Salicylic Acid and NIM1/NPR1-Independent Gene Induction by Incompatible *Peronospora parasitica* in Arabidopsis

Gregory J. Rairdan, Nicole M. Donofrio, and Terrence P. Delaney


To identify pathogen-induced genes distinct from those involved in systemic acquired resistance, we used cDNA-amplified fragment length polymorphism to examine RNA levels in *Arabidopsis thaliana* wild type, *nim1-1*, and salicylate hydroxylase-expressing plants after inoculation with an incompatible isolate of the downy mildew pathogen *Peronospora parasitica*. Fifteen genes are described, which define three response profiles on the basis of whether their induction requires salicylic acid (SA) accumulation and NIM1/NPR1 activity, SA alone, or neither. Sequence analysis shows that the genes include a calcium binding protein related to TCH3, a protein containing ankyrin repeats and potential transmembrane domains, three glutathione S-transferase gene family members, and a number of small, putatively secreted proteins. We further characterized this set of genes by assessing their expression patterns in each of the three plant lines after inoculation with a compatible *P. parasitica* isolate and after treatment with the SA analog 2,6-dichloroisonicotinic acid. Some of the genes within subclasses showed different requirements for SA accumulation and NIM1/NPR1 activity, depending upon which elicitor was used, indicating that those genes were not coordinately regulated and that the regulatory pathways are more complex than simple linear models would indicate.

Additional keywords: calmodulin, CXC750, ECS1, gene expression profiling, NahG plants, ORFX.

Plants are in frequent contact with potential pathogens and consequently have evolved effective mechanisms to resist infection. These include passive or mechanical barriers to infection, preformed chemical defenses, and resistance mechanisms that are activated by exposure to pathogens. In many cases, products of host resistance (*R*) genes mediate pathogen recognition and activate pathways that control induced local defenses such as the hypersensitive response (*HR*), a rapid, programmed cell death response, and the oxidative burst, which is caused by the production of activated oxygen species at the infection site (Hammond-Kosack and Jones 1996). Pathogen exposure also can induce systemic defense responses, which render the plant more resistant to infection by a wide variety of virulent pathogens. The best understood of these responses is systemic acquired resistance (SAR) (Ryals et al. 1996; Sticher et al. 1997). SAR is correlated with the accumulation of transcripts from a few dozen pathogenesis-related (*PR*) genes, including acidic and basic β-1,3-glucanases, chitinases, and a wide array of other genes of unknown function (Linthorst 1991; Ward et al. 1991). Only a few *PR* genes have been demonstrated to contribute to resistance in vivo (Alexander et al. 1993; Broglie et al. 1991; Liu et al. 1994), and no single *PR* gene has been shown to be essential for SAR, suggesting that the coordinate expression of these genes is needed to produce resistance.

Despite intensive research, to date only two molecules, salicylic acid (SA) and the NIM1/NPR1 protein, have been shown convincingly to be required for SAR. SA was first implicated as important for the induction of SAR by observations that plants treated with acetylsalicylic acid expressed SAR (White 1979) and that SA accumulation accompanied the biological induction of SAR (Malamy et al. 1990; Métraux et al. 1990). SA accumulation was shown to be required for expression of SAR in studies of transgenic tobacco and *Arabidopsis thaliana* plants that expressed a bacterial salicylate hydroxylase (nahG) gene. These NahG plants were unable to accumulate SA, impaired in the induction of PR transcripts in response to pathogen exposure, failed to express SAR, and more susceptible to virulent and avirulent pathogens (Delaney et al. 1994; Gaffney et al. 1993). The Arabidopsis NIM1/NPR1 gene was identified in four independent mutant screens that looked for disruption of plant defense responses induced by SA or the chemical analog 2,6-dichloroisonicotinic acid (INA) or sought mutants that displayed enhanced disease symptoms (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). Like NahG Arabidopsis, *nim1/npr1* mutants show reduced accumulation of many *PR* gene transcripts and do not develop resistance to pathogens after SA treatment. Because *nim1/npr1* mutants accumulate wild-type levels of SA yet do not respond to exogenously applied SA, it seems likely that the NIM1/NPR1 protein acts downstream of SA in the signal transduction pathway leading to SAR (Delaney et al. 1995).

Because the induction of SAR requires SA accumulation and the NIM1/NPR1 signal transduction pathway, neither NahG plants nor *nim1/npr1* mutants are able to express SAR. Compared to *nim1/npr1* mutants, however, NahG plants have a more severe defense-impaired phenotype, suggesting that SA-responsive yet NIM1/NPR1-independent defense path-
ways also play important roles in defense. Evidence for these SAR-independent defense pathways is seen following infection of wild-type, NahG, and nim1/npr1 mutants with virulent strains of the oomycete *Peronospora parasitica* or bacterium *Pseudomonas syringae*. NahG plants support more pathogen growth than nim1-1 plants and significantly more than wild-type hosts (Delaney et al. 1994; Delaney et al. 1995; Donofrio and Delaney 2001). Resistance gene-mediated resistance also is disrupted in NahG plants because some normally avirulent *P. syringae* strains grow well in these plants and some avirulent *P. parasitica* isolates also show growth on NahG hosts (Delaney et al. 1994; Hammond-Kosack and Jones 1996; McDowell et al. 2000; G. J. Rairdan and T. P. Delaney, unpublished results). NIM1/NPR1 also contributes to full expression of some R-gene-mediated resistance, as indicated by the moderate susceptibility exhibited by nim1-1 plants to normally avirulent *P. parasitica* isolates (Delaney et al. 1995; G. J. Rairdan and T. P. Delaney, unpublished results). Together, these observations suggest that SA plays a larger role in resistance signaling than just as an activator of the NIM1/NPR1 pathway. In addition, because NahG plants show compromised but not abolished resistance to several normally avirulent pathogens, other defense mechanisms must exist that are neither dependent upon SA accumulation nor the NIM1/NPR1 pathway. The existence of SA-dependent yet NIM1/NPR1-independent resistance pathways also has been implicated previously by studies of constitutive-defense mutants such as cpr5, cpr6, acd6, and ssi1, all of which retain their resistance phenotypes in an nim1/npr1 mutant background but not in a NahG background (Bowling et al. 1997; Clarke et al. 1998; Rate et al. 1999; Shah et al. 1999).

In the work described here, our goal was to identify and characterize genes and, by extension, signal transduction pathways, which are induced by pathogens independent of SA, NIM1/NPR1, or both. We selected an avirulent pathogen inducer for these screens on the assumption that a R-gene-mediated defense was likely to be supported by multiple resistance pathways on the basis of our earlier observations that nim1-1 or NahG plants show a reduced but not absent expression of gene-for-gene resistance. All plants used were derived from *A. thaliana* accession Wassilewskija (Ws-0), which carries three paralogous TIR-NBS-LRR-class resistance genes at the RPP1 locus (Botella et al. 1998), which are responsible for resistance to *P. parasitica* isolate Noco2. We used the cDNA–amplified fragment length polymorphism (AFLP) technique (Bachem et al. 1996) to profile mRNA production in wild-type, nim1-1, and NahG plants after inoculation with Noco2. This is a robust and sensitive technique to identify differentially expressed transcripts and has a number of advantages over hybridization-based methods such as subtractive hybridization or microarray analysis. Because it involves polymerase chain reaction (PCR), cDNA–AFLP is sensitive and specific and can discriminate between closely related but polymorphic mRNA species. Furthermore, unlike microarray approaches, cDNA–AFLP can lead to gene discovery without prior isolation of the sequence as a cDNA or genomic clone.

Pathogen-induced genes were classified into three groups on the basis of their pattern of expression in the three host genotype plants tested. One class is maximally induced only in wild-type plants. Such genes are, therefore, dependent upon SA accumulation and NIM1/NPR1 activity, as are a number of well-known genes associated with SAR. A second class of genes is induced independently of SA and NIM1/NPR1, and a third class requires SA but not NIM1/NPR1 for induction. The latter group of genes clearly demonstrates the existence of an SA-responsive pathway that acts independently of the NIM1/NPR1 pathway. We also examined the expression of these genes in response to INA treatment or inoculation with a compatible *P. parasitica* isolate. These agents showed similar inductive properties to the avirulent pathogen inducer but also revealed interesting differences, indicating that the elicitors tested have shared properties but are not equivalent.

**RESULTS**

We used cDNA–AFLP to identify cDNA fragments derived from transcripts that are more abundant in Noco2-treated Arabidopsis plants than in water-treated control plants. By comparing the cDNA–AFLP profiles between Ws-0 (wild

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**Fig. 1.** cDNA–amplified fragment length polymorphism (AFLP) analyses of *Peronospora parasitica* isolate Noco2-induced gene expression. Wild-type, nim1-1, and NahG Arabidopsis plants (accession Wassilewskija; Ws-0) were treated with water or inoculated with a *P. parasitica* isolate Noco2 conidiospore suspension. Aerial tissues were collected 1 and 4 days after inoculation, as indicated, mRNA was extracted, and the tissues were analyzed by cDNA–AFLP. Arrows indicate cDNA fragments derived from genes expressed in a, SAR, b, NPR, and c, SIR pattern. a-c, Fragments shown were derived from SAR1, NIR1, and SIR1 genes, respectively (see below). d, Arrow shows a cDNA fragment derived from the hygromycin-B-phosphotransferase gene used for selection of nahG transgenic plants and serves as a cDNA–AFLP positive control.
type), nim1-1, and NahG hosts, genes could be classified on the basis of whether their induction required SA accumulation, NIM1/NPR1 activity, or neither (Fig. 1). In follow-up experiments, we used RNA gel blots to confirm the expression patterns of these genes in plants following Noco2 exposure and to examine their expression after infection with a virulent P. parasitica strain or treatment with INA, a chemical inducer of SAR.

**Genes identified by cDNA–AFLP.**

We analyzed approximately 7,000 cDNA–AFLP fragments derived from two independent cDNA double digestions (Nco1/Taq1 and Vsp1/Taq1; see below for details) and identified 15 pathogen-induced genes, nine of which previously have not been assigned putative function based on sequence similarity to known genes (Table 1). Each was assigned to one of three classes, depending upon their expression in wild-type, nim1-1, or NahG plants (Fig. 1). **SAR**-class genes require SA and NIM1/NPR1 for induction. **NIR**-class genes require SA but not NIM1/NPR1 for expression, and SA and **NPR1** independent response (**SIR**)-class genes require neither SA nor NIM1/NPR1 for induction. If a gene previously had not been characterized, we assigned it a provisional name on the basis of its expression pattern (e.g., **NIR1**; Table 1).

We found a single **SAR**-class gene, **SAR1**, which showed strong expression only in wild-type plants, thus requiring SA and NIM1/NPR1 for full induction. The predicted protein product of this gene is 16.7 kDa, contains a centrally located transmembrane motif, and has strong similarity to ORFX, a product of this gene is 16.7 kDa, contains a centrally located transmembrane motif, and has strong similarity to ORFX, a product of this gene.

**Table 1. Characteristics of SAR, NIR, and SIR genes identified by cDNA–amplified fragment length polymorphism**

<table>
<thead>
<tr>
<th>Name</th>
<th>Expression</th>
<th>DDBJ/EMBL/GenBank accession no.</th>
<th>Size (kDa)</th>
<th>Signal peptide</th>
<th>Structural motifs</th>
<th>Closest informative database match (e-value)a</th>
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</thead>
<tbody>
<tr>
<td>SAR1</td>
<td>SAR</td>
<td>AA792331.1</td>
<td>16.7</td>
<td>TMD</td>
<td></td>
<td>Tomato ORFX (3e-41)</td>
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<tr>
<td>NIR1</td>
<td>NIR</td>
<td>BAB10494.1</td>
<td>8</td>
<td>SP</td>
<td>Four TMDs</td>
<td>Arabidopsis thaliana CxC750 (3e-06)</td>
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<tr>
<td>NIR2</td>
<td>NIR</td>
<td>AAD20710.1</td>
<td>10.7</td>
<td>CP</td>
<td>None</td>
<td>A. thaliana CxC750 (9e-09)</td>
</tr>
<tr>
<td>NIR3</td>
<td>NIR</td>
<td>AAC0734.1</td>
<td>16.8</td>
<td></td>
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<td>Drosophila mechanosensory transduction channel</td>
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<tr>
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<td>CAB78482.1</td>
<td>74</td>
<td></td>
<td></td>
<td>NOMPC (3e-13)</td>
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<tr>
<td>CaBP22</td>
<td>NIR</td>
<td>AAD12002.1</td>
<td>21.7</td>
<td>Four EFs</td>
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<td></td>
<td>Citrus jambhiri blight-associated protein p12 (3e-18)</td>
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<td>BAB01152.1</td>
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<td>β-Lectin domain</td>
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<td>Cladrastis kentukea lectin precursor (9e-19)</td>
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<td>SIR</td>
<td>CAB86639.1</td>
<td>65</td>
<td></td>
<td></td>
<td>Nicotiana tabacum Nt-gh3 deduced protein (e-159)</td>
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<td>SIR4</td>
<td>SIR</td>
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<td>Black currant pRIB5 protein (7e-49)</td>
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<td>GRP3</td>
<td>SIR</td>
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<td>Glycine rich</td>
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<td>GST1</td>
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</table>

a SAR: Systemic acquired resistance expression pattern; NIR: NIM1/NPR1-independent response expression pattern; SIR: Salicylic acid and NIM1/NPR1-independent response expression pattern.

b Corresponds to predicted protein encoded by the gene.

c As predicted by TargetP (Emanuelsson et al. 2000). SP, secretory pathway; CP, chloroplast targeted.
d As predicted by SMART (Schultz et al. 2000) and/or Pfam (Bateman et al. 2000). TMD, transmembrane domain; EF, EF-hand protein.

e National Center for Biotechnology Information (Bethesda, MD, U.S.A.) Basic BLASTP values and default parameters (17 January 2001).
rus blight-associated protein contain regions with significant similarity to expansins. The SIR2 product is predicted to be secreted and has similarity to lectins, a class of proteins that binds glucans and includes proteins with antifungal and anti-herbivore properties (Chrispeels and Raikhel 1991). SIR3 encodes a protein that is homologous to GmGH3 and NtGH3, which are auxin-inducible proteins from soybean and tobacco, respectively (Hagen et al. 1984; Roux and Perrot-Rechenmann 1997). SIR4 shows strong similarity to the blackcurrant pRIB5 gene, which is regulated differentially during fruit ripening (Woodhead et al. 1998) and is related closely to three other tandemly repeated Arabidopsis genes on chromosome 3.

Five other SIR-class genes were found, which previously had been characterized by others, and are thus named accordingly. GRP3 encodes an 11.5-kDa glycine-rich polypeptide that also contains an amino-terminal secretory signal peptide. This gene was identified independently in yeast two-hybrid screens as encoding a protein that interacts with the receptor domain of the Arabidopsis wall-associated receptor kinase-1,

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**Fig. 2.** RNA gel blot analysis of SAR, NIR, and SIR-class transcript accumulation elicited by inoculation with the incompatible Peronospora parasitica isolate Noco2. Wild-type, nim1-1, and NahG Arabidopsis plants (accession Wassilewskija; Ws-0) were treated with water or inoculated with a P. parasitica isolate Noco2 conidiospore suspension. Aerial tissues were collected 1 and 4 days after inoculation, total RNA was extracted from these tissues, and 5 or 15 µg (*) of extracted RNA was used for RNA gel blot analysis. Hybridization probes are listed on the left. rRNA: 18s rRNA; a representative blot is shown.

**Fig. 3.** Alignments of NRI1 and NRI2 DNA and protein sequences. A, Alignment of NRI1 and NRI2 genomic loci. Vertical bars indicate identical nucleotides, gaps are shown as dots, coding sequence is printed in capital letters, and introns are italicized. A possible TATA box is in bold. Underlining indicates the XB box, which is a PR-1 LS10 promoter element (Lebel et al. 1998). B, Alignment of predicted NRI1 and NRI2 proteins. Identical amino acids are indicated by vertical bars; gaps are indicated by hyphens. A predicted signal peptide is in bold.
WAK1, which has been implicated in defense and shown to promote tolerance to normally toxic levels of SA when expressed in transgenic plants (He et al. 1998; Kohorn 2000; Park et al. 2001; B. Kohorn, personal communication). pEARLI-4 is a gene identified previously in a screen for aluminum-responsive transcripts (Richards et al. 1995). We also identified three glutathione S-transferase genes, GST1 (also known as ERD11), GST2 (also known as AIPM24.1), and GST11 (Edwards et al. 2000; Zettl et al. 1994; Zhou and Goldsbrough 1993), all of which were regulated in a SIR-like manner. The identification of these family members illustrates an advantage of the cDNA–AFLP method compared with most hybridization-based screens to identify differentially expressed genes. We were able to demonstrate that each of these GST family members was induced by pathogen exposure in wild-type, nim1-1, and NahG hosts, even though the genes are very similar to each other. In hybridization assays, these genes would almost certainly cross-react, complicating expression analysis. Indeed, Zhou and Goldsbrough (1993) used Arabidopsis genomic DNA gel blots to demonstrate that a full-length GST2 cDNA probe hybridized under stringent conditions to a number of gene family members. Our ability to independently visualize the three GST genes described was the result of the specificity of cDNA–AFLP, which can discriminate between gene family members with as little as a single base pair polymorphism.

Expression analysis of pathogen-induced genes.

To characterize the regulatory pathways that control the expression of genes identified by cDNA–AFLP, we used RNA gel blot analyses to assess transcript accumulation in plants after inoculation with incompatible or compatible P. parasitica isolates or after treatment with INA (Figs. 2, 4, and 5). In addition to testing genes identified in our screens, we included two other Arabidopsis genes for RNA gel blot analyses: PR-1, a well characterized SAR-class gene and thus a control (Uknes et al. 1992), and ECS1 because, like NIR1 and NIR2, it encodes a small, secreted, proline-rich protein that is induced by exposure to X. campestris (Aufsatz et al. 1998). Templates used for probes included full-length cDNAs (SAR1, NIR1, and GRP3), PCR products (NIR2 and NIR3), or cloned cDNA–AFLP fragments (see below).

Gene expression induced by the incompatible P. parasitica isolate Noco2.

To confirm our cDNA–AFLP results, we used RNA gel blot analysis to examine the same RNA samples that were used to create the cDNA templates. RNAs were extracted from plants 1 and 4 days postinoculation (dpi) with the incompatible P. parasitica isolate Noco2. RNA gel blots were prepared with 5 or, where needed, 15 µg of total RNA to provide sensitivity, because some probes produced weak hybridization signals. In every case where a strong signal was observed with a RNA blot, its pattern in the three host genotypes was similar to that seen in the cDNA–AFLP analysis (i.e., SAR, NIR, or SIR profiles; Fig. 2). For specific genes, a number of quantitative comparisons between genotypes could be made from RNA gel blot analyses that were not obvious from cDNA–AFLP. For example, SAR1 showed some induction by Noco2 in nim1-1 and NahG, although this induction was significantly less than that seen in wild-type plants. In addition, Noco2 induction of SIR2 and GST11 was stronger in NahG plants than in Ws-0 or nim1-1, suggesting that SA accumulation may repress the induction of these genes by Noco2. Four gene probes produced very weak signals on RNA gel blots (CaBP-22, GST1, SIR3, and SIR4), whereas cDNA–AFLP showed unambiguous Noco2-induced RNA accumulation. When we used RNA blot analysis, we were unable to detect SIR4 induction by Noco2, even with 15 µg of RNA loaded onto the blotted gel. The signal seen on the SIR4 autoradiogram is likely a result of cross-hybridization of the probe to a related, constitutively expressed gene because two bands can be observed to hybridize to the SIR4 probe on RNA blots that examined Emco5-
induced expression (Fig. 4). We are confident, however, that the four genes are induced by Noco2 because they were identified in cDNA–AFLP screens that identified other Noco2-induced transcripts and are induced by the compatible *P. parasitica* isolate Emco5 (Fig. 4).

**Gene expression induced by the compatible *P. parasitica* isolate Emco5.**

Most pathogen-induced genes characterized to date are responsive to compatible and incompatible pathogens, although induction by the former usually shows slower induction kinetics and a lower magnitude response (Bell et al. 1986; Kiedrowski et al. 1992). By contrast, rapid gene expression induced by incompatible pathogens is associated with effective defense because the rapid response presumably is mediated by *R*-gene signals that are activated in plants exposed to incompatible pathogens.

To test whether genes identified in this study require *R*-gene action for induction, we used RNA gel blot analysis to assess RNA accumulation in plants inoculated with the compatible *P. parasitica* isolate Emco5. We also tested whether induction of the genes had similar requirements for NIM1/NPR1 and SA after inoculation with either compatible or incompatible *P. parasitica* isolates. RNA was prepared from wild type, nim1-1, and NahG plants at 1, 3, and 6 dpi with Emco5 and was used for RNA blot analysis. By 6 dpi, all three host genotypes were heavily colonized with sporulating *P. parasitica*. Most genes that exhibited a NIR or SIR-like pattern in an incompatible interaction were induced in a similar manner in the compatible interaction (Fig. 4). *EC31* and *SAR1*, however, had slightly different expression patterns than those seen in response to the incompatible *P. parasitica* (compare Figs. 2 and 4). Whereas *EC31* expression in response to incompatible Noco2 strictly requires SA and NIM1/NPR1, the virulent Emco5 elicited a modest *EC31* induction in nim1-1 mutants, suggesting that Emco5 can induce this gene through a NIM1/NPR1-independent pathway that is not activated by the incompatible pathogen. Similarly, *SAR1* showed a weak induction in NahG and nim1-1 plants after exposure to Noco2, whereas Emco5 elicited a strong induction. The induction of *SAR1* by Emco5 was similar in magnitude in nim1-1 and NahG plants, suggesting that this response is not the result of leakiness of the nim1-1 mutation or NahG transgene but rather is a response independent of SA and NIM1. The fact that *PR-1* was not induced by Emco5 at 6 dpi in the two defense-impaired genotypes also supports this interpretation.

**Gene expression induced by INA.**

Because SA or its synthetic analog INA induces many *PR* genes in a NIM1/NPR1-dependent manner, we tested the genes identified in our screen for their response to INA and whether any observed induction required NIM1/NPR1. RNA was collected from wild-type, nim1-1, and NahG plants at 1 and 4 days after application of INA, and RNA accumulation levels in each sample were analyzed by probing RNA gel blots with radiolabeled probes corresponding to each identified gene (Fig. 5). In most cases, transcript levels increased in wild-type and NahG plants after application of INA. Neither *SAR1* nor *PR-1* were induced in nim1-1 plants, a result consistent with our observations of Noco2-treated plants and previous work with PR-1 (Delaney et al. 1995). *NIR1* and *NIR2* were induced by INA in all three genotypes but their expression levels were lower in nim1-1 plants compared with wild-type or NahG, suggesting that NIM1/NPR1 plays some role in the expression of these genes. About half of the *SIR*-class genes were induced by INA. Surprisingly, whereas Noco2 induction of *GST2*, *GST11*, *SIR1*, and *pEARLI-4* did not require a NIM1/NPR1 function, induction of these genes by INA did require it. An exception was the *GRP3* gene, which showed significant induction by INA in nim1-1 plants, clearly demonstrating the existence of a NIM1/NPR1-independent response to INA. For most of the genes in all classes, INA-induced RNA accumulation was similar in NahG and wild-type plants, an anticipated occurrence given previous observations that INA induces *SAR* and *SAR* genes such as *PR-1* in NahG plants (Vernooij et al. 1995). INA induction of the *EC31* gene, however, was significantly stronger in wild-type plants compared with NahG, indicating that INA cannot fully complement SA deficiency in NahG plants (Fig. 5).
DISCUSSION

We used cDNA–AFLP to identify Arabidopsis genes induced by exposure to an avirulent P. parasitica isolate. This method proved to be sensitive, allowing us to identify genes that were not expressed at high levels, and specific, permitting closely related mRNA species to be observed in isolation. For example, we are able to document that mRNAs derived from three conserved glutathione S-transferase genes are upregulated by pathogen exposure. We describe 15 genes identified in our screens that show three general classes of regulation according to their requirement for SA and NIM1/NPR1 activity, SA alone, or neither. Some of the genes may be important for plant defense on the basis of their similarity to other previously characterized genes. Two of these genes, CaBP-22 and NIR4, may play regulatory roles in signaling pathways, and many are predicted to encode small, secreted proteins, characteristics shared with a variety of proteins suggested to have antimicrobial properties (Otvo 2000).

We examined approximately 7,000 cDNA–AFLP bands and studied the expression of 15 genes that were identified as clearly up-regulated in response to pathogen exposure. The fraction of Noco2-induced genes described (approximately 0.2% of the total set) is not a measure of the prevalence of pathogen-induced genes in Arabidopsis for a variety of reasons. First, we established stringent criteria for the inclusion of genes in this study and describe only genes that showed differential expression by cDNA–AFLP and RNA gel blot analyses. A large number of differentially expressed fragments were likely to be discarded because clear RNA hybridization results were not obtained. For example, differentially expressed genes that are members of conserved, highly expressed gene families may appear constitutively expressed upon gel blot analysis as the result of cross-hybridization. Furthermore, because cDNA–AFLP is likely to be more sensitive than RNA blot hybridization methods, some weakly expressed genes may be visualized only by the former method and excluded from this study. In addition, because cDNA–AFLP is restriction enzyme based, it will fail to identify those pathogen-induced genes whose transcripts lack the appropriate six-base endonuclease recognition site (here, Nco1 or Vsp1), a requirement that biases the screen against smaller transcripts, which many defense-regulated genes produce. Moreover, 7,000 fragments are not representative of 7,000 individual genes. In many cases, at least two Nco1–Tag1 (or Vsp1–Tag1) fragments will be generated from each double digest. We found that several of the described differentially regulated genes were recovered more than once in our screens. For these reasons, we emphasize the regulatory properties of the described genes rather than the abundance of Noco2-induced genes.

CaBP-22 encodes a putative CaM-like protein, with the greatest similarity to Arabidopsis CaM2/3/5 isoforms (65% identical amino acids), and is a member of the CLAT subfamily of EF-hand protein (Kawasaki et al. 1998; R. Kretzinger, personal communication). Like plant CaMs, CaBP-22 contains four EF-hand domains involved in calcium binding (Ling and Zielinski 1993). CaBP-22 is distinguished from plant CaMs by an unusual three amino acid deletion in the flexible hinge region that joins the second and third Ca2+-binding domains and by a 45 amino acid carboxyl-terminal extension similar (57% identical, 77% similar) to that in one other known protein, the Arabidopsis CaM-related protein-3/TCH3 (Sistrunk et al. 1994), which is induced by mechanical stimuli. A variety of evidence indicates that calcium–CaM signaling is an important component of plant defense responses. For example, isolated parsley cells treated with pathogen-derived elicitors show a dramatic increase in extracellular Ca2+ uptake, which is required to induce a number of elicitor-responsive genes (Nurnberger et al. 1994). Furthermore, increases in intracellular Ca2+ levels were shown to precede the HR elicited in cowpea by a fungal pathogen, and Ca2+ antagonists interfered with HR formation (Xu and Heath 1998). A role for CaM in defense signaling also was suggested by the constitutive defense phenotype displayed by transgenic tobacco plants that expressed either of two soybean fungal elicitor-induced CaM genes (Heo et al. 1999). In our work with intact plants, we observed a strong induction of CaBP-22 mRNA accumulation after infection with the virulent P. parasitica isolate in wild-type and nim1-1 but not NahG plants. The avirulent P. parasitica isolate produced a similar but much less marked induction, and INA produced a modest induction in each plant genotype. It will be interesting to determine whether the haustral invasion of plant cells induces CaBP-22 expression on the basis of its similarity to the touch-responsive TCH3 gene.

The pathogen-induced NIR4 gene may potentially play a role in signal transduction, given that it encodes a predicted protein that contains ankyrin repeats and putative transmembrane domains. Ankyrin repeats are found in a diverse set of proteins and may be involved in protein–protein interactions (Bork 1993). Among these are a number of signaling proteins such as the Drosophila cactus and vertebrate IkB proteins that interact with transcription factors Dorsal and NF-kB, respectively (Belvin and Anderson 1996); the Arabidopsis NIM1/NPR1 protein, which plays an essential role in the regulation of SAR (Cao et al. 1997; Ryals et al. 1997); and the Drosophila mechanosensory transduction channel NOMPC, which contains, in addition to ankyrin repeats, several transmembrane domains (Walker et al. 2000).

NIR1, NIR2, and ECS1 genes are predicted to encode a family of small, secreted, proline-rich proteins. Whereas these features provide few clues to their function, some secreted proline-rich proteins such as Drosophila drosocin and metchnikowin have potent antimicrobial activity (Charlet et al. 1996; Levashina et al. 1995; Otvo 2000). The primary structures of the proline-rich NIR-class genes we describe are not similar to the Drosophila peptides or to other characterized antimicrobial peptides. However, even the two Arabidopsis NIR1 and NIR2 paralogs, which show extensive similarity at the nucleotide level, have considerable variation in their amino acid sequences, suggesting that these proteins may be subject to a diversifying selection (Kretzman and Akashi 1995). The gene structures of NIR1 and NIR2 are particularly interesting. The DNA sequences of these two unlinked loci are highly conserved outside of the coding regions (Fig. 3a), whereas the open reading frame sequences corresponding to the predicted processed proteins are quite variable, predictably manifesting itself as a low level of similarity between the two proteins (Fig. 3b). Thus, the promoter regions, 5′ and 3′ UTR sequences, and sequences encoding putative signal peptides for these two genes are 86% identical, whereas the sequences predicted to encode the processed proteins align poorly, possibly as a result of the many small deletions or insertions within...
these genes. The significance of this surprising gene structure is unclear but may suggest a transposition or recombination hot spot or very strong diversifying selection for these genes. Despite the poor conservation of the predicted NIR1 and NIR2 proteins, the coding sequences do have similarities: the genes contain a single intron in corresponding positions and both proteins are predicted to have similar amino-terminal signal sequences, two cysteine residues, and an abundance of proline residues.

Our analysis of the regulation of genes identified in this screen illustrates the complexity of plant responses to pathogens. Some of these features and pathways implicated by this study are modeled (Fig. 6) such that SAR, NIR, and SIR pathways are indicated by solid arrows and dashed and dotted lines correspond to redundant or alternate pathways that lead to the induction or repression of particular genes outside of their primary pathway assignment. Although we assigned genes into three categories, genes within a group were not always strictly coordinately regulated. This is apparent when comparing the induction of SAR1 and PR-1 by virulent Emco5 and avirulent Noco2 isolates of P. parasitica. Both genes require SA and NIM1/NPR1 for full induction after exposure to Noco2, whereas the induction of SAR1 by Emco5 does not require SA accumulation or NIM1/NPR1 activity. This difference may result from the two P. parasitica isolates activating distinct signaling pathways that differ in their influence on each gene’s promoter. SAR1 induction by INA does seem to be highly NIM1/NPR1 dependent, however, so it seems likely that the pathogen induction of SAR1 in wild-type plants is the cumulative result of SA-dependent and -independent pathways converging on the SAR1 promoter. The observation that full INA induction of ECS1 requires endogenous salicylic acid is interesting because the response of other genes to INA is similar in wild-type and NahG plants. Because INA induction of ECS1 also requires NIM1/NPR1, it is possible that ECS1 expression relies on a SA- and NIM1/NPR1-dependent amplification loop. Such feedback loops have been proposed to play a role in the induction of gene expression and other defense responses (Delaney 1997; Jirage et al. 1999; Weymann et al. 1995) and provide one possible explanation for the unusual regulation pattern exhibited by ECS1.

Induction of NIR class genes by P. parasitica is dependent upon SA accumulation yet does not require NIM1/NPR1. Full induction of these genes by INA, however, was not observed in nim1-1 plants, suggesting that NIR gene induction occurs by NIM1/NPR1-independent and -dependent signaling. The NIR1 and NIR2 gene promoter regions contain elements with striking similarity to the PR-1 gene promoter LSU1 motif, which has significant similarity to the kB box and shown to be important for INA-dependent induction of PR-1 (Lebel et al. 1998). Although NIR1 and NIR2 differ from PR-1 in their overall regulation pattern, full induction of each by INA requires NIM1/NPR1, suggesting that this shared promoter element may mediate the NIM1/NPR1-dependent responses of NIR1 and NIR2. The nine SIR class genes also show differences in their responses to elicitors. SIR2 and SIR4 appear unresponsive to INA, whereas the other SIR-class genes are induced. Induction of SIR1, GST2, and GST11 by INA is at least partially dependent upon NIM1/NPR1, whereas GRP3 shows nearly wild-type induction in nim1-1 plants.

These data demonstrate the existence of an array of pathogen-responsive regulatory pathways that control plant defense gene expression. cDNA microarray experiments performed by Maleck et al. (2000) revealed even more regulatory complexity controlling the subset of genes that were analyzed in both studies. Eight of the fifteen genes we identified by cDNA–AFLP also were analyzed by microarray (Maleck et al. 2000) and, although the specific treatments and genotypes analyzed in that work differed from ours, a number of interesting comparisons and contrasts can be made between the two sets of experiments. For instance, SAR1 (EST 141F18T7) (Maleck et al. 2000) indeed showed a NIM1/NPR1-dependent induction in both sets of experiments, even though the microarray data was obtained with secondary (uninfected) tissue that had been induced by an avirulent Pseudomonas spp. strain on other plant parts. Interestingly, SAR1 is one of the few genes in the microarray analysis that had dramatically altered expression in the NIM1/NPR1-overexpression line tested, which suggests that its expression is very sensitive to NIM1/NPR1-regulated cues. In both studies, NIR1 (EST 103C7T7) expression was much reduced in NahG plants. In the microarray analysis, this gene may have had a high basal level of expression in the reference wild-type plants, inferred by the large negative fold-induction values observed in the NahG plants tested (Maleck et al. 2000). Pathogen induction of NIR1 was affected only modestly by a nim1-1 mutation in the other study, which also is consistent with our data involving a nim1-1 allele. Perhaps most interesting are the contrasting results seen between studies, with respect to the glutathione S-transferase genes analyzed by both groups. The microarray analysis demonstrated that systemic induction by avirulent Pseudomonas spp. of GST1 (EST 206N21T7), GST2 (EST 242J18T7), and GST11 (EST 248O1T7) requires SA and NIM1/NPR1, an observation
that is different from our results that show local *P. parasitica* induction of these genes to be independent of, if not enhanced by, the absence of SA and mutation of *NIM1/NPR1*. Moreover, on the basis of microarray-determined expression profiling, Maleck et al. (2000) placed the GST genes within the PR-1 regulon, whereas our analysis indicates that the GST genes should be considered part of a distinct expression class. These differences may result from the different pathogens and host genotypes used in each study, or the microarray study may be complicated by cross-hybridization between GST family members. Alternatively, the contrasting results obtained in the two studies may be the result of real differences in the regulation of GST genes in local versus systemic tissues.

It is apparent from studies of various Arabidopsis mutants with altered disease resistance that plant defense signaling is not controlled by a small number of simple linear pathways. Our data confirm that this also is true at the transcriptional level for pathogen-induced genes, suggesting that a number of independent yet interacting pathways regulate various genes. This complexity in plant defense responses may be the result of an “evolutionary arms race” with pathogens, with new host defense strategies being layered over old in order to adapt to recurring selective pressures imposed by pathogens.

In this study, we describe three sets of Arabidopsis genes that differ in inducibility in wild-type, *nim1-1*, and NahG plants after inoculation with *P. parasitica* or treatment with INA. The range of induction patterns reveals the existence of distinct regulatory pathways defined by their requirement for SA accumulation and the function of the NIM1/NPR1 protein. These genes may define pathways that support important arms of the defense machinery in plants, help to define the complexity of pathogen-induced responses, and point to new avenues for engineering disease resistance in plants. Genes with the NIR pattern of regulation may be useful markers for the salicylate-dependent, SAR-independent resistance mechanisms that have been implicated in studies of mutants and NahG plants. For example, the constitutive defense mutants *cpr5*, *cpr6*, *accd*, and *ssi1* express SA-dependent, NIM1/NPR1-independent resistance (Bowling et al. 1997; Clarke et al. 1998; Clarke et al. 2000; Rate et al. 1999; Shah et al. 1999). In *cpr5*, NIM1/NPR1-independent resistance is not associated with expression of *PR-1*, suggesting that this mutant expresses a resistance system distinct from SAR (Bowling et al. 1997). Because *SIR*-class genes are induced by pathogens independent of SA and NIM1/NPR1, they may identify resistance pathways independent of SA accumulation such as those implicated by observations of SA-independent gene-for-gene resistance (Bittner-Eddy and Beynon 2001; Brading et al. 2000; McDowell et al. 2000).

We emphasize that our results do not show function for the genes described; that information will require manipulation of gene expression in combination with pathogen susceptibility assays. *SIR* and *NIR* genes, however, may be useful markers to monitor expression of novel and potentially important defense pathways and thus can be employed in the design of mutant screens aimed at disrupting master regulatory genes of these pathways. This approach was fruitful in the study of SAR, resulting in the discovery of *nim1/npr1* alleles, and may prove to be effective in the isolation of *sir* and *nir* mutants. Because regulatory mutants such as *nim1/npr1* have a more severe phenotype than do plants with altered expression of individual potential effector genes (such as *PR-1*), *sir* or *nir* regulatory mutants may reveal the existence of novel defense pathways by their susceptibility phenotype.

**MATERIALS AND METHODS**

**Plants and growth conditions.**

Ws-0 was obtained from the Ohio State University Arabidopsis Biological Resource Center (Columbus, OH, U.S.A.). Ws *nim1-1* plants were described previously (Delaney et al. 1995), and the Ws-NahG line (Molina et al. 1998) was provided by Syngenta (Research Triangle Park, NC, U.S.A.). Plants were grown at 22°C in short-day conditions (14 h of light and approximately 150 µE fluence provided by cool-white fluorescent lamps), with approximately 60% relative humidity on mesh-covered Cornell soil mix (Boddy and Sheldrake 1977) composed of 0.34 stere of vermiculite, 0.22 stere of peat moss, 0.11 stere of perlite, 2.27 kg of lime, and 1.81 kg of Micromax micronutrient blend (Sierra Chemical, Milpitas, CA, U.S.A.).

**Biological and chemical elicitation.**

*P. parasitica* isolate Noco2 (Crute et al. 1992) was provided by J. Parker (The Sainsbury Laboratory, Norwich, U.K.) and Emco5 (Holub and Beynon 1996) was provided by J. Dangl (University of North Carolina, Chapel Hill, U.S.A.). Noco2 and Emco5 were maintained on Col-0 or Ws-0 hosts, respectively, as described in Uknes et al. (1992). Inoculum was prepared from 7-dpi plants by placing heavily sporulating leaves into water, gently vortexing, and centrifuging the liquid to collect the conidiospores, which were resuspended in water (6 × 10^5 conidiospores per ml). The spore suspension was misted onto 17- to 21-day-old Arabidopsis plants with a Preval compressed air paint sprayer (Precision Valve, Yonkers, NY, U.S.A.), and plants were covered with a clear-plastic dome to maintain high humidity, which is optimal for *P. parasitica* germination and growth. Chemical induction of SAR was achieved by misting plants with a 0.33 mM suspension of INA (0.25 mg per ml of a formulation containing 25% INA plus wettable powder), which was obtained from Syngenta. INA treatment, as described, did not elicit cell death or produce visible changes in plant appearance.

**RNA extraction and analysis.**

Aerial plant tissue was cut off at the described time points and immediately frozen in liquid nitrogen, and RNA was extracted as described by Lagrimini (1987). The methodology for cDNA–AFLP was performed as described by Bachem et al. (1996), with Taq1 as the frequent-cutting endonuclease and *Nco1* or *Vsp1* as the rare-cutting enzyme. The poly(A)^+ mRNA used to construct cDNA was extracted from 100 µg of total RNA with a biotinylated d(T)~25~ oligonucleotide and Streptavidin Magnasphere paramagnetic beads (Promega, Madison, WI, U.S.A.). The first and second cDNA strands were synthesized on paramagnetic beads as described by Lambert and Williamson (1997). Restriction endonuclease digestion of the bead-coupled cDNA liberated cDNA fragments, which were then removed from the paramagnetic beads. Adaptors ligated to the digested cDNA were 5′-ctgtagactctgac-3′ and 3′-ctgacgctgtac-5′, for *Nco1*; 5′-ctgtagactctgac-3′ and 3′-ctgacgctgtac-5′, for
To PCR amplify the adapter-ligated cDNA fragments, 2.0 µl of a 40-µl ligation was used as template in a 20-µl PCR with primers complementary to corresponding adaptors (NcoI amplification primer 5'-cttctatt-3' and Vsp1 primer 5'-gacgatgagtcctgac-3'). The reaction conditions for this PCR were 1× PCR buffer, 1.5 mM MgCl₂, 0.25 mM deoxynucleoside triphosphate (dNTP), 0.25 mM primer each, and 1 U of Taq DNA polymerase (GIBCO-BRL, Carlsbad, CA, U.S.A.), with 30 PCR cycles (94°C, 30 s; 50°C, 30 s; and 72°C, 60 s) in a DNA Engine thermal cycler (MJ Research; Waltham, MA, U.S.A.). After the initial amplification, the DNA concentrations were normalized between samples by running 8.0 µl of the 20 µl reaction on a 2% agarose gel and diluting the remaining 12 µl 10- to 20-fold for use in the selective amplification. Then, to selectively amplify a subset of the cDNA fragments, pairs of selective primers identical to the initial amplification primers but containing two arbitrary 3' selective nucleotides (256 primer combinations for each enzyme pair) were used. To permit visualization of the reaction products by autoradiography, the selective amplification was performed with a ³²P end-labeled selective primer corresponding to the rare-coupling restriction enzyme (NcoI or Vsp1). Rare-cutter selective primers were labeled with ³²P ATP using T4 polynucleotide kinase, as recommended by the enzyme provider (New England Biolabs, Boston, MA, U.S.A.). The reaction conditions for selective amplification were 1× PCR buffer, 1.5 mM MgCl₂, 0.05 µM labeled primer, 20 µM unlabeled primer, 0.2 µM dNTP, and 1 U of Taq DNA polymerase in 10-µl volume. Thermal cycling was performed as described in Bachem et al. (1996), as was subsequent analysis of radiolabeled PCR products with polyacrylamide gel electrophoresis and autoradiography. A more detailed protocol is available upon request.

Gel slices containing differentially expressed bands of interest were identified by alignment with the autoradiogram, excised from the polyacrylamide gel, and boiled for 15 min in water covered with mineral oil. To PCR amplify the desired band, 2.0 µl of the eluted DNA was used as template using the selective primers that identified the DNA as well as the conditions described above. The resulting PCR product was purified with a Qiaquick PCR purification kit (Qiagen, Valencia, CA, U.S.A.) and sequenced directly at the Cornell University BioResource Center with the selective NcoI or Vsp1 primer.

The resulting sequence data allowed us to identify the corresponding genes and cDNA clones with genomic databases and the Arabidopsis cDNA Sequence Analysis Project (a joint project at the University of Minnesota and Michigan State University). For RNA gel blot analysis, a variety of sources were used to generate probes. PCR-amplified AFLP bands were used as probes if sequence analysis showed that they consisted almost entirely of a single DNA species. To ensure that hybridization results were not the result of contaminating PCR products, a second probe made from the corresponding cDNA clone obtained from the Ohio State Stock Center was used to verify the results. Other probes were made from cloned PCR products, which were sequenced to verify that they corresponded to the predominant DNA species in the PCR product. Results obtained with cloned PCR products were often superior to those with cDNA clones because they were smaller and thus presumably less likely to cross-hybridize with paralogous genes. cDNA clones were, therefore, not obtained if we had a PCR product clone corresponding to that gene. cDNA inserts from Escherichia coli clones were amplified by PCR with primers corresponding to the T7 and SP6 sites that flank the insert. When necessary, PCR products were cloned into a pBluescript KS+ T-vector (Stratagene, La Jolla, CA, U.S.A.), prepared as described in Marchuk et al. (1991). These TA clones were amplified as described for cDNA clones with T7 and T3 primers, sequenced with T7 primer, and radio labeled as a hybridization probe. Because the NIR3 cDNA–AFLP fragment was small, we amplified a larger fragment from genomic DNA to use as a probe. We also designed a gene-specific NIR2 probe to minimize cross-hybridization with NIR1. Probes used were SAR1: Michigan State University (MSU) EST cDNA clone 141F18T7; PR1: full-length cDNA (Uknes et al. 1992); ECSI: MSU EST cDNA 124K13T7; NIR1: MSU EST cDNA clone 111M12T7; NIR2: amplified gene-specific fragment from genomic DNA with 5'-tctggcgcagcaggaagc and 5'-tgttcccgggagagagagagagagagagagagagaga primers (189 bp); NIR3: designed primers 5'-atactcatgctttcctcagc and 5'-ataataactctgctatcaccac (429 bp); NIR4: cloned cDNA–AFLP fragment 5'-cgagat-ga-3' (273 bp; ellipses indicates region not shown); CabBP-22: cloned cDNA–AFLP fragment 5'-gcgtggttt-3' (181 bp); SIR1: cloned cDNA–AFLP fragment 5'-atgccagtttccggagctattcgc-3' (344 bp); SIR3: cloned cDNA–AFLP fragment 5'-atgagaaaacttatcatcgtcggagcatc-3' (128 bp); SIR4: cloned cDNA–AFLP fragment 5'-agtctgtttgcagttcataagaagatgctc-3' (165 bp); GRP3: MSU EST cDNA clone 162H7T7; pEARLI-4: cloned cDNA–AFLP fragment 5'-caggtcagtcagcagttcataagaagatgctc-3' (114 bp); GST1: cloned cDNA–AFLP fragment 5'-gcaagctattccgagcccctgttattcgc-3' (120 bp); GST2: cloned cDNA–AFLP fragment 5'-aatcaggaagagctgtcctgttattcgc-3' (131 bp); GST3: cloned cDNA–AFLP fragment 5'-aatcaggaagagctgtcctgttattcgc-3' (237 bp). The template DNA for the 18S rRNA probe was excised from clone JHD2-15A (CD3-197; Arabidopsis Biological Resource Center).

RNA gel blot analysis.

RNA gel blot analysis was performed essentially as described in Uknes et al. (1993). Approximately 5 and 15 µg of RNA per sample was fractionated by electrophoresis on denaturing 1.2% agarose gels (1× MGE [20 mM MOPS[3(N-morpholino)propanesulfonic acid], 5 mM EDTA, pH = 7.0], 3% vol/vol of formaldehyde), RNA transferred overnight in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to Nytran nylon membranes (Schleicher & Schuell, Dassel, Germany), and cross-linked to the membrane with a UV Stratalinker 1800 (Stratagene). Probes were made using α-³²P dCTP with a random primer labeling system (GIBCO-BRL). Overnight hybridizations and washes were performed at 65°C as described by Church and Gilbert (1984). Radioactivity was detected with either a phosphor screen and Storm 840 Phosphorimag (Molecular Dynamics, Sunnyvale, CA, U.S.A.) or autoradiography film (LabScientific, Livingstone, NJ, U.S.A.).

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