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 uridylylation 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
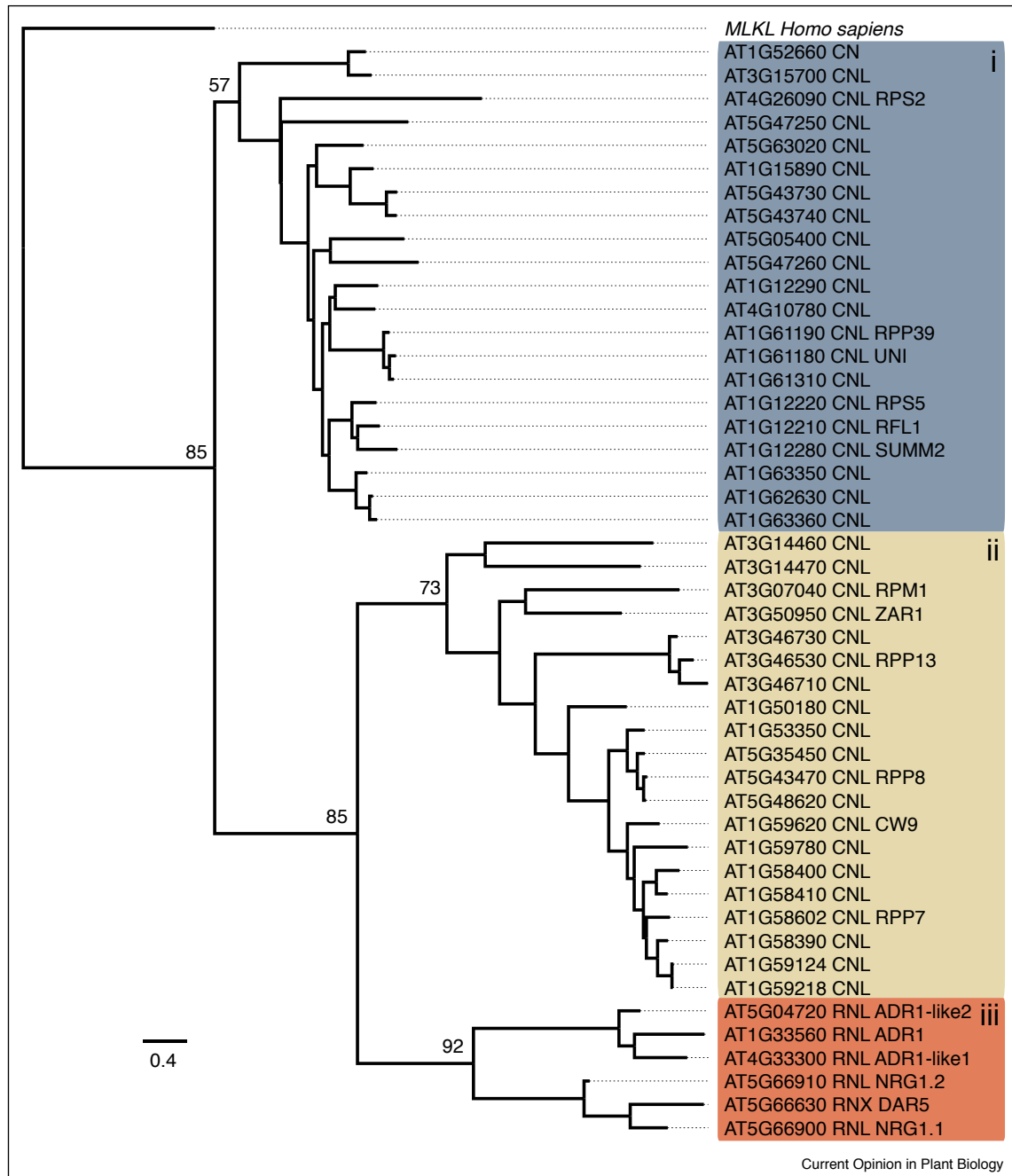
N-terminal end domain maximum-likelihood phylogenetic tree of Arabidopsis coiled-coil and RPW8 domain-containing NLR proteins and Human mixed lineage kinase domain-like pseudokinase protein.

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
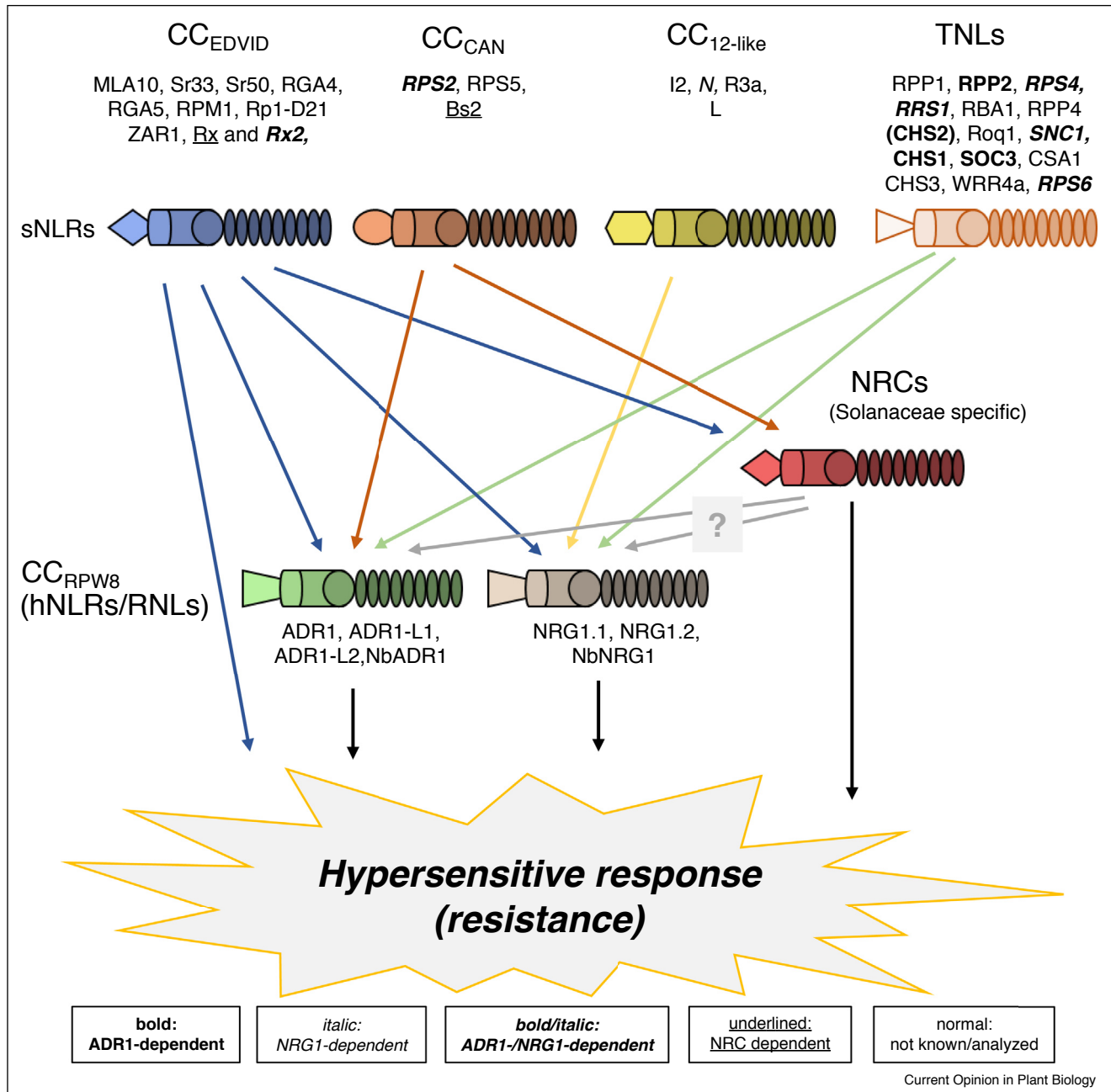
Signaling network of plant sensor and helper NLRs.

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$^{\bullet\bullet}$]. 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$^{\bullet\bullet}$,36$^{\bullet\bullet}$]. 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$^{\bullet\bullet}$,35$^{\bullet\bullet}$,36$^{\bullet\bullet}$,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$^{\bullet}$], 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$^{\bullet}$,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
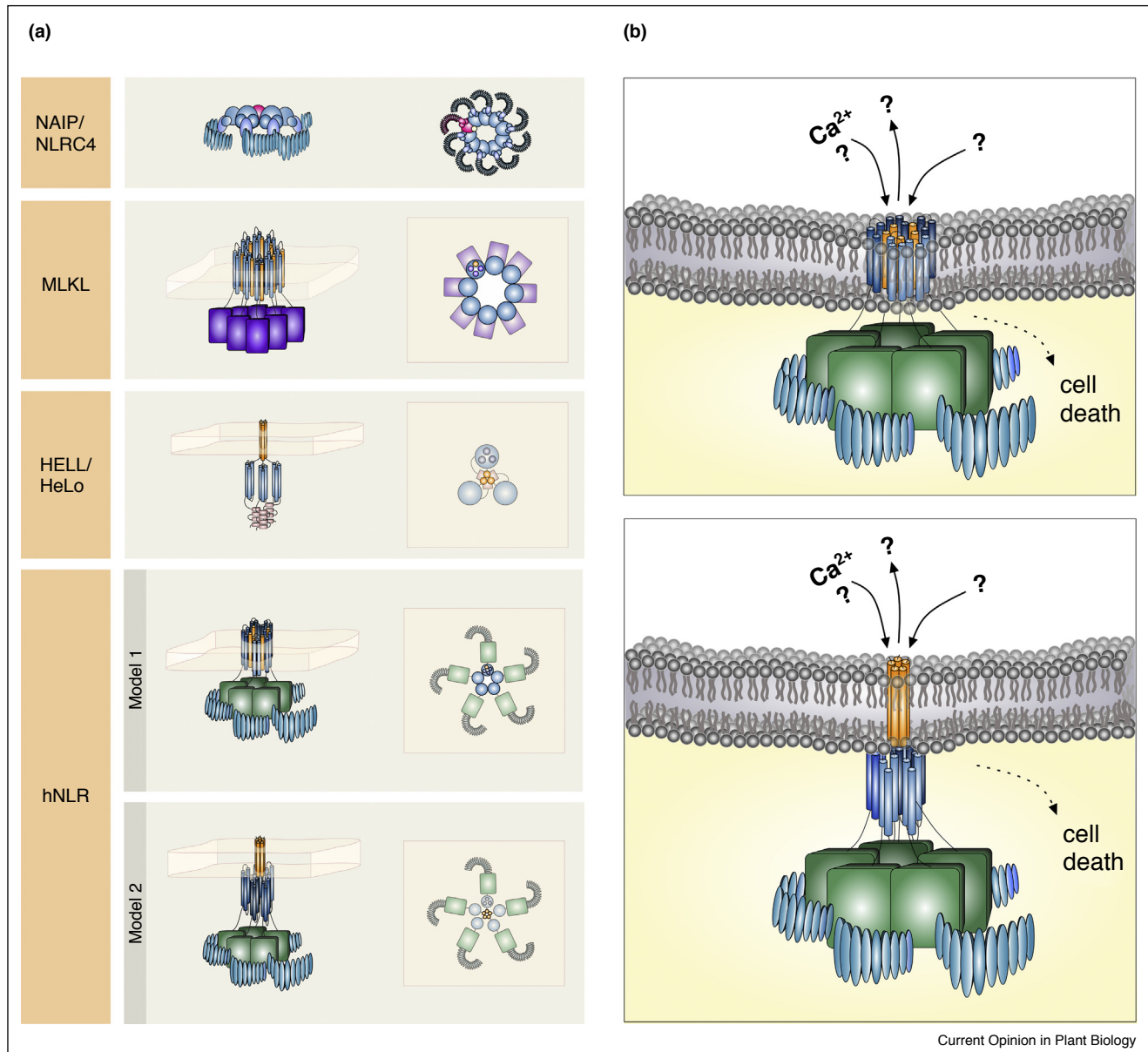
Current Opinion in Plant Biology

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

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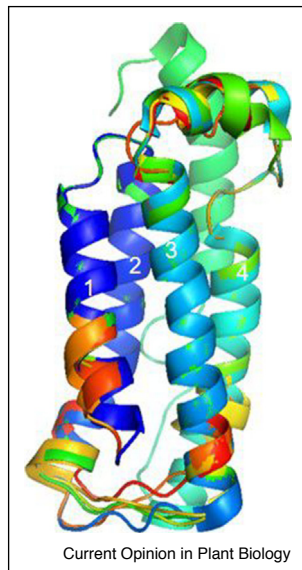
An overlay of the N-terminal MLKL (2msv) and the predicted CC$_R$ domains of ADR1 ADR1-L1, ADR1-L2, NRG1.1, and NRG1.2. The predicted CC$_R$ domain models were produced by threading onto the 2msv structure by Phyre2. All CC$_R$ domains adopt highly similar 4HB structure very similar to the known 4HB 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 phosphatidy-linositol 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.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest
•• of outstanding interest

1. Dangl JL, Horvath DM, Staskawicz BJ: **Pivoting the plant immune system from dissection to deployment**. *Science* 2013, **341**:746-751.

2. Jones JD, Dangl JL: **The plant immune system**. *Nature* 2006, **444**:323-329.

3. Jones JD, Vance RE, Dangl JL: **Intracellular innate immune surveillance devices in plants and animals**. *Science* 2016, **354**.

4. Bonardi V, Cherkis K, Nishimura MT, Dangl JL: **A new eye on NLR proteins: focused on clarity or diffused by complexity?** *Curr Opin Immunol* 2012, **24**:41-50.

5. Bernoux M, Burdett H, Williams SJ, Ellis JG, Newell K, Anderson P, Dodds PN, Kobe B, Lawrence G, Zhang X: **Comparative analysis of the flax immune receptors L6 and L7 suggests an equilibrium-based switch activation model**. *Plant Cell* 2016, **28**:146-159.

6. Wang J, Wang J, Hu M, Qi J, Wang G, Han Z, Qi Y, Wang H-W,
•• Zhou J-M, Chai J: **Ligand-triggered allosteric ADP release primes a plant NLR complex**. *Science* 2019, **364**:eaav5868.
Wang *et al.* provide the first full length structure of a plant NLR in resting and intermediate pre-activated states. The two uridylyl moieties of $PBL2^{UMP}$ interact with and stabilize the activation segment of RKS1, consequently resulting in a conformational outward rotational change of about 60 degrees only in the ZAR1 NB domain. Thus, uridylylated PBL2 allosterically induces release of ADP from the ZAR1-RKS1-$PBL2^{UMP}$ complex.

7. Wang J, Hu M, Wang J, Qi J, Han Z, Wang G, Qi Y, Wang H-W,
•• Zhou J-M, Chai J: **Reconstitution and structure of a plant NLR resistosome conferring immunity**. *Science* 2019, **364**:eaav5870.

Wang *et al.* provide the first full length structure of a plant NLR and the first biochemical explanation of indirect recognition of non-self via a preformed NLR heterodimeric complex. An effector-poised ZAR1-RKS1-uridylylated PBL2 intermediate monomer is activated by ATP, causing major structural rearrangements to generate an active pentamer. Pentamerization drives re-localization to the plasma membrane and causes the N-terminal CC domain helix to rearrange, forming a potential pentameric pore.

8. Thomma BP, Nürnberger T, Joosten MH: **Of PAMPs and effectors: the blurred PTI-ETI dichotomy**. *Plant Cell* 2011, **23**:4-15.

9. Leibman-Markus M, Pizarro L, Bar M, Coaker G, Avni A: **NRC proteins-a critical node for pattern and effector mediated signaling**. *Plant Signal Behav* 2018, **13**.

10. Leibman-Markus M, Pizarro L, Schuster S, Lin ZD, Gershony O, Bar M, Coaker G, Avni A: **The intracellular nucleotide binding leucine-rich repeat receptor–SlNRC4a enhances immune signaling elicited by extracellular perception**. *Plant Cell Environ* 2018, **41**:2313-2327.

11. Bonardi V, Tang S, Stallmann A, Roberts M, Cherkis K, Dangl JL: **Expanded functions for a family of plant intracellular immune receptors beyond specific recognition of pathogen effectors**. *Proc Nat Acad Sci U S A* 2011, **108**:16463-16468.

12. Baggs E, Dagdas G, Krasileva K: **NLR diversity, helpers and integrated domains: making sense of the NLR IDentity**. *Curr Opin Plant Biol* 2017, **38**:59-67.

13. Halter T, Navarro L: **Multilayer and interconnected post-transcriptional and co-transcriptional control of plant NLRs**. *Curr Opin Plant Biol* 2015, **26**:127-134.

14. Newman TE, Lee J, Williams SJ, Choi S, Halane MK, Zhou J, Solomon P, Kobe B, Jones JD, Segonzac C: **Autoimmunity and effector recognition in *Arabidopsis thaliana* can be uncoupled by mutations in the RRS 1-R immune receptor**. *New Phytol* 2018, **222**:954-965.

15. Ma Y, Guo H, Hu L, Martinez PP, Moschou PN, Cevik V, Ding P, Duxbury Z, Sarris PF, Jones JD: **Distinct modes of derepression of an Arabidopsis immune receptor complex by two different bacterial effectors**. *Proc Nat Acad Sci U S A* 2018, **115**:10218-10227.

16. Cesari S, Thilliez G, Ribot C, Chalvon V, Michel C, Jauneau A, Rivas S, Alaux L, Kanzaki H, Okuyama Y *et al.*: **The rice resistance protein pair RGA4/RGA5 recognizes the *Magnaporthe oryzae* effectors AVR-Pia and AVR1-CO39 by direct binding**. *Plant Cell* 2013, **25**:1463-1481.

17. Dong OX, Ao K, Xu F, Johnson KC, Wu Y, Li L, Xia S, Liu Y, Huang Y, Rodriguez E: **Individual components of paired typical NLR immune receptors are regulated by distinct E3 ligases**. *Nat Plants* 2018, **4**:699.

18. Tong M, Kotur T, Liang W, Vogelmann K, Kleine T, Leister D, Brieske C, Yang S, Ludke D, Wiermer M *et al.*: **E3 ligase SAUL1 serves as a positive regulator of PAMP-triggered immunity and its homeostasis is monitored by immune receptor SOC3**. *New Phytol* 2017, **215**:1516-1532.

19. Liang W, van Wersch S, Tong M, Li X: **TIR-NB-LRR immune receptor SOC 3 pairs with truncated TIR-NB protein CHS 1 or TN 2 to monitor the homeostasis of E3 ligase SAUL 1**. *New Phytol* 2018, **221**:2054-2066.

20. Deng Y, Zhai K, Xie Z, Yang D, Zhu X, Liu J, Wang X, Qin P, Yang Y, Zhang G: **Epigenetic regulation of antagonistic receptors confers rice blast resistance with yield balance**. *Science* 2017, **355**:962-965.

21. Grant MR, McDowell JM, Sharpe AG, de Torres Zabala M, Lydiate DJ, Dangl JL: **Independent deletions of a pathogen-resistance gene in Brassica and Arabidopsis**. *Proc Nat Acad Sci U S A* 1998, **95**:15843-15848.

22. Carter ME, Helm M, Chapman A, Wan E, Restrepo Sierra AM, Innes R, Bogdanove AJ, Wise RP: **Convergent evolution of effector protease recognition by Arabidopsis and barley**. *Mol Plant-Microbe Interact* 2018 http://dx.doi.org/10.1094/MPMI-07-18-0202-FI.

23. Schultink A, Qi T, Bally J, Staskawicz B: **Using forward genetics**
• **in *Nicotiana benthamiana* to uncover the immune signaling pathway mediating recognition of the *Xanthomonas perforans* effector XopJ4**. *New Phytol* 2018, **221**:1001-1009.
This paper establishes forward genetics in *N. benthamiana*, and it shows that the diverse effector recognition platform of ZAR1 and class XII RLCKs is conserved in distant plant species.

24. Lewis JD, Lee AH-Y, Hassan JA, Wan J, Hurley B, Jhingree JR, Wang PW, Lo T, Youn J-Y, Guttman DS: **The Arabidopsis ZED1 pseudokinase is required for ZAR1-mediated immunity induced by the *Pseudomonas syringae* type III effector HopZ1a**. *Proc Nat Acad Sci U S A* 2013, **110**:18722-18727.

25. Seto D, Koulena N, Lo T, Menna A, Guttman DS, Desveaux D:
• **Expanded type III effector recognition by the ZAR1 NLR protein using ZED1-related kinases**. *Nat Plants* 2017, **3** http://dx.doi.org/10.1038/nplants.2017.1027.
This paper further demonstrates that ZAR1 can use multiple ZED1-related kinases to recognize a diverse set of effectors. The authors show that together ZAR1 and ZRK3 mediate HopF2a resistance.

26. Wang G, Roux B, Feng F, Guy E, Li L, Li N, Zhang X, Lautier M, Jardinaud M-F, Chabannes M: **The decoy substrate of a pathogen effector and a pseudokinase specify pathogen-induced modified-self recognition and immunity in plants**. *Cell Host Microbe* 2015, **18**:285-295.

27. Helm M, Qi M, Sarkar S, Yu H, Whitham SA, Innes RW:
• **Engineering a decoy substrate in soybean to enable recognition of the Soybean Mosaic Virus NIa protease**. *Mol Plant Microbe Interact* 2019 http://dx.doi.org/10.1094/MPMI-12-18-0324-R.
This preprint furthers NLR engineering in a crop species. The authors show that a PBS1 decoy protein modified to contain a cleavage site for the Soybean mosaic virus (SMV) NIa protease can trigger cell death when cleaved.

28. Kim SH, Qi D, Ashfield T, Helm M, Innes RW: **Using decoys to expand the recognition specificity of a plant disease resistance protein**. *Science* 2016, **351**:684-687.

29. Zhang J, Li W, Xiang T, Liu Z, Laluk K, Ding X, Zou Y, Gao M, Zhang X, Chen S: **Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector**. *Cell Host Microbe* 2010, **7**:290-301.

30. Qi T, Seong K, Thomazella DPT, Kim JR, Pham J, Seo E, Cho M-J,
•• Schultink A, Staskawicz BJ: **NRG1 functions downstream of EDS1 to regulate TIR-NLR-mediated plant immunity in *Nicotiana benthamiana***. *Proc Nat Acad Sci U S A* 2018, **114**: E10979-E10987.
The authors show via Co-IP that NbNRG1 self-associates and interacts with NbEDS1 in *N. benthamiana*. Authors also suggest that EDS1 functions upstream of NRG1s based on cell death activity of NbNRG1 in an eds1 mutant *N. benthamiana*.

31. Wu CH, Abd-El-Haliem A, Bozkurt TO, Belhaj K, Terauchi R,
•• Vossen JH, Kamoun S: **NLR network mediates immunity to diverse plant pathogens**. *Proc Natl Acad Sci U S A* 2017, **114**:8113-8118.
These authors reveal a complex genetic network of helper NRC proteins that function with the evolutionary-related sNLRs in Solanaceae. The authors propose a model, in which NLR networks increase the robustness of immune signaling to counteract rapidly evolving pathogens.

32. Wu CH, Belhaj K, Bozkurt TO, Birk MS, Kamoun S: **Helper NLR proteins NRC2a/b and NRC3 but not NRC1 are required for Pto-mediated cell death and resistance in *Nicotiana benthamiana***. *New Phytol* 2016, **209**:1344-1352.

33. Collier SM, Hamel LP, Moffett P: **Cell death mediated by the N-terminal domains of a unique and highly conserved class of NB-LRR protein**. *Mol Plant Microbe Interact* 2011, **24**:918-931.

34. Bonardi V, Tang S, Stallmann A, Roberts M, Cherkis K, Dangl JL: **Expanded functions for a family of plant intracellular immune receptors beyond specific recognition of pathogen effectors**. *Proc Natl Acad Sci U S A* 2011, **108**:16463-16468.

35. Castel B, Ngou PM, Cevik V, Redkar A, Kim DS, Yang Y, Ding P,
•• Jones JD: **Diverse NLR immune receptors activate defence via the RPW 8-NLR NRG 1**. *New Phytol* 2018, **222**:966-980.

The authors test the ability of a wide array of TNL sensors to mediate resistance in double nrg1 Arabidopsis background and nrg1 *N. benthamiana*. NRG1 is shown to be required for the hypersensitive cell-death response and full oomycete resistance mediated by TNLs, but NRG1 is not required for salicylic acid induction or bacterial resistance.

36. Wu Z, Li M, Dong OX, Xia S, Liang W, Bao Y, Wasteneys G, Li X:
•• **Differential regulation of TNL-mediated immune signaling by redundant helper CNL s**. *New Phytol* 2018.
The authors demonstrate that some sensor TNLs differentially use the ADR1 and NRG1 helper families to transduce downstream defense signals. Importantly, the authors also created a plant line devoid of all RNL helper NLRs that can be used to test for synergistic effects of ADR1s and NRG1s in plant immunity.

37. Peart JR, Mestre P, Lu R, Malcuit I, Baulcombe DC: **NRG1, a CC-NB-LRR protein, together with N, a TIR-NB-LRR protein, mediates resistance against tobacco mosaic virus**. *Curr Biol* 2005, **15**:968-973.

38. Gabriëls SH, Vossen JH, Ekengren SK, van Ooijen G, Abd-El-Haliem AM, van den Berg GC, Rainey DY, Martin GB, Takken FL, de Wit PJ *et al.*: **An NB-LRR protein required for HR signalling mediated by both extra- and intracellular resistance proteins**. *Plant J* 2007, **50**:14-28.

39. Li Y, Zhang Y, Wang QX, Wang TT, Cao XL, Zhao ZX, Zhao SL, Xu YJ, Xiao ZY, Li JL: **RESISTANCE TO POWDERY MILDEW 8.1 boosts pattern-triggered immunity against multiple pathogens in Arabidopsis and rice**. *Plant Biotechnol J* 2018, **16**:428-441.

40. Zhong Y, Cheng ZM: **A unique RPW8-encoding class of genes that originated in early land plants and evolved through domain fission, fusion, and duplication**. *Sci Rep* 2016, **6** http://dx.doi.org/10.1038/srep32923.

41. Meyers BC, Kozik A, Griego A, Kuang H, Michelmore RW: **Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis**. *Plant Cell* 2003, **15**:809-834.

42. Collier SM, Moffett P: **NB-LRRs work a "bait and switch" on pathogens**. *Trends Plant Sci* 2009, **14**:521-529.

43. Shao ZQ, Xue JY, Wu P, Zhang YM, Wu Y, Hang YY, Wang B, Chen JQ: **Large-scale analyses of angiosperm nucleotide-binding site-leucine-rich repeat genes reveal three anciently diverged classes with distinct evolutionary patterns**. *Plant Physiol* 2016, **170**:2095-2109.

44. Zhang Y, Xia R, Kuang H, Meyers BC: **The diversification of plant NBS-LRR defense genes directs the evolution of microRNAs that target them**. *Mol Biol Evol* 2016, **33**:2692-2705.

45. Zhang Q, Ma C, Zhang Y, Gu Z, Li W, Duan X, Wang S, Hao L, Wang Y, Wang S: **A single-nucleotide polymorphism in the promoter of a hairpin RNA contributes to *Alternaria alternata* leaf spot resistance in apple (Malus× domestica)**. *Plant Cell* 2018, **30**:1924-1942.

46. Rairdan GJ, Collier SM, Sacco MA, Baldwin TT, Boettrich T, Moffett P: **The coiled-coil and nucleotide binding domains of the Potato Rx disease resistance protein function in pathogen recognition and signaling**. *Plant Cell* 2008, **20**:739-751.

47. Brendolise C, Martinez-Sanchez M, Morel A, Chen R, Dinis R, Deroles S, Peeters N, Rikkerink EHA, Montefiori M: **NRG1-mediated recognition of HopQ1 reveals a link between PAMP and Effector-triggered Immunity**. *bioRxiv* 2018 http://dx.doi.org/10.1101/293050.

48. Dong OX, Tong M, Bonardi V, El Kasmi F, Woloshen V, Wunsch LK, Dangl JL, Li X: **TNL-mediated immunity in Arabidopsis requires complex regulation of the redundant ADR1 gene family**. *New Phytol* 2016, **210**:960-973.

49. Roberts M, Tang S, Stallmann A, Dangl JL, Bonardi V: **Genetic requirements for signaling from an autoactive plant NB-LRR intracellular innate immune receptor**. *PLoS Genet* 2013, **9** http://dx.doi.org/10.1371/journal.pgen.1003465.

50. Grech-Baran M, Witek K, Szajko K, Witek AI, Morgiewicz K, Wasilewicz-Flis I, Jakuczun H, Marczewski W, Jones JD, Hennig J: **Extreme resistance to potato virus Y in potato carrying the Ry gene is mediated by a TIR-NLR immune receptor**. *bioRxiv* 2018 http://dx.doi.org/10.1101/445031.

51. Meyers BC, Morgante M, Michelmore RW: **TIR-X and TIR-NBS proteins: two new families related to disease resistance TIR-NBS-LRR proteins encoded in Arabidopsis and other plant genomes**. *Plant J* 2002, **32**:77-92.

52. Nishimura MT, Anderson RG, Cherkis KA, Law TF, Liu QL,
• Machius M, Nimchuk ZL, Yang L, Chung EH, El Kasmi F *et al.*: **TIR-only protein RBA1 recognizes a pathogen effector to regulate cell death in Arabidopsis**. *Proc Natl Acad Sci U S A* 2017, **114**: E2053-E2062.
The authors isolated the TIR-only protein RBA1 which confers resistance to HopBA1. Both dimerization-interfaces, as in canonical TNLs, are required for RBA1-mediated cell death signaling activity. This paper furthered the understanding about how 'truncated' NLRs function in immunity.

53. Loquet A, Saupe SJ: **Diversity of amyloid motifs in NLR signaling in fungi**. *Biomolecules* 2017, **7** http://dx.doi.org/10.3390/biom7020038.

54. Daskalov A, Habenstein B, Martinez D, Debets AJ, Sabaté R, Loquet A, Saupe SJ: **Signal transduction by a fungal NOD-like receptor based on propagation of a prion amyloid fold**. *PLoS Biol* 2015, **13** http://dx.doi.org/10.1371/journal.pbio.1002059.

55. Le H, Harton J: **Pyrin- and CARD-only proteins as regulators of NLR functions**. *Front Immunol* 2013, **4** http://dx.doi.org/10.3389/fimmu.2013.00275.

56. Zhang X, Bernoux M, Bentham AR, Newman TE, Ve T, Casey LW,
• Raaymakers TM, Hu J, Croll TI, Schreiber KJ: **Multiple functional self-association interfaces in plant TIR domains**. *Proc Nat Acad Sci U S A* 2017, **114**:E2046-E2052.
This paper demonstrates that the two interfaces of TIR domains are important for signaling and self-association. The authors show that mutations in either the AE-interface or DE-interface region of the TIR domain disrupt cell-death signaling activity in all tested cases, and self-association in some instances.

57. Casey LW, Lavrencic P, Bentham AR, Cesari S, Ericsson DJ, Croll T, Turk D, Anderson PA, Mark AE, Dodds PN: **The CC domain structure from the wheat stem rust resistance protein Sr33 challenges paradigms for dimerization in plant NLR proteins**. *Proc Nat Acad Sci U S A* 2016, **113**:12856-12861.

58. Daskalov A, Paoletti M, Ness F, Saupe SJ: **Genomic clustering and homology between HET-S and the NWD2 STAND protein in various fungal genomes**. *PLoS One* 2012, **7** http://dx.doi.org/10.1371/journal.pone.0034854.

59. Seuring C, Greenwald J, Wasmer C, Wepf R, Saupe SJ, Meier BH, Riek R: **The mechanism of toxicity in HET-S/HET-s prion incompatibility**. *PLoS Biol* 2012, **10** http://dx.doi.org/10.1371/journal.pbio.1001451.

60. Robinson R: **Prion folding sends a death signal in fungus**. *PLoS Biol* 2015, **13** http://dx.doi.org/10.1371/journal.pbio.1002058.

61. Daskalov A, Habenstein B, Sabaté R, Berbon M, Martinez D, Chaignepain S, Coulary-Salin B, Hofmann K, Loquet A, Saupe SJ: **Identification of a novel cell death-inducing domain reveals that fungal amyloid-controlled programmed cell death is related to necroptosis**. *Proc Natl Acad Sci U S A* 2016, **113**:2720-2725.

62. Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS, Zhang J-G, Alvarez-Diaz S, Lewis R, Lalaoui N, Metcalf D, Webb AI: **The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism**. *Immunity* 2013, **39**:443-453.

63. Quarato G, Guy CS, Grace CR, Llambi F, Nourse A, Rodriguez DA, Wakefield R, Frase S, Moldoveanu T, Green DR: **Sequential engagement of distinct MLKL phosphatidylinositol-binding sites executes necroptosis**. *Mol Cell* 2016, **61**:589-601.

64. Su L, Quade B, Wang H, Sun L, Wang X, Rizo J: **A plug release mechanism for membrane permeation by MLKL**. *Structure* 2014, **22**:1489-1500.

65. Hildebrand JM, Tanzer MC, Lucet IS, Young SN, Spall SK, Sharma P, Pierotti C, Garnier J-M, Dobson RC, Webb AI: **Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and**

necroptotic cell death. *Proc Nat Acad Sci U S A* 2014, **111**:15072-15077.

66. Bentham AR, Zdrzałek R, De la Concepcion JC, Banfield MJ: **Uncoiling CNLs: structure/function approaches to understanding CC domain function in plant NLRs**. *Plant Cell Physiol* 2018, **59**:2398-2408.

67. Cesari S, Moore J, Chen C, Webb D, Periyannan S, Mago R, Bernoux M, Lagudah ES, Dodds PN: **Cytosolic activation of cell death and stem rust resistance by cereal MLA-family CC–NLR proteins**. *Proc Natl Acad Sci U S A* 2016, **113**:10204-10209.

68. Hao W, Collier SM, Moffett P, Chai J: **Structural basis for the interaction between the potato virus X resistance protein (Rx) and its cofactor Ran GTPase-activating protein 2 (RanGAP2)**. *J Biol Chem* 2013, **288**:35868-35876.

69. Shigematsu H, Iida K, Nakano M, Chaudhuri P, Iida H, Nagayama K: **Structural characterization of the mechanosensitive channel candidate MCA2 from *Arabidopsis thaliana***. *PloS One* 2014, **9** http://dx.doi.org/10.1371/journal.pone.0087724.

70. Wang W, Wen Y, Berkey R, Xiao S: **Specific targeting of the Arabidopsis resistance protein RPW8.2 to the interfacial membrane encasing the fungal Haustorium renders broad-spectrum resistance to powdery mildew**. *Plant Cell* 2009, **21**:2898-2913.

71. Kim H, O'Connell R, Maekawa-Yoshikawa M, Uemura T, Neumann U, Schulze-Lefert P: **The powdery mildew resistance protein RPW8.2 is carried on VAMP721/722 vesicles to the extrahaustorial membrane of haustorial complexes**. *Plant J* 2014, **79**:835-847.

72. Berkey R, Zhang Y, Ma X, King H, Zhang Q, Wang W, Xiao S: **Homologues of the RPW8 resistance protein are localized to the extrahaustorial membrane that is likely synthesized de novo**. *Plant Physiol* 2017, **173**:600-613.

73. Dondelinger Y, Declercq W, Montessuit S, Roelandt R, Goncalves A, Bruggeman I, Hulpiau P, Weber K, Sehon CA, Marquis RW *et al.*: **MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates**. *Cell Rep* 2014, **7**:971-981.

74. Huang D, Zheng X, Wang ZA, Chen X, He WT, Zhang Y, Xu JG,
•• Zhao H, Shi W, Wang X *et al.*: **The MLKL channel in necroptosis is an octamer formed by tetramers in a dyadic process**. *Mol Cell Biol* 2017, **37**.
The authors demonstrate the MLKL channel is an octamer comprised two previously identified tetramers, and the octamer is the agent of necroptosis. Because of the inability to detect the N or C termini on the outside of the cells, the authors propose MLKLs span the membrane with their termini inside the cell.

75. El Kasmi F, Chung EH, Anderson RG, Li J, Wan L, Eitas TK, Gao Z, Dangl JL: **Signaling from the plasma-membrane localized plant immune receptor RPM1 requires self-association of the full-length protein**. *Proc Natl Acad Sci U S A* 2017, **114**:E7385-E7394.

76. Mestre P, Baulcombe DC: **Elicitor-mediated oligomerization of the tobacco N disease resistance protein**. *Plant Cell* 2006, **18**:491-501.

77. Roberts M, Tang S, Stallmann A, Dangl JL, Bonardi V: **Genetic requirements for signaling from an autoactive plant NB-LRR intracellular innate immune receptor**. *PLoS Genet* 2013, **9**:e1003465.

78. Kofoed EM, Vance RE: **Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity**. *Nature* 2011, **477**:592-595.

79. Halff EF, Diebolder CA, Versteeg M, Schouten A, Brondijk TH, Huizinga EG: **Formation and structure of a NAIP5-NLRC4 inflammasome induced by direct interactions with conserved N- and C-terminal regions of flagellin**. *J Biol Chem* 2012, **287**:38460-38472.

80. McDowell JM, Dangl JL: **Signal transduction in the plant immune response**. *Trends Biochem Sci* 2000, **25**:79-82.

81. Cui H, Tsuda K, Parker JE: **Effector-triggered immunity: from pathogen perception to robust defense**. *Annu Rev Plant Biol* 2015, **66**:487-511.

82. Yuan P, Jauregui E, Du L, Tanaka K, Poovaiah BW: **Calcium signatures and signaling events orchestrate plant-microbe interactions**. *Curr Opin Plant Biol* 2017, **38**:173-183.

83. Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J: **The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death**. *Plant J* 2000, **23**:441-450.

84. Nanson JD, Kobe B, Ve T, Death: **TIR, and RHIM: self-assembling domains involved in innate immunity and cell-death signaling**. *J Leukoc Biol* 2018, **105**:363-375.

85. Essuman K, Summers DW, Sasaki Y, Mao X, DiAntonio A,
•• Milbrandt J: **The SARM1 toll/interleukin-1 receptor domain possesses intrinsic NAD(+) cleavage activity that promotes pathological axonal degeneration**. *Neuron* 2017, **93**:1334-1343 e1335.
The authors demonstrate that a diverse set of TIR domains from bacteria and archea have NAD+-consuming enzymatic activity. Multiple bacterial and archaeal TIR domains cleave NAD+ into Nam and ADPR, while a subset of TIR domains generate cADPR. Together, these findings strongly suggest that the primordial function of the TIR domain is to cleave NAD+.

86. Essuman K, Summers DW, Sasaki Y, Mao X, Yim AKY, DiAntonio A, Milbrandt J: **TIR domain proteins are an ancient family of NAD(+)-consuming enzymes**. *Curr Biol* 2018, **28**:421-430 e424.

87. Pajuelo D, Gonzalez-Juarbe N, Tak U, Sun J, Orihuela CJ,
•• Niederweis M: **NAD(+) depletion triggers macrophage necroptosis, a cell death pathway exploited by mycobacterium tuberculosis**. *Cell Rep* 2018, **24**:429-440.
The authors show that NAD+ hydrolysis by tuberculosis necrotizing toxin induces necroptosis in mammalian macrophages. However, NAD+ depletion alone is sufficient to activate RIPK3 and MLKL, bypassing the canonical toxin-mediated initiation of the pathway. Importantly, cell death caused by NAD+ depletion is RIPK3- dependent and MLKL-dependent, demonstrating these two proteins to be key mediators of necroptosis.

88. Hunt L, Lerner F, Ziegler M: **NAD–new roles in signalling and gene regulation in plants**. *New Phytol* 2004, **163**:31-44.

89. Zhang Q, Zmasek CM, Cai X, Godzik A: **TIR domain-containing adaptor SARM is a late addition to the ongoing microbe-host dialog**. *Dev Comp Immunol* 2011, **35**:461-468.

90. Durner J, Wendehenne D, Klessig DF: **Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose**. *Proc Natl Acad Sci U S A* 1998, **95**:10328-10333.

91. Petriacq P, de Bont L, Tcherkez G, Gakiere B: **NAD: not just a pawn on the board of plant-pathogen interactions**. *Plant Signal Behav* 2013, **8**:e22477.

92. Zhang X, Bernoux M, Bentham AR, Newman TE, Ve T, Casey LW, Raaymakers TM, Hu J, Croll TI, Schreiber KJ: **Multiple functional self-association interfaces in plant TIR domains**. *Proc Nat Acad Sci U S A* 2017, **114**:E2046-E2052.