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Elicitor-Mediated Oligomerization of the Tobacco N Disease Resistance Protein [W] [OA]

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Plant nucleotide binding site–leucine-rich repeat (NBS-LRR) proteins are similar to the nucleotide binding oligomerization domain (NOD) protein family in their domain structure. It has been suggested that most NOD proteins rely on ligand-mediated oligomerization for function, and we have tested this possibility with the N protein of tobacco (*Nicotiana tabacum*). The *N* gene for resistance to *Tobacco mosaic virus* (TMV) is a member of the Toll-interleukin receptor (TIR)-NBS-LRR class of plant disease resistance (*R*) genes that recognizes the helicase domain from the TMV replicase. Using transient expression followed by immunoprecipitation, we show that the N protein oligomerizes in the presence of the elicitor. The oligomerization was not affected by silencing *Nicotiana benthamiana* ENHANCED DISEASE SUSCEPTIBILITY1 and N REQUIREMENT GENE1 cofactors of *N*-mediated resistance, but it was abolished by a mutation in the P-loop motif. However, loss-of-function mutations in the RNBS-A motif and in the TIR domain retain the ability to oligomerize. From these results, we conclude that oligomerization is an early event in the N-mediated resistance to TMV.

INTRODUCTION

Plant disease resistance (R) proteins are components of a plant surveillance system that recognize pathogen-derived elicitors and trigger signal transduction cascades, leading to defense. The largest class of plant R proteins contains a nucleotide binding site (NBS) and C-terminal leucine-rich repeats (LRRs) with either a Toll-interleukin receptor domain (TIR) or a loosely defined coiled coil (CC) at their N terminus (Dangl and Jones, 2001). These proteins are similar to the mammalian NOD (for nucleotide binding oligomerization domain) protein family, which functions in inflammation and apoptosis (Inohara and Nunez, 2003). R proteins and NOD proteins are alike in their domain structure, in that their N-terminal domains have been implicated in signaling, and in their role in the innate recognition of microorganisms (Inohara and Nunez, 2003).

By analogy with their animal protein homologues, it seemed likely that NBS-LRR proteins would oligomerize in response to pathogen elicitors. It is possible that, like NOD proteins interacting with ligands, they would oligomerize through their NBS domain (Inohara and Nunez, 2003). In addition, at least with TIR R proteins, it seemed possible, based on a comparison with Toll-like receptors (TLRs) and members of the interleukin-1 receptor superfamily (IL-1Rs), that the N-terminal domains would oligomerize. TLRs and IL-1Rs are transmembrane receptors with a cytoplasmic TIR domain and extracellular LRR and Ig domains, respectively (Silverman and Maniatis, 2001). After interaction with an extracellular ligand, these receptor proteins oligomerize; in turn, there are homotypic protein–protein interactions between the intracellular TIR domains. Activation of the signaling pathway follows from the oligomerization of the TIR domains (Xu et al., 2000; Hu et al., 2004; Sun et al., 2004).

However, in a study that had the potential to detect elicitor-mediated oligomerization of NBS-LRR R proteins, there was no evidence for homotypic protein–protein interactions. This study involved the CC-NBS-LRR protein Rx that confers resistance to *Potato virus X* (PVX) upon recognition of the PVX coat protein (CP) (Bendahmane et al., 1999). Interactions of the CC and LRR domains were detected, but they were heterotypic: CC interacted with the NBS-LRR domains, whereas LRR interacted with CC-NBS (Moffett et al., 2002). Moreover, these interactions were disrupted rather than induced by the elicitor. Based on these findings, it was proposed that the activation of Rx involves conformational changes, as with NOD proteins, but that oligomerization was not required.

Here, we describe further investigations of R protein interactions and oligomerization using the tobacco (*Nicotiana glutinosa*) N protein. N is unlike Rx in that it is a TIR-NBS-LRR rather than a CC-NBS-LRR protein (Whitham et al., 1994). It mediates recognition of the helicase domain in the tobacco mosaic virus (TMV) replicase (Erickson et al., 1999) and activates a resistance response requiring several known general cofactors of disease resistance: ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) (Peart et al., 2002a), SUPPRESSOR OF G-2 ALLELE OF SKIP1 (SGT1) (Peart et al., 2002b), REQUIRED FOR Mla12 RESISTANCE1 (RAR1) (Liu et al., 2002b), HEAT SHOCK PROTEIN90

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(HSP90) (Liu et al., 2004a; Lu et al., 2003), members of the COP9 signalosome (Liu et al., 2002a), and protein kinases (Jin et al., 2003; Liu et al., 2004b). Additionally, N REQUIREMENT GENE 1 (NRG1), a CC-NBS-LRR protein, has been shown to be specifically involved in the N-mediated response (Peart et al., 2005). Using transient expression of epitope-tagged proteins, we show that early events in the pathway leading to TMV resistance are oligomerization and stabilization of the N protein. Based on these results, we propose that elicitor-mediated activation of N, and possibly of other NBS-LRR proteins, is similar to the ligand-mediated triggering of NOD proteins.

RESULTS

Functional Transient Expression of Epitope-Tagged N Protein

The N-mediated response can be observed in tobacco (*Nicotiana tabacum*) cv Samsun (NN) as a hypersensitive response (HR) after transient expression of the TMV P50 elicitor (Figure 1A). In N-transgenic *Nicotiana benthamiana* plants (line 310A, carrying N under the control of its native promoter), there is no HR but the N response is manifested as resistance against a green fluorescent protein–tagged version of TMV (TMV:GFP). TMV:GFP induces green fluorescent foci on the inoculated leaves of nontransgenic plants that are not produced on 310A (Figure 1B).

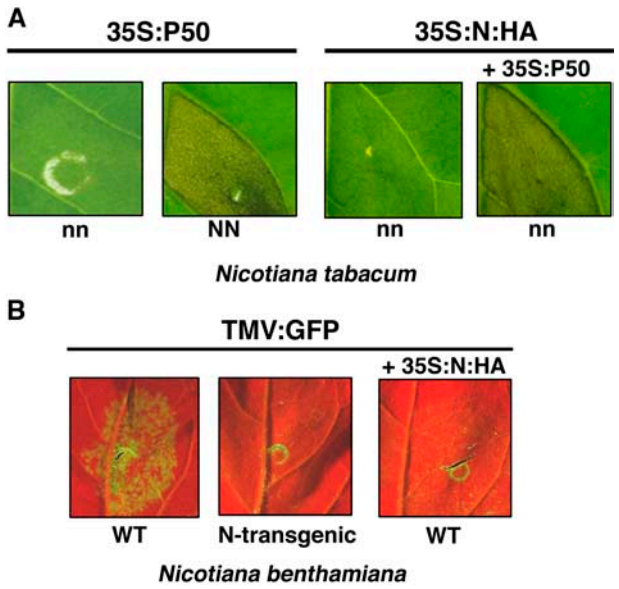

Figure 1. Functional Analysis of Transiently Expressed N Protein.

(A) *Agrobacterium tumefaciens*–mediated expression of the P50 protein from TMV into leaves of TMV-susceptible tobacco (nn; first panel from left) and N-bearing tobacco (NN; second panel from left). The third panel shows the expression of an HA-tagged version of the N protein (35S:N:HA) in TMV-susceptible tobacco leaves, and the fourth panel shows the coexpression of P50 and 35S:N:HA in TMV-susceptible tobacco leaves. Photographs were taken at 2 d after infiltration.

(B) *Agrobacterium*-mediated expression of a GFP-tagged version of TMV (TMV:GFP) into leaves of *N. benthamiana* (left panel) and N-transgenic *N. benthamiana* (center panel). The right panel shows the coexpression of TMV:GFP and 35S:N:HA in *N. benthamiana* leaves. Photographs were taken under UV light at 5 d after infiltration.

Transiently expressed hemagglutinin (HA)- and myc-tagged versions of the N protein under the control of the cauliflower mosaic virus (CaMV) 35S promoter and terminator were also functional in the transient expression assay. Thus, transient expression of HA-tagged *N* genomic sequence in tobacco cv Petite Havana (nn) generated a P50-dependent HR (Figure 1A), and in nontransformed *N. benthamiana* it suppressed TMV:GFP (Figure 1B). The HR response was not visible when HA-tagged N was expressed in tobacco cv Petite Havana (nn) in the absence of P50 (Figure 1A). The same results were obtained with a myc-tagged version of the protein and with similar constructs coupled to the promoter from *N*. However, except where stated, the experiments described below were with the 35S promoter constructs. In all instances, the constructs had the 35S rather than the N transcriptional terminator; nevertheless, the encoded wild-type N proteins were functional mediators of TMV resistance. This finding is in contrast with the previous report that the N genomic 3′ sequence is required for proper N function (Dinesh-Kumar and Baker, 2000). A possible explanation for the difference is our use of a transient assay rather than stable transformation for the expression of N.

N Protein Oligomerizes in Response to Elicitor

We transiently coexpressed HA- and myc-tagged versions of N to determine whether N can self-associate. These constructs were expressed with either the TMV P50 elicitor or, as a control, the CP from *Potato virus Y*, which does not elicit any type of *N*-mediated response. In the presence of the control CP or with N-HA expressed alone, N-HA did not coimmunoprecipitate with N-myc and vice versa (Figures 2A and 2C). However, in the presence of the P50 elicitor (Figure 2A), the two forms of N coimmunoprecipitated. This N protein coprecipitation is not an artifact of 35S overexpression, because HA- and myc-tagged constructs under the control of the N native promoter interacted similarly in the presence of P50 (Figure 2B). There was no interaction of N with a functional HA-tagged version of NRG1 (Figure 2C) in these assays or with a functional GFP-tagged version of the P50 elicitor (Figure 2D).

Next, we addressed the elicitor-mediated oligomerization of N in different genetic backgrounds in which the N-mediated response was compromised. To do so, we performed our experiments in plants silenced for *EDS1*, *NRG1*, and *SGT1*, genes required for N-mediated resistance (Peart et al., 2002a, 2002b, 2005). *Tobacco rattle virus* (TRV)-induced silencing of *EDS1* and *NRG1* had no effect on the P50-dependent interactions of N protein. The N-HA and N-MYC proteins coimmunoprecipitated in extracts of TRV:EDS1- or TRV:NRG1-infected plants, as in plants infected with an empty TRV (TRV:00) (Figure 3A). These results were consistent in three independent experiments, and additional experiments confirmed the predicted loss of TMV resistance in plants infected with the EDS1- and NRG1-silencing constructs (Peart et al., 2002a, 2002b, 2005). Therefore, from these results, we can rule out the possibility that oligomerization

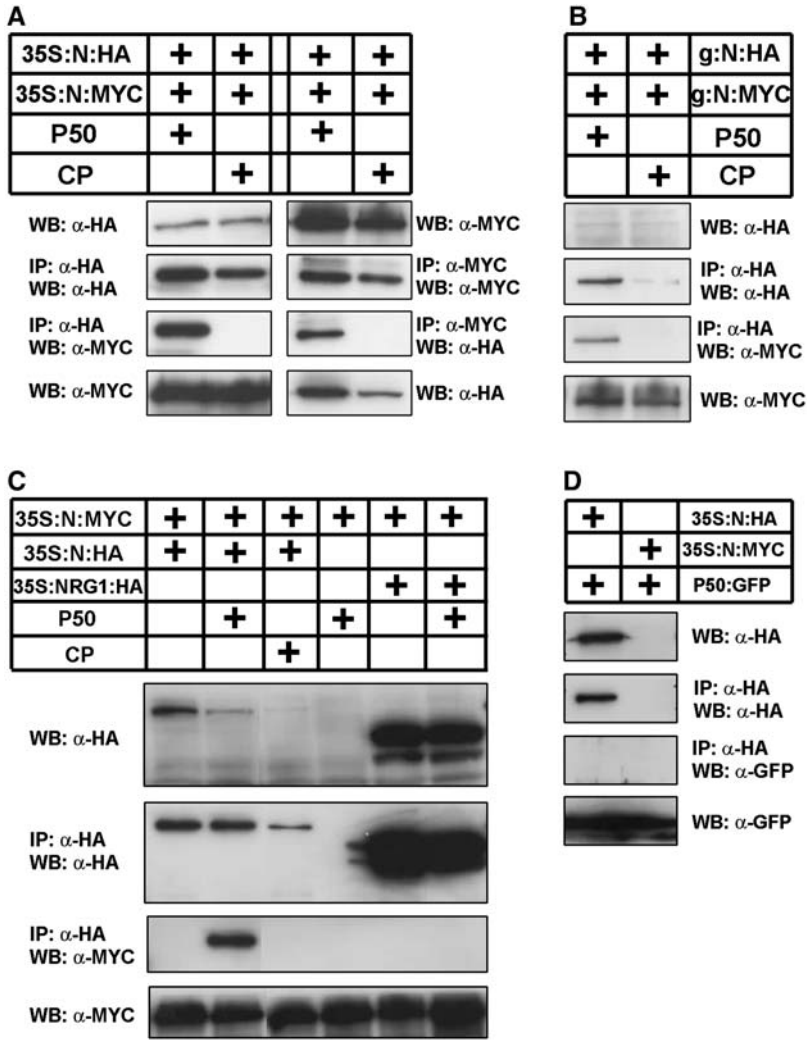

Figure 2. N Protein Oligomerizes in Response to Elicitor.

(A) HA- and myc-tagged versions of N were coexpressed together with the TMV-derived elicitor (P50) or, as a control, the CP from PVY into leaves of *N. benthamiana* plants. Two days later, protein extracts were subjected to immunoprecipitation experiments using anti-HA (IP: α-HA) or anti-myc (IP: α-MYC) agarose beads. The expressed proteins and immune complexes were analyzed by SDS-PAGE followed by protein gel blotting with anti-HA (WB: α-HA) and anti-myc (WB: α-MYC) antibodies. N constructs consisted of the complete N genomic sequence (from start to stop codons) plus the epitope tag under the control of the CaMV 35S promoter and terminator. Results are representative of at least three independent experiments.
(B) Same as in **(A)**, but with the N-derived clones under the control of the native promoter and terminator. Experiments were performed as described for **(A)**, only with anti-HA agarose beads (IP: α-HA).
(C) HA- and myc-tagged versions of N were coexpressed either alone or together with P50 or CP in *N. benthamiana*. myc-tagged N was also coexpressed with a HA-tagged version of NRG1 in the presence or absence of the P50 elicitor. NRG1 is a CC-NBS-LRR protein required for N-mediated resistance. myc-tagged N coexpressed with P50 was used as a control. Two days later, protein extracts were subjected to immunoprecipitation with anti-HA agarose beads and protein gel blotting as described for **(A)**. Results are representative of three independent experiments.
(D) HA- and myc-tagged versions of N were expressed together with a GFP-tagged version of P50 (P50:GFP). Two days later, protein extracts were subjected to immunoprecipitation with anti-HA agarose beads and protein gel blotting with anti-HA (WB: α-HA) and anti-GFP (WB: α-GFP) antibodies. Results are representative of two independent experiments. P50:GFP retained its function as elicitor and induced the oligomerization of N protein.

is a consequence of the resistance response, and we conclude that elicitor-mediated oligomerization of N protein is upstream of EDS1 and NRG1 in the N-mediated response to TMV.

The level of N protein in the P50-elicited samples was consistently higher than in nonelicited samples (CP) (see Figures 2, 3, 4C, and 5C), suggesting that N is stabilized or solubilized in the presence of the P50 elicitor. However, after silencing of SGT1 with TRV:SGT1, in contrast with the results with TRV:EDS1 and TRV:NRG1, there were lower levels of soluble N and the P50-induced N oligomerization could not be detected (Figure 3A), even after overexposure of the protein gel blot (Figure 3B). The levels of N were so low that we cannot draw any conclusions

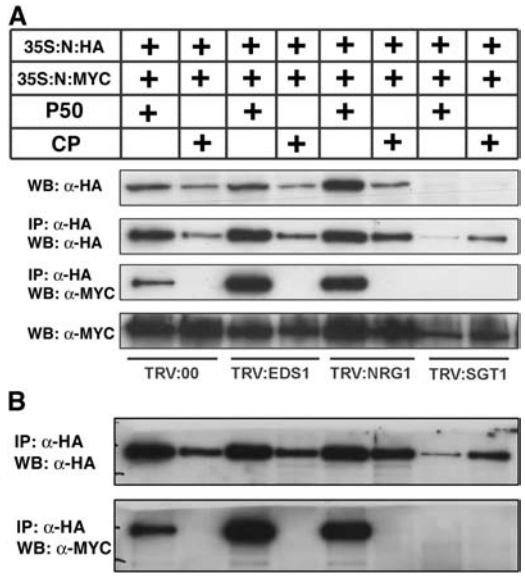

Figure 3. Oligomerization Is a Specific Early Event in the N-Mediated Response.

(A) HA- and myc-tagged versions of N were coexpressed either alone or together with P50 or CP in *N. benthamiana* plants silenced for *EDS1* (TRV:EDS1), *NRG1* (TRV:NRG1), or *SGT1* (TRV:SGT1). N-mediated resistance is not effective in these silenced plants. *N. benthamiana* plants inoculated with an empty silencing vector (TRV:00) were used as controls. Proteins were expressed 21 d after infection with the TRV-derived constructs, and 2 d later protein extracts were subjected to immunoprecipitation with anti-HA agarose beads followed by protein gel blotting. Results are representative of three independent experiments. The lower levels of immunoprecipitated proteins present in the TRV:00-infected samples compared with the TRV:EDS1- and TRV:NRG1-infected samples are most likely attributable to the resistance response taking place at the infiltrated leaves of TRV:00-infected plants.
(B) Longer exposure of protein gel blots of immune complexes from **(A)**.

about the role of SGT1 in N oligomerization. However, these results indicate that SGT1, either directly or indirectly, plays a role in the stabilization of N.

Mutations in Conserved Motifs Affect P50 Elicitor-Mediated Oligomerization and N Protein Stabilization

The P-loop motif (Figure 4A) in the NBS is likely involved in nucleotide binding in NBS-LRR proteins (Tameling et al., 2002) and is necessary for their function in disease resistance (Dinesh-Kumar et al., 2000; Bendahmane et al., 2002). To test the P-loop role in N self-association, we created a 35S promoter N construct with the mutation GK221,222AA. The mutant protein was stable and, as expected, failed to trigger a resistance response against TMV (Figure 4B) in either the HR or TMV:GFP resistance assay method. This mutant also lost the ability to oligomerize or coprecipitate with the wild-type protein in the presence of the elicitor (Figure 4C) and did not increase in abundance after P50 elicitation. From these results, we conclude that elicitor-mediated oligomerization and stabilization of N requires the presence of an intact P-loop motif. Furthermore, our data show that the CP does not reduce the abundance of the N protein (Figure 4C).

By contrast, a second conserved motif, RNBS-A, does not affect the coprecipitation of N, although it is required for N protein function in resistance assays. RNBS-A is located between the P-loop and kinase-2 motifs, and its consensus sequence differs between TIR and non-TIR R proteins (Meyers et al., 1999, 2003). Close inspection of an alignment of known functional TIR R proteins identified the presence of a putative LXXLL motif (Leo and Chen, 2000) inside the RNBS-A motif (Figure 5A). Because LXXLL motifs are known to participate in protein–protein interactions, we reasoned that this motif might play a role in the N-mediated oligomerization.

We created a 35S-driven N construct with the RNBS-A mutation LL270,271AA (N$_{AA}$; Figure 5). The same mutation in the equivalent LXXLL motif of the mammalian NOD protein CTIIA caused loss of function and affected its oligomerization ability (Sisk et al., 2001). N$_{AA}$ produced a stable protein but failed to mediate resistance against TMV (Figure 5B). Although this construct occasionally caused a very weak HR in response to P50 in tobacco, it consistently allowed virus multiplication in the TMV:GFP resistance assay. Unlike wild-type N, this mutant protein in the elicited sample (P50) was consistently less abundant than in the nonelicited sample (CP) (Figure 5C), indicating that the RNBS-A motif influences the elicitor-mediated protein stabilization. However, the RNBS-A mutant retained the ability to oligomerize in response to the P50 elicitor (Figure 5C). Thus, neither elicitor-mediated protein stabilization nor resistance is an automatic consequence of N protein oligomerization.

TIR Domain Interactions

To further investigate the N interactions, we coexpressed epitope-tagged versions of the TIR, NBS, LRR, TIR-NBS, and NBS-LRR domains of N in the presence and absence of P50. In some instances (e.g., with NBS), the domains were unstable and protein could barely be detected (see Supplemental Table 1 and Supplemental Figure 1 online); however, of the heterotypic combinations of stable domains, none formed a functional complex, as described previously for domains of Rx (Moffett et al., 2002) (P. Mestre, unpublished data). However, there was a homotypic interaction of TIR domains (Figure 6B). Coimmunoprecipitation of TIR domains did not require and was not affected by the presence of P50 (see Supplemental Figure 2 online). The TIR coprecipitation was specific because the TIR of N interacted only weakly or did not interact with the TIR domains of the RPS4 or Bs4 NBS-LRR proteins (see Supplemental Figure 3 online). A summary of all of the homotypic and heterotypic interactions tested in the resistance and pull-down assays is presented in Supplemental Tables 2 and 3 online.

We tested the significance of the TIR interactions by mutation of TIR domain amino residues at predicted solvent-exposed sites that may play a role in signaling and protein–protein interactions (Figure 6A). All nine mutants produced stable proteins, and three of them (R24A, S80A, and P110Y) were compromised in the HR assay in tobacco leaves and in the resistance assay against TMV:GFP in *N. benthamiana* (Figure 6C; data not shown).

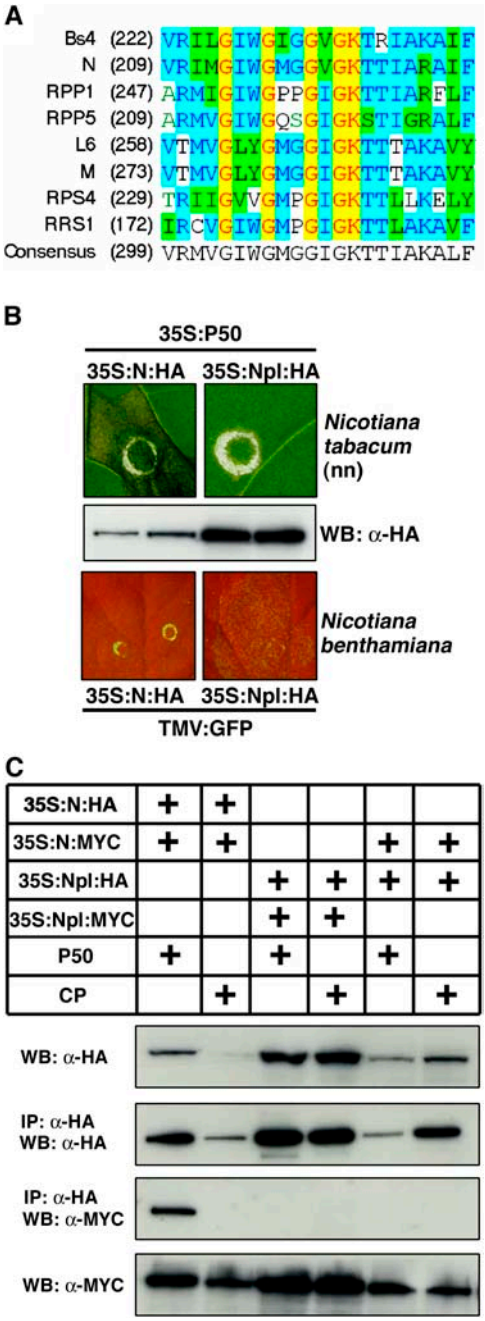

Figure 4. N Protein with a Mutated P-Loop Motif Does Not Oligomerize in Response to Elicitor.

(A) Alignment of the P-loop motif of selected TIR-NBS-LRR R proteins from pepper (*Capsicum annuum*; Bs4), tobacco (N), flax (*Linum usitatissimum*; L6 and M), and *Arabidopsis* (RPP1, RPP5, RPS4, and RSS1). Red lettering on yellow background indicates identity; blue on cyan indicates conservation in at least 50% of the sequences; black on green indicates blocks of similarity. In our analysis, the Gly and Lys at positions 221 and 222 in the N protein were mutated to Ala (GK221,222AA).

(B) HA-tagged N protein (35S:N:HA) and mutant GK221,222AA (35S:Npl:HA) were coexpressed with the P50 elicitor in tobacco leaves (top panel) and with TMV:GFP in *N. benthamiana* leaves (bottom panel). The HA-tagged proteins were also expressed in *N. benthamiana*, and two independent samples per construct were subjected to protein gel blotting with anti-HA antibodies (middle panel). Protein samples were taken at 2 d post-agroinfiltration (dpa). Photographs of tobacco leaves were taken at 2 dpa, and those of *N. benthamiana* leaves were taken under UV light at 5 dpa.

(C) Combinations of HA- and myc-tagged versions of N and Npl were coexpressed together with P50 or CP in *N. benthamiana* plants. Two days later, protein extracts were subjected to immunoprecipitation followed by protein gel blotting. Results are representative of two independent experiments.

In the HR assay, the R24A mutant was completely inactive and the S80A and P110Y mutants induced a very weak HR. All three mutants completely failed to induce resistance in the TMV:GFP assay. The corresponding mutants of full-length N retained their ability to oligomerize in response to the P50 elicitor (Figure 6D), although the interaction was weakest for mutant R24A. Similarly, the isolated TIR domain mutants exhibited homotypic interactions, although, as with the full-length proteins, the TIR domain of R24A was the weakest interactor (Figure 6E). These results are as predicted if the coprecipitation of full-length N is mediated by homotypic interactions of the TIR domain. They also are the predicted outcomes if oligomerization of N is an early event in the elicitor-mediated activation of the disease resistance pathway.

DISCUSSION

Here, we describe elicitor-mediated oligomerization and stabilization of the N protein as novel processes associated with resistance against TMV (Figures 2 to 4). The oligomerization and stabilization appear to be separate processes because they are differentially affected by mutations in the RNBS-A motif of N (Figure 5). We infer that oligomerization is functionally significant because, like the N resistance response, it is dependent on an intact P-loop motif (Figure 4). Moreover, there was a correlation between N oligomerization and the elicitation of resistance in that activation of resistance with either mutant or wild-type N was always associated with oligomerization. However, oligomerization is not sufficient to trigger the resistance response (Figure 5), and we deduce that additional interactions of oligomerized N are required for N function. In addition, because silencing of a presumed downstream signaling component in the N signaling pathway (EDS1) has no effect on either the oligomerization or the stabilization of N (Figure 4), it is likely that these are early events associated with the elicitor activation of N. A model of N oligomerization-induced activation of disease resistance is described in more detail below.

The results from the mutational analysis of the TIR domain are in agreement with the proposed signaling function of the TIR. The R24A, S80A, and P110Y mutations have evidently resulted in loss of function in the signaling domain but have caused only partial loss (R24A) or no loss of the oligomerization function. This proposed separation of oligomerization and signaling functions is reinforced by our analysis of an RNBS-A mutant (Figure 5). It is also consistent with the conclusion from EDS1 and NRG1 silencing (Figure 3) that N oligomerization is an early event in the sequence of events leading to P50-elicited disease resistance.

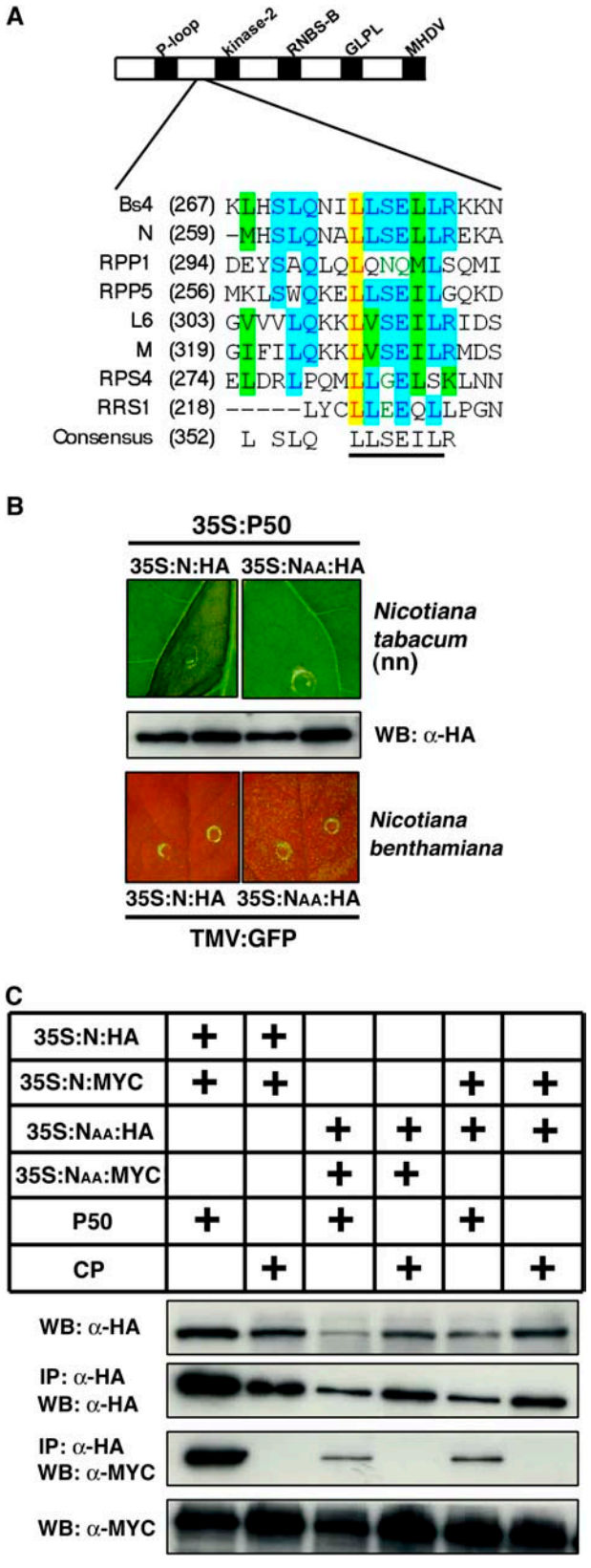

Figure 5. A Mutant in the RNBS-A Motif Retains Oligomerization Ability.

(A) Alignment of the region surrounding the putative LXXLL motif (underlined in the consensus sequence) inside the RNBS-A motif. R proteins and color coding are as in Figure 4A. The scheme at the top shows the relative position of the sequence in the NBS domain. The nomenclature of the motifs of the NBS domain is as described by Meyers et al. (1999). In our analysis, the Leu residues at positions 270 and 271 in the N protein were mutated to Ala (LL270,271AA).

(B) HA-tagged N protein (35S:N:HA) and mutant LL270,271AA (35S:NAA:HA) were coexpressed with the P50 elicitor in tobacco leaves (top panel) and with TMV:GFP in *N. benthamiana* leaves (bottom panel). The HA-tagged proteins were also expressed in *N. benthamiana*, and two independent samples per construct were subjected to protein gel blotting with anti-HA antibodies (middle panel). Protein samples were taken at 2 dpa. Photographs of tobacco leaves were taken at 2 dpa, and those of *N. benthamiana* leaves were taken under UV light at 5 dpa.

(C) Combinations of HA- and myc-tagged versions of N and NAA were coexpressed together with P50 or CP in *N. benthamiana* leaves. Two days later, protein extracts were subjected to immunoprecipitation followed by protein gel blotting. Results are representative of two independent experiments.

Our finding that the silencing of SGT1 resulted in low levels of N soluble protein is consistent with, although does not prove, the possibility that SGT1 is involved in the elicitor-induced stabilization of N. SGT1 interacts with the RAR1 and HSP90 cofactors of disease resistance, and it has been proposed that RAR1 and SGT1 are cochaperones of HSP90 in the folding of R proteins (Shirasu and Schulze-Lefert, 2000; Schulze-Lefert, 2004). This idea is supported by several lines of evidence: RPM1 levels are reduced in *Arabidopsis rar1* or *hsp90.2* mutants (Tornero et al., 2002; Hubert et al., 2003); the amount of RPS2 is reduced in *Arabidopsis rar1* (Belkhadir et al., 2004); silencing of HSP90 in *N. benthamiana* causes reduced levels of Rx (Lu et al., 2003); RAR1 controls the steady state level of Mla proteins in barley (*Hordeum vulgare*) (Bieri et al., 2004).

However, R protein stabilization, SGT1 function, and disease resistance are not always associated in the same way. The RPM1 NBS-LRR protein, for example, which confers resistance against *Pseudomonas syringae* strains, is unlike N in that it is destabilized when resistance is elicited (Boyes et al., 1998). In this instance, the role of SGT1 may be as a cofactor in a RAR1-dependent degradation mechanism (Holt et al., 2005) rather than as a R protein stabilizer, as implied by our analysis of N. To reconcile these apparently conflicting results, we propose that SGT1 and associated proteins are part of a system for controlling the level of R proteins through either positive or negative regulation.

A model of N activation is shown in Figure 7. The initial events are changes to the conformation of N. We considered the possibility that these changes could be analogous to the disruption of CC and the LRR domain interactions that are associated with the elicitation of Rx-mediated resistance (Figure 7, left branch). However, in an extensive analysis (see Supplemental Tables 2 and 3 online), there was no evidence for intramolecular interactions by coexpression of N domains. Perhaps the intramolecular interactions in N are weaker than in Rx and are not effective for proteins expressed in trans. Alternatively, it is possible that elicitor-mediated activation of N involves interactions with other as yet unidentified proteins or, perhaps, a change in subcellular localization of the protein (Figure 7, right branch).

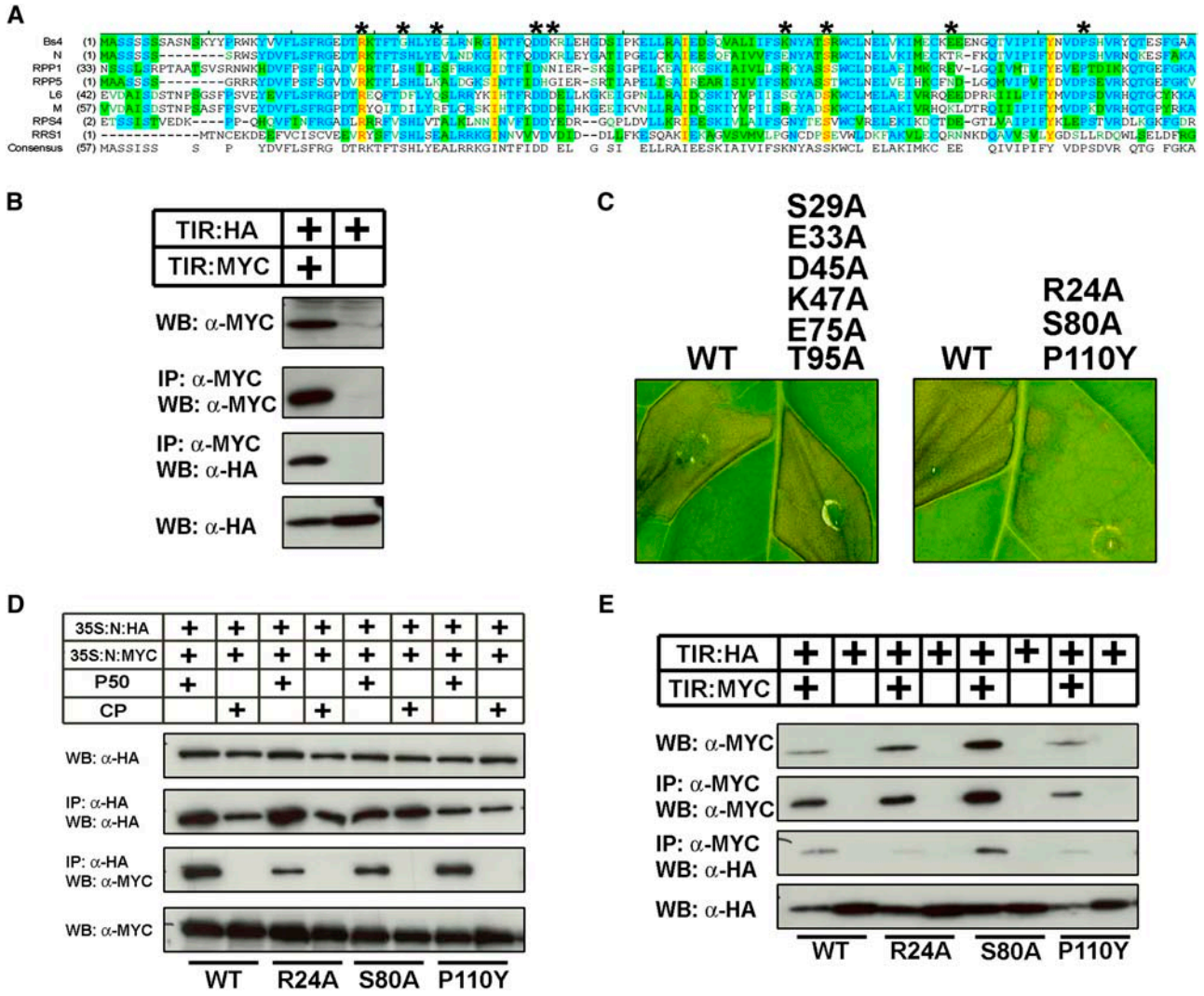

Figure 6. Loss-of-Function Mutants in the TIR Oligomerize in Response to Elicitor.

(A) Alignment of the sequence corresponding to the N-terminal 123 amino acids of the TIR domain from N. R proteins and color coding are as in Figure 4A. Amino acids mutated in our study are marked with asterisks.

(B) HA- and myc-tagged versions of the TIR domain (residues 1 to 150) from N were coexpressed in *N. benthamiana*. HA-tagged TIR expressed alone was used as a control. Two days later, protein extracts were subjected to immunoprecipitation experiments using anti-myc (IP: α-MYC) agarose beads followed by protein gel blotting. Results are representative of three independent experiments.

(C) HA-tagged N protein (WT) and mutants were coexpressed with the P50 elicitor in tobacco leaves. Each mutation indicates an individual mutant, and each panel illustrates representative data with mutants giving a wild-type (left) or loss-of-function (right) phenotype. Photographs were taken at 2 dpa. The mutants in each category are listed above the panels. The photograph in the right panel corresponds to mutant S80A.

(D) HA- and myc-tagged versions of N protein were coexpressed together with P50 or CP in *N. benthamiana* leaves. Two days later, protein extracts were subjected to immunoprecipitation with anti-HA beads followed by protein gel blotting. The mutation in the N sequence is indicated at the bottom. Results are representative of three independent experiments. The HA- and myc-tagged N constructs were both from the wild-type or the mutant versions, as indicated below the bottom panel.

(E) HA-tagged TIRs from N (WT) and from loss-of-function mutants were coexpressed with their corresponding myc-tagged versions in *N. benthamiana* leaves. Two days later, protein extracts were subjected to immunoprecipitation with anti-myc beads followed by protein gel blotting. The mutation in the TIR sequence is indicated at the bottom. Results are representative of three independent experiments.

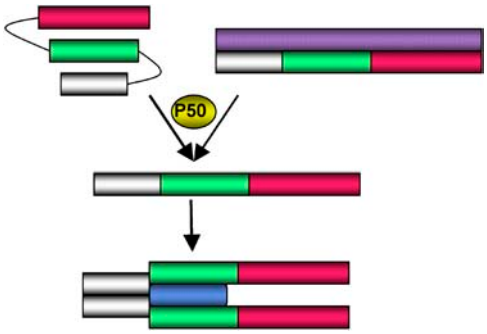

Figure 7. Model for the Activation of N.

Scheme of a proposed mechanism for the activation of N based on the results presented in this work. In the absence of elicitor, N adopts an inactive conformation by means of intramolecular interactions and/or interaction with other proteins (purple box). Recognition of the P50 elicitor causes the release of N from the inactive complex, allowing its oligomerization through, at least, the TIR domain. Recruitment of additional components (blue box) via their interaction with the NBS stabilizes the complex and renders it active. Gray boxes, TIR; green boxes, NBS; red boxes, LRR.

Our model proposes that these initial conformational changes would cause N to oligomerize in complexes that are required for the activation of the EDS1-dependent and perhaps other response pathways, leading to virus resistance and HR (Figure 7). In principle, the TIR interactions (Figure 6) could mediate the oligomerization process if they are exposed by the elicitor-induced changes to N. Presumably, the isolated TIR domains, being free of the rest of the N protein, would not be masked by a subcellular location or other domains in N and would be available to interact even in the absence of elicitor. We show the homotypic N interactions in Figure 7 as being direct, but we emphasize that they could be indirect and dependent on host factors that have not yet been identified. It is also possible that domains of N, in addition to the TIR, may be involved in the oligomerization process. It is possible that, as with Toll and TLRs, the TIR domain interactions are secondary to oligomerization at other more C-terminal domains (Xu et al., 2000; Hu et al., 2004; Sun et al., 2004). Unfortunately, the isolated NBS domains of N were not stable in the transient assay, and we could not assay their potential for homomeric interactions.

How could oligomerization of N activate the virus resistance and HR pathways? It is unlikely to be simple induced proximity of TIR domains, because the isolated TIR domains interact but do not induce an HR. Accordingly, as with other members of the NOD family, expression of the N-terminal domain alone does not activate response pathways (Inohara and Nunez, 2003). Perhaps, as described for the NOD protein CTIIA (Sisk et al., 2001, and references therein), the NBS domain in the N oligomer is a scaffold for components of the signaling pathway (Figure 7). This possibility is consistent with our finding that an RNBS-A mutant oligomerizes in response to elicitor but does not trigger a resistance response (Figure 5): the role of RNBS-A would be to interact with signaling components and not in oligomerization.

The elicitor-induced oligomerization and stabilization of N, being upstream of EDS1 (Figure 3), are the earliest identified molecular features of the N resistance pathway; therefore, they are useful for positioning other processes in the sequence of recognition and response mechanisms. Here, for example, we have shown that the CC-NBS-LRR protein NRG1 is likely to act downstream of the elicitor recognition process because elicitor-induced oligomerization/stabilization of N occurs in the NRG1-silenced plants. As overexpression of NRG1 induces responses that are not dependent on EDS1 (Peart et al., 2005), it is likely that NRG1 is downstream or, perhaps more likely, independent of EDS1 in the N pathway.

We were able to detect the N oligomerization in our transient assay system because the elicitor-induced cell death is absent. In other experimental systems, including the Rx CC-NBS-LRR protein, the elicitor-induced cell death is rapid and prevented us from detecting the transiently expressed proteins (Moffett et al., 2002). The Rx cell death response can be suppressed, for example, in an SGT1-silencing background or with P-loop mutants (Moffett et al., 2002), but these conditions are not suitable for the detection of N oligomerization (Figures 3 and 4). Therefore, it is an open question whether other NBS-LRR proteins oligomerize in response to elicitor. Clearly, to explore the similarity of plant NBS-LRR R proteins with NOD proteins of animals, it will be necessary to extend the analyses of protein interactions to a range of other R proteins. It would also help to have more detailed analysis of N and more information about structure and structure–function relationships.

METHODS

Plant Material

Wild-type *Nicotiana benthamiana*, *N*-transgenic *N. benthamiana* line 310A (Bendahmane et al., 1999), tobacco (*Nicotiana tabacum*) cv Samsun (NN), and tobacco cv Petite Havana (nn) plants were grown in glasshouses under controlled light and temperature.

Plasmid Construction

Two pBIN61-derived vectors (Bendahmane et al., 2002) were made for the transient expression of epitope-tagged versions of N protein and fragments of it. A triple HA tag was amplified by PCR from pACTAG2 (Charest et al., 1995) using a forward primer carrying a *Spe*I site plus extra restriction sites and a reverse primer with an *Xma*I restriction site. The PCR product was digested with *Spe*I and *Xma*I and cloned into pBIN61 digested with *Xba*I and *Xma*I, resulting in the vector we refer to as pHAN, which comprises a linker (*Xho*I-*Pml*I-*Avr*II) between the 35S promoter and the triple HA tag. Vector pMYCN was obtained using the same procedure, but the myc tag (five copies) was amplified from plasmid pCS2+MT (Rupp et al., 1994).

To clone N fragments into pHAN and pMYCN, we amplified the different fragments from an N genomic clone (see below) using forward and reverse primers carrying *Xho*I and *Avr*II restriction sites, respectively. The amplified products were cloned directionally into pHAN and pMYCN digested with *Xho*I and *Avr*II. A list of the different clones obtained, the N sequences they comprise, and the primers used for amplification is shown in Supplemental Table 1 online. Primer sequences are available on request. All PCRs were performed using Pfu polymerase, and the identities of all clones obtained were confirmed by sequencing.

Full-length HA-tagged N (35S:N:HA) consists of the complete N genomic sequence (from start to stop codons) plus the HA tag under the

control of the CaMV 35S promoter and terminator. It was built from several N fragments as follows. pHAN:LRR1 and pHAN:LRR2 were digested with *Bam*HI. The insert derived from pHAN:LRR1 and the backbone derived from pHAN:LRR2 were gel-purified and ligated to obtain pHAN:LRR4. Next, this construct and pHAN-TNBL were digested with *Sal*I and *Dra*III. The insert derived from pHAN:TNBL and the backbone derived from pHAN:LRR4 were gel-purified and ligated to obtain 35S:N:HA. Exactly the same procedure, but with pMYCN-based constructs, was used to create 35S:N:MYC.

To place MYC-tagged N under the control of the N native promoter (gNMYC), a 12.3-kb DNA fragment containing N coding sequence, introns, 4.3 kb of 5′ flanking sequence, and 1.3 kb of 3′ flanking sequence was transferred from plasmid pTG34 (Whitham et al., 1994) to pBIN19 (Bevan, 1984) using *Xho*I, creating pBIN19:N. A 5xMYC tag was amplified from pSC2+MT using a reverse primer with an *Avr*II site and a forward primer with an overhang containing the last 20 nucleotides of N coding sequence, which contains a unique *Sac*I site. The amplified product was digested with *Avr*II. Next, the 1.3 kb of 3′ flanking sequence was amplified using forward and reverse primers carrying *Avr*II and *Xho*I sites, respectively, and the PCR product was digested with *Avr*II, ligated to the *Avr*II-digested MYC tag, gel purified, and then digested with *Sac*I and *Xho*I. Finally, pBIN19:N was digested with *Xho*I and *Sac*I and the released fragment was used in a three-way ligation with the 5xMYC 3′-untranslated region *Sac*I-*Xho*I fragment and *Xho*I-digested pBIN19. To obtain gNHA, the *Sac*I-*Avr*II fragment of gNMYC was replaced with its HA counterpart.

All mutants of N described in this work were obtained by PCR methods. Forward N primers starting at ATG and including an *Xho*I site (primer Nup) were used with reverse primers including the mutation, whereas forward primers including the mutation were used together with a downstream reverse primer (NBSdw; see Supplemental Table 1 online). Both PCR fragments were gel-purified, subjected to five cycles of PCR without primers, and then amplified with primers Nup and NBSdw. The final amplified fragment contained a *Stu*I site that was always located downstream of the mutation. The PCR product was digested with *Xho*I and *Stu*I and replaced into 35S:N:HA and 35S:N:MYC. The sequences of primers used for mutagenesis are available on request. All PCRs were performed using Pfu polymerase, and the identities of all clones obtained were confirmed by sequencing.

Mutants in the context of the TIR domain were obtained by PCR methods. Forward N primers starting at ATG and including an *Xho*I site (primer Nup) were used with reverse primers including the mutation, whereas forward primers including the mutation were used together with a downstream reverse primer (TIRdw; see Supplemental Table 1 online). Both PCR fragments were gel-purified, subjected to five cycles of PCR without primers, and then amplified with primers Nup and TIRdw. The PCR product was digested with *Xho*I and *Avr*II and cloned directionally into pHAN and pMYCN.

To obtain 35S:NRG1:HA, the NRG1 sequence was amplified from a cDNA clone (Peart et al., 2005) using forward and reverse primers carrying *Xho*I and *Avr*II restriction sites, respectively. After digestion, the amplified product was cloned directionally into pHAN digested with *Xho*I and *Avr*II.

The sequence of the P50 elicitor (nucleotides 2082 to 3418 from TMV, which correspond to the helicase domain of the viral replicase) (Erickson et al., 1999) was amplified from the U1 strain from TMV using forward and reverse primers with *Sal*I and *Xma*I restriction sites, respectively. The resulting product was digested with these enzymes and cloned into pBINY53 (Mestre et al., 2000).

To obtain P50:GFP, the same sequence described above was amplified with forward and reverse primers with *Sal*I and *Xba*I restriction sites, respectively. The resulting product was digested and cloned into a pBINY53-derived binary vector containing the GFP4 sequence (P. Mestre, unpublished data). Primer sequences are available upon request. The other constructs used in this work have been described elsewhere: CP (Mestre et al., 2000); TMV:GFP, TRV:00, and TRV:EDS1 (Peart et al., 2002a); TRV:SGT1 (Peart et al., 2002b); and TRV:NRG1 (Peart et al., 2005).

Agrobacterium tumefaciens–Mediated Transient Expression

Binary constructs were transiently expressed in *N. benthamiana* and tobacco leaves as described (Mestre et al., 2000). In brief, *Agrobacterium* cells were inoculated into 5 mL of L medium supplemented with 50 μg/mL kanamycin and 2.5 μg/mL tetracycline and grown at 28°C. After centrifugation, cells were resuspended in 5 mL of a solution containing 10 mM $MgCl_2$ and 150 μM acetosyringone. The cultures were incubated at room temperature for 2 to 3 h before infiltration. N-derived cultures were infiltrated at 0.2 OD_{600}. P50 and CP cultures were infiltrated at 0.1 OD_{600}. TMV:GFP was infiltrated at a 50-fold dilution from 1 OD_{600}.

Protein Extraction, Immunoprecipitation, and Immunoblotting

All procedures were performed exactly as described previously (Moffett et al., 2002). Anti-HA (3F10) agarose beads (Sigma-Aldrich) and anti-myc (9E10) agarose beads (Santa Cruz Biotechnology) were used for immunoprecipitation. Protein gel blot analysis was performed with anti-HA 3F10 antibodies (Roche) and anti-myc A-14 antibodies (Santa Cruz Biotechnology). Coomassie Brilliant Blue R 250 staining of the membranes after protein gel blot analysis was used to confirm equal loading. The intensity of the Ig bands on the protein gel blots of immunoprecipitated samples was used as an additional loading control. These bands are not included in the figures for reasons of clarity. The Ig bands corresponding to Figures 4C and 5C are shown in Supplemental Figure 4 online.

Virus-Induced Gene Silencing

Virus-induced gene silencing experiments were performed using a TRV vector as described elsewhere (Ratcliff et al., 2001). Briefly, *N. benthamiana* plants were infiltrated with *Agrobacterium* carrying the different TRV-based constructs, and plants were used for agroinfiltration 3 weeks later.

Sequence Analysis

Alignments were performed using the AlignX application of VectorNTI suite 9. Predictions of TIR secondary structure and solvent accessibility were performed with PHD and PROF (Rost and Sander, 1993).

Accession Numbers

Sequence data for the genes used in this work can be found in the GenBank/EMBL data libraries under the following accession numbers: *NbSGT1* (AF516180), *NbEDS1* (AF479625), *NRG1* (DQ054580), and *N* (Q40392). The accession numbers for the EMBL/GenBank protein sequences of the resistance proteins shown in the alignments are as follows: Bs4 (AAR21295), RPP1 (AAC72977), RPP5 (AAF08790), L6 (AAA91022), M (AAB47618), RPS4 (CAB50708), and RRS-1 (Q9FH83).

Supplemental Data

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

Supplemental Table 1. Clones Expressing HA-Tagged Fragments of the N Protein.

Supplemental Table 2. Combinations of N Domains Tested for Transcomplementation.

Supplemental Table 3. Combinations of N and N Domains Assayed for Physical Interaction by Immunoprecipitation.

Supplemental Figure 1. Domain Structure of the N Protein and Expression Levels of HA-Tagged Versions of N-Derived Protein Fragments.

Supplemental Figure 2. Effect of the Presence of P50 on TIR Domain Coimmunoprecipitation.

Supplemental Figure 3. Specificity of the TIR Domain Coimmunoprecipitation.

Supplemental Figure 4. Loading Controls for Protein Gel Blots from Figures 4 and 5.

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