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The Arabidopsis *NIM1* Protein Shows Homology to the Mammalian Transcription Factor Inhibitor IκB

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The *NIM1* (for noninducible immunity) gene product is involved in the signal transduction cascade leading to both systemic acquired resistance (SAR) and gene-for-gene disease resistance in Arabidopsis. We have isolated and characterized five new alleles of *nim1* that show a range of phenotypes from weakly impaired in chemically induced pathogenesis-related protein-1 gene expression and fungal resistance to very strongly blocked. We have isolated the *NIM1* gene by using a map-based cloning procedure. Interestingly, the NIM1 protein shows sequence homology to the mammalian signal transduction factor IκB subclass α. NF-κB/IκB signaling pathways are implicated in disease resistance responses in a range of organisms from Drosophila to mammals, suggesting that the SAR signaling pathway in plants is representative of an ancient and ubiquitous defense mechanism in higher organisms.

INTRODUCTION

Systemic acquired resistance (SAR) is one component of the complex system that plants use to defend themselves from pathogens (Hunt and Ryals, 1996; Ryals et al., 1996). SAR is a particularly important aspect of plant–pathogen responses because it is a pathogen-inducible, systemic resistance against a broad spectrum of infectious agents, including viruses, bacteria, and fungi. When the SAR signal transduction pathway is blocked, plants become more susceptible to pathogens that normally cause disease, and they also become susceptible to some infectious agents that normally would not cause disease (Delaney et al., 1994, 1995; Gaffney et al., 1993; Cao et al., 1994; Bi et al., 1995; Mauch-Mani and Slusarenko, 1996; Delaney, 1997). These observations indicate that the SAR signal transduction pathway is critical for maintaining plant health.

Little is known about the steps that lead to the activation of SAR. Conceptually, the response can be divided into two phases. In the initiation phase, a pathogen infection is recognized, and a signal is released that travels through the phloem to distant tissues. This systemic signal is perceived by target cells, which react by expressing both SAR genes and disease resistance. The maintenance phase of SAR refers to the period of time, from weeks up to the entire life of the plant, during which the plant is in a quasi steady state and disease resistance is maintained (Ryals et al., 1996).

Salicylic acid (SA) accumulation appears to be required for SAR signal transduction. Plants that cannot accumulate SA due to treatment with specific inhibitors, epigenetic repression of phenylalanine ammonia–lyase, or transgenic expression of salicylate hydroxylase, which specifically degrades SA, also cannot induce either SAR gene expression or disease resistance (Gaffney et al., 1993; Delaney et al., 1994; Maher et al., 1994; Mauch-Mani and Slusarenko, 1996; Pallas et al., 1996). Although it has been suggested that SA might serve as the systemic signal, this is currently controversial, and to date, all we know for certain is that if SA cannot accumulate, then SAR signal transduction is blocked (Vernooij et al., 1994; Shulaev et al., 1995; Pallas et al., 1996).

Recently, Arabidopsis has emerged as a model system for studying SAR (Uknes et al., 1992, 1993; Cameron et al., 1994; Mauch-Mani and Slusarenko, 1994; Dempsey and Klessig, 1995; Delaney, 1997). It has been demonstrated that SAR can be activated in Arabidopsis by both pathogens and chemicals, such as SA, 2,6-dichloroisonicotinic acid (INA), and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Uknes et al., 1992; Vernooij et al., 1995; Lawton et al., 1996). After treatment with either INA or BTH or pathogen infection, at least three pathogenesis-related (PR) protein genes, namely, *PR-1*, *PR-2*, and *PR-5*, are coordinately induced concomitant with the onset of resistance (Uknes et al., 1992, 1993).

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A number of Arabidopsis mutants have been isolated that have modified SAR signal transduction (Delaney, 1997). The first of these mutants were the so-called *lsd* (for lesions simulating disease) and *acd2* (for accelerated cell death) (Dietrich et al., 1994; Greenberg et al., 1994) mutants. These mutants all have some degree of spontaneous necrotic lesion formation on their leaves, elevated levels of SA, mRNA accumulation for the SAR genes, and significantly enhanced disease resistance. At least seven different *lsd* mutants have been isolated and characterized (Dietrich et al., 1994; Weymann et al., 1995). Another interesting class of mutants is *cim* (for constitutive immunity) mutants (Lawton et al., 1993). Like *lsd* and *acd2* mutants, these mutants have elevated SA and SAR gene expression and resistance; however, in contrast to *lsd* or *acd2*, they do not display detectable lesions on their leaves. *cpr1* (for constitutive expresser of PR genes) may be a type of *cim* mutant; however, because the presence of microscopic lesions on the leaves of *cpr1* has not been ruled out, *cpr1* might be a type of *lsd* mutant (Bowling et al., 1994).

Mutants have also been isolated that are blocked in SAR signaling. *ndr1* (for non-race-specific disease resistance) is a mutant that allows growth of both *Pseudomonas syringae* containing various avirulence genes and also normally avirulent isolates of *Peronospora parasitica* (i.e., causal agent of downy mildew disease) (Century et al., 1995). Apparently, this mutant is blocked early in SAR signaling. *npr1* (for nonexpresser of PR genes) is a mutant that cannot induce expression of the SAR signaling pathway after INA treatment (Cao et al., 1994). *eds* (for enhanced disease susceptibility) mutants were isolated based on their ability to support bacterial infection after inoculation of a low bacterial concentration (Glazebrook et al., 1996; Parker et al., 1996). Certain *eds* mutants are phenotypically very similar to *npr1*, and recently, *eds5* and *eds53* have been shown to be allelic to *npr1* (Glazebrook et al., 1996). Previously, we isolated a mutant called *nim1* (for noninducible immunity) that would support *P. parasitica* growth after INA treatment (Delaney et al., 1995). Although *nim1* can accumulate SA after pathogen infection, it cannot induce SAR gene expression or disease resistance, suggesting that the mutation blocks the pathway downstream of SA. *nim1* is also impaired in its ability to respond to INA or BTH, suggesting that the block exists downstream of the action of these chemicals (Delaney et al., 1995; Lawton et al., 1996).

In this study, we report on the isolation of five new mutants of the *NIM1* gene. The alleles appear to fall into three classes of relative strength with respect to their ability to block signal transduction through the *NIM1* pathway. To understand better the function of NIM1 in SAR signal transduction, we isolated the gene by using a map-based approach. Interestingly, the NIM1 protein shares significant homology to the IκBα subclass of transcription inhibitors, suggesting that NIM1 may interact with an NF-κB–related transcription factor to induce SAR gene expression and trigger disease resistance.

RESULTS

Characterization of Additional *nim1* Mutants

Previously, we reported the isolation and characterization of an Arabidopsis mutant, *nim1-1*, from 80,000 plants of a T-DNA–tagged Arabidopsis ecotype Wassilewskija (Ws-0) population (Delaney et al., 1995). This mutant was isolated by spraying 2-week-old plants with 0.33 mM INA followed by inoculation with *P. parasitica*. Plants that supported fungal growth after INA treatment were selected as putative mutants. Along with the isolation of *nim1-1*, we reported the isolation of >70 other putative Nim⁻ mutants by screening 280,000 M_2 plants from an ethyl methanesulfonate–mutagenized Ws-0 population. After progeny testing, we selected five of these putative mutants for further characterization. To determine whether the mutants were dominant or recessive, we used Ws-0 plants as pollen donors to cross to each of the mutants later designated *nim1-2* through *nim1-6*. The F_1 plants were then scored for their ability to support fungal growth after INA treatment. As shown in Table 1, for *nim1-2*, *nim1-3*, *nim1-4*, and *nim1-6*, all F_1 plants were phenotypically wild type, indicating a recessive mutation in each line. Interestingly, *nim1-5* showed the Nim1⁻ phenotype in all 35 F_1 plants, indicating that this particular mutant is dominant. For verification, the reciprocal cross was conducted using *nim1-5* as the pollen donor to fertilize Ws-0 plants. In this case, all 18 F_1 plants were phenotypically Nim⁻, confirming the dominance of the *nim1-5* mutation.

To determine whether the mutations were allelic to the previously characterized *nim1-1* mutation, pollen from *nim1-1*

Table 1. Genetic Segregation of *nim* Mutants

Mutant	Generation	Female	Male	Phenotype: Wild Type[a]	Phenotype: *nim1*[b]
nim1-1[c]	F_1	Wild type[d]	*nim1-1*	24	0
nim1-2	F_1	*nim1-2*	Wild type	3	0
nim1-3	F_1	*nim1-3*	Wild type	3	0
nim1-4	F_1	*nim1-4*	Wild type	3	0
nim1-5	F_1	*nim1-5*	Wild type	0	35
	F_1	Wild type	*nim1-5*	0	18
nim1-6	F_1	*nim1-6*	Wild type	3	0
nim1-2	F_1	*nim1-2*	*nim1-1*	0	15
nim1-3	F_1	*nim1-3*	*nim1-1*	0	10
nim1-4	F_1	*nim1-4*	*nim1-1*	0	15
nim1-5	F_1	*nim1-5*	*nim1-1*	0	14
nim1-6	F_1	*nim1-6*	*nim1-1*	0	12

[a] Number of plants with elevated PR-1 mRNA accumulation and absence of *P. parasitica* after INA treatment.

[b] Number of plants with no PR-1 mRNA accumulation and presence of *P. parasitica* after INA treatment.

[c] Data from Delaney et al. (1995).

[d] Wild type denotes the wild-type Ws-0 strain.

was used to fertilize *nim1-2* through *nim1-6*. Because *nim1-1* carried resistance to kanamycin, F_1 progeny were identified by antibiotic resistance. In all cases, the kanamycin-resistant F_1 plants were Nim^-, indicating that they are all allelic to the *nim1-1* mutant. Because the *nim1-5* mutant is dominant and apparently homozygous for the mutation, it was necessary to analyze *nim1-1* complementation in the F_2 generation. If *nim1-1* and *nim1-5* were allelic, then the expectation would be that all F_2 plants have a Nim^- phenotype. If not, then 13 of 16 F_2 plants would have been expected to have a Nim^- phenotype. Of 94 plants, 88 clearly supported fungal growth after INA treatment. Six plants showed an associated phenotype of black specks on the leaves, reminiscent of a lesion mimic phenotype, and supported little fungal growth after INA treatment. Further genetic analysis is necessary to understand whether the "black speck" phenotype is somehow modifying *nim1*. Because *nim1-5* carries a point mutation in the *NIM1* gene (data presented in a later section), we consider it to be a *nim1* allele.

To determine the relative strength of the different *nim1* alleles, each mutant was analyzed for the growth of *P. parasitica* under normal growth conditions and after pretreatment with SA, INA, or BTH. As shown in Figure 1, during normal growth, *nim1-1*, *nim1-2*, *nim1-3*, *nim1-4*, and *nim1-6* all seemed to more or less support the same rate of fungal growth, which was somewhat faster than the rate with the Ws-0 control. The exception was the *nim1-5* plants in which fungal growth was delayed by several days relative to both the other *nim1* mutants and the Ws-0 control, but eventually all of the *nim1-5* plants succumbed to the fungus. After SA treatment, the mutants could be grouped into three classes: *nim1-4* and *nim1-6* showed a relatively rapid fungal growth; *nim1-1*, *nim1-2*, and *nim1-3* plants exhibited a somewhat slower rate of fungal growth; and fungal growth in *nim1-5* plants was even slower than in the untreated Ws-0 controls. After either INA or BTH treatment, the mutants also seemed to fall into three classes: *nim1-4* was the most severely compromised in its ability to restrict fungal growth after chemical treatment; *nim1-1*, *nim1-2*, *nim1-3*, and *nim1-6* were all moderately compromised; and *nim1-5* was only slightly compromised. In these experiments, Ws-0 did not support fungal growth after INA or BTH treatment. Thus, with respect to inhibition of fungal growth after chemical treatment, the mutants fall into three classes, with *nim1-4* being the most severely compromised, *nim1-1*, *nim1-2*, *nim1-3*, and *nim1-6* showing an intermediate inhibition of fungus, and *nim1-5* being only slightly impaired in fungal resistance.

The accumulation of *PR-1* mRNA was also used as a criterion to characterize the different *nim1* alleles. RNA was extracted from plants 3 days after either water or chemical treatment or 14 days after inoculation with a compatible fungus (*P. parasitica* isolate Emwa). The RNA gel blot in Figure 2 shows that *PR-1* mRNA accumulated to high levels after treatment of wild-type plants with SA, INA, or BTH or infection by *P. parasitica*. In the *nim1-1*, *nim1-2*, and *nim1-3* plants, *PR-1* mRNA accumulation was dramatically reduced

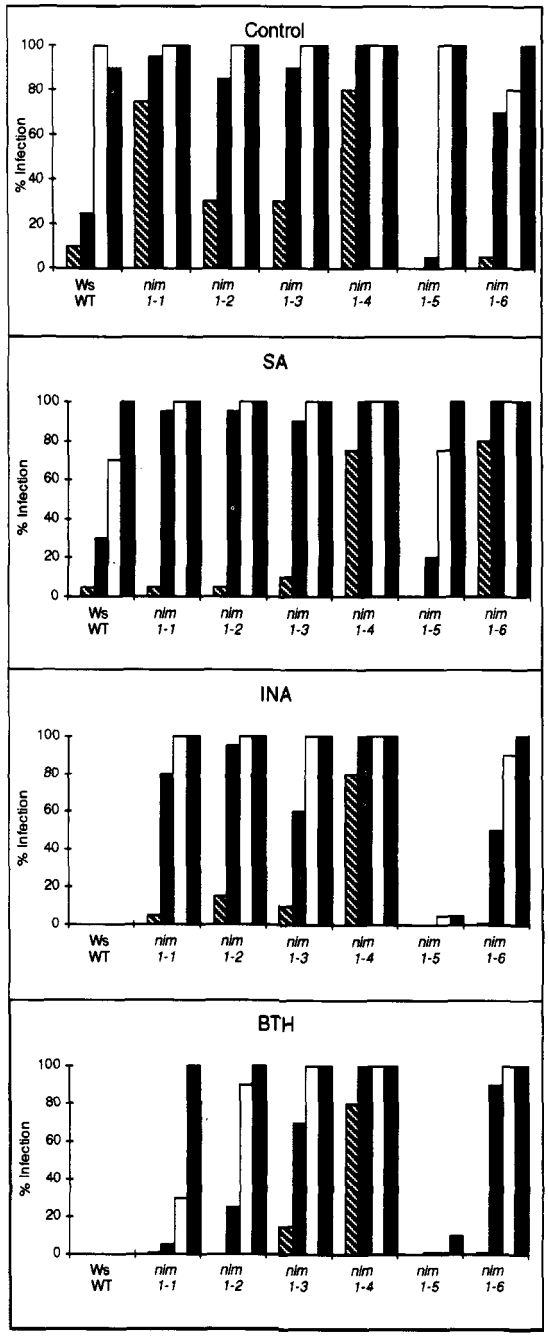

Figure 1. Fungal Infection of *nim1* Alleles.

P. parasitica was inoculated onto plants 3 days after water or chemical treatment. Plants were scored for infection 5 days (striped bars), 6 days (stippled bars), 7 days (open bars), and 11 days (solid bars) after inoculation. The percentage of infection is determined as the percentage of plants in each experiment showing some surface conidia on at least one leaf. Ws WT, Ws-0 wild type.

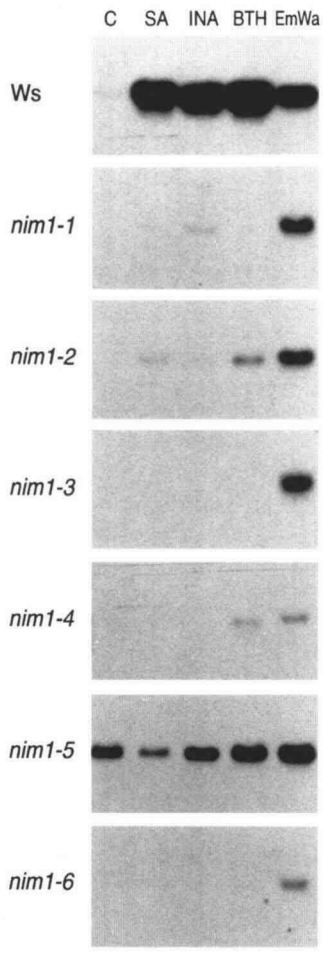

Figure 2. Accumulation of *PR-1* mRNA after Pathogen Infection or Chemical Treatment.

Plants containing various *nim1* alleles and Ws-0 (Ws) were treated with water (C), SA, INA, or BTH 3 days before RNA isolation. The EmWa sample consists of RNA isolated from plants 14 days postinoculation with *P. parasitica* isolate Emwa. Blots were hybridized using an Arabidopsis *PR-1* cDNA as a probe (Uknes et al., 1992).

relative to the wild type after chemical treatment. *PR-1* mRNA was also reduced after *P. parasitica* infection, but there was still some accumulation in these mutants. In the *nim1-4* and *nim1-6* plants, *PR-1* mRNA accumulation was more dramatically reduced than in the other alleles after chemical treatment (evident in longer exposures), and significantly less *PR-1* mRNA accumulated after *P. parasitica* infection, supporting the idea that these could be particularly strong *nim1* alleles. Interestingly, *PR-1* mRNA accumulation was elevated in the *nim1-5* mutant but was only mildly induced after chemical treatment or *P. parasitica* infection. Based on both *PR-1* mRNA accumulation and fungal infection, the mutants fall into three classes: severely compromised alleles (*nim1-4* and *nim1-6*), moderately compromised alleles (*nim1-1*, *nim1-2*, and *nim1-3*), and a weakly compromised allele (*nim1-5*).

Fine Structure Mapping of the *nim1* Mutation

To determine a rough map position for *NIM1*, 74 F_2 Nim$^-$ plants from a cross between *nim1-1* (Ws-0) and Landsberg *erecta* (L*er*) were identified for their susceptibility to *P. parasitica* and lack of accumulation of *PR-1* mRNA after INA treatment. After testing a number of simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994), *nim1-1* was found to lie ~8.2 centimorgans (cM) from nga128 and 8.2 cM from nga111 on the lower arm of chromosome 1. In a subsequent analysis, *nim1-1* was found to lie between nga111 and ~4 cM from the SSLP marker ATHGENEA.

For fine structure mapping, 1138 Nim$^-$ plants from an F_2 population derived from a cross between *nim1-1* and L*er* DP23 were identified based on both their inability to accumulate *PR-1* mRNA and their ability to support fungal growth after INA treatment. DNA was extracted from these plants and scored for zygosity at both ATHGENEA and nga111. As shown in Figure 3A, 93 recombinant chromosomes were identified between ATHGENEA and *nim1-1*, giving a genetic distance of ~4.1 cM (93 of 2276), and 239 recombinant chromosomes were identified between nga111 and *nim1-1*, indicating a genetic distance of ~10.5 cM (239 of 2276). Informative recombinants in the ATHGENEA to nga111 interval were further analyzed using amplified fragment length polymorphism (AFLP) analysis (Vos et al., 1995).

Initially, 10 AFLP markers between ATHGENEA and nga111 were identified, and these were used to construct a low-resolution map of the region (Figures 3A and 3B). The AFLP markers W84.2 (1 cM from *nim1-1*) and W85.1 (0.6 cM from *nim1-1*) were used to isolate yeast artificial chromosome (YAC) clones from the CIC (for Centre d'Etude du Polymorphisme Humain, INRA and CNRS) library (Creusot et al., 1995). Two YAC clones, CIC12H07 and CIC12F04, were identified with W84.2, and two YAC clones, CIC7E03 and CIC10G07 (data not shown), were identified with the W85.1 marker (Figure 3B). However, it was determined that there was a gap between the two sets of flanking YAC clones. From this point, bacterial artificial chromosome (BAC) and P1 clones that overlapped CIC12H07 and CIC12F04 were isolated and mapped, and three sequential walking steps were then performed extending the BAC/P1 contig toward *NIM1* (Figure 3C; Choi et al., 1995; Liu et al., 1995). At various times during the walk, new AFLPs were developed that were specific for BAC or P1 clones, and these were used to determine whether the *NIM1* gene had been crossed. It was determined that *NIM1* had been crossed when BAC and P1 clones were isolated that gave rise to both AFLP markers L84.6a and L84.8. The AFLP marker L84.6a found on P1 clones P1-18, P1-17, and P1-21 identified three recombinants, and L84.8 found on P1 clones P1-20, P1-22, P1-23, and P1-24 and BAC clones BAC-04,

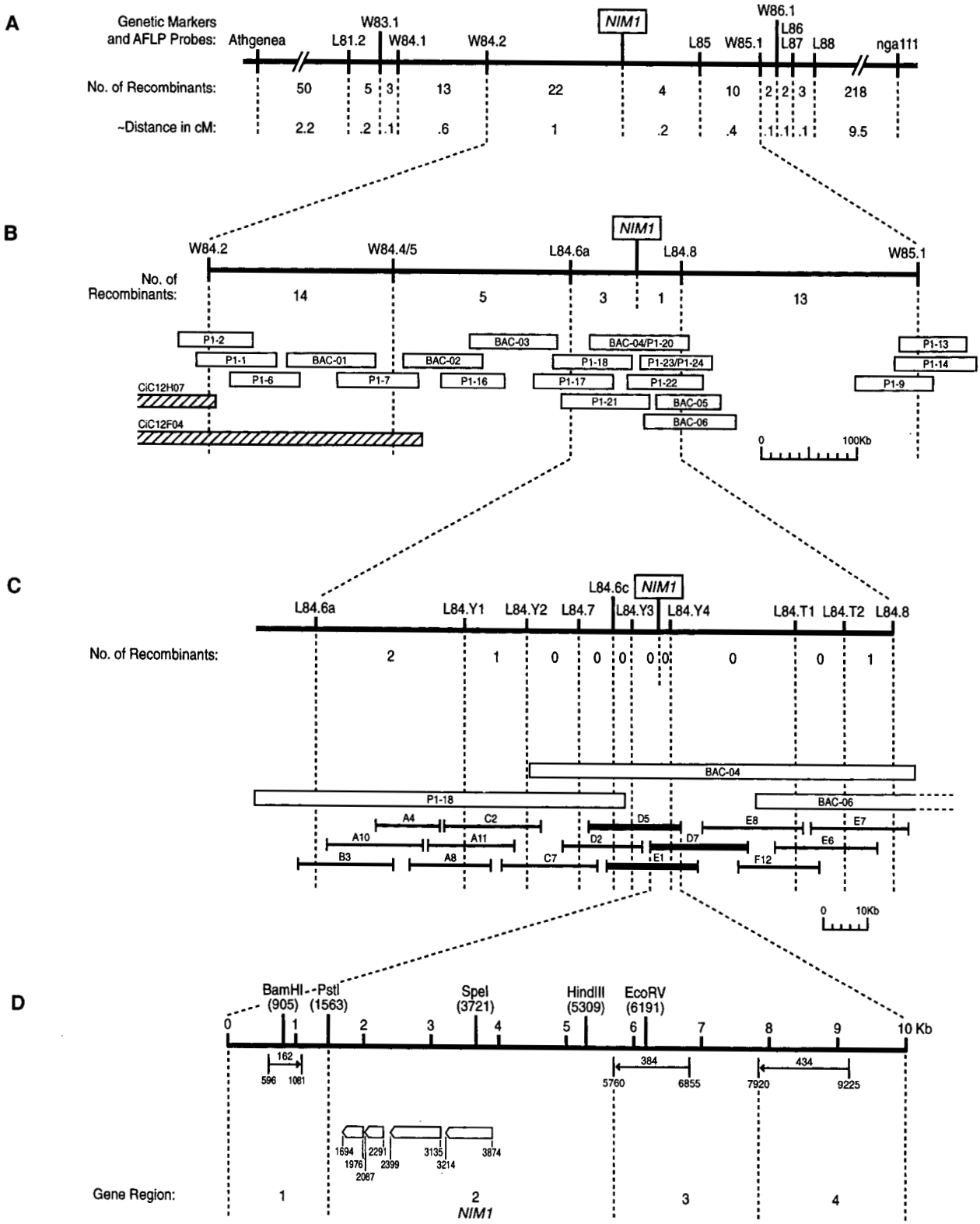

Figure 3. Fine Structure Mapping of the *NIM1* Locus.

Shown is the mapping of the *NIM1* gene at increasing levels of resolution.

(A) Map position of *NIM1* on chromosome 1. The total number of gametes scored was 2276.

(B) Yeast artificial chromosome (striped), bacterial artificial chromosome (BAC), and P1 clones used to clone *NIM1*.

(C) Cosmid clones that cover the *NIM1* locus. The three cosmids that complemented *nim1-1* are shown as thicker lines.

(D) The four putative gene regions on the smallest fragment of complementing genomic DNA (see Results). The four open reading frames that comprise the *NIM1* gene are indicated by the open bars. The arrows indicate the direction of transcription. Numbering is relative to the first base of Arabidopsis genomic DNA present in cosmid D7.

BAC-05, and BAC-06 identified one recombinant. Because these clones overlap to form a large contig (>100 kb) and include AFLP markers that flank *nim1*, the gene was located on the contig. The BAC and P1 clones that comprised the contig were used to generate eight additional AFLP markers, which showed that *nim1* was located between L84.Y1 and L84.8, representing a gap of ~0.09 cM.

A cosmid library was constructed in the Agrobacterium-compatible T-DNA cosmid vector pCLD04541 by using DNA from BAC-06, BAC-04, and P1-18. A cosmid contig was developed using AFLP markers derived from these clones. Physical mapping showed that the physical distance between L84.Y1 and L84.8 was >90 kb, giving a genetic to physical distance of ~1 megabase per cM. To facilitate the later identification of the *NIM1* gene, the DNA sequence of BAC-04 was determined.

Isolation of the *NIM1* Gene

To identify which cosmids contained the *NIM1* gene, we transformed the 12 cosmids listed in Table 2 into *nim1-1*, and transformants were evaluated for their ability to complement the mutant phenotype. Cosmids D5, E1, and D7 were all found to complement *nim1-1*, as determined by the ability of the transformants to accumulate *PR-1* mRNA after INA treatment. The ends of these cosmids were sequenced and found to be located on the DNA sequence of BAC-04. There were 9918 bp in the DNA region shared by D7 and D5, which contained the *NIM1* gene. As shown in Figure 3D, four putative gene regions were identified in this 10-kb sequence. Region 1 potentially could encode a protein of 19,105 D, region 3 could encode a protein of 44,554 D, and region 4 could encode a protein of 52,797 D. Region 2 had four open reading frames of various sizes located close together, suggesting a gene with three introns. Analysis using the NetPlantGene program (Hebsgaard et al., 1996) indicated a high probability that the open reading frames could be spliced together to form a large open reading frame encoding a protein of 66,039 D.

To ascertain which gene region contained the *NIM1* gene, we took gel blots containing RNA isolated from leaf tissue of Ws-0 and the different *nim1* mutants after either water or chemical treatment and probed them with DNA derived from each of the four gene regions. In these experiments, care was taken to label probes to high specific activity, and autoradiographs were exposed for more than 1 week. In our past experience, these conditions would identify RNA at concentrations of approximately one copy per cell. The only gene

Table 2. Complementation of the *nim* Phenotype by Cosmid Clones

Clone Name	No. of Transformants	No. of Plants with INA-Induced PR-1/ Total No. of Plants Tested	% of Plants with INA-Induced PR-1
A8	3	0/3	0
A11	18	4/18	22
C2	10	1/10	10
C7	33	1/33	3
D2	81	7/81	8
D5	6	5/6	83
E1	10	10/10	100
D7	129	36/36	100
E8	5	0/5	0
F12	6	0/6	0
E6	1	0/1	0
E7	34	0/4	0
Ws control[a]	NA[b]	28/28	100
nim control	NA	0/34	0

[a] Ws, Ws-0 wild type.
[b] NA, not applicable.

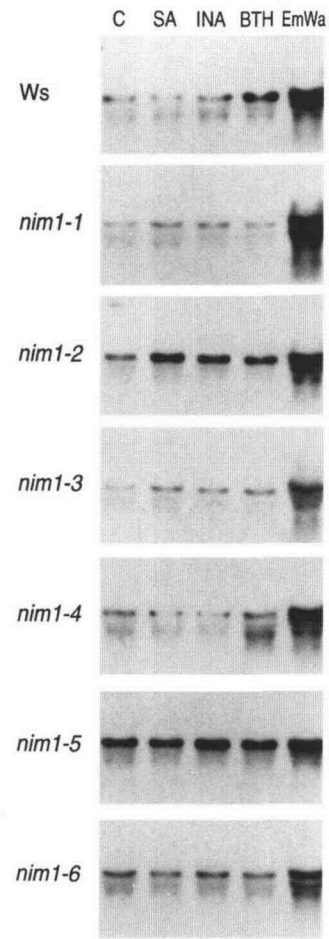

Figure 4. Accumulation of *NIM1* RNA in Chemical- and Pathogen-Treated Plants.

The RNA gel blots in Figure 2 were probed for expression of RNA by using a probe derived from 2081 to 3266 in the sequence shown in Figure 5. Abbreviations are as given in the legend to Figure 2.

region that produced detectable RNA was gene region 2. As shown in Figure 4, the mRNA identified by the gene region 2 probe was induced by BTH treatment of wild-type plants but not in any of the mutants. Furthermore, RNA accumulation was elevated in all of the plants after *P. parasitica* infection, indicating that this particular gene is induced after pathogen infection.

To establish further the gene region encoding *NIM1*, the DNA sequence from each of the four gene regions was determined for each of the *nim1* alleles and compared with the corresponding gene region from Ws-0. No mutations were detected between Ws-0 and the mutant alleles in either gene regions 3 or 4, and only a single change was found in gene region 1 in the *nim1-6* mutant. However, a single base pair mutation was found in each of the alleles relative to Ws-0 for region 2. The DNA sequence of gene region 2 is shown in Figure 5. As shown in Table 3 and Figure 5, in *nim1-1*, a single adenosine was inserted at position 3579 that would cause a frameshift, resulting in a change in seven amino acids and a deletion of 349 amino acids. In *nim1-2*, there is a cytidine-to-thymidine transition at position 3763 that changes a histidine to a tyrosine. In *nim1-3*, a single adenosine is deleted at position 3301, causing a frameshift that altered 10 amino acids and deleted 412 from the predicted protein. Interestingly, both *nim1-4* and *nim1-5* have a guanosine-to-adenosine transition at position 4160, changing an arginine to a lysine, and in *nim1-6*, there is a cytosine-to-thymidine transition, resulting in a stop codon causing the deletion of 255 amino acids from the predicted protein. Although the mutation in *nim1-4* and *nim1-5* alters the consensus donor splice site for the mRNA, reverse transcription–polymerase chain reaction (RT-PCR) analysis indicates that this mutation does not lead to an alteration of mRNA splicing (data not shown).

The gene region 2 DNA sequence was used in a BLAST search (Altschul et al., 1990) and identified an exact match with the Arabidopsis expressed sequence tag T22612 and significant matches to the rice expressed sequence tags S2556, S2861, S3060, and S3481. A DNA probe covering base pairs 2081 to 3266 was used to screen an Arabidopsis cDNA library, and 14 clones were isolated that correspond to gene region 2. From the cDNA sequence, we could confirm the placement of the exon/intron borders shown in Figure 5. Rapid amplification of cDNA ends (RACE) by PCR was conducted using RNA from INA-treated Ws-0 plants, and the likely transcriptional start site was determined to be the A at position 2588 in Figure 5.

The Protein Encoded in Gene Region 2 Has Strong Structural Homology to IκBα

The sequence of gene region 2 was used in BLAST searches, and matches were identified based on homology to ankyrin domains found in a number of proteins such as spectrins, ankyrins, NF-κB, and IκB (Michaely and Bennett, 1992). Beyond the ankyrin motif, conventional computer analysis did not detect other strong homologies. Pair-wise visual inspections between the protein encoded by gene region 2 and 70 known ankyrin-containing proteins were conducted, and striking similarities were found to members of the IκBα class of transcription regulators (Baeuerle and Baltimore, 1996; Baldwin, 1996). As shown in Figure 6, amino acid identities are highlighted in black, and similarities in either dark or light gray are based on strong or moderate conservation by using a modified Dayhoff table (Schwartz and Dayhoff, 1979; Gribskov and Burgess, 1986). This analysis indicates that the protein encoded by gene region 2 shares significant homology with IκBα.

DISCUSSION

Does Gene Region 2 Encode the *NIM1* Gene?

We conclude that gene region 2 encodes the *NIM1* gene based on several lines of evidence. From fine structure mapping, we know the *NIM1* gene must lie in the 90-kb region between AFLP markers L84.Y1 and L84.8. Complementation data from cosmids comprising a contig of this region show that the *NIM1* gene lies in the 9918 bp shared between cosmids D7 and D5. Within this region, there are only four potential genes that could encode *NIM1*. Of these four, only gene 2 has detectable mRNA in leaf tissue. Because the SAR signaling pathway is functional in leaf tissue, genes encoding factors involved in signal transduction should be expressed in these tissues. Furthermore, gene 2 mRNA accumulates after pathogen infection, which would be consistent with a gene involved in SAR signaling. The most compelling evidence, however, comes from DNA sequence analysis of the various mutants relative to the Ws-0 wild type. Each of the gene regions were sequenced in their entirety, and no mutations were found between Ws-0 and the different alleles in gene regions 3 or 4. In gene region 1, there was only a single change between the six alleles and Ws-0, and that was found in *nim1-6*. However, in gene region 2, there was a single base pair change leading to a change in the predicted protein sequence for each allele relative to Ws-0. Based on these data, we conclude that gene region 2 encodes *NIM1*.

Is *NIM1* a Structural Homolog of IκBα?

In Figure 6, the alignment between the NIM1 protein and mouse, human, and rat IκBα is compelling despite the gaps that were introduced to optimize homology. Interestingly, NIM1 contains several important structural domains of IκBα throughout the entire length of the protein, including ankyrin domains (indicated by the dashed underscoring in Figure 6),

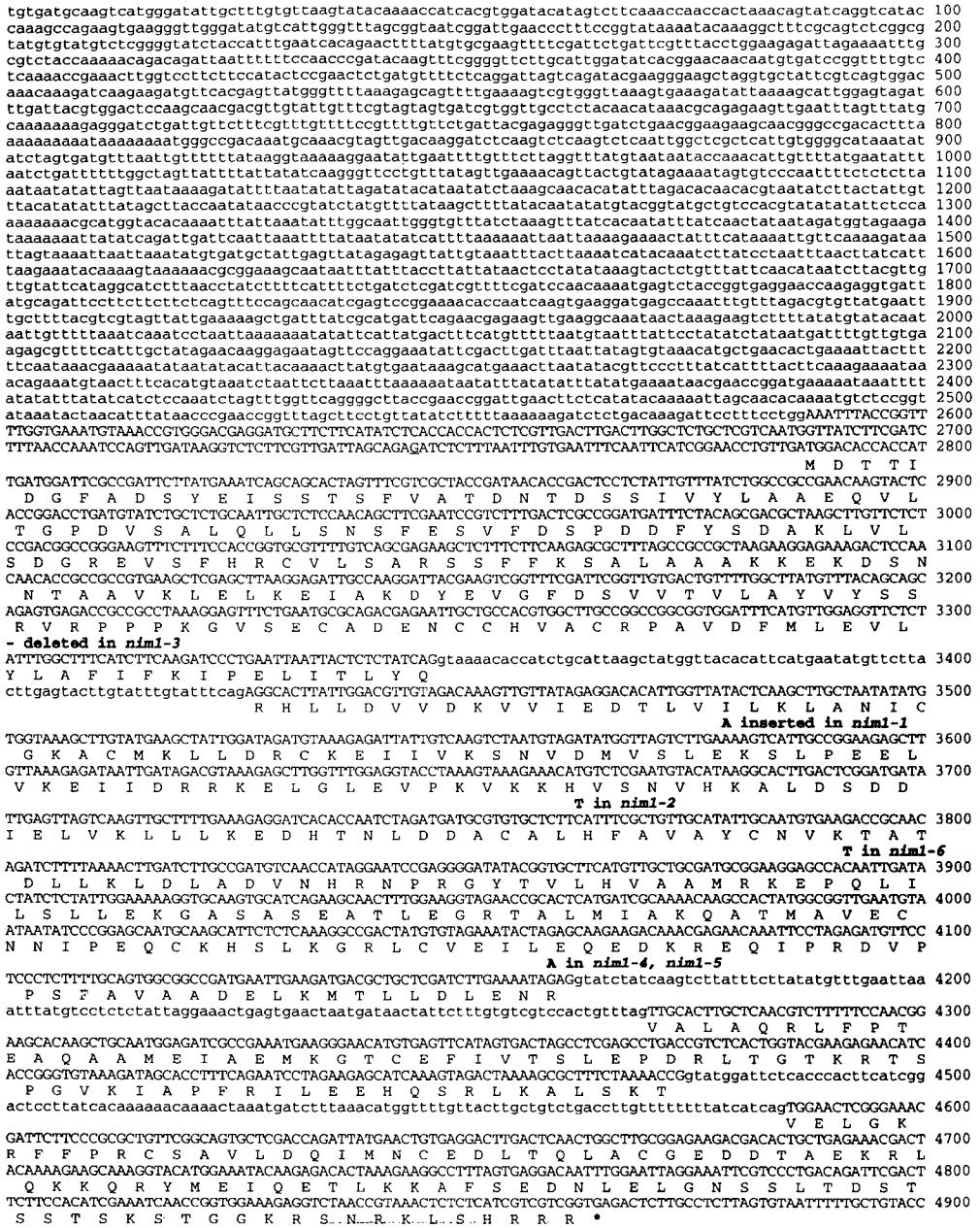

Figure 5. DNA Sequence of the *NIM1* Gene from Arabidopsis Accession Col-0.

Exon regions of the gene are shown in capital letters, and introns and 5′ and 3′ flanking sequences are shown in lowercase letters. The deduced protein sequence is shown below the corresponding DNA sequence. The positions of mutations in the *nim1* alleles are shown in boldface above the DNA sequence. The underlined G nucleotide indicates the start of the longest cDNA, and the asterisk indicates the stop codon. The GenBank accession number is UG7794.

Table 3. Sequence Changes in *nim1* Alleles[a]

Allele or Ecotype	Allele-Specific DNA Changes/Location	Resulting Change to NIM1 Protein
nim1-1	A nucleotide inserted at 3579[b]	Seven amino acids changed and deletion of 349 amino acids[c]
nim1-2	C nucleotide changed to T at 3763	Histidine changed to tyrosine
nim1-3	A nucleotide deleted at 3301	Ten amino acids changed and deletion of 421 amino acids
nim1-4	G nucleotide changed to A at 4160	Arginine changed to lysine
nim1-5	G nucleotide changed to A at 4160	Arginine changed to lysine
nim1-6	C nucleotide changed to T at 3893	Glutamine changed to a Stop and deletion of 255 amino acids
Ws-0	C nucleotide changed to A at 2536	None (in intron)
	C nucleotide changed to T at 2570	None (in intron)
	T nucleotide changed to A at 2689	None (in intron)
	G nucleotide changed to A at 3064	Serine changed to asparagine
	G nucleotide changed to A at 3072	Alanine changed to threonine
	GCC triplet deleted at 3111	Deletion of alanine
	C nucleotide changed to G at 3668	Serine changed to tryptophan
	A nucleotide changed to C at 4082	Glutamine changed to proline
	T nucleotide changed to G at 4218	None (in intron)
	T nucleotide changed to C at 4955	Isoleucine changed to leucine

[a] Nucleotides that are altered in respect to the Columbia wild-type sequence are shown. In addition to allele-specific changes, all mutant alleles are in the Ws ecotype and have the Ws-0 changes as well as the mutation indicated.
[b] Positions of altered nucleotides refer to nucleotides numbered in Figure 5.
[c] Amino acid changes are derived from the predicted NIM1 protein sequences as shown in Figure 5.

two N-terminal serines (amino acids 55 and 59 of NIM1), a pair of lysines (amino acids 98 and 99 in NIM1), and an acidic C terminus. Overall, NIM1 and IκBα share identity at 30% of the residues and conservative replacements at 50% of the residues. Thus, there is homology between IκBα and NIM1 throughout the proteins with an overall similarity of 80%.

The IκBα protein functions in signal transduction by binding to the transcription factor NF-κB and preventing it from entering the nucleus (Baeuerle and Baltimore, 1996; Baldwin, 1996). When the signal transduction pathway is activated, IκBα is phosphorylated at two serine residues (amino acids 32 and 36 of mouse IκBα). The phosphorylation programs ubiquitination at a double lysine (amino acids 21 and 22 of mouse IκBα). After ubiquitination, the NF-κB/IκB complex is routed through the proteosome, where IκBα is degraded and NF-κB is released to the nucleus. The phosphorylated serine residues important in IκBα function are conserved in NIM1 within a large contiguous block of conserved sequence from amino acids 35 to 84 (Figure 6). In contrast to IκBα, in which the double lysine is located ~15 amino acids toward the N terminus of the protein, in NIM1, a double lysine is located ~40 amino acids toward the C-terminal end. Furthermore, a high degree of homology exists between NIM1 and IκBα in the serine/threonine–rich C-terminal region, which has been shown to be important in basal turnover rate (Sun et al., 1996). Based on the analysis of structural homology and the presence of elements known to be important for IκBα function, NIM1 may function like the IκBα subclass of proteins.

Conventional computer programs were unable to identify the homology to IκBα. In fact, the principal match that was found by conventional analysis was to many ankyrin-containing proteins, and this was based on one rather highly conserved domain shown in Figure 6. Considerably more time and effort were required to recognize the IκBα alignment, and this should be considered when trying to match plant proteins to the heavily mammalian sequence–biased data bases.

Although we were able to isolate six *nim1* alleles with phenotypes ranging from severe to weak, the analysis of the mutation in each of these genes does not offer much insight into the function of NIM1. For example, the mutation in *nim1-3* causes the deletion of 412 amino acids from the protein, which would be expected to have a dramatic effect on a protein, but a more severe phenotype is caused by a single point mutation in *nim1-4* causing an arginine-to-lysine substitution. A more detailed understanding of NIM1-dependent signaling will lead to a better understanding of the effect of mutations on NIM1 function.

It is important to point out that the mutation in *nim1-4* is the same as that in *nim1-5*. However, these alleles have different phenotypes with respect both to chemically induced SAR and to their mode of inheritance, with *nim1-4* being

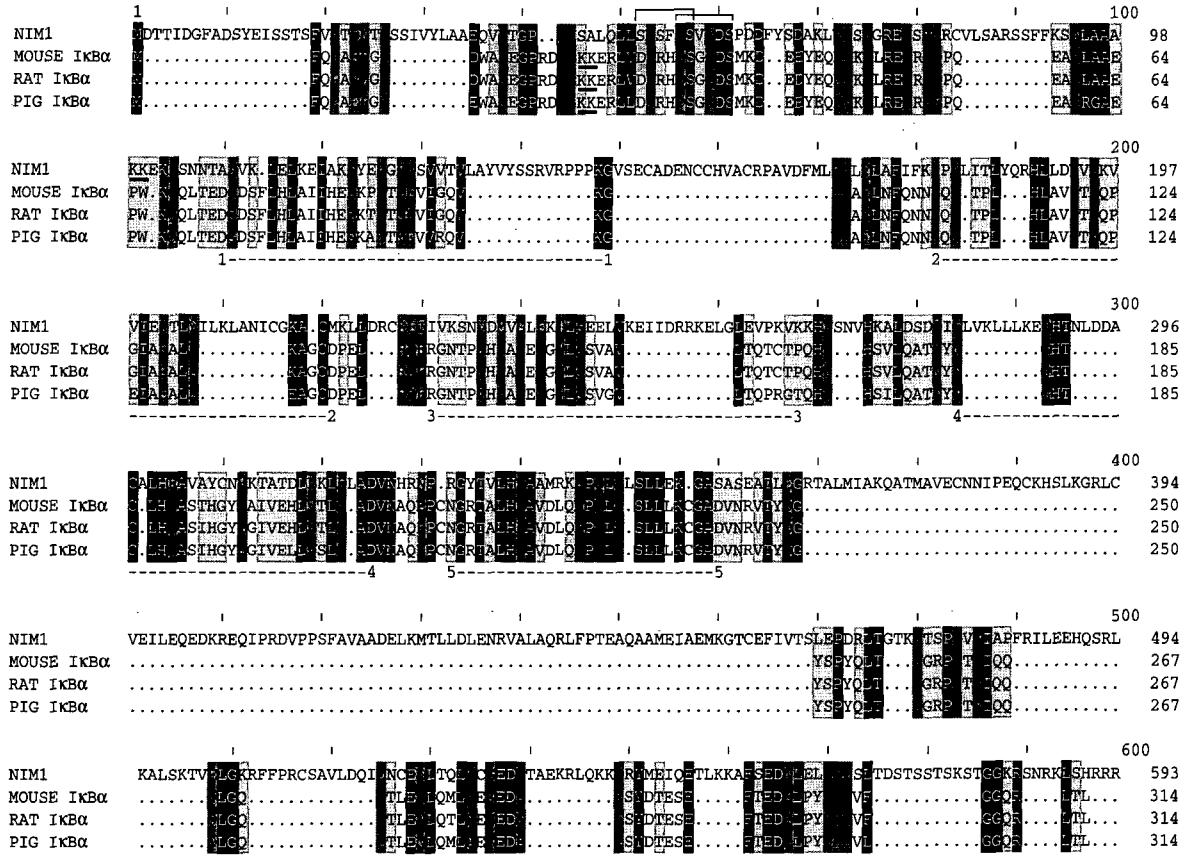

Figure 6. Alignment of the NIM1 Protein Sequence to IκBα from Mouse, Rat, and Pig.

Black bars indicate amino acid identity between NIM1 and the IκBα sequences (matrix score equals 1.5), dark gray bars indicate a similarity score >0.5, light gray bars indicate a similarity score <0.5 but >0.0, and a score <0.0 indicates no similarity and is not highlighted (see Methods). Locations of the mammalian IκBα ankyrin domains were identified according to de Martin et al. (1995). The dots in a sequence indicate gaps between NIM1 and IκBα proteins. The five ankyrin repeats in IκBα are indicated by the dashed lines under the sequence. The lysines required for ubiquitination of IκBα are underlined, and the phosphorylation sites in IκBα are bracketed (see Discussion). Amino acids are numbered relative to the NIM1 protein, with gaps introduced where appropriate. Vertical dashes are positioned every 10 amino acids.

recessive and *nim1-5* being apparently dominant. This strongly suggests that the mutation in *nim1-5* does not fully account for the phenotype. It seems more likely that *nim1-5* has a mutation, in addition to that in the *NIM1* gene, which may modify the phenotype of this mutant line.

NF-κB and IκB in Disease-Related Pathways in Other Organisms

In mammals, NF-κB/IκB signal transduction can be induced by a number of different stimuli, including exposure of cells to lipopolysaccharide, tumor necrosis factor, interleukin 1 (IL-1), or virus infection (Baeuerle and Baltimore, 1996; Baldwin, 1996). The activated pathway leads to the synthesis of a number of factors involved in inflammation and immune responses, such as IL-2, IL-6, IL-8, and the granulocyte/macrophage–colony stimulating factor (de Martin et al., 1993). In transgenic mouse studies, the knockout of NF-κB/IκB signal transduction leads to a defective immune response, including enhanced susceptibility to bacterial and viral pathogens (Baeuerle and Baltimore, 1996; Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996). In Arabidopsis, SAR is functionally analogous to inflammation in that normal resistance processes are potentiated after SAR activation, leading to enhanced disease resistance (Gaffney et al., 1993; Cao et al., 1994; Delaney et al., 1994, 1995; Bi et al., 1995; Mauch-Mani and Slusarenko, 1996; Delaney, 1997). Furthermore, inactivation of the pathway leads to enhanced susceptibility to bacterial, viral, and fungal pathogens. Interestingly, SA has been reported to block

NF-κB activation in mammalian cells (Kopp and Ghosh, 1994), whereas in the case of Arabidopsis, SA activates signal transduction.

Bacterial infection of Drosophila activates a signal transduction cascade, leading to the synthesis of a number of antifungal proteins such as cercropin B, defensin, diptericin, and drosomycin (Ip et al., 1993; Lemaitre et al., 1996). This induction is dependent on the gene product of *dorsal* and *dif*, two NF-κB homologs, and is repressed by *cactus*, an IκB homolog, in the fly. Mutants that have decreased synthesis of the antifungal and antibacterial proteins have dramatically lowered resistance to infection. Similarly, *nim1* mutants are blocked in pathogen-dependent induction of SAR gene expression, which includes the synthesis of a number of antimicrobial proteins such as β-1,3-glucanase and *PR-5*, and the mutants have significantly depressed pathogen resistance (Ryals et al., 1996). Thus, the Arabidopsis SAR pathway shows interesting functional parallels to the NF-κB/IκB regulation scheme in both mammals and flies.

How Could an NF-κB/IκB Signal Transduction Pathway Function in SAR?

The NF-κB/IκB signal transduction pathways are conserved in both mammals and flies. A stimulus such as IL-1 treatment or bacterial inoculation leads to activation of a signal transduction pathway because of the degradation of IκB or its homolog and the release of the NF-κB transcription factor to the nucleus to stimulate transcription (Baeuerle and Baltimore, 1996; Baldwin, 1996). Activation of the SAR pathway in Arabidopsis by SA or pathogen infection leads to enhanced SAR gene expression and resistance, which is similar to that in mammals and flies. However, in contrast to these organisms, mutations in the IκB component (i.e., NIM1), which should eliminate the inhibitor and cause nuclear localization of the transcription factor, result in inhibition of the SAR signal transduction pathway. This suggests that the transcription factor targeted by NIM1 serves as a repressor of SAR gene expression and disease resistance either by direct or indirect action.

Interestingly, the plant disease resistance genes *N* (Whitham et al., 1994), *L6* (Lawrence et al., 1995), and *Rpp5* (Bent, 1996) have significant similarity to both the IL-1 receptor and the Toll protein. These receptors are involved in NF-κB/IκB signaling in humans and Drosophila, respectively, suggesting involvement of an NF-κB/IκB pathway in plant disease resistance gene activation. Direct involvement of NIM1 in disease resistance gene activation is not clear and requires further investigation.

All higher organisms must defend themselves from a variety of pathogens. In mammals and flies, a component of the pathogen defense system is activated by a signal transduction system that is dependent on NF-κB/IκB transcription activation. In plants, the SAR signal transduction pathway also seems to involve at least one factor, NIM1, that is homologous to IκB, suggesting that SAR is representative of a conserved defense pathway used in many organisms.

METHODS

Plant Material and Isolation of Genetic Recombinants

nim1 mutants were isolated from two *Arabidopsis thaliana* ecotype Wassilewskija (Ws-0) plant populations as previously described by Delaney et al. (1995). The *nim1-1* line was isolated from a T-DNA–transformed population, and the other *nim1* mutants were isolated from an M_2 ethyl methanesulfonate–mutagenized population. Plants were grown in conditions described by Uknes et al. (1992). *nim1-1* plants were crossed to Landsberg *erecta* (*Ler*) or the *Ler* marker line DP23 (Patton et al., 1991), and F_2 progeny were scored for the Nim^- phenotype and analyzed molecularly for recombination. The selected F_2 progeny were allowed to self-pollinate, and F_3 progeny were rescreened. Simple sequence length polymorphism (SSLP) markers described by Bell and Ecker (1994) were used to identify plants that had recombinations in the vicinity of the *NIM1* locus.

Determination of the Nim^- Phenotype

Plants were assayed using two methods to determine the phenotype. Plants at the four-true-leaf stage were misted with 330 μM 2,6-dichloroisonicotinic acid (INA). Three days later, a leaf was harvested for RNA extractions and analysis. Plants were then sprayed with a *Peronospora parasitica* isolate Emwa conidial suspension as described previously (Delaney et al., 1995). Seven to 10 days after inoculation, plants were scored for fungal growth and compared with control Ws-0 plants (exhibiting very little or no growth) and *nim1-1* plants (supporting high levels of growth). The Nim^- phenotype was also determined by analyzing the extracted RNA for *PR-1* gene expression. A Nim^- phenotype is indicated by a lack of *PR-1* gene expression and growth of fungus despite INA treatment.

Analysis of Chemical Responsiveness in *nim1* Mutants

Ws-0 and *nim1* mutant plants were grown to the four-true-leaf stage and then treated with water, 5 mM salicylic acid (SA), 330 μM INA, or 300 μM benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), as described previously (Uknes et al., 1992). After three days, plants were divided into two groups. Tissue from one group was harvested for RNA extraction, and the second group was inoculated with *P. parasitica* isolate Emwa (Delaney et al., 1995). At various time points after inoculation, plants were analyzed microscopically to determine the percentage of plants per pot showing sporulation.

Isolation of Nucleic Acids from Arabidopsis and Clones

For mapping the *nim1-1* locus, we extracted DNA from F_2 and F_3 plants by using a hexadecyltrimethyl-ammonium bromide method (Rogers and Milliman, 1984). Cosmid DNA was isolated using a modification of the ammonium acetate method (Ausubel et al., 1987). RNA was isolated from single leaves for Nim^- phenotype determination

by using a rapid procedure described by Verwoerd et al. (1989). Other RNA extractions and RNA blot hybridizations were performed, as described previously, using pools of treated tissue (Ausubel et al., 1987; Lagrimini et al., 1987). Blots were probed with the indicated ^{32}P-labeled cDNAs or polymerase chain reaction (PCR) products made with a random primer labeling system (Gibco BRL).

Identification of Markers and Genetic Mapping of the *NIM1* Locus

The primers and conditions for SSLP mapping at the ATHGENEA and nga111 loci were described previously (Bell and Ecker, 1994). To identify molecular markers closer to the *NIM1* gene, we generated amplified fragment length polymorphism (AFLP) markers by using the selective restriction fragment amplification method described by Vos et al. (1995). Initially, a population of recombinant inbred lines derived from a cross between L*er* and Columbia (Col-0) (Lister and Dean, 1993) was used for AFLP marker screening by using an EcoRI-MseI primer combination. Nine of the AFLP markers mapped using the recombinant inbred lines were identified as being informative for mapping the *NIM1* locus by using the recombinant plants from the *nim1-1* × L*er* cross. Other primer combinations were used to generate additional AFLP markers for screening the yeast artificial chromosome (YAC), P1, and bacterial artificial chromosome (BAC) clones. (Detailed protocols listing all of the primer combinations used can be provided upon request.)

Identification of YAC, BAC, and P1 Clones

An Arabidopsis ecotype Col-0 library (CIC library; Creusot et al., 1995) was screened for YAC clones in the *NIM1* region. DNA was isolated from pools of YAC clones, and each pool was screened with two of the AFLP markers identified as being informative. Four YAC clones were found and further fingerprinted by AFLP markers using additional primers. The results indicated that there was a gap between the two largest YACs, and the *NIM1* gene could not be positioned on either of these YAC clones. A Col-0 P1 library (Liu et al., 1995) and Col-0 BAC library (Choi et al., 1995) were then screened using flanking AFLP markers. Two sets of nonoverlapping P1 contigs were identified flanking the *NIM1* region. Using various P1 or BAC clones as probes, we identified new clones extending ~550 kb from the closest centromeric AFLP marker. New AFLP markers were generated to characterize the P1/BAC contig extension, and these markers were analyzed on informative recombinants. *NIM1* was found to be located on a DNA segment estimated to be 90 kb in length and contained in clone BAC-04 or P1-18.

Construction of a Cosmid Contig and Transformation into Agrobacterium

DNA was isolated from BAC-04, BAC-06, and P1-18 and partially digested with Sau3A. The 20- to 25-kb fragments were purified using a sucrose gradient, pooled, and filled in with dATP and dGTP by using standard procedures (Sambrook et al., 1989). The vector pCLD04541 (kindly provided by C. Dean, John Innes Centre, Norwich, UK) was cleaved with XhoI, filled in with dCTP and dTTP, and ligated with the prepared fragments. Cosmid DNA was isolated from resulting positive clones and analyzed by AFLP markers to determine the order of the cosmid clones. A set of 15 overlapping cosmids was selected spanning the *NIM1* region. The cosmids were moved into *Agrobacterium tumefaciens* AGL-1 (a kind gift from R. Ludwig, University of California, Santa Cruz, La Jolla) through conjugative transfer in a triparental mating with helper strain HB101/pRK2013.

Generation and Analysis of Arabidopsis Transformants

Twelve of the cosmids in Agrobacterium were transformed into a kanamycin-sensitive *nim1-1* line by using vacuum infiltration (Bechtold et al., 1993; Mindrinos et al., 1994). Seed from the infiltrated plants were harvested several weeks later and allowed to germinate on GM agar plates containing 50 μg/mL kanamycin (Weymann et al., 1995). Surviving seedlings were transferred to soil ~2 weeks after plating, grown for 1 week in soil, and then tested for the Nim$^-$ phenotype.

DNA Sequencing

BAC-04 DNA was randomly sheared in a nebulizer to generate fragments with an average length of 2 kb. Ends of the sheared fragments were repaired, and the fragments were purified. Prepared DNA was ligated with EcoRV-digested pBRKanf4 (a derivative of pBRKanf1 [Bhat, 1993]). We selected 1600 resulting kanamycin-resistant colonies for plasmid isolation by using the Wizard Plus 9600 Miniprep System (Promega). Plasmids were sequenced using dye terminator chemistry (Applied BioSystems, Foster City, CA), with primers designed to sequence both strands of the plasmids (M13-21 forward and T7 reverse; Applied BioSystems). Data were collected on ABI377 DNA sequencers (Applied BioSystems). Sequences were edited and assembled into contigs by using Sequencher 3.0 (GeneCodes Corp., Ann Arbor, MI) and the Staden genome assembly programs Phred, Phrap, and Crossmatch (Phil Green, Washington University, St. Louis, MO) and Consed (David Gordon, Washington University). DNA from the cosmids found to complement the *nim1-1* mutation was sequenced using primers designed by Oligo 5.0 Primer Analysis Software (National Biosciences, Inc., Plymoth, MN). Sequencing of DNA from Ws-0 and the *nim1* alleles and cDNAs was performed essentially as described above.

Sequence Analysis

Sequence homology searches were performed using BLAST (Altschul et al., 1990). The multiple sequence alignment was constructed using Clustal V (Higgins and Sharp, 1989) as part of the Lasergene Biocomputing Software package from DNASTAR (Madison, WI). The sequences used in the alignment were NIM1, mouse IκBα (GenBank accession number 1022734), rat IκBα (GenBank accession numbers 57674 and X63594; Tewari et al., 1992), and pig IκBα (GenBank accession number Z21968, de Martin et al., 1993; GenBank accession number 517193, de Martin et al., 1995). Parameters used in the Clustal analysis were gap penalty of 10 and gap length penalty of 10. Evolutionary divergence distances were calculated using the PAM250 weight table (Dayhoff et al., 1979). Residue similarity was calculated using a modified Dayhoff table (Schwartz and Dayhoff, 1979; Gribskov and Burgess, 1986).

Isolation of a *NIM1* cDNA

An Arabidopsis cDNA library made in the λYES expression vector (Elledge et al., 1991) was screened with ^{32}P-labeled PCR products generated from gene region 2 (Figure 1) by using standard procedures (Sambrook et al., 1989). Positive plaques were purified, and plasmid DNA was recovered. The cDNA inserts were sequenced using either vector-specific primers or primers internal to the insert. Rapid amplification of cDNA ends (RACE) analysis was performed using a 5′ RACE kit (Gibco BRL) according to the manufacturer's instructions.

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NOTE ADDED IN PROOF

The BamHI (bp 905) to HindIII (bp 5309) fragment shown in Figure 3D containing only the *NIM1* open reading frame also complements the *nim1-1* allele. The amino acid sequence of NPR1 is identical to that of NIM1, suggesting that these two genes are allelic (**Cao, H., Glazebrook, J., Clark, J.D., Volko, S., and Dong, X.** [1997]. The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88,** 57–63).