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The Arabidopsis *NPR1* Gene That Controls Systemic Acquired Resistance Encodes a Novel Protein Containing Ankyrin Repeats

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Summary

The Arabidopsis *NPR1* gene controls the onset of systemic acquired resistance (SAR), a plant immunity, to a broad spectrum of pathogens that is normally established after a primary exposure to avirulent pathogens. Mutants with defects in *NPR1* fail to respond to various SAR-inducing treatments, displaying little expression of pathogenesis-related (*PR*) genes and exhibiting increased susceptibility to infections. *NPR1* was cloned using a map-based approach and was found to encode a novel protein containing ankyrin repeats. The lesion in one *npr1* mutant allele disrupted the ankyrin consensus sequence, suggesting that these repeats are important for *NPR1* function. Furthermore, transformation of the cloned wild-type *NPR1* gene into *npr1* mutants not only complemented the mutations, restoring the responsiveness to SAR induction with respect to *PR*-gene expression and resistance to infections, but also rendered the transgenic plants more resistant to infection by P. syringae in the absence of SAR induction.

Introduction

Plants respond in a variety of ways to pathogenic microorganisms (Lamb et al., 1989; Lamb, 1994). Systemic acquired resistance (SAR) is a defense response that can be triggered by a local hypersensitive response (HR) to an avirulent pathogen, which renders uninfected parts of the plant resistant to a variety of normally virulent pathogens (Ross, 1961a; Kuc, 1982). SAR is thought to be the consequence of the concerted activation of many genes that are often referred to as pathogenesis-related (*PR*) genes (Van Loon and Van Kammen 1970; Ward et al., 1991; Yalpani et al., 1991; Uknes et al., 1992). Certain chemicals, such as salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA), and benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH), have been shown to induce SAR when applied exogenously to plants (White, 1979; Metraux et al., 1991; Görlach et al., 1996). Several lines of evidence indicate that endogenous SA production induced after an HR is involved in the signal transduction pathway(s) coupling the HR with the onset of SAR (Malamy et al., 1990; Metraux et al., 1990; Rasmussen et al., 1991; Gaffney et al., 1993; Delaney et al., 1994). The SAR-inducing activity of the synthetic chemicals INA and BTH is probably due to the similarities between their molecular structures and that of SA.

Although SA has been shown to be a required signal molecule in SAR, the mechanism by which SA induces SAR and *PR* gene expression is unknown. In tobacco, an SA-binding protein with catalase activity has been purified (Chen et al., 1993). Chen et al. have proposed that SA functions by increasing the intracellular level of H_2O_2, which may be involved in the activation of *PR* genes and in the induction of SAR. However, attempts to induce SAR using exogenous H_2O_2 have not been successful (Bi et al., 1995; Neuenschwander et al., 1995), prompting questioning of whether catalase is directly involved in the induction of SAR.

We conducted a mutant screen in Arabidopsis aimed at identifying the regulatory genes of the SAR pathway. A transgenic Arabidopsis line was generated that contained a β-glucuronidase reporter gene (*GUS*) driven by the SA- and INA-responsive *BGL2* gene promoter, and mutants were identified that failed to activate *BGL2-GUS* expression in the presence of the SAR inducers SA and INA (Cao et al., 1994). The *npr1-1* (nonexpresser of *PR* genes) mutant exhibits almost complete lack of expression of the *BGL2-GUS* reporter gene and of the endogenous *PR1*, *BGL2*, and *PR5* genes in response to SA, INA, and avirulent pathogen treatments (Cao et al., 1994) . Further characterization of the *npr1-1* mutant showed that a single, recessive mutation in the *NPR1* gene blocks the induction of SAR. In *npr1-1* plants pretreated with SA or INA, or infected with an avirulent pathogen, subsequent growth of virulent pathogens such as the bacterial pathogen Pseudomonas syringae pv. maculicola (Psm) ES4326 (Dong et al., 1991) and the obligate biotrophic fungal pathogen Peronospora parasitica (P. parasitica) strain NOCO (Parker et al., 1993) was not inhibited as it was in the parental line carrying the wild-type *NPR1* gene. Such *npr1*-like mutants have been repeatedly isolated using different genetic screening strategies (Delaney et al., 1995; Glazebrook et al., 1996; D. F. Klessig, personal communication). Two of these mutants were identified on the basis that they were more susceptible to infection by Psm ES4326 than wild-type plants, and genetic complementation tests showed that both mutants (named *npr1-2* and *npr1-3*) are allelic to *npr1-1*. Klessig et al. have shown that a mutant they isolated, *sai1*, is also allelic to *npr1-1*.

The *NPR1* gene not only controls the onset of systemic resistance but also affects local acquired resistance (LAR), the ability of plants to restrict the spread of virulent pathogen infections (Ross, 1961b). In *npr1*-mutant plants, the virulent pathogen Psm ES4326 grows to a greater extent, spreads further beyond the initial site of

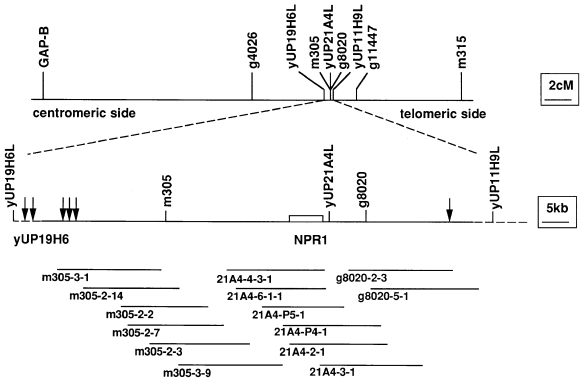

Figure 1. Map of the *NPR1* Locus on Chromosome I

Six heterozygotes were detected for marker g4026 among the 23 F_3 families that were heterozygous at GAP-B, whereas none were found among the seven F_3 families that were heterozygous at m315. Therefore, g4026 is 5.92 cM on the centromeric side of the *NPR1* gene. For marker g11447, 436 *npr1-1* F_3 families were tested and 17 heterozygotes were discovered. These heterozygotes were all homozygous Col-0 at the GAP-B locus, indicating that the g11447 marker was 1.95 cM on the telomeric side of the *NPR1* gene. No heterozygotes for marker m305 located between g4026 and g11447 were found among the 305 *npr1-1* families examined. A YAC contig covering m305 was identified and the left-end fragments of the YAC clones yUP19H6, yUP21A4, and yUP11H9 were used for further mapping. Among the GAP-B recombinants on the centromeric side of the *NPR1* gene, five were recombinant (shown as vertical arrows) at the yUP19H6L end-probe. Among the 17 recombinants for g11447 on the telomeric side of the *NPR1* gene, one was recombinant at the yUP11H9L end-probe. Since yUP11H9L hybridizes with the yUP19H6 YAC clone, these results showed that the *NPR1* gene is located on yUP19H6. The cosmid contig derived from yUP19H6 was shown to contain *NPR1* since three recombinants were found using an RFLP marker from cosmid m305-3-1 on the centromeric side of the contig and a single recombinant was detected using a RFLP marker from cosmid g8020-2-3 at the telomeric side of the contig. The 7.5 kb region shared by the complementing cosmids (21A4-4-3-1, 21A4-6-6-1, 21A4-P5-1, 21A4-P4-1, and 21A4-2-1) is indicated by a rectangular box.

invasion, and forms larger, less defined yellow lesions than in the wild-type plants (Cao et al., 1994; Glazebrook et al., 1996).

Here we report map-based cloning of the Arabidopsis *NPR1* gene. The sequence data reveal that *NPR1* encodes a novel protein containing ankyrin repeats. The mutations that abolish *NPR1* function include disruption of the ankyrin repeat consensus sequence, conversion of a cysteine to a tyrosine, and truncations of the protein.

Results

Mapping the *NPR1* Gene

The *NPR1* gene was mapped by crossing the *npr1-1*/*npr1-1* mutant, which is in the Columbia ecotype (Col-0, carrying the *BGL2-GUS* reporter gene), to the *NPR1*/*NPR1* Landsberg ecotype (La-*er*, also carrying the *BGL2-GUS* reporter gene). Initially, a rough map position for *NPR1* was obtained by assaying the F_3 *npr1-1*/*npr1-1* progeny for the genotypes of various CAPS (codominant amplified polymorphic sequences) (Konieczny and Ausubel, 1993) and RFLP (restriction fragment length polymorphism) markers. This analysis showed that the *NPR1* gene lies on chromosome I, between the CAPS marker GAP-B (22.7 cM on the centromeric side of the *NPR1* gene) and the RFLP marker m315 (7.58 cM on the telomeric side of the *NPR1* gene) (Figure 1). Subsequent fine mapping of the *NPR1* gene was carried out as described in the legend to Figure 1. The data showed that *NPR1* is very tightly linked to the RFLP marker m305. The m305 marker is contained within a YAC contig (yUP19H6, yUP21A4, and yUP11H9) generated in J. Ecker's laboratory at the University of Pennsylvania and published electronically on the World Wide Web (http://cbil.humgen.upenn.edu/~atgc/ATGCUP.html). Using YAC-end probes from this contig, the *NPR1* gene was found to be located on the YAC yUP19H6. In addition to m305, markers yUP21A4L and g8020 were also shown to be very closely linked to the *NPR1* gene, as no recombinants were identified. m305, yUP21A4L, and g8020 all hybridize to the yUP19H6 YAC clone, further supporting the conclusion that yUP19H6 contains the *NPR1* gene.

Complementation of *npr1* Mutations

A complementation testing strategy was used to delimit the position of *NPR1* within yUP19H6. A cosmid library was constructed by cloning genomic DNA from the yeast strain carrying yUP19H6 into the binary vector pCLD-04541 (Bent et al., 1994). The cosmid library was probed with markers m305, yUP21A4L, and g8020, and a single contig spanning approximately 80 kb was generated (Figure 1). From this contig, fourteen cosmids were chosen to transform *npr1* mutant plants (Bechtold et al., 1993). The *npr1-2* mutant was used as one of the recipients, because, unlike the *npr1-1* mutant, it is not kanamycin-resistant. This permitted the kanamycin resistance conferred by the neomycin phosphotransferase (*NPTII*) gene in pCLD04541 to be used for selection of transformants.

Transformants carrying each cosmid were tested for SA and INA induction of *PR1* expression by RNA blot analysis (examples are shown in Figure 2A). As expected, the wild-type line showed high levels of *PR1* gene induction by SA and INA, whereas the *npr1-2* mutant exhibited only a minor induction. Transformants of the *npr1-2* mutant containing cosmids 21A4-6-1-1, 21A4-P5-1, 21A4-4-3-1, and 21A4-2-1 showed strong induction of *PR1* by SA and INA, while those containing the other clones displayed little induction. These results demonstrated that *NPR1* lies within the 7.5 kb region that is common to the four complementing clones (Figure 1). Transformants of cosmid 21A4-P4-1 were not available when the experiment described above was conducted. However, based on its relative position, it is expected that this clone would also complement the *npr1-2* mutation.

Because the *npr1-1* plants are kanamycin-resistant, owing to the presence of the *NPTII* gene introduced with the *BGL2-GUS* reporter gene, transformants carrying the cosmid clones could not be selected using kanamycin. Instead, transformants of the *npr1-1* line carrying cosmids that complement the *npr1-1* mutation were selected directly by growing potentially transformed seeds in the presence of a high concentration of SA (0.5 mM).

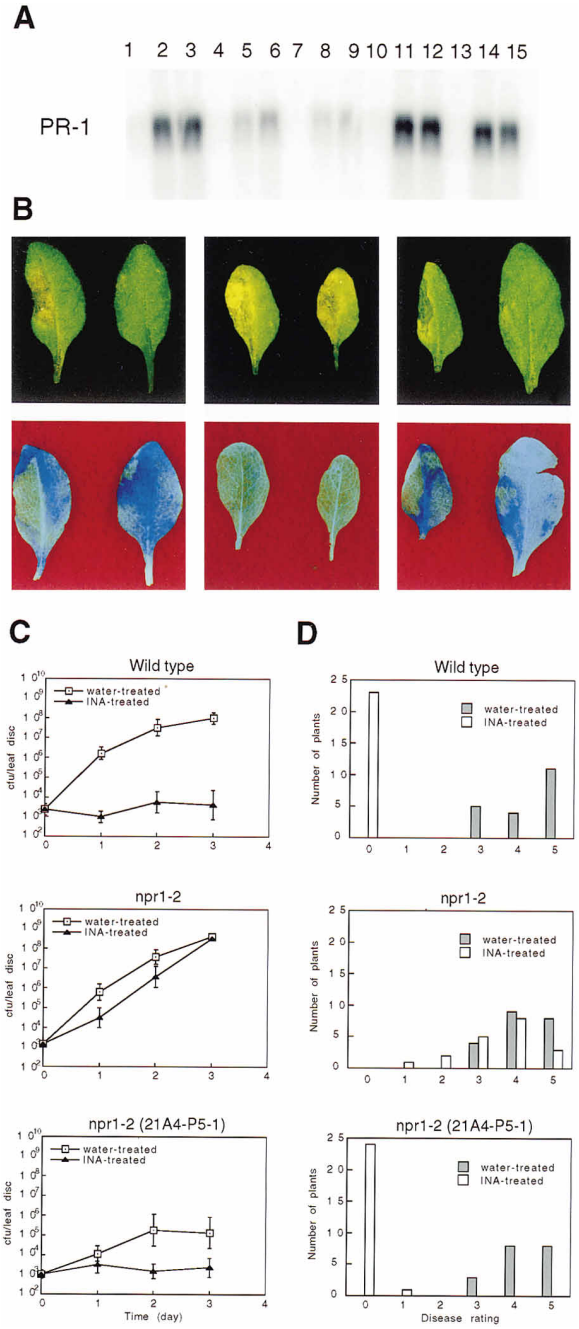

Figure 2. Phenotypic Analysis of Complementation

(A) Northern blot analysis of expression of the *PR-1* gene in wild type (Col-0, lanes 1–3), *npr1-2* (lanes 4–6), and *npr1-2* transformants with a noncomplementing cosmid (m305-2-7, lanes 7–9) and with complementing cosmids (21A4-P5-1, lanes 10–12, and 21A4-6-1-1, lanes 13–15). RNA samples were prepared from 15-day-old seedlings grown on MS media (lanes 1, 4, 7, 10, and 13), MS with 0.1 mM INA (lanes 2, 5, 8, 11, and 14), and MS with 0.1 mM SA (lanes 3, 6, 9, 12, and 15).

(B) Disease symptoms (top panels) and *BGL2-GUS* expression (bottom panels) induced by Psm ES4326 on wild type (left panels), *npr1-1* (middle panels), and an *npr1-1* transformant with a complementing cosmid (21A4-4-3-1, right panels). Psm ES4326 suspension (OD_{600} = 0.001, the leaf samples on the left; OD_{600} = 0.0001, the leaf samples on the right) was infiltrated into the left side of the leaves and symptoms were examined 3 days after infection. GUS staining was carried out according to Jefferson et al. (1987).

This selection scheme was devised based on the reduced tolerance to SA observed in *npr1* mutants; when grown on medium containing a high concentration of SA, the growth of mutant plants is arrested at the cotyledon stage and the seedlings are bleached, whereas wild-type plants grow normally under the same conditions. Those transformed *npr1-1* plants that developed green leaves in 0.5 mM SA were transplanted and complementation was confirmed by assaying the activity of the *BGL2-GUS* reporter gene (examples are shown in Figure 2B). In these experiments, *npr1-1* transformants carrying cosmids 21A4-4-3-1, 21A4-P5-1, 21A4-2-1, and 21A4-P4-1 were obtained, consistent with the idea that *NPR1* lies in the 7.5 kb genomic region defined by the complementation analysis in the *npr1-2* mutant background (Figure 1).

In addition to reduced *PR* gene expression, plants with *npr1* mutations display susceptibility to virulent pathogens even after SAR induction. These mutant phenotypes are also complemented by the cosmids described above. As shown in Figure 2B, infection by the bacterial pathogen Psm ES4326 caused visible disease symptoms three days after infection. The symptoms in the wild-type plants and the complemented *npr1-1* transformants were well confined to the site of infiltration (the left side of the leaf), whereas the lesions in the *npr1-1* plants spread significantly beyond the site of infiltration. Furthermore, when the dosage of the infecting bacteria was reduced 10-fold, severe symptoms were only observed in the *npr1-1* mutant (leaves on the right). This experiment shows that 21A4-4-3-1 complemented the enhanced susceptibility to Psm ES4326 displayed by *npr1-1*. The expression of the *BGL2-GUS* gene was analyzed in the same leaves after examination of the symptoms. Strong GUS expression (blue staining) was detected in the marginal regions of the well-confined lesions in the wild-type plants but was absent from the diffuse lesions in the *npr1-1* plants. Reporter gene expression was restored in the complemented transformants. In addition to these visual observations, bacterial growth was measured quantitatively (Figure 2C). Complete inhibition of Psm ES4326 growth was observed in the wild-type plants following an INA treatment

(C) Growth of Psm ES4326 in wild type, *npr1-2* , and an *npr1-2* transformant with a complementing cosmid (21A4-P5-1). Plants were treated with 0.65 mM INA 72 hr prior to Psm ES4326 infection (OD_{600} = 0.001). Samples were taken 0, 1, 2, and 3 days after infection. Error bars represent 95% confidence limits of log-transformed data (Sokal and Rohlf, 1981). Six to eight samples were taken for each time point. cfu, colony forming units.

(D) Disease rating of P. parasitica NOCO infection in wild type, *npr1-2,* and an *npr1-2* transformant with a complementing cosmid (21A4-P5-1). INA treatment (0.65 mM) was carried out 72 hr 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 plant (20 to 25 plants were examined for each genotype with each treatment). The scales are defined as follows: 0, no conidiophores on the plant; 1, no more than 5 conidiophores per infected leaf; 2, 6–20 conidiophores on a few infected leaves; 3, 6–20 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. The data were analyzed using Mann-Whitney U tests (Sokal and Rohlf, 1981).

```
1    MDTTIDGFADSYEISSTSFVATDNTDSSIVYLAAEQVLTG
41   PDVSALQLLSNSFESVFDSPDDFYSDAKLVLSDGREVSFH
81   RCVLSARSSFFKSALAAAKKEKDSNNTAAVKLELKEIAKD
121  YEVGFDSVVTVLAYVYSSRVRPPPKGVSECADENCCHVAC
161  RPAVDFMLEVLYLAFIFKIPELITLYQRHLLDVVDKVVIE
201  DTLVILKLANICGKACMKLLDRCKEIIVKSNVDMVSLEKS
241  LPEELVKEIIDRRKELGLEVPKVKKHVSNVHKALDSDDIE
281  LVKLLLKEDHTNLDDACALHFAVAYCNVKTATDLLKLDLA
321  DVNHRNPRGYTVLHVAAMRKEPQLILSLLEKGASASEATL
361  EGRTALMIAKQATMAVECNNIPEQCKHSLKGRLCVEILEQ
401  EDKREQIPRDVPPSFAVAADELKMTLLDLENRVALAQRLF
441  PTEAQAAMEIAEMKGTCEFIVTSLEPDRLTGTKRTSPGVK
481  IAPFRILEEHQSRLKALSKTVELGKRFFPRCSAVLDQIMN
521  CEDLTQLACGEDDTAEKRLQKKQRYMEIQETLKKAFSEDN
561  LELGNSSLTDSTSSTSKSTGGKRSNRKLSHRRR*
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Figure 3. Deduced Amino Acid Sequence of NPR1
The ankyrin repeats are underlined.

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A
NPR1 (323)  NHRNPRGYTVLHVAAMRKEPQLILSLLEKGASASEATLEGRTALMIAKQ
            N +   GYT LH AA +    +I  LL+  AS +E T+ G TAL IA++
ANK3 (740)  NAKTKNGYTALHQAAQQGHTHIINVLLQNNASPNELTVNGNTALAIARR

NPR1 (262)  KVKKHVSNVHKALDSDDIELVKLLLKED
            K K  +S +H A   D +  V+LLL+ +
ANK3 (313)  KTKNGLSPLHMATQGDHLNCVQLLLSRN

B
1st repeat  (265)  KHVSNVHKALDSDDIELVKLLLKEDHTNLDDAC  (297)
2nd repeat  (294)  DDACALHFAVAYCNVKTATDLLKLDLADVNHRN  (326)
3rd repeat  (328)  RGYTVLHVAAMRKEPQLILSLLEKGASASEATL  (360)
4th repeat  (361)  EGRTALMIAKQATMAVECNNIPEQCKHSLKGRL  (393)

ANK consensus

(Michaely and       G TPLHLAAR GHVEVVKLLLD GADVNA TK
 Bennett)              A  I SQ NNLDIAEV  K   NPD  D
                          V  K    T M R   Q   SI   N
                                        E

(Bork)              t otLHhAh  tt thht LLt t t
```

Figure 4. Sequence Similarities of NPR1 to Ankyrin

(A) Alignment of NPR1 amino acid sequence with mouse ankyrin 3 (ANK3). Two regions producing the highest scoring pairs (smallest sum probability = 0.0004) generated using a BLAST search are shown. The sequence identities between NPR1 (amino acids 323–371 and 262–289) and ANK3 (amino acids 740–788, 313–340) are 42% and 35%, and the sequence similarities are 59% and 57%. The identical and the similar amino acids (+) are highlighted in bold letters.

(B) Alignment of the ankyrin repeats in NPR1 with the ankyrin repeat consensus derived by Michaely and Bennett (1992) and by Bork (1993). Since there are a few nonoverlapping amino acids between the two derived consensus sequences, both are presented. In the consensus derived by Bork, the conserved features are indicated: t, turn-like or polar; o, S/T; h, hydrophobic; capitals, conserved amino acids. Those amino acids identical to the consensus are highlighted in bold letters.

three days prior to infection, whereas at most only a 10-fold decrease in Psm ES4326 growth was observed in the *npr1-2* mutant subjected to the same treatment. The growth of Psm ES4326 was also completely halted in the complemented transformants after the INA treatment. Interestingly, lower bacterial growth (as much as 10^3-fold) was observed even in the water-treated transformants, as compared to the water-treated wild type (Figure 2C) and the water-treated transformants carrying noncomplementing cosmids (data not shown). Such enhanced resistance could result from the increased *NPR1* mRNA levels in these complemented transformants (data not shown). Similarly, a test of resistance to a fungal pathogen, P. parasitica NOCO, was performed to verify complementation of the *npr1* mutation. Conidiophore formation was scored to assess the severity of the infection. As shown in Figure 2D, INA-induced resistance to P. parasitica NOCO was restored in the transformants with the complementing cosmids.

Isolation of the *NPR1* cDNA and Characterization of the *NPR1* Gene

DNA fragments covering the 7.5 kb region shared by the complementing cosmids were sequenced and used to detect transcripts on a blot containing poly(A)+ RNAs isolated from wild-type plants and *npr1-1*, *npr1-2*, and *npr1-3* mutants after treatment with H_2O or 0.65 mM INA and 2 mM SA. Only one ~2 kb transcript was detected by probes made from either a 1.96 kb HindIII or a 0.5 kb HindIII fragment. The intensity of the transcript was approximately 2-fold greater in the INA/SA-induced samples than in the H_2O-treated controls. Thus, the expression of this transcript, which was subsequently proven to be the *NPR1* transcript, is induced by INA/SA treatment. No significant differences in the induction patterns of this transcript were detected among the wild-type or any of the three *npr1* mutants (data not shown).

To isolate an *NPR1* cDNA clone, a cDNA library was screened using a probe made from the 1.96 kb HindIII fragment. One clone, designated pKExNPR1, was isolated, and the 2.1 kb cDNA insert was shown to contain a full-length coding region (Figure 3) by DNA sequencing. Comparison of the cDNA sequence and the genomic sequence revealed that there are four exons and three introns in the *NPR1* gene.

A BLAST search of GenBank showed that the *NPR1* gene encodes a novel protein containing two regions with significant homology to ankyrin in the ankyrin repeats, the highest scoring segment pair being with the mammalian *ankyrin 3* gene (Figure 4A) (Peters et al., 1995). The ankyrin repeat consensus has been identified in a diverse group of proteins involved in cell structure, transcription regulation, cell differentiation, and enzymatic and toxic activities (Michaely and Bennett, 1992; Bork, 1993). These repeats, which occur in at least four consecutive copies, have been found to fold cooperatively and to be involved in protein–protein interactions. Using the consensus sequence defined by Michaely and Bennett, and the one defined by Bork, two additional ankyrin repeats were identified in NPR1 (Figure 4B).

To further characterize the *NPR1* gene, the mutations in *npr1-1*, *npr1-2*, *npr1-3*, and *npr1-4* were identified by DNA sequencing. The mutant *npr1-4* is a new *npr1* allele that we identified in the Col-0 (*BGL2-GUS*) background (Cao et al., 1994) based on its enhanced susceptibility to Psm ES4326. Each mutant allele was found to contain a single base-pair change. The *npr1-1*, *npr1-2*, *npr1-3*, and *npr1-4* alleles, respectively, altered the highly conserved histidine (residue 334) in the third ankyrin-repeat consensus sequence to a tyrosine, changed a cysteine (residue 150) to a tyrosine, introduced a nonsense codon (residue 400) that should result in a truncated protein

lacking 194 amino acids of the C-terminal end of the protein, and destroyed the acceptor site of the third intron junction. All of these point mutations are GC-to-AT transitions, consistent with the mode of action of the mutagen—ethyl-methanesulfonate (EMS)—used for the generation of these mutants.

Discussion

In the transgenic *npr1* lines that contain complementing cosmids, all *npr1* mutant phenotypes were restored to the wild type (Figure 2). Intriguingly, expression of the wild-type *NPR1* gene in the mutant background rendered the transgenic plants more resistant to Psm ES4326 infection than the wild type in the absence of SAR induction. These results demonstrate that the *npr1* phenotypes are caused by mutations in a single gene and that NPR1 is a positive regulator of acquired resistance responses.

Analysis of genomic sequences of four mutant *npr1* alleles not only confirmed the identity of the *NPR1* gene but also highlighted some of the regions and amino acids in the protein that are critical to the protein function. The *npr1-2* mutation converts a cysteine residue to a tyrosine, suggesting that some of the 17 cysteine residues in NPR1 may participate in intramolecular or intermolecular disulfide bonds that are important for the activity of the protein. However, the effect of this mutation is incomplete, as demonstrated by the residual inducible *PR1* expression observed in the *npr1-2* mutant (Figure 2A). The *npr1-3* and *npr1-4* mutations cause a premature stop codon and a splice site disruption, respectively, indicating that the C-terminal region of NPR1 is important for its function. The most intriguing mutation discovered is the single mutation in the *npr1-1* allele that alters the highly conserved histidine residue in the third ankyrin-repeat consensus to a tyrosine (Figure 4B). A study based on pattern searches and systematic database screening has discovered hundreds of ankyrin repeats in eukaryotic, prokaryotic, and viral proteins (Bork, 1993). Though proteins that contain these repeats have diverse functions, in many cases these repeats have been shown to be involved in protein–protein interactions. The crystal structure of the ankyrin repeats contained in 53BP2, a protein that binds to the tumor suppressor p53, has been examined and shown to form an L-shaped structure, with β hairpins as the bottom of the letter L, followed by two anti-parallel α helices as the top. The conserved histidine in the consensus has been shown to reside in the first α helix and to play a critical role in the folding of the L-shaped repeats by making a pair of hydrogen bonds to the β-hairpin backbone of the next repeat (Gorina and Pavletich, 1996). Therefore, it is probable that the histidine-to-tyrosine change in the third ankyrin repeat in *npr1-1* causes destablization of the protein.

Some well-studied ankyrin repeat–containing proteins are transcription-regulating proteins, including the NF-κB precursor p105 and the inhibitor I-κB. The nuclear translocation of the NF-κB complex is induced by a large array of agents including bacterial and viral pathogens, immune and inflammatory cytokines, UV radiation, and oxidants such as H_2O_2 (Thanos and Maniatis, 1995). This translocation has also been shown to be inhibited by sodium salicylate and aspirin. These anti-inflammatory drugs act by blocking the degradation of the NF-κB inhibitor, I-κB, causing retention of the transcription factor in the cytosol (Kopp and Ghosh, 1994). Speculation that an NF-κB-like regulatory mechanism is involved in SA signaling to turn on SAR was prompted by comparisons of the SAR response in plants to animal immune responses, which revealed superficially parallel pathways (Chen et al., 1993). It was proposed that elevation of the endogenous level of SA after an HR would lead to an increase in the intracellular level of reactive oxygen species, such as H_2O_2, through the inhibition of a catalase by SA, and that reactive oxygen species may be involved in the activation of *PR* genes and in the induction of SAR through the function of NF-κB-like proteins. Is NPR1 an ortholog of NF-κB or I-κB in plants? Though there has been no evidence indicating that NPR1 is a transcription factor, NPR1 may affect the expression of *PR* genes indirectly by positively regulating the nuclear localization or the activity of a transcription factor through protein-protein interaction mediated by the ankyrin repeat motifs.

Is NPR1 an SA receptor? The activity of NPR1 may be regulated either by direct binding of SA or by an indirect effect of elevation in the endogenous level of SA, such as a change in the intracellular redox state resulting from the inhibitory effect of SA on catalase. This idea is consistent with the observation that *npr1* mutants are sensitive to elevated SA concentrations in the growth media. Presumably, wild-type plants have a mechanism to ameliorate the toxic effects of SA, either by a detoxification mechanism or by feedback regulation of SA synthesis. Some such protective mechanism must be disabled in *npr1* mutants, resulting in the SA-sensitive phenotype. If NPR1 is a receptor of SA, the SA-sensitive phenotype of the *npr1* mutants is easily explained by postulating that *npr1* mutants are unable to detect elevated SA levels and therefore do not respond appropriately. However, NPR1 cannot be the only physiologically relevant SA receptor in Arabidopsis because *NPR1* mRNA levels still increase in *npr1* mutants in response to SA and INA treatment. Alternatively, if NPR1 acts downstream of SA recognition, NPR1 activity must lead to induction of both defense genes and genes involved in adaptation to high levels of SA.

The proposed roles for NPR1 as a transcription regulator or an SA receptor are not mutually exclusive. NPR1 could function as a transcription regulator whose activity is affected by the binding of SA. In the induced state, NPR1 could activate a transcription factor to translocate to the nucleus, induce expression of *PR* genes, and down-regulate the SA level, thereby reducing the toxic effects of SA. Through a different mechanism, the expression of the *NPR1* gene itself is also up-regulated by SA, amplifying the response to the signal. Future studies of NPR1 will focus on testing these hypotheses.

Experimental Procedures

Markers Used in Mapping of the *NPR1* Gene

Cosmids g4026 and g8020 and YAC-ends yUP19H6L, yUP11H9L, and yUP21A4L were used to detect polymorphisms between Col-0

and La-*er*. PCR-based CAPS markers were generated from RFLP markers g11447 and m305. Detailed information about these markers will be provided upon request.

DNA and RNA Analysis

Plant genomic DNA samples were isolated from frozen leaf tissues and used in the PCR reactions and Southern blot analysis following standard procedures (Dellaporta et al., 1983; Ausubel et al., 1993). Blot analysis of total RNA and transcript quantitation were carried out according to procedures described previously (Cao et al., 1994). The *PR1* probe was prepared using a PCR product amplified from Arabidopsis genomic DNA using *PR1*-specific primers (Glazebrook et al., 1996).

Construction of the Cosmid Contig Spanning *NPR1*

Genomic DNA was prepared from a yeast strain containing the YAC clone yUP19H6, partially digested with the restriction enzyme TaqI, size-selected on a 10%–40% sucrose gradient, and cloned into the ClaI site of the binary vector pCLD04541 (Bent et al., 1994). The cosmid clones were packaged into bacteriophage lambda particles using a commercial packaging extract (Gigapack XL, Stratagene), and introduced into E. coli strain DH5α (BRL) according to the instructions of the supplier. The library was probed with markers m305, yUP21A4L, and g8020. DNA preparations from the positive clones were analyzed by HindIII restriction digestion and Southern hybridization using the probes described above.

Isolation of the *NPR1* cDNA Clone

A cDNA library constructed by Dr. F. Katagiri (Mindrinos et al., 1994) was plated (60,000 cfu/plate) on LB medium, and the plates were incubated at 37°C for 4.5 hr. Colonies were lifted onto Colony/Plaque Screen membranes (NEN Research Product) and then the membranes were placed on an LB plate, with the colony side up. Both plates were incubated at 30°C for 12 hr. Hybridization was carried out with a probe made from the 1.96 kb HindIII fragment (Ausubel et al., 1993).

Infection with Bacterial and Fungal Pathogens

The infections of Arabidopsis by the bacterial pathogen Psm ES4326 and the fungal pathogen P. parasitica strain NOCO were performed as previously described (Bowling et al., 1994; Cao et al., 1994; Glazebrook et al., 1996).

DNA Sequence Analysis

Sequencing of the 7.5 kb genomic region was carried out using pBluescript SK+ clones of the HindIII fragments as templates, and for the *NPR1* cDNA the pKExNPR1 plasmid was used. To analyze the mutant alleles, PCR products ($\sim$400 bp) were generated using the enzyme AmpliTaq Gold (Perkin-Elmer), and sequenced directly using an ABI automated sequencer. The sequence data were analyzed using the software DNA Strider, MacVector 4.0.1, and GeneFinder. Sequence data were also submitted for a BLAST search of GenBank and compared to the TIGR AtDB.

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