

Arabidopsis NDR1 Is an Integrin-Like Protein with a Role in Fluid Loss and Plasma Membrane-Cell Wall Adhesion^{1[W][OA]}

Caleb Knepper, Elizabeth A. Savory, and Brad Day*

Graduate Program in Genetics (C.K., B.D.), Department of Energy-Plant Research Laboratory (C.K.), and Department of Plant Pathology (E.A.S., B.D.), Michigan State University, East Lansing, Michigan 48824

Arabidopsis (*Arabidopsis thaliana*) NON-RACE-SPECIFIC DISEASE RESISTANCE1 (NDR1), a plasma membrane-localized protein, plays an essential role in resistance mediated by the coiled-coil-nucleotide-binding site-leucine-rich repeat class of resistance (R) proteins, which includes RESISTANCE TO *PSEUDOMONAS SYRINGAE*2 (RPS2), RESISTANCE TO *PSEUDOMONAS SYRINGAE* PV *MACULICOLA*1, and RPS5. Infection with *Pseudomonas syringae* pv *tomato* DC3000 expressing the bacterial effector proteins AvrRpt2, AvrB, and AvrPphB activates resistance by the aforementioned R proteins. Whereas the genetic requirement for NDR1 in plant disease resistance signaling has been detailed, our study focuses on determining a global, physiological role for NDR1. Through the use of homology modeling and structure threading, NDR1 was predicted to have a high degree of structural similarity to Arabidopsis LATE EMBRYOGENESIS ABUNDANT14, a protein implicated in abiotic stress responses. Specific protein motifs also point to a degree of homology with mammalian integrins, well-characterized proteins involved in adhesion and signaling. This structural homology led us to examine a physiological role for NDR1 in preventing fluid loss and maintaining cell integrity through plasma membrane-cell wall adhesions. Our results show a substantial alteration in induced (i.e. pathogen-inoculated) electrolyte leakage and a compromised pathogen-associated molecular pattern-triggered immune response in *ndr1-1* mutant plants. As an extension of these analyses, using a combination of genetic and cell biology-based approaches, we have identified a role for NDR1 in mediating plasma membrane-cell wall adhesions. Taken together, our data point to a broad role for NDR1 both in mediating primary cellular functions in Arabidopsis through maintaining the integrity of the cell wall-plasma membrane connection and as a key signaling component of these responses during pathogen infection.

Defense signaling in plants following pathogen infection results in the activation of multiple, often parallel, signaling pathways. To accomplish this, the activation of plant resistance signaling requires the utilization of numerous preformed defense responses, systemic and cell-to-cell signaling, and, in many cases, the initiation of gene-for-gene resistance (Knepper and Day, 2010). While examples of the activation and regulation of parallel processes have been described (van Wees et al., 2000), there are numerous instances in which bifurcation exists (Wiermer et al., 2005). In plants, it is now evident that regulation and specificity often have overlapping nodes; the guard hypothesis is one such example (for review, see Chisholm et al.,

2006; Jones and Dangl, 2006). In short, host resistance (R) proteins are responsible for the indirect recognition of pathogen effectors by means of “guarding” a virulence target and initiating an effective defense response.

The largest class of R genes found in plants encode for nucleotide-binding site-Leu-rich repeat (NB-LRR) signaling molecules (Moffett, 2009). NB-LRR R proteins have been historically divided into two subgroups, based on the N-terminal presence of either a coiled-coil (CC) domain or a domain with similarity to the Toll IL-1 receptor (TIR) family of proteins (for review, see Takken and Tameling, 2009). While R proteins play a central role in the activation of resistance following pathogen perception, R proteins alone are not sufficient for the initiation of resistance. As such, numerous ancillary proteins and chaperones serving as coactivators of resistance have also been identified (Azevedo et al., 2002; Muskett et al., 2002; Tornero et al., 2002; Hubert et al., 2003; Belkhadir et al., 2004). For example, ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1; Parker et al., 1996) has been shown to mediate defense signaling through the activation of the TIR domain-containing R proteins, while NON-RACE-SPECIFIC DISEASE RESISTANCE1 (NDR1; Century et al., 1995, 1997; Coppinger et al., 2004) has been shown to be required for R proteins that contain

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* Corresponding author; e-mail bday@msu.edu.

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the CC domain (Aarts et al., 1998). There are, however, exceptions to this rule; several CC-NB-LRR R proteins that specify resistance to the oomycete pathogen *Hyaloperonospora arabidopsidis* function independently of NDR1 (McDowell et al., 2000; Bittner-Eddy and Beynon, 2001). Thus, while there appears to be conservation in terms of specificity, function, and activation of signaling through both EDS1 and NDR1 in terms of R protein structure, the full mechanism(s) associated with these pathways is unknown.

Of the two primary signaling components required for the activation of R protein-mediated resistance, the role of EDS1 in defense signaling is best understood. EDS1 serves as a central regulatory protein involved in both biotic and oxidative stress signaling (for review, see Wiermer et al., 2005). In this capacity, EDS1, through its interaction with PHYTOALEXIN-DEFICIENT4 and SENESCENCE-ASSOCIATED GENE101, is required for elicitation of the hypersensitive response (HR) during bacterial infection (Feys et al., 2001, 2005). EDS1 has also been shown to function in halting post-invasive growth of nonpathogenic fungi (Yun et al., 2003) as well as functioning in oxidative stress signaling (Wiermer et al., 2005). Thus, EDS1 can be classified as a broad-spectrum signaling mediator required for the activation of resistance to multiple pathogen types (Hu et al., 2005).

In contrast, the function of NDR1 within the CC-NB-LRR signaling pathway remains unclear. NDR1 is a plasma membrane-localized protein (Century et al., 1997) that undergoes multiple posttranslational modifications, including C-terminal processing (i.e. glycosylphosphatidylinositol anchoring) and N-linked glycosylation (Coppinger et al., 2004). Unlike most glycosylphosphatidylinositol-anchored proteins, NDR1 appears to be unique in that the anchor at the C terminus is resistant to cleavage by phospholipase C and therefore possibly positions NDR1 within the plasma membrane as a "double anchored" protein (Coppinger et al., 2004). This positioning further raises the possibility that NDR1 may play a role in signaling from within the apoplast, transducing the signal from the extracellular space to within the cell, possibly via its association with RPM1-INTERACTING PROTEIN4 (RIN4; Day et al., 2006); however, this hypothesis remains untested. NDR1 has been demonstrated to associate with RIN4 (Day et al., 2006), which has been shown to be required for both the regulation and activation of resistance mediated by at least two members of the CC-NB-LRR class of R proteins (Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003; Belkhadir et al., 2004; Day et al., 2005; Chisholm et al., 2006). The association of RIN4 with the resistance proteins RESISTANCE TO *PSEUDOMONAS SYRINGAE* PV *MACULICOLA*1 (RPM1; Grant et al., 1995) and RESISTANCE TO *PSEUDOMONAS SYRINGAE*2 (RPS2; Kunkel et al., 1993) is believed to maintain the proteins in a signaling-incompetent state (Ellis and Dodds, 2003; Chisholm et al., 2006). The association of NDR1 with a key negative regulator of R protein activation (RIN4) may

in fact point to a role for NDR1 as a signal recognition element upstream of the activation of specific R protein signaling (Belkhadir et al., 2004) and, moreover, may signify a role for NDR1 in broader defense and stress-associated responses requiring both NDR1 and RIN4.

The activation of defense signaling through ligand-receptor interactions (e.g. pathogen recognition receptor-pathogen associated molecular pattern [PAMP]) is likely preceded by the mechanical stimulation of responses at the onset of pathogen invasion. For example, in mammalian innate immune and stress signaling, integrins have been characterized as a central signaling component of the mechanosensing apparatus of cells (Kumamoto, 2008). Integrins are ubiquitous plasma membrane receptors that recognize extracellular glycoproteins (e.g. collagen and fibronectins) via the conserved, solvent-exposed Arg-Gly-Asp (RGD) motif (Gee et al., 2008). On the cytoplasmic side, integrins connect the extracellular matrix to the cytoskeleton, and following signal recognition, they can initiate signaling responses associated with cell differentiation (Streuli, 2009) and growth and proliferation (Huvneers et al., 2007). In plants, peptides containing the RGD motif have been shown to block numerous processes, including fungal toxin penetration (Manning et al., 2008), mechanoperception (for review, see Telewski, 2006), cell wall adhesion to the plasma membrane (Canut et al., 1998), and abiotic stress responses (Zhu et al., 1993; for review, see Gao et al., 2007). Similarly, RGD-containing peptides have also been shown to interfere with cell signaling responses initiated during interactions with microorganisms, particularly those involving fungal and oomycete pathogens (Kiba et al., 1998; Mellersh and Heath, 2001; Senchou et al., 2004). While bona fide integrin proteins have not been identified in plants, high-affinity RGD-binding sites have been identified (Canut et al., 1998), and it is hypothesized that these sites mediate plasma membrane-cell wall adhesion.

In this work, we describe a role for *Arabidopsis* (*Arabidopsis thaliana*) NDR1 in both defense and stress response signaling based on a predicted structure of NDR1 with homology to biotic and abiotic stress-responsive proteins. Using this predicted structural homology and the known positioning of NDR1 in defense signaling, we demonstrate using molecular-genetic, physiological, and cell biology approaches that NDR1 plays a role in electrolyte release in response to bacterial pathogen infection as well as in maintenance of the plasma membrane-cell wall junction. Mutational analysis of NDR1 supports a role for the RGD-like motif, NGD (Asn-Gly-Asp), as a key motif in NDR1 involved in defense signaling following *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000) infection. Taken together, our data suggest a role for NDR1 in coordinating broader cellular processes in response to stress and bacterial pathogen infection.

RESULTS

NDR1 Shares Predicted Structural Homology with LEA14, an Integrin-Like Protein

Two independent methods were used to generate a predicted structure for NDR1 (Fig. 1A). First, the protein fold recognition server PHYRE (Protein Homology/analogy Recognition Engine; Kelley and Sternberg, 2009) was used to generate a predicted structural model. In the second approach, we utilized the automated homology-modeling server I-TASSER to generate five predicted structures for NDR1 (Supplemental Fig. S1). Both PHYRE and I-TASSER generated similar homology model predictions for NDR1. The outputs of these predictions were then used as a template to guide the structure-function analyses described in this study. Homology modeling and structure threading were used to predict a tertiary fold of NDR1. Once a secondary structure for NDR1 was predicted using PHYRE, homology modeling was performed using MODELER (Martí-Renom et al., 2000) and Chimera (Pettersen et al., 2004). The output of this analysis was then threaded onto the solved structure of the Arabidopsis LATE EMBRYOGENESIS ABUNDANT14 (LEA14) protein (Fig. 1B, pdb_1yy; Singh et al., 2005), a member of the late embryogenesis family of proteins.

Further analysis of the predicted structure was performed in a domain-by-domain manner, from which we identified several striking similarities with mammalian proteins involved in signal perception and innate immune responses (Supplemental Fig. S2). For example, the large β -sheet torus (Fig. 1A, blue arrows) resembles the core structure of type III fibronectins (Potts and Campbell, 1994). Further modeling (Supplemental Fig. S2) suggests that the primary core structure of NDR1 shares strong structural similarity with the membrane-bound subunit (i.e. fibronectin

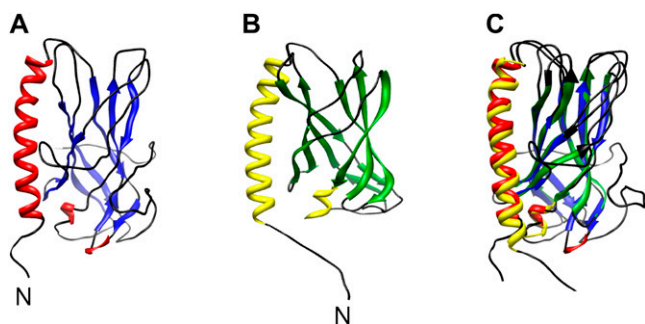


Figure 1. Homology modeling of NDR1 with the integrin-like protein LEA14. A, Predicted structure of NDR1. B, The solved structure of LEA14 (pdb_1yy; Singh et al., 2005). C, NDR1 predicted structure threaded onto the solved structure of LEA14, highlighting the predicted structural homology. N denotes N terminal. PHYRE analysis returned an estimated structural homology precision of 95%, with an E-value of 0.008. Additional predicted models are shown in Supplemental Figure S1.

FNIII domain) of integrin, with the putative transmembrane domains of both NDR1 and integrin connected to large single β -sheets (Hynes, 2009). Adjacent to the three-amino acid α -helix (Fig. 1B, small yellow helix; compare the homologous position in Fig. 1C), we identified the presence of a solvent-exposed RGD-like motif (i.e. NGD) at amino acid positions 178 to 180. In host-fungus interactions, the role of RGD motifs in defense signaling has been characterized as a potential ligand-binding site involved in cell wall-plasma membrane adhesion (Manning et al., 2008; for review, see Dodds et al., 2009). Moreover, RGD sites have also been postulated as both a target and a mode of action for pathogen effector proteins and secretion systems, presumably to facilitate pathogen entry through disrupting cell wall-plasma membrane focal adhesions (Jiménez-Soto et al., 2009; Wang et al., 2009).

NDR1 Plays a Role in Limiting Electrolyte Leakage in Response to *Pst* DC3000 Infection

Based on the proposed function of LEA proteins in Arabidopsis, we hypothesized that NDR1 may play a broad role in regulating fluid loss. While the precise function of the LEA family of proteins remains elusive, numerous studies have linked LEAs to a variety of biotic and abiotic stress responses (De Meutter et al., 2005; Goyal et al., 2005; Battaglia et al., 2008). In support of this, Singh et al. (2005) proposed LEAs to have a role in stopping or slowing the process of fluid loss in response to wounding or dehydration. Based on the predicted structural homology of NDR1 to LEA14, we hypothesized that defense signaling may be regulated, in part through NDR1, as a function of controlling nutrient availability during pathogen infection (Katagiri et al., 2002). When inoculated with the phytopathogenic bacterium *Pst* DC3000, *ndr1-1* plants show altered electrolyte leakage compared with ecotype Columbia (Col-0) plants. These data are in agreement with similar plant-pathogen studies that have utilized electrolyte leakage as a physiological marker for the HR as well as the activation of defense signaling in plants (Baker et al., 1991; Mackey et al., 2002). While previous methods used to measure electrolyte leakage relied on simple conductance measurements over a relatively short time scale (i.e. 24 h; Supplemental Fig. S3; Supplemental Appendix S1), we chose to examine the changes in leakage in response to disease progression, and not simply in correlation to the HR, by utilizing a method previously applied to abiotic stress (Gilmour et al., 1988). When *ndr1-1* plants were dip inoculated with *Pst* DC3000, the leakage measurements observed paralleled those of Col-0 (Fig. 2A). These data are consistent with previously published bacterial growth data and the enhanced disease susceptibility phenotype(s) (Century et al., 1995) while also indicating a possible alteration in membrane permeability in the *ndr1-1* mutant. When plants were inoculated with *Pst* DC3000 expressing the Cys protease effector protein AvrRpt2 (Axtell et al., 2003), the

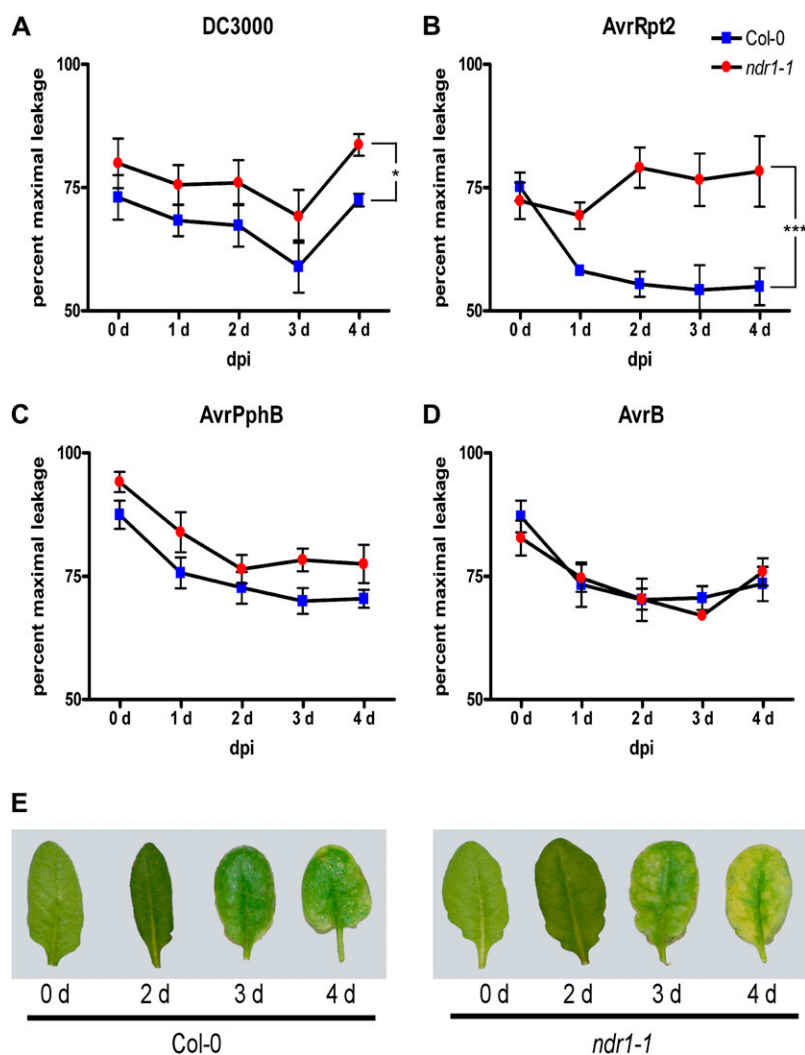


Figure 2. A to D, Enhanced electrolyte leakage in the *ndr1-1* mutant following inoculation with *Pst* DC3000. Levels of electrolyte leakage from Col-0 and *ndr1-1* plants in response to DC3000 inoculation are displayed as percentage maximal leakage. Treatments include *Pst* DC3000 expressing vector control (A), AvrRpt2 (B), AvrPphB (C), and AvrB (D). The dramatic increase in leakage observed in *ndr1-1* as compared with Col-0 when inoculated with *Pst* DC3000 correlates with the onset of disease symptoms. dpi, Days postinoculation. Error bars display SD from four technical replicates from two to three biological replicates. Significance was determined using two-way ANOVA, where asterisks represent statistically significant differences between Col-0 and *ndr1-1*: * $P < 0.05$, *** $P < 0.001$. E, Col-0 and *ndr1-1* leaves at 0, 2, 3, and 4 d postinoculation with *Pst* DC3000 expressing AvrRpt2.

difference in measured electrolytes between *ndr1-1* and Col-0 were in greater contrast, with the most striking difference observed at 2 d postinoculation (Fig. 2B). This result provides further evidence for an alteration in membrane integrity in the *ndr1-1* mutant, as a simple increase in programmed cell death cannot explain the dramatic difference in leakage observed; *ndr1-1* does not undergo HR in response to AvrRpt2 (Century et al., 1995). The large observed difference in leakage also correlates with the onset of disease symptoms in *ndr1-1* (Fig. 2E). To this end, while *ndr1-1* does not exhibit a significant increase in leakage, our findings would suggest that the *ndr1-1* mutant is unable to restrict leakage induced following *Pst* DC3000-AvrRpt2 inoculation. This apparent loss in restricting leakage may be a primary mechanism through which AvrRpt2 is able to enhance susceptibility in *ndr1-1*. Indeed, this hypothesis is further supported by previous results from Freeman and Beattie (2009), which show that Arabidopsis responds to the effector AvrRpm1 by restricting vascular flow to the infected

area. Related to the work presented herein, it is noteworthy that both AvrRpm1 and AvrRpt2 are known to target RIN4 (Mackey et al., 2002, 2003), the only previously identified interacting partner of NDR1 (Day et al., 2006). *Pst* DC3000 expressing the effector proteins AvrPphB (Shao et al., 2003) and AvrB (Mackey et al., 2002) did not elicit a statistically significant difference in the electrolyte release response in *ndr1-1* plants compared with Col-0 (Fig. 2, C and D).

The *ndr1-1* Mutant Displays Altered mRNA Expression of Several Biotic and Abiotic Stress-Responsive Genes

Given the differences between the disease phenotypes in the *ndr1-1* mutant versus Col-0 plants as well as the observation of enhanced electrolyte leakage, we hypothesized that a relationship between these phenotypes and gene expression may exist. We reasoned that altered expression of genes that are known to be associated with nutrient and water stress, as well as other general abiotic stresses associated with drought

tolerance, may be indicators of an altered stasis in *ndr1-1*. Based on the general role of LEA proteins in both biotic and abiotic stress responses as well as the predicted similarity of NDR1 to LEA14 (Fig. 1), we hypothesized that NDR1 may also play a role in abiotic stress tolerance or may be functionally affected by these stresses. We compiled a candidate list of genes from publicly available microarray and expression data (Sato et al., 2007) whose expression profiles may correlate with our observed electrolyte phenotypes. Two members of the LEA family met our search criteria and were investigated for altered mRNA expression in *ndr1-1* plants. LEA group 1 domain-containing protein (At1g32560) showed a nearly 3-fold increase in expression in *ndr1-1* mutant plants as compared with Col-0 when inoculated with *Pst* DC3000 (Fig. 3A). LEA group 1 domain-containing protein also showed altered levels of expression in *ndr1-1* plants when inoculated with *Pst* DC3000 expressing AvrRpt2, but when inoculated with *Pst* DC3000 expressing AvrPphB, a rapid induction was observed in *ndr1-1*, leading to a nearly 4-fold increase in mRNA expression at 48 h postinoculation (hpi; Fig. 3A). The expression patterns of LEA14 were nearly identical between Col-0 and *ndr1-1* under all conditions, with the exception of *Pst* DC3000 expressing AvrRpt2, which showed a nearly 3-fold increase in mRNA at 48 hpi in *ndr1-1* plants (Fig. 3A). This differential expression in LEA family genes observed between Col-0 and *ndr1-1* suggests an increase in cellular stresses occurring within an *ndr1-1* cell following pathogen inoculation and may also indicate a similar role for NDR1 in cellular stress responses as that of LEA14.

Genes associated with water regulation and ion release (e.g. aquaporin homologs; *AtPIP1;3*, *AtPIP1;4*, *AtPIP1;5*, *AtPIP2;5*, and *AtPIP2;6*) were also examined. No significant differences in expression were observed between Col-0 and *ndr1-1* at 0 hpi (Fig. 3B). However, two of the aquaporin homologs (*AtPIP1;4* and *AtPIP2;5*) showed a rapid induction in *ndr1-1* compared with Col-0 when infected with *Pst* DC3000 alone or expressing AvrRpt2 (Fig. 3B). This finding provides further evidence that NDR1 may play a role in mediating fluid loss, as *AtPIP1;4* and *AtPIP2;5* have been previously demonstrated to be up-regulated in response to drought stress (Alexandersson et al., 2005). Consistent with our observed electrolyte leakage data, we did not detect any significant differences in the aquaporin homologs tested between Col-0 and *ndr1-1* following inoculation with *Pst* DC3000 expressing either AvrB or AvrPphB (Supplemental Fig. S4). These data further suggest increased water stress within the cells of *ndr1-1* plants following inoculation with *Pst* DC3000. Interestingly, we observed an increase in *PDF1.2* mRNA expression at 24 hpi with *Pst* DC3000 expressing AvrB in the *ndr1-1* mutant (Supplemental Fig. S5), which is consistent with previously published observations in RIN4-associated mutants (Cui et al., 2010).

NDR1 Self-Associates in Planta via the Formation of Oligomers

One of the primary characteristics of integrins, and integrin-like proteins, is their association in vivo as dimers (Zhao and Newman, 2001). Based on the predicted structural similarity of NDR1 to integrin-like proteins, we hypothesized that NDR1 exists as multimers in planta. To test this, differentially epitope-tagged (e.g. T7 and hemagglutinin [HA] epitope) NDR1 constructs were expressed in *Nicotiana benthamiana* using *Agrobacterium tumefaciens*-mediated transient expression. As shown in Figure 4A, using epitope-tagged NDR1 constructs, we detected a self-association in reciprocal coimmunoprecipitation pull downs, providing another example of integrin-like structure. The band intensity observed for the reciprocal self-associations correlated with differences in total tagged NDR1 protein detection rates (Supplemental Fig. S6).

The NGD Motif Is Not Required for the Interaction of NDR1 with RIN4 or for Self-Association as a Multimeric Protein Complex

RIN4 has been shown to interact with NDR1 (Day et al., 2006). Based on the similarity of the NGD motif to known ligand-binding sites (i.e. the RGD site found in integrins), the possibility exists that this site serves to stabilize the NDR1-RIN4 interaction or as an additional point of interaction between the two associated proteins. Coimmunoprecipitation experiments were performed to isolate the NDR1-RIN4 protein complex (Fig. 4D). T7-tagged NDR1, as well as variants with mutations within the NGD motif (i.e. 178AAA180 and 178RGD180), were tested for association with HA-tagged RIN4. In all cases, an interaction between NDR1 and RIN4 was detected, despite alterations to the NGD motif (Fig. 4, E and F). These data suggest that the NGD motif is not required for the NDR1 association with RIN4 in planta, in agreement with the previous finding that the association occurs within the N-terminal region of NDR1 (Day et al., 2006). Based on the proposed topology of NDR1 (Coppinger et al., 2004), our results are not surprising; moreover, they are consistent with published data describing integrin dimerization and protein association (van der Flier and Sonnenberg, 2001; Zhao and Newman, 2001).

We also tested the impact of mutations in the NGD motif on the NDR1-NDR1 self-association identified in this study. Unlike the NDR1-RIN4 association, the specific residues or motifs required for the formation of multimeric complexes of NDR1 are unknown. HA epitope-tagged NDR1 along with various T7-tagged NDR1 proteins (i.e. 178NGD180, 178AAA180, and 178RGD180) were transiently expressed in *N. benthamiana* using *Agrobacterium*-mediated transient expression. Complexes consisting of NDR1 along with NDR1 variants containing an altered NGD site could successfully be isolated using coimmunoprecipitation (Fig. 4, B and C). These data also demonstrate that the

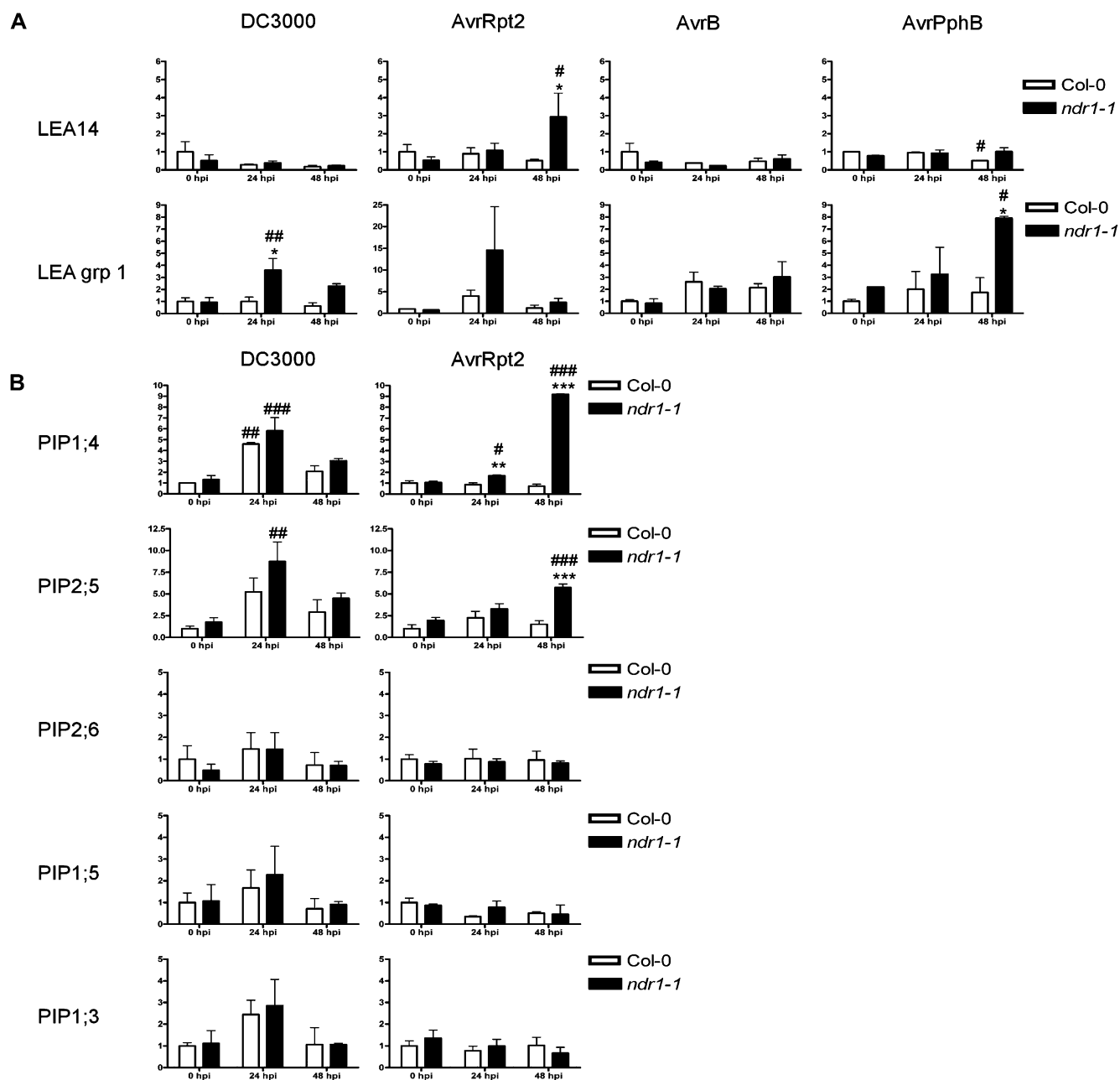


Figure 3. Relative mRNA expression of biotic and abiotic responsive genes in *ndr1-1* following *Pst* DC3000 inoculation. A, Altered levels of expression in wild-type Col-0 and *ndr1-1* mutant plants of LEA family genes in response to *Pst* DC3000 or *Pst* DC3000 expressing AvrRpt2, AvrB, or AvrPphB. B, Expression levels of aquaporin homologs known to be drought responsive in Arabidopsis when inoculated with *Pst* DC3000 or *Pst* DC3000 expressing AvrRpt2. Error bars display SD from one to two technical replicates from two biological replicates. Samples were taken at 0, 24, and 48 hpi. Expression is displayed as 0-h average fold for Col-0. Significance was determined using two-way ANOVA, where asterisks represent statistically significant differences between Col-0 and *ndr1-1* and pound signs represent statistically significant changes over time: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

NGD site is not required for the formation of self-associated multimeric NDR1 protein complexes.

The *ndr1-1* Mutation Has a Reduced Response to the PAMP flg22

While it has long been known that *ndr1-1* mutant plants are compromised in resistance to *Pst* DC3000 as

well as to strains expressing several specific bacterial effector proteins (Century et al., 1995), the underlying mechanisms of this process have never been explored. In this study, we explored this through the analysis of the expression of *FLG22-INDUCED RECEPTOR-LIKE KINASE1* (*FRK1*), an early marker of mitogen-activated protein kinase (MAPK) pathway activation

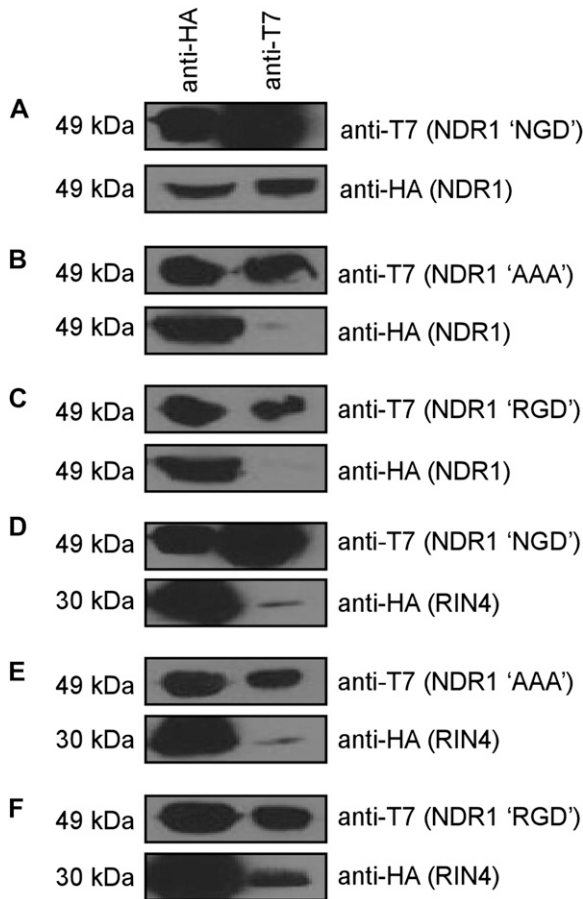


Figure 4. NDR1 associates with itself and RIN4 in planta, and this association is unaffected by mutations in the NGD site. *Agrobacterium* expressing HA:NDR1 or HA:RIN4, along with *Agrobacterium* expressing T7:NDR1 with substitutions in the NGD motif (wild type, NGD, RGD, or AAA), was infiltrated into *N. benthamiana*. Tagged proteins were immunoprecipitated from samples taken 48 h after infiltration using anti-HA (NDR1 or RIN4; right lane) or anti-T7 (NDR1; left lane) antibody. Proteins were detected by blotting with anti-T7 (top blot) or anti-HA (bottom blot) HRP-conjugated antibody. A, T7:NDR1 NGD-HA:NDR1. B, T7:NDR1 AAA-HA:NDR1. C, T7:NDR1 RGD-HA:NDR1. D, T7:NDR1 NGD-HA:RIN4. E, T7:NDR1 AAA-HA:RIN4. F, T7:NDR1 RGD-HA:RIN4. Western blots in Supplemental Figure S6 show relative detection limits.

(Asai et al., 2002), in both Col-0 and *ndr1-1* plants treated with the PAMP flg22. As shown in Figure 5B, the *ndr1-1* mutant showed a significantly weakened response to flg22 treatment, as measured by MAPK activation. Furthermore, total protein levels of MAPK3 and MAPK6, both of which are required for priming of the biotic stress response (Beckers et al., 2009), were reduced in *ndr1-1* plants treated with flg22 as compared with Col-0 (Fig. 5C).

Mutation of the NGD Site in NDR1 May Compromise Resistance to *Pst* DC3000

Our experimental approach assessing the role of the NGD site in mediating protein-protein interactions revealed no detectable impact on the NDR1-RIN4 or

NDR1-NDR1 association (Fig. 4), so the question remains whether mutations in this site alter the overall resistance response to *Pst* DC3000. To test this, we generated stable T7-tagged transgenic lines in the *ndr1-1* mutant background expressing the two mutant variants of NDR1 (i.e. RGD and AAA) at positions 178 to 180 as well as an NDR1 complemented line (i.e. NGD). As shown in Figure 6A, the NGD complemented line displayed resistance to *Pst* DC3000 and *Pst* DC3000 expressing each of the three effector proteins described above at a level comparable to wild-

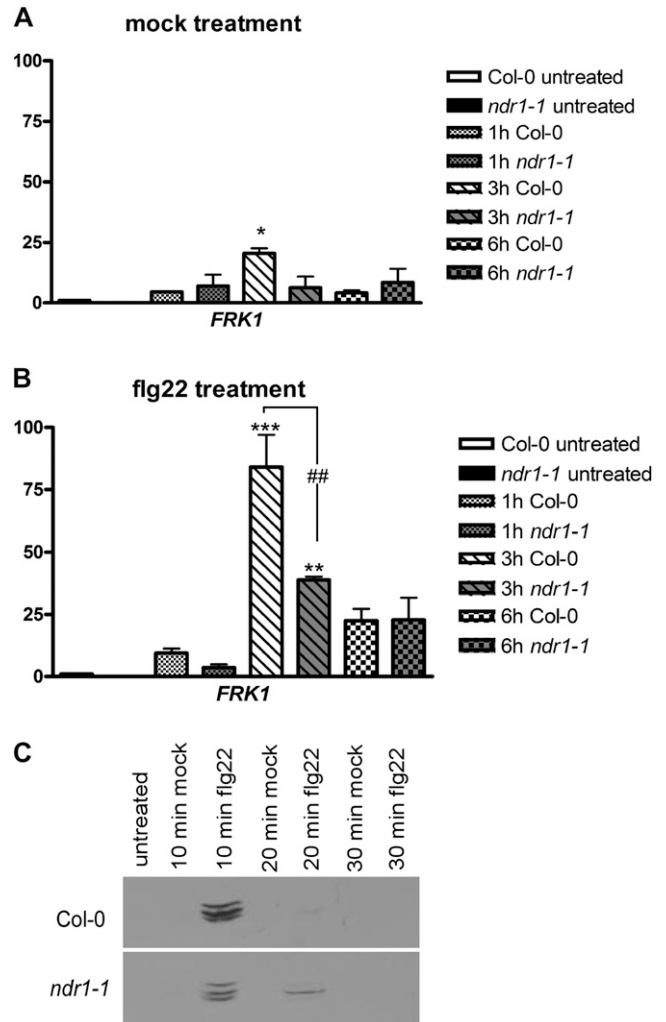


Figure 5. *ndr1-1* exhibits altered PAMP responses. A and B, The expression levels of *FRK1* mRNA was analyzed by qRT-PCR in Col-0 and *ndr1-1* mutant plants in response to flg22 or mock treatment. Error bars display SD from two technical replicates from one biological replicate. Expression is displayed as untreated average fold for Col-0. Statistical significance was determined using one-way ANOVA followed by Tukey's test, where asterisks represent statistically significant differences as compared with untreated Col-0 and pound signs represents statistically significant differences between Col-0 and *ndr1-1*: * $P < 0.05$; **,# $P < 0.01$; *** $P < 0.001$. C, Western blot analysis of MAPK3/6 in Col-0 and *ndr1-1* mutant plants in response to flg22 or mock treatment.

type Col-0. Similarly, conversion of the NGD motif to RGD also resulted in a near wild-type level of resistance to all *Pst* DC3000 strains tested (Fig. 6B). When the NGD motif was changed to AAA, a complementation of resistance to *Pst* DC3000 overexpressing any of the three effector genes was observed; however, only an intermediate level of resistance to *Pst* DC3000 was detected (Fig. 6C). The 1 log increase in bacterial growth observed in the AAA lines as compared with Col-0, while not statistically significant under our assigned *P* value ($P < 0.05$; Supplemental Table S3),

is nonetheless an interesting finding and strengthens the case for the NGD site of NDR1 as playing a critical role in specific defense responses. *Pst* DC3000 demonstrates an increased growth on *ndr1-1* plants as compared with Col-0 (Century et al., 1995). This, together with our observations of the AAA complemented line, suggests that NDR1 may be involved in mediating PAMP-triggered, or basal, immunity. Indeed, this hypothesis is further supported by the alteration observed in the MAPK pathway response to the PAMP flg22 in the *ndr1-1* mutant (Fig. 5). The inability of the

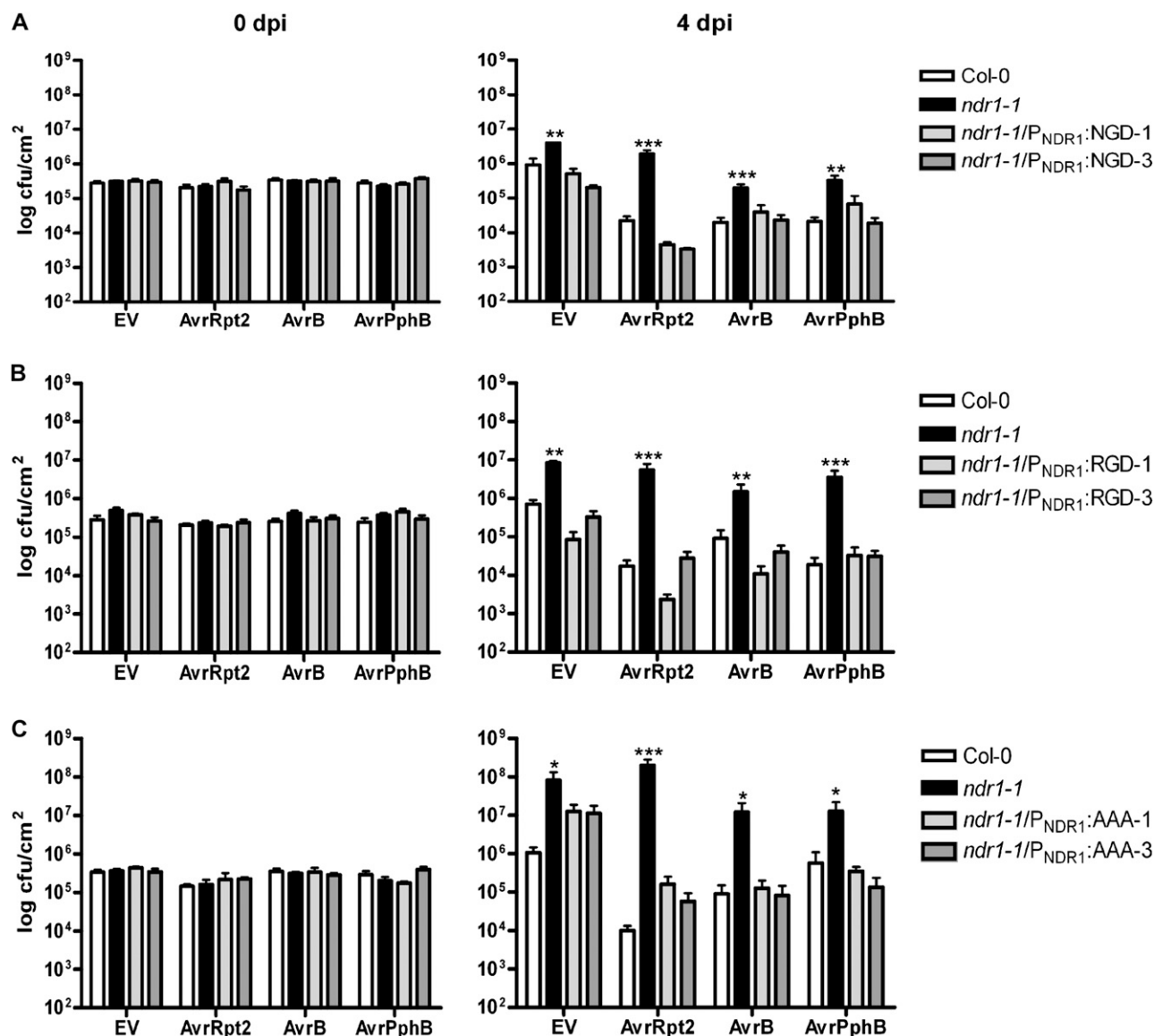


Figure 6. Growth of *Pst* DC3000 in Arabidopsis is altered by mutations to the NGD site of NDR1. Bacterial growth assay of *Pst* DC3000 (EV) and *Pst* DC3000 expressing AvrRpt2, AvrB, or AvrPphB dip inoculated on *ndr1-1* lines complemented with native *NDR1* promoter with *NDR1* NGD (*ndr1-1/P_{NDR1}:NGD*; A), *NDR1* RGD (*ndr1-1/P_{NDR1}:RGD*; B), or *NDR1* AAA (*ndr1-1/P_{NDR1}:AAA*; C). Growth was assayed at 0 and 4 d postinoculation. Growth is expressed as log cfu cm⁻². Error bars display SD from three technical replicates from two biological replicates. Statistical significance was determined using unbalanced two-way ANOVA (model included in Supplemental Fig. S7), where asterisks represent statistically significant differences between Col-0 and *ndr1-1*: * $P < 0.1$, ** $P < 0.01$, *** $P < 0.0001$ (for *P* values, see Supplemental Table S3).

AAA complemented lines to fully complement resistance would suggest that this site may in fact play a role in resistance signaling to *Pst* DC3000, possibly through mediating association with an as yet unidentified ligand, similar to the function of integrin RGD-binding sites (Plow et al., 2000).

ndr1-1 Mutant Plants Have Altered Cell Wall Adhesions

Several pieces of evidence point to a role for NDR1 in the adhesion of the plasma membrane to the cell wall: predicted structural similarity to integrins as well as the putative orientation of NDR1 within the plasma membrane (Coppinger et al., 2004). To test this hypothesis, 8-d-old *ndr1-1* and Col-0 hypocotyls were visualized before and after CaCl₂-induced plasmolysis to assess the integrity of plasma membrane-cell wall adhesion. As shown in Figure 7A, a distinctive concave shape was observed in the membranes of Col-0 hypocotyl cells undergoing plasmolysis, with obvious attachments to the cell wall still visible (Fig. 7A, arrows). These data are in agreement with previously observed results using hypocotyls or suspension-cultured cells (Canut et al., 1998; Gouget et al., 2006). Surprisingly, CaCl₂-induced plasmolysis of the *ndr1-1* mutant was significantly altered (Fig. 7, A and C), resulting in the complete detachment of the plasma membrane from the cell wall, yielding spherical protoplasts with no remaining attachments (convex plasmolysis). Complementation of the *ndr1-1* mutant with a constitutively expressed (i.e. 35S) GFP:NDR1 fusion protein restored the cell wall attachment phenotype to the wild type (Fig. 7B).

Disruption of plasma membrane-cell wall adhesion has been previously correlated with the function of the RGD motif (Canut et al., 1998). Moreover, it is well established that the conversion from concave to convex plasmolysis can be induced in cells with the addition of exogenous RGD-containing or RGD-binding peptides (Canut et al., 1998; Mellersh and Heath, 2001; Senchou et al., 2004; Gouget et al., 2006). As shown in Figure 8, addition of exogenous peptides did not alter the plasmolysis phenotype in *ndr1-1* hypocotyls. Interestingly, the addition of the VNGDG peptide prior to the addition of CaCl₂ was able to alter the plasmolysis pattern of Col-0 hypocotyls from concave to convex in the same manner as the addition of the VRGDG peptide, suggesting that the NGD motif might function similarly to the RGD motif. In addition, complemented *ndr1-1* lines expressing either P_{NDR1}:NGD or P_{NDR1}:RGD behaved similarly to Col-0 without peptide treatment as well as when treated with exogenous peptides. Conversely, P_{NDR1}:AAA complemented lines responded in the same manner as *ndr1-1* under both treatment conditions (Fig. 8), strengthening the case for the NGD motif functioning in adhesion in NDR1.

DISCUSSION

NDR1 was identified more than 15 years ago in a screen for enhanced disease susceptibility mutants in

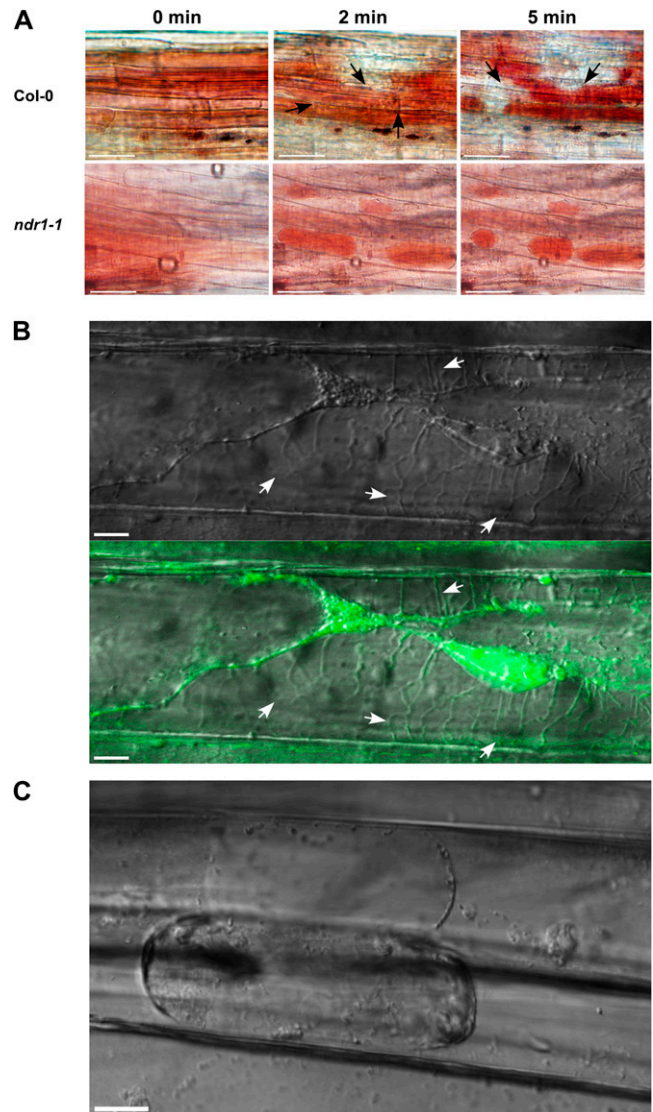


Figure 7. *ndr1-1* mutant plants exhibit altered plasmolysis and plasma membrane-cell wall focal adhesions. A, Time course (left to right: 0 [before], 2, and 5 min after treatment) of 8-d-old Col-0 or *ndr1-1* CaCl₂-plasmolyzed hypocotyls. In contrast to plasmolyzed wild-type Col-0 hypocotyl cells, which reveal plasma membrane-cell wall attachments (arrows), the plasma membrane of *ndr1-1* cells quickly loses adhesion following the induction of plasmolysis, yielding spherical protoplasts within the cell wall. Bars = 25 μ m. B, Complemented *ndr1-1* mutant line constitutively expressing a GFP:NDR1 protein exhibits wild-type plasma membrane-cell wall adhesions (arrows). Differential interference contrast (top panel) and confocal-differential interference contrast overlay (bottom panel) images of CaCl₂-induced plasmolysis are shown. Arrows indicate Hechtian strand formation, illustrating significant physical linkages with the plasma membrane and cell wall. Bars = 30 μ m. C, Hechtian strands are absent, or significantly reduced, in the *ndr1-1* mutant plant. Bar = 10 μ m.

Arabidopsis following *Pst* DC3000 inoculation (Century et al., 1995). Numerous studies have speculated on a role for NDR1 in defense signaling (Belkhadir et al., 2004; Coppinger et al., 2004; Zhang et al., 2004; Day

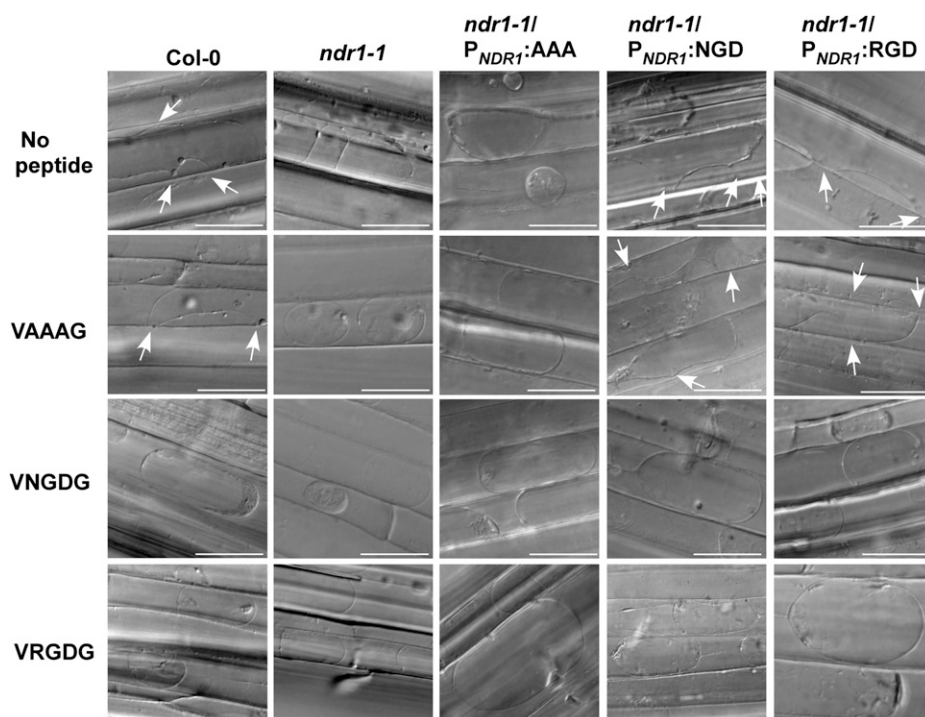


Figure 8. Application of exogenous peptides can alter the plasma membrane-cell wall adhesion in wild-type Col-0. Application of exogenous VNGDG or VRGDG peptide results in reduced plasma membrane-cell wall adhesions in Col-0, *ndr1-1/P_{NDR1}:NGD*, and *ndr1-1/P_{NDR1}:RGD* hypocotyl cells. Both *ndr1-1* and *ndr1-1/P_{NDR1}:AAA* hypocotyls are unaltered in adhesion phenotypes upon addition of purified VRGDG, VNGDG, and VAAAG peptides. Arrows indicate cell wall adhesion points. Differential interference contrast microscopy images were collected 5 min after the induction of plasmolysis. Bars = 25 μ m.

et al., 2006); however, a functional role has remained elusive. Our primary goal in this study was to look beyond the general defense signaling processes with which *NDR1* is known to be associated and focus on determining a broader physiological role for *NDR1*. This knowledge would both give insight into the general role of *NDR1* and also enable us to hypothesize on its function in plant disease resistance signaling.

Studies have shown that *NDR1* is required for a specific subset of resistance signaling pathways in plants (Innes, 1998). A conceptual model has evolved that suggests that a biochemical role for *NDR1* must encompass a multitude of specific signaling pathways associated with the activation of resistance. For example, *NDR1* is required for the activation of a majority of CC-NB-LRR R proteins in Arabidopsis (Aarts et al., 1998). However, there are exceptions to this requirement (McDowell et al., 2000; Bittner-Eddy and Beynon, 2001). With this in mind, how do we broadly assign a role for *NDR1* in defense signaling while also explaining exceptions to the rule?

The association of *NDR1* with *RIN4* provides a possible mechanism through which *NDR1* participates in defense signaling (Day et al., 2006). As a required component of signaling mediated by three CC-NB-LRR resistance proteins (e.g. *RPM1*, *RSP2*, and *RPS5*), association with *RIN4* physically links *NDR1* to two of these R proteins: *RPM1* and *RPS2* (Axtell and Staskawicz, 2003; Mackey et al., 2003). Day et al. (2006) speculated that the *NDR1*-*RIN4* association might serve as a protein scaffold, directly or indirectly linking multiple signaling components at the plasma membrane. The association of *NDR1* with *RIN4*, as

well as the association of *RIN4* with both *RPM1* and *RPS2*, provides the potential for the assembly of a multiprotein complex at the plasma membrane.

The control of electrolyte release during host-pathogen interactions (Baker et al., 1991; Mackey et al., 2002) as well as the desiccation responses induced in plants as a result of pathogen infection (Wright and Beattie, 2004) led us to hypothesize that *NDR1* may play a role in similar, associated responses. This proved successful, as our data suggest a role for *NDR1* in electrolyte release following infection with *Pst* DC3000 (Fig. 2, A–D). However, our data do not fully define the precise role of *NDR1* in the general process of nutrient release. The simplest explanation, given the proposed role of *NDR1* in membrane adhesion, may be that the absence of *NDR1* results in a weakened membrane system, one that is unable to prevent or restrict leakage in response to a virulent pathogen. Alternatively, *NDR1* may be associated with additional proteins, such as membrane-associated transporters and/or regulatory processes, in the host plants, which themselves are involved in nutrient release-associated mechanisms. In support of this, recent work by Liu et al. (2009) identified several interacting partners of *RIN4*, thereby potentially linking *NDR1* to a multitude of host cell processes, including guard cell dynamics and membrane potential.

The predicted structural homology of *NDR1* with *LEA14* (Fig. 1) and, by inference, with integrins (Supplemental Fig. S2) prompted us to investigate a general role for *NDR1* in stress responses that are regulated by the *LEA* family of proteins. In plants, *LEA* proteins have been described as playing a role in

a broad range of biotic and abiotic responses, including salt tolerance, as well as cold, heat, and drought stress (De Meutter et al., 2005; Goyal et al., 2005; Battaglia et al., 2008). Our observation of an increase in *LEA14* mRNA expression in *ndr1-1* plants in response to *Pst* DC3000 infection may in fact suggest that *ndr1-1* plants undergo increased water stress during pathogen infection. We reason that this increase in *LEA14* expression may be an attempt by the plant to compensate for the increased electrolyte leakage observed in *ndr1-1* plants. Therefore, we hypothesize that NDR1 plays a role in limiting fluid loss. A similar role was proposed for *LEA14* in response to wounding and dehydration (Singh et al., 2005). Changes in expression patterns of *LEA* group 1 domain-containing transcripts further strengthen the argument for NDR1 in the regulation of abiotic stress tolerance, while increases in the expression of *PIP1;4* and *PIP2;5* (Fig. 3) demonstrate an increase in drought stress within the cells of *ndr1-1* mutant plants inoculated with *Pst* DC3000 expressing AvrRpt2. Linking the physiological and phenotypic observations with the mRNA expression data provides a more complete picture of the role NDR1 plays in processes associated with electrolyte release, water movement, drought tolerance, and pathogen response.

In mammals, integrins are viewed as the primary signal response elements, having critical roles in adhesion signaling (Huvener and Danen, 2009). Based on the predicted structural homology with the fibronectin protein fold, we hypothesized that NDR1 may be involved with the plasma membrane-cell wall network. Based on the double anchor model (Coppinger et al., 2004), we hypothesized that NDR1 may function by mediating signaling processes or play a role in plasma membrane-cell wall adhesion. During compatible or incompatible plant-pathogen interactions, adhesion between the plasma membrane and cell wall can be disturbed or strengthened, respectively, and elicitation of defense responses is dependent on adherence (Mellersh and Heath, 2001). Our work shows that *ndr1-1* mutant plants lack observable adhesion points between the plasma membrane and cell wall under CaCl_2 -induced plasmolysis (Fig. 7A). Interestingly, we observed that addition of peptides with the NDR1 motif NGD (i.e. VNGDG) to Col-0 hypocotyls prior to plasmolysis resulted in conversion from the concave to the convex plasmolysis phenotype. This is in agreement with previous work described by Canut et al. (1998), who characterized the role of RGD peptides in the disruption of plasma membrane-cell wall adhesion. Furthermore, complemented *ndr1-1* lines expressing *NDR1* with the wild type NGD, or mutant RGD, site behave, as one would predict, with both NDR1 variants able to complement the mutation and thus restore normal plasmolysis. Conversely, substituting AAA into the NGD site mirrored the lack of adhesion and convex plasmolysis observed in *ndr1-1* mutant plants.

In support of our proposed role for NDR1 in mediating these processes, there are numerous examples

describing the role of plasma membrane-cell wall adhesion in basic plant physiology (Gouget et al., 2006), mediating resistance during host-pathogen interactions (Mellersh and Heath, 2001), as well as a potential virulence target during pathogen invasion (Pieterse et al., 1992; Senchou et al., 2004). In each of these instances, exogenous application of RGD peptides altered host cell membrane dynamics. In this study, the addition of exogenous peptides (e.g. VNDGG, VRGDG, or VAAAG), while significantly altering plasma membrane-cell wall adhesion, did not alter the HR observed in either Col-0 or *ndr1-1* plants (Supplemental Table S1). This finding indicates that the role of NDR1 in adhesion does not fully account for the *ndr1-1* mutant's loss of HR in response to the effector AvrRpt2. Based on the role(s) NDR1 plays in defense signaling processes initiated by a diverse array of plant pathogens, we hypothesize that the role of *NDR1* in these processes are in (1) mediating plasma membrane-cell wall adhesion, (2) regulation of fluid movement in response to pathogen infection, (3) the perception of mechanical stimuli initiated at the plasma membrane-cell wall interface, and (4) the transmission of this signal from the apoplast to/through the plasma membrane either via its association with RIN4 or other associated proteins.

The proposed role of NDR1 in mediating leakage may partially explain the differential requirements for NDR1 in CC-NB-LRR-mediated resistance to bacterial pathogens and to the oomycete pathogen *H. arabidopsidis* (McDowell et al., 2000; Bittner-Eddy and Beynon, 2001). Wright and Beattie (2004) have previously shown that limiting leaf water availability results in restricted *P. syringae* growth, thereby supporting our hypothesis that the inability of *ndr1-1* mutants to restrict leakage provides a more suitable environment to support bacterial growth. Given the different mechanisms of infection and disease progression between a bacterial pathogen and an obligate oomycete pathogen such as *H. arabidopsidis*, it is not surprising that NDR1 would have a much stronger effect on defense responses associated with a specific subset of CC-NB-LRR proteins.

While much work remains to fully define the role of NDR1 in plant defense signaling, this work provides a foundation for moving forward with additional biochemical analyses. To date, the genetic role of *NDR1* in defense signaling has been narrowly defined without having a biochemical function for NDR1. As such, the role of *NDR1* has been defined based almost exclusively on the absence (e.g. *ndr1-1* mutant) of the protein. In total, our work proposes a role for *NDR1* in mediating electrolyte leakage and, in part, regulating processes associated with desiccation and drought tolerance, processes that are primary tenets of plant disease resistance and abiotic stress response. A role for *LEA14* may be the preservation of nutrient and fluid loss during biotic and abiotic stresses (Singh et al., 2005), and NDR1 may have a similar role while also having evolved parallel functionality as an integral

signaling component of the plant defense network, possibly linking both effector-triggered immunity- and PAMP-triggered immunity-induced signaling following *P. syringae* infection.

MATERIALS AND METHODS

Homology Modeling and Structure Threading

The primary amino acid sequence of NDR1 (AT3G20600) was submitted to the PHYRE protein fold recognition server (<http://www.sbg.bio.ic.ac.uk/phyre/>). The predicted structure was analyzed using the MODELER comparative homology-modeling software (Martí-Renom et al., 2000). The output of this analysis, a predicted model of all nonhydrogen atoms based on spatial restraints, was then viewed using Chimera (Pettersen et al., 2004). Image overlays were performed using the solved NMR structure of LEA14 (AT1G01470; Iyyca; Singh et al., 2005). Further analysis (Supplemental Fig. S1) was performed by submitting the primary amino acid sequence of NDR1 to I-TASSER (<http://zhanglab.cmb.med.umich.edu/I-TASSER/>), an internet-based structure prediction service.

Plant Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were grown at 20°C under a 12-h/12-h light/dark cycle at 60% relative humidity in a Bio Chambers model FLX-37 growth chamber. *Nicotiana benthamiana* plants were grown under the same conditions.

DNA Cloning and Mutagenesis

Cloning of DNA constructs was performed using standard protocols. Site-directed mutagenesis of NDR1 was performed according to previously published protocols (Day et al., 2005) modified from the QuikChange PCR Mutagenesis Kit (Stratagene). Two site-directed mutations within the NGD site of NDR1, located at amino acids 178 to 180, were constructed. A template plasmid (i.e. pTOPO-NDR1) containing the open reading frame of NDR1 flanked by *SalI* (5') and *SacI* (3') restriction enzyme sites was used in combination with the DNA oligonucleotide primer sets labeled RGD and AAA in Supplemental Table S1. Following 18 cycles (95°C for 1 min, 55°C for 3 min, 68°C for 5 min) on a Bio-Rad MyCycler thermal cycler (Bio-Rad Laboratories) using *Pfu* Turbo DNA polymerase (Clontech), the product was then treated with *DpnI* for 1 h at 37°C. Five microliters of the digested DNA reaction was transformed into *Escherichia coli* DH5 α cells and grown on Luria-Bertani medium containing 100 μ g mL⁻¹ kanamycin overnight at 37°C. Mutant NDR1 constructs were cloned into either the native promoter vector pDDNDR (Coppinger et al., 2004) or the 35S binary vector pMD-1 with an N-terminal T7 epitope tag (Day et al., 2005). To make pDDNDR native promoter constructs, primers were designed to add a 5' T7 epitope tag and *SalI* site and a 3' *SpeI* site (Supplemental Table S2). Amplicons from the site-directed mutant constructs were ligated into pGEM T-EASY vector (Promega) and subsequently digested with *SalI* and *SpeI* and ligated into pDDNDR (Coppinger et al., 2004). DNA plasmids were transformed and maintained in *E. coli* DH5 α cells. The fidelity of all DNA constructs was confirmed by DNA sequencing (ABI 3730 Genetic Analyzer; Applied Biosystems). *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Lazo et al., 1991) and strain C58C1 (Tai et al., 1999; for *N. benthamiana* expression) were transformed with wild-type and NDR1 mutant constructs by electroporation.

All native, wild-type NDR1 T7 and HA fusion constructs were previously demonstrated to be functional and to fully complement the *ndr1-1* mutation (Coppinger et al., 2004; Day et al., 2006).

Cloning of NDR1 Mutant Constructs

Two site-directed mutations within the NGD site of NDR1, located at amino acids 178 to 180, were constructed using a quick-change PCR approach. A template plasmid (i.e. pTOPO-NDR1) containing the open reading frame of NDR1 flanked by *SalI* (5') and *SacI* (3') restriction enzyme sites was used in combination with the DNA oligonucleotide primer sets labeled RGD and AAA in Supplemental Table S1. Following 18 cycles (95°C for 1 min, 55°C for 3

min, 68°C for 5 min) on a Bio-Rad MyCycler thermal cycler using *Pfu* Turbo DNA polymerase (Clontech), the product was then treated with *DpnI* for 1 h at 37°C. Five microliters of the digested DNA reaction was transformed into *E. coli* DH5 α cells and grown on Luria-Bertani medium containing 100 μ g mL⁻¹ kanamycin overnight at 37°C. The resultant site-directed mutant constructs were digested with *SalI* and *SacI* restriction enzymes and ligated into the respective sites in the 35S binary vector pMD-1-T7, which incorporates a 5' T7 epitope tag (Day et al., 2005). To make pDDNDR native promoter constructs, primers were designed to add a 5' T7 epitope tag and *SalI* site and a 3' *SpeI* site (Supplemental Table S1). Amplicons from the site-directed mutant constructs were ligated into pGEM T-EASY vector (Invitrogen) and subsequently digested with *SalI* and *SpeI* and ligated into pDDNDR (Coppinger et al., 2004). DNA plasmids were transformed and maintained in *E. coli* DH5 α cells. *A. tumefaciens* strain GV3101 (pMP90) (Lazo et al., 1991) and strain C58C1 (Tai et al., 1999; for *N. benthamiana* expression) were transformed with wild-type and NDR1 mutant constructs by electroporation.

All native, wild-type NDR1 T7 and HA fusion constructs were previously demonstrated to be functional and to fully complement the *ndr1-1* mutation (Coppinger et al., 2004; Day et al., 2006).

Arabidopsis Transformation

Flowering Arabidopsis plants were transformed and selected for homozygosity, as described by Clough and Bent (1998), on Murashige and Skoog medium containing 1% Bacto agar and 25 μ g mL⁻¹ kanamycin.

Pathogen Inoculation and Growth Assays

Pseudomonas syringae pv *tomato* DC3000 strains containing pVSP61 (empty vector) or pVSP61-containing AvrRpt2, AvrB, or AvrPphB were described previously (Kunkel et al., 1993; Simonich and Innes, 1995). To assay for bacterial growth, 4-week-old plants were dip inoculated in bacterial suspensions of 3×10^7 colony-forming units (cfu) mL⁻¹. Leaves were preselected and marked to ensure that analyses were performed on developmentally similar leaves. At 0 and 4 d postinoculation, three leaf discs of 0.7 cm diameter were collected from a single plant into a microcentrifuge tube containing 1 mM MgCl₂ + 0.1% Triton X-100. Bacterial growth assays were performed as described by Tornero and Dangl (2001) with the modification of plating 5 μ L instead of 2 μ L, as described by Tian et al. (2009). Results were analyzed for significance using SAS (version 9.2; SAS Software) using an ANOVA model modified from Tsuda et al. (2008; Supplemental Fig. S7). Log₁₀-transformed bacterial titer counts were compared using Tukey's test.

Electrolyte Leakage

Electrolyte leakage was measured in 4- to 5-week-old plants using a protocol modified from Gilmour et al. (1988). Leaves were preselected and marked based on similarity in size before plants were dip inoculated at 3×10^7 cfu mL⁻¹. After inoculation, plants were covered with a clear plastic dome for 30 min before the 0-h time point, when leaves were removed. The remaining pots were left covered for another 2.5 h. A single leaf was removed from a plant and a disc (0.7 cm diameter) was harvested using a number 3 cork borer. Excised leaf discs were floated in a bath of sterile deionized water and quickly swirled before being placed in a tube containing 3 mL of sterile deionized water. Four plants were used for each replicate. Tubes containing leaf discs were shaken on an orbital rocker at 35 rpm for 3 h. After 3 h, leaf discs were removed and the solution was assayed for conductance using a conductance meter (Traceable 23226-505; VWR Scientific). Leaf discs were frozen at -80°C for 1 h. After the freeze cycle, the leaf punch was returned to the original sample tube and rocked for an additional 3 h at room temperature. After 3 h, the leaf punch was removed and the conductance was measured, recorded as total leakage. Electrolyte leakage was recorded and calculated as percentage leakage of total (i.e. first reading/second reading) adjusted to percentage maximal.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from leaves using the RNeasy Plant Mini Kit (Qiagen). First-strand cDNA was synthesized from 1 μ g of total RNA using SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed on a Mastercycler ep Realplex real-time PCR system (Eppendorf) using HotStart-IT SYBR Green qPCR Master Mix (2 \times ;

USB). Cycle time for all replicates was 95°C for 2 min followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. Data were analyzed by two-way ANOVA using Prism 4 (GraphPad Software) with outliers removed by Grubb's test ($\alpha = 0.05$) utilizing the QuickCalcs online outlier calculator (GraphPad Software; <http://www.graphpad.com/quickcalcs/Grubbs1.cfm>). Aquaporin homolog primers were used as described (Alexandersson et al., 2010). All primer sets utilized are listed in Supplemental Table S2.

HR Assay

The HR was assayed as described by Century et al. (1995), slightly modified, by hand infiltration of Arabidopsis leaves with *Pst* DC3000 using a needleless syringe at a concentration of 1×10^7 cfu cm⁻², following a 6- or 12-h pretreatment by hand infiltration of 5 mM VAAAG, VNGDG, or VRGDG peptide solution in 1 mM MgCl₂ buffer or a mock control of only buffer. The leaves were evaluated for tissue collapse 20 h after infiltration of the bacteria.

Plasmolysis

Plasmolysis experiments were performed based on the methods of Gouget et al. (2006) using 8-d-old etiolated hypocotyls with the cotyledons and roots removed. Hypocotyl sections were immersed in 50 mM Tris (pH 8.0) for 1 h at room temperature, rinsed in sterile distilled water, and then stained with 0.05% neutral red for 5 to 30 min. Sections were rinsed in sterile distilled water and mounted on a coverslip in 15 μ L of sterile water. A second coverslip was placed on top of the section, offset to the first coverslip. To plasmolyze the cells, 15 μ L of 1.0 M CaCl₂ was placed on the sample where the second coverslip overlapped the first, allowing the solution to cover the sample via capillary action. Stained, plasmolyzed hypocotyls were observed with an Olympus IX-71 inverted microscope, and images were acquired with an Olympus DP70 camera. Images were processed and adjusted for contrast using Canvas X (ACD Systems). Approximately 100 hypocotyls of each genotype were observed in total from more than five biological replicates. For peptide addition experiments, the same method as described above was followed, with the exception that the hypocotyls were mounted in 15 μ L of 5 mM peptide in distilled water. Peptides (VRGDG, VAAAG, and VNGDG) were synthesized by EZ-Biolabs at a purity of greater than 99%.

Tagged Protein Constructs and Coimmunoprecipitation

Coimmunoprecipitation experiments were performed as described by Day et al. (2006), with slight modifications. In brief, *A. tumefaciens* strains expressing the epitope-tagged (e.g. T7 or HA) NDR1, RIN4, and NDR1 mutant constructs fused to a 35S promoter were infiltrated into 5-week-old leaves on *N. benthamiana* at a final, individual concentration of 4×10^8 cells mL⁻¹. Leaves were incubated at room temperature for 40 h, after which time 16 1-cm² leaf discs were harvested into liquid nitrogen and held at -80°C until processing. Samples were processed according to Day et al. (2005).

T7 epitope monoclonal and T7 horseradish peroxidase (HRP)-conjugated antibodies were purchased from Novagen. HA epitope monoclonal antibody was purchased from Covance. HA HRP-conjugated antibody and protease inhibitors were purchased from Roche.

MAPK Western Blotting

To detect MAPK3/6 activity, 40 μ g of total protein was loaded onto a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane, followed by incubation for 1 h with an antibody specific for anti-pTEpY (catalog no. 9101S; Cell Signaling Technology). An anti-rabbit HRP secondary antibody was used for detection on film.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At3g20600 (*NDR1*), At1g01470 (*LEA14*), At1g32560 (*LEA* group 1 domain-containing protein), At2g39010 (*PIP2;6*), At3g54820 (*PIP2;5*), At4g23400 (*PIP1;5*), At4g00430 (*PIP1;4*), At1g01620 (*PIP1;3*), At2g14610 (*PR1*), At5g44420 (*PDF1.2*), At3g03600 (*RPS2*), and At2g19190 (*FRK1*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Predicted structural models of Arabidopsis NDR1 using I-TASSER and PHYRE homology-modeling servers.

Supplemental Figure S2. Homology modeling of the predicted β -sheet torus of NDR1 with LEA14 and the fibronectin type III domain of human integrin β -4 (10.2210/pdb2yrz/pdb).

Supplemental Figure S3. Electrolyte leakage conductance measurements.

Supplemental Figure S4. Expression of aquaporin homolog family mRNAs following inoculation with *Pst* DC3000 expressing AvrB or AvrPphB.

Supplemental Figure S5. Expression of *NDR1*, defense marker genes, and *RPS2* in Arabidopsis following inoculation with *Pst* DC3000.

Supplemental Figure S6. Expression levels of the HA- and T7-tagged NDR1 constructs utilized.

Supplemental Figure S7. Statistical model for the analysis of bacterial growth assays.

Supplemental Table S1. The effect of exogenous peptide application on the HR in Col-0 and *ndr1-1* mutant plants.

Supplemental Table S2. Primers used for qRT-PCR as well as primer sets for the development of the RGD and AAA site-directed mutant constructs.

Supplemental Table S3. *P* values of *Pst* DC3000 growth assays.

Supplemental Appendix S1. Electrolyte leakage: supplemental conductance method.

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