The Coiled-Coil and Nucleotide Binding Domains of the Potato Rx Disease Resistance Protein Function in Pathogen Recognition and Signaling

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Plant genomes encode large numbers of nucleotide binding and leucine-rich repeat (NB-LRR) proteins, some of which mediate the recognition of pathogen-encoded proteins. Following recognition, the initiation of a resistance response is thought to be mediated by the domains present at the N termini of NB-LRR proteins, either a Toll and Interleukin-1 Receptor (TIR) or a coiled-coil (CC) domain. In order to understand the role of the CC domain in NB–LRR function, we have undertaken a systematic structure–function analysis of the CC domain of the potato (Solanum tuberosum) CC-NB-LRR protein Rx, which confers resistance to Potato virus X. We show that the highly conserved EDVID motif of the CC domain mediates an intramolecular interaction that is dependent on several domains within the rest of the Rx protein, including the NB and LRR domains. Other conserved and nonconserved regions of the CC domain mediate the interaction with the Ran GTPase–activating protein, RanGAP2, a protein required for Rx function. Furthermore, we show that the Rx NB domain is sufficient for inducing cell death typical of hypersensitive plant resistance responses. We describe a model of CC-NB-LRR function wherein the LRR and CC domains coregulate the signaling activity of the NB domain in a recognition-specific manner.

INTRODUCTION

Plant disease resistance (R) genes confer immunity to pathogens possessing corresponding avirulence (Avr) genes. As this type of defense involves paired genetic components, it is referred to as gene-for-gene, or race-specific, resistance (Flor, 1971). The protein products of most R genes mediate the recognition of Avr gene products and initiate the signaling events associated with gene-for-gene resistance. Plant R gene–mediated disease resistance results in a strong host response, often culminating in a type of programmed cell death known as the hypersensitive response (HR) (Heath, 2000). Different plant R genes confer specific recognition to one or more of the myriad structurally unrelated Avr proteins from diverse pathogens, including viruses, bacteria, oomycetes, fungi, nematodes, and insects. The proteins encoded by R genes, however, are assigned to a limited number of protein classes based on the organization of their structural domains, the most numerous type being the nucleotide binding and leucine-rich repeat (NB-LRR) proteins (Martin et al., 2003). Plant genomes contain hundreds of genes encoding NB-LRR proteins that are highly variable both within and between species. NB-LRR proteins are so named because they possess a central NB domain and a C-terminal LRR domain. Between these two domains is a region of homology known as the ARC (for Apaf1, R proteins, and CED4) domain, and the NB and ARC domains together are often referred to collectively as the NB-ARC or NBS domain (van der Biezen and Jones, 1998). Molecular modeling and structure–function experiments suggest that the ARC domain can be further divided into two structural units, ARC1 and ARC2, that have distinct functions (Albrecht and Takken, 2006; McHale et al., 2006; Rairdan and Moffett, 2006).

There are two major classes of NB-LRR proteins that are distinguished by the domains present at their N termini: those that possess a TIR (for Toll and Interleukin-1 Receptor homology) domain and those that do not. In place of a TIR domain, many NB-LRR proteins possess an N-terminal domain of ~120 to 200 amino acids that is often predicted to contain a coiled-coil (CC) motif. In many NB-LRR proteins, this domain does not conform to CC prediction programs, but the proteins show a clear phylogenetic relationship with those that do. As such, the CC-NB-LRR class of proteins can be defined primarily by characteristic motifs present in the NB and ARC domains (Meyers et al., 1999). Some CC-NB-LRR proteins possess a CC domain in conjunction with, or replaced by, other N-terminal domains such as the solana- ceous domain or a predicted BED DNA binding domain, whereas others have little or no sequence N-terminal to the NB domain (Bai et al., 2002; Mucyn et al., 2006; Tuskan et al., 2006).

Multiple domains of NB-LRR proteins appear to act together to convert the recognition of Avr proteins into a signal initiation event. This is mediated, at least in part, through intramolecular interactions. For example, the function of the potato (Solanum tuberosum) and pepper (Capsicum annuum) CC-NB-LRR proteins Rx and Bs2 can be reconstituted when expressed as

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fragments of either CC-NB-ARC plus LRR or CC plus NB-ARC-LRR. The same fragment pairs undergo physical interactions, alterations of which are associated with the activation of NB-LRR proteins (Moffett et al., 2002; Leister et al., 2005; Raidan and Moffett, 2006). Similar interdomain interactions appear to take place in TIR-NB-LRR proteins (Ueda et al., 2006).

Recently, several proteins have been shown to interact with the N termini of CC-NB-LRR proteins, including the interactions of RIN4 with RPM1 and PBS1 with RPS5 in Arabidopsis thaliana, Pto with Prf in tomato (Solanum lycopersicum), and the Ran GTPase-activating protein RanGAP2 with the potato proteins Rx and Gpa2 (Mackey et al., 2002; Mucyn et al., 2006; Ade et al., 2007; Sacco et al., 2007; Tameling and Baulcombe, 2007). RIN4, PBS1, and Pto also interact with the Avr determinants of their cognate CC-NB-LRR interaction partner (Mackey et al., 2002; Mucyn et al., 2006; Ade et al., 2007). Thus, it would appear that these CC-interacting proteins act to mediate recognition rather than signaling. The barley (Hordeum vulgare) Mla proteins bind to WRKY transcription factors through their CC domains (Shen et al., 2007), although it is not clear whether this interaction is involved in recognition or signaling. A role for the N terminus of NB-LRR proteins in recognition is further supported by a recent report suggesting an indirect interaction between the TIR domain of the tobacco (Nicotiana tabacum) N protein and its Avr determinant (Burch-Smith et al., 2007).

We have performed a structure–function analysis of the CC domain of the potato Rx protein, a typical CC-NB-LRR protein that confers resistance to Potato virus X (PVX) through recognition of the PVX coat protein (CP) (Bendahmane et al., 1999). Rx-mediated resistance is dependent on RanGAP2, which interacts with the Rx CC domain (Sacco et al., 2007; Tameling and Baulcombe, 2007). By assessing Rx CC mutants for their ability to confer a CP-dependent HR and PVX resistance, as well as their ability to undergo both intramolecular and intermolecular interactions, we have defined the function of the various regions of this domain. We define the EDVID motif as one of the few broadly conserved CC motifs and show that it is required for Rx activity due to its role in mediating an intramolecular interaction, whereas separate and overlapping regions of the CC domain mediate an interaction with RanGAP2. Most inactivating mutations in the Rx CC domain disrupted either the intramolecular interaction or the interaction with RanGAP2, suggesting that these are the major functions of the Rx CC domain. Although CC domains have been proposed to be signaling domains, we found no evidence to support this in the case of Rx. Rather, we show that the NB domain of Rx is sufficient to initiate an HR when overexpressed. These results led us to propose a mechanism to explain how NB-LRR proteins are able to translate Avr perception into the initiation of defense signaling.

**RESULTS**

**Deletion and Substitution Analysis of the Rx CC Domain**

Aside from interactions with recognition cofactors, little is known about how the CC domain enables R protein function. Since the CC domains of NB-LRR proteins do not show strong primary sequence conservation, we systematically mutated the Rx CC domain and tested the mutants for their ability to induce CP-dependent responses and to undergo both intramolecular and intermolecular interactions. We took advantage of the fact that the Rx CC domain undergoes a physical and functional interaction with the NB-ARC-LRR fragment when expressed in trans (Moffett et al., 2002). We generated N-terminal and C-terminal deletion variants of the Rx CC domain as fusions with enhanced green fluorescent protein (EGFP) in order to stabilize smaller fragments. These constructs were then coexpressed in Nicotiana benthamiana leaves with an NB-ARC-LRR construct and the PVX CP via Agrobacterium tumefaciens–mediated transient expression (agroexpression) to assess their ability to elicit an HR. All deletions from the N terminus disabled CC functionality (Figures 1A and 1B). However, C-terminal deletion fragments C1 and C2 were still functional in this assay (Figures 1A and 1B), suggesting that the N-terminal 86 residues of the CC domain are necessary and sufficient to initiate a CP-dependent HR. We refer to this fragment as the minimal functional region of the CC domain.

Although the HR is often associated with R gene–mediated viral resistance, if a virus is able to replicate and escape the initial infection site before the onset of an HR, the latter response will not be sufficient to contain the virus. In such a case, the viral Avr protein is able to accumulate locally and systemically, eventually inducing more extensive HR responses (Farnham and Baulcombe, 2006; Raidan and Moffett, 2006). As such, the ability to suppress the accumulation of PVX is a more stringent test of Rx functionality, in that some Rx variants capable of mediating a CP-dependent HR are unable to confer complete PVX resistance (Raidan and Moffett, 2006). To test the ability of CC deletion fragments to confer PVX resistance, we agroexpressed Rx NB-ARC-LRR and a subset of the CC deletion variants along with a dilute suspension of Agrobacterium carrying a binary vector encoding an infectious clone of PVX:GFP (see Methods). In this assay, all cells in the infiltrated patch were transformed with the Rx fragments, whereas only isolated individual cells were infected with PVX:GFP. The ability of PVX:GFP to accumulate and spread was manifested by spreading patches of green fluorescence that eventually merge (Figure 1C, bottom left). Upon coexpression of wild-type Rx CC and NB-ARC-LRR fragments, an efficient antiviral resistance response was initiated and no green fluorescence was observed (Figure 1C, top right). As expected, CC fragments N1 and C3, which did not elicit an HR, were likewise unable to mediate PVX resistance (Figures 1B and 1C). The C1 fragment was able to condition both an HR and PVX resistance, whereas the C2 fragment induced an HR but not PVX resistance, suggesting that the C2 fragment is impaired in a quantitative manner (Figures 1B and 1C).

EGFP:HA–tagged CC deletion constructs were also tested for their ability to coimmunoprecipitate the fusion protein Rx NB-ARC-LRR:Myc. In this assay, fragments lacking up to 37 amino acids from the N terminus (N3) were capable of binding NB-ARC-LRR, as was a C-terminal deletion mutant comprising residues 1 to 67 (C4) (Figure 1D). To test whether a fragment encompassing the region shared by the N3 and C4 fragments (amino acids 38 to 67) was sufficient to bind NB-ARC-LRR, this region (N3-C4) was fused to EGFP:HA and tested for NB-ARC-LRR coimmunoprecipitation. This fragment was able to bind NB-ARC-LRR, albeit more weakly than larger fragments (Figure 1D). These results implicate the region encompassing N3-C4 as being
important for NB-ARC-LRR binding, although flanking residues appear to stabilize the interaction.

To identify essential internal regions within the minimal functional region of the CC domain, we systematically replaced groups of six residues with the amino acid sequence NAAIRS in the context of full-length Rx. The NAAIRS motif is found in both α-helices and β-sheets (Wilson et al., 1985), suggesting that it may be less likely to cause gross disruptions in secondary structure, and it has been used successfully to identify critical regions of the Arabidopsis SNI1 protein (Mosher et al., 2006). Only two NAAIRS replacements, B and J, affected the ability of Rx to effect an HR (Figure 2A). However, both of these derivatives accumulated poorly (see Supplemental Figure 1 online). We also generated small internal deletions by replacing 12 residues with a single NAAIRS sequence in the context of the full CC fragment (residues 1 to 144). The internal deletion derivatives of CC were tested for their ability to interact functionally and physically with Rx NB-ARC-LRR (Figures 2B and 2C). In agreement with the N-terminal deletion analysis (Figure 1), internal deletion mutants 1 and 2 were nonfunctional for inducing an HR but were still able to interact with NB-ARC-LRR. Internal deletion mutants 3 to 6, 8, and 9 consistently interacted with NB-ARC-LRR. Although some variation in binding strength was observed for internal deletion 8, this was not consistent between experiments. The nonfunctional internal deletion mutants 6 and 8, but not 7, retained the ability to interact with NB-ARC-LRR (Figures 2B and 2C). Together with the deletion analysis shown in Figure 1, this suggests that the region encompassing internal deletion mutant 7 is required for binding NB-ARC-LRR.

The EDVID Motif of the CC Domain

We performed an alignment of the N termini of CC-NB-LRR resistance proteins to identify conserved motifs that might be
important for function (see Supplemental Figure 2 online). We included only those R proteins that have been characterized as conferring resistance to a known pathogen and, in cases of highly similar R proteins, included a representative protein (e.g., HRT represents RPP8 and RCY-1). Within the region encompassing internal deletion mutant 7 (Figure 2B), the sequence VRELAYDAEDVID emerged as being strongly conserved (Figure 3A). This sequence corresponds to the WVxxIRELAYDIEDIVDxY sequence described in rice (Oryza sativa) NB-LRR proteins as the non-TIR motif (Bai et al., 2002). Since NB-LRR proteins lacking a TIR domain may encode other conserved motifs, and some may lack a VRELAYDAEDVID sequence, we refer to this motif as the EDVID motif. The EDVID motif was conserved among all CC-NB-LRR resistance proteins analyzed with the exception of RPS2, RPS5, and Dm3, which we were unable to align with the EDVID consensus, and belong to a different evolutionary clade than CC-NB-LRRs that encode an EDVID motif (Meyers et al., 1999). The EDVID motif is ~110 to 130 residues N-terminal to the P-loop (PL) of most NB-LRR proteins, including R proteins that contain an extended N-terminal solanaceous domain such as Prf, Mi1.2, and Hero (Mucyn et al., 2006).

The acidic residues of the EDVID motif appeared particularly well conserved, with all CC domains possessing at least one acidic residue (Figure 3A). Since none of the individual Rx NAAIRS substitutions replaced all three acidic residues (Figure 2A), we analyzed the Rx EDVID motif in greater detail by altering it from TEDMVD to TAAMVD and TAAMVD, as well as introducing the TAAMVD into the Rx CC:HA construct. When the CC variants were coexpressed with NB-ARC-LRR and CP, a quantitative effect was observed: greater loss-of-function phenotypes correlated with greater alteration of this motif (Figure 3B). Neither the TAAMVD nor the NAAIRS replacement CC derivatives were functional in trans, although the TAAMVD replacement in full-length Rx was able to produce a weak HR (Figure 3B). These results suggest that the EDVID motif plays an important structural role in Rx function.

![Figure 2. NAAIRS Scanning Mutagenesis of the Rx CC Domain.](image)

(A) Functional analysis of NAAIRS substitutions. The minimal sequence of the Rx CC domain sufficient to induce an HR (construct C2 in Figure 1A) was systematically mutated by replacing consecutive blocks of six residues with the amino acid sequence NAAIRS for analysis within the context of the full-length Rx clone. The lines below the Rx sequence underscore the amino acids that were replaced by NAAIRS in Rx variants, designated A to N. Plus signs indicate that a CP-dependent HR was seen within 48 h of agroexpression in N. benthamiana leaves; W indicates that an HR was observed, but at a significantly later time compared with the wild type, typically presenting a visibly weaker response at 72 to 96 h after infiltration. (B) Functional analysis of NAAIRS internal deletions. The minimal sequence of the Rx CC domain sufficient to induce an HR was systematically mutated by replacing consecutive blocks of 12 residues with the amino acid sequence NAAIRS within the context of the full (amino acids 1 to 144) CC:HA construct. Each internal deletion is underscored and indicated by a number below. Plus signs indicate that a CP-dependent HR was seen within 48 h of coexpression of the indicated variants with NB-ARC-LRR in N. benthamiana leaves; W indicates that an HR was observed, but at a significantly later time compared with the wild type, typically presenting a visibly weaker response at 72 to 96 h after infiltration. (C) NB-ARC-LRR binding properties of NAAIRS internal deletions. NB-ARC-LRR binding by NAAIRS variants was assessed by coexpressing the indicated CC:HA variant with NB-ARC-LRR:Myc in N. benthamiana leaves followed by immunoprecipitating (IP) the CC:HA variant with anti (α)-HA antibody–conjugated beads. The immunoprecipitates or total proteins were then immunoblotted (IB) with the indicated antibodies.
Figure 3. The EDVID Motif.

(A) Identification of a conserved EDVID motif. N-terminal sequences from characterized CC-NB-LRR resistance proteins were aligned to the region of Rx defined by internal deletion 7 in Figure 2B. Amino acids are grouped by color according to conserved side chain chemical properties. Residues conserved among CC domains are indicated below the alignment. A consensus sequence encompassing the EDVID motif (underlined) is shown below the sequence. Residues are colored according to shared physicochemical properties.

(B) Effects of the HR phenotype of site-directed mutations within the EDVID motif. Individual amino acids within the Rx EDVID motif (TEDMVD in Rx) were mutated individually or in combinations as underlined. The resulting mutants were coexpressed in the context of either full-length Rx or the Rx CC:HA plus NB-ARC-LRR in N. benthamiana leaves and assessed for their ability to elicit a CP-dependent HR. HR reactions were seen within 24 h (+++), 48 h (++), 72 h (+), or not at all (−/−), nd, not done.

(C) PVX resistance phenotype of EDVID variants. The same constructs used in (B) were coexpressed in N. benthamiana leaves with a dilute suspension of Agrobacterium possessing a binary vector containing an infectious PVX:GFP clone. Incomplete resistance to PVX:GFP was indicated by the visualization of GFP fluorescence at 7 d after infiltration. As a control, the PVX:GFP was coexpressed with EGFP, which is not excited by the 365-nm light used to visualize PVX:GFP.

(D) NB-ARC-LRR binding by EDVID mutants. The indicated CC:HA derivatives were coexpressed with NB-ARC-LRR:Myc in N. benthamiana leaves followed by immunoprecipitation (IP) of the CC:HA variant with anti (α)-HA antibody–conjugated beads. Immunoprecipitates and input proteins were subsequently immunoblotted (IB) with the indicated antibodies.
We tested the ability of the Rx EDVID variants to confer resistance to PVX:GFP. The EDVID motif substitution mutants were transiently coexpressed in N. benthamiana leaves with a dilute suspension of Agrobacterium harboring an infectious PVX:GFP clone. Six days after inoculation, the accumulation of PVX:GFP was assessed by visualizing green fluorescence under UV illumination. As with the HR assay, the Rx EDVID mutants showed reduced virus resistance correlating with increased alteration of the EDVID motif (Figure 3C).

Because the EDVID motif lies within the minimal region required for CC binding to NB-ARC-LRR (Figures 1 and 2), we tested the effect of EDVID substitutions on this interaction. Substitutions in the EDVID motif compromised CC binding to NB-ARC-LRR: TEDMVA bound poorly, TAAMVD bound very poorly, and TAAMVA showed no detectable binding (Figure 3D). These results correlate with the functional assays (Figures 3B and 3C) and provide further evidence that the EDVID motif is required for the intramolecular interaction involving the CC domain.

Interaction between the CC Domain and RanGAP2

We and others have previously shown that the CC domain of Rx interacts with RanGAP2 and that RanGAP2 is required for Rx function (Saccone et al., 2007; Tameling and Baulcombe, 2007). We thus investigated the correlation between CC function and interaction with St RanGAP2. By communoprecipitation, we found that the C1 fragment retained robust interaction with RanGAP2 (Figure 4A), whereas the negligible binding of C2 to RanGAP2 likely explains why it is only partially active (cf. Figures 1 and 4A). The N-terminal CC deletion fragments N1, N2, and N3 also interacted with RanGAP2, albeit much less efficiently than the wild-type CC (Figure 4A), which may explain their lack of function. These results suggested that the entire region encompassed by the first 115 residues of the CC domain is required for optimal interaction with RanGAP2. The region delimited by N4 and C1 encompasses the EDVID motif that is required for CC interaction with NB-ARC-LRR (Figure 2C). We investigated the involvement of the EDVID motif in RanGAP2 interaction using a subset of internal deletion constructs of the full CC:HA (Figure 2B) that flank the EDVID motif. Internal deletion mutants 5, 6, and 8, which flank the EDVID motif, showed little or no interaction with RanGAP2 (Figure 4B). By contrast, internal deletion mutant 7, which deletes the EDVID motif (Figure 2B), showed robust interaction with RanGAP2 (Figure 4B). Likewise the TEDMVD-to-TAAMVA mutant of Rx CC:HA also showed robust interaction with RanGAP2 (Figure 4B). These results suggest that the regions of CC that bind NB-ARC-LRR and RanGAP2 may be overlapping but separate. As such, the HR activity of CC mutants correlates with the ability to bind both NB-ARC-LRR and RanGAP2 (cf. Figures 2 and 4).

Characterization of the Rx NB-ARC-LRR/CC Interaction Interface

We next sought to identify a CC binding region within the Rx NB-ARC-LRR fragment. Mutation of the PL motif of the NB domain disrupts CC binding (Moffett et al., 2002), suggesting that the CC might bind to this domain. However, the Myc-tagged Rx CC domain did not communoprecipitate either NB-ARC:HA or NB:EGFP:HA (Figure 5A). Therefore, using a series of Ala substitutions in NB-ARC motifs shown previously to compromise Rx function, but not the LRR/CC-NB-ARC interaction (Rairdan and Moffett, 2006), we tested the effect of these substitutions on the physical and functional interactions between CC and NB-ARC-LRR. Structural modeling suggests that the region of the NB domain N-terminal to the PL is required for nucleotide binding (Albrecht and Takken, 2006). Therefore, we also included in our analysis an Rx fragment lacking the first 20 amino acids of NB-ARC-LRR (Δ1-158). As expected, the pattern of HR induced by coexpressing Rx CC and NB-ARC-LRR mutants plus CP (Figure 5B) was very similar to that seen when CC-NB-ARC mutants were expressed with LRR plus CP (Rairdan and Moffett, 2006). However, in this case, HR induction correlated strictly with the ability of the NB-ARC-LRR derivatives to interact physically with the CC domain (Figure 5B). Only the FE307/318AA and SY378AA NB-ARC-LRR mutants showed weak CC binding and weak HR induction.

The Rx D460V and Y712H mutations in the MHDV motif and LRR domain, respectively, induce autoactive phenotypes such that an HR is elicited in the absence of CP (Bendahmane et al.,...
Figure 5. Mutations within the NB, ARC, and LRR Domains Impair Binding of the Rx CC Domain.

(A) The CC does not bind NB or NB-ARC fragments. Rx CC:Myc was coexpressed with NB-ARC-LRR:HA, NB:EGFP:HA, NB-ARC:HA, or EGFP:HA followed by immunoprecipitation (IP) with anti-Myc (αMyc) antibody–conjugated beads. The immunoprecipitates and total extracts were immuno- blotted (IB) with the indicated antibodies.

(B) and (C) CC binding of Rx NB-ARC-LRR:HA variants. The indicated mutants (see text) or Gpa2/Rx chimera (GGRRR) NB-ARC-LRR:HA constructs (described in Methods) were coexpressed in N. benthamiana leaves with CC:Myc. Immunoprecipitation with anti-Myc antibody–conjugated beads was performed 2 d later. The immunoprecipitates and total extracts were subsequently immunoblotted with the indicated antibodies. The ability to produce an HR within 48 h when the various construct combinations were coexpressed in N. benthamiana leaves with or without CP is indicated by a plus sign. W indicates that an HR was observed, but at a significantly later time compared with the wild type, typically presenting a visibly weaker response at 72 to 96 h after infiltration.

(D) Inactive Rx LRR mutants bind Rx CC-NB-ARC. Inactive LRR:Myc constructs identified by error-prone PCR mutagenesis (see Supplemental Table 1 online) were coexpressed with Rx CC-NB-ARC:HA in N. benthamiana leaves and immunoprecipitated with anti-Myc antibody–conjugated beads 2 d later. The immunoprecipitates or total extracts were then immunoblotted with the indicated antibodies. The ability to produce a CP-dependent HR within 48 h when the same construct combinations were coexpressed in N. benthamiana leaves is indicated by a plus sign.

(E) Effect of LRR mutations on CC binding. NB-ARC-LRR:HA constructs incorporating the same LRR mutations as in (D) were coexpressed in N. benthamiana leaves with CC:Myc and immunoprecipitated 2 d later with anti-HA antibody–conjugated beads. The immunoprecipitates and total protein were then immunoblotted with the indicated antibodies.
2002; Farnham and Baulcombe, 2006). Likewise, experiments with Rx and its closely related homolog Gpa2 (Bendahmane et al., 2000; van der Vossen et al., 2000) have shown that autoactivity results when the Rx LRR and the CC-NB-ARC of Gpa2 are combined either in trans or in cis in a full-length chimeric protein (GGRRR) (Rairdan and Moffett, 2006). Surprisingly, when introduced into the context of CC plus NB-ARC-LRR, none of the three autoactive variants induced an HR except in the presence of CP (Figure 5C). Coimmunoprecipitation assays showed that NB-ARC-LRR(Y712H) bound the CC, while the D460V and the GGRRR derivatives did not.

Mutations throughout the NB and ARC domains abrogate CC binding to NB-ARC-LRR (Figure 5B), suggesting that this region is important for CC binding. However, the observation that the CC domain does not bind the NB or NB-ARC domains alone (Figure 5A) raised the possibility that the LRR may be required for CC binding. To test this, we generated several nonfunctional LRR variants. Briefly, a library of LRR mutants was generated using error-prone PCR, and candidate LRRs were coexpressed in N. benthamiana leaves with Rx CC-NB-ARC and CP. Then, LRR variants that were stably expressed but nonfunctional (see Supplemental Table 1 online) were tested for their ability to interact with CC-NB-ARC. All three variants tested (mut1, mut2, and mut3) retained the ability to bind CC-NB-ARC (Figure 5D). These variants were then introduced into Rx NB-ARC-LRR and tested for CC binding activity. Indeed, all three variant LRRs strongly compromised binding between the CC and NB-ARC-LRR fragments (Figure 5E), demonstrating a requirement for a functional LRR in this interaction.

**The Rx NB Domain Is Sufficient to Initiate Programmed Cell Death**

Overexpression of NB-LRR protein fragments in plant tissue has been used in a number of cases to delimit the regions of the protein responsible for the induction of resistance responses. Overexpression of full-length Rx induces an HR only in very young N. tabacum leaves, and deletion of the Rx ARC and LRR domains results in a protein (CC-NB) with enhanced HR-inducing activity (Bendahmane et al., 2002). We found that fusion of Rx fragments with HA-tagged EGFP results in greatly increased protein levels, such that Rx CC-NB:EGFP:HA accumulates to levels higher than CC-NB:HA (Figure 6A). Furthermore, expression of CC-NB:EGFP:HA induces a robust HR response within 48 h in older tobacco and N. benthamiana leaves, whereas CC-NB:HA does not (Figure 6B) (Bendahmane et al., 2002). Expression of an Rx CC:EGFP:HA fusion protein does not induce an HR, despite similar levels of protein accumulation (Figures 6A and 6B). This may be due to a requirement for interaction with the NB domain. However, the CC domain does not interact with the NB domain in trans (Figure 5A). Introduction of the PL mutation into CC-NB:EGFP:HA, which abrogates the intramolecular interaction (Moffett et al., 2002), had no effect on the induction of the HR (Figure 6B). The PL mutation would also be predicted to abrogate any putative NB–NB domain interactions (Mestre and Baulcombe, 2006), diminishing the possibility that the Rx NB domain might interact with any endogenous NB-LRR proteins in this manner. Fusion of the NB domain alone to EGFP:HA also resulted in the stabilization of this protein fragment such that it accumulated to levels similar to CC-NB:EGFP:HA (Figure 6A). Transient expression of both NB:EGFP:HA and NB:EGFP:HA(PL) induced HR responses of equal intensity to CC-NB:EGFP:HA (Figure 6B), suggesting that the NB domain is sufficient to initiate downstream responses. In a separate experiment, we compared the accumulation levels of Rx:HA and NB:EGFP:HA and found that the latter accumulated to higher levels than the full-length protein, similar to the difference in accumulation between CC-NB:HA and CC-NB:EGFP:HA (Figure 6A). Thus, protein fragment accumulation appears to correlate positively with activity, in addition to the previously reported negative regulatory role played by the C-terminal regions of Rx (Bendahmane et al., 2002). Regardless, these results suggest that the region of the Rx protein that ultimately initiates defense signaling lies within the NB domain.

Rx-mediated responses are compromised in plants in which Sgt1 (for Suppressor of G2 allele of skp1) protein levels have been reduced by virus-induced gene silencing (Moffett et al., 2002; Peart et al., 2002b). To determine whether the HR induced by NB:EGFP:HA shares the same signaling components as full-length Rx, we silenced Sgt1 in N. benthamiana plants transgenic for Rx (Lu et al., 2003). As an additional negative control, we silenced the ENHANCED DISEASE SUSCEPTIBILITY1 gene (Eds1), which compromises N gene–mediated resistance to Tobacco mosaic virus but not Rx-mediated resistance to PVX (Peart et al., 2002a). The HRs induced by NB:EGFP:HA, Rx(D460V), and Rx plus CP were compromised in Sgt1-silenced plants but unaffected in plants infected with the empty virus–induced gene-silencing vector (TV:00) or in Eds1-silenced plants (Figure 6C). Immunoblotting showed that NB:EGFP:HA accumulated to similar levels in Sgt1-silenced plants compared with plants silenced with a vector containing a β-glucuronidase (GUS) insert (Figure 6D). The genetic requirement for Sgt1 suggests that the HR induced by NB:EGFP:HA is unlikely to be caused by nonspecific toxicity of the NB fragment.

**DISCUSSION**

**The Role of the Rx CC Domain**

The CC domain has been proposed to initiate signaling, but a mechanism for such activity is lacking. At the same time, recent reports suggest that the N termini of several NB-LRR proteins play roles in recognition (Mackey et al., 2002; Mucyn et al., 2006; Ade et al., 2007; Burch-Smith et al., 2007), and we have proposed a similar role for RanGAP2 (Sacco et al., 2007). We have found that the majority of characterized CC-NB-LRR resistance proteins possess an EDVID motif (Figure 3A). In fact, the EDVID motif is more consistently present in CC-NB-LRR proteins than a predicted CC, and its presence may serve as a signature motif for certain classes of NB-LRR proteins. The EDVID motif appears to mediate the intramolecular NB-ARC-LRR–CC interaction (Figures 2C and 3D), whereas RanGAP2 appears to interact with regions flanking the EDVID motif (Figure 4B). This latter interaction may involve more than one binding surface, as it also appears to involve residues at the extreme N terminus of Rx (Figure 4A). It is tempting to speculate that opposed or juxtaposed surfaces of the CC domain may interact with NB-ARC-LRR and
RanGAP2 such that the interaction with RanGAP2 could modulate the interaction between CC and NB-ARC-LRR or vice versa. Such a scenario would provide a mechanism for molecular events involving RanGAP2 to be communicated through the CC domain to the rest of the Rx protein. Although we do not rule out the possibility of interactions with other cellular proteins, our results are consistent with an emerging model wherein the N termini of NB-LRR proteins function in indirect recognition rather than initiate downstream signaling.

Interaction between Rx Domains

The intramolecular interaction involving the Rx CC domain appears to be context-specific. The CC domain does not bind the...
NB or NB-ARC fragments alone (Figure 5A) and is not sufficient for interaction with the LRR (Raidan and Moffett, 2006). However, mutations throughout the NB-ARC-LRR disrupt the interaction with the CC domain (Figure 5). We suggest that these different mutations affect some general property of Rx NB-ARC-LRR that allows it to bind to the CC domain. Two NB-LRR proteins have been shown to bind and hydrolyze ATP (Tameling et al., 2002, 2006). Mutations in motifs predicted to participate in nucleotide binding, such as the PL, K2, GLPL, and MHDV domains and the first 20 amino acids of the NB domain (Albrecht and Takken, 2006), would almost certainly impair nucleotide binding and any tertiary structure that might be induced upon nucleotide binding. The requirement for the LRR in CC binding suggests that this domain may also affect the structure of the NB-ARC domain, possibly influencing nucleotide binding.

The MHDV motif of the ARC2 domain is thought to be analogous to the sensor II motif found in AAA+-ATPases (Albrecht and Takken, 2006; Takken et al., 2006). This motif appears to be involved in nucleotide-dependent conformational changes (Riedl et al., 2005). Autoactivated molecules can be generated by mutating the MHDV motif or by incompatible ARC2/LRR pairings (Bendahmane et al., 2002; Raidan and Moffett, 2006). All three NB-ARC-LRR variants derived from autoactive molecules elicited CP-dependent, but not CP-independent, HR when coexpressed with the CC domain (Figure 5C). The CC domain does not bind NB-ARC-LRR fragments derived from Rx(D460V) or the GRRR chimera (Figure 5C). This result suggests that, upon translation, these NB-ARC-LRR fragments rapidly adopt a conformation resembling a postactivation state and that this conformation is no longer capable of binding to a CC domain. This conformation would already be achieved by the time the NB-ARC-LRR fragments come into contact with a CC domain in trans, whereas the presence of the CC domain in cis would presumably allow the necessary interdomain interaction to occur before autoactivation.

Since mutations that disrupt nucleotide binding also result in a lack of CC binding, it is conceivable that the postactivation state adopted by these autoactivated fragments no longer binds nucleotides. Recognition of CP by Rx may initiate additional conformational changes allowing the interdomain interactions to realign, consistent with our previous suggestion that recognition of CP by Rx enables it to undergo multiple rounds of activation (Raidan and Moffett, 2006). This may involve resetting the NB-ARC-LRR fragment to a nucleotide-bound state, allowing it to undergo a subsequent interaction with a CC domain in trans. The NB-ARC-LRR fragment derived from Rx(Y712H) behaves phenotypically similarly to Rx(D460V) in HR assays but is able to bind the CC domain (Figure 5C). Thus, although Rx(Y712H) appears to require resetting by CP recognition as well, it may arrest in a distinct conformational state, possibly bound to ATP or ADP.

**Signal Initiation by Rx**

Transient expression studies have shown that HRs can be induced by overexpression of the CC-NB fragment of Rx and by the CC-NB-ARC fragments of RPS2 and RPS5 but not by any of the respective CC domains (Tao et al., 2000; Bendahmane et al., 2002; Ade et al., 2007). Likewise, the TIR-NB-ARC fragments, but not the TIR domains, of RPS4 and RPP1a are sufficient to induce overexpression-mediated HR (Zhang et al., 2004; Weaver et al., 2006). A role for the TIR domain in signaling remains a possibility, as the TIR domain of the flax (*Linum usitatissimum*) L10 protein initiates a weak HR when transiently expressed in tobacco (Frost et al., 2004). The CC domain of the atypical NB-LRR protein Nrg1 initiates an HR when overexpressed (Pearl et al., 2005). However, the Nrg1 gene was isolated as an R gene signaling component, and its CC domain is distinctive in being most homologous with the cytoplasmic domain of the transmembrane protein RPW8 (Xiao et al., 2001). We know of no reports demonstrating that a CC domain derived from an EDVID-class R protein is able to induce defense responses alone.

Accumulation of the Rx NB domain causing HR elicitation requires its fusion to EGFP (Figure 6). An innate instability may explain why the overexpression of NB domains has not been reported for other R proteins. Alternatively, the activity of CC-NB-ARC or TIR-NB-ARC fragments of other R proteins may reflect a role for the CC and TIR domains in stabilizing or enhancing the activity of the NB domain. For example, interaction with the LRR domain is a prerequisite for an Rx CC-NB-ARC fragment containing an autoactivating mutation to be competent for Avr-independent signaling (Moffett et al., 2002). Thus, although the expression of the Rx NB domain allows for deregulated activity, an interaction with the CC domain may normally be required to allow NB domains to be competent for signaling.

Such a scenario may explain, in part, why a functional PL is not necessary for the isolated NB domain to induce an HR. Nucleotide binding likely plays an important structural role in allowing the NB domain to adopt a signaling-competent state in the full-length molecule, and nucleotide hydrolysis may play a role in relieving negative regulatory interactions and/or exposing the signaling motif upon activation. In the isolated NB domain, these constraints appear to be overcome, and thus nucleotide binding is no longer necessary. Furthermore, this suggests that an NB motif other than the PL is likely to interact with downstream signaling factors.

We propose that CC-NB-LRR proteins require a series of events that prime the molecule to be competent for activation by a triggering event. The LRR domain must interact with the ARC domain for Rx function (Raidan and Moffett, 2006). We suggest that this interaction may be necessary, in part, because it is a prerequisite for the NB domain to become competent for ATP binding. Binding of ATP would allow the binding of the CC domain, which in turn would be required for the NB domain to adopt a conformation competent for engaging downstream signaling components. This priming mechanism may also involve other proteins, as HRs can be induced either by overexpression of RanGAP2 with Rx (Sacco et al., 2007) or by overexpression of the NB-LRR protein Prf with its recognition cofactor Pto (Mucyn et al., 2006). Upon priming, the intramolecular interactions appear to form a perfect fit, in that alterations associated with recognition cause the protein to lose its autoinhibition (Raidan and Moffett, 2006).

We further propose that in cases of indirect recognition, Avr proteins are initially perceived through CC-bound recognition cofactors and that this perception is transmitted through the CC to the rest of the NB-LRR protein. Whether or not such perception results in a loss of autoinhibition, however, may still depend
on the LRR. Such a scenario could explain why recognition co-factors, such as RIN4, PBS1, and Pto, have been shown to interact with the N-terminal domains of NB-LRR proteins (Mackey et al., 2002; Mucyn et al., 2006; Ade et al., 2007) even though molecular genetic studies show that recognition specificity is determined by the LRR domain (Ellis et al., 1999; Shen et al., 2003; Dodds et al., 2006; Qu et al., 2006; Raidan and Moffett, 2006). This scenario would predict that multiple Avr proteins may activate different R proteins through a common cofactor and that variation at the LRR–ARC interface would determine which interactions resulted in the activation of the NB-LRR protein. As such, the evolution of new R gene specificities would not necessarily require the NB-LRR protein to gain the ability to bind directly to either a new recognition cofactor or a new Avr protein.

Understanding how the NB domain initiates signaling will require further study. However, the identification of the signaling moiety of Rx represents an important step in developing strategies to do so. Furthermore, our results suggest that the CC and LRR domains both act to regulate the signaling capacity of the NB domain in a recognition-dependent manner. This has important implications for models of NB-LRR function and the evolution of their recognition specificities.

METHODS

Plasmid Construction

All site-directed mutants and NAAIRS substitutions described in this article were generated by extension-overlap PCR (Vallejo et al., 2003) using KOD high-fidelity thermostable polymerase according to the manufacturer’s instructions (Novagen). For the NAAIRS substitutions, the two PCR products to be joined either terminated or started with the DNA sequence AACGGAGT or the antisense thereof, such that these two products would anneal and allow extension to form a template for further rounds of amplification using distal primers. For example, the TEDMV-to-NAAIRS replacement used the following two primers: 5′-ACTCCGGATTGCTGCGTTTGTGTATGCTACCTCTACGATTTC-3′ and 5′-ACGGACGGATCCTCGGATTGCTGCGTTTGTGTATGCTACCTCTACGATTTC-3′. To simplify cloning methods, the CC deletion constructs were made similarly using extension-overlap PCR, with larger portions of the CC being replaced with NAAIRS; therefore, CC deletion constructs are fused to the NAAIRS moiety of Rx represents an important step in developing strategies to do so. Furthermore, our results suggest that the CC and LRR domains both act to regulate the signaling capacity of the NB domain in a recognition-dependent manner. This has important implications for models of NB-LRR function and the evolution of their recognition specificities.

Transient Protein Expression and Analysis

Binary vectors were transformed into Agrobacterium tumefaciens strain C58C1 carrying the virulence plasmid pCH32, and Agrobacterium-mediated transient expression (agroexpression) was performed as described previously (Bendahmane et al., 2000) at OD600 = 0.2. All proteins were coexpressed in Nicotiana benthamiana or Nicotiana tabacum leaves under the control of the P3SS promoter of the pBIN61 vector (Bendahmane et al., 2002). HR phenotypes generally presented at 24 to 48 h. The PVX:GFP resistance assay consisted of agroinfiltrating XbaI and ligated into pBIN61-Rx:Myc digested with the same enzymes followed by transformation of Agrobacterium. Individual clones were then transiently expressed in N. benthamiana with pBIN61-Rx:HA (CC-NB-ARC) and PVX CP. Clones that did not induce an HR were assessed for stable expression by immunoblot analysis. Constructs that were nonfunctional but were stably expressed were selected for further study.

Virus-Induced Gene Silencing

Gene silencing experiments were performed essentially as described (Pearl et al., 2002b). Briefly, 14-d-old N. benthamiana plants transgenic for both the Rx and N genes (Lu et al., 2003) were infiltrated with Agrobacterium carrying pBINTRA6 and a pTV vector (Ratcliff et al., 2001) containing fragments of Nb Eds1 and Nb Sgt1 (Pearl et al., 2002a, 2002b), the GUS gene (Tameling and Baulcombe, 2007), or no insert, and plants were used for agroexpression experiments 3 to 4 weeks later.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: CAB50786 (Rx), AAF89751 (PVX CP), AM411448 (RamGAP2), AF516810 (Nb Sgt1), AF479625 (Nb Eds1), CAB55838 (GPA2), AAF09256 (Bs2), AAF36987 (HRT), AAF42831 (RPP13), AAVS7531 (TM2-3), Q93214 (RPM1), BAA58975 (Pi-b), AAQ01784 (Lr10), CAC29241 (Ma6b), BAB91344 (Pl-ta), AAD27815 (I2), AAR19096 (RPG1-b), AAX89382 (Rps1-k1), ABO28718 (RB), AAQ61548 (Ram3B), AAS79233 (Rp3), AAP74647 (Lr21), AAP81261 (Rp1), BAA325086 (Xa1), AAC67238 (Ni1.2), CAD29728 (HERO), AAG31014 (Sw5), AAC49408 (PRF), and AAL39063 (R1).

Supplemental Data

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

Supplemental Figure 1. Immunoblotting of Single NAAIRS Substitution Derivatives.
Supplemental Figure 2. Alignment of CC-NB-LRR N-Terminal Domains.

Supplemental Table 1. Rx LRR Mutants Generated by Error-Prone PCR.

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