Structure and Function of Resistance Proteins in Solanaceous Plants

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Abstract

Gene-for-gene resistance in plants is based on the presence of a resistance (R) gene in the host and a matching Avirulence (Avr) gene in the pathogen. Many R genes have been cloned over the past two decades, mostly from the Solanaceae. The gene products, called R proteins, display modular domain structures. R protein function has recently been shown to require dynamic interactions between the various domains. In addition to these intramolecular interactions, R proteins interact with other proteins to form signaling complexes that are able to activate an innate immune response that arrests proliferation of the invading pathogen, thereby conferring disease resistance. In this review, we summarize current understanding of R protein structure and function, as well as the molecular mechanisms underlying the activation of defense signaling processes. As well as being a rich source for R genes, Solanaceae are a leading model system in which to study inter- and intramolecular interactions of R proteins.

INTRODUCTION

Solanaceae, a family of flowering dicot plants, likely derived the name from the Latin word *sol*, sun, because the flowers of the most prominent genus in this family, *Solanum*, resemble the sun and its rays. The family includes some prominent crops such as potato (*Solanum tuberosum*), tomato (*S. lycopersicum*), pepper (*Capsicum* spec.), and eggplant (*S. melongena*). Like all other plants, solanaceous plants are attacked by a wide range of pathogens including oomycetes, viruses, bacteria, fungi, nematodes and insects such as white fly and aphids that cause significant crop losses (137). In response to these attackers, plants have evolved passive and active defense mechanisms. Active defense responses can be subdivided into adaptive and innate immunity. Adaptive immunity in plants appears to be restricted to antiviral defense responses that depend on an RNAi-like mechanism (164). The innate immune system is more general and responds to a wide variety of plant pathogens. Innate immunity relies on specialized receptors that can be roughly divided into two groups: the Pathogen or Pattern Recognition Receptors (PRRs) and the Resistance (R) proteins. PRRs recognize Microbe- or Pathogen-Associated Molecular Patterns (MAMPs/PAMPs) that are often highly conserved molecules shared between microorganisms of the same class (105, 183). PRRs allow plants to recognize distinct invaders using a limited set of receptors (23, 183). In contrast to PRRs, R proteins respond to molecules (called avirulence proteins or elicitors) that are generally not conserved between species or even between isolates of a given pathogen. Accordingly, R proteins are encoded by large gene families, numbering several hundreds of genes per genome (95). Because of the one-to-one relationship between a plant *R* gene and the matching *avirulence (Avr)* gene in a pathogen, this type of immunity was called gene-for-gene resistance (41). Resistance mediated by R proteins is often associated with the appearance of localized cell death at the infection site, a phenomenon called the hypersensitive response (HR). This is distinct from the resistance response mediated by PRR receptors, as these generally do not induce an HR response upon pathogen recognition (61).

In this review, we provide an overview of the R genes that have been cloned from the Solanaceae. We describe the encoded proteins and their predicted structures. Furthermore, we discuss the data available on the intra- and intermolecular interactions of R proteins from Solanaceae in the context of other model systems. The interaction patterns together with the identified downstream signaling components provide new insights into R protein function and downstream signaling.

MAMP/PAMP: Microbe/Pathogen-Associated Molecular Pattern

Elicitor: pathogen-derived molecule that triggers defense responses resulting in enhanced resistance to the pathogen. When recognition is mediated by an R (resistance) protein, it is also referred to as an Avr (avirulence) protein and when recognized by a PRR (Pathogen Recognition Receptor), as a PAMP (Pathogen-Associated Molecular Pattern)

Hypersensitive response (HR): gene-for-gene resistance is often associated with a local cell death response at the site of infection. This form of programmed cell death is referred to as the hypersensitive response

NB-ARC: nucleotide binding (NB) domain shared between human APAF-1 (Apoptotic Protease-Activating Factor 1), some plant R proteins, and CED-4 (*Caenorhabditus elegans* Dead protein 4)

LRR: leucine-rich repeat

R PROTEIN CLASSIFICATION

Over 55 *R* genes have now been cloned from different monocot and dicot plant species [(87) and Solanaceous R genes listed in (**Table 1**)]. Although *R* genes confer resistance to very different pathogens, the encoded proteins share a limited number of conserved elements. Based on these domains R proteins can be divided into four classes (**Figure 1**). The vast majority contain a central nucleotide-binding (NB) subdomain as part of a larger entity called the NB-ARC domain, which is present in the human apoptotic protease-activating factor 1 (APAF-1), R proteins, and the CED-4 protein of *Caenorhabditus elegans* (154). C-terminal to the NB-ARC domain lies a leucine-rich repeat (LRR) domain, which is sometimes followed by an extension of variable length. Hence, this group of R proteins is collectively referred to as NB-LRR proteins. These NB-LRR proteins are divided into two classes on the basis of their N-terminal region. If this region shows homology to a protein domain found in the *Drosophila* Toll and human Interleukin-1 Receptor (IL-1R), it is called the TIR domain (168) and these proteins are referred to as TIR-NB-LRR or TNL proteins (TNL class).

Table 1 Cloned solanaceous plant disease resistance genes

Gene[a]	Plant species	Ref[b]	Avr gene	Pathogen	Ref[b]
TNL class					
Bs4	*Solanum lycopersicum*	(129)	AvrBs4, Hax4	*Xanthomonas campestris*	(15, 67)
Gro1-4	*Solanum tuberosum*	(108)		*Globodera rostochiensis*	
N	*Nicotiana tabacum*	(168)	Helicase	TMV	(37)
CNL class					
Bs2	*Capsicum annuum*	(140)	AvrBs2	*Xanthomonas campestris*	(139)
Gpa2 (Rxh1)	*Solanum tuberosum*	(160)		*Globodera pallida*	
Hero[c]	*Solanum lycopersicum*	(38)		*Globodera rostochiensis*, *G. pallida*	
I-2	*Solanum lycopersicum*	(106, 134)		*Fusarium oxysporum*	
Mi-1.2[c]	*Solanum lycopersicum*	(96, 165)		*Meloidogyne incognita*, *M. arenaria*, *M. javanica*, *Bemisia tabacci*	
Prf[c]	*Solanum lycopersicum*	(128)	AvrPto, AvrPtoB	*Pseudomonas syringae*	(2, 124)
R1[c]	*Solanum tuberosum*	(11)		*Phytophthora infestans*	
R3a	*Solanum tuberosum*	(53)	Avr3a	*Phytophthora infestans*	(4)
Rpi-blb1 (RB)	*Solanum bulbocastanum*	(135, 158)		*Phytophthora infestans*	
Rpi-blb2[c]	*Solanum bulbocastanum*	(159)		*Phytophthora infestans*	
Rx1	*Solanum tuberosum*	(13)	CP	PVX	(13)
Rx2	*Solanum acaule*	(14)	CP	PVX	(14)
Sw-5[c]	*Solanum lycopersicum*	(18)		Tospovirus	
Tm-2	*Solanum lycopersicum*	(76)	MP	ToMV	(20)
Tm-2-2	*Solanum lycopersicum*	(75)	MP	ToMV	(20)
RLP class					
Cf-2	*Solanum lycopersicum*	(32)	Avr2	*Cladosporium fulvum*	(84)
Cf-4	*Solanum lycopersicum*	(148)	Avr4	*Cladosporium fulvum*	(62)
Cf-4A (Hcr9-4E)	*Solanum lycopersicum*	(143)	Avr4E	*Cladosporium fulvum*	(167)
Cf-5	*Solanum lycopersicum*	(31)		*Cladosporium fulvum*	
Cf-9	*Solanum lycopersicum*	(60)	Avr9	*Cladosporium fulvum*	(161)
Cf-9B (Hcr9-9B)	*Solanum lycopersicum*	(111, 112)		*Cladosporium fulvum*	

(*Continued*)

Table 1 (*Continued*)

Gene[a]	Plant species	Ref[b]	Avr gene	Pathogen	Ref[b]
Ve1	*Solanum lycopersicum*	(66)		*Verticillium albo-atrum*	
Ve2	*Solanum lycopersicum*	(66)		*Verticillium albo-atrum*	
Other					
Asc-1	*Solanum lycopersicum*	(17)	-	*Alternaria alternata*	

[a]Synonymous names in parentheses.
[b]References that describe cloning of the gene.
[c]The R proteins encoded by these genes contain an extended N-terminus.

NB-LRR: R protein containing an NB-ARC domain coupled to a LRR domain

CC: coiled coil motif

eLRR: extracellular LRR

Because some non-TIR proteins contain predicted coiled-coil structures (CC) in their N-terminal domain, non-TIR NB-LRR proteins are collectively referred to as CC-NB-LRR or CNL proteins (CNL class). CNL proteins are subdivided into two groups depending on the presence of a short or an extended N terminus (see below). Phylogenetic analyses of the NB-ARC domains of NB-LRR proteins revealed separate clustering of TNL and CNL proteins. This finding suggests coevolution of the N-terminal and NB-ARC domains and is indicative of an ancient segregation of these two classes, providing an extra basis for the subdivision of NB-LRR R proteins (90). All NB-LRR proteins are believed to act intracellularly. A limited number of R proteins act extracellularly, and they contain a predicted extracellular LRR (eLRR) domain at their N terminus. This eLRR is connected via a transmembrane domain to a variable cytoplasmic C-terminal region. When the cytoplasmic domain contains a protein kinase domain the R protein is placed in the RLK class, that of Receptor-Like Kinases. If no such domain is present, it is placed in the RLP class, that of Receptor-Like Proteins. One solanaceous R protein (Asc1) does not fit into any of these four classes.

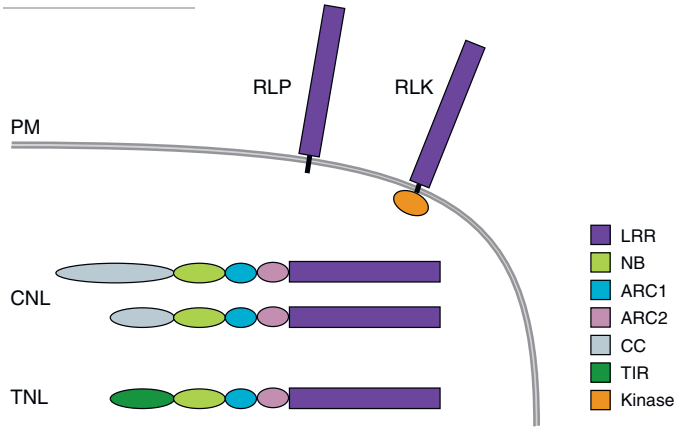

Figure 1

Schematic representation of typical members of the four R protein classes. Protein domains and putative cellular localization are indicated. The Receptor-Like Protein (RLP) and the Receptor-Like Kinase (RLK) classes of R proteins span the plasma membrane (PM) and contain an extracellular leucine rich repeat (LRR) domain. R proteins of the RLK class have not been identified in Solanaceae. The CNL and TNL classes of R proteins are located intracellularly (cytoplasmic, nuclear, or membrane-bound) and contain a central NB-ARC domain (consisting of NB, ARC1, and ARC2 subdomains) coupled to an LRR domain. TNLs carry an N-terminal TIR domain, while CNLs contain either a CC or an extended CC domain.

In addition to the genes that have been isolated and confirmed to function as an *R* gene, numerous *R* gene homologues have been identified in genome sequencing and annotation programs. In *Arabidopsis*, TNLs form the largest group of NB-LRR proteins (94), whereas this class is absent in monocots (94, 110). This difference could reflect differences in host/pathogen coevolution in mono- and dicots. Most solanaceous NB-LRR proteins belong to the CNL class (**Table 1**), whereas only three TNLs have been identified: the tomato *Bs4*, the potato *Gro1-4*, and the tobacco *N* gene conferring resistance to

Xanthomonas campestris, *Globodera rostochiensis* and Tobacco Mosaic Virus (TMV), respectively (**Table 1**).

The RLP class contains the tomato Cf and Ve proteins that confer resistance to *Cladosporium fulvum* and *Verticillium albo-atrum*, respectively (66, 122). The two Ve proteins differ from the Cf proteins by the presence of a putative C-terminal endocytosis signal (66). No R proteins that belong to the RLK class have yet been identified in the Solanaceae. Except for the rice *Xa21* gene, this group contains PRRs: the *Arabidopsis* EF-tu and flagellin FLS2 receptors (22, 44, 184) and the tomato *Le*EIX (123). Tomato *Asc1* could not have been placed in any of the 4 classes because it does not encode a typical R protein involved in gene-for-gene resistance: resistance to *Alternaria alternata* is brought about by reduced sensitivity to the AAL mycotoxin rather than by specific recognition of the pathogen (17). The tomato Pto kinase confers, together with the CNL Prf, resistance to *Pseudomonas syringae* (100). In this review, we refer to Prf as the R protein and Pto as a regulator of Prf function.

AVR PROTEINS AND R PROTEIN-MEDIATED RECOGNITION

It seems inadequate for a pathogen to disclose its presence to a plant by secreting *Avr* gene products that are recognized by an R protein. Obviously, *Avr* genes did not evolve to serve this purpose, and indeed evidence accumulates that many Avr proteins are virulence factors (61). Plant pathogenic bacteria deliver approximately 15–30 proteins into host cells using a specialized type III secretion system (TTSS) (19). Whether these effectors suppress or trigger host defense depends on the host being attacked (163). Effectors appear to manipulate many signaling processes, but only for a small number of effectors has a host target been identified (46). Clues on how they interfere with signal transduction processes of the host can sometimes be obtained from structural similarity to other proteins. For instance, members of the XopJ/AvrRxv effector family appear to encode SUMO proteases, suggesting interference with SUMO signaling (107), whereas AvrPtoB is an active ubiquitin E3 ligase likely interfering with specific protein degradation in the host (58). Avr proteins such as AvrRpt2 and AvrPphB act as proteases and cleave specific host proteins [(6, 69, 132, 133; for recent reviews on bacterial effectors, see 1, 46, 101)].

Less is known about fungal effectors. Four *Avr* genes have been cloned from the fungus *Cladosporium fulvum* (**Table 1**), all of which encode small cysteine-rich extracellular proteins found in the tomato apoplast. Avr2 acts as a protease inhibitor (125), whereas Avr4 binds to chitin present in fungal cell walls, thereby protecting it from degradation by plant chitinases (153). The targets of the remaining Avr products of *C. fulvum* are still unknown, but they are probably located in the apoplast (63, 149). Other plant pathogenic fungi, such as rusts and powdery mildews, create more intimate contacts with their hosts by forming specialized feeding structures called haustoria. These specialized feeding structures penetrate the host plant cell wall but remain separated from the host cytoplasm by the host cell envelope. Recently, several *Avr* genes have been identified from haustoria-forming fungi (21, 33, 119). These genes encode proteins with a predicted signal peptide for secretion into the extrahaustorial matrix, but nonetheless act inside the host cell where they are perceived by matching NB-LRR proteins. How these Avr proteins are taken up by the host is not yet clear [(33, 119; for a recent review on fungal Avr proteins and secreted proteins, see 118)].

The Avr3a protein from the oomycete *Phytophthora infestans* also acts inside the host cell as, in the absence of the *R3* resistance gene, it is able to suppress cell death triggered by the elicitin INF1 of *P. infestans* (16). Avr3a and two other oomycete Avr genes cloned from *Hyaloperonospora parasitica* share a conserved sequence motif, RxLR (65). This motif might

Receptor-Like Protein (RLP): an eLRR protein with a short cytoplasmic domain lacking homology to a protein kinase domain

Receptor-Like Kinase (RLK): an eLRR plasma membrane-spanning protein with a cytoplasmic protein kinase domain

APAF-1: apoptotic protease-activating factor 1

TIR: protein domain with homology to the *Drosophila* Toll and human Interleukin-1 receptor

TNL: TIR-NB-LRR protein, an R protein containing a central NB-ARC domain fused to an N-terminal TIR domain and a C-terminal LRR domain

CNL: CC-NB-LRR protein, an R protein containing a central NB-ARC domain fused to an N-terminal non-TIR domain and a C-terminal LRR domain

Effector: pathogen-derived molecule that manipulates the host cell thereby facilitating infection. When an effector is recognized by a plant and triggers defense it is called an elicitor

TPR: tetratricopeptide repeat

be the signal for uptake into the host cell from the extrahaustorial space.

Based on the existence of monogenic resistance, metazoan pathogens such as nematodes and insects are also predicted to contain *Avr* genes (165, 172). Unfortunately, no metazoan *Avr* genes have yet been identified (64, 172).

Because in all cases described above corresponding R and Avr proteins colocalize, one interpretation of the gene-for-gene relation assumes a direct interaction between the two proteins. Using yeast two-hybrid and in vitro pull-down approaches, a direct interaction was indeed implicated in a few cases, i.e., between Pita/AvrPita (59), L/AvrL567 (33), PopP2/RRS-1 (27) and the TMV p50 helicase with the NB-ARC-LRR part of the tobacco N protein (152). Direct interaction between the latter, however, was not found by others using in vivo pull-down assays (92, 114), but rather an indirect interaction was detected (18a). A direct interaction was recently also proposed for the Bs4/AvrBs4 pair (130) since AvrBs4 has a tetratricopeptide repeat (TPR)-like structure also found in the I-2 interacting protein phosphatase 5 (PP5) (25).

Because most bacterial Avr proteins probably act as effector proteins that target host cellular components, R proteins might sense the presence of an Avr protein by monitoring the state of the host target. This indirect interaction model is called the guard hypothesis (24, 155). Although a guard that waits until its guardee is killed may seem counterintuitive, this theory has gained support. The best-studied example of modification of a host target by Avr proteins, resulting in R protein activation, is the phosphorylation or cleavage of *Arabidopsis* Rin4 protein by AvrB, AvrRpm1, and AvrRpt2, respectively (69, 86). These events trigger activation of the NB-LRR proteins Rpm1 and Rps2. Also, the cleavage of the Pbs1 kinase bound to Rps5 by the avrPphB protease, thereby triggering Rps5 activation, supports the guard hypothesis (132).

Three additional examples in support of the guard hypothesis have been described in solaneceous pathosystems. First is the interaction between the AvrPto and AvrPtoB effectors of *Pseudomonas syringae* and the tomato kinase Pto, that is sensed by NB-LRR protein Prf. The Avr proteins interact directly with their target Pto (70, 146), whereas Pto constitutively binds to the N terminus of Prf (100). Although the presence of a tertiary complex could not be shown, interaction of all three components is essential for the induction of defense signaling. Upon binding of AvrPto or AvrPtoB to Pto, a conformational change of this protein is proposed to trigger the defense signaling potential of the interacting Prf R protein (100).

A second example of indirect pathogen perception in tomato is the *Avr2/Cf-2* system. *Cf-2* resistance is strictly dependent on the tomato cysteine protease Rcr3 that is inhibited by the Avr2 protein from *C. fulvum*. This Rcr3-Avr2 complex is thought to trigger Cf-2 mediated responses (125). The Rcr3 sequence from cultivated tomato (*Solanum lycopersicum* L.) differs slightly from the Rcr3 in *S. pimpinellifolium*, and *Sl*Rcr3 induces Cf-2 dependent necrosis in the absence of any Avr. This observation suggests that a small conformational difference in the *Sl*Rcr3 protein mimics the Avr2-inhibited *Sp*Rcr3 state so that it activates Cf-2 (125).

Evidence of indirect Avr binding was also obtained for the TNL protein N of tobacco. Using coimmunoprecipitations and bimolecular fluorescence complementation (BiFC) experiments, it was shown that N and p50, the part of the helicase protein that is required for its Avr function, associate in vivo. This association is mediated by an unknown protein binding to the TIR domain, called N interacting protein (NIP1). NIP1 is a good candidate to be the guardee of N (S. P. Dinesh-Kumar, personal communication). In addition to the examples described above, indirect Avr recognition has been proposed for the tomato Mi-1.2 protein. Mi-1.2 is intriguing in that it confers resistance to highly diverse animal pathogens: the root-knot nematode *Meloidogyne incognita*, the potato aphid *Macrosiphum*

euphorbiae, and the whitefly *Bemisia tabaci* (97, 104, 165). Mi-1.2-mediated resistance to nematodes is accompanied by an HR, while an HR is not observed in the interaction with whitefly and the potato aphid (171). A mutant screen identified *Rme-1*, which is unlinked to *Mi-1.2* but is required for Mi-mediated resistance against all three pathogens. As Rme-1 is not involved in other R gene pathways and acts upstream (or at the same step) of Mi-1.2 in the signaling cascade, it is a candidate for the Mi-1.2 guardee (88). However, cloning of the *Rme-1* gene is needed to confirm that Rme-1 functions as the Mi-1.2 guardee.

The examples for direct and indirect Avr perception are still too few for any firm conclusion to be drawn as to whether there is a prevalence for one over the other. During evolution, R protein recognition specificities are likely to be generated at random, some targeting Avr products directly, and others recognizing host factor modifications. The group targeting Avr proteins directly can relatively easily be overcome by mutations in the Avr protein that abolish the interaction, but not its virulence function. Resistance based on indirect recognition is (at least theoretically) more difficult to overcome, because mutations avoiding recognition will also affect virulence.

STRUCTURAL FEATURES OF R PROTEIN DOMAINS

As described above, most R proteins can be classified based on their protein domain architecture. **Figure 1** schematically presents the 4 structural classes of R proteins. Unfortunately, crystal structures have not yet been obtained for any domain of plant R proteins. However, crystal structures of LRR and NB-ARC domains are available from evolutionarily related proteins. These crystal structures have been used for 3-D structure modelling of plant LRR and NB-ARC domains (90, 142, 157). These studies have provided insight into some structural features of these domains, and they are utilized to explain the effect of certain mutations on R protein activation and downstream signaling (90, 142).

The Leucine-Rich Repeat Domain

The LRR domain is the only domain present in all R proteins listed in **Table 1**. LRRs are present in many receptors of virtually any organism, where this domain is involved in ligand recognition (36). The LRR domain consists of 2 to 42 repeats, each comprising a β-sheet with the core consensus xxLxLxx (36). In plant LRRs, individual repeats are formed by 24–28 residues and contain a core of 14 residues with the consensus sequence LxxLxxLxLxxC/Nxx. This core forms the β-sheet and the attached loop-regions. Each core is separated from the next by a spacer of variable length. Crystal structures of over 20 LRR proteins have revealed differences in overall structure, but in all proteins a series of parallel β-strands form a right-handed superhelical β-sheet (36, 72). The only plant LRR protein for which the crystal structure was solved is PGIP2, an eLRR protein that binds and inhibits polygalacturonases from fungi such as *Fusarium moniliforme* and *Aspergillus niger* (77). The PGIP2 structure is characterized by the presence of a second β-sheet (β2) in each repeat, with consensus NxLxGx, connecting the first β-sheet (β1) with an α-helix in the spacer (29).

In plant R proteins there are several differences between intra- and extracellular LRR domains. In the LRRs of TNLs and CNLs no clear subdomains are apparent, although a conserved motif is present in the third LRR (95). Mutation of the D in this so-called VLDL motif in the potato Rx protein to an E produced a constitutively active protein (12), whereas a mutation adjacent to this motif in the *Arabidopsis* CNL Rps5 had inhibitory epistatic effects on resistance (166). The role of this motif in NB-LRR protein function is not clear, but the sequence fits the consensus for a leucine-rich nuclear export signal (12, 74).

Plant eLRR proteins are characterized by a longer repeat consensus sequence than intracellular LRRs; a 24-residue motif LxxLxxLxLxxNxLxGxIPxxLGx instead of the general 14-residue consensus (40). The consensus of the PGIP2 β2 is conserved in the Cf proteins and since ligand binding presumably involves only β1, β2 might be involved in binding other proteins (40, 122) or in homo- or heterodimerization with other eLRRs. The Cf and Ve eLRR domains can be subdivided into three subdomains, which in Cf proteins are referred to as C1, C2, and C3 (122). C1 forms the major part of the eLRR domain and consists of 21–28 hypervariable repeats. C2 forms a spacer domain separating C1 from C3. C3 consists of three to four relatively conserved repeats (122). R protein eLRR domains, unlike the LRR domains in NB-LRR proteins, have many putative N-linked glycosylation (NGS) sites in the exposed regions. For Cf-9, the NGS sites in the C1 are essential for Cf-9 function, whereas introduction of NGS sites in the C3 disrupts its function (157). Another unique feature of eLRRs is the presence of N- and C-terminal cysteine-rich "capping" domains that flank the LRR domain and shield the hydrophobic core of the last LRR from the solvent. For the small tomato eLRR protein LRP (Leucine Rich Protein), capping domains were shown to form disulfide bonds that are necessary to protect the eLRR from proteolysis (73).

The NB-ARC Domain

The recently published crystal structures of the NB-ARC domains of APAF-1 and CED-4 show that the NB-ARC domain consists of clearly distinguishable subdomains. Besides the NB subdomain, the NB-ARC of APAF-1 contains three additional subdomains (ARC1–ARC3) that form a four-helix bundle, a winged-helix fold and a helical bundle, respectively (120). Subdomain ARC3 is absent in plant R proteins and CED-4, but ARC1 and ARC2 are conserved (**Figure 2**)

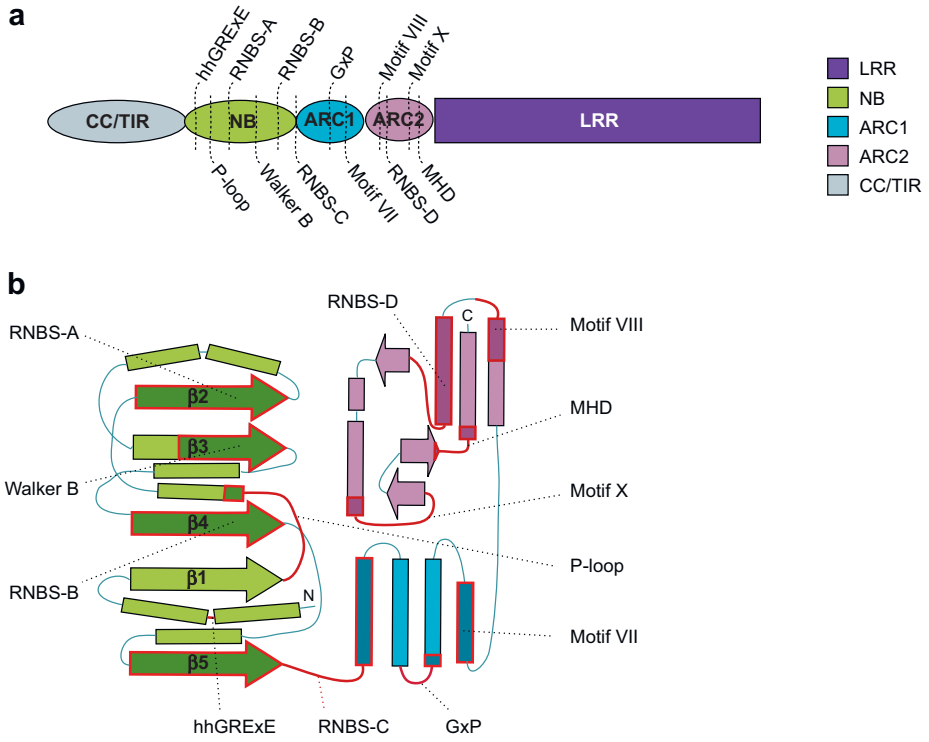

Figure 2

Proposed protein topology of the NB-ARC domain. (*a*) The position of conserved motifs in the NB, ARC1, and ARC2 subdomains is indicated in the schematic representation of a typical NB-LRR R protein. (*b*) Conserved motifs are marked (*in darker colors and red outlines*) in a close-up of the protein topology of the NB-ARC domain. Arrows and bars represent β-strands and α-helices, respectively.

(3, 175). Proteins containing an NB-ARC domain are evolutionarily related to the mammalian NACHT-LRR (NAIP, CIITA, HET-E, and TP1) protein family, of which many members function in innate immunity (3, 57, 78). ARC1, -2, and -3 of APAF-1 correspond to the NAD1–3 subdomains of the NACHT-LRR proteins (3).

The NB-ARC and NACHT-LRR proteins belong to the STAND superfamily of ATPases (Signal Transduction ATPases with Numerous Domains). The nucleotide-binding domain of these proteins is proposed to function as a molecular switch, where NTP-hydrolysis induces a conformational change required to regulate signal transduction (78). The NB subdomains of both NB-ARC and NACHT domains form an NTP binding fold consisting of a parallel five-stranded β-sheet flanked by α-helices (142), placing these proteins in the large group of P-loop NTPases (162). As shown in **Figure 2**, the β-sheet is five-stranded and assumes a 2-3-4-1-5 topology in which β1 is located between β4 and β5. The strands β1 and β3 encompass the most conserved motifs of the NB subdomain: the P-loop itself (also called Walker A motif) and the Walker B motif. The P-loop is defined by the consensus sequence GxxxxGKS/T in which the Lysine binds the β- and γ-phosphates of the nucleotide, and the Ser and Thr residues coordinate the Mg^{2+}-ion in the binding pocket. The Walker B motif is defined by the hhhhDD/E consensus site (where h represents a hydrophobic residue); in R proteins this motif is often hhhhDD. The first Glu residue (D) is important for the coordination of the Mg^{2+}-ion whereas the second acidic residue (D/E) is thought to act as a catalytic base during nucleotide hydrolysis (48, 78).

Several other motifs have also been identified in the NB-ARC domain (**Figure 2**), including Resistance NBS motifs RNBS-A, -B, -C, and -D; the GxP motif; and motifs VII and VIII (**Figure 2**) [for an overview of synonymous names for these motifs, see (142)]. The hhGRExE motif is part of a linker region connecting the NB subdomain with the N terminus. In the ARC2 subdomain, a highly conserved motif is present called the MHD-motif. Many residues in the conserved motifs are essential for NB-LRR protein function and are predicted to map at positions where they are able to interact with the nucleotide (142). For instance, the conserved His residue in the APAF-1 MHD motif interacts directly with the β-phosphate of dATP (120). Mutation of this motif in the R proteins I-2, Mi-1.2, L6, and Rx results in autoactivation (25, 52, 98; G.v.O., unpublished), possibly by interfering with nucleotide binding.

The NB-ARC domains of I-2, Mi-1.2, and N have been shown to specifically bind (d)ATP, similar to APAF-1 and CED4 (68, 144, 152, 175). The NB-ARC domain of these R proteins also catalyzes the hydrolysis of ATP to ADP (see Sidebar ATP Hydrolysis By NB-ARC Domains).

STAND: Signal Transduction ATPases with Numerous Domains

ATP HYDROLYSIS BY NB-ARC DOMAINS

In APAF-1, binding of cytochrome c results in hydrolysis of ATP by the NB-ARC domain, followed by replacement of the formed ADP by ATP (68). ATP hydrolysis and nucleotide exchange are both essential to produce the activated state. Like APAF-1, CED-4 requires ATP binding for function. However, hydrolysis or nucleotide exchange has not been observed for CED-4 (175).

Specific binding and hydrolysis of ATP was recently observed for the solanaceous R proteins I-2, Mi-1.2, and N (144, 152). For I-2, biochemical analysis of two autoactivating mutants revealed that these mutants are affected in ATP hydrolysis but not in nucleotide binding (145). This observation suggests that in the active, HR-signaling state, ATP is bound to the NB-ARC. Mutations in the P-loop of several other R proteins were shown to abolish function (30, 147, 151). These data indicate that nucleotide binding is essential for R protein signaling. To define more exactly the effect of the nucleotide binding status of R proteins on R protein function, biochemical analyses of NB-ARC domains, or preferably entire R proteins, is inevitable.

The N-Terminal Domain

Diversifying selection: adaptive evolution based on positive selection of new amino acid sequence variants. Opposite of purifying selection that favors sequence conservation

As described above, the two NB-LRR classes are distinguished by the presence or absence of a TIR domain at the N terminus. The TIR domain is conserved in metazoan Toll and IL-1R proteins (174), which are involved in innate immunity. Crystal structures have been published for human Toll-like receptors TLR1 and TLR2. Structures of plant TIR domains have not yet been resolved, but structures similar to animal family members are proposed, as amino acids shared between the TNL N and the metazoan Toll or IL-1R protein were shown to be essential for functioning of both (30). The topology of the TIR domain consists of a parallel five-stranded β-sheet, five α-helices, and connecting loops. The loop between β2 and α2 contains several highly conserved surface-exposed residues that are important for receptor signaling by recruiting interacting proteins (174).

CNL proteins often contain predicted coiled-coil motifs in the N-terminal domain (α-helix-rich domain that contains seven-residue repeat sequences). In **Figure 1** and **Table 1**, we distinguish two types of N-terminal domains: a short CC domain and an extended CC domain. This extended domain has been found only in Solanaceae and might be specific for this family. Recently, a novel protein-protein interaction domain was identified in the extended N terminus of tomato Prf. This domain has been found in solanaceous R proteins with an extended N terminus only, and was called the Solanaceous Domain (SD) (100).

A recent sequence analysis has revealed a short conserved motif in the middle of the N-terminal domain. This so-called non-TIR or nT motif was originally identified due to its high conservation in monocot CNLs (10). The motif is less conserved in dicots, but a core EDxxD motif can be identified in many CNL proteins and is one of the few motifs that can be identified as being broadly conserved across the N terminus of CNLs of different families of plants (G. J. Rairdan & P. Moffett, unpublished data). This EDxxD motif was shown to be required for Rx function, due at least in part to its involvement in an intramolecular interaction (G. J. Rairdan & P. Moffett, unpublished data).

R PROTEIN DOMAIN FUNCTIONALITY AND INTERPLAY

As detailed above, R proteins are composed of several (sub)domains that appear to function consecutively during signal perception. In this section we address the following questions: (*a*) What is the specific function of each (sub)domain? (*b*) How do the domains interact? (*c*) How does this result in a protein that is able to recognize Avr proteins and trigger activation of defense responses?

Many studies have shown that the LRR domain is under diversifying selection, and specifically the surface-exposed residues in the β–sheet that are putatively involved in ligand recognition (93, 99, 103). Diversifying selection is compatible with a receptor surface that might be involved in Avr perception (99). The prediction that the LRR domain confers Avr-specific recognition has indeed been confirmed experimentally, and this specificity can be altered with a few minor changes into a new one (33, 35, 59, 116, 156, 173). A significant degree of diversifying selection was also observed in the N-terminal region of some NB-LRR proteins, suggesting that this region is also important for the origination of new recognition specificities (99), which has been confirmed experimentally (35, 83). In addition to a role in recognition specificity, the N-terminal domain may be involved in the recruitment of downstream signaling components. A strong indication that the N terminus is involved in downstream signaling is the differential requirement of downstream signaling components by CNLs and TNLs [reviewed in (87)].

With the LRR and the N-terminal domain conferring pathogen recognition and

downstream signaling, the central NB-ARC is thought to act as the molecular switch that controls the activation state of the protein (142). The "on" and "off" state of the switch is defined by the nucleotide that is bound to the NB-ARC domain (ATP versus ADP).

Important progress has been made recently in elucidation of the intramolecular interactions in NB-LRR R proteins. R protein signaling by NB-LRRs appears to depend on dynamic interactions between the three major domains. Positive and negative regulatory regions are scattered over the entire protein as demonstrated by many autoactivating and loss-of-function mutations (7, 12, 30, 142, 147, 151). These mutations suggest that compatibility between the different domains is required to keep NB-LRR proteins in an auto-inhibited but activatable state. Support for such harmonization between domains is provided by domain swap experiments between homologous R proteins in which nonharmonized domains are fused. For instance, LRR exchange between homologues at the maize *Rp1* locus results in a necrotic phenotype (138). Likewise, domain swaps between Mi-1.2 and its paralogue Mi-1.1 resulted in autoactivating incompatibilities between the LRR of Mi-1.2 and either the first 161 amino acids of the amino terminal domain (NT-1), or the next 751 residues encompassing the NB-ARC domain of Mi-1.1 (55). Autoactivating fusions were also obtained by swapping different, nonoverlapping, LRR sequences in Rx for the equivalent region of Gpa2 (116). These experiments suggest that the different domains interact with each other and that mispairing can result in spontaneous activation of the protein.

Studies on the potato protein Rx provided the first example of direct physical and functional intramolecular interactions between the different protein domains found in NB-LRR proteins (98). Coexpression of either the CC and NB-ARC-LRR fragments or the CC-NB-ARC and LRR fragments reconstituted Avr-dependent HR. This transcomplementation involved physical interaction between the domains, which was disrupted by coexpression of the PVX coat protein, the activator of Rx (98, 116). The interaction between the CC domain and the NB-ARC-LRR fragment requires a functional EDxxD motif in the CC domain (G.J. Rairdan & P. Moffett, unpublished data). For tobacco TNL protein N, the LRR domain was found to interact in vitro with the TIR-NB-ARC fragment, and this interaction was lost when the elicitor was added (152). The latter observation, however, could not be confirmed in vivo, as no intramolecular interactions were detectable upon coexpression of N domains. Perhaps the intramolecular interactions in N are weaker than in Rx or are not effective for proteins expressed in trans (92). Immunoprecipitation studies with the R protein Bs2 also showed an intramolecular interaction between the CC-NB-ARC and LRR parts, but instead of disrupting this interaction, the interaction was enhanced when the elicitor (AvrBs2) was coexpressed. For Bs2 no interaction could be observed between the CC fragment and NB-ARC-LRR.

To investigate the contribution of the various subdomains of the NB-ARC to the intramolecular interactions with the N- and C-terminal domains, a series of domain swaps between Rx and Gpa2 were made. These studies showed that the Rx ARC1 subdomain is necessary for the interaction between the CC-NB-ARC and LRR fragments (116). Binding to LRR domains seems to be a general property of the ARC1 subdomain. For instance, the CC-NB-ARC part of Rx was able to interact with the LRR domains of several other NB-LRRs (i.e., Bs2 and *Arabidopsis* HRT LRR domains). However, these interactions did not reconstitute a functional R protein capable of activating defense responses (neither autoactivation nor elicitor-dependent activation) (116). Point mutations in the ARC1 subdomain had only a quantitative effect on the binding affinity for the LRR domain, and reduced LRR binding affinity did not necessarily compromise functionality of the R protein. This observation supports an extensive and partially conserved

interaction surface between the ARC1 and the LRR domain with individual contacts quantitatively contributing to the interaction. Subsequent deletion studies with the LRR of Rx revealed that the entire LRR domain is necessary for the interaction with the CC-NB-ARC part, suggesting that the interaction surface is scattered over the LRR domain (116).

A function for the ARC2 subdomain became apparent when the Gpa2 ARC2 subdomain was swapped with the Rx ARC2 domain, resulting in a constitutively active protein. The LRR is believed to have a positive regulatory role in this activation process, as R proteins are generally inactive in the absence of the cognate LRR when expressed at endogenous levels (116, 117). The ARC2 domain of Gpa2 and the LRR domain of Rx apparently are sufficiently compatible to execute the positive regulatory role of the LRR and to induce HR, but the combination lacks the structural constraints to prevent autoactivation. Rairdan & Moffett therefore concluded that the ARC2 subdomain relays pathogen recognition mediated by the LRR domain into changes in R protein conformation, unleashing its downstream signaling potential (116). A transducing role for the ARC2 subdomain fits with the fact that mutations in the ARC2 subdomain often result in an autoactivating protein (142). These observations suggest fine-tuning and coevolution between the LRR domain and the ARC2 subdomain in NB-LRR proteins. This idea might be tested by a survey of polymorphisms in NB-LRR proteins.

A model in which Avr perception alters the interaction between the LRR and ARC2 subdomains, repositioning critical motifs and thereby allowing the molecule to progress to an active conformation, is in agreement with domain-swap experiments between Mi-1.2 and Mi-1.1 (56). These swaps demonstrated autoactivating incompatibilities between the LRR and a region encompassing the ARC2 (56). Additional intramolecular interactions for Mi-1.2 were suggested based on mutational analysis. A single amino acid replacement in the first half of the Mi-1.2 LRR by the Mi-1.1 residue (R961D) leads to autoactivation, suggesting that this residue is needed for autoinhibition of HR signaling. This autoactivation phenotype was suppressed in trans by overexpression of a subdomain of the Mi-1.1 N terminus (NT1), suggesting that HR signaling mediated by the LRR is normally suppressed by the NT1 (55). The necessity of compatibility between the NT1 domain and residue 961 was also shown by swapping the Mi-1.1 NT1 into a Mi-1.2 background, which results in autoactivation. This effect might be specific for the subclass of CNLs with an extended N terminus, since the NT1 domain is absent in R proteins with a shorter N terminus (**Table 1**).

The ability of NB-LRR proteins to establish intramolecular interactions depends on the conformation of the NB subdomain, that is likely regulated by the nucleotide bound. For instance, mutations in the P-loop of Rx that are predicted to disrupt nucleotide binding, abolished the interaction between the CC fragment and the NB-ARC-LRR fragment, whereas the LRR and CC-NB-ARC interaction was not lost (98). Also in Bs-2 the latter interaction does not require a functional P-loop (79), whereas in N it does in vitro (152). Direct support for a conformational change in the NB-ARC domain upon binding different nucleotides is provided by the tomato CNL protein I-2. The ADP-bound state of this protein displayed increased affinity for the nucleotide compared to the ATP-bound state (145), indicating a different conformation of the protein. In addition, two autoactivation mutants of I-2 were shown to have a normal nucleotide binding affinity but a reduced ATPase activity. These data suggested that the ADP conformation reflects a resting state, whereas the ATP conformation represents the activated state of the I-2 protein (145). For the N protein a model has been proposed in which the ATP-bound state reflects the resting state (152). Upon Avr perception by N, its ATPase activity is stimulated, resulting in hydrolysis of the nucleotide and transition to the

activated, ADP-bound state of the protein. Unfortunately, the I-2 and N data are not directly comparable as both were obtained using truncated proteins, containing either the CC-NB-ARC (I-2) or the NB-ARC-LRR (N). The crucial next step to reconcile the observed differences will be to perform nucleotide binding and hydrolysis experiments using full-length R proteins.

The NB-ARC domains of the mammalian NB-ARC protein APAF-1 and its DARK1 and CED4 analogues in *Drosophila* and *C. elegans* have been shown to homo-oligomerize upon activation and to form wheel-like structures of, respectively, 7, 8, and 4 molecules. These "wheels of death" provide a platform for binding and subsequent activation of downstream procaspases (175, 177, 178).

Recently, oligomerization upon recognition of the Avr was also observed for the tobacco TNL protein N (92). Oligomerization and resistance both require an intact P-loop, hence presumably the ability to bind nucleotides. Mutation of the RNBS-A motif does not affect elicitor-dependent oligomerization but still abolishes resistance (92), indicating that resistance is not an automatic consequence of oligomerization. The N TIR domain can also oligomerize on its own (92). However, oligomerization of the TIR domain was independent of the Avr protein. Mutation of three of the predicted solvent exposed residues in the TIR domain abolished HR signaling coinciding with a weaker homotypic interaction of the TIR domain. However, when these mutations were introduced in the full-length protein, oligomerization still occurred. This suggests that the elicitor-triggered oligomerization of the full-length R protein mainly involves NB-ARC::NB-ARC interactions, similar to the CED-4, APAF-1, and DARK oligomers. The N NB-ARC domain alone was not sufficiently stable upon transient expression to test this hypothesis (92). A major question still remaining is whether oligomerization is a general feature of NB-LRR R proteins or whether it is unique for N or possibly the TNL class.

INTERMOLECULAR INTERACTIONS OF R PROTEINS

In addition to the intramolecular interactions described above, R proteins interact with other proteins to form large, dynamic, multimeric protein complexes. Yeast two-hybrid screens and recently coimmunoprecipitation experiments have identified R protein-interacting proteins.

For solanaceous NB-LRR proteins entire proteins as well as the different domains have been used as baits. For the LRR domain, thought to be the main specificity determinant, this did not reveal putative "guardees" and/or Avr products. Rather, LRR interactors were found to be chaperones and chaperone-associated proteins. For example, the LRRs of N and I-2 physically interact with heat shock protein 90 (Hsp90) (25, 80). Hsp90 is a chaperone mainly involved in folding receptor proteins into a signaling-competent state (115). The N-terminal part of the LRR domain of I-2 interacts with Hsp90, whereas the C-terminal part was found to bind specifically to protein phosphatase 5 (PP5) (25). Binding to PP5 is not exclusive for I-2 since in yeast two-hybrid assays PP5 was also found to interact with the solanaceous R proteins Mi-1.2 and Rx, and *Arabidopsis* Rps5 and Rpm1 (25) as well as with N (S. P. Dinesh-Kumar, unpublished data). PP5 interacts not only with the LRR domain of NB-LRR proteins, but through its TPR domain also with the C terminus of Hsp90. The biological function of the single-copy gene PP5 in disease resistance remains elusive, since neither knock-down in tomato nor knock-out in *Arabidopsis* affects disease resistance (25). In contrast, silencing of the different Hsp90 homologs showed a requirement for Hsp90 in disease resistance and HR activation for a large number of NB-LRR and RLP R proteins (25, 42, 54, 64, 80, 82, 141).

Another TPR-containing Hsp90-interacting cochaperone is Sgt1. The role of Sgt1 and its interaction partner Rar1 in disease

resistance has been strongly established (5, 9, 113). Several studies have demonstrated physical interaction between Hsp90, Sgt1, and Rar1 (54, 80, 141). A role for Sgt1 as a cochaperone in Solanaceae is illustrated by the N and Rx proteins, whose accumulation depends on Sgt1 (8, 92) and the tomato Bs2 protein that requires Sgt1 for intramolecular interactions (79). Also outside the Solanaceae the combined activities of Rar1, Sgt1, and cytosolic Hsp90 are required to modulate R protein accumulation and signaling competence (50). Originally, Sgt1 was identified in yeast where it is involved in the regulation of the cell cycle and kinetochore assembly, and interacts with E3 ubiquitin ligases. Sgt1 is part of SCF E3 ubiquitin ligases via an interaction with SKP1 (71). SCF E3 ligase complexes target proteins for degradation by the 26S proteasome (170). The SCF complex can associate with the COP9 signalosome (85, 131), and interactions of COP9 and SCF subunits with either Sgt1 or Rar1 have been demonstrated in planta (9, 81). Silencing of subunits of the SCF complex or COP9 signalosome impaired resistance mediated by N (81). Hence, as well as being a chaperone, Sgt1 could also be involved in R protein-mediated signaling by targeting negative regulators for degradation via the SCF complex.

Despite extensive research, no proteins have been identified that directly interact with the NB-ARC domain of NB-LRR proteins, which could mean that the NB-ARC is solely involved in intramolecular interactions. For the N-terminal domains of NB-LRR proteins, however, interactors have been identified. This proposed "downstream signaling" domain was found to bind most of the guardees mentioned above, which supports the involvement of this domain in Avr perception. An example of an *Arabidopsis* guardee that interacts with the N-terminal domain of an R protein is the Ser/Thr kinase Pbs1 that interacts with the CC domain of the *Arabidopsis* CNL Rps5 (132). A tertiary complex of Pbs1 and Rps5 with the elicitor AvrPphB has been shown in which cleavage of Pbs1 leads to activation of the R protein Rps5 (2a, 132). Another example is the interaction of guardee Rin4 with the CC domain of the CNL proteins Rpm1 and Rps2. In addition to the interaction with Rin4, the N-terminal domains of Rpm1 and Rpp5 (the CC-NB-ARC part) also interact with an ortholog of TIP49 (51). TIP49a is part of the transcriptional machinery and interacts with the TATA binding protein (TBP) complex. Reduction of mRNA levels of *Arabidopsis* TIP49a revealed that it does not affect Rpm1 function but acts as a negative regulator of Rpp5 (51).

Within the Solanaceae an example of indirect Avr binding is provided by the TNL protein N of tobacco, which binds the Avr protein via an unknown protein (18a). In tomato, the guardee Pto interacts not only with the N terminus of CNL Prf and with AvrPto (100), but also with a set of plant proteins called Pto interacting proteins (Pti's). Pti-1 is a Ser/Thr kinase related to Pto (182). Pti-4, -5, and -6 are ethylene-responsive transcription factors that bind specifically to the GCC box in the promoter regions of a large number of genes encoding "pathogenesis-related" (PR) proteins (47, 181).

Although some NB-LRR proteins have been shown to associate with membrane structures, some interactions (TIP49, Pti-4, -5, -6) described above provide a direct link between R proteins and regulation of gene expression. An indirect link between R proteins and the nucleus is provided by Rx. Two research groups independently identified RanGAP as an interactor with the Rx N terminus (W.I.L. Tameling & D.C. Baulcombe; M. Sacco & P. Moffett, personal communications). In plants, the function of RanGAP is not clear, but in mammalian cells RanGAP associates with RAN, a Ras-related small GTPase that has an important function in nucleo-cytoplasmic trafficking and is part of a nuclear cargo-importing complex at the nuclear envelope (91, 126). In mammalian cells this complex is involved in the translocation of receptor-Hsp90-immunophilin complexes. The TPR-containing immunophilins (such as

PP5) are required to link the heteromeric complex to a motor protein (such as dynein) for directional movement along microtubules toward the nuclear pores (115). Two components of the plant nuclear pore complex, the importin alpha homologue MOS6 (AtImpα3) and a nucleoporin 96 homologue MOS3-1, were recently identified in a suppressor screen to be required for the snc1 phenotype (109, 180). The snc1 gene encodes a mutated NB-LRR protein that triggers constitutive activation of defense signaling (179). A mos3-1 single mutant is not only compromised in the snc1 phenotype but also in both basal resistance and R protein-mediated resistance (180).

Together, the data above imply that some R proteins may require nuclear localization for their function and that these proteins could be directly involved in transcriptional regulation. Recently, nuclear localization has been reported for two TNL proteins (Rrs1 and N from *Arabidopsis* and tobacco, respectively) (18a, 28) and two CNL proteins (Mla1 and Mla10 from barley) (133a). Rrs1 is unique in that it bears a WRKY transcription factor domain at its C terminus (28), providing a direct link between R protein function and transcription regulation. Activation of Rrs1 occurs upon binding to the Avr protein PopP2, and both proteins are nuclearly localized (27). The Mla1, Mla10, and N proteins are present both in the cytoplasm and the nucleus. Nuclear localization is required for N and Mla10 function, since equipping them with nuclear export signals inactivates them. These observations indicate that N and Mla10 have a function in the nucleus, similar to Rrs-1 (18a, 133a). Likewise, Mla10 interacts with WRKY transcription factors upon recognition of the cognate Avr protein (133a).

An analogous type of nuclear localization was found for the prototypic animal counterpart of R proteins, the MHC class II transactivator CIITA (136). Upon viral infection, CIITA is activated and translocated to the nucleus where it activates transcription by binding to DNA binding proteins that bind to MHC-II promoter regions (89, 150). Both translocation and transactivation of CIITA require a functional NB subdomain (49). The ARC2 subdomain of CIITA adopts a winged-helix fold similar to that predicted in NB-LRR proteins (3). Such a structure is characteristic of several DNA-binding transcription factors, suggesting that both R proteins and CIITA might also be directly involved in DNA binding (3). If NB-LRR proteins do indeed act directly as transcriptional regulators, this might explain the small number of putative downstream signaling partners that have been identified so far and the relatively large number of components from the transcriptional machinery among them. Many putative transcription factors that bind R proteins in yeast two-hybrid screens might also have been discarded as autoactivators.

To find interactors for the membrane-spanning RLP class of R proteins, the cytoplasmic tail of Cf-9 has been used as bait in yeast two-hybrid screens. A thioredoxin homologue, CITRX (Cf-9 Interacting ThioRedoxin), was identified in this way as a negative regulator of Cf-9/Avr9-mediated cell death and defense responses (121). Silencing of *CITRX* in tomato and *N. benthamiana* resulted in an accelerated Cf-9/Avr9-triggered HR accompanied by the induction of defense-related genes (121). This interaction seems to be Cf-9 specific as no interaction was found in in vitro pull-downs with the related Cf-2 protein and Cf-2 function was not affected upon *CITRX* silencing (121). Recently, the protein kinase ACIK1 (Avr9/Cf-9 Induced Kinase) was shown to interact with CITRX (102). Silencing experiments suggested that this protein is a positive regulator of Cf-9/Avr9 function and is required for full Cf-9 disease resistance (127).

PARTNERS IN R PROTEIN SIGNALING

As detailed above, the fishing expeditions for R protein interactors have thus far yielded

Virus-induced gene silencing (VIGS): a gene transcript suppression technique to identify plant gene function. A chimeric plant virus, containing a fragment of a plant gene, is used to infect a plant. As part of the antiviral response, mRNAs from the endogenous plant gene are specifically degraded

only small numbers of components that may be involved in R protein signaling. In contrast, forward genetic screens using virus-induced gene silencing (VIGS) have been highly successful and have identified a relatively large number of candidate genes. A VIGS screen of 2400 cDNAs from a normalized cDNA library in *Nicotiana benthamiana* revealed that Pto-dependent HR was compromised in 3% of the cases (26). In a similar VIGS screen using 4992 cDNAs, Pto-dependent HR was compromised in 1.6% of the cases (82). Alternative screens were performed with custom-made libraries made from cDNA-AFLP fragments that were selected based on their differential expression pattern upon Avr perception. To identify genes involved in Cf-4/Avr4-dependent HR, 192 cDNA-AFLP fragments were selected that are differentially expressed upon expression of an *Avr4* transgene. These fragments were subsequently used for VIGS in *N. benthamiana*, and 20 of them were found to correspond to genes required for Cf-4/Avr4–mediated HR (42). A similar approach using Cf-9/Avr9-induced genes resulted in the identification of 4 genes of the tested 43 that affect Cf-9-mediated HR (127).

Although these VIGS screens identified relatively large numbers of genes whose silencing suppresses HR, only for a small number does silencing also abolish disease resistance. For instance, only 6 of 79 candidates identified in one of the Pto screens are required for *Pto*-mediated resistance to *P. syringae* pv *tabaci*, 3 of them are derived from *Hsp90* genes (82). Likewise, of the 20 gene fragments that affect Cf-4-mediated HR, only 6 are essential for resistance to *Cladosporium fulvum* (42).

The set of genes required for disease resistance can be divided roughly into five groups. The first group consists of chaperones such as Hsp90 that have also been identified in other studies as R protein interactors (42, 82). The second group, found in each screen, consists of ribosomal proteins such as L19 (42, 82). The third group consists of proteins that are likely to be involved in specific signaling pathways like MAP kinases (34) and protein kinases like ACIK1 (127). The fourth group contains proteins involved in protein degradation via the 26S proteasome. In addition to Sgt1, other examples are the two U-box proteins ACRE74/*Nt*CMPG1 and ACRE276, which are both ubiquitin E3 ligases (45, 176), and ACRE189, an F-box protein (127). The fifth and final group consists of two NB-LRR proteins. The CNL NRG1 (N-requirement gene 1) appears to be specifically required for *N* function and not for other NB-LRR proteins (114). This indicates that CNL proteins may not only function as resistance proteins but might also be involved in resistance signaling mediated by TNL proteins. Similarly, the CNL NRC-1 (NB-LRR protein Required for Cf-4 function) was found to be required for resistance mediated by the RLPs Cf-4 and Cf-9 (43). NRC-1 has also been shown to be required for HR mediated by the CNL R proteins Prf, Rx, and Mi-1.2 and by the RLK *Le*Eix (43). The presence of more than one R protein-like factor in a single resistance pathway could indicate that resistance pathways are interwoven. Additional support for crosstalk of eLRR, TNL, and CNL pathways comes from genetic studies. For instance, Cf-4 depends on EDS1, a lipase-like protein mainly required for resistance responses mediated by TNL proteins (169), hinting at the presence of a TNL in this pathway as well. The function of the TNL N was also found to be dependent on EDS1, whereas the CNL NRG1, which acts downstream of N, is not. Both are dependent on Sgt1, again showing the general importance of this protein for R signaling. Together, these data show that some resistance proteins require downstream NB-LRRs for cell death signaling (114).

MODEL FOR NB-LRR R PROTEIN FUNCTION

Based on the data above, a "generalized" model for the function of NB-LRR R proteins can be proposed (**Figure 3**). In the resting

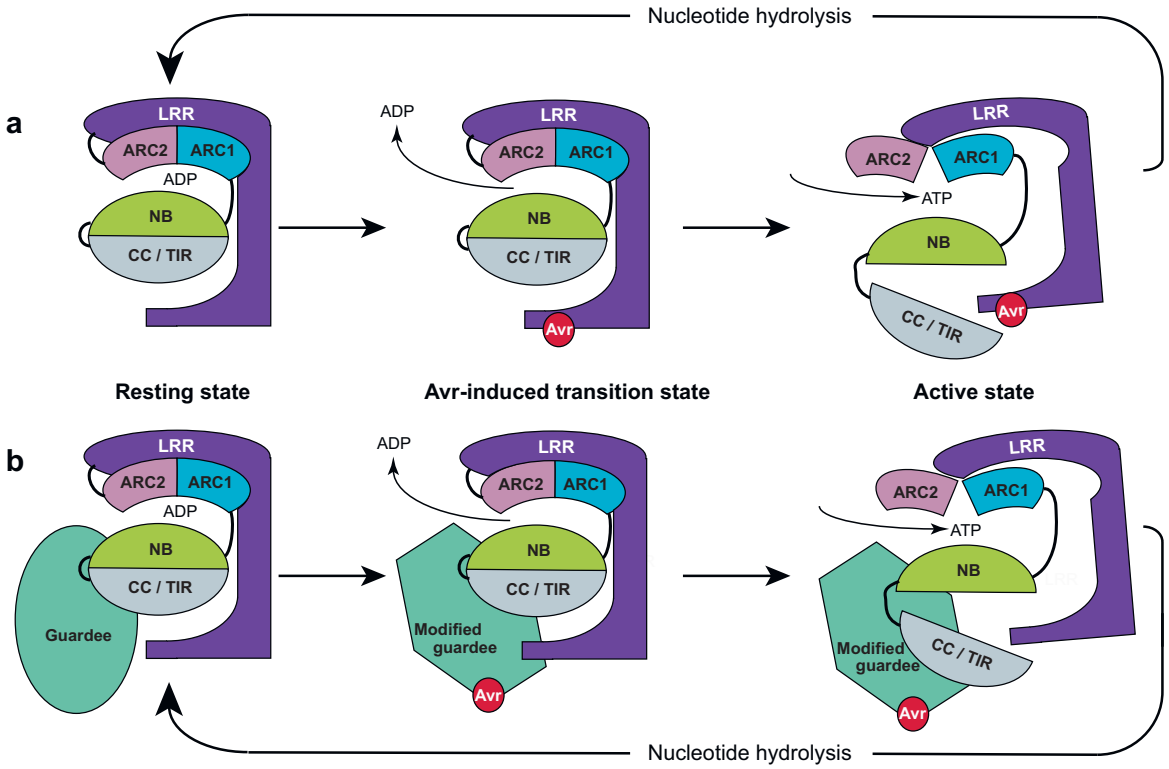

Figure 3

A current model for NB-LRR R protein function and activation. (*a*) Direct Avr-interaction model. In the absence of a pathogen, the NB-LRR protein is in the ADP-bound resting state. Upon binding of the Avr protein, the interaction between the LRR and the ARC1 and 2 is altered (transition state), resulting in nucleotide exchange and a different conformation of the protein. This altered ATP-bound conformation represents the active state of the NB-LRR protein. Hydrolysis of ATP returns the protein into its resting state. (*b*) Guard model. Rather than binding directly to the R protein, the Avr protein modifies a guardee bound to the N terminus of the R protein. This modification is detected by the LRR altering its interaction with the ARC1 and ARC2.

state R proteins are autoinhibited, and the NB-ARC domain interacts with both the LRR and the CC or TIR domains. This complex is molded and/or preserved in a signaling-competent state by its interaction with the chaperone Hsp90 and one or more co-chaperones such as Sgt1, Rar1, and PP5. Perturbation of the LRR domain (either by mutations or by Avr recognition) allows transition to the active state. Avr proteins can be recognized either directly, possibly by direct binding to the LRR, or indirectly by modification of a guardee that is bound to the N terminus (CC/TIR). In the latter case, the modification of the guardee could be detected by the LRR. Such a model for indirect recognition is supported by the current data on the TNL protein N. The Avr protein (p50 helicase) is bound via an unkown protein to the TIR domain (18a). In vitro, p50 can also interact directly with the LRR domain of N (152). One interpretation of these data is that the LRR domain recognizes binding of p50 to the "guardee" bound to the TIR domain. Conceivably, after (in)direct Avr recognition by the LRR, its interaction

with the ARC2 subdomain changes, resulting in a different conformation of the NB-ARC that allows nucleotide exchange (145) or hydrolysis (152). The exact intramolecular conformation at this stage is still unknown (e.g., dissociation of the N terminus) and could very well differ between various R proteins (79, 98, 116, 152). The activated R protein then either directly recruits downstream signaling components or could require oligomerization first, in analogy to the "wheel-of-death." These downstream signaling components could well be transcriptional regulators (133a), as at least some R proteins need to be nuclearly localized for their function, and one of the earliest defense signaling responses is change in gene expression (39).

Hydrolysis of ATP by the NB-ARC could return activated R proteins to the autoinhibited state. Such a mechanism would explain why hydrolysis mutants are autoactivating, and why defense signaling is initiated only when the Avr concentration in the cell is sufficiently high. Future research will put this model to the test and determine whether it applies to all or only a subset of NB-LRR proteins.

SUMMARY POINTS

1. R proteins can be grouped into two intracellular and two extracellular classes. The CNL class is the largest class in the Solanaceae, with 15 of the 27 identified R proteins.
2. Four R proteins have been shown to bind Avr proteins directly, while four others bind either indirectly or recognize the modification the Avr protein exerts on a plant target protein. For most R proteins, it is not known how they mediate Avr recognition.
3. Indirect recognition of Avr proteins is in at least some cases mediated by the "guardee" that binds to the N-terminal domain of the NB-LRR protein. Since the LRR confers recognitional specificity, this observation suggests that modifications of the bound guardee can be monitored by the LRR.
4. Activation of NB-LRR proteins likely requires a series of conformational changes, possibly mediated via nucleotide exchange/hydrolysis by the central nucleotide binding site.
5. Pull-down experiments revealed only a small number of proteins directly interacting with NB-LRR proteins. Besides (potential) guardees, these are mostly chaperones and co-chaperones. For extracellular R proteins (Cf-9) only one direct interactor has been identified so far.
6. Genetics screens (often based on VIGS) have identified a large number of genes required to induce HR. Only a subset (±10–20%) of these are required for disease resistance. The latter group encodes, among others, (co-)chaperones, proteins involved in specific protein degradation and ribosomal subunits.
7. Evidence accumulates that a number of NB-LRR proteins require nuclear localization for their function.

FUTURE DIRECTIONS

1. Determine the 3-D structure of NB-LRR and RLP proteins to improve understanding of the molecular mechanisms underlying their function.

2. Identify the virulence targets for Avr proteins as these are likely the Achilles' heel of plant immune systems. These studies could also determine whether particular R proteins recognize Avr proteins directly or indirectly.
3. Visualize dynamics of the subcellular localization of R proteins upon pathogen attack to understand where R proteins exert their function. Couple these data to the nucleotide binding state of the NB-ARC domain to elucidate the role of ATP binding and hydrolysis for R protein function.
4. Analyze the conformational dynamics of R protein complexes and the proteins interacting with the different conformational states to gain insight into the signal transduction processes.

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