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Help wanted: helper NLRs and plant immune responses

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Plant nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins function as intracellular receptors in response to pathogens and activate effector-triggered immune responses (ETI). The activation of some sensor NLRs (sNLR) by their corresponding pathogen effector is well studied. However, the mechanisms by which the recently defined helper NLRs (hNLR) function to transduce sNLR activation into ETI-associated cell death and disease resistance remains poorly understood. We briefly summarize recent examples of sNLR activation and we then focus on hNLR requirements in sNLR-initiated immune responses. We further discuss how shared sequence homology with fungal self-incompatibility proteins and the mammalian mixed lineage kinase domain like pseudokinase (MLKL) proteins informs a plausible model for the structure and function of an ancient clade of plant hNLRs, called RNLs.

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Background

Plants are abundant sources of nutrients and water, and thus attractive hosts to microbial communities. To defend against microbial pathogens, plants have evolved a two-tiered immune system [1]. In the first tier, extracellular pattern recognition receptors (PRRs), typically leucine-rich repeat kinases and lysine motif kinases, recognize conserved microbial-associated molecular pattern (MAMP) ligands, triggering MAMP-triggered immunity (MTI) [1]. In response, pathogens have evolved virulence effectors that delay or suppress MTI [1]. Plants, as a counter measure, have evolved a second tier of defense, initiated by a family of nucleotide-binding leucine-rich repeat (NLR) protein receptors that are activated by recognizing effector presence directly or indirectly [1–4].

In plants, typical NLRs have a variable N-terminal domain: a Toll/interleukin-1 receptor (TIR) domain or a non-TIR domain, which resembles a coiled-coil domain (CC), followed by a central nucleotide binding (NB-ARC) and a C-terminal leucine-rich repeat (LRR) domain [1–4]. NLRs with CC domains are hereafter abbreviated CNLs and NLRs possessing TIR domains are abbreviated TNLs. NLRs are thought to be activated through the exchange of ADP (resting state) for ATP (active state) by the ATPase activity of the NB-ARC domain itself or by NLR-interacting proteins, acting as nucleotide-exchange factors [4,5,6••,7••]. Effector-mediated NLR activation results in a largely re-amplified MTI response termed effector-triggered immunity (ETI) that is typically associated with a hypersensitive cell death response (HR) at the infection site [1–4]. The demarcation between MTI and ETI is increasingly blurred and the action of extracellular receptors can require intracellular receptors [8,9]. For instance, SlNRC4a plays a non-canonical role as a positive regulator of LeEIX2/EIX-mediated MTI defense responses [10]. NLRs that either recognize the effector directly or recognize the effector's action on a host target or decoy of a target are collectively called sensor NLRs (sNLRs) [11]. NLRs have been actively reviewed in this forum [12,13] and we focus on selected new publications.

Sensor NLRs

ID sensor NLR pairs

Some of the functionally defined sNLRs that serve as excellent experimental models are tightly genetically linked to, and form a protein complex with, a co-regulated partner NLR. This functions as a signaling 'executor' module that transduces the effector recognition by the sNLR to initiate an immune response. We consider these pair-specific executors distinct from the helper NLRs later discussed. Some of the sNLR partners in these linked pairs contain an 'integrated domain' (ID) that is likely a decoy of an effector target and binding of an effector to the ID activates the complex. The two most

studied ID pairs are the Arabidopsis TNL RPS4/RRS1 pair and the rice CNL RGA4/RGA5 pair [12,14,15]. In the Arabidopsis RPS4/RRS1 pair, RRS1 carries an integrated WRKY transcription factor domain targeted by two unrelated bacterial effectors AvrRps4 and PopP2 [14,15]. In the rice RGA4/RGA5 pair, RGA5 carries an integrated RATX1 domain targeted by *Magnaporthe oryzae* (Rice blast fungus) effectors Avr-Pia and AVR1-CO39 [12,16]. RPS4 and RGA4 are the executor NLRs of their respective pairs.

Recent progress has been made in the mechanistic understanding of repression and activation of RPS4/RRS1. Deletion of the RRS1 WRKY domain or the WRKY adjacent domain 4 (DOM4) results in an allele that triggers constitutive RPS4-dependent defense activation suggesting that the WRKY domain contributes to maintaining an inactive complex [15]. AvrRps4 interaction with the WRKY domain disrupts WRKY association with a specific domain, leading to activation of the complex [15]. Furthermore, some mutations in RPS4 and RRS1 compromise PopP2 but not AvrRps4 recognition, suggesting that AvrRps4 and PopP2 derepress the complex differently [15].

Sensor partners without IDs

sNLR partners without IDs have been described in both Arabidopsis and rice. Three Arabidopsis TNL sNLRs named SIDEKICK SNC1 1 (SIKIC1), SIKIC2, and SIKIC3 are redundantly required for SNC1-mediated defense [17]. Further, SIKIC2 physically associates with SNC1 [17]. Interestingly, the TNLs are under regulation by distinct E3 ligases, with SNC1 levels regulated by CPR1 while the SIKICs are regulated by MUSE1 and MUSE2 [17]. SOC3 can partner with either of the genetically linked CHS1 or TN2 to monitor the homeostasis of E3 ligase SAUL1. SOC3 appears to be the executor module [18,19].

In addition to RGA4/RGA5, other sNLR partners (without IDs) in rice have been described. The CNL PigmR confers broad-spectrum resistance to *M. oryzae* [20]. The CNL PigmS competitively attenuates PigmR homodimerization by heterodimerizing with PigmR to suppress PigmR-mediated resistance specifically during rice seed development [20].

Orthologous sNLRs

In some cases, sequence divergent sNLRs from distantly related species can be activated by the same effector; these interactions appear to have convergently evolved [21,22]. Few true NLR orthologs have been identified. One example is the discovery of NbZAR1, a likely true ortholog of the *Arabidopsis thaliana* protein ZAR1 (AtZAR1), by a forward genetic screen to identify components required for perception of the bacterial acetyltransferase effector XopJ4 [23•]. In Arabidopsis, ZAR1 serves roles in recognition of multiple bacterial effectors that each have different biochemical activities. In addition to ZAR1, immune activation requires class XII receptor-like cytoplasmic kinases (RLCKs) and additional kinase or pseudokinase targets or decoys [24]. In conjunction with the pseudokinase ZED1, AtZAR1 mediates an ETI response to the acetyltransferase effector HopZ1a [24]. AtZAR1 and the ZRK3 pseudokinase are required for HopF2a-induced disease resistance [25•]. Further, AtZAR1 also functions with the RLCK RKS1 to mount an ETI response to the effector AvrAC. In the recently solved ZAR1 structure, AtRKS1 interacts with AtZAR1 and helps to keep the NLR in a monomeric inactive ADP-bound complex. AvrAC-induced uridylyation of the RCLK PBL2 specifically enables $PBL2^{UMP}$ to bind to RKS1 in the preformed ZAR1–RKS1 complex. This binding induces a conformational change in RKS1, which simultaneously leads to a dislocation of the ZAR1 NB domain and the release of ADP [6••,7••]. This nucleotide-free ZAR1–RSK1–PBL^{UMP} complex, oligomerizes upon ATP binding to form a pentameric complex, a resistosome, that is required for resistance and cell death signaling [6••,7••,26]. PBL2 uridylylation and binding to RKS1 are specifically required for AvrAC triggered immunity [6••,7••,26]. It remains unknown whether NbZAR1 also has evolved as a required resistance component against various type III effectors. However, recognition of XopJ4 by NbZAR1 also requires a class XII RLCK dubbed JIM2 [23•]. Collectively, these data suggest that ZAR1 has evolved to be a flexible platform to monitor RLCK homeostasis and further suggests that RCLKs are a battleground constantly targeted by diverse effectors.

Engineering decoys of sNLRs

With further understanding of the modes of action of specific sNLRs, attention turns to efforts to engineer the decoys that sNLRs guard [27•,28]. The bacterial effector protease AvrPphB cleaves the kinase PBS1, and this activates the Arabidopsis CNL RPS5 [28]. Like BIK1 and PBL2, PBS1 is a member of the RLCK subfamily VII [29]. The precise PBS1 cleavage site is defined, and can be substituted with cleavage sites for other pathogen virulence factor proteases, enabling RPS5 to be activated by these proteases [28]. Further, a modified soybean PBS1 decoy protein containing a cleavage site for the Soybean mosaic virus (SMV) NIa protease triggers cell death in soybean protoplasts when cleaved by this protease. Thus, RPS5 can be coopted to act as a resistance gene against SMV infection. PBS1 decoy engineering appears to be viable in at least one crop, in which endogenous PBS1 and analogous RPS5 proteins exist [27•].

Helper NLRs

In addition to the sensor and executor NLRs noted above, there is emerging evidence that many NLR-mediated immune responses require the presence and activity of so called 'helper' NLRs (hNLRs) [30••,31••,32–

34,35••,36••]. hNLRs seem to serve as downstream signaling hubs for a diverse array of sNLRs [30••,31••,32,33,35••,36••]. We summarize findings demonstrating that hNLRs function downstream of sNLR activation, and we speculate as to how their activity might be regulated. Drawing upon sequence homology of hNLRs to animal cell-death executing proteins and localization, we discuss plausible hNLR roles in ETI, and, specifically, in HR.

RNLs are an ancient and conserved hNLR clade

There are three described hNLR families, all encoding CNLs: the *ACTIVATED DISEASE RESISTANCE 1* (ADR1) family [34], the *N REQUIRED GENE 1* (NRG1) family [37], and *NB-LRR protein required for HR-associated cell death* (NRC) family [38]. In this review, we focus on the ADR1 and the NRG1 hNLRs, which are a unique subclade of CNLs defined by the relatedness of their N-terminal domain to an understudied immune system protein called RPW8; therefore, the ADR1 and NRG1 hNLRs are termed RNLs [33]. RPW8 and its paralogs contribute to powdery mildew resistance and upregulation of MTI responses [39]. We direct readers to Wu *et al.* [32] and Wu *et al.* [36••] for details about the *Solanaceae*-limited CNL NRC family and their functions as hNLRs. It is as yet unclear if and how the RNLs and NRC proteins intersect functionally.

The *ADR1* family and the *NRG1* family are small, ancient, and related RNL families found in all analyzed plant genomes [40]. The *ADR1* and *NRG1* gene families are sister clades in trees built from either their unique N-terminal coiled coil RPW8-like domain (CC_R) (Figure 1) or their CNL-A subclass NB-ARC [33,41,42]. In comparison to the large expansion of TNLs and CNLs, the RNL family expansion has been strikingly limited [33,43,44]. In addition to their limited expansion within genomes, RNLs, like sNLRs, are the targets of regulatory RNAi silencing machinery [13,44,45].

The CC domain most closely related to the CC_R is the CC_{EDVID} subclade (Figure 1), where the EDVID is a conserved amino acid motif in the CC [46]. When the NB-ARC is used as the basis for phylogenic comparison, the CC_{EDVID} CNLs do not cluster with the RNLs suggesting that they do not share a common ancestor. Thus, we hypothesize that the CC_{EDVID} domain and the CC_R domain may be experiencing selection to maintain an amino acid sequence pattern with similar general function at the structural level. Furthermore, there is a lack of evidence that CC_{EDVID} NLRs require CC_R hNLRs to function.

In Arabidopsis, there are four paralogs of ADR1: ADR1, ADR1-L1, ADR1-L2, and a largely N-terminally truncated ADR1-L3 [34]. Arabidopsis contains three NRG1 paralogs, NRG1.1, NRG1.2, and a severely N-terminally truncated NRG1.3. [35••,36••,37,47]. The Arabidopsis genome also encodes a protein with the structure CC_R-NB-ARC-LIM, named DAR5. *Nicotiana benthamiana* possesses a single copy of ADR1 and two NRG members NRG1 and NRG2, where the N-terminally truncated and poorly expressed NRG2 is postulated to be a pseudogene [35••,36••,37,47].

ADR1 and NRG1 families possess both distinct and redundant functions

Redundant functions

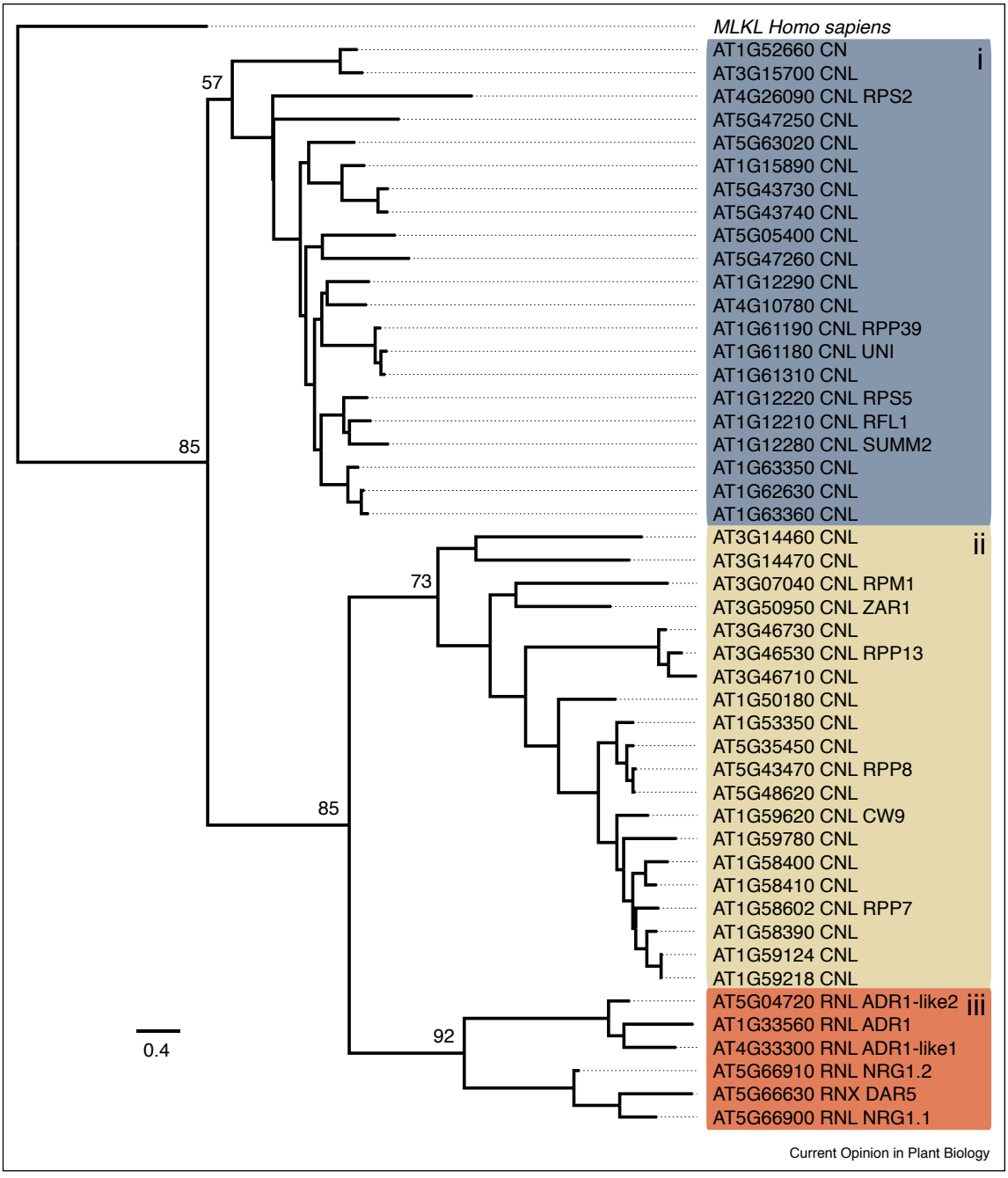
ADR1 and NRG1 function as redundant downstream hubs for a diverse array of sNLRs, not unlike the network observed for sNLR activation mediated by members of the NRC protein family in the *Solanaceae* [31••,32] (Figure 2). For instance, the ADR1 family members are redundantly required downstream of both CNL and TNL sNLRs RPS2, RPP2, RPP4, RPS4/RRS1 for full effector-driven ETI, and ADR1s are positive regulators of some NLR auto-immune mutants [33,34,35••,36••,48] (Figure 2). Similarly, the NRG1 family members function redundantly downstream of all tested TNLs [30••,33,35••,36••] (Figure 2).

The broad functional redundancy among the ADR1 and NRG1 families, respectively, was one driver in the creation of a 'helperless' Arabidopsis plant, a *sextuple* hNLR mutant (*adr1 adr1-L1 adr1-L2 nrg1.1 nrg1.2 nrg1.3*) [33,34,35••,36••,48]. Before the creation of the *sextuple* plant, it was not possible to determine if the ADR1 and NRG1 families were functionally redundant, with respect to each other. To date, the helperless plant has only been challenged with two bacterial pathogens, *Psm* ES4326 and *Pst* DC3000 expressing *AvrRps4* or *HopA1*. When challenged with *Psm* ES4326, enhanced disease susceptibility was observed in the *sextuple* compared with that of its parents, *nrg1 triple*, *adr1 triple*, and wild type Col-0, suggesting that ADR1s and NRG1s have a synergistic effect on basal defense. Further, when the mutant genotypes were challenged with DC3000 *AvrRps4* or DC3000 *HopA1*, TNL-mediated defense of the *sextuple* was significantly more compromised compared to *adr1 triple* and *nrg1 triple* as measured by bacterial growth and increased disease symptoms [36••]. The susceptibility of the *sextuple* mutant to the virulent *Psm* ES4326 strain was significantly enhanced in comparison to the immune-compromised *eds1-2* mutant, suggesting that basal defense, and thus also MTI, initiated by extracellular receptors, might depend on RNL activity or presence. This finding further supports the idea of a regulatory interplay between MTI and ETI.

Functions requiring both ADR1 and NRG1 RNLs

The creation of a helperless plant opens the avenues for addressing several lines of inquiry. It is now possible to determine whether the ADR1 and NRG1 families are functionally redundant with respect to multiple NLRs,

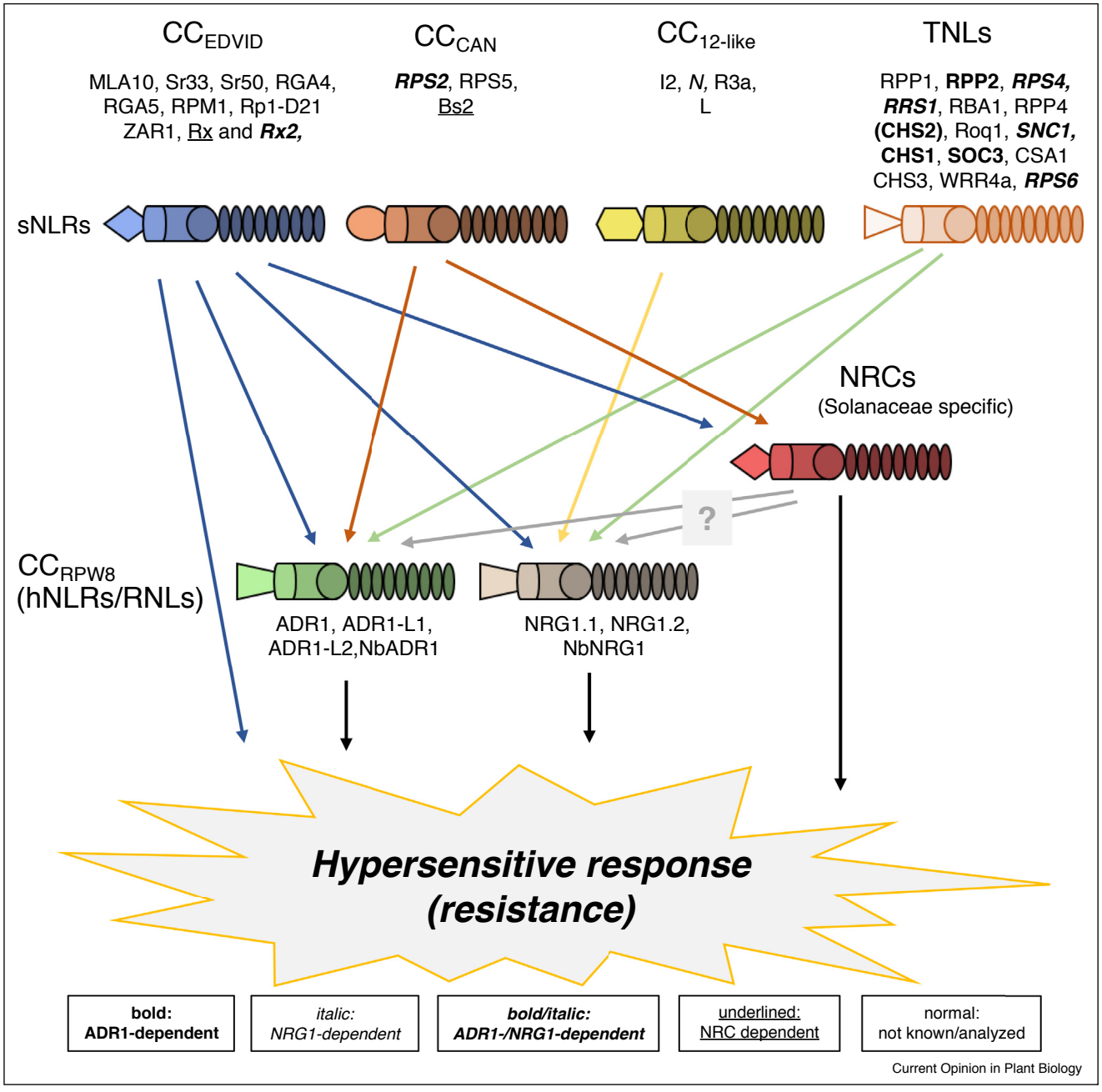
Figure 1



N-terminal end domain maximum-likelihood phylogenetic tree of Arabidopsis coiled-coil and RPW8 domain-containing NLR proteins and Human mixed lineage kinase domain-like pseudokinase protein.
Amino acids 1–180, corresponding to the CC, RPW8, and MLKL N-terminal domain regions, were extracted and aligned iteratively using MUSCLE and CLUSTALW. Several sequences were removed to reduce gaps in the alignment. The Jones, Taylor, Thornton model, with frequencies, three discrete Gamma categories and a 100-bootstrap test of phylogeny, was used to generate the tree. Bootstrap support of major nodes is indicated on the phylogeny. Scale bar indicates amino acid substitutions per site. C = coiled coil, R = RPW8, N = NB, L = LRR, X = other. Clade i) contain no EDVID motifs, clade ii) contain EDVID motifs and clade iii) are the RPW-NLRs and they do not contain EDVID motifs.

besides RPS4/RRS1 or RPS6. For instance, it has already been determined through TRV silencing that Rx2-mediated resistance requires both ADR1 and NRG1 families for function in *N. benthamiana* [33]. Of special interest, is whether RPM1 mediated HR will be lost in a helperless plant, as RPM1 signaling is not negatively affected either by the loss of the *ADR1* or *NRG1* family individually [34,35••].

Figure 2



Signaling network of plant sensor and helper NLRs.
Experimentally proven signaling-dependencies are indicated by colored arrows and listing of NLRs; bold = ADR1-dependent, italic = NRG1-dependent, bold/italic = ADR1-dependent and NRG1-dependent, underlined = NRC-dependent, normal = not known/analyzed. Whether the solanaceous NRCs also require the hNLRs for cell death and resistance signaling is not yet clear, however very likely as Rx2-mediated resistance in *N. benthamiana* was shown to require the hNLRs (Collier *et al.* [33]) and its paralog Rx requires the three NRCs - NRC2,3,4 - for cell death induction (Wu *et al.* [31••]).

Distinct functions

ADR1 family members function upstream of salicylic acid (SA) accumulation and subsequent activation of SA-dependent responses in ETI initiated by multiple sNLRs, including CNLs and TNLs (Figure 2) [34,36••,49]. ADR1-L2 may be thus far distinguished from sensor NLRs by the dispensability of its P-loop for helper function in sensor-NLR mediated and executor-NLR mediated immunity [25•,31••,34]. It is unknown if the two other ADR1 paralogs also possess a dispensable

P-loop for their helper functions. The necessity of the NRG1 family members' P-loops has also been examined. The *chs3-2D* mutant requires the presence of At*NRG1* family members for the *chs3-2D* auto-immune dwarf phenotype. AtNRG1.1 and AtNRG1.2 do not require their P-loop activity to contribute to the *chs3-2D* auto-immune dwarf phenotype [36••]. However, NbNRG1 does require its P-loop for HR auto-activity following over-expression in *N. benthamiana* [37].

While ADR1 proteins contribute to both CNL-mediated and TNL-mediated immunity [34], NRG1 proteins are thus far not required for CNL signaling [30••,36••]. By contrast, NRG1 proteins are required for disease resistance initiated by the TNL proteins N, Roq1, RPS4/RRS1, RPP1, SOC3/CHS1, CSA1/CHS3, and WRR4A [30••,35••,36••,37] (Figure 2). Interestingly, NRG1 is required for both TIR-mediated HR response and for a TNL-mediated 'extreme resistance' phenotype that lacks HR [50]. The requirement for NRG1 function by TNLs is supported by evolutionary evidence that NRG1 and TNL genes were simultaneously lost in (most) monocots and the *Lamiales* family [33]. While TNLs seem to have been lost in these lineages, TIR only domains (T), TIR-NB-ARC proteins (TN), or TIR-uncharacterized domain proteins (TX), have been found in some monocots [51]. It remains unknown if these truncated T, TN, TX proteins function in immunity, like the Arabidopsis TIR only RBA1 [52•], and if they do, whether they require hNLRs.

Pore-forming fungi HeLo and HELL domains resemble the RNL CC_R domain

RNLs collectively serve as downstream signaling hubs to a diverse array of sNLRs, yet the precise function of the RNLs in mediating ETI remains unknown. However, shared sequence homology and predicted structural homology with fungal and animal proteins provide valuable insights into their possible mode(s) of action.

Cell death initiated by fungal cells during heterokaryon incompatibility requires an 'NLR-like' protein that activates a downstream cell death executing protein [53,54]. While the fungal 'NLRs' contain a variable N-terminal domain and an NB-ARC or a NACHT domain, they do not contain a LRR domain [53], which is commonly replaced with other repeating superstructures, such as TPR (tetratricopeptide repeats), WD repeats or ANK (ankyrin) repeats [53]. Thus, following precedent in fungal NLR literature [53], we adhere to the NLR designation for these proteins. Fungal proteins acting downstream of fungal NLRs are commonly referred to as effector proteins or as heterokaryon incompatibility proteins, and are composed of an amyloid folding domain and a variable N-terminal domain [53]. To avoid confusion with bacterial effectors, we refer to the fungal proteins as incompatibility proteins.

While both mammalian and plant sNLRs are activated through oligomerization, fungal NLR systems instead operate through amyloid fold templating by the NLR onto the incompatibility protein [53,55,56•,57]. Consider for instance, the NACHT and WD Repeat Domain Containing 2 (NWD2) and Heterokaryon incompatibility protein S (Het-S). In this highly conserved fungal system, the NLR NWD2, upon binding its ligand, activates the downstream Het-S pore-forming protein by converting its prion-forming region into the β-solenoid fold [54,58]. Upon refolding into the β-solenoid fold, Het-S's HeLo domain forms a pore in the membrane leading to cell death (Figure 3) [59,60]. HeLo-like (HELL) domains have been characterized in other filamentous fungi. For instance, the HeLo-like protein (HELLP), possessing an N-terminal HELL domain, behaves analogously to Het-S [61]. Remarkably, the HeLo and HELL domains share sequence homology to both the four helical bundle (4HB) of the N-terminus of the mammalian cell death executor protein MLKL and the plant CC_R domains that define the RNL subclass of hNLRs [61].

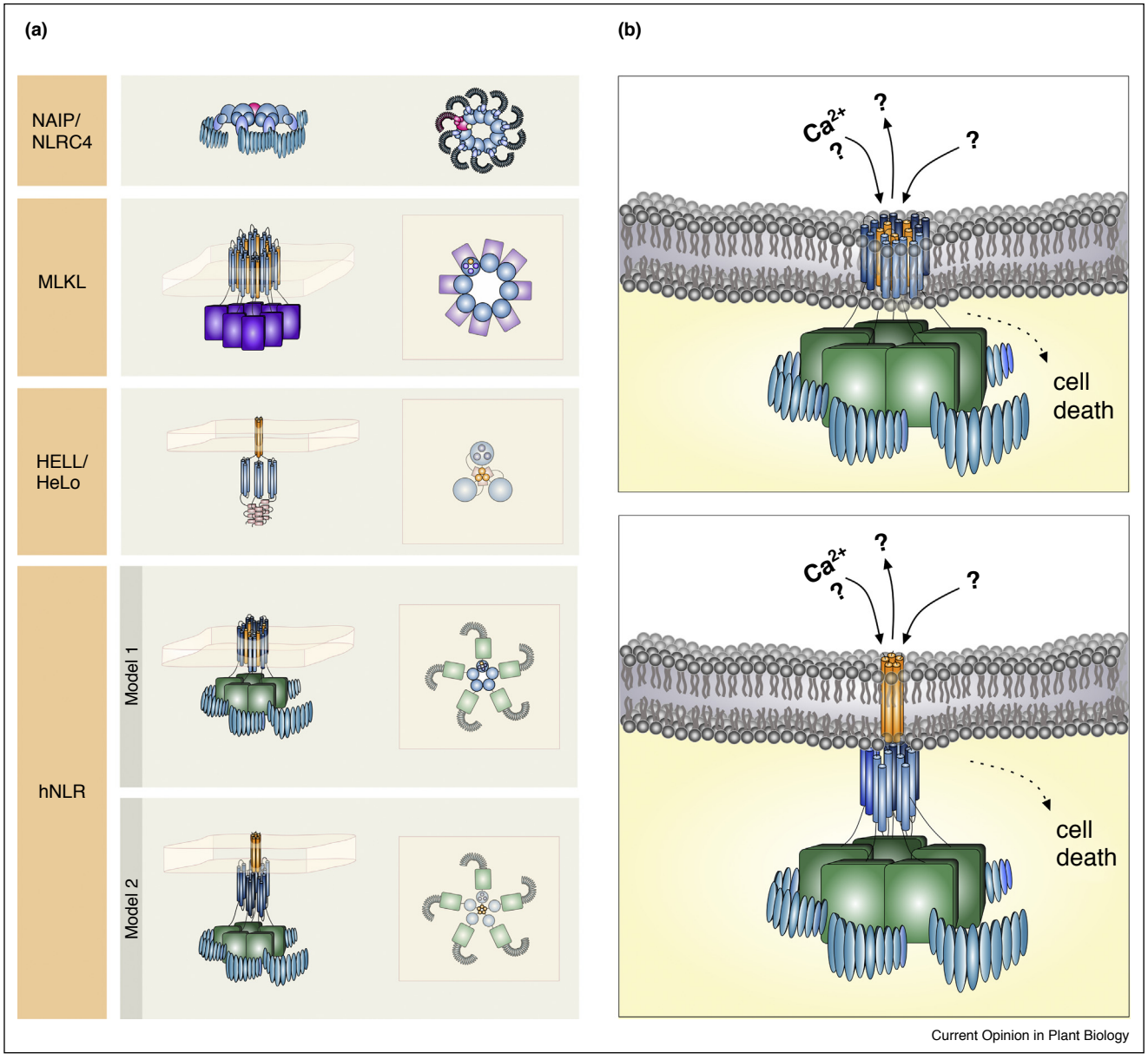
The pore-forming protein MLKL shares proposed structural homology with the CC_R domain

Given the sequence homology between the 4HB of MLKL and the CC_R domain [61], we examined how MLKL structure and function might impact the structure and function of RNLs. MLKL is required for the activation of necroptosis [62]. The 4HB domain of MLKL is required for insertion into the plasma membrane following activation and oligomerization which leads to subsequent pore formation (Figure 3) [63–65].

A structural homology search using Phyre2 revealed that all five Arabidopsis CC_R domains are predicted to fold into a 4HB with strong similarity to MLKL, as noted independently for ADR1 [66]. Further, we could thread with high confidence all AtRNLs onto the MLKL structure (Figure 4). Thus, based on structural homology and the high confidence of secondary structure prediction, we speculate that the CC_R domain of the RNLs adopt a MLKL-like 4HB fold.

We also tested whether canonical CNLs N-terminal domains would potentially fold into similar 4HBs. However, CC domains can vary significantly in terms of residue conservation. Thus, we limited our examination to only canonical CC domains that most closely resemble the CC_R domain sequence. In accordance with the phylogeny of the CC domain of the Arabidopsis CNLs, the CC_{EDVID} subclade most resembles the CC_R domain (Figure 1). We built an hmm profile using an alignment of MLKL, HELL and HET domains from [61] as a guide. Performing an hmm-search against the Arabidopsis genome using our hmm as the query, we hit multiple CC_{EDVID} NLRs, such as ZAR1 (Table 1). The hmm-

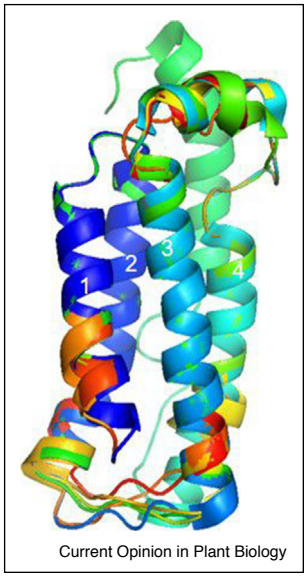
Figure 3



Signalosome and membrane pore formation by regulators of immunogenic cell death in different kingdoms.
A Mammalian innate immune receptors NAIP (pink) and NLRC4 (blue) oligomerize to form a signaling competent inflammasome upon PAMP-detection. The fungal HELL/HeLo-domain containing NLR-like proteins interact upon activation and form a tetrameric complex inserting the first a-helix of their 4HB HELL domain into membranes to initiate hybrid incompatibility. The animal MLKL protein oligomerizes after phosphorylation by RIPK3 forming an amyloid-like structure followed by translocation to the plasma membrane causing cell membrane disruption, which is thought to mediate necroptosis. In plants, the hNLRs might function in a similar way, forming pores with their RPW8 domains in cell membranes after being activated by effector-activated or auto-activated sNLRs. B Two models of RNL mediated pore formation are possible. Model 1: Similar to MLKL pore formation the full 4HB of the RPW8-domain is inserted into membranes allowing membrane deterioration and/or pore formation leading to changes in ion fluxes. Model 2: Only the first alpha-helix of the RPW8-domain is inserted into membranes upon activation and oligomerization of the RNL proteins, similar to HELL/Het-domain containing protein pore formation.

search hit many CC_{EDVID} CNLs, confirming our CC-built phylogenetic tree. Moreover, when Phyre2 is used to predict the folding of the N-terminal regions of ZAR1, MLKL is indicated as one of the top four folding templates. This also fits very well with the recently published cryo-EM structure of full-length AtZAR1 [6••,7••]. The ZAR1 CC domain in the inactive ZAR1 forms a monomeric 4HB structure similar to the published Rx and Sr33 CC structures [57,67,68]. Thus, based on sequence homology, structural folding software predictions and

Figure 4



An overlay of the N-terminal MLKL (2msv) and the predicted CC_R domains of ADR1 ADR1-L1, ADR1-L2, NRG1.1, and NRG1.2. The predicted CC_R domain models were produced by threading onto the 2msv structure by Phyre2. All CC_R domains adopt highly similar 4HB structure very similar to the known 4HB MLKL, despite the low shared residue identity (ADR1 20% ID, 97.5% confidence; ADR1-L1 11% ID, 99% confidence; ADR1-L2 14% confidence, 98.6% confidence, NRG1.1 14% ID, 98%; NRG1.2 13% ID, 98.1% ID). Helices predicted to form the 4HB are numbered 1 through 4.

Table 1

Hmm-search results from query of the Arabidopsis genome. We built an hmm profile using an alignment from Ref. [53] as a guide. Performing an hmm-search against the Arabidopsis genome using our hmm as the query, we hit multiple CC_{EDVID} NLRs

NLRs

Atg	Description	Structure	E value
AT5G66900	NRG1.1	RNL	0.034
AT1G58390	unnamed NLR	CC_EDVID-NL	0.039
AT3G50460	HR2	RPW8	0.047
AT1G58807	unnamed NLR	CC_EDVID-NL	0.094
AT1G59124	unnamed NLR	CC_EDVID-NL	0.094
AT5G04720	ADR1-L2	RNL	0.17
AT5G05400	unnamed NLR	CNL	0.024
AT1G58400	unnamed NLR	CC_EDVID-NL	0.33
AT1G58410	unnamed NLR	CC_EDVID-NL	0.45
AT3G50950	ZAR1	CC_EDVID-NL	0.74
AT1G58848	unnamed NLR	CC_EDVID-NL	0.94
AT1G59218	unnamed NLR	CC_EDVID-NL	0.94

Other identified proteins of interest

Atg	Description	E value
AT2G18860	Syntaxin/t-SNARE family protein	0.059
AT2G17780	MCA2; mechanosensitive Ca channel	0.066
AT1G50970	Membrane trafficking VPS53 family protein	0.17
AT1G48240	Putative plant snare 12	0.85

solved structures of plant CC domains it is strongly suggested that CC_R domains and CC_{EDVID} domains may broadly adopt a 4HB fold, supposedly similar to MLKL or other pore-forming and oligomerizing proteins.

In addition to CC_{EDVID} NLRs, our hmm-search hit At2g17780 with high confidence (Table 1). At2g17780 encodes the Arabidopsis protein Mid1-Complementing Activity 2 (MCA2). MCA2 (and likely its paralog MCA1) forms a homotetramer, in which a single N-terminal helix from each subunit is arranged to form a plasma membrane Ca^{2+} channel [69]. Considering the hmmer hit to MCA2 in combination with the structural modeling of the RNLs onto MLKL, we speculate that RNLs, and potentially other CNLs, oligomerize to form pores in plant membranes, similar to the suggested ZAR1 CC domain pore. We present two possible models for activation (Figures 3 and 4). In model one, the RNLs N-termini generally fold into a 4HB like MLKL, and these oligomerize to form a large multimeric tertiary structure in which the entire 4HB of each subunit is embedded into the membrane. In model two, only the first helix of the 4HB of each subunit inserts into the membrane to form a putative cation channel, as suggested for ZAR1 or MCA2. Additionally, HELL and HeLo domains are predicted to have a single helix flip out to cause the pore [59,61]. Mutation of the GxxxG glycine zipper prevents cell death [61]. Interestingly, the GxxxG motif is conserved in the NRG1 proteins [33]. More experimentation, and plant RNL structures, are needed to determine the predictive accuracy of the RNL 4HB and the mode in which the RNLs trigger HR.

Subcellular localization of RNLs suggests membrane-specific function

The subcellular localization of proteins is important for their function. However, NLR and, in particular, hNLR localization has not been intensively studied. First insights into hNLR localization have recently been provided by Wu *et al.* [36••], who reported a similar steady-state as well as defense-activated localization of AtNRG1.1-mNeonGreen and AtNRG1.2-mNeonGreen transiently expressed from their native promoters in a heterologous system [36••]. Both AtNRG1 proteins localized to the cytosol and partially co-localized with the ER-marker HDEL-mRFP. In addition, AtNRG1.1 also partially co-localized with a PM-marker and was detected at some intracellular ER-associated puncta. An attractive hypothesis would posit that RNLs would be activated at various subcellular locations and translocate to membranes, reminiscent of the defense-associated functions of RPW8 and MLKL.

In response to powdery mildew infection, RPW8.2 is targeted to the extrahaustorial membrane (EHM) via VAMP722/721-mediated vesicle trafficking [70,71]. Once incorporated into the EHM, RPW8.2 is thought to trigger

localized immune responses [70]. In addition to RPW8.2, its paralogs HR1, HR2, and HR3 are also transported to the EHM upon powdery mildew infection to induce post-penetration resistance [72]. Thus, pathogen-induced membrane localization of RPW8.2 and its paralogs contributes to powdery mildew resistance.

The membrane localization of activated MLKL is important for its executor function during necroptosis. Upon necroptosis induction, the N-terminal 4HB of activated MLKL oligomerizes to form a complex consisting of eight protomers which translocate to the PM via phosphatidylinositol phosphate binding. This causes membrane deterioration resulting in necroptotic cell death [73,74••]. However, the exact underlying mechanism by which MLKL causes membrane disruption is still largely unknown. Considering the recent localization data and structural homology between RNLs and MLKL (Figure 4), it is plausible that RNL function is coupled to membrane integrity. However, sophisticated cell biological approaches are required to better understand where RNLs localize pre-activation and post-activation and how they function at membranes.

P-loop function, RNL membrane localization, and helper function

A functional P-loop is important for membrane localization and cell death function of certain NLRs like RPM1, Roq1 or N [30••,75,76]. Interestingly, RNLs (AtADR1-L2, NbNRG1) can require an intact P-loop for their auto-activity and, for ADR1-L2, in the propagation of the unregulated runaway cell death phenotype of the Arabidopsis *lsd1* mutant [30••,37,77]. Considering the possible membrane localization of activated hNLRs, it is possible that P-loop mutant hNLRs lose membrane localization, and, therefore, the ability to induce auto-activity mediated cell death. Equally possible would be that P-loop mutant hNLRs also lack the ability to oligomerize, since a functional P-loop was shown to be important for NLR self-association [30••,75].

In contrast, Arabidopsis RNLs (AtADR1-L2, AtNRG1.1, AtNRG1.2) do not require an intact P-loop for their helper function downstream of either effector-activated or specific auto-active sNLRs [34,36••]. In these cases, the P-loop mutant RNLs might rather act as a scaffold to initiate or support oligomerization with sNLRs or other functional RNLs, leading to their activation and subsequent membrane translocation. This would be consistent with their observed redundancy and could be tested by analyzing complementation of a 'helperless' mutant plant with P-loop mutant hNLR variants. A similar P-loop independent function of the mammalian hNLR NLRC4 was found in pyroptosis, a form of programmed cell death. However, the dispensability of the NLRC4 P-loop for oligomerization of NLRC4 and interaction with the sNLRs NAIP2 and NAIP5 is not yet clear [78,79] (Figure 3).

Interestingly, the helper function of the *N. benthamiana* NbNRG1 in Roq1 (TNL) mediated cell death requires a functional NbNRG1 P-loop [30••]. This suggests that the single NbADR1 cannot compensate for the loss-of-function of NbNRG1 function. It would be very interesting to see if the two different hNLR families would indeed form heteromeric complexes and whether these are biologically functional, and if the Arabidopsis helperless plant can be complemented by the *N. benthamiana* RNLs and vice versa. The generation of a helperless *N. benthamiana* plant is eagerly awaited.

RNLs and CNLs could form membrane-disrupting pores or ion-channels necessary for ETI

Changes in anion and cation fluxes across plant membranes into to the cytosol are a very early response to pathogen attack and a hallmark of MTI and ETI [80,81]. Calcium (Ca^{2+}) is probably the major ion and second messenger involved in mounting a successful immune response in both animals and plants. Although, the activation of specific Ca^{2+} permeable channels and pumps that lead to the early increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]$cyt) is well characterized for MTI, we still have not unequivocally identified the Ca^{2+} channels or pumps and the mechanisms of their activation in ETI [82]. The timing of the observed second increase in the biphasic $[Ca^{2+}]$cyt burst upon infection of Arabidopsis with different avirulent *Pseudomonas* DC3000 strains correlates with the timing of HR that is specific for the corresponding activated sNLR [83]. It is plausible that effector-recognition by sNLRs leads to the activation of RNLs, resulting in their oligomerization and translocation to cellular membranes, where they might form pores with their CC domains that enable or support ion fluxes, for example of Ca^{2+} ions (Figure 3B). Such a pore-forming function is also suggested for effector-activated AtZAR1 [7••]. Here, the most N-terminal alpha helix is rearranged upon activation and a pentamer of these single helices is formed as the full length protein pentamerizes. Mutations on the inside face of this helix lose activity but retain oligomerization and plasma membrane re-localization, suggesting a specific function for this potential pore forming structure. It is possible that some CNLs, like Arabidopsis RPM1 and ZAR1, might have retained (or gained) the ability to form pores in membranes, like the plasma membrane, explaining the lack of a documented hNLR requirement [34,35••]. How sNLRs activate hNLRs is still not clear and evidence for physical interaction between effector-activated sNLRs with hNLRs is also eagerly awaited [32,37].

Could TIR activation result in RNL oligomer formation?

All analyzed TNL-mediated immune responses require hNLR function, or more specifically RNL function [30••,34,35••,36••], but no interaction between a TNL and RNL is thus far documented [37]. Nevertheless, a tempting speculation is that all TIR-dependent signaling converges onto RNLs and, given the above discussion, these form membrane pores to drive cell death.

TIR domains in Toll-like receptors (TLR) of animals have a scaffolding function for oligomerization and protein complex formation [84]. Working models suggested that this was the case for plant TNLs as well. However, recent findings reported an ancient NAD^+ (nicotinamide adenine dinucleotide)-consuming enzymatic activity for prokaryotic TIR domains and the TIR-domain of the mammalian SARM1 (sterile alpha and TIR motif containing 1) protein, a TLR adaptor protein that executes pathological axon degeneration [85••,86]. Interestingly, canonical innate immune system TIR domains from Toll-like receptors have lost this activity [85••,86]. Thus, a specific clade of TIR domains cleaves NAD^+ into ADP-ribose (ADPR), cyclic ADRP and nicotinamide and thereby depletes cells of NAD^+. NAD^+ depletion in macrophages and human T lymphocytes can trigger RIPK3 (receptor-interacting serine-threonine kinase 3) and MLKL-dependent necroptosis [87••], suggesting that MLKL or RIPK3 could somehow detect either the NAD^+ depletion or the accumulation of one or more cleavage products.

cADPR and ADPR belong to the adenine-containing second messenger family involved in regulation of cellular Ca^{2+} homeostasis, releasing calcium from intracellular storages like the ER or vacuole in animals and plants [88]. Given the relationship between plant TIR domains and the bacterial or mammalian SARM1 TIR domain [89], it is tempting to speculate that a similar mechanism might be employed by plants, and might include, by analogy, initiation of cell death by RNL-mediated pore formation in cellular membranes. Also, while cADPR can activate defense gene expression in *N. benthamiana*, no clear function of NAD^+ cleavage-products in plant immunity has been demonstrated [90,91]. An alternative hypothesis is informed by the findings of the importance of the TIR domain homodimerization for TNL function [52•,92]. TIR homodimers could recruit RNLs and initiate their oligomerization in an EDS1-dependent manner.

Conclusions

Although the first plant NLR proteins were identified and cloned 25 years ago, we still do not fully understand how their activity is regulated and how their activation is transmitted to a successful immune response. However, it is becoming clearer that most, if not all, NLRs do not function individually, but rather depend on an integrated regular network of sensor NLRs, executer NLRs, and helper NLRs [32,34,35••,36••,48]. Detailed analyses of the two ancient RNL-families, the ADR1s and the NRG1s, will reveal how they cooperate with sNLRs and with each other to transduce-specific immune responses upon effector recognition, and how this activation leads to cell death and disease resistance. The outstanding missing links for the NLR community are structural information for exemplars of various resting state and activated NLR classes and reliable, well controlled cell biology data derived from fluorescence protein-tagged full-length wild type NLRs expressed from their native promoters in appropriate homologous systems.

Conflict of interest statement

Nothing declared.

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