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Structure, biochemical function, and signaling mechanism of plant NLRs

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ABSTRACT

To counter pathogen invasion, plants have evolved a large number of immune receptors, including membrane-resident pattern recognition receptors (PRRs) and intracellular nucleotide-binding and leucine-rich repeat receptors (NLRs). Our knowledge about PRR and NLR signaling mechanisms has expanded significantly over the past few years. Plant NLRs form multi-protein complexes called resistosomes in response to pathogen effectors, and the signaling mediated by NLR resistosomes converges on Ca^{2+}-permeable channels. Ca^{2+}-permeable channels important for PRR signaling have also been identified. These findings highlight a crucial role of Ca^{2+} in triggering plant immune signaling. In this review, we first discuss the structural and biochemical mechanisms of non-canonical NLR Ca^{2+} channels and then summarize our knowledge about immune-related Ca^{2+}-permeable channels and their roles in PRR and NLR signaling. We also discuss the potential role of Ca^{2+} in the intricate interaction between PRR and NLR signaling.

Key words: plant immunity, PRR, NLR, resistosome, Ca^{2+}-permeable channels, Ca^{2+} signaling, second messenger

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INTRODUCTION

The plant immune system mainly relies on two types of receptors to mediate immune responses. One type is cell-surface-located pattern recognition receptors (PRRs) sensing the conserved signatures of invading pathogens, called pathogen-associated molecular patterns (PAMPs) or host-derived damage-associated molecular patterns (DAMPs), to initiate pattern-triggered immunity (PTI) (Yu et al., 2017; DeFalco and Zipfel, 2021). The other type is intracellular nucleotide-binding (NB), leucine-rich repeat (LRR) receptors (NLRs). Plant NLRs mediate direct or indirect recognition of race-specific pathogen effectors delivered into plant cells, initiating effector-triggered immunity (ETI) (Cui et al., 2015; Jones et al., 2016; Zhou and Zhang, 2020). Despite their different structures and subcellular localizations, PRRs and NLRs share a suite of downstream defense responses, including Ca^{2+} influx; bursts of reactive oxygen species (ROS); activation of mitogen-activated protein kinase (MAPK) cascades; production of phytocytokines and defense hormones, including salicylic acid (SA) and ethylene; and massive transcriptional reprogramming (Ngou et al., 2022; Figure 1). Probably because of their similarity, PTI and ETI are tightly connected and mutually potentiate (Ngou et al., 2021; Yuan et al., 2021). However, PTI and ETI differ in timing, amplitude, and duration of defense. Compared with PTI, ETI involves prolonged and more robust immune responses and is frequently accompanied by a hypersensitive response (HR), a form of localized programmed cell death associated with pathogen restriction or killing. The pathogen resistance and cell death activity of the plant HR can be physiologically, genetically and temporally uncoupled (Künstler et al., 2016).

Overview of PRRs and PTI signaling

Plant PRRs are primarily receptor-like kinases (RLKs) or receptor-like proteins (RLPs). RLKs and RLPs have a tripartite domain organization, containing an extracellular domain (ECD), a

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transmembrane domain, and a cytoplasmic serine/threonine kinase domain in RLKs or a short cytoplasmic tail in RLPs (Boutrot and Zipfel, 2017; Wang and Chai, 2020b; Lee et al., 2021; Song et al., 2021). For example, FLAGELLIN-SENSITIVE 2 (FLS2) is an LRR-RLK because its ECD encodes LRRs that recognize the bacterial PAMP flg22 (Felix et al., 1999; Gomez-Gomez and Boller, 2000), whereas the LRR-RLKs PEP RECEPTOR 1,2 (PEPR1,2) are receptors of the phytocytokine Pep1 and homologous peptides in *Arabidopsis thaliana* (hereafter called *Arabidopsis*) (Huffaker et al., 2006; Yamaguchi et al., 2006, 2010; Krol et al., 2010). The ECDs of PRRs are necessary and sufficient for PAMP/DAMP recognition (Liu et al., 2012, 2016a; Sun et al., 2013, 2022; Tang et al., 2015; Xiao et al., 2019; Xu et al., 2022b). Ligand binding induces *trans*-phosphorylation of cytoplasmic kinase domains in the PRR complexes (Figure 1). BRI1-ASSOCIATED KINASE 1 (BAK1; also called SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 [SERK3]) belongs to the SERK family of LRR-RLKs and acts as a co-receptor of various LRR-RLKs, including FLS2 and PEPR1,2 (Chinchilla et al., 2007; Schulze et al., 2010; Sun et al., 2013; Tang et al., 2015). Because of the lack of the C-terminal kinase domain for signal transduction, LRR-RLPs constitutively interact with the LRR-RLK SUPPRESSOR OF BIR-1 (SOBIR1), forming binary RLKs (Gust and Felix, 2014; Albert et al., 2015; Bi et al., 2016) to recruit BAK1.

Receptor-like cytoplasmic kinases (RLCKs) are direct downstream components of PRRs (Figure 1). *Lycopersicon esculentum* (tomato) AVR9/CF-9 INDUCED KINASE 1 (ACIK1) is the first RLCK identified to participate in PRR-mediated immunity (Rowland et al., 2005), and its *Arabidopsis* ortholog BOTRYTIS-INDUCED KINASE 1 (BIK1) has a crucial role in PTI signaling (Zhang et al., 2010a; Lu et al., 2010). BIK1 and its homologous protein PBS1-LIKE PROTEIN 1 (PBL1), which belong to the PBL family, constitute a key signaling node downstream of multiple PRR complexes. Activated RLCKs phosphorylate multiple downstream immune signaling components to transduce immune signals (Liang and Zhou, 2018). For example, BIK1 phosphorylation of different residues of RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD) is critical for PAMP-induced ROS burst and antibacterial immunity (Kadota et al., 2014; Li et al., 2014). BIK1 can also phosphorylate and activate Ca^{2+} channels, including CYCLIC NUCLEOTIDE-GATED CHANNEL (CNGC) 2-CNGC4 (Tian et al., 2019) and REDUCED HYPEROSMOLALITY INDUCED CA^{2+} INCREASE 1.3 (OSCA1.3) (Thor et al., 2020), to trigger Ca^{2+} influx and stomatal closure, respectively. Ca^{2+} as a secondary messenger activates downstream components, such as Ca^{2+}-dependent protein kinases (CPKs), including CPK4/5/6/11, which, in turn, phosphorylate and activate RBOHD for ROS production (Boudsocq et al., 2010; Dubiella et al., 2013). Two MAPK cascade signaling pathways, MAPK Kinase Kinase (MAPKKK or MEKK) 1-MAPK Kinase (MAPKK or MKK) 1/2-MPK4 and MAPKKK3/5-MKK4/5-MPK3/6, participate in PTI responses (Meng and Zhang, 2013). A recent study has shown that the RLCK VII-4 subfamily member PBL19 directly phosphorylates MEKK1 and MAPKKK5 to activate MPK4 and MPK3/6, respectively (Yamada et al., 2016; Wang et al., 2017; Bi et al., 2018).

Overview of NLRs and ETI signaling

NLRs are conserved in plants and animals. Plant and animal NLRs belong to the signal transduction adenosine triphosphatases (ATPases) with numerous domains (STAND) family and share a similar domain organization (Wang and Chai, 2020a; Duxbury et al., 2021). NLRs are characterized by a variable N-terminal domain, a central conserved nucleotide binding and oligomerization domain (NOD), and a C-terminal LRR domain (Figure 1). The NOD module consists of three subdomains: nucleotide-binding domain (NBD), helical domain 1 (HD1), and winged-helix domain (WHD). A major biochemical function of the NOD is to act as a nucleotide switch regulating NLR oligomerization (Yang et al., 2019; Wang and Chai, 2020a; Duxbury et al., 2021; Forderer et al., 2022). Based on the domain structures of their N termini, plant NLRs are mainly classified into three groups: coiled-coil (CC) NLRs (CNLs), Toll/interleukin-1 receptor (TIR) NLRs (TNLs), and CC_R-NLRs (RNLs). RNLs possess a CC domain similar to the membrane-anchored resistance protein RESISTANCE TO POWDERY MILDEW 8 (RPW8), hence known as CC_R (Collier et al., 2011). Overexpression of the CC or TIR domain is sufficient to recapitulate pathogen-induced immune responses of full-length NLRs (Swiderski et al., 2009; Krasileva et al., 2010; Bernoux et al., 2011; Collier et al., 2011; Maekawa et al., 2011; Cesari et al., 2016; Schreiber et al., 2016; Baudin et al., 2017). Atypical NLRs, such as C-terminally truncated NLRs, also exist in plants. For instance, *Arabidopsis* RESPONSE TO THE BACTERIAL TYPE III EFFECTOR PROTEIN HOPBA1 (RBA1) is a TIR-only protein (Nishimura et al., 2017), and *Arabidopsis* TN2 (TIR-NB 2) lacks the LRR domain (Zhao et al., 2015). Functionally, plant NLRs can be divided into sensor NLRs and helper NLRs, which are responsible for recognition of pathogen effectors and immune signal outputs, respectively (Jubic et al., 2019; Feehan et al., 2020). Examples of helper NLRs include ACTIVATED DISEASE RESISTANCE 1 (ADR1), N REQUIREMENT GENE 1 (NRG1) (Peart et al., 2005; Bonardi et al., 2011; Collier et al., 2011; Qi et al., 2018; Castel et al., 2019; Lapin et al., 2019; Wu et al., 2019), and NB-LRR PROTEIN REQUIRED FOR HR-ASSOCIATED CELL DEATH (NRC) (Gabriels et al., 2007; Wu et al., 2016, 2017). There is also a class of singleton NLRs in plants that can recognize effectors and execute downstream signaling. Representative members of singleton NLRs include the *Arabidopsis* CNLs RESISTANCE TO P. SYRINGAE PV MACULICOLA 1 (RPM1) (Grant et al., 1995) and HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) (Lewis et al., 2010; Wang et al., 2015) and the *Triticum monococcum* (wheat) CNL STEM RUST RESISTANCE 35 (Sr35) (Salcedo et al., 2017). In some cases, effector recognition requires two genetically linked NLRs (called paired NLRs) with one functioning as the sensor and the other as the executor. Well-characterized paired NLRs include the CNL pairs R-GENE ANALOG (RGA) 5/RGA4 (Cesari et al., 2013) in *Oryza sativa* (rice) and the *Arabidopsis* TNL pair RESISTANCE TO RALSTONIA SOLANACEARUM 1 (RRS1)/RESISTANT TO P. SYRINGAE 4 (RPS4) (Le Roux et al., 2015; Sarris et al., 2015).

Recognition of pathogen effectors frequently involves the C-terminal LRR domain, which results in oligomerization of plant NLRs and formation of large NLR-containing complexes called resistosomes (Wang et al., 2019a; Ma et al., 2020; Martin et al., 2020; Förderer et al., 2022; Zhao et al., 2022) (Figure 1). CNL resistosomes, such as ZAR1 in *Arabidopsis*, function as Ca^{2+}-permeable influx channels (Bi et al., 2021). Signaling mediated by CNLs typically requires NON RACE-SPECIFIC DISEASE

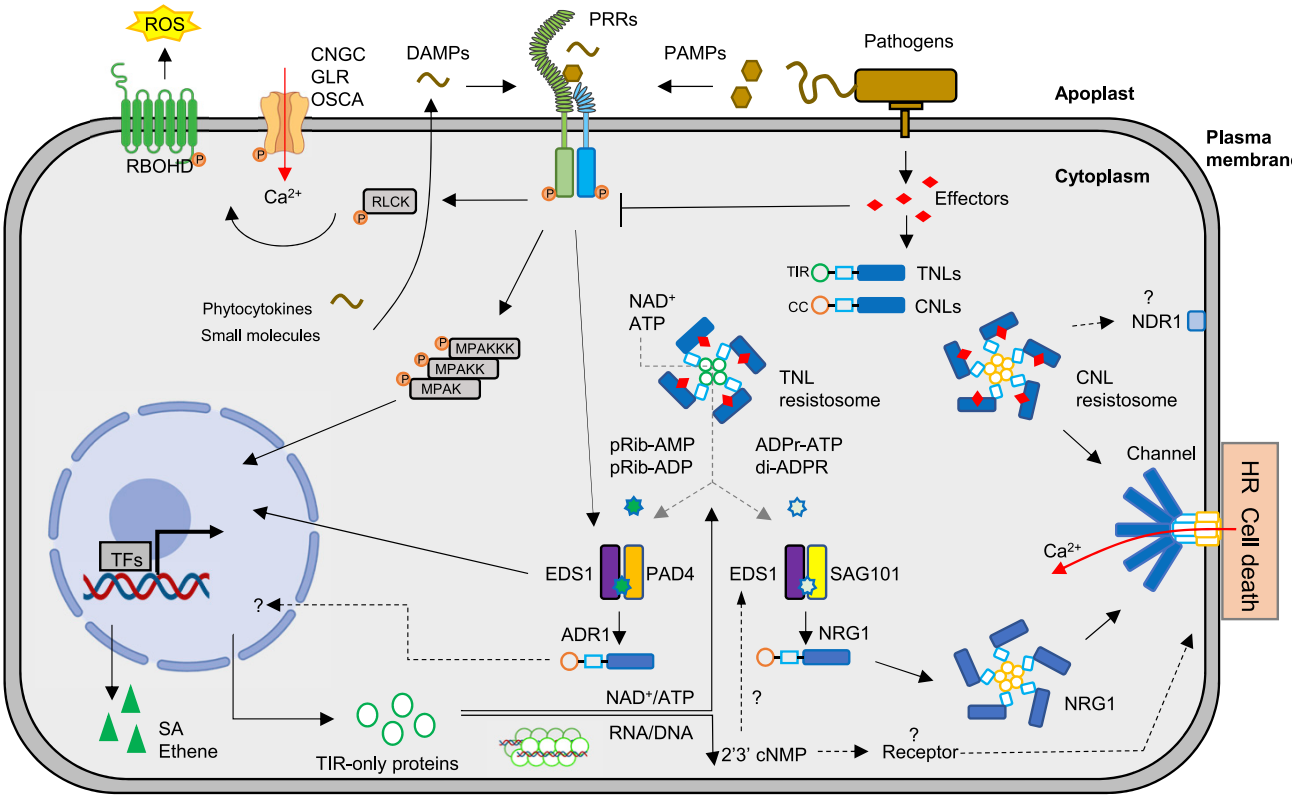

Figure 1. Plant immune system and signaling network.

Recognition of pathogen PAMPs or host DAMPs by cell surface immune receptor PRRs leads to pattern-triggered immunity (PTI). Activation of PRRs triggers phosphorylation of intracellular kinase RLCKs and MAPKs. The activated RLCKs phosphorylate and activate membrane-residing calcium channels (CNGCs, GLRs, and OSCA1.3) and the NADPH oxidase RBOHD to induce Ca^{2+} influx and ROS burst, respectively. MAPK phosphorylation cascades induce massive transcriptional reprogramming, leading to expression of defense-related genes. Pathogens deliver race-specific effector proteins to suppress host PTI in various modes to facilitate infection. Under these circumstances, intracellular NLR immune receptors, including CNLs and TNLs, sense these pathogen effectors and lead to another layer of plant immunity known as ETI. Recognition of effectors by singleton CNLs leads to formation of oligomeric CNL resistosomes, which can function as a calcium-permeable channel to mediate ETI signaling. The membrane-localized NDR1 has been demonstrated to participate in CNL-induced cell death, but the detailed mechanism remains elusive. TNL resistosomes activated by perception of pathogen effectors typically function as NADase holoenzymes to produce the secondary signaling messengers pRib-AMP/ADP and ADPr-ADP/di-ADPR, which are, respectively, recognized by EDS1-PAD4 and EDS1-SAG101 in *Arabidopsis*. Activated EDS1-PAD4 and EDS1-SAG101 interact with the helper RNLs ADR1 and NRG1, respectively. EDS1-activated ADR1 and NRG1 form resistosomes and function as Ca^{2+}-permeable channels to induce HR cell death. EDS1-activated ADR1 plays roles in regulation of resistance via an unknown mechanism. PTI also activates EDS1-PAD4 complex-mediated ETI. TIR-only proteins can also function as 2′3′-cNMP synthetases with dsRNA as the substrate to promote EDS1 signaling through an unknown mechanism.

RESISTANCE 1 (NDR1) (Century et al., 1997; Day et al., 2006; Knepper et al., 2011). However, the mechanism of how NDR1 contributes to CNL signaling remains unclear. NRC helpers are required for many CNLs in solanaceous plants to mediate immune responses (Wu et al., 2016, 2017). NRCs have been proposed to form resistosomes upon activation (Adachi et al., 2019; Duggan et al., 2021; Ahn et al., 2022; Contreras et al., 2022). In contrast with the CNL resistosomes, TNL resistosomes have nicotinamide adenine dinucleotide (NAD) nucleosidase (NADase) activity encoded in the N-terminal TIR domains (Horsefield et al., 2019; Wan et al., 2019; Ma et al., 2020; Martin et al., 2020). All tested TNLs require the RNLs ADR1 and NRG1 (Lapin et al., 2022) and the lipase-like protein ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and its two paralogs, PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE-ASSOCIATED GENE 101 (SAG101), in *Arabidopsis* (Bhandari et al., 2019; Gantner et al., 2019; Lapin et al., 2019; Dongus and Parker, 2021). Like the ZAR1 resistosome, self-activating mutants of ADR1 and NRG1 act as Ca^{2+}-permeable channels (Jacob et al., 2021).

RESISTOSOMES: STRUCTURE AND BIOCHEMICAL FUNCTION

Assembly of resistosomes is required for plant NLR signaling

Oligomerization is a common theme of signaling mediated by NLRs (Wang and Chai, 2020a; Duxbury et al., 2021). This notion is well exemplified by assemblies of apoptosomes (Qi et al., 2010; Zhou et al., 2015) and NLR inflammasomes (Hu et al., 2015; Zhang et al., 2015), which mediate apoptosis and pyroptosis in animals, respectively. Cytochrome *c* binding induces oligomerization of APOPTOTIC PEPTIDASE ACTIVATING FACTOR 1 (Apaf-1) in the presence of ATP or

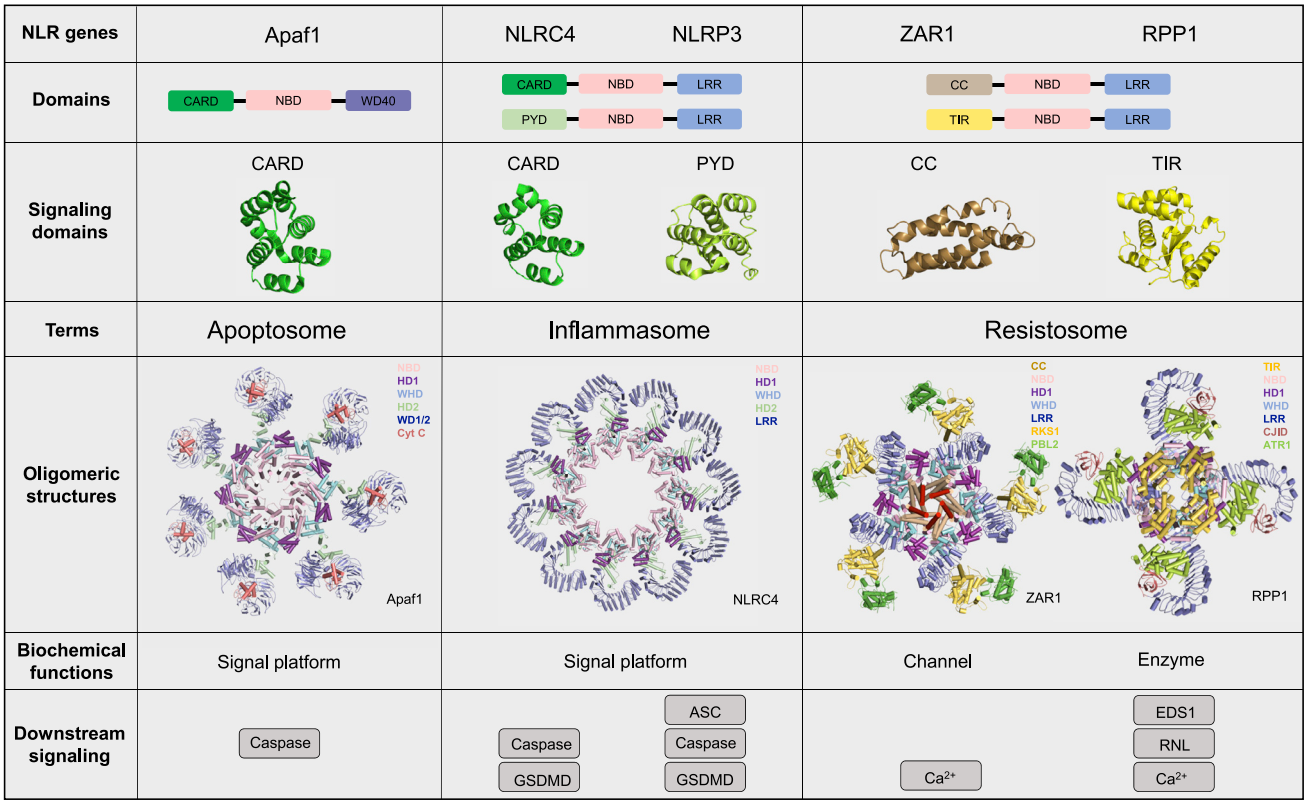

Figure 2. Structures and functions of the apoptosome, inflammasome, and resistosome.
Domain organization, N-terminal signaling domain structures, oligomeric structures, biochemical functions, and downstream signaling components of the NLR proteins Apaf-1, NLRC4, NLRP3, ZAR1, and RPP1. The heptameric Apaf-1 apoptosome contains cytochrome *c* (salmon) and Apaf-1 (domains are colored differently). The Apaf-1 apoptosome functions as a platform to recruit caspase-9 through CARD-CARD interaction. The NLRC4 inflammasomes are decamers or undecamers (NAIP:NLRC4 1:9 or 1:10) and function as a platform to recruit caspase-1/11. The CNL ZAR1 resistosome, containing the pathogen effector AvrAC-uridylylated PBL2 (green), RKS1 (light yellow), and ZAR1 (domain-based colors), is pentameric and functions as a PM Ca^{2+}-permeable channel to trigger ETI signaling. The tetrameric TNL RPP1 resistosome contains the pathogen effector ATR1 (green) and RPP1 (domain-based colors) and functions as an NADase holoenzyme to produce immune molecules recognized by EDS1 family proteins to activate helper NLRs (RNLs).

deoxyadenosine triphosphate (dATP), forming a large protein complex called the Apaf-1 apoptosome. The Apaf-1 apoptosome, mainly heptameric, recruits the downstream cysteine protease caspase-9, leading to formation of the Apaf-1/caspase-9 holoenzyme to mediate activation of the cysteine protease (Li et al., 2017). A highly conserved paradigm has been demonstrated in NLR signaling in animals (Hu and Chai, 2016; Wang et al., 2021b). A well-studied example of this is NLR FAMILY CARD DOMAIN CONTAINING 4 (NLRC4), which recognizes bacterial flagellin and the type III secretion system component PrgJ through NEURONAL APOPTOSIS INHIBITOR PROTEIN 5/6 (NAIP5/6) and NAIP2, respectively (Kofoed and Vance, 2011; Zhao et al., 2011; Tenthorey et al., 2017; Yang et al., 2018). Flagellin or PrgJ binding induces NAIP interaction with NLRC4, resulting in assembly of substoichiometric NAIP-NLRC4 complexes, called NLRC4 inflammasomes, with a stoichiometry of 1:9 or 1:10 between NAIP and NLRC4 (Hu et al., 2015; Zhang et al., 2015). The inflammasomes directly interact with caspase-1 or through the adaptor ASC (APOPTOSIS-ASSOCIATED SPECK-LIKE PROTEIN CONTAINING A CARD) to proteolytically mature the protease. The Apaf-1 apoptosome and NLRC4 inflammasomes act as platforms for protease activation (Figure 2).

NLRs have been known to function as a nucleotide switch for many years, but the underlying mechanism was not demonstrated until recently. ZAR1 recognizes the *Xanthomonas campestris* pv. *campestris* uridylylase effector protein AvrAC to mediate ETI (Wang et al., 2015). In unchallenged cells, ZAR1 forms a constitutive complex with the RLCK RESISTANCE-RELATED KINASE 1 (RKS1). Upon *X. campestris pv. campestris* infection, AvrAC uridylylates another RLCK member, PBL2. The modified PBL2 ($PBL2^{UMP}$) associates with RKS1 and consequently activates ZAR1-mediated immune signaling. The ZAR1-RKS1-$PBL2^{UMP}$ complex reconstituted using purified proteins is momoneric (Wang et al., 2019b). Structural comparison between ZAR1-RKS1 and ZAR1-RKS1-$PBL2^{UMP}$ reveals a striking conformational change in the ZAR1 NBD, which is predicted to impair adenosine diphosphate (ADP) binding as confirmed by biochemical data. This would facilitate ZAR1 ATP binding because of its much higher concentrations in cells. $PBL2^{UMP}$, however, makes no direct contact with the ZAR1 NBD, suggesting an allosteric mechanism of $PBL2^{UMP}$-induced impairment of the ADP binding activity of ZAR1 (Wang et al., 2019b). As observed in other inactive NLRs (Hu et al., 2013; Maekawa et al., 2016; Sharif et al., 2019; Steele et al., 2019; Hochheiser

et al., 2022) and the NLR-like Apaf-1 (Riedl et al., 2005; Reubold et al., 2011), ADP is deeply bound in inactive ZAR1 in the cryoelectron microscopy structure of ZAR1-RKS1, making it difficult, if not impossible, for ADP to be directly replaced by ATP or dATP for activation. Ligand-induced allosteric substitution of ADP by ATP can also avoid accidental activation of NLRs under resting conditions.

dATP/ATP induces oligomerization of the reconstituted monomeric ZAR1-RKS1-PBL2^{UMP} complex (Wang et al., 2019a), similar to dATP-induced assembly of the Apaf-1 apoptosome (Zhou et al., 2015). The oligomeric ZAR1 complex, called the resistosome, forms a wheel-like pentamer entirely mediated by ZAR1. A remarkably conserved structure is also found for the Sr35 resistosome containing the wheat CNL Sr35, its cognate effector AvrSr35, and ATP molecules (Förderer et al., 2022; Zhao et al., 2022), suggesting that the pentameric assembly may be conserved among plant CNL channels (Figure 2). Some sensor CNLs, such as Rpi-amr1/3 (Rpi, R genes against *Phytophthora infestans*; amr, AVRamr from *P. infestans*) and RESISTANCE TO POTATO VIRUS X (Rx) appear not to require oligomerization for activation of the NRC helper (Ahn et al., 2022; Contreras et al., 2022), but the precise active forms of these CNLs remain unclear. Mutations disrupting the ZAR1 or Sr35 resistosomes substantially suppress the HR cell death and resistance activities of the two CNLs (Wang et al., 2019a; Förderer et al., 2022; Zhao et al., 2022). Formation of the ZAR1 resistosome has been demonstrated in plant cells (Hu et al., 2020; Bi et al., 2021). These data support the biological relevance of the CNL resistosomes.

The TNLs RECOGNITION OF PERONOSPORA PARASITICA 1 (RPP1) from *Arabidopsis* (Ma et al., 2020) and RECOGNITION OF XOPQ 1 (ROQ1) from *Nicotiana benthamiana* (Martin et al., 2020) have been shown to form resistosomes. Mutations disrupting the RPP1 and ROQ resistosomes abolish the cell death activity, supporting the biological significance of these two TNL resistosomes. RPP1 and ROQ1 recognize the oomycete effector ATR1 and bacterial pathogen effector XopQ, respectively. The RPP1-ATR1 (RPP1 resistosome) and ROQ1-XopQ (ROQ1 resistosome) complexes were purified from insect cells and *Nicotiana benthamiana* plants, respectively. Despite the difference, these two TNL resistosomes form highly conserved tetramers (Figure 2). A conserved C-terminal domain called C-terminal jelly-roll and IG-like domain is directly involved in RPP1 and ROQ1 recognition of ATR1 and XopQ, respectively (Ma et al., 2020; Martin et al., 2020). In contrast with the ATP-bound ROQ1, ZAR1, and Sr35resistosomes, the RPP1 resistosome binds ADP in the P-loop region of RPP1. The unexpected nucleotide binding of active RPP1 results from acquisition of an additional β2-α2 loop, which promotes an inter-protomer NBD-WHD interaction and compensates for the loss of interactions mediated by the γ-phosphate group of ATP (Ma et al., 2020).

NLR resistosomes function as Ca^{2+}-permeable channels or NADase holoenzymes

CNL and TNL resistosomes form wheel-like structures similar to those of the Apaf-1 apoptosome and the NLR inflammasomes. However, striking differences exist in their N-terminal signaling domains among these large protein complexes (Figure 2). In contrast with the flexible N-terminal caspase recruitment domains (CARDs) in the Apaf-1 apoptosome (Zhou et al., 2015; Li et al., 2017) and the NLRC4 inflammasomes (Hu et al., 2015; Zhang et al., 2015), the CC domains of the CNL resistosomes (Wang et al., 2019a; Förderer et al., 2022; Zhao et al., 2022) or the TIR domains of the TNL resistosomes (Ma et al., 2020; Martin et al., 2020) are well defined, suggesting that the CNL and TNL resistosomes may use different mechanisms for signal transduction.

In the ZAR1 resistosome, the N-terminal helix α1 forms a funnel-shaped structure, which is the only exposed portion of the CC domain (Figure 2). These structural observations suggest that the funnel-shaped structure is important for ZAR1 resistosome function. Simultaneous mutations of two negatively charged residues (Glu11 and Glu18) at the inner surface of the funnel-shaped structure abolish ZAR1-mediated immune responses, suggesting that the ZAR1 resistosome may have pore- or channel-related activity (Wang et al., 2019a). Protein fractionation analysis shows AvrAC-induced ZAR1 association with the plasma membrane (PM). PM localization has been demonstrated for the CNLs RESISTANT TO P. SYRINGAE 2 (RPS2) (Elmore et al., 2012) and RPM1 (Gao et al., 2011; El Kasmi et al., 2017). The ZAR1 resistosome is formed in *Arabidopsis* protoplasts and displays calcium-permeable cation-selective channel activity in lipid bilayers (Bi et al., 2021). ZAR1 activation in the plant cell triggers Ca^{2+} influx, perturbation of subcellular structures, and immune responses (Bi et al., 2021). These data support PM channel activity of the ZAR1 resistosome (Figure 2).

The available data suggest that the Ca^{2+}-permeable channel activity may be evolutionarily conserved in CNLs of different plant species. The Sr35 resistosome from wheat not only has a structure strikingly similar to the ZAR1 resistosome (Figure 2) but also displays Ca^{2+}-permeable channel activity when expressed in *Xenopus* oocytes (Förderer et al., 2022; Zhao et al., 2022). In contrast with that of the ZAR1 resistosome, however, the channel activity of the Sr35 resistosome appears to be independent of acidic residues predicted to line the inner surface of the channel formed by the α1 helix in the Sr35 resistosome. It may be that the N termini of the Sr35 resistosome are structurally and functionally distinct from those of the ZAR1 resistosome. Auto-active RNL NRG1 and ADR1 also act as Ca^{2+}-permeable channels. Like those from the α1 helix of ZAR1, the negatively charged residues at the α1 helix of NRG1 and ADR1 are required for Ca^{2+} influx and cell death (Jacob et al., 2021). Bioinformatics data show that the ZAR1 α1 helix is conserved among CNLs of distantly related plant species, with ~20% of them including the helper NLRs NRCs sharing the consensus "MADA" motif (Adachi et al., 2019). An N-terminal 29-residue peptide of NRC4 with yellow fluorescent protein (YFP) fused to the C terminus is sufficient to induce HR cell death when expressed in *Nicotiana benthamiana*, providing direct evidence of the biological function of α1 in a CNL. However, many *Solanaceae* CNLs have a large domain called the Solanaceae domain prior to their CC domain. Some of them cooperate with NRCs to mediate immune responses (Wu et al., 2016, 2017).

The CC domain of many CNL/RNLs is sufficient to induce HR-like cell death when expressed in plant cells (Collier et al., 2011;

Maekawa et al., 2011; Cesari et al., 2016; Baudin et al., 2017). In the structures of the ZAR1 and Sr35 resistosomes, the inter-CC domain interaction results in formation of a similar helical barrel structure, which is stabilized by the conserved NOD (Wang et al., 2019a; Förderer et al., 2022; Zhao et al., 2022). High concentrations of the domain protein might bypass the requirement of the NOD for oligomerization, but direct evidence of whether the CC domain alone can form a channel structure like that from the ZAR1 or Sr35 resistosomes is lacking.

The TIR domain is a conserved immune module in animals, plants, and bacteria (Lapin et al., 2022). TIR domains were first found as intracellular domains of animal transmembrane immune receptor interleukin-1 receptors (IL-1Rs) and Toll-like receptors (TLRs) and function as scaffolds (Gay et al., 2014). Recognition of immune signals by the ECDs of TLRs or IL-IRs induces receptor dimerization, thereby forming intracellular TIR domain homo-dimers and recruiting downstream cytoplasmic TIR adaptor proteins (Yin et al., 2015). The biochemical function of plant TIR domains was not identified until recent biochemical and structural studies (Horsefield et al., 2019; Wan et al., 2019; Ma et al., 2020; Martin et al., 2020). The discovery of NADase activity of plant TIR domains was inspired by the animal STERILE ALPHA AND TIR MOTIF CONTAINING 1 (SARM1) TIR domain (Gerdts et al., 2015; Essuman et al., 2017). SARM1 is the only animal TIR adaptor protein coupled with enzymatic activity. Plant TNL- and TIR-only proteins shared the conserved NADase catalytic residue glutamate (Horsefield et al., 2019; Wan et al., 2019). Biochemical studies have demonstrated the NADase activity of plant TIR domain proteins (Horsefield et al., 2019; Wan et al., 2019) and TNL resistosomes (Ma et al., 2020; Figure 2). Mutations of the catalytic glutamate residue abrogate TNL- and TIR-mediated immunity and cell death (Horsefield et al., 2019; Wan et al., 2019; Ma et al., 2020; Martin et al., 2020), indicating that the NADase activity is essential for TNL and TIR domain signaling in plants. Assembly of the TNL resistosomes promotes the TIR-encoded NADase activity (Ma et al., 2020; Martin et al., 2020). Tetrameric assembly of the TIR domain in the TNL resistosomes results in formation of two composite catalytic sites, each of which is formed by an asymmetric TIR dimer (Ma et al., 2020; Martin et al., 2020). A similar mechanism is also involved in activation of the NADase activity of SARM1 (Shi et al., 2022), the bacterial TIR domain proteins *Acinetobacter baumannii* TIR (Manik et al., 2022) and TIR-SAVED (SMODS-associated and fused to various effector domain) (Hogrel et al., 2022), and the endonuclease activity of the bacterial NLRs Avs (antiviral STAND) and Avs4 (Gao et al., 2022).

UNIFIED MECHANISMS IN PLANT NLR SIGNALING

Ca^{2+}-permeable channel activity is a unified mechanism of plant NLR signaling

Despite their variations in signaling input, composition, and structure, NLR inflammasomes converge on inflammatory caspases for activation of a conserved suite of immune responses (Cao et al., 2022; Ohto, 2022). The underlying mechanism has been well documented. Assembly of NLR inflammasomes, including NLR FAMILY PYRIN DOMAIN CONTAINING 3 (NLRP3) and NLRC4, induces proximity of their N-terminal pyrin domains or CARDs for recruitment and activation of caspase-1 or another inflammatory caspase directly or via the adaptor ASC through homotypic interactions (Swanson et al., 2019). Activated caspase-1 proteolytically matures the cytokines IL-1β and IL-18 and cleaves the gasdermin D (GSDMD) substrate to release its N-terminal pore-forming domain (He et al., 2015; Kayagaki et al., 2015; Shi et al., 2015). The GSDMD pores formed in the PM facilitate secretion of mature inflammatory cytokines and other DAMPs (Liu et al., 2016b; Ding et al., 2016; Ruan et al., 2018; Xia et al., 2021) and trigger initiation of lytic cell death, called pyroptosis (Figure 3).

Like that of animal NLRs, signaling mediated by CNL/RNLs and TNLs also shares similar immune outputs (Figure 3). This observation is rationalized by TNL-activated ADR1 and NRG1 of the RNL class (Peart et al., 2005; Bonardi et al., 2011; Collier et al., 2011; Qi et al., 2018; Castel et al., 2019; Lapin et al., 2019; Wu et al., 2019, 2021; Sun et al., 2021; Huang et al., 2022; Jia et al., 2022). Ca^{2+}-permeable channel activity appears to be evolutionarily conserved among CNL/RNLs and NRCs (Figure 3; Bi et al., 2021; Jacob et al., 2021; Ahn et al., 2022; Contreras et al., 2022; Feehan et al., 2022; Förderer et al., 2022), suggesting that Ca^{2+}-permeable channel activity is likely to be a unified mechanism for plant NLR signaling. However, the mechanisms of how NLR-induced Ca^{2+} influx is decoded remain poorly understood. Presumably, calcium-binding proteins, including calmodulins (CaMs), CAM-like proteins (CMLs), calcineurin B-like proteins (CBLs), and CPKs, translate the elevated cytosolic Ca^{2+} levels into a downstream immune response (Luan and Wang, 2021).

The notion that CNL resistosomes act as Ca^{2+} channels is of substantial significance for understanding NLR signaling because it suggests that extracellular Ca^{2+} influx into plant cells is a major NLR-activated signal to initiate host defense and cell death (Figure 3). Multiple lines of evidence support NLR-activated Ca^{2+} influx as a trigger for immune signaling. The ZAR1 resistosome forms hours before loss of PM integrity (an indicator of HR cell death) (Bi et al., 2021), suggesting that the resistosome is a trigger but not a direct executor of the HR cell death. Gain of function of the CNGC20 mutant with increased Ca^{2+} influx activity constitutively activates EDS1- and SA-dependent *Arabidopsis* immunity (Zhao et al., 2021), indicating that elevations in cytosolic Ca^{2+} accumulation can be sufficient to induce immune signaling. Like Ca^{2+} influx, ROS production is also one of the earliest events of plant immune responses (Thordal-Christensen et al., 1997) and is largely dependent on the activity of the PM-localized nicotinamide adenine dinucleotide phosphate (NADPH) oxidase RBOHD (Torres et al., 2002). ROS act as second messengers to amplify immune signaling. Pharmacological and genetic studies suggested that Ca^{2+} functions upstream of ROS during ETI (Grant et al., 2000). The NADPH oxidase inhibitor diphenylene iodonium chloride has little effect on ETI signaling (Grant et al., 2000). In contrast, treatment with lanthanum chloride ($LaCl_3$), an extracellular calcium antagonist, markedly suppresses ETI immune responses, including increases in cytosolic Ca^{2+} concentration, H_2O_2 accumulation, and HR cell death (Gao et al., 2013). The pathogen effector AvrRps4 strongly induces ion leakage activity of *Arabidopsis* leaves but not ROS and HR cell death (Ngou et al.,

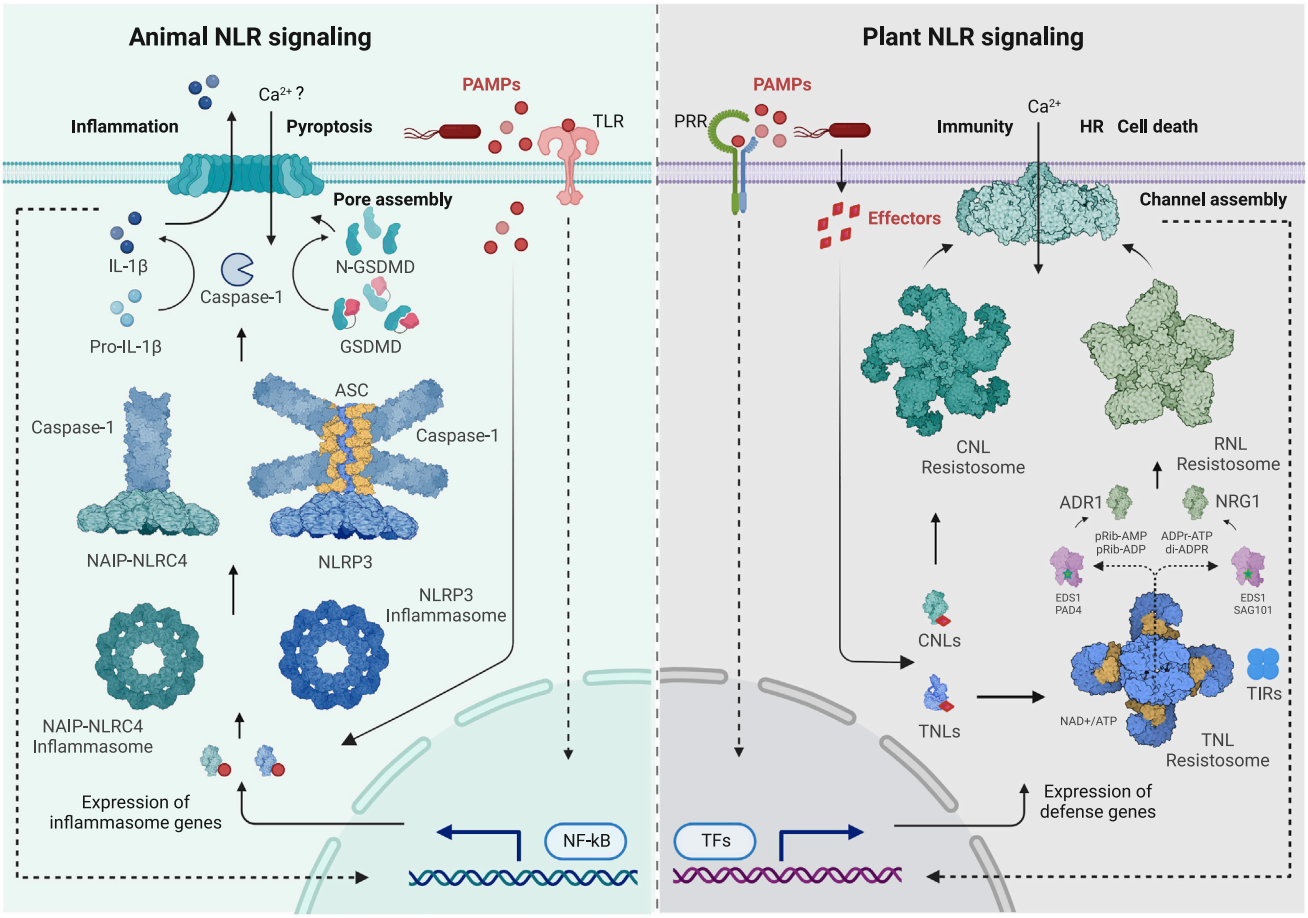

Figure 3. NLR signaling mechanisms in animals and plants.

Cartoon representations of NLR signaling in animals (left) and plants (right). Left: recognition of PAMPs or DAMPs induces assembly of NLRs, such as NLRC4 and NLRP3 inflammasomes. In the case of NLRP3, transcriptional upregulation of inflammasome-related genes by Toll-like receptors (TLRs) is needed to activate the inflammasome. The NLR inflammasomes interact with the ASC adaptor protein, which, in turn, recruits and activates caspase-1. When activated, caspase-1 proteolytically processes gasdermin D (GSDMD) to release its N-terminal pore-forming domain, which forms pores on the PM to execute pyroptosis. Caspase-1 also cleaves pro-IL-1β and pro-IL-18 into IL-1β and IL-18, which are released through the GSDMD pores. The NLRC4 inflammasomes can also directly recruit caspase-1 for activation. Right: plant CNL recognition of pathogen effectors induces pentameric resistosomes that act as PM Ca^{2+}-permeable channels to induce ETI. Recognition of pathogen effectors by plant TNLs results in formation of tetrameric resistosomes that function as NADase holoenzymes to catalyze production of the secondary messengers pRib-AMP/ADP and ADPr-ATP/di-ADPR, which bind to and activate EDS1-PAD4 and EDS1-SAG101, respectively. The second messenger-activated EDS1-PAD4 and EDS1-SAG101 allosterically induce the PM Ca^{2+}-permeable channel activity of helper NLRs (RNLs), including ADR1 and NRG1, triggering TNL-mediated ETI signaling. Activation of PRRs by PAMPs facilitates NLR signaling by upregulation of ETI-related genes.

2020). It remains unknown whether CNL resistosomes also have conductivity to deliver other signaling molecules. The pore size of the funnel-shaped structure in the ZAR1 resistosome is less than 10 Å in diameter (Wang et al., 2019a), which does not seem to be large enough to pass large signaling molecules. The possibility cannot be excluded that conformational changes in the pore during ETI allow passage of non-ion signaling molecules.

The increase in cytosolic Ca^{2+} during plant immunity can also be caused by release from internal Ca^{2+} pools (Spalding and Harper, 2011; Edel et al., 2017; Resentini et al., 2021; Koster et al., 2022). Supporting a role of Ca^{2+} released from these pools in plant immunity, inhibition of Ca^{2+} release from intracellular compartments by ruthenium red (RR) blocks HR cell death induced by the pathogen effectors AvrRpt2 or AvrRpm1 (Gao et al., 2013). However, RR inhibition appears to be less efficient than $LaCl_3$ (Gao et al., 2013), supporting the idea that Ca^{2+} influx is a trigger for ETI responses. NLR-activated Ca^{2+} influx as a signaling trigger agrees with the cell death activity of CNL resistosomes in animal cells. Co-expression of Sr35 and AvrSr35, but not either alone, triggers cell death in insect cells (Förderer et al., 2022). Similar cell death activity has also been demonstrated for auto-active ADR1 and NRG1 mutants in human HeLa cells (Jacob et al., 2021). These results suggest that unregulated channel activity is sufficient to recapitulate plant CNL/RNL-mediated cell death in eukaryotic cells. Although the mechanisms underlying the cell death mediated by plant CNL/RNLs in animal and insect cells remain unknown, these findings support the idea that the Ca^{2+} ion can act as a cell death trigger to kill animal cells (Orrenius et al., 2003). Ca^{2+} as a trigger of cell death was first suggested to be involved in the cardiac pathology that occurs after ischemia

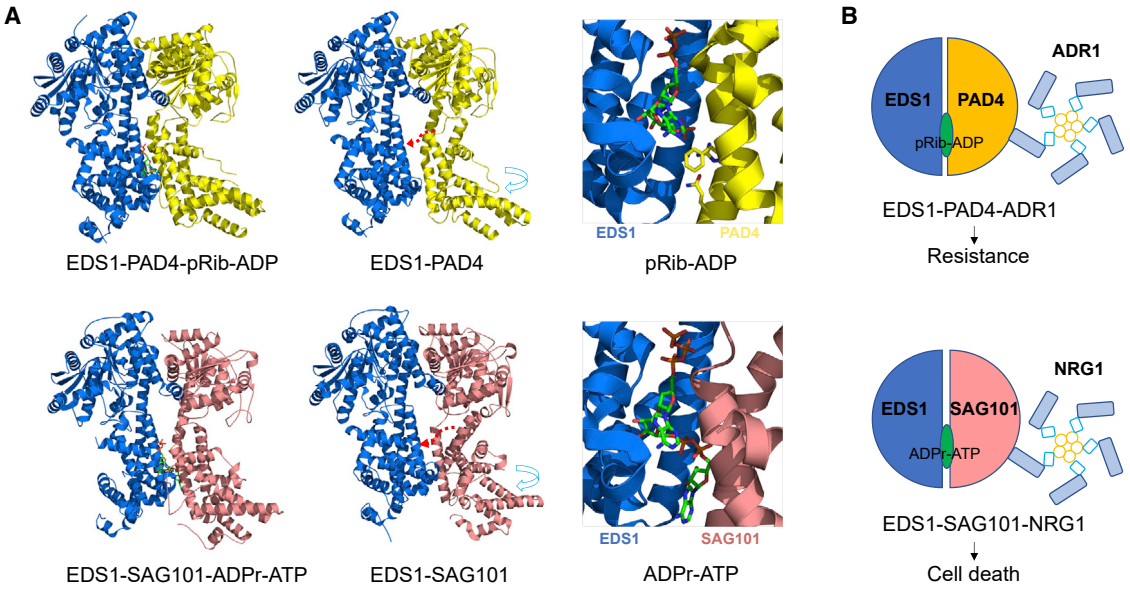

Figure 4. EDS1 signaling activated by TIR-catalyzed small molecules.
(A) The plant TIR-catalyzed small molecules pRib-ADP and ADPr-ATP bind specifically to EDS1-PAD4 and EDS1-SAG101 dimers, respectively, triggering conformational changes in PAD4 and SAG101 EP domains. EDS1, PAD4, and SAG101 are shown in blue, yellow, and pink, respectively. pRib-ADP and ADPr-ATP are shown in green. The small molecule binding grooves of the EDS1 complexes are highlighted.
(B) Small molecule binding allosterically induces EDS1-PAD4 and EDS1-SAG101 complex interaction with ADR1 and NRG1, respectively.

(Fleckenstein et al., 1974). Now it is well established that the Ca^{2+} ion has a central role in activating distinct parts of apoptosis through itself or in conjunction with other apoptotic programs to kill the cell (Orrenius et al., 2003).

ETI involves massive transcriptional reprograming. How can this be reconciled with the PM channel activity of CNL/RNLs? One might expect that Ca^{2+} itself enters the nucleus and activates Ca^{2+}-dependent proteins in the nucleus. Consistent with this possibility, many Ca^{2+} sensors, including CaMs, CPKs, and even transcriptional factors such as CALMODULIN-BINDING PROTEIN 60g (CBP60g) (Li et al., 2021) and CaM-binding transcription activator (CAMTA) 3 (Jacob et al., 2018; Sun et al., 2020), are nucleus localized. Increases in nuclear free Ca^{2+} concentrations have been reported in response to various stresses (van Der Luit et al., 1999; Pauly et al., 2001; Xiong et al., 2004). However, this model is not supported by the observation that dynamic cyto-nuclear trafficking is required for the disease resistance activity of many NLRs, including CNLs (Gu et al., 2016, 2017; Ludke et al., 2022). An alternative model for the transcriptional activity of CNLs/RNLs may be that they adopt different structural forms from those of the resistosomes to mediate immunity in the nucleus. The CC domain of ZAR1 adopts different fold architectures in its inactive and active structures (Wang et al., 2019a), a phenomenon called protein metamorphosis (Bryan and Orban, 2010), that can confer them different functions. Several CNLs have been shown to interact with transcriptional factors (Wang et al., 2021a), suggesting that CNLs may directly regulate transcriptional programming. Given that overexpression of the CC domain is sufficient to induce pathogen resistance (Wroblewski et al., 2018), it is reasonable to assume that this structural domain is involved in interaction with CNL-interacting partners for regulation of transcriptional programming. The CC domain of the *Hordeum vulgare* (barley) CNL MILDEW-A (MLA) 10 interacts with the transcriptional factors WRKY DNA-BINDING PROTEIN (WRKY) 1/2 (Shen et al., 2007). A recent study shows that NRG1 is PM and nucleus localized upon activation, but only the PM-resident NRG1 forms oligomers (Feehan et al., 2022), suggesting oligomerization-independent NRG1 functions.

TIR-catalyzed small molecules function as second messengers to link TNL signaling to RNLs

EDS1, PAD4, and SAG101 constitute a plant-specific family sharing similar domain structures, including an N-terminal lipase-like domain and a highly conserved C-terminal EDS1-PAD4 (EP) domain (Wagner et al., 2013). EDS1 forms exclusive dimers with PAD4 and SAG101. Genetic and biochemical data support functional cooperation of EDS1-PAD4 with ADR1 and EDS1-SAG101 with NRG1 (Peart et al., 2005; Bonardi et al., 2011; Collier et al., 2011; Qi et al., 2018; Castel et al., 2019; Lapin et al., 2019; Wu et al., 2019, 2021; Sun et al., 2021). However, the mechanism of how the EDS1 dimers integrate signals from TNLs to activate ADR1 and NRG1 of the RNL class have remained elusive until recently. The TIR NADase activity produces a variant cyclic adenosine diphosphate ribose (v-cADPR) that is a potential immune signaling molecule (Wan et al., 2019). Variant cyclic ADPR formed by 1″-2′ glycosidic linkage between the two riboses of NAD^+ (2′cADPR) is generated by plant TIR domain proteins (Leavitt et al., 2022; Manik et al., 2022). Our recent findings demonstrate RPP1 resistosome-induced specific EDS1-PAD4 interaction with ADR1 and EDS1-SAG101 interaction with NRG1 in insect cells (Huang et al., 2022; Jia et al., 2022; Figure 4). Similar activity also exists in the TIR domain of the *Arabidopsis* TNL RPS4 (RPS4-TIR). The RPP1 resistosome- or

RPS4-TIR-catalyzed small molecules bind to and stimulate EDS1 heterodimer interactions with ADR1 or NRG1. With high-resolution mass spectrometry, crystallography, and cryoelectron microscopy, the small molecules have been identified as structurally related 2-(5″-phosphoribosyl)-5″-adenosine monophosphate (pRib-AMP), pRib-ADP, ADP-ribosylated ATP (ADPr-ATP), and ADP-ribosylated ADPR (di-ADPR). Chemically synthesized pRib-AMP/ADP induce strong EDS1-PAD4 interaction with ADR1 but weak EDS1-SAG101 interaction with NRG1. In contrast, ADPr-ATP/di-ADPR are highly specific for promoting formation of the EDS1-SAG101-NRG1 complex. The overlapping but distinct activity of the TIR-catalyzed products is consistent with the shared and specific immune outputs mediated by EDS1-PAD4-ADR1 and EDS1-SAG101-NRG1 (Dongus and Parker, 2021). Thus, specific immune functions of the two EDS1 heterodimers could be conferred by their recognized TIR-catalyzed small molecules. TIR-induced assemblies of the EDS1-PAD4-ADR1 and EDS1-SAG101-NRG1 signaling complexes explain the functional incompatibility between EDS1-SAG101 and NRG1 in different plant species (Gantner et al., 2019; Lapin et al., 2019).

Structural studies reveal the mechanisms of selective EDS1-SAG101 activation by ADRr-ATP/di-ADPR and preferential EDS1-PAD4 activation by pRib-ADP/AMP. pRib-ADP and ADPr-ATP interact with a similar groove formed by the C-terminal EP domains of EDS1-PAD4 and EDS1-SAG101, respectively (Huang et al., 2022; Jia et al., 2022; Figure 4). The pRib-ADP-contacting residues of PAD4 are largely conserved in SAG101. In contrast, SAG101 residues for interacting with the ADPR moiety of ADPr-ATP are variable in PAD4. These variations result in a much smaller ligand binding groove in EDS1-PAD4, explaining why the complex is unable to recognize the comparatively bulkier ADPr-ATP/di-ADPR. Mutations of amino acids at the ligand binding sites reduce EDS1-PAD4 and EDS1-SAG101 association with ADR1 and SAG101, respectively, and compromise immunity functions *in vivo* (Lapin et al., 2019; Sun et al., 2021; Dongus et al., 2022). These results support the biological significance of the EDS1-PAD4-ADR1 and EDS1-SAG101-NRG1 complexes. pRib-ADP and ADPr-ATP become nearly completely buried after binding to EDS1 heterodimers. Their binding induces similar conformational changes in the C-terminal EP domains of PAD4 and SAG101. These results suggest that these two small molecules allosterically induce EDS1 heterodimer interactions with downstream ADR1 and NRG1. However, whether this NRG1/ADR1 activation mechanism is conserved among different plant species remains unknown. One recent study indicates that nuclear accumulation of EDS1 complexes is sufficient for ROQ1-induced cell death in *N. benthamiana* (Zonnchen et al., 2022), suggesting different activation mechanisms of EDS1.

The available data support the four TIR-catalyzed small molecules as second messengers (Figure 3). A second messenger is produced by an enzyme whose formation is regulated by a first messenger, induces specific effectors to exert specific biological effects, and is removed by a signal-terminating system (Seifert, 2015). pRib-AMP and pRib-ADP are rapidly degraded in *N. benthamiana* extracts (Huang et al., 2022), suggesting the existence of mechanisms involved in eliminating TIR-catalyzed products to terminate their signaling. The enzymes responsible for this remain unknown, but Nudix hydrolases, which hydrolyze a wide range of organic pyrophosphates with varying degrees of substrate specificity (McLennan, 2006), can be potential candidates. TIR-catalyzed small molecules are likely conserved in different plant species. *Brachypodium distachyon* TIR domain-containing protein (BdTIR), triggers cell death when expressed in *N. benthamiana* (Wan et al., 2019) and catalyzes small molecules that can activate *Arabidopsis* EDS1-SAG101 interaction with NRG1 *in vitro* (Jia et al., 2022).

In addition to being NADases, plant TIR domain proteins also have ADPR-transferase activity (Jia et al., 2022). In contrast to the canonical poly-ADP-ribosylation (PAR) polymerases (Alemasova and Lavrik, 2019), however, TIRs catalyze transfer of ADPR from NAD^+ to small molecules. When NAD^+ is the only substrate, the NAD^+-derived ADPR is transferred to the NAD hydrolyzed product ADPR, resulting in formation of di-ADPR. ADPr-ATP can be formed when NAD^+ and ATP are used as the substrates (Figure 5). It will be of interest to investigate whether TIRs can catalyze transfer of ADPR to other small molecules. The configuration of C'' (arrangement of its covalently linking atoms) from ADPr of ADPr-ATP or di-ADPR remains the same as that of NAD^+. This contrasts with ADPR chain extension catalyzed by PAR polymerases (Alemasova and Lavrik, 2019). Thus, di-ADPR, when generated by hydrolysis of PARs, is an enantiomer of that catalyzed by TIRs.

TIR-catalyzed production of di-ADPR and ADPr-ATP strongly supports the ribosyl-transferase activity of plant TNLs and TIR domain-containing proteins (Jia et al., 2022). *In vitro* biochemical data suggest that hydrolysis of these two compounds leads to production of pRib-AMP or pRib-ADP (Jia et al., 2022). This could be a mechanism by which plants balance ADR1 and NRG1 signaling. Regardless of the biosynthetic pathways of these nucleotide derivatives, plant TIR domain proteins are multi-functional enzymes. It will be of interest to investigate whether these TIR-catalyzed products can be metabolized to generate novel signaling molecules.

TIR domain proteins are 2′,3′-cyclic AMP (cAMP)/cyclic guanosine monophosphate (cGMP) synthetases

In addition to the enzyme activities discussed in the last section, plant TIR domains, including the *Arabidopsis* TIR-only protein RBA1 and the TIR domain of the flax TNL L7, also display 2′,3′-cAMP/cGMP synthetase activity with double-stranded RNA (dsRNA) or double-stranded DNA (dsDNA) as substrates (Yu et al., 2022) (Figure 1). *In vitro*, the two TIR domain proteins strongly prefer dsRNA as a substrate for production of these two non-canonical cyclic nucleotide monophosphates (cNMPs), although *in vivo* substrates remain to be identified. In contrast, ATP and guanosine triphosphate are not *in vitro* substrates of TIRs to generate 2′,3′-cAMP/cGMP, suggesting that nuclease activity is required for the synthetase activity. The nuclease activity, however, is not sufficient for TIR-mediated cell death in *N. benthamiana* because a mutation of RBA1 Cys83 has little effect on nuclease activity but substantially suppresses the cell death activity in *N. benthamiana* (Yu et al., 2022). 2′,3′-cNMPs were initially identified as intermediates of RNA cleavage by RNases, which also generate 2′,3′-cyclophosphate-terminated RNA oligonucleotides (Jackson, 2017). In contrast with these RNases, no similar products are produced by plant TIR domains when dsRNA is used as the substrate.

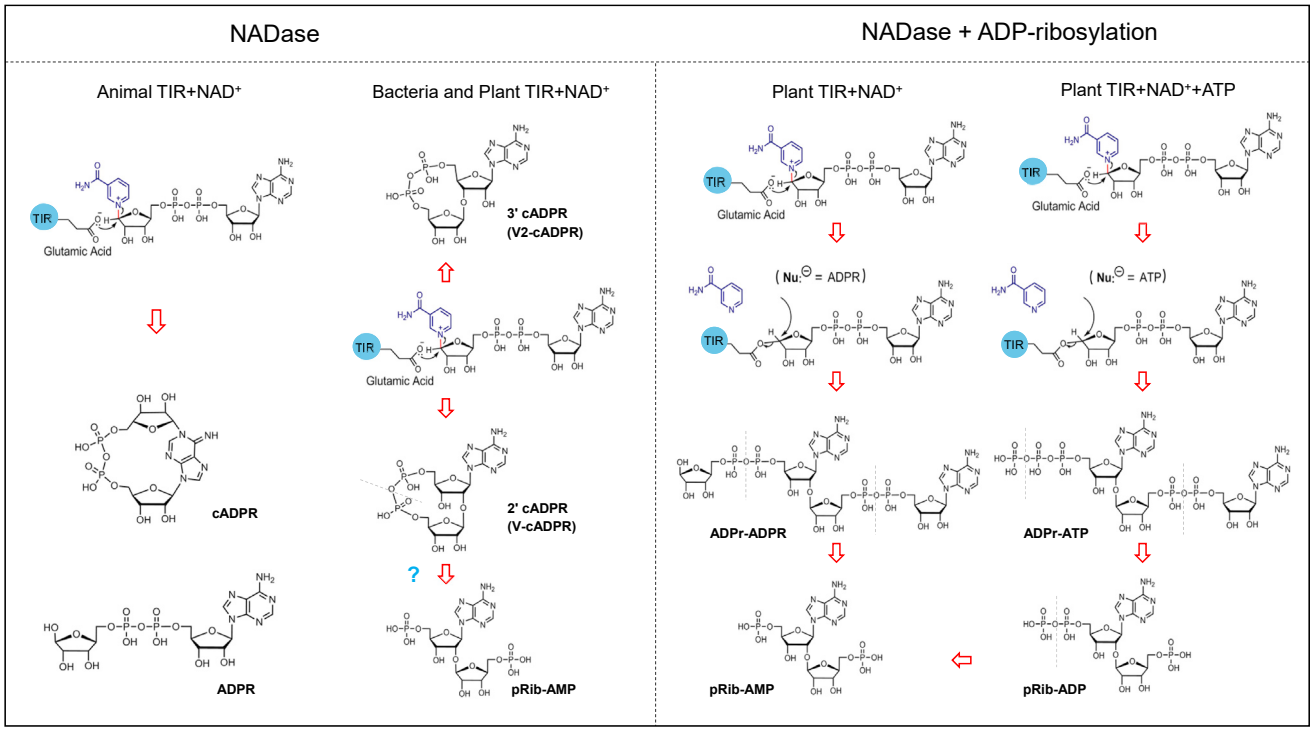

Figure 5. Production of immune signal molecules by TIR-mediated NAD$^+$ degradation in different species.
The SARM1 TIR domain hydrolyzes NAD$^+$ to produce ADPR and cADPR, whereas bacterial and plant TIRs hydrolyze NAD$^+$ to produce ADPR, 2′ cADPR, and 3′ cADPR. On the other hand, plant TIRs can use NAD$^+$ or NAD$^+$/ATP as substrates to produce pRib-AMP/pRib-ADP and di-ADPR/ADPr-ATP, respectively via their NADase and ribosyl-transferase activities. Production of pRib-ADP/AMP likely occurs through hydrolysis of ADPr-ATP/di-ADPR. pRib-AMP might also be produced by hydrolysis of 2′ cADPR. Plant TIR domains can also catalyze dsRNA hydrolysis to generate 2′,3′-cAMP/cGMP. The TIR domains are shown in filled blue circles. The conserved catalytic residue glutamic residue is indicated.

RBA1 Cys83 is highly conserved among plant TIR domain proteins, and mutations of this RBA1 residue or its equivalents in other TIRs significantly impair the cell death activity in *N. benthamiana* (Bernoux et al., 2011; Williams et al., 2016). RBA1 C83A is greatly reduced in 2′,3′-cAMP/cGMP synthetase activity but still retains wild-type-like NADase activity, supporting a critical role of 2′,3′-cAMP/cGMP in RBA1-mediated cell death (Yu et al., 2022). Accumulation of these two non-canonical 2′,3′-cNMPs in *N. benthamiana* plants expressing the wild type but not the catalytic mutant E86A of RBA1 is significantly enhanced. Evidence of the significance of 2′,3′-cAMP/cGMP in the cell death activity also comes from the nucleotide hydrolase NUDIX HYDROLASE HOMOLOG 7 (NUDT7), a negative regulator of EDS1 signaling in *Arabidopsis* (Bartsch et al., 2006; Ge et al., 2007). NUDT7 hydrolyzes 2′,3′-cAMP/cGMP but not 3′,5′-cAMP/cGMP, and the hydrolysis activity is completely abolished by the catalytic mutation E154Q (Yu et al., 2022). Co-expression with wild-type NUDT7 but not the E154Q mutant greatly suppresses RBA1-mediated cell death activity in *N. benthamiana*. These results suggest that TIR-catalyzed 2′,3′-cAMP/cGMP are upstream of EDS1. However, expression of RBA1 in *eds1* mutants of *N. benthamiana* induces no pronounced accumulation of 2′,3′-cAMP/cGMP. To reconcile these data, a model is proposed where positive feedback is formed between 2′,3′-cAMP/cGMP and EDS1 in EDS1 signaling (Yu et al., 2022).

Interestingly, asymmetric TIR dimers similar to those in the RPP1 and ROQ1 resistosomes required for NADase activity are not present in the filament structure of L7-TIR bound by dsDNA (Figure 6). The TIR tetramer in the TNL resistosomes is incompatible with the oligomeric TIR in the filament structure, suggesting that TNL resistosomes might not have synthetase activity. RPP1 alone, but not the RPP1-ATR1 resistosome, exhibits 2′,3′-cAMP/cGMP synthetase activity *in vitro* (D.Y and J.C., perosnal communication, D.Y and J.C). A more recent study shows that a mutation of Cys90 in the *Arabidopsis* TNL SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1 (SNC1), predicted to be important for 2′,3′-cAMP/cGMP synthetase activity, has no effect on immune response, but the enzymatic activity of the TNL remains untested (Tian et al., 2022). It currently remains unclear whether and how the 2′,3′-cAMP/cGMP synthetase activity contributes to TNL function. It is possible that *TIR*-only genes are induced during TNL activation and that 2′,3′-cAMP/cGMP synthesized by these TIR-only proteins function as signal amplifiers in TNL signaling. Alternatively spliced TIR domain proteins are required for full function of some TNLs, suggesting that coordinated expression of alternative and regular transcripts of truncated TNLs and full-length TNLs is required for their full immune activity (Jordan et al., 2002).

RBA1 mutant C83A is compromised in 2′,3′-cAMP/cGMP synthetase activity but retains wild-type NADase activity (Yu et al., 2022). However, the RBA1 mutant displays activity in inducing EDS1-SAG101 interaction with NRG1 (Jia et al., 2022). This is in concert with the observations that NADase activity is not sufficient for TIR signaling (Horsefield et al., 2019; Wan et al., 2019; Duxbury et al., 2020) and that inducible expression of

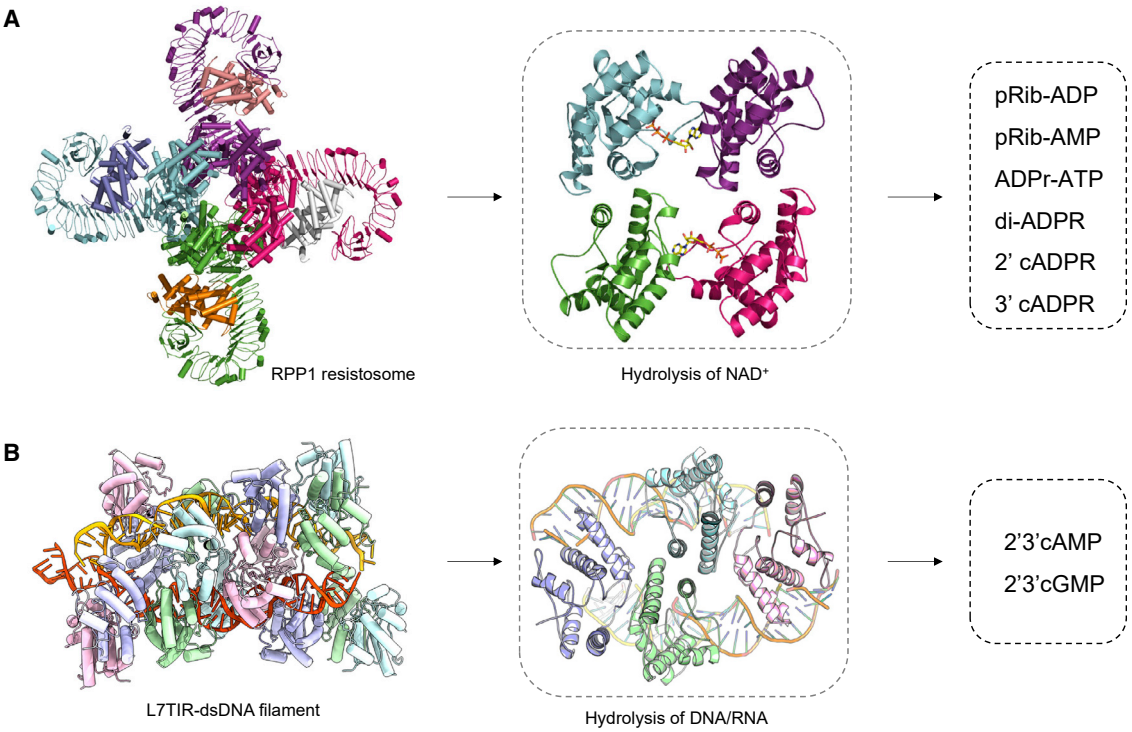

Figure 6. Plant TIR domains form different oligomers to hydrolyze NAD$^+$ and dsDNA/RNA.
(A) TIR domains from the RPP1 resistosome (left) form two asymmetric TIR dimers, each of which forms a composite active site. The two active sites are important for NAD$^+$ hydrolysis to produce the small molecules pRib-ADP/AMP, ADPr-ATP/di-ADPR, 2′cADPR, and 3′cADPR.
(B) Left: the L7 TIR domains bound by dsDNA (left). Right: a tetrameric L7 TIR in the complex. The symmetric TIR dimer required for formation of the composite active sites in the RPP1 resistosome is not present in the L7 TIR complex. Formation of the L7 TIR-dsDNA or, presumably, L7 TIR-dsRNA is important for production of 2′,3′cAMP/cGMP.

AvrRps4 induces no cell death and MAPK activation (Ngou et al., 2020). How can these *in vitro* and *in vivo* data be reconciled? It should be borne in mind that all components have to be in place for ligands to activate receptor signaling *in vivo*. This is well exemplified by activation of the animal NLR NLRP3, which requires a priming signal for transcriptional up-regulation of *NLRP3* and pro-IL-1β in addition to NLRP3-specific stimuli (Swanson et al., 2019). 2′,3′-cAMP induces many stress-related genes in *Arabidopsis* (Kosmacz et al., 2018; Chodasiewicz et al., 2022), raising the possibility that this non-canonical cNMP and probably 2′,3′-cGMP as well might upregulate genes required for activation of EDS1 signaling.

TIR domains are multi-functional enzymes

As discussed above, plant TIR domains encode multiple enzymatic activities catalyzing metabolism of NAD$^+$, NAD$^+$+ATP, and nucleic acids, resulting in production of structurally diversified nucleotide metabolites, including pRib-ADP/AMP, di-ADPR/ADPr-ATP, and 2′,3′-cAMP/cGMP. These nucleotide compounds function to induce EDS1 signaling. Besides these TIR-catalyzed products, plant TIR domain proteins, such as *B. distachyon TIR domain-containing protein*, can also produce 2′cADPR and 3′cADPR (Bayless et al., 2022; Manik et al., 2022). However, the biological function of these two nucleotide derivatives in plant immune signaling remains unclear. Hydrolysis of 2′cADPR has been proposed to produce pRib-AMP (Manik et al., 2022; Figure 5). However, this model cannot explain how TIR-catalyzed pRib-ADP is produced. Alternatively, it is formally possible that 2′ cADPR results from circularization of pRib-AMP via phosphate-phosphate linkages. Bacterial TIR domains, such as *A. baumannii TIR* and Thoeris B from the bacterial antiphage defense system, also have the function of catalyzing production of 2′cADPR and 3′cADPR (Ofir et al., 2021; Manik et al., 2022; Figure 5). 3′cADPR and, to a lesser extent, 2′cADPR activate the NADase activity of Thoeris A, depleting cellular NAD$^+$ and causing death of bacteria. The phytobacterial TIR domain-containing effector HopAM1 can generate 3′cADPR when hydrolyzing NAD$^+$ (Eastman et al., 2022; Manik et al., 2022). Production of this compound is associated with HopAM1 suppression of plant immunity, but it cannot be excluded that HopAM1 also produces other small molecules for virulence. The mechanism of how HopAM1 inhibits plant immunity remains elusive, but NAD$^+$ depletion could have a role in this process. The mammalian TIR domain-containing protein SARM1 can hydrolyze NAD$^+$ into nicotinamide, ADPR, or cADPR (Shi et al., 2022). The NADase activity of SARM1 is essential to induce axon degeneration. The underlying mechanism may be direct NAD$^+$ depletion or production of signaling molecules to kill cells indirectly.

CALCIUM SIGNALING IN PLANT IMMUNITY

Ca^{2+} is a shared trigger for PTI and ETI signaling

It is becoming increasingly evident that PRRs and NLRs share many signaling components, including calcium channels,

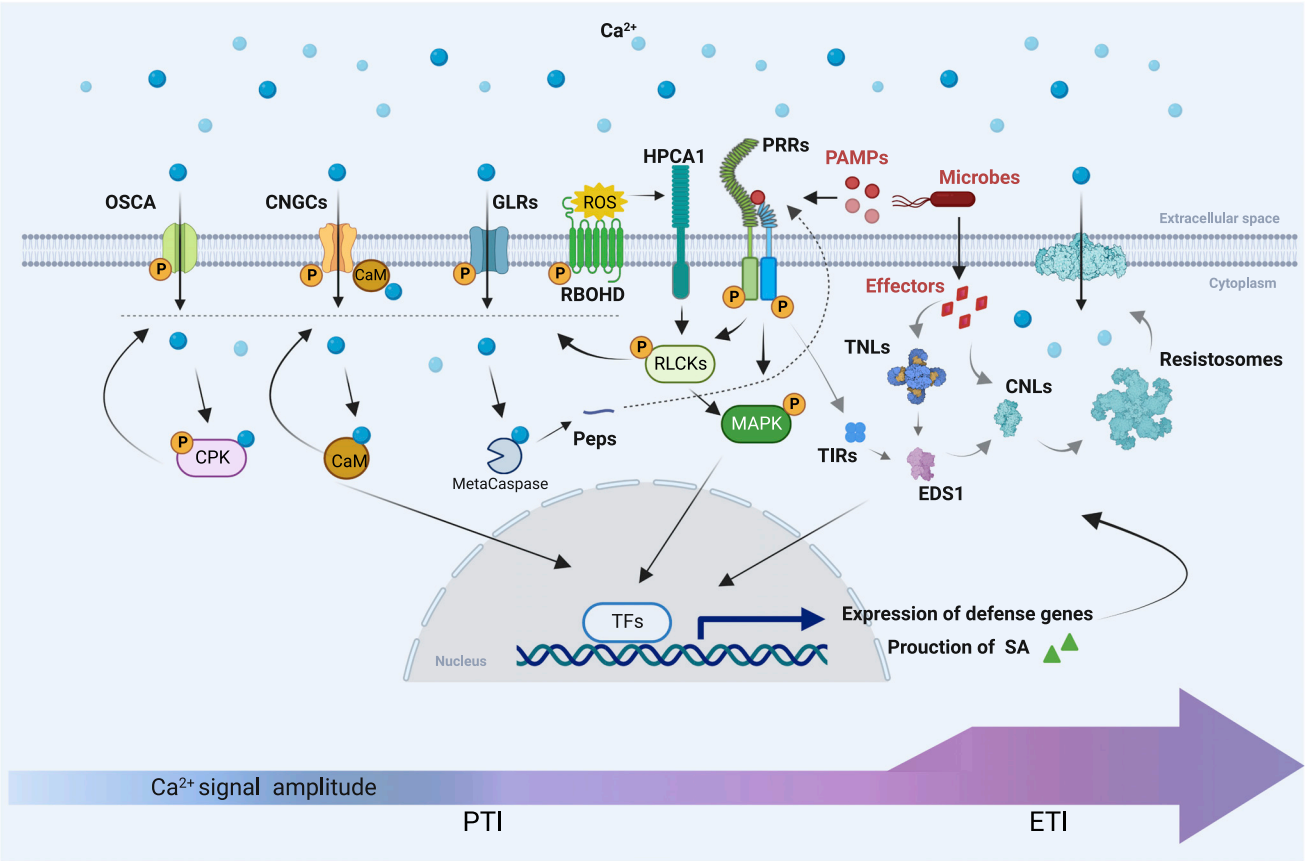

Figure 7. Calcium signaling in plant immunity.
In PTI, PAMP-activated PRRs trigger phosphorylation of RLCKs and MAPK cascades. Activation of RLCKs leads to phosphorylation and activation of PM-localized Ca^{2+} channels, including CNGCs, GLRs, and OSCAs, for Ca^{2+} influx. In ETI, pathogen effectors are perceived by the intracellular NLRs. Pathogen effector-activated CNLs or TNL-activated RNLs form resistosomes. CNL/RNL resistosomes act as PM Ca^{2+}-permeable channels to allow extracellular Ca^{2+} influx. Calcium transporters such as ACAs mediate calcium efflux to counterbalance Ca^{2+} signaling. Ca^{2+} signaling is involved in many amplification loops, including Ca^{2+}-ROS, Ca^{2+}-SA, Ca^{2+}-Ca^{2+}, and PTI-ETI. The CPK, CaM, RBOHD, CAMTA3, and CBP60g transcription factors; metacaspase; and TIR proteins play critical roles in these amplification loops. Compared with PTI, ETI triggers strong and long-lasting cytosolic Ca^{2+} influx, leading to HR cell death.

NADPH oxidases, and MAPKs (Cui et al., 2015; Yu et al., 2017; Zhou and Zhang, 2020; DeFalco and Zipfel, 2021). This is supported by transcriptional profiling of activated NLRs, including the barley CNL MLA1 (Jacob et al., 2018), the *Arabidopsis* TNL RPS4 (Sohn et al., 2014), and various PRRs (Bjornson et al., 2021), which show significant overlap in early response genes. For example, two pathogen-induced transcription factors, SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1) and CBP60g, are a convergent point in PTI and ETI signaling (Huang et al., 2021b; Li et al., 2021). These results suggest that PTI and ETI signaling may converge somewhere to mediate activation of shared downstream components.

Many PAMPs and DAMPs have been shown to be sufficient to induce rapid Ca^{2+} signals in plant cell culture (Atkinson et al., 1996; Levine et al., 1996; Gelli et al., 1997; Zimmermann et al., 1997) and *Arabidopsis* plants (Ranf et al., 2008, 2011; Vadassery et al., 2009; Krol et al., 2010). Treatment with $LaCl_3$ completely abrogates the PAMP/DAMP-induced Ca^{2+} signals, suggesting that PM-localized, calcium-permeable channels are required for the Ca^{2+} signals. Supporting this conclusion, inhibition of Ca^{2+} release from intracellular compartments by RR barely affects Pep-13-triggered immune responses in *Petroselinum crispum* (parsley) cells (Blume et al., 2000). *rbohd* mutants only show a slight defect in elicitor-triggered Ca^{2+} signals in *Arabidopsis* seedlings (Ranf et al., 2011). In contrast, elicitor-induced ROS production is severely attenuated by treatment with Ca^{2+} channel blockers (Ranf et al., 2011). These results suggest that Ca^{2+} signals act upstream of ROS production to trigger PTI signaling. Pharmacological data also support involvement of Ca^{2+} signaling upstream of MAPK pathways in plant immunity (Lebrun-Garcia et al., 1998; Romeis et al., 1999; Link et al., 2002). Several classes of PM-localized channels, including CNGCs (Tian et al., 2019), OSCAs (Thor et al., 2020), and glutamate-like receptors (GLRs) (Kwaaitaal et al., 2011; Bjornson et al., 2021), implicated in conducting Ca^{2+} during plant immunity, have been identified.

Because Ca^{2+} is a trigger for the PTI and ETI signaling pathways (Figure 7), and many components are also shared in these two processes, how can they lead to different biological outcomes? The different origins and amplitudes of calcium signals could be important in determining the different physiological responses of PTI and ETI signaling. Although PAMPs/DAMPs induce a transient rise in cytosolic Ca^{2+} (Tian et al., 2019), a sustained increase in

cytosolic Ca^{2+} is observed in NLR-mediated ETI (Bi et al., 2021; Jacob et al., 2021). These data appear to support the Ca^{2+} signature hypothesis, where spatial and temporal characteristics of stimulus-specific Ca^{2+} signals contribute to the specificity of the biological outcome (Kim et al., 2022; Koster et al., 2022). However, strong evidence of specific information encoded by Ca^{2+} signatures leading to a specific outcome is scarce (Hetherington and Brownlee, 2004). Different PAMPs/DAMPs induce distinct cytosolic Ca^{2+} signatures (Ranf et al., 2011) despite conserved downstream components of PTI signaling. There is evidence of an alternative model to explain the specificity of Ca^{2+} signals (Scrase-Field and Knight, 2003). This model hypothesizes that Ca^{2+} simply acts as a switch to activate Ca^{2+}-dependent components and that the signaling specificity is dictated by components other than Ca^{2+}. The switch model can also explain the differences in PTI and ETI responses. For example, it may be that PTI- and ETI-activating channels associate with different Ca^{2+}-sensing partners to decode the different origins of Ca^{2+} signals, leading to different physiological responses. The signature and switch mechanisms might operate in PTI and ETI, depending on the circumstances in plant cells.

In addition to Ca^{2+} influx (Spalding and Harper, 2011; Edel et al., 2017; Resentini et al., 2021), Ca^{2+} efflux mediated by active transporters is involved in shaping Ca^{2+} signals (Figure 7). Ca^{2+} in the cytoplasm can also be pumped to the intracellular Ca^{2+} stores, including the vacuole and endoplasmic reticulum (ER), through Ca^{2+} pumps and Ca^{2+}/H^{+} exchangers (CAXs). The coordinate action of Ca^{2+} channels and transporters generates stimulus-specific signals. In addition to CAXs, active Ca^{2+} transporters include auto-inhibited Ca^{2+}-ATPases (ACAs) and ER Ca^{2+}-ATPases (Geisler et al., 2000; Shigaki and Hirschi, 2000; Garcia Bossi et al., 2020). Double knockout of the PM-localized ACA8 and its homolog ACA10 compromises flg22-induced Ca^{2+} signals and resistance to *Pseudomonas syringae* infection (Frei dit Frey et al., 2012) and stomatal closure upon PAMP perception (Yang et al., 2017). Besides PM-localized ACA8/10, ACA4/11 on tonoplasts and ACA1/2/7 on the ER are also involved in PTI-triggered Ca^{2+} signaling, although the mechanism remains unclear (Hilleary et al., 2020; Rahmati Ishka et al., 2021). CNGCs and ACAs are subject to regulation by CaM and phosphorylation; for example, to control Ca^{2+} transport across the PM. Binding of CaM can activate and inactivate CNGCs (Hua et al., 2003). CaM7-gated CNGC2-CNGC4 channel activity has been demonstrated recently in *Arabidopsis*. Overexpression of CaM7 substantially compromises flg22-induced PTI signaling (Tian et al., 2019). The biological significance of CaM7 inhibition of CNGC2-CNGC4 remains unclear, but it might provide negative feedback restricting Ca^{2+} flux into plant cells. CaM stimulates the activity of ACA pumps by preventing their auto-inhibition. The activity of ACAs can also be inhibited by phosphorylation in the N-terminal domain (Geisler et al., 2000). However, more studies are needed to connect ACAs and CAXs to the immunity signaling pathways.

Ca^{2+} signals and amplification of plant immune responses

Formation of high-order assembly is a common strategy adopted by immune receptors in animals for amplification of immune signaling (Cai et al., 2017). For example, filament formation of ASC seeded by NLR inflammasomes can markedly improve the efficiency of caspase activation by recruiting a large number of inactive caspase molecules (Lu et al., 2014). Although higher-order oligomers (Yu et al., 2022) and phase separation (Huang et al., 2021a) have been shown to be involved in plant immune signaling, the plant immune system appears to rely more on signal amplification loops to mount quick and robust immune responses. These amplification loops typically involve synergistic interactions between Ca^{2+}, ROS, and SA. In this section, we discuss plant immune signaling amplification involving Ca^{2+}.

ROS and intracellular calcium signaling interact with and amplify each other, forming the ROS-Ca^{2+} self-amplifying loop (Figure 7). The major ROS-producing enzyme RBOHD, during plant immunity, can be activated by directly binding cytosolic Ca^{2+} with its N-terminal EF-hand motif or phosphorylation by Ca^{2+}-binding proteins (Ogasawara et al., 2008). CPK5 has been shown to activate ROS signaling by phosphorylating RBOHD (Boudsocq et al., 2010; Dubiella et al., 2013). CBL1/CBL9-interacting protein kinase 26 phosphorylates RBOHF to produce ROS (Drerup et al., 2013; Kimura et al., 2013). Abrogation of ROS accumulation in the *rbohD* mutant or through inhibitor application leads to loss of a second Ca^{2+} peak during ETI, demonstrating a feedback effect of ROS on Ca^{2+} signaling (Gao et al., 2013). In addition, ROS can be sensed by the PM-anchored RLK HYDROGEN-PEROXIDE-INDUCED CA^{2+} INCREASES 1 (HPCA1), leading to downstream signaling, including activation of Ca^{2+} influx (Tian et al., 2019; Wu et al., 2020). A more recent study depicts a role of HPCA1 in this ROS-Ca^{2+} self-amplifying loop by showing that HPCA1 triggers an increase in cytosolic Ca^{2+} levels via the calcium-permeable channel MECHANOSENSITIVE ION CHANNEL-LIKE 3 (MSL3). HPCA1 is required for systemic cell-to-cell ROS and calcium signaling in response to local bacterial infection and a broad range of abiotic stresses (Fichman et al., 2022).

Ca^{2+} influx and SA can form self-amplification loops to enhance immune response. For instance, Ca^{2+} influx modulates the activity of the calcium-binding transcription factor CAMTA3 and CBP60g, which results in further upregulation of expression of SA-biosynthesis genes, including ISOCHORISMATE SYNTHASE 1 (*ICS1*), ENHANCED DISEASE SUSCEPTIBILITY 5 (*EDS5*), and AVRPPHB-SUSCEPTIBLE 3 (*PBS3*) (Zhang et al., 2010b; Sun et al., 2015; Huang et al., 2020; Figure 7). SA perception by NONEXPRESSER OF PR GENES (NPR) 1 or NPR3/NPR4 induces a plethora of defense-related genes, including a number of PRRs, NLRs, and their signaling components (Ding et al., 2018). In SA biosynthesis- or perception-defective mutants, PRR- and NLR-mediated immunity is compromised (Zhang and Li, 2019). These data indicate that calcium influx of PRR or NLR immune signaling promotes accumulation of SA, which, in turn, enhances calcium signaling and facilitates PTI and ETI responses.

Elevations in the cytosolic concentrations of Ca^{2+} can activate Ca^{2+}-dependent metacaspases (Figure 7), which mature and release an endogenous elicitor, Pep1, in *Arabidopsis* (Hander et al., 2019; Shen et al., 2019). The DAMP initiates a feedback mechanism to amplify the original PTI signaling, including Ca^{2+} influx. Ca^{2+} signals can also be amplified by positive regulation of Ca^{2+} transporters and channels, including ACAs, CNGCs, and GLRs modulated by CaMs or CPKs (Geisler et al., 2000; Zhou et al., 2014; Pan et al., 2019). Amplification of initial

Ca^{2+} signals by secondary Ca^{2+} signals generated by endomembrane Ca^{2+} channels has been reported (Swarbreck et al., 2013), although the identities of the secondary channels remain unknown.

Activation of ETI requires participation of PRRs and their co-receptors, and ETI can further amplify PTI, indicating that PRRs and NLRs cooperatively potentiate each other to enhance plant immune responses (Ngou et al., 2021; Pruitt et al., 2021; Tian et al., 2021; Yuan et al., 2021). Given that Ca^{2+} is a shared trigger for plant immunity, this raises the question of whether Ca^{2+} has a role in mutual potentiation of PTI and ETI. ETI-bolstered PTI is proposed to occur through elevation in intracellular Ca^{2+} concentrations (Bjornson and Zipfel, 2021). This model is consistent with Ca^{2+}-dependent transcriptional regulation of immune genes during ETI (Gao et al., 2013). Ca^{2+} influx promotes expression of TIR domain protein-encoding genes during PTI, and activation of these TIRs is important for PTI signaling in *Arabidopsis* (Pruitt et al., 2021; Tian et al., 2021). Activation of TIR signaling presumably results in Ca^{2+}-permeable channel activity of ADR1 and NRG1 (Jacob et al., 2021). These results indicate a critical role of self-amplification of Ca^{2+} signals in PTI. However, it cannot be excluded that activation of TIRs leads to Ca^{2+}-unrelated activity to promote PTI signaling. It currently remains unknown whether Ca^{2+} has a role in potentiating ETI by PTI, but there is evidence indicating that the ETI-potentiating signals seem not to be PTI specific. The intensity of cell death of the RPS4-expressing *N. benthamiana* plants is strictly correlated with expression levels of the TNL protein (Zhang et al., 2004). This result suggests that elevation in protein concentrations can have a role similar to PTI signaling in promoting RPS4-mediated cell death.

PERSPECTIVES

Ca^{2+} is a universal second messenger involved in diverse biological processes, including development and immunity (Tian et al., 2020). Many findings in the past few years highlight a critical role of Ca^{2+}-permeable channels in triggering initiation of PTI and ETI signaling (Xu et al., 2022a; Kim et al., 2022; Koster et al., 2022). These discoveries started to decipher the mechanisms underlying pathogen-induced Ca^{2+} influx, and some PM-localized channels involved in conducting Ca^{2+} during plant immunity were identified. Particularly, recent findings regarding plant resistosomes revealed that CNLs and TNLs can converge on Ca^{2+} signals to mediate ETI responses. However, CNLs resistosomes are non-canonical Ca^{2+}-permeable channels, and little is known about their channel properties. It remains completely unknown whether they are subjected to regulation like CNGCs and other canonical calcium channels. Biophysical and electrophysiological characterization of CNL resistosomes and Ca^{2+} channels involved in PTI signaling will facilitate our understanding of their gating properties. Such studies may provide insight into the quick and transient Ca^{2+} currents during PTI and sustained Ca^{2+} currents during ETI. Future studies directed toward deciphering how Ca^{2+} influx is translated into downstream transcriptional signals will advance our understanding of immune signaling. Many Ca^{2+} sensors, particularly CaMs and CMLs, are encoded in plant genomes. Therefore, it remains a challenge to define the biological role of a specific Ca^{2+} sensor in immune signaling. Valuable information to dissect specific functions of CaM/CMLs could be gained through analyses of gene expression, subcellular localization of proteins, and identification of their targets. It would be also challenging to disentangle the interplay among different Ca^{2+} channels and the relationship between Ca^{2+} channels and Ca^{2+} transporters. Such studies may provide clues about how Ca^{2+} signaling specificity is achieved. PTI and ETI inter-potentiate in plant immunity to achieve resistance against pathogens, but the underlying molecular mechanisms remain largely unknown.

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