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Plant NB-LRR signaling: upstreams and downstreams

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Plant disease resistance proteins commonly belong to the nucleotide binding-leucine rich repeat (NB-LRR) protein family. These specialized immune proteins mediate recognition of diverse pathogen-derived effector proteins and initiate potent defense responses. NB-LRRs exhibit a multidomain architecture and each domain appears to have discrete functions depending on the stage of NB-LRR signaling. Novel proteins that were found to interact with the core HSP90 chaperone complex regulate accumulation and activation of NB-LRR immune receptors. Recent studies have also advanced our understanding of how accessory proteins contribute to NB-LRR activation. The dynamic nature of NB-LRR localization to different subcellular compartments before and after activation suggests that NB-LRRs may activate immune responses in multiple parts of the cell. In this review we highlight recent advances in understanding NB-LRR function.

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Introduction

Plants use a sophisticated innate immune surveillance system to recognize pathogens (reviewed in [1]). One part of the system uses integral plasma membrane proteins with extracellular receptor domains to perceive conserved pathogen associated molecular patterns (PAMPs) that are presented by pathogens during infection. Another branch of the immune monitoring system uses primarily intracellular Resistance (R) proteins to recognize the presence of specific pathogen effector proteins in host cells. In susceptible plant genotypes, pathogen effectors promote virulence by targeting plant proteins or processes to manipulate host cell physiology to be more amenable to pathogen growth and replication [2]. Recognition of effectors by R proteins in resistant plant genotypes results in activation of effector-triggered immunity (ETI) that is often associated with programmed cell death at sites of infection, termed the hypersensitive response (HR) [3].

Most *R* genes cloned to date encode nucleotide-binding leucine-rich repeat (NB-LRR) proteins that mediate recognition of diverse effectors from all classes of plant pathogens. It is accepted that two general modes of effector recognition exist: (1) direct physical interaction between the immune receptor and effector and (2) indirect interaction mediated by additional plant proteins that the immune receptor associates with and monitors for effector-induced modifications. These indirect recognition 'accessory' proteins may be either genuine virulence targets of the effector (the guard model) or decoy proteins that the plant has evolved to mimic *bona fide* effector targets (the decoy model) [1,4]. A third model for effector recognition is a hybrid of direct and indirect modes [5]. In this model, the accessory protein serves as 'bait' that the effector associates with before direct interaction with the NB-LRR and this two-step process activates immune signaling [5]. Although R proteins were originally thought to recognize effectors in a 'gene-for-gene' manner, emerging data suggest that some NB-LRRs can function in pairs, at least genetically and perhaps biochemically, to establish a full immune response (reviewed in [6]). Activation of ETI signaling results in massive reprogramming of the cell to defense responses, and therefore must be tightly regulated. Many factors control NB-LRR signaling upstream and downstream of activation. In this review we highlight the latest advances in understanding NB-LRR biology.

Main text of review

Multi-talented domain architecture

NB-LRRs belong to a subfamily of proteins within the STAND (signal transduction ATPase with numerous domains) superfamily that also contains regulators of immunity, inflammation, and apoptosis in animals [7,8]. Plant NB-LRRs contain a variable N terminus, a central nucleotide binding pocket (NB-ARC domain), and a C-terminal LRR domain. The N-terminal domain usually exhibits either a toll/interleukin-1 receptor (TIR) domain or a coiled coil domain (CC), which influences the requirement for distinct downstream signaling components [9]. TIR-NB-LRRs require Enhanced Disease Susceptibility 1 (EDS1), while most CC-NB-LRRs require Non-race specific Disease Resistance 1 (NDR1) for activation of immune responses. Furthermore, the CC domain of many NB-LRRs is required for interaction with accessory proteins [7], while the TIR domain has been implicated in accessory protein binding, effector recognition specificity, and initiation of the HR [7,10] see note added in the

proof. The NB-ARC domain mediates nucleotide binding and exchange/hydrolysis and might also serve additional downstream signaling functions [5,7]. For instance, the NB domain of the CC-NB-LRR Rx has recently been shown to be sufficient for HR induction [11]. The C-terminal LRR domain is predicted to form a curved intra-molecular and/or inter-molecular protein interaction surface and evidence exists for positive (i.e. recognition specificity) and negative (i.e. autoinhibition) roles in signaling [5,7]. This domain probably provides a platform that keeps NB-LRRs in a primed, signaling competent state in the absence of effector stimulus, yet is labile enough to allow conformational changes upon effector-induced perturbations [5,7].

A universal paradigm for the respective roles of individual protein domains during activation of NB-LRR signaling has not been established. It is likely that single domains have multiple, discrete functions depending on stage of signaling (i.e. pre-activation or post-activation). Furthermore, functions of similar domains may differ depending on the context of the NB-LRR to which they belong, its interacting protein partners, and mode of effector recognition. In-depth reviews of NB-LRR domain function, molecular dynamics, and models of activation have been published recently [5,7,12,13].

Upstreams: accumulation and activation–new regulators revealed

Plant NB-LRRs require a conserved chaperone complex for proper folding, accumulation and regulation. Major components of this complex include Heat shock protein 90 kDa (HSP90) and its co-chaperones Required for MLA12 Resistance 1 (RAR1) and Suppressor of the G2 allele of SKP1 (SGT1) (reviewed in [14]). Each component interacts pairwise with the other two, forming a stable complex [14,15•]. HSP90 functions as a homodimer and it has been proposed that RAR1 bridges the N-termini of each HSP90 monomer to regulate 'open' and 'closed' conformational states that regulate NB-LRR stabilization [15•,16•]. HSP90 and RAR1 generally function as positive regulators of NB-LRR accumulation [14]. By contrast, SGT1 proteins can influence NB-LRR protein levels both positively and negatively [14,17,18]. The seemingly dual functions of SGT1 and the finding that it can associate with components of the SCF (Skp1-Cullin-F-box) ubiquitin ligase complex has led to the hypothesis that SGT1 can also regulate NB-LRR protein turnover via the proteasome [18,19].

SRFR1 – another player in NB-LRR accumulation

Recent studies have identified SRFR1 (Suppressor of *rps4-RLD* 1) as an additional negative regulator of NB-LRR accumulation [20,21••,22••]. *SRFR1* mutants in the Col-0 background exhibit constitutive defense responses mediated by overaccumulation and ectopic activation of the SNC1 (Suppressor of *npr1*, Constitutive 1) NB-LRR [21••,22••]. However, in *srfr1/snc1* double knockout mutants, defense-associated genes are upregulated and other NB-LRRs are detected at increased levels, suggesting that SRFR1 can also regulate defenses independent of SNC1 [21••,22••]. SRFR1 is a highly conserved eukaryotic protein containing N-terminal tetratricopeptide repeats (TPR) and a conserved C-terminal domain of unknown function [20]. Interestingly, the SRFR1 TPR domain interacts with the TPR domains of SGT1a and SGT1b [22••]. Similar to *srfr1* mutants, increased amounts of several NB-LRR proteins were observed in *sgt1b* mutants [18,22••], suggesting that both proteins may share related function(s). SRFR1 can associate in complex with the SNC1 and/or RPS4 NB-LRRs [21••]. Presumably, SGT1 also resides in this complex.

At what level(s) of NB-LRR accumulation does SRFR1 operate? Owing to their mutual interaction and related phenotypes, it is possible that SRFR1 functions with SGT1 in proteasome-mediated NB-LRR turnover [22••]. Thus, it will be important to establish whether SRFR1 interaction with SGT1 is necessary for its regulation of NB-LRR accumulation. Notably, both SGT1 and SRFR1 localize to the cytoplasm and nucleus [20,21••,23]. SRFR1 exhibits similarity with eukaryotic transcriptional repressors and has been hypothesized to function as a negative regulator of defense gene expression [20,21••]. Enhanced defense-associated gene expression in the *srfr1/snc1*double knockout might also be explained by weak activation of additional overaccumulating NB-LRRs. It will be interesting to determine if SRFR1 protein levels change upon NB-LRR activation. SRFR1 degradation might cause deregulation of additional NB-LRRs (e.g. SNC1) that could result in an amplification of immune responses.

CRT1 – an early component of NB-LRR protein activation

CRT1 (Compromised Recognition of TCV), a member of the GHKL (Gyrase, Hsp90, histidine kinase, MutL) ATPase/kinase superfamily, was originally identified in a genetic screen as being required for HRT-mediated defense against turnip crinkle virus [24]. However, CRT1 function is not limited to the HRT NB-LRR. Genetic analyses demonstrate that *CRT1* and its close homologs are required for immune responses mediated by both TIR-NB-LRRs and CC-NB-LRRs. Furthermore, CRT1 can bind the NB domains of multiple NB-LRRs, indicating that CRT1 may generally facilitate R protein function [24,25•]. CRT1 also interacts with HSP90, but not RAR1 or SGT1. Unlike HSP90 and its co-chaperones (and SRFR1 above), CRT1 does not affect NB-LRR steady-state levels [25•]. Interestingly, CRT1 cannot be co-immunoprecipitated with activated forms of several NB-LRRs. This finding suggests that CRT1 probably functions early in NB-LRR folding and/or activation and then dissociates from actively signaling NB-LRRs [25•]. As CRT1 localizes to endosomal compartments in the cytoplasm [25•], it

remains to be determined how it might regulate diverse NB-LRRs with different localization patterns.

Midstream: the recognition event

Effector recognition is hypothesized to induce conformational changes in NB-LRRs, releasing inhibition, and freeing the NB-LRR to activate downstream signaling [5,7,12,13]. At least two general strategies for effector recognition by NB-LRRs exist. Direct physical interactions between the fungal effectors AvrPita, AvrL567, and AvrM with the corresponding NB-LRRs Pi-ta, L, and M, respectively, are likely to occur, and in these cases activation of ETI correlates absolutely with effector/NB-LRR interactions in yeast two-hybrid assays [26–28]. Likewise, co-immunoprecipitation experiments *in planta* between alleles of Arabidopsis RPP1 and its cognate *Hyaloperonospora arabidopsidis* effector ATR1 demonstrate that (1) association of the RPP1 LRR with ATR1 is necessary for activation of ETI and (2) mutations in the TIR or NB domains abolish ETI but not ATR1 binding [29•]. These observations are consistent with the idea that during direct recognition events, the LRR confers recognition specificity, while the TIR and NB domains function in activation and downstream signaling [5,7,29•]. In contrast to the above examples of direct binding to eukaryotic effectors, recognition of most bacterial effectors is mediated indirectly through an accessory host protein that interacts with both the effector and NB-LRR. It remains to be established how NB-LRRs function in combination with their accessory proteins to establish a functional immune recognition complex and whether this complex changes dynamically during recognition events.

Indirect recognition – RIPK modifies RIN4 to trigger RPM1 activation

Immune responses regulated by the conserved plant protein RIN4 have been instrumental in understanding how NB-LRRs indirectly perceive effectors. *Arabidopsis* RIN4 is targeted by multiple bacterial effectors (AvrRpt2, AvrRpm1, AvrB, and HopF2) and is monitored for effector-induced modification by at least two NB-LRRs (RPS2 and RPM1) [30–33]. AvrRpt2 is a protease that directly cleaves RIN4, a modification that activates RPS2-ETI [31,32,34]. HopF2 is an ADP-ribosyltransferase that can modify RIN4 and RIN4 is required for HopF2 virulence activity [33,35]. Although AvrB and AvrRpm1 exhibit no sequence homology to known kinases, both effectors associate with and induce phosphorylation of RIN4 [30]. It is hypothesized that RPM1 recognizes RIN4 phosphorylation and initiates ETI. However, the kinase(s) involved remained elusive and direct evidence for this model has been lacking.

Recent studies confirm that effector-induced phosphorylation of specific residues of RIN4 can activate the RPM1 NB-LRR [36••,37••]. Phosphomimetic mutations demonstrate that phosphorylation of a single amino acid residue, RIN4 T166, is sufficient to activate RPM1-mediated HR when expressed in *N. benthamiana* and *Arabidopsis* [36••]. Phosphorylation of T166 increases when AvrB and AvrRpm1 are delivered into plant cells by bacteria [36••,37••]. Furthermore, *Arabidopsis* RIPK (RPM1-induced protein kinase) interacts with and phosphorylates RIN4 at multiple residues (T21, S160, and T166) [37••]. RIPK also interacts with AvrB and *ripk* knockout plants exhibit reduced AvrB-induced RIN4 phosphorylation and defects in RPM1-mediated immune responses, illustrating the importance of RIPK in RPM1-mediated immune responses [37••]. RIPK is a member of the large receptor-like cytoplasmic kinase (RLCK) family and RPM1-mediated ETI is not completely abolished in the *ripk* mutant. It is likely that other related kinases can also interact with AvrB to induce RIN4 phosphorylation and contribute to RPM1-ETI. Taken together, these data support a model in which AvrB induces RIN4 phosphorylation via host kinases and modification of a specific RIN4 residue activates RPM1 (Figure 1). Despite the attempts of multiple laboratories, no kinase activity has been detected for AvrB. However, it is also possible that AvrB itself mimics host kinase activity to phosphorylate RIN4, or AvrB may work with host kinases to induce target protein phosphorylation.

Downstreams: events post NB-LRR activation

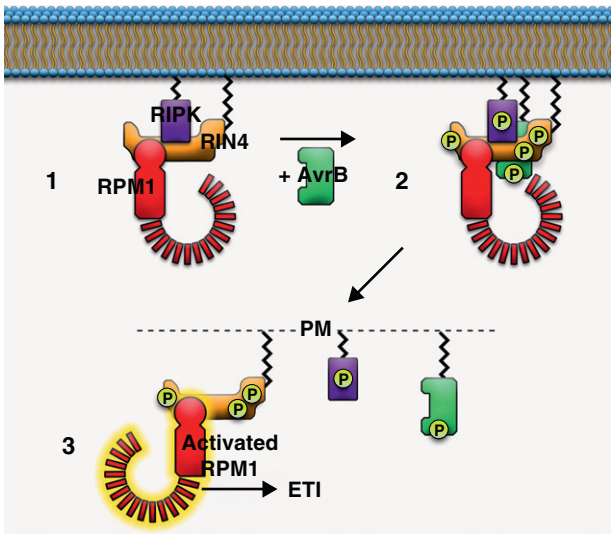
NB-LRRs in the nucleus

A clear model of the signaling events that link NB-LRR activation to downstream immune responses remains elusive. Several recent studies suggest that nuclear activity of some NB-LRRs is necessary to trigger defense responses (Table 1). The CC-NB-LRR MLA10 (barley) and TIR-NB-LRRs RRS1-R (*Arabidopsis*), N (tobacco), RPS4 (*Arabidopsis*), and SNC1 (*Arabidopsis*) require nuclear localization and accumulation for full activation of immunity [38–42] (Table 1). Genetic screens looking for modifiers of the *snc1-1* autoactive NB-LRR allele (*mos* mutants) have revealed several nuclear processes that are important for NB-LRR-mediated immune responses including nuclear protein export and import, mRNA export, and regulation of transcription [38,43,44]. Moreover, the presence of a WRKY DNA-binding domain in RRS1-R and the association of MLA10 and N with plant WRKY and SPL transcription factors, respectively, have led to the hypothesis that NB-LRRs have a direct role in regulating immune related transcriptional changes [12]. This idea is supported by the demonstration that upon recognition of the effector AvrA10, MLA10 associates with WRKY transcription factors to relieve repression of defense genes [39]. Thus, a major recent focus of R protein study has been to understand the specific functions of other nuclear-localized NB-LRRs.

SNC1 interacts with transcriptional regulators to initiate defenses

Recently, SNC1 was found to associate with the transcriptional corepressor TPR1 (Topless Related 1) to

Figure 1



Model for RPM1 activation. **(1)** At steady state, RIPK, RIN4, and RPM1 form a complex. Additional proteins (not shown) may associate with this complex. **(2)** When delivered into the cell, AvrB associates with both RIN4 and RIPK, inducing RIPK-mediated phosphorylation of both AvrB and RIN4. RIN4 is phosphorylated at amino acid residues T21, S160, and T166. **(3)** RIN4 phosphorylation alters interaction dynamics of complex members. AvrB and RIPK dissociate from phosphorylated RIN4. RPM1 perceives RIN4 phosphorylation at amino acid residue T166 and initiates signal cascades culminating in effector triggered immunity.

control gene expression during immune responses. Null mutations in *TPR1* partially suppress the *snc1-1* autoactive immune phenotype [45•]. The TIR domain of SNC1 interacts directly with TPR1 to repress expression of Defense no Death 1 (DND1) and Defense no Death 2 (DND2), two cyclic nucleotide-gated ion channels that are known negative regulators of plant immunity [45•–47]. Genetic analysis demonstrated that *TPR1* and its close homologs are required for defenses mediated by other TIR-NB-LRRs, but not immunity controlled by the CC-NB-LRR RPS2 [45•]. These findings suggest that some TIR-NB-LRRs interact with transcriptional co-repressors to inhibit the expression of negative immune regulators to trigger defense responses. As expression of only the TIR domain from several NB-LRRs can elicit the HR [10], it will be important to determine if interaction with TPR-like proteins is required for this response.

Importance of nucleocytoplasmic distribution of immune regulators: the case of Rx

Emerging evidence is revealing that NB-LRRs can activate defense mechanisms in multiple subcellular compartments. The CC-NB-LRR Rx, which recognizes the coat protein of Potato *virus X* (PVX), localizes to the cytoplasm and nucleus and both pools contribute to full immunity [48••,49••] (Table 1). Experiments using either a nuclear export signal or a nuclear localization signal fused to Rx, the PVX coat protein, or RanGAP2 (a Rx cytoplasmic retention factor necessary for Rx function) demonstrate that the PVX coat protein activates Rx in the cytoplasm, and forced nuclear hyperaccumulation of Rx suppresses immune responses [48••,49••]. However, nuclear export signal-mediated expulsion of Rx from the nucleus moderately reduced resistance, indicating that the nuclear pool of Rx also functions in immunity [48••]. This is the first demonstration of both nuclear and cytoplasmic pools of a NB-LRR being required for resistance.

Intriguingly, stabilization of Rx in the cytoplasm by overexpressing RanGAP2 actually increased resistance [49••], suggesting that cytoplasmic Rx is predominately responsible for limiting PVX replication. The cytoplasmic pool of Rx probably activates several immune signaling cascades. These could stimulate antiviral mechanisms in the cytoplasm such as translational inhibition of viral RNAs [50]. The nuclear pool of Rx could serve additional functions such as activation of defense gene expression. It is certainly conceivable that cytoplasmic Rx activates signaling cascades that are transmitted to the nucleus, and nuclear Rx contributes redundantly to these immune signals. The likelihood of Rx activating cytoplasm-localized antiviral defenses also raises questions regarding commonalities in resistance mechanisms downstream of NB-LRR activation. Thus, it appears possible that NB-LRRs conferring resistance to different pathogen types

Table 1

Importance of nucleocytoplasmic partitioning in NB-LRR function

NB-LRR (Localization)	Effector (Localization)	Subcellular compartment required for defense	Interactors (Localization)	References
RPS4 (C, N)	AvrRps4 (C)	N	Unknown	[40]
N (C, N)	p50 helicase (C, N)	N (HR)	NRIP1 (CP, C, N)	[42,51]
SNC1 (C, N)	N/A	N	TPR1 (N)	[38,45•]
RRS1-R (N)	PopP2 (N)	N/A	PopP2 (N)	[41]
MLA10 (C, N)	AvrA10 (N/A)	N	WRKY1 and WRKY2 (N)	[39]
Rx (C, N)	PVX coat protien (C, N)	C + N	RanGAP1 and RanGAP2 (C)	[48••,49••]

Abbreviations: N = nucleus, C = cytoplasm, CP = chloroplast, N/A = data not available, HR = hypersensitive response.

may utilize fundamentally different mechanisms. The nature of NB-LRR-mediated cytoplasmic and nuclear immune signaling and their relative importance is not clear. Future experiments addressing the importance of these findings in Rx and other NB-LRRs will be an important area of research.

Conclusions

Plant NB-LRR R proteins are highly diverse in terms of pathogen proteins recognized and interacting plant protein partners. NB-LRRs are subject to multiple levels of regulation in order to prevent erroneous activation of potent and metabolically costly immune responses. Newly discovered proteins that regulate NB-LRR accumulation and activation appear to associate with the core HSP90-SGT1-RAR1 chaperone complex. Knowledge of how these components are integrated into the complex may lead to the elucidation of specific biochemical mechanisms of NB-LRR stabilization, activation, and turnover. Furthermore, identification of all components of these macromolecular complexes *in planta* coupled to structural analyses will be required to gain a firm understanding of how these crucial protein complexes regulate plant immunity.

Many NB-LRRs exhibit complex patterns of subcellular localization pre-activation and post-activation. Recent studies demonstrating contributions of both cytoplasmic and nuclear localizations of the Rx NB-LRR to immune signaling suggests that a subset of NB-LRRs can activate defenses in multiple subcellular compartments. It is plausible that cytoplasmic defense signaling could be sufficient for effective immunity in certain situations, and that NB-LRR nuclear activity is a way for the plant to 'hedge its bet' against some pathogens. Being able to measure the exact nature of NB-LRR signaling in the cytoplasm and nucleus is the first step to understanding the relative contribution of each to disease resistance. The complexity of NB-LRR function and associated signaling can only by matched by their importance in plant-microbe interactions.

Note Added in Proof

After the completion of this manuscript, the first crystal structures of R protein CC (from barley MLA10) and TIR (from flax L6) domains were published [52,53]. These studies suggest that NB-LRRs can homodimerize via their N-termini. Moreover, homodimerization of only the CC or TIR domain appears to be both necessary and sufficient to trigger cell death in *planta* [52,53].

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