**Tansley insight**

Con-Ca\(^{2+}\)-tenating plant immune responses via calcium-permeable cation channels

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**Summary**

Calcium serves as a second messenger in a variety of developmental and physiological processes and has long been identified as important for plant immune responses. We discuss recent discoveries regarding plant immune-related calcium-permeable channels and how the two intertwined branches of the plant immune system are intricately linked to one another through calcium signalling. Cell surface immune receptors carefully tap the immense calcium gradient that exists between apoplast and cytoplasm in a short burst via tightly regulated plasma membrane (PM)-resident cation channels. Intracellular immune receptors form atypical calcium-permeable cation channels at the PM and mediate a prolonged calcium influx, overcoming the deleterious influence of pathogen effectors and enhancing plant immune responses.

**I. Introduction**

Plants use plasma membrane (PM) localised receptors to recognise extracellular pathogen-associated molecular patterns (PAMPs) and trigger PAMP-triggered immunity (PTI). PAMP-triggered immunity is characterised by a transient immune response that is sufficient to restrict the growth of nonadapted microbes. Virulent pathogens, however, have evolved virulence effectors that are capable of modulating PTI, rendering it inefficient. Plants counteract the effector disruptions of PTI by an arsenal of intracellular sensor nucleotide binding-leucine rich (NB-LRR) receptors (NLRs) that recognise pathogen effectors and boost the immune response to effectively bypass the effector-blocked PTI response (Jones & Dangl, 2006). Although PTI and effector-triggered immunity (ETI) are initiated by different receptors, pattern recognition receptor (PRR) and NLR signalling potentiate a largely overlapping defence response but with different amplitudes (Tian et al., 2021; Yuan et al., 2021). ‘Sensor’ NLRs possess either a N-terminal coiled-coil (CC) domain or Toll-interleukin-1 receptor/resistance (TIR) domains. Coiled-coil-domain NLRs (CNLs) are functionally self sufficient and TIR-domain NLRs (TNLs) require ‘helper’ CC\(^{RWP8}\)-containing NLRs (RNLs) to function (Jubic et al., 2019). Effector-triggered immunity mediated by NLRs is a high amplitude and lasting immune response that
often culminates in the death of the host cell (Jones et al., 2016). Recent studies have revealed the molecular function of at least some CNLs and RNLs as calcium-permeable cation channels (Bi et al., 2021; Jacob et al., 2021). This discovery highlights the importance of Ca\(^{2+}\) in defence and allows us to describe plant immune signalling from the perspective of the receptor’s molecular function.

II. Regulation of calcium levels under normal conditions

Ca\(^{2+}\) is an essential nutrient that also acts as a potent secondary messenger for all aspects of plant physiology including development, abiotic stress and defence (Luan & Wang, 2021). Under normal, nonstimulating conditions, free cytosolic Ca\(^{2+}\) levels are kept at a low level (around 0.1 µM), which prevents Ca\(^{2+}\) cytotoxicity and enables minute Ca\(^{2+}\) level changes to act as signals (Thor, 2019). Low cytoplasmic Ca\(^{2+}\) levels are maintained via Ca\(^{2+}\) export to the apoplast and sequestration into the vacuole or chloroplast by H\(^+\)/Ca\(^{2+}\) antiporters and Ca\(^{2+}\)-ATPases that results in an immense buildup of Ca\(^{2+}\) (up to 10 mM) in apoplast and organelles (Thor, 2019; Hilleary et al., 2020). Upon activation, Ca\(^{2+}\) channels produce specific Ca\(^{2+}\) signatures defined by the frequency and amplitude of the Ca\(^{2+}\) level variation they support. The Ca\(^{2+}\) signature determines which Ca\(^{2+}\)-regulated factors will respond to the stimulus and for how long (Whalley & Knight, 2013). The regulation of cytoplasmic Ca\(^{2+}\) levels during the plant immune response involves a large number of channels (please refer to Fig. 1).

III. PTI and ETI converge on cytosolic Ca\(^{2+}\) regulation

PAMP-triggered immunity is typically initiated by ligand recognition and a consequent phosphorylation cascade. Pattern recognition receptors (PRRs) are either kinases themselves, receptor-like kinases (RLKs) that function directly with RLK or RLP co-receptors and/or function with receptor-like cytoplasmic kinases, receptor-like proteins (RLPs) (Macho & Zipfel, 2014). Pattern recognition receptor-initiated phosphorylation cascades regulate the activity of plasma membrane-resident Ca\(^{2+}\) channels that trigger rapid Ca\(^{2+}\) bursts and the transient activation of a mitogen-activated protein kinase (MAPK) cascade (Tian et al., 2019; Yu et al., 2019; Thor et al., 2020). Effector-triggered immunity responses mediated by the sensor CNL HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) or helper RNLs trigger the formation of Ca\(^{2+}\) permeable channels that consist of structured oligomers of the NLR receptors themselves (Wang et al., 2019; Bi et al., 2021; Jacob et al., 2021). This results in a lasting Ca\(^{2+}\) influx and sustained activation of the MAPK cascade, possibly through the action of calcium-dependent protein kinases (CDPKs or CPKs) (Bredow & Monaghan, 2019). Constitutive increases in either cytoplasmic Ca\(^{2+}\) levels or MAPK activity are sufficient to activate a strong immune response and ectopic cell death (Yoshioka et al., 2006; Genot et al., 2017; Hilleary et al., 2020; Zhao et al., 2021). However, in the absence of a PRR-triggered kinase cascade, Ca\(^{2+}\) influx resulting from transient NLR activation is not sufficient to trigger defence and cell death (Ngou et al., 2021; Yuan et al., 2021). This suggests that, whereas PRR-induced phosphorylation cascade and transient Ca\(^{2+}\) burst activate an immune response, sustained NLR-driven Ca\(^{2+}\) influx heightens its intensity (Fig. 1).

IV. Regulation of calcium levels by PRR signalling

Cation channels belonging to the glutamate receptor-like (GLR), the reduced hyperosmolality induced Ca\(^{2+}\) increases (OSCAs) and the cyclic nucleotide-gated channels (CNGCs) families are involved in PRR signalling (Tian et al., 2019) (Fig. 2a). Loss of function of CNGC2 or CNGC4 triggers the so-called ‘defence no death’ (DND) phenotype characterised by the constitutive activation of defence and the loss of hypersensitive cell death (Clough et al., 2013). A recent study demonstrated that the GLR2 channel family is additionally regulated during the NLR-mediated hypersensitive response (Yuan et al., 2021). CNGC11/12 are found to be directly regulated by NLRs (Bi et al., 2021). The modulation by BRASSINOSTEROIDS of GLR2.8 and CNGC20/19 facilitates rapid Ca\(^{2+}\) responses (Yu et al., 2019). Glutamate receptor-like channels also function as Ca\(^{2+}\)-permeable cation channels in the plasma membrane (Macho & Zipfel, 2014). However, in the absence of a PRR-triggered kinase cascade, Ca\(^{2+}\) influx resulting from transient NLR activation is not sufficient to trigger defence and cell death (Ngou et al., 2021; Yuan et al., 2021). This suggests that, whereas PRR-induced phosphorylation cascade and transient Ca\(^{2+}\) burst activate an immune response, sustained NLR-driven Ca\(^{2+}\) influx heightens its intensity (Fig. 1).

![Diagram of calcium channels and their regulation](image-url)
et al., 2000). CNGC2 and CNGC4 form heteromeric channels that are important for proper Ca\(^{2+}\) nutrition and for preventing overaccumulation of Ca\(^{2+}\) in the apoplast and the cell wall (Wang et al., 2017; Tian et al., 2019). Under low extracellular Ca\(^{2+}\) conditions, the defence no death phenotype is reverted (Tian et al., 2019). Therefore, the CNGC2/4 heteromer is not required for NLR signalling. Rather, in dnd mutants, elevated extracellular Ca\(^{2+}\) levels heighten defence activation and inhibit cell death through an unknown mechanism that may involve SA (Zavaliev et al., 2020).

However, biochemical evidence links CNGC2/4 to PRR signalling. CNGC2/4 is activated by the receptor-like cytoplasmic kinase (RLCK) Botrytis-induced kinase 1 (BIK1) and is required for PAMP-induced Ca\(^{2+}\) burst and defence against the effector delivery-deprived *Pst* DC3000 ΔHrcC (Tian et al., 2019). Like the defence no death phenotype, the requirement for CNGC2/4 in PRR signalling is suppressed under low Ca\(^{2+}\) conditions. Perhaps overaccumulation of extracellular calcium leads to the inhibition or degradation of Ca\(^{2+}\) channels that would otherwise compensate for the loss of CNGC2/4 in PRR signalling. Consistent with this hypothesis, the activity of CNGCs themselves is tightly regulated by Ca\(^{2+}\) levels via calmodulins (CaMs) (DeFalco et al., 2016; Dietrich et al., 2020). In addition, CNGCs can be regulated at the
level of protein abundance. CNGC20 forms a heteromeric channel with CNGC19 that is regulated by PRR signalling and required for defence against *Piriformospora indica* (Yu et al., 2019; Jogawat et al., 2020). CNGC20 abundance is negatively regulated by the PRR co-receptor BRI1-ASSOCIATED RECEPTOR KINASE (BAK1) under normal conditions. In the absence of BAK1, CNGC20 overaccumulates and triggers ectopic defence and cell death. (Fig. 1).

Redundancy of PRR-regulated Ca²⁺ channels is important. CNGC2/4 and CNGC20/19 only contribute a fraction of the PRR-triggered Ca²⁺ influx (Tian et al., 2019; Jogawat et al., 2020). The GLR cation channels are also important contributors to the PRR-induced calcium spike as evidenced by the use of GLR inhibitors and loss-of-function mutants (Manzoor et al., 2013). GLR3.3 is required for half of oligogalacturonide-triggered calcium influx (Manzoor et al., 2013). The triple mutant gbr2.7 gbr2.8 gbr2.9 showed a very slight but significant reduction in three different PRR-associated calcium bursts (Bjornson et al., 2021). Some channels are cell-type specific, as the BIK1-regulated Ca²⁺ channels OSCA1.3 and OSCA1.7, which significantly contribute to the PAMP-triggered Ca²⁺ influx in guard cells but do not impact mesophyll cell responses (Thor et al., 2020). Although they are required for stomatal closure, the osca1.3 osca1.7 double mutant still retains some Ca²⁺ influx upon PAMP treatment indicating that other channels are involved in stomatal defence.

### V. Regulation of calcium levels by NLR signalling

Diverse virulence effectors inhibit PRR-mediated Ca²⁺ influx during infection with virulent pathogens (Lammertz et al., 2019). Recently, the CNL ZAR1 and the RNLs ACTIVATED DISEASE RESISTANCE 1 (ADR1) and N REQUIREMENT GENE 1.1 (NRG1.1) were shown to encode Ca²⁺-permeable channels enriched at the PM (Bi et al., 2021; Jacob et al., 2021). Both ZAR1 and RNLs were shown to transport Ca²⁺ and other cations via electrophysiology in artificial membrane (ZAR1) and human HEK293 cells (RNLs), in the absence of any putative plant ion channels partners. They were both found to be impermeable to chloride anions or larger molecules such as tetraethylammonium ions, suggesting they form a narrow and cation-specific channels (Bi et al., 2021; Jacob et al., 2021). Other CNLs like RPS2 and RPM1 trigger a long-lasting Ca²⁺ influx that is required for defence and cell death (Grant et al., 2000; Gao et al., 2013; Jubic et al., 2019). The direct promotion of cytosolic Ca²⁺ influx by NLRs provides a new mechanism, independent of PRR-mediated calcium influx, required for NLR-mediated cell death responses in the presence of effectors (Fig. 2b).

ZAR1 and RNLs are not typical Ca²⁺ channels. In the resting state, ZAR1 and RNLs are absent from the PM and activation leads to channel assembly and PM insertion. The fact that the NLR-driven calcium influx is long lasting and can lead to cell death suggests that NLR channel activities may not be under Ca²⁺/CaM regulation as found for the CNGGs (Tian et al., 2019; Bi et al., 2021; Jacob et al., 2021). In fact, NLRs are apparently only regulated at the channel assembly level, in response to effectors, and by protein turnover. Indeed, the SNIPER1 and SNIPER2 E3 ligases regulate broadly NLR protein levels by targeting the shared NB domains of sensor NLRs (Wu et al., 2020). This differential mode of regulation may explain why NLRs, in contrast with RR-regulated channels, overcome effector suppression of PTI and trigger cell death.

Pattern recognition receptor and NLR signalling may cooperate to regulate calcium levels. CNGC11/12 are apparently not required for PRR signalling but do contribute to resistance to either *Hyaloperonospora arabidopsidis* Emw1 or *Pseudomonas syringae* DC3000(avrRpt2), which both trigger specific NLRs (Yoshioka et al., 2006; Moeder et al., 2011). However, CNGC11/12 are negatively regulated by CaM in the presence of high calcium levels, which is inconsistent with the steady and lasting elevation of cytosolic calcium levels during NLR signalling. Also, CNGC11/12 are not required for cell death induction during NLR signalling, so it is unclear if CNGC11/12 contribute to calcium influx during NLR signalling or potentiate NLR activation in the context of PRR signalling (Ngou et al., 2021; Yuan et al., 2021). By contrast, NLRs are involved in signalling and defence priming triggered by some PRRs. RNLs of the ADR1 family are involved in some PRR signalling and are required for some PRR-driven defence priming (Pruitt et al., 2021; Tian et al., 2021). It remains unknown how ADR1s are activated by PRR signalling and how they impact cytosolic Ca²⁺ levels during PRR signalling.

### VI. Translating cytosolic calcium to defence signalling

Various calcium-binding proteins such as CaM, CAM-like proteins (CMLs), calcineurin B-like proteins (CBLs), Ca²⁺-dependent protein kinases (CDPKs or CPKs), phospholipase D and respiratory burst oxidase homologue (RBOH) NADPH oxidases, among others, translate cytoplasm Ca²⁺ levels into downstream transcriptional defence signals (Luan & Wang, 2021). CaM-binding transcriptional activator (CAMTA) 3 acts as a mediator of convergent transcriptional regulation from NLR- and PRR-mediated signalling, as demonstrated by a dominant-interfering camta3 mutant that exhibited compromised PTI- and ETI-mediated transcriptional responses in Arabidopsis (Jacob et al., 2018). CAMTA3 directly binds to promoters of CALMODULIN-BINDING PROTEIN 60g (CBP60g) and SYSTEMIC ACQUIRED RESISTANCE DEFICIENT1 (SARD1) and loss of CAMTA3 leads to constitutive activation of immune responses suggesting its function as transcriptional repressors (Sun et al., 2020). CAMTA3 appears to be targeted by virulence effectors and is guarded by two TNLs, DSC1 and DSC2 (Lolle et al., 2017). Defence upregulation in *camta* mutants is thus induced by constitutive NLR activation.

CBP60 family transcription factors include both positive and negative immune regulators (Li et al., 2021). CBP60a is known as a negative regulator while positive regulators like CBP60b or CBP60g bind and activate promoters of defence genes such as isochorismate synthase 1 (ICS1), an essential enzyme for synthesis of SA (Li et al., 2021), which induces the expression of pathogenesis-related genes and plays critical roles in plant immunity (Zhang & Li, 2019). CBP60b is also likely a guarder of the TNL SNC1, like CAMTAs illustrating the importance of Ca²⁺-responsive factors in defence (Li et al., 2021).

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Arabidopsis CBL1 and CBL9 recruit CBL-interacting protein kinase (CIPK) 26 to the PM where it phosphorylates NADPH oxidase. This produces reactive oxygen species (ROS) that may be toxic to invading pathogens and can act as potent defence signalling molecules (Ma et al., 2020). By contrast, CIPK6 has been shown to negatively regulate ROS production in PTI and ETI (Ma et al., 2020). CPK1/2/4/11 have also been shown to activate ROS signalling by phosphorylating NADPH oxidases (Gao et al., 2013). In addition to ROS, direct phosphorylation of a specific subgroup of defence-related WRKY transcription factors has been reported upon sustained activation of Arabidopsis CPK4/5/6/11 (Gao et al., 2013). Calcium-dependent protein kinases also connect calcium signalling to MAPK pathways, as they are capable of phosphorylating MAP kinases (Bredow & Monaghan, 2019).

How Ca\(^{2+}\) regulates CNL- and RNL-mediated cell death is not fully understood. Due to its cytotoxicity, Ca\(^{2+}\) overaccumulation could be directly disrupting cellular homeostasis and triggering cell death. Alternatively, cell death could result from NLR-mediated pore formation, leading to the activation of Ca\(^{2+}\)-responsive proteins that would specifically respond to the precise Ca\(^{2+}\) signatures. These are not mutually exclusive. Cell death and defence can be dissociated during NLR signalling (so-called ‘extreme resistance’; Ross et al., 2021) and some CPKs contribute to cell death induction, arguing for an active cell death process (Gao et al., 2013; Laflamme et al., 2020).

Overall, intricate networks of diverse calcium-binding proteins translate elevated cytosolic Ca\(^{2+}\) levels into transcriptional reprogramming through MAPKs, WRKY, CAMTA and CPB60 transcription factors (Gao et al., 2013; Bredow & Monaghan, 2019). Simultaneous activation of SA and ROS signalling pathways fine-tunes the transcription machinery toward defence responses, rather than other Ca\(^{2+}\)-dependent processes, putatively via antagonistic actions of SA (Moeder et al., 2010). The exact nature of the defence response may differ depending on the immediate composition of the calcium decoders. By regulating the expression of the calcium-binding proteins, PTI potentiates and shapes ETI and vice versa (Ngou et al., 2021; Yuan et al., 2021). We currently lack the precise understanding of the calcium signatures generated by NLRs and whether individual NLRs generate unique signatures that determine the eventual outcome, or, alternatively, whether the outcome depends on the downstream calcium decoders.

### VII. Conclusions

The discovery that ZAR1 and RNLs are calcium-permeable cation channels significantly altered our understanding of the intricate relationship between the CNL and TNL branches of NLR immune receptor function. Although PRR and NLR signalling both converge on the cytoplasmic Ca\(^{2+}\) influx, the PRR-driven Ca\(^{2+}\) influx is tightly regulated by phosphorylation and CaM or related proteins. Pattern recognition receptor signalling is essentially transient, which is important to avoid the negative impact of immune system activation on growth. The distinct long-lasting calcium influx generated by NLRs suggests a much lower or even a total lack of calcium-dependent negative feedback on the channel activity (Grant et al., 2000; Bi et al., 2021; Jacob et al., 2021). This differential regulation may explain why NLR signalling cannot be dampened by the pathogen virulence effectors that inhibit PRR signalling, even though both PRRs and NLRs signal through largely similar downstream signal transduction cascades. Alternatively, as PRR signalling is required together with NLR signalling to trigger strong immunity (Yuan et al., 2021), it should be considered that NLR signalling overcomes effector action by boosting defence before effectors can effectively suppress it (Fig. 2). The initial PRR signalling activates a fast and transient phosphorylation cascade and Ca\(^{2+}\) burst, starting a few minutes after PAMP recognition (Fig. 2a). Together, these events orchestrate the transient accumulation of ROS, SA and defence molecules, among which are Ca\(^{2+}\)-responsive transcription factors. Over the first few hours following infection, depending on the pathogen, virulent pathogens inject intracellular effectors that dampen PRR signalling. After delivery, effectors render the cell refractory to PAMP stimulation (Fig. 2c), but at early time points 1–4 h after infection, PRR signalling is still actively upregulating defence-related genes that include Ca\(^{2+}\)-responsive elements (Fig. 2b). In this primed state, effector-activated NLRs can outpace or bypass effector-suppressed PRR signalling by delivering overwhelming Ca\(^{2+}\), which rapidly leads to NLR-mediated cell death and, likely, ETI.

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