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Review

NLR signaling in plants: from resistosomes to second messengers

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Nucleotide binding and leucine-rich repeat-containing receptors (NLRs) have a critical role in plant immunity through direct or indirect recognition of pathogen effectors. Recent studies have demonstrated that such recognition induces formation of large protein complexes called resistosomes to mediate NLR immune signaling. Some NLR resistosomes activate Ca^{2+} influx by acting as Ca^{2+}-permeable channels, whereas others function as active NADases to catalyze the production of nucleotide-derived second messengers. In this review we summarize these studies on pathogen effector-induced assembly of NLR resistosomes and resistosome-mediated production of the second messengers of Ca^{2+} and nucleotide derivatives. We also discuss downstream events and regulation of resistosome signaling.

Highlights

Pathogen effector-induced assembly of resistosomes has been established as an important event for nucleotide binding and leucine-rich repeat-containing receptor (NLR) signaling in plants.

The pentameric coiled-coil domain-containing NLR (CNL) resistosomes act as Ca^{2+}-permeable channels, whereas the tetrameric Toll-interleukin 1-like receptor (TIR) NLR (TNL) resistosomes are NADase holoenzymes.

TNL resistosomes catalyze the production of nucleotide-derived second messengers to activate the downstream helper NLRs activated disease resistance 1 (ADR1) and N requirement gene 1 (NRG1) of the CNL class. Thus, CNLs and TNLs converge on Ca^{2+} signals to trigger plant immunity.

NLR signaling cross-talks with pattern-triggered immunity (PTI) signaling pathways.

NLR signaling pathways in plants are negatively regulated by both hosts and pathogens.

The plant immune system

Plants rely on multiple receptors to detect invading microbial pathogens and mount immune responses [1,2]. One subfamily of plant immune receptors are **pattern-recognition receptors (PRRs)** (see Glossary) at the cell surface [1–3]. PRRs recognize **pathogen-associated molecular patterns (PAMPs)** or host-derived **damage-associated molecular patterns (DAMPs)**, leading to **pattern-triggered immunity (PTI)**.

PTI constitutes the first line of inducible plant defense against pathogens. Some pathogens can breach this layer of defense by secreting **effector proteins** into plant cells to dampen PTI. To counteract the virulence activity of the pathogen effectors, plants have evolved a second subfamily of immune receptors: intracellular NLRs. NLRs specifically recognize effector proteins, inducing **effector-triggered immunity (ETI)** and confer race-specific resistance to pathogens at the site of pathogen entry [1,2,4].

PRRs and NLRs have different structures and subcellular localizations but mediate conserved downstream immune responses, including Ca^{2+} influx, bursts of reactive oxygen species (ROS), production of phytocytokines and defense phytohormones, and transcriptional reprogramming [1,2]. Probably for this reason, PTI and ETI are tightly connected [5,6]. However, PTI and ETI differ in timing, amplitude, and duration of defense, which could be important in determining their different physiological outcomes. In addition to these responses, ETI also includes a hypersensitive response (HR), a form of rapid localized programmed cell death at the site of infection [7].

NLRs are the largest intracellular immune receptors with hundreds of distinct members in different plant species [4]. NLRs have two conserved domains: a central nucleotide-binding and oligomerization domain (NOD), and a C-terminal leucine-rich repeat (LRR) domain. A variable Toll-interleukin 1-like receptor (TIR) or coiled-coil (CC) domain is attached at the N terminus, resulting in TIR-NLR (TNL) or CC-NLR (CNL), respectively [8,9]. In addition to pathogen-sensing NLRs,

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there are some **helper (h) NLRs** which function to translate signals from pathogen-sensing NLRs into ETI responses [10,11]. Examples of hNLRs include activated disease resistance 1 (ADR1) and N requirement gene 1 (NRG1) of the Resistance to Powdery Mildew 8 (RPW8) CNL family [12–15] (Figure 1) and NLRs required for cell death (NRCs) [16,17]. More recent studies have demonstrated that ETI signaling, mediated by many **sensor NLRs** in members of the Solanaceae, depends on NRCs which form **resistosomes** upon activation [18,19].

In this review we summarize the activation and assembly of NLR resistosomes and discuss their downstream **second messengers**, including calcium ion and nucleotide derivations. We also review the crosstalk between ETI and PTI signaling pathways and negative regulation of NLR signaling by pathogen effectors and host regulators.

Assembly of NLR resistosomes

NLRs can recognize effectors directly or indirectly. For example, direct interaction of the *Nicotiana benthamiana* TNL Roq1 (recognition of XopQ 1) with its recognized effector XopQ (*Xanthomonas* outer protein Q) [20,21], the arabidopsis TNL RPP1 (recognition of *Peronospora parasitica* 1) with effector ATR1 (*Arabidopsis thaliana* recognized 1) [22,23], and the wheat CNL Sr35 (stem rust resistance gene 35) with effector AvrSr35 [24–26] confer resistance against *Xanthomonas* spp, *Hyaloperonospora parasitica*, and *Puccinia graminis tritici*, respectively. Some NLRs recognize their cognate effectors by monitoring effector-mediated perturbations of host targets. One example of this is the arabidopsis CNL ZAR1 (HOPZ-ACTIVATED RESISTANCE 1) [27], which exists in a preformed complex with a host kinase RKS1 (resistance-related kinase 1) in normal conditions and recognizes the *Xanthomonas* effector AvrAC indirectly through uridylylation of another host kinase PBL2 (PBS1-like protein 2) [28,29]; AvrAC-uridylylated PBL2 associates with RKS1 and consequently activates ZAR1-mediated immunity. Alternatively, in some cases, NLRs may not be necessarily activated by pathogen effectors. For example, the arabidopsis TNL CHS3 (CHILLING SENSITIVE 3)/CSA1 (CONSTITUTIVE SHADE-AVOIDANCE 1) pair detects perturbations of the PRR coreceptor BAK1 (BRASSINOSTEROID INSENSITIVE 1-associated kinase 1) [30], whereas the malectin-like receptor-like kinase LET1 activates autoimmunity by the CNL SUMM2 (SUPPRESSOR OF MKK1 MKK2) via MEKK2 (MAP/ERK kinase kinase-2) scaffolding [31].

Effector recognition leads to NLR oligomerization and the formation of large protein complexes termed resistosomes (Figure 1). Cryoelectron microscopy (cryoEM) analyses show that the ZAR1 resistosome containing ZAR1, RKS1, and uridylylated PBL2 ($PBL2^{UMP}$) forms a wheel-like pentameric complex [32] (Figure 1, left). Pentamerization of the ZAR1 resistosome is mediated mainly by the NOD module of ZAR1, with RKS1 and $PBL2^{UMP}$ being presented at the rim of the wheel (Figure 2). In contrast to ZAR1, Sr35 directly recognizes its cognate effector AvrSr35, but the resulting Sr35 resistosome is also a pentameric complex with structure remarkably similar to that of the ZAR1 resistosome [24,25]. A more recent study showed that oligomeric NRG1 resistosomes are likely to be formed at the plasma membrane (PM) [33]. Interestingly, PTI signaling is required for the formation of NRG1 resistosomes, but the underlying mechanisms await further elucidation [33].

By comparison, direct binding of ATR1 to the C-terminal end of RPP1 results in the formation of a tetrameric RPP1 resistosome [22] (Figure 1, right). A similar assembly of the Roq1 resistosome is induced by direct XopQ binding to Roq1 [21]. As seen in the CNL resistosomes, oligomerization of the two TNL resistosomes is primarily mediated by the NOD module (Figure 2). Structural and modeling studies showed that NOD is sequestered from oligomerization in inactive NLRs [22,24,25,32], suggesting that recognition of pathogens results in release of the NOD module for NLR oligomerization. However, it remains unknown whether oligomerization of all plant NLRs leads to assembly of resistosomes.

Glossary

Damage-associated molecular patterns (DAMPs): conserved molecules generated by damage to the host. Damaged plant cells release DAMPs, which are detected by PRRs that trigger defense responses to limit further damage. Examples of DAMPs include ATP, host-derived peptides, and extracellular DNA.

Effector proteins: proteins produced by pathogens that are secreted into host cells. Pathogen effectors can disturb host defense responses and promote infection. They can also be recognized by plant resistance (R) proteins, leading to the activation of defense responses.

Effector-triggered immunity (ETI): a second type of plant immunity. ETI is activated upon the recognition of pathogen effectors by plant intracellular resistance (R) proteins. ETI signaling can also activate Ca^{2+} influx, bursts of reactive oxygen species (ROS), production of phytocytokines and defense phytohormones, and transcriptional reprogramming. ETI culminates in a hypersensitive response (HR), a form of programmed cell death at the site of attempted penetration of pathogens.

Helper NLRs (hNLRs): these do not have effector recognition capacity and they act downstream of sensor NLRs for signal transduction. Examples of hNLRs include ADR1s and NRG1s.

NADase: an essential enzyme that plays a crucial role in cellular processes by catalyzing the hydrolysis of NAD^+ into nicotinamide and ADP-ribose, or other breakdown products. Through this enzymatic activity, NADase contributes to the regulation of various biological pathways, including aging, stress responses, and disease pathways.

Paired NLRs: two NLRs in which one functions as the sensor for effector perception and the other as the executor for signal transduction. The two NLRs typically form a complex to mediate immune signaling. Examples of paired NLRs include RRS1/RPS4 in arabidopsis and RGA4/RGA5 in rice.

Pathogen-associated molecular patterns (PAMPs): conserved molecules of pathogens – such as bacteria, fungi, and viruses – that can be recognized by PRRs to trigger defense responses. Examples of PAMPs include flagellin, chitin, and bacterial lipopolysaccharides.

Pattern-recognition receptors (PRRs): specialized plant receptors located at the cell surface that can

Many NLRs act in pairs, with the sensor NLR recognizing pathogen effectors and the executor NLR initiating immune signaling [34]. Whether and how **paired NLRs** form resistosomes represent a challenge for understanding their signaling mechanisms.

NLR resistosomes converge on the second messenger Ca^{2+}

PM-localization and Ca^{2+} permeability of CNL resistosomes

In the cryoEM structure of the ZAR1 resistosome, the five N-terminal α1-helices of ZAR1 form a solvent-exposed structure shaped like a channel or pore [32] (Figure 2). Functional data support a critical role for the solvent-exposed structure in ZAR1-mediated ETI. Importantly, electrophysiological evidence supports the ZAR1 resistosome function as a Ca^{2+}-permeable channel to mediate immune response [35] (Figure 1, bottom left). Single molecule imaging showed that the ZAR1 resistosome forms hours before the loss of PM integrity [35]. These results suggest that Ca^{2+} influx mediated by the ZAR1 resistosome acts as a trigger for ZAR1 signaling.

ZAR1 α1 is conserved in many CNLs from distantly related plant species [36], suggesting that Ca^{2+}-channel activity may be conserved among CNL resistosomes. Indeed, similar activity has been demonstrated for the wheat CNL Sr35 resistosome, which bears a highly similar structure to that of the ZAR1 resistosome [25]. However, in contrast to that in the ZAR1 resistosome, the functionally essential α1 helix is not well-defined in the Sr35 resistosome. It may be that a membrane environment is required for Sr35 to form a funnel-shaped structure in the Sr35 resistosome.

PM localization has been shown for the arabidopsis CNLs RPM1 (resistance to *Pseudomonas syringae* pv. *maculicola* 1) [37] and RPS2 (resistance to *P. syringae* 2) [38], and the *N. bethamiana* CNL Tm-22 [39], but whether these CNLs can form ZAR1-like resistosomes remains to be examined. The TNL-activated hNLRs, NRG1s, and ADR1s can also form resistosomes at the PM and display similar Ca^{2+}-permeable channel activity in their autoactive forms [33,40], indicating that CNLs and TNLs converge on Ca^{2+} signals (Figure 2).

A large domain called Solanaceae domain (SD) before the CC domain is found in many noncanonical CNLs in members of the Solanaceae [41], suggesting that these CNLs themselves may not form oligomeric structures. Recent studies showed that some of these noncanonical CNLs function to activate NRC resistosomes [18,19]. However, it remains undetermined whether these NRC resistosomes have Ca^{2+}-permeable channel activity.

CNL resistosome-mediated extracellular Ca^{2+} influx

Multiple lines of functional evidence support extracellular Ca^{2+} influx as a trigger of ETI signaling [42,43]. Elevation in intracellular Ca^{2+} concentrations is one of the earliest events during ETI. Gain-of-function mutations of CNGC19/20 (CYCLIC NUCLEOTIDE GATED CHANNEL19/20) with increased Ca^{2+} influx activity constitutively activate EDS1 (enhanced disease susceptibility 1)- and SA (salicylic acid)-dependent arabidopsis immunity [44,45]. Unregulated channel activity of the Sr35, NRG1, and ADR1 resistosomes is sufficient to recapitulate plant CNL-mediated cell death in eukaryotic cells [25,40]. Although Ca^{2+} released from internal pools can contribute to ETI signaling, pharmacological study showed that blocking of Ca^{2+} release from intracellular compartments by ruthenium red (RR) is less efficient for inhibition of HR cell death than by the Ca^{2+} influx blocker $LaCl_3$ [46], supporting the notion that Ca^{2+} influx is a major trigger of ETI responses.

Non-PM cellular localization of CNLs

Nuclear localization is required for the disease resistance activity of many NLRs [47], but the mechanism of how the cellular localization of NLRs is associated with this activity remains

detect conserved pathogen-derived molecules or host-derived damage signals to initiate defense responses.
Pattern-triggered immunity (PTI): one form of plant immunity triggered by PRR recognition of PAMPs or DAMPs. PTI signaling activates a cascade of events, including Ca^{2+} influx, bursts of reactive oxygen species (ROS), activation of the mitogen-activated protein kinase (MAPK) pathway, production of phytocytokines and defense phytohormones, and transcriptional reprogramming.
Resistosome: a large oligomeric complex formed on direct or indirect recognition of pathogen effectors by plant NLRs; resistosomes trigger ETI to limit pathogen infection. Resistosomes play a crucial role in plant innate immunity. Nomenclature of resistosomes is based on the names of the involved NLRs. Resistosomes can be divided into CNL and TNL types. The CNL resistosomes activate Ca^{2+} influx via acting as Ca^{2+}-permeable channels, whereas the TNL resistosomes function as NADase holoenzymes to produce nucleotide-derived second messengers to trigger EDS1 signaling.
Second messenger: typically, small molecules that transmit signals from extracellular stimuli to the interior of a cell. A second messenger functions by binding to and activating various intracellular proteins or receptors, inducing cellular responses such as gene expression, enzyme activity, or ion channel opening/closing. Examples of second messengers include Ca^{2+}, cAMP, cGMP, and IP3.
Sensor NLR: an NLR responsible for the recognition of pathogen effectors that typically partners with other NLRs to mediate immune signaling.

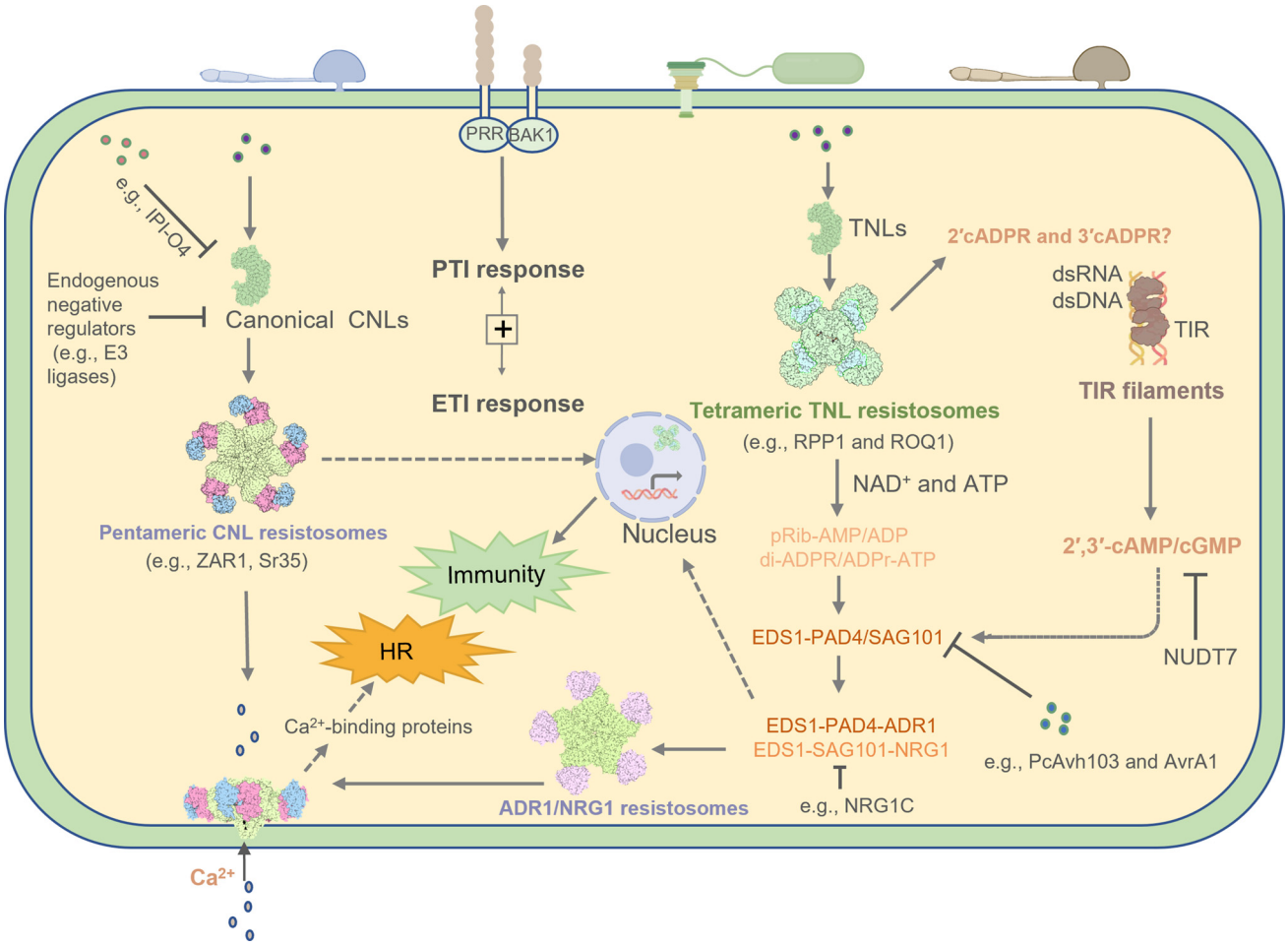

Figure 1. Overview of nucleotide binding and leucine-rich repeat-containing receptor (NLR) signaling in plants. Upon recognition of pathogen effectors, plant NLRs form large protein complexes called resistosomes. The pentameric coiled-coil domain-containing NLR (CNL) resistosomes translocate to the plasma membrane (PM) and function as Ca^{2+}-permeable channels to allow Ca^{2+} influx into the plant cell. In comparison, the tetrameric Toll-interleukin 1-like receptor (TIR) NLR (TNL) resistosomes act as NADase holoenzymes to catalyze production of nucleotide-derived second messengers pRib-AMP/ADP and di-ADPR/ADPr-ATP. The structurally related second messengers bind to and stimulateenhanced disease susceptibility 1 (EDS1)–phytoalexin deficient 4 (PAD4) and EDS1–senescence-associated gene101 (SAG101) interaction with activated disease resistance 1 (ADR1) and N requirement gene 1 (NRG1), respectively, activating the ADR1 and NRG1 resistosomes (the reseda portion in ADR1/NRG1 resistosomes represents ADR1 or NRG1 and the pink portion at the outside of the resistosome represents the lipase-like proteins) and their Ca^{2+}-permeable channel activity. TIR domain proteins can also catalyze production of 2′,3′-cAMP/cGMP to promote EDS1 signaling. Ca^{2+}-binding proteins can be important to translate the NLR resistosome-based Ca^{2+} signals into effector-triggered immunity (ETI), including the hypersensitive response (HR) and transcriptional reprogramming of defense-related genes. ETI and pattern-triggered immunity (PTI) signaling can mutually potentiate, as indicated by the framed plus symbol. Plant NLR signaling can be negatively regulated by pathogen effectors and host-derived components such as E3 ligases and NRG1C. Abbreviations: AvrA1, avirulence effector A1; ds, double stranded; IPI-O4, in planta-induced gene O4; NUDT7, NUDIX HYDROLASE HOMOLOG 7; PcAvh103, *Phytophthora capsici* avirulence homolog effector 103; PRR, pattern-recognition receptor; ROQ1, recognition of XopQ 1; RPP1, recognition of Peronospora parasitica 1; Sr35, stem rust resistance gene 35; ZAR1, HOPZ-ACTIVATED RESISTANCE 1.

enigmatic. Several CNLs have been shown to interact with transcriptional factors [48], suggesting that CNLs may directly regulate transcriptional programming in the nucleus. PTI and ETI signaling activate transcription of a similar set of genes, suggesting that other mechanisms can also be involved in NLR-mediated transcriptional reprogramming. NRG1A is both PM- and nucleus-localized upon activation, but only the PM-resident NRG1A forms oligomers [33], suggesting an oligomerization-independent NRG1 function.

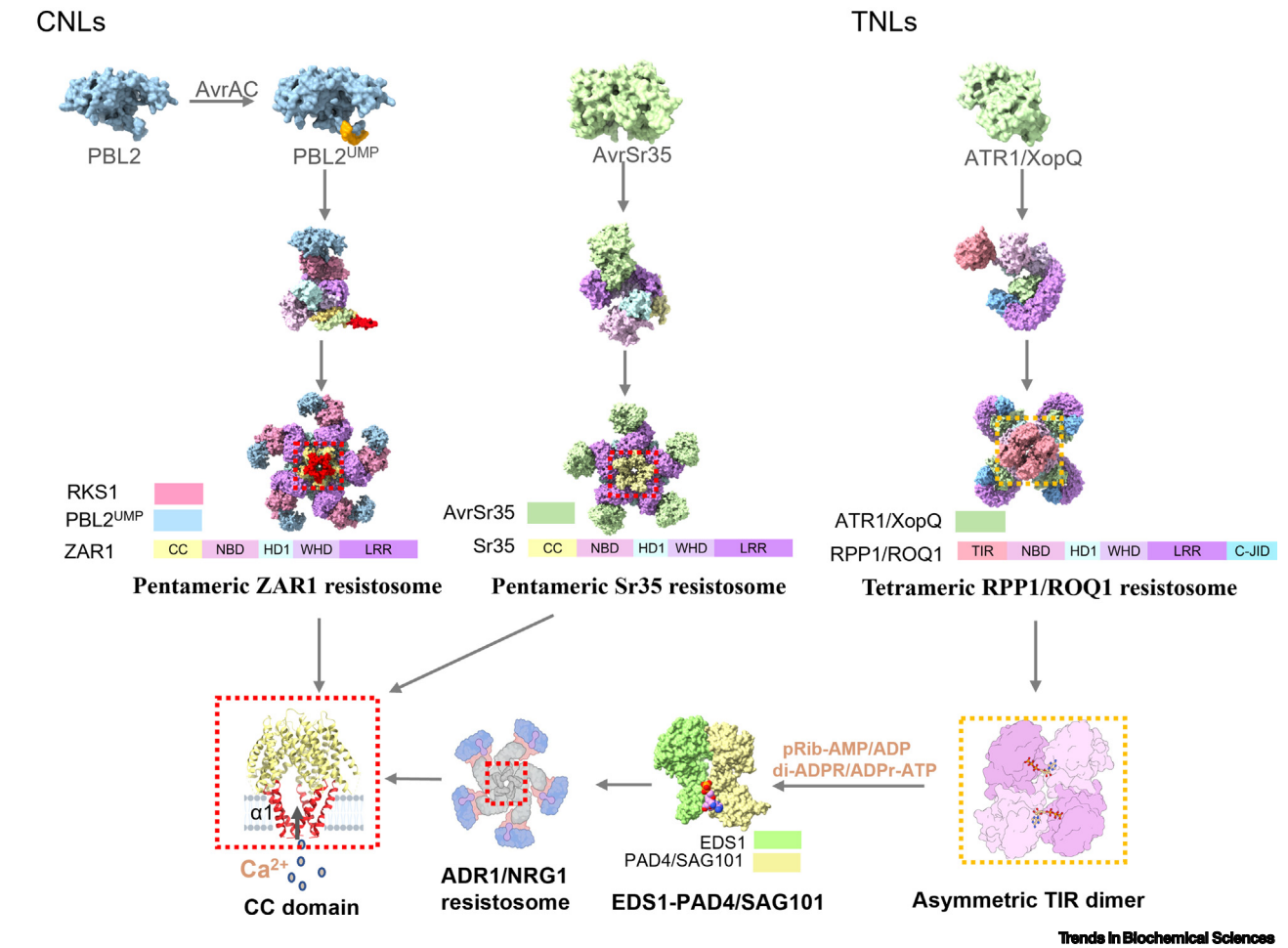

Figure 2. Mechanisms of nucleotide binding and leucine-rich repeat-containing receptor (NLR) resistosome-mediated production of Ca^{2+} and nucleotide-derived second messengers. Avirulence effector AC (AvrAC) from *Xanthomonas campestris* pv. *Campestris* and avirulence effector Sr35 (AvrSr35) from *Puccinia graminis* f. sp. *tritici* induce conformational changes in ZAR1 and Sr35, respectively. This results in the formation of the pentameric HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) and stem rust resistance gene 35 (Sr35) resistosomes. The five N-terminal α1-helices in the ZAR1 resistosome form a funnel-shaped structure, which can integrate into the plasma membrane (PM) and act as a Ca^{2+}-permeable channel. Note that equivalent α1-helices in the Sr35 resistosome are not well defined, presumably due to the lack of a membrane environment under conditions for structural determination. The pathogen effector ATR1 (*Arabidopsis thaliana* recognized 1) from the downy mildew pathogen *Hyloperonospora arabidopsidis* and XopQ (*Xanthomonas outer* protein Q) from the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* induce tetrameric assembly of the RPP1 (recognition of *Peronospora parasitica 1)* and ROQ1 (recognition of XopQ1) resistosomes, respectively. The four Toll-interleukin 1-like receptor (TIR) domains in the resistosomes form two asymmetric dimers (two horizontal dimers), which contain two composite active sites to catalyze production of pRib-AMP/ADP and di-ADPR/ADPr-ATP. These four small molecules bind to and stimulate EDS1 (enhanced disease susceptibility 1) heterodimer interaction with activated disease resistance 1 (ADR1) or N requirement gene 1 (NRG1), activating their resistosome and Ca^{2+}-permeable channel activities. The nucleotide-binding and oligomerization domain (NOD) module discussed in the main text comprises nucleotide-binding domain (NBD), helical domain 1 (HD1), and winged-helix domain (WHD). Abbreviations: C-JID, C-terminal jelly-roll and Ig-like domain; CNL, coiled-coil domain-containing NLR; $PBL2^{UMP}$, AvrAC-uridylylated PBS1-like protein (PBL)2; TNL, tetrameric Toll-interleukin 1-like receptor (TIR) NLR. The images in the figure are not drawn on the same scale.

Increases in nuclear free Ca^{2+} concentrations have been reported in response to various stresses [49–51]. It should be kept in mind that spatial distribution of Ca^{2+} in a cell is not uniform, and concentrations of Ca^{2+} can have steep gradients a few nanometers away from the Ca^{2+} channel [52]. Thus, Ca^{2+} transported by the PM-localized resistosomes and/or other PM-localized Ca^{2+}-permeable channels may not reach the nucleus. It remains possible that CNLs form resistosomes at the nucleus or at the continuum of the nucleus and another organelle such as endoplasmic

reticulum (ER). Such a cellular localization may result in resistosome-mediated Ca^{2+} signaling in the nucleus. An example for this has been demonstrated for the Ca^{2+}-permeable channel CNGC15 during symbiotic Ca^{2+} oscillation [53].

Downstream signaling of TNL resistosomes

While CNLs and TNLs converge on Ca^{2+} signals, they have different signaling mechanisms in that TNLs, but not CNLs, rely on EDS1 to activate ETI. In contrast to the CNL resistosomes, TNL resistosomes are **NADases**. Assembly of the ATR1-induced RPP1 resistosome significantly enhances RPP1 NADase activity, indicating that the resistosome acts as an NADase holoenzyme [22]. The enzymatic activity is encoded in the N-terminal TIR domain of TNLs [54,55], which is required for immune signaling mediated by TNLs [22] and TIR-only proteins such as response to the bacterial type III effector protein HopBA1 (RBA1) in arabidopsis [56]. Tetramerization results in the formation of two composite active sites in the TIR domains of TNL resistosomes [21,22] (Figure 2). A similar mechanism has also been demonstrated for activation of TIR domain proteins from other species [57–59].

TNL signaling depends on EDS1 and its analogs PAD4 (phytoalexin deficient 4) and SAG101 (senescence-associated gene101) in arabidopsis. EDS1 forms exclusive dimers with PAD4 and SAG101. The hNLRs, ADR1s and NRG1s, are also required for TNL signaling [60,61]. Genetic and biochemical data showed that EDS1–PAD4 and EDS1–SAG101 cofunction with ADR1s and NRG1s, respectively [12–15,62–66]. Recent studies have revealed the mechanism of how TNL signals are relayed to activate downstream components (Figure 1) [67–70]. Structural biology coupled with high-resolution mass spectrometry identified structurally related 2′-(5″-phosphoribosyl)-5′-adenosine monophosphate (pRib-AMP), pRib-adenosine diphosphate (pRib-ADP), ADP-ribosylated ATP (ADPr-ATP), and ADP-ribosylated ADPR (di-ADPR) as RPP1 resistosome-catalyzed products in insect cells [69,70]. Enzymatic activity of producing these four nucleotide-derived small molecules was also shown for the TIR domain of the arabidopsis TNL RPS4. *In vitro* enzyme activity assays indicated that plant TIR domain containing proteins use a dual substrate of NAD^+ and ATP to generate these nucleotide-derivatives [69]. To produce the four structurally related nucleotide derivatives, plant TIR domain proteins require ADPR transferase activity, which catalyzes transfer of the ADPR moiety from NAD^+ to ADPR for the production of di-ADPR or to ATP for the production of ADPr-ATP [69]. pRib-ADP and pRib-AMP likely result from hydrolysis of di-ADPR or ADPr-ATP. However, *in vivo* metabolic pathways for the production of these nucleotide derivatives remain to be explored.

Biochemical data show that preferential binding of pRib-AMP/ADP induces EDS1–PAD4 interaction with ADR1s [70], whereas specific binding of di-ADPR/ADPr-ATP stimulates EDS1–SAG101 interaction with NRG1s [69] (Figure 1). Formation of the EDS1–SAG101–NRG1 and EDS1–PAD4–ADR1 complexes has been demonstrated *in vivo* [33,65,66]. TIR-induced assemblies of the EDS1–PAD4–ADR1 and EDS1–SAG101–NRG1 signaling complexes in *arabidopsis* confer specific immune functions of the two EDS1 heterodimers [63,71].

Specific interaction of EDS1–SAG101 with NRG1s affords an explanation of why *arabidopsis* EDS1–SAG101 cannot cofunction with *N. benthamiana* NRG1. Structural analyses reveal the mechanisms by which EDS1–SAG101 and EDS1–PAD4 recognize ADRr-ATP/di-ADPR and pRib-ADP/AMP, respectively [68–70]. Upon binding to EDS1 heterodimers, pRib-ADP or ADPr-ATP become almost completely buried. Binding of these nucleotide derivatives induces similar conformational changes in the C-terminal EP domains (shared by EDS1, PAD4, and SAG101) of PAD4 and SAG101. Together, these results indicate that the four TIR-catalyzed small molecules act as second messengers to induce EDS1 heterodimer interactions with downstream ADR1s and NRG1s, presumably leading to assembly of the ADR1 and NRG1 resistosomes, respectively. Interestingly, however, oligomeric NRG1 at the PM contains neither

EDS1 nor SAG101, suggesting that these two lipase-like proteins may dissociate from EDS1–SAG101–NRG1 following NRG1 oligomerization [72].

Plant TIR domain proteins can also produce 2′cADPR and 3′cADPR when NAD^+ is used as the substrate [58,73,74]. Notably, the TIR-containing effector proteins HopBY and HopAM1 of *P. syringae* can efficiently hydrolyze NAD^+ to generate 2′cADPR and 3′cADPR respectively to promote virulence activity of the bacterial pathogen [75,76]. These results suggest that these two small molecules are less likely to trigger plant immunity.

In addition to the NADase, transferase, and cyclase activities, plant TIR domains can also act as 2′,3′-cyclic adenosine monophosphate (cAMP)/cyclic guanosine monophosphate (cGMP) synthetases when double-stranded RNA/double-stranded DNA (dsRNA/dsDNA) is used as the substrate [77] (Figure 1). Expression of wild-type but not the catalytic mutant E86A of RBA1 strongly promotes accumulation of these two noncanonical cyclic nucleotides in *N. benthamiana* plants. Furthermore, NUDIX HYDROLASE HOMOLOG 7 (NUDT7), a negative regulator of EDS1 signaling in arabidopsis [78–80], hydrolyzes 2′,3′-cAMP/cGMP but not 3′,5′-cAMP/cGMP, and the hydrolysis activity is required for suppression of RBA1-mediated cell death in *N. benthamiana*. Additionally, the *Xanthomonas euvesicatoria* effector XopQ, which suppresses EDS1-dependent cell death in *Nicotiana* species [81], displays the activity of 2′,3′-cAMP/cGMP hydrolysis. These data support the biological significance of 2′,3′-cAMP/cGMP in EDS1 signaling, but the underlying mechanism remains poorly understood. It was suggested that 2′,3′-cAMP/cGMP function as signal amplifiers to transcriptionally upregulate components in TNL signaling [77]. CryoEM analysis shows that the TIR domain of the flax TNL L7 bound by dsDNA forms filaments. Organization of L7 TIR domains in the filaments is incompatible with TIR tetramerization in the TNL resistosomes, suggesting that TNL resistosomes might not have the 2′,3′-cAMP/cGMP synthetase activity. This is in concert with the observation that the synthetase activity is not required for immune signaling mediated by the arabidopsis TNL SNC1 (Suppressor of *npr1-1*, constitutive 1) [82].

These data indicate that both CNL and TNL signaling converge on Ca^{2+}, suggesting that Ca^{2+}-permeable channel activity is a unified mechanism for plant NLR signaling. How NLR-activated Ca^{2+} influx is translated into downstream immune responses is currently poorly understood. In addition to the NLR resistosomes, other Ca^{2+}-permeable channels such as CNGCs also have a role in ETI signaling. It will be of interest to investigate whether and how these channels coordinate with NLR resistosomes to mediate ETI. Ca^{2+}-binding proteins – including calmodulins (CaMs), CaM-like proteins (CMLs), calcineurin B-like proteins (CBLs), Ca^{2+}-dependent protein kinases (CPKs), and transcriptional factors such as CAMTA3 and CBP60g – are likely to play a critical role in this process [83] (Figure 1). Some of these Ca^{2+} sensors have been shown to be important for the decoding of Ca^{2+} signals during PTI [78]. How they achieve the specificity of ETI and PTI is an interesting question for the future.

Crosstalk between ETI and PTI signaling pathways

Recent studies have demonstrated an intricate connection between the PTI and ETI pathways [5,6]. Activation of ETI strongly promotes expression and protein accumulation of many PTI signaling components, including PRRs and their coreceptors. Interestingly, intact PRR signaling is required for ETI-promoted expression of PTI-related components. Transcriptome analyses showed that PTI activation, in turn, induces expression of genes coding NLRs and TIR signaling components, and promotes NLR protein accumulation [84–86]. Thus, transcriptional and translational preparations of signaling components are critical to copotentiation of ETI and PTI. This mechanism can be important for PTI-promoted assembly of the NRG1s resistosomes at the PM [33]. A similar mechanism has been revealed for the activation of animal NLRs in which

upregulated expression of NLR inflammasome components by the cell surface Toll-like receptors (TLRs) bolsters NLR signaling [87]. PTI-promoted expression and activation of several TIR-containing proteins enhances PTI signaling [85]. In arabidopsis, RLP (receptor-like protein)-mediated PTI requires signaling of EDS1–PAD4–ADR1 [88], but it remains unknown whether a resistosome-like activity is needed for the ADR1-containing complex. EDS1 is not required for PTI response in *N. benthamiana* [89], indicating complexity of EDS1 signaling in different plant species. Immune responses induced by the CNL RPS2 and the paired TNL RRS1 (resistance to *Ralstonia solanacearum* 1)/RPS4 (resistance to *P. syringae* 4) are impaired in arabidopsis mutants deficient in PRR signaling components [6], further supporting a critical role for PTI in boosting ETI. In further support of this PTI-potentiated model, the PAMP flg22 (an N-terminal 22-residue peptide from bacterial flagellin) promotes TIR-mediated cell death in *N. benthamiana* [90].

Currently, the mechanism underlying mutual potentiation of PTI and ETI remains poorly understood. Considering that Ca^{2+} is a shared trigger for ETI and PTI, it will be of interest to investigate whether Ca^{2+} signals have a role in their mutual potentiation. ETI-bolstered PTI is proposed to be through the elevation in intracellular Ca^{2+} concentrations [91]. This model is consistent with Ca^{2+}-dependent transcriptional regulation of immune genes during ETI [92]. Furthermore, Ca^{2+} influx promotes expression of TIR domain protein-encoding genes during PTI, and activation of these TIR proteins is important for PTI signaling in arabidopsis [85,88,92]. Activation of TIR signaling presumably results in Ca^{2+}-permeable channel activity of ADR1 and NRG1 resistosomes [40]. However, there exists evidence implicating that ETI-potentiating signals are not limited to PTI. For example, HR cell death activity of the transgenic plants expressing the bacterial effectors AvrRpt2 [93], AvrRps4 [94], or the TIR domain of the flax TNL L6 [95] can be potentiated by simply enhancing their protein expression levels [96]. This appears to agree with the notion that HR cell death is a consequence of surpassing immune signaling thresholds [97].

Negative regulations of NLR signaling

ETI responses have a critical role in mediating plant immunity. It is therefore conceivable that pathogens must have evolved strategies to dampen these responses to establish infection (Figure 1). The potato CNL Rpi-blb1 (also known as RB) is derived from *Solanum bulbocastanum* and confers resistance to most of the potato late blight pathogen *Phytophthora infestans* strains via recognition of members of the pathogen effector family IPI-O, such as IPI-O1 and IPI-O2 [98]. However, some members of this pathogen family (like IPI-O4) can block RB recognition of IPI-O1, disabling RB-mediated programmed cell death [99]. Biochemical data showed that both IPI-O1 and IPI-O4 interact with the RB CC domain [100], suggesting that IPI-O4 may outcompete IPI-O1 in blocking assembly of the potential RB resistosome. A recent effectoromics screen identified several pathogen effectors from the cyst nematode *Globodera rostochiensis* and *P. infestans* that suppress immune signaling of helper NRCs through different mechanism [101]. One of these effectors, SS15 (SPRYSEC15) from *G. rostochiensis*, directly binds the NOD module of NRC2 and NRC3 and suppresses NRC oligomerization [19,101]. In contrast to SS15, the effector AVRcap1b from *P. infestans* likely targets host proteins downstream of these cell death executor NLRs [101]. Pathogens also evolved effectors targeting EDS1 to promote their virulence activity. A *P. capsici* effector PcAvh103 interacts with the lipase domain of EDS1 and promotes dissociation of the EDS1–PAD4 but not EDS1–SAG101 interaction [102]. This is expected to specifically block assembly of the ADR1 resistosome. Similarly, two EDS1-like proteins in soybean, *Gm*EDS1a and *Gm*EDS1b, interact with the *P. syringae* effector AvrA1 required for virulence [103]. But whether and how the interaction impacts *Gm*EDS1 association with *Gm*PAD4 remain unexamined.

ETI responses are robust and often lead to host-cell death, yielding growth penalty (Box 1). Thus, NLR signaling must be tightly controlled in the absence of pathogens to avoid their aberrant activation

Box 1. Connecting NLR resistosomes to pathogen infection and crop yield

Strong immune responses triggered by NLR resistosomes are often associated with reduction in crop growth and yield, which is described as fitness costs. Finely controlled resistosome assembly and activity could be conducive to maximize NLR-mediated defense capacity while limiting the costs of NLR resistance.

(Figure 1). The arabidopsis E3 ligases SNIPER1 and its homolog SNIPER2 have a crucial role in broadly controlling the levels of NLR immune receptors by ubiquitinating the NB domains of these NLRs [104]. Expression of some NLR genes is suppressed by secondary phased small interfering RNAs (phasiRNAs) under normal conditions to avoid autoimmune responses and to save energy for plant growth [105]. In arabidopsis, HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 15 (HOS15) and HISTONE DEACETYLASE 9 (HDA9) physically associate with many NLR genes and repress their expression [106]. A recent study demonstrated that the N-terminally truncated hNLR NRG1C antagonizes ETI immunity mediated by the full-length NRG1A or NRG1B [107]. This study presents evidence that NRG1C associates with EDS1–SAG101, which may outcompete full-length NRG1A or NRG1B interaction with the EDS1 heterodimer and consequently block assembly of the NRG1A/B resistosomes. Interestingly, an *N. benthamiana* NLR called NRCX with a nonfunctional N-terminal α1 belongs to the NRC helper family but negatively regulates NRC2/NRC3-mediated HR cell death, although the underlying mechanism remains unclear [108]. Another example of negative regulation of NLR signaling is provided by the rice *Pigm* locus, which confers durable resistance to the rice blast fungus *Magnaporthe oryzae* [109]. The *Pigm* locus encodes a cluster of NLRs, including a resistance NLR, PigmR, and a susceptible NLR, PigmS. PigmR–PigmS dimerization prevents formation of PigmR homodimers that are likely to be required for defense activation. Increased *PigmS* expression reduces *PigmR*-mediated resistance and increases seed production to counteract the yield cost induced by *PigmR*.

Concluding remarks

The past few years have witnessed significant progress in elucidation of the NLR signaling mechanism. A central established concept is that plant NLR signaling converges on Ca^{2+}. Addressing the question of how NLR-activated Ca^{2+} signals are decoded will be a critical step toward further dissection of NLR signaling. A challenge for this is that elevations in cytosolic Ca^{2+} concentrations during ETI can result from NLR resistosome-activated Ca^{2+} influx and Ca^{2+} release from internal pools. Disentanglement of the differently originated Ca^{2+} signals would be conducive to unraveling the intrinsically complicated networks involved in NLR signaling. Ca^{2+} sensors are presumably important for decoding the NLR-activated Ca^{2+} signals. Much research work is expected to advance our understanding of downstream NLR signaling events, including HR cell death and transcriptional reprogramming (see Outstanding questions). Addressing these and other questions may offer new opportunities for crop protection strategies and hence more sustainable agriculture.

Outstanding questions

Do the NLR resistosomes have direct interacting partners regulating their channel or enzyme activity?

Do the TNL resistosomes have enzymatic activity other than those already known?

Can the known TNL resistosome-generated nucleotide-derived second messengers be metabolized into signaling molecules? Do the nucleotide-derived second messengers generated by arabidopsis TNL resistosomes function in other plant species?

How is the nuclear localization of NLRs associated with their transcriptional reprogramming activity?

What are the sensors of the resistosome-activated Ca^{2+} signals? How do the sensors translate the Ca^{2+} signals into HR cell death and transcriptional reprogramming?

Ca^{2+} signals are a major trigger of both ETI and PTI signaling. How is the signaling specificity achieved?

How do paired NLRs function at the structural level?

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Declaration of interests

No interests are declared.

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