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Identification and Cloning of a Negative Regulator of Systemic Acquired Resistance, SNI1, through a Screen for Suppressors of

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Summary

Systemic acquired resistance (SAR) is a plant immune response induced after a local infection by necrotizing pathogens. The gene is a positive regulator of SAR, essential for transducing the SAR signal salicylic acid (SA). Mutations in the gene abolish the SA-induced expression of pathogenesis-related ( ) genes and resistance to pathogens. To identify additional regulators of SAR, we screened for suppressors of . In the background, the (suppressor of _ , inducible 1) mutant shows near wild-type levels of expression and resistance to pathogens after induction. Restoration of SAR in by the recessive mutation indicates that wild-type SNI1 may function as a negative regulator of SAR. We cloned the gene and found that it encodes a leucine-rich nuclear protein.

Introduction

Systemic acquired resistance (SAR) is a general plant defense response that can be triggered after a local infection by pathogens causing necrotic lesions. This response is long lasting and effective against a variety of pathogens, including fungi, bacteria, and viruses (Ryals et al., 1996). Induction of SAR is correlated with upregulation of several well-characterized pathogenesis-related ( ) genes (Van Loon and Van Kammen, 1970; Ward et al., 1991; Yalpani et al., 1991; Uknes et al., 1992). In particular, and β-1,3-glucanase (; also known as) have been widely used as molecular markers for SAR (Uknes et al., 1992; Bowling et al., 1994; Cao et al., 1994). Convincing evidence has shown that salicylic acid (SA) is a necessary and sufficient signal for SAR induction. The onset of SAR is accompanied by an increase in the endogenous levels of SA (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991) and can be brought on by exogenous application of SA (White, 1979) or analogs such as 2,6-dichloroisonicotinic acid (INA; Métraux et al., 1991) and benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH; Görlach et al., 1996). Furthermore, transgenic plants expressing the SA-degrading enzyme salicylate hydroxylase are unable to accumulate SA and are compromised in SAR (Gaffney et al., 1993).

Several genetic screens have been conducted in to identify the regulatory components of the signaling pathway leading to SAR. From the SAR-compromised mutants, (also known as) is the only locus identified that affects a function downstream of the SA signal (Cao et al., 1994, 1997; Delaney et al., 1995; Glazebrook et al., 1996; Ryals et al., 1997; Shah et al., 1997). The mutants are impaired in the ability to accumulate mRNA or to mount an SAR response in the presence of an SAR inducer. The gene encodes a novel protein containing at least four ankyrin repeats (Cao et al., 1997; Ryals et al., 1997), which are found in proteins of diverse functions (Bork, 1993), including IκB and Cactus, which regulate animal immune responses (Thanos and Maniatis, 1995; Lemaitre et al., 1996). The functional importance of these ankyrin repeats in NPR1 has been clearly demonstrated by the isolation of several mutants that contain lesions in the ankyrin repeat consensus (Cao et al., 1997; Ryals et al., 1997). Specifically, in the mutant, where a highly conserved histidine (residue 334) in the third ankyrin repeat is changed to a tyrosine, the SA- or INA-induced expression of a reporter gene, (a fusion gene composed of the 5′ untranslated region of and the coding region of β-glucuronidase), and the endogenous genes is abolished (Cao et al., 1997).

To further dissect the SAR pathway, we conducted a suppressor screen in the background to look for mutants that are able to restore the inducible gene expression. These suppressors would most likely harbor mutations in genes that function downstream of or parallel to in the SAR pathway. Here we report the isolation and characterization of (suppressor of _ , inducible 1), a recessive mutant that restores wild-type levels of SA- and INA-inducible gene expression and disease resistance. The gene was cloned using a map-based approach and found to encode a novel, leucine-rich nuclear protein. The sequence data also reveal that the mutation causes missplicing of the mRNA, resulting in frameshift and truncation in the N-terminal region of the protein. Based on the genetic and molecular characterization of , a working model is proposed for SNI1 as a negative regulator of SAR expression.

Results

Identification of the Mutant as a Suppressor of

Seeds of containing the reporter gene were mutagenized with ethyl methanesulfonate (EMS), and suppressors of the mutation were screened in the M2 population for restored expression of the SAR-responsive in the presence of the SAR inducer INA (0.1 mM). Of 7000 M2 plants examined, 13 (representing at most 11 loci based on the complementation and recombination tests) showed an increase in

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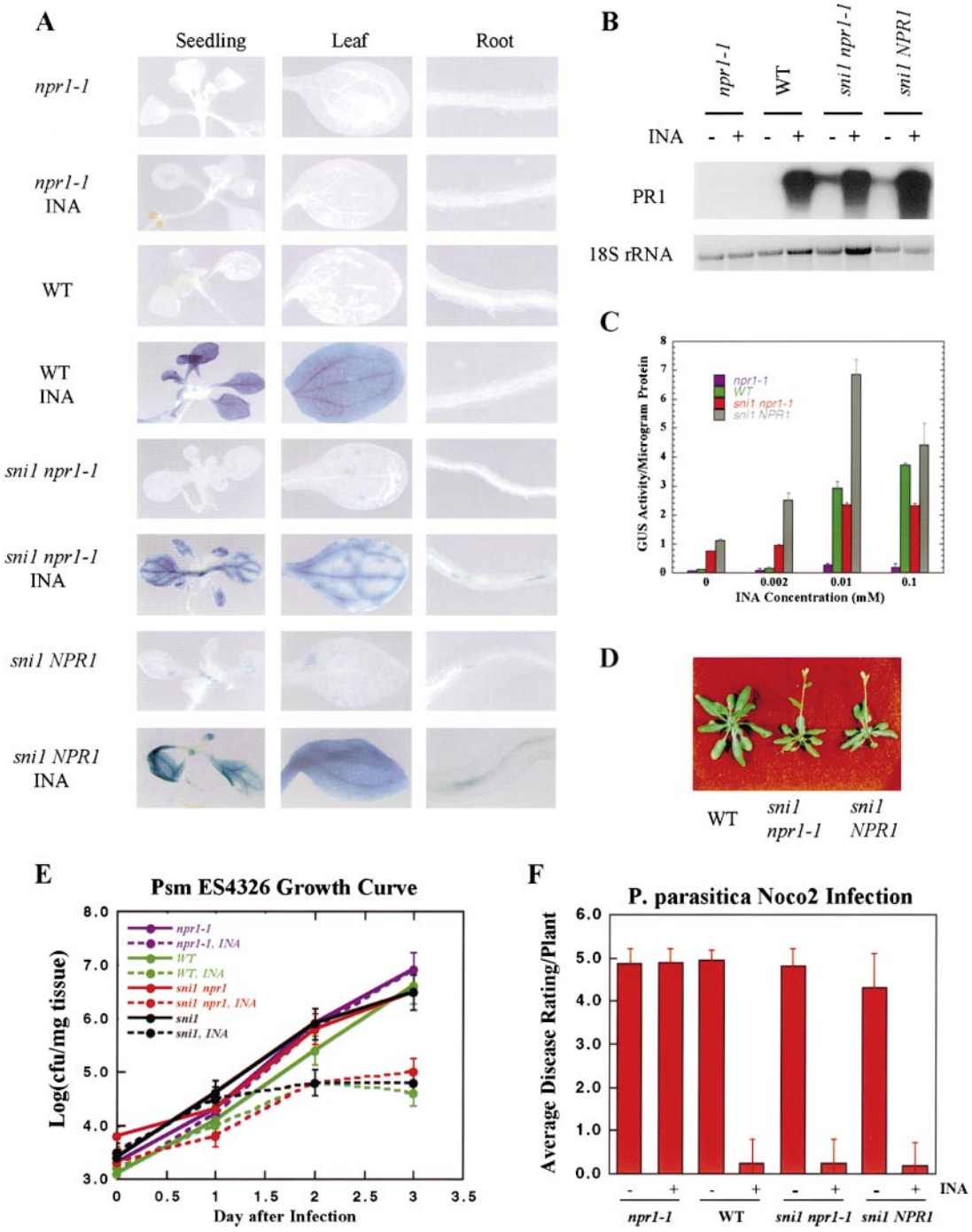

Figure 1. Characterization of the Mutant

(A) Expression of the reporter gene in wild-type (WT), , , and plants. Two-week-old seedlings grown on MS media in the presence or absence of INA (0.1 mM) were stained for GUS activity according to Jefferson et al. (1987).

(B) RNA blot analysis of gene expression in wild-type (WT), , , and plants. RNA samples were prepared from 2-week-old seedlings grown on MS media or MS with 0.1 mM INA. For each sample, 10 μg of total RNA was loaded. The probe for was made as described previously (Bowling et al., 1997). As loading controls, the 18S rRNA bands detected by ethidium bromide staining were used.

(C) Induction of by different concentrations of INA in wild-type (WT), , , and plants. Ten 2-week-old seedlings grown on MS media containing 0, 0.002, 0.01, and 0.1 mM INA were collected and GUS activity measured (Jefferson et al., 1987). The values represent the average of three replicates ± SE. GUS activity is given as absolute fluorescence units per minute per microgram of protein.

(D) Morphological phenotypes of wild-type (WT), , and plants. The plants were grown on soil for 4 weeks before the photograph was taken.

expression after induction by INA and were designated mutants. In particular, the mutant plants exhibited sporadic, weak GUS staining under uninduced conditions but consistent, strong GUS staining after induction by INA (Figure 1A). GUS activity in was detected most strongly in the veins of both leaves and roots, whereas in wild-type, uniform GUS staining was detected in leaves while no GUS staining was seen in roots.

To confirm the effects of mutations on the expression of endogenous genes and to rule out possible mutants of the reporter gene, RNA blot analysis was performed on all mutants to identify those that restored inducible gene expression in the background. Among these mutants, only expressed the gene at levels similar to the wild type under INA induction (Figure 1B). Consistent with reporter gene expression (Figure 1A), a higher background expression of was detected in the mutant without induction.

Genetic Characterization of

The double mutant ( ) was backcrossed with ( ), and the resulting F1 progeny lost the inducible expression, suggesting that is recessive. These F1 plants were then allowed to self-fertilize, and the F2 plants were assayed for the presence or the absence of INA-induced GUS expression. Of 112 F2 plants examined, 27 had strong GUS staining, while the rest had no staining, demonstrating that the phenotype results from a single nuclear mutation ($\chi 2 = 0.047$; $P > 0.5$). Genetic crosses revealed that is also capable of suppressing other mutant alleles (data not shown). Complementation tests showed that is not allelic to other mutants (data not shown).

The double mutant was also crossed with wild-type plants ( ) containing the reporter gene, and the F1 progeny lost the background expression of under uninduced conditions but showed wild-type GUS staining after INA induction. The F2 plants were also examined for segregation of inducible expression to determine whether the phenotype is caused by a reversion of or by a second-site mutation. If is linked to , all F2 progeny should show inducible expression. However, of 181 plants tested, 148 showed strong GUS staining, while 33 displayed no detectable GUS staining after INA treatment, indicating that is not linked to (expected ratio: 13:3; $\chi 2 = 0.032$; $P > 0.5$).

The single mutant expressed and at a higher level (3-fold) than the double mutant under induced conditions (Figures 1A, 1B, and 1C). This difference in the INA-induced expression between and implies that NPR1 function is still required for high-level gene induction in the mutant. Ectopic GUS staining was also detected in the roots of the plants (Figure 1A), as seen in .

Both and plants are smaller than wild type (Figure 1D). In seedlings, the emerging first pair of true leaves are much narrower than those of the wild type (data not shown). These phenotypes were found to cosegregate with and shown later by complementation analysis to be caused by . In mutant plants, no macroscopic or microscopic lesions were detected by trypan blue staining, even after an INA treatment (data not shown), indicating that cell death is not involved in causing the phenotype.

The Effects of on SA Accumulation and Sensitivity

The endogenous levels of SA in were measured under noninducing conditions and after infection by an SAR-inducing pathogen pv. ES4326/ Rpt2 (ES4326/ Rpt2). The results showed that both the baseline and the induced levels of SA found in (0.4 ± 0.004 μg/g and 1.3 ± 0.45 μg/g, respectively) are comparable to those of the wild type (0.2 ± 0.008 μg/g and 1.7 ± 0.69 μg/g, respectively).

The sensitivity of and mutants to SA and INA was measured by a quantitative assay of the reporter gene expression. As shown in Figure 1C, wild type displayed the highest level of expression at 0.1 mM INA, a concentration normally used for SAR induction, while remained nonresponsive to INA. However, in the and mutants, maximum levels of expression were reached at a concentration (0.01 mM) ten times lower than in the wild type. Similar results were obtained when SA was used as the inducer (data not shown). In comparing and , higher levels of were detected for , indicating that a functional NPR1 is required for maximum induction of the reporter gene.

(E) Growth of ES4326 in wild-type (WT), , , and plants. Four-week-old soil-grown plants were treated with 0.65 mM INA 2 days prior to infection by dipping the plants into a ES4326 suspension ($OD_{600} = 0.2$) in 10 mM $MgCl_2$ and 0.01% Silwet L-77 (Bowling et al., 1994). Eight leaves were excised for each phenotype, treatment, and time point (0, 1, 2, and 3 days), rinsed with H_2O, weighed, and bacteria extracted and plated (Bowling et al., 1994). Error bars represent 95% confidence limits of log-transformed data from four replicates (Sokal and Rohlf, 1981). cfu, colony-forming units.

(F) Average disease rating of Noco2 infection in wild-type (WT), , , and plants. Two-week-old seedlings were treated with 0.65 mM INA 2 days prior to infection with a spore suspension (3×10^4 spores/ml). The disease symptoms were scored 7 days after the infection with respect to the number of conidiophores observed on each seedling (30 seedlings were examined for each phenotype and treatment). The scales are defined as follows: 0.0, no conidiophores on the plants; 1.0, no more than 5 conidiophores per infected leaf; 2.0, 6–20 conidiophores on a few infected leaves; 3.0, 6–20 conidiophores on most infected leaves; 4.0, 5 or more conidiophores on all infected leaves; 5.0, 20 or more conidiophores on all infected leaves. Average disease ratings were calculated by summing the scores of all 30 seedlings and dividing by 30. The error bars represent standard errors.

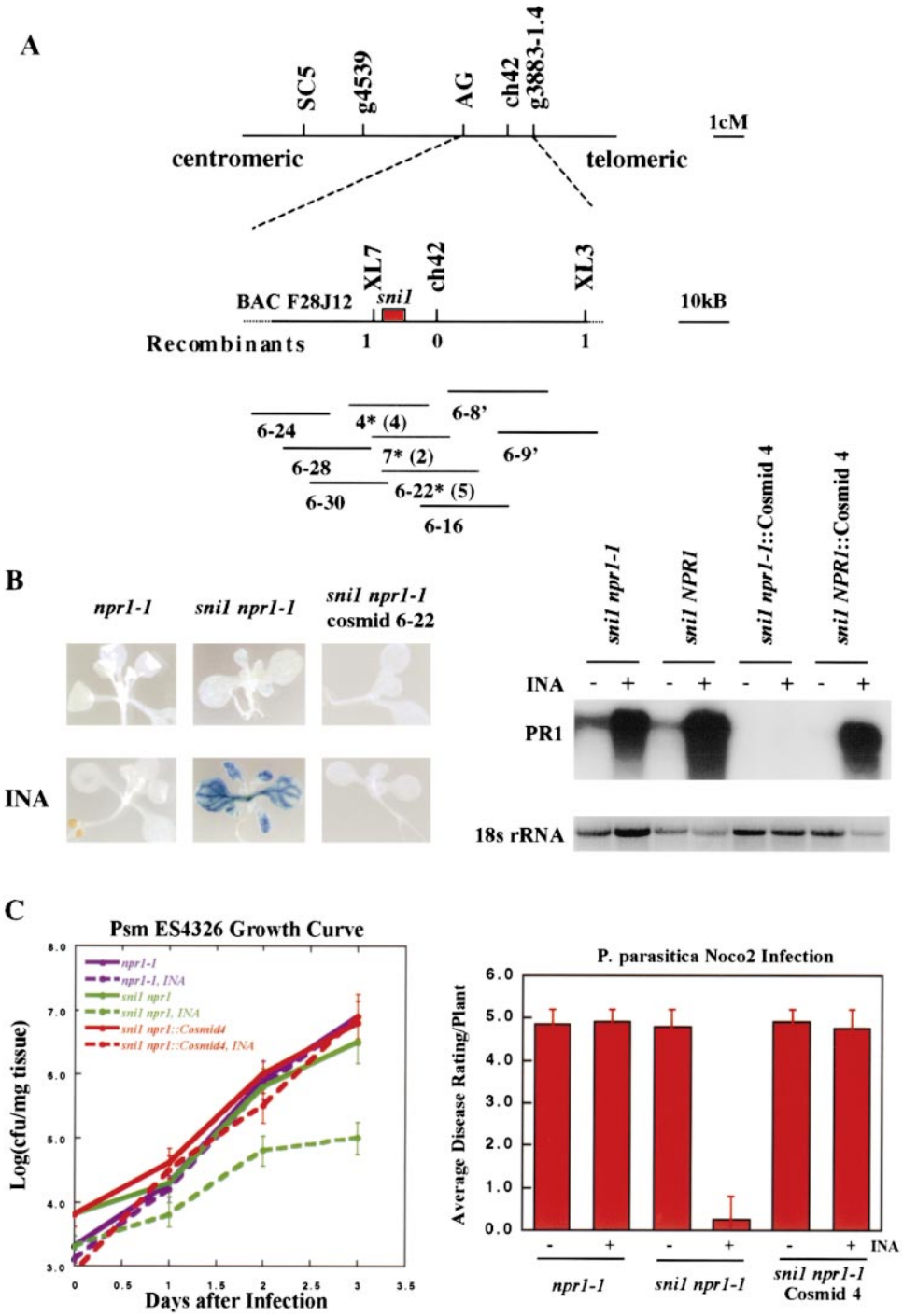

Figure 2. Map-Based Cloning of

(A) Map of the locus on chromosome IV. Fifty-eight F2 progeny homozygous for were used first to determine the crude map position of . Two and zero heterozygotes were detected using CAPS markers SC5 and AG, respectively, indicating that SC5 is ~1.7 cM on the centromeric side of and AG is closely linked to . For fine mapping, CAPS markers g4539 and g3883-1.4 were used to examine 718 F2 progeny homozygous for . Twenty-three heterozygotes were discovered by g4539 (~1.6 cM) and twenty-two by g3883-1.4 (~1.5 cM). Because the heterozygotes found by these two markers were mutually exclusive, was determined to be flanked by these two markers. No heterozygotes were discovered using the marker ch42 located between g4539 and g3883-1.4. CAPS markers XL7 and XL3 were then generated according to the sequence information for the interval between g4539 and g3883-1.4. Among the g4539 heterozygotes, one was found to be a heterozygote for XL7. From the g3883-1.4 heterozygotes, one was discovered to be a heterozygote for XL3. A cosmid contig covering the interval between XL7 and XL3 was generated by subcloning the BAC F28J12. Cosmids 4 and 7 were made in pCLD04541, transformed into a mutant lacking the gene, and selected by resistance to kanamycin (50 μg/ml). The rest of the cosmids were made in pSLJ75516, transformed into carrying the reporter gene, and selected by resistance to the herbicide basta (0.04%). The cosmids that complemented the mutation were labeled by an asterisk, and the number of independent transformants tested is shown in parentheses.

Resistance of to Pathogens

The effect of on pathogen resistance in the or background was tested using both the bacterial pathogen ES4326, which causes leaf spots, and the oomycete pathogen Noco2, which results in downy mildew on uninduced wild-type plants. After INA induction, wild-type plants became resistant to these two pathogens, while remained susceptible (Figures 1E and 1F). Strikingly, the double mutant displayed induced resistance similar to that of wild type, indicating that the mutation restored SAR in . This dramatic effect of on resistance was, however, undetectable in the wild-type background, as shown by the wild-type-like pathogen growth profiles observed in . Although background expression of genes is elevated in both and , this is evidently not enough to confer resistance against the two pathogens at the concentrations used in these infection experiments. Induction by INA or SA is required to render plants resistant to ES4326 and Noco2.

Map-Based Cloning of

To map the locus, was crossed with wild type ( ) in a polymorphic genetic background. In the resulting F2 population, progeny homozygous at the locus, identified using the morphological phenotypes of , were examined using various CAPS (codominant leaved mplified olymorphic equences) markers (Konieczny and Ausubel, 1993). The locus was mapped to chromosome IV between markers AG and g3883-1.4. It was shown to be tightly linked to marker ch42, with which no recombinants were discovered among the 718 progeny examined (Figure 2A). The ch42 sequence is located in the middle of the bacterial artificial chromosome (BAC) clone F28J12, which has been completely sequenced. Thus, more CAPS markers were generated on both sides of ch42 according to the sequence information, and markers XL7 and XL3 were used to narrow down the interval containing to a 43.3 kb region.

Two cosmid libraries covering the 43.3 kb region were generated from F28J12 using the binary vector pCLD04541 (carrying the kanamycin resistance for selection) or pSLJ75516 (carrying the herbicide glufosinate ammonium, or "basta," resistance for selection), and a cosmid contig spanning the region was generated (Figure 2A). Complementation of the mutation was observed when cosmid clones 4, 7, and 6-22 were transformed into mutants. In these transformants, which are , expression of and the reporter was completely inhibited, as seen in the mutant (Figure 2B). The transformants also displayed susceptibility to both ES4326 and Noco2 even after INA induction (Figure 2C). When the cosmids containing were transformed into the single mutant, the background expression of genes was repressed; the plants behaved like wild type, showing inducible gene expression (Figure 2B). The T2 progeny of the complementing lines all segregated for the and phenotypes, which corresponded to the presence and absence of the selective markers, respectively. This indicated that these were true complementing transformants rather than contaminants from either or wild type. Transgenic plants containing cosmids other than clones 4, 7, and 6-22 showed the mutant phenotype.

Sequence Analysis of

The genomic insert in cosmid 4 was analyzed and the sequences of wild type and compared. In the entire 15 kb region represented by the insert, only one mutation (G to A) was identified. A number of PCR primer pairs were then used to perform RT-PCR to identify the cDNA sequence in the region flanking the mutation. The resulting RT-PCR fragment was labeled and used to screen a cDNA library (Kieber et al., 1993). From 10^6 plaques screened, one cDNA clone was isolated and shown to contain an almost full-length coding sequence for , missing only 11 nucleotides of the 5′ end that were later determined by RT-PCR and sequencing. A full-length cDNA clone was generated by PCR, and the functionality of this cDNA clone was confirmed by its ability to complement the mutation when it was expressed, under the control of the 35S promoter of cauliflower mosaic virus, in stable transformants (data not shown).

Sequence analysis predicted that encodes a novel protein of 432 amino acids (Figure 3A) with a molecular weight (MW) of 48.8 kDa. Hydropathy plot analysis indicated that SNI1 is a soluble protein with no obvious transmembrane domains. A survey of the amino acid sequence showed that SNI1 is a leucine-rich protein (12.7%). A database search for homologs yielded one EST clone isolated from cotton (accession number AI054954), implying that SNI1 may be conserved in plants. Even though no substantial homology was found between SNI1 and any known proteins, a short stretch of homology was discovered with the mouse retinoblastoma (Rb) protein (Bernards et al., 1989; Figure 3B), a tumor suppressor that represses the transcription regulated by transcription factors such as E2F (Nevins, 1992).

Comparison of the cDNA sequence and the genomic sequence revealed that the gene consists of 15 exons. The G to A mutation in occurred in the 3′ intron acceptor site at the junction between the fifth intron and the sixth exon. RT-PCR and sequence analysis of the mutant showed that the mutation caused an 11 nucleotide deletion in the cDNA, resulting in a frameshift early in the coding region (Figure 3A, amino

(B) Complementation of in gene expression. Using protocols described in Figure 1, GUS staining was performed on , , and transformed with the complementing cosmid 6-22, while RNA blot analysis was carried out in and transformed with the complementing cosmid 4.

(C) Complementation of in response to pathogen infection. Using the protocols described in Figure 1, , , and transformed with cosmid 4 were infected by ES4326 and Noco2. The growth curves for ES4326 and the average disease ratings for Noco2 are presented.

A

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CTTCTCGTTTTCTTCTTTGGTGCTG CTGAGAAGAAATTAGTGAAATTGTG AAAGAGAAGATGTCGAAAGAGACGA AGGGTAATAACAATACAAGCAGAGT  100
                                                                  M  S  K  E  T   K  G  N  N  N  T  S  R  V   14

GATGAGTGGCTATGGCGGTAGCTTG GAAGCTAACACCTTGGCTATGATTG ATTCCACCGGAGCTAAAGACAGTCG CGACGCTAACGAAGATCGTTTGCAG  200
  M  S  G  Y  G  G  S  L   E  A  N  T  L  A  M  I   D  S  T  G  A  K  D  S  R   D  A  N  E  D  R  L  Q    47

TATCTGGAAGCTGTTCGTGCTGCTT CACTTGTACCCGAAAATGGAATTCC TCCAACCAACAAAATGTACCAAGCG ATTTTCAGGATATTGAGATTTGGTA  300
 Y  L  E  A  V  R  A  A   S  L  V  P  E  N  G  I  P   P  T  N  K  M  Y  Q  A   I  F  R  I  L  R  F  G     80

AAACATTGGAACTTATCACAGCAAG TTTCCAGCTTTTGACTCAATTACAT CAGCGGTTTCCTTGGGTTTATGTAT CTGATTCAGCTGATCAGTTGGACAT  400
K  T  L  E  L  I  T  A  S   F  Q  L  L  T  Q  L  H   Q  R  F  P  W  V  Y  V   S  D  S  A  D  Q  L  D  I  114

CGTTGACGAGGCTTGGTCACCGTTT AATTTCGGGTCTGATGTTGATTCTG ATGAAAAGGATTTATCAGTGAGAAG CTTATTTTTGCAACAGCTGATTCAG  500
  V  D  E  A  W  S  P  F   N  F  G  S  D  V  D  S   D  E  K  D  L  S  V  R  S   L  F  L  Q  Q  L  I  Q   147

AACATGAACAAAAGAGTTAATGAGT CTGAGGAATCAGATTTAAAGATCCT TGGAAATATGTTTCTGTTCAAGTAT CTTGCTCATGTTCTTAAGCTAGATT  600
 N  M  N  K  R  V  N  E   S  E  E  S  D  L  K  I  L   G  N  M  F  L  F  K  Y   L  A  H  V  L  K  L  D   180

TCACACCCCGAAATCAAGTGTATGA AGAAACTATGAACTGGAGTCTCTTA AAGGAATCTTTTCTGAATCTACTTC TGGCTTCAAGAAAAGTGAATTTCAA  700
F  T  P  R  N  Q  V  Y  E   E  T  M  N  W  S  L  L   K  E  S  F  L  N  L  L   L  A  S  R  K  V  N  F  K  214

ACTTCTAATGAAAGATTATCTATCA ACAATGTGTGCATCCATTGATGCTG ATGAAAAGTCTATCAGTTTGGTAGA ATTGCACAAGGACATGCTTACTGCT  800
  L  L  M  K  D  Y  L  S   T  M  C  A  S  I  D  A   D  E  K  S  I  S  L  V  E   L  H  K  D  M  L  T  A   247

ATGAAAGAACTTCTAGTAATGATCA TGGAGCTTGATACATCAAAGAAGAA AGCTGACTTAGAAGGGATTACCTCT AGAGGAGATGGCGTAAGGACCCCTG  900
 M  K  E  L  L  V  M  I   M  E  L  D  T  S  K  K  K   A  D  L  E  G  I  T  S   R  G  D  G  V  R  T  P    280

CAATGGAGATCATTCTCGACGAGCT GACTTATGATGGATACTTGCTGTCA AAATTTCTTCAGGTTTTCGATGATC CTAAATGGAAGCTAGAGATTGTTCT 1000
A  M  E  I  I  L  D  E  L   T  Y  D  G  Y  L  L  S   K  F  L  Q  V  F  D  D   P  K  W  K  L  E  I  V  L  314

CCAATACCTTACTAAATACATTCCT AAGCCTGTTGTACGTACCCGAAGAA CAACTGTTCCTCAAGCAGAGGATTC GAAAACACTAAATGGGATCTTGAAG 1100
  Q  Y  L  T  K  Y  I  P   K  P  V  V  R  T  R  R   T  T  V  P  Q  A  E  D  S   K  T  L  N  G  I  L  K   347

ACGTTTTCAAATGGCACAAATCCAA AGAACATCACTAAAAAGATAGGACC TGACATTGTTCAGATCCTCATCGGC CATGCCTTTCTGGCTCGGCTTACAT 1200
 T  F  S  N  G  T  N  P   K  N  I  T  K  K  I  G  P   D  I  V  Q  I  L  I  G   H  A  F  L  A  R  L  T    380

TCTCTGACCCTCACGAAGGAGACTC TATTTCAGAGATATGCAGTAGTATC ATCTCTGCATTTACTAGTCTAAAGC GAGTAGATCAGAAAATCGAGATTCT 1300
F  S  D  P  H  E  G  D  S   I  S  E  I  C  S  S  I   I  S  A  F  T  S  L  K   R  V  D  Q  K  I  E  I  L  414

ACCGTTTGGGAAAGAAGTGTTGTTT ACTGCAGGAATGGTACTCAAGGCAA AAGCTTAAAGCATCTAGGAATCAAG ATTTTAACAAATCTAGAGACTTCAA 1400
  P  F  G  K  E  V  L  F   T  A  G  M  V  L  K  A   K  A  *                                                 432

TTGGATTAGAAGCAAAGAAGTTACA ATGTAATGTAGGAATTAAGAGTTAC AGATCAATGAATTATGAATTTATAT ATT
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B

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SNI1 : 226 ASIDADEKSISLVELHKDMLTAMKELLVMIMELDTSKK---------------------- 263
           A++D DE   +  EL K + T++ +   ++ E+DTS K
RB   : 100 AAVDLDEMPFTFTELQKSIETSVYKFFDLLKEIDTSTKVDNAMSRLLKKYNVLCALYSKL 159

SNI1 : 264 --KADLEGITSRGDGVRTPAMEIILDELTYDGYLLSK--FLQVFDDPKWKLEI---VLQY 316
              +L  +T     + T    +++ ++++  +LL+K   LQ+ DD     ++   V+ Y
RB   : 160 ERTCELIYLTQPSSALSTEINSMLVLKISWITFLLAKGEVLQMEDDLVISFQLMLCVVDY 219

SNI1 : 317 LTKYIPKPVVRTRRTTVPQAEDSKTLNGILKTFSNGTN-PKNITKKIGPD--IVQIL 370
             K+ P  ++R      P    +  +NG  +T   G N    I K++  D  I+++L
RB   : 220 FIKFSPPALLRE-----PYKTAAIPINGSPRTPRRGQNRSARIAKQLENDTRIIEVL 271
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Figure 3. Sequence Analysis of

(A) cDNA sequence and deduced amino acid sequence. The 11 nucleotides that are deleted in the mRNA as a result of the mutation are underlined.

(B) Sequence homology between SNI1 and mouse retinoblastoma (Rb). Using a BLAST search of the GenBank, SNI1 was found to share 22% amino acid sequence identity and 42% amino acid sequence similarity with Rb in the N domain (Hensey et al., 1994), with an E value (the expect value) of 1.1. An E value is a parameter that describes the number of hits one can expect to see by chance when searching a database of a particular size. An E value of 1.1 indicates marginal homology.

acid residue 140). This misspliced mRNA was the predominant transcript made from the gene because no wild-type transcript was detected using RT-PCR in the mutant.

Expression and Subcellular Localization of

RNA blot analysis using poly A^+ RNA detected a very faint band of approximately 1500 nucleotides in both uninduced and induced wild-type plants (data not shown). Transgenic lines were generated carrying a promoter fusion to the reporter gene. Very weak GUS staining was detected mainly in the veins. INA treatment seemed to have no effect on the staining pattern (data not shown).

To determine the subcellular localization of the SNI1 protein, the green fluorescent protein ( ) gene was fused to either the 5′ or the 3′ end of . The resulting fusion genes were driven by the constitutive 35S promoter of the cauliflower mosaic virus. The resulting fusion proteins had a MW of 75 kDa, which is above the size exclusion limit (40–60 kDa) for passive diffusion of protein through the nuclear pores (Raikhel, 1992). When either or was delivered into onion epidermal cells by particle bombardment,

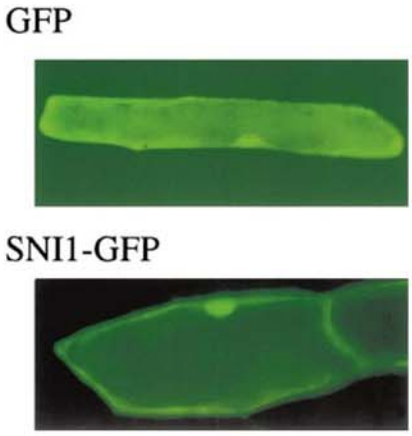

Figure 4. Nuclear Localization of SNI1-GFP

The and DNA preparations (20 μg) were delivered separately into onion epidermal cells using particle bombardment. After 12 hr of incubation, GFP fluorescence was observed using a fluorescence microscope with an excitation wavelength of 488 nm. The nuclei in the cells are visible in the bright-field images (data not shown). The was used as a control.

GFP fluorescence was observed predominantly in the nuclei (Figure 4). Weak fluorescence was also detected in the cytoplasm. When was transiently expressed, the smaller GFP protein (26 kDa) was found to be evenly distributed throughout the cells.

Discussion

To identify additional regulators of SAR, a screen for suppressors of was performed. Even though is a missense mutation, it completely abolishes SA- or INA-induced gene expression and resistance, providing a clean background for the suppressor screen. The recessive mutation, which causes a frameshift in the N-terminal region of the protein and most likely a complete knockout of the SNI1 function, restores inducible gene expression and pathogen resistance in (Figure 1), suggesting that the wild-type SNI1 protein is a negative regulator of SAR. Indeed, expression of the wild-type gene in results in repression of SAR, abolishing INA-induced gene expression and resistance (Figures 2B and 2C). The lack of SAR induction in plants and restoration of SAR in plants suggest that the wild-type SNI1 protein represses SAR and the role of NPR1 in SAR is probably to remove the SNI1 repression. However, we cannot rule out the possibility that SNI1 represents a redundant pathway independent of NPR1. The mutation may somehow render the plants supersensitive to INA and SA induction, resulting in restoration of SAR in the mutant background. In this scenario, the wild-type SNI1 is still a negative regulator of SAR, which functions to dampen the effects of INA and SA. The elevated background gene expression in (Figures 1A and 1B) and the reduced threshold levels of SA and INA that are required to induce gene expression (Figure 1C) provide further evidence that the mutation removes a negative control of genes and SAR.

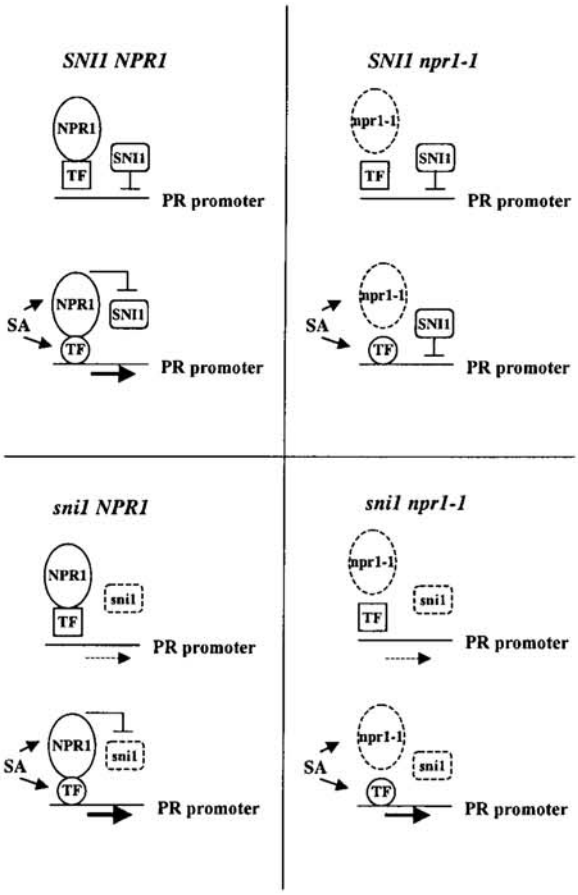

Figure 5. Proposed Model for the Regulation of SAR by SNI1 and NPR1

In wild type ( ), SNI1 is a repressor of genes (a blocked line) and, therefore, a negative regulator of SAR. Induction of gene expression (arrow) and SAR requires both activation of a positive regulator, presumably a TF, by SA and derepression (a blocked line) of SNI1 by the SA-activated NPR1. In a mutant, the compromised NPR1 function (dashed oval) results in a failure to derepress SNI1 and a lack of inducible gene expression and resistance. In a double mutant, the function of SNI1 is compromised (dashed rectangle) and the repression by SNI1 is released even in the absence of an SAR inducer, leading to a background level expression of genes (dashed arrow). Activation of the TF is still required for a full-scale induction of gene expression and SAR. In a single mutant, the lack of SNI1 function results in a background level of gene expression.

How SNI1 functions to repress gene expression remains to be determined. Analysis of the SNI1 sequence showed no obvious functional motifs or homology to any known transcription factors. However, transcriptional repression may be achieved by different strategies (Cowell, 1994). SNI1 may represent a novel repressor of transcription that directly binds to a specific DNA sequence and inhibits the transcriptional machinery. Because no apparent DNA-binding domain has been found in SNI1, it is more likely that SNI1 binds to DNA indirectly through interaction with a DNA-binding protein. In support of this hypothesis, a element, which contains the consensus of a binding site for the plant-specific transcription factor WRKY family, was found in the promoter and shown to negatively

regulate the expression of the gene (Lebel et al., 1998). Mutations in this element result in an expression pattern of similar to that observed in the mutant, that is, an elevated background expression and an enhanced response to INA induction. Equally plausible, the SNI1 protein may affect transcription by sequestering a transcriptional activator. The stretch of sequence homology detected between SNI1 and the tumor suppressor Rb is intriguing because Rb negatively regulates gene expression by interacting with transcription factors such as E2F (Nevins, 1992). The biological function of the homologous region in Rb, known as the N domain (Hensey et al., 1994), is still unclear even though it is highly conserved among Rb homologs isolated from many organisms. Cell culture and in vitro experiments indicate that this domain is dispensable for Rb function (Jacks et al., 1992; Lee et al., 1992, 1994; Fung et al., 1993). However, deletion mutants of this domain failed to rescue Rb mutant mice, suggesting that this domain may in fact be of functional importance (Riley et al., 1997).

The mechanism by which NPR1 may inactivate SNI1 is also not clear. In a yeast two-hybrid analysis, no interaction between NPR1 and SNI1 was detected (data not shown), suggesting that NPR1 may not directly bind to SNI1. This is consistent with the fact that can suppress other mutant alleles besides . It is possible, however, that NPR1 and SNI1 are members of a protein complex of which other components have yet to be identified.

Our observation that gene expression and SAR in the double mutant are inducible, rather than constitutive, suggests the existence of an activation step that is independent of NPR1 and SNI1 and required for the induction of SAR. This proposed parallel activation was detected only when the functions of both NPR1 and SNI1 were abolished. SA is required for this induction event because expression of the SA-degrading salicylate hydroxylase gene ( ) in the mutant prevents gene induction by the exogenous application of SA (data not shown). This activation event probably does not require the function of NPR1 but may be facilitated by NPR1 because in the gene induction is less dramatic (by 3-fold) than in . Indeed, in a yeast two-hybrid screen, NPR1 was found to bind specifically to a subclass of bZIP transcription factors (Zhang et al., 1999). These bZIP transcription factors may represent the parallel, SA-dependent, positive regulators of the gene expression and SAR postulated above. In support of this, mutations in the binding site for the bZIP transcription factors in the promoter abolish the inducibility of by SA and INA (Lebel et al., 1998). The consequence of the NPR1–bZIP transcription factor interaction has yet to be revealed. Binding of NPR1 to the bZIP transcription factors may facilitate the activation of these transcription factors or recruit NPR1 to its functional location.

Taking all the data together, a working model is proposed to explain the signal transduction pathway leading to the activation of gene expression and to illustrate the roles of SNI1 and NPR1 in the induction of SAR (Figure 5). In wild-type plants, induction of genes and SAR by SA or INA may require two separate events: inactivation of SNI1 by SA through a function of NPR1 and activation of a positive regulator, presumably the bZIP transcription factor, by SA through a mechanism that does not require NPR1 but may be facilitated by NPR1. In an mutant, where SNI1 repression cannot be removed due to the lack of NPR1 activity, induction of SAR is blocked. However, in the double mutant, where gene expression is no longer repressed due to the mutation, the requirement for NPR1 is eliminated and background levels of gene expression are observed under uninduced conditions. The lack of SNI1 repression may also explain the elevated basal expression of genes in the single mutant. When a wild-type gene was transformed into and , this background expression of genes was repressed and the phenotype of the transformants changed back to wild type and , respectively. Alternative models could also be derived to explain the data represented in this paper. More experiments will be carried out to test our working model.

Identification and cloning of enables us to further unravel the regulatory mechanism of gene expression and SAR. Our data suggest that induction of SAR may involve both activation and derepression events. The significance of SNI1 repression of SAR is underlined by the leakiness in gene regulation observed in mutants under noninducing conditions and by the reduced plant size of these mutants. Identification of also suggests a possible means of engineering plants with enhanced disease resistance. Our data have shown that disruption of SNI1 function results in enhanced sensitivity to SA and INA. Future experiments will be aimed at determining whether this enhanced sensitivity to induction leads to an enhanced resistance response to pathogens.

Experimental Procedures

Mutant Screen and Analysis

seeds (40,000) homozygous for the mutation and carrying the reporter gene were mutagenized using 0.3% EMS as previously described (Bowling et al., 1994). Two-week-old M2 seedlings grown on Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) containing 0.1 mM INA (MS-INA) were tested for GUS activity using 4-methylumbelliferyl β-D-glucuronide (MUG) as substrate (Bowling et al., 1994). Those seedlings that showed positive GUS activity were transferred to soil to set seeds. The same screen was then performed on the M3 progeny. The presence of in the suppressed isolates was confirmed by an NlaIII restriction digestion polymorphism generated by this mutation. PCR primers (F4: 5′GAAGCTATTGGATAGATG3′ and R5: 5′GTTGAGCAAGTG CAACT3′) were used to amplify a 770 bp genomic fragment containing the locus.

The double mutant was crossed with , and 112 F2 progeny grown on MS-INA (for 2 weeks) were examined for GUS activity using 5-bromo-4-chloro-3-indolyl glucuronide as substrate (Jefferson et al., 1987). The obtained segregation ratio for GUS staining was used to determine the Mendelian characters of . In the F2 population, morphological phenotypes were found cosegregating with the mutation, namely reduced plant size and pointed first pair of true leaves. The double mutant was also crossed with wild type (also contains the reporter gene), and the segregation ratio for GUS staining in the F2 progeny showed that is not linked to . From the same F2 population, was identified using the reduced-plant-size and the pointed-first-true-leaf phenotypes of to detect homozygotes and the PCR–NlaIII digestion method to detect

homozygotes. All the genetic data were analyzed with chi-square tests for goodness of fit (Sokal and Rohlf, 1981).

Plant genomic DNA was extracted for characterization using a previously described protocol (Dellaporta et al., 1983). Total RNA preparation used for RNA blot analysis was made according to Cao et al. (1994). Poly A^+ RNA was prepared using Dynabeads (Dynal Inc., Rochester, NY).

SA Measurement

Leaf tissues were harvested from 4-week-old, soil-grown plants, ground in liquid N_2, and weighed. SA extraction was performed using a modified protocol derived from Raskin et al. (1989). Three milliliters of methanol (90%) was added to the ground tissues, and the resulting mixture was vortexed and sonicated for 20 min. After centrifugation at 7500 g for 10 min, the supernatant was collected and the pellet was reextracted with 2 ml of methanol (100%) and recentrifuged at 7500 g for another 10 min. The supernatants from both extractions were combined and air dried in a water bath (60°C). The dried samples were resuspended in 2.5 ml 5% trichloroacetic acid (TCA), vortexed, sonicated for 5 min, and centrifuged at 7500 g for 10 min. The supernatants were collected and extracted in 100/99/1 (vol) ethylacetate/cyclopentane/isopropanol by vigorous vortexing for 10 min. The top organic phase was removed and air dried in a water bath (60°C). The dried extract was resuspended in 250 μl of mobile phase (0.2 M KAc, 0.5 mM EDTA [pH 5]), vortexed, sonicated for 5 min, and spun through a 0.22 μm nylon filter (Costar, Greenwich, CT). To measure the amount of extracted SA, the samples were separated through a 100 × 4.6 sperisorb DDS2 column (Keystone Scientific Inc., Bellesonte, PA) with a particle size of 3 μm and a pore size of 80 Å at a mobile-phase flow rate of 1 ml/min. Fluorescent detection was performed on an HPLC spectrofluorescence detector equipped with a Xenon-mercury arc lamp at an excitation/emission wavelength of 295/405 nm. This procedure had a 25% recovery rate as determined by extracting known amounts of SA.

Pathogen Infection

Infection of wild-type and mutant with ES4326 and Noco2 was carried out as previously described (Bowling et al., 1994). For the ES4326 infection, four samples (eight leaves/sample) were taken for each genotype, treatment, and time point to determine the in planta growth of the bacteria, and the 95% confidence limits of the log-transformed data were calculated (Sokal and Rohlf, 1981). For the Noco2-infected plants, a disease rating was determined for each plant according to Cao et al. (1998). For each genotype and treatment, 30 plants were examined and an average disease rating calculated.

Map-Based Cloning of

The double mutant that is in the Columbia (Col) ecotype was crossed into wild type in the Landsberg ecotype (La- ; also carries the reporter gene). The F2 progeny homozygous for were identified in 2-week-old plate-grown seedlings using the morphological phenotypes associated with . The homozygosity of those progeny critical for determining the map position of was later confirmed in the F3 generation using the - specific reporter gene expression pattern. Various CAPS markers were used to survey the collected progeny to determine the chromosomal position of the locus. Among the CAPS markers used (http://genome-www.stanford.edu/arabidopsis/aboutcaps.html), g4539 and g3883-1.4 of chromosome IV were found to flank . Subsequently, a marker (ch42) mapped between g4539 and g3883-1.4 was used. Among the 718 homozygous F2 plants examined using marker ch42, no recombinants were discovered, indicating that ch42 is closely linked to . Sequence information (http://www.mips.biochem.mpg.de/proj/thal/) was then used to generate CAPS markers XL3 and XL7 on either side of ch42. XL3 was amplified using primers 5′CTGGCATCCGTGAAAC3′ and 5′GCAGGACTTGATGTATCC3′, and the polymorphism between Col and La- was detected by NdeI restriction digestion. XL7 was amplified using primers 5′CCATCCAAAGGCGATAAC3′ and 5′CCAAAC TACTACCGGATG3′ and the polymorphism revealed by DdeI digestion. The interval containing was further narrowed down to a 43.3 kb region between XL7 and XL3, with which one recombinant on the centromeric side and another on the telomeric side of were identified. This 43.3 kb region is contained in BAC F28J12. F28J12 was obtained from the Arabidopsis Biological Resource Center at the Ohio State University, and the BAC DNA was prepared according to the protocol provided with the clone. To subclone the BAC, 2 μg of the DNA was partially digested with the TaqI restriction endonuclease for 5 min at 60°C and ligated into the ClaI site of binary vector pCLD04541, which carries NPTII conferring kanamycin resistance, or pSLJ75516, which encodes the herbicide basta resistance. The ligated products were then packaged using Gigapack XL (Stratagene) and used to infect (DH5α). The resulting two libraries were probed with PCR fragments generated using the following primers: XL4 (F: 5′GTGATGGTGAGGGCTTC3′; R: 5′CGTC GGGATCTACAGG3′); XL7; ch42; XL11 (F: 5′GATGGCAATTGCTGGA G3′; R: 5′CTAATGGGATGCGACTC3′); and XL1 (F: 5′GATGAGATGT GCTGAG3′; R: 5′CATCGATTTCGCCGTTC3′). The positive clones were then analyzed by restriction digestion using XhoI and HindIII. Because the region was completely sequenced, a contig was easily constructed from both libraries by analyzing the restriction patterns of the clones. The contig made with pCLD04541 was transformed (Clough and Bent, 1998) into a line that had lost the reporter gene and was therefore kanamycin sensitive. The transformants from pCLD04541 clones were selected on MS medium containing 50 μg/ml kanamycin. The contig made with pSLJ75516 was transformed into and transformants selected by spraying 10-day-old soil-grown plants with 0.04% basta (in 0.01% Silwet L-77; Union Carbide, Danbury, CT). Complementation of was determined first by the restoration of wild-type morphology and then by the loss of inducible expression of the reporter gene or the endogenous gene. Segregation of these phenotypes in the T2 generation was monitored to distinguish true complementation from contamination by . PCR was also performed to confirm the presence of the cosmid clone in the complementing transformants using the vector-specific primers.

The 15 kb genomic region in the mutant corresponding to the insert of the complementing cosmid 4 was PCR amplified and sequenced using an ABI automated sequencer. The mutation was identified by aligning the obtained sequence with that of the wild type. In this entire 15 kb region, only one mutation (G to A) was found. The PCR primers used for sequencing were then combined to perform RT-PCR (GeneAmp Kit; Perkin Elmer, Norwalk, CT), and the cDNA sequence in the region flanking the locus was determined. The partial cDNA sequence was then used as a probe to screen a cDNA library made in λ ZAPII containing 1–3 kb inserts (Kieber et al., 1993). From the 10^6 plaques screened, one cDNA clone was isolated. The cDNA clone was shown to contain an almost full-length coding sequence, missing only 11 nucleotides of the 5′ end that were determined by RT-PCR and sequencing. A full-length cDNA clone was generated by PCR, put under the control of the constitutive 35S promoter of cauliflower mosaic virus, transformed (Clough and Bent, 1998) into , and shown to complement the mutation. By comparing the genomic and cDNA sequences, introns were identified in the gene. The G-to-A mutation in was found to be in a 3′ intron acceptor site, and the effect of this mutation on splicing was detected by RT-PCR of the mutant mRNA and sequence analysis.

Sequence data were analyzed with different programs available through the Internet (http://www.expasy.ch/).

Subcellular Localization of SNI1

The gene carried by the plasmid pRT2ΔN-mGFP (Stacey et al., 1999) was fused to either the 5′ or the 3′ end of the gene by inserting the coding sequence into the BglII and NcoI sites of the plasmid, respectively. The resulting plasmids were purified using a Qiagen Midiprep Kit (Valencia, CA), and 20 μg of DNA was bombarded into onion epidermal cells according to a protocol by von Armim and Deng (1994). GFP expression was observed using a fluorescence microscope.

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