A Gain-of-Function Mutation in a Plant Disease Resistance Gene Leads to Constitutive Activation of Downstream Signal Transduction Pathways in suppressor of npr1-1, constitutive 1

Yuelin Zhang, Sandra Goritschnig, Xinnian Dong, and Xin Li

IntroducTion

Plants have evolved sophisticated defense mechanisms against pathogen infections, during which resistance (R) genes play central roles in recognizing pathogens and initiating defense cascades. Most of the cloned R genes share two common domains: the central domain, which encodes a nucleotide binding adaptor shared by APAF-1, certain R proteins, and CED-4 (NB-ARC), plus a C-terminal region that encodes Leu-rich repeats (LRR). In Arabidopsis, a dominant mutant, suppressor of npr1-1, constitutive 1 (snc1), was identified previously that constitutively expresses pathogenesis-related (PR) genes and resistance against both Pseudomonas syringae pv maculicola ES4326 and Peronospora parasitica Noco2. The snc1 mutation was mapped to the RPP4 cluster. In snc1, one of the TIR-NB-LRR-type R genes contains a point mutation that results in a single amino acid change from Glu to Lys in the region between NB-ARC and LRR. Deletions of this R gene in snc1 reverted the plants to wild-type morphology and completely abolished constitutive PR gene expression and disease resistance. The constitutive activation of the defense responses was not the result of the overexpression of the R gene, because its expression level was not altered in snc1. Our data suggest that the point mutation in snc1 renders the R gene constitutively active without interaction with pathogens. To analyze signal transduction pathways downstream of snc1, epistasis analyses between snc1 and pad4-1 or eds5-3 were performed. Although the resistance signaling in snc1 was fully dependent on PAD4, it was only partially affected by blocking salicylic acid (SA) synthesis, suggesting that snc1 activates both SA-dependent and SA-independent resistance pathways.

1To whom correspondence should be addressed. E-mail xinli@interchange.ubc.ca; fax 604-822-6089.

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sis-related (PR) gene expression and resistance induced by avirulent pathogens or SAR-inducing agents such as salicylic acid (SA) (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). snc1 (suppressor of npr1-1, constitutive1) is an Arabidopsis mutant isolated from a screen for suppressors of npr1-1 (Li et al., 2001). The snc1 mutation results in constitutive PR gene expression and resistance to *Pseudomonas syringae pv maculicola* (P.s.m.) ES4326 and *P. syringae pv syringae* Noco2 in the npr1-1 background. snc1 plants are smaller than wild-type plants, accumulate high levels of SA, and often have curly leaves. Although snc1/SNC1 plants constitutively express PR genes, their morphology is indistinguishable from that of wild-type plants. It appears that both copies of SNC1 must be mutated for the mutant morphological phenotypes to be visible.

Previously, the snc1 mutation was mapped to a 120-kb region on chromosome 4. This region contains a cluster of RPP5 orthologs, including the recently cloned RPP4 (Parker et al., 1997; van der Biezen et al., 2002). The snc1 mutation phenotypes are suppressed completely by eds1, but not by ndr1, suggesting that SNC1 is located upstream of EDS1 and that one of the RPP5 homologs is activated constitutively in snc1 (Li et al., 2001). Here, we report the identification of snc1 and discuss the molecular mechanism by which this mutation affects R gene-mediated defense.

RESULTS

**snc1 Contains a Mutation in At4g16890**

To identify the molecular lesion in snc1, PCR fragments covering the entire 120-kb region to which snc1 was mapped were amplified from snc1 DNA and sequenced. The sequence then was compared with that of the wild type, and a single G-to-A mutation was found in the coding region of At4g16890 (Figure 1), suggesting that SNC1 is At4g16890. The cDNA sequence of the gene was obtained by sequencing reverse transcriptase-mediated (RT) PCR fragments and found to be consistent with the annotation of At4g16890. At4g16890 encodes a putative protein of 1468 amino acids that is highly similar to RPP4 (62% identical) and RPP5 (68% identical), two closely related R genes of the Toll-Interleukin1 Receptor (TIR)-NB-LRR class. As shown in Figure 1, the TIR and NB-ARC domains are encoded by exon 1 and 2, respectively. A stretch of 80 to 90 amino acids is encoded by exon 3. The snc1 mutation is located in this region, which recently was named NL linker (Meyers et al., 2003). The remaining exons towards the 3' end encode the LRRs. The snc1 mutation converts the Glu at position 552 to a Lys in the NL linker.

To investigate the relationship between SNC1 and other TIR-NB-LRR-type R proteins, full-length amino acid sequences of R proteins that were shown previously to be functional were used to generate a phylogenetic tree (Figure 2A). Alignment of the amino acid sequences encoded by exon 3 of SNC1 and the corresponding sequences from other TIR-NB-LRR R proteins also was performed (Figure 2B). Although Glu-552 is conserved among SNC1, RPP4, and RPP5, other TIR-NB-LRR-type R proteins contain different residues at this position. However, closely related R proteins tend to have the same corresponding residues. Among the aligned R proteins, all except one have a charged amino acid at this position, suggesting potential functional conservation of these residues.

To compare the mutation in the snc1 protein with other gain-of-function R protein variants, the location of these mutations is summarized in Figure 2C. In the tomato Mi protein, there is an unusually long N-terminal extension. Replacing the N-terminal 161 amino acids with the corresponding region of a nonfunctional homolog causes localized HR when expressed transiently in *Nicotiana benthamiana* leaves (Hwang et al., 2000). Several Rx mutations also were found to constitutively activate HR in transient assays (Bendahmane et al., 2002). These mutations are located in either the NB or the LRR region. Both Mi and Rx encode coiled-coil (CC)-NB-LRR-type R proteins. The lesion mimic *Rp1-D-21* mutant also contains a rearrangement in the LRR-encoding region of a *Rp1-D* haplotype, but it is unclear whether the lesion mimic phenotype is actually caused by this mutant haplotype (Sun et al., 2001). Recently, a mutation in the NB region of a TIR-NB-LRR-type R protein was found to cause spontaneous lesions in *ssd4* (Shirano et al., 2002). The mutation in snc1 is the only one located in the NL linker region, and it is the only confirmed R gene mutation that causes constitutive disease resistance in the absence of cell death.

**Deletions in At4g16890 Revert the snc1 Mutant to Wild Type**

To confirm that the mutation in At4g16890 is responsible for the snc1 phenotypes, we performed a genetic screen to identify revertant mutations of the snc1 gene that restored wild-type morphology. Seeds of snc1 npr1-1 were mutagenized by fast neutron bombardment, and M2 plants were screened for mutants with wild-type morphology. M3 seeds from the candidate mutants were planted again to test for segregation of the wild-type and snc1 morphologies. Because snc1 is a dominant mutation and snc1/SNC1 heterozygous plants have wild-type morphology, mutants that produce segregating progeny are likely to have defects in one copy of the snc1 mutant gene.

Indeed, when nine such mutants were analyzed by PCR for mutations in At4g16890, eight had either large deletions or rearrangements detectable by agarose gel electrophoresis. All eight mutations affected at least part of the At4g16890 coding region, with some also affecting neighboring genes. In one case, the entire exon 3 that contains the original snc1 mutation was deleted (snc1-r2; Figure 1). In the only mutant with no large deletion detected, a small deletion of 8 bp (snc1-r1; Figure 1) was identified in the first exon by direct sequencing of snc1-r1.

We selected snc1-r1 npr1-1 and snc1-r2 npr1-1 for further characterization because the deletions in these two mutants were within At4g16890. As shown in Figure 3A, snc1-r1 npr1-1 and snc1-r2 npr1-1 plants were larger than snc1 npr1-1 plants and no longer exhibited curly leaves. Both snc1-r1 npr1-1 and snc1-r2 npr1-1 had completely lost the constitutive expression of the *pBGL2*-β-glucuronidase (*GUS*) reporter gene (Figure 3B). RT-PCR analysis revealed that constitutive expression of the endogenous *BGL2* (*PR-2*) gene also was abolished in these plants (Figure 3C).
susceptibility to P.s.m. ES4326, a phenotype similar to that of npr1-1 plants (Figure 3D). Overall, these data indicate that deletions in the mutated At4g16890 reversed the phenotypes of the snc1 npr1-1 mutant to those of npr1-1. SNC1/snc1 heterozygous plants were shown previously to constitutively express the pBGL2-GUS reporter gene (Li et al., 2001). To determine whether snc1 plants containing deletions in one copy of the snc1 gene (referred to hereafter as snc1/−) still express pBGL2-GUS, seeds from heterozygous revertants were plated on MS medium (Murashige and Skoog, 1962) and the seedlings were stained for expression of the GUS reporter gene. We found that 36 of 47 plants from seeds of snc1/snc1-r1 did not stain and 23 of 28 plants from seeds of snc1/snc1-r2 did not stain, suggesting that snc1/− plants do not constitutively express pBGL2-GUS. This is consistent with the finding that no pBGL2-GUS expression was observed in the leaves of heterozygous snc1 revertants (data not shown).

The snc1 Phenotype Is Not Attributable to Overexpression of At4g16890

Previously, bal plants, as well as Arabidopsis transgenic plants overexpressing At4g16890, were shown to constitutively express PR genes (Stokes et al., 2002). Given this finding, we analyzed the expression level of At4g16890 in snc1 npr1-1, snc1, and wild-type plants to determine whether the snc1 mutant phenotype is caused by the overexpression of At4g16890. RT-PCR analysis showed that the expression level of At4g16890 was not altered in the mutant plants (Figure 4). Similar results also were obtained using real-time quantitative RT-PCR (data not shown). Thus, the snc1 phenotype is caused by the point mutation in At4g16890 rather than by overexpression of the gene. It is unclear whether the snc1 mutant protein accumulates to a higher level than the wild-type protein.

To test whether the expression of At4g16890 carrying the snc1 mutation would confer the snc1-like phenotype to wild-type plants, genomic clones containing either wild-type At4g16890 or a mutant At4g16890 containing the snc1 point mutation were transformed into wild-type plants. The snc1-like morphology was observed in 15 of 21 plants (>70%) transformed with the mutant At4g16890. On the other hand, only 5 of 23 plants (<25%) transformed with the wild-type gene developed snc1-like morphology, which could be the result of the overexpression of the At4g16890 transgene in these plants. Based on these data, we conclude that SNC1 is At4g16890 and that the Glu-552-to-Lys-552 mutation causes the constitutive activation of this R protein homolog.

The snc1 Phenotype Is Fully Dependent on PAD4

Previously, we showed that the snc1 mutant phenotype is suppressed completely by eds1. This finding is consistent with the fact that SNC1 belongs to the TIR-NB-LRR class of R genes, which usually require EDS1 as a downstream signaling component (Aarts et al., 1998). PAD4 interacts with EDS1 in vivo (Feys et al., 2001). To determine whether PAD4 also is required for the manifestation of the snc1 phenotype, a double mutant was constructed between snc1 and pad4-1 (Glazebrook et al., 1996; Jirage et al., 1999). As shown in Figure 5A, pad4-1 completely suppressed the morphological phenotypes of snc1. The double mutant also lost constitutive pBGL2-GUS expression (Figure 5B) and resistance to both P.s.m. ES4326 and P. parasitica NoCo2 (Figures 5C and 5D). These data suggest that PAD4 is fully required for downstream signaling in snc1.

snc1 Activates Both SA-Dependent and SA-Independent Defense Pathways

To test whether the increased SA level in snc1 is required for the activation of downstream defense pathways, a double mutant was constructed between snc1 and eds5-3, a mutant defective in pathogen-induced SA synthesis (Nawrath and Métraux, 1999; Nawrath et al., 2002). As shown in Figures 6A and 6B, snc1 eds5-3 had a similar amount of SA as eds5-3. snc1 eds5-3 plants were slightly smaller than wild-type plants, and the leaves of snc1 eds5-3 were still curly (Figure 6C). Constitutive expression of pBGL2-GUS in snc1 was not affected by eds5-3 (Figure 6D). RT-PCR analysis of PR-2 (BGL2) expression confirmed that eds5-3 did not affect the expression of PR-2 in the double mutant (Figure 6E). To determine whether the resistance of snc1 was affected by the decreased SA level, growth of the virulent bacterial pathogen P.s.m. ES4326 on snc1 eds5-3 also was determined (Figure 6F). Although snc1 npr1 was resistant to P.s.m. ES4326, snc1 eds5-3 was more susceptible than wild-type

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**Figure 1.** Gene Structure of SNC1 (At4g16890).

Exons (rectangles) and introns (lines) are drawn to proportion to their lengths. Exons encoding the TIR (exon 1), NB-ARC (exon 2), and LRR (exons 4 to 7) domains are indicated. The position of the G-to-A mutation in snc1 is indicated by the arrow. The two revertant mutations (snc1-r1 and snc1-r2) are shown as triangles.
DISCUSSION

To identify the mutation that causes constitutive PR gene expression and pathogen resistance in snc1 plants, we sequenced the complete region to which the snc1 mutation was mapped and identified a single mutation in At4g16890, an NB-LRR-class R gene that is highly similar to RPP4 and RPP5. We showed that deletions of this mutated R gene revert the mutant plants to the wild-type phenotype. In addition, we observed the snc1-like phenotype in >70% of wild-type plants transformed with a genomic clone containing the snc1 mutation. These data indicate that At4g16890 encodes SNC1 and that the point mutation we identified in SNC1 is a gain-of-function mutation that renders this R protein constitutively active. Unlike bal plants, snc1 plants do not overexpress At4g16890. In addition, snc1 is genetically stable but bal is metastable (Stokes et al., 2002). In our effort to identify revertants of snc1, we obtained nine revertants as well as a small number of recessive mutants (Y. Zhang and X. Li, unpublished data) by screening M2 plants from ~4000 M1 families. This is quite different from the results of the genetic screen that Stokes et al. (2002) performed to identify revertants of bal, in which a high frequency of revertants (~7% of the M2 plants) was observed in the progeny of mutagenized bal plants.

Mutational analysis of the NB-LRR class of R genes has suggested that both the NB and LRR domains are essential for the functions of these R proteins. A large number of mutations in both the NB and LRR domains have been found to inactivate the R proteins (Warren et al., 1998; Dinesh-Kumar et al., 2000; Tao et al., 2000, Axtell et al., 2001; Tornero et al., 2002), implying that the mutated residues are important for R protein function. Although negative regulation of this group of R proteins may play a crucial role in R gene–mediated resistance, as suggested by the data that RPS2 can be activated constitutively by the elimination of its negative regulator RIN4 (Axtell et al., 2001; Tornero et al., 2002), implying that the mutated residues are important for R protein function. Although negative regulation of this group of R proteins also may contribute to resistance to the bacterial pathogen.

A model is proposed to explain how the mutation in snc1 results in the constitutive activation of defense responses (Figure 7). In wild-type plants, SNC1 interacts with an R PROTEIN BINDING PROTEIN (RBP) equivalent to RIN4. This interaction involves the NL linker region in SNC1, and amino acid Glu-552 is one of the critical residues for this interaction. Changing the negatively charged Glu-552 to the positively charged Lys-552 results in reduced binding affinity between snc1 and the negative regulator and dissociation of snc1 from the complex, which then leads to the constitutive activation of the downstream resistance pathways.
Our model explains the phenotypic differences between 
\( \text{snc1}/\text{SNC1} \), \( \text{snc1}/- \), and \( \text{snc1}/\text{snc1} \) plants. In \( \text{snc1}/\text{SNC1} \) plants, both the wild-type and mutant proteins bind to the negative regulator of SNC1. As a result of reduced binding affinity between snc1 and RBP, a portion of the snc1 protein dissociates from the negative regulator and partially activates the downstream signal transduction pathways. Because there is no wild-type protein in \( \text{snc1}/- \) plants, the excess amount of RBP sequesters all of the snc1 protein and blocks the activation of downstream defense responses. Because \( \text{snc1}/\text{snc1} \) plants most likely contain more snc1 protein than \( \text{snc1}/\text{SNC1} \) plants, the phenotype is more dramatic in \( \text{snc1}/\text{snc1} \) plants. The constitutive PR gene expression in transgenic plants overexpressing SNC1 (At4g16890) also can be explained by the excess amount of SNC1 protein unbound to the negative regulator of SNC1. Furthermore, the existence of a negative regulator of SNC1 equivalent to RIN4 is supported by the finding that a loss-of-function mutation in \( \text{BON1}/\text{CPN1} \) (Hua et al., 2001; Jambunathan et al., 2001) leads to the constitutive activation of SNC1-dependent resistance pathways (J. Hua, personal communication).

How downstream signaling pathways are activated by snc1 protein dissociated from its negative regulator is unclear. Recently, different domains of Rx have been shown to interact with each other in vivo (Moffett et al., 2002). It was proposed that disruption of these intramolecular interactions leads to the activation of downstream pathways. It is possible that dissociation of the snc1 protein from its negative regulator also induces conformational changes of the protein, which subsequently triggers either the release or recruitment of active effectors, as suggested previously (Moffett et al., 2002).

Analysis of residues that correspond to Glu-552 in SNC1 revealed significant divergence of the residues among different TIR-NB-LRR–type R proteins (Figure 2B). RPS4 and RRS1-R
actually contain a Lys at this position, indicating that the Glu-to-Lys difference at this position does not necessarily render other R genes constitutively active. The effects of this change may depend strictly on the nature of the interactions between the R proteins and their interacting partners. Compared with mutations in other constitutively active R gene variants, the mutation in snc1 is unique. The region in which the snc1 mutation is located is different from the regions of other R proteins in which HR-inducing mutations were found (Figure 2C). The chimeric Mi proteins that induce localized HR contain replacements in the N-terminal extension (Hwang et al., 2000), whereas mutations in Rx that lead to constitutive HR are localized either in the NB or the LRR (Bendahmane et al., 2002). In addition, both Mi and Rx are CC-NB-LRR–type R genes. The only reported TIR-NB-LRR–type R gene mutation that causes spontaneous lesion formation is ssi4, which also is found in the NB region (Shirano et al., 2002).

Unlike ssi4, snc1 plants do not develop spontaneous lesions. snc1 is the first confirmed R gene mutant that activates downstream resistance pathways in the absence of HR, suggesting that R gene–mediated resistance can be uncoupled from cell death. Transgenic plants overexpressing the snc1 mutant gene do not have spontaneous lesions either (data not shown). The lack of HR-like lesions in snc1 plants and the unique location of the snc1 mutation suggest that the mutation may activate the R protein by a mechanism different from those in the constitutive HR-inducing mutants. We hypothesize that the activation of downstream defense pathways in snc1 is attributable to the disruption of intermolecular interactions, whereas the HR induction by constitutively active Rx, Mi, and SSI4 variants is caused by the disruption of intramolecular interactions by the mutations, as suggested previously (Hwang et al., 2000; Moffett et al., 2002).

The absence of spontaneous lesions in snc1 also makes it a very useful tool for studying resistance pathways downstream of R genes without the interference of cell death. This is demonstrated by the analysis of the snc1 pad4-1 and snc1 eds5-3 double mutants. Because SNC1 encodes an R protein belonging to the TIR-NB-LRR class and EDS1 is required for resistance conferred by this group of R genes, it is not surprising that eds1 completely suppressed the snc1 mutant phenotypes. Resistance conferred by the TIR-NB-LRR class of R genes often is only partially dependent on PAD4 (Glazebrook et al., 1996; Feys et al., 2001; van der Biezen et al., 2002). It is surprising that pad4 completely suppressed the snc1 mutant phenotype. One of the main differences between resistance in snc1 and resistance conferred by other TIR-NB-LRR R genes such as RPP4 and RPP5 is that HR is not involved in the resistance

Figure 5. Analysis of snc1 pad4-1.

(A) Morphology of snc1 pad4-1 plants. The photograph shows 4-week-old plants grown on soil.

(B) Suppression of constitutive pBGL2-GUS reporter gene expression in snc1 by pad4-1. Twenty-day-old seedlings grown on MS medium were stained for GUS activity.

(C) Enhanced susceptibility of the snc1 pad4-1 double mutant to P. s. m. ES4326. The leaves of 4-week-old soil-grown plants were infiltrated with a suspension of P. s. m. ES4326 in 10 mM MgCl2 (OD600 = 0.00005). The photograph was taken 3 days after infection. At this dose, wild-type (WT) Arabidopsis normally is resistant to the bacterium.

(D) Susceptibility of snc1 pad4-1 to P. parasitica Noco2. Two-week-old seedlings were sprayed with Noco2 spores at a conidiospore suspension concentration of 5 x 10⁷ spores per milliliter of water. The infection was rated as follows on 20 plants at 6 days after infection by counting the number of conidiophores per infected leaf: 0, no conidiophores on the plants; 1, no more than 5 conidiophores per infected leaf; 2, 6 to 20 conidiophores on a few of the infected leaves; 3, 6 to 20 conidiophores on most of the infected leaves; 4, 5 or more conidiophores on all infected leaves; 5, 20 or more conidiophores on all infected leaves. Col, Columbia wild type.
Figure 6. Epistasis Analysis of snc1 and eds5-3.

(A) and (B) Free SA (A) and total SA (B) levels in the mutants. Leaf tissue was harvested 4 weeks after germination and used for SA extraction. Each treatment had four replicates. Col, Columbia wild type.

(C) Morphology of snc1 eds5 compared with snc1 and the wild type (WT).

(D) GUS staining of the pBGL2-GUS reporter gene in snc1 and snc1 eds5. Staining was performed on 20-day-old plants grown on MS medium.

(E) PR-2 expression in the mutants. RT-PCR was used to analyze the expression of PR-2 as described for Figure 3.

(F) Bacterial growth of P.s.m. ES4326 in the mutants. Two leaves of each plant were infiltrated with the bacteria (OD600 = 0.00005). Leaf discs within the inoculated areas were taken after 0 and 3 days of infiltration. Four replicates were taken for each treatment. Error bars represent 95% confidence limits of log-transformed data. cfu, colony-forming units.
in snc1 because snc1 plants do not form spontaneous lesions. As suggested previously by Feys et al. (2001), most likely, EDS1 and PAD4 both are fully required for the HR-independent resistance responses, whereas EDS1 encodes additional functions for the generation of HR. Because the morphological phenotype of snc1 is not nearly as dramatic as that of the transgenic plants overexpressing this gene (Stokes et al., 2002), SNC1 probably is only partially activated by snc1. This effect also may contribute to the full dependence of the snc1 phenotype on PAD4.

Another interesting observation is that eds5-3 had no effect on constitutive PR-2 expression in snc1, whereas the expression of the SA-degrading enzyme NahG in snc1 suppressed the pBGL2-GUS reporter gene (Li et al., 2001). Because catechol, the product of NahG, induces disease susceptibility in plants (van Wees and Glazebrook, 2003), the suppression of pBGL2-GUS by NahG might be caused by the accumulation of catechol rather than by the reduction of SA. Alternatively, SA synthesized independent of EDS5 might be required for constitutive PR-2 expression, and the lack of PR-2 expression in snc1 NahG might be the result of the hydrolysis of SA synthesized independent of EDS5.

A simplified model is proposed in Figure 8 to describe the resistance pathways activated by snc1. The activation of downstream pathways appears to require both EDS1 and PAD4, because mutations in EDS1 and PAD4 completely suppressed the snc1 phenotype. On the other hand, increased SA levels were only partially responsible for the snc1 phenotypes, because snc1 eds5-3 double mutants with wild-type levels of SA had intermediate sizes, curly leaves, and constitutive PR-2 expression. Thus, both SA-dependent and SA-independent pathways exist downstream of snc1. Previously, it was shown that the induction of PR-2 by avirulent pathogens was not affected in the inoculated leaves of eds5 mutants (Nawrath and Métraux, 1999). The expression levels of PR-2 in cpr1 and cpr5 also were not affected by eds5 (Clarke et al., 2000). Thus, the constitutive expression of PR-2 appears to be a hallmark of the SA-independent resistance pathway.

Although snc1 eds5-3 plants are susceptible, snc1 npr1 plants are resistant to the bacterial pathogen P.s.m. ES4326, indicating that both NPR1-dependent and NPR1-independent pathways are activated downstream of SA. The SA-dependent and NPR1-independent pathway appears to be the major contributor to the resistance to P.s.m. ES4326 in snc1 npr1 plants. On the other hand, SA alone cannot induce resistance to P.s.m.

Figure 7. Model for the Activation of SNC1 by the snc1 Mutation.

(A) In wild-type plants, SNC1 is sequestered by the proposed negative regulator RBP and is inactive.
(B) In snc1/SNC1 plants, some snc1 protein dissociates from its negative regulator and activates downstream defense pathways.
(C) In heterozygous revertant snc1/− plants, all snc1 protein is sequestered as a result of the excess amount of the negative regulator.

Figure 8. Model for Pathways Activated in snc1 Plants.
ES4326 in the npr1 background (Cao et al., 1994), suggesting that the SA-independent pathway(s) also is required for resistance to the bacterial pathogen in snc1 npr1 plants.

METHODS

Screening for snc1 Revertants

The snc1 npr1-1 mutant seeds of Arabidopsis thaliana were treated by fast-neutron bombardment at a dose of 60 Gray by Andrea Koydm (Agriculture and Biotechnology Laboratory, International Atomic Energy Agency, Vienna, Austria). M1 plants were grown on soil and allowed to self-pollinate. M2 seeds from 10 to 20 plants were pooled upon harvest. M2 plants were grown on soil at 22°C under 16-h-light/8-h-dark cycles. Approximately 40,000 M2 plants from ~4000 M1 families were screened for those with wild-type size and morphology. Seeds from putative mutants were collected and planted again. Lines producing progeny with both wild-type (approximately three-fourths) and snc1 (approximately one-fourth) morphology were analyzed further for the presence of deletions in At4g16890 by PCR and sequence analysis.

Mutant Characterization

To test pBGL2-GUS reporter gene expression, seeds were surface-sterilized and plated on MS medium (Murashige and Skoog, 1962). The plates were incubated in a TC16 plant growth chamber from Conviron (Winnipeg, Canada) at 22°C under 16-h-light/8-h-dark cycles. After 20 days, GUS staining was performed using a protocol described previously (Bowling et al., 1994). Infection of the plants with Pseudomonas syringae pv maculicola ES4326 and Peronospora parasitica Noco2 was performed as described previously (Li et al., 2001). Salicylic acid (SA) was extracted from fresh leaf tissue of 4-week-old plants and measured as described previously (Li et al., 1999).

Expression Analysis

To analyze the level of gene expression, seeds by reverse transcriptase–mediated PCR, total RNA samples were prepared from 20-day-old plants grown on MS medium using the Totally RNA kit from Ambion (Austin, TX). Reverse transcription was performed using the RT-for-PCR kit from Clontech (Palo Alto, CA). Real-time PCR was performed using the QuantiTect SYBR Green PCR kit to avoid amplification of genomic DNA. The primers used to amplify PR-2

Reverse Complementation

A 7.2-kb Pat1-BamHI genomic fragment containing the wild-type At4g16890 gene was first cloned from BAC clone F5D3 to pGEM3Z (from Perkin-Elmer). The same fragment then was cloned from pGEM3Z to pGreen229 (Helias et al., 2000) to obtain pG229-SNC1. A 1.2-kb KpnI-AvrII genomic fragment containing the snc1 mutation was amplified by PCR from the genomic DNA of snc1 plants and used to replace the wild-type fragment in pG229-SNC1. The resulting clone was named pG229-snc1. Both pG229-SNC1 and pG229-snc1 then were used to transform the wild-type plants by the floral-dip method (Clough and Bent, 1998).

Creating the snc1 pad4-1 Double Mutant

To create the snc1 pad4-1 double mutant, the original snc1 npr1-1 (as female) was crossed with pad4-1. The resulting F1 plants had wild-type morphology and were grown to set seeds. In the F2 population, 36 seedlings were grown on soil and 7 of the 36 plants showed the distinct snc1 morphology of small stature and curly leaves. The F3 seeds of these seven snc1-like plants were collected and replanted. Among them, three had approximately one-quarter large wild-type plants segregating out. The plants with wild-type morphology were potential snc1 pad4-1 double mutants. These lines were plated on MS plates with 0.2 mM SA to check for NPR1 homozygosity, because npr1 mutants bleach to death on high concentrations of SA that wild-type plants can survive (Cao et al., 1997). These lines also were plated on MS medium containing 50 µg/mL kanamycin to check for the presence of the pBGL2-GUS reporter gene. The lines that were both NPR1 and pBGL2-GUS homozygous were used for further characterization. The resulting lines were backcrossed with snc1 to confirm the presence of the snc1 mutation, and as expected, the F1 plants had snc1 morphology. The presence of pad4-1 in the final mutant was confirmed by sequencing analysis of the pad4-1 locus.

Creating the snc1 eds5-3 Double Mutant

The strategy for obtaining the snc1 eds5-3 double mutant was similar to that used to isolate snc1 pad4-1. The original snc1 npr1-1 (as female) was crossed with eds5-3, and the resulting F1 plants had wild-type morphology and were grown to set seeds. In the F2 population, 72 seedlings were grown on soil and 19 of the 72 plants showed the distinct curly leaves of snc1. Among these 19 plants, 3 were larger, whereas the other 16 remained small. The F3 seeds of four small snc1-like plants were collected and replanted. Among them, two had approximately one-quarter large plants with curly leaves. The large plants with curly leaves were potential snc1 eds5-3 double mutants. These lines were plated on MS plates with 0.2 mM SA to check for NPR1 homozygosity. Lines that were NPR1 or npr1-1 homozygous were used for further characterization. The resulting lines were backcrossed with snc1 to confirm the presence of the snc1 mutation, and as expected, the F1 plants had snc1 morphol-


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