whether *SSI1*-induced expression of *PDF1.2* in the *ssi1* mutant bypasses the requirement of JA and ethylene. However, inhibition of JA and ethylene biosynthesis by SA could partly explain the inability of exogenous SA to induce *PDF1.2* expression in wild-type plants.

npr1-5 nahG plants. Interestingly, our studies of the *ssi1* mutant suggest that NPR1 negatively regulates *PDF1.2* expression. *PDF1.2* expression was repeatedly observed to be higher in *ssi1 npr1-5* plants compared with *ssi1 NPR1* plants (Figures 1B and 7B). Similarly, Bowling et al. (1997) have observed elevated steady state levels of *PDF1.2* transcript in the *npr1-1* mutant grown on agar plates compared with wild-type plants.

If SSI1 is involved in the expression of both the *PR* genes and *PDF1.2*, why does the application of SA or BTH fail to induce *PDF1.2* expression in plants homozygous for the wild-type *SSI1* gene? One possible explanation is that a second signal, in addition to SSI1, is required for *PDF1.2* expression in SA/BTH–treated wild-type plants. In *ssi1* plants, the mutant SSI1 protein might be able to bypass the need for this second signal. An alternative, although not mutually exclusive, explanation is that SA, which is required for *PR* expression, inhibits the synthesis of JA (Peña-Cortés et al., 1993) and ethylene (Pennazio et al., 1985; Leslie and Romani, 1988). Both JA and ethylene, along with their corresponding signal transduction pathways, are known to be required for pathogen-induced activation of the *PDF1.2* gene. Supporting this possibility, defensin accumulation after *A. brassicicola* infection was shown to be higher in *nahG* transgenic Arabidopsis plants than in nontransgenic plants (Penninckx et al., 1996). Similarly, *PDF1.2* expression was repeatedly observed to be higher in heterozygous *ssi1* plants (Figure 1), which accumulate twofold lower levels of total SA, than in the homozygous *ssi1* plants (Figure 3). The *ssi1* mutant might constitutively accumulate high levels of JA and/or ethylene as well as SA. Elevated levels of JA and ethylene would lead to activation of *PDF1.2* expression in *ssi1* plants. In support of this possibility, the JA-responsive thionin (*THI2-1*), lipoxygenase (*LOX2*), and vegetative storage protein (*VSP*) genes and the ethylene-responsive basic *PR-3* gene are constitutively expressed at elevated levels in the *ssi1* mutant (J. Shah and D.F. Klessig, unpublished results). Interestingly, the *acd2* mutant of Arabidopsis, which like *ssi1* plants spontaneously develops lesions, accumulates high levels of SA, and constitutively expresses both *PR* and *PDF1.2* genes (Greenberg et al., 1994; Penninckx et al., 1996), also accumulates ninefold higher levels of JA than do wild-type plants (Penninckx et al., 1996).

An interesting outcome of our study with the *ssi1* mutant is the finding that the SA-responsive and the JA- and ethylene-responsive defense pathways do not appear to function completely independently of each other. Rather, each might regulate the temporal expression and/or amplitude of the other pathway. The *SSI1* gene might encode an important switch that, depending on the input signal(s), may differentially regulate these pathways. Several lines of evidence support the existence of cross-talk between the SA-mediated pathway and the JA- and ethylene-dependent, wounding-responsive pathway(s). For example, whereas many wounding responses are mediated by JA and ethylene, overexpression of the rice Ras-like G protein gene *rgp1* renders SA accumulation and *PR* gene expression wounding responsive in tobacco (Sano et al., 1994). Similarly, overexpression of the tobacco WIPK (for wounding-induced protein kinase) mitogen-activated protein (MAP) kinase gene leads to elevated levels of SA and *PR* gene expression upon wounding (Seo et al., 1995). In addition, the tobacco MAP kinase SIPK (for SA-induced protein kinase) is rapidly and transiently activated by wounding as well as SA (Zhang and Klessig, 1998), further suggesting the existence of shared components between these different defense pathways. Finally, *NPR1* has recently been shown to be required for the JA- and ethylene-mediated activation of systemic resistance induced by *P. fluorescens* (Pieterse et al., 1998). Thus, cloning the *SSI1* gene and identifying suppressors of the *ssi1* mutation should help elucidate not only the signaling components associated with these different defense pathways but also the mechanisms through which they interact.

METHODS

Growth Conditions for Plants and Bacteria

Plants (*Arabidopsis thaliana*) were grown in soil at 22°C in growth chambers programmed for a 16-hr-light (8000 to 10,000 lux) and 8-hr-dark cycle, unless otherwise stated. *Pseudomonas syringae* pv *tomato* DC3000 carrying a plasmid-borne avirulence *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

Infection of plants with *P. s. tomato* DC3000 carrying a plasmid-borne *avrRpt2* gene (Bent et al., 1994) was performed as described previously (Shah et al., 1997). Four leaves per plant were infiltrated with a suspension ($OD_{600\ nm}$ of 0.001) in 10 mM $MgCl_2$. Twelve leaf discs, 0.5 cm in diameter (0.20 cm^2), were harvested at the indicated times and processed for bacterial counts and RNA extraction, as described previously (Shah et al., 1997). The average bacterial count in the leaves immediately after infection was 2000 colony-forming units per leaf disc.

Chemical Treatment of Plants

Three-week-old plants were sprayed and subirrigated with a solution of salicylic acid (SA; 500 μM) or benzothiadiazole (BTH; 100 μM active ingredient) in water, as previously described (Shah et al., 1997). Wherever possible, another set of control plants was similarly treated with water. Leaves were harvested at the indicated times after treatment and quick frozen in liquid nitrogen. Leaf samples were stored at −80°C. For analysis of individual plants, two fully expanded leaves were harvested before any chemical treatment. This sample served as the untreated control.

RNA Extraction and Gel and Dot Blot Analyses

Large-scale preparation of RNA from Arabidopsis was conducted according to Das et al. (1990). Small-scale extraction of RNA from one or two leaves was performed in the TRIzol reagent (Gibco BRL, Gaithersburg, MD), following the manufacturer's instructions. RNA gel blot analysis and synthesis of random primed probes for *PR-1*, *BGL2*, *PR-5*, and rDNA were done as described previously (Shah et al., 1997). Probes specific for the *nahG* and *PDF1.2* gene transcripts were synthesized by random primed ^{32}P-labeling of gel-purified DNA fragments containing the *nahG* insert and a 400-bp polymerase chain reaction (PCR) fragment of *PDF1.2* (Penninckx et al., 1996). RNA gel blot hybridization and dot blot analysis were performed according to Sambrook et al. (1989).

Histochemistry and Microscopy

Leaf samples for trypan blue staining and epifluorescence microscopy were obtained from 3-week-old plants. Samples were processed and analyzed as described by Bowling et al. (1997).

SA and SA Glucoside Estimations

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

Mutagenesis and Selection of *ssi* Mutants

Five thousand seeds from plants homozygous for the *npr1-5* mutant allele (ecotype Nössen) were mutagenized with 0.3% ethyl methanesulfonate (EMS; Sigma), as previously described (Shah et al., 1997). M_2 seeds were harvested as pools; each pool contained M_2 seeds derived from ~10 EMS-mutagenized M_1 seeds. Approximately 80 M_2 seeds from each pool were germinated in soil in four 144-cm^2 pots (20 seeds per pot). Leaf samples from the 20 plants in each pot were pooled, and RNA was extracted using the TRIzol reagent. Five micrograms of total RNA was analyzed on a gel blot for expression of the *PR-1* gene. Pools with high constitutive levels of *PR-1* transcript were identified, and RNA was extracted from each individual plant in these pools. Five micrograms of total RNA from these pools was analyzed for *PR-1* expression by RNA gel blot analysis. Individual plants constitutively expressing the *PR-1* gene were thus identified and allowed to set seed. The mutant phenotype was confirmed in the M_3 generation.

Cleaved Amplified Polymorphic Sequence Analysis for *npr1-5*

The EMS-induced *npr1-5* mutation caused a C-to-T transition in the *NPR1* gene (H. Cao, J. Shah, D.F. Klessig, and X. Dong, unpublished results), resulting in the substitution of serine for proline at amino acid 342 in the mutant NPR1 protein. This single base pair mutation also abolished an NlaIV restriction site present in the wild-type *NPR1* gene. PCR primers were designed to amplify a 691-bp region covering amino acids 200 to 430 (5′-GAGGACACATTGGTTATACTC-3′; 5′-CAAGATCGAGCAGCGTCATCTTC-3′). Restriction analysis of the PCR-amplified products with NlaIV generated two fragments of 266 and 425 bp for the *npr1-5* allele and three fragments of 182, 243, and 266 bp for the wild type and *nim1-3* allele. PCR amplifications were performed as described by Konieczny and Ausubel (1993).

Genetic Analysis

Backcrosses were performed by pollinating flowers of the *npr1-5* parental line (*SSI1 npr1-5*) with pollen from an *ssi1 npr1-5* double mutant. For all other genetic analyses, progeny from a backcrossed line homozygous for the *ssi1* and *npr1-5* mutant alleles was used. To generate *ssi1* plants homozygous for the *NPR1* wild-type allele, pollen from an *ssi1 npr1-5* double mutant was used to pollinate flowers from Arabidopsis ecotype Nössen line 1/8 E/5 (Shah et al., 1997), that is, wild type at both the *SSI1* and *NPR1* loci. Likewise, to generate *ssi1* plants homozygous for the *nim1-3* mutant allele, pollen from an *ssi1 npr1-5* double mutant was used to pollinate flowers from an *SSI1 nim1-3* plant (ecotype Wassilewskija). Success of the cross was confirmed by cleaved amplified polymorphic sequence (CAPS) analysis on F_1 plants for heterozygosity at the *NPR1* locus. Segregation of the *ssi1* mutant allele was monitored in the F_2 progeny by RNA gel blot or dot blot analysis for constitutive *PR-1* gene expression. CAPS analysis was performed on DNA from these phenotypically *ssi1* plants to identify plants homozygous for the wild-type *NPR1* or the *nim1-3* mutant allele. For mapping analysis, pollen from an *ssi1 npr1-5* double mutant (ecotype Nössen) was used to pollinate flowers from a wild-type plant of ecotype Columbia. F_2 progeny plants from the above cross were monitored for spontaneous lesion and constitutive *PR-1* expression phenotype by dot blot analysis. DNA for PCR was isolated from leaf tissue by the method of Konieczny and Ausubel (1993) and used for CAPS or simple sequence length polymorphism (SSLP) marker analysis, as described previously (Konieczny and Ausubel, 1993; Bell and Ecker, 1994).

Crosses with Arabidopsis Plants Expressing the *nahG* Gene

Transgenic NahG plants of ecotype Nössen were generated by using the *Agrobacterium tumefaciens*–mediated whole plant infiltration protocol (Bechtold et al., 1997). After three generations of selfing, a transgenic line having the T-DNA integrated at a single locus was identified and used for all experiments. Pollen from this transgenic NahG plant was used to pollinate flowers from an *ssi1 npr1-5* plant. Success of the cross was confirmed by analyzing expression of the *nahG* gene in the F_1 plants.

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