Dual Regulatory Roles of the Extended N Terminus for Activation of the Tomato Mi-1.2 Resistance Protein

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Submitted 26 November 2011. Accepted 12 April 2012.

Plant resistance (R) proteins mediate race-specific immunity and initiate host defenses that are often accompanied by a localized cell-death response. Most R proteins belong to the nucleotide binding-leucine-rich repeat (NB-LRR) protein family, as they carry a central NB-ARC domain fused to an LRR domain. The coiled-coil (CC) domain at the N terminus of some solanaceous NB-LRR proteins is extended with a solanaceae domain (SD). Tomato *Mi-1.2*, which confers resistance against nematodes, white flies, psyllids, and aphids, encodes a typical SD-CNL protein. Here, we analyzed the role of the extended N terminus for *Mi-1.2* activation. Removal of the first part of the N terminus (Nt1) induced *Mi-1.2*-mediated cell death that could be suppressed by overexpression of the second half of the N-terminal region. Yet, autoactivating NB-ARC-LRR mutants require in *trans* coexpression of the N-terminal region to induce cell death, indicating that the N terminus functions both as a negative and as a positive regulator. Based on secondary structure predictions, we could link both activities to three distinct subdomains, a typical CC domain and two novel, structurally-conserved helical subdomains called SD1 and SD2. A negative regulatory function could be assigned to the SD1, whereas SD2 and the CC together function as positive regulators of *Mi-1.2*-mediated cell death.

Besides a basal immune system that can be triggered by a wide variety of potential pathogens, plants also evolved a specific immune mechanism that is activated only by particular races of specialized pathogens. This second type of immunity, called effector-triggered immunity (ETI), is initiated after recognition of pathogen-produced effector proteins by matching host resistance (R) proteins (Jones and Dangl 2006). In order to restrict invading pathogens, activated R proteins induce defense responses that are often accompanied by a hypersensitive response (HR). This HR can often be visualized by the occurrence of local cell death around the infection zone (Heath 2000). Since R proteins can trigger host cell death, their activities must

be tightly controlled, a process mediated by inter- and intramolecular interactions (Lukasik and Takken 2009).

The majority of R proteins belong to the intracellular multi-domain nucleotide binding-leucine-rich repeat (NB-LRR) protein family. The roles of the distinct domains for NB-LRR functioning have been studied by analyzing their interaction patterns and via structure-function analyses (Lukasik and Takken 2009; van Ooijen et al. 2007). The most conserved domain among NB-LRR proteins is the NB domain, which is shared by human Apaf-1, plant R proteins, and the *Caenorhabditis elegans* Ced-4 protein and is also called the NB-ARC domain. The crystal structures of the NB-ARC domains from Apaf-1 and Ced-4 have been resolved, allowing modeling of the homologous NB-ARC domain of plant R proteins (Riedl et al. 2005; Takken et al. 2006; Yan et al. 2005). The 3D model revealed that plant NB-ARC domains are actually subdivided into three subdomains, the NB, ARC1, and ARC2, that together form the NB pocket (Albrecht and Takken 2006; Takken et al. 2006). Biochemical studies using tomato I-2 and *Mi-1.2*, flax M and L6, and the barley Mla27 R proteins revealed that they, indeed, bind nucleotides (Maekawa et al. 2011; Tameling et al. 2002; Williams et al. 2011). Both tomato R proteins have ATPase activity, and the observation that ATPase mutants become autoactivating formed the basis for the molecular ‘switch’ model (Tameling et al. 2006). This model proposes that the NB state of the NB-ARC determines the switch between a resting (ADP bound) and an activated (ATP bound) state of an R protein (Takken et al. 2006; Tameling et al. 2006). Additional support for this model comes from the observation that activated M copurifies specifically with ATP, while the inactive state is ADP bound (Williams et al. 2011).

The NB-ARC is involved in intramolecular interactions with its neighboring domains (Lukasik and Takken 2009). At the C terminus, the NB-ARC domain is fused to a LRR. The LRR domain is proposed to form a horseshoe-shaped structure forming a potential interaction surface (Padmanabhan et al. 2009). Due to its highly polymorphic nature and its ability to interact with a subset of effectors, it has been proposed to determine recognition specificity of NB-LRR proteins (Padmanabhan et al. 2009). Besides pathogen recognition, the LRR domain negatively modulates NB-LRR protein activity in the absence of a pathogen (Ade et al. 2007; Raidan and Moffett 2006; Tao et al. 2000; van Ooijen et al. 2008a; Weaver et al. 2006).

The type of N-terminal domain divides the NB-LRR proteins into two distinct classes: i) those carrying a toll and human interleukin-1 receptor (TIR) domain called the TIR-NB-ARC-LRR or TNL class, and ii) the others, often carrying a domain with predicted coiled-coil (CC) structures. The latter

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*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures are published online and that Figures 1 through 6 appear in color online.

are classified as CC-NB-ARC-LRR or CNL proteins (Pan et al. 2000). Recently, the crystal structures of a TIR and a CC domain of a plant TNL and CNL have been resolved (Bernoux et al. 2011; Maekawa et al. 2011). Notably, both domains were found to form homo-dimers and their overexpression in planta is sufficient to induce HR (Bernoux et al. 2011; Collier et al. 2011; Frost et al. 2004; Krasileva et al. 2010; Maekawa et al. 2011; Swiderski et al. 2009). Based on this property, the N terminus of these proteins was proposed to be the smallest functional module required for induction of host defense. This observation is surprising, as earlier it was shown for the potato R protein Rx1 that overexpression of its NB domain is sufficient to induce cell death (Rairdan et al. 2008). Either NB-LRR proteins rely on different subdomains to transduce downstream HR signaling or, alternatively, they form heteromeric complexes in which overexpression of a single domain triggers activation of an intact but inactive NB-LRR. N-terminal domains have also been shown to be involved in pathogen perception by interacting with plant proteins that are targeted by pathogens (Lukasik and Takken 2009).

Whereas most N termini of CNL proteins are relatively short, in some CNL, they are extended with extra domains or regions (Tameling and Takken 2008). A domain frequently present at extended N termini is the solanaceae domain (SD), called after its presence in a subset of solanaceae CNL proteins (Mucyn et al. 2006). The significance of an extended N terminus for SD-CNL protein function has mainly been studied for the tomato Prf protein, which carries also an additional N-terminal extension. This additional extension was shown to be required for oligomerization and interaction with signaling components like the kinase Pto and effector protein; the function of its SD domain, however, remains elusive (Gutierrez et al. 2010; Mucyn et al. 2006).

To study the role of an extended N terminus of an SD-CNL protein, we focus here on the tomato Mi-1.2 protein, which confers resistance to tomato psyllids (*Bactericera cockerelli*), root-knot nematodes (*Meloidogyne* spp.), potato aphid (*Macrosiphum euphorbiae*), and the sweetpotato whitefly (*Bemisia tabaci*) (Casteel et al. 2006; Milligan et al. 1998; Nombela et al. 2003; Rossi et al. 1998; Vos et al. 1998). Many aspects of Mi-1.2 activation regulation have been addressed using biochemical and genetic approaches (Hwang and Williamson 2003; Hwang et al. 2000; Tameling et al. 2002; van Ooijen et al. 2008a). The N-terminal half of Mi-1.2, e.g., lacking the LRR domain, has been produced and purified from *Escherichia coli*, and this fragment was demonstrated to bind and hydrolyze ATP in vitro (Tameling et al. 2002). To link biochemical properties to activation states of Mi-1.2, specific point mutations were made in the NB-ARC domain, and these were found to confer either autoactivity or loss-of-function in full-length Mi-1.2 (van Ooijen et al. 2008a and b). The same mutants were used to examine physical and functional interactions between the CC-NB-ARC and the C-terminal LRR domain. To this end, both parts were coexpressed in *Nicotiana benthamiana* leaves using agroinfiltration (van Ooijen et al. 2008a). Whereas a physical interaction between the LRR domain and the rest of the protein was found for all tested (mutant) NB-ARC domains, in *trans* autoactivity was only restored for specific combinations, indicating that Mi-1.2 activation is a multistep process. Besides these experiments, domain-swapping experiments between Mi-1.2 and a nonfunctional homolog Mi-1.1 have been performed to identify the role of specific Mi-1.2 domains. LRR domain swaps revealed that this domain is mainly involved in pathogen recognition specificity and signal transduction (Hwang and Williamson 2003; Hwang et al. 2000). To analyze the function of the extended Mi-1.2 N terminus, it was arbitrarily divided into two regions based on the

presence of a *NcoI* site that is conserved in Mi-1.1 and Mi-1.2. The first 161 amino acids (aa) of the N terminus (represent the Nt1) and the second half of the N-terminal region that extends from aa 162 to the start of the NB-ARC domain form the Nt2 region. The chimaeras in which these regions have been exchanged between Mi-1.2 and Mi-1.1 revealed that negative regulation of cell death is mediated by interplay of the Nt1 region and residue 961 of the LRR domain (Hwang and Williamson 2003; Hwang et al. 2000).

This extensive body of data and the availability of tools makes Mi-1.2 a good model to study, at the molecular level, how HR signaling is regulated. In this study, Mi-1.2 deletion constructs were used to identify the minimal unit required to induce cell death. Secondary structure predictions of the extended N terminus revealed the presence of three distinct subdomains that could also be distinguished in other solanaceous CNL resistance proteins. The role of these specific N-terminal subdomains in regulating Mi-1.2 activity was analyzed using overexpression and *trans*-complementation assays in *N. benthamiana* leaves.

RESULTS

The N terminus as negative regulator of Mi-1.2 function.

To further explore the proposed regulatory role of the N terminus of Mi-1.2 on its function (Hwang and Williamson 2003; Hwang et al. 2000), constructs encoding wild-type protein as well as truncated versions were overexpressed transiently from a 35S promoter, by agroinfiltration in *N. benthamiana* leaves. All proteins except Nt2 and Nt1-Nt2 were equipped with either a tandem affinity purification (TAP) or a hemagglutinin (HA) tag (discussed below) to aid their detection on Western blots. As expected in the absence of an elicitor, expression of wild-type protein (Nt1-Nt2-NB-ARC-LRR, encoded by construct 1; Fig. 1A) gave no necrotic response, nor could cell death be visualized by trypan blue staining (Fig. 1B). In contrast, the protein lacking the Nt1 domain (Nt2-NB-ARC-LRR, construct 2) induced a cell-death response 48 h postinfiltration (hpi) (Fig. 1B). On a Western blot of total protein lysates of agroinfiltrated leaf material, the truncated protein, along with many degradation products, is readily detected (Fig. 1C, lane 2) using Mi-1.2 antibodies (van Ooijen et al. 2008b), whereas full-length Mi-1.2 protein is not visible (Fig. 1C, lane 1b). However, upon prolonged exposure, a clear band was detected at the predicted size of Mi-1.2 (145 kDa) (Fig. 1C, lane 1a), excluding the possibility that the absence of a response for the full-length protein is explained by lack of expression. This observation supports the hypothesis that Nt1 has an inhibitory effect on the autoactivity of full-length Mi-1.2 (Hwang and Williamson 2003; Hwang et al. 2000). Next, we tested in the same way a number of additional truncated versions of Mi-1.2 (Fig. 1A), notably the LRR domain alone (LRR, encoded by construct 3), NB-ARC-LRR (construct 4), the NB domain (construct 5), NB-ARC (construct 6), Nt1-Nt2-NB-ARC (construct 7), Nt1-Nt2 (construct 8), and both Nt1 (construct 9) and Nt2 (construct 10) alone. None of the constructs 3 to 9 induced cell death, as would be visible upon trypan blue staining (Fig. 1B). Overexpressed proteins were detected on a Western blot probed with Mi-1.2 antibody raised against the Nt2-NB-ARC part of Mi-1.2 (van Ooijen et al. 2008b) (Fig. 1C, lanes 3 to 8). Recognition of the TAP-tagged LRR domain was mediated by the generic immunoglobulin G-binding property of the ProtA domain in the TAP tag. For the Nt1 domain, an HA tag was (N terminally) attached to enable detection of the tagged protein using an HA antibody (Fig. 1C, lane 9). Infiltration of Nt2 or HA-tagged Nt2 did not result in a visible reaction in leaves, nor could these proteins be detected on Western blots (data not shown).

To investigate whether the negative regulatory function of the Nt1 can be exerted in *trans* as well, Nt2-NB-ARC-LRR and Nt1 were expressed in overlapping circles on *N. benthamiana* leaves. In this way, the effect of expression of the separate constructs as well as the combination of the two can be compared on a single leaf. Coexpression of β -glucuronidase (GUS) and Nt2-NB-ARC-LRR constructs served as control. Figure 2A shows that expression of Nt1 or GUS (upper circles) did not induce visible cell death on the leaves 2 days after infiltration. Cell death could also not be detected upon trypan blue staining. Expression of Nt2-NB-ARC-LRR (lower circles), however, did induce clear cell death. Coexpression (overlapping areas) of either Nt1 or GUS with Nt2-NB-ARC-LRR did not reduce or prevent cell death. Also, at 3 days after infiltration, no effect of the Nt1 on the autoactivity of Nt2-NB-ARC-LRR was observed (Fig. 2A, far right panel). In contrast, when the Nt1-Nt2 was used instead of Nt1, a clear suppression of Nt2-NB-ARC-LRR-induced cell death was observed (Fig. 2B). Nt1-Nt2-mediated suppression was not absolute and could be reversed by overexpression of the Nt2-NB-ARC-LRR or by long lag times between subsequent infiltrations (discussed below). The suppression effect prompted us to test the Nt2 alone as well, despite the fact that we were unable to detect expression of this subdomain before. Surprisingly, expres-

sion of Nt2 together with Nt2-NB-ARC-LRR also resulted in suppression of the autoactivity of Nt2-NB-ARC-LRR (Fig. 2C), indicating that the expression level of Nt2, although too low to be detected on Western blot, was sufficient to downregulate induction of cell death. The same results were obtained when we used HA-tagged Nt2 (Fig. 2D). As shown in Figure 2E, modulation of autoactivity by the Nt1, Nt2, and Nt1-Nt2 is not due to altered accumulation of the Nt2-NB-ARC-LRR. Taken together, these results suggest that Nt1 in *cis* and Nt2 in *trans* act as negative regulators of cell death induced by an autoactive Nt2-NB-ARC-LRR protein. Furthermore, the observation that Nt2-NB-ARC-LRR induced a necrotic response, whereas NB-ARC-LRR did not (Fig. 1B), suggests that the Nt2 acts as a positive regulator.

The N terminus as positive regulator of Mi-1.2 function.

Previously, we identified three specific, single amino acid substitutions in the NB-ARC domain of full-length Mi-1.2 that render the protein autoactive (van Ooijen et al. 2008a) (Supplementary Fig. S1). Substitution of the T at position 557 by an S (T557S) or the D at position 360 by an E (D360E) in the P-loop and the Walker B motif in the NB subdomain are both predicted to impair hydrolysis activity of the protein, thereby conferring autoactivity to the protein. Substitution H840 to an

Fig. 1. Deleting the first part of the N-terminus (Nt1) region of Mi-1.2 results in induction of a cell-death response. **A**, Schematic representation of the constructs: 1, Nt1-Nt2-NB-ARC-LRR-TAP (1 to 1,258 amino acids [aa]); 2, TAP-Nt2-NB-ARC-LRR (162 to 1,258 aa); 3, TAP-LRR (900 to 1,258 aa); 4, TAP-NB-ARC-LRR (513 to 1,258 aa); 5, TAP-NB (513 to 678 aa); 6, TAP-NB-ARC (513 to 899 aa); 7, Nt1-Nt2-NB-ARC-TAP (1 to 899 aa); 8, Nt1-Nt2 (1 to 539 aa); 9, HA-Nt1 (1 to 161 aa); and 10, Nt2 (162 to 539 aa). The different domains in the Mi-1.2 protein are indicated using different colors: Nt1 (yellow); Nt2 (orange); NB (red); ARC1 (pink); ARC2 (blue); LRR (green). The sizes of each part are approximately scaled. The lines are spaced every 10 kDa, and every 50 kDa is marked with a dotted line. The ability to induce (+) or not induce (-) cell death is indicated for the nine proteins that could be detected on an immunoblot. **B**, *Nicotiana benthamiana* leaves agroinfiltrated to express the first nine constructs corresponding to the proteins listed in A. At 5 days post agroinfiltration (dpi) a representative leaf was photographed (left panel) and stained with trypan blue to visualize cell death (right panel). **C**, *N. benthamiana* leaves agroinfiltrated to express constructs corresponding to proteins 1 to 9 in A were harvested 1 dpi. Protein extracts were subjected to immunoblotting, and detection was done either with anti-Mi-1.2 (van Ooijen et al. 2008b) for proteins 1 to 8 (lane 1a and b, 2 to 8) or anti-HA for Nt1 (lane 9). Lane 1a is a prolonged exposure (30 min) of lane 1b (5 min). Construct 3 (TAP-LRR) is detected by the intrinsic immunoglobulin G-binding property of the protA domain in the TAP-tag. Equal protein loading of the blots was confirmed by Ponceau-S staining of Rubisco (lower panel). Apparent molecular masses (kDa) are indicated at the left.

A in the MHD motif of subdomain ARC2 has been proposed to affect the interaction with the nucleotide, resulting to a more open conformation, thereby activating the protein (van Ooijen et al. 2008b). Hwang and Williamson (2003) found that replacement of R961 with a D in the LRR also confers autoactivity, possibly due to a disturbed interaction between the LRR and the Nt1.

To elaborate on the positive regulatory role exerted by the N-terminal domain on autoactivation, the Nt1-Nt2 and NB-ARC-LRR were coexpressed in *N. benthamiana* leaves. For the NB-ARC-LRR fragment, both nontagged (Fig. 3A to D, left leaf halves) and N-terminally TAP-tagged (Fig. 3A to D, right leaf halves, and E, both leaf halves) proteins were tested. Neither wild type nor the mutated NB-ARC-LRR constructs induced cell death when expressed alone (Fig. 3A to E, lower circles). Likewise, Nt1-Nt2 alone did not induce cell death either (upper circles). Coexpression of Nt1-Nt2 with NB-ARC-LRR constructs restored autoactivity only for mutant T557S, as judged from the observed cell death and trypan blue-stained cells in the overlapping coinfiltrated area (Fig. 3B). The presence of the N-terminal TAP tag did not affect this *trans* complementation (Fig. 3B, right leaf half). For the other mutants, when coexpressed in *trans* with Nt1-Nt2, no restoration of the

cell death-inducing phenotype was observed (Fig. 3C to E). Western blot analysis of protein lysates of agroinfiltrated leaf material shows that all constructs were expressed (Fig. 3F). From this, we conclude that the autoactive mutants require the Nt1-Nt2 domain in *cis* to induce cell death, except for the T557S P-loop mutant, for which autoactivity could also be restored when the Nt1-Nt2 was coexpressed in *trans*. This result indicates that Nt1-Nt2 is essential for Mi-1.2-mediated cell death.

Next, we coexpressed NB-ARC-LRR (T557S) (Fig. 4A to C, lower circles) with subdomains Nt1 and Nt2, both separately and together (Fig. 4A to C, upper circles, left leaf halves) next to Nt1-Nt2 as positive control (Fig. 4A to C, upper circles right leaf halves). Except for the controls (Fig. 4A to C, right leaf halves), none of the combinations resulted in induction of cell death, suggesting that an intact Nt1-Nt2 domain is required for restoration of the T557S autoactive mutant phenotype. The Nt2 region contains the sequence KDVID, representing the conserved EDVID motif (Mazourek et al. 2009; Raidan et al. 2008). This motif is found in most CC-NB-LRR proteins, in which it is located approximately 110 to 130 residues N-terminal to the P loop. To examine a possible role for this conserved motif in the regulatory function exerted by the Mi-1.2

Fig. 2. The cell-death response triggered by expression of the Nt2-NB-ARC-LRR fragment can be suppressed by coexpression of either the Nt1-Nt2 or the Nt2 alone. The constructs indicated in the schematic figures (first panel) were agroinfiltrated as overlapping circles. At 2 days postinfiltration (dpi), representative *Nicotiana benthamiana* leaves were photographed (second panel) and were stained with trypan blue to visualize cell-death (third panel). For **A**, an additional leaf was photographed 3 dpi (fourth panel). The lower circles reflect the areas expressing TAP-Nt2-NB-ARC-LRR, while the upper circles represent infiltration of **A**, HA:Nt1 (left) and TAP:GUS (right); **B**, Nt1-Nt2 (left) and TAP:GUS (right); **C**, HA:Nt1 (left) and Nt2 (right); **D**, HA:Nt1 (left) and HA:Nt2 (right). **E**, Immunoblot showing the accumulation of TAP-tagged Nt2-NB-ARC-LRR coinfiltrated with extraction buffer EB (lane 1) or Nt1 (lane 2), Nt2 (lane 3), Nt1-Nt2 (lane 4), or green fluorescent protein (GFP) (lane 5). GFP was used instead of TAP:GUS because the tag from the latter cross-reacts with the secondary antibody used for detection of the Mi-1.2 antibody.

N terminus, the lysine (K) and the two asparagine (D) residues were replaced by alanines (A). In contrast to the wild-type Nt1-Nt2 domain, Nt1-Nt2(AAVIA) did not restore the autoactive phenotype when coexpressed with NB-ARC-LRR (T557S) (Fig. 4D), despite that Nt1-Nt2(AAVIA) accumulated at similar levels as the nonmutated variant (Supplementary Fig. S2). Thus the KDVID motif in the Nt2 region is required for the positive regulatory function of the Mi-1.2 N terminus (Nt1-Nt2) when expressed in *trans*.

The autoactivating P-loop mutation (T557S) is localized in the NB subdomain, showing the importance of this domain for induction of cell death. Therefore, we analyzed whether this domain itself can induce cell death when expressed alone or in *trans* with Nt1-Nt2. Although expressed, neither the NB-ARC (T557S) nor the NB (T557S) proteins alone induced cell death (Fig. 4E to F; lower circles, left half of the leaves). Also, co-expression of these domains with Nt1-Nt2 in a *trans*-complementation assay did not result in cell death (Fig. 4E to F; overlapping area, left half of the leaves). From this, we conclude that the presence of the LRR domain is required for the Nt1-Nt2 to reconstitute in *trans* the autoactive phenotype of a T557S mutant.

Prediction of N-termini secondary structure reveals two distinct subdomains.

The extended N-terminal domain of Mi-1.2 was found to exert both positive- and negative regulation on Mi-1.2-induced cell death. The division of the N-terminal domain at aa position 161 into the regions Nt1 and Nt2 revealed that these segments play different roles in regulation of the Mi-1.2 cell-death signaling (Figs. 1 through 4). To obtain a better insight into the structure of the extended N terminus of Mi-1.2 and to see if distinct subdomains could be delineated, we performed secondary-structure predictions for the Nt1-Nt2 domain. The extended N-terminal domains of a subset of SD-CNL proteins (Mi-1.2, Mi-1.1, Rpi-blb2, Hero, and Prf [starting from aa 493]) and three selected CNL proteins lacking an extended N terminus (I-2, Gpa2, and Mla10) were aligned using ClustalW. Rpi-blb2 from *Solanum bulbocastanum* is a close homolog (82% identity in the SD-CC region) of Mi-1.2 and confers resistance to *Phytophthora infestans* (van der Vossen et al. 2005). The tomato protein Hero provides broad-spectrum resistance against the potato cyst nematode *Globodera rostochiensis* and contains an N-terminal domain that shares 36.3% identity with Mi-1.2 (Ernst et al. 2002). Prf mediates *Pseudomonas syringae*

Fig. 3. The autoactive phenotype of the T557S mutant can be reconstituted by coexpression of the N-terminal domain (Nt1-Nt2) with the nucleotide binding-ARC-leucine-rich repeat (NB-ARC-LRR). *Nicotiana benthamiana* leaves were agroinfiltrated to express the constructs indicated in the left panels. Representative leaves were photographed 3 days post agroinfiltration (dpi) (middle panels). Cell death was visualized by trypan blue staining of the same leaf (right panels). The upper circles mark the regions of Nt1-Nt2 infiltration and the lower circles of the NB-ARC-LRR variants: **A**, NB-ARC-LRR (left side) and TAP-NB-ARC-LRR (right side); **B**, NB-ARC-LRR T557S (left side) and TAP-NB-ARC-LRR T557S (right side); **C**, NB-ARC-LRR D630E (left side) and TAP-NB-ARC-LRR D630E (right side); **D**, NB-ARC-LRR H840A (left side) and TAP-NB-ARC-LRR H840A (right side); **E**, TAP-NB-ARC-LRR R961D (both sides). **F**, Protein expression levels of TAP-tagged NB-ARC-LRR variants and Nt1-Nt2 are shown on the immunoblot of total protein extracts as detected using anti-Mi-1.2 antibody.

resistance and is an atypical SD-CNL, in that it carries an additional N-terminal extension as compared with the other R proteins. In the aligned segment it is less homologous to Mi-1.2 than the other SD-CC domains (16.3% identity). To compare the CC domain of these selected SD-CNL proteins with the CC domain of classical CNL proteins, we included Gpa2 from *Solanum tuberosum* subsp. *andigena* conferring resistance to the potato cyst nematode *G. pallida* (van der Vossen et al. 2000). Gpa2 is a close homolog of Rx1, both of whose CC domains have been the subject of several functional studies (Rairdan et al. 2008; Smit et al. 2010). In addition, the CC domain of the barley Mla10 is included in the alignment, as it is the first CC domain for which an experimentally determined structure has been published (Maekawa et al. 2011). The alignment shows that the C-terminal part of the N-terminal domains of the SD-CNL proteins is indeed homologous to the CC domain found in classical CNL proteins (Fig. 5). Also, in the five SD-CNL proteins, the conserved EDVID motif (Mazourek et al. 2009) was present in the segment homologous to the CC (Fig. 5; marked by the consensus sequence of the extended motif WLxxVRELAYDAEDVLDx above the alignment). The identification of a CC subdomain in Mi-1.2 made it possible to test whether this domain alone is capable of inducing cell death, analogous to the CC of Mla10 (Maekawa et al. 2011).

As is shown in Supplementary Figure S3, expression of the CC alone in *N. benthamiana* does not trigger cell death. Extension of the CC with the NB, NB-ARC, or NB-ARC-LRR regions also does not result in an autoactive phenotype. These data support our previous observation that the Nt2-NB-ARC-LRR fragment is the minimal region required to induce a cell-death response (Fig. 1).

The protein domain prediction program DomPred identifies the SD domain and the conserved CC as likely separate subdomains. The secondary structure of the N termini was predicted using the Phyre prediction server (Bennett-Lovsey et al. 2008; Kelley and Sternberg 2009). The secondary structure and disorder of Mla10 are not based on Phyre predictions but are derived from the actual crystal structure (Potato Database 3QFL [Maekawa et al. 2011]). Phyre applies three independent high-accuracy secondary-structure predictors (Psi-Pred, SSPro and Jnet2 [Cole et al. 2008; Cuff and Barton 2000; Jones 1999; Pollastri et al. 2002]) to create a consensus secondary structure in which each residue is assigned one of three possible states: alpha helix, beta strand, or coil (which we refer to as 'loop' to avoid confusion with the term coiled coil) (Bennett-Lovsey et al. 2008; Kelley and Sternberg 2009). In the alignment (Fig. 5), the predicted state is depicted when the averaged confidence value is greater than 5. The same division in three alpha heli-

Fig. 4. The N-terminal domain (Nt1-Nt2) carrying an intact EDVID motif is required for in *trans* complementation of the T557S Mi-1.2 mutant. *Nicotiana benthamiana* leaves were agroinfiltrated with the constructs indicated in the left panels. At 3 days postinfiltration (dpi), representative leaves were photographed (middle panels) and were stained with trypan blue to visualize cell death (right panels). On the right half of each leaf Nt1-Nt2 (upper circles) and TAP-NB-ARC-LRR T557S (lower circles) were coexpressed as a positive control. **A to D**, On the left half of the leaves the TAP-NB-ARC-LRR T557S (bottom circle) was coexpressed in *trans* with **A**, Nt1, **B**, Nt2, **C**, Nt1 together with Nt2 and **D**, Nt1-Nt2 AVIAA mutant. **E**, and **F**, Nt1-Nt2 (upper circle) was coexpressed with either **E**, TAP-NB-ARC T557S (bottom circle) or **F**, TAP-NB T557S (bottom circle).

ces that was found in the Mla10 CC domain can also be observed in the predicted secondary structures of the CC of Mi-1.2 (382 to 510 aa) and the other R proteins. Like the CC domain, the extended N-terminal domains are predicted to consist predominantly of alpha helices separated by short loop regions. Longer loop regions separate the CC domain from the NB subdomain and the SD. In the middle of the SD (aa 200 to 215 in Mi-1.2), a relatively long loop region is found close to the division between Nt1 and Nt2 (aa 161). The Disopred software (Ward et al. 2004) predicts that this loop region is natively disordered, which could indicate that it forms a flexible linker between two more structured regions (Fig. 5). Notably, both halves of the SD domain (called SD1 and SD2) contain regions predicted to form coils like those found in the CC (Fig. 5, black bars) (COILS, 21 aa window [Lupas et al. 1991] and Paircoil2 [McDonnell et al. 2006]). To conclude, the secondary-structure prediction for R proteins with extended N termini reveals a clear separation between the N-terminal SD domain and the C-terminal CC domain. The high conservation suggests a similar structure for N-terminal domains of other SD-CNL proteins. The position at which the N-terminal domain of Mi-1.2 was divided into Nt1 and Nt2 is close to a predicted flexible loop region that suggests that the SD domain consists of two distinct subdomains (SD1 and SD2).

The SD2 and CC subdomains are the minimal regions required for *in trans* complementation of the NB-LRR (T557S) fragment.

The secondary-structure predictions suggest that the N-terminal domain of Mi-1.2 can be divided into three subdomains that are connected by flexible linkers (Fig. 5). The predicted secondary structure reveals that the arbitrary division into Nt1 and Nt2

does not match that of the SD1, SD2, and CC subdomains. The division between Nt1 and Nt2 (aa 161) is located approximately 40 aa upstream of the linker separating the SD1 and SD2 (aa 200 to 215). Nt1 encompasses most of SD1, while Nt2 includes the remaining fragment of SD1 together with the complete SD2 and CC domains. This implies that the Nt1 and Nt2 constructs encode partially truncated subdomains, which could affect their stability and functioning. Hence, we designed a new set of constructs in which we divided the subdomains according to the predicted linker regions. To test the roles of the predicted SD1, SD2, and CC domains for Mi-1.2 functioning, a set of constructs was created encoding the single subdomains (SD1, SD2, CC) divided at positions in the predicted linkers (SD1/SD2, aa 204; SD2/CC, aa 380; CC/NB, aa 524) (Fig. 6A). In addition, combinations of flanking subdomains (SD1-SD2, SD2-CC, SD1-SD2-CC) were analyzed in comparison with the Nt2 (Fig. 6A). All proteins were equipped with a C-terminal green fluorescent protein (GFP)-4HA (-gh) tag and, for most constructs except SD2 and SD1-SD2-CC, untagged versions were created as controls. The constructs were transiently coexpressed with the Mi-1.2 NB-LRR (T557S) P-loop mutant to test if they could complement its ability to induce cell death. Both the full-length N-terminal domain (SD1-SD2-CC-gh) and the SD2-CC construct reconstituted Mi-1.2 functionality as shown by the induction of a cell-death response (Fig. 6B). The functionality of the SD2-CC construct was surprising, as, at its N terminus, it is 43 aa shorter than the Nt2 construct that does not complement the cell death phenotype. Coexpression of the individual SD1, SD2, or CC domain and of the SD1-SD2 construct did not complement the ability of the Mi-1.2 NB-LRR (T557S) protein to induce cell death. However, coexpression of combinations of subdomains, either SD1-SD2 with the CC or SD1, SD2,

Domain divisions in constructs

Fig. 5. Secondary structure prediction for several N termini of nucleotide binding-leucine-rich repeat (NB-LRR) proteins. The secondary structures of the N termini of tomato I-2, potato Gpa2, barley Mla10, and several NB-LRR proteins carrying a solanaceae domain (SD): Mi-1.2, Mi-1.1, Rpi-blb2, Hero, and Prf (amino acids [aa] 493 to 1,131) were predicted using Phyre. The locations of α -helices (red), β -strands (dark blue), and loops (green) are indicated in the alignment. The locations of predicted coiled-coils regions (COILS, 21-aa window, $P > 0.25$) are marked by a black bar below the secondary structure prediction. The location of the conserved EDVID motif is indicated by the consensus sequence WLxxVRELAYDAEDVLDx shown above the alignment and by a white box. The lower panel represents the disorder prediction (blue) for the N termini of the depicted proteins mapped on the same alignment. Proposed borders dividing the various SD: SD1, SD2, CC, and NB are illustrated with gray bars. The division of the Mi-1.2 N terminus in the first and second parts of the N-terminus (Nt1 and Nt2) domain and the start of the NB segment are indicated by a schematic drawing and by black arrows listing the exact aa position. In a similar way, the positions are marked for the division in SD1, SD2, CC, and NB domains.

and the CC along with the NB-LRR (T557S) construct did result in a clear cell death (Fig. 6C). Combination of the SD2 and CC constructs with NB-LRR (T557S) leads to a weaker but consistent induction of cell death.

On an anti-GFP Western blot, all GFP-tagged constructs were detected at the expected height in the gel and accumulate to similar levels (Fig. 6D). Interestingly, the untagged Nt2 could not be detected using the anti-Mi antibody, while untagged

Fig. 6. The SD2 and CC domains are the minimal region of the Mi-1.2 N terminus required for *trans*-complementing the Mi-1.2 nucleotide binding-leucine-rich repeat (NB-LRR) (T557S) P-loop mutant construct. **A**, Schematic overview of Mi-1.2 N-terminal domain constructs based on the updated subdomain divisions derived from the secondary structure prediction. The subdomains were named SD1 (amino acids [aa] 1 to 204), SD2 (aa 204 to 380) and CC (aa 380 to 524). The scale denotes the aa sequence of Mi-1.2 and the borders of Nt1 and Nt2 are indicated. **B**, Complementation assay of Mi-1.2 NB-LRR (T557S). The NB-LRR (T557S) construct was coexpressed via agroinfiltration with N-terminal domain SD1-SD2-CC constructs. Both green fluorescent protein-hemagglutinin (GFP-4HA) tagged (-gh) and untagged versions (when available) were used. Reconstitution of Mi-1.2 (T557S) autoactivity could be observed by the development of a cell-death response at the infiltration spot. The images show the phenotype 7 days after infiltration. **C**, Complementation assay of Mi-1.2 NB-LRR (T557S). The NB-LRR (T557S) construct was coexpressed via agroinfiltration with N-terminal domain SD1-SD2-CC constructs. Both green fluorescent protein-hemagglutinin (GFP-4HA) tagged (-gh) and untagged versions (when available) were used. Reconstitution of Mi-1.2 (T557S) autoactivity could be observed by the development of a cell-death response at the infiltration spot. The images show the phenotype 7 days after infiltration. **D**, Immunoblots of the Mi-1.2 N-terminal domain constructs. The left blot shows the GFP-4HA (-gh) tagged versions of the N-terminal domain constructs detected by anti-GFP in a total leaf extract. The right anti-Mi-1.2 blot compares constructs based on the Nt1/Nt2 division and constructs based on the secondary structure predicted divisions. The SD2-CC and SD2-CC-gh constructs are included, as they represent the minimal segment of the Mi-1.2 N terminus that is required to functionally complement Mi-1.2 NB-LRR (T557S) (6B). Below each blot the RuBisCO band on the Coomassie-stained blot is shown as loading control.

SD2-CC and SD2-CC-gh covering nearly the same domain, were readily detectable by Western blot (Fig. 6D). As the main difference between these constructs is the additional 43 aa at the N terminus of Nt2, we concluded that these have an adverse effect on its stability and functionality.

DISCUSSION

Induction of HR, often visible as localized cell death, is a common mechanism exploited by plant NB-LRR resistance proteins to ward-off pathogen attack. Despite extensive dissection of the Mi-1.2 protein, we were unable to identify a single domain of which in planta overexpression resulted in cell death. The smallest unit triggering cell death is the Nt2-NB-ARC-LRR fragment, which indicates that, except for the Nt1 region, all other domains are required for this activity. This region contains the NB subdomain, which is, for Rx1, sufficient to trigger cell death upon overexpression (Rairdan et al. 2008). Also, unlike what has been reported for Mla10 (Maekawa et al. 2011), cell death could also not be mediated by the CC domain alone. Thus, unlike the N terminus of Mla10 and the TIR domains of three TIR-NB-LRR resistance proteins from *Arabidopsis*, flax, and tobacco (Frost et al. 2004; Krasileva et al. 2010; Maekawa et al. 2011; Swiderski et al. 2009; Weaver et al. 2006), the N terminus of Mi-1.2 alone is unable to trigger cell death upon overexpression in planta.

Autoactivity of Mi-1.2 triggered by deletion of its Nt1 region, implies that this region has a negative regulatory function. Based on domain-swapping experiments, this region has previously been proposed to be involved in autoinhibition of Mi-1.2 activity (Hwang and Williamson 2003; Hwang et al. 2000). Similar to the deletion of the Nt1 region (Fig. 1), the exchange of the Mi-1.2 Nt1 region with the corresponding region from the nonfunctional homolog Mi-1.1 (MiDS3) induces autoactivity. Coexpression of the Mi-1.2 Nt1 region could suppress the autoactive response of the MiDS-3 variant in *trans* (Hwang et al. 2000). Since deletion of the Nt1 resulted in a strong increase of the accumulation of the Mi-1.2 fragment (Fig. 1C), it is tempting to speculate that Nt1 exerts its negative regulatory function by controlling Mi-1.2 turnover. A Mi-1.2 construct in which the Nt1 is deleted might be less prone to degradation, resulting in increased accumulation resulting in autoactivity. This hypothesis would also explain why swapping the Mi-1.2 Nt1 with an incompatible Nt1 domain of Mi1.1 gives autoactivation, as the chimera might adopt a similar 'stable' conformation. Apparently, this conformation is reversible, as in *trans* overexpression of the Mi-1.2 Nt1 can suppress the autoactivation phenotype (Hwang et al. 2000). Surprisingly, Nt1 cannot suppress autoactivity of the truncated Nt2-NB-ARC-LRR protein (Fig. 2). These differences might be attributable to technical differences in conducting the experiments. In both experiments, the *Nt1* gene was expressed using a 35S promoter, whereas the *Nt2-NB-ARC-LRR* gene was expressed from a 35S promoter and the *MiDS-3* gene from its native promoter (Hwang et al. 2000). Possibly, the expression level of the autoactive variants in relation to that of the Nt1 region determines whether cell death was suppressed.

Extended N-terminal truncations of the autoactive Nt2-NB-ARC-LRR protein abolished its ability to trigger cell death (Fig. 1). This observation suggests that the Nt2 region is required for positive regulation of Mi-1.2 activity, which was confirmed in a *trans*-complementation assay with NB-ARC-LRR variants carrying autoactivating mutations (Fig. 3). Only the T557S substitution in the NB subdomain was able to *trans*-complement when coexpressed with the intact N terminus (Fig. 3). This *trans*-complementation pattern is similar to that observed for Nt1-Nt2-NB-ARC fragments coexpressed with

the LRR domain in which T557S was also the strongest auto-activator (van Ooijen et al. 2008a). In that study, the D630E substitution in the NB subdomain was identified as a weak activator, whereas we did not observe cell death for the Nt1-Nt2 and NB-ARC-LRR D630E combination. Mutations in the ARC2 (H840A) domain did not functionally *trans*-complement in either study, while the LRR domain mutant (R961D) has not been analyzed before (Fig. 3) (van Ooijen et al. 2008a). Notably, for Rx1, coexpression of its CC N terminus with autoactivating NB-ARC-LRR variants carrying mutations in either the ARC or LRR domain also did not result in autoactivation but restored its normal elicitor-dependent HR activity (Rairdan et al. 2008). Unfortunately, we cannot test whether the non-cell death inducing combinations would induce elicitor-mediated cell death, as the Mi-1.2 elicitor remains unknown. Since autoactivity of Mi-1.2 T557S is restored when the NB-ARC-LRR is coexpressed in *trans* with the N-terminal domain, these findings imply distinct functions for the particular subdomains and imply that Mi-1.2 activation is a multistep process of which some steps require the Nt1-Nt2 and others do not.

Our deletion studies suggest that the positive regulation by the Mi-1.2 N terminus is associated with its Nt2 region and, more specifically, with the SD2-CC region. The SD2-CC subdomains can physically be separated to complement in *trans* (Fig. 6). The fact that a combination of SD2 and the CC domain is required for this positive regulatory role, suggests that these two distinct domains may form a common interface involved in activation. Apparently, the formation of this surface is somehow disrupted in the Nt2 fragment, as it is unable to complement the T557S mutation. The main difference between Nt2 and SD2-CC is the presence of a small part of SD1. So, this region may either interfere with the exposure of the interaction surface of the SD2 and CC or contribute to protein instability of the Nt2 domain due to improper folding caused by the disrupted SD1 structure.

To pinpoint the positive regulatory role to the CC region, we mutated the conserved EDVID motif located in Nt2. The triple KDVID mutant (AAVIA) abolished the ability of the Nt1-Nt2 domain to *trans*-complement the NB-ARC-LRR T557S mutant (Fig. 4). The conserved EDVID motif was shown previously to be required for function and intramolecular interactions of the CNL Rx1 (Rairdan et al. 2008). Here, we demonstrate that the EDVID motif is also important for the function of SD-CNL resistance proteins and, based on its functional conservation, it is tempting to speculate that the CC subdomain exerts its positive regulatory function by interacting with the NB-ARC-LRR, like the CC of Rx1 does (Rairdan et al. 2008).

The Nt2 region is required for cell-death induction (Figs. 1, 3, and 4), yet its overexpression in a *trans*-complementation assay suppressed Nt2-NB-ARC-LRR-induced cell death (Fig. 2). Excess amounts of Nt2 might affect Mi-1.2-mediated cell death by competing for downstream signaling components or by dimerization with the larger protein fragment, thereby preventing the formation of a signaling-competent complex. The negative regulation of the Mi-1.2 activity by Nt2 seems to be subsequent to Nt1-mediated negative regulation, as autoactivation induced by removal of Nt1 can be suppressed by overexpression of Nt2. These findings are in agreement with those obtained from Mi-1.2 and Mi-1.1 swapping experiments (Hwang and Williamson 2003; Hwang et al. 2000). First, induction of cell death by expression of Mi-DS5 (Nt2-NB-ARC of Mi-1.1 swapped in an Mi-1.2 background) suggests that one or both the Nt2 and NB-ARC domains have a negative regulatory role on Mi-1.2 activation. Secondly, cell death induced by expression of Mi-DS3 (only Nt-1 of Mi-1.1 swapped in a Mi-1.2 background) or Mi-DS5 (Nt2-NB-ARC of Mi-1.1 swapped in an Mi-1.2 background) developed more slowly than cell death

induced by expression of Mi-DS4 (Nt1-Nt2-NB-ARC of Mi-1.1 swapped in an Mi-1.2 background). Possibly, the faster development of cell death caused by expression of Mi-DS4 is due to the cumulative effect of removal of a two-step negative regulation process mediated by Nt1 and Nt2. Perhaps, this two-step negative regulation is functionally related to the structural SD domain division into two subdomains, SD1 and SD2 (Fig. 5). Also for the positive regulatory function of the N terminus, there is a clear distinction in function between SD1 and SD2.

To summarize, our data are consistent with a model in which activation of SD-CNL R proteins is a multistep process. Upon pathogen recognition by the LRR domain, the inhibition conferred by the SD1 domain on NB-ARC is relieved, allowing the SD2-CC-NB-ARC-LRR part to induce host defenses including cell death. It will be interesting to perform similar deletion and *trans*-complementation studies on other SD-CNL proteins as, based on the high overall similarity in the predicted secondary structure (Fig. 5), our findings might also apply to those proteins.

MATERIALS AND METHODS

Construction of binary vectors.

Wild-type Mi-1.2 and mutants T557S, D630E, H840A cloned in a binary vector (CTAPi) have been described before (Gabriels et al. 2007; van Ooijen et al. 2008a and b, 2010). These constructs together with the Mi-1.2 CC-NB-ARC construct (van Ooijen et al. 2008a) served as polymerase chain reaction (PCR) templates to create the new constructs described below. All PCR primers (Table 1) used were purchased from MWG Biotech (Ebersberg, Germany). Note, that, when a stop codon is incorporated in the reverse primer, it prevents translational fusion of the protein to any C-terminal tag present in the vector. The Nt1-Nt2 (1 to 539 aa) construct was amplified from Mi-1.2 CC-NB-ARC (van Ooijen et al. 2008a) using primers FP764 and FP1051. Gateway attB flanks were added in a second amplification step using FP872 and FP873. The resulting PCR product was transferred into binary vector CTAPi (Rohila et al. 2004) by the Gateway one-tube protocol for cloning attB-PCR products directly into destination vector (Invitrogen, Carlsbad, CA, U.S.A.). The construct HA:Nt1 (1 to 161 aa) was made using primer combination FP764 and FP978 with pG74 (MHD) as template and pGWB415 as destination vector (Nakagawa et al. 2007). This fusion resulted in an N-terminal protein fusion to an HA tag. To generate Nt2 (162 to 539 aa), FP976 and FP1051 primers were used in combination with the Mi-1.2 CC-NB-ARC (van Ooijen et al. 2008a) template, followed by a second PCR to add the attB flanks. The obtained product was recombined into pDONR207 (Invitrogen) and, subsequently, the plasmid was recombined by LR clonase (Invitrogen) into destination vectors. Use of CTAPi as destination vector resulted in an untagged Nt2 version, whereas recombination into pGWB415 generates an N-terminally HA-fused NT2 protein. Nt1-Nt2-NB-ARC fused C-terminally to the TAP tag was created using primers FP764 and FP858 on pG74 (van Ooijen et al. 2008b). Again, attB flanks were added in second PCR reactions, and this product was recombined via pDONR into CTAPi vector. To create Nt2-NB-ARC-LRR (162 to 1,258 aa), a similar approach was followed but using primers FP976 and FP766 and destination vector NTAPi (Rohila et al. 2004). In this vector, the expressed protein is fused to an N-terminal TAP tag. A similar cloning strategy was followed to create the NB (513 to 678 aa) (FP2085 and FP2527) and NB-ARC (513 to 899 aa) (FP2085 and FP859) constructs in NTAPi (Rohila et al. 2004). Depending on the PCR template used, PG54, PG98 (van Ooijen et al. 2010), or PG96 (Gabriels et al. 2007), the wild type, H840A, or the T557S mutant was

created. All NB-ARC-LRR constructs (513 to 1,258 aa) except for the R960D mutant were generated by two-step Gateway recombination into CTAPi (untagged) or NTAPi (TAP tagged) (Rohila et al. 2004) destination vector. The PCR products used for this Gateway cloning were amplified with FP2085 and FP766 primers on PCR template PG74 or the corresponding full-length clones of Mi-1.2 mutants described before (Gabriels et al. 2007; van Ooijen et al. 2008a and b, 2010). The R960D mutant was generated by circular mutagenesis (Hemsley et al. 1989), using template Mi-1.2 NB-ARC-LRR in pGEXT-4 (van Ooijen et al. 2008b) using FP1098 and FP1101. An *Eco*72I/*Bsp*119I restriction fragment carrying the mutation was exchanged with the same fragment in pG74 (van Ooijen et al. 2008b) to obtain a full-length Mi-1.2 R961D mutant. To place the NB-ARC-LRR R961D mutant in NTAPi (Rohila et al. 2004), a *Bst*XI/*Bsp*119I restriction fragment was exchanged between the NB-ARC-LRR R961D pGEXT-4 construct and the wild-type NB-ARC-LRR in NTAPi (described above).

The AAVIA mutation in Nt1-Nt2 required several PCR steps. Two PCR products generated using FP2309 and FP2418 and FP976 with FP2419 were mixed and were used as template in a PCR with FP976 and FP2309. The last amplification step combined both products into one product carrying the AAVIA mutation. Subsequently, an *Eco*R72I/*Spe*I restriction fragment carrying the mutation was exchanged with the corresponding region in the above-described Nt1-Nt2 construct to generate Nt1-Nt2^{AAVIA}.

Creation of a TAP-equipped Mi-1.2 LRR domain (van Ooijen et al. 2008a) and the TAP-tagged GUS protein (van Ooijen et al. 2010) were described previously. The GFP construct was created by ligating a GFP-encoding PCR fragment amplified with FP2223 and FP2223 from pGWB451 into an *Xba*I-*Sac*I-digested pGWB451 vector. The CC-NB-ARC-LRR, CC-NB-ARC, CC-NB, and CC-encoding fragments were PCR-amplified with *Pfu*I Turbo (Stratagene, La Jolla, CA, U.S.A.) from the Mi-1.2 Nt2-NB-ARC-LRR template, using the FP2965 forward primer in combination with, respectively, the FP766, FP859, FP2527 or FP1051 reverse primer. Gateway attB-recombination sites were added in a second PCR, using primers FP872 and FP873. The CC-NB-ARC-LRR construct in NTAPi was created using the Gateway one-step protocol for cloning attB-PCR products directly into destination vectors (Invitrogen). For the other constructs, entry clones were generated via a Gateway BP reaction and recombination of purified PCR products with pDONR207 (Invitrogen). The binary plasmids were subsequently generated via a LR reaction of entry clones with the destination vector NTAPi (Rohila et al. 2004; van Ooijen et al. 2010).

During cloning, we noted that many of the constructs encoding the Mi-1.2 NB subdomain were unstable in *E. coli*, resulting in low transformation frequencies and aberrant sizes of the inserts of the obtained transformants. Sequencing of a subset of these clones revealed the presence of an *E. coli* insertion sequence 10 (IS10) or parts of it in our constructs (data not shown). Although these insertions were clearly independent events, all IS10 elements inserted in the sequence ACA ACT CGA GAA AAG GAA GTG GCT TTG CAT GGA AAG CTG AAC, which corresponds to aa EVAL at position 661 to 664 in the NB subdomain. The DNA sequence fits the consensus sequence found for IS10 insertion sites, thereby forming a 'hot spot' for integration (Bender and Kleckner 1992; Kovarik et al. 2001). Nevertheless, unaffected constructs could also be obtained and, since *Agrobacterium* sp. strain GV3101 does not carry *Tn10*, the Mi-1.2 clones were stable once transformed to the *agrobacterium*. To confirm absence of *Tn10* in these plasmids, all *agrobacterium* transformants were checked using a PCR with a Mi-1.2 forward primer (FP1987) and an IS10-spe-

cific reverse primer (FP2683). Also the region containing the hot spot was amplified, using Mi-1.2 primers (FP1987 and FP2432) to check whether it had the expected size, and if so, the product was sequence-verified.

The constructs used for the SD1-SD2-CC subdivision were amplified using the following primer sets: MiD01For and MiD01Rev for SD1-SD2-CC, MiD01For and MiSD1Rev for SD1, MiD01For and MiSD2Rev for SD1-SD2, MiSD2For and MiSD2Rev for SD2, MiSD2For and MiD01Rev for SD2-CC, and MiCCFor and MiD01Rev for CC. The forward primers introduce a *NcoI* site overlapping with a start codon at the 5' end. The reverse primers introduced a *BamHI* site followed by a stop codon and a *KpnI* site. For in-frame fusion with GFP-4HA the *NcoI-BamHI* sites were used. For the untagged versions of the constructs the *NcoI-KpnI* sites were used, in which case, the stop codon introduced by the reverse primer is retained. These constructs were introduced in the pRAP:GFP vector as described previously (Slootweg et al. 2010). The complete expression cassette (35S promoter, Tnos terminator) was cloned to the pBIN+ binary vector (van Engelen et al. 1995) via the *AscI-PacI* restriction sites.

Agroinfiltrations: agrobacterium-mediated transient transformation.

Agroinfiltrations were performed as described (van Ooijen et al. 2008b), with modifications reported by Ma and associates (2012) and Takken and associates (2000). Binary vectors were transformed to *Agrobacterium tumefaciens* GV3101, except for constructs seen in Figure 7, which were introduced in strain MOG101 (Koncz and Schell 1986). *Agrobacteria* were grown to an optical density at 600 nm (OD_{600}) = 0.8 in Luria Bertani medium (10 g of tryptone, 5 g of yeast extract, and 2.5 g of NaCl per liter) supplemented with 20 μ M acetosyringone and 10 mM morpholineethanesulfonic acid (MES), pH = 5.6. Pelleted cells were resuspended in infiltration medium (1 $\times$ Murashige-Skoog salts, 10 mM MES, pH=5.6; 2% [wt/vol] sucrose; 200 μ M acetosyringone) and were syringe-infiltrated at OD_{600} = 0.4 (for *trans*-complementation assays) or OD_{600} = 0.8 (for protein extraction) into 4-week-old *N. benthamiana* leaves. To visualize cell death, the infiltrated leaves were photographed and stained with trypan blue (van Ooijen et al. 2008b). Each phenotype was confirmed independently at least three times. When no visible cell death developed, the agroinfiltrated leaf was monitored for a maximum of five days. For protein extraction, three agroinfiltrated leaves were harvested 24 hpi and were frozen in liquid nitrogen. Protein isolation and detection was performed as described (Ma et al. 2012; van Ooijen et al. 2008b), with small modifications. In short, after grinding in liquid nitrogen, 250 mg of tissue was allowed to thaw in 2 ml of extraction buffer (25 mM Tris, pH = 7.5, 1 mM EDTA, 150 mM NaCl, 5 mM dithiothreitol, 1 $\times$ Roche complete protease inhibitor cocktail, 2% polyvinylpyrrolidone; 2.5% glycerol, and 0.1% NP-40). Extracts were cleared by centrifugation (12,000 $\times$ g, 20 min. at 4°C), and the supernatant was passed through G25 Sephadex columns. Equal amounts of protein samples (25 μ g) were mixed with Laemmli sample buffer and were loaded on 8% sodium dodecyl sulfate-polyacrylamide gel. Protein gels were semi-dry blotted on polyvinylidene fluoride membranes and were assayed for equal loading by Ponceau S staining. Blots were blocked with 5% skimmed milk, and Mi-1.2 was detected using either a 1:3,000 Mi-1.2 antibody (Ma et al. 2012; van Ooijen et al. 2008b) as first antibody followed by a goat antirabbit linked to horseradish peroxidase (Rockland Immunochemicals, Inc., Gilbertsville, PA, U.S.A.) in a 1:4,000 dilution or HA antibody in a 1:4,000 dilution followed by goat antirat linked to horseradish peroxidase. The GFP-4HA-tagged constructs were detected by a

1:2,000 dilution of the anti-GFP antibody (AB290 Abcam, Cambridge, MA, U.S.A.), followed by the donkey antirabbit peroxidase-conjugated antibody 711-035-152 (1:10,000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.). Detection was done using ECL plus (GE Healthcare, Piscataway, NJ, U.S.A.).

Measuring cell-death suppression using the *trans*-complementation assays required extensive optimization of the agroinfiltration procedure, as the outcome is highly sensitive to the experimental conditions applied. The cell-death development is not completely suppressed by the N terminus but merely delayed. Cell-death suppression was not observed upon overexpression of the cell death-inducing constructs or when the subsequent infiltration was done at a late timepoint. For agroinfiltrations in which two constructs were coexpressed, N-terminal parts or GUS with the Nt2-NB-ARC-LRR, an OD_{600} = 0.25 was used per bacterial strain. A too-high OD_{600} value of strains carrying constructs encoding Mi-1.2 N termini occasionally causes nonspecific suppression of cell death (data not shown). After infiltrating the upper circles for expression of N-terminal regions or GUS protein, the leaves were dabbed dry with tissue and were left for 6 to 7 h to absorb the infiltrated

Table 1. Oligonucleotide sequences

Primer name	Sequence
764	AAAAAGCAGGCTCTATGGAAAAACGAAAAGATA TT
766	AGAAAGCTGGGTCTACTTAAATAAGGGGATAT TCTT
858	AGAAAGCTGGGTAGAATGCCTTTTCTTATTTGA ATCG
859	AGAAAGCTGGGTCTAAGAATGCCTTTTCTTATT TGAA
872	GGGGACAAGTTTGTACAAAAAAGCAGGCT
873	GGGGACCACCTTTGTACAAGAAAGCTGGGT
976	AAAAAGCAGGCTCTATGGGGTTGATACTGAATG GTTGC
978	AGAAAGCTGGGTTTAAATGGAAATCTCTTATGTT GCCACA
1,051	AGAAAGCTGGGTCTATCCACTGGTGAGCTTTCT AAG
1,098	CATTTGAGGTACTTAAGAATTGACACACAAGTTA AATATCTGCC
1,101	GGCAGATATTTAACTTGTGTGCAATTCTTAAGT ACCTCAAATG
1,986	GGACCCGCAGATTTAGATGTC
2,085	AAAAAGCAGGCTCTACCATGGAGAGAAAGTCAT TGACAACTG
2,223	CGCGTCTAGAAATGAGTAAAGGAGAAGAACT
2,224	CGCGCAGAGCTCTTATTTGTATAGTTCATCCA
2,309	CCTCCACTAGTCACCACTT
2,418	GTGGCTTATGAGGCAGcAGcTGTCATAGcTTCAAT TATTGTTTCG
2,419	CGAACAATAATTGAAGCTATGACAgCTgcTGCCTC ATAAGCCAC
2,432	TCCGTCTTTCCACAAATCC
2,527	AGAAAGCTGGGTTTCACAATCGAAGGTCAAGAG GATCAG
2,683	GCCAACGGTATACAGCGAGT
2,965	AAAAAGCAGGCTTCTTCATGCATCTTCTACACA
2,223	CGCGTCTAGAAATGAGTAAAGGAGAAGAACT
2,224	CGCGCAGAGCTCTTATTTGTATAGTTCATCCA
MiD01For	GGCTAGCTCCATGGAAAAACGAAAAGATATTGA AGAAGCA
MiD01Rev	CGGTACCTTAGGATCCACTAGTACCTACAATTAT TTTATCAGTTGTCAATGACTTTC
MiCCFor	GCCATGGCTGATGGACCACTTTCATGC
MiSD1Rev	CGGTACCTTAGGATCCGAGCCGAGAGTCTTCATC AGTCTG
MiSD2For	GCCATGGAGCTAGATGAGGATGAACACAATG
MiSD2Rev	CGGTACCTTAGGATCCTTGAGATGAATTGGGGC TTTC

liquid. Subsequently, the lower circles were infiltrated to express the Nt2-NB-ARC-LRR construct. Extended periods between the infiltration of upper and lower circles frequently resulted in nonspecific suppression of the Nt2-NB-ARC-LRR autoactivity (data not shown). Leaves were harvested when necrosis became visible, which normally occurred between 24 to 48 hpi. Late harvesting can result in lack of visible suppression of Nt2-NB-ARC-LRR-mediated cell death (data not shown). Since the age of the leaf influences onset and severity of cell death (Ma et al. 2012), only the second and third fully developed leaves were infiltrated on a plant that carries four to five leaves. A result was considered to be significant when at least one of the two infiltrated leaves per plant showed specific suppression of Nt2-NB-ARC-LRR-mediated cell death of four plants analyzed.

ACKNOWLEDGMENTS

We thank to H. Lemereis, T. Hendrix, and L. Tikovsky for plant care. G. van Ooijen is acknowledged for help with experiments and construction of the full-length Mi-1.2 R961D mutant, F. Gawehns-Bruning, L. Ma, L. Furci, and M. Hijdra for help with optimization of agroinfiltration assays in the greenhouse, J. Roosien for making the constructs, and R. Pomp for the immuno blots shown in Figure 6. We thank the two anonymous reviewers and the editor for their useful comments. The EC Integrated Project BIOEXPLOIT CT-2005-513959 supported E. Lukasik-Shreepathy, A. Goverse and F. Takken, and E. Slootweg was financed by The Technological Topinstitute Green Genetics (TTI GG).

LITERATURE CITED

Ade, J., DeYoung, B. J., Golstein, C., and Innes, R. W. 2007. Indirect activation of a plant nucleotide binding site-leucine-rich repeat protein by a bacterial protease. *Proc. Natl. Acad. Sci. U.S.A.* 104:2531-2536.

Albrecht, M., and Takken, F. L. 2006. Update on the domain architectures of NLRs and R proteins. *Biochem Biophys Res Commun* 339:459-462.

Bender, J., and Kleckner, N. 1992. IS10 transposase mutations that specifically alter target site recognition. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:741-750.

Bennett-Lovsey, R. M., Herbert, A. D., Sternberg, M. J., and Kelley, L. A. 2008. Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre Prot. 70:611-625.

Bernoux, M., Ve, T., Williams, S., Warren, C., Hatters, D., Valkov, E., Zhang, X., Ellis, J. G., Kobe, B., and Dodds, P. N. 2011. Structural and functional analysis of a plant resistance protein TIR domain reveals interfaces for self-association, signaling, and autoregulation. *Cell Host Microbe* 9:200-211.

Casteel, C. L., Walling, L. L., and Paine, T. D. 2006. Behavior and biology of the tomato psyllid, *Bactericera cockerelli*, in response to the *Mi-1.2* gene. *Entomol. Exp. Appl.* 121:67-72.

Cole, C., Barber, J. D., and Barton, G. J. 2008. The Jpred 3 secondary structure prediction server. *Nucleic Acids Res.* 36:W197-201.

Collier, S. M., Hamel, L. P., and Moffett, P. 2011. Cell death mediated by the N-terminal domains of a unique and highly conserved class of NB-LRR protein. *Mol. Plant-Microbe Interact.* 24:918-931.

Cuff, J. A., and Barton, G. J. 2000. Application of multiple sequence alignment profiles to improve protein secondary structure prediction. *Proteins* 40:502-511.

Ernst, K., Kumar, A., Kriseleit, D., Kloos, D. U., Phillips, M. S., and Ganai, M. W. 2002. The broad-spectrum potato cyst nematode resistance gene (*Hero*) from tomato is the only member of a large gene family of NBS-LRR genes with an unusual amino acid repeat in the LRR region. *Plant J.* 31:127-136.

Frost, D., Way, H., Howles, P., Luck, J., Manners, J., Hardham, A., Finnegan, J., and Ellis, J. 2004. Tobacco transgenic for the flax rust resistance gene *L* expresses allele-specific activation of defense responses. *Mol. Plant-Microbe Interact.* 17:224-232.

Gabriels, S. H., Vossen, J. H., Ekengren, S. K., van Ooijen, G., Abd-El-Halim, A. M., van den Berg, G. C., Rainey, D. Y., Martin, G. B., Takken, F. L., de Wit, P. J., and Joosten, M. H. 2007. An NB-LRR protein required for HR signalling mediated by both extra- and intracellular resistance proteins. *Plant J.* 50:14-28.

Gutierrez, J. R., Balmuth, A. L., Ntoukakis, V., Mucyn, T. S., Gimenez-Ibanez, S., Jones, A. M., and Rathjen, J. P. 2010. Prf immune complexes of tomato are oligomeric and contain multiple Pto-like kinases

that diversify effector recognition. *Plant J.* 61:507-518.

Heath, M. C. 2000. Hypersensitive response-related death. *Plant Mol. Biol.* 44:321-334.

Hemsley, A., Arnheim, N., Toney, M. D., Cortopassi, G., and Galas, D. J. 1989. A simple method for site-directed mutagenesis using the polymerase chain reaction. *Nucleic Acids Res.* 17:6545-6551.

Hwang, C. F., and Williamson, V. M. 2003. Leucine-rich repeat-mediated intramolecular interactions in nematode recognition and cell death signaling by the tomato resistance protein Mi. *Plant J.* 34:585-593.

Hwang, C. F., Bhakta, A. V., Truesdell, G. M., Pudlo, W. M., and Williamson, V. M. 2000. Evidence for a role of the N terminus and leucine-rich repeat region of the *Mi* gene product in regulation of localized cell death. *Plant Cell* 12:1319-1329.

Jones, D. T. 1999. Protein secondary structure prediction based on position-specific scoring matrices. *J. Mol. Biol.* 292:195-202.

Jones, J. D., and Dangl, J. L. 2006. The plant immune system. *Nature* 444:323-329.

Kelley, L. A., and Sternberg, M. J. 2009. Protein structure prediction on the Web: A case study using the Phyre server. *Nat. Protoc.* 4:363-371.

Koncz, C., and Schell, J. 1986. The promoter of TI-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vectors. *Mol. Gen. Genet.* 204:383-396.

Kovarik, A., Matzke, M. A., Matzke, A. J., and Koulakova, B. 2001. Transposition of IS10 from the host *Escherichia coli* genome to a plasmid may lead to cloning artefacts. *Mol. Genet. Genomics* 266:216-222.

Krasileva, K. V., Dahlbeck, D., and Staskiewicz, B. J. 2010. Activation of an Arabidopsis resistance protein is specified by the *in planta* association of its leucine-rich repeat domain with the cognate oomycete effector. *Plant Cell* 22:2444-2458.

Lukasik, E., and Takken, F. L. 2009. STANDING strong, resistance proteins instigators of plant defence. *Curr. Opin. Plant Biol.* 12:427-436.

Lupas, A., Van Dyke, M., and Stock, J. 1991. Predicting coiled coils from protein sequences. *Science* 252:1162-1164.

Ma, L., Lukasik, E., Gawehns, F., and Takken, F. L. 2012. The use of agroinfiltration for transient expression of plant resistance and fungal effector proteins in *Nicotiana benthamiana* leaves. *Method Mol. Biol.* 835:61-74.

Maekawa, T., Cheng, W., Spiridon, L. N., Toller, A., Lukasik, E., Saijo, Y., Liu, P., Shen, Q. H., Micluta, M. A., Somssich, I. E., Takken, F. L., Petrescu, A. J., Chai, J., and Schulze-Lefert, P. 2011. Coiled-Coil domain-dependent homodimerization of intracellular barley immune receptors defines a minimal functional module for triggering cell death. *Cell Host Microbe* 9:187-199.

Mazourek, M., Cirulli, E. T., Collier, S. M., Landry, L. G., Kang, B. C., Quirin, E. A., Bradeen, J. M., Moffett, P., and Jahn, M. M. 2009. The fractionated orthology of Bs2 and Rx/Gpa2 supports shared synteny of disease resistance in the Solanaceae. *Genetics* 182:1351-1364.

McDonnell, A. V., Jiang, T., Keating, A. E., and Berger, B. 2006. Pair-coil2:improved prediction of coiled coils from sequence. *Bioinformatics* 22:356-358.

Milligan, S. B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., and Williamson, V. M. 1998. The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. *Plant Cell* 10:1307-1319.

Mucyn, T. S., Clemente, A., Andriotis, V. M., Balmuth, A. L., Oldroyd, G. E., Staskiewicz, B. J., and Rathjen, J. P. 2006. The tomato NB-ARC-LRR protein Prf interacts with Pto kinase in vivo to regulate specific plant immunity. *Plant Cell* 18:2792-2806.

Nakagawa, T., Suzuki, T., Murata, S., Nakamura, S., Hino, T., Maeo, K., Tabata, R., Kawai, T., Tanaka, K., Niwa, Y., Watanabe, Y., Nakamura, K., Kimura, T., and Ishiguro, S. 2007. Improved Gateway binary vectors: High-performance vectors for creation of fusion constructs in transgenic analysis of plants. *Biosci. Biotechnol. Biochem.* 71:2095-2100.

Nombela, G., Williamson, V. M., and Muniz, M. 2003. The root-knot nematode resistance gene *Mi-1.2* of tomato is responsible for resistance against the whitefly *Bemisia tabaci*. *Mol. Plant-Microbe Interact.* 16:645-649.

Padmanabhan, M., Cournoyer, P., and Dinesh-Kumar, S. P. 2009. The leucine-rich repeat domain in plant innate immunity: A wealth of possibilities. *Cell Microbiol.* 11:191-198.

Pan, Q., Wendel, J., and Fluhr, R. 2000. Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. *J. Mol. Evol.* 50:203-213.

Pollastri, G., Przybylski, D., Rost, B., and Baldi, P. 2002. Improving the prediction of protein secondary structure in three and eight classes using recurrent neural networks and profiles. *Proteins* 47:228-235.

Rairdan, G. J., and Moffett, P. 2006. Distinct domains in the ARC region of the potato resistance protein Rx mediate LRR binding and inhibition of activation. *Plant Cell* 18:2082-2093.

- Rairdan, G. J., Collier, S. M., Sacco, M. A., Baldwin, T. T., Boettrich, T., and Moffett, P. 2008. The coiled-coil and nucleotide binding domains of the Potato Rx disease resistance protein function in pathogen recognition and signaling. *Plant Cell* 20:739-751.
- Riedl, S. J., Li, W., Chao, Y., Schwarzenbacher, R., and Shi, Y. 2005. Structure of the apoptotic protease-activating factor 1 bound to ADP. *Nature* 434:926-933.
- Rohila, J. S., Chen, M., Cerny, R., and Fromm, M. E. 2004. Improved tandem affinity purification tag and methods for isolation of protein heterocomplexes from plants. *Plant J.* 38:172-181.
- Rossi, M., Goggin, F. L., Milligan, S. B., Kaloshian, I., Ullman, D. E., and Williamson, V. M. 1998. The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. *Proc. Natl. Acad. Sci. U.S.A.* 95:9750-9754.
- Slootweg, E., Roosien, J., Spiridon, L. N., Petrescu, A. J., Tameling, W., Joosten, M., Pomp, R., van Schaik, C., Dees, R., Borst, J. W., Smant, G., Schots, A., Bakker, J., and Govers, A. 2010. Nucleocytoplasmic distribution is required for activation of resistance by the potato NB-LRR receptor Rx1 and is balanced by its functional domains. *Plant Cell* 22:4195-4215.
- Swiderski, M. R., Birker, D., and Jones, J. D. 2009. The TIR domain of TIR-NB-LRR resistance proteins is a signaling domain involved in cell death induction. *Mol. Plant-Microbe Interact.* 22:157-165.
- Takken, F. L., Luderer, R., Gabriels, S. H., Westerink, N., Lu, R., de Wit, P. J., and Joosten, M. H. 2000. A functional cloning strategy, based on a binary PVX-expression vector, to isolate HR-inducing cDNAs of plant pathogens. *Plant J.* 24:275-283.
- Takken, F. L., Albrecht, M., and Tameling, W. I. 2006. Resistance proteins: Molecular switches of plant defence. *Curr. Opin. Plant Biol.* 9:383-390.
- Tameling, W. I., Elzinga, S. D., Darmin, P. S., Vossen, J. H., Takken, F. L., Haring, M. A., and Cornelissen, B. J. 2002. The tomato R gene products I-2 and Mi-1 are functional ATP binding proteins with ATPase activity. *Plant Cell* 14:2929-2939.
- Tameling, W. I., Vossen, J. H., Albrecht, M., Lengauer, T., Berden, J. A., Haring, M. A., Cornelissen, B. J., and Takken, F. L. 2006. Mutations in the NB-ARC domain of I-2 that impair ATP hydrolysis cause autoactivation. *Plant Physiol.* 140:1233-1245.
- Tameling, W. I. L., and Takken, F. L. W. 2008. Resistance proteins: Scouts of the plant innate immune system. *Eur. J. Plant Pathol.* 121:243-255.
- Tao, Y., Yuan, F., Leister, R. T., Ausubel, F. M., and Katagiri, F. 2000. Mutational analysis of the Arabidopsis nucleotide binding site-leucine-rich repeat resistance gene *RPS2*. *Plant Cell* 12:2541-2554.
- van der Vossen, E. A., van der Voort, J. N., Kanyuka, K., Bendahmane, A., Sandbrink, H., Baulcombe, D. C., Bakker, J., Stiekema, W. J., and Klein-Lankhorst, R. M. 2000. Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: A virus and a nematode. *Plant J.* 23:567-576.
- van der Vossen, E. A., Gros, J., Sikkema, A., Muskens, M., Wouters, D., Wolters, P., Pereira, A., and Allefs, S. 2005. The *Rpi-blb2* gene from *Solanum bulbocastanum* is an *Mi-1* gene homolog conferring broad-spectrum late blight resistance in potato. *Plant J.* 44:208-222.
- van Engelen, F. A., Molthoff, J. W., Conner, A. J., Nap, J. P., Pereira, A., and Stiekema, W. J. 1995. pBINPLUS: An improved plant transformation vector based on pBIN19. *Transgenic Res.* 4:288-290.
- van Ooijen, G., van den Burg, H. A., Cornelissen, B. J., and Takken, F. L. 2007. Structure and function of resistance proteins in solanaceous plants. *Annu. Rev. Phytopathol.* 45:43-72.
- van Ooijen, G., Mayr, G., Albrecht, M., Cornelissen, B. J., and Takken, F. L. 2008a. Transcomplementation, but not physical association of the CC-NB-ARC and LRR domains of tomato R protein Mi-1.2 is altered by mutations in the ARC2 subdomain. *Mol. Plant* 1:401-410.
- van Ooijen, G., Mayr, G., Kasiem, M. M., Albrecht, M., Cornelissen, B. J., and Takken, F. L. 2008b. Structure-function analysis of the NB-ARC domain of plant disease resistance proteins. *J. Exp. Bot.* 59:1383-1397.
- van Ooijen, G., Lukasik, E., Van Den Burg, H. A., Vossen, J. H., Cornelissen, B. J., and Takken, F. L. 2010. The small heat shock protein 20 RS12 interacts with and is required for stability and function of tomato resistance protein I-2. *Plant J.* 63:563-572.
- Vos, P., Simons, G., Jesse, T., Wijbrandi, J., Heinen, L., Hogers, R., Frijters, A., Groenendijk, J., Diergaarde, P., Reijans, M., Fierens-Onstenk, J., de Both, M., Peleman, J., Liharska, T., Hontelez, J., and Zabeau, M. 1998. The tomato *Mi-1* gene confers resistance to both root-knot nematodes and potato aphids. *Nat. Biotechnol.* 16:1365-1369.
- Ward, J. J., McGuffin, L. J., Bryson, K., Buxton, B. F., and Jones, D. T. 2004. The DISOPRED server for the prediction of protein disorder. *Bioinformatics* 20:2138-2139.
- Weaver, M. L., Swiderski, M. R., Li, Y., and Jones, J. D. 2006. The Arabidopsis thaliana TIR-NB-LRR R-protein, RPP1A: Protein localization and constitutive activation of defence by truncated alleles in tobacco and Arabidopsis. *Plant J.* 47:829-840.
- Williams, S. J., Sornaraj, P., deCourcy-Ireland, E., Menz, R. I., Kobe, B., Ellis, J. G., Dodds, P. N., and Anderson, P. A. 2011. An autoactive mutant of the M flax rust resistance protein has a preference for binding ATP, whereas wild-type M protein binds ADP. *Mol. Plant-Microbe Interact.* 24:897-906.
- Yan, N., Chai, J., Lee, E. S., Gu, L., Liu, Q., He, J., Wu, J. W., Kokel, D., Li, H., Hao, Q., Xue, D., and Shi, Y. 2005. Structure of the CED-4-CED-9 complex provides insights into programmed cell death in *Caenorhabditis elegans*. *Nature* 437:831-837.

AUTHOR-RECOMMENDED INTERNET RESOURCES

COILS, a coiled-coil prediction server:
www.ch.embnet.org/software/COILS_form.html
 DomPred protein domain prediction program server:
bioinf.cs.ucl.ac.uk/dompred
 The Phyre prediction server: www.sbg.bio.ic.ac.uk/phyre