NHL25 and NHL3, Two NDR1/HIN1-Like Genes in Arabidopsis thaliana with Potential Role(s) in Plant Defense

Anne Varet, Jane Parker, Pablo Tornero, Norbert Nass, Thorsten Nürnberger, Jeffery L. Dangl, Dierk Scheel, and Justin Lee

1Institute of Plant Biochemistry, Department of Stress- and Developmental Biology, Weinberg 3, D-06120, Halle/Saale, Germany; 2 Max Planck Institute for Plant Breeding Research, Department of Molecular Phytopathology, D-50829, Cologne, Germany; 3 Department of Biology and 4 Department of Microbiology and Immunology, and Curriculum in Genetics, Coker Hall CB#3280, University of North Carolina, Chapel Hill, 27599-3280 U.S.A.

Submitted 17 December 2001. Accepted 5 March 2002.

The Arabidopsis genome contains 28 genes with sequence homology to the Arabidopsis NDR1 gene and the tobacco HIN1 gene. Expression analysis of eight of these genes identified two (NHL25 and NHL3 for NDR1/HIN1-like) that show pathogen-dependent mRNA accumulation. Transcripts did not accumulate during infection with virulent Pseudomonas syringae pv. tomato DC3000 but did accumulate specifically when the bacteria carried any of the four avirulence genes avrRpm1, avrRpt2, avrB, or avrRps4. Furthermore, expression of avrRpt2 in plants containing the corresponding resistance gene, RPS2, was sufficient to induce transcript accumulation. However, during infection with an avirulent oomycete, Peronospora parasitica isolate Cala-2, only NHL25 expression was reproducibly induced. Salicylic acid (SA) treatment can induce expression of NHL25 and NHL3. Studies performed on nahG plants showed that, during interaction with avirulent bacteria, only the expression of NHL25 but not that of NHL3 was affected. This suggests involvement of separate SA-dependent and SA-independent pathways, respectively, in the transcriptional activation of these genes. Bacteria-induced gene expression was not abolished in ethylene- (etr1-3 and ein2-1) and jasmonate- (coi1-1) insensitive mutants or in mutants impaired in disease resistance (ndr1-1 and pad4-4). Interestingly, NHL3 transcripts accumulated after infiltration with the avirulent hrcc mutant of Pseudomonas syringae pv. tomato DC3000 and nonhost bacteria but not with the virulent Pseudomonas syringae pv. tomato DC3000, suggesting that virulent bacteria may suppress NHL3 expression during pathogenesis. Hence, the expression patterns and sequence homology to NDR1 and HIN1 suggest one or more potential roles for these genes in plant resistance.

Keywords: gene-for-gene, wounding.

Plants are constantly exposed to potential pathogens and can resist most attacks by activating defense mechanisms. The first crucial step to mounting defense reactions is the recognition of the pathogen (Dangl and Jones 2001). This relies on sophisticated sensing mechanisms for signal molecules that could be pathogen-derived or generated during the infection process (Bent 1996). Defense reactions initiated are usually multicomponent and complex but often occur as a rapid localized cell death at the site of infection (referred to as hypersensitive response [HR]) to contain the pathogen, as well as a systemic acquired resistance (SAR) throughout the plant (Grant and Mansfield 1999).

The “gene-for-gene” hypothesis, demonstrated to control race- and cultivar-specific plant-pathogen interaction, relies on the presence or the absence of an avirulence (avr) gene in the pathogen and a corresponding resistance (R) gene in the plant (Flor 1971). A majority of R genes isolated encode proteins with putative nucleotide binding sites (NBS) and contain leucine-rich repeats (LRR). These NBS-LRR resistance proteins harbor in their amino terminus either a leucine zipper (LZ) or a so-called TIR domain that has homology to the Drosophila Toll and human interleukin-1 receptors (Dangl and Jones 2001).

Genetic analysis in Arabidopsis has been instrumental in unraveling the complex signal transduction in disease resistance, and simplified signaling models have been proposed (Glazebrook 2001). Various signaling pathways can lead to expression of defense-related genes after pathogen attack, and mutant analyses suggest these pathways are required for successful resistance. One pathway is salicylate-dependent and requires genes like EDS1, PAD4, and NPR1. Two other pathways are dependent on jasmonate and ethylene: one pathway does not require NPR1 and is defined by mutants like coi1, ein2, and etr1, and one functions during interaction with nonpathogenic rhizobacteria and requires NPR1. These pathways are not strictly independent of each other, and a complex network may be activated in response to a particular pathogen (Feys and Parker 2000).

Three different networks of R gene-mediated signaling have also been proposed through the analysis of Arabidopsis mutants (Feys and Parker 2000). R genes known to require EDS1 and PAD4 for mediating resistance belong to the TIR-NBS-LRR class, while R genes known to require NDR1 and PBS2 belong to the LZ-NBS-LRR class (Aarts et al. 1998; Glazebrook et al. 1997; Warren et al. 1999). There are, however, exceptions from this general classification indicating existence of one or more other signaling networks. The RPP8 gene does not require EDS1 or NDR1, while EDS1 and NDR1 in combination appear to mediate full RPP7 function (McDowell et al. 2000). Like RPP8, RPP13 encodes an LZ-NBS-LRR protein, and RPP13-mediated resistance functions independently of NDR1, PBS2, EDS1, and PAD4 (Bittner-Eddy and Beynon 2001). Another
exception is RPW8, a broad-spectrum R gene with low similarity to the NBS-LRR genes that requires salicylate and EDS1 (Xiao et al. 2001). The EDS1 and PAD4 proteins share sequence similarity with lipases, although enzyme activity has not been shown (Falk et al. 1999; Jirage et al. 1999). The sequence of NDR1 does not provide any clue to possible biochemical function, but it is predicted to be a membrane protein (Century et al. 1997). Furthermore, NDR1 has limited sequence similarity to the tobacco HIN1 protein, and its transcript also accumulates upon pathogen attack (Century et al. 1997). The HIN1 gene is activated by the bacterial elicitor harpin and bacteria with a functional hrp gene cluster (Gopalan et al. 1996). A partial sequence of the tobacco gene, NG2, identified as an inducer of HR-like cell death (Karrer et al. 1998) also shares sequence similarity with HIN1.

The Arabidopsis genome contains 28 genes that display similarities to NDR1 and HIN1 (The Arabidopsis Genome Initiative 2000). These have been tentatively designated as NHL1-28 (NDR1/HIN1-like) and grouped into several subclasses on the basis of sequence similarities (Dörmann et al. 2000). Like NDR1, one to two putative transmembrane domains are predicted for these NHL proteins although, in some cases, these domains also coincide with predicted cleavage sites of signal peptides. However, similarities to NDR1/HIN1 are restricted to short amino acid stretches. This suggests that the NHLs might represent protein families sharing common structural motifs but not necessarily similar biochemical properties or signaling roles. We postulate that those members having a role in pathogen response may share similar expression patterns with NDR1 and HIN1. Therefore, we have screened some of these NHL genes for their capacity to respond to bacterial infection. Two out of eight genes studied showed transcript accumulation specifically during an incompatible interaction, and detailed expression studies suggested that they potentially define two new response pathways to pathogen infection.

**RESULTS**

**Differential expression of a subset of the NHL genes.** To screen for pathogen-responsive members of the NHL gene family, we infiltrated Arabidopsis Columbia leaves with phytopathogenic bacteria and analyzed gene expression by RNA gel blot and reverse transcription-polymerase chain reaction (RT-PCR). Only a subset of the NHL sequences were available in the public databases when this project was initiated; thus, only eight of these genes (NHL3, NHL9, NHL19, NHL23, NHL24, NHL25, NHL26, and NHL27) (Dörmann et al. 2000) have been investigated. The use of RT-PCR allowed us to distinguish between related cross-hybridizing sequences as well as increasing the detection of low expression levels. In one case, RT-PCR and DNA sequencing allowed the detection of an unpredicted 80-bp intron in NHL25 (accession number...
NM123055). In all other cases, the sizes of the RT-PCR amplified bands corresponded well to the predicted values, confirming a lack of introns in the other seven genes studied.

Increased transcript levels of two genes (NHL3 and NHL25, accession numbers NM120715 and NM123055) were detected upon infiltration with Pseudomonas syringae pv. tomato DC3000 (Pst DC3000) strains that carried an avirulence gene (avrRpm1, avrRpt2, avrB, or avrRps4), all of which initiate incompatible interactions (Fig. 1A). Depending on the type of avirulence gene involved, transcript accumulation began about 6 to 12 h after infiltration and decreased after 24 to 48 h (Fig. 1A). While expression of both genes is induced, the overall levels are quite different. NHL25 transcripts accumulate to much lower levels than NHL3 transcripts and are detectable only with RT-PCR. Reprobing the blots (data not shown) revealed very similar expression patterns of NDR1 (Century et al. 1997). However, gene expression was not induced by infiltration with a 10 µM solution of the HrpZ-encoded harpin over a period of 24 h (data not shown). Thus, the expression pattern of these two genes resembles that of NDR1 but not of HIN1. The virulent Pst DC3000 strain did not lead to increased expression of either of the two genes within the time period tested (Fig. 1A). Interestingly, the timing of NHL25 and NHL3 transcript accumulation preceded HR development and the expression of PRI transcripts, which were first detected 12 to 24 h postinfection (not shown).

Since increased expression of both NHL25 and NHL3 appears to be confined to incompatible interactions, it is likely that this relies on the recognition of the avirulence gene products. Indeed, transgenic plants containing the avirulence gene avrRpt2 under the control of an estradiol-inducible promoter (Tornero et al. 2002) expressed both NHL25 and NHL3 when treated with estradiol (Fig. 1B). This induced expression is dependent on a functional RPS2 gene for recognition of avrRpt2 gene product, as is evident from the lack of NHL25 and NHL3 expression in rps2 mutant plants after estradiol treatment. Hence, expression of an avirulence gene in plant cells containing the corresponding R gene is sufficient to activate one or more signal cascades leading to increased NHL25 and NHL3 expression.

In order to verify if the enhanced gene expression was restricted to bacterial pathogens, we analyzed the incompatible interaction between Arabidopsis thaliana ecotype Col-0 and the oomycete pathogen Peronospora parasitica isolate Cala-2. Interestingly, RT-PCR analysis revealed a clear accumulation of NHL25 mRNA, whereas no significant changes in NHL3 transcript levels were detected (Fig. 1C).

Salicylic acid accumulation is required for bacteria-mediated expression of NHL25 but not of NHL3.

Since salicylic acid (SA) has been shown to play a central role in the activation of defense genes and SAR (Glazebrook 2001), we tested the effect of SA on NHL25 and NHL3 expression. Levels of NHL3 transcripts increased rapidly in a biphasic manner, while NHL25 transcripts started to accumulate only 12 h after SA treatment (Fig. 2). Therefore, SA is sufficient for inducing NHL25 and NHL3 expression. To investigate if SA is also necessary for pathogen-induced expression of NHL25 and NHL3, SA-deficient Arabidopsis (Col-0) plants expressing the salicylate hydroxylase gene (nahG) (Delaney et al. 1995) were infiltrated with Pst DC3000 (avrRpm1). Expression of NHL25 in these plants was reduced compared with wild-type plants, whereas expression of NHL3 was not affected (Fig. 3A). Taken together, the results demonstrate that while SA treatment is sufficient to induce transcript accumulation of both genes, it is not necessary for the enhancement of NHL3 expression during incompatible interactions with bacterial pathogens.

Ethylene and jasmonate are not required for bacteria-induced expression of NHL25 and NHL3.

Jasmonate and ethylene are, like SA, potential signaling molecules involved in the regulation of many defense-related genes (Reymond and Farmer 1998). To investigate the role of ethylene, the bacteria-induced expression of NHL25 and NHL3 was tested in ethylene response mutants. Mutant etr1 (Bleecker et al. 1988) is altered in its ability to perceive and react to ethylene due to a dominant mutation in the ETR1 gene that encodes an ethylene receptor (Chang et al. 1993). This mutation does not significantly affect the NHL25 and NHL3 transcript levels after avirulent bacterial treatment (data not shown). To eliminate the possibility that the ‘leaky’ phenotype of etr1 (Chang and Shockey 1999) may mask any requirement for ethylene, we analyzed the effect of another ethylene response mutant, ein2 (Guzman and Ecker 1990). The enhanced expression of NHL25 and NHL3 during the incompatible interaction is, as in etr1, not affected by ein2 (Fig. 3A). Hence, sensitivity to ethylene is not essential for induction of NHL25 and NHL3 expression after infiltration with avirulent bacterial pathogens.

Exogenous application of methyl jasmonate failed to induce transcript accumulation of NHL25 or NHL3, although transcripts of other jasmonate-responsive genes, such as Atjrg21 (Bau 2001), accumulated (data not shown). To exclude a possible role of endogenous jasmonate, we studied NHL25 and NHL3 expression levels after pathogen challenge in the coi1-1 mutant. The coi1-1 mutant is insensitive to methyl jasmonate and impaired in the jasmonate (JA) signaling pathway (Feyes et al. 1994). As shown in Figure 3A, this mutation does not significantly affect NHL25 and NHL3 transcript levels after infection with avirulent bacteria. Thus, jasmonate is not an essential signal molecule in mediating NHL25 and NHL3 mRNA accumulation.

NHL25 and NHL3 expression during bacterial incompatible interaction is independent of NDR1, EDS1, and PAD4.

The ndr1-1 mutant is susceptible to Pst DC3000 carrying any one of the four avirulence genes avrB, avrRpm1, avrRpt2, or avrRphB (Century et al. 1995), and defines a specific R gene-dependent signaling pathway (Aarts et al. 1998). Since NHL25 and NHL3 display some sequence homology with NDR1 and respond similarly to infection with avirulent bacteria, the dependence of NHL25 and NHL3 transcript accumulation on NDR1 was investigated. Neither NHL25 expression nor

Fig. 2. Salicylic acid-mediated mRNA accumulation of NHL25 and NHL3. Plants were sprayed with 100 µM salicylic acid (SA) and analyzed by reverse transcription-polymerase chain reaction (NHL25) or RNA gel blots (NHL3). Equal loading of RNA was checked by amplifying a constitutively expressed gene (EF1α) or by methylene-blue staining of the ribosomal bands, respectively. Similar results were obtained in two other independent experiments.
NHL3 expression were found to be affected by the ndr1-1 mutation during the avrRpm1-RPM1 interaction (Fig. 3A).

EDSI and PAD4 are required for resistance mediated by TIR domain-containing R genes (Aarts et al. 1998). The increased expression of NHL25 and NHL3 was not abolished after infiltration of the pad4-1 mutant (Glazebrook et al. 1996) with Pst DC3000 (avrRps4), which stimulates the TIR RPS4-mediated resistance pathway (Gassman et al. 1999) (Fig. 3B). In agreement with the finding that EDS1 and PAD4 interact in plant cells (Feys et al. 2001), the eds1-1 mutation (Parker et al. 1996) also did not block the induction of NHL25 and NHL3 expression after infiltration with Pst DC3000 (avrRps4) (data not shown). However, the induction of NHL25 transcript accumulation appears to be slightly delayed in the pad4-1 mutant (Fig. 3B).

**Virulent Pst DC3000 may suppress the bacteria-induced NHL3 expression.**

Inoculation with virulent bacteria did not lead to increased expression of NHL25 and NHL3 (Fig. 1A). From these data, it seems unlikely that the expression of these genes is mediated by general elicitors such as surface components of the bacterium. Accordingly, NHL25 and NHL3 expression was not altered after infiltration of Arabidopsis leaves with the flagellin peptide elicitor flg15 (Felix et al. 1999) (data not shown). However, it remains possible that the virulent bacterial strains produce suppressor molecules that block NHL25 and NHL3 expression. Thus, we analyzed NHL25 and NHL3 expression after challenge with a Pst DC3000 hrcC Type III-secretion deficient mutant that is incapable of delivery of effector proteins (Roine et al. 1997) as well as with the nonhost bacteria P. syringae pv. phaseolicola Race 6 and its corresponding hrpA pilus mutant (Lee et al. 2001). NHL25 expression was not significantly induced by these bacteria (Fig. 4). Interestingly, elevated levels of NHL3 mRNAs were observed after treatment with the Pst DC3000 hrcC mutant strain, P. syringae pv. phaseolicola and P. syringae pv. phaseolicola hrpA mutant (Fig. 4). Hence, NHL3 expression can be induced by an unknown elicitor that may be common to at least the two tested phytopathogenic bacteria. More importantly, this activity appears to be suppressed by virulent Pst DC3000.

**Fig. 3.** Pathogen-induced expression of NHL25 and NHL3 in A, salicylic acid (SA)-deficient (nahG), ethylene-insensitive (ein2-1), jasmonate-insensitive (coi1-1), ndr1-1 mutants and B, pad4-1 mutants. Plants were treated and analyzed by reverse transcription-polymerase chain reaction (NHL25) or RNA gel blots (NHL3). Equal loading of RNA was checked by amplifying a constitutively expressed gene (EF1α) or by methylene-blue staining of the ribosomal bands, respectively. Similar results were obtained in two other independent experiments. Note that four more polymerase chain reaction cycles were necessary to visualize the low level of NHL25 expression in nahG plants (top middle panel) compared with wild-type Columbia (Col-0) plants.
**NHL3 transcripts accumulate rapidly in local and systemic tissues after wounding.**

In some experiments, NHL3 transcript levels also increased transiently in leaves infiltrated with a solution of 10 mM MgCl₂ (data not shown). This rapid but transient induction may be due to a wounding effect. Indeed, after wounding of leaves with for- ceps, NHL3 transcripts rapidly accumulated locally in wounded leaves but also systemically in the unwounded leaves (Fig. 5). Dissection of the components of both signaling cascades leading to expression of NHL3 may provide insights into how wound and pathogen signaling overlap. We therefore investigated the possible involvement of some of the signals ascribed to pathogen attack and wounding, such as SA, jasmonate, and ethylene.

The wound-induced expression pattern was unaltered in SA-deficient nahG plants (Fig. 5). Arabidopsis exhibits a transient increase in ethylene production after wounding (Rojo et al. 1999), but no effect of the etr1 mutation or of the ein2 mutation was detectable on the NHL3 expression pattern in response to wounding (Fig. 5). Similarly, the wounding response of NHL3 was also not significantly affected in the jasmonate-insensitive coil-1 mutant (Fig. 5). Hence, none of the hormone signals studied were found to be essential for wound-mediated expression of NHL3.

**DISCUSSION**

On the basis of sequence homology, NDR1/HIN1-like (NHL) genes have been identified in the Arabidopsis genome (Dörmann et al. 2000). The encoded proteins are thought to be possible mediators of pathogen defense (Dörmann et al. 2000). It is, however, not known if all of them are required for R function or are involved in defense gene activation. Several genes encoding important defense signal components including NDR1 (Century et al. 1997) or EDS1 and PAD4 (Falk et al. 1999; Jurage et al. 1999) are also pathogen-responsive, probably as part of a signal feedback amplification loop. Pathogen-responsive members of the NHLs are thus likely to be involved in the defense response. We have identified two members of this NHL family (NHL25 and NHL3) that show transcript accumulation after pathogen attack. Transcripts of six other genes from this gene family that were studied did not accumulate after inoculation with virulent or avirulent bacteria. As yet, we cannot rule out the possibility that these six genes might also play significant roles in the plant’s response to other pathogens. We focused on the characterization of two NHL genes that are clearly responsive to the tested pathogens.

Figure 6 shows a model summarizing the findings of this work. We found that interaction with bacteria expressing four different avirulence genes and also the expression of the avirulence gene avrRpt2 in planta led to the increased expression of NHL25 and NHL3 (Fig. 1). Our studies involved genetic interactions of R genes comprising both the TIR (RPS4, RPP2) and the LZ (RPM1, RPS2) types with their matching avr genes (Fig. 1). The results showed that the bacteria-induced expression of NHL25 and NHL3 can be mediated through either structural class of R protein. Recognition of the avirulence gene product by the R gene product, whether direct or indirect, is sufficient to evoke one or more signal events leading to NHL25 and NHL3 expression. The increased expression of both genes correlated with the subsequent appearance of HR. In this respect, it is interesting that the tobacco NG2 gene (Karrer et al. 1998), which has some sequence homology to the NDR1 and HIN1 gene family, has been isolated through a functional screen for HR induction in tobacco. Furthermore, transgenic plants expressing NHL25 ectopically led to ‘light-dependent speck-like’ disease symptoms and elevated levels of PRI expression (Dörmann et al. 2000). Hence, it is possible that expression of genes such as NHL25 and NHL3 may be involved in PRI gene expression or HR development, or both.

Interestingly, only NHL25 reproducibly showed mRNA accumulation after infection with *P. parasitica* isolate Cala2 (Figs. 1C and 6). Another difference between the two genes is the differential requirement of SA for enhanced gene expression after avirulent bacterial challenge (Fig. 3). Thus, after recog- nition of the potential pathogen, signal events diverged into SA-dependent and SA-independent pathways for NHL25 and NHL3, respectively (Fig. 6).

Depending on the avr and R genes involved, the transcript accumulation of NHL25 occurs as early as 6 h after pathogen challenge (Fig. 1A) and requires SA (Fig. 3). Thus, it is sur- prising that NHL25 transcripts accumulate relatively late after SA treatment, which is not an uptake problem since expression of NHL3 is already induced (Fig. 2). This suggests that SA is not the intermediate signal between infection and induction of NHL25 expression. More likely, one or more unknown factors generated in incompatible plant-pathogen interactions acts in synergy with SA to induce NHL25 expression (Factor X in Fig. 6). This factor X alone would be insufficient to induce NHL25 expression in the nahG plants, and SA alone would only lead to late transcript accumulation in the absence of this factor (Fig. 6).

SA treatment leads to a biphasic accumulation of NHL3 transcripts (Fig. 2). This is reminiscent of the biphasic pattern recently described for the SA-induced physical interaction between NPR1/NIM1 and TGA2 transcription factor within the plant nucleus (Subramaniam et al. 2001); these proteins are known to mediate response to SA (Despres et al. 2000). Interestingly, the NHL3 promoter contains two in- verted TGACG sequence elements. TGACG motifs were found to be required for the binding of TGA-hZIP transcription factors (Schindler et al. 1992) and were shown to be im- portant within the PRI promoter for response to treatment with the SAR-inducing chemical, INA (Lebel et al. 1998). It is thus tempting to speculate that the inverted TGACG se- quences within the NHL3 promoter may be involved in the response to SA treatment.

Strikingly, inoculation with nonhost bacteria *P. syringae* pv. *phaseolicola* and its Type III plus (hrpA) mutant led to enhanced NHL3 (but not NHL25) expression (Fig. 4). This indicates that delivery of avr or effector proteins into the plant cell via the Type III secretion system (TTSS) is not necessary for the nonhost bacteria-induced expression of NHL3. Furthermore, this suggests the existence in *P. syrin-
ylene. It remains to be determined if electrical, hydraulic, or wounding. However, we can rule out the role of SA, JA, or ethylene to clarify the nature of the systemic signal that activates this wound response was independent of ethylene and SA are also similar to that of NHL3 after wounding. We can rule out the role of SA, JA, or ethylene. It remains to be determined if electrical, hydraulic, or cytosolic acidification events (Bowles 1998; Herde et al. 1999) are wound signals for activation of NHL3.

With the aid of Arabidopsis mutants, we attempted to dissect the signal pathways involved in regulating expression of NHL25 and NHL3. Neither gene required ethylene and jasmonate for increased expression (Fig. 3). They therefore belong to a different set of pathogen-responsive genes than e.g. plant defensins that require these hormones for induced expression (Penninckx et al. 1998). Enhanced NHL25 expression depends on the recognition of Avr proteins by the matching plant R proteins (Fig. 1). Interestingly, this expression, mediated through either TIR-NBS-LRR or LZ-NBS-LRR R genes, was not abolished in the ndr1-1 mutant and only slightly delayed in the pad4-1 mutant (Fig. 3). Thus, the signal events leading to the induction of NHL25 belong to either a pathway upstream of or parallel to these mediated by NDR1 or PAD4 and EDS1. In the case of NHL3, the induced expression during incompatible plant-bacteria interaction clearly defines a novel pathway, as no such pathway independent of SA, ethylene, and jasmonate has been described.

Although NHL25 and NHL3 share sequence homology and similar expression patterns after inoculation with avirulent bacteria, they are differentially regulated by virulent and nonhost bacteria and by wounding (Figs. 1A, 4, 5, and 6). We propose that NHL25 may be used as a specific marker gene for incompatible interactions with pathogens and possibly for HR development. NHL3 expression, on the other hand, can be triggered by multiple biotic and abiotic stresses in addition to regulation by avirulent bacteria in a gene-for-gene manner.

Most pathogen-related genes studied to date show transcript accumulation in response to both avirulent and virulent pathogens although, in the latter case, a delayed reaction of lower intensity has frequently been observed (Kombrink and Somssich 1997). Genes that are exclusively induced during interaction with avirulent pathogens are likely to be implicated in mediating resistance. Here, we describe one gene (NHL25) that fits this description for gene-for-gene interactions. NHL3 is also such a defense-related gene but, in addition, is induced after inoculation with nonhost bacteria and appears to be suppressed by virulent bacteria in a TTSS-dependent manner (Fig. 4). This suggests the importance of the gene product in counteracting susceptibility to bacteria. Unfortunately, we failed to isolate any “knock-out” lines for

**Fig. 5.** Wound-induced expression of NHL3 in Arabidopsis and mutant plants. Plants were wounded with forceps and analyzed by RNA blots. Unwounded plants were also included as untreated controls (top panel). Internal controls for RNA levels were checked by methylene-blue staining of the ribosomal bands.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Col-0</th>
<th>nahG</th>
<th>etr1-3</th>
<th>ein2-1</th>
<th>col1-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0 ½ 1 3</td>
<td>½ 1 3</td>
<td>½ 1 3</td>
<td>½ 1 3</td>
<td>½ 1</td>
</tr>
<tr>
<td>Local</td>
<td>0 ½ 1 3</td>
<td>½ 1 3</td>
<td>½ 1 3</td>
<td>½ 1 3</td>
<td>½ 1</td>
</tr>
<tr>
<td>Systemic</td>
<td>0 ½ 1 3</td>
<td>½ 1 3</td>
<td>½ 1 3</td>
<td>½ 1 3</td>
<td>½ 1</td>
</tr>
</tbody>
</table>
NHL25 and NHL3, even after screening three different T-DNA or transposon insertion libraries.

In conclusion, the exclusive expression patterns and their homology to the resistance-mediating gene, NDR1, are suggestive that these two genes may be involved in resistance.

MATERIALS AND METHODS

Plant and growth conditions.

All experiments were performed with Arabidopsis thaliana ecotype Columbia (Col-0). The Col-0 ett-1-3 and the Col-0 ein2-1 plants were obtained from the Ohio State University Arabidopsis Biological Resource Center (Columbus, OH, U.S.A.). Plants were grown in a phytochamber (Heraeus Voetsch, Balingen, Germany) at 22°C, either under short-day conditions (8 h light and 16 h darkness) for infection experiments or under long-day conditions (16 h light and 8 h darkness) for seed set in a potting mixture consisting of soil and sand (2:1).

Bacterial strains and plasmids.

The bacterial pathogen P. syringae pv. tomato DC3000, its corresponding avirulent strains expressing the avirulence genes avrRpt2, avrRpm1, avrB, and avrRps4 and Pst DC3000 hrcC mutant have been described previously (Debener et al. 1991; Hinsch and Staskawicz 1996; Staskawicz et al. 1987; Whalen et al. 1991; Yuan and He 1996). Generation of the hra4 mutant of P. syringae pv. phaseolicola Race 6 was described in Lee and associates (2001). Bacteria were grown at 28°C in King’s B medium (King et al. 1954) containing 50 µg of rifampicin per ml and the appropriate antibiotics required for plasmid maintenance. The avrRpt2, avrRpm1, avrB, and avrRps4 genes were expressed in P. syringae strains on plasmids pV288, pK48, pVB01 (Kunkel et al. 1993), and pVSP61 (Hinsch and Staskawicz 1996), respectively.

Bacterial and oomycete inoculation.

Plants were infected by infiltration with bacterial suspensions of 10^8 CFU per ml in 10 mM MgCl₂ (optical density at 600 nm of 0.2) as described previously (Kiedrowski et al. 1992). Peronospora Cala2 infections were performed as described by Aarts and associates 1998. Leaf material was harvested at the indicated time points, frozen in liquid N₂, and stored at -70°C. For each time point, four leaves per plant from three individual plants were pooled.

Treatment with salicylic acid and methyl jasmonate.

For hormone treatments, leaves were sprayed with a 100 µM solution of salicylic acid (SERVA, Heidelberg, Germany) or methyl jasmonate (ZENON Corporation, Tokyo). Methyl jasmonate treatment was also performed by floating excised leaves at 25°C under constant light conditions (120 µmol/m²/s). For harpin treatment, leaves were infiltrated with 10 µM harpin (gene product of HrpZ) diluted in 5 mM MES pH5.5 (Lee et al. 2001).

RNA extraction and analysis.

Plant material was ground in liquid N₂, and RNA was isolated using TRIZOL-reagent (Gibco Life Technologies, Karlsruhe, Germany) according to the manufacturer’s instructions. RNA was quantified by UV-spectroscopy and 10 µg or 0.2 µg were used for RNA blot or RT-PCR analyses, respectively. RT-PCR was performed using the Ready-to-Go RT-PCR system (Amersham Pharmacia Biotech, Freiburg, Germany). First-strand cDNA synthesis was primed using oligo-dT primer for 30 min at 42°C. The reaction was divided into two tubes. One reaction was used as internal control by amplifying a fragment of translation elongation factor 1-alpha, EF-1α (Curie et al. 1993). The second reaction was used for amplifying the gene of interest by adding the corresponding primer combinations. PCR was initiated at 95°C (4 min), followed by 25 cycles of 10 s denaturation at 94°C, 10 s annealing at a suitable Tₐ for the primer pairs, and 40 s synthesis at 72°C. A final step of synthesis at 72°C for 10 min was used to complete the reaction. Preliminary experiments were used to verify that PCR conditions were not saturated.

For quantification, the PCR-amplified products were separated on agarose gels, transferred onto nylon-membranes (HybondN+, Amersham Pharmacia Biotech) and hybridized to ³²P-labeled DNA probes. DNA probes used in RNA and DNA

Fig. 6. Model summarizing the deduced pathways of treatments that induce NHL25 and NHL3 expression. Avirulent bacteria induce mRNA accumulation of NHL25 and NHL3 in a gene-for-gene-dependent manner but via two different signaling pathways (SA-dependent and SA-independent pathways). Although salicylic acid (SA) is sufficient in inducing NHL25 and NHL3 expression and is required for NHL25 expression, it is probably not the intermediate signal; an unknown factor (X) has to be proposed. Transcript accumulation of NHL25 is further induced by Peronospora parasitica isolate Cala2, while NHL3 is responsive to nonhost bacteria and wounding. Virulent Pst DC3000 probably suppresses the NHL3 transcript accumulation during compatible interactions via an unknown effector Y in a Hrp-dependent manner. The pathogen responsiveness, homology to NDR1, and suppression of NHL3 induction by virulent Pst DC3000 suggest potential roles of these two NHL genes in defense response.
analyses were synthesized using the Megaprime kit (Amersham Pharmacia Biotech) and α-32P-dATP. Hybridization was performed in 5x SSPE (1x SSPE is 0.18 M NaCl, 10 mM NaPO4, and 2 mM EDTA [pH 7.4]), 5x Denhardt’s reagent (Denhardt 1966), 0.1% sodium dodecyl sulfate (SDS), 100 µg of denatured salmon sperm DNA per ml, and 50% formamide at 42°C overnight, and the blots were washed twice at 60°C in 0.5x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS for 20 min and in 0.1x SSC and 0.1% SDS for 10 min. Radioactivity was visualized and quantified using a PhosphorImager (STORM, Molecular Dynamics, Amersham Pharmacia Biotech). For quantitative analysis of RNA gel blots, a 26S-rDNA probe was used to normalize differences in loading.

Primers used for amplifications are as follows: 5′-TCACTAT-CAACATTTGTTGTAATTGC-3′ and 5′-TGGATCTGTGAAGGAGGG-3′. The Arabidopsis Genome Initiative 2000. Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature 408:796-815.


