

# Calcium and Reactive Oxygen Species Rule the Waves of Signaling<sup>1</sup>

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Calcium ( $\text{Ca}^{2+}$ ) is an important second messenger with diverse functions not only in mammals but also in plants. It is released in response to a variety of stimuli like biotic and abiotic stresses and facilitates a tight regulation of response reactions as well as of developmental processes (Sanders et al., 2002; Steinhorst and Kudla, 2012).  $\text{Ca}^{2+}$  accumulation events are characterized by distinct temporal and spatial features, and they can vary in terms of amplitude, frequency, and duration (Webb et al., 1996; Scrase-Field and Knight, 2003; Dodd et al., 2010; Kudla et al., 2010). Spatially defined  $\text{Ca}^{2+}$  signals can be generated due to the especially slow diffusion rate of the  $\text{Ca}^{2+}$  ion in the cytoplasm in combination with tightly regulated release and uptake from and into different intracellular stores and the apoplast. Together, these characteristics encode information about particular stimuli, for example, drought stress that is presented to the cell as so-called  $\text{Ca}^{2+}$  signatures (Webb et al., 1996). This information has to be decoded and transmitted by a signaling machinery in order to initiate adequate response reactions, for example, stomatal closure (Allen et al., 2000, 2001; Sanders et al., 2002).  $\text{Ca}^{2+}$  signatures can be sensed by proteins that bind  $\text{Ca}^{2+}$  via helix-loop-helix EF-hand motifs. *Arabidopsis* (*Arabidopsis thaliana*) possesses at least 250 putative EF-hand proteins, 100 of which have been classified as  $\text{Ca}^{2+}$  sensor proteins (Day et al., 2002; Hashimoto and Kudla, 2011). Given that each member of this intricate set of  $\text{Ca}^{2+}$  sensor proteins can exhibit characteristic expression and subcellular localization profiles as well as distinct  $\text{Ca}^{2+}$  affinities, plants are equipped with a complex signal-decoding machinery to process a wide range of different  $\text{Ca}^{2+}$  signals (Batistić and Kudla, 2004; Batistić and Kudla, 2010).  $\text{Ca}^{2+}$  functions in concert with other important second messengers like reactive oxygen species (ROS). ROS can be generated in a controlled manner by several types of enzymes, such as NADPH oxidases, in order to contribute to pathogen defense and cell signaling. Recent findings point to direct connections between ROS and  $\text{Ca}^{2+}$  signaling pathways that enable

cell-to-cell communication and thereby long-distance transmission of signals in plants. In this Update, we focus on new findings in the field of plant  $\text{Ca}^{2+}$  signaling during the past 3 years since the status of the field was discussed in comprehensive reviews (Dodd et al., 2010; Kudla et al., 2010; Mazars et al., 2011; Reddy et al., 2011) and put special emphasis on the contribution of a plant-specific  $\text{Ca}^{2+}$  signaling network to deciphering defined  $\text{Ca}^{2+}$  signals and its integration with ROS signaling.

### GENERATION OF CALCIUM SIGNALS: NEW INSIGHTS

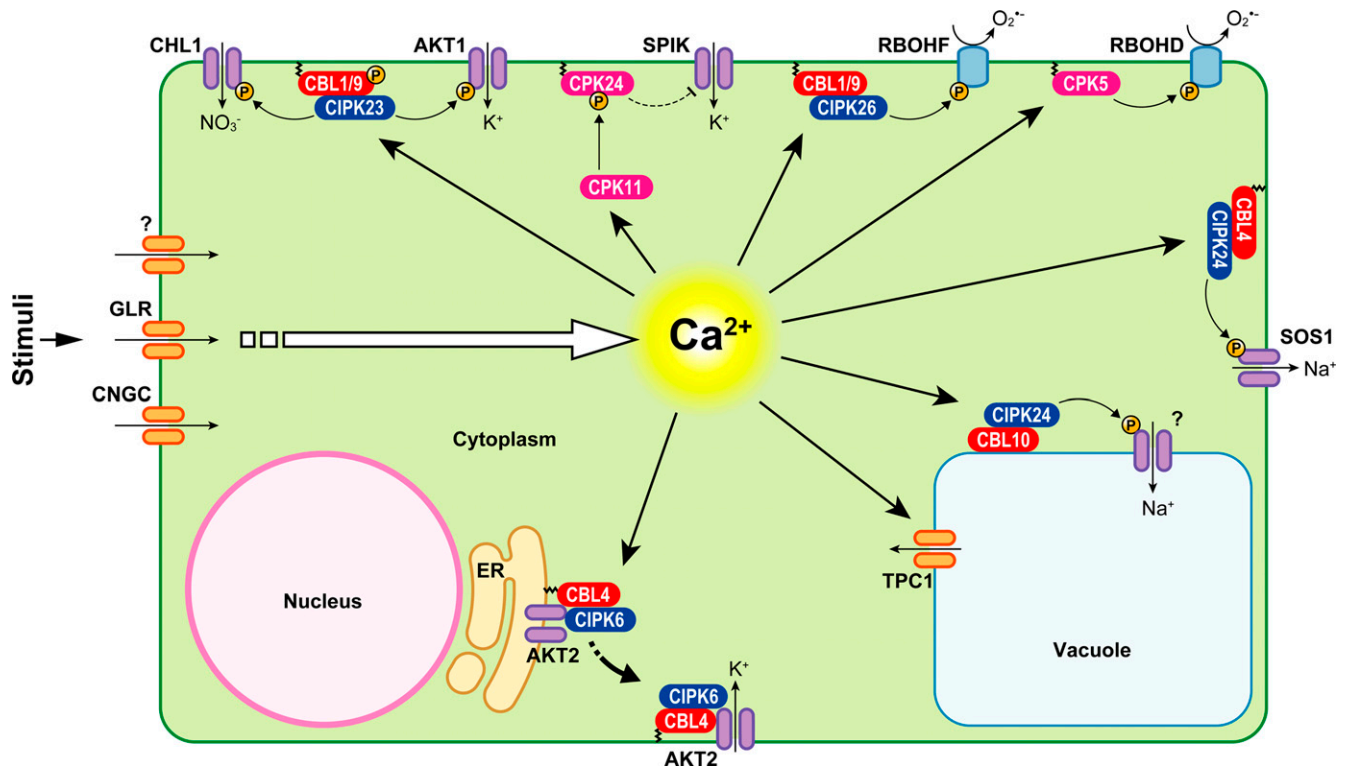
To form a  $\text{Ca}^{2+}$  signal, fluxes of  $\text{Ca}^{2+}$  across membranes have to occur in order to increase and subsequently decrease the cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ). In contrast to components responsible for  $\text{Ca}^{2+}$  extrusion out of the cytosol, like P-type  $\text{Ca}^{2+}$ -ATPases and  $\text{Ca}^{2+}$ /proton antiporters, the molecular identity of  $\text{Ca}^{2+}$ -specific influx channels has remained unknown or controversial for a long time. However, recently, for two families of the group of nonspecific ligand-gated ion channels, increasing evidence is emerging that supports these candidates as being responsible for the generation of  $\text{Ca}^{2+}$  signals in response to pathogens, abiotic stresses, and during polar growth processes. These protein families are the glutamate-like receptor homologs (GLRs) and the cyclic nucleotide-gated ion channels (CNGCs; Fig. 1).

### GLRs Function in $\text{Ca}^{2+}$ Signaling

Members of the GLR family in plants are involved in many biological processes, such as carbon/nitrogen balance, photosynthesis, abscisic acid (ABA) sensing, responses to abiotic stresses, root morphogenesis, plant-pathogen interaction, and regulation of ionic currents including  $\text{Ca}^{2+}$  (Kim et al., 2001; Kang and Turano, 2003; Kang et al., 2004; Qi et al., 2006; Stephens et al., 2008; Cho et al., 2009; Price et al., 2012; Vincill et al., 2013). The importance of GLRs for the generation of  $\text{Ca}^{2+}$  currents during distinct cellular processes has recently been demonstrated for GLR1.2. GLR1.2 is a channel that facilitates  $\text{Ca}^{2+}$  influx across the plasma membrane in growing pollen tubes and thereby contributes to the modulation of the tip-focused  $\text{Ca}^{2+}$  gradient (Michard et al., 2011). Mutant analyses revealed that this channel

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**Figure 1.** Schematic model of  $\text{Ca}^{2+}$ -mediated signal transduction processes in a plant cell. Examples of signal transduction processes mediated by CDPKs and CBL/CIPK complexes from Arabidopsis together with their respective target proteins are shown. For further details, see text. ER, Endoplasmic reticulum; P, target phosphorylation.

is crucial for proper  $\text{Ca}^{2+}$  fluxes to ensure pollen tube growth and full fertility. This holds true not only with respect to tube morphogenesis but also to pollen tube guidance, since GLR1.2 activity is regulated through the abundance of D-Ser in the surrounding pistil tissue.  $\text{Ca}^{2+}$  conductance of pollen GLRs has been demonstrated with  $\text{Ca}^{2+}$ -specific vibrating probe measurements combined with GLR agonists (D-Ser) or antagonists (Michard et al., 2011). However, direct evidence for amino acid-gated  $\text{Ca}^{2+}$  conductance of plant GLRs was still missing at that time, since the expression of plant GLRs in *Xenopus laevis* oocytes for voltage-clamp analyses was not successful, possibly due to wrong targeting (Li et al., 2006a; Price et al., 2012). Animal ionotropic glutamate receptors (iGluRs), however, could be analyzed with this method (Hollmann et al., 1989; Vincill et al., 2012), and it has been found that they form heterotetramers with varying conductances for sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ), and  $\text{Ca}^{2+}$  (Kandel et al., 2000; Davenport, 2002; Furukawa et al., 2005; Traynelis et al., 2010). Therefore, they were classified as nonselective cation channels. Studies conducted thus far suggest that also plant GLRs are nonselective cation channels (Kim et al., 2001; Roy et al., 2008; Tapken and Hollmann, 2008; Price et al., 2012). However, none of these studies provided direct proof for amino acid-gated activity of a plant GLR.

Importantly, in a recent study, Vincill et al. (2012) established HEK cells as a system for studying GLR function. In their work, they found that AtGLR3.4 is an amino acid-gated channel that is suggested to be highly selective to  $\text{Ca}^{2+}$  and is capable of inducing cytosolic  $\text{Ca}^{2+}$  peaks in response to Asn, Gly, or Ser. In a more recent study, it was found that GLR3.4 together with GLR3.2 can form functional heteromers in planta and that this heteromerization is favored over homomerization of each of the two GLRs (Vincill et al., 2013). However, these heteromers exhibited the same  $\text{Ca}^{2+}$  conductance properties in response to Asn as GLR3.4 homomers. GLR3.2 did not form functional homomers in this study. In this regard, future research is needed to analyze in more detail the conductance properties of differently composed GLR heteromers to elucidate, for example, comprehensive agonist profiles or different kinetic properties (Vincill et al., 2013). Mutant analyses of GLR3.4 and GLR3.2, which are expressed in the apical root phloem of Arabidopsis, revealed that both GLRs are involved in the regulation of lateral root formation. This finding of GLR involvement in root developmental processes is especially interesting considering that primordium production induced by mechanical bending of primary roots is known to depend on  $\text{Ca}^{2+}$  signaling (Richter et al., 2009; Vincill et al., 2013). In order to extend our understanding about the function of GLRs in lateral root formation, precise subcellular localization analyses and

analyses of expression patterns during developmental stages in combination with live-cell imaging of  $\text{Ca}^{2+}$  signals in response to amino acids are needed. Moreover, not much is known so far about the regulation of plant GLR at the posttranslational level, such as phosphorylation. Animal Glu receptors can be subject to regulation by phosphorylation that modulates receptor trafficking and receptor channel properties (Chen and Roche, 2007). Interestingly, several GLRs from Arabidopsis were identified as potential 14-3-3 client proteins in a proteomics study by Chang et al. (2009), which hints at interactions with other signaling proteins, including kinases and phosphatases (Price et al., 2012). Hence, activation or deactivation of GLRs by  $\text{Ca}^{2+}$ -regulated kinases could represent a mechanism of positive or negative feedback regulation, respectively. Therefore, it would be most interesting to test whether plant GLRs are also subject to phosphorylation and if this phosphorylation can be brought about by  $\text{Ca}^{2+}$ -regulated kinases, such as CBL-interacting protein kinases (CIPKs) or  $\text{Ca}^{2+}$ -dependent protein kinases (CDPKs).

### The Contribution of CNGCs to $\text{Ca}^{2+}$ -Regulated Processes

CNGCs are nonspecific cation channels that form a family of 20 members in Arabidopsis and are regulated by cyclic nucleotides such as cAMP or cyclic GMP (Mäser et al., 2001; Talke et al., 2003; Ward et al., 2009). Accumulating evidence suggests that CNGCs are required for  $\text{Ca}^{2+}$  fluxes across the plasma membrane and thereby contribute to  $\text{Ca}^{2+}$  signaling in the context of developmental processes, abiotic stress responses, pathogen defense responses, and regulation of pollen tube tip growth as well as in the establishment of thermotolerance (Frietsch et al., 2007; Chaiwongsar et al., 2009; Ma and Berkowitz, 2011; Tunc-Ozdemir et al., 2013a, 2013b). First evidence that linked CNGC function to polar growth processes in plants was provided by mutant analyses that revealed that CNGC18 function is required for pollen tube tip growth and consequently is crucial for male fertility (Frietsch et al., 2007). In addition to CNGC18, also CNGC7 and CNGC8 have been implicated in the regulation of pollen tube tip growth. Both proteins seem to fulfill overlapping functions, since mutant analyses of single and double mutant alleles revealed a reduced pollen transmission efficiency and bursting pollen tubes only for the double mutant (Tunc-Ozdemir et al., 2013a). Unfortunately, these studies did not address potential changes in  $\text{Ca}^{2+}$  dynamics of *cngc* mutant pollen. Therefore, possible alternative explanations for the observed pollen phenotypes could be, for example, a disrupted coordination of growth cycles or a disturbed turgor pressure regulation. The latter would be in agreement with the fact that CNGCs are also conductive for  $\text{K}^+$ , as, for example, AtCNGC3 and AtCNGC10 (and other CNGCs) can compensate for  $\text{K}^+$  uptake in *akt1* mutants (which lack the Shaker-like potassium channel AKT1; Caballero et al., 2012).

The importance of CNGC function for pollen fertility has been further corroborated lately in that AtCNGC16 was found to convey thermotolerance to (germinating) pollen by connecting cyclic nucleotide signaling to the transcriptional heat-stress response (Tunc-Ozdemir et al., 2013b). Pollen transmission of the *cngc16* mutant was significantly reduced under temperature stress and also under drought stress conditions, and this phenotype appeared to be linked to an attenuated expression of key stress-responsive genes. The function of CNGCs in plant thermotolerance appears not to be restricted to pollen. The cation channel CNGC6 is ubiquitously expressed in Arabidopsis and mediates heat stress-induced  $\text{Ca}^{2+}$  influx, which affects the thermotolerance of vegetative plant tissues and also leads to induction of a heat-shock protein (HSP; Gao et al., 2012). *cngc6* mutant plants exhibited decreased thermotolerance and reduced expression of several HSPs compared with the wild type. Furthermore, it was found that the level of cytosolic cAMP was increased by mild heat shock in the wild type. CNGC6 in turn was activated by cytosolic cAMP, and application of exogenous cAMP promoted the expression of HSP genes. Together, these findings support the concerted action of  $\text{Ca}^{2+}$  and cyclic nucleotide monophosphates (cNMPs) in plant thermotolerance. The role of CNGCs and  $\text{Ca}^{2+}$  signaling in establishing acquired thermotolerance appears to be quite complex and evolutionarily conserved. Mutants of Arabidopsis CNGC2 as well as the putative ortholog *cngcb* mutant of the moss *Physcomitrella patens* exhibited a hyperthermosensitive phenotype accompanied by a hyperthermoresponsive  $\text{Ca}^{2+}$  influx, giving rise to a heat stress response and acquired thermotolerance at significantly milder heat treatments than in wild-type plants (Finka et al., 2012). In these plants, the responsiveness to temperature was shifted by 5°C. Moreover, patch-clamp recordings on *P. patens* protoplasts revealed that deletion of one CNGC affected the open probability of remaining CNGC. This phenomenon points to complex compensatory and feedback regulatory mechanisms that may involve the regulated formation of CNGC heteromultimers and the modulation of cNMP and  $\text{Ca}^{2+}$ -calmodulin (CaM) signaling. In all CNGCs studied so far, the binding site for CaM overlaps with the cNMP-binding site (Talke et al., 2003). Accordingly, it was well accepted that the binding of  $\text{Ca}^{2+}$ -activated CaM to CNGCs interferes with cNMP-dependent activation. A new level of  $\text{Ca}^{2+}$ -CaM-mediated CNGC regulation has been recently uncovered by the characterization of a conserved IQ domain, which is separated from the cyclic nucleotide-binding domain (Fischer et al., 2013). In CNGC20, this IQ domain interacts with CaM in a  $\text{Ca}^{2+}$ -dependent manner. This novel finding represents an additional CaM-binding mode, increasing the complexity of CNGC regulation that deserves further studies.

In addition to GLRs and CNGCs, plant annexins have been found to be related to changes in cellular  $\text{Ca}^{2+}$  concentrations (Mortimer et al., 2008; Laohavisit et al.,

2009). A recent study reported that *Arabidopsis annexin1* mediates radical-activated plasma membrane  $\text{Ca}^{2+}$ -permeable conductance in root cells (Laohavisit et al., 2012). Analyses of *annexin1* loss-of-function mutants revealed a lack of the root hair and epidermal  $\text{OH}^\bullet$ -activated  $\text{Ca}^{2+}$  and  $\text{K}^+$  conductances, a finding that has so far not been obtained in studies of animal annexins (Laohavisit et al., 2012). Although plant annexins are without doubt involved in regulating  $\text{Ca}^{2+}$ -related processes in plants, care should be taken to declare them as novel  $\text{Ca}^{2+}$ -permeable transporters, and clearly, more research is needed to clarify their mode of function. The idea that annexins could be  $\text{Ca}^{2+}$  channels originated from early studies of mammalian annexins in artificial lipid bilayers (Rojas et al., 1992; Hawkins et al., 2000; Kourie and Wood, 2000). Additional studies revealed a second mode of function for animal annexins in that they modulate the activity of  $\text{Ca}^{2+}$  channels (Díaz-Muñoz et al., 1990; Gerke and Moss, 2002). While the modulatory function of animal annexins in regulating ion channels stood the test of time, today their function as bona fide  $\text{Ca}^{2+}$  channels has been severely challenged. In addition to conceptual problems with how peripherally membrane-associated annexins could conduct  $\text{Ca}^{2+}$  across the membrane, accumulating evidence indicated that annexin attachment to (artificial) membrane bilayers perturbs the organization of lipids in the bilayer to efficiently electroporate the membrane and, therefore, permit  $\text{Ca}^{2+}$  entry (Demange et al., 1994; Gerke and Moss, 2002; Gerke et al., 2005). These conclusions may be considered when interpreting experimental results on plant annexin function.

In contrast to the situation at the plasma membrane, much less is known about channels that could mediate  $\text{Ca}^{2+}$  release from internal stores. An abundant channel at the tonoplast is the slow vacuolar channel that was originally described as a voltage-dependent cation channel that is regulated by cytoplasmic  $\text{Ca}^{2+}$  concentrations (Hedrich and Neher, 1987). In addition, a  $\text{Ca}^{2+}$ -release capability of this channel was reported (Allen and Sanders, 1994). This channel was molecularly identified as two-pore channel1 (TPC1; Fig. 1). A *tpc1* knockout mutant lacks functional slow vacuolar channel activity and is defective in both the ABA-induced repression of germination and in the response of stomata to extracellular  $\text{Ca}^{2+}$  (Peiter et al., 2005). TPC1 is the only vacuolar  $\text{Ca}^{2+}$ -permeable channel cloned to date. However, due to its complex regulation and its low selectivity among cations, the role of this channel in  $\text{Ca}^{2+}$  signaling is still debated (Peiter, 2011).

## SENSING AND TRANSMISSION OF CALCIUM SIGNALS

Plants are equipped with an elaborate set of  $\text{Ca}^{2+}$  sensor proteins that sense defined  $\text{Ca}^{2+}$  signals and transmit them to initiate adequate responses to specific stimuli. In addition to the ubiquitous eukaryotic  $\text{Ca}^{2+}$  sensor calmodulin (CaM), they possess further families

of EF-hand  $\text{Ca}^{2+}$  sensor proteins. These are the calmodulin-like proteins, the family of CDPKs, and the calcineurin B-like protein (CBL) family. CDPKs are  $\text{Ca}^{2+}$  sensor proteins that can bind  $\text{Ca}^{2+}$  via four EF-hand motifs in their C-terminus (Kudla et al., 2010). Binding  $\text{Ca}^{2+}$  leads to activation of the kinase domain located at the N-terminus and thereby facilitates direct sensing and transmission of a  $\text{Ca}^{2+}$  signal. In contrast, CBLs represent solely  $\text{Ca}^{2+}$  sensors without an enzymatic function. CBLs can bind  $\text{Ca}^{2+}$  with their four EF-hands, but in order to convert the signal into phosphorylation events, interaction with members of the CIPK family is required (Shi et al., 1999). Interaction between CBLs and CIPKs is mediated via the NAF domain, a conserved motif within the regulatory C-terminal domain of the kinase, and releases the kinase from autoinhibition (Albrecht et al., 2001). In the following, we will discuss the latest findings and new emerging mechanisms associated with CDPK and CBL-CIPK signaling networks as well as their interconnection in the context of ROS signaling.

## New Emerging Functions and Mechanistic Principles of the CDPK Network

CDPKs (also abbreviated as CPKs) represent versatile  $\text{Ca}^{2+}$ -sensing effector proteins that are involved in the regulation of many processes, such as abiotic and biotic stress signaling, pathogen defense, and hormone-regulated developmental processes in different plant cell types and in some protists (Cheng et al., 2002; Harper and Harmon, 2005; Liese and Romeis, 2013). In this section, we focus only on the most significant new findings on CDPK function and regulation in the context of their potential interplay with other  $\text{Ca}^{2+}$  signaling systems. In *Arabidopsis*, the family of CDPK protein kinases encompasses 34 members with both overlapping and distinct functions. Recently, substantial advances have been made in analyzing the characteristics and diverse functions of CDPKs. One example is CPK21, which was found to be involved in the regulation of responses to osmotic stress (Franz et al., 2011). Seedlings of a loss-of-function allele displayed increased stress responses when subjected to osmotic stress, and this phenotype could be rescued by expression of the active kinase. Considering that opposite phenotypes have been reported for mutants of CBLs and CIPKs like *cb11* and *cipk1* (Albrecht et al., 2003; D'Angelo et al., 2006), this raises the possibility that, depending on the actual cellular  $\text{Ca}^{2+}$  concentration, both signaling systems may function antagonistically during osmotic stress conditions. Investigation of  $\text{Ca}^{2+}$ -binding properties of the four EF-hands of CPK21 and the impact on kinase activity by analyzing different EF-hand mutant enzymes revealed differences between the two EF-hand pairs, suggesting that this feature could contribute to the specificity of CPK21 function (Franz et al., 2011).

Concerning the regulation of CDPKs by  $\text{Ca}^{2+}$  binding, an advanced model has recently been discussed (Liese

and Romeis, 2013) that extends the hitherto existing model (Harper and Harmon, 2005) in terms of understanding the molecular mechanisms of autoinhibition and activation (Ojo et al., 2010; Wernimont et al., 2010, 2011). Accumulating evidence points to an important role of the N-terminal region of CDPKs as a targeting determinant that is crucial for establishing functional specificity *in vivo* (Asai et al., 2013). Membrane targeting of CDPKs appears to involve lipid modifications, since the majority of CDPKs contain potential N-terminal myristoylation and S-acylation sites that are required for membrane targeting of, for example, CPK2 and CPK3 from *Arabidopsis* (Martín and Busconi, 2000; Mehlmer et al., 2010). Also, the plasma membrane localization of StCPK5 depends on myristoylation and palmitoylation of the kinase N-terminus, and this targeting is essential for the phosphorylation of StRBOHB (for respiratory burst oxidase homolog B) *in vivo* (Asai et al., 2013). Similarly, myristoylation of *Arabidopsis* CPK5 seems to be a prerequisite for plasma membrane targeting of this CDPK (Lu and Hrabak, 2013). Since distinct lipid modification patterns could result in targeting to defined membrane subdomains, this targeting mechanism likely contributes to establishing specificity of CDPKs in plants. Moreover, since targeting of CBL-CIPK complexes, like the targeting of CDPKs, largely relies on similar lipid modification by myristoylation and S-acylation (see below), it appears conceivable that both signaling systems function simultaneously in the same membrane domains and may regulate the same target proteins.

A new mechanistic principle of CDPK function and regulation that may largely affect our concepts of  $Ca^{2+}$  signaling has very recently emerged from a study of the concerted regulation of a  $K^+$  channel by a pair of CDPKs in pollen (Zhao et al., 2013). So far, CDPKs (and CBL-CIPK complexes) have been considered as one-step relays that convey  $Ca^{2+}$  signals directly into phosphorylation events on substrate proteins. In the study by Zhao et al. (2013), the pollen-specific Shaker-type  $K^+$  channel SPIK was found to be negatively regulated by the concerted and subsequent action of CPK11 and CPK24. Disruption of *CPK11* or *CPK24* completely impaired the  $Ca^{2+}$ -dependent inhibition of inward  $K^+$  currents and enhanced pollen tube growth. Moreover, the *cpk11/cpk24* double mutant exhibited similar phenotypes to the corresponding single mutants, suggesting that these two CDPKs function in the same signaling pathway. Moreover, CPK11 interacted with CPK24 *in vivo* and phosphorylated the N-terminus of CPK24 *in vitro*, suggesting that these two CDPKs work together as part of a kinase cascade. It will be interesting to address the question of whether this mechanism of kinase cascades also applies for other CDPK-target combinations or even allows for cross-regulatory interconnections with CIPKs. In the past, there has been steadily increasing evidence that CDPKs directly contribute to the modulation of ROS signaling via the regulation of RBOH NADPH oxidases (Kudla et al., 2010). Previous studies in potato (*Solanum tuberosum*) suggested that two

CDPKs (StCDPK4 and StCDPK5) phosphorylate NADPH oxidases and thereby positively regulate the production of ROS (Kobayashi et al., 2007). Accordingly, ectopic expression of a constitutively active mutant version of StCDPK5 provoked ROS production in *Nicotiana benthamiana* leaves, and this CDPK-mediated ROS production was disrupted by knockdown of StCDPK5. Moreover, a quadruple loss-of-function mutant of the *Arabidopsis* CDPKs CPK4, CPK5, CPK6, and CPK11 has been reported to exhibit impaired pathogen responsiveness involving a lack of ROS production, indicative of disturbed RBOH activation (Boudsocq et al., 2010). However, whether these CDPKs directly target RBOH proteins that mediate ROS production during pathogen responses remained to be addressed.

Subsequent integrated functional genomic and biochemical genetic screens identified six closely related CDPKs as contributing to immune signaling. Of these, CPK1/CPK2/CPK4/CPK11 were reported to phosphorylate NADPH oxidases *in vitro* and were implemented as crucial components for the production of ROS during immune signaling (Gao et al., 2013). In a most recent breakthrough study, Tina Romeis and colleagues discovered that the plasma membrane-localized CPK5 positively regulates the NADPH oxidase RBOHD during pathogen defense in *Arabidopsis* (Dubiella et al., 2013; Fig. 1). They demonstrated that activation of RBOHD occurs via CPK5-dependent phosphorylation of several Ser residues within the oxidase N-terminus *in vivo*. Most remarkably, the regulation of RBOHD by CPK5 enables signal propagation upon pathogen-associated molecular pattern (PAMP) stimulation to distal parts of the plant via an apoplastic ROS wave. Accordingly, CPK5 and RBOHD have been proposed to represent key components of a self-propagating activation circuit mediating cell-to-cell communication (Dubiella et al., 2013). Specifically, this work identified six Ser residues at which phosphorylation was increased in CPK5-overexpressing lines but was unaltered or reduced in *cpk5* mutants. Interestingly, this work also provided evidence for the contribution of other protein kinases to RBOHD regulation, since phosphorylation of two additional Ser residues was enhanced in the *cpk5* mutant and reduced in CPK5-overexpressing lines. These findings suggest that other protein kinases (e.g. CIPKs) may participate in the regulation of RBOHD phosphorylation *in vivo* (Dubiella et al., 2013). These exciting new facets of  $Ca^{2+}$  and ROS signaling will be discussed in more detail at the end of this review.

### The CBL-CIPK Network: A Versatile Signaling Machinery

Evaluation of genome sequences identified a set of 10 CBLs and 26 CIPKs in *Arabidopsis* and a set of 10 CBLs and 30 CIPKs in rice (*Oryza sativa*; Kolukisaoglu et al., 2004; Weinl and Kudla, 2009). Phylogenetic analyses of the evolutionary development of CBLs and their interacting protein kinases revealed that the extensive network they build up in higher plants such as *Arabidopsis*

is indeed restricted to higher plants. This complex network appears to have developed from a founder pair of a single CBL protein and a single CIPK that can still be detected in green algae like *Ostreococcus tauri* and the related species *Ostreococcus lucimarinus* and *Chlorella* spp. (Batistič and Kudla, 2009, 2012; Weigl and Kudla, 2009). In contrast, genomes of lower plants like the moss *P. patens* encode four CBLs and seven CIPKs (Batistič et al., 2010), and the lycophyte *Selaginella moellendorffii* possesses a complement of four CBL proteins and five CIPKs (Weigl and Kudla, 2009). Evolution of the complex Ca<sup>2+</sup> signal-decoding network of CBLs and CIPKs as it is found in Arabidopsis presumably occurred concurrently with the evolution of increasing morphological and developmental sophistication of land plants as a result of the colonization of new fluctuating habitats (Weigl and Kudla, 2009). Interestingly, the CBL-CIPK network is not limited to the plant lineage, since putative CBL and CIPK proteins were identified in the genomes of the protozoan species *Trichomonas vaginalis* and *Naegleria gruberi* (Batistič and Kudla, 2009, 2012; Weigl and Kudla, 2009). The discovery of these otherwise plant-specific components of the CBL-CIPK network in human pathogens, on the one hand, argues for an ancient CBL-CIPK network in nonplant species and, on the other hand, provides a potential starting point for medical research to combat human pathogens.

#### N-Terminal Lipid Modification Facilitates CBL Membrane Targeting

CBL proteins can be divided into two groups, according to their N-termini. CBL1, CBL4, CBL5, CBL8, and CBL9 constitute one group with a comparatively short N-terminus, whereas CBL2, CBL3, CBL6, CBL7, and CBL10 possess extended N-termini (Kolukisaoglu et al., 2004; Batistič et al., 2008, 2010). Except for CBL8, all members of the first group are subject to lipid modifications that determine their targeting to the plasma membrane. The N-termini of CBL1, CBL4, CBL5, and CBL9 harbor an MGXXX(S/T) motif that allows for the attachment of myristic acid to the Gly (Ishitani et al., 2000; Batistič et al., 2008). In addition to myristoylation, CBL1 has to be N-terminally S-acylated in order to be functional (Batistič et al., 2008). Further analyses conducted on CBL1 revealed that its N-terminus is S-acylated by both palmitate and stearate and that this dual lipid modification is crucial for plasma membrane targeting. CBL2, CBL3, CBL6, and CBL10, members of the second group of CBL proteins, possess extended N-termini that are responsible for targeting to the tonoplast (Batistič and Kudla, 2009; Batistič et al., 2010). CBL10 is an exception in this group, since its N-terminus contains a potential transmembrane domain that is thought to be important for localization at the tonoplast (Kim et al., 2007). In contrast, tonoplast targeting of CBL2 is brought about by S-acylation of three Cys residues in its N-terminus (Batistič et al., 2012). The vacuolar localization of CBL2 and CBL3 has also been confirmed in Arabidopsis

(Tang et al., 2012). It seems likely that palmitoylation of CBLs is conducted by protein S-acyltransferases (PATs). In Arabidopsis, 24 PATs have been characterized, two of which have been detected at the tonoplast and nine at the plasma membrane (Batistič, 2012). These PATs represent candidates that could bring about the targeting of CBLs and lipid-modified CDPKs into defined membrane domains. The first evidence for the importance of PAT activity for CBL targeting has been recently presented by CBL localization analyses in a *pat10* mutant line (Zhou et al., 2013) that suggested that the tonoplast-localized PAT10 is responsible for tonoplast targeting of CBL2, CBL3, and CBL6. Future research is needed to advance our understanding of the mechanisms by which PATs target CBL and CDPK proteins to membranes and how this might contribute to creating specific membrane microdomains that functionally combine different Ca<sup>2+</sup> and hormone signaling proteins (Demir et al., 2013).

#### Subcellular Targeting of CBL-CIPK Complexes: A Mechanism for the Transmission of Spatially Defined Signals

While most CBL proteins are localized at membranes, their interacting kinases are mainly present in the cytosol and in the nucleus when expressed alone (Batistič et al., 2010). In planta protein-protein interaction analyses using the bimolecular fluorescence complementation (BiFC) system have greatly advanced our understanding of CBL and CIPK localization and targeting (Hu et al., 2002; Kerppola, 2006; Waadt and Kudla, 2008; Waadt et al., 2008; Batistič et al., 2010). BiFC interaction analyses of CIPK23 and CIPK24, for example, demonstrated that these two kinases both interact with CBL1 and CBL9 at the plasma membrane (Xu et al., 2006; Waadt et al., 2008). These results, among others, firmly established that CBL proteins determine the localization and site of action of CBL-CIPK complexes. A special case was observed for the interaction of the cytosolic calcium sensor CBL8 with the kinase CIPK14. Interaction between these two proteins was exclusively detected at the plasma membrane (Batistič et al., 2010). Considering that CBL8 is not lipid modified at its N-terminus and thereby differs from the lipid-modified plasma membrane-targeted CBL1, CBL4, CBL5, and CBL9, this points to another not yet identified targeting mechanism of CBL8-CIPK complexes. Other CIPK14 interactors are the tonoplast-localized CBL2 and CBL3, which target CIPK14 to the vacuole in planta, indicating that one and the same kinase can be recruited to different subcellular destinations by interacting with different CBLs located at different membranes. Further examples detected with the BiFC method that support the idea of alternative targeting routes for CIPKs are CBL-CIPK24 complexes at the plasma membrane such as CBL1-CIPK24 and CBL5-CIPK24 and at the tonoplast such as CBL2-CIPK24 and CBL10-CIPK24 (Waadt et al., 2008; Batistič et al., 2010). However, the BiFC method artificially stabilizes protein interactions and does not allow one to

follow where the noninteracting fraction of proteins remains within the cell. Therefore, coexpression of fluorophore-tagged CBLs and CIPKs was applied as a complementary approach (Schlücking et al., 2013). Such studies revealed that GFP-tagged CIPK5 is no longer present in the cytosol but targeted to the tonoplast instead, when CBL3-mCherry is coexpressed in *N. benthamiana*. In this way, it became evident that CBL proteins are indeed able to efficiently trap interacting CIPKs out of the cytosol and recruit them to membranes.

This method of coexpression and colocalization analysis should allow us in the future to compare the interaction strengths of different CBL-CIPK pairs or preferential complex formation by expressing a CIPK together with two differently located interacting CBLs and monitoring competitive recruitment of the CIPK. Moreover, this should allow us to address the dependence of alternative CBL-CIPK interaction on the  $\text{Ca}^{2+}$  concentration in the cytosol. It appears likely that different CBL-CIPK complexes require individual  $\text{Ca}^{2+}$  concentrations to form and that this dependence relates to the decoding of specific  $\text{Ca}^{2+}$  signals. Moreover, the  $\text{Ca}^{2+}$ -binding capacities of CBL proteins can change upon interaction with a distinct CIPK, as has been shown by crystallization studies of CBL4-CIPK24 as well as of CBL2-CIPK14 complexes (Sánchez-Barrena et al., 2013). This adds another layer of complexity to the dependence of CBL-CIPK complex formation on cytosolic  $\text{Ca}^{2+}$  concentrations. Taken together, all these variable parameters of CBL-CIPK complex formation appear to act in concert and thereby contribute to decoding distinct  $\text{Ca}^{2+}$  signals at defined locations within the cell, enabling the generation of specific and adequate responses.

### Phosphorylation of CBLs Is Important for Target Regulation

A novel regulatory mechanism of CBL-CIPK complexes that has been recently uncovered is the phosphorylation of CBL proteins by their interacting CIPKs (Mahajan et al., 2006; Lin et al., 2009; Du et al., 2011; Hashimoto et al., 2012). Importantly, it was found that phosphorylation of AtCBL1 and AtCBL9 by their interacting kinase CIPK23 is absolutely required for the activation of the  $\text{K}^+$  channel AKT1 in *X. laevis* oocytes. It was shown that phosphorylation of the CBL protein increased the activity of the CBL-CIPK complex toward its substrate by an unknown mechanism. Most likely, phosphorylation of the flexible C-terminus of a CBL leads to conformational changes that in some way enhance the specificity and activity of the complex toward its target. Phosphorylation of the analyzed CBLs from Arabidopsis was found to occur at a Ser residue within a conserved C-terminal FPSF motif. This finding was also supported by another work in which the respective region was named the PFPF motif (Du et al., 2011). In some cases, the phosphorylation

status of CBL proteins appears to impact on the stability of a CBL-CIPK complex (Du et al., 2011; Hashimoto et al., 2012). Clearly, this issue deserves further investigation to better understand its meaning and consequences.

### CBL-CIPK Complexes Regulate a Broad Range of Targets

Targets regulated by CBL-CIPK complexes have so far mainly been identified at the plasma membrane, while not much is known about the functions of vacuolar CBL-CIPK complexes. However, in the past 2 years, major advances have been made linking tonoplast-localized CBL proteins to the regulation of vacuolar pH,  $\text{K}^+$  homeostasis, and ABA signaling (Batistič et al., 2012; Tang et al., 2012; Liu et al., 2013). Recently, it has been shown that CBL2 targeting to the tonoplast depends on N-terminal S-acylation (Batistič et al., 2012). Mutant analyses with a *cbl2* knockout line revealed that CBL2 function is required for appropriate ABA responses during seed germination. A *cbl2* mutant line was impaired in germination on ABA-containing medium compared with the wild type. Importantly, proper targeting to the tonoplast was absolutely required for full functionality of CBL2, pointing to a function of a tonoplast-localized  $\text{Ca}^{2+}$  sensor in regulating ABA responses by decoding  $\text{Ca}^{2+}$  signatures emanating from the vacuole. However, the mechanism by which CBL2 regulates ABA responses at the vacuole is still enigmatic. Since the *cbl2* single mutant exhibits an ABA-hypersensitive phenotype, it is assumed that CBL3, the closest homolog of CBL2, is not redundant in this context, although these two proteins are 92% identical and exhibit overlapping expression patterns (Batistič et al., 2012). Similarly, it has been reported that CBL3 but not CBL2 is involved in the regulation of  $\text{K}^+$  homeostasis and that CBL3 together with CIPK9 functions in  $\text{K}^+$  homeostasis under low- $\text{K}^+$  stress (Liu et al., 2013). Only *cbl3* mutant plants exhibited a low- $\text{K}^+$ -tolerant phenotype and increased  $\text{K}^+$  content under low- $\text{K}^+$  conditions, leading to the assumption that only CBL3 together with CIPK9 but not CBL2 is functionally included in this particular pathway. Thus, quite remarkably despite their high degree of similarity, CBL2 and CBL3 appear to fulfill distinct functions. Nevertheless, CBL2 and CBL3 can also have overlapping functions (Tang et al., 2012).

A recent work demonstrated that only a *cbl2/cbl3* double mutant, but not the respective single mutants, exhibits several developmental defects related to a reduced vacuolar ATPase activity accompanied by compromised ionic tolerance and micronutrient accumulation. However, evidence for a direct connection between CBL2/CBL3 function and the regulation of vacuolar ATPase activity is still pending and requires further investigation. In contrast to the rather enigmatic situation at the tonoplast, several ion transport processes have been described at the plasma membrane that are subject to regulation by CBL-CIPK complexes,

such as the salt overly sensitive (SOS) pathway for salt tolerance or  $K^+$  channel regulation and the modulation of  $NO_3^-$  uptake in plants. CBL4-CIPK24 (SOS3-SOS2) complexes at the plasma membrane, for example, can activate the  $H^+/Na^+$  antiporter SOS1 and thereby contribute to the regulation of  $Na^+$  extrusion (Liu and Zhu, 1998; Halfter et al., 2000; Qiu et al., 2002; Quintero et al., 2002, 2011; Fig. 1). The kinase CIPK23, on the other hand, is targeted to the plasma membrane by the two highly related  $Ca^{2+}$  sensors CBL1 and CBL9 (Xu et al., 2006; Cheong et al., 2007), where these complexes can regulate the activity of the Shaker-like potassium channel AKT1 (Li et al., 2006b; Xu et al., 2006; Hashimoto et al., 2012; Fig. 1). Furthermore, CIPK23 can also regulate the activity of the plasma membrane nitrate transporter CHL1 (also named NRT1.1; Ho et al., 2009; Fig. 1). In addition to its function as a CBL1-CIPK23 complex in AKT1 and CHL1 regulation, CBL1 also plays a role in drought and osmotic stress responses together with CIPK1 (Albrecht et al., 2003; D'Angelo et al., 2006).

Recently, a proteomic analysis of a *cbl1* mutant revealed that in response to salt stress, several protein expression levels are altered compared with the wild type, further strengthening CBL1-involvement in salt stress responses (Shi et al., 2011). The affected proteins are predicted to function in various processes, such as signal transduction, ROS scavenging, energy production, carbon fixation, metabolism, mRNA and protein processing, and structural stability. Furthermore, additional roles for CBL1 are beginning to emerge, such as regulation of cold response together with CIPK7 or modulation of glucose- and GA-mediated responses (Huang et al., 2011; Li et al., 2013a, 2013b). Another example for a plasma membrane CBL functioning in salt stress signaling is CBL5. Overexpression of CBL5 renders plants more resistant to salt and osmotic stresses during seed germination independent from ABA signaling, suggesting a role as a positive regulator for CBL5 in salt and drought stress responses (Cheong et al., 2010). Future research is needed to identify targets and to unravel the underlying mechanisms for this observation. CIPKs that in addition to SOS2/CIPK24 have recently been implicated in salt stress responses are CIPK16 and CIPK6 (Tripathi et al., 2009; Chen et al., 2013; Roy et al., 2013). CIPK16 was identified by quantitative trait locus mapping as being linked to  $Na^+$  exclusion, as it was up-regulated under salt stress conditions. Furthermore, overexpression of CIPK16 leads to an improved salt tolerance. Comparably, also overexpression of CIPK6 increases plant tolerance to salt stress, while a *cipk6* knockdown line exhibited hypersensitive responses to salt stress in addition to several developmental defects as well as reduction of auxin transport processes (Tripathi et al., 2009; Chen et al., 2013). Moreover, CIPK26 has very recently been identified as an interactor of the RING-type E3 ligase Keep on Going (KEG), a negative regulator of ABA signaling (Lyzenga et al., 2013). Interaction with KEG probably leads to the degradation of CIPK26 via the 26S proteasome. Overexpression of CIPK26 induces an ABA-

hypersensitive phenotype, suggesting CIPK26 as a positive regulator of ABA signaling, possibly by interaction with components of ABA signaling such as ABI1, ABI2, and ABI5. Interestingly, KEG targets ABI5 for degradation, while CIPK26 appears to phosphoregulate this ABA signaling component, suggesting that KEG is acting to counteract and balance ABA responses that are promoted by CIPK26 and ABI5. These findings point to a tight regulation of CBL and CIPK function at the protein level that clearly deserves further investigation.

An interesting new twist in the exploration of the CBL-CIPK network is the discovery of its participation in the regulation of polar growth processes such as pollen germination and tube elongation (Mähs et al., 2013). CBL1 and CBL9 function is crucially required for proper pollen germination as well as for pollen tube growth. It appears that these two plasma membrane CBLs have overlapping functions in this context and that they contribute to  $K^+$  homeostasis. Analyses of mutant alleles and overexpression lines suggested a positive regulatory function in the course of  $K^+$  uptake across the plasma membrane. CIPKs implicated in this process, as well as their targets, still have to be determined, but it seems likely that potential targets in pollen are represented by  $K^+$  channels, such as AKT5 or SPIK, considering that a comparable regulatory mechanism has been described for AKT1 in other cell types (Li et al., 2006b; Xu et al., 2006).

### Mechanisms of Target Regulation

Targets such as ion channels can be subject to phosphorylation and thereby activated or deactivated by CIPKs that have been targeted by their interacting CBL partners to the respective membrane subdomain. This has been demonstrated, for example, for the potassium channel AKT1 and for the nitrate transporter CHL1 (Li et al., 2006b; Xu et al., 2006; Cheong et al., 2007; Ho et al., 2009). Both become phosphorylated and thereby activated by CIPK23 when the kinase is targeted to the plasma membrane by CBL1 or CBL9. Furthermore, AKT1 can be dephosphorylated by AKT1-INTERACTING PP2C1, which means that the action of CIPKs can be counteracted by phosphatases (Lee et al., 2007; Sánchez-Barrena et al., 2013). This observation points to an even more elaborate regulatory mechanism, taking into account that also CIPKs can directly interact with PP2Cs (Ohta et al., 2003). This has been shown for SOS2/CIPK24, which interacts with the PP2C-type protein phosphatase ABI2 through the C-terminal protein phosphatase interaction motif (Ohta et al., 2003). In this regard, two independent mechanisms of channel regulation that have already been postulated for slow anion channel1 (SLAC1) regulation by the protein kinase Open Stomata1 (OST1) and by the protein phosphatase PP2CA appear conceivable (Lee et al., 2009b). On the one hand, the phosphatase could block the kinase activity by interaction with the kinase. This would



terminate the activation of the channel. Additionally, the channel could be dephosphorylated and thereby directly inactivated by the phosphatase. Indeed, biochemical data indicated direct dephosphorylation of SLAC1 by the PP2C ABI1 (Brandt et al., 2012). The anion channel SLAC1 appears to represent a hub of guard cell regulation and, consequently, is subject to regulation by multiple kinases that involve SNF1-related kinase2s (SnRK2s), CDPKs, and receptor-like kinases (Geiger et al., 2009, 2010; Brandt et al., 2012; Hua et al., 2012). In several of these studies, kinase interaction with PP2Cs appears to reduce SLAC1 channel activation. It will be interesting to elucidate if CIPKs also contribute to the regulation of this channel.

Interaction of CIPKs with CBLs via the NAF domain and with phosphatases via the protein phosphatase interaction domain are assumed to be mutually exclusive (Sánchez-Barrena et al., 2013). However, there is also the possibility that phosphatases might additionally interact with the kinase domain of CIPKs (Lan et al., 2011). Moreover, interactions between PP2C phosphatases and CBLs might even further increase the complexity of the mechanism of target phosphoregulation. Remarkably, in addition to the regulation of targets via phosphorylation, recent research uncovered that CBL-CIPK complexes are also able to modulate channel activity independently of phosphorylation events. This turned out to be the case for K<sup>+</sup> channel AKT2 regulation by CBL4-CIPK6 complexes (Held et al., 2011). Here, the interaction with the kinase is required for channel activation, but instead of phosphorylating the channel, the CBL4-CIPK6 complex serves as a translocation mediator delivering the channel from the endoplasmic reticulum to the plasma membrane, where it can fulfill its function (Fig. 1). Whether this targeting mechanism also applies for other targets regulated by CBL-CIPK complexes remains to be explored. Another potential mechanism of target regulation has recently emerged for AKT1. It was found that AKT1 directly interacts with CBL10 (Ren et al., 2013). This interaction apparently does not rely on a kinase and may give rise to AKT1 inactivation by outcompeting activating CBL-CIPK23 complexes. Together, these examples show that many variations exist in the mechanisms that contribute to target regulation.

### The CBL-CIPK Network Is Conserved in Many Plant Species

Several recent studies indicate that the function of the CBL-CIPK network in abiotic stress and ABA signaling is not restricted to Arabidopsis. The diverse spectrum of plants in which CBL and CIPK function was analyzed encompasses species such as rice, rape (*Brassica napus*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), apple (*Malus domestica*), cotton (*Gossypium hirsutum*), and poplar (*Populus* spp.; Yang et al., 2008; Piao et al., 2010; Zhang et al., 2011, 2013; Chen et al., 2012; Wang et al., 2012; Deng et al., 2013; He et al., 2013). Moreover, lately,

five novel CIPKs and two CBLs have been discovered in kidney bean (*Phaseolus vulgaris*) and 43 putative CIPK genes that are closely related to rice CIPKs have been identified in maize (*Zea mays*; Hamada et al., 2009; Chen et al., 2011). A function of a rice CIPK has been reported for OsCIPK31, which was found to modulate responses to abiotic stresses during seed germination and seedling growth (Piao et al., 2010), and OsCIPK23, which turned out to be a multistress-induced gene likely regulating signaling pathways during pollination and drought stress responses (Yang et al., 2008). Another example for a multifunctional rice CIPK is OsCIPK15, which has been implicated in mediating oxygen deficiency tolerance as well as being involved in various PAMP-induced immune responses together with OsCIPK14 (Lee et al., 2009a; Kurusu et al., 2010). Comparative structural analyses of CBLs and CIPKs from Arabidopsis, pea (*Pisum sativum*), and rice revealed a high degree of conservation, and additional functional analyses suggested comparable mechanisms underlying signal transduction via CBL-CIPK complexes from these species (Mahajan et al., 2006; Tuteja and Mahajan, 2007).

In terms of target regulation, it seems that findings from Arabidopsis can also be transferred to other plant species, such as the regulation of potassium channels. The Shaker potassium channel VvK1.1 from grape (*Vitis vinifera*), which is the counterpart of AtAKT1, could be stimulated by coexpression of AtCIPK23-AtCBL1 in *X. laevis* oocytes (Cuéllar et al., 2010). Accordingly, it appears likely that this channel is regulated by a related CBL-CIPK complex in grape. In a subsequent study, a second Shaker potassium channel (VvK1.2) was characterized and analyzed for conductance in oocytes together with CBL-CIPK pairs from grape (Cuéllar et al., 2013). It was found that VvK1.2 could be stimulated by the two different VvCBL-VvCIPK pairs VvCIPK04-VvCBL01 and VvCIPK03-VvCBL02 (Cuéllar et al., 2013). Moreover, the SOS pathway for salt tolerance that was originally identified in Arabidopsis is also conserved in rice (Martínez-Atienza et al., 2007). Taken together, these findings suggest that the CBL-CIPK network fulfills conserved functions in many different plant species. However, it will be interesting to study the function of CBL-CIPK complexes in lower plants that have less complex CBL-CIPK networks, like algae and mosses, to uncover their original function.

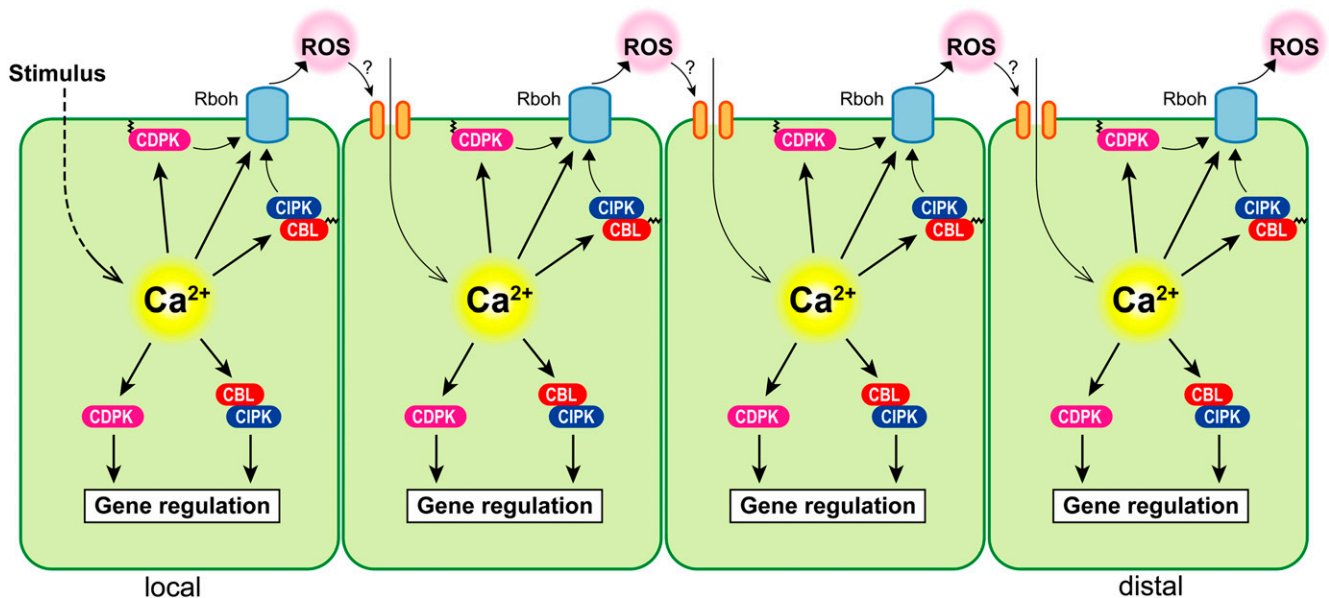
### CALCIUM AND ROS SIGNALING DIRECTLY INTERACT

Elevation of [Ca<sup>2+</sup>]<sub>cyt</sub> and the synthesis of ROS are fundamental constituents of rapid immune responses to pathogen attacks and other signaling pathways in plants and animals. Both are prerequisite for establishing pathogen resistance (Schwessinger and Zipfel, 2008; Boller and Felix, 2009; Dubiella et al., 2013) but are also important for ABA signaling in guard cells, seed germination, pollen tube growth, and root elongation as

well as in the regulation of plant  $\text{Na}^+/\text{K}^+$  homeostasis upon salt stress (Kwak et al., 2003; Potocký et al., 2007, 2012; Liu et al., 2009; Ma et al., 2012). Moreover, several lines of evidence support a mutual interconnection between  $\text{Ca}^{2+}$  and ROS elevation, because it has been reported that ROS activate  $\text{Ca}^{2+}$  channels in guard and root cells (Pei et al., 2000; Demidchik et al., 2003; Hua et al., 2012), and vice versa,  $\text{Ca}^{2+}$  enhances cellular ROS accumulation (Takeda et al., 2008). The most-studied ROS-producing enzymes in plants are the RBOHs (Suzuki et al., 2011; Marino et al., 2012). RBOH enzymes are integral membrane proteins that can generate superoxide anions that are rapidly converted to hydrogen peroxide. RBOH proteins possess two  $\text{Ca}^{2+}$ -binding EF-hands and multiple phosphorylation sites in their N-termini (Keller et al., 1998; Dubiella et al., 2013). The first evidence for phosphoregulation of an RBOH by a  $\text{Ca}^{2+}$ -regulated kinase was provided in potato with the identification of StRBOHB phosphorylation by StCPK5 (Kobayashi et al., 2007; Asai et al., 2013). Studies conducted in HEK293T cells revealed that AtRBOH activity is modulated by  $\text{Ca}^{2+}$  binding to the EF-hands as well as by N-terminal phosphorylation (Ogasawara et al., 2008; Takeda et al., 2008; Kimura et al., 2012). In addition to these findings, the observation that  $\text{Ca}^{2+}$  binding alone does not lead to the activation of RBOHs when kinases are inhibited in HEK cells points to a fundamental importance of RBOH phosphoregulation (Kimura et al., 2012).

Recently, the activation of RBOHF by plasma membrane-localized CBL1/CBL9-CIPK26 complexes has been described (Drerup et al., 2013; Fig. 1). Analyses in HEK293T cells and in vitro phosphorylation assays uncovered that CBL1/CBL9-CIPK26 complexes phosphorylate and thereby activate RBOHF. Furthermore, this study was the first to demonstrate an interaction

between a CIPK and an RBOH in planta using the BiFC system in *N. benthamiana*. Important insights for in planta regulation of RBOH proteins by phosphorylation were provided by the finding that activation of RBOHD by CPK5 enables signal propagation upon PAMP stimulation to distal parts of the plant via an apoplastic ROS wave (Dubiella et al., 2013). Previous studies by Mittler et al. (2011) had already established that ROS waves can propagate through plants with a speed of up to  $8.4 \text{ cm min}^{-1}$  and that these waves are crucial for long-distance signaling. Moreover, it has been reported that the NADPH oxidase RBOHD plays a critical role in the formation and propagation of such ROS waves (Miller et al., 2009). In addition, classical and newer studies have provided evidence that also  $\text{Ca}^{2+}$  waves occur in plants (Trewavas et al., 1996; Trewavas, 1999; Gilroy, 2013). Major insights into the mechanisms that interconnect  $\text{Ca}^{2+}$  and ROS waves have been provided by the recent study of Dubiella et al. (2013), who found a mechanistic link between  $\text{Ca}^{2+}$  and ROS signaling in the context of cell-to-cell signal propagation. In the model resulting from this work,  $\text{Ca}^{2+}$  activates  $\text{Ca}^{2+}$ -dependent kinases that lead to RBOH activation that, in turn, forms apoplastic ROS. These ROS induce  $\text{Ca}^{2+}$  release in neighboring cells that, in turn, again activates  $\text{Ca}^{2+}$ -dependent kinases. In this way, cell-to-cell propagation of a signal is achieved. Since accumulating evidence suggests that also other protein kinases than CPK5 contribute to the regulation of RBOHD, it is tempting to speculate that also the CBL-CIPK network is involved in generating ROS waves for long-distance signal propagation. This is supported by recent work that has shown that CBL1/CBL9-CIPK26 complexes can activate the NADPH oxidase RBOHF (Drerup et al., 2013), and also RBOHD appears to be subject to regulation by CBL-CIPK complexes (P. Köster and J. Kudla, unpublished



**Figure 2.** Schematic model of  $\text{Ca}^{2+}$ - and ROS-mediated cell-to-cell signal propagation in plants. For further details, see text.

data). Moreover, several other stimuli are known to induce RBOHD-generated apoplastic ROS waves as well, such as wounding and local heat stress application (Miller et al., 2009). Therefore, it appears likely that ROS waves forward signals not only in PAMP-triggered responses.

Taking this information together, all these data suggest a new model in which the cytosolic  $\text{Ca}^{2+}$  signal leads to the activation of RBOHD by  $\text{Ca}^{2+}$ -regulated kinases, subsequent ROS generation, and propagation of the signal by the activation of  $\text{Ca}^{2+}$  channels of the neighboring cells (Fig. 2). Despite the convincing evidence for this model with regard to pathogen responses, it will be important to investigate the overall significance of the  $\text{Ca}^{2+}$ -dependent phosphoregulation of ROS-producing NADPH oxidases for other physiological processes in the near future. As mentioned before, local heat stress application was found to induce systemic heat stress responses through ROS waves generated by the NADPH oxidase RBOHD, which is activated by  $\text{Ca}^{2+}$ -regulated kinases (Miller et al., 2009). Combining this information with the novel finding that CNGCs can facilitate  $\text{Ca}^{2+}$  influx in response to heat stress (Finka et al., 2012; Gao et al., 2012; Tunc-Ozdemir et al., 2013b), it is tempting to speculate whether CNGCs might be responsible for  $\text{Ca}^{2+}$  influx in response to a local heat stress signal and thereby enable signal propagation through the activation of RBOHD, resulting in systemic heat stress tolerance. It will be interesting to investigate mechanistic details of this novel long-distance signal propagation in the future.

## CONCLUSION AND PERSPECTIVES

In recent years, the exploration of  $\text{Ca}^{2+}$  signal transduction in plants has significantly moved forward. Major advances have been made in the investigation of components contributing to  $\text{Ca}^{2+}$  signal generation, particularly in the field of GLRs, as well as components responsible for the translation of  $\text{Ca}^{2+}$  signals into physiological responses. Two of the latter components are represented by CDPKs and by the CBL-CIPK network, which both appear to fulfill a broad range of tasks crucial for plant survival. Recent studies uncovered new mechanisms influencing the CBL-CIPK-mediated signal transmission, such as PAT-mediated lipid modification of CBLs and phosphorylation of CBLs by their interacting kinases. These mechanisms require deeper analysis for better understanding. Likewise, the mechanism of differential CBL-CIPK complex formation and its dependence on  $[\text{Ca}^{2+}]_{\text{cyt}}$  deserves further investigation. In addition to target phosphoregulation by CBL-CIPK complexes, additional types of target regulation are now emerging, such as phosphorylation-independent translocation, adding another layer to the functional complexity of this regulatory network. A new direction for the investigation of the  $\text{Ca}^{2+}$  signal-decoding machinery has opened with the finding that the regulation of ROS-generating RBOHDs by  $\text{Ca}^{2+}$ -regulated kinases is an essential part of cell-to-cell

communication and the establishment of systemic responses to local stimuli. Based on existing models for apoplastic ROS and cytosolic  $\text{Ca}^{2+}$  waves (Trewavas et al., 1996; Trewavas, 1999; Torres et al., 2005; Miller et al., 2009; Mittler et al., 2011; Dubiella et al., 2013), we here postulate a new potential model that combines these two important second messengers and also involves the CBL-CIPK network. Nevertheless, despite major advancements, we are merely beginning to understand how  $\text{Ca}^{2+}$  signals are generated and processed. One unsolved issue, for example, is the question of how apoplastic ROS signals are sensed and converted into cytoplasmic  $\text{Ca}^{2+}$  signals. Also, the identity of  $\text{Ca}^{2+}$  channels responsible for ROS-induced  $\text{Ca}^{2+}$  influx has to be uncovered. Future research is needed to address these kinds of fundamental questions and to elucidate the exact mechanisms underlying CBL-CIPK-mediated signal transduction and how these mechanisms are linked to generate specificity in  $\text{Ca}^{2+}$  signal-response coupling.

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