

Resistosome and inflammasome: platforms mediating innate immunity

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The nucleotide-binding domain (NBD) and leucine-rich repeat (LRR) containing (NLR) proteins are intracellular immune receptors that sense pathogens or stress-associated signals in animals and plants. Direct or indirect binding of these stimuli to NLRs results in formation of higher-order large protein complexes termed inflammasomes in animals and resistosomes in plants to mediate immune signaling. Here we focus on plant NLRs and discuss the activation mechanism of the ZAR1 resistosome from *Arabidopsis thaliana*. We also outline the analogies and differences between the ZAR1 resistosome and the NLR inflammasomes, and discuss how the structural and biochemical information available on these two large types of protein complexes sheds light on signaling mechanisms of other plant NLRs.

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Introduction

The nucleotide-binding domain (NBD) and leucine-rich repeat (LRR) containing (NLR) proteins are a family of critical intracellular immune receptors in both animals and plants [1,2]. NLRs contain a conserved tripartite domain structure typically carrying an N-terminal signaling domain, a central nucleotide-binding and oligomerization domain (NOD) and a C-terminal leucine-rich repeat (LRR) domain. Similar domain structure is also present in the apoptotic protein Apaf-1. The NOD module can be further divided into an NBD, helical domain 1 (HD1) and a winged helical domain (WHD). Helical domain 2 (HD2) follows NOD in animal NLRs but is

lacking in plant NLRs. Most plant NLRs contain either a coiled-coil (CC) or toll/interleukin 1 receptor/resistance (TIR) domain at their N-termini. NLRs belong to the STAND (signal-transduction ATPases with numerous domains) ATPases of the AAA+ superfamily.

NLRs in animals detect pathogen-associated molecular patterns (PAMPs), host-derived damage-associated molecular patterns (DAMPs) or pathogen-derived effectors in the cytosol to activate innate immunity [3,4]. In response to ligand perception, animal NLRs oligomerize and then recruit pro-caspase-1 directly or through the adaptor molecule apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). This leads to the formation of cytosolic multiprotein complexes termed inflammasomes mediating caspase-1 activation [5–7]. Once activated, caspase-1 matures IL-1 β and IL-18 and cleaves the gasdermin D (GSDMD) substrate to induce pyroptosis, an inflammatory form of cell death [8–11]. The best characterized NLR inflammasomes are the NAIP-NLRC4 pairs [12^{**}, 13^{**}, 14, 15]. NLRs in plants confer specific recognition of pathogen-derived effectors, initiating effector-triggered immunity (ETI) characterized by localized cell death at the site of infection known as hypersensitive response (HR) [1, 16–18]. While sharing conserved structure and function, CC-NLRs and TIR-NLRs vary in their mechanisms of activating the resistance response. EDS1 and the CC-NLRs NRG1s and ADR1/ADR1-Ls function downstream of signaling mediated by TIR-NLRs [19–26], whereas NDR1 is important for signaling mediated by some CC-NLRs [27–30]. Furthermore, the TIR domain of TIR-NLRs was recently shown to have NADase activity, which is required for HR cell death [31, 32]. In contrast, there has been no enzymatic activity assigned to the CC domain. Despite the differences, both CC and TIR domains function as a signaling module to mediate immune responses [22, 33–36]. A recent study showed that the CC-NLR ZAR1 (HOPZ-ACTIVATED RESISTANCE 1) from *Arabidopsis thaliana* forms a higher-order complex called ‘resistosome’ comparable to the NLR inflammasomes [37^{**}]. ZAR1 forming a constitutive complex with RKS1 recognizes the *Xanthomonas campestris* effector AvrAC, which uridylylates PBL2 and enables the modified protein (PBL2^{UMP}) to interact with RKS1 in the preformed complex to activate ZAR1-mediated immunity [38, 39].

In this review, we outline on the structural and biochemical aspects of plant NLRs and discuss the analogies and differences between the ZAR1 resistosome and NLR inflammasomes in their activation.

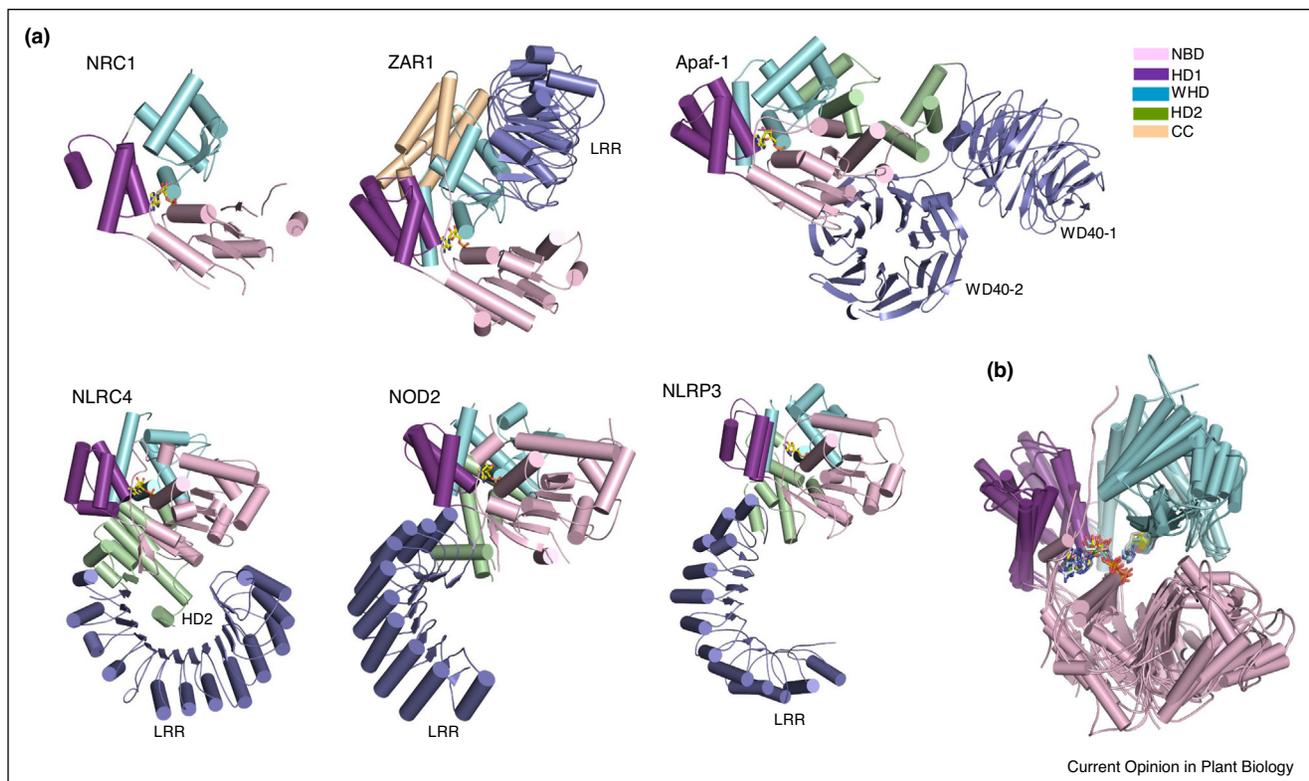
Autoinhibited and activated conformations of NLRs

Thus far, several structures of NLR proteins including ZAR1 [40^{**}], NRC1 [41], NLRC4 [42^{**}], NLRP3 [43^{**}], and NOD2 [44^{*}] in their inactive states have been solved. The NOD modules of these structures are highly conserved with an ADP molecule bound (Figure 1). The bound ADP forms a conserved set of interactions with NBD, HD1, and WHD. Notably, the interaction between WHD and ADP is mediated by a conserved histidine residue (Figure 1), which is from the ‘MHD’ motif of plant NLRs [45]. Mutations of this residue resulted in constitutively active NLRs [45–51], demonstrating an essential role of this interaction in NLR autoinhibition. The inactive conformation structure of NRC1, which only contains NOD, demonstrates that other domains are not required for autoinhibition of the NLR. In contrast with the subdomains of NOD, the C-terminal domains of these proteins (except NRC1) are variably positioned. Nonetheless, structural comparison showed that the C-terminal LRR domains of ZAR1 [40^{**}] and NLRC4 [42^{**}] and C-terminal WD40 domain of Apaf-1

[52^{*}] similarly function to sequester the three proteins in a monomeric state. Unlike the disordered CARDs from the inactive NLRC4 and Apaf-1, the CC domain of ZAR1 (ZAR1^{CC}) contacts with HD1 and WHD. The interaction likely acts to keep the CC domain inactive, because ZAR1^{CC} but not ZAR1^{CC-NBD} is immune-active when expressed in *Nicotiana benthamiana* [53]. HD2 which is lacking in the plant NLRs also contributes to autoinhibition of NLRC4, NLRP3 [43^{**}] and NOD2 [44^{*}].

Structural comparison of ZAR1 [37^{**}], NLRC4 [12^{**},13^{**}] and Apaf-1 [54^{*}] in their active states showed conserved positioning of the three subdomains of NOD (Figure 2). By comparison, the C-terminal domains of these three proteins are presented to strikingly different positions. Structural alignment between inactive and active states of the three proteins revealed that structural remodeling occurs to them during activation [12^{**},13^{**},37^{**},54^{*}]. In all the three cases, the C-terminal segment rotates around the hinge region between WHD and HD1. Our recent cryo-EM studies [37^{**},40^{**}] indicated the existence of an intermediate state between the inactive and active state

Figure 1

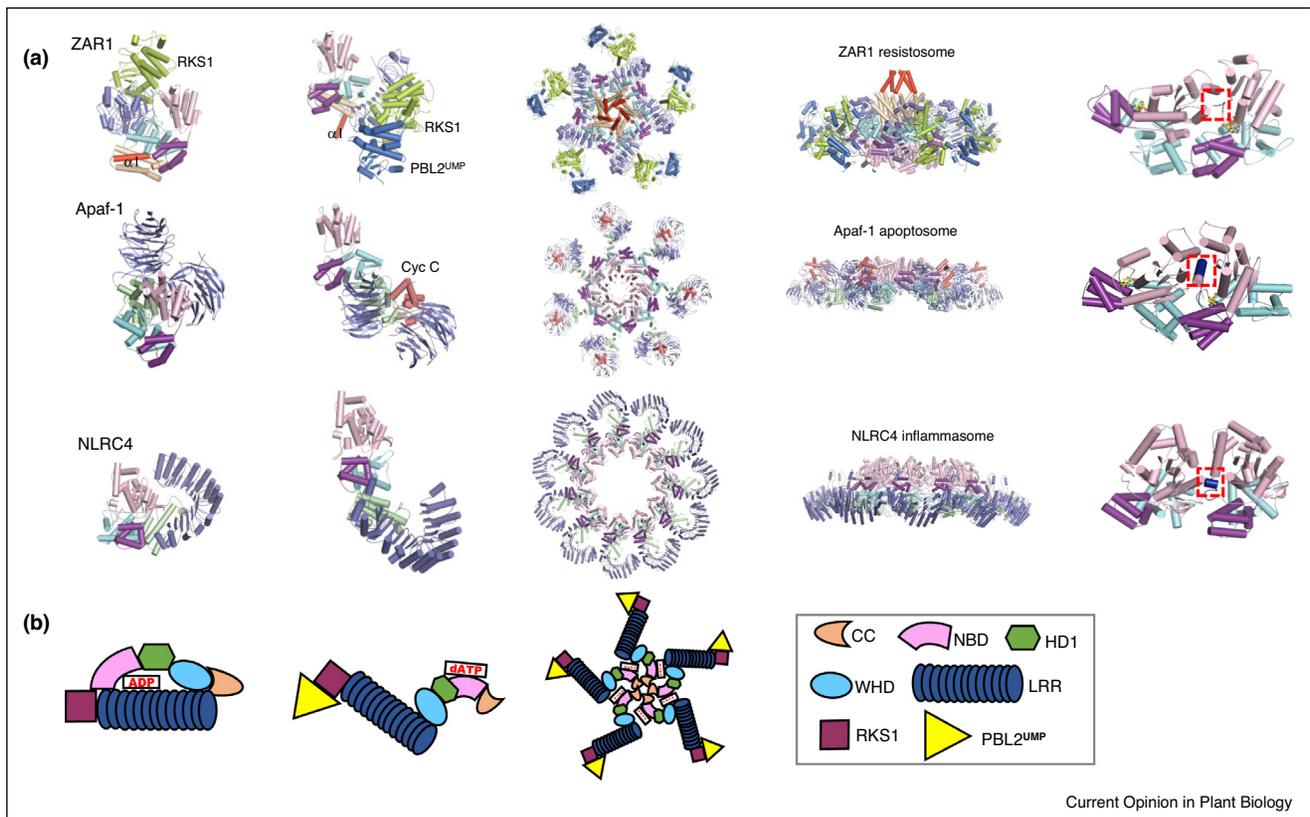


Structural comparison of NLR proteins in their inactive state.

(a) The structures indicated are shown in the same orientation with their NOD (NBD-HD1-WHD) modules aligned. Color codes for some subdomains are indicated at the upper right corner. NBD: nucleotide binding domain; HD1: helical domain 1; WHD: winged helix domain; CC: coiled-coil; LRR: leucine-rich repeat; WD40: also known as beta-transducin repeats with ~40 amino acids in length, often terminating in a Trp-Asp (W-D) dipeptide.

(b) Structural alignment of the NOD modules is shown in (a). The bound ADP molecules and the conserved histidine residues from WHD are shown in stick.

Figure 2



Activation of the ZAR1 resistosome, the Apaf-1 apoptosome and the NLRC4 inflammasome.

(a) The first, second, and third rows show different states of ZAR1, Apaf-1, and NLRC4, respectively. The first and the second columns show the inactive and active states of the three proteins, respectively. Shown in the third and fourth columns are structures of oligomers of the three proteins in two orientations. The last column shows lateral dimeric NODs of ZAR1 (first row), Apaf-1 (second row), and NLRC4 (third row) in the same orientation and the N-terminal segment involved in the homodimerization is highlighted in blue. Color codes are the same as those in Figure 1a.

(b) Cartoon illustration of ligand-induced ZAR1 activation. The first, second, and third panels represent inactive, active, and oligomerized states of ZAR1, respectively. Color codes for proteins and structural domains are shown in the right frame.

of ZAR1. Compared to that in inactive ZAR1, NBD in this state rotates outward about 60 degrees induced by ligand (the uridylylated form of PBL2) binding, which acts to promote exchange of ADP with ATP or dATP. ATP or dATP binding likely restores the conformation of NBD in the intermediate state and triggers conformational changes in the C-terminal segment of ZAR1, thus converting the intermediate state into a fully active one. Thus, sequential conformational changes in ZAR1 are required for its activation. A similar mechanism is likely used by Apaf-1 for formation of the Apaf-1 apoptosome [54^{*}]. Activation of NLRC4 may differ from that of ZAR1 and Apaf-1. (d)ATP does not seem to be essential for formation of the NLRC4 inflammasome. Consistently, the arginine residue required for (d)ATP binding by ZAR1 (Arg297) and Apaf-1 (Arg265) is substituted with threonine. Furthermore, the active conformation of

NLRC4 is self-propagated once activated [12^{**},13^{**}]. These results suggest that NLRC4 activation may not involve formation of an intermediate state.

(d)ATP binding and hydrolysis of NLR proteins

Many NLRs have been demonstrated to require an intact P-loop for their functions *in vivo* [47,50,55–58]. Structural evidence for this comes from studies of ZAR1 and Apaf-1. ATP or dATP is absolutely required for formation of the ZAR1 resistosome [37^{**}] and the Apaf-1 apoptosome [54^{*}]. Structures of active ZAR1 and Apaf-1 showed that dATP/ATP acts to stabilize the active conformation of ZAR1 or Apaf-1 via interaction of the γ -phosphate of the bound dATP/ATP with linker between HD1 and WHD. A double mutation of the two arginine residues coordinating to the γ -phosphate group of the bound dATP resulted in loss of AvrAC-induced ZAR1 oligomerization,

immune response and HR cell death [37**]. In contrast with ZAR1 and other P-loop demanding NLRs, some NLRs do not require an intact P-loop for their function. For example, mutations of P-loop had little effect on the pathogen-induced immune activity of the NLRs RGA5 [59], RRS1 [60] ADR1-L2 [21], and NRG1 [26]. Similarly, flagellin-induced assembly of the NAIP5-NLRC4 inflammasome was not affected by mutations in the P-loop of NAIP5 [61]. Nonetheless, an ATP molecule was found to bind to NAIP5 from the flagellin-induced NLRC4-NAIP5 heterodimeric complex [62**]. However, unlike that of the ZAR1-bound or Apaf-1-bound dATP, the γ -phosphate of the NAIP5-bound ATP does not interact with other domains than NAIP5^{NBD} and NAIP5^{HD1}. This structural observation indicates that the ATP molecule is not involved in stabilization of the active conformation of NAIP5 and provides an explanation for the dispensable role of the P-loop in formation of the NAIP5-NLRC4 inflammasome. In contrast with NAIP5, NLRC4 from the NAIP5-NLRC4 heterodimeric complex or the NLRC4 inflammasome was observed not to bind ATP [62**,63**]. While in an active conformation, NLRC4 in the NAIP5-NLRC4 heterodimeric complex is much less well defined than NAIP5, suggesting that NLRC4 self-association can stabilize its active conformation.

Some of NLRs have been shown to catalyze breakdown of ATP into ADP and free phosphate ion. The biochemical reaction presumably switches NLRs from an active into an inactive conformation. However, whether the NLR proteins are in an active form was not reported in these studies. This is important since inactive Apaf-1 but not Apaf-1 from the apoptosome possesses ATPase activity [64]. This can be case with ZAR1, because the five dATP molecules in the ZAR1 resistosome remain intact. Notably, the ATP-binding site of the Apaf-1/CED-4 apoptosomes [54*,65*] or the ZAR1 resistosome [37**] is composed of residues from the same subunit, whereas those of canonical ATPase comprise residues from two adjacent subunits [66]. Further investigations using active NLR proteins will assist in defining the role of ATPase activity of NLRs in their activation.

Biochemical function of ligand binding of NLRs

Modes of ligand recognition by plant NLRs are diversified including direct and indirect recognition, recognition through paired NLRs and integrated domains [1,67,68]. Regardless of the recognition mechanisms, ligand binding was hypothesized to induce conformational changes in an NLR and consequently promote its oligomerization. Indeed, this hypothesis is supported by cryo-EM studies of ZAR1 [37**,40**] and Apaf-1 [54*], though the two proteins employ different mechanisms for ligand recognition. ZAR1-RKS1-PBL2^{UMP} or Apaf-1-cytochrome c oligomerizes into a large protein complex only in

the presence of ATP or dATP, indicating that either of the two small molecules functions to induce oligomerization. Unexpectedly, PBL2^{UMP} binding induces conformational changes only in the NBD of ZAR1. Supporting the structural observation, PBL2^{UMP} binding to RKS1 substantially impaired the ADP-binding activity of ZAR1 through an allosteric mechanism. This would favor ZAR1 association with ATP or dATP, which in turn induces further conformational changes in ZAR1 for formation of the ZAR1 resistosome. Thus, RKS1 in the preformed ZAR1-RKS1 complex can be understood as an inactive nucleotide exchange factor (NEF), which is activated by PBL2^{UMP} binding. Whether other NLR-guarded host proteins can act as a NEF to mediate effector-triggered NLR activation remains further to be investigated. It is of interest to note that NEFs of Hsp70s are highly diversified in their structures and mechanisms of catalyzing nucleotide exchange despite their conserved biochemical function [69]. Biochemical data showed that cytochrome c functions similarly to promote exchange of ADP with ATP or dATP to induce formation of the Apaf-1 apoptosome [70], suggesting that ligand itself can act as a NEF to activate NLRs. This idea agrees with the equilibrium model proposed for plant NLRs [71], wherein effector binding functions to tilt the equilibrium between inactive and active states of resting NLRs toward the latter form.

NAIPs and NLRC4 are the paired NLRs in which NAIPs specifically recognize bacterial components to induce activation of NLRC4 [72,73]. In contrast with ZAR1 and Apaf-1, ligand binding induces no oligomerization of NAIP5. Cryo-EM studies showed that flagellin-binding functions to stabilize the active conformation of NAIP5 [62**,63**], which interacts with inactive NLRC4 and consequently induces self-activation to form the NAIP5-NLRC4 inflammasome with a stoichiometry of 1:9 or 1:10 between NAIP5 and NLRC4. These results indicate that ligand binding does not necessarily result in oligomerization of an NLR, and the flagellin-activated NAIP5 acts to seed oligomerization of NLRC4. Given that the P-loop is not required for the activation of the NLRC4 inflammasome, these results suggest that ligands of NAIPs are unlikely to act as a NEF. The reason for this might be that NLRC4 employs an induced self-activation mechanism for inflammasome assembly, whereas ZAR1 and Apaf-1 need to be in a fully activated state before assembled into the resistosome and the apoptosome, respectively. Many plant NLRs have been shown function in pairs to perceive effector proteins with one acting as the sensor to recognize ligand and the other as the helper to mediate immune signaling [74], similar to the paired NAIP-NLRC4. In contrast with NAIPs, however, some sensor NLRs in plants have been shown to inhibit the activation of their paired helper NLRs [59,75,76], suggesting that plant and animal paired NLRs might differ in their activation mechanisms. Determination of stoichiometry between the sensor and

the helper NLR would be critical to dissect how plant paired NLRs are activated.

Oligomerization of NLRs

Structures of activated ZAR1, NLRC4 and Apaf-1 [12^{••},13^{••},37^{••},54^{••},65^{••}] have been solved. Despite their low sequence similarity, all the three proteins are capable of forming wheel-like structures (Figure 2). It is noteworthy to mention that, in addition to wheel-like structures, helical and curved structures have been observed for NLR proteins [77–79]. The conserved NOD module has a critical role in assembly of the ZAR1 resistosome, the NLRC4 inflammasome and the Apaf-1 apoptosome. In the three large protein complexes, one side of NBD from one protomer stacks against the opposite side of NBD from a neighboring protomer, resulting in asymmetric and lateral packing of NBDs and formation of the ring-like structures (Figure 2). Interestingly, a non-conserved segment N-terminal to NBD wedges into two neighboring NBDs, making extensive interactions stabilizing the NBD–NBD contacts (Figure 2). Supporting an indispensable role of the N-terminal segment in oligomerization, mutation of Trp150 from the region completely disrupted ZAR1 pentamerization *in vitro* and ZAR1-mediated HR and disease resistance [37^{••}]. The ring-like structures formed by NBDs are further strengthened by alternating contacts between HD1 and WHD1 from adjacent subunits in the resistosome and the apoptosome. In addition to autoinhibition and ligand recognition, the LRR domain participates in oligomerization of ZAR1. A similar role of the LRR domain of NLRC4 was also demonstrated in formation of the NLRC4 inflammasome [12^{••},13^{••}]. A more recent study suggested that the LRR domain is required for NLRP3 activation [43^{••}]. In contrast with the disordered CARDs in the NLRC4 inflammasome and the Apaf-1 apoptosome, the CC domains in the ZAR1 resistosome pack against each other and form an α -helical barrel interacting with the chamber formed by ZAR1^{WHD} and ZAR1^{LRR}. This structural difference implies that ZAR1 and animal NLRs may have distinct signaling mechanisms. Formation of the α -helical barrel structure by ZAR1^{CC} agrees with self-association of ZAR1^{CC} [53]. Thus, all the subdomains are involved in ZAR1 oligomerization. Oligomerization of the CC-NLR RPP7 induced by HR4 (a homolog of RPW8) was recently demonstrated *in vivo* [80]. Interestingly, the LRR domain was found to be required for the HR4-induced RPP7 oligomerization, although the underlying mechanism remains unclear. In addition to RPP7, a series of CC-NLRs oligomerization were also reported *in planta*, including RPM1, Prf, RPS5 [81–83]. Homo-dimerization and hetero-dimerization of TIR domains required for defense signaling is consistent with oligomerization-mediated activation of TIR-NLRs [34,60,84]. Consistently, oligomerization of the TIR-NLRs RPP1 [85], N protein [86], and ROQ1

[23] has been shown before, though how oligomerization is associated with their activation is unclear.

Signaling roles of the N-terminal CC and TIR domains of plant NLRs

Overexpression of TIR or CC domains of many plant NLRs is both necessary and sufficient for HR cell death, indicating that the TIR or CC domain is a signaling module to mediate immune activation. Recent studies provided significant insight into the biochemical functions of these two types of structural domains. Multiple lines of evidence showed that the N-terminal amphipathic helices in the ZAR1 resistosome form a funnel-shaped structure, which is required for ZAR1 association with the plasma membrane (PM) and ZAR1-mediated immune signaling [37^{••}]. The ZAR1 resistosome was therefore proposed to function as a channel or a pore to trigger HR and resistance. Several CC-containing proteins were demonstrated to possess pore-forming activity for cell death [87,88], suggesting that pore or channel formation might be a conserved function of some CC-NLRs. Indeed, a more recent study showed that the N-termini of many CC-NLRs are functionally conserved because the N-terminal amphipathic helix of ZAR1 and the N-termini of several distantly related CC-NLRs can functionally replace that of the CC-NLR NRC4 (NLR required for cell death 4) [89[•]]. However, whether these CC-NLRs can form a structure similar to that of the ZAR1 resistosome for activation of immunity remains unknown. Furthermore, a large percentage (~80%) of CC-NLRs do not carry the function-critical ‘MADA’ motif present in ZAR1 and some other CC-NLRs [89[•]]. Recent studies showed that NRCs act as helper NLRs for activation of this type of CC-NLRs in *Solanaceae* [90–92]. Although further studies are required to understand the underlying mechanism, these studies represent a significant step toward understanding how non-MADA-containing CC-NLRs are activated. In the inactive ZAR1, the pore-forming activity of the N-terminal amphipathic helix is inhibited by contacts with ZAR1^{WHD} and ZAR1^{LRR}. Conformational changes and fold switch are therefore required to release the autoinhibited pore-forming activity of the CC domain [37^{••},40^{••}].

A biochemical function of the TIR domain of plant NLRs was recently revealed by two independent studies [31,32], both of which presented evidence that the TIR domain from several TIR-NLRs or TIR only proteins possess NAD-cleaving activity. Mutations of the putative catalytic residue abolished HR cell death in *N. benthamiana*, suggesting that the enzymatic activity is required to activate downstream signaling. However, these plant TIR proteins caused no depletion of NAD when expressed in plants. The ADPR and v-cADPR (variant cADPR) products were therefore suggested to act as a trigger for HR cell death signaling. But the possibility still remains that other chemicals can also

be the substrates of plant TIR proteins. The NADase activity of TIR domains can be induced by pathogen, suggesting that oligomerization may be important for the enzymatic activity. This is consistent with the observation that mutations disrupting self-interaction interface of TIR resulted in loss of the NADase activity [31,32].

Cellular localizations of plant NLRs

Plant NLRs are intracellular and can localize to the PM, endoplasmic reticulum (ER), Golgi apparatus and tonoplast, chloroplast, nucleus, or cytoplasm [17]. The diverse subcellular localizations of NLRs can presumably allow plants to detect the presence of different pathogen effectors. Coordination of nuclear and cytoplasmic pools of some NLRs such as the CC-NLR Rx in potato has been shown to be important for establishment of HR and immunity [93–95]. Notably, some NLRs alter their subcellular localizations following activation. For example, activation of ZAR1 by AvrAC converts the cytosolic NLR into a PM-localized protein, which is required for ZAR1-mediated HR and immunity [37**]. Another example is the TIR-NLR RPS4, which primarily resides in the endomembranes in unchallenged conditions [96] but becomes nucleus-localized to activate immune responses [97] in response to the AvrRps4 effector. In the case of ZAR1, simultaneous mutation of two negatively charged residues located at the inner surface of the funnel-shaped structure nearly abolished ZAR1-mediated immune responses [37**]. Interestingly, however, the double mutation had little effect on the AvrAC-induced PM localization of ZAR1. Detection of cellular localization of NLRs could be hindered by HR cell death induced by their activation. Therefore, similar mutations if identified in other CC-NLRs would be a useful tool for study of cellular localizations of these NLRs. The putative catalytic residue is important for the NADase activity of TIR domains and activation of downstream signaling [31,32]. Mutations of this residue could serve a similar purpose for investigation of the localizations of TIR-NLRs.

Perspective

During the past ten years, structural and biochemical studies significantly advanced our understanding of the mechanisms of action of NLR proteins. The knowledge gained from these studies sheds light on signaling mediated by plant NLRs. Many important yet challenging questions concerning plant NLRs, however, remains to be answered. One of these is to provide evidence of whether the ZAR1 resistosome functions as a channel or pore to mediate immune signaling. It is still unknown if the resistosome is a trigger or an executor for HR cell death. It is equally important to know whether the mechanisms of the ZAR1 resistosome can be applied to other CC-NLRs. Although NAIP-NLRC4 may act as a template for understanding of plant paired NLRs, our knowledge of how these plant NLRs are activated is still limited. Thus far, there has been no structural

evidence for ligand-induced oligomerization of TIR-NLRs. It will be of interest to know whether and how oligomerization are important for the NADase activity of TIR domains. Moreover, how the enzymatic activity is associated with activation of downstream components such as EDS1 and NRG1 still remains enigmatic. Identification of the product(s) of TIR NADases would be a key step toward addressing this question. NRG1s and ADR1/ADR1-Ls have overlapping but not identical functions [19,21–26]. Future studies will be needed to clarify what determines the divergent functions of these conserved CC-NLRs.

Conflict of interest statement

Nothing declared.

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