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H. H. FLOR DISTINGUISHED REVIEW

New Biochemical Principles for NLR Immunity in Plants

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While working for the United States Department of Agriculture on the North Dakota Agricultural College campus in Fargo, North Dakota, in the 1940s and 1950s, Harold H. Flor formulated the genetic principles for coevolving plant host-pathogen interactions that govern disease resistance or susceptibility. His 'gene-for-gene' legacy runs deep in modern plant pathology and continues to inform molecular models of plant immune recognition and signaling. In this review, we discuss recent biochemical insights to plant immunity conferred by nucleotide-binding domain/leucine-rich-repeat (NLR) receptors, which are major gene-for-gene resistance determinants in nature and cultivated crops. Structural and biochemical analyses of pathogen-activated NLR oligomers (resistosomes) reveal how different NLR subtypes converge in various ways on calcium (Ca^{2+}) signaling to promote pathogen immunity and host cell death. Especially striking is the identification of nucleotide-based signals generated enzymatically by plant toll-interleukin 1 receptor (TIR) domain NLRs. These small molecules are part of an emerging family of TIR-produced cyclic and noncyclic nucleotide signals that steer immune and cell-death responses in bacteria, mammals, and plants. A combined genetic, molecular, and biochemical understanding of plant NLR activation and signaling provides exciting new opportunities for combatting diseases in crops.

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Plants rely on an innate immune system for protection against pathogens and pests. Harold H. Flor's ground-breaking gene-for-gene hypothesis predicted that plant disease resistance to different infectious microbial strains is conferred by simply inherited matching gene pairs—resistance (*R*) genes in the host and avirulence (*AVR*) genes in the pathogen (Flor 1971). This genetic foundation for pathogen race–specific disease resistance in plants paved the way to cloning the first nucleotide-binding (NB) domain/leucine-rich-repeat (LRR) receptor (*NLR*) genes many decades later. NLR-containing receptors are the most prevalent but not the only *R* gene–encoded proteins characterized in model plants and crops (Jones et al. 2016; Sanchez-Martin and Keller 2021). NLR proteins possess a central conserved NB domain for ADP/ATP exchange fused to C-terminal LRRs of varied lengths and generally one of two N termini, namely, a toll-interleukin 1 receptor (TIR) or coiled-coil (CC) domain for downstream signaling (Jones et al. 2016). CC- and TIR-type NLRs are known as CNLs and TNLs, respectively, and both classes have expanded in number and diversified pathogen recognition capacities during land plant evolution (Jones et al. 2016; Liu et al. 2021; Saur et al. 2021). Later studies of NLR functions in plants and mammals and, indeed, NLR-like immunity proteins in bacteria reveal how the NLR multidomain architecture lends itself exquisitely to being a molecular switch for activating immunity and cell-death pathways across cellular kingdoms of life (Gao et al. 2022; Kibby et al. 2022; Ngou et al. 2022b; Xiong et al. 2020).

During an intensive phase of plant NLR biology research in the early 2000s, it became clear that NLR-recognized pathogen *AVR* gene products are members of extensive and often highly variable virulence factor (effector) families (Dodds and Rathjen 2010). Effectors are delivered to host cells by pathogens in order to promote infection, often by disabling pattern-triggered immunity (PTI) mediated by cell-surface pattern recognition receptors (PRRs). Detection of particular effectors by NLR receptors transcriptionally reinstates and potentiates PTI defenses in a pro-

cess called effector-triggered immunity (ETI) (Cui et al. 2015; Ngou et al. 2022a; Rhodes et al. 2022). In *Arabidopsis thaliana*, PTI-ETI cross-potentiation leads to ETI-associated host cell death, called a hypersensitive response (HR), which helps to limit pathogen spread (Ngou et al. 2021; Yuan et al. 2021a). While some NLRs are activated by direct pathogen effector binding (the simplest molecular interpretation of Flor's gene-for-gene model), various iterations of indirect NLR-effector recognition also exist, in which NLRs monitor or 'guard' effector-targeted defense components (baits) or decoys of bait proteins the host has evolved to betray pathogen interference (Dodds and Rathjen 2010; Ngou et al. 2022a). Both direct and indirect NLR effector detection mechanisms are important drivers of host-pathogen coevolution (Buscaill and van der Hoorn 2021; Ngou et al. 2022b; Saur et al. 2021).

Only a few additional (non-NLR) ETI components were identified in initial mutant screens, which were mostly performed in *Arabidopsis*. However the genetic characterization of *Non–Race Specific Resistance1* (*NDR1*), encoding a plasma membrane–tethered integrin-like protein required for resistance triggered by several CNLs (Century et al. 1997; Knepper et al. 2011), and *Enhanced Disease Susceptibility1* (*EDS1*), encoding a lipase-like protein essential for TNL mediated ETI (Aarts et al. 1998; Falk et al. 1999), already indicated mechanistic differences between CNL and TNL receptor signaling, despite gene expression studies in *Arabidopsis* indicating that CNL, TNL, and PRR-triggered responses to bacterial pathogen attack produce qualitatively similar transcriptional outputs (Cui et al. 2015). Thus, different immune receptor signaling systems converge at one or more points prior to nuclear transcription. Notably, immune signaling mediated by different NLRs in animals converges on initiator caspases (Hu and Chai 2023). Further important components of NLR immunity emerged after testing combinatorial mutants, as it became apparent that part of the robustness of ETI in plants is its recruitment of parallel, compensatory defense pathways to limit pathogen growth (Cui et al. 2015). So when one ETI-stimulated pathway or node is compromised genetically or by pathogen disturbance, other functional paralogs or pathways can often step in to maintain resistance.

In this review, we focus principally on biochemical insights to NLR (gene-for-gene) immunity gained over the last few years. The new information helps to establish fundamental working principles for NLR-effector recognition and signaling and creates opportunities for NLR engineering and deployment towards a more sustainable agriculture. Huge technological advances in resolving the structures of protein complexes and molecules, coupled with impressive next-generation genome sequencing and analytical approaches, have transformed understanding of NLR receptor and microbial effector functions that shape plant host-pathogen interactions.

Pathogen Effector–Induced CNL Resistosome Ion Channels

In 2015, two landmark studies using cryo-electron microscopy (cryo-EM) reported the first structures of a pathogen-activated mammalian NLR complex, called an inflammasome, formed between a sensor NLR (NAIP2) and a signaling (or helper) NLR (NLRC4) (Fig. 1) (Hu et al. 2015; Zhang et al. 2015). Upon NAIP2-specific binding of PrgJ, a component of the bacterial type III secretion system (Kofoed and Vance 2011; Zhao et al. 2011), the NAIP2 NLR undergoes a conformational change that triggers self-activation of NLRC4 and the oligomerization of multiple NLRC4 protomers to form an ordered 10- or 11-mer cart wheel-like assembly with a stoichiometry of 1:9 or 1:10 between NAIP2 and NLRC4. The substoichiometric NAIP2-NLRC4 hetero-oligomers bring NLRC4 N-terminal caspase recruitment domains together to form a scaffold for binding initiator caspase enzymes to promote signaling cascades leading to an inflammatory response (Ahn et al. 2023; Hu and Chai 2023). A later cryo-EM-based study showed that a similar mechanism controls activation of the NAIP5-NLRC4 inflammasomes (Tenthorey et al. 2017). More recently, the NLR family pyrin domain containing three (NLRP3) inflammasomes were also found to contain 10 or 11 protomers sharing remarkable structural similarity to the NAIP-NLRC4 inflammasomes (Xiao et al. 2023) (Fig. 1).

Recent cryo-EM approaches resolved the structures of a monomeric (pre-activation) and bacterial effector-activated *Arabidopsis* CNL receptor HopZ-Activated Resistance1 (ZAR1) (Fig. 1) (Wang et al. 2019a, b). In vitro and in vivo ZAR1 biochemical assays revealed that indirect effector recognition results in formation of a pathogen-induced ZAR1 homo-pentamer (called a ZAR1 resistosome). Strikingly, ZAR1 pentamerization promoted NLR association with the plasma membrane and realigned the five N-terminal α-helices in the ZAR1 resistosome to create a nonselective calcium (Ca^{2+})-permeable ion channel (Bi et al. 2021; Wang et al. 2019a). A similar homo-pentameric NLR architecture with autonomous Ca^{2+}-permeable ion channel activity was determined for the wheat CNL receptor Sr35 (Förderer et al. 2022; Zhao et al. 2022), which recognizes the devastating Ug99 isolate of wheat stem rust fungus *Puccinia graminis* f. sp. *tritici* (Salcedo et al. 2017). Like that of ZAR1, the N-terminal α1 helix is indispensable for Sr35-mediated ETI signaling, suggesting a conserved role of this region in CNL functions. Unlike ZAR1, Sr35 is conformationally activated by direct fungal effector binding to the receptor LRR domain (Förderer et al. 2022). The effector binding results in a steric clash of this structural domain with the NB domain, which in turn facilitates nucleotide exchange and consequent pentamerization of Sr35 (Förderer et al. 2022). It is striking that a similar mechanism underlies ZAR1 activation through its indirect effector recognition mode (Wang et al. 2019a, b).

Hence, the ZAR1 and Sr35 homo-pentameric structures likely reveal a common working principle for CNL receptor constraint and specific activation, irrespective of whether effector direct binding (Sr35) or effector-modified host components (ZAR1) drive NLR conformational changes necessary for receptor signaling. It is presumed that CNL receptor-mediated Ca^{2+} influx to the plant cytoplasm promotes Ca^{2+}-dependent signaling cascades that reprogram nuclear transcription for pathogen resistance and, ultimately, host localized cell death. The ZAR1 and Sr35 resistosome structures reveal an entirely new class of homomeric $Ca2^{+}$ channel that can act as a trigger for ETI. This new class of channel may work together with canonical $Ca2^{+}$ channels in the plant immune response, such as cyclic nucleotide-gated $Ca2^{+}$ channels (CNGCs) and other channel families that contribute to PTI and ETI (Koster et al. 2022; Luan and Wang 2021; Parker et al. 2022; Xu et al. 2022). Nuclear localization is required for the disease resistance activities of some CNLs (Lolle et al. 2020), which could directly regulate transcriptional programming in the nucleus via interaction with transcriptional factors (Wang et al. 2021). It is also conceivable that such CNLs form Ca^{2+} channels at the nuclear membrane or at the continuum of the nucleus and another organelle, such as endoplasmic reticulum (ER) (Charpentier et al. 2016; Luan and Wang 2021; Tipper et al. 2023).

CNL Heteromeric Complexes as ETI Sensors and Helpers

A number of other molecularly characterized CNLs (and indeed TNLs) function as cooperative, interacting NLR pairs in

which one NLR acts as a 'sensor' protein and its partner a signal transducer (Xi et al. 2022). Genes for co-functioning NLR pairs can be closely linked in the plant genome and harbor noncanonical integrated domains (IDs). The rice sensor CNLs RGA5 and Pik-1 are well-characterized members of diversified CNL families in cereals that contain IDs and are functionally paired with helper CNLs (Białas et al. 2021; Cesari et al. 2013, 2014; Guo et al. 2018). The ID can serve as a decoy registering effector interference with authentic host targets, thereby converting effector immunity suppression to NLR activation and resistance (Xi et al. 2022). Embedding of IDs within the NLR is part of a host-pathogen coevolutionary dynamic to assert resistance by the host and disarm resistance by new microbial strains (Shimizu et al. 2022; Xi et al. 2022). The genome-enabled discovery and structure-function dissection of NLRs harboring IDs provide crucial positional coordinates for engineering NLR modules with new or broader recognition capacities (Cesari et al. 2022; Kourelis et al. 2023; Maidment et al. 2023; Zdrzalek et al. 2023). It is interesting that rice RGA5 with its helper CNL RGA4 or the related Pik-1 with Pik-2 stay bound to each other after pathogen effector activation, perhaps as a two-tiered decamer or a substoichiometric heterocomplex resembling the human NAIP2/5-nucleated NLRC4 inflammasomes (Hu and Chai 2023). It is not known whether effector-activated CNL pairs have ion channel activities similar to ZAR1 and Sr35.

Further knowledge of CNL sensor-helper functional relationships has come from analyses of the NLR Required for Cell Death (NRC) CNL family containing sensor and helper CNL members that have greatly expanded in asterid clades, which include the Solanaceae (Wu et al. 2017). In *Nicotiana benthamiana*, helper *NRCs* (*NRC1* to *NRC4*) are genetically required for pathogen resistance and host cell death triggered by various sensor CNLs in a partially overlapping manner (Adachi et al. 2019; Ahn et al. 2023; Derevnina et al. 2021; Wu et al. 2017). This fits with the deployment of alternative NRCs in ETI to compensate for the disabling of one or another NRC node by pathogen effectors (Derevnina et al. 2021). The NRC-family proteins possess predicted N-terminal α1-helices that model closely onto and, in two tested cases (NRC3 and NRC4), can be functionally interchanged with the corresponding ZAR1 α1-helical portion

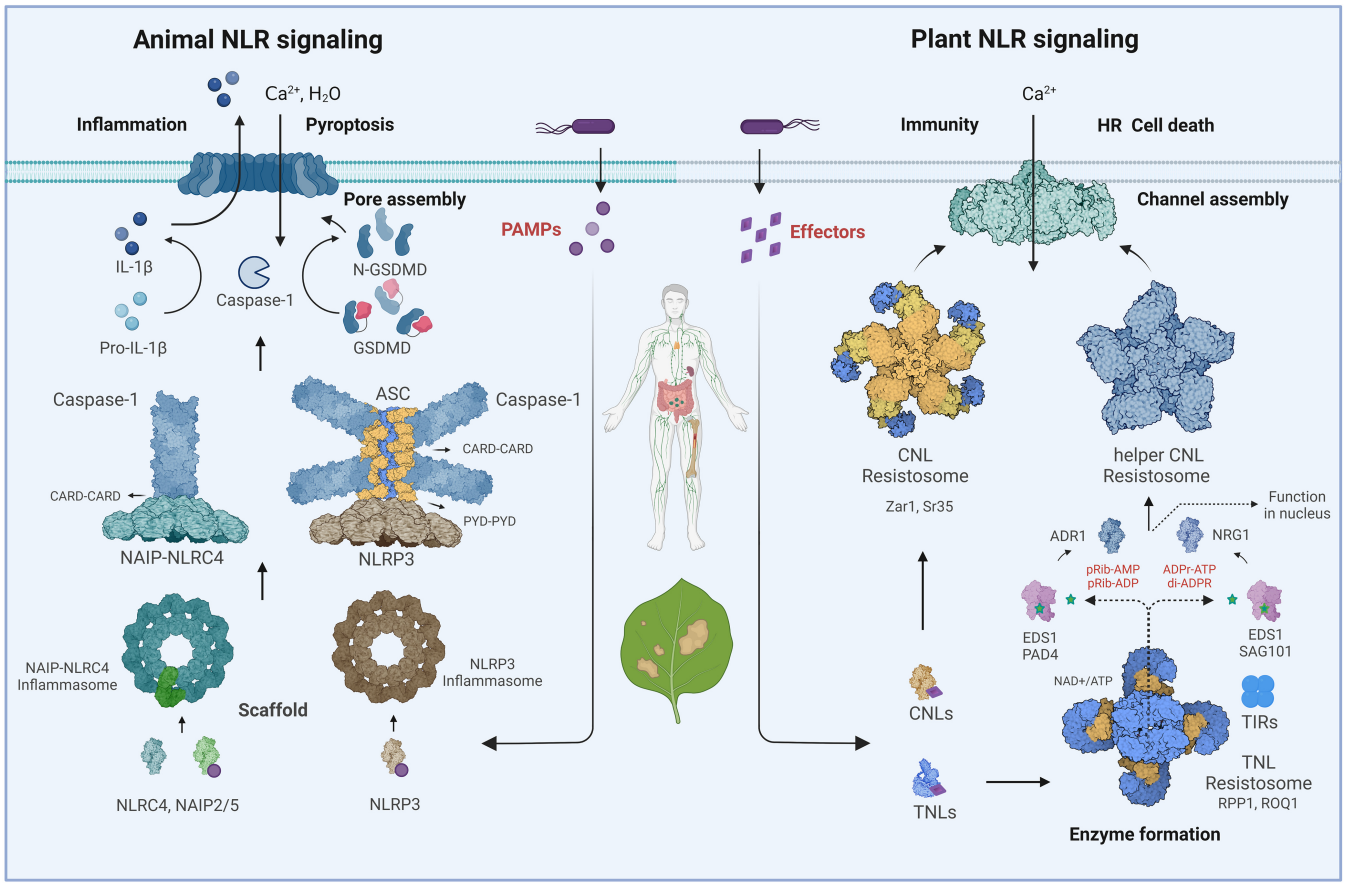

Fig. 1. Unifying concepts for plant and animal NLR (nucleotide-binding domain/leucine-rich-repeat) signaling in NLR immune signaling in animals (left) and plants (right). As illustrated on the left, perception of specific ligands (pathogen-associated molecular patterns [PAMPs]) induces activation and oligomerization of animal NLRs such as NAIP2/5, NLRC4, and NLRP3, resulting in the formation of large protein complexes called inflammasomes. NAIP2/5-NLRC4 and NLRP3 inflammasomes directly or indirectly (mediated by ASC adaptor protein) recruit and mediate the activation of proinflammatory caspases (such as caspase-1). Activated caspase enzymes proteolytically cleave gasdermin D (GSDMD) to release its N-terminal pore-forming domain. This leads to pores at the cell membrane that eventually promote lytic cell death, called pyroptosis. Mature cytokine molecules IL-1β and IL-18, which are processed by caspases, as well as extracellular Ca^{2+} and H_2O pass through the GSDMD pore to promote immunity and cell death. As illustrated on the right, pathogen-delivered effectors induce activation and oligomerization of plant NLRs, leading to the formation of oligomeric NLR complexes called resistosomes. Assembly of the pentameric coiled-coil (CC)-type NLR (CNL) resistosomes ZAR1 and Sr35 stimulates Ca^{2+}-permeable channel activity via exposed N-terminal CC domains that directly target the cell membrane and mediate Ca^{2+} influx to trigger an immune response and host cell death. Assembly of toll-interleukin 1 receptor (TIR)-type NLR (TNL) tetrameric resistosomes orientates the N-terminal TIR domains to form an NADase holoenzyme. The TNL resistosome catalyzes production of small molecules pRib-AMP/ADP and ADPr-ATP/di-ADPR, which, respectively, bind to and activate heteorodimers of the lipase-like protein EDS1 with its exclusive paralog partners PAD4 and SAG101. Small molecule–activated EDS1-PAD4 and EDS1-SAG101 dimers allosterically induce their associations with and Ca^{2+}-permeable channel activities of helper CNLs (ADR1s and NRG1s), leading to pathogen resistance and host cell death.

(Adachi et al. 2019; Kourelis et al. 2022). It is therefore likely that NRCs have ZAR1-related ion channel activities. This is further supported by observed sensor CNL-activated NRC2 and NRC4 self-association and formation of high–molecular weight resistosome-like complexes in vivo (Ahn et al. 2023; Contreras et al. 2023). Intriguingly, two tested effector-activated sensor CNLs that signal via NRCs—Rpi-amr3, recognizing effectors produced by the potato blight disease pathogen *Phytophthora infestans*, and Rx, recognizing the coat protein of potato virus X, were found not to be stably part of the induced NRC complexes (Ahn et al. 2023; Contreras et al. 2023). Hence, an 'activation and release' model was proposed for certain sensor CNLs that signal via helper NRC proteins. It is possible that non-integration of the sensor NLR into an NRC resistosome is energetically favorable. This might be especially important for low concentrations of activated sensor NLR to then amplify defense signals via induced helper NRC resistosomes.

In summary, the current picture of pathogen-induced sensor and helper CNL complexes is of several conformational variations around a unifying model of sensor or helper CNL pentameric resistosome assembly, functional membrane association, and Ca^{2+} permeable ion channel activity to promote ETI resistance and cell death (Fig. 1).

CNL-Like Helper NLRs Contribute to TNL Receptor-Mediated ETI

Like CNLs, the TNLs *Arabidopsis* RPP1 (Recognition of *Peronospora parasitica*1) and *N. benthamiana* Roq1 (Recognition of XopQ1) form resistosomes in response to pathogen effectors (Ma et al. 2020; Martin et al. 2020), suggesting that induced resistosome activity is a conserved property of plant NLRs. In contrast with the pentameric CNL resistosomes, the TNL resistosomes were found to be tetrameric and their assembly to be required for activation of TNL signaling (Fig. 1) (Ma et al. 2020; Martin et al. 2020). Members of two related CNL-like helper NLR subgroups, called Activated Disease Resistance1 (ADR1) and N Requirement Gene1 (NRG1), were discovered to be signaling components for pathogen-sensing TNL receptors (Bonardi et al. 2011; Peart et al. 2005; Qi et al. 2018). These two helper NLR subtypes are phylogenetically distinct from CNLs (Lapin et al. 2019, 2022) but have structurally related CC^{HeLo} (or CC^{R}) N-terminal domains that facilitate their plasma membrane association and oligomerization to form potential Ca^{2+}-permeable ion channels or pores (Feehan et al. 2023; Jacob et al. 2021; Saile et al. 2021). It is interesting that the CC^{HeLo} domain is present in a number of other immunity and cell death–promoting, membrane-associated components in fungi, plants, and mammals, suggesting that this domain is broadly recruited across kingdoms (Feehan et al. 2020; Maekawa et al. 2023).

In *Arabidopsis*, three genetically redundant *ADR1*-family genes, *ADR1*, *ADR1-L1*, and *ADR1-L2*, contribute to various tested TNL ETI responses against different pathogens (Castel et al. 2019; Lapin et al. 2019; Saile et al. 2020; Sun et al. 2021; Wu et al. 2019). The *ADR1*s cooperate with *EDS1*-family immune regulators *EDS1* and *Phytoalexin Deficient4* (*PAD4*) in a pathway that promotes transcriptional defenses and pathogen resistance (Lapin et al. 2020). By contrast, two redundant *Arabidopsis NRG1*-family genes, *NRG1.1* and *NRG1.2*, work exclusively with *EDS1* and a third *EDS1*-family gene *Senescence-Associated Gene101* (*SAG101*) to confer TNL ETI related transcription and host cell death (Lapin et al. 2020). Notably, a third *Arabidopsis NRG1* gene (*NRG1.3*) encodes an N-terminally truncated NRG1 isoform that dampens NRG1.1/NRG1.2–mediated signaling and, thus, probably provides a natural brake on TNL ETI-related host cell death (Wu et al. 2022). Similar homeostatic control of *N. benthamiana* NRC2 immunity signaling by a truncated NRC-X protein was reported (Adachi et al. 2023), underscoring the need to fine-tune helper NLR pathways.

In vivo protein interaction assays and TNL ETI reconstitution experiments in *N. benthamiana*, showed that EDS1-PAD4 heterodimers associate specifically with ADR1 proteins in TNL-activated tissues, whereas EDS1-SAG101 dimers form exclusive TNL-dependent complexes with NRG1s (Sun et al. 2021; Wu et al. 2021). The induced assembly of *Arabidopsis* EDS1-PAD4 complexes with ADR1s and EDS1-SAG101 with NRG1s determines two distinct and partially compensating EDS1 immunity branches (or nodes) (Dongus and Parker 2021). Phylogenomic distribution patterns for *TNL*, *CC^{HeLo} NLR* subclass and *EDS1*-family genes across seed plants (gymnosperms and angiosperms) support a dedicated role of the EDS1-SAG101-NRG1 node in TNL ETI signaling that is restricted to dicot species, because these genes have been lost from monocot and certain dicot lineages (Lapin et al. 2019, 2020; Liu et al. 2021). By contrast, *EDS1*, *PAD4*, and *ADR1* genes occur in all examined seed plant genomes including monocots (cereals and grasses) and a few dicot lineages, which do not contain TNLs but do have TIR-containing proteins (Johanndrees et al. 2023; Lapin et al. 2019, 2020; Liu et al. 2021). This distribution fits with an observed broader defense potentiation role of EDS1-PAD4 with ADR1s in TNL, CNL, and certain cell-surface PRR-triggered immune responses (Bhandari et al. 2019; Dongus and Parker 2021; Pruitt et al. 2021; Tian et al. 2021).

In summary, two related CC^{HeLo}-domain helper NLR subtypes form complexes with EDS1-family non-NLR proteins to execute sensor NLR-triggered immune responses. The existing molecular and structure-guided functional data suggest a quite simple model in which TNL-activated EDS1 dimers facilitate the assembly of CC^{HeLo} NLR pentameric resistosomes with Ca^{2+} permeable ion channel activities at the plasma membrane or endomembranes (Fig. 1) (Jia et al. 2023). In this scenario, EDS1 dimer-enabled CC^{HeLo} NLR oligomers could achieve essentially the same immunity output as ZAR1 or Sr35 CNLs alone and, potentially, asterid NRC helper CNLs primed by sensor CNLs. While this is a compelling model, it does not explain recorded nuclear roles of EDS1 and SAG101 in immunity or the accumulation of a nuclear EDS1-SAG101-NRG1 pool in TNL-triggered tissues (Feehan et al. 2023; Garcia et al. 2010; Zönnchen et al. 2022). It is possible there is more than one subcellular site of action for CC^{HeLo}-NLRs—at the plasma membrane and inside or close to nuclei. These activities might be dynamically regulated within and between cells, depending on inputs and available signaling components.

Versatile TNL Receptor and TIR Protein NADase Enzymes

The TIR domain is a conserved immune module in animals, plants, and bacteria (Jia et al. 2023; Johanndrees et al. 2023; Locci et al. 2023). In animals, as demonstrated for cell-surface toll-like receptor–mediated immune signaling, TIR domains mainly function as adaptors via homotypic interactions (O'Neill and Bowie 2007). Recent ground-breaking studies showed that the animal TIR domain–containing protein SARM1 (sterile alpha and TIR motif containing 1) possesses NAD^+ hydrolyzing and cyclase activities. Human SARM1 catalyzes the production of ADPR and cyclic ADPR (cADPR) to promote neuronal degeneration, likely through depletion of cellular NAD^+ (Essuman et al. 2017, 2018). Inspired by these findings, plant (Horsefield et al. 2019; Wan et al. 2019) and bacterial (Ofir et al. 2021) TIR domain proteins were demonstrated to have related TIR

enzymatic activities. In contrast with SARM1, however, these TIR domains produce two noncanonical isomers of cADPR with 1″-2′ or 1″-3′ *O*-glycosidic bonds linking the two ribose moieties in ADP, which were named 2′cADPR and 3′cADPR, respectively (Bayless et al. 2023; Hulin et al. 2023; Leavitt et al. 2022; Manik et al. 2022). In bacteria, 3′cADPR produced by the TIR-domain enzymatic protein ThsB has an important role in bacterial defense against phage infection through its generation of 3′cADPR, which then activates a ThsA executor NADase in the Thoeris anti-phage system (Leavitt et al. 2022).

Functions of the two above noncanonical cyclic nucleotides in plants remain less well-defined (Jia et al. 2023; Locci et al. 2023). Interestingly, 2′cADPR and 3′cADPR can be efficiently produced by the *Pseudomonas syringae* pv. *tomato* TIR-domain effectors HopAM1 (Eastman et al. 2022) and HopBY (Hulin et al. 2023), respectively. Both cyclic nucleotides are associated with inhibition of plant immunity. Although mechanisms underlying bacterial TIR effector immune-inhibitory activities remain elusive, these results argue against an immunity-triggering role of the 2′cADPR and 3′cADPR cyclic nucleotides in plants. This model is consistent with the observation that a TIR protein (AbTir) from *Acinetobacter baumannii* bacteria catalyzes production of 2′cADPR but fails to induce *EDS1*-dependent cell death in *N. benthamiana* (Duxbury et al. 2020).

Given the indispensable role of TIR-domain NADase activity and EDS1 dimers in TNL and TIR-protein signaling (Lapin et al. 2022), it was widely postulated that plant TIR-catalyzed nucleotide molecules activate EDS1 dimers to promote CC^{Helo}-domain helper NLR-dependent signaling. The chemical identities of these hypothesized molecules remained unknown until recently. Four structurally related noncyclic compounds—phophoribosyl adenosine mono (pRib-AMP), phophoribosyl diphosphate (ADP), ADP-ribosylated-ADPR (di-ADPR), and ADP-ribosylated ATP (ADPr-ATP)—were identified as products of the TIR domain from TNL RPS4 and effector-activated full-length TNL RPP1 (Huang et al. 2022; Jia et al. 2022). Besides NADase activity, an ADP-ribosyl transferase activity is also required for TIR-mediated production of these four nucleotide derivatives. pRib-AMP/ADP and di-ADPR/ADPr-ATP are specifically recognized by EDS1-PAD4 and EDS1-SAG101, respectively (Fig. 1). Structural and biochemical data showed that pRib-AMP/ADP and di-ADPR/ADPr-ATP specific binding allosterically induces EDS1-PAD4 interaction with ADR1 and EDS1-SAG101 with NRG1, respectively (Huang et al. 2022; Jia et al. 2022), presumably leading to activation of helper NLR resistosomes with Ca^{2+}-permeable channel activities (Feehan et al. 2023; Jacob et al. 2021). In vivo studies verified that small molecule binding sites in the two *Arabidopsis* EDS1 heterodimers are indeed responsible for their induced associations with co-functioning CC^{Helo}-domain helper NLRs and for immunity (Dongus et al. 2022). Thus, EDS1-PAD4 and EDS1-SAG101 are receptors for endogenous TIR-catalyzed ribosylated nucleotide second messengers (Fig. 1).

The TIR/TNL-produced nucleotide derivatives enable specific recruitment of different helper NLRs by EDS1 heterodimers, resulting in distinctive immune outputs (Castel et al. 2019; Dongus et al. 2022; Lapin et al. 2019; Qi et al. 2018; Wu et al. 2019). Importantly, a TIR-only protein (BdTIR) from the monocot *Brachypodium distachyon*, which lacks TNLs, NRG1, and SAG101, also induced *Arabidopsis* EDS1-PAD4 interaction with ADR1-L1 and EDS1-SAG101 interaction with NRG1A when expressed in insect cells (Huang et al. 2022; Jia et al. 2022). This suggests that TIR-catalyzed production of second messengers is conserved across seed plant species. Notably, expression of the *P. syringae* pv. *tomato* HopAM1 effector failed to induce EDS1-PAD4 interaction with ADR1-L1 in an in-vitro assay (Huang et al. 2022), consistent with phytobacteria-generated cADPR molecules inhibiting rather than stimulating plant immunity (Eastman et al. 2022; Hulin et al. 2023). In addition to the NADase and cyclase activities, TIR domain proteins such as the *Arabidopsis* TIR-only Response to HopBA1 (RBA1) and the TIR domain of the TNL L7 (L7 TIR) also displayed an activity of degrading nucleic acids, particularly double-stranded RNA (dsRNA) (Yu et al. 2022), although it remains unknown whether dsRNA is an in vivo substrate. TIR-catalyzed hydrolysis of dsRNA/dsDNA resulted in the production of 2′,3′-cAMP/cGMP, which boosted EDS1 signaling (Yu et al. 2022). 2′,3′-cAMP was shown to induce expression of many stress-related *Arabidopsis* genes (Chodasiewicz et al. 2022). Thus, 2′,3′-cAMP/cGMP could serve as a signal to upregulate expression of genes required for EDS1 signaling and other stress responses.

Self-association is important for TIR domain functions (Lapin et al. 2022; Zhang et al. 2017). The cryo-EM structures of the RPP1 and Roq1 resistosomes (Ma et al. 2020; Martin et al. 2020) provided a first glimpse of how TIR self-association enables their enzymatic activity. In the two TNL oligomeric structures NB domain–mediated tetramerization brings the four TIR domains into proximity, forming two head-to-tail TIR homodimers to create two composite catalytic sites (Ma et al. 2020; Martin et al. 2020). Notably, a similar mechanism was also discovered for the activation of SARM1 (Shi et al. 2022) and bacterial TIR proteins AbTIR (Manik et al. 2022) and TIR-SAVED (Hogrel et al. 2022), suggesting a conserved arrangement of TIR domains for NADase activity. By contrast, a head-to-tail dimerization mode was not observed in filaments formed by L7-TIR bound by dsDNA (Yu et al. 2022). It was proposed that plant TIR domain proteins act like Lego bricks for different enzymatic activities (Jia et al. 2023), and it would be of interest to determine whether TIR domain proteins from other species have this remarkable property. The arrangement of L7 TIR domains in filaments is incompatible with that of TIR domains in the TNL resistosomes, suggesting that TNL resistosomes do not have 2′,3′-cAMP/cGMP synthetase activity. Indeed, a predicted cyclic synthetase activity of *Arabidopsis* TNL protein SNC1 was found to be dispensable for immune signaling (Tian et al. 2022).

Although the biosynthesis pathways for TIR/TNL-catalyzed nucleotide derivatives remain poorly defined, available data indicate that plant TIR domains are versatile enzymes with capacities for NADase, cyclase, ADP-ribosyl transferase, and nuclease activities. Current evidence also suggests that different TIR-catalytic nucleotide-based products are utilized by bacteria, mammals, and plants.

Outlook

Since Flor's formulation of the gene-for-gene model highlighting the importance of specific recognition events for effective disease resistance, many plant NLRs and their cognate pathogen effectors have been identified and characterized. Genetic, genomic and, more recently, biochemical advances have laid the foundation for understanding NLR immunity mechanisms and the evolutionary dynamics underlying host-pathogen interactions in nature and agriculture. Despite progress over the past decades, there are numerous outstanding questions in NLR biology. So far, only a small fraction of cognate pathogen effectors have been identified for agronomically important plant NLRs. Thus, many NLRs remain "orphan receptors." Identification of their matching effectors will enhance our understanding of NLR signaling processes and provide more templates for engineering of NLRs with novel specificities. Also, it is unknown whether CNL resistosomes, as noncanonical Ca^{2+}-permeable channels, are subject to similar regulation as CNGCs and other canonical Ca^{2+} channels contributing to plant immune responses. In this regard, phosphorylation found to regu-

late the activation of the *Arabidopsis* RRS1-R/RPS4 TNL pair (Guo et al. 2020) points to additional layers of NLR regulation. The available data suggest that NLR resistosomes converge on Ca^{2+} signaling cascades for promoting pathogen resistance and host cell death. Elucidating how NLR-activated Ca^{2+} signals are decoded will be a critical step in the dissection of NLR signaling. Many CNLs have atypical and likely non–channel forming CC domains whose modes of action remain to be resolved. A further burning question is how nuclear localization of some NLRs is associated with their transcriptionally reprogramming activities. Also, TIRs and TNLs catalyze the production of many nucleotide derivatives, including second messengers. The extent to which different nucleotide molecules cooperate in determining plant immunity outputs and whether their actions are spatially regulated across cells and tissues (Jacob et al. 2023; Yu et al. 2022; Zavaliev et al. 2020) will be important to resolve. Given that EDS1 is a hub for stress responses in different seed plant species, it is also worth exploring whether nucleotide-based small molecules feeding directly or indirectly to EDS1 have broader roles in plant resilience to environmental stresses. In *Arabidopsis*, ETI and PTI were recently demonstrated to cross-potentiate each other for mounting a strong immune response (Yuan et al. 2021b). How these receptor systems communicate and whether this phenomenon is widespread in plants is still unclear. The field of plant innate immunity is positioned to address these and other questions using a combination of genetics, biochemistry, and structural biology, with a horizon to design new sustainable crop protection strategies firmly in sight.

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