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A Gain-of-Function Mutation in an Arabidopsis Toll Interleukin1 Receptor–Nucleotide Binding Site–Leucine-Rich Repeat Type R Gene Triggers Defense Responses and Results in Enhanced Disease Resistance

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In a screen for suppressors of *npr1-5*–based salicylic acid (SA) insensitivity, we isolated a semidominant gain-of-function mutation, designated *ssi4*, that confers constitutive expression of several *PR* (pathogenesis-related) genes, induces SA accumulation, triggers programmed cell death, and enhances resistance to bacterial and oomycete pathogens. Through map-based cloning, *ssi4* was identified and found to encode a putative protein belonging to the TIR-NBS-LRR (Toll Interleukin1 Receptor–Nucleotide Binding Site–Leu-Rich Repeat) class of R (resistance) proteins. Comparison between *ssi4* and the corresponding wild-type sequence revealed a single amino acid substitution in the NBS. Epistasis analysis indicated that SA and *EDS1* are required for *ssi4*-induced *PR-1* expression and enhanced disease resistance; they also are required for the increased accumulation of *SSI4* and *EDS1* transcripts detected in the *ssi4* mutant. Although high levels of *ssi4* transcripts correlate with the appearance of the mutant phenotype, overexpression of the wild-type *SSI4* gene failed to induce stunting, spontaneous lesion formation, or increased *PR-1* expression associated with the *ssi4* mutation. Thus, the *ssi4* phenotype does not appear to be caused by overexpression of this *R* gene; rather, we propose that the NBS substitution generates a constitutively activated R protein. Furthermore, because SA treatment induced the expression of *SSI4* and the closely related TIR-NBS-LRR genes *RPP1* and *RPS4* but had little effect on the expression of the coiled-coil NBS-LRR genes *RPM1* and *RPS2*, we suggest that SA not only functions as a critical signal for downstream resistance events but also upregulates the expression of certain *R* genes.

INTRODUCTION

For eons, battles have raged between plants and pathogenic microbes intent on using them as a nutritional resource. To ward off infection, plants activate a variety of defense responses. In the infected leaf, these defenses include strengthening of cell walls, activation and/or synthesis of antimicrobial compounds, and expression of many defense-associated proteins, including the pathogenesis-related (PR) proteins (Durner et al., 1997; Dempsey and Klessig, 1999). A hypersensitive response, characterized by the formation of necrotic lesions and the restriction of the pathogen to the cells within and immediately surrounding these lesions, also frequently develops. After these local responses, the uninoculated portions of the plant usually exhibit increased *PR* gene expression and the appearance of an enduring resistance to a broad spectrum of pathogens known as systemic acquired resistance (SAR). For some plant–pathogen interactions, the signal transduction cascade leading to disease resistance is activated by the direct or indirect interaction between the products of a plant *R* (resistance) gene and a pathogen *Avr* (avirulence) gene (Flor, 1971; Keen, 1990). Based on this gene-for-gene model, if the cognate gene from either the plant or the pathogen is missing, plant defense responses either fail to be activated or are induced too weakly and/or too late to prevent pathogen colonization.

A variety of studies have demonstrated that salicylic acid (SA) plays a critical role in the defense signaling pathway. In many plant species, SA levels increase in conjunction with the activation of *PR* gene expression and disease resistance (Malamy et al., 1990; Métraux et al., 1990; Uknes et al., 1993). Furthermore, plants unable to accumulate SA as a

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result of the expression of the bacterial *nahG* gene fail to develop SAR and exhibit increased susceptibility to pathogen infection (Delaney et al., 1994; Vernooij et al., 1994). Analysis of various genetic mutants, including the *cpr* (Bowling et al., 1994, 1997; Clarke et al., 1998; Silva et al., 1999; Yoshioka et al., 2001), *cim* (Ryals et al., 1996), *acd* (Greenberg et al., 1994; Rate et al., 1999), *ssi* (Shah et al., 1999, 2001; Kachroo et al., 2001), and *lsd* (Dietrich et al., 1994; Weymann et al., 1995) mutants, also has revealed a correlation between increased SA levels, constitutive *PR* expression, and SAR. By contrast, mutations in the *NPR1* gene cause a SA-insensitive phenotype, which is characterized by heightened disease susceptibility and the inability to develop SAR after SA treatment (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997).

During the past few years, many *R* genes have been cloned. Based on their predicted protein structures, R proteins can be divided into several groups. The largest group contains a nucleotide binding site (NBS) and a Leu-rich repeat (LRR) region (Ellis et al., 2000a). NBS domains have been identified in many prokaryotic and eukaryotic proteins, in which their ability to bind ATP or GTP is essential for their biological activity (Saraste et al., 1990). The ability of plant R proteins to bind nucleotides has yet to be demonstrated; however, they contain several conserved motifs, such as the P loop, which are known to be important for this function. LRR domains are known to mediate protein–protein interactions in diverse proteins (Jones and Jones, 1996). Based on the high degree of divergence between the LRRs of R protein homologs, they are thought to play a role in pathogen recognition (Bent, 1996; Jones, 1996; Ellis et al., 2000b; Nimchuk et al., 2001). The NBS-LRR class of R proteins can be subdivided further into two classes based on the secondary structure of the N terminus. The first class contains a putative coiled-coil region at the N terminus (CC-NBS-LRR). The second class contains an N-terminal TIR region, which shares homology with the Toll protein of *Drosophila* and the Interleukin1 receptor (IL-1R) of mammals (TIR-NBS-LRR) (Ellis et al., 2000a). Because the Toll protein and IL-1R play roles in activating innate immunity, the TIR domain has been proposed to transduce a signal that activates plant defense responses (Staskawicz et al., 1995; Baker et al., 1997).

Recent evidence indicates that the signals from the TIR- and CC-containing classes of R proteins are transduced through separate pathways (Aarts et al., 1998). The TIR-NBS-LRR proteins appear to induce disease resistance via a pathway that requires the *EDS1* gene (Parker et al., 1996). By contrast, the CC-NBS-LRR proteins frequently use a pathway that requires the *NDR1* gene (Century et al., 1995). However, a few CC-NBS-LRR proteins have been identified that require neither EDS1 nor NDR1 (McDowell et al., 2000; Bittner-Eddy and Benyon, 2001). Thus, a third defense signaling pathway(s) that is independent of these proteins also appears to exist.

The ability of different R proteins to activate a specific defense pathway suggests that diverse signal transduction strategies are used. Currently, the mechanisms by which a pathogen is perceived and the resistance signal is initiated and transduced are poorly understood. However, several recent studies have demonstrated that overexpression of *R* genes can result in enhanced disease resistance. For example, overexpression of the *Pto* gene in tomato induces constitutive *PR* gene expression, increased levels of SA, spontaneous lesion formation, and enhanced disease resistance (Tang et al., 1999). Broad-spectrum disease resistance also is induced by overexpression of *Prf*, a CC-NBS-LRR *R* gene whose product works in conjunction with Pto (Oldroyd and Staskawicz, 1998). In addition, overexpression of the Arabidopsis TIR-NBS-LRR *R* gene *At4g16890* results in enhanced disease resistance (Stokes et al., 2002). It has been hypothesized that R proteins are found normally in a complex with a guard protein; while sequestered in this complex, they are unable to activate defense responses (Dangl and Jones, 2001). After interaction with the appropriate Avr protein, a change in the R protein would allow the complex to dissociate and a signal transduction event would be initiated. Thus, if R proteins are present normally in rate-limiting amounts, their overexpression might be sufficient to activate the defense signaling pathway.

To further investigate the SA signaling pathway leading to plant disease resistance, we used a genetic screen to isolate suppressors of the *npr1-5* mutation. One mutant, designated *ssi4*, was identified based on its ability to constitutively express several *PR* genes. Analysis of the cloned *ssi4* gene revealed that the predicted open reading frame (ORF) encodes a TIR-NBS-LRR protein with a single amino acid substitution in the NBS domain. Consistent with the presence of an altered TIR-NBS-LRR *R* gene, *ssi4* mutants exhibited enhanced resistance to *Pseudomonas syringae* pv *maculicola* ES4326 and *Peronospora parasitica* biotype Emco5. Furthermore, both SA and *EDS1* were required for *ssi4*-induced constitutive *PR-1* expression and enhanced disease resistance. All plants exhibiting the *ssi4* mutant phenotype contained increased levels of *ssi4* transcripts. However, because *SSI4*-overexpressing plants failed to express *PR-1* or to display enhanced disease resistance, the NBS mutation, rather than overexpression of this TIR-NBS-LRR gene, appears to be responsible for signaling defense responses. Interestingly, SA treatment induced the expression of *SSI4* and several other TIR-NBS-LRR *R* genes. This finding suggests that SA is involved in a positive feedback loop that regulates the expression of a variety of defense-associated genes, including certain *R* genes.

RESULTS

The *ssi4* Mutation Confers Constitutive *PR* Gene Expression

To identify signaling components of the SA defense pathway, 3- to 4-week-old M2 progeny of ethyl methanesulfon-

ate–mutagenized *npr1-5* seeds (ecotype Nö) were screened for individuals that exhibited constitutive *PR* gene expression. One individual, designated *ssi4* (suppressor of salicylic acid insensitivity of *npr1-5*), was found to express the *PR-1*, *PR-2*, and *PR-5* genes (Figure 1A). By contrast, the jasmonic acid– and ethylene-inducible *PDF1.2* gene was not expressed (data not shown). The *ssi4* mutant also exhibited several morphological abnormalities, including stunted growth, severe chlorosis, and the development of spontaneous lesions (Figure 1B). Because these lesions contained large areas of dead cells (Figure 1C) and were associated with the presence of autofluorescent material (data not shown), the cell death induced by *ssi4* is similar to that seen during the hypersensitive response.

ssi4 Is a Monogenic Gain-of-Function Mutation

To investigate the nature of the *ssi4* mutation, we analyzed the progeny of a backcross between the *ssi4 npr1-5* double mutant and the *SSI4 npr1-5* parent. All of the F1 progeny exhibited constitutive *PR* gene expression and the stunted morphology associated with the *ssi4* mutation. However, all of the *ssi4*-associated phenotypes were less pronounced in these heterozygous plants, suggesting that *ssi4* is a semidominant mutation (data not shown). Analysis of F2 progeny confirmed this finding (Figures 1A and 1B).

To determine whether the *ssi4* mutation suppresses the *npr1-5* phenotype by functioning as an intragenic suppressor of the *npr1-5* allele, *ssi4 npr1-5* and wild-type (ecotype Nö) plants were crossed. *ssi4* homozygous F2 progeny that also were homozygous for *NPR1* were identified using a codominant cleaved amplified polymorphic sequence marker for *npr1-5* (Shah et al., 1999). The *ssi4 NPR1* plants showed the same phenotypes as *ssi4 npr1-5* plants (data not shown). Thus, the *ssi4* mutation is not a simple revertant of *npr1-5*, and the effects of this mutation are independent of the *npr1-5* mutation.

We next determined whether the *ssi4* mutation is attributable to a dominant gain of function or to haploinsufficiency by crossing *ssi4* homozygous plants with Columbia tetraploid plants (CS3432). RNA gel blot analysis of the resulting triploid F1 progeny revealed that they constitutively expressed *PR-1*, and these plants also displayed extensive leaf chlorosis and formed lesions spontaneously (data not shown). This result suggests that the *ssi4* phenotype is caused by a gain-of-function mutation rather than by haploinsufficiency.

Positional Cloning of *ssi4*

To determine the map position of the *ssi4* locus, we crossed *ssi4* plants with wild-type plants from ecotype Landsberg. As expected, the F2 progeny segregated in a 3:1 ratio

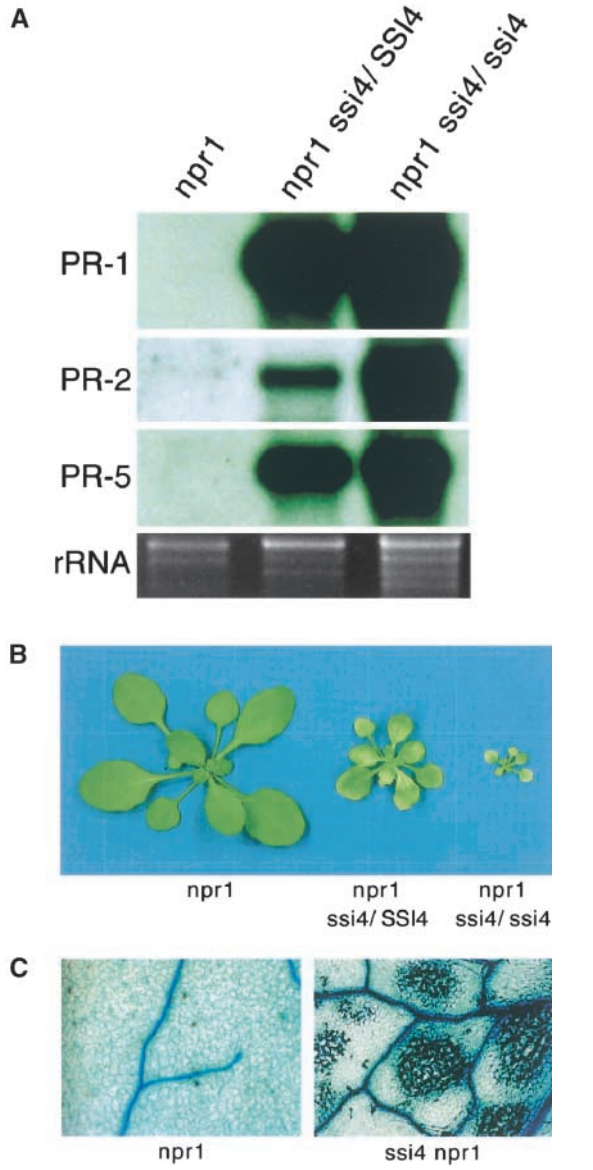

Figure 1. Phenotypic Effects of the *ssi4* Mutation.

(A) Expression of the *PR-1*, *PR-2*, and *PR-5* genes in *npr1-5*, *npr1-5 ssi4/SSI4*, or *npr1-5 ssi4/ssi4* plants. RNA gel blot analysis was performed on 10 μg of total RNA extracted from 3-week-old soil-grown plants. Ethidium bromide staining of rRNA (bottom gel) was used as a loading control.

(B) Comparison of the morphological phenotypes displayed by plants heterozygous or homozygous for the *ssi4* mutant allele in the *npr1-5* background. Plants were grown on soil and photographed when 3 weeks old.

(C) Microscopy of trypan blue–stained leaves from *npr1-5* and *ssi4 npr1-5* plants. Leaves from *ssi4 npr1-5* plants contain intensely stained areas of dead cells.

(*PR-1*$^+$:*PR-1*$^-$) when scored for constitutive *PR-1* gene expression. Because of the semi-dominant nature of the *ssi4* mutation, we determined map position by analyzing F2 plants exhibiting the wild-type (*PR-1*$^-$) phenotype. A total of 1179 wild-type–like F2 plants were analyzed; *SSI4* was mapped to a 132-kb region of chromosome V. This region corresponds to 37 kb at the 3′ of the P1 clone MBK23, the entire region covered by overlapping clones MUF8 and K16L22, and 2 kb of MJC20 beyond the region of overlap between K16L22 and MJC20, as defined by the Arabidopsis sequencing project (Figure 2A).

Because *ssi4* is a gain-of-function mutation, complementation analysis could be performed only using the *ssi4* gene. Thus, a transformation-competent artificial chromosome (TAC) library containing *ssi4* genomic DNA was constructed with an average insert size of 10 to 20 kb. After screening 100,000 colonies, those clones spanning the *ssi4*-containing region were isolated and organized into a contig (Figure 2A). Each clone was introduced into wild-type (ecotype Nö) plants by vacuum infiltration. Hygromycin-resistant T1 plants then were screened for the *ssi4* phenotype. Only plants containing clone 94, which encompasses the MUF8.2 reading frame, exhibited stunted growth and extensive leaf chlorosis (data not shown). These plants also displayed constitutive *PR-1* gene expression (Figure 2B), confirming that this clone contains the *ssi4* mutation.

Sequence analysis of the MUF8.2 ORF from TAC clone 94, as well as reverse transcriptase (RT)–mediated PCR of the *ssi4* mutant and *SSI4* wild-type mRNAs, identified the *ssi4* mutation as a G-to-A transition at codon 422 of MUF8.2, resulting in a Gly-to-Arg amino acid substitution. Sequencing of PCR-generated fragments using genomic DNA or cDNA from mutant or wild-type plants as the template produced the same result. Because the *ssi4* mutation does not alter any restriction enzyme site, a derived cleaved amplified polymorphic sequence marker was created to monitor the segregation of mutant genes in transgenic plants containing clone 94. T2 analysis of these plants further confirmed that the *ssi4* mutation is responsible for the mutant phenotype; the *ssi4* transgene cosegregated with constitutive *PR-1* gene expression and the stunted, chlorotic morphology exhibited by *ssi4* plants.

Analysis of TAC clone 94 revealed that it contains a single ORF of 3434 nucleotides. Comparison of RT-PCR–generated *SSI4* or *ssi4* cDNA with genomic DNA indicated that this gene contains three introns that are spliced out to produce a transcript with a predicted protein of 1055 amino acids. A search of the GenBank database indicated that *SSI4* is highly similar to several genes that encode functional R proteins, including *N* (Whitham et al., 1994), *L6* (Lawrence et al., 1995), *RPS4* (Gassmann et al., 1999), members of the *RPP5* gene family (Parker et al., 1997; van der Biezen et al., 2002), and *RPP1-WsA* (Botella et al., 1998). Like the R proteins encoded by these genes, SSI4 contains a TIR-NBS-LRR structure (Figure 2C). Downstream of the TIR domain, SSI4 contains seven conserved motifs that are associated with the NBS domains of all other TIR-NBS-LRR R proteins (Meyers et al., 1999). The Gly-to-Arg substitution in *ssi4* is located in a nonconserved region of the NBS just upstream of the resistance nucleotide binding site D TIR motif. The C-terminal portion of SSI4 contains an LRR domain consisting of 13 imperfect LRRs that range in length from 20 to 25 amino acids.

ssi4 Encodes an Activated TIR-NBS-LRR Protein

The discovery that *SSI4* encodes a TIR-NBS-LRR protein raised the possibility that other defense responses, in addition to *PR* gene expression, might be activated by the NBS mutation. To assess this possibility, we assayed *ssi4* mutants for enhanced disease resistance and increased levels of SA. SA levels in *ssi4 npr1* homozygous plants were 300-fold higher than those detected in wild-type plants or in the parental *npr1-5* mutant, and SA glucoside (SAG) levels were >200-fold greater (Figure 3A). Plants heterozygous for *ssi4* in the *npr1-5* background exhibited intermediate levels of SA and SAG. By contrast, *ssi4* homozygous and heterozygous mutants carrying the *NPR1* allele accumulated substantially lower levels of SA and SAG than the corresponding double mutants.

To determine whether *ssi4* plants exhibit enhanced disease resistance, inoculations were performed using the bacterial pathogen *P. syringae* pv *maculicola* ES4326 and the oomycete pathogen *P. parasitica* biotype Emco5. As a result of the extreme stunting of *ssi4* homozygous plants, inoculations with *P. syringae* pv *maculicola* ES4326 were performed only on *ssi4* heterozygous plants. By 3 days after inoculation with *P. syringae* pv *maculicola* ES4326, *ssi4*/*SSI4* *npr1* plants contained ~15- or 20-fold less bacteria than either wild-type or *npr1-5* parental plants, respectively (Figure 3B). *ssi4*-induced resistance was even higher in the *NPR1* background; these plants contained 150-fold less bacteria than wild-type plants. Similar results were observed after inoculation with *P. parasitica* biotype Emco5. At 7 days after inoculation, the level of sporulation on the cotyledons of *ssi4*/*ssi4* *npr1* or *ssi4*/*ssi4* *NPR1* plants was reduced greatly compared with that detected on wild-type or *npr1-5* single-mutant plants (Figure 3C).

ssi4 Induces Most Defense Responses via an SA- and *EDS1*-Dependent Pathway

After activation, R proteins stimulate many defense responses via an SA-dependent pathway(s). To determine whether SA is required for *ssi4*-induced *PR* gene expression and disease resistance, a cross between *ssi4* and *nahG* plants (ecotype Nö) was performed. None of the resulting F1 progeny constitutively expressed the *PR-1* gene (Figure 4A), became chlorotic (Figure 4B), or exhibited enhanced resis-

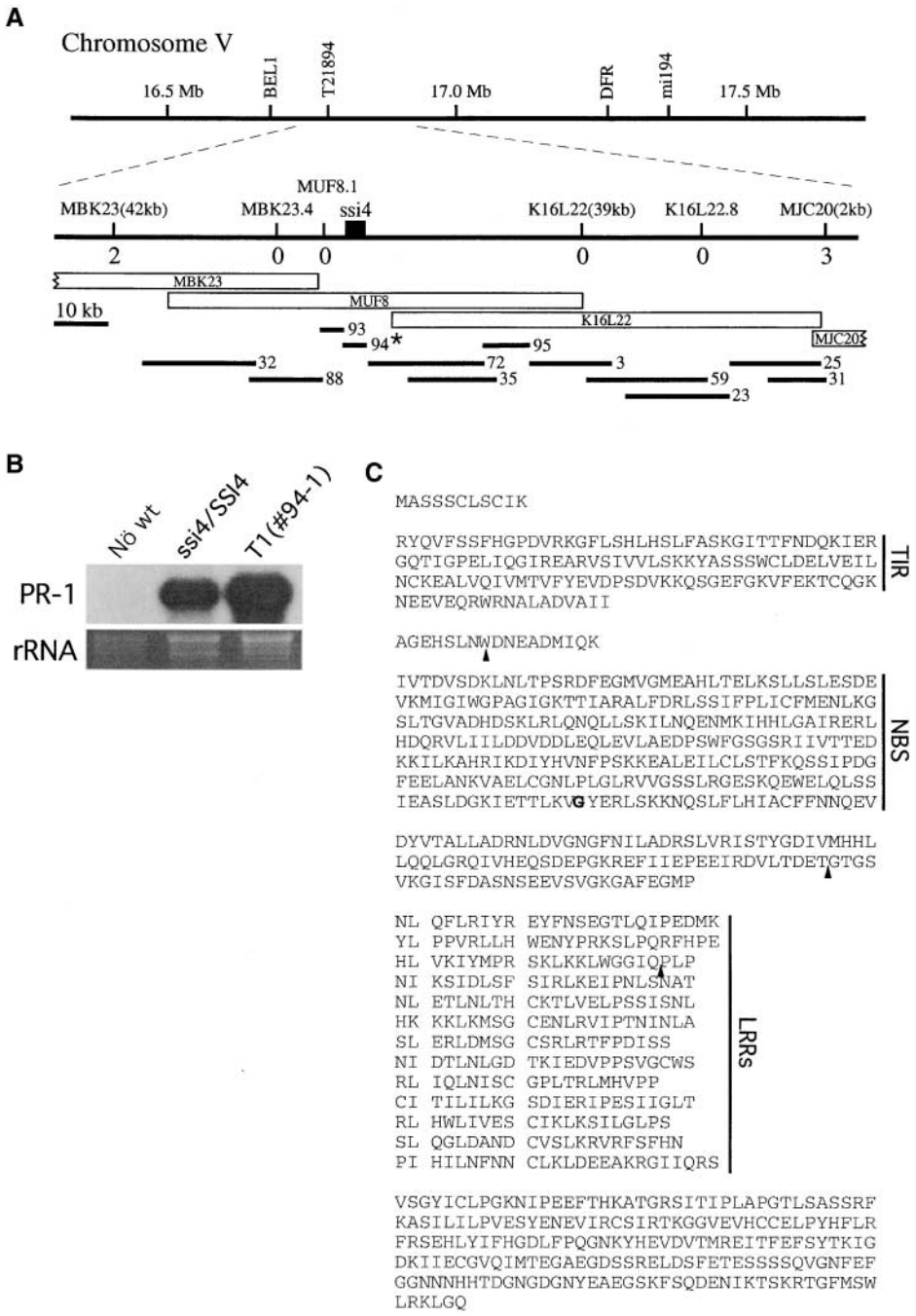

Figure 2. Isolation and Identification of the *ssi4* Mutation.

(A) Genetic and physical map of the *ssi4* region of chromosome V. The top line represents chromosome V, with the broken lines showing the points of recombination. The middle line represents a larger scale of the region within the recombination breakpoints, with markers indicated above it, the number of recombination events for each marker designated below it, and the MUF8.2 ORF (*ssi4*) denoted as a black box. P1 and TAC clones spanning this region are represented as labeled boxes (MBK23, MUF8, K16L22, and MJC20). Directly beneath these boxes are the numbered TAC-based clones representing the contig of this region generated from *ssi4* genomic DNA. Clone 94, marked with an asterisk, contains the *ssi4* mutation and maps to the MUF8.2 ORF.

(B) *PR-1* expression in wild-type (Nö wt), *ssi4/SSI4*, and T1 (#94-1) plants. Ten micrograms of total RNA was used for RNA gel blot analysis. rRNA stained with ethidium bromide served as a loading control.

(C) Amino acid sequence of SSI4. Basic Local Alignment Search Tool (BLAST) analysis of the predicted SSI4 protein sequence revealed three conserved regions identified by the vertical lines: TIR, NBS, and LRR. The *ssi4* mutation (G422R) is indicated in boldface type. The arrowheads indicate the intron positions in the corresponding genomic *SSI4* sequence. The aligned spaces in the LRR region indicate the putative β-turn/β-strand motif as aligned with the porcine ribonuclease inhibitor structure.

tance to *P. syringae* pv *maculicola* ES4326 (Figure 3B). Thus, SA is required for these *ssi4*-mediated phenomena. However, *nahG*-expressing *ssi4* heterozygous plants exhibited some stunting, and their leaves were curled (Figure 4B). At the microscopic level, trypan blue staining revealed a significant number of dead cells surrounding the leaf vascular area (data not shown). Thus, *ssi4* appears to induce stunting and spontaneous lesion formation via a pathway that is at least partly independent of SA. Although the residual level of SA in *ssi4 nahG* plants was not determined, it is likely to be similar to that in wild-type plants, because *PR-1* gene expression was no longer constitutive and previous studies of *ssi1 nahG* and *ssi2 nahG* plants indicated that *nahG* in the ecotype Nö background was able to suppress SA accumulation to basal levels (Shah et al., 1999, 2001).

Previous studies have demonstrated that several members of the TIR-NBS-LRR class of *R* genes activate disease resistance via an *EDS1*-dependent pathway, whereas most members of the CC-NBS-LRR class use *NDR1* (Aarts et al., 1998). To determine whether *ssi4* induces defense responses via either of these signal transducers, *ssi4* plants were crossed with *eds1-1* and *ndr1-1* plants. Analysis of the resulting *ssi4 ndr1* double mutants revealed that they were nearly as stunted and chlorotic as the *ssi4* single mutant in the *npr1-5* background (Figure 4C). In addition to these abnormalities, *ssi4 ndr1* double mutants developed spontaneous lesions and constitutively expressed *PR-1* (Figure 5A and data not shown). Furthermore, after inoculation with *P. syringae* pv *maculicola* ES4326 or *P. parasitica* biotype Emco5, *ssi4 ndr1* plants exhibited greater resistance than *ndr1* single mutants (Figures 3B and 3C). However, they were much less resistant than *ssi4* single mutants; this result suggests that *NDR1* either plays some role in the enhanced resistance conferred by the *ssi4* mutation or has an additive effect on resistance. By contrast, *ssi4 eds1* double mutants exhibited wild-type morphology, even in the *ssi4* homozygous condition (Figure 4D). Furthermore, these plants did not develop spontaneous lesions, failed to constitutively express *PR-1* (Figure 5A and data not shown), and exhibited heightened susceptibility to infection by *P. syringae* pv *maculicola* ES4326 and *P. parasitica* biotype Emco5 (Figures 3B and 3C). Based on these results, *ssi4*, like other TIR-NBS-LRR genes, requires a functional *EDS1* gene to signal defense responses and disease resistance.

ssi4 Induces Increased *ssi4* and *EDS1* Gene Expression

It was demonstrated previously that overexpression of the *R* genes *Pto*, *Prf*, and *At4g16890* induces constitutive *PR* gene expression, spontaneous cell death, increased SA levels, and/or enhanced disease resistance (Oldroyd and Staskawicz, 1998; Tang et al., 1999; Stokes et al., 2002). Because the *ssi4* mutant exhibits a similar phenotype, we tried to determine whether the presence of the NBS substitution alters the expression of this gene. RNA gel blot analysis revealed that transcripts for the *ssi4* gene accumulate to moderate levels in *ssi4* mutant plants (Figure 5A). By contrast, the level of *SSI4* transcripts in wild-type plants was extremely low and could be detected only by RT-PCR (Figures 5A and 5B). This increase in *ssi4* expression does not appear to be caused by a promoter mutation, because analysis of 981 base pairs 5′ of the translational start site revealed no difference between the *ssi4* and wild-type sequences. *ssi4* transcripts also were readily detectable in *ssi4 npr1* and *ssi4 ndr1* mutant plants, but not in *ssi4 eds1* (Figure 5A) and *ssi4 nahG* mutant plants (Figure 4A). These results suggest that increased expression of *ssi4* requires a functional *EDS1* gene and is SA dependent.

Because *ssi4*-induced defense responses appear to be activated via an *EDS1*-dependent pathway, we tested whether *ssi4* plants also constitutively express *EDS1*. Correlating with *ssi4* expression, transcripts for *EDS1* were detected readily in *ssi4* and *ssi4 npr1* plants as well as in the *ssi4 ndr1* double mutant (Figure 5A). By contrast, few or no transcripts were detected in wild-type plants or in the *ssi4 eds1* double mutant. Because *eds1-1* contains a point mutation resulting in a nonfunctional protein (Falk et al., 1999), the lack of *EDS1* transcripts in *ssi4 eds1* plants suggests that a functional EDS1 protein is required for the upregulation of both *ssi4* and *EDS1* expression. Analysis of *PAD4*, which encodes a protein that forms a complex with EDS1 (Feys et al., 2001), revealed that it, like *ssi4* and *EDS1*, is expressed at readily detectable levels only in *ssi4*, *ssi4 npr1*, and *ssi4 ndr1* plants (Figure 5A). Strikingly, transcripts for *RPP1*, a closely related TIR-NBS-LRR gene, also accumulate to increased levels in *ssi4* plants (Figure 5B). Thus, the *ssi4* mutation causes increased expression of a wide variety of genes involved in plant defense.

It is possible that the high levels of SA found in the *ssi4* mutant induce the expression of *ssi4*, *EDS1*, and *PAD4*. Consistent with this possibility, *EDS1* was shown to be induced by SA treatment (Falk et al., 1999). To determine whether SA also induces *SSI4* expression, we treated wild-type (ecotype Nö) plants with SA. As expected, SA treatment strongly induced *PR-1* expression by 6 h after treatment, and a very weak band corresponding to *SSI4* also was detected at this time (data not shown). Thus, SA treatment induces *SSI4* expression, although to a much lower level than that detected in *ssi4* mutant plants. We then tested whether other TIR-NBS-LRR genes could be induced by SA treatment. Because *R* genes frequently are expressed at very low levels, this analysis was performed using RT-PCR. Consistent with our RNA gel blot analysis, transcripts for *SSI4* increased until 6 h after SA treatment and then decreased gradually during the remainder of the time course (Figure 5B). The same trend was seen with *RPP1*, another TIR-NBS-LRR gene (Figure 5B). By contrast, the CC-NBS-LRR gene *RPM1* exhibited little or no induction by SA treatment. In addition, *RPS4*, a TIR-NBS-LRR gene, was activated by SA, whereas *RPS2*, another CC-NBS-LRR gene, was not (data not shown).

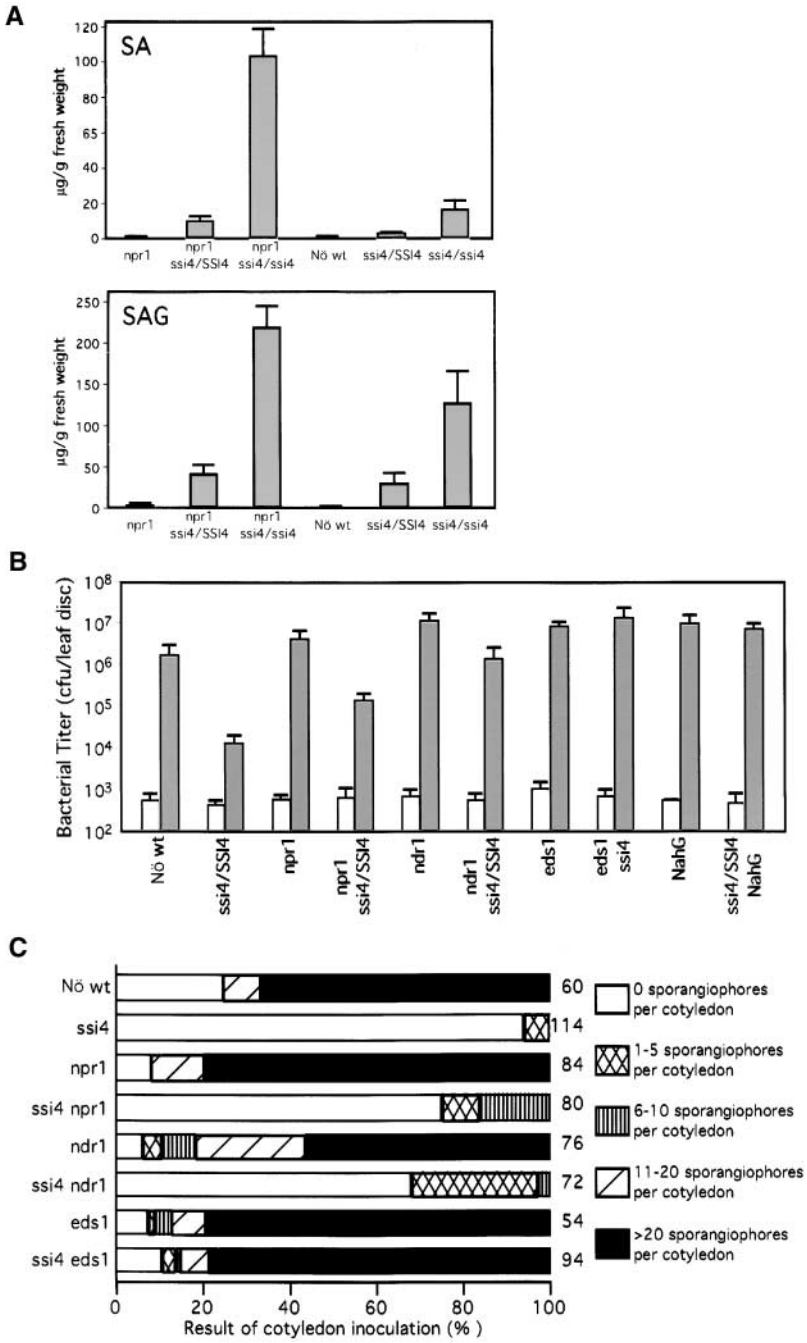

Figure 3. The ssi4 Protein Stimulates SA Accumulation and Enhanced Disease Resistance.

(A) SA and SAG levels in the leaves of 3-week-old, soil-grown *npr1* single mutants, *npr1* mutants heterozygous or homozygous for *ssi4*, wild-type (Nö wt) plants, and *ssi4* heterozygous and homozygous single mutants. The values presented are averages of four replicates.

(B) Growth of *P. syringae* pv *maculicola* ES4326. Leaves of different *ssi4* plant genotypes were infiltrated with *P. syringae* pv *maculicola* ES4326 (OD_{600} = 0.002). Four leaf discs were collected immediately after inoculation (white bars), and six leaf discs were collected at 3 days after inoculation (gray bars). Colony-forming units (cfu) per leaf disc are expressed ±SD and represent averages of four or six samples.

(C) Growth of *P. parasitica* ecotype Emco5. Cotyledons of 7-day-old seedlings from the various plant genotypes listed at left were inoculated by applying a drop of conidiospore suspension (10^5 spores/mL). There was no difference in the size of cotyledons between wild-type and mutant plants at the time of infection. Pathogen growth was assayed by counting the number of sporangiophores per cotyledon at 7 days after inoculation. The shade of each box indicates the severity of infection, based on the number of sporangiophores per cotyledon (see key at right). Numbers to the right of the sample boxes indicate the number of cotyledons assayed.

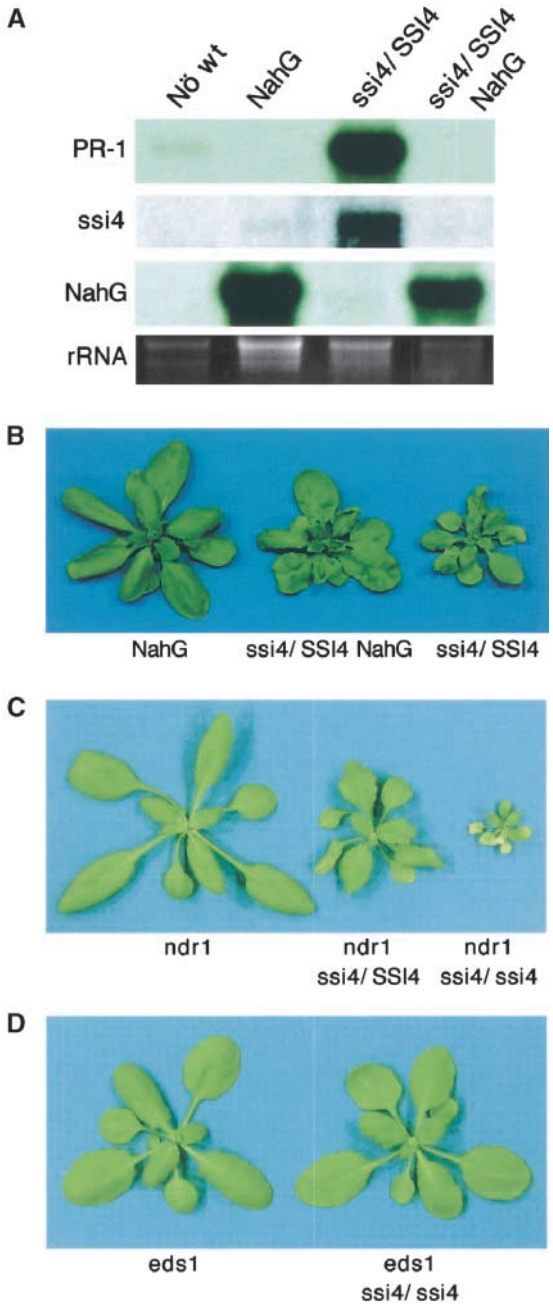

Figure 4. Most *ssi4* Phenotypes Are Activated via an SA- and *EDS1*-Dependent Pathway.

(A) Expression of the *PR-1*, *nahG*, and *ssi4* genes in wild-type (Nö wt), *nahG*, *ssi4*/*SSI4*, and *ssi4*/*SSI4 nahG* plants. Ten micrograms of total RNA was used for RNA gel blot analysis, and ethidium bromide staining of rRNA was used as a loading control.

(B) The phenotypes of 3-week-old soil-grown *nahG*, *ssi4*/*SSI4 nahG*, and *ssi4*/*SSI4* plants.

(C) Phenotypes of *ndr1* plants and *ndr1* plants heterozygous or homozygous for the *ssi4* mutation. Plants were grown in soil and photographed when 3 weeks old.

(D) Phenotypes of *eds1* and *ssi4 eds1* plants. Plants were grown in soil and photographed when 3 weeks old.

Overexpression of *SSI4* Does Not Constitutively Activate Defense Responses

The correlation between increased *ssi4* expression and the constitutive activation of defense responses suggested that overexpression of this TIR-NBS-LRR gene may be responsible for these effects. To assess this possibility, we transformed ecotype Nö plants with an *SSI4* cDNA fused to the 35S promoter of *Cauliflower mosaic virus* in a pBI121 binary vector. Kanamycin-resistant T1 plants were isolated, and their level of *SSI4* expression was determined by RNA gel blot analysis for eight independent T1 plants (Figure 6). None of the T1 plants, including those with highly increased levels of *SSI4* transcripts, developed the severe *ssi4* phenotype or constitutively expressed *PR-1* (Figure 6 and data not shown). This was confirmed further by analyzing ~30 to 40 T2 plants of T1 lines 9, 15, 16, and 21. Interestingly, overexpression of *SSI4* resulted in a corresponding increase in *EDS1* transcripts, suggesting that the increased levels of *EDS1* mRNAs in *ssi4* plants are attributable in part to overexpression of the *ssi4* gene. However, increased levels of *SSI4* and *EDS1* transcripts in *35S*::*SSI4* T2 plants did not confer enhanced resistance to the oomycete pathogen *P. parasitica* biotype Emco5; ~200 to 300 T2 plants of T1 lines 9, 15, and 16 were tested and found to be as susceptible as nontransformed control plants (data not shown). These results suggest that the constitutively activated defense responses exhibited by *ssi4* mutant plants are not simply the result of overexpression of the *ssi4* gene; rather, they are caused by the amino acid substitution in the NBS.

DISCUSSION

To elucidate the components involved in defense signaling, a screen for suppressor mutations of *npr1-5* was performed. One semidominant gain-of-function mutant, designated *ssi4*, was identified based on its ability to constitutively express several *PR* genes. In addition, *ssi4* plants exhibited a stunted, chlorotic morphology and spontaneously developed lesions. Map-based cloning of the *ssi4* gene revealed that it encodes a TIR-NBS-LRR protein. Thus, *ssi4* exhibits structural similarity to a large class of plant *R* genes; nearly 100 members have been identified in the Arabidopsis genome (Dangl and Jones, 2001). Comparison with the wild-type *SSI4* sequence revealed that the *ssi4* gene contains a point mutation leading to a single amino acid substitution in the NBS domain. Although the crystal structures of a TIR domain and several LRR domains, all from other organisms,

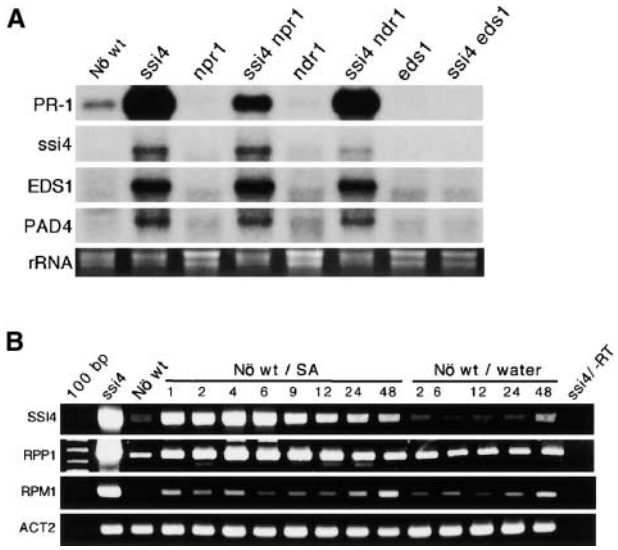

Figure 5. *ssi4* Expression Requires *EDS1* and Is SA Inducible.

(A) Expression of the *PR-1*, *SSI4*, *EDS1*, and *PAD4* genes in wild-type (Nö wt) plants, *ssi4*, *npr1*, *ndr1*, and *eds1* single mutants, and *ssi4 npr1*, *ssi4 ndr1*, and *ssi4 eds1* double mutants (all of the mutant genes were present in the homozygous state). RNA gel blot analysis was performed using 10 μg of total RNA, and ethidium bromide staining of rRNA was used as a loading control.

(B) RT-PCR analysis of wild-type plants treated with 500 μM SA or sterile water. Plants were sprayed with SA or water, and leaves were harvested at the times indicated after treatment. RT-PCR was performed using total RNA and gene-specific primers; the products were visualized on an ethidium bromide–stained agarose gel. The level of *Actin2* (ACT2) was used as an internal control to normalize the amount of cDNA template. A control with RNA from *ssi4* processed without the addition of reverse transcriptase (−RT) is shown at right.

were solved recently, structural information on the NBS domain remains elusive (Xu et al., 2000; Kobe and Kajava, 2001). Recent sequence analysis of many R protein NBS domains has identified several conserved TIR-NBS-LRR–specific motifs that are dispersed between highly divergent nonconserved regions (Meyers et al., 1999). The Gly-to-Arg substitution caused by the *ssi4* mutation was detected in a nonconserved region just upstream of the resistance NBS D motif. Thus, it is unclear whether this mutation affects the putative NBS of ssi4 or some other activity performed by this domain.

The combined discoveries that *SSI4* encodes a TIR-NBS-LRR–type R protein and that the Gly-to-Arg substitution in its NBS confers constitutive *PR* gene expression led us to suspect that ssi4 is a constitutively activated R protein. Consistent with this hypothesis, *ssi4* plants accumulated high levels of SA and SAG and exhibited enhanced resistance to both *P. syringae* pv *maculicola* ES4326 and *P. parasitica* biotype Emco5. Analysis of *ssi4 nahG* plants revealed that most of the *ssi4*-induced phenotypes, including constitutive *PR* gene expression, enhanced disease resistance, and chlorosis, require increased levels of SA. Because the *ssi4* mutation was isolated in the *npr1-5* background, all of these SA-dependent defenses must be activated via an *NPR1*-independent pathway. Analyses of several other Arabidopsis mutants also have revealed an SA-dependent, *NPR1*-independent pathway(s) that mediates *PR* gene expression and/or disease resistance (Bowling et al., 1997; Clarke et al., 1998; Li et al., 1999, 2001; Rate et al., 1999; Shah et al., 1999, 2001; Kachroo et al., 2001; Yoshioka et al., 2001). Because SA alone does not induce *PR* expression or enhanced resistance in *npr1* plants, the activation of the SA-dependent, *NPR1*-independent pathway(s) is thought to require a second signal that works in conjunction with SA. As has been proposed by others, this second signal could be generated by pathogen infection or by various mutations, such as *ssi4*.

It is interesting that although *NPR1* is not required for the *ssi4*-mediated activation of defense responses, it appears to play an important role in regulating SA accumulation. SA and SAG levels were substantially greater in *ssi4 npr1-5* double mutants than in wild-type plants. SA and SAG levels in the *ssi4* single mutant also were increased with respect to wild-type plants, although they were lower than those in the double mutant. A similar phenomenon was observed in *snc1*, *cpr5*, *cpr6*, and *ssi2* single mutants, which accumulate less SA than *snc1 npr1*, *cpr5 npr1*, *cpr6 npr1*, and *ssi2 npr1* double mutants (Clarke et al., 2000; Li et al., 2001; Shah et al., 2001). Together, these results suggest that *NPR1* not only transduces the SA signal but also plays a role in down-regulating SA levels, as was proposed previously by Clarke et al. (2000).

Most R proteins activate disease resistance via either an *EDS1*- or an *NDR1*-dependent pathway, with the structure of the R protein determining which pathway is used (Aarts et al., 1998). Like other TIR-NBS-LRR proteins, *ssi4* requires *EDS1* to activate resistance to *P. syringae* pv *maculicola* ES4326 and *P. parasitica* biotype Emco5. Indeed, *ssi4 eds1* double mutants were indistinguishable from wild-type plants, demonstrating that *EDS1* is required for all of the *ssi4*-induced phenotypes. In addition to a strong requirement for *EDS1*, *NDR1* also appears to play a minimal role in signaling the *ssi4* phenotype. *ssi4 ndr1* double mutants exhibited less pronounced levels of *PR* gene expression, stunting, and resistance to *P. syringae* pv *maculicola* ES4326 compared with *ssi4* single mutants. Similarly, the presence of an *ndr1* allele reduced *snc1*- and *RPS4*-mediated resistance to strains of *P. syringae* and decreased *RPP*-mediated resistance to various *P. parasitica* isolates (Aarts et al., 1998; Li et al., 2001). Thus, these *EDS1*-dependent *R* genes also may incrementally activate defense responses via an *NDR1*-dependent pathway.

There have been several reports that *R* gene overexpression results in enhanced disease resistance (Oldroyd and Staskawicz, 1998; Tang et al., 1999; Stokes et al., 2002).

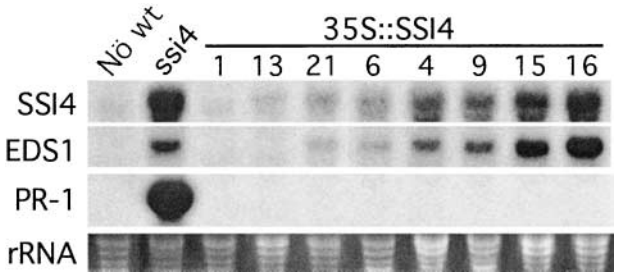

Figure 6. Defense Gene Expression in Transgenic Plants Carrying the *SSI4* cDNA Fused to the 35S Promoter of *Cauliflower mosaic virus*.

Ten micrograms of total RNA extracted from 4-week-old T1 plants was subjected to RNA gel blot analysis. The blot was probed sequentially for the *SSI4*, *EDS1*, and *PR-1* gene transcripts. The numbers at top correspond to the different T1 plants. Ethidium bromide staining of rRNA served as a loading control.

Consistent with this possibility, increased levels of *ssi4* transcripts correlated with constitutive SA accumulation, *PR* gene expression, and enhanced disease resistance. However, because overexpression of the wild-type *SSI4* gene did not lead to stunting, constitutive *PR-1* expression, or enhanced resistance to *P. parasitica* biotype Emco5, the Gly-to-Arg substitution in the NBS, rather than or in addition to overexpression of this *R* gene, appears to be required to activate defense responses. There are several possible explanations for how the NBS substitution in ssi4 can lead to constitutive defense response activation. For example, the Gly-to-Arg mutation might generate an aberrant protein that stresses the cell, thereby activating a nonspecific pathway for defense responses. Consistent with this possibility, previous studies have demonstrated that expression of various foreign or endogenous transgenes can induce *PR* gene expression and SAR (Durner et al., 1997). However, because SSI4 is a TIR-NBS-LRR–type R protein and ssi4 requires EDS1 but not NDR1 for defense response activation, it seems unlikely that this protein activates defenses via a nonspecific pathway. An alternative possibility is that the NBS mutation abolishes ssi4's ability to bind a guard protein that sequesters (and thereby silences) SSI4 in the absence of the appropriate Avr protein. Arguing against this possibility is the finding that overexpression of the *SSI4* gene, which should titrate out the guard protein (as may have happened with overexpressed *Pto*, *Prf*, and *At4g16890*), did not induce defense responses. A more likely explanation is that the NBS mutation produces a constitutively activated R protein that constantly stimulates the defense signaling pathway, causing increased levels of SA, constitutive *PR* gene expression, and enhanced disease resistance. The observation that *ssi4* is a semidominant gain-of-function mutation is consistent with this model. Note that the latter two models are not mutually exclusive.

In addition to stimulating downstream defense responses, the increased SA levels in the *ssi4* mutant appear to enhance the expression of the *ssi4* gene. Supporting this conclusion, *ssi4* plants that express the *nahG* gene failed to accumulate increased levels of *ssi4* transcripts. Analysis of the wild-type *SSI4* gene and the closely related TIR-NBS-LRR *R* genes *RPP1* and *RPS4* suggests that their expression also is upregulated by SA. All of these genes were induced by SA treatment. Additionally, transcripts for *RPP1* accumulated to higher levels in the *ssi4* mutant than in wild-type plants. Another TIR-NBS-LRR *R* gene, *162J11T7*, was shown recently to be highly induced by treatment with the SA analog BTH and to a lesser extent by infection with bacterial or oomycete pathogens (Maleck et al., 2000). Thus, increased levels of SA, such as those generated by pathogen infection, may upregulate the expression of at least some TIR-NBS-LRR genes. By contrast, SA treatment did not induce the CC-NBS-LRR genes *RPM1* or *RPS2*, which suggests that upregulation of their expression either does not occur or involves a different mechanism.

Together, our results suggest that a positive feedback loop links SA accumulation and the expression of several TIR-NBS-LRR *R* genes, including *SSI4*. In this model, pathogen infection would lead to activation of the SSI4 protein; the activated protein then would transduce the resistance signal primarily via an *EDS1*-dependent signaling pathway. Increased SA levels generated during this response would not only activate downstream defenses directly but also would upregulate the expression of some *R* genes, thereby amplifying the defense response. Because defense signaling would not be initiated until the SSI4 protein was activated (presumably by a direct or indirect interaction with the pathogen Avr protein), plants that overexpress the *SSI4* gene would not accumulate SA or display constitutive defense responses. By contrast, plants that express a constitutively activated R protein, such as ssi4, would accumulate increased levels of SA, which would stimulate additional expression of the *ssi4* gene and activate the resistance signaling pathway.

METHODS

Isolation of the *ssi4* Mutant and Genetic Analysis

Mutagenesis and screening for the *ssi4* mutant were conducted as described previously (Shah et al., 1999). Briefly, 5000 seeds from *Arabidopsis thaliana* plants homozygous for the *npr1-5* allele were mutagenized with 0.3% ethyl methanesulfonate (Sigma), and the M2 seeds from these plants were harvested in pools of ~10 ethyl methanesulfonate–mutagenized M1 plants. Pooled M2 plants were screened for putative *PR-1* overexpressors. Backcrosses were performed by pollinating flowers of the *npr1-5* parental line (*SSI4 npr1-5*) with pollen from the *ssi4 npr1-5* mutant. To isolate *ssi4* mutants homozygous for the *NPR1* wild-type allele, *ssi4 npr1-5* mutant plants were crossed with wild-type ecotype Nö plants. Cleaved amplified polymorphic sequence (CAPS) marker analysis of 36 F2 plants then

was used to identify plants that contained only the wild-type *NPR1* allele. The genotype at the *ssi4* locus in these F2 plants was determined by monitoring the segregation of *PR* gene expression in the F3 population using RNA gel blot analysis. To isolate *ssi4 ndr1* and *ssi4 eds1* double mutants, *ndr1-1* (ecotype Columbia) or *eds1-1* (ecotype Wassilewskija) plants were crossed with *ssi4* homozygous plants. PCR markers for the *ndr1-1*, *eds1-1*, and *ssi4* (see below) alleles then were used to identify the genotypes of the F2 progeny.

Map-Based Cloning of *ssi4*

ssi4 homozygous plants were crossed with a wild-type Landsberg plant, and F2 progeny lacking constitutive *PR* gene expression (homozygous for the *SSI4* allele) were identified by RNA gel blot analysis. CAPS and simple sequence length polymorphism marker analysis performed on these F2 plants localized the *ssi4* mutation to chromosome V, between *PHYC* and *DFR*. Sequence information (www.kazusa.or.jp/kaos/) then was used to generate novel CAPS and simple sequence length polymorphism markers in this region. Of 1179 F2 plants examined using these markers, two recombination events were identified using MBK23.42kb and three recombination events were detected using MJC20.2kb. No recombination events were identified using additional markers within this 132-kb region. Thus, *ssi4* is located within a region spanning the latter half of P1 clone MBK23, the entire region of P1 clone MUF8, and the entire region of transformation-competent artificial chromosome (TAC) clone K16L22, as defined previously (www.kazusa.or.jp/kaos/).

An *ssi4* TAC library was created as described by Liu et al. (1999). Briefly, genomic DNA from *ssi4* homozygous mutant plants was partially digested with HindIII, and 10- to 20-kb fragments were purified by agarose gel electrophoresis and cloned into pYLTAC7 (Liu et al., 1999). The library was screened using the K16L22 TAC clone and PCR fragments from P1 clones MBK23 and MUF8 as probes. A contig was constructed based on the results of end-sequencing positive clones with vector-based primers. Two gaps in the contig were identified and filled by PCR-generated clones (Advantage-HF2; Clontech, Palo Alto, CA). Clones representing the entire contig then were transformed into wild-type ecotype Nö plants using the vacuum infiltration method (Bechtold and Pelletier, 1998). Transformants were selected on Murashige and Skoog (1962) medium containing 15 μg/mL hygromycin, and the presence of the entire T-DNA region was confirmed by PCR analysis using primers for the *sacB* and hygromycin resistance genes, indicating the left and right borders of the vector, respectively (Liu et al., 1999).

Segregation analysis of the *ssi4* mutation among F2 and T2 plants was conducted by creating a derived CAPS marker. An ~100-bp fragment was amplified using primers MUF8.2BglIIF (5′-GGTTCATCTTTGCGTGGGGAGAGCAAGCAA-3′) and MUF8.2BglIIR (5′-GTGAAGGAATAGAGATTGATTTTTCTTCGACAATCTTTCAGATC-3′) and digested with BglII. This generated either a 140-bp undigested fragment in the amplified product from the wild-type plants or 100- and 40-bp fragments in the amplified product from the *ssi4* plants.

RNA Extraction and RNA Gel Blot Analysis

Total RNA was extracted from 3- to 4-week-old leaves using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). RNA was separated on a 1.2% denaturing agarose gel, transferred onto a Hybond-NX membrane (Amersham Pharmacia), and hybridized with radiolabeled probes that were generated as described previously (Shah et al., 1997). PCR-amplified products of *SSI4* (SSI4F, 5′-CTCAAGAGAGTATGCTTCTCTTTCCATAACCC-3′; SSI4R, 5′-CTGGTTTGGTCTTCATGAGACTCCATGAG-3′), *EDS1* (EDS3F, 5′-GGATAGAAGATGAATACAAGCC-3′; EDS1R, 5′-ACCTAAGGTTCAGGTATCTGT-3′), and *PAD4* (PAD4F, 5′-ATGGACGATTGTCGATTCGAG-3′; PAD4R, 5′-CTAAGTCTCCATTGCGTCACT-3′) were used as probes to detect these transcripts by RNA gel blot analysis.

Trypan Blue Staining

Leaf samples were taken from 2-week-old plants grown on soil. Trypan blue staining was performed as described previously (Bowling et al., 1994).

Salicylic Acid and Salicylic Acid Glucoside Measurement

Salicylic acid (SA) and SA glucoside were extracted and measured from 0.25 g (fresh weight) of leaf tissue as described previously (Bowling et al., 1994).

Pathogen Infection

Infection with *Pseudomonas syringae* pv *maculicola* ES4326 was performed on 4-week-old soil-grown plants as described previously (Shah et al., 1997). Leaves were infiltrated with a bacterial suspension (OD_{600} = 0.002) in 10 mM $MgCl_2$. For each genotype, four leaf discs (4 mm in diameter) were harvested at day 0 and six discs were harvested at day 3; the discs were homogenized in 10 mM $MgCl_2$, and colony-forming units were counted. Infection with *Peronospora parasitica* biotype Emco5 was performed by spraying or applying a single drop of asexual inoculum suspension (10^5 conidiosporangia/mL) per cotyledon of 7-day-old seedlings. The seedlings were grown at 19°C and >90% RH with an 8-h photoperiod. *P. parasitica* biotype Emco5 growth was assayed visually by counting the number of sporangiophores per cotyledon at 7 days after inoculation.

SA Treatment of Plants

Three-week-old plants were sprayed and irrigated for 10 min with a solution of SA (500 μM) or water. Leaves were harvested at the times indicated (in Figure 5B), and RNA was extracted as described above.

Reverse Transcriptase–Mediated PCR

cDNA for reverse transcriptase (RT)–mediated PCR was generated using SuperScript Reverse Transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions. PCR was performed using Advantage 2 polymerase (Clontech). The gene-specific primers used for RT-PCR analysis were as follows: for *SSI4*, SSI4F (5′-CTCAAGAGAGTATGCTTCTCTTTCCATAACCC-3′) and SSI4R (5′-CTGGTTTGGTCTTCATGAGACTCCATGAG-3′); for *RPP1*, RPP1F (5′-GTGGAGCTCCCCGCTATCGAGAATGCGAC-3′) and RPP1R (5′-GCAAGGGAATCTGGAAGTTGGGGGAGTGATACC-3′); for *RPM1*, RPM1F (5′-GCATACATGGGACCTAGGTTGCGTTTTGCACAAGG-3′) and RPM1R, (5′-GCCTTGGCCGCCTAAGATGAGAGGCTCAC-3′);

and for *Actin2*, ACT2F (5′-CTAAGCTCTCAAGATCAAAGGCTT-AAAAAGCTGGGG-3′) and ACT2R (5′-CTTATACAATACTTATAT-TAACATTGCAAAGAGTTTCAAGGT-3′).

Construction of *SSI4*-Overexpressing Plants

An *SSI4* cDNA of 3226 bp was amplified from Arabidopsis wild-type ecotype Nö RNA by RT-PCR using primers MUF8.2F (5′-CTT-TCTTTCAAGCATTTGTGATCTCTCATGGCTTCT-3′) and MUF8.2R (5′-CTGGTTTGGTCTTCATGAGACTCCATGAG-3′) and cloned into the pCR2.1-TOPO vector (Invitrogen Life Technologies). The cDNA clone was sequence verified, and the *SSI4* coding sequence then was subcloned into the XbaI and SacI sites of pBI121 (Clontech), which placed the *SSI4* gene under the control of the 35S promoter of *Cauliflower mosaic virus*. This construct then was transformed into wild-type ecotype Nö plants by vacuum infiltration (Bechtold and Pelletier, 1998). Transformants were selected on Murashige and Skoog (1962) medium containing 50 μg/mL kanamycin for 2 weeks, transferred to soil, and grown for 2 additional weeks before analysis of *SSI4* expression.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Number

The GenBank accession number for the *SSI4* genomic sequence is AY179750.

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A Gain-of-Function Mutation in an Arabidopsis Toll Interleukin1 Receptor–Nucleotide Binding Site–Leucine-Rich Repeat Type R Gene Triggers Defense Responses and Results in Enhanced Disease Resistance

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