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A new biochemistry connecting pathogen detection to induced defense in plants

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Summary

Plant cell surface and intracellular immune receptors recognizing pathogen attack utilize the same defense machineries to mobilize resistance. New genetic, protein structural and biochemical information on receptor activation and signaling is transforming understanding of how their shared defense network operates. We discuss the biochemical activities of two classes of intracellular nucleotide-binding/leucine-rich repeat (NLR) receptor – one forming a Ca^{2+} channel, the other an NADase enzyme, which define engagement of enhanced disease susceptibility 1 (EDS1)-family heterodimers and co-functioning helper NLRs (RNLs) to connect receptor systems and amplify defenses. Toll-Interleukin-1 Receptor (TIR) domain NLR receptors and TIR-domain proteins, with a capacity to produce NAD^+ derived small molecules, require EDS1 dimers and RNLs for defense induction. The TIR-driven EDS1/RNL modules emerge as central elements in Ca^{2+} based immunity signaling initiated by receptors outside and inside host cells.

Key words

immune receptor, RNL, effector-triggered immunity, pattern-triggered immunity, disease resistance, EDS1, TIR, NADase

I. Introduction

The plant immune system deploys two interconnected receptor layers which detect microbial molecules or host 'damage' signals (patterns) and trigger resistance to disease. Panels of plasma membrane (PM)-anchored pattern recognition receptors (PRRs), including receptor-like kinases (RLKs) and receptor-like proteins (RLPs), activate a basal resistance response called pattern-triggered immunity (PTI), which is often sufficient to prevent invasion by non- or poorly adapted microbes. Inside cells, nucleotide-binding/leucine-rich repeat (NLR) receptors sense activities of virulence factors (effectors) that are delivered into host cells by adapted pathogen strains, often to disable PTI. NLR-effector recognition leads to effector-triggered immunity (ETI) which frequently culminates in host localized cell death (the hypersensitive response).

There has been a surge of new information over the last 3 years on biochemical processes underpinning PRR and NLR activation and downstream signaling. PRRs trigger early defenses against microbe colonization, and NLRs provide a mechanism to reinstate and transcriptionally amplify PTI-related defenses that are breached during infection. Hence, PRRs and NLRs converge on qualitatively similar transcriptional and metabolic outputs, such as NADPH-oxidase generated reactive oxygen species (ROS), Ca^{2+} ion fluxes, MAP kinase (MAPK) cascades and stress hormone network reprogramming, the latter often leading to boosted salicylic acid (SA) defense (Yuan et al., 2021a). Studies in *Arabidopsis* published earlier this year show that these two receptor layers potentiate each other to strengthen the immune response and therefore should be viewed more as interlinked barriers to disease than separate entities (Ngou et al., 2021; Yuan et al., 2021b). Here we consider some of the newest molecular and biochemical insights to signaling in ETI and its connectivity to PTI. A clearer picture emerges of intersecting defense pathways downstream of pathogen receptors activated in different cell compartments.

II. Functional significance of NLR oligomeric scaffolds

NLR receptor activation, via direct pathogen effector binding or indirect detection of effector-induced host modifications, defines current plant ETI models (Saur et al., 2021). Fusion of a central nucleotide-binding (NB)/oligomerization domain with C-terminal LRRs (of varying lengths) and N-terminal coiled-coil (CC) or Toll-Interleukin-1 receptor (TIR) domains creates an effective ADP/ATP-driven conformational switch. It is therefore perhaps not surprising that similar NLR architectures evolved in animals for innate immunity and cell death regulation (Xiong et al., 2020; Saur et al., 2021). In 2019, the cryo-EM structures of *Arabidopsis* ZAR1 (a CC-domain NLR, abbreviated to CNL) before and after indirect bacterial effector activation, set the stage for understanding plant NLR modes of action (Wang et al., 2019a; Wang et al., 2019b). Activated ZAR1 assembles into a wheel-like pentamer in which the first α -helices of five CC-domains are exposed to form a funnel that contacts the PM (**Fig. 1A**). Single-molecule imaging and ion flux measurements of a pre-assembled ZAR1 oligomer show it forms an autonomous Ca^{2+} -permeable cation channel at the PM (Bi et al., 2021). This has huge significance for the field because it suggests a direct CNL receptor mechanism for promoting Ca^{2+} influx into cells, thereby likely increasing Ca^{2+} -regulated kinase and transcription factor activities to mediate host defense and cell death (Seybold et al., 2014; Yuan et al., 2021a). In contrast to transient cytosolic Ca^{2+} elevation which occurs within minutes of cell-surface PRR activation in PTI, CNL-triggered Ca^{2+} influx can last several hours (Grant et al., 2000; Bi et al., 2021), similar to sustained MAPK signaling which orchestrates ETI transcriptional defense (Tsuda et al., 2013; Yuan et al., 2021a). Hence, a major CNL sensor output is elevated Ca^{2+} inside host cells which induces anti-pathogen defenses and, ultimately, cell death (see **Box 1**).

Last year, the cryo-EM structures of two pathogen-activated TIR-domain NLR receptors (called TNLs) - *Arabidopsis* RPP1 which directly binds an oomycete effector, and wild tobacco (*Nicotiana benthamiana*; *Nb*) Roq1 binding a bacterial effector, were resolved and functionally characterized (Ma et al., 2020; Martin et al., 2020). The activated TNLs oligomerize into stable tetramers which, in contrast to CNL ZAR1, assemble two asymmetrically aligned N-terminal TIR-domain pairs. This creates an NAD^+ hydrolyzing enzyme which initiates signaling (Ma et al., 2020; Martin et al., 2020) (**Fig. 1A**). The contrasting outputs of CNL and TNL activation - one an induced membrane channel, the other a holoenzyme - help to explain why these two NLR classes have different signaling requirements in disease resistance. Genetic studies in *Arabidopsis* and *Nb* tobacco show that TNL, but not CNL sensor NLRs, require a small conserved family of HET-S/LOB-B (HeLo)-domain NLRs (referred to as helper NLRs, or RNLs) and the EDS1 family of three lipase-like proteins to confer local pathogen resistance and host cell death (Feehan et al., 2020; Dongus and Parker, 2021) (**Fig. 1A**).

Oligomerization of CNL and TNL receptors into multimeric platforms broadly resembles mammalian NLR inflammasome assemblies which serve as scaffolds for concentrating signaling components, such as caspase enzymes, to initiate pro-inflammatory and cell death responses (Xiong et al., 2020). Hence, a shared mechanistic principle for NLR oligomers across kingdoms appears to be the construction of a multimeric signaling scaffold. The same logic applies to the need for tight NLR constraint when not required, since their mis-activation leads to autoimmunity with serious fitness consequences.

III. TNL and TIR-domain small molecule products

TIR domains have an ancient origin and self-association properties (Bayless and Nishimura, 2020). In animal immunity they are often employed as homotypic signaling adaptors which lack NADase activity. However, the mammalian TIR-domain containing protein SARM1 has NADase activity which causes neuronal death by hydrolyzing and depleting cellular NAD⁺ (Essuman et al., 2017; Bayless and Nishimura, 2020). TIR domains of many plant TNL receptors and truncated TIR-domain proteins contain a conserved NADase catalytic glutamic acid (Glu) residue, and a number of these proteins possess Glu-dependent NADase activity after self-association (Horsefield et al., 2019; Wan et al., 2019a). In contrast to SARM1, *in vitro* biochemical assays and *in vivo* cell death phenotyping suggest that plant TIR domains signal by generating one or more NAD⁺ hydrolysis products (Horsefield et al., 2019; Wan et al., 2019a; Bayless and Nishimura, 2020). Several candidate TIR-domain NAD⁺ derived molecules, such as c-ADPR and a variant form v-cADPR, accumulate in plant tissues but these are not sufficient to elicit cell death (Bayless and Nishimura, 2020; Duxbury et al., 2020). The fact that NADase-active TNLs and TIR-only proteins require *EDS1* to elicit plant cell death (Wan et al., 2019a; Bayless and Nishimura, 2020; Wu et al., 2021), is in line with a TIR/NAD⁺ produced SM signaling *via* EDS1 to promote immunity. A new set of TIR-domain synthesized cyclic nucleotides (2',3'-cAMP and 2,3-cGMP) were identified recently which lead to immune-related cell death (Yu, 2021), suggesting that multiple TIR-generated SMs converge on EDS1. In various studies, EDS1 was found to associate with TNLs (Lapin et al., 2020). Maybe the interaction creates a concentrated microenvironment for EDS1 and/or other components to receive a TIR-generated SM and amplify the defense signal. In this context, it is interesting that TNL RPP1 NADase activity is itself stimulated by Ca²⁺ ions *in vitro* (Ma et al., 2020) and therefore TNL/SM generation could be further boosted by calcium influx to cells.

IV. EDS1-family dimers transduce NLR signals: connecting some dots

A plausible link between TNL and EDS1 *via* one or more SM intermediates is brought into sharp focus by recent structure-function insights to the EDS1-family of three proteins: EDS1, PAD4 and SAG101. EDS1-family genes are found in gymnosperms and angiosperms (seed plants) and therefore post-date the origins of NLRs, RNLs and core phytohormone pathways in plant evolution (Lapin et al., 2020) (see **Fig. 2**). The EDS1-family is characterized by fusion of an N-terminal α/β -hydrolase (class 3-lipase) domain with a unique C-terminal α -helical bundle 'EP' domain (Lapin et al., 2020). *Arabidopsis* EDS1 forms exclusive, stable heterodimers with SAG101 or PAD4 mediated by similar N-terminal domain interactions and independent of lipase catalytic residues (Wagner et al., 2013). The dimer N-terminal interfaces stabilize weaker associations between partner EP-domains to create an essential signaling surface around a cavity (Wagner et al., 2013; Bhandari et al., 2019; Dongus and Parker, 2021).

Although EDS1-SAG101 and EDS1-PAD4 dimers are similar, they do not have identical roles in the plant immune response. In *Arabidopsis* and *Nb* tobacco, EDS1-SAG101 signals preferentially in ETI conferred by TNL receptors where it promotes defense and host cell death (Castel et al., 2019; Lapin et al., 2019; Wu et al., 2019; Sun et al., 2021). By contrast, EDS1-PAD4 promotes a PTI-like basal immune response which contributes to varying extents to TNL and CNL ETI (Cui et al., 2017; Cui et al., 2018). *Arabidopsis* EDS1-PAD4 controlled basal immunity slows infection of virulent pathogens in the absence of ETI-related host cell death (Cui et al., 2017). Closer analysis of ETI conferred by an *Arabidopsis* TNL pair (RRS1 – RPS4) to *Pseudomonas* bacteria, revealed that EDS1-PAD4 (via its EP-domains) promotes rapid transcriptional up-regulation of defense genes (Bhandari et al., 2019). In addition to amplifying expression of numerous NLRs, RNLs and immunity components, *Arabidopsis* EDS1-PAD4 effectively prioritizes SA resistance over SA-antagonizing jasmonic acid (JA) pathways (Cui et al., 2018; Bhandari et al., 2019). In both EDS1 dimers, several positively charged EP-domain residues bordering the cavity are crucial for function (Wagner et al., 2013; Gantner et al., 2019; Lapin et al., 2019), consistent with the dimer EP-domains potentially accommodating a TIR-generated polar SM (**Fig. 1B**).

V. Rationalizing pathogen-induced EDS1 dimer - RNL associations

At this point it's useful to revisit the HeLo-domain containing RNLs (helper NLRs) mentioned above which, in *Arabidopsis* and *Nb* tobacco, are additional essential elements in TNL mediated ETI (Qi et al., 2018; Feehan et al., 2020). RNLs divide into two small sub-families, ADR1s and NRG1s. HeLo-domains are present in fungal cell death regulators and mammalian Mixed Lineage Kinase-Like (MLKL) immunity executors which can form pores at host membranes (Feehan et al.,

2020; Mahdi et al., 2020). Moreover, HeLo-domains have a similar topology to the N-terminal coiled-coils of plant CNL receptors (Feehan et al., 2020; Jacob et al., 2021). In fact, an *Arabidopsis* NRG1 autoactive variant forms an oligomer which associates with cell membranes in a mode potentially similar to the activated CNL ZAR1 Ca²⁺ permeable ion channel (**Fig. 1A**) (Jacob et al., 2021). Does this mean that RNLs are functional equivalents of CNL oligomers for promoting Ca²⁺ influx into cells?

The answer might be more nuanced. Studies of *Arabidopsis* *ADR1*- and *NRG1*-family CRISPR mutants show that NRG1-type RNLs cofunction with EDS1-SAG101 in TNL ETI, and *ADR1*s with EDS1-PAD4 in TNL ETI and basal immunity, as two genetically separate branches or nodes (Lapin et al., 2019; Feehan et al., 2020; Saile et al., 2020; Sun et al., 2021). The co-occurrence of *NRG1*s with *SAG101*, and of *ADR1*s with *EDS1* and *PAD4* genes, in seed plant genomes lends further support to EDS1-SAG101-NRG1 and EDS1-PAD4-ADR1 cooperation as two nodes (Van Ghelder et al., 2019; Lapin et al., 2020). Indeed, TNL-triggered immunity and cell death could be reconstituted in an *Nb* tobacco CRISPR mutant lacking all *EDS1*-family members by transiently co-expressing an *Arabidopsis* EDS1-SAG101 dimer with *Arabidopsis* NRG1 (Lapin et al., 2019). Since *Arabidopsis* NRG1 could not be substituted by tobacco or tomato (solanaceous) NRG1 or *Arabidopsis* ADR1 in these assays, it seems that molecular co-functions of particular RNL subtypes with EDS1 dimers within clades underpin two genetically hard-wired resistance mechanisms. Importantly, EDS1 dimer – RNL cooperation depends on specific associations between node components, as borne out by immunoprecipitation studies in TNL-induced *Arabidopsis* and tobacco (*Nb*) leaf extracts (Sun et al., 2021; Wu et al., 2021). It is striking that induced interaction of EDS1-SAG101 with NRG1s and the immune response require TNL activation, a functional EDS1-SAG101 dimer EP-domain ‘cavity’ surface and an oligomerization-competent form of NRG1 (Sun et al., 2021) (**Fig. 1B**). Hence, pathogen-activated TNL receptors mobilize ETI defense through induced EDS1 dimer - RNL complexes.

EDS1-family/RNL cooperation and association needs to be rationalized with proposed RNL activities as Ca²⁺ permeable channels at host membranes (Jacob et al., 2021). If the prime function of ADR1- and NRG1-family RNLs is as CNL receptor-like oligomers forming membrane pores for Ca²⁺ signal relay, we speculate that (i) an EDS1-family heterocomplex binds to and induces RNL oligomerization to enhance or alter its channel properties, or (ii) EDS1 dimer-RNL complex formation is a transient but essential step, after which a mature RNL oligomer is released to function at membranes. Both scenarios prompt the question whether an EDS1-assisted RNL pore is equivalent to that made by CNL sensor NLRs or has different properties.

Analysis of membrane pores in mammalian immune responses reveals a considerable range of sizes and signaling outcomes, as well as recruitment of additional components for membrane permeabilization (Ros et al., 2017; Flores-Romero et al., 2020; Kayagaki et al., 2021). Another unknown is the extent to which RNL, and indeed CNL, ion channel activities relate to other Ca²⁺ channel families contributing to PTI and/or ETI responses (**BOX 1**).

VI. Convergence of NLR and PRR signaling at the EDS1-PAD4-ADR1 node

Two recent studies of *Arabidopsis* cell-surface PRR (RLP23) basal immunity (PTI) reinforce the view that the EDS1-SAG101-NRG1 and EDS1-PAD4-ADR1 nodes make distinctive contributions to plant immunity (Pruitt et al., 2021; Tian et al., 2021). RLP23 is activated by an oomycete PAMP and signals via two membrane-bound RLKs, SOBIR1 and BAK1, and a small family of mobile receptor-like cytoplasmic kinase (RLCKs) (Albert et al., 2015; Wan et al., 2019b), of which PBL31 is the most prominent (Pruitt et al., 2021). RLP23 induced pathogen immunity has a strong genetic requirement for *EDS1-PAD4* (but not *EDS1-SAG101*), and uses the same dimer EP-domain cavity surface as engaged by NLRs in ETI (Pruitt et al., 2021). These findings highlight a role of the EDS1-PAD4 dimer at an intersection between NLR and PRR signaling, perhaps to mediate ETI – PTI potentiation (**Fig. 1C**) (Dongus and Parker, 2021; Yuan et al., 2021a).

Protein interaction assays suggest that the RLP23-SOBIR1 co-receptor associates constitutively with pools of EDS1-PAD4 and ADR1s at the inner side of the plasma membrane (Pruitt et al., 2021), maybe to launch signaling from the host cell surface. A related study implicates a number of transcriptionally induced TIR-domain and TNL genes as components of *EDS1-PAD4* dependent PRR basal immunity, so TNLs and/or TIR-domain proteins are potential amplifiers of PRR-triggered defense (Tian et al., 2021) (**Fig. 1C**). The close structural relatedness of the two EDS1 dimers, combined with evidence that TNL and/or TIR-protein generated SMs drive EDS1 dimer - RNL associations, prompts the speculation that RNLs have become effective NLR immune receptors for TIR/SM-activated EDS1 dimers during seed plant evolution (**Fig. 2**).

VII. Conclusions

Advances made over the last 2-3 years have transformed the picture of immune receptor functions and defense network evolution in land plants. We present our impression of this in five incremental stages from an early 'core' general stress response system, through PRRs that became recruited and tuned to biotic stress, to an increasingly elaborate defense network involving CNLs, TNLs and RNLs connected by EDS1-family proteins which potentiate ETI - PTI outputs (**Fig. 2**). The new information also brings a fresh set of challenges. Of these, the most

important seems to be determining whether one or more SMs generated by TIR-domains of TNLs and TIR proteins define recruitment of the two EDS1 nodes in NLR and PRR triggered immunity. A recent preprint article identifies additional TIR-domain enzymatic activities and nucleotide products which cause immune-related cell death (Yu, 2021). A further question is whether the role of EDS1 dimers is simply to activate RNLs which then proceed alone or as a large multi-protein complex to signal as CNL-like membrane-associated ion channels. Alternatives we have considered are that EDS1 - RNL association changes the character of the RNL oligomer and/or that EDS1 - RNL complexes have additional roles in the plant immune response. Although not covered in this perspective, we shouldn't ignore that fact that in *Arabidopsis* a nuclear EDS1 pool confers NLR signaling (Lapin et al., 2020). Is this resistance function tied to RNLs or do nuclear EDS1 dimers have a different activity? Certainly, *Arabidopsis* PAD4 can multitask in biotic stress signaling (Dongus et al., 2020), suggesting these regulators have more than one mode of action, perhaps defined by their subcellular locations and partners. Finally, it is tempting to speculate that TNLs and TIR-domain proteins all signal through EDS1-family members in ETI and PTI, but it remains to be established whether this is the case and how, molecularly and spatially, it would work.

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Figure 1. Modes of NLR and PRR activation and signaling.

A. Pathogen-activated coiled-coil (CC)-domain NLR (CNL) ZAR1 assembles into a pentamer with five exposed CC-domain α -helices forming a funnel (orange) that contacts the plasma membrane (PM) and creates a Ca^{2+} -permeable inward cation channel (Wang et al., 2019a; Bi et al., 2021). Pathogen-activated Toll-Interleukin-1 Receptor (TIR)-domain NLR (TNL) receptors RPP1 and Roq1 oligomerize into similar tetramers, in which two asymmetrically aligned TIR-domain pairs form an active NAD⁺ hydrolase (NADase) enzyme (yellow) (Ma et al., 2020; Martin et al., 2020). In both NLR oligomers, the central nucleotide-binding/oligomerization (NB)- and C-terminal leucine-rich repeat (LRR)-containing domains are shown in grey. ZAR1-activating host proteins PBL2^{UMP} and RSK1 and RPP1-activating pathogen effector ATR1 are in green. The EDS1-family of lipase-like proteins and HeLo-domain helper NLRs (RNLs) are necessary for signaling downstream of TNL receptors to confer resistance and cell death in effector-triggered immunity (ETI). By contrast, the membrane-bound CNL ZAR1 oligomer probably autonomously induces local resistance and cell death by promoting Ca^{2+} -dependent processes and transcriptional defense inside host cells. Protein structures were drawn from protein data base PDBs 7CRC (RPP1: <https://www.rcsb.org/3d-view/7CRC>) and 6J5T (ZAR1: <https://www.rcsb.org/structure/6J5T>).

B. Direct pathogen effector binding leads to TNL (RPP1 and Roq1) receptor tetramerization, creating an active NADase enzyme which produces an NAD⁺ derived small molecule (SM, yellow star) (Ma et al., 2020; Martin et al., 2020). In the speculative model shown, an SM binds to the EP-domain cavity surface of an EDS1-SAG101 dimer which promotes its association with oligomerization-competent NRG1-family RNLs. It is possible that an RNL oligomer associates with multiple EDS1-SAG101 dimers. Induced EDS1-RNL interaction is a necessary but perhaps transient step in TNL mediated ETI signaling (Lapin et al., 2019; Sun et al., 2021; Wu et al., 2021).

C. A model depicting the EDS1-PAD4-ADR1 node in *Arabidopsis* operating at an intersection between ETI initiated by TNL receptors inside cells and pattern-triggered immunity (PTI) initiated by pattern recognition receptors (PRRs) at the cell surface. *Arabidopsis* PRR RLP23 forms a

complex with RLKs SOBIR1 and BAK1 (Albert et al., 2015; Wan et al., 2019b) and signals primarily via cytoplasmic receptor-like kinase PBL31 and the EDS1-PAD4-ADR1 node to promote defences and pathogen immunity (Pruitt et al., 2021; Tian et al., 2021). Pools of EDS1-PAD4, ADR1 and PBL31 are found in close proximity to the plasma membrane and together with SOBIR, suggesting that EDS1-PAD4-ADR1 controlled basal immunity might be launched from PRR receptor sites at the plasma membrane (Pruitt et al., 2021). *Arabidopsis* TNL receptors utilize the EDS1-SAG101-NRG1 and EDS1-PAD4-ADR1 nodes to varying extents in ETI signaling. As indicated, it is likely that PRR-induced TNLs and/or truncated TIR-domain proteins contribute to defense amplification via EDS1-RNL nodes (Yuan et al., 2021a). MAMP, microbe-associated molecular pattern; RLP, receptor-like protein.

Figure 2. Incremental stages in developing a plant immune receptor signaling network.

We present here a 5-stage model depicting the gradual elaboration of an immune receptor signaling network that now functions in seed plants. Stage 1: Reactive oxygen species (ROS), mitogen-activated protein kinases (MAPKs), Ca^{2+} and transcription factors (TFs) are general stress components constituting a core system for responding to plant stress. Stage 2: Pattern recognition receptors (PRRs) evolved in plants to activate existing ROS, MAPK and Ca^{2+} signaling and adapt or tune the core stress response machinery for anti-pathogen immunity. This feature is supported by PRR general stress-related transcriptomes (Jacob et al., 2018; Bjornson et al., 2021). Stage 3: In response to pathogen effector manipulation, plants evolved CC-domain NLRs (CNL) and HeLo-domain RNLs to form effector-triggered oligomeric pores with Ca^{2+} permeable ion channel activity to boost Ca^{2+} cascades and amplify PRR immunity, resulting in a more robust response called effector-triggered immunity (ETI). Stage 4: In seed plants (eg. *Arabidopsis*), RNLs became recruited by TNL receptors via EDS1-PAD4 or EDS1-SAG101 dimers to broaden the pathogen resistance spectrum and assist ETI-PTI cross-potential. Stage 5: In *Arabidopsis*, LRR-RP receptor RLP23 (potentially representing multiple PRRs) recruited the EDS1-PAD4-ADR1 module through unknown TIR/TNL components to extend the NLR - PRR defense network and strengthen systemic resistance. P/S, PAD4 or SAG101. Black arrows indicate positive regulation and orange arrows indicate enhanced activation.

Box 1 Calcium (Ca^{2+}) channels in immunity

One of the earliest events in pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) is a rapid increase of cytosolic Ca^{2+} (Yuan et al., 2021a). Inside the cell, Ca^{2+} ions function

as second messengers to transmit and amplify defense cascades mediated by Ca²⁺-binding proteins and their downstream targets. Immune-related transport of Ca²⁺ into plant cells is controlled by several types of Ca²⁺ permeable channel, including cyclic nucleotide-gated channels (CNGCs), OSCA1.3 and 1.7 and glutamate-like receptors (GLRs). *Arabidopsis* CNGC2 and CNGC4 mediate PAMP-induced increases in cytosolic Ca²⁺ in *Arabidopsis* leaves under conditions of high external [Ca²⁺] (Tian et al., 2019). A mis-regulated *Arabidopsis* CNGC20 channel leads to increased Ca²⁺ inside cells and both PTI and ETI responses (Zhao et al., 2021). OSCA1.3 and 1.7 are essential mediators of PAMP-induced stomatal immunity (Thor et al., 2020). Members of a GLR2 clade (GLR2.7, 2.8 and 2.9) also contribute to PAMP-induced Ca²⁺ influx in *Arabidopsis* (Bjornson et al., 2021). In contrast to the transient rise in cytosolic Ca²⁺ induced by PAMPs, a sustained increase in cytosolic Ca²⁺ is observed in NLR mediated ETI (Grant et al., 2000; Bi et al., 2021). These distinctive cytosolic Ca²⁺ signatures might be key factors determining ETI strength compared to PTI. They also suggest that different Ca²⁺ channel types contribute to NLR mediated ETI. Indeed, cryo-EM structural studies and biochemical activity assays of *Arabidopsis* activated CNL receptor ZAR1 show that it makes a pore at the PM with cation selective Ca²⁺-permeable inward ion channel activity (Bi et al., 2021). Protein structural and biochemical evidence also point to ADR1- and NRG1-type RNLs oligomerizing and forming pores associated with increased Ca²⁺-permeable cation channel activity at the PM (Jacob et al., 2021). ZAR1 and NRG1 promote a sustained increase of cytosolic Ca²⁺, fitting with Ca²⁺ signatures observed in ETI. Possibly, most CNLs and RNLs have the capacity to form Ca²⁺-permeable channels to directly regulate cytosolic Ca²⁺ levels. Nevertheless, mutation of CNGC2 or CNGC4 genes in *Arabidopsis* impairs NLR-triggered HR cell death (Clough et al., 2000) (Balague et al., 2003), indicating that various Ca²⁺ channels involved in PTI also contribute to Ca²⁺ influx in ETI. It will be important to determine how different ion channel mechanisms are regulated and coordinated for immune responses across host cells and tissues.

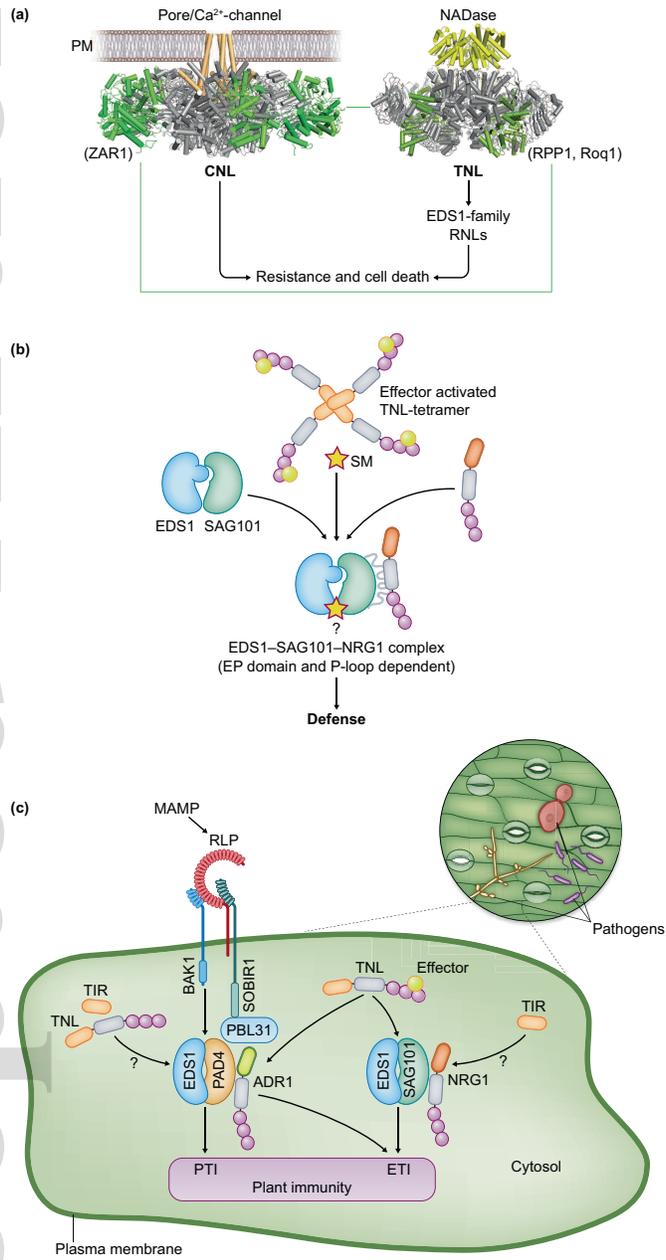


Figure 1
Tansley Insight 38072

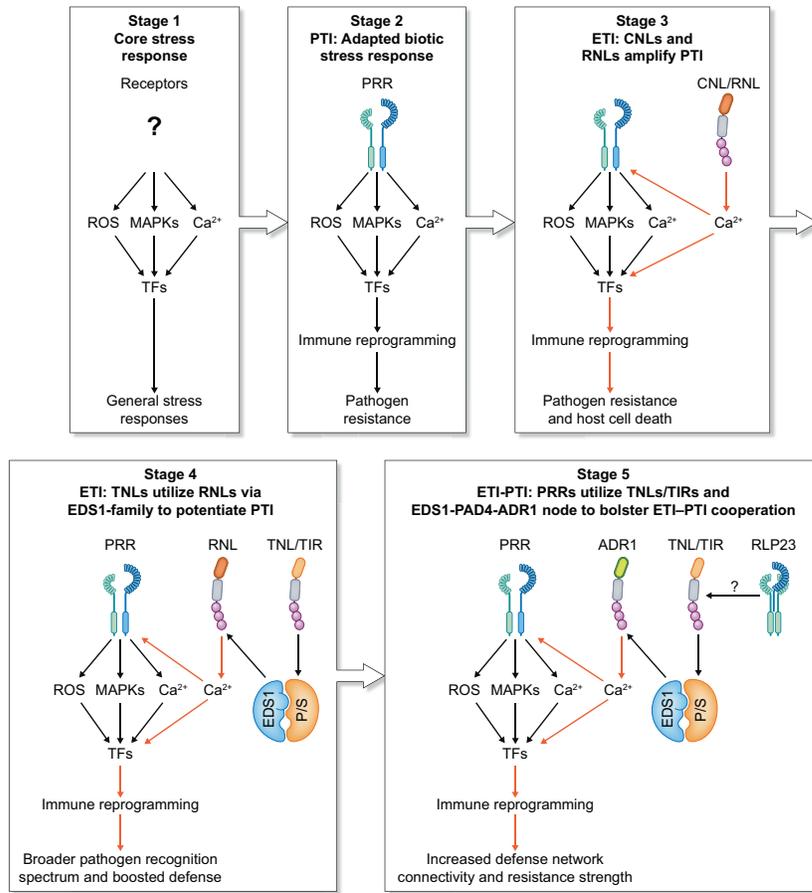


Figure 2
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