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New insights in plant immunity signaling activation

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Abstract

Plant disease resistance can be triggered by specific recognition of microbial effectors by plant nucleotide binding-leucine rich repeat (NB-LRR) receptors. Over the last few years, many efforts have greatly improved the understanding of effector and NB-LRR function, but have left a lot of questions as to how effector perception activates NB-LRR induction of defense signaling. This review describes exciting new findings showing similarities and differences in function of diverse plant NB-LRR proteins in terms of pathogen recognition and where and how resistance proteins are activated. Localization studies have shown that some NB-LRRs can activate signaling from the cytosol while others act in the nucleus. Also, the structural determination of two NB-LRR signaling domains demonstrated that receptor oligomerization is fundamental for activation of resistance signaling.

INTRODUCTION

Understanding plant immunity mechanisms will provide a crucial input for improving disease control measures to protect agricultural production. Plant immunity relies on two major levels of resistance [1–3]. The first level is triggered by the recognition of conserved microbial molecules called Pathogen Associated Molecular Patterns (PAMPs) by cell surface located receptors PRRs (Pattern Recognition Receptors). This type of resistance is referred to as PTI for PAMP Triggered Immunity. PRRs generally consist of transmembrane proteins with an extracellular Leucine Rich Repeat (LRR) domain. Pathogens adapted to specific host plants avoid and/or suppress PTI through the action of virulence effectors. Host plants have evolved a second level of surveillance known as Effector-Triggered-Immunity (ETI) to counteract adapted pathogens. ETI is mediated by Resistance (R) proteins that directly or indirectly perceive pathogen effectors, then called Avirulence (Avr) proteins. This recognition is often characterized by a local cell death at the pathogen infection site termed the Hypersensitive Response (HR). Most ETI receptors (R proteins) are intracellular and belong to the conserved family of NB-LRR proteins containing a C-terminal LRR and a central nucleotide binding (NB) domain which is often referred to as the NB-ARC region (Nucleotide-Binding adaptor shared by Apaf-1, Resistance proteins and CED-4) [4]. Apaf-1 and CED-4 are part of the animal nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family, whose members also function as regulators of innate immune responses and apoptosis [5]. Evidence suggests that the NB-ARC domain can bind and hydrolyse nucleotides which may act as a molecular switch to regulate R protein activity

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upon pathogen perception [6]. Plant NB-LRRs can be divided in two subclasses depending on their N-terminal extremity [7]. The first class contains a Toll-Interleukin-1 Receptor (TIR) domain also found in the membrane-bound Toll-like receptor (TLR) family of animal immune receptors. The “non-TIR” class contains either a coiled-coil (CC) domain or a variable domain of unknown function [8].

Although our knowledge of plant immunity has improved considerably over the last ten years, the mechanisms by which effector perception is linked to NB-LRR activation remain elusive. What are the signals inducing receptor activation? Where and how do they trigger defense signaling? Here, we review the most recent findings providing new insights on NB-LRR function from pathogen perception to immune signaling activation.

1. Pathogen recognition: what is the signal leading to receptor activation and signaling?

Plant NB-LRR proteins can recognize pathogen effectors either by direct physical interaction [9–13], or indirectly by detecting modifications of host target proteins that are induced by the effector [14–17]. Recent advances described below, have demonstrated in two model-systems how effector enzymatic activity and/or effector-mediated modifications can be involved in both direct and indirect recognition events.

The canonical example of indirect recognition involves the Arabidopsis RIN4 protein which acts as an accessory protein to two NB-LRR immune receptors, RPS2 and RPM1. RPS2 activates resistance in response to loss of RIN4 due to cleavage by the bacterial protease avrRpt2 [14,15]. RPM1 activates resistance in response to either of two unrelated effectors, avrB and avrRpm1. RIN4 becomes phosphorylated in the presence of these effectors, leading to the hypothesis that RPM1 is activated by phosphorylated RIN4, although neither effector has a detectable kinase activity [16]. Two recent studies have now confirmed this hypothesis [18,19] and identified a host protein kinase that phosphorylates RIN4 [19] (Figure 1). Both studies identified the RIN4 Threonine residue in position 166 as a critical phosphorylation site required to activate RPM1. Antibodies raised against a RIN4 peptide containing phosphothreonine 166 could detect *in vivo* phosphorylation of this site in response to AvrB and AvrRpm1 induction. Alanine substitutions at this site prevented RPM1 activation, while aspartate or glutamate substitutions, which act as phosphomimics due to their negative charge, caused effector-independent activation of RPM1. Liu et al [19] identified the host protein kinase RIPK (RPM1-Induced Protein Kinase) in a pull down assay as a RIN4 interactor in the presence of AvrRpm1. The authors showed that RIPK is able to phosphorylate RIN4 *in vitro* on threonine 166, and also to bind AvrB, suggesting that AvrB binding to RIN4 and RIPK induces RIPK-mediated phosphorylation of RIN4, leading to activation of RPM1. However, a RIPK knock-out mutant only partially affected RIN4 phosphorylation and RPM1 mediated resistance, suggesting that other kinases may also contribute to these events.

The Arabidopsis RRS1-R resistance protein confers resistance to the causal agent of bacterial wilt *Ralstonia solanacearum* containing the effector PopP2 [12]. The two R/Avr partners physically interact in yeast and this interaction has been recently confirmed in the nucleus of living plant cells using Fluorescence Lifetime Imaging (FLIM) [20]. Mass spectrometric analysis revealed that PopP2 displays an autoacetyl-transferase activity *in vitro* that acetylates a conserved Lysine residue at position 383. This enzymatic activity depends on the PopP2 predicted catalytic core residues. Mutation of the K383 residue compromised RRS1-R mediated immunity without disrupting PopP2/RRS1 association in the nucleus, suggesting that RRS1 activation requires PopP2 enzymatic activity in addition to its physical contact. However no PopP2-mediated acetylation of its known interacting partners RRS1 and the Cysteine protease RD19 [21] could be detected *in planta*, so it is not yet clear how PopP2 acetylation activity contributes to its recognition and what are its

substrate(s). A second resistance protein, RPS4, acts in concert with RRS1 to confer resistance to different plant pathogens [22,23], and may also be involved in recognition of enzymatically active PopP2.

2. Immune receptor localization: where does the action take place?

In the past five years, a number of studies have demonstrated the importance of nucleocytoplasmic trafficking of immune receptors and immune components for disease resistance activation [24–26]. Indeed, effector-triggered nuclear accumulation of nucleocytoplasmic NB-LRRs such as Barley MLA10, Tobacco N, and Arabidopsis RPS4 and SNC1 is required for efficient induction of defense responses [27–30] For instance, in the presence of the corresponding barley mildew effector AvrA10, MLA10 was found to accumulate in the nucleus and to associate with two WRKY family proteins that act as transcriptional repressors of PTI [28]. These observations led to a model where pathogen perception induces immune receptor accumulation in the nucleus where they activate immune signaling responses through transcriptional reprogramming [31].

However, two recent studies demonstrated that the nucleocytoplasmic Potato NB-LRR Rx protein activates immune responses from the cytosol even though it requires both cytosolic and nuclear pools for correct regulation of its activity [32**,33**] (Figure 1). Rx interacts with RanGAP2, a small cytosolic GTPase Ran required for Rx function [34,35], and this interaction appears to control Rx nucleocytoplasmic equilibrium. Overexpression of RanGAP2 sequesters Rx in the cytosol, while expression of a modified version of RanGAP2 fused to a nuclear localization signal (NLS) leads to Rx accumulation in the nucleus [33**]. Both versions lead to increased accumulation of Rx protein, but nuclear retention inhibits Rx function, while cytosolic accumulation leads to enhanced Rx function including a weak autoactivity. Similar results were observed for autoactive mutants of Rx, indicating that defense signaling is mediated by the cytoplasmic pool of Rx. Similarly, fusion of an NLS directly to Rx significantly compromised its activity, while a nuclear exclusion signal had a minimal effect [32**]. Furthermore, Rx is not activated when the Potato Virus X coat protein (CP), its corresponding elicitor, is forced to accumulate in the nucleus, suggesting that both pathogen recognition and resistance signaling have to take place in the cytoplasm [32**]. Thus, nuclear accumulation of Rx may be a negative regulatory mechanism to limit its activation in the absence of the CP.

Not all NB-LRRs show nuclear localization, such as the plasma membrane-associated RPM1. Interestingly, RPM1 remains membrane-associated in the presence of the autoactivating RIN4 T166E mutant [18], as does the autoactive RPM1 D505V mutant [36*]. Gao et al [36*] further showed that nuclear exclusion, or direct membrane tethering did not compromise RPM1 activity. These results strongly suggest that RPM1 activation and signaling occurs at the plasma membrane and initiates a cytosolic signaling pathway (Figure 1). Some NB-LRRs contain membrane anchors or palmitoylation or myristoylation sites, which direct the protein to specific intracellular membrane locations. For instance the flax rust resistance proteins L6, M and P2 are respectively targeted to Golgi membranes, tonoplast and the cytosol (Takemoto et al., unpublished). N-terminal domain swap experiments between L6, M and P2, showed that membrane attachment is important for L6 resistance protein function as well as for effector-independent signaling by the autoactive L6 TIR domain (M. Bernoux, unpublished). This suggests that L6 early signaling occurs at the Golgi membrane (Figure 1).. To date no common early R protein signaling partners have been identified and it is possible that different resistance signaling pathways may be activated from either cytosolic or nuclear locations.

One key component of the immune signaling is EDS1, which is required downstream of TIR-NB-LRR resistance proteins, including L6 [37,38]. Garcia et al [39**] showed that both

cytosolic and nuclear pools of EDS1 are required for its function in coordinating immune responses. Upon pathogen perception, defense associated transcriptional reprogramming is dependent on EDS1 nuclear accumulation. However, fusion of a nuclear export signal or a cytoplasmic retention glucocorticoid receptor concentrated EDS1 in the cytosol but only partially compromised pathogen resistance compared to an *eds1* mutant. This indicated that a cytoplasmic pool of EDS1 is maintained and required to allow complete resistance activation after pathogen perception. The role of the EDS1 cytoplasmic pool is not yet clear, but this observation also suggests that resistance signaling pathways may operate in both cellular compartments.

3. Receptor oligomerization: a common feature for signaling activation?

One of the key unanswered questions in plant immunity is how NB-LRR receptors are activated to initiate the resistance signal? Current models predict that effector recognition induces NB-LRR protein changes in intramolecular interactions and conformation associated with nucleotide exchange by the NB domain [40] thus exposing an N-terminal signaling domain to activate downstream defense response [7]. While there has been limited biochemical data available to confirm these general propositions, Williams et al [41*] recently showed that wildtype M resistance protein from flax binds preferentially to ADP, while an autoactive mutant preferentially binds to ATP. This is consistent with the proposition that the ADP-bound protein represents the signaling off state and the ATP-bound protein the on state. Activation and signaling of animal NLRs and TLRs have been characterized in detail and serve as a useful comparative model. Once activated, animal NLRs oligomerize through their central nucleotide binding domain generating a wheel-shaped oligomeric platform [42]. This scaffold allows proximity-induced association of their N-terminal effector domains which then recruit signaling partners to activate immune or apoptotic responses [43]. Similarly, structural and biochemical data from animal TLRs indicate that PAMP perception by the extracellular LRR region leads to the homodimerization of the cytosolic TIR domain [44]. This dimerisation provides a new scaffold that binds to adaptor proteins to initiate downstream immune signaling [45]. A few studies indicate that plant NB-LRR proteins can also oligomerise. The Arabidopsis RPS5 and Tomato Prf CC-NB-LRRs exist as oligomeric complexes prior to pathogen perception [46,47], while the Tobacco N TIR-NB-LRR protein forms TIR-dependent oligomers upon perception of the TMV p50 protein [48]. However, how these associations are linked to signaling activity is not well understood.

Protein structural studies of plant NB-LRRs are now coming to the front stage with the recent determination of the first crystal structures of two NB-LRR signaling domains, the CC domain of Barley MLA10 [49**] and the TIR domain of Flax L6 [50**]. These N-terminal domains are each sufficient to autonomously trigger defense signaling *in planta* and form homodimers in solution. Each protein fragment crystallized as a dimer and site directed mutations in the dimer interface disrupted both dimerization *in vitro* and signaling activity *in planta*, which strongly supports that oligomerization of the signaling domain is required to activate defense responses [49**,50**]. Similarly to Prf and RPS5, MLA1 (a closely related allelic variant of MLA10) self-associates *in planta* in the absence of the pathogen effector, and occurs in a 300–400kDa complex in mildew infected as well as in uninfected plants [49**]. According to size exclusion chromatography and BN-PAGE, the Prf complex fits with a Prf dimer associated with two molecules of the accessory Pto kinase protein [46,51], but it is not clear whether other proteins are present in the MLA1 complex. Interestingly purified full length MLA27 protein behaved as a monomer in vitro. Gel filtration chromatography coupled to multiangle laser light scattering (MALLS) together with Analytical Ultra Centrifugation demonstrated that the autoactive L6 TIR domain involves two molecules *in vitro* [50**]. However, non autoactive L6 protein fragments including the

NB-ARC domain or the full length protein do not self-associate in yeast. This suggests that, like the Tobacco N protein, L6 needs to be activated to oligomerize, although there is no evidence for changes in L6 oligomerisation state *in planta*. Further mutational analyses of the L6 TIR domain identified a signaling region independent of the dimerization interface. Mutations in this region disrupt L6 TIR signaling activity *in planta* but not dimerization in yeast, suggesting that it may be involved in the recruitment of signaling partners subsequent to dimerization (figure 1).

Thus, oligomerization seems to be required for signaling activity for at least five different plant immune receptors. In the case of the three CC-NB-LRRs, an inactive dimer is present prior to activation, while dimerisation of the two TIR-NB-LRRs appears to be associated with activation (figure 1). This may reflect a basic mechanistic difference between these two classes of protein, but further analysis of additional examples of each will be required to test this. It is not yet clear whether higher order oligomeric complexes may also form after R protein activation, as is the case for the animal NLRs, although the MLA1 gel filtration profile remains unchanged after pathogen challenge suggesting no change in oligomerisation state [49**].

The steps following NB-LRR activation leading to signaling also remain obscure. So far very few studies have described signaling components interacting with plant immune receptors. Mutations in the MLA10 CC dimer interface not only affect signaling but also its interaction with the WRKY1 defense repressor [28,49**]. Although this interaction requires only the first 45 residues of MLA CC domain, which does not dimerize by itself, the whole MLA10 CC dimer unit may provide the right scaffold allowing WRKY factor recruitment to derepress defense genes transcription. The Arabidopsis Topless-related 1 (TPR1) protein has been identified in a genetic screen to suppress the *snc1* mutation, an autoactive TIR-NB-LRR protein. Overexpression of TPR1 activates immune responses and this protein associates *in vitro* with SNC1 TIR domain [52]. However, knocking out TPR1 only partially affects SNC1 mediated resistance response, indicating that TPR1 is not the only component controlling defense signaling activation. However there is no evidence that WRKY1 and TPR1 are more generally involved in other R protein signaling pathways. Interestingly, although the CC or TIR domains from a number of different NB-LRRs show an autoactive phenotype when overexpressed, others do not [53,54]. Since these assays are mostly performed in *Nicotiana benthamiana* or *N. tabacum*, this may suggest the involvement of species-specific direct signaling partners. Purification of protein complexes containing activated receptors should provide further information on oligomerisation and early signaling partners initiating cascades downstream of R proteins.

Conclusion

The common structural and functional domain patterns of plant NB-LRRs suggest that these proteins rely on similar mechanisms for activation and signaling. However, although NB-LRRs clearly adopt some common strategies like recognition of effector-mediated enzymatic activity or receptor oligomerization for signaling activation, there are also significant functional differences related to their subcellular localization, mode of activation and interaction with specific signaling partners. The studies described here stand at the dawn of understanding resistance protein function and require further analyses to deepen these hypotheses and identify common and specific routes used by these receptors. For instance, studying and comparing the subcellular localization patterns of diverse NB-LRRs using real time microscopy techniques prior to and upon effector elicitation or pathogen infection will be crucial to understand the early stages of pathogen recognition and defense activation. Further detailed structural and biochemical approaches, such as solving the 3D structure of other plant NB-LRR domains, will be required to define structurally the intramolecular

interactions and conformational changes that are involved in R protein activation. Identifying interacting partners of activated receptors will also be necessary to understand how activated resistance proteins induce defense signaling and whether common signaling routes are shared between NB-LRRs.

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HIGHLIGHTS

- Effector-mediated enzymatic activity can be required in both direct and indirect R/Avr recognition systems.
- Some NB-LRRs can activate defense signaling from the cytosolic compartment.
- Crystal structures of two functional NB-LRRs signaling domains have been determined for the first time.
- Receptor oligomerisation is a prerequisite for signaling.

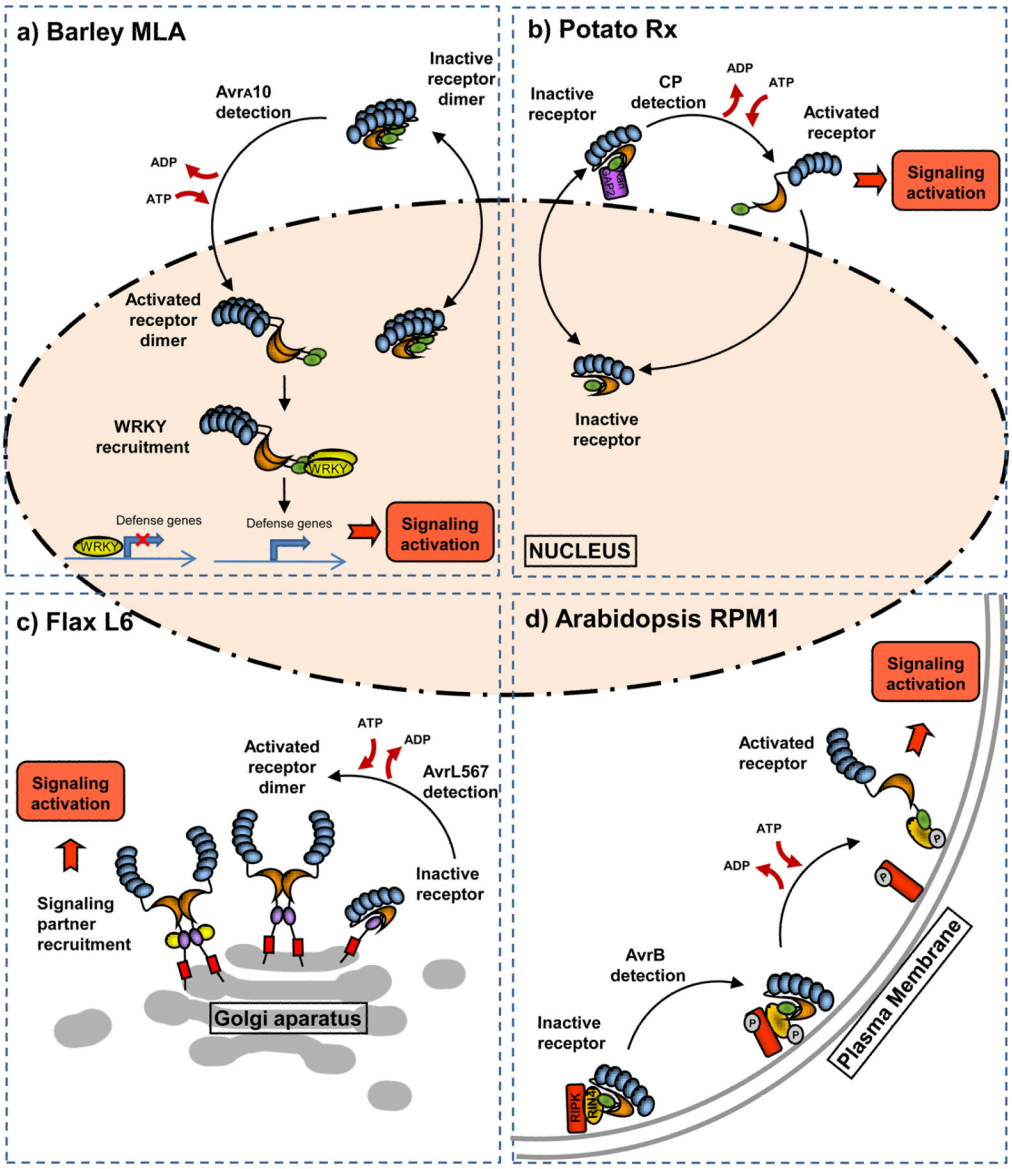

Figure 1. Different models for NB-LRR signaling activation

Models for activation and signalling are presented for several R proteins to illustrate the potential variability in the mechanism and subcellular location of these events. The R protein N-terminal TIR and CC domains are represented by purple and green ovals respectively, the NB-ARC domain by an orange crescent and the Leucine Rich Repeats by a series of blue ovals.

a) In a resting state, Barley MLA navigates between the cytosol and the nucleus as an inactive homodimer interacting through the CC domain. The presence of its corresponding Barley mildew effector AvrA10 induces the accumulation of MLA in the nucleus, nucleotide exchange and conformational changes allowing the interaction of the CC domain with WRKY factors to derepress defense activation. b) The potato CC-NB-LRR Rx is also present in both the nucleus and cytosol. Its nucleocytoplasmic partitioning depends on the trafficking regulator RanGAP2 (purple), which acts as a cytoplasmic retention factor of Rx. The Potato Virus X Coat Protein (CP) is recognized in the cytosol and signaling is activated in this location. The Rx nuclear pool is required for correct regulation of resistance function.

This protein is shown as a monomer as no direct evidence of its oligomerisation state is available. c) In the absence of pathogen, L6 is attached to the Golgi membrane through its N-terminal signal anchor (red rectangle). The protein is kept in an inactive state where the TIR domain dimerization interface is not exposed. Upon recognition of the flax rust effector AvrL567, nucleotide exchange and conformational change exposes the TIR domain for homodimerization and interaction with signaling proteins (yellow circles) to activate defense signalling. d) The Arabidopsis RPM1 protein is kept at the plasma membrane in a complex with the effector target RIN4 (yellow) and the protein kinase RIPK (red). The presence of the Pseudomonas effector AvrB induces RIPK and RIN4 phosphorylation. RIN4 modification leads to RPM1 activation and signaling at the plasma membrane. Again, in the absence of direct evidence otherwise, this protein is shown as a monomer..