



Life-or-death decisions in plant immunity

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Upon pathogen challenge, plant cells can mount defense not only by triggering programmed cell death (PCD) to limit pathogen growth, but also by secreting immune signals to activate subsequent organism-scale defense responses. Recent advances in the study of plant immune mechanisms have found that pathogen-induced oligomerization of immune receptors is a common 'on' switch for the normally self-inhibitory proteins. The resulting 'resistosome' triggers PCD through the formation of a calcium channel or a NADase. Synergy between different receptor-mediated signaling pathways appears to be required for sustained immune induction to trigger PCD of infected cells. In the neighboring cells, PCD is inhibited through the production of immune signal salicylic acid (SA) which mediates degradation of PCD-inducing immune components in biomolecular condensates. Future work is required to connect the resistosome-mediated channel formation and the NADase activity to the downstream regulation of immune execution.

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The advent of molecular study of innate immune mechanisms in both plants and animals was marked by the cloning of innate immune receptors in 1990s. The surprising conservation of specific domains found in these receptors from both kingdoms, such as the Toll/interleukin-1 receptor (TIR) domain and the leucine-rich repeat (LRR) domain, has inspired scientists to search for potential commonalities in pathogen sensing, signal transduction and immune execution in organisms across kingdoms. However, unlike the animal immune systems, plants do not have specialized immune cells, a plant immune response has to be balanced with normal physiological activities. There are also distinctions in the downstream signaling transduction. Moreover, direct

homologs of caspases critical in the execution of immunogenic PCD (e.g. pyroptosis) in animals are missing in the plant lineage, begging for the question how PCD of the infected plant cell is mediated by the intracellular nucleotide-binding domain (NBD) and LRR receptors (NLRs). Here we highlight some of the recent breakthroughs in the study of plant immune mechanisms which provide us a much clearer view on the basic elements in mounting a full immune response.

Oligomerization of immune receptors as a common activation mechanism

The plant immune receptors can be roughly distinguished by the signals that they recognize, with the membrane-localized pattern-recognition receptors (PRRs) recognizing functionally conserved microbe-associated molecular patterns (MAMPs) leading to pattern-triggered immunity (PTI), whereas the intracellular NLRs detecting either the pathogen-specific effectors delivered through various secretory systems or the perturbations of host targets caused by the effectors resulting in effector-triggered immunity (ETI). This is different from the animal immune receptors. With the Toll-like receptors (TLRs) recognizing MAMPs on the membrane, the cytosolic NLRs have members to perceive MAMPs as PRRs and also members to detect the invasion of pathogen effectors [1,2].

Regardless of the signals that they recognize, the structural similarities in NLRs from both lineages are intriguing and suggestive. Besides the C-terminal LRR domain that provides the ligand specificity, and the middle NBD, later named as nucleotide-binding oligomerization domain (NOD), the plant NLRs also have a variable N-terminus, based on which they are classified into three groups: coiled-coil domain (CC)-containing NLR (CNL), TIR domain-containing NLR (TNL) and resistance to powdery mildew 8 (RPW8)-like CC domain (CC-R)-containing NLR (RNL) [3].

Studies of animal receptors discovered a tendency of interaction between the same type of domains leading to the assembly of novel functional modules or proximity of working domains such as caspase recruitment domain (CARD) to enhance sensitivity for a limited level of pathogen stimuli [4]. For example, the mammalian intracellular NLRs can form inflammasomes through the NODs to allow N-terminal recruitment of pro-caspase-1 via a direct interaction between CARD domains in the case of the CARD-containing NLRs (i.e. NLRC4) or with the help of the apoptosis-associated speck-like protein containing a CARD (ASC) for other NLRs [1,5].

Recent breakthroughs in cryo-EM structural studies of plant NLRs discovered that they switch from the auto-inhibitory monomer to the active oligomer following effector-mediated activation of the LRR domains [6–8,9^{••},10^{••},11^{••},12^{••}]. The first structure reported by Wang *et al.* [9^{••}] showed that the *Arabidopsis* PBL1-like protein 2 (PBL2) uridylated by the *Xanthomonas* effector AvrAC becomes associated with resistance-related kinase 1 (RKS1) to activate the CNL receptor ZAR1 (HOPZ-activated resistance 1) by forming the ZAR1-RKS1-PBL2^{UMP} pentameric ‘ZAR1 resistosome’ (Figure 1). This is accomplished by repositioning of the NOD in ZAR1 to allow oligomerization, followed by the CC domain self-association with a dramatic allosteric change at the very N-terminal helix to form a funnel-shaped structure that penetrates the plasma membrane. Based on the chemical nature of residues involved and the diameter of this pentameric funnel (10–30 Å), the authors proposed that it may serve as a cation channel [9^{••},10^{••},13^{••}].

If the funnel structure formed by the CC domain of ZAR1 is functionally important, the logical question to ask is how does the TNL class of NLRs initiate immune signaling in the absence of the CC domain? The back-to-back publications by Ma *et al.* [11^{••}] and Martin *et al.* [12^{••}] showed that two TNL receptors, the *Arabidopsis* RPP1 (recognition of *Peronospora parasitica* 1) against the *Hyaloperonospora* effector ATR1 and the *Nicotiana benthamiana* ROQ1 (Recognition of Xopq 1) against the *Xanthomonas* effector XopQ, are activated through direct binding of the effectors to the C-terminal LRR domain and the polymorphic jelly roll/Ig-like domain (C-JID). The result is the formation of the tetrameric ‘RPP1 resistosome’ and ‘ROQ1 resistosome’, respectively. The conformational change that occurs within the NOD brings the functional end of the NLRs (i.e. the TIR domain) together to form an active NADase enzymatic pocket (Figure 1) [11^{••},12^{••}]. This provided the structural basis for the detection of NADase activity from the TIR domain-containing proteins such as the mammalian SARM1 protein and the plant immune receptors RBA1 (Response to the bacterial type III effector protein HopBA1), RPS4 (Resistant to *Pseudomonas syringae* 4) and RPP1, and the requirement of the enzymatic activity for triggering PCD by these proteins [14^{••},15^{••}].

Though not all TIR domains have the NADase enzymatic activity, they all have the ability to oligomerize. For example, the TIR domain of TLRs in the mammalian innate immunity dimerizes upon MAMP recognition and recruits more TIR domain-containing adaptors to oligomerize for the assembly of the downstream functional modules such as the death domain (DD) or kinase domain (K) to activate the kinase cascade and subsequent transcription factors (TFs) for the production of proinflammatory cytokines (Figure 1) [16–18]. Instead of forming

higher order oligomer, plant PRRs are activated by forming heterodimer with the co-receptors to initiate the kinase cascade and transduce the signal [19].

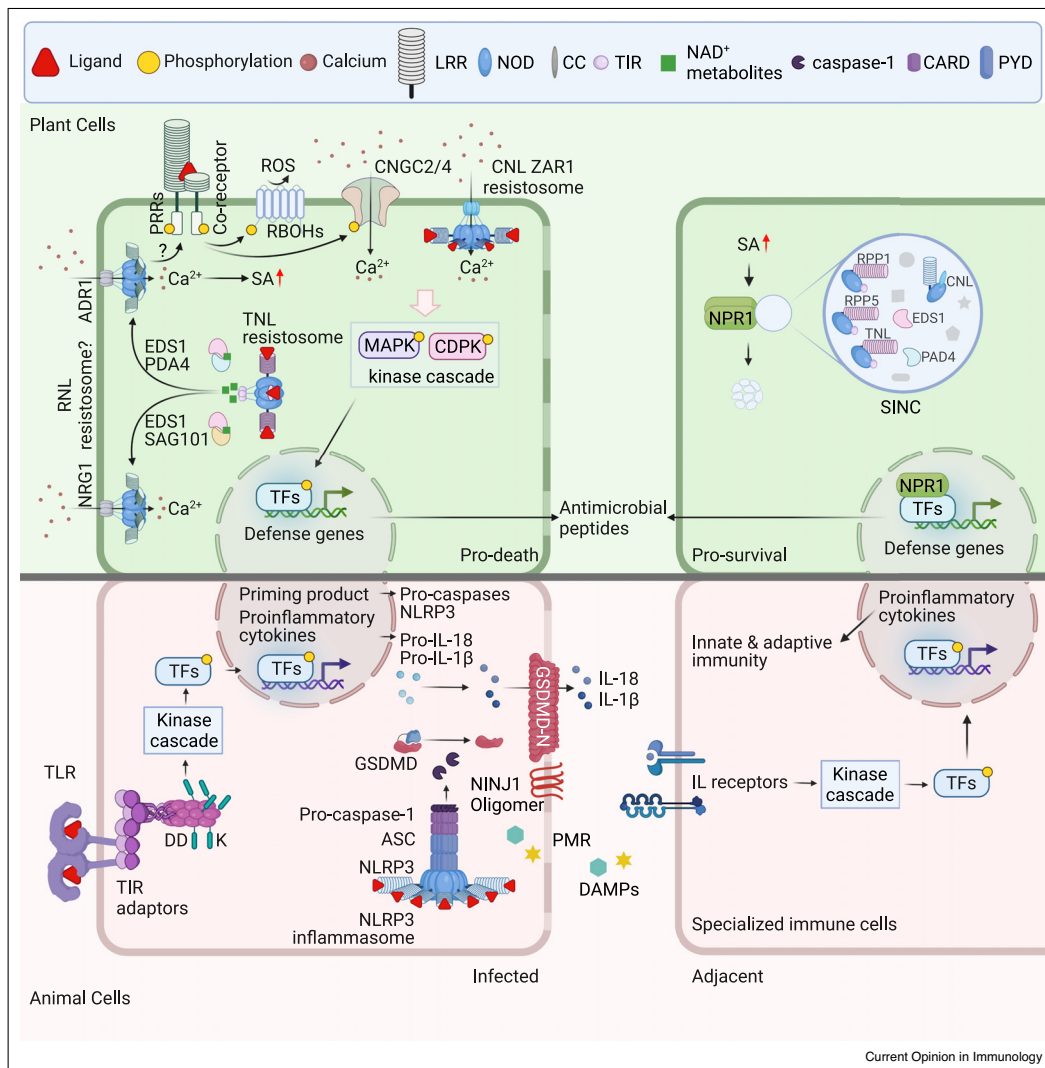
The significance of using oligomerization as a common activation mechanism for innate immune induction is the enhanced sensitivity to pathogen signals which are likely to be present in a meager amount. The simultaneous involvement of multiple components may also ensure that the response, which often leads to cell death, is tightly controlled and robust.

Pore formation as the trigger, instead of the executor, of immune-associated PCD in plants

In both plant and animal innate immune responses, PCD of the infected cell is a common strategy used to restrict pathogen growth as well as to release immune signals for the subsequent defense responses in the organisms. However, NLR-triggered PCD in plants occurs without direct homologs of caspases as in mammalian cells where inflammatory caspases, such as caspase-1 activated by the inflammasome, cleave gasdermin D (GSDMD) to release its N-terminal fragment (GSDMD-N) to form membrane pores, leading to pyroptosis [20]. The plasma membrane anchoring of the plant ZAR1 resistosome through its N-terminal funnel suggests that in plants, NLR-induced PCD also involves the formation of membrane pores. However, the size of this pore is much smaller than that formed by GSDMD-N (~215 Å) [21] and there is limited cell swelling, suggesting that the pore formed by the ZAR1 resistosome is unlikely the final executor of cell death, but rather a cation-permeable channel that triggers cell death-inducing signaling (Figure 1). To follow-up on the structural breakthrough, Bi *et al.* showed that activation of ZAR1 indeed leads to a calcium influx that is dependent on the key channel residue Glu11 and that calcium serves as an essential messenger for the subsequent reactive oxygen species (ROS) burst, organelle disturbance and the final plasma membrane rupture (PMR) [13^{••}]. The massive transcriptional reprogramming due to an increase in the nuclear pore permeability has been proposed to be involved in PCD execution [22]. Intriguingly, recent identification of *Ninjurin 1* (NINJ1) in macrophage PMR, whose mutant exhibited a loss of cell viability but persistent ballooned morphology, genetically separated GSDMD pore-triggered pyroptotic cell death from the ultimate cell lysis accompanied by the massive release of danger-associated molecular patterns (DAMPs) [23]. Whether a NINJ1-like protein is required for PMR in plants needs future investigation.

Although the TNL resistosome does not lead to pore formation directly, the NAD⁺ metabolic products generated by their NADase activity, such as variant of cyclic adenosine diphosphate ribose (v-cADPR), may serve as signals to activate RNLs to form membrane pores. This

Figure 1



An overview of plant and animal immune mechanisms.

In plant, a potential pathogen is first recognized by the cell surface-localized PRR/co-receptor complex to induce PTI. An adapted pathogen can also deliver virulence-promoting effectors into the host cells, which can be detected by the intracellular NLRs to induce ETI. These immune responses share some cellular events with distinct temporal patterns and intensities which include the PTI-mediated calcium influx channelled by CNGC2/4 upon PRR-induced phosphorylation and the ETI-mediated calcium increase due to the oligomerization of CNLs (e.g. ZAR1 resistosome) triggered directly by the effector or RNLs (NRG1 and ADR1) activated indirectly via TNL resistosome-generated NAD⁺ metabolites and EDS1-SAG101 or EDS1-PAD4 through an unknown mechanism. Elevated calcium level in the cytoplasm activates the MAPK and CDPK cascades leading to the phosphorylation of RBOHs (NADPH oxidase/respiratory burst oxidase homologs) and TFs to induce ROS production and defense gene expression, respectively. EDS1-PAD4, together with the RNL ADR1, have been found to be required for the production of the immune signal SA and defense gene expression to confer resistance. This complex is also reported to be associated with specific PRR complexes to boost PTI through SA synthesis and induction of TNL genes. Prolonged cytosolic calcium increase and ROS production are required for PCD of the infected cell during ETI. Beyond the infection site, cell death is inhibited through the SA-responsive NPR1 protein which promotes cell survival through induction of antimicrobial genes in the nucleus and the formation of SA-induced NPR1 condensates (SINCs) in the cytoplasm to sequester and degrade multiple signaling components involved in cell death to switch on the pro-survival immune strategy.

In animals, cell surface-localized TLRs detect extracellular MAMPs and trigger transcriptional changes through sequential and co-operative assembly of protein domains such as TIR and DD to bring the kinase domain (K) into proximity to initiate the kinase cascade that ultimately leads to the activation of TFs. The increases in pro-caspases and NLRP3 expression prime the cell for agonist-triggered formation of inflammasomes, such as by the intracellular NLRP3, the CARD-containing and pyrin domain (PYD)-containing adaptor ASC, and pro-caspase-1. The matured caspase-1 not only processes the proinflammatory cytokines but also cleaves GSDMD to release GSDMD-N to form membrane pores causing pyroptotic cell death. The secreted proinflammatory cytokines, such as IL-18 and IL-1β, bind to their receptors on the surface of adjacent specialized immune cells to induce further innate or adaptive immune responses. NINJ1-mediated plasma membrane rupture (PMR) is the last step of pyroptotic cell lysis, through which DAMPs are massively released.

Common features are as noted at the top of the graph.

hypothesis is based on the observation that CC-R domain-containing RNLs are essential for TNL-mediated cell death [24,25]. In the recent work by Jacob *et al.* [26^{••}], the authors performed structural modeling of the *Arabidopsis* RNL, *N*requirement gene 1.1 (NRG1.1), and found that this ‘helper NLR’ shares a structural similarity with the N-terminal 4-helix bundle of the *mixed lineage kinase domain-like* (MLKL) protein which has been shown to oligomerize and serve as a cation permeable channel in mammalian cell necroptosis [27,28]. Based on the structural model, genetic and functional analyses of key residues in NRG1.1 and another RNL, *activated disease resistance 1* (ADR1), supported the prediction that oligomerization and channel function of these helper NLRs are critical for direct regulation of cytoplasmic Ca^{2+} levels and subsequent cell death (Figure 1). In addition to their essential roles for TNL resistance, helper NLRs also facilitate resistance mediated by several CNLs. The level of the immune response is modulated between the ‘sensor NLRs’ and the helper NLRs. Whereas the helper NLR mutant *nrg1* completely abolishes cell death induced by the sensor TNLs, it only attenuates cell death triggered by the sensor CNL RPS2 and has no effect on the CNL RPS5-mediated or MLA7-mediated PCD [24], indicating an interplay between the NLRs in reaching the signaling threshold and that NLR signaling pathways converge on the formation of pores as well as the subsequent calcium flux for cell death commitment.

What the activation signal is for the helper NLRs remains to be investigated. The secondary position of these NLRs in the signaling cascade is reminiscent of that for the animal NLRP3 inflammasome which is activated by a loss of host cell homeostasis such as K^+ efflux, lysosomal rupture, calcium mobilization and production of ROS [29]. NAD^+ metabolites, such as v-cADPR, produced by TNL resistosome may be good candidates for signals that activate helper NLRs. Moreover, the signaling protein *enhanced disease susceptibility 1* (EDS1) has long been known to be required for TNL-mediated immune response. Its complexes with homologs *senescence associated gene 101* (SAG101) and *phytoalexin deficient 4* (PAD4) contribute to ETI-cell death and SA-mediated inhibition of pathogen growth by preferential interaction with the two helper NLRs, NRG1 and ADR1, respectively (Figure 1) [30,31]. The discoveries of AvrRPS4 effector-dependent association between EDS1-SAG101 and NRG1 and the stimulation of the association between EDS1-PAD4 with ADR1-L1 by the overexpression of the TIR-containing receptor RBA1 immediately bring on the following questions: (1) What other sensor NLRs can trigger EDS1-SAG101-NRG1 and EDS1-PAD4-ADR1 assembly? (2) What is the structural basis for triggering the complexes assembly and the RNL resistosome formation? (3) How does the interplay between the activated sensor CNLs and the helper NLRs happen mechanistically? and (4) Whether NAD^+ metabolites generated by

TNL resistosome are directly involved in the complex assembly or indirectly through cellular changes such as calcium flux [32,33]?

Crosstalk between different receptor-mediated pathways in mounting a full immune response

A major question in the innate immune study is how the system distinguishes pathogens from commensal and beneficial microbes through recognizing MAMPs which have conserved functions. One possible way to prevent false alarms is through the crosstalk between PTI mediated by the cell surface PRRs and ETI by the intracellular NLRs. Overlapping cellular events between PTI and ETI have been found in calcium mobilization, the involvement of kinase cascade including both *mitogen-activated protein kinase* (MAPK) and *calcium-dependent protein kinase* (CDPK), ROS production, as well as defense gene induction (Figure 1), although with distinct temporal patterns and intensities [34]. Calcium influx is considered the initial event which is channeled by cyclic *nucleotide-gated channel 2/4* (CNGC2/4) in PTI and activated by a CC-containing NLR resistosome in ETI [13^{••},26^{••},35]. More calcium-permeable channels have been reported in establishing the PTI-calcium signature, such as the *hyperosmolality-induced calcium increase 1.3/1.7* (OSCA1.3/1.7) in guard-cells to trigger their closure to block pathogen entry [36], as well as the *glutamate receptors* (GLRs), inhibition of which with antagonists compromised MAMP-induced calcium influx [37]. Whether additional channels participate in the ETI-calcium signature needs further exploration. Once PTI is triggered by MAMPs, ETI is enhanced as well, and *vice versa*. Recent studies by Ngou *et al.* and Yuan *et al.* more firmly established the reliance of full ETI on the early signaling components of PTI and the feedback boost of PTI by ETI [38^{••},39^{••}]. Studies from Pruitt *et al.*, and Tian *et al.* further linked the TNL signaling components, EDS1, PAD4 and ADRs, to PTI [40,41]. These findings indicate that only pathogens carrying the MAMP signals and having the ability to deliver effectors into the plant cell can trigger a full immune response. In animals, extracellular *lipopolysaccharide* (LPS) detection by TLR4 on the cell surface only serves the priming function through induced gene expression of proinflammatory cytokines, pro-caspases, and also NLRP3, whereas detection of cytosolic LPS, as a result of bacterial invasion, leads to the inflammasome activation, assembly of GSDMD-N membrane pores, execution of pyroptotic cell death, as well as maturation and secretion of active cytokines (Figure 1) [42–45]. With development of single-cell level analyses and application of transient gene function perturbation in plants, more spatial and temporal information will be provided to explain whether the crosstalks observed in the genetic experiments occur within the same cells or between cells.

Biomolecular condensates in promoting cell survival

Oligomerization of immune receptors is the trigger for immune-associated PCD. However, PCD of infected cells is not the final 'goal', but rather a commonly used sacrifice to promote survival of the organism through induction of other immune responses. In animals, GSDMD-formed pore in pyroptosis is required for the release of proinflammatory cytokines such as interleukin (IL)-1 β and IL-18 [20] which bind to their receptors and transduce the signal through a kinase cascade to systemically activate and regulate specialized immune cells including T cells and B cells involved in adaptive immunity (Figure 1) [46], while the final cell lysis leads the massive release of DAMPs to alert the bystander cells [23]. However, even activation of GSDMD is not an irreversible path to cell death because the calcium influx through the pore can also activate the ESCRT (endosomal sorting complexes required for transport)-mediated membrane repairment of the infected cells [20], indicating that even for specialized immune cells, death is tightly controlled.

In the absence of professional immune cells, plants synthesize and release immune signals such as SA during the local PTI and ETI responses to promote survival of neighboring cells and activate broad-spectrum systemic acquired resistance (SAR) through secreting an array of antimicrobial peptides [47]. A recent study by Zavaliev *et al.*, found that the master immune regulator, non-expressor of pathogenesis-related gene 1 (NPR1) is not only required in the nucleus for expressing genes in defense peptide production and secretion, but also in the cytoplasm for the formation of SA-induced NPR1 condensates (SINCs) enriched with many NLRs such as RPP1 and RPP5, signaling components such as EDS1 and PAD4, together with protein degradation machineries in cells surrounding the PCD site [48**]. Therefore, in addition to the mechanisms in controlling NLR homeostasis that have been reported previously, from chromatin structure of the NLR gene loci [49,50], the nucleocytoplasmic distributions of NLR proteins [51] to ubiquitin/proteasome-mediated degradation [52,53], sequestration and degradation of the entire signaling pathway components through the formation of SINCs may be a more effective mechanism to shut down PCD and switch on the survival-promoting immune strategy, SAR (Figure 1). A similar live-or-die checkpoint has been reported by Samir *et al.* in the animal system where competition for the ATP-dependent RNA helicase DDX3X (DEAD-box helicase 3X-linked) required for the formation of the cytosolic stress granules and the NLRP3 inflammasome-associated ASC specks determines whether the stressed cell survives or undergoes pyroptotic cell death [54]. Prior induction of stress granule formation leads to a decrease in NLRP3 inflammasome assembly and a reduction in active caspase-1 and secreted cytokine levels.

Similarly, pre-treating plants with SA blocks NLR-triggered PCD [48**].

In the past few years, several major missing pieces have been found in our understanding of plant immune mechanisms (Figure 1). With the big picture coming into focus, some of the immanent questions to address are: (1) How do TNL resistosomes activate helper NLRs? What is the molecular activity of EDS1/SAG101 and EDS1/PAD4 in this signal relay? (2) What are the signaling events downstream of resistosome-induced calcium influx? (3) How is PCD executed during ETI? (4) What are the biogenesis mechanism and the structure of SINC in promoting cell survival? More than ever, we are closer to understanding how plants thrive under constant pathogen challenges.

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Conflict of interest statement

X.D. is a cofounder of Upstream Biotechnology, Inc., and a Scientific Advisory Board member of Inari Agriculture.

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