Ca\textsuperscript{2+} signals in plant immunity

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**Abstract**

Calcium ions function as a key second messenger ion in eukaryotes. Spatially and temporally defined cytoplasmic Ca\textsuperscript{2+} signals are shaped through the concerted activity of ion channels, exchangers, and pumps in response to diverse stimuli; these signals are then decoded through the activity of Ca\textsuperscript{2+}-binding sensor proteins. In plants, Ca\textsuperscript{2+} signaling is central to both pattern- and effector-triggered immunity, with the generation of characteristic cytoplasmic Ca\textsuperscript{2+} elevations in response to potential pathogens being common to both. However, despite their importance, and a long history of scientific interest, the transport proteins that shape Ca\textsuperscript{2+} signals and their integration remain poorly characterized. Here, we discuss recent work that has both shed light on and deepened the mysteries of Ca\textsuperscript{2+} signaling in plant immunity.

**Keywords** calcium; channel; ETI; immunity; PTI

**Subject Categories** Microbiology, Virology & Host Pathogen Interaction

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**The plant immune system**

All eukaryotes use immune systems to protect themselves against potential pathogens. The plant immune system consists of two characterized perception layers: one that utilizes cell-surface pattern recognition receptors (PRRs) to perceive extracellular immunogenic patterns, and another that relies on intracellular nucleotide-binding leucine-rich repeat (NLR) receptors that recognize pathogenic effectors inside the cell (Jones & Dangl, 2006).

In the first layer of the plant immune system, apoplastic immunogenic elicitors such as pathogen-, microbe-, damage-, or herbivore-associated molecular patterns (PAMPs, MAMPs, DAMPs, or HAMPs, respectively) or immune-modulating peptide phytohormones are recognized by PRRs, which leads to defense responses termed pattern-triggered immunity (PTI) (Boller & Felix, 2009; Yu et al, 2017; DeFalco & Zipfel, 2021). All plant PRRs described to date are receptor kinases (RKs) or receptor proteins (RPs) (Boutron & Zipfel, 2017; Albert et al, 2020). RKs are characterized by a domain structure reminiscent of metazoan receptor tyrosine kinases (RTKs) (DeFalco & Zipfel, 2021); namely, a ligand-binding extracellular domain (ECD), a single-span transmembrane helix (TM) and a cytosolic protein kinase domain (Jamieson et al, 2018), while RPs lack a cytoplasmic kinase domain and instead form functional bipartite receptors with adapter RKs (Liebrand et al, 2013; Albert et al, 2015; Postma et al, 2016). Because of their domain architecture, plasma membrane (PM)-localized PRRs (or their complexes) allow extracellular ligand binding to be communicated across the membrane into cytosolic signaling events. The molecular nature of elicitors varies, including proteins, lipids, and carbohydrates, and can be derived from either the potential pathogen or herbivore (e.g., MAMPs, PAMPs, or HAMPs) or the host plant, as in the case of macromolecules released upon cell damage (DAMPs) or secreted peptide phytohormones (Gust et al, 2017). PRR ECDs are characterized by a variety of subdomains, including leucine-rich repeat (LRR), epidermal growth factor-like (EGF), lectin, and lysin motif (LysM) domains (Boutron & Zipfel, 2017). The best-studied PRRs to date are the LRR-RKs FLAGELLIN-SENSING 2 (FLS2) and EF-TU RECEPTOR (EFR), which perceive the bacterial PAMPs flg22 and elf18, respectively (Gómez-Gómez & Boller, 2000; Zipfel et al, 2006). Both FLS2 and EFR form stable ligand-dependent complexes with common LRR-RK co-receptors of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family, such as BRASSINOSTEROID-INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1, also called SERK3) (Chinchilla et al, 2007; Heese et al, 2007; Roux et al, 2011). Complex formation between PRRs and co-receptors leads to phosphorylation events within the cytoplasmic kinase domains and the activation of receptor-like cytoplasmic kinases (RLCKs), which directly phosphorylate and regulate target proteins in order to activate PTI (Liang & Zhou, 2018; DeFalco & Zipfel, 2021) (Fig 1A).

Pathogens introduce effectors into the host cytoplasm that promote pathogenicity, often by disturbing PTI (Jones & Dangl, 2006). To counteract this, plants rely on a second layer of immunity, in which intracellular NLR-type receptors sense effectors and/or their activity, leading to effector-triggered immunity (ETI). Interestingly, plant NLRs share a common architecture with those of animals, featuring a conserved nucleotide-binding domain (NBD) and LRR domain, with variable accessory domains at both N and C termini (DeYoung & Innes, 2006; Jones et al, 2016; Baggs et al, 2017; van Wersch et al, 2020). NLRs are categorized based on their N-terminal domains: coiled-coil (CC)-NLRs (CNLs), toll/interleukin-related (TIR)-NLRs (TNLs), or RPW8-NLRs (RNLs). Of these NLRs, CNLs and TNLs function as sensors while RNLs function as helpers downstream of TNLs (Baggs et al, 2017; Wu et al, 2017; Jubic et al, 2019; Feehan et al, 2020). NLRs can be present in an inactive state, in which the LRR domain is likely autoinhibitory, and adenosine
diphosphate (ADP) is bound to their NBD (Williams et al., 2011; Bernoux et al., 2016). Upon activation, ADP is exchanged to adenosine triphosphate (ATP) and autoinhibition is released (Fig 1A). In animals, NLR activation often leads to oligomerization via N-terminal domains and the formation of large multimeric structures (Danot et al., 2009). A similar oligomerization mechanism has been long hypothesized for plant NLRs, but has only been recently corroborated by structural data that are discussed in detail below.

PTI and ETI have traditionally been viewed as independent pathways; however, at least some signaling components are shared by both layers of immunity (Thomma et al., 2011). Activation of either layer of the immune system triggers numerous overlapping cell signaling events, including Ca²⁺ fluxes, production of apoplastic reactive oxygen species (ROS), mitogen-activated protein kinase (MAPK) cascades, transcriptional reprogramming, and phytohormone biosynthesis (Cui et al, 2015; Yu et al, 2017; Zhou & Zhang, 2020; DeFalco & Zipfel, 2021). ETI is generally also accompanied by a form of programmed cell death termed the hypersensitive response (HR) at the site of infection (DeYoung & Innes, 2006; Jones & Dangl, 2006), although HR-like cell death is also induced by some forms of PTI (Wang et al, 2020). Recent work has further demonstrated that PTI and ETI are linked at transcriptional and/or molecular levels (Ngou et al, 2021; Pruitt et al, 2021; Tian et al, 2021; Yuan et al, 2021); however, the exact mechanisms governing linkage of

**Figure 1.** PTI and ETI induce cytoplasmic Ca²⁺ elevations.

RKs and RPs are PRRs residing at the PM. They form complexes with co-receptors upon perception of molecular patterns originating from microbes, viruses, herbivores, parasitic plants, or damaged host cells. In turn, RLCKs are activated and released from the complexes to activate downstream signaling to induce pattern-triggered immunity, of which Ca²⁺ release within few minutes after ligand perception is one facet. Microbes introduce effector proteins into host cells to disturb and overcome immune responses. Cytoplasmic NLRs sense the presence or activity of effectors to induce ETI. To this end, autoinhibition is released, ADP is changed to ATP and oligomerization of NLRs occurs, leading to downstream signaling and finally ETI (A). A significant cytoplasmic Ca²⁺ increase has been reported to occur in Arabidopsis leaves starting 1.5 h and peaking at about 2 h after infection with avirulent bacteria (B). Schematic Ca²⁺ signatures of Arabidopsis plants induced by bacterial infection as reported by Grant et al (2000) (B). RK: receptor kinase; co-RK: coreceptor kinase; RP: receptor protein; RLCK: receptor like cytoplasmic kinase; NLR: nucleotide-binding leucine-rich repeat receptor; CC: coiled-coil; TIR: toll/interleukin-related; CNLs: CC-NLRs; TNLs: TIR-NLRs; RNLs: RPW8-NRLs; NBS: nucleotide binding site; LRR: leucine-rich repeats; PTI: pattern-triggered immunity; ETI: effector-triggered immunity, c[Ca²⁺]: cytoplasmic free Ca²⁺ concentration.
these immune pathways remains to be elucidated fully. As changes in intracellular Ca²⁺ levels have been well documented downstream of both PRR and NLR activation, Ca²⁺ signaling is thought to be key to both layers of the plant immune system (Seybold et al., 2014; Moeder et al., 2019).

Ca²⁺ in immunity

Ca²⁺ is a universal second messenger in eukaryotes (Clapham, 2007). Owing to its cytotoxicity, cytosolic Ca²⁺ levels must be maintained at low (~10⁻⁸ to 10⁻⁷ M) levels in living cells, and thus Ca²⁺ is sequestered in intracellular stores (in plants, primarily the vacuole and the endoplasmic reticulum, but also the vesicular compartments, the chloroplasts and mitochondria) or the apoplast via active transport, generating enormous electrochemical potential gradients across membranes (Clapham, 2007; Edel et al., 2017; Costa et al., 2018). Ca²⁺-permeable channels can therefore generate rapid, transient increases in Ca²⁺ concentrations, which are in turn interpreted by a large suite of Ca²⁺-binding sensor proteins that regulate diverse cellular processes (DeFalco et al., 2010). Ca²⁺ signaling is thus summarized in three steps: encoding (via stimulus-triggered Ca²⁺ fluxes), decoding (via Ca²⁺ sensor proteins), and responses (via regulation of downstream cellular processes).

In plants, Ca²⁺ signaling is involved in all aspects of life, including growth regulation, development, abiotic stress responses, and reproduction (Kudla et al., 2018), as well as the establishment of beneficial plant-microbe interactions (Tian et al., 2020). In this review, we focus on how cytoplasmic Ca²⁺ signals are encoded via transport across the PM during immune signaling.

Ca²⁺ influx and the oxidative burst (Doke, 1983, 1985; Apostol et al., 1989; Kepper et al., 1989) were among the first cellular responses to pathogen infection or elicitor treatment to be described (Atkinson et al., 1996; Levine et al., 1996; Zimmermann et al., 1997; Lecourieux et al., 2002). ROS production during the oxidative burst was eventually attributed to the activity of PM-localized NADPH oxidases of the RESPIRATORY BURST OXIDASE HOMOLOGUE (RBOH) family (Torres et al., 2002); in the model plant Arabidopsis thaliana (hereafter, Arabidopsis), a single member, RBOHD, is responsible for ROS production in response to elicitors (Nühse et al., 2007; Zhang et al., 2007). In contrast, the molecular nature of the Ca²⁺ channel(s) involved in plant immunity remained comparably elusive for many years (Seybold et al., 2014).

Cytosolic Ca²⁺ signals evoked by treatment with various immunogenic elicitors were first measured in plant cell culture using Ca²⁺ radioisotopes, Ca²⁺-sensitive dyes, or electrophysiological approaches (Atkinson et al., 1996; Levine et al., 1996; Gelli et al., 1997; Zimmermann et al., 1997). The development of genetically encoded Ca²⁺ indicators (GECIs) greatly expanded the possibilities for real-time, kinetic analysis of Ca²⁺ fluxes in intact tissues upon infection or elicitor treatment. The first GECI deployed in plants was aequorin (AEQ) from Aequoria victoria (Knight et al., 1991), which forms a holo-enzyme with its cofactor coelenterazine and emits light upon Ca²⁺-binding. When challenged with either virulent or avirulent strains of the pathogenic bacterium Pseudomonas syringae, Arabidopsis plants expressing AEQ showed a first Ca²⁺ signal peak after ~10 min. A second, stronger, more persistent Ca²⁺ signal was seen after 1.5–2 h only with avirulent, ETI-activating P. syringae (Grant et al., 2000; Kang et al., 2010; Hung et al., 2014). The similar kinetics of early Ca²⁺ elevation induced by P. syringae and that triggered by elicitors (Blume et al., 2000; Lecourieux et al., 2002) and the biphasic nature of the response to ETI-inducing bacteria suggested that PTI and ETI may induce distinct Ca²⁺ signals (Fig 1B).

Subsequent analyses of AEQ-expressing Arabidopsis plants have shown perception of diverse elicitors, including PAMPs, DAMPs, and phytocytokines, to be sufficient to elicit rapid Ca²⁺ signals (Ranf et al., 2008, 2011; Vadassery et al., 2009; Krol et al., 2010). Such PTI Ca²⁺ signaling requires functional PRRs and downstream signaling components, including RLCKs such as the RLCK-VII/ AVRPPHB SUSCEPTIBLE 1 (PBS1)-LIKE (PBL) family members BOTRYTIS-INDUCED KINASE 1 (BIK1) and PBL1 (Li et al., 2014; Ranf et al., 2014; Monaghan et al., 2015). More recently, the deployment of fluorescent GECIs in plants has allowed for the analysis of elicitor-induced Ca²⁺ signals at the cellular level. Such fluorescent GECIs include ratiometric (e.g., yellow cameleons) and intensiometric (e.g., GCaMPs and GECOs) sensors (Grenz et al., 2021b; Waadt et al., 2021). Fluorescent GECIs have been utilized to show that elicitor-induced Ca²⁺ signals in leaves are oscillatory at the single-cell level (Thor & Peiter, 2014; Keinath et al., 2015) and that in roots both elicitor application and laser ablation-induced cell damage lead to the formation of Ca²⁺ transients (Keinath et al., 2015; Marhavý et al., 2019; Waadt et al., 2020).

ROS and Ca²⁺—tightly linked second messengers

There is extensive interplay between Ca²⁺ and ROS signaling (Gilroy et al., 2016); however, the initial PTI-related Ca²⁺ signal triggered by P. syringae was shown to be only mildly reduced by treatment with the NADPH oxidase inhibitor DPI or catalase, while there was no effect on the longer-term, effector-triggered signal (Grant et al., 2000). Similarly, rbohd mutants showed a slight, quantitative defect in elicitor-triggered Ca²⁺ signals when measured in seedlings (Ranf et al., 2011). In contrast, elicitor-induced ROS production can be severely attenuated by treatment with Ca²⁺ channel blockers (Ranf et al., 2011). Elicitor perception can directly activate RBOHD via phosphorylation by BIK1 (Kadota et al., 2014; Li et al., 2014), suggesting a complex relationship between Ca²⁺ and ROS in immune signaling and a model wherein, upon elicitor perception, initial activation of RBOHD through PRR-mediated phosphorylation primes the system for subsequent activation through Ca²⁺ signaling (Kadota et al., 2015) (Fig 2). Ca²⁺ not only activates RBOHD directly via its cytoplasmic Ca²⁺-binding EF-hand domains but also indirectly via Ca²⁺-regulated kinase-mediated RBOHD phosphorylation (Ogasawara et al., 2008; Dubiella et al., 2013). Interestingly, BIK1 and CALCIUM DEPENDENT PROTEIN KINASE 5 (CPK5) activate RBOHD through phosphorylation at distinct sites (Dubiella et al., 2013; Kadota et al., 2014; Li et al., 2014). While target residues have been described to be strictly required for PTI-induced ROS bursts (Nühse et al., 2007), individual contribution from other phosphorylation sites and the impact of certain phosphorylation patterns remain to be uncovered.

A recent AEQ-based screen for impaired H₂O₂-induced Ca²⁺ signaling identified an LRR-RK, HYDROGEN PEROXIDE INDUCED Ca²⁺ INCREASE 1 (HPCA1), as a putative ROS sensor (Wu et al., 2020a). Interestingly, HPCA1 was independently identified as CANNOT RESPOND TO DMBQ 1 (CARD1), which showed a loss of
response to the quinone compound 2,6-dimethoxy-1,4-benzoquinone (DMBQ), which regulates interactions with parasitic plants and also triggers HPCA1/CARD1-dependent Ca\(^{2+}\) signaling (Laohavisit et al., 2020). Both the nature of the channel(s) that are regulated by HPCA1/CARD1, as well as the exact role of ROS in regulating Ca\(^{2+}\) signaling via such sensor(s) remain unclear. Interestingly, AEQ-measured calcium signals in response to H\(_2\)O\(_2\) were reduced in cngc2 and cngc4 mutants (Tian et al., 2019), suggesting that these channels may function downstream of ROS perception.

Shaping immune signals via Ca\(^{2+}\) efflux

Ca\(^{2+}\) signals are generated via the coordinated action of channels and active transporters and involve influx from the apoplast and release from intracellular stores (Spalding & Harper, 2011; Edel et al., 2017; Resentini et al., 2021). In addition, plants possess three major families of proteins that mediate active Ca\(^{2+}\) transport out of the cytosol: Ca\(^{2+}\)/H\(^+\) exchangers (CAXs), autoinhibited Ca\(^{2+}\)-ATPases (ACAs) and ER Ca\(^{2+}\)-ATPases (Geisler et al., 2000; Shigaki & Hirschi, 2000; García Bossi et al., 2020). ACA autoinhibitory can be relieved by Ca\(^{2+}\)/CaM-binding, which allows for rapid feedback regulation of Ca\(^{2+}\) signals (Geisler et al., 2000). The PM-localized ACA8 and its homolog ACA10 were identified as interactors of FLS2, and aca8 aca10 mutants displayed quantitative defects in flg22-induced calcium signals and compromised resistance to P. syringae infection (Frei dit Frey et al., 2012), as well as disturbed stomatal closure upon PAMP perception (Yang et al., 2017), suggesting that Ca\(^{2+}\) efflux across the PM to the apoplast shapes Ca\(^{2+}\) signaling during PTI.

Two tonoplast-localized ACAs, ACA4 and ACA11, have also been implicated in immunity, as aca4 aca11 mutants display autoimmune phenotypes and spontaneous cell death (Boursiac et al., 2010). Although aca4 aca11 mutants have wildtype total calcium content (Boursiac et al., 2010), subsequent work has revealed that basal cytosolic calcium levels are elevated in aca4 aca11 (Hilleary et al., 2020). Elicitor-induced calcium signals also show elevated peaks in aca4 aca11 mutants (Fig 3), which can be rescued by mislocalization of PM ACAs to the tonoplast (Hilleary et al., 2020), indicating that transport of Ca\(^{2+}\) into the vacuole is critical to maintain Ca\(^{2+}\) homeostasis and modulate signaling during PTI.

Plasma membrane-localized Ca\(^{2+}\) channels involved in immunity

Extensive work has demonstrated that elicitor-induced Ca\(^{2+}\) signals strictly require PM-localized, Ca\(^{2+}\)-permeable channels, as treatment with blockers such as Gd\(^{3+}\) or La\(^{3+}\) abolishes such signals (Blume et al., 2000; Grant et al., 2000; Lecourieux et al., 2002; Kwaaitaal et al., 2011; Ranf et al., 2011; Maintz et al., 2014; DeFalco et al., 2017). While such studies clearly implicate Ca\(^{2+}\)-permeable channels as components of immune signaling, their nature has remained hidden. However, recent work has started to decipher how Ca\(^{2+}\) signals are generated upon immune activation, and the defense-related roles of several classes of plant Ca\(^{2+}\) channels have begun to be characterized. Below, we discuss immunity-related channel candidates by their phylogenetic groups rather than following a chronological order of identification or a strict PTI/ETI dichotomy.

CNGCs—from strong phenotypes to complex regulation

One of the first families of potential Ca\(^{2+}\) channels identified in plants were the tetrameric cyclic nucleotide-gated channels (CNGCs) (Köhler & Neuhaus, 1998). Plant CNGCs comprise large gene
families (e.g., 20 members in Arabidopsis) (Mäser et al., 2001) and are named for their topology and domain organization, which are reminiscent of mammalian cyclic nucleotide-gated (CNG) and hyperpolarization-activated cyclic nucleotide-modulated (HCN) families (Kaupp & Seifert, 2002; Matulef & Zagotta, 2003). Individual CNGCs have six transmembrane helices and cytosolic N and C termini, with the cyclic nucleotide-binding domain (CNBD) located within the CNGC C terminus (Kaplan et al., 2007). While previous reports have indicated that the CNBDs of plant CNGCs may bind cyclic nucleotides (Baxter et al., 2008), and some electrophysiological analyses have indicated that application of cAMP or cGMP can promote CNGC activity (Leng et al., 2002; Zhang et al., 2007; Gao et al., 2014, 2016; Meena et al., 2019), it remains unclear whether cyclic nucleotides are bona fide agonists for plant CNGCs in planta. Furthermore, the existence of guanylate and adenylate cyclases (GCs and ACs) in plant proteomes is still under debate and will not be discussed in detail here. Indeed, while studies suggest multiple plant proteins, including RKs, to display GC activity (Qi et al., 2010; Turek & Irving, 2021), the low determined in vitro activities of the putative GCs and the position of their putative active sites within the kinase domains of RKs argues against a physiological relevance for such potential GC activity (Ashton, 2011; Bojar et al., 2014).

Nevertheless, extensive electrophysiological work over the past two decades has shown that at least some CNGCs form Ca$^{2+}$-permeable, non-selective cation channels (Jarratt-Barnham et al., 2021). CNGCs are directly regulated by the conserved Ca$^{2+}$ sensor calmodulin (CaM), with one or more CaM-binding domains (CaMBDs) found within the cytosolic C termini of all CNGCs examined to date (Arazi et al., 1999; Köhler & Neuhaus, 2000; Hua et al., 2003; Fischer et al., 2013, 2017; DeFalco et al., 2016a) as well as the N terminus of some CNGC isoforms (DeFalco et al., 2016a). Ca$^{2+}$/CaM regulation of CNGCs is complex (DeFalco et al., 2016b) as a Ca$^{2+}$-independent IQ motif CaMBD at the C-terminal end of the channel is essential for CNGC function (DeFalco et al., 2016a; Pan et al., 2019), with additional Ca$^{2+}$-dependent CaMBDs providing negative (feedback) regulation (DeFalco et al., 2016a; Pan et al., 2019; Tian et al., 2019).

Plant CNGCs are divided into four subfamilies based on phylogeny, with group IV CNGCs further divided into groups IVa and IVb (Mäser et al., 2001). The best-studied CNGCs to-date are the two Arabidopsis group IVb members, CNGC2 and CNGC4, which were first isolated as the defense, no death (dnr) or HR-like lesion mimic (hlm) mutants dnd1 and dnd2/hlm1 (null mutants of CNGC2 and CNGC4, respectively) (Clough et al., 2000; Balagué et al., 2003; Jurkowski et al., 2004). The dnd mutants were initially described to be defective in the induction of HR, despite still being able to carry out ETI to avirulent pathogens (Yu et al., 1998). These dnd mutants display numerous phenotypic defects, including dwarf morphology, delayed flowering, elevated concentrations of the phytohormone salicylic acid (SA), spontaneous cell death, and dis-regulated auxin signaling (Clough et al., 2000; Balagué et al., 2003; Chan et al., 2003; Jurkowski et al., 2004; Chin et al., 2013; Chakraborty et al., 2021). In keeping with the immune-related phenotypes of dnd1/cngc2 mutants, CNGC2 was also suggested to be a mediator of Ca$^{2+}$ fluxes in plant immunity, as production of the signaling molecule nitric oxide (NO) was reported to be reduced in cngc2 mutants compared to WT plants after treatment with the PAMP lipopolysaccharide (LPS) (Ali et al., 2007). The same study used pharmacological inhibitors to implicate CaM, Ca$^{2+}$ channels, and a NO synthase (NOS)-type protein to be required for this process. Given the lack of mammalian-type NOS enzymes in land plants (Santolini et al., 2017) and the myriad functions of CaM (DeFalco et al., 2010), results from such pharmacological studies must however be interpreted cautiously. Subsequent work using AEQ reporter lines suggested that CNGC2 is required for full Ca$^{2+}$ signals in response to some but not all elicitors (Ma et al., 2012). Given the convergence of signaling
downstream of diverse PRRs (Couto & Zipfel, 2016; Bjornson et al., 2021), it remains unclear how such specificity may be achieved. Interestingly, virus-induced gene silencing (VIGS) of IVb isoforms in tomato compromised ROS production in response to flg22, further suggesting that these CNGCs may positively regulate PTI (Saand et al., 2015).

Recently, loss-of-function cngc2 and cngc4 mutants were each isolated in an AEQ-based forward genetic screen for compromised Ca²⁺ signaling upon flg22 treatment (Tian et al., 2019). Both mutants displayed defects in Ca²⁺ influx and ROS production after treatment with flg22 and exhibited compromised resistance to P. syringae. Remarkably, these phenotypes were however strictly dependent on high Ca²⁺ concentrations in the growth media, as cngc2 and cngc4 responses under low Ca²⁺ growth were indistinguishable from those of WT plants. Interestingly, PRR signaling mutants, such as bik1, do not display such conditional phenotypes (Li et al., 2014; Ranf et al., 2014; Monaghan et al., 2015). Detailed electrophysiological characterization of the heterologously expressed channels in Xenopus laevis oocytes found the single subunits to be inactive, while CNGC2-CNGC4 heteromers produce strong currents (Tian et al., 2019), in keeping with a model wherein these channel subunits function together (Chin et al., 2018). CNGC2-CNGC4 currents were inhibited by CaM; further experiments suggested that phosphorylation of the CNGC4 C terminus by BIK1 can partially release this negative regulation (Tian et al., 2019) (Fig 4A). This work further highlights the complex regulation to which CNGCs are likely subject, including by CaM, phosphorylation, and, potentially, ligand-binding (Jarratt-Barnham et al., 2021).

Both cngc2 and cngc4 mutants are hypertensive to Ca²⁺ concentration in growth media (Chan et al., 2003; Chin et al., 2013), and their pleiotropic dry phenotype has been suggested to be caused by the mutant’s inability to take up Ca²⁺ from the apoplast into the cells in the vicinity of vasculature (Wang et al., 2017). Over-accumulation of apoplastic Ca²⁺ and the resulting perturbations of both tissue- and cellular Ca²⁺ homeostasis may thus (at least partially) cause cngc2 (and cngc4) phenotypes, though this will require further study to resolve fully. Given that PTI is not affected in cngc2 and cngc4 mutants grown at low Ca²⁺ concentrations (Tian et al., 2019), at such growth conditions—under which no growth defects also occur—other, currently unknown Ca²⁺ channels must also contribute to PTI (Dietrich et al., 2020). Recent studies reported a member of CNGC subfamily II, AtCNGC6, to be involved in the generation of Ca²⁺ signals during immunity after perception of the DAMP eATP, supporting the possibility of diverse CNGC subunits playing specific roles in plant immune responses (Duong et al., 2022).

CNGCs and cell death

A genetic screen in rice (Oryza sativa, Os) recently identified loss-of-function mutants of OsCNGC9 (a group III CNGC and homolog of Arabidopsis CNGC18) that displayed compromised resistance to rice blast disease and lesion-mimic phenotypes after flowering (Wang et al., 2019b). PAMP-induced Ca²⁺ currents across the PM were found to be strongly diminished in OsCNGC9 mesophyll cells compared to WT controls and, using an elegant heterologous reconstitution assay in mammalian cell culture, the authors demonstrated activation of the channel by OsRLCK185, a rice member of the RLCK-VII/PBL family that functions downstream of chitin perception (Wang et al., 2019b) (Fig 4B). The autoimmune phenotypes of OsCNGC9 mutants are reminiscent of Arabidopsis cngc2 and cngc4, it will therefore be interesting to determine whether such autoimmune phenotypes are due to these channels being guarded by NLRs and/or through perturbed Ca²⁺ homeostasis.

In contrast to the loss-of-function mutants described above, several gain-of-function CNGC mutants have also been isolated from genetic screens. These include several instances of (semi-) dominant gain-of-function mutations that trigger autoimmunity such as cpr22 (caused by expression of an in-frame CNGC11/12 chimera) (Yoshio et al., 2006; Uruhart et al., 2007) and cngc20-4 (caused by a leucine to phenylalanine mutation within one of the transmembrane helices of CNGC20) (Zhao et al., 2021) of Arabidopsis and the brush mutant of Lotus japonicus (hereafter, Lotus), which is caused by an N-terminal glycine to glutamic acid mutation in the Lotus homolog of Arabidopsis CNGC19 (Chiaison et al., 2017) (Fig 4C). While such gain-offunction mutations must be interpreted cautiously, detailed study of these mutants suggests that dis-regulated CNGCs can induce Ca²⁺- and SA-dependent immunity and HR-like cell death (Yoshioka et al., 2006; Uruhart et al., 2007; DeFalco et al., 2016a; Zhao et al., 2021), suggesting possible roles in ETI signaling and immunity more generally (Moeder et al., 2019).

CNGC20 has also recently been identified as a positive regulator of a specific form of autoimmunity (Yu et al., 2019). Loss of the SERK family co-receptors BAK1/SERK3 and BAK1-LIKE 1 (BKK1/SERK4) triggers constitutive cell death and seedling lethality (He et al., 2007; Kemmerling et al., 2007; Schwessinger et al., 2011). VIGS-based screen revealed that this cell death is dependent on CNGC20 and to a lesser extent its close IVa homolog CNGC19 (Yu et al., 2019). This study further proposed a mechanism wherein SERKs phosphorylate the C terminus of CNGC20 to destabilize the channel in the absence of immunogenic stimuli, thereby precluding detrimental Ca²⁺ influx and cell death (Yu et al., 2019), adding an interesting component to the regulation of Ca²⁺ fluxes in immunity (Fig 4C). Whether or not recruitment of BAK1 into PRR complexes after elicitor recognition permits CNGC20 phosphorylation and therefore induces CNGC20 activity should be addressed in future studies. Recent work suggests a more complex role for Ca²⁺ in BAK1-related cell death, as NLRs that mediate bkk1 bkk1 autoimmune mutation have been identified, including the NLR CONSTITUTIVE SHADE-AVOIDANCE 1 (CSA1) (preprint: Schulze et al., 2021) and helper NLRs of the ACTIVATED DISEASE RESISTANCE 1 (ADR1)-like family (Wu et al., 2020b). Strikingly, these ADR1-type helper NLRs have recently been proposed to themselves act as Ca²⁺-permeable cation channels (see detailed discussion below).

The other CNGC-IVa member in Arabidopsis, CNGC19, was identified in a screen for genes whose expression was upregulated by mechanical wounding, and cngc19 mutants were found to be more susceptible to Spodoptera littoralis caterpillars, likely due to impaired jasmonate (JA) and alipathic glucosinolate production (Meena et al., 2019). Interestingly, Ca²⁺ signals in response to the DAMP AtPep1 were also reduced in cngc19 mutants (Meena et al., 2019), though other work using cngc19 cngc20 proteolasts expressing GCaMP3 suggested that group IVa CNGCs are not required for the Ca²⁺ signals in response to flg22 (Yu et al., 2019). CNGC19 was also implicated in responses to the root-colonizing mutualist endophytic fungus Piriformospora indica (Jogawat et al., 2020). cngc19 mutants display clear phenotypes with respect to the symbiosis-
induced gain in growth rate; however, only minor defects in cytoplasmic Ca\(^{2+}\) rises after treatment with cell wall extracts from *P. indica* have been reported. This indicates the involvement of additional channel(s) (Jogawat et al., 2020).

An intriguing aspect of plant immune signaling is the propagation of electrical and second messenger-based signals through the plant body, despite the obvious lack of any neurons or nervous system tissues in plants. In addition to reduced AtPep1 responses, *cngc19* mutants also displayed reduced systemic Ca\(^{2+}\) signals after mechanical wounding in the vasculature (Meena et al., 2019). Such signaling has long been associated with glutamate receptor-like (GLR) channels, which are discussed below.

**GLRs – the long road to plant immunity**

GLRs form a family of PM-localized, ligand-gated Ca\(^{2+}\) channels. Plant GLRs are named for their homology to metazoan ionotropic glutamate receptors (iGluRs), which are ligand-regulated, homo- or heterotetrameric cation channels functioning in animal nervous systems. In *Arabidopsis*, GLRs form a 20-member family that is subdivided into three clades: GLR1s, GLR2s and GLR3s; (Lam et al., 1998); individual members of the family have been implicated in various physiological processes (Wudick et al., 2018). GLRs feature a large, extracellular N-terminal domain, which perceives amino acid ligands, three transmembrane helices and a short, cytosolic C terminus (Alfieri et al., 2020).

GLRs were first implicated in the generation of Ca\(^{2+}\) signals upon elicitor perception by pharmacological experiments using iGluR inhibitors, which reduced PAMP-induced Ca\(^{2+}\) signals (Kwaaitaal et al., 2011). *Arabidopsis glr3.3* mutants were subsequently found to have compromised immunity toward the oomycete pathogen *Hyaloperonospora arabidopsidis*. In the same study, however, Ca\(^{2+}\) measurements in AEQ-expressing *glr3.3* lines did not show

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reduced signals after treatment with oligogalacturonides (OGs), products of hydrolyzed host cell walls that act as DAMPs during *H. arabidopsis* colonization (Manzoor *et al*, 2013). Similarly, *glr3.3* was also found to be more susceptible to *P. syringae* (Li *et al*, 2013), although formation of Ca\(^{2+}\) signals was not analyzed in that study. Together, such findings suggested a role for GLR3.3 in plant immunity which is distinct from its role in mediating the formation of the early Ca\(^{2+}\) signal. Indeed, ground-breaking work from the labs of Ted Farmer and Simon Gilroy instead unraveled the role of GLR3s in the propagation of long-distance signals and the formation of systemic immune responses. Multiple clade 3 GLRs were initially found to be required for the generation of electrical signals necessary for the induction of defense responses in distal tissues of plants after mechanical wounding or larval feeding on local leaves (Mousavi *et al*, 2013). Using *Arabidopsis* plants stably expressing GCaMP3, another study found that GLR3.3 and GLR3.6 are required for the generation of Ca\(^{2+}\) signals that propagate through the vasculature upon wounding or feeding (Toyota *et al*, 2018), and, correspondingly, *glr3.3 glr3.6* plants were shown to be more susceptible to *S. littoralis* (Nguyen *et al*, 2018). Simultaneous measurements of the electrical signals and cytoplasmic Ca\(^{2+}\) concentrations revealed that membrane depolarization preceded the rise of Ca\(^{2+}\) levels, a temporal sequence that was also observed in mesophyll cells after perception of flg22 (Nguyen *et al*, 2018; Li *et al*, 2021). Such results highlight the specific role of clade 3 GLRs in systemic signaling. Taken together, those studies support a model wherein interconnected electrical, Ca\(^{2+}\) and ROS signals, as well as activity of the tonoplast-localized cation channel TWO-PORE CHANNEL 1 (TPC1) are required for effective long-distance signal propagation in plants (Steinhorst & Kudla, 2014; Evans *et al*, 2016; Choi *et al*, 2017; Farmer *et al*, 2020; Johns *et al*, 2021).

It remains unclear whether clade 3 (or other) GLRs are also direct or indirect targets of PRR-activated signaling pathways. It has been proposed that OG perception involves RKs of the WALL-ASSOCIATED KINASE (WAK) family of epidermal growth factor (EGF)-motif containing RKs (Brutus *et al*, 2010; Kohorn & Kohorn, 2012), while local Ca\(^{2+}\) signals in response to aphid feeding are BAK1 as well as GLR3.3- and GLR3.6-dependent (Vincent *et al*, 2017). This suggests that the PRR signaling machinery may regulate GLR activity. Extracellular glutamate, which can act as a DAMP upon cell disruption, is also capable of inducing Ca\(^{2+}\) signals that are abolished in *glr3.3 glr3.6* mutants (Toyota *et al*, 2018; Shao *et al*, 2020), suggesting apoplastic amino acid(s) may act as agonist ligands for GLRs. The direct binding of glutamate to GLRs was further resolved through structural analysis of GLR3 ectodomains (Alfieri *et al*, 2020; Gangwar *et al*, 2021; Green *et al*, 2021). The role of clade 3 GLRs in systemic signaling has been recently reviewed in detail (Grenzi *et al*, 2021a), while the details of how ligand-gating and/or PRR signaling coordinate the activation of GLR3 channel activity remain to be fully resolved.

While clade 3 GLRs and their specific role in intercellular and long-distance signaling are to date the best studied, other GLRs have also been recently found to play roles in the immune system. The *Arabidopsis* clade 2 GLRs GLR2.7 and GLR2.9 were recently identified in a large-scale transcriptive analyses as so-called “core immunity response” (CIR) genes, which were transcriptionally upregulated in response to a panel of elicitors but not abiotic stresses ( Bjornson *et al*, 2021) (Fig 5). GLR2.7 and GLR2.9 form a tandemly-arranged, closely-related cluster along with GLR2.8, and *glr2.7 glr2.8 glr2.9* triple mutants displayed defects in Ca\(^{2+}\) responses upon treatment with a variety of elicitors and reduced immunity against *P. syringae* ( Bjornson *et al*, 2021). In keeping with their identification as CIR genes, these GLR2s were not found to contribute to Ca\(^{2+}\) signals during abiotic stress, suggesting that PTI involves common signaling components downstream of diverse elicitor/PRR complexes, but distinct from those involved in abiotic stress responses. As with GLR3s, how PRR complex activation mechanistically triggers rapid Ca\(^{2+}\) fluxes involving these GLR2s remains to be uncovered, as does the potential role for amino-acid binding in this process.

**OSCA s, Ca\(^{2+}\) and stomatal gatekeeping**

While the CNGC and GLR families of proteins were annotated shortly following release of the first sequenced plant genomes, the REDUCED HYPEROSMOLALITY, INDUCED Ca\(^{2+}\) INCREASE (OSCA) family was only recently identified. OSCAs have nine transmembrane helices, with a short extracellular N terminus and a larger C terminus, and constitute a 15-member family in *Arabidopsis* (Yuan *et al*, 2014). OSCA1.1 was identified in an AEQ-based screen for regulators of Ca\(^{2+}\) signaling in response to osmotic stress (Yuan *et al*, 2014) and its homolog OSCA1.2 (also named CALCIUM PERMEABLE STRESS-GATED CATION CHANNEL 1, CSCI) was identified through a heterologous screening of uncharacterized *Arabidopsis* transmembrane proteins for Ca\(^{2+}\) channel activity (Hou *et al*, 2014). Both OSCA1.1 and OSCA1.2/CSCI were shown to be Ca\(^{2+}\)-permeable channels (Hou *et al*, 2014; Yuan *et al*, 2014), while...
subsequent structural, electrophysiological, and bioinformatic studies have revealed that OSCAs represent an evolutionarily conserved family of mechanosensitive, Ca\(^{2+}\)-permeable cation channels (Jojoa-Cruz et al., 2018; Liu et al., 2018; Murthy et al., 2018).

In addition to systemic and long-distance immune signaling, Ca\(^{2+}\) signaling also occurs at the single cell level in stomatal immunity. Stomata are gas-exchange pores in the leaf epidermis that are formed by pairs of guard cells, with stomatal aperture controlled by changes in guard cell turgor (Lawson & Matthews, 2020). Aside from controlling gas exchange, stomata also serve as key points of entry for foliar pathogens (Melotto et al., 2017), and elicitor perception leads to rapid stomatal closure (Melotto et al., 2006; Desikan et al., 2008; Zeng & He, 2010). Stomatal closure is controlled by activation of SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) and/or SLAC1 HOMOLOGUE 3 (SLAH3), which mediate guard cell anion efflux, and which can be activated by Ca\(^{2+}\)-dependent or -independent phosphorylation cascades (reviewed in (Jezek & Blatt, 2017)). PTI signaling involves Ca\(^{2+}\) influx in guard cells (Thor & Peiter, 2014), and recently Arabidopsis osca1.3 osca1.7 loss-of-function mutants were found to be defective in elicitor-induced stomatal closure (Thor et al., 2020). The mechanism of the underlying core signaling pathway was duly unraveled, as OSCA1.3 was identified as a direct substrate of BIK1, which phosphorylates the channel on its N-terminal cytosolic loop, providing a direct molecular connection from the activated PRR complex to the Ca\(^{2+}\) signal generation in guard cells (Fig 6A). PAMP treatment triggered phosphorylation of this BIK1-dependent phosphosite (Benschop et al., 2007; Thor et al., 2020), and phosphorylation was found to promote the channel activity of OSCA1.3 in heterologous electrophysiological measurements (Thor et al., 2020). Ca\(^{2+}\) signaling defects in osca1.3 osca1.7 mutants were also specific to guard cells, as signals in seedlings and epidermal tissues were unaffected. This study indicates a specific role of OSCA1.3 and OSCA1.7 in guard cells and stomatal immunity, and future studies may reveal whether other members of this family play additional roles in immunity, as well as how their potential mechano-regulation contributes to such functions. Remarkably, another route of PRR signaling required for stomatal immunity was recently identified. Upon perception of chitin by CHITIN-ELICITOR RECEPTOR KINASE 1 (CERK1)/LYSING, CONTAINING RECEPTOR-LIKE KINASE 5 (LYK5) complexes, PBL27 directly phosphorylates the anion channel SLAH3 (Liu et al., 2019) (Fig 6B). Why different elicitors activate specific pathways to

**Figure 6.** PRR signaling controls Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent pathways leading to stomatal immunity.

Perception of bacterial flg22 leads to activation of BIK1 and phosphorylation of the Ca\(^{2+}\) channel OSCA1.3. Subsequent Ca\(^{2+}\) influx into the cytosol is required for guard cell closure. This closure is likely achieved through the activation of Ca\(^{2+}\)-regulated kinases, which in turn phosphorylate SLAC1 or SLAH3 anion channels. Upon channel activation of those channels, resulting ion fluxes cause turgor loss in the guard cell and stomatal closure (A). Perception of chitin by CERK1/LYK5 complexes activates the RLCK PBL27, which directly phosphorylates SLAH3, leading to stomatal closure (B).
achieve the same physiological response, and whether the chitin induced pathway indeed functions without contribution of Ca\(^{2+}\) signaling, remains to be resolved.

In addition to the osca mutants defective in elicitor-induced Ca\(^{2+}\) influx, disturbance of Ca\(^{2+}\) signals through loss of the ACA 8 and 10 cause loss of pathogen-induced stomatal closure (Yang et al., 2017). Mutations in either of those Ca\(^{2+}\) pumps or their interactor BONZAI 1 (BON1) caused enhanced steady state Ca\(^{2+}\) signals and additionally failed to generate stimulus dependent stomatal Ca\(^{2+}\) oscillations due to retarded Ca\(^{2+}\) efflux after initial influx (Yang et al., 2017). Interestingly, the effect on guard cell Ca\(^{2+}\) fluxes in osca1.3 osca1.7 mutants after flg22 application was quantitative (Thor et al., 2020), in contrast to the near-complete loss of flg22-induced stomatal closure in these mutants, while the defects in ACA8 and ACA10 activity still allowed the generation of Ca\(^{2+}\) signals but nevertheless prevented stomatal closure (Yang et al., 2017). Together, these studies suggest that minor perturbations in Ca\(^{2+}\) signals can trigger detrimental effects on downstream physiological processes.

**ANNs—atypical Ca\(^{2+}\) channels?**

Annexins (ANNs) are small proteins occurring in both prokaryotes and eukaryotes and form a family of eight members in Arabidopsis (Laohavisit & Davies, 2011; Clark et al., 2012). Unlike other Ca\(^{2+}\)-permeable channels, ANNs are soluble proteins that lack transmembrane helices and instead reversibly bind negatively charged phospholipids, a process that is controlled by Ca\(^{2+}\) (Laohavisit & Davies, 2011). ANNs have previously been suggested either to regulate Ca\(^{2+}\) fluxes or provide Ca\(^{2+}\) transport activity themselves in response to H\(_2\)O\(_2\) and salt stress (Laohavisit et al., 2012; Ma et al., 2019).

Recently, Arabidopsis ANN1 was identified as a positive regulator of local and systemic Ca\(^{2+}\) responses that are induced upon mechanical wounding and perception of *S. littoralis* oral secretions, with ANN1 loss-of-function or overexpression lines displaying enhanced or decreased susceptibility toward *S. littoralis*, respectively (Malabarba et al., 2021). Furthermore, *ann1* mutants were compromised in both transcriptional responses and JA production—phenotypes remarkably reminiscent of those reported for *cngec19* mutants (Meena et al., 2019). In this context, it will be an interesting target of future studies to parse how ANN1- and CNGC19-mediated Ca\(^{2+}\) influx is able to distinguish between the induction of local and long-distance signals.

In addition to those wound-induced signals, ANN1 was also found to be involved in the generation of Ca\(^{2+}\) signals upon treatment of *Arabidopsis* with eATP (Mohammad-Sidik et al., 2021), which is perceived as a DAMP by the L-type lectin RK DOES NOT RESPOND TO NUCLEOTIDES 1/P2 RECEPTOR KINASE 1 (DORN1/2PK1) (Choi et al., 2014). The quantitative defect in eATP-induced Ca\(^{2+}\) in *ann1* mutants suggests ANN1 as part of the signaling pathway downstream of PRR activation; however, it remains unclear whether ANN1 itself acts as a Ca\(^{2+}\) transporter, as well as how such activity is regulated. Furthermore, ANN1 was reported to interact with the chitin-perceiving PRR CERK1 and thereby connects chitin perception and salt stress responses, a process in which ANN1 was previously characterized (Laohavisit et al., 2013; Espinoza et al., 2017). However, the underlying molecular mechanism remains to be resolved. Interestingly, ANN1 was independently identified as a mediator of *Arabidopsis* cold stress tolerance and was shown to positively regulate Ca\(^{2+}\) signals after cold shock (Liu et al., 2021). In this case, ANN1 Ca\(^{2+}\) transport activity was documented using electrophysiological characterization in *X. laevis* oocytes, with phosphorylation by the kinase OST1 having a positive effect on this activity (Liu et al., 2021). Whether similar regulatory phosphorylation of ANNs occurs in the context of immune signaling remains to be discovered, as does the mechanism by which activity of ANNs and GLRs are coordinated in the formation of long-distance Ca\(^{2+}\) signals upon wounding.

**Ankyrin repeat domain proteins—a new class of Ca\(^{2+}\)-permeable channels in immunity?**

Recently, LR14a, a wheat six-transmembrane PM intrinsic protein with a N-terminal cytoplasmic domain containing 12 ankyrin repeats was found to confer resistance to leaf rust in wheat (Kolodziej et al., 2021). Silencing of LR14a led to increased growth of the causal fungal pathogen *Puccinia triticina* and reduced induction of HR flecks. Interestingly, LR14a shares structural similarity with the mammalian protein TRANSIENT RECEPTOR POTENTIAL CHANNEL SUBFAMILY A MEMBER1 (TRPA1) (Suo et al., 2020; Kolodziej et al., 2021). TRPs are Ca\(^{2+}\)-permeable cation channel, suggesting a similar function of LR14a. LR14a was found to be required for the transcriptional induction of 160 genes upon infection with *P. triticina* which were associated with the gene ontology term “Ca\(^{2+}\)-binding”. Overexpression of LR14a in *Nicotiana benthamiana* leaves induced a water-soaking like phenotype indicative for osmotic disbalance, which could be prevented by the application of the Ca\(^{2+}\) channel blocker La\(^{3+}\) (Kolodziej et al., 2021). These findings support the possibility that LR14a acts as a Ca\(^{2+}\) channel, although electrophysiological characterization of the protein remains lacking.

Interestingly, another ankyrin repeat domain containing protein, *Arabidopsis ACCELERATED CELL DEATH 6* (ACD6), is a positive regulator of cell death, as multiple *acd6* alleles were found to induce varying degrees of autoimmunity and have been subject of research for over 20 years (Rate et al., 1999; Lu et al., 2003). While the molecular basis of ACD6 action remained largely elusive, a recent study has documented ACD6-induced ion channel activity upon heterologous expression in *X. laevis* oocytes (preprint: Zhu et al., 2021). Furthermore, autoimmunity of the *acd-1* allele could be abolished by growth at low [Ca\(^{2+}\)], suggesting similar perturbations of Ca\(^{2+}\)* homeostasis as reported for the *dnd* mutants (Chen et al., 2003; Chin et al., 2013; Wang et al., 2017; preprint: Zhu et al., 2021). ACD6 had been previously found to be associated with multiple RKs (Tateda et al., 2014; Zhang et al., 2017); however if and how this contributes to its regulation during immune responses has not been resolved.

**NLRs—wheels of death**

PTI signaling immediately downstream of elicitor perception by PRRs involves a characteristic rapid and transient Ca\(^{2+}\) signal. Understanding elicitor-triggered Ca\(^{2+}\) fluxes has been the focus of most studies of immunity-related Ca\(^{2+}\) channels. ETI signaling, by contrast, involves long-term, sustained Ca\(^{2+}\) signals (as discussed above). As outlined previously, PTI and ETI induce qualitatively similar signaling outputs, some of which (e.g., ROS, MAPK activation) have been shown to involve the same molecular components in both pathways (Kadota et al., 2019). It was thus reasonable to expect that similar channels were involved in Ca\(^{2+}\) signaling during both PTI and ETI, a supposition reinforced by the ETI-like autoimmune and cell death phenotypes.
of several Ca²⁺ channel mutants, as discussed above. However, the landscape of Ca²⁺ channels in immunity was recently revealed to be more complex than previously thought. Plant NLRs have been long hypothesized to form large, multi-meric complexes (as it the case in animals, (Jones et al., 2016)). This was finally shown to be the case with structural analysis of the complex of the CNL HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) and its RLCK interactors RESISTANCE-RELATED KINASE 1 (RKS1) and PBL2 (Wang et al., 2019a, 2019c). Using cryo-electron microscopy (cryo-EM), the authors were able to resolve how activation of ZAR1 via uridylation of the decoy PBL2 by the bacterial effector AvrAC triggers subsequent exchange of ADP to dADP in the ZAR1 NBD, leading the complex to take a radially symmetrical, pentameric structure, termed a resistosome (Fig 7). Interestingly, the N-terminal α-helical domains of ZAR1 formed a funnel-like domain within the resistosome, which was hypothesized to embed into membranes (Wang et al., 2019a). The overall resistosome structure resembled that of mammalian inflammasomes, and of the fungal toxin HET-S, both of which create pores in membranes upon activation through terminal helical domains and thereby allow ion transport (highlighted in Dangl & Jones, 2019; Mermigka & Sarris, 2019), suggesting that this may be the case in plants. Subsequent work using a combination of detailed electrophysiological characterization and in planta Ca²⁺ measurements revealed the nature of the ZAR1 resistosome as a non-selective cation-channel with permeability to Ca²⁺ (Bi et al., 2021). This ion permeability is required for ZAR1-induced cell death, which occurs through disintegration of the PM and cellular rupture (Bi et al., 2021).

In addition to the CNL ZAR1, TNLs have since been shown to also assemble into resistosome-like structures (Ma et al., 2020; Martin et al., 2020), though no evidence yet indicates that these also form pores in membranes. Instead, helper NLRs of the RNL type such as ADR1 and NRG1.1, which function downstream of TNL sensors, were recently found to form oligomers and constitute ion pores through assembly of their α-helical N-terminal domains (Jacob et al., 2021). Auto-activated forms of both NRG1.1 and ADR1 were found to function as Ca²⁺-permeable channels in planta and to induce cell death upon controlled over-expression. Similar to what had been reported for the ZAR1 resistosome (Bi et al., 2021), the cation permeability of NRG1.1 and ADR1 was dependent of the presence of negatively charged residues within the pore region of the protein complexes (Jacob et al., 2021).

The striking overall similarities found in the ZAR1 resistosome and the channels formed by NRG1.1 and ADR1 raise the question if the formation of ion-permeable pores is indeed the general function of several Ca²⁺ channel mutants, as discussed above. However, the landscape of Ca²⁺ channels in immunity was recently revealed to be more complex than previously thought. Plant NLRs have been long hypothesized to form large, multi-meric complexes (as it the case in animals, (Jones et al., 2016)). This was finally shown to be the case with structural analysis of the complex of the CNL HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) and its RLCK interactors RESISTANCE-RELATED KINASE 1 (RKS1) and PBL2 (Wang et al., 2019a, 2019c). Using cryo-electron microscopy (cryo-EM), the authors were able to resolve how activation of ZAR1 via uridylation of the decoy PBL2 by the bacterial effector AvrAC triggers subsequent exchange of ADP to dADP in the ZAR1 NBD, leading the complex to take a radially symmetrical, pentameric structure, termed a resistosome (Fig 7). Interestingly, the N-terminal α-helical domains of ZAR1 formed a funnel-like domain within the resistosome, which was hypothesized to embed into membranes (Wang et al., 2019a). The overall resistosome structure resembled that of mammalian inflammasomes, and of the fungal toxin HET-S, both of which create pores in membranes upon activation through terminal helical domains and thereby allow ion transport (highlighted in Dangl & Jones, 2019; Mermigka & Sarris, 2019), suggesting that this may be the case in plants. Subsequent work using a combination of detailed electrophysiological characterization and in planta Ca²⁺ measurements revealed the nature of the ZAR1 resistosome as a non-selective cation-channel with permeability to Ca²⁺ (Bi et al., 2021). This ion permeability is required for ZAR1-induced cell death, which occurs through disintegration of the PM and cellular rupture (Bi et al., 2021).

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of all helper NLRs. The physiological role of those channels will have to be analyzed in detail in future studies to resolve several open questions regarding channel-like NLR functions in immunity. After strong, induced overexpression of (auto-) activated helper NLRs, massive ion fluxes and rapid cell death have been documented (Jacob et al., 2021). This cell death is likely to be a consequence of the loss of ion homeostasis and resulting PM destabilization rather than Ca$^{2+}$ signaling per se. It therefore remains to be seen whether, under natural infection conditions, effector-triggered activation of NLR-formed Ca$^{2+}$ channels induces bona fide Ca$^{2+}$ signals that are perceived by Ca$^{2+}$ sensors to in turn induce physiological responses other than HR. Similarly, it will be critical to resolve how the channel-like activities of NLRs are interwoven with those of classical Ca$^{2+}$ channels, given both the ETI-like phenotypes of numerous channel mutants and the interdependence of the BAK1-related cell death on both ADR1-type RNLs (Wu et al., 2020b) and CNGC20 (Yu et al., 2019).

Our understanding of NLR function continues to evolve rapidly, and recent parallel studies have reported NADase activity of the TIR domain of TNLs upon their activation (Horsefield et al., 2019; Wan et al., 2019; Ma et al., 2020). The mechanistic basis of this activity has been resolved with the structure of the TNL RPP1, wherein the tetrameric protein complex was found to form a holoenzyme (Ma et al., 2020). A similar tetramerization upon activation was also been recently reported for the N. benthamiana NLR RECOGNITION OF XopQ 1 (ROQ1) (Martin et al., 2020). How NADase activity regulates downstream signaling pathways remains to be fully characterized; however, it will be of great interest to determine if and how the resulting products (nicotinamide, adenosine diphosphate ribose (ADPR), and a variant of cyclic ADPR (v-cADPR)) may modulate and/or induce Ca$^{2+}$ fluxes. The same holds true for another recently-reported enzymatic activity of TIR domain containing proteins: RESPONSE TO THE BACTERIAL TYPE III EFFECTOR PROTEIN HOPB1 (RBA1) was recently found to produce 2',3'-cAMP/cGMP through hydrolysis of RNA and DNA molecules (preprint: Yu et al., 2021). Production of 2',3'-cAMP/cGMP appears to be required for TIR mediated signaling and cell death, but the exact function of those molecules will require further study.

Recently, plant genomes were found to encode proteins with similarities to necroptosis-inducing MIXED LINEAGE KINASE-DOMAIN LIKE (MLKL) proteins (Mahdi et al., 2020). In animals, those MLKL proteins are phosphorylated upon necroptosis to induce oligomerization. This causes them to translocate through membrane insertion of an N-terminal four helix bundle called HeLo domain, which ultimately disturbs membrane integrity and causes cell death (Petrie et al., 2019). Interestingly, Arabidopsis MLKL3 and 4 were found to form tetramers, and loss of MLKL function led to severe defects in immunity toward the obligate biotrophic fungus Golovinomyces orontii via a TNL-dependent pathway that does not involve the induction of cell death (Mahdi et al., 2020). Remarkably, chemical oligomerization of MLKL HeLo domains was found to be sufficient for the induction of cell death in Arabidopsis (Mahdi et al., 2020). How Arabidopsis MLKLs are regulated during immune responses, if their action also induces Ca$^{2+}$ fluxes across the PM, and to what extent their functional mechanism is similar to that of the ZAR1 resistosome or the ADR1 type RNLs will be interesting topics for future studies.

Conclusions and outlook: answers, yet more questions

The molecular basis of Ca$^{2+}$ signaling during immune responses has been a major scientific question within plant biology for decades. As outlined in this review, numerous candidate channel proteins have been identified in recent years as contributing to PTI and/or ETI. However, despite this rapid increase in knowledge, critical questions remain unanswered, and the fact remains that the channel(s) responsible for the early Ca$^{2+}$ transient during PTI is/are still largely unknown.

The study of immunity and Ca$^{2+}$ signaling continues to benefit from tool development, and the modern, ever-growing GECI repertoire has allowed for ever-more detailed analyses of Ca$^{2+}$ signals in vivo. However, we must remember that our conceptualization of Ca$^{2+}$ signaling is at least partially defined by the GECIs we use, and may be too broad. It is possible that loss of individual Ca$^{2+}$ channels evokes loss of individual Ca$^{2+}$ signals within micro- and nanodomains, which are simply not resolved even by state-of-the-art Ca$^{2+}$ measurements.

It is remarkable that numerous channels from different families appear to contribute quantitatively to the rapid Ca$^{2+}$ signal upon elicitor perception. These results beg the question of whether PRR-mediated signaling cascades indeed target and regulate such a high number of individual channels. One possibility is that such a dividing and reuniting signaling architecture may allow for genetic robustness, although this remains to be explored. It is also possible that individual channels function in cell type-specific manners, as has been at least suggested in the case of OSCAs in guard cells. It is also possible that each of the Ca$^{2+}$ channels currently identified are indeed either quantitative contributors and/or regulators, while the channel(s) mediating the major influx still await identification. Indeed, the recent identification of channel families (e.g., OSCAs) or novel characterization of known proteins as potential channels (e.g., NLRs, ankyrin repeat domain proteins) indicates that there remains much to be discovered regarding Ca$^{2+}$-permeable channels in plants.

With regards to NLRs functioning as cation channels in ETI, future studies will have to find if they indeed generate Ca$^{2+}$ signals that evoke specific downstream responses, or if their channel activity rather represents the loss of membrane impermeability, with Ca$^{2+}$ influx just being a fellow traveler of cell death’s onset. In either case, it will be as well critical to resolve the role of “classical” Ca$^{2+}$ channels in ETI signaling.

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Author contributions
Philipp Köster: Visualization; Writing—original draft; Writing—review & editing. Thomas A DeFalco: Visualization; Writing — original draft; Writing—review & editing. Cyril Zipfel: Funding acquisition; Writing—review & editing.

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The authors declare that they have no conflict of interest.

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