The role of Fusarium oxysporum effector protein Avr2 in resistance and pathogenicity

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CHAPTER 2

Molecular basis of effector recognition by plant NB-LRR proteins

Abstract

Gene-for-gene resistance in plants is based on the presence of an avirulence gene in the pathogen and a matching resistance (*R*) gene in the host. Many cloned *R* genes encode the proteins belonging to the family of nucleotide binding-leucine rich repeat (NB-LRR) proteins. NB-LRRs mediate recognition of the pathogen-derived effector (avirulence) proteins to subsequently activate host innate immune responses. Structure-function analyses and the recent elucidation of the 3D structures of subdomains of NB-LRR have provided new insight in how these different functions are combined and what the contribution is of the individual subdomains. Besides interdomain contacts, interactions with chaperones, the proteasome and effector baits are required to keep NB-LRRs in a signaling-competent, yet auto-inhibited state. In this review we explore the structural features of subdomains in NB-LRR proteins, assembly of these subdomains into a signaling-competent NB-LRR as well as their stabilization and accumulation. In addition, effector recognition by NB-LRRs and the subsequent induction of defense signaling will be discussed.

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Introduction

Pathogens attack plants in attempts to assimilate nutrients from them. These pathogens include bacteria, viruses, nematodes, fungi and oomycetes. All current plant species have been successful in surmounting pathogen attack. Nonetheless the struggle between plant pathogens and their hosts is still continuing. This co-evolutionary battle has equipped plants with sophisticated defence mechanisms and cognate pathogens with a corresponding arsenal of counter strategies to overcome them (Bent *et al.*, 2007; Chisholm *et al.,* 2006).

Plants rely on passive and induced defence mechanisms to resist pathogen infection (Jones *et al.*, 2006). Examples of passive defences are physical and biochemical barriers such as trichomes, epidermal wax layers, the cell wall and the production of antimicrobial compounds (Chisholm *et al.*, 2006; Heath, 2000). Adapted pathogens able to breach these passive defences barriers are confronted with induced defence responses that are orchestrated by a multilayered innate immune system (Segonzac *et al.*, 2011). The first layer of the induced immune system utilizes membrane-localized pattern-recognition receptors (PRRs) that sense microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs). Recognition of these invariant and essential molecules by PRRs initiates PAMP-triggered immunity (PTI). Specialised pathogens evolved strategies to overcome PTI by the production of effector proteins. To counteract the virulence activities of effectors, resistant plants have evolved resistance (R) proteins. R proteins represent the key component of the second layer of the induced immune system. Upon effector recognition by an R protein defence responses are initiated that often culminate into a hypersensitive response (HR); a localised cell death response around the infection site (Heath, 2000; Thomma *et al.*, 2011). Effectors recognized by R proteins are called avirulence (Avr) factors, and the resistance reaction initiated is known as Effector-Triggered Immunity (ETI) (Dodds *et al.*, 2010; Jones *et al.*, 2006). This specific recognition phenomenon was first described by Flor in 1942 as the gene-for-gene hypothesis (Flor, 1971). In those days the gene pairs involved were not known, but nowadays a large number of *Avrs* has been cloned and in 1992 also the first *R* gene was isolated: the *Hm1* from maize that confers race-specific resistance to the fungus *Cochliobolus carbonum* (Johal *et al.*, 1992). Now, two decades later, over 70 *R* genes have been cloned from both monocot and dicot plant species conferring resistance to a wide variety of pathogens (Liu *et al.*, 2007; Martin *et al.*, 2003). Based on the presence of conserved domains and their presumed location in the cell R proteins can be classified into different families (reviewed in (Martin *et al.*, 2003; van Ooijen *et al.*, 2007)). The large majority of cloned *R* genes encode intracellular NB-LRR proteins, with a conserved nucleotide-binding (NB) domain and a C-terminal leucine-rich repeat (LRR) domain.

In this chapter we will focus on the recognition of effectors by R proteins that contain

an NB domain and a LRR domain. Their multi-domain structure allows them to simultaneously act as pathogen sensor, molecular switch, and as a response factor to induce downstream signalling. The recent elucidation of the 3D structures of particular subdomains, combined with structure-function studies, provided new insight in how these distinct activities are combined into a NB-LRR protein. The combination of interdomain contacts, interactions with chaperones and the 26S proteasome appears to keep NB-LRRs in a signalling-competent, yet auto-inhibited state. Here, we describe NB-LRR building blocks, their assembly into signalling competent proteins as well as their stabilization and accumulation. In addition different effector recognition models will be discussed.

Building blocks of NB-LRRs; Classification and structural features of subdomains

The domain most conserved in NB-LRR proteins is the central nucleotide-binding domain. Besides the NB domain the region connecting the NB and LRR domains is conserved in all NB-LRRs and is defined as the ARC domain. This domain is named after its presence in the mammalian Apoptosis activating factor 1 (Apaf-1), plant R proteins and Cell death protein 4 (CED-4) of *C. elegans* (Takken *et al.*, 2006; van der Biezen *et al.*, 1998a). Together the NB and ARC domain are defined as NB-ARC domain. NB-ARC proteins form a subclass of the STAND protein family (Signal Transduction ATPases with Numerous Domains), a class of molecular switches involved in processes such as immunity, apoptosis (e.g. APAF1 and CED4) and transcriptional regulation (Danot *et al.*, 2009). Like R proteins all STAND proteins have a modular architecture allowing them to function simultaneously as sensor, as switch, and as response factor.

Whereas the N- and C-terminal domains can be highly variable in STAND proteins, only a limited number of domains are found in R proteins. As the name NB-LRR indicates their C-terminus is invariantly an LRR domain. Based on the N-terminal domain the NB-LRR R proteins are divided into two classes: TIR-NB-LRRs (TNLs) and non-TNLs (Meyers *et al.*, 1999; Meyers *et al.*, 2003) (Figure 1). This division is based on either the presence or absence of an N-terminal domain with homology to the *Drosophila* Toll and the human Interleukin-1 Receptor protein called the TIR domain. The Arabidopsis RRS1-R TNLs is C-terminally extended with a WRKY domain, a protein domain found in a subset of plant specific transcription factors. With increasing numbers of plant genome sequences becoming available, more genes encoding TNLs are identified and some of them encode proteins with alternative domain arrangements. For example, in poplar and Arabidopsis *TNL*s have been found encoding proteins with a TIR-NB-LRR-TIR domain arrangement (Kohler *et al.*, 2008; Meyers *et al.*, 2003; Pan *et al.*, 2000; Radwan *et al.*, 2008). However, it remains to be shown that these *TNL*s represent functional *R*-genes.

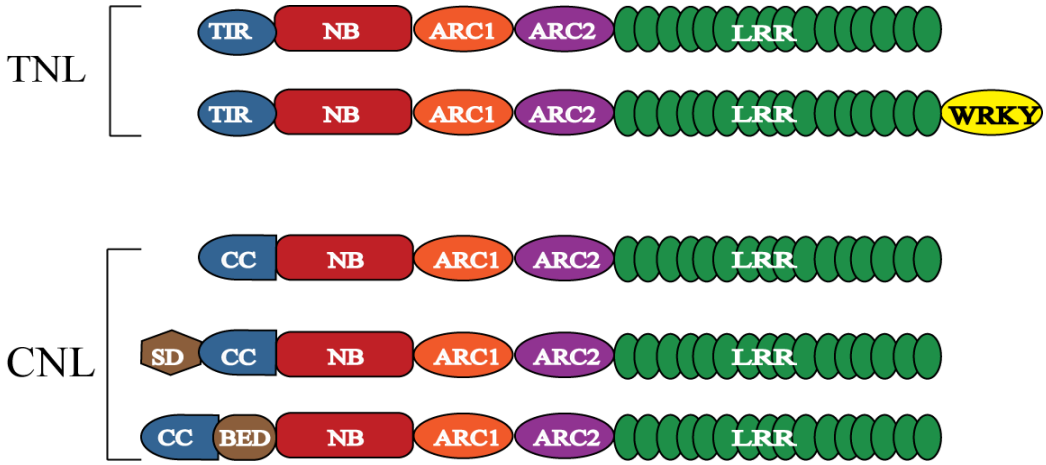

Figure 1. **Schematic representation of TNL and CNL classes of NB-LRR proteins**. The (sub)domains are depicted as colored boxes. TIR (blue): Toll/Interleukin-1 Receptor; CC (blue): Coiled Coil; NB (red): Nucleotide Binding; ARC1 (orange) and ARC2 (purple): APAF1, R protein and CED4; LRR (green): Leucine Rich Repeat; SD (brown): Solanaceous Domain; BED (brown): BEAF/DREAF zinc finger domain and WRKY (yellow): WRKY transcription factor.

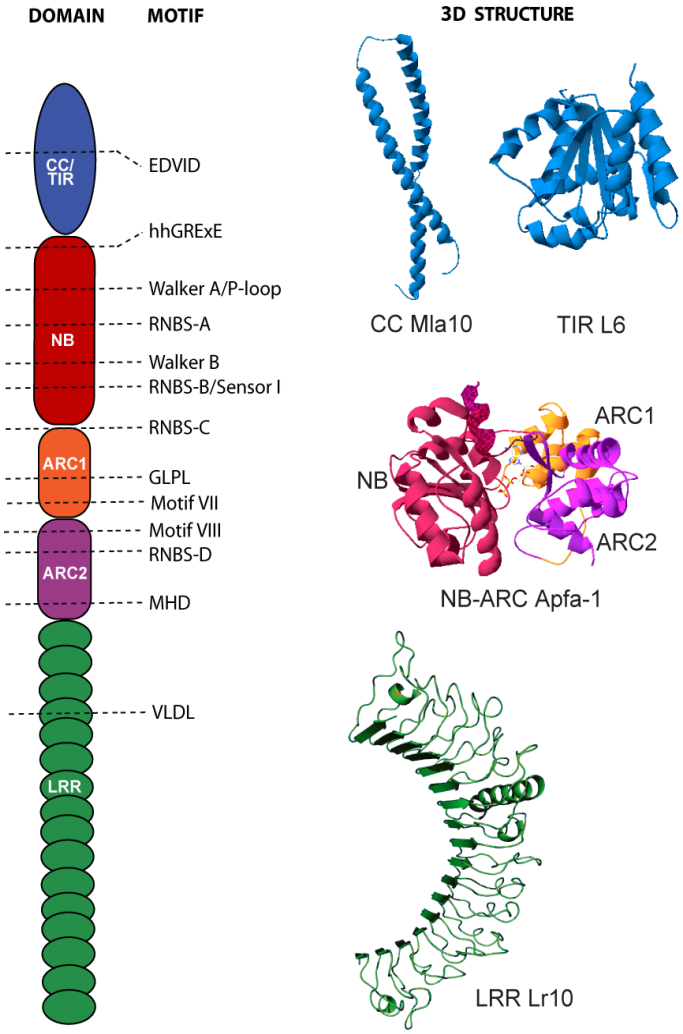

Figure 2. Schematic representation of a typical NB-LRR protein. The (sub) domains are shown as colored boxes; CC/TIR domain (blue), NB (red), ARC1 (Orange), ARC2 (purple) subdomains and LRR domain (green). Conserved motifs in subdomains are indicated. The 3D structures of the domains are shown on the right. The 3D structure models of the CC and TIR subdomain are based on the crystal structures of Mla10 (PDB id 3QFL) and L6 (PDB id 3OZI), respectively. The ADP-bound NB-ARC structure from the human Apaf-1 (PDB id 1Z6T) is shown. The LRR structure is based on a probabilistic three-dimensional model using joint fragment remote homology modeling.

By far the largest group of non-TNL R proteins carries a Coiled-Coil (CC) N-terminal domain and this group is collectively referred to as CC-NB-LRRs (CNLs) (Figure 1). The CC domain is sometimes joined by additional domains such as a so-called Solanaceae domain (SD) or a BED (BEAF/DREF) zinc finger DNA-binding domain, that is located in between the CC and NB domains (Bhaskar *et al.*, 2008; Heise *et al.*, 2007; Mucyn *et al.*, 2006). Analysis of sequenced plant genomes, e.g. those of rice, Arabidopsis and poplar, reveals CNLs with domain arrangements different from the ones described above (Figure 1). Some CNLs contain two NB domains, and others have the CC domain replaced by the BED domain. Again others encode CNLs lacking the N-terminal CC domain, but show NB domain sequence similarity with 'true' CNLs (Kohler *et al.*, 2008; Meyers *et al.*, 2003; Zhou *et al.*, 2004). Here again, it remains to be shown that these CNL variants function as R proteins. So most NB-LRR R proteins have a classical tri-partite domain architecture with an N-terminal TIR/CC, a central NB-ARC, and a C-terminal LRR, and hence in the subsequent chapter we focus only on these conserved domains.

TIR domain

The TIR domain is widely present in animal and plant proteins involved in immune responses. The crystal structure of TIR domains of human Toll-like receptors (TLR) 1 and 2 were the first to be elucidated (Xu *et al.*, 2000). Recently, the crystal structure of the TIR domain of a first plant TNL R protein, the flax L6 protein conferring resistance to the fungus *Melampsora lini*, was published (Figure 2). The 3D structure models of these TIR domains showed a similar global fold consisting of a five-stranded parallel beta-sheet that is surrounded by five alpha helices (Bernoux *et al.*, 2011b; Chan *et al.*, 2010). The TIR domain of human TLR exhibits low affinity for self-association in solution (Xu *et al.*, 2000). The TIR domain of L6 can also self-associate as shown in a yeast two-hybrid assay. In the crystal structure two TIR-TIR interfaces can be distinguished. Interface 1 involves residues from αD1, αD3, and αE helices, the βE strand, and the DE and EE loops. Interface 2 has a hydrophobic core consisting of I104, L108, L109, and W131. Mutational analysis revealed that interface 1 is responsible for the self-interaction while interface 2 could be involved in downstream signalling (Bernoux *et al.*, 2011b). Interestingly, the surface-exposed residues in interface 1 are conserved among mammalian and plant TNLs including the tobacco N and Arabidopsis RPS4 protein (Dinesh-Kumar *et al.*, 2000; Swiderski *et al.*, 2009). This conservation suggests that the role of this interface, dimerisation, is conserved between TNLs. Collectively, these data suggest that TIR monomers can from NB-LRR proteins form compact dimers. Whereas one of the conserved surface patches of the TIR is involved in dimerization the other conserved surface patch is likely involved in signalling or activity

regulation.

CC domain

CC is a rather common domain found in a wide range of proteins involved in different biological processes including signal transduction, mechanical stability of cells, and in intracellular transport (Burkhard *et al.*, 2001). The structure of the CC domain consists of two to five alpha helices that form a super-coil (Mason *et al.*, 2004). Excitingly, the crystal structure of the CC domain of the CNL-type R protein MLA10 from barley has recently been reported (Maekawa *et al.*, 2011). The monomeric structure forms a helix-loop-helix structure, in which two long antiparallel α-helices are linked by short loops. In crystals the CC domain dimerizes forming and extended (90 Å) rod-shaped structure with a helical bundle at each end (Maekawa *et al.*, 2011). In most characterized CNL CC domains, except for RPS2, RPS5 and Dm3, there is a very conserved motif referred to as the EDVID motif. This motif in the potato Rx protein determines the interaction between the CC and the NB-LRR moieties (Figure 2) (Mazourek *et al.*, 2009; Rairdan *et al.*, 2008). In crystals of the MLA10 CC domain, the EDVID motif is located in the middle of helix α2a in a position exactly opposite of the ‘‘hinge’’ region formed by the helix α2a and α2b from the other CC domain (Maekawa *et al.*, 2011). Structure modelling of the RPM1 CC domain based on the MLA CC structure reveals that RPM1 CC domain might form dimers similar to MLA (Maekawa *et al.*, 2011), suggesting that the self-association property of the CC-domain might be conserved for at least a subset of CNLs.

NB-ARC domain

The NB-ARC domain is the most conserved domain in NB-LRRs. In most STAND proteins the NB-ARC domain is composed of four sub-domains i.e. NB, ARC1, ARC2, and ARC3. The ARC3 subdomain is absent in plant NB-LRRs. Instead, there is a short linker connecting ARC2 and LRR domain (Meyers *et al.*, 2003; Takken *et al.*, 2006). Crystal structures of a NB-ARC domain from plant NB-LRR proteins have not yet been elucidated, but those of Apaf-1 and CED-4 have been reported. The structure reveals that the NB domain forms a parallel β-sheet flanked by α-helices, ARC1 forms a four-helix bundle, ARC2 consists of a winged-helix fold, and ARC3 also forms a helical bundle. Together these domains form a nucleotide-binding pocket. At the interface between the NB, ARC1 and ARC2 subdomains a nucleotide is bound; ADP for Apaf-1 and ATP for CED-4, respectively (Riedl *et al.*, 2005; Yan *et al.*, 2005). Whereas the ADP-bound Apaf-1 complex has a compact globular conformation (Figure 2) the CED4-ATP structure reveals a much more open configuration in which NB, ARC1 and ARC2 are repositioned relative to each other (Riedl *et al.*, 2005;

Takken *et al.*, 2006).

Plant NB-ARC domains have many conserved peptide motifs in common with other STAND proteins in which the residues located in these motifs play crucial roles in NTP-binding and hydrolysis and in triggering conformational changes (Figure 2) (Danot *et al.*, 2009; Takken *et al.*, 2006). Within the β-sheet of the NB subdomain, strands β1 and β3 are part of the Walker A (P-loop), and Walker B motif, respectively. The Walker-A is defined by the consensus sequence GxxxxGKS/T, in which the Lys (K) residue binds the β- and γ-phosphates of ADP/ATP, and Ser (S) and Thr (T) residues are important for coordinating an Mg^{2+} ion (Takken *et al.*, 2006). The Walker B is characterized on the consensus hhhhDD/E sequences (where h indicates a hydrophobic residue). The conserved Asp (D) residue plays a critical role in binding a Mg^{2+} ion via a water molecule, whereas the second acidic residue Asp/Glu (D/E) is thought to serve as a catalytic base in the process of ATP hydrolysis (Hanson *et al.*, 2005; Leipe *et al.*, 2004; Takken *et al.*, 2006). Another conserved motif in the NB subdomain is the RNBS-B motif, which is defined by the sequence hhhhToR (where o represents an alcoholic residue) in strand β4. The Arg (R) residue is predicted to fulfil the function of the sensor I motif in detecting the γ–phosphate of NTP (Ogura *et al.*, 2001; van Ooijen *et al.*, 2008b; van Ooijen *et al.*, 2007). A similar sensor function is proposed for the highly conserved MHD-motif, represented by the sequences hxhHD, located in the ARC2 sub-domain (Takken *et al.*, 2006). In Apaf-1 the His (H) residue interacts directly with the β-phosphate of NTP (Riedl *et al.*, 2005). Auto activation mutations in this motif have been found in several NB-LRRs, including I-2, Mi-1.2, L6 and Rx, indicating that this motif has a crucial role in controlling R protein activity, and it has been proposed that it fulfils the same function as the sensor II motif in AAA+ proteins (Bendahmane *et al.*, 2002; Howles *et al.*, 2005; van Ooijen *et al.*, 2008b). Besides these motifs, other conserved motifs identified within the NB-ARC domain are the hhGRExE, RNBS-A, C and D, and motifs VII and VIII. Many of these motifs are also conserved in STAND proteins suggesting functional conservation. Indeed mutations in many of these motifs have been linked to either autoactivation or loss-of-function phenotypes in NB-LRR R proteins (Takken *et al.*, 2006). So, although no crystal structure of a plant NB-ARC protein has been elucidated, its global fold can be predicted with high confidence by remote homology modelling using the CED4 and Apaf-1 3D structures.

Like Apaf-1 and CED4 also plant NB-LRR R proteins form stable complexes with ADP or ATP as shown for the flax M and L6 proteins, the barley MLA27 protein, the tobacco N protein, and the tomato Mi-1.2 and I-2 proteins (Maekawa *et al.*, 2011; Tameling *et al.*, 2002; Ueda *et al.*, 2006; Williams *et al.*, 2011). Except for MLA27 it has been shown that these R proteins are also capable of hydrolysing bound ATP into ADP similar as Apaf-1

(Tameling *et al.*, 2002; Ueda *et al.*, 2006; Williams *et al.*, 2011). If nucleotide exchange in plant NB-ARC domains results in a similar dramatic change in conformation as observed for ADP-Apaf-1 and ATP-CED4 this domain would be perfectly equipped to act as a molecular switch. The change from a closed conformation with ADP bound to an open ATP bound conformation will have a major effect on the intramolecular interactions between the different domains and thereby rearrange the global conformation of R proteins.

LRR domain

The first LRR domain was identified in the alpha2-glycoprotein from human serum (Takahashi *et al.*, 1985). Proteins with LRR domains are present in all organism including viruses, prokaryotes and eukaryotes. Many of them act as receptors, in which the LRR domain is involved in ligand perception (Enkhbayar *et al.*, 2004; Martin *et al.*, 2003). In general LRR domains consist of 2 to 42 tandem repeats, each containing a consensus core sequence LxxLxLxx-N, where L is a leu residue, N is either an asp, thr, ser or cys residue, and x is any residue (Bella *et al.*, 2008; Enkhbayar *et al.*, 2004; van Ooijen *et al.*, 2007). Although differences exist in overall structures of LRRs, all share the same essential features, in that a single repeat represents a wedge-shaped loop that together with other repeats forms a curved super-helix (Bella *et al.*, 2008; Kobe *et al.*, 2001). In plant NB-LRRs each repeat consists of 24-28 residues with the core consensus sequence LxxLXXLxLXXC/Nxx. These repeats are connected by a variable spacer (Padmanabhan *et al.*, 2009; van Ooijen *et al.*, 2007). Genome-wide analysis revealed that Arabidopsis NB-LRRs have an average of 14 LRRs, each containing a repeat with on average 24 residues (Meyers *et al.*, 2003). A crystal structure of an LRR domain from a plant NB-LRR protein has not been elucidated yet. Remote homology modelling using the available LRR sequences in the protein databases has been hampered by their highly irregular structures, with varying repeat lengths and non-canonical LRR motifs. Recently, however, a method was described in which irregular LRRs can be modelled with high confidence. This method is based on joint-fragment remote homology modelling where individual repeats are modelled on the best fitting template allowing the construction of a composite LRR model (Sela *et al.*, 2011; Slootweg *et al.*, 2010). Such a model for the LRR domain of Lr10, a CNL from wild emmer wheat, revealed a compact horseshoe-like structure that is divided into two distinct halves (Sela *et al.*, 2011). Whereas the N-terminal part on its surface carries a cluster of positively charged residues the surface of the C-terminal part is enriched in aromatic amino acids. Both halves are separated by an irregular repeat forming a nick in the structure dividing the LRR into two subdomains. It remains to be investigated whether this is a generic property of NB-LRR proteins, but the division into two halves suggests discrete functions for each subdomain. No conserved

motifs have been identified in the C-terminal half of LRR domains, but in the N-terminal half of LRRs from both TNLs and CNLs a conserved "VLDL" motif is identified in the third LRR (Figure 2). This motif was first described in RPS5, a CNL from Arabidopsis (Meyers *et al.*, 2003; Padmanabhan *et al.*, 2009). Mutations in or adjacent to this motif in RPS5 and Rx interfere with their function suggesting biological relevance for this motif (Bendahmane *et al.*, 2002; Padmanabhan *et al.*, 2009; Warren *et al.*, 1998). In summary, 3D structural modelling of the LRR of NB-LRR proteins suggests a compact horseshoe-like structure likely consisting of two distinct moieties.

Putting the parts together: combining the domains to build a signalling competent NB-LRR protein

Combining the structure models discusses above with the physical interactions reported between these domains enables the development of a model on how these domains dock and together form a functional R protein. For Rx, Bs2, RPS5 and Mi-1.2 the LRR domain has been shown *in planta* to associate with the CC-NB-ARC part of the R protein (Ade *et al.*, 2007; Leister *et al.*, 2005; Moffett *et al.*, 2002; van Ooijen *et al.*, 2008a). The observation that co-expression of the LRR and CC-NB-ARC of Rx and Bs2 provided a functional "R" protein able to recognise the cognate effector, resulting in CP- and AvrBs2-dependent HR, respectively, also implies a physical interaction between the LRR and the CC-NB-ARC (Leister *et al.*, 2005; Moffett *et al.*, 2002). Mutational analysis indicated that the ARC1 subdomain of Rx is responsible and essential for LRR binding (Moffett *et al.*, 2002). Two papers report an interaction between the CC domain and other domains: the RPS5 CC domain interacts with both the NB-ARC and LRR domain, while the Rx CC domain interacts with the entire NB-ARC-LRR domain (Ade *et al.*, 2007; Moffett *et al.*, 2002). For Bs2 no association between its CC domain and NB-ARC-LRR *in planta* could be detected although co-expression of both domains complemented Bs2 function implying a functional interaction (Leister *et al.*, 2005). So, although only few cases have been reported, these findings imply the presence of intramolecular interactions in NB-LRRs.

The model that emerges is that in the absence of a pathogen the NB-ARC domain interacts with the LRR, keeping the protein in a closed conformation (Ade *et al.*, 2007; Moffett *et al.*, 2002; van Ooijen *et al.*, 2008a). The positively charged patch found in the N-terminal half of the Lr10 LRR suggests that an electrostatic interface might stabilises this closed conformation (Sela *et al.*, 2011). Since this region is conserved among NB-LRR proteins, this charge interaction could be an intrinsic feature of the LRR. Whereas the N-terminus of the LRR domain binds the NB-ARC the C-terminus might be exposed and able to detect changes in the R protein complex. The extended length of the CC (~ 90 Å)

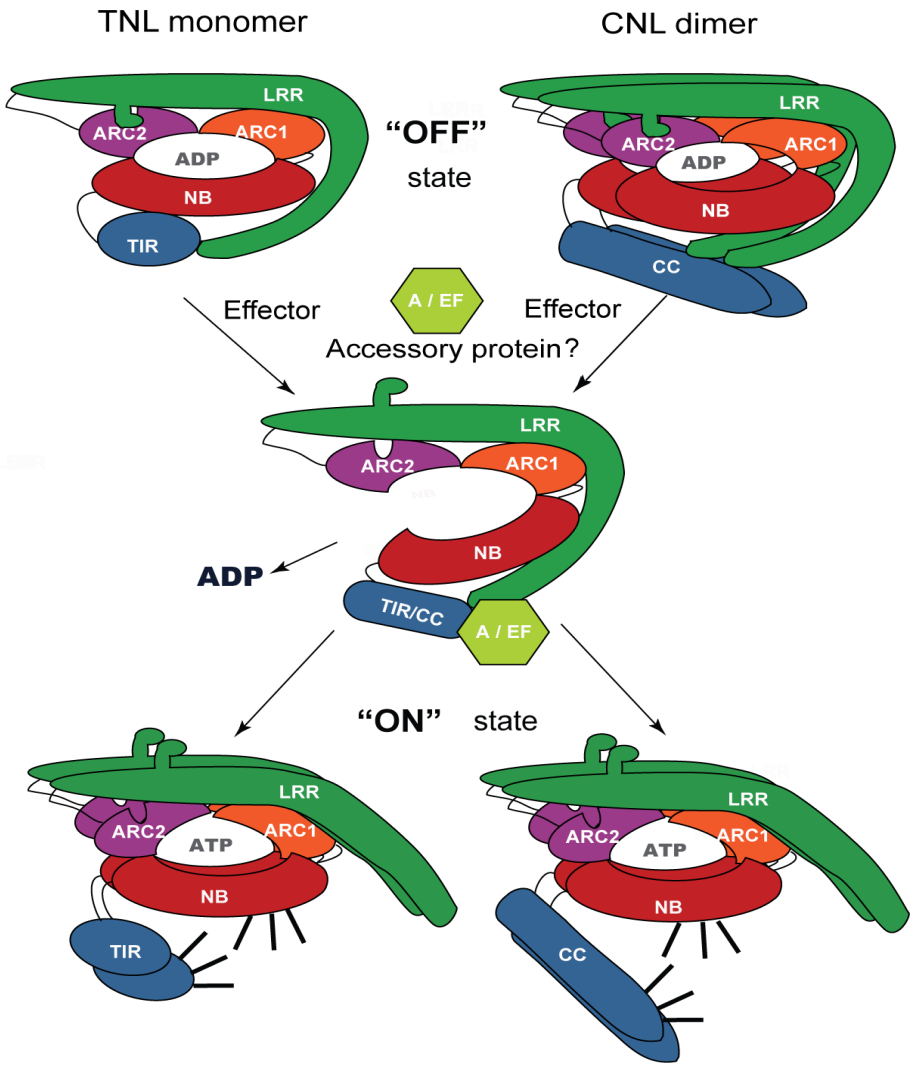

Figure 3. Model for NB–LRR protein activation. In the resting state an NB-LRR protein is kept in a closed and auto inhibited state in which the LRR and N-terminal domain (CC/TIR) fold back on the NB-ARC core. Effector recognition - sometimes aided by an accessory protein – likely occurs by an interface formed by the C-terminal half of the LRR and the CC/TIR domain. Effector recognition results in a conformational change that is transduced via the N-terminal part of the LRR to the ARC2. This change allows exchange of ADP for ATP thereby triggering a second conformational in the NB-ARC resulting in a more open structure in which interfaces on either the NB or N-terminal domain (CC/TIR) become exposed and activate defence signalling. In this model self-association of CNLs occurs prior to elicitor-dependent activation while TNLs multimerise after effector recognition; in both cases the activated state is represented by an oligomer.

allows this rod-like domain to interact at the same time with both the NB-ARC and LRR domain resulting in a compact protein structure in the resting state. A similar compact structure is predicted for TNL proteins carrying a globular TIR domain. In both models the LRR C-terminus is near the N-terminal TIR/CC domain (Figure 3). Collectively, these data are consistent with a model that in the absence of a pathogen the protein the R protein has a

compact conformation that is stabilised by many interdomain interactions keeping it in an inactive resting state.

Stabilization and accumulation of NB-LRR proteins: their maturation and stabilisation

Besides the interdomain interaction described above two other tightly linked processes are involved in keeping NB-LRRs in a signalling-competent, but auto-inhibited state in the absence of a pathogen. These are chaperone-assisted protein maturation and targeted protein degradation. The ATP-operated molecular chaperone Hsp90 (heat shock protein 90) and its co-chaperones Rar1 (Required for MLA12 Resistance 1), PP5 (Protein Phosphatase 5) and Sgt1 (Suppressor of the G2 allele of Skp1) are involved in NB-LRR maturation. Hsp90 is composed of three domains: an ATPase domain, a client-binding domain and a dimerization domain. Plant Hsp90s function as constitutive dimers and they associate with numerous NB-LRRs. Inhibition of Hsp90 function compromises disease resistance conferred by many NB-LRRs (Kadota *et al.*, 2011; Kadota *et al.*, 2010) Compromised *Hsp90* expression reduces protein levels of many R proteins such as Rx, RPM1, RPS5 and I-2 (Holt *et al.*, 2005; Hubert *et al.*, 2003; Lu *et al.*, 2003; Shirasu, 2009; van Ooijen *et al.*, 2010). Hsp90 interacts with co-chaperones such as Rar1, PP5, and Sgt1. In a *rar1* mutant background steady-state protein levels of many, but not all, NB-LRR R proteins are reduced (Bieri et al., 2004; Holt *et al.*, 2005; Muskett *et al.*, 2002). PP5 interacts with the LRRs from the CNLs I-2, Mi-1.2, and RPM1 and also with the Hsp90 dimerization domain (De La Fuente Bentem van *et al.*, 2005; Golden *et al.*, 2008). Although silencing of PP5 in tomato did not compromise I-2-mediated resistance, virus induced gene silencing in *N. benthamiana* resulted in compromised I-2 signalling concomitant with a reduction in I-2 protein accumulation (van Ooijen *et al.*, 2010). These data show that *PP5, Rar1* and *Hsp90* act as positive regulators of steady-state protein levels of many NB-LRRs by ensuring proper folding and stabilisation. Recently also an ATP independent chaperone was identified to be required for NB-LRR accumulation and stabilisation. The small heat shock protein RSI2 interacts with the tomato CNL protein I-2 and silencing *RSI*2 in *N. benthamiana* compromises *I-2* function concomitant with a reduced accumulation of the R protein (van Ooijen *et al.*, 2010).

Sgt1 appears to have a dual role in controlling the accumulation of NB-LRR proteins. It acts as a positive regulator of *RPP4*-, *RPP31*-, and *RPS5*-mediated HR, but as a negative regulator for RPS5 accumulation (Elmore *et al.*; Holt *et al.*, 2005). Sgt1 acts as Hsp90 specific adaptor for NB-LRRs but simultaneously it binds to the SCF (Skp1-Cullin-F-box) complexes (Catlett *et al.*, 2006; Zhang *et al.*, 2008). SCF complexes are ubiquitin E3 ligases that mark proteins for 26S proteasome-mediated degradation. Their specificity is conferred

by F-box proteins (Petroski *et al.*, 2005), such as CPR1 (Constitutive expresser of *PR* 1, also known as CPR30). This Arabidopsis F-box protein was found to control accumulation of the TNL proteins SNC1 (suppressor of npr1-1, constitutive 1) and RPS2 (Cheng *et al.*, 2011; Gou *et al.*, 2012; Kim *et al.*, 2002). A *CPR1* mutant has increased SNC1 and RPS2 protein levels, resulting in an autoimmune phenotype (Cheng *et al.*, 2011). Whereas CPR1 acts as negative regulator of RPS2 and RPM1 resistance, it only mildly affects resistance mediated by RPS5, RPP2 and RPS4 (Cheng *et al.*, 2011). Possibly other F-box proteins are involved in controlling protein levels of these NB-LRRs. It remains to be resolved whether Sgt1 and CPR1 act together to direct ubiquitinylation of NB-LRRs by the SCF^{CPR1} complex or whether these are separate processes. Another link between Sgt1 and the SCF machinery is the interaction between Rar1-Sgt1 and components of the COP9 signalosome; a complex that regulates SCF activity via NEDDylation (Azevedo *et al.*, 2002; Liu *et al.*, 2002). Together a picture emerges in which Sgt1 links NB-LRRs to the SCF-mediated machinery to control turnover of (mis)folded proteins and meanwhile links NLRs to the Hsp90-Sgt1-Rar1 complex to ensure proper NB-LRR folding. Together these processes appear to fine-tune the steady state protein levels of signalling-competent NB-LRRs.

Two other genes have recently been identified that act as a negative regulator of NB-LRR activity: *SRFR1* (Suppressor of *rsp4*-RLD 1) and *CRT1* (Compromised Recognition of TCV) (Kim *et al.*, 2010; Kwon *et al.*, 2009; Li *et al.*, 2010). *SRFR1* was originally identified in a gain-of-resistance screen against *P. syringae* carrying *avrRsp4* (Kwon *et al.*, 2004). In *Col-0* the *srfr1* mutant exhibits constitutive defence signalling that correlates with increased expression and accumulation of TNLs SNC1, RPS2 and RPS4 (Kim *et al.*, 2010; Li *et al.*, 2010). *In vivo* SRFR1 interacts with TNLs SNC1, RPS4 and RPS6, but not with CNL RPM1. Besides these interactions it also forms complexes with EDS1 (Enhanced disease resistance 1) and SGT1 (Bhattacharjee *et al.*, 2011; Li *et al.*, 2010). How SRFR1 regulates the steady-state levels of these TNL proteins is as yet unclear, but its interaction with Sgt1 provides another link to SCF-mediated protein degradation pathway. CRT1 is remotely related to the Hsp90 superfamily and is required for the proper function of a subset of TNLs and CNLs (Kang *et al.*, 2008; Kang *et al.*, 2010). The protein interacts with the NB domain of NLRs and also with Hsp90, but not with Sgt1 or Rar1. Since the steady state levels of NLRs are not affected in a *crt1* mutant this suggest that CRT1, is not strictly required for NB-LRR protein accumulation. Its interaction with NLRs seems restricted to non-activated NLRs suggesting that CRT1 might play a role in converting inactive NB-LRRs into activation-competent proteins.

In conclusion, maturation of R proteins in signalling-competent molecules is a multi-step process in which many generic and conserved proteins are involved. There

appears to be a fine-tuned balance between folding and the accumulation of these proteins in the cell to prevent undesirable induction of defence signalling in the absence of a pathogen.

Pathogen recognition, how are effectors detected by NB-LRRs?

Two mechanistic models exist that explain effector recognition by NB-LRRs; direct and indirect perception. The first, also known as the receptor-ligand model, is based on a direct and physical interaction between the NB-LRR and its cognate effector (Ellis *et al.*, 2007). Indirect recognition assumes an accessory protein (co-factor) that interacts with both the NB-LRR and the effector (Figure 3).

There are a few examples fitting the direct interaction model. The first direct interaction described is the one between the rice resistance protein Pi-ta and the AvrPita effector from *Magnaporthe grisea.* Both proteins interact in a yeast two-hybrid assay and *in vitro* (Jia *et al.*, 2000). Also in yeast an interaction between the flax rust effectors AvrL456 and the NB-LRR part of the L proteins was found (Dodds *et al.*, 2006; Ellis *et al.*, 2007). In addition, the Arabidopsis RRS1-R protein that confers resistance to *Ralstonia solanacearum* physically interacts with its cognate effector protein PopP2 (Deslandes *et al.*, 2003). Recently, an interaction *in planta* of the LRR domain of Arabidopsis RPP1 with the downy mildew effector ATR1 has been reported (Krasileva *et al.*, 2010). These examples show that NB-LRRs and effectors can directly interact and that for this interaction the LRR domain is essential. This latter observation is in agreement with the above proposed role of this domain in recognition specificity.

For most NB-LRRs and effectors a direct interaction could not be detected and therefore an alternative model was proposed in which NB-LRR proteins indirectly detect the interaction or modification of a host protein by the cognate effector. A few examples will be provided, for an extensive review, sees Collier et al. (2009) and Lukasik et al. (2009) (Collier *et al.*, 2009; Lukasik *et al.*, 2009). The indirect recognition model originates from the perception mechanism of the *P. syringae* effectors AvrPto and AvrPtoB by the tomato proteins Pto (a kinase) and Prf (an NB-LRR) (van der Biezen *et al.*, 1998b). Pto mimics the kinase domain of activated PRR receptors, which is targeted by the effector proteins AvrPto and AvrPtoB (Mucyn *et al.*, 2006). Pto is bound to the N-terminus of Prf, and interaction of Pto with bacterial effectors is perceived by Prf resulting in its activation. Another well-characterized example of indirect recognition is provided by the Arabidopsis RIN4 protein. RIN4 interacts with three different effector proteins from *P. syringae,* notably AvrRpm1, AvrB, and AvrRpt2, and interacts with the CC domain of the NB-LRR proteins RPM1 and RPS2 (Axtell *et al.*, 2003; Mackey *et al.*, 2003; Mackey *et al.*, 2002). Phosphorylation of RIN4 mediated by AvrB or AvrRPM1 activates RPM1, while

AvrRpt2-mediated cleavage of RIN4 triggers activation of RPS2. Hence, modification of RIN4 by these effectors is crucial to activate the interacting NB-LRR R protein. Similar to AvrRpt2 the effector AvrPphB is a protease, AvrRpt2 cleaves the Arabidopsis PBS1 kinase that binds to the CC of the NB-LRR RPS5, resulting in its activation (Shao *et al.*, 2003).

Depending on the identity of the protein targeted by the effector the interaction is referred to as guard, decoy, refined-switch or bait-and-switch model (Collier *et al.*, 2009; Dodds *et al.*, 2010; Lukasik *et al.*, 2009; van der Hoorn *et al.*, 2008). The guard model assumes that all "guardees" are virulence targets, while the decoy model proposes that these co-factors have co-evolved to mimic virulence targets without actually being one. Since there is no convincing proof to prefer one of these two models to the other the "switch" models were proposed. These models do not assign a specific function for the effector target, other than describing it as a co-factor (molecular bait) required for the NB-LRR to perceive an effector.

The "bait" proteins identified so far all interact with the N-terminal domain of their respective NB-LRR. For example, RIN4, PBS1 and RanGAP2 interact with the CC domain of RPM1, RPS2, RPS5, Rx and Gpa2, NRIP1 interacts with the TIR domain of N, and Pto interacts with the SD (Solanaceae domain) domain of Prf (Collier *et al.*, 2009; Eitas *et al.*, 2010). As depicted in the schematic NB-LRR model described in Figure 3 this means that the bait is in close proximity of C-terminal half of the LRR domain. Hence, the LRR is perfectly located to monitor effector-induced alterations of the bait such as its cleavage, phosphorylation or any other modification. Taken together, the LRR seems the main determinant for effector recognition. Effector recognition can occur either via a direct interaction with the NB-LRR protein or indirect via a "bait" protein bound to its N-terminal domain that is manipulated by the effector.

When the pathogen attacks: perception and signalling by NB-LRR proteins

In the previous paragraph the assembly of a signalling competent but auto inhibited NB-LRR R protein is described. The next question is how (in)direct effector perception releases auto inhibition to allow the NB-LRR protein to switch from its resting state into an activated state that induces defence signalling. In this multistep process the different subdomains seem to fulfil distinct functions.

Pathogen sensing

As detailed above the LRR domain plays a key role in effector recognition. Domain swapping experiments between highly similar NB-LRRs identify the C-terminal half of the LRR as the main determinant for pathogen recognition specificity (Ellis *et al.*, 2007; Rairdan

et al., 2008; Shen *et al.*, 2007; Zhou *et al.*, 2006). Such a role is in accordance with the LRR being the most variable part of an R protein with many hyper-variable residues under positive selection (Bendahmane *et al.*, 1999; Dodds *et al.*, 2006; Farnham *et al.*, 2006; Seeholzer *et al.*, 2010). This positive selection is proposed to accelerate the evolution of new interaction specificities (Ellis *et al.*, 2007). Mapping these variable residues on the predicted Lr10 LRR 3D structure shows that they are located on the concave β-sheet face and are likely solvent exposed, consistent with a role in pathogen recognition (McDowell *et al.*, 2008; Meyers *et al.*, 1998; Mondragón-Palomino *et al.*, 2002; Noël *et al.*, 1999; Padmanabhan *et al.*, 2009). Whereas the C-terminal part is involved in recognition, the N-terminal part primarily seems to be involved in intramolecular signalling as mismatches in this part frequently result in auto active or loss-of-function phenotypes (Lukasik *et al.*, 2009). Another indication for negative regulation conferred by the LRR correspond with the auto active phenotype observed upon its deletion as shown for RPS5, RPS2 and RPSA1 (Ade *et al.*, 2007; Tao *et al.*, 2000; van Ooijen *et al.*, 2008a; Weaver *et al.*, 2006). Such a dual function for the LRR, both autoinhibition and effector recognition, is in agreement with the presence of two distinct subdomains in the predicted structure of the Lr10 LRR (Sela *et al.*, 2011).

Activation of NB-LRRs

After effector recognition by the LRR its negative regulation is lifted, possibly by a conformational change in the interface between the N-terminal half of the LRR and the NB-ARC domain (Figure 3). Biochemical studies using the tomato I-2 and Mi-1.2 proteins, the flax L6 and M proteins and barley MLA27 have shown that these proteins in their auto inhibited state are bound to ADP. In this state the NB-ARC is proposed to have the same closed conformation as that observed of ADP bound Apaf-1 (Maekawa *et al.*, 2011; Tameling *et al.*, 2002; Williams *et al.*, 2011) Release of the negative regulation of the LRR is proposed to trigger a conformational change in the NB-ARC allowing the exchange of ADP for ATP. This hypothesis is based on the observation that I-2 mutants that are unaffected in nucleotide binding, but compromised in ATP hydrolysis, become constitutively active, suggesting that the ATP state is the active state (Tameling *et al.*, 2002). Recent support for this model comes from the analysis of the flax rust protein M. Whereas the wild type protein co-purifies specifically with ADP, an auto active variant, carrying a mutation in the MHD motif, was preferably bound to ATP (Williams *et al.*, 2011). Exchange of ADP for ATP is predicted to shift the closed ADP-bound conformation to an open conformation in analogy to the open configuration of the ATP-CED4 crystal structure in which NB, ARC1 and ARC2 domains are repositioned relative to each other. Such a major

conformational change in the core of a NB-LRR protein is predicted to have a major effect on the overall conformation and especially on the N terminal domain and the C-terminal LRR sensor domain tethered to the NB-ARC domain. Additional indications for major conformational changes that correlate with the nucleotide binding state of an NB-LRR protein are provided by the recent analysis of the interaction patterns of I-2 mutants with two interacting tomato proteins. Whereas the CC interactor mainly bound to mutants locked in the ATP state the other interactor mainly bound to the ADP state suggesting distinct conformations exposing distinct binding interfaces for these mutants (Lukasik-Shreepaathy *et al.*, 2012).

Defence signalling

Which domain or domains activate downstream signalling in an activated R protein remains an open question. For different R proteins different domains have been reported to be required and sufficient to trigger HR and/or resistance. For the potato Rx protein its NB domain has been shown to be sufficient to induce an hypersensitive response (Rairdan *et al.*, 2008). For the TNL proteins L6, RPP1A and RPS4 their TIR domains are sufficient to induce an HR, whereas for MLA10 its CC domain suffices (Bernoux *et al.*, 2011b; Maekawa *et al.*, 2011; Swiderski *et al.*, 2009; Weaver *et al.*, 2006). Interestingly, mutations that abolish the ability of the CC to dimerise also abolish its HR inducing capacity. Also full length MLA forms constitutive dimers and mutations in the CC that abolish MLA dimerization also eradicate its resistance function (Maekawa *et al.*, 2011). These data imply that a dimer is the minimal functional unit required to trigger defence. Constitutive multimerisation of CNL proteins in the absence of a pathogen has also been described for the Arabidopsis RPS5 and the tomato Prf protein (Gutierrez *et al.*, 2010) and might indicate a generic propensity of CNL proteins (Figure 3).

Although the TIR domain of L6 crystallises as a dimer, the full-length L6 and N TNL proteins are reported to dimerise only after pathogen perception (Bernoux *et al.*, 2011a; Mestre *et al.*, 2006). Although there are only few studied examples, the observed difference between monomeric and multimeric NB-LRR proteins in the absence of an effector could imply a fundamental mechanistic difference between TNL and CNL protein activation. The common theme is that in both cases the activated state of the NB-LRR protein is represented by an oligomer. How and where in the cell activated NB-LRR protein triggers host defence is an unanswered question that falls outside the scope of this chapter and is discussed in chapter 6. For some NB-LRRs, such as Rx, N, and MLA nucleocytoplasmic trafficking is required for their function suggesting distinct activities in the different subcellular compartments (Burch-Smith *et al.*, 2007; Slootweg *et al.*, 2010; Tameling *et al.*, 2010). A

hint to these functions comes from a recent study on RPS4 where induction of HR is associated with its cytoplasmic localization, whereas a nuclear localization for RPS4 is associated with defence signalling and resistance (Heidrich *et al.*, 2011). Future studies should reveal where in the cell pathogens are perceived and in which subcellular compartment the different braches of the defence system (HR and defence) are activated by NB-LRRs (Heidrich *et al.*, 2011).

Concluding remarks

Much progress has been made in our understanding of the molecular mechanism underlying NB-LRR R protein function. Their multi-domain structure allows these proteins to act simultaneously as sensor, switch and response factor. NB-LRR R protein activation is a multistep process that requires fine-tuned intramolecular interactions between compatible subdomains. The different domains in NB-LRR proteins fulfil different functions during the various activation states of the protein. Whereas the N-terminal domain frequently plays a role in effector recognition via its interaction with an effector target (bait), the same domain is being used to activate downstream signalling as shown for MLA10 and different TNL proteins. The LRR is required to keep the protein inactive in the absence of a pathogen, but it is also the major recognition specificity determinant. The NB-ARC domain acts both as a nucleotide-operated switch, to control the activation state of the NB-LRR protein, but for Rx it can also induce HR signalling. Since unintentional activation of signalling competent NB-LRR proteins can have deleterious effects on the plant, their steady state levels are tightly controlled by the balanced interaction between chaperones and the proteasome.

The models proposed here are based on studies of a limited number of NB-LRR proteins, which means that our knowledge is fragmentary. More biochemical and structural studies are required to validate and possibly extend these models. Elucidation of the 3D structure of intact R proteins in both their resting and activated state will be essential to define structural determinants explaining how changes in conformation and intramolecular interactions regulate their activity. Furthermore, identification of interaction partners of R proteins is needed to truly understand how NB-LRRs perceive pathogens. In line, we need to deduce where in the cell activated R proteins induce host defences.

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