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

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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 *pad4-1*). 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 *NPRI*. Two other pathways are dependent on jasmonate and ethylene: one pathway does not require *NPRI* and is defined by mutants like *coi1*, *ein2*, and *etr1*, and one functions during interaction with nonpathogenic rhizobacteria and requires *NPRI*. 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

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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

Fig. 1. Pathogen-induced mRNA accumulation of two *NHL* genes. **A**, *Pseudomonas syringae* pv. *tomato* DC3000 infiltration. Six-week-old plants were infiltrated with magnesium chloride (MgCl₂), *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000), and *Pst* DC3000 carrying the indicated avirulence genes. Leaf tissues (including two untreated controls [UC]) were collected at the indicated time points and analyzed by reverse transcription-polymerase chain reaction (RT-PCR) (*NHL25*) or RNA gel blots (*NHL3*). Equal loading of RNA was checked by amplifying a constitutively expressed gene (translation elongation factor 1 alpha, *EF1α*) or by methylene-blue staining of the ribosomal bands, respectively. Similar results were obtained in two other independent experiments. **B**, Transgenic plants expressing *avrRpt2* under the control of an estradiol-inducible promoter were sprayed with 10 μM estradiol to express *avrRpt2* in plants containing a mutated (*rps2*) or a functional *RPS2* gene (*RPS2*). RNA was extracted and analyzed by RT-PCR or RNA gel blot as described above. **C**, *Peronospora parasitica* infection. *Arabidopsis* plants were sprayed with spores of *P. parasitica* (*Pp*) isolate Cala-2 resuspended in water. Leaf tissues were collected at the indicated time points and were analyzed by RT-PCR. The expression levels of *NHL25* and *NHL3* are shown as the ratio of transcript levels in *Pp*-inoculated to water-treated tissues (normalized to the 16 h time point). Bars represent the means and standard errors of three independent experiments.

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 μ M 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 *PR1* 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).

Fig. 2. Salicylic acid-mediated mRNA accumulation of *NHL25* and *NHL3*. Plants were sprayed with 100 μ M 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 (*EF1 α*) or by methylene-blue staining of the ribosomal bands, respectively. Similar results were obtained in two other independent experiments.

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 (Feys 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 *avrPphB* (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

NHL3 expression were found to be affected by the *ndr1-1* mutation during the *avrRpm1-RPM1* interaction (Fig. 3A).

EDS1 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 forceps, *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 *coi1-1* 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; Jirage 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 for 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.

Fig. 4. *Pseudomonas syringae*-induced mRNA accumulation of *NHL25* and *NHL3*. Plants were treated with the indicated bacterial strains 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 parallel experiments. P. s. ph. = *Pseudomonas syringae* pv. *phaseolicola*.

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 *NHL2* ectopically led to 'light-dependent speck-like' disease symptoms and elevated levels of *PR1* expression (Dörmann et al. 2000). Hence, it is possible that expression of genes such as *NHL25* and *NHL3* may be involved in *PR* 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 recognition 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 surprising 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 inverted TGACG sequence elements. TGACG motifs were found to be required for the binding of TGA-bZIP transcription factors (Schindler et al. 1992) and were shown to be important within the *PR1* 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 sequences 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 pilus (*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-*

gae pv. *phaseolicola* of an elicitor that does not require the TTSS to induce *NHL3* mRNA accumulation. The nature of this elicitor is unknown, but it is unlikely to be the flg15 peptide (Felix et al. 1999), since this peptide did not trigger *NHL3* expression (data not shown). Such an elicitor could also be responsible for the *NHL3* induction obtained with the *Pst* DC3000 *hrcC* mutant (Fig. 4). The absence of *NHL3* transcript accumulation after treatment with the virulent strain *Pst* DC3000 suggests that the bacteria secrete via the TTSS one or more effector proteins that interfere with *NHL3* mRNA accumulation (Fig. 6, effector Y). By contrast, although this suppressor is still present during incompatible interactions, coinjected Avr proteins such as AvrRpm1, AvrRpt2, AvrB, or AvrRps4 may overcome this suppressor effect (Figs. 1 and 4). It has been shown that animal and plant bacterial pathogens are able to suppress host defense mechanisms by means of effector proteins secreted by the TTSS (Brown et al. 1995; Jackson et al. 1999; Orth et al. 2000; Tsiamis et al. 2000). Hence, the possibility that virulent *Pst* DC3000 may suppress *NHL3* transcript accumulation and the fact that avirulent as well as nonhost bacteria induced *NHL3* expression suggest a potential role for *NHL3* in the *Arabidopsis* general resistance against bacteria. This is reminiscent of the suppression of defense gene expression and phytoalexin accumulation in bean by virulent *P. syringae* pv. *phaseolicola* (Jakobek et al. 1993; Jakobek and Lindgren 1993).

NHL3 showed a rapid wound-inducible expression in local and systemic leaves (Figs. 5 and 6). It is conceivable that pathogens may take advantage of wound sites for entry into plants and, hence, the rapid activation of wound and pathogen-responsive genes such as *NHL3* may serve as an early attempt to elevate the defense status against possible infections. The tomato *Twil* gene, encoding a putative glycosyl transferase (O'Donnell et al. 1998), shows an expression pattern strikingly similar to *NHL3*. This gene responded rapidly to wounding, pathogen-derived factors, and also SA. The time frame of local and systemic gene activation after wounding and the fact that this wound response was independent of ethylene and SA are also similar to that of *NHL3*. Further studies are needed to clarify the nature of the systemic signal that activates *NHL3* after wounding. However, 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.

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 *etr1-3* 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 *hrpA* 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 10⁸ 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 (ZEON 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 T_m 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 $\alpha^{32}\text{P}$ -dATP. Hybridization was performed in 5 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaPO₄, and 2 mM EDTA [pH 7.7]), 5 $\times$ 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 0.5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS for 20 min and in 0.1 $\times$ 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'-TCACATCAACATTGTGGTCATTGGC-3' and 5'-TTGATCTGGTCAAGAGCCTCAAG-3' for *EF-1 α* , 5'-CCAAGACACAGCAAGCAGCACC-3' and 5'-CCCGAGTTTGATCCGAACCG-3' for *NHL25* (NM_123055); 5'-ATGGCGGACTTAAACGGTGC-3' and 5'-TCAAAAGTCAACGTCACACTTGGTCGG-3' for *NHL3* (NM_120715), 5'-CAGCCACTCATCATGCAACCG-3' and 5'-TCCAACGAAGTCGACCGTCG-3' for *NHL23* (NM_120716), 5'-GCCCAAGCTAGAATTGATGCCG-3' and 5'-GTTAGGCTGCGACAAAGAGACCG-3' for *NHL24* (NM_122192), 5'-ACGTCTCCGGCAATCCATCG-3' and 5'-GCTGATCGTCAAGATACAAGGCG-3' for *NHL27* (accession number AB005237, nucleotide 9667-10146), 5'-GGCGAACGGATTAAACGGCG-3' and 5'-TCAACGTGGCACTTGGTGGG-3' for *NHL9* (NM_129098), 5'-CATTGCGC-CAGAAAAGGAGGG-3' and 5'-TATGCACCCGAGACCCATGTC-3' for *NHL26* (NM_124752), 5'-AAGCAGACGACGCAGCCAAAGC-3' and 5'-CGTCCAGTCTTAATCGCACGAC-3' for *NHL19* (NM_116371).

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