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# The integration of reactive oxygen species (ROS) and calcium signalling in abiotic stress responses

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## Abstract

Reactive oxygen species (ROS) and calcium ( $\text{Ca}^{2+}$ ) signalling are interconnected in the perception and transmission of environmental signals that control plant growth, development and defence. The concept that systemically propagating  $\text{Ca}^{2+}$  and ROS waves function together with electric signals in directional cell-to-cell systemic signalling and even plant-to-plant communication, is now firmly imbedded in the literature. However, relatively few mechanistic details are available regarding the management of ROS and  $\text{Ca}^{2+}$  signals at the molecular level, or how synchronous and independent signalling might be achieved in different cellular compartments. This review discusses the proteins that may serve as nodes or connecting bridges between the different pathways during abiotic stress responses, highlighting the crosstalk between ROS and  $\text{Ca}^{2+}$  pathways in cell signalling. We consider putative molecular switches that connect these signalling pathways and the molecular machinery that achieves the synergistic operation of ROS and  $\text{Ca}^{2+}$  signals.

## KEYWORDS

calcium sensor proteins, crosstalk, signal transduction

## 1 | INTRODUCTION

The sessile nature of plants requires that they perceive environmental challenges that may pose threats to their viability. Rapid signalling and communication from individual cells that perceive potential threats to neighbouring cells is a prerequisite for survival, driven by evolutionary necessity. Sensitive detection of physical and chemical changes in the environment occurs at level of the plasma membrane (PM) and also in the systems that produce and use energy. These detection systems incorporate fluctuations in reactive oxygen species (ROS) and cytoplasmic calcium ( $\text{Ca}^{2+}$ ) that are interlinked to a raft of pre-emptive adaptation mechanisms that respond to external threats. ROS are oxidative signals that arise from primary and secondary metabolism and provide essential information on

metabolism and the energy state of the different cell compartments. They are thus considered to be major “pro-life” survival signals. The different ROS forms [singlet oxygen ( $^1\text{O}_2$ ), superoxide ( $\bullet\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), hydroxyl radicals ( $\bullet\text{OH}$ )] trigger different signalling pathways (Foyer and Hanke, 2022). The oxidation of protein cysteinyl (Cys) thiols that reversibly changing in protein characteristics and functions, is a major route of  $\text{H}_2\text{O}_2$ -mediated signal transduction that regulates plant growth and defence (Considine & Foyer, 2021). Recent studies have identified a number of redox-sensors in plants, including HPCA1 ( $\text{H}_2\text{O}_2$ -induced  $\text{Ca}^{2+}$ -increases 1) and QSOX1 (quiescin sulphhydryl oxidase homolog; Chae et al., 2021; Wu et al., 2020). QSOX1 contains a protein disulphide isomerase-like oxidoreductase with an active CxxC motif within a thioredoxin (TRX)-like fold as well as a sulphhydryl oxidase

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mitochondrial ERV (Essential for Respiration and Vegetative growth)/ALR (Augmenter of Liver Regeneration)-related domain with two CxxC motifs and a flavin adenine dinucleotide-binding motif (Chae et al., 2021). These receptor proteins perceive extracellular H<sub>2</sub>O<sub>2</sub> and transmit information to regulate downstream redox responses through different signalling pathways.

The spatiotemporal increase in rapidly diffusing extracellular and intracellular signalling molecules and ions is often called 'wave generation' (Choi et al., 2014; Mittler et al., 2011; Trewavas, 1999). This process allows rapid signalling in a stimulus-dependent manner to bring about appropriate responses in gene expression (Assmann and Romano, 1999; Newton et al., 2016). It is easy to imagine how the apoplastic ROS burst (hypersensitive response) became the basis of cell-to-cell communication and formed the foundation for a robust type of systemic response that we now call systemic acquired acclimation (SAA; to abiotic stress) and systemic acquired resistance (SAR; to biotic threats). This response is characterized by a rapid, directional spread of signal(s), which can reach systemic tissues that have not experienced the threat within minutes of the first stress perception. The ROS wave model of cell-to-cell signalling incorporates intracellular Ca<sup>2+</sup> waves via the influx of Ca<sup>2+</sup> ions, as well as electric signals. Furthermore, it has been shown that the Ca<sup>2+</sup> channels called glutamate receptor-like (GLR)3.3 and GLR3.6 play an important role in regulating systemic electric and Ca<sup>2+</sup> signals after wounding (Mousavi et al., 2013; Nguyen et al., 2018; Shao et al., 2020; Toyota et al., 2018). Wound signalling molecules that are released by damaged cells bind to corresponding receptors that trigger the propagation of slow wave potentials (SWPs). The SWPs initiate the transmission of cytoplasmic calcium Ca<sup>2+</sup> signals and so activate jasmonate synthesis and expression of defence-responsive genes. The wounding response includes mechanisms for the integration and propagation of rapid systemic signals, leading to SAA and SAR. Moreover, the electric signal induced in response to wounding or high light stress in dandelion leaves was recently shown to be transmitted from plant to plant by direct contact, in a manner that involved changes in the ion channel activity and ROS waves (Szechyńska-Hebda et al., 2022).

Exposure to environmental stress induces rapid local increases in cytosolic Ca<sup>2+</sup> levels that are sensed and relayed inside the cell, as well as activating the respiratory burst oxidase homolog D, (RBOHD) proteins and generate an apoplastic ROS burst. In the case of high light stress this activation also involves phytochrome (phyB; Devireddy et al., 2020). The ROS and Ca<sup>2+</sup> waves depend on a continuous chain of cell-to-cell signalling events that extends from the initial tissue that senses the stress (the local tissue) to the entire plant (systemic tissues) through a positive feedback loop mechanism. A crucial feature of the signalling process is that it involves the coordination and integration of apoplastic and symplastic signalling pathways so that they function synchronously to drive directional signalling.

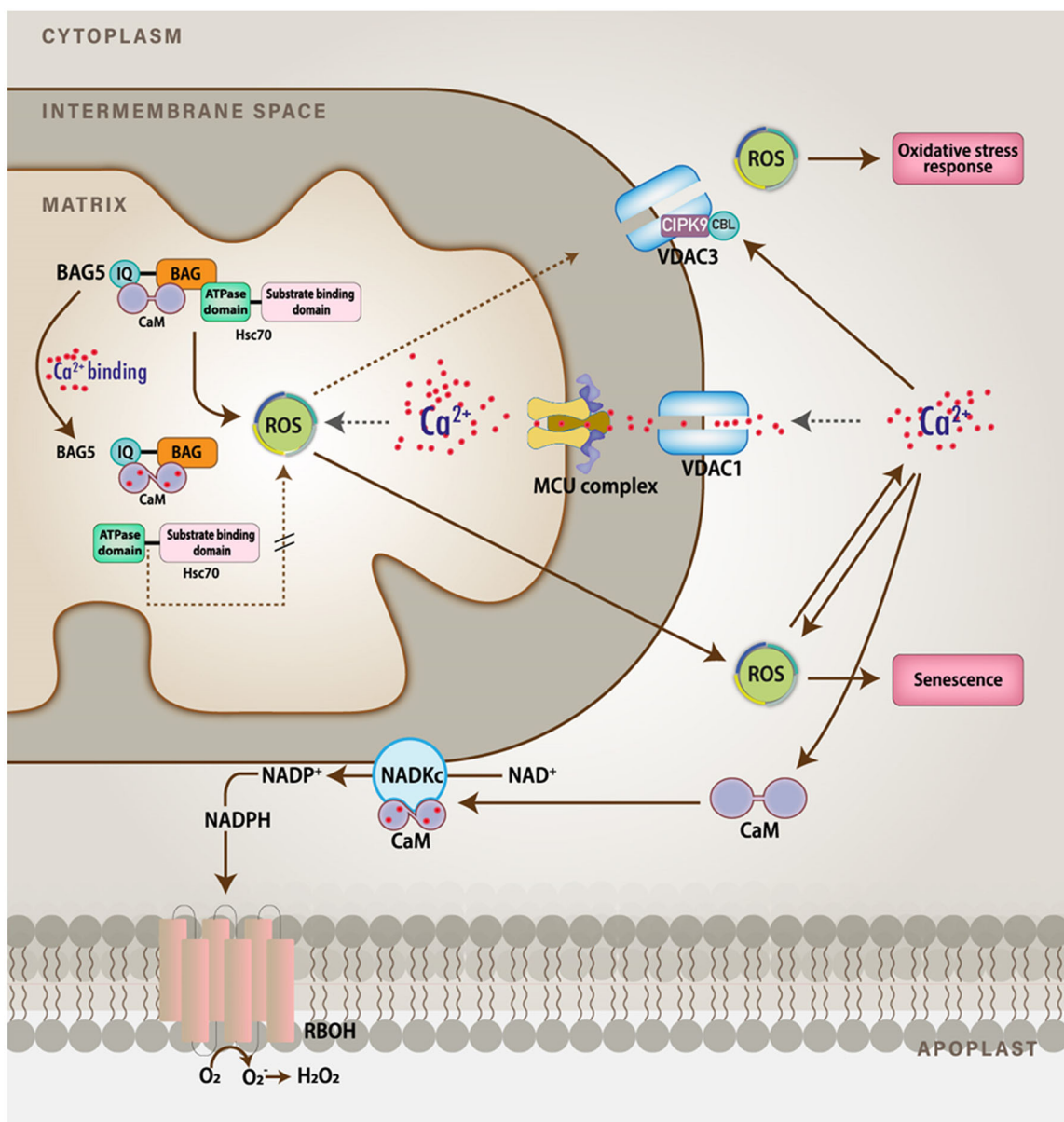
All ROS molecules potentially have signalling functions. Periods of high ROS accumulation in specific cellular compartments are required to effectively transmit signals, often related to energy

metabolism. Each cellular compartment contains low molecular weight antioxidants such as ascorbate and glutathione, antioxidant enzymes and ROS processing enzymes that limit ROS accumulation and signalling (Foyer and Noctor, 2009; Noctor and Foyer, 2016). It is likely that the concentrations of ascorbate and glutathione, the activities of antioxidant enzymes and abundance of ROS processing enzymes are regulated in the different intracellular compartments to allow appropriate regulation of the cell-to-cell ROS signalling wave. Many developmental, biotic, and abiotic signals are primarily perceived at the PM by receptor proteins. However, crosstalk between the signalling pathways is triggered by extracellular and intracellular receptors. This occurs, for example, in the co-activation of plant immune responses involving a branched innate immune response system. Plant PM-localized pattern-recognizing receptors (PRRs) detect conserved pathogen molecular patterns, activating pattern-triggered immunity (PTI). Some microbes are able to bypass PTI by transferring effector protein and molecules that trigger susceptibility in plant cells. In response to these effectors, a second branch of immune responses called effector triggered immunity (ETI) is activated (Jones & Dangl, 2006). Both PTI and ETI cause significant transcriptional reprogramming in plants. PTI and ETI are likely to result in stronger signalling than either system alone (Ngou et al., 2021). The crosstalk between Ca<sup>2+</sup> and ROS signalling pathways in response to biotic stresses and plant immune responses has been reviewed recently (Marcec and Tanaka, 2021; Marcec et al., 2019). Hence, the following discussion will focus largely on Ca<sup>2+</sup>-ROS interactions during abiotic stress responses.

In the following discussion, we summarize recent findings concerning the regulatory proteins involved in Ca<sup>2+</sup> and ROS interactions and we consider the fine-tuning of ROS and Ca<sup>2+</sup> waves in the establishment of SAA and SAR. The organellar signalling events that mediate this integration are discussed with particular reference to stress responses. Much of the information presented here and used to illustrate current concepts is shown in Figure 1 (Dell'Aglio et al., 2019; Godbole et al., 2003; Israelson et al., 2007; Kanwar et al., 2021; Li et al., 2016). While much of the information shown in Figure 1 has been derived from studies in Arabidopsis, similar findings are now being reported in other species, as outlined below. Our aim is to provide a current perspective of the intracellular regulators and Ca<sup>2+</sup> sensor proteins that are involved in Ca<sup>2+</sup> and ROS interactions during stress signalling.

## 2 | APOPLASTIC SIGNALLING

Stress-induced cell signalling requires the perception of stimuli, often by membrane-bound receptors, that trigger Ca<sup>2+</sup> fluxes and lead to the activation of RBOHs resulting in ROS accumulation in the apoplast that in turn triggers MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) signalling cascades and other similar pathways that regulate nuclear gene expression. These pathways are also triggered in response to biotic stress, for example in the sensing of pathogen-, microbe-, or damage-associated molecular patterns by PM-localized



**FIGURE 1** A hypothetical model for the regulation of cytosol-mitochondria communication via  $\text{Ca}^{2+}$ -ROS cross-talk. The VDACS and the MCU complex are gatekeepers that mediate cytosol-mitochondrial communication. In animals, VDACS such as VDAC1 function in  $\text{Ca}^{2+}$  transport (Israelson et al., 2007). Similarities in VDACS between plant and animal systems (Godbole et al., 2003) suggest that plant VDACS may also be involved in  $\text{Ca}^{2+}$  signalling. We propose that plant VDACS might regulate  $\text{Ca}^{2+}$  passage in mitochondria and thus influence mitochondrial  $\text{Ca}^{2+}$  levels and ROS homeostasis. However, excessive  $\text{Ca}^{2+}$  accumulation can trigger ROS accumulation in mitochondria and affect respiration. The  $\text{Ca}^{2+}$ -signalling kinase CIPK9 phosphorylates VDAC3. We suggest that this process allows transport of ROS from the mitochondria to the cytosol through a mechanism yet to be defined. In this way,  $\text{Ca}^{2+}$  signalling may modulate oxidative stress responses, as depicted by the dashed line. Mitochondrial ROS homeostasis is also controlled by the  $\text{Ca}^{2+}$ -mediated CaM-Hsc70-BAG5 complex and NADKc, which interacts with  $\text{Ca}^{2+}$  activated CaM to increase NADP(H) synthesis to drive ROS production by RBOH proteins (Dell’Aglia et al., 2019; Li et al., 2016). This diagram illustrates possible interactions between  $\text{Ca}^{2+}$  and ROS that may occur either directly (solid lines) or indirectly (dashed lines). BAG5, BCL-2-associated athanogene;  $\text{Ca}^{2+}$ , Calcium; CaM, calmodulin; CBL, calcineurin B-like proteins; CIPK9, CBL-interacting protein kinase 9; Hsc70, heat shock cognate protein 70; MCU, mitochondrial calcium uniporter; NADKc,  $\text{NAD}^+$  kinase; NADP(H), nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; VDAC, voltage dependent anion channel. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

receptors leading to the regulation of genes involved in pathogen defence (Nguyen et al., 2021). The apoplastic ROS burst is a fundamental response of plant cells to the perception of physical and chemical signals. The ROS burst involves the generation of

superoxide by the RBOHs, which are the plant equivalent of the mammalian NADPH (nicotinamide adenine dinucleotide phosphate) oxidases (NOX).  $\text{H}_2\text{O}_2$  is then generated from superoxide by the action of superoxide dismutases (SODs). A paradigm-shift in current

understanding occurred when it became clear that this response is intrinsic to the cell-to-cell transmission of long-distance cell-to-cell signals in plants, as well as plant to plant and microorganism to plant communication (Fichman et al., 2019, 2021; Mittler et al., 2011, 2022; Zandalinas, Fichman, Devireddy, et al., 2020; Zandalinas, Fichman, & Mittler, 2020; Zhu, 2016). Several RBOH forms, particularly RBOHD and RBOHF, were identified as essential sources of the apoplastic ROS generation that is essential for this process (Fichman and Mittler, 2020; Fichman et al., 2019, 2021; Mittler et al., 2022; Zandalinas, Fichman, Devireddy, et al., 2020; Zandalinas, Fichman, & Mittler, 2020; Miller et al., 2009). Similarly, the PM-associated leucine-rich-repeat receptor-like kinase (LRR-RLK) called HPCA1 (Wu et al., 2020 [also called CANNOT RESPOND TO DMBQ 1; CARD1]), which is an important receptor for extracellular H<sub>2</sub>O<sub>2</sub> (Fichman et al., 2022) and quinones (Laohavisit et al., 2020) was shown to be required for the propagation of cell-to-cell ROS signals (Fichman et al., 2022).

HPCA1 mediates the H<sub>2</sub>O<sub>2</sub>-induced activation of Ca<sup>2+</sup> channels in guard cells leading to stomatal closure (Wu et al., 2020). According to the model of Wu et al. (2020) the kinase activity of HPCA1 is low in the absence of H<sub>2</sub>O<sub>2</sub> so the Ca<sup>2+</sup> influx channel is closed. HPCA1 has two additional pairs of cysteine residues in the extracellular domain that are subject to covalent modification in the presence of H<sub>2</sub>O<sub>2</sub>, leading to autophosphorylation of the HPCA1 protein. The autophosphorylation of amino acid residues in the cytosolic domain facilitates gating of Ca<sup>2+</sup> channels, leading to an increase in cytosolic Ca<sup>2+</sup> levels. HPCA1 is required for systemic transmission of ROS and Ca<sup>2+</sup> signals (also termed the 'Ca<sup>2+</sup> wave') in all cell types but not for electric signals and related systemic membrane depolarization responses (Fichman et al., 2022). Moreover, the systemic Ca<sup>2+</sup> signals mediated by HPCA1 recruit the Ca<sup>2+</sup>-permeable channel called Mechanosensitive ion channel like 3 (MSL3). Some downstream components of the Ca<sup>2+</sup>-dependent signalling cascade are identified including components such as the calcineurin B-like Ca<sup>2+</sup> sensor (CBL4), the CBL-interacting protein kinase 26 (CIPK26), and the sucrose-non-fermenting-1-related protein kinase 2.6 (SnRK2.6, also termed 'open stomata 1', OST1; Fichman et al., 2022). The serine residues S343 and S347 on RBOHD are putative phosphorylation targets of OST1. In this way, HPCA1 links the accumulation of apoplastic H<sub>2</sub>O<sub>2</sub> to Ca<sup>2+</sup> signalling and the further activation of superoxide production by RBOHs in a positive feedback loop (Wu et al., 2020). HPCA1 is therefore a key component in coordinating the systemic cell-to-cell ROS and Ca<sup>2+</sup> signals that are required for plant resilience to stress.

While much of the above information has been derived from studies in Arabidopsis, RBOH-mediated apoplastic ROS generation has been shown to be important in systemic signalling leading to stress tolerance in crop species such as tomato. For example, the expression of SIRBOH1 is induced under elevated atmospheric CO<sub>2</sub> (eCO<sub>2</sub>) levels leading to increased H<sub>2</sub>O<sub>2</sub> accumulation in the vascular tissues of roots, stems and leaf petioles. eCO<sub>2</sub>-induced increases in salt tolerance were abolished in SIRBOH1-silenced plants, suggesting that salt tolerance was increased under eCO<sub>2</sub> in an apoplastic

ROS-dependent manner (Yi et al., 2015). Further work revealed that eCO<sub>2</sub> triggers apoplastic H<sub>2</sub>O<sub>2</sub>-dependent auxin production in tomato shoots, a process that facilitates systemic signalling that results in strigolactone biosynthesis in the roots and improved associations with arbuscular mycorrhizal fungi (AMF). This was accompanied by enhanced nutrient acquisition and carbon, nitrogen and phosphate cycling (Zhou et al., 2019). Thus, a RBOH-dependent-auxin-strigolactone systemic signalling cascade was involved in the regulation of eCO<sub>2</sub>-induced AMF symbiosis and phosphate uptake (Zhou et al., 2019). Moreover, systemic signalling via RBOH-mediated apoplastic ROS generation was shown to be important in the light signalling-dependent regulation of photosynthesis by the tomato shoot apex. A series of grafting experiments was used to demonstrate that information regarding red light perception at the shoot apex triggers phytochrome B-mediated auxin production leading to RBOH-mediated apoplastic ROS generation in systemic leaves to allow adaptation to the light environment (Guo, Wang, et al., 2016). Reciprocal grafting experiments were also used to investigate the role of apoplastic ROS signalling in the shoot/root crosstalk that controls the synthesis of jasmonates (JAs) in the defence responses of tomato plants to root knot nematodes (RKN; Wang, Zhang, et al., 2019). Grafting stem sections from mutants lacking GLR3.5 or the mutants deficient in RBOH1 attenuated resistance to RKN and abolished RKN-induced electrical signals, as well as associated ROS and JA accumulation in the upper stems and leaves. Similarly, silencing the tomato MPK1 or MPK2 abolished RKN-induced accumulation of JAs and associated resistance (Wang, Hu, et al., 2019). Such studies demonstrate the presence of systemic signalling systems in crop plants that integrate electrical, ROS and phytohormone signals to modulate plant defences in distal organs via cell-to-cell and root-shoot-root communication.

### 3 | SYMPLASTIC SIGNALLING

Plasmodesmata (PD) are required for the rapid transmission of RBOHD-generated ROS signals and the orchestration of cell-to-cell systemic communication that leads to SAA in response to high light stress (Fichman et al., 2021). PD are intercellular concentric pores that cross cell walls and mediate the local and systemic transport of signalling molecules to coordinate developmental and environmental responses. These pores have an outer specialized membranous domain that is a continuation of the PM and an inner desmotubule, which connects the endoplasmic reticulum (ER) of neighbouring cells. Symplasmic transport occurs through the cytoplasmic sleeve, which is the space between the PM and the desmotubule (Paniagua et al., 2021). The size of the PD pore is increased in response to RBOHD-generated apoplastic ROS accumulation in a manner that is dependent on PD-localized protein 1 (PDLP1) and PDLP5. In addition, PD amplify the systemic signal in each cell along the path of the systemic ROS wave, as well as participating in the establishment of local and systemic acclimation (Fichman et al., 2021). The regulation of PD is targeted by pathogenic effectors to facilitate



cell-to-cell movement (Li et al., 2021). Plant cells respond to the perception of pathogens by depositing callose in the paramural space around the PD to constrict the space available for transport (Wu et al., 2018).  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent activities, phytohormones and receptor-like kinases participate in the control of callose synthase activity, callose deposition and PD closure (Leijon et al., 2018). For example, the regulation of PD closure via flagellin 22 (flg22)-induced callose deposition involves the  $\text{Ca}^{2+}$  signalling component called CML41 (Calmodulin-like proteins). Cytosolic  $\text{Ca}^{2+}$ -dependent control of PD pores has been reported in response to pathogens (Tilsner et al., 2016; Xu et al., 2017).

The mechanisms by which RBOHD-generated ROS counteract such stress responses and so allow systemic signalling to occur are unknown. However, this regulation may involve substantial cellular reprogramming and protein re-localisation. PD also house clusters of receptor-like activities that may function together with PM receptor proteins to orchestrate developmental and stress signals. For example, the PM-localized Leucine Rich Repeat Receptor Like Kinases (LRR-RLKs) QSK1 (Qīān Shǒu kinase) and IMK2 (Inflorescence Meristem Kinase2) are relocated to PD intercellular pores in response to osmotic stress (Grison et al., 2019). Such findings indicate that the reorganization of RLKs to PD and other intracellular sites is an important feature of plant stress responses.

#### 4 | ORGANELLAR SIGNALLING SYSTEMS

Different types of second messengers are involved in the relay of stress signals in the cytosol (e.g., cyclic nucleotides and other metabolites), cell membranes (e.g., lipid-based second messengers), as well as within or between cellular compartments (metabolites) and between cells {ROS ( $\bullet\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ ) and volatile compounds}. Several signalling components and pathways have been identified but the mechanisms that integrate different signalling pathways to elicit appropriate responses to the various signals that occur simultaneously in distinct cellular compartments remain poorly understood (Jeandroz et al., 2013; Mazars et al., 2010; Smékalová et al., 2014; Xia et al., 2015; Xuan et al., 2020).

Signals arising from central energy metabolism (photosynthesis and respiration) are crucial for cell survival in changing environmental conditions. Superoxide and  $\text{H}_2\text{O}_2$  are produced by the photosynthetic and respiratory electron transport chains in a semiregulated manner (Foyer and Hanke, 2022). ROS, metabolite and associated signals operate in multiple overlapping retrograde pathways that are collectively often called 'organelle-to-nucleus', 'chloroplast-to-nucleus', or 'mitochondria-to-nucleus retrograde' signalling, act in a co-ordinated manner. For example, mitochondria-to-nucleus signalling, which involves two key transcription factors belonging to Arabidopsis NAC (NAM, ATAF and CUC) domain containing family: ANAC013 and ANAC017, is linked to chloroplast-to-nucleus signalling (Shapiguzov et al., 2019). The ANAC013 and ANAC017 transcription factors are released from the ER upon perception of appropriate signals and translocated to the nucleus, where they activate the

expression of a specific set of genes called mitochondrial dysfunction stimulon (MDS) genes that include the alternative oxidases, *SOT12* (*sulphotransferase12*) and ANAC013 (De Clercq et al., 2013). The enhanced expression of ANAC013 provides positive feedback regulation of the signalling pathway. In addition, SOT12 belongs to the group of MDS genes that overlap with the genes induced by the SAL1, 3'-phosphoadenosine 5'-phosphate (PAP) chloroplast retrograde signalling pathway (Van Aken, & Pogson, 2017).

Retrograde signalling networks are vital for organelle biogenesis and functions. They are particularly important in the adaptation to changing environmental cues such as light. Interestingly, the organelle to nucleus signalling pathways also incorporate the regulation of PD in 'organelle-nucleus-PD signalling (ONPS)'. Redox signals from chloroplasts can modify PD-mediated intercellular trafficking (Ganusova et al., 2020).

Perturbations in the energy-generating systems of the chloroplast serve as a marker by which the nucleus perceives environmental stress (Foyer and Hanke, 2022). Nuclear-encoded plastid proteins are differentially expressed in response to changes in photosynthetic energy metabolism. These include the light harvesting chlorophyll a/b binding proteins (LHCB) and the small subunit (SSU) of ribulose-1, 5-bisphate carboxylase/oxygenase (Mayfield & Taylor, 1984; Oelmüller & Mohr, 1986; Pogson et al., 2008). The activation of MAPK signalling pathways in response to abscisic acid (ABA)-induced  $\text{H}_2\text{O}_2$  generation might also involve chloroplast signals (Zhang et al., 2006) through the participation of a chloroplast CAS ( $\text{Ca}^{2+}$  sensor protein; Guo, Feng, et al., 2016). The *CAU1* gene, which encodes H4R3sme2 (for histone H4 Arg 3 with symmetric dimethylation)-type histone methylase protein arginine methyltransferase5/Shk1 binding protein1, regulates the expression of  $\text{Ca}^{2+}$  signalling genes such as CAS. H4R3sme2 binds to the CAS promoter and regulates the CAS chromatin's H4R3sme2-type histone methylation. As cytosolic  $\text{Ca}^{2+}$  levels increase, H4R3sme2 methylation is decreased, alleviating the repression of CAS expression in the control stomatal closure and drought tolerance (Fu et al., 2013, 2018). CAS-mediated cytosolic  $\text{Ca}^{2+}$  fluctuations activate MAPK signalling cascades. The 14-3-3w  $\text{Ca}^{2+}$  binding proteins function as protein scaffolds for the MKK4/MKK5-MPK3/MPK6 complex that participates in retrograde signalling pathways. For example, the ABI4 (ABA-insensitive 4) transcription factor can be phosphorylated by MPK3, MPK4, and MPK6, a posttranslational modification that leads to decreased expression of *LHCB* (light harvesting chlorophyll a/b binding) genes (Guo, Feng, et al., 2016). Such findings demonstrate that chloroplast-localised processes participate in cellular  $\text{Ca}^{2+}$  signalling events.

While the interplay between  $\text{Ca}^{2+}$  and ROS signals is likely to be conserved in unicellular organisms and multicellular plants, the molecular components involved in these interactions are likely to be different. For example, calredoxin (CRX), which is the only CML identified in *Chlamydomonas reinhardtii*, is a potential site of integration of ROS and  $\text{Ca}^{2+}$  signals. CRX are chloroplast-localised proteins that contain CaM domain, 4  $\text{Ca}^{2+}$  binding EF- hands and a TRX domain. CRX is involved in both redox and  $\text{Ca}^{2+}$  signalling in the chloroplasts (Hochmal et al., 2016). CRX interacts with chloroplast

2-Cys peroxiredoxin 1 (PRX1). The activity of the TRX domain is influenced by  $\text{Ca}^{2+}$  binding, which is crucial for the downstream binding and reduction of PRX1 in oxidative stress responses. ROS and  $\text{Ca}^{2+}$  signalling pathways are linked in the regulation of lipid accumulation in response to a combination of nitrogen limitation and oxidative stress in *Chlorella sorokiniana* (Liufu et al., 2023). The two RBOH isoforms in *Marchantia polymorpha* have conserved C-terminal phosphorylation sites, which may serve as sites of  $\text{Ca}^{2+}$ -dependent regulation (Bánfi et al., 2004; Kimura et al., 2020; Marchetti et al., 2021).

$\text{Ca}^{2+}$ -ROS crosstalk regulates metabolic and energetic functions in chloroplasts and mitochondria, as well as retrograde signalling. For example, the VDACs (voltage dependent anion channels) located on the outer mitochondrial membrane (OMM) and the MCU (mitochondrial  $\text{Ca}^{2+}$  uniporter) complex located on the inner mitochondrial membrane (IMM) are central gatekeepers in animal systems that may also fulfil similar roles in plants, particularly by enabling cytosol-mitochondrial communication. In Arabidopsis, six putative MCU orthologs have been identified. Of these MCU1, MCU2 and MCU3 are reported to mediate mitochondrial  $\text{Ca}^{2+}$  uptake (Ruberti et al., 2022). The MCU complex in mammalian systems contains the essential MCU regulator (EMRE) and three mitochondrial  $\text{Ca}^{2+}$  uptake (MICU) proteins with EF-hands that serve as regulatory components (Garg et al., 2021; Liu et al., 2020; Xing et al., 2019). While only the MICU1 protein was reported to have a conserved regulatory function in Arabidopsis (Wagner et al., 2015), MCU complex proteins are required for mitochondrial  $\text{Ca}^{2+}$  homeostasis (Ruberti et al., 2022; Zhang, Ma, Bao, et al., 2022). VDACs are the most abundant proteins present in the outer mitochondrial membranes (OMM) of all eukaryotes. Four VDAC isoforms have been identified in Arabidopsis and it has been suggested that they might regulate the fluxes of ions including  $\text{Ca}^{2+}$  (Ravi et al., 2021; Wagner et al., 2016). Interestingly, the VDAC1 proteins in mammals also reported to contain  $\text{Ca}^{2+}$  binding sites (Israelson et al., 2007).

The MCU complex and VDACs are considered to participate in two-way communication across the mitochondrial membrane via  $\text{Ca}^{2+}$  and ROS signals. The MCU complex and VDACs influence  $\text{Ca}^{2+}$  uptake and hence mitochondrial  $\text{Ca}^{2+}$  levels (Wagner et al., 2016). They also influence ROS homeostasis (Sanyal et al., 2020; Zhang et al., 2019) and contribute to  $\text{Ca}^{2+}$ -ROS crosstalk by interacting with either  $\text{Ca}^{2+}$  or ROS signalling components (Kanwar et al., 2021; Li et al., 2013; Wen et al., 2013). Mitochondrial  $\text{Ca}^{2+}$ -ROS crosstalk might be facilitated by the  $\text{Ca}^{2+}$  signalling kinase CIPK9 and VDAC3 in a phosphorylation-dependent manner. The MCU complex also participates in  $\text{Ca}^{2+}$  uptake at the inner mitochondrial membrane (Ruberti et al., 2022; Wagner et al., 2015; Wang and Teng, 2018). The MCU complex and VDACs act as ROS sensors during hypoxia and inflammation-mediated oxidative stress in animals (Dong et al., 2017). However, the precise functions of these systems in regulating ROS in plant mitochondria remains to be demonstrated.

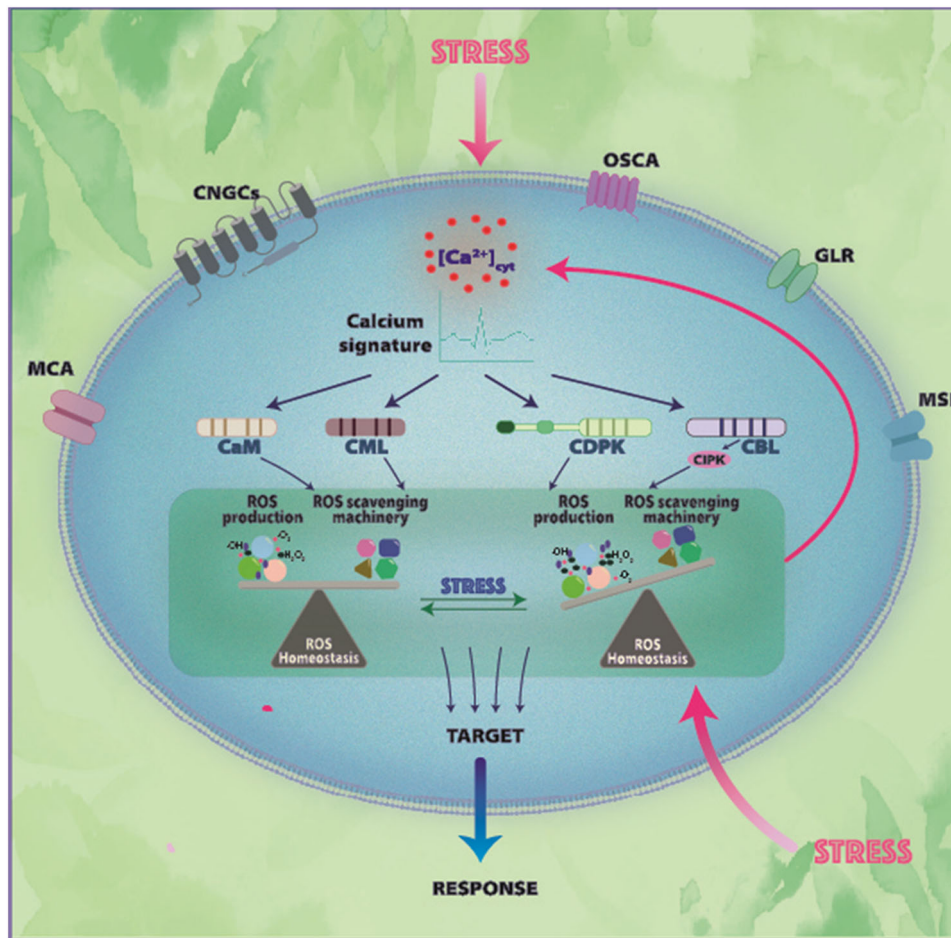
The Arabidopsis CaM-dependent NAD<sup>+</sup> kinase (NADKc) that is localised on the mitochondrial periphery participates in the

elicitor-induced oxidative burst, suggesting NADKc serves to link  $\text{Ca}^{2+}$  sensing through CaM and ROS generation by the RBOH proteins. The cytosolic NADP(H) pool drives RBOH activity to produce the ROS burst (Pugin et al., 1997). CaM recruitment to NADKc and the formation of the NADKc-CaM complex occurs as a result of a cytosolic  $\text{Ca}^{2+}$  influx, for example in response to flg22, which is a potent elicitor of plant immune responses that is recognised by the membrane bound leucine-rich repeat-receptor kinase called flg-Sensitive 2. The influx of cytosolic  $\text{Ca}^{2+}$  results in NADKc activation and NADPH production, as system that may serve to amplify the ROS burst in response to the elicitor (Dell'Aglio et al., 2019). While the role of mitochondria in these pathways is unknown, we may speculate that NADKc-CaM interactions may activate additional mitochondrial targets or signalling processes that perturb mitochondrial homeostasis enhancing ROS production by the respiratory electron transport chain. These signals may eventually lead to programmed cell death (PCD) or fine-tuning of other redox signalling pathways (Sies et al., 2017) as illustrated in Figure 1, which incorporates current concepts regarding ROS-  $\text{Ca}^{2+}$  crosstalk. However, many components of this system remain to be identified and the processes must be defined more clearly in order for the mechanistic elements of the crosstalk to be fully understood.

Although the nucleus has never been considered to be a source of ROS signals, it is clear that oxidation of the nucleus is an early response to environmental stresses such as heat (Babbar et al., 2021) and inhibitors of chloroplast and mitochondrial functions (Karpinska et al., 2017). The nucleus serves as a source of  $\text{Ca}^{2+}$  signals in plant-microbe interactions. For example, the nuclear-membrane localised potassium-permeable channel called DMI1 (does not make infections1) and the type IIA  $\text{Ca}^{2+}$ -ATPase isoforms (SERCA-type  $\text{Ca}^{2+}$  ATPase, ECA) called MCA8 (mid1-complementing activity 8) are required for the  $\text{Ca}^{2+}$  oscillations underpinning symbiosis in *Medicago truncatula* (Charpentier et al., 2016). Nuclear and perinuclear  $\text{Ca}^{2+}$  oscillations were induced by Nod (nodulation) factors and Myc (mycorrhizal signalling) factors that are released by rhizobia bacteria and arbuscular mycorrhizal fungi, respectively. The interface between the DMI1 and RCK (regulator of conductance of K<sup>+</sup>) domains forms a locked channel (Liu et al., 2022).

## 5 | ROLE OF $\text{Ca}^{2+}$ SENSOR PROTEINS IN CROSSTALK WITH $\text{Ca}^{2+}$ AND ROS DURING ABIOTIC STRESS

An increase in intracellular  $\text{Ca}^{2+}$  is one of the earliest signalling events that occurs in response to a wide range of abiotic stresses (Xu et al., 2022). The above discussion has highlighted the integration and close co-operation  $\text{Ca}^{2+}$  and ROS signalling that is mediated by components such as  $\text{Ca}^{2+}$ -induced ROS production (CIRP) and ROS-induced  $\text{Ca}^{2+}$ -release (RICR) (Gilroy et al., 2014). Crucially, the RBOH proteins are synergistically activated by the binding of  $\text{Ca}^{2+}$  to EF-hand motifs as well as  $\text{Ca}^{2+}$ -dependent phosphorylation (Gilroy



**FIGURE 2** A hypothetical model of calcium-sensing proteins involved in  $\text{Ca}^{2+}$ -ROS crosstalk. Abiotic stresses such as heat, salinity, drought, and cold stress induce increases in cytosolic  $\text{Ca}^{2+}$  levels, each generating a  $\text{Ca}^{2+}$  signature that is decoded and translated into a biological signal by  $\text{Ca}^{2+}$  sensor proteins (CaMs, CMLs, CDPKs and CBLs) that interact with different target proteins to elicit an appropriate response. These  $\text{Ca}^{2+}$  signalling pathways are coordinated with different signalling pathways, including ROS signalling. Abiotic stresses induce oxidative signalling through increased ROS production, which in turn might activate  $\text{Ca}^{2+}$  signalling pathways in plants.  $\text{Ca}^{2+}$  signalling can activate apoplastic ROS production leading to systemic signalling as illustrated in Figure 3. Apoplastic ROS production can further increase cellular  $\text{Ca}^{2+}$  levels that activates the antioxidant and ROS-processing systems to decrease ROS within the cytoplasm. In this way, ROS and  $\text{Ca}^{2+}$  interplay might govern plant responses to abiotic stress. [ $\text{Ca}^{2+}$ ]<sub>cyt</sub>, cytosolic calcium; CaM, Calmodulin; CBL, calcineurin B-like proteins; CDPK,  $\text{Ca}^{2+}$ -dependent protein kinases; CIPK, CBL-interacting protein kinase; CML, Calmodulin-like proteins; CNGCs, cyclic nucleotide gated channels; GLR, glutamate receptor-like;  $\text{H}_2\text{O}_2$ , hydrogen peroxide; MCA, mid1-complementing activity; MSL, mechanosensitive ion channel like; OSCA, hyperosmolality-gated calcium permeable channel; Rboh, respiratory burst oxidase homolog; ROS, reactive oxygen species;  $\bullet\text{O}_2^-$ , superoxide anion;  $\bullet\text{OH}$ , hydroxyl radical. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

et al., 2014; Kimura et al., 2012). Similarly, the expression of antioxidant and redox proteins is regulated through  $\text{Ca}^{2+}$ -dependent pathways (Siddiqui et al., 2020; Xue et al., 2018; Yang, Gao, et al., 2022) under stress conditions. The ROS-dependent activation of a wide variety of inwardly-, outwardly-rectifying, and voltage-independent groups of ion channels is crucial to cell signalling that underpins growth as well as defence processes (Demidchik, 2018). The CNGCs (cyclic nucleotide gated channels) and mechanosensitive ion channels may function as ROS ( $\text{H}_2\text{O}_2$ )-activated  $\text{Ca}^{2+}$  channels that mediate  $\text{Ca}^{2+}$ -influxes in such responses (Fichman et al., 2022; Wang et al., 2013). Some components of the  $\text{Ca}^{2+}$  sensing machinery may therefore function in  $\text{Ca}^{2+}$ -ROS crosstalk.

The  $\text{Ca}^{2+}$  sensors and responders such as Calmodulin (CaM), CMLs, calcineurin B-like proteins (CBLs),  $\text{Ca}^{2+}$ -dependent protein kinases (CPKs/CDPKs) and  $\text{Ca}^{2+}$  relay/responders are central to the regulation of ROS production in response to stress, as illustrated in Figure 2 (Li et al., 2008). They contain a common structural motif with conserved helix-loop-helix structure (EF-hand) that binds to  $\text{Ca}^{2+}$  (DeFalco et al., 2010). These proteins are grouped into sensor-relays (CaMs, CMLs, CBLs) and sensor-responder (CDPKs) systems because of their catalytic activity. There are 34 CDPKs, 7 CaM, 50 CMLs and 10 CBLs in Arabidopsis (Kolukisaoglu et al., 2004; McCormack & Braam, 2003; McCormack et al., 2005; Shi et al., 2018).  $\text{Ca}^{2+}$  sensor proteins are crucial components of stress signalling pathways



(Ma et al., 2020; McCormack et al., 2005; Reddy et al., 2011; Singh et al., 2018).

## 6 | CDPKS: A SENSOR-RESPONDER SYSTEM FOR $\text{Ca}^{2+}$ AND ROS CROSSTALK DURING ABIOTIC STRESS

A raft of  $\text{Ca}^{2+}$  sensors including the CDPK family of  $\text{Ca}^{2+}$  binding regulatory protein kinases decode  $\text{Ca}^{2+}$  signals in each cellular compartment. The CDPKs fulfil a diverse range of essential regulatory functions, including the differential expression of antioxidant enzymes in response to a range of abiotic stresses. For example, *LeCDPK1* (tomato), 12 CDPKs (wheat), *BnaCDPK6* and *BnaCDPK14* (rapeseed) are required for activation of antioxidant defences in response to wounding, oxidative stress and drought (Ahmadi et al., 2022; Chico et al., 2002; Li et al., 2008).  $\text{Ca}^{2+}$  sensing proteins and antioxidant enzymes were also prominent in the transcript signature of peanut plants subjected to drought stress (Thoppurathu et al., 2022), winter rapeseed subjected to cold stress (Wei et al., 2022) and rice in response to salinity (Arefian et al., 2022). The CDPK/CPK network functions together with the RBOH proteins to regulate ROS homeostasis and drive stress signalling (Suzuki et al., 2011). Some examples of this type of regulation include the CPK5-dependent phosphorylation of RBOHD that activates ROS production (Dubiella et al., 2013), the CPK8-dependent phosphorylation of CAT3 (catalase) at S261 that regulates ABA signalling in stomatal guard cells (Zou et al., 2015) and the CDPK10-dependent phosphorylation of the *Poncirus trifoliata* PtrAPX (ascorbate peroxidase) that regulates drought stress responses (Meng et al., 2020). Additionally, the *Brassica napus* CPK2 interacts with RBOHD to regulate ROS production and PCD (Wang et al., 2018). Moreover, phosphorylation of the tomato APX2 protein by CPK28 at threonine residues T59 and T164 enhances the activity of the enzyme in response to heat stress (Hu et al., 2021). The intensity and systemic spread of the ROS wave is influenced by the levels of antioxidants such as APX (Fichman et al., 2019). This involves a  $\text{Ca}^{2+}$  signalling component that regulates RBOH phosphorylation in most reports. Moreover, redox modifications of Cysteine32 in the APX protein leading to S-nitrosylation and S-sulfhydration also increases the catalytic activity of the enzyme (Aroca et al., 2015; Yang et al., 2015). Therefore, the posttranslational modification of enzymes such as APX plays an important role in ROS- $\text{Ca}^{2+}$  interactions.

The  $\text{Ca}^{2+}$ -dependent phosphorylation of the potato RBOH enzymes occurs at the S82 and S97 residues at the N-terminus of the protein. These sites are phosphorylated by CDPK4 and CDPK5 in a  $\text{Ca}^{2+}$ -dependent manner (Kobayashi et al., 2007). StCDPK5-mediated ROS generation is important in plant defence against potato blight pathogens (Kobayashi et al., 2012). The wheat RBOH called TaNOX7 was shown to interact with CDPK13 in luciferase complementation, BiFC (Bimolecular fluorescence complementation), and co-immunoprecipitation assays (Hu et al., 2020). This interaction regulated ROS production, drought tolerance and fertility in wheat

(Hu et al., 2020). A genome-wide characterization of *Dendrobium officinale* revealed 24 genes encoding CDPKs. Of these, CDPK9 and CDPK20 were shown to interact with RBOHD and RBOHH in a yeast two-hybrid assay system (Yang, Chen, et al., 2022). Further studies have revealed information on other components involved in the ROS or  $\text{Ca}^{2+}$  signalling systems but not ROS- $\text{Ca}^{2+}$  interactions. For example, the overexpression of *CPK4* in rice conferred salt and drought tolerance (Campo et al., 2014). Similarly, a drought tolerant canola (*Brassica napus*) cultivar had higher levels of *BnaCDPK6* and *BnaCDPK14* transcripts than a drought sensitive variety. Antioxidant enzymes such as catalase (CAT) and SOD were highly expressed in the drought tolerant variety, suggesting that CDPK activates antioxidant defences in response to drought in *Brassica napus* (Ahmadi et al., 2022). The apple CPK1 was shown to regulate  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  accumulation through the modulation of antioxidant enzyme activities in relation to cold tolerance in *Nicotiana benthamiana* (Dong et al., 2020). Hence, CDPKs can regulate both ROS production by RBOH enzymes and the extent of ROS accumulation through the control of antioxidant enzymes.

## 7 | CAM AND CML CONNECT $\text{Ca}^{2+}$ AND ROS SIGNALLING IN RESPONSE TO ABIOTIC STRESS

CaM and CMLs are  $\text{Ca}^{2+}$  sensors that ensure tight coupling of  $\text{Ca}^{2+}$  and ROS signalling pathways in response to abiotic stress through the mediation of protein kinase and phosphatase activities (Zeng et al., 2015). For example, CaMK [(CaM-dependent protein kinase); also known in CCaMK ( $\text{Ca}^{2+}$ /CaM-dependent protein kinase) in maize] regulates brassinosteroids (BR)-induced antioxidant defence mechanisms. BR activates two positive feedback loop mechanisms involving  $[\text{Ca}^{2+}]_{\text{cyt}}$  that regulate cellular  $\text{H}_2\text{O}_2$  levels in maize leaves. In this system, ZmCCaMK is activated by BR to increase intracellular  $\text{Ca}^{2+}$  accumulation, which in turn regulates  $\text{H}_2\text{O}_2$  production in the apoplast by the RBOH enzymes. The activation of antioxidant enzymes such as APX and SOD thereafter limits intracellular ROS accumulation (Yan et al., 2015). Similarly, the rice OsCam1-1 protein is important in the regulation of salt tolerance by the modulation of antioxidant enzymes (Chaicherdsakul et al., 2017). The heterologous expression of the cyclophilin OsCYP2 was found to enhance tolerance to several abiotic stresses in a similar manner (Kumari et al., 2009). Exposure to abiotic stress and auxin signalling trigger OsCYP2 interactions with CaM to initiate defence responses (Roy et al., 2022). Peroxidases (POD) may serve as a node for CaM-mediated  $\text{Ca}^{2+}$ -ROS crosstalk. For example, a  $\text{Ca}^{2+}$ -dependent POD called *Euphorbia characias* peroxidase (ELP) is activated by CaM (Mura et al., 2005). CaM was linked to  $\text{H}_2\text{O}_2$  signalling in *Panax ginseng* in a pathway that involved ABA-induced increases in antioxidant defence. Blocking CaM functions by the application of antagonists or the addition of ROS scavengers prevented the expression of *PgCaM* and abolished the increased expression of antioxidants. The expression of potato CaM2 in tobacco (Raina et al., 2021) and the *Eleusine coracana*

CaM in Arabidopsis (Jamra et al., 2021) decreased intracellular ROS accumulation and enhanced drought tolerance. Such CaM-mediated regulation may operate in the control of plant development as well as plant responses to a wide range of abiotic and biotic stresses (Parvin et al., 2012). For example,  $\text{Ca}^{2+}$  and CaM synchronise ROS production in the formation of adventitious roots (Niu et al., 2017).  $\text{Ca}^{2+}$  acts together with nitric oxide (NO) to promote adventitious root formation and regulate antioxidant functions in response to osmotic stress (Niu et al., 2017).

ABA-induced ROS production is fine-tuned by the  $\text{Ca}^{2+}$  sensor CML20 in the regulation of stomatal closure in response to water stress. APX2 expression and intracellular ROS accumulation are decreased in an ABA-independent manner, suggesting that CML20 serves as a negative regulator of ABA-induced ROS production in guard cells (Wu et al., 2017). This system has been analysed using either 'gain-of-function' approaches (OsCML4, OsDSR1 (Drought stress response-1), *Solanum habrochaites* CML44) or the ectopic expression of CMLs (*Millettia pinnata* CML40 in Arabidopsis, *Medicago sativa* CML46 in tobacco) (Du et al., 2021; Munir et al., 2016; Yin et al., 2015, 2017; Zhang et al., 2021). Together, the data obtained from these studies demonstrates that CML homologs are also positive regulators of stress tolerance via regulation of antioxidant systems. Similarly, the overexpression of the CaM binding transcription activator (CAMTA) in chickpea resulted in the enhanced expression of antioxidant enzymes such as APX, CAT, GST and so forth. and decreased intracellular  $\text{H}_2\text{O}_2$  accumulation in response to drought and salt stress (Meenakshi et al., 2022). The expression of GmCAMTA genes was found to be decreased by  $\text{H}_2\text{O}_2$  treatment in *Glycine max* (Wang et al., 2014).

Crosstalk between ROS and MAPK signalling pathways is central to plant stress responses (Jalmi and Sinha, 2015; Nakagami et al., 2006; Son et al., 2011, 2013). The binding of CaM to MPK8 in a  $\text{Ca}^{2+}$  dependent manner is required for enzyme activation, which in turn, controls the expression of RBOHD genes, thereby negatively regulating apoplastic-ROS production in Arabidopsis in response to mechanical wounding (Takahashi et al., 2011).  $\text{Ca}^{2+}$  and CaM signalling pathways are also linked to ROS signalling in plant responses to heat stress. For example, Arabidopsis and yeast mutants that are defective in phosphomannomutase (PMM), an enzyme in the ascorbate synthesis pathway (Wheeler et al., 1998) show enhanced sensitivity to heat stress because of a lower antioxidant capacity.  $\text{Ca}^{2+}$  pretreatment was found to activate the ascorbate synthesis pathway (Ding et al., 2014). CML10 interacts with PPM to regulate ascorbate synthesis and  $\text{Ca}^{2+}$  mediated ROS signalling in Arabidopsis (Cho et al., 2016). These signalling pathways are also important in the regulation of stress-induced PCD, a process that can be activated by decreases in the expression of antioxidant enzymes. For example, the ROS regulated CaM binding protein called AtBAG5 (BCL-2-associated anthanogene) is important in the regulation of PCD in Arabidopsis (Kang et al., 2005). Group I members of the BAG protein family (viz., SIBAG4, SIBAG7 and SIBAG9) in tomato have an isoleucine glutamine (IQ) CaM binding motif. These proteins are differentially expressed in response to a wide range of abiotic stress

conditions. The heterologous expression of tomato SIBAG9 in Arabidopsis decreased the expression and activities of Fe-SOD1 (FSD1) and CAT1, resulting in intracellular  $\text{H}_2\text{O}_2$  accumulation and increased stress sensitivity (Jiang et al., 2022).

The  $\text{Ca}^{2+}$  signalling network also interacts with the Hsc70 (heat shock cognate protein 70) chaperone system via CaM (apo- or holo) to regulate senescence in Arabidopsis (Li et al., 2016). Hsc70 interacts with AtBAG5 only at low  $\text{Ca}^{2+}$  concentrations to promotes ROS generation. The AtBAG5 protein binds to Hsc70 ( $K_d = 0.24 \mu\text{M}$ ) and apo-CaM ( $0.17 \mu\text{M}$ ) in absence of  $\text{Ca}^{2+}$ . In presence of  $\text{Ca}^{2+}$ , the CaM and BAG5 interactions show a higher binding affinity ( $3.36 \mu\text{M}$ ) than the BAG5/holo-CaM complex with Hsc70 ( $K_d = 53.3 \mu\text{M}$ ). The binding affinity of bag5-Hsc70 complex is greatly reduced for holo-CaM ( $21.2 \mu\text{M}$ ) in presence of  $\text{Ca}^{2+}$ , compared to the apo-CaM ( $0.39 \mu\text{M}$ ). High mitochondrial  $\text{Ca}^{2+}$  concentrations alter CaM characteristics and limit the ability of the Hsc70 protein to bind to AtBAG5. The disruption of AtBAG5 binding to Hsc70, frees Hsc70 from the CaM-Hsc70-BAG5 complex in the mitochondria, reducing ROS levels and vice versa (Fu et al., 2017; Li et al., 2016).

## 8 | THE CBL-CIPK MODULE AS A NODE CONNECTING ROS AND $\text{Ca}^{2+}$ SIGNALLING EVENTS

CBLs are  $\text{Ca}^{2+}$  sensors that work together with interacting CIPKs to regulate a multitude of signalling processes (Luan, 2009; Pandey, 2008; Sanyal et al., 2015; Tang et al., 2020). The CBL-interacting protein kinases (CIPKs) are differentially expressed in *rbohB* mutants (Shi et al., 2020). The  $\text{Ca}^{2+}$ -dependent activation of RBOH enzymes by protein phosphorylation is regulated by the CBL-CIPK module in a  $\text{Ca}^{2+}$  dependent manner. For example, CIPK26 was shown to interact with the N-terminal region of AtRBOHF, which contains the EF-hands (Kimura et al., 2013). The RBOHF-mediated production of ROS increases together with increases in the  $[\text{Ca}^{2+}]_{\text{cyt}}$  concentration. ROS generation is strongly enhanced by co-expression of CBL1 (or CBL9) with CIPK26. Together with CIPK26, CBL1 and CBL9 were found to enhance ROS production through the synergistic activation of RBOHF. This was facilitated by  $\text{Ca}^{2+}$  binding to the EF-hands and  $\text{Ca}^{2+}$ -induced phosphorylation by CBL1 or the CBL9-CIPK26 complex in HEK293T cells (Drerup et al., 2013). It is possible that  $\text{Ca}^{2+}$  binding at the N-terminal causes structural changes that activate and recruit the CBL-CIPK module. Increases in  $[\text{Ca}^{2+}]_{\text{cyt}}$  that occur in response to hormones such as ABA (Hua et al., 2012), can also result in the activation of the CBL-CIPK26 module, thus enhancing RBOHF activity and ROS production that leads to stomatal closure.

Drought stress induces the expression of various members of the rice CIPK family, including CIPK11 (Xiang et al., 2007). For example, overexpression of CIPK11 increased the extent of ROS accumulation in Arabidopsis under drought stress (Ma, Cao, et al., 2019). CIPK11 is reported to phosphorylate RBOHF at the N-terminal, functioning as an alternative pathway to CIPK26 in the regulation of ROS

production in *Arabidopsis* (Han et al., 2019). In contrast, however, the expression of CIPK11 from the halophyte *Nitraria tangutorum* in *Arabidopsis thaliana* conferred salt tolerance, a finding was linked to increased proline accumulation and lower ROS levels (Lu et al., 2021). CIPK11 may therefore serve different functions in response to a range of environmental cues.

The salt tolerance pathway, also known as the salt overly sensitive (SOS) pathway, includes SOS2 (CIPK24), a protein kinase that is activated by interactions with SOS3 or CBL4 in a  $\text{Ca}^{2+}$ -dependent manner. The interaction between SOS2 and NDPK2 (nucleotide diphosphate kinase 2) in *Arabidopsis* modulates CAT2 and CAT3 activities and decreases ROS accumulation in plants exposed to salt stress (Verslues et al., 2007). Similarly, the wheat TaCIPK29 protein functions as a positive regulator of salt stress, regulating ROS homeostasis in transgenic tobacco lines (Deng, Hu, et al., 2013).

The *Brachypodium distachyon* CIPK31 protein activates ROS scavenging systems in response to abiotic stress. CIPK31-expressing tobacco plants had lower malondialdehyde (MDA) and  $\text{H}_2\text{O}_2$  levels with increased CAT, POD, SOD and GST activities (Luo et al., 2017). Moreover, the BdCIPK31 protein functions as a positive regulator of cold and osmotic stress tolerance (Luo et al., 2018). The expression of an apple CIPK MdSOS2L1 (MdCIPK24-like1) protein in transgenic apple and tomato lines conferred salt tolerance through lower ROS accumulation. MdSOS2L1 was found to interact with *MdCBL1*, *MdCBL4* and *MdCBL10* in yeast two-hybrid systems. In addition, proteome analysis and LC/MS showed the involvement of antioxidant pathway genes in the enhanced salt tolerance of the transgenic lines overexpressing MdSOS2L1 (Hu et al., 2016).

CIPK2 interacts with TaCBL1, 2, 3 and 4 and CBL1, 4, 5 and 8 in wheat and grapevines, respectively (Wang et al., 2016; Xu et al., 2020). Overexpression of CIPK2 in tobacco resulted in lower levels of ROS accumulation and higher CAT and SOD activities, as well as enhanced drought tolerance (Wang et al., 2016). Moreover, VaCIPK2 overexpression in *Arabidopsis* decreased ABA-induced ROS accumulation and altered ABA-dependent stomatal movements (Xu et al., 2020). Similarly, the *Capsicum annuum* L. CIPK3 regulates drought responses through the activation of antioxidant defences and the methyl jasmonate pathway. Overexpression of CaCIPK3 in tomato leads to drought tolerance through increases in CaSOD, CaPOD and CaCAT activities (Ma, Li, et al., 2021). Increases in ROS accumulation induce the expression of the  $\text{Ca}^{2+}$  sensor CBL10, which functions in abiotic stress-induced  $\text{Ca}^{2+}$  signalling pathways. For example,  $\text{Ca}^{2+}$ , CBL10, BR and ROS are linked in drought stress responses (Kang and Nam, 2016).

MdCIPK13 regulates salt tolerance in apples by phosphorylating the sucrose transporter MdsUT2.2, a process that enhances transport activity and promotes sugar-associated osmo-regulatory compound accumulation (Ma, Sun, et al., 2019). CaCIPK13 knock-down lines show enhanced cold sensitivity with increased  $\text{H}_2\text{O}_2$  accumulation and reduced CAT, POD and SOD activity during cold stress in pepper. CaCIPK13 interacts with CBL1, 6, 7 and 8 (Ma et al., 2022). Such interactions suggest that  $\text{Ca}^{2+}$  and ROS interact to enhance abiotic stress tolerance through the  $\text{Ca}^{2+}$ -CBL-CIPK complex-dependent regulation of ROS levels.

ROS and  $\text{Ca}^{2+}$  might also synergistically regulate salt stress responses. For example, the synthetic strigolactone (SL) analogue GR24 mediates salt tolerance in cucumber through the regulation of  $\text{Ca}^{2+}$  and ROS. SLs increase antioxidant enzyme activity under salt stress (Ling et al., 2020; Zheng et al., 2021). Exposure to salt stress increases the expression of RBOHA-F, together with intracellular  $\text{Ca}^{2+}$  contents and the levels of CaMKs, CaM, CDPKs and  $\text{Ca}^{2+}$ -ATPase in the cucumber seedlings and leaves (Jakubowska et al., 2015; Niu et al., 2018) and all these parameters further increased with the addition of SL analogue GR24. The addition of EGTA,  $\text{LaCl}_3$ , DPI or DMTU reversed the effects of GR24 on key parameters such as the levels of ROS and  $\text{Ca}^{2+}$ , antioxidant enzyme activities, as well as CaMKs, CaM, CDPKs and  $\text{Ca}^{2+}$ -ATPase. Together, these findings indicate that  $\text{Ca}^{2+}$  and ROS signalling pathways are interconnected in a feedback loop mechanism (Zhang, Ma, Zhang, et al., 2022). *Arabidopsis* mutants lacking functional CBL9 and CIPK23 proteins have lower levels of *APX1*, *CAT1*, *CAT3* transcripts and increased  $\text{H}_2\text{O}_2$  levels (Nath et al., 2019).

GhCIPK6 is required for salt tolerance in *Gossypium hirsutum* (He et al., 2013) and mediates crosstalk between  $\text{Ca}^{2+}$ , ROS scavenging and MAPK pathways (Su et al., 2020). For example, SiCIPK6 phosphorylates SiRd2 (responsive to desiccation 2). Overexpression of Rd2 resulted in lower ROS accumulation through the mediation of SiCIPK6. This protein regulates the oxidative burst through activation of RBOHB in response to biotic stress (de la Torre et al., 2013). These findings suggest that *SiRd2* functions as a negative regulator of the CBL10-CIPK6-RBOHB interactions that control ROS production (Gutiérrez-Beltrán et al., 2017). These studies highlight the importance of CIPK-dependent protein phosphorylation in response to ROS signalling. TaCIPK14 is an abiotic stress responsive gene, that regulates a wide range of stress responses. The expression of TaCIPK14 in tobacco cells exposed to cold and salt stress increased CAT activity and decreased  $\text{H}_2\text{O}_2$  levels. Increases in ROS levels trigger the expression of *CIPK14*, which in turn regulates the activities of antioxidant enzymes (Deng, Zhou, et al., 2013). Moreover, both CIPK6 and CIPK14 negatively regulates of plant immune response to *Pseudomonas syringae* in *Arabidopsis* (Ma, Chen, et al., 2021; Sardar et al., 2017).

The discovery of CBLs and CIPKs, which belong to the sensor-relay protein family, revolutionised concepts concerning plant  $\text{Ca}^{2+}$  signalling. CBLs and CIPKs directly regulate ROS-producing enzymes. They exert positive and negative controls over RBOHs activity and regulate the expression of ROS regulating genes. They are also involved in other signalling cascades with stress regulators such as ABA, BR, JA and SL. In association with CIPK, they fine-tune downstream responses. For example, CBL1 and CBL9 interact with CIPK26 to regulate RBOHF and ROS signalling. In association with CIPK23, CBL1 and CBL9, they also regulate AKT1 (inward-rectifying  $\text{K}^+$  channels) and SLAC1, which regulates ion homeostasis in *Arabidopsis* (Lee et al., 2009; Xu et al., 2006). The CBLs and CIPKs fulfil indispensable functions in cell-to-cell communication and ROS wave propagation. A list of  $\text{Ca}^{2+}$  sensing proteins that are crucial to  $\text{Ca}^{2+}$ -ROS crosstalk is provided in Table 1.

**TABLE 1** A list of calcium sensing and signalling proteins that have been shown to be involved in ROS/Ca<sup>2+</sup> crosstalk in abiotic stress responses.

Calcium sensing proteins	Identified homolog	Organism	Mechanism of regulating ROS pathways	Mode of detection	Functional relevance
Ca <sup>2+</sup> -dependent protein kinases (CDPK/CPK)	CDPK1	Tomato	Differential expression by ROS	Northern blot analysis	Plant defence to wound and biotic stress [1]
	CDPK6, CDPK12	Rapeseed	Differential gene expression and changed antioxidant enzymes activity	Real time PCR	Response to drought stress [2]
	CDPK family homologs	Wheat	Differential gene expression due to H <sub>2</sub> O <sub>2</sub> and abiotic stress conditions	-	Response to biotic and abiotic stress [3]
	CDPKs/CIPKs family homologs	Rice	Differential expression in <i>rbohB</i> mutant	Transcriptome profile analysis	Role of RbohB in drought tolerance in rice [4]
	CPK8	Arabidopsis	Phosphorylate CAT3 at Ser261	In-vitro and in-planta protein phosphorylation	Regulates ABA mediated drought responses and ROS homeostasis [5]
	CPK2	Rapeseed	Interacts with RbohD	Mating-based split ubiquitin assay and BIFC	Putative role in ROS and cell death regulation [6]
	CDPK10	<i>Poncirus trifoliata</i>	Interacts and phosphorylates APX, its overexpression shows reduced ROS	Yeast-two-hybrid and in-vitro kinase assay	Modulates ROS homeostasis and functions during drought tolerance [7]
	CDPK 4, CDPK5	Potato	Phosphorylates RbohB at Ser82, Ser97 and induces ROS production	Mass spectrometry analysis and ectopic expression in <i>Nicotiana benthamiana</i>	Regulates oxidative burst [8]
	CDPK13	Wheat	Interacts with NOX7	Luciferase complementation, BIFC and co-immunoprecipitation assay	Regulates ROS homeostasis during wheat development and fertility as well as drought tolerance [9]
	CDPK9, CDPK20	<i>Dendrobium officinale</i>	Interacts with RbohD and RbohH	Yeast-two-hybrid	Regulates ROS production and stomatal closure in response to SA/JA [10]
	CDPK6, CDPK14	Rapeseed	Induction of ROS scavenging machinery	Antioxidant enzymatic assays	Drought resistance [2]
	CPK1	Apple	Reduced MDA and ROS level, increased antioxidant enzyme activities	Ectopic expression in <i>N. benthamiana</i>	Cold tolerance response in plants [11]
Calmodulin (CaM) and Calmodulin-like proteins (CMLs)	CCaMK	Maize	Induced expression and activity in response to BR, regulates BR-induced antioxidant defence	Transient expression in maize protoplast and complementation in rice	Functions in BR regulation Ca <sup>2+</sup> and ROS loop [12]
	CaM1	Rice	Increased antioxidant enzyme activity and reduced ROS content	Transgenic overexpression in rice	Salt tolerance in rice [13]
	CaM	Rice	Interacts with CYP2	-	Drought and salt tolerance in rice [14]

(Continues)



TABLE 1 (Continued)

Calcium sensing proteins	Identified homolog	Organism	Mechanism of regulating ROS pathways	Mode of detection	Functional relevance
ELP	<i>Euphorbia characias</i>		CaM binding protein regulated by Ca <sup>2+</sup> /CaM	CaM binding assays	CaM mediated Ca <sup>2+</sup> /ROS crosstalk [15]
CaM	<i>Panax ginseng</i>		Induce expression followed by increased antioxidant enzyme activity under ABA and H <sub>2</sub> O <sub>2</sub> , which is abolished by CaM blocker and ROS scavengers	CaM blocker for example, TFP and ROS scavengers such as DMTU	ABA mediated ROS production regulated through CaM [16]
CaM2	Potato		Overexpression depicts decreased ROS accumulation	Ectopic expression in <i>N. benthamiana</i>	Drought tolerance [17]
CaM	<i>Eleusine coracana</i>		Overexpression shows reduced oxidative load	Ectopic expression in Arabidopsis	Drought and salt tolerance [18]
BAG9	Tomato		Overexpression shows reduced antioxidant enzymes expression level as well as activity	Overexpression in Arabidopsis	Increased sensitivity to multiple abiotic stress [19]
CML20	Arabidopsis		CaM binding protein activity, upregulated stress responsive genes, down regulated antioxidant scavenging genes, increased ROS levels	In-vitro CaM binding assays,	ABA induced ROS production during drought [20]
CML40	Pongamia		Overexpression lines shows increased proline and lower MDA content	Overexpression in Arabidopsis	Salt tolerance [21]
CML4	Rice		Increased ROS scavenging enzymes activity and expression	Rice transgenic lines overexpressing CML4	Drought tolerance in ABA independent manner [22]
DSR1	Rice		Increased ROS scavenging enzymes activity and expression	Rice transgenic lines overexpressing DSR1 and DSR1 RNA interference lines	Drought tolerance in rice [23]
CaM	Arabidopsis		CaM binds to MPK8 and controls expression of RbohD genes	CaM binding assays	Ca <sup>2+</sup> and ROS interplay during wound signalling pathways [24]
CML10	Arabidopsis		Interacts with PPM	Yeast-two-hybrid, BiFC and co-immunoprecipitation assay	Regulates oxidative stress through ascorbic acid pathway [25]
CML44	<i>Solanum habrochaites</i>		Differential expression of ROS scavenging machinery and reduced ROS content	Enhance antioxidant enzyme activity and reduced ROS load	Transgenic tomatoes overexpressing CML44 [26]
CML46	Alfalfa		Enhanced ROS scavenging enzymes activity and reduced ROS content	Ectopic expression in <i>N. benthamiana</i>	Tolerance to abiotic stress [27]
CAMTA	Chickpea		Enhanced ROS scavenging enzymes activity and reduced expression of stress marker genes	Chickpea transgenic lines overexpressing CAMTA	Regulates salt and drought stress [28]
CAMTA	Soybean		Differential expression under various abiotic stress and H <sub>2</sub> O <sub>2</sub> treatment	RT-PCR	Regulates ROS in response to stress [29]

TABLE 1 (Continued)

Calcium sensing proteins	Identified homolog	Organism	Mechanism of regulating ROS pathways	Mode of detection	Functional relevance
Calcineurin B-like proteins (CBL) and CBL-interacting protein kinases (CIPKs)					
	CBL1/9-CIPK26	Arabidopsis	Interacts and phosphorylates RbohF, ROS production is increased in CBL9, CIPK26 and RbohF co expression	Yeast-two-hybrid, in-vitro phosphorylation and co-expression analysis in HEK293T cells	CIRP and RICEP in plants [30]
	CIPK11	Arabidopsis	Overexpression lines shows increased ROS content, it also phosphorylates RbohF	Overexpression in Arabidopsis, in-vitro phosphorylation assay	Response to drought stress [31, 32]
	CIPK11	<i>Nitraria tangutorum</i>	Increased antioxidant enzymatic activity and decreased ROS content	Ectopic expression in Arabidopsis	Response to salt stress [33]
	SOS2 (CIPK24)	Arabidopsis	Interacts with NDPK2, CAT2 and CAT3	Yeast-two-hybrid	Salt stress responses [34]
	CIPK31	<i>Brachyosium distachyon</i>	Enhanced ROS scavenging enzymes activity and reduced ROS content	Transgenics overexpressing CIPK31 in <i>N. benthamiana</i>	Drought and Salt stress responses [35]
	CIPK31	<i>Brachyosium distachyon</i>	Improved ROS detoxification	Ectopic expression in <i>N. benthamiana</i>	Cold and osmotic stress responses [36]
	CBL10	Arabidopsis	ROS and BR regulated induced CBL10 expression	RT-PCR	Response to drought stress [37]
	SOS2L1	Apple	Involvement of antioxidant pathway gene	LC/MS performed in transgenic lines overexpressing CBL10 in apple and tomato	Salt stress responses [38]
	CIPK29	Wheat	Enhanced ROS scavenging enzymes activity and reduced ROS content	Transgenics overexpressing CIPK29 in <i>N. benthamiana</i>	Salt stress responses [39]
	CIPK2	Wheat	Enhanced ROS scavenging enzymes activity	Transgenics overexpressing CIPK2 in <i>N. benthamiana</i>	Response to drought stress [40]
	CIPK2	Amur grape	Reduced ABA induced ROS level	Overexpression in Arabidopsis	ABA dependent stomatal movement and ROS homeostasis during drought stress [41]
	CIPK3	<i>Capsicum annuum</i>	Altered ROS scavenging enzymes activity and pathway	Ectopic expression in tomato	Drought tolerance [42]
	CIPK13	Pepper	Altered ROS scavenging enzymes activity and ROS levels	Knockdown lines in pepper	Cold stress responses [43]
	SOS3	<i>Tamarix hispida</i>	Altered ROS scavenging enzymes activity and ROS levels	Transgenic <i>T. hispida</i> lines as well as overexpression in Arabidopsis	Salt stress responses [44]
	CBL9, CIPK23	Arabidopsis	Altered ROS scavenging enzymes activity and ROS levels	Cbl9 and cipk23 mutant in Arabidopsis	Salt stress responses [45]
	CBL10, CIPK6	<i>Gossypium hirsutum</i>	Induced expression in response to drought, salt and ABA treatment	Overexpression in Arabidopsis	Multiple abiotic stress tolerance [46]

(Continues)

TABLE 1 (Continued)

Calcium sensing proteins	Identified homolog	Organism	Mechanism of regulating ROS pathways	Mode of detection	Functional relevance
	CBL10, CIPK6	Tomato	CIPK6 phosphorylates Rd2 whose overexpression shows reduced ROS	In-vitro phosphorylation assay, Transgenics overexpressing Rd2 in <i>N. benthamiana</i>	Oxidative stress signalling [47]
	CIPK9	Arabidopsis	CIPK9 phosphorylates VDACC3 and alters ROS levels	In-vitro and in-planta phosphorylation assays, Genetic evidence	Oxidative stress signalling [48]
	CIPK14	Wheat	Altered ROS scavenging enzymes activity and ROS levels	Tobacco cell expression	Cold and salt stress response

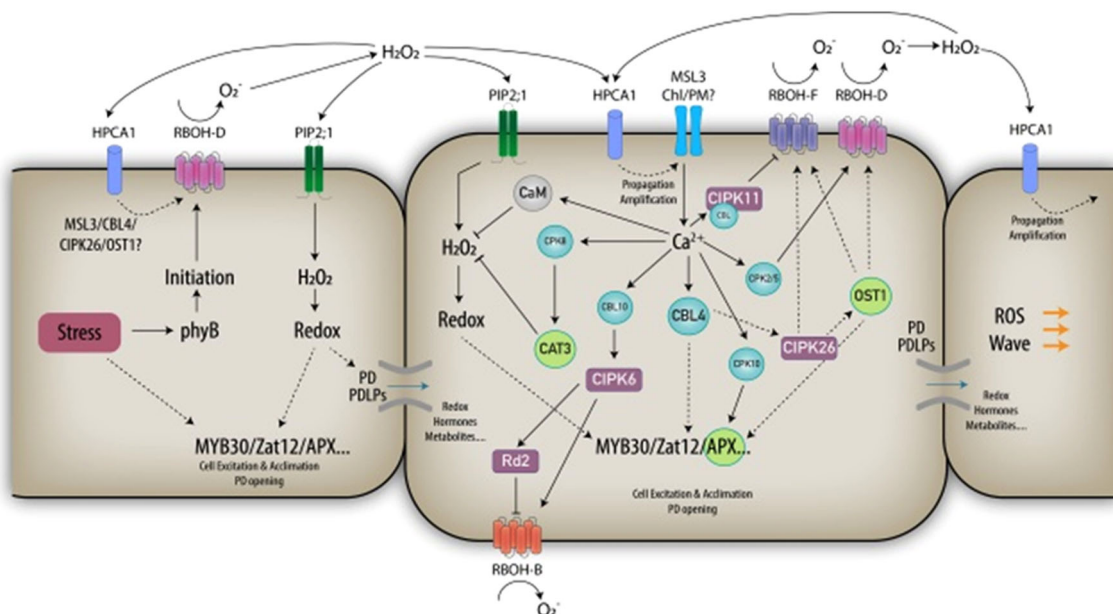
Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; BAG, Bcl-2 associated athanogene; BiFC, bimolecular fluorescence complementation;  $Ca^{2+}$ , calcium; CAMTA, calmodulin binding transcription activator; CAT, catalase; CCaMK, calcium/calmodulin dependent protein kinases; CIRP,  $Ca^{2+}$  induced ROS production; DMTU, dimethylthiourea; DSR, drought stress response; ELP, *Euphorbia characias* peroxidase;  $H_2O_2$ , hydrogen peroxide; JA, jasmonic acid; PPM, phosphomannomutase; Rboh, respiratory burst oxidase homolog; Rd, responsive to desiccation; RICP, ROS  $Ca^{2+}$  induced production; ROS, reactive oxygen species; SA, salicylic acid; SOS, salt overly sensitive; TFP, trifluoperazine; VDACC3, voltage dependent anion channel.

## 9 | CONCLUSIONS AND PERSPECTIVES

While plants lack of a nervous system, they still have the ability to transmit environmental signals systemically from the site of perception to distal tissues. Within this context,  $Ca^{2+}$  and ROS are versatile signalling components that operate individually or synchronously to transmit information concerning environmental conditions to facilitate appropriate adjustments in plant growth and defence. Membrane-associated channels and pumps shuttle  $Ca^{2+}$  ions between the extracellular space, the cytosol, organelles and the nucleus, and multiple systems generate oxidative (ROS) signals in the extracellular and intracellular compartments to regulate protein functions, metabolism and gene expression. The changes in  $Ca^{2+}$  ion distribution control the membrane potential, modulate mitochondrial and photosynthetic ATP production and regulate the activities of proteins such as CaM, which have a diverse range of functions. The evidence discussed above highlights the central role of  $Ca^{2+}$  and ROS interplay in the regulation of plant growth, development and defence, particularly in relation to environmental triggers (Marcec and Tanaka, 2021; Marcec et al., 2019).

The recent characterisation of the ROS wave pathway of cell-to-cell signalling in Arabidopsis and other species has led to a steep change in current concepts of how the  $Ca^{2+}$ -ROS interplay is regulated at a mechanistic level, as illustrated in Figure 3. In summary, the perception of changes in apoplastic ROS accumulation by HPCA1 activates a CPK-dependent signal transduction pathway that involves many  $Ca^{2+}$ -signalling proteins including CBL4, CIPK26 and OST1. A number of these  $Ca^{2+}$ -regulated proteins activate RBOHD and RBOHF and amplify apoplastic ROS production, which is then sensed by the cell following in the chain, via its own HPCA1. The repetition of these processes from cell-to-cell forms a positive amplification loop that drives the ROS signal from cell-to-cell (Fichman et al., 2019, 2022). In this concept, CIPK26 activates RBOHF and OST1 (Drerup et al., 2013), and in turn OST1 activates RBOHD and RBOHF (Wang et al., 2020). Perception of apoplastic ROS by HPCA1 also leads to the opening of aquaporins such as PIP2;1 (Maurel et al., 2021; Mittler et al., 2022) that facilitate the transfer of apoplastic ROS into the cytosol. In this way, the redox state of signalling components in the cytosol can be changed to regulate multiple proteins and transcription factors such as MYB30 and ZAT12 (Mittler et al., 2022) to enhance stress tolerance (Fichman et al., 2021; Zandalinas, Fichman, Devireddy, et al., 2020; Zandalinas, Fichman, & Mitler, 2020b; Fichman and Mittler, 2021; Mittler et al., 2022). The systemic transmission of ROS signals is also dependent on a range of  $Ca^{2+}$ -permeable channels, including MSL3, that function downstream of HPCA1 (Fichman et al., 2022).

The systemic cell-to-cell transmission of  $Ca^{2+}$  and ROS signals is also linked to an 'electric wave' that involves rapid depolarization of the PM and is dependent on the function of GLRs (Fichman and Mittler, 2021). While the systemic cell-to-cell changes in  $Ca^{2+}$  and ROS signals are suppressed in *hPCA1* mutants, the rapid local and systemic membrane depolarization signal is unaffected (Fichman



**FIGURE 3** A simple model illustrating how the waves of  $Ca^{2+}$  and reactive oxygen species (ROS) may facilitate cell-to-cell signalling. Any physical or chemical shock to the plasma membrane induces a ROS burst in the apoplast by the activation of RBOH proteins. Many factors are likely to be involved in the perception and transmission of environmental and metabolic signals that promote RBOHD activation in local tissues. The example illustrated here is the requirement for phytochrome B (phyB) in the plant response to high light. RBOH-mediated ROS production is crucial for in cell-to-cell signalling and long-distance communication, as described by Fichman et al. (2022). In this model, HPCA1 senses changes in apoplastic ROS levels and initiates cell signalling pathways including cytosolic  $Ca^{2+}$  fluctuations mediated at least in part through MSL3. Thereafter,  $Ca^{2+}$  sensor proteins govern downstream signalling and activate RBOH-dependent ROS generation as well as the antioxidant and ROS processing machinery. The regulation of PD size by PDLP enhances the symplastic communication between cells allowing the spread of the cell-to-cell signals. PD functions are regulated by  $Ca^{2+}$ , callose synthase activity and callose deposition. Many components of this signalling cascade remain to be identified. Examples of signalling systems that are less well characterised are indicated by dashed lines. APX, ascorbate peroxidase; CaM, calmodulin; CAT, catalase; CBL, calcineurin B-like proteins; CIPK, CBL-interacting protein kinase; CPK, Calcium dependent protein kinase;  $H_2O_2$ , hydrogen peroxide; HPCA1,  $H_2O_2$ -induced  $Ca^{2+}$  increase; MSL3, mechanosensitive ion channel like 3; MYB, myeloblastosis domain protein; OST1, open stomata 1; PD, plasmodesmata; PDLP, PD localised protein; PhyB, Phytochrome B; PIP2:1, plasma membrane intrinsic protein; RBOH, respiratory burst oxidase homologs; RD2, responsive to desiccation; Zat12, zing finger of *Arabidopsis thaliana*. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

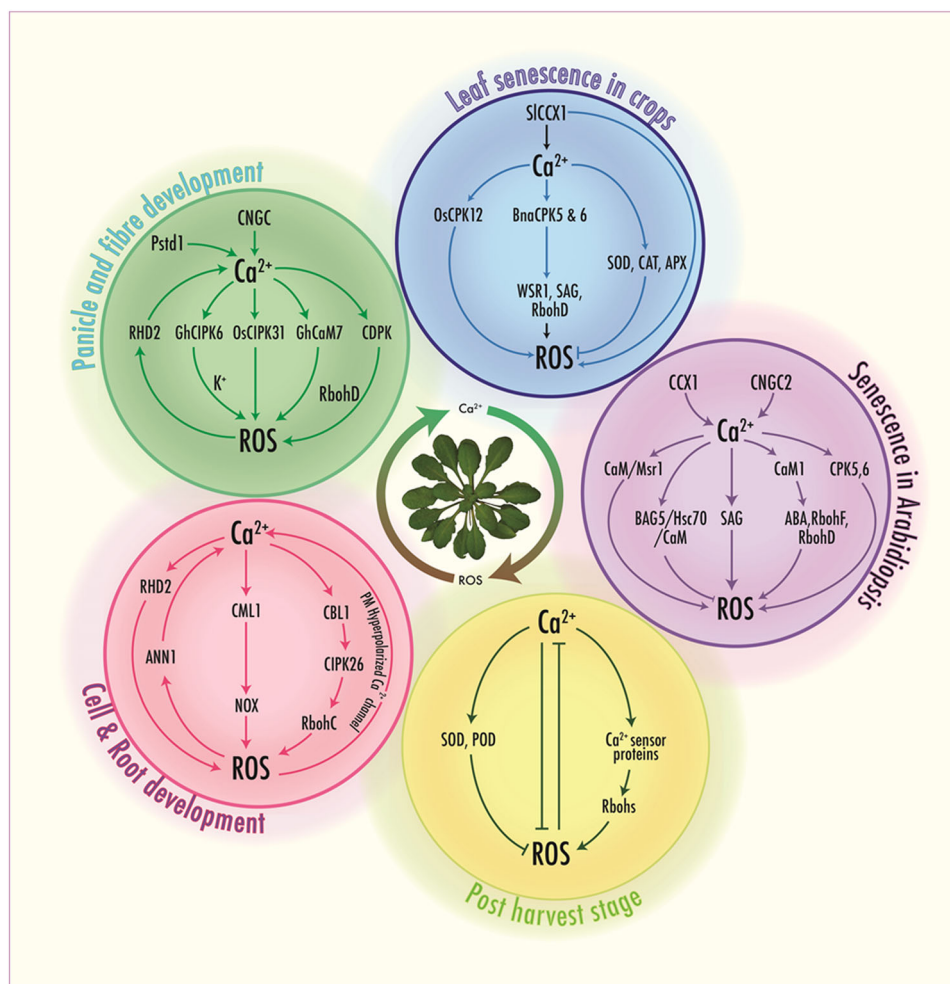
et al., 2022), demonstrating that the waves of different signals can operate independently. While the signalling system illustrated in Figure 3 is intrinsic to plant stress responses, there is as yet little indication of how specificity is entrained in the signalling pathways. The signalling-induced increases in apoplastic and cytosolic  $H_2O_2$  accumulation are likely to be accompanied by stress-induced fluctuations in  $H_2O_2$  accumulation in mitochondria and chloroplasts that may serve to add specificity to the signal but little is known about the mechanisms that integrate signals from the different cellular compartments. Moreover, the movement of other signalling molecules through the PD is likely to be key to honing the specific nature of signal transmission.

The molecular machinery that achieves the synergistic operation of ROS and  $Ca^{2+}$  signals remains poorly understood. Redox posttranslational modifications (PTMs) are likely to operate synchronously with protein phosphorylation and other PTMs to modulate a wide range of signalling protein functions (Spoel, 2018), as is the case with HPCA1 and many more. The subsequent increase in intracellular

$Ca^{2+}$  levels, followed by the activation of  $Ca^{2+}$  sensing proteins, regulates a range of PTMs and other functions either by direct regulation (CDPKs and CBL-CIPKs) or by activating other signalling cascades (mostly but not exclusively governed by CaM and CMLs). Many questions remain to be addressed, particularly concerning how the ROS- $Ca^{2+}$  interplay regulates key processes such as the cell cycle and epigenetic controls that allow adaptation to prevailing environmental conditions. In vivo imaging systems that will allow the direct measurement of key parameters such as  $Ca^{2+}$ , glutathione and  $H_2O_2$  in different cellular compartments using genetically encoded biosensors will be essential to investigate the dynamic nature of such controls (Kostyuk et al., 2020).

For simplicity, the above discussion has focussed on the proteins and processes that facilitate interconnections between ROS and  $Ca^{2+}$  signalling pathways in response to abiotic stress. It is important to emphasise, however, that these and similar proteins and systems are likely to form nodes for ROS/ $Ca^{2+}$  signalling that regulate many aspects of plant biology, as illustrated in Figure 4.





**FIGURE 4** Schematic representation of the many possible roles of calcium sensor proteins and ROS/Ca<sup>2+</sup> interactions in plant growth and development. Ca<sup>2+</sup> sensor proteins such as CPK5, CPK6, CPK12, CaM1, Msr1, CaM7, CML1, CIPK31, CIPK6 and the CBL1-CIPK26 complex have been shown to influence ROS signalling pathways during a wide range of plant growth and developmental responses (Cui et al., 2020; He et al., 2013; Jiang et al., 2017; Kimura et al., 2013; Luo et al., 2018; Tang et al., 2014; Wang, Zhang, et al., 2019; Yang et al., 2018). Hence, crosstalk between ROS and Ca<sup>2+</sup> may be considered to regulate nearly every stage of plant development, from germination to senescence, and to even extend to the postharvest physiology of fruits and vegetables. ABA, abscisic acid; ANN1, annexin 1; BAG, Bcl-2-associated athanogene; Ca<sup>2+</sup>, calcium; CaM, calmodulin; CCM1, Cation/Ca<sup>2+</sup> exchanger; CNGC, cyclic nucleotide gated channel; Hsc, heat shock cognate; Hvc2d1, *Hordeum vulgare* C2-domain; MSR, methionine sulfoxide reductase; NOX, NADPH oxidases; Rboh, respiratory burst oxidase homolog; RHD, root hair development; ROS, reactive oxygen species; SAG, senescence-associated gene; WSR1, WRKY regulating SA and ROS 1. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

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#### DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no data sets were generated or analysed during the current study.

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