



Review

Analysis of calcium signaling pathways in plants 

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ABSTRACT

Background: Calcium serves as a versatile messenger in many adaptation and developmental processes in plants. Ca^{2+} signals are represented by stimulus-specific spatially and temporally defined Ca^{2+} signatures. These Ca^{2+} signatures are detected, decoded and transmitted to downstream responses by a complex toolkit of Ca^{2+} binding proteins that function as Ca^{2+} sensors.

Scope of review: This review will reflect on advancements in monitoring Ca^{2+} dynamics in plants. Moreover, it will provide insights in the extensive and complex toolkit of plant Ca^{2+} sensor proteins that relay the information presented in the Ca^{2+} signatures into phosphorylation events, changes in protein–protein interaction or regulation of gene expression.

Major conclusions: Plants' response to signals is encoded by different Ca^{2+} signatures. The plant decoding Ca^{2+} toolkit encompasses different families of Ca^{2+} sensors like Calmodulins (CaM), Calmodulin-like proteins (CMLs), Ca^{2+} -dependent protein kinases (CDPKs), Calcineurin B-like proteins (CBLs) and their interacting kinases (CIPKs). These Ca^{2+} sensors are encoded by complex gene families and form intricate signaling networks in plants that enable specific, robust and flexible information processing.

General significance: This review provides new insights about the biochemical regulation, physiological functions and of newly identified target proteins of the major plant Ca^{2+} sensor families. This article is part of a Special Issue entitled Biochemical, biophysical and genetic approaches to intracellular calcium signaling.

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1. Introduction

Calcium ions (Ca^{2+}) were adopted as a second messenger and now represent a most versatile signaling molecule in all eukaryotic organisms. In plants, spatially and temporally distinct changes in cellular Ca^{2+} concentrations, designated as “ Ca^{2+} signatures” that are evoked in response to different stimuli like drought, salt or osmotic stresses, temperature, light and plant hormones represent a central mechanistic principle to present defined stimulus-specific information. These specific “ Ca^{2+} signatures” are formed by the tightly regulated activities of channels and transporters at different membranes and cell organelles [1–3]. While the identity and function of components of the Ca^{2+} extrusion system are rather well understood in plant cells, the molecular identity of Ca^{2+} specific influx channels has remained unknown. However, non-specific influx of Ca^{2+} mediated by ligand gated cation channels like cyclic nucleotide gated channels and glutamate receptor -like proteins contribute to different Ca^{2+} mediated cellular functions like the response to pathogens, pollen tube growth and abiotic stress [2]. The components and mechanisms that generate Ca^{2+} signals in plants have been extensively discussed in several very informative reviews recently [1,2,4] to which we refer the interested reader. Remarkably, while the well characterized

prototypical animal Ca^{2+} channels like the inositol (1,4,5)-triphosphate receptors appear to be absent from land plants, they were identified in the genomes of the evolutionary basal lineage of chlorophyta algae [5]. This suggests that the loss of these channels in higher plants during evolution resulted in the development of “plant specific” Ca^{2+} signaling mechanisms. This also involved the adaptation of the “ Ca^{2+} toolkit” on the level of the Ca^{2+} binding proteins, which decode the information presented in Ca^{2+} signatures and transduce the Ca^{2+} signal to downstream responses.

A major group of Ca^{2+} sensor proteins possesses classical helix-loop-helix EF hand motifs, which bring about Ca^{2+} binding of these proteins that result in Ca^{2+} -dependent conformational changes. The genome of *Arabidopsis thaliana*, the most studied model plant, encodes at least 250 EF hand proteins [6]. Although several of these proteins can be categorized into protein families that are also present in animals, others acquired specific structural rearrangements or appear to be specific to plants and lower protists [7,8]. The large number of the Ca^{2+} binding proteins compared to other organisms, the unique structural composition of Ca^{2+} binding proteins and the complexity of the target proteins regulated by the Ca^{2+} sensors allows the plant to tightly control the appropriate adaptation to its ever changing environment. It is actually the still not well understood interface of information presentation by a specific Ca^{2+} signal and initiation of information decoding by Ca^{2+} sensors that represent a most critical step in specific information processing. Consequently, in the following we first reflect on advancements in monitoring Ca^{2+} dynamics in plants. Subsequently, we discuss the

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recently gained knowledge about the biochemical regulation, physiological functions and newly identified target proteins of the major plant Ca^{2+} sensor families.

2. Analyzing Ca^{2+} dynamics in plants

First attempts to determine the cytosolic Ca^{2+} concentrations and their dynamics in plant cells were undertaken using Ca^{2+} selective microelectrodes or by microinjecting aequorin [9,10]. Such studies resulted in the first report that in the green alga *Chara* stimulus specific changes of cytosolic Ca^{2+} indicated a function of Ca^{2+} as a second messenger in plants [9]. Moreover, experiments with microelectrodes already enabled the measurement of the cytosolic resting levels and recorded oscillatory changes of the Ca^{2+} concentration after the application of the phytohormone auxin [11,12]. However, the usage of the electrode was technically difficult in higher plants because of their small cell size and since in most cells the cytoplasm is confined to a small strip between the plasma and vacuolar membrane. Therefore, the uncertainty of the location of the tip hampered further detailed analysis. Other methods, like Ca^{2+} precipitation techniques (e.g. antimonite staining) allowed to determine the Ca^{2+} storage capacity of organelles in plant cells [13]. In addition, single wavelength fluorescent dyes like chlorotetracyclin and Calcium-Green 1 allowed to visualize Ca^{2+} within the cell [14–16] but were less suitable for determining the actual Ca^{2+} concentrations within the cell. Also radioactive Ca^{2+} was used but only allowed the determination of tissue distributions of this ion [17–19].

The improvement of ratiometric fluorescent dyes (Quin-2, Fura-2 and Indo-1) allowed investigations of Ca^{2+} concentrations and their dynamics via their changes in excitation and emission spectra upon Ca^{2+} binding [20,21]. This allowed to determine the resting levels within the cytosol of the plant cell, the vacuole (e.g. Fura-2 is taken up by the plant vacuole) and of other plant organelles in various plant species [22–25]. These experiments revealed the existence of steep concentration differences between the cytosol (30–90 nM; [22]) and organelles like the ER (3 μM ; [23]) and the vacuole (>5 μM , [22]). However, differences in the recorded resting levels of intact cells and protoplasts were also observed in these studies. This finding was further supported in a recent study, showing that intact cells and protoplasts behave differently in adjusting their Ca^{2+} levels [26]. Further investigations revealed that the Ca^{2+} concentrations within plant cells change in response to application of different chemicals [22] and by the release of caged inositol (1,4,5)-triphosphate [27]. Several groups reported that defined changes of cytosolic Ca^{2+} concentration are triggered by cellular second messengers like NAADP, IP₃, IP₆, Sphingosine-1-Phosphate and cADPR [28–31]. Very importantly, such studies also for the first time established that physiological stimuli like phytohormones [32] and red light [33] resulted in defined and stimulus specific changes in cytosolic Ca^{2+} concentrations. Fluorescent dyes also enabled further analysis of the sub-cellular distribution of Ca^{2+} , as for example the accumulation during mitosis at spindle poles [34] or the polar (tip high) distribution within growing pollen tubes [35]. Further analyses with these dyes revealed that during the growth of the pollen tube the tip-high gradient fluctuates (oscillates) [16,36], and showed that pollen tubes that underwent a self-incompatibility response do not oscillate at the tip but at the shank of the tube [37]. Oscillatory changes of cellular Ca^{2+} concentrations were also recorded by applying different concentrations of extracellular Ca^{2+} or the phytohormone abscisic acid (ABA) to guard cells of *Commelina* [38,39], as well as in response to the Nod factor in Legumes which is important for the symbiosis between the Rhizobia and the plant root system [40]. Moreover, these analyses established that the Ca^{2+} level in plants is also controlled by membrane voltage [41], implicating that Ca^{2+} influx processes in plants and animals are regulated in a similar way. Together all these findings supported the notion that in plants the identity and intensity of a specific stimulus-impulse results in stimulus-specific and dynamic alterations of cytosolic Ca^{2+} concentration [31,38]. These

defined increases in cytosolic free Ca^{2+} ion concentration with regard to duration, amplitude, frequency and spatial distribution prompted A. M. Hetherington and coworkers to formulate the concept of “ Ca^{2+} signatures” [42]. According to this concept a specific Ca^{2+} signature that is defined by precise control of spatial, temporal and concentration parameters of alterations in cytosolic Ca^{2+} concentration would distinctively encode signal information that is specific for its inducing stimulus. Although the use of fluorescent dyes allowed to significantly advance the understanding of Ca^{2+} signaling in plants, the inherent limitations of applying these dyes (loading difficulties, dye sequestration, high buffer capacity) underscored the need to develop more sophisticated Ca^{2+} measurement systems [43,44].

This demand was met with the introduction of the reporter protein aequorin in stably transformed plant lines by the group of A. Trewavas [45]. The use of recombinant aequorin in transgenic lines increased the spatial resolution of Ca^{2+} analyses since aequorin could be expressed in special cell types [46–48] and could be targeted to specific organelles. The latter allowed to analyze the release of Ca^{2+} directly at the point of influx [49–51]. The use of aequorin allowed researchers to determine which of all the biotic and abiotic stimuli influencing the plants life induce Ca^{2+} influx into the cytosol [52–68]. Moreover, it enabled to determine from which source Ca^{2+} was released (extracellular or intracellular) [51,69,70] and to analyze the long-term periodic (circadian rhythmic) release of Ca^{2+} within the plant cell [49]. Calcium release signatures were described as oscillatory [46,50], monophasic with a single increase in the recorded luminescence [46,57], and very often biphasic with a large, rapid first increase and a second smaller but sustained increase [46,54,61,71,72] (summarized in Table 1). However, due to the very low fluence rate of aequorin, in most cases the measurements had to be performed using whole seedlings or larger parts (whole tissues) of plants. Therefore the interpretation of these signatures has to be handled with caution, as they do not represent measurements of single cells [73]. This notion was exemplified by mathematic simulations of oscillations of several thousand single cells [55]. The resulting output of the cell population exhibited a biphasic shape as experimentally recorded indicating that the amplitude of the initial increase in luminescence rather reflects the total number of cells within the population starting to oscillate at the same time (in phase oscillation) than the actual increase in Ca^{2+} . Accordingly, the drop of the luminescence recorded with aequorin would represent the out-of phase oscillation of the cell population. Consequently this would suggest that single cells respond mainly with oscillations to a stimulus instead of single monophasic or biphasic increases. Therefore, the limited resolution of aequorin measurements required a further improvement of the detection system, which combines the ability to record Ca^{2+} transients at a high resolution together with the ability to express the Ca^{2+} indicator at desired tissues or intracellular locations.

This tool was provided with the development of the ratiometric cameleon Ca^{2+} reporter proteins. This Ca^{2+} monitoring system is based on fluorescence resonance energy transfer between two green fluorescent protein variants that are fused to calmodulin and the binding-peptide M13, which interact in a Ca^{2+} dependent manner [74,75]. The use of cameleon in plants was pioneered by G. Allen and co-workers [76]. By using cameleon expressing plants it became possible to clearly dissect the role of Ca^{2+} oscillations for the closure of guard cells in response to the phytohormone ABA, to a cold stimulus or to oxidative stress [77]. Further experiments established that Ca^{2+} signals with a defined oscillation range or “signature” are required for the long term closure of guard cells, while the rapid closure itself is independent of the oscillation kinetics [73]. Also it became evident that different symbiotic interactions between plants and Rhizobia or mycorrhizal fungi depend on differential Ca^{2+} oscillations, which were recorded using a cytoplasmic cameleon [78]. In addition, nuclear targeted cameleon allowed to visualize dynamic changes of Ca^{2+} concentration within the nucleus [79]. Plants expressing the improved version of cameleon 3.1 [80] were used to record cell type specific Ca^{2+} oscillations evoked by extracellular nucleoside-triphosphates [81].

Table 1Examples of Ca^{2+} signatures recorded in plant cells.

Stimulus	Signature	Reporter protein	Plant material	Reference
Osmotic stress				
Salt	Mono-phasic	Aequorin	<i>Arabidopsis</i> seedlings	[60]
Mannitol	Mono-phasic	Aequorin	<i>Arabidopsis</i> seedlings	[60]
Hypo-osmotic	Bi-phasic	Aequorin	Tobacco cell suspension culture	[54]
Temperature				
Cold	Mono-phasic	Aequorin	<i>Arabidopsis</i> seedlings	[51]
Cold	Oscillatory	Aequorin	<i>Nicotiana plumbaginifolia</i> seedlings	[50]
Heat	Mono-phasic	Aequorin	Tobacco seedlings	[65]
Light				
Red light	Mono-phasic	Fluo-3	Wheat protoplasts	[33]
Blue light	Mono-phasic	Aequorin	<i>Arabidopsis</i> seedlings	[53]
Pathogen and symbiotic interactions				
Pep13	Bi-phasic	Aequorin	Parsley suspension culture	[64]
Nod factor	Oscillations	YC2.1	<i>Medicago</i> root hairs	[78]
Mycorrhiza	Oscillations	YC2.1	<i>Medicago</i> root hairs	[78]
Hormones				
Salicylic acid	Mono-phasic	Aequorin	Tobacco suspension culture	[66]
ABA	Oscillations	YC2.1	<i>Arabidopsis</i> epidermal strips	[73]
Methyl-jasmonate	Oscillations	YC3.6	<i>Arabidopsis</i> epidermal strips	[184]
Mechanical stimulation				
Touch	Mono-phasic	YC3.6	<i>Arabidopsis</i> root tissue	[84]
Bending	Bi-phasic	YC3.6	<i>Arabidopsis</i> root tissue	[84]

Type of stimulus and recorded signature is given, together with the reporter protein used to monitor changes in cellular Ca^{2+} concentrations as well as the organism and type of material used within the experiments.

toxic metals [82], or in growing root hair tips [83] and mechanically stimulated roots [84].

A further advantage of the cameleon system is the ease to adapt the Ca^{2+} binding properties of the indicator. This recently enabled investigations of Ca^{2+} dynamics in plant organelles which contain much higher Ca^{2+} concentrations, like within peroxisomes [85] and the ER [86]. The concurrent expression of differentially targeted cameleon reporters has very recently allowed simultaneous recordings of mitochondrial and nuclear Ca^{2+} dynamics in *Arabidopsis* (Alex Costa, personal communication). Moreover, specific targeting of cameleon proteins to either the plasma membrane or to the vacuolar membrane nowadays allows to investigate the contribution of the major plant Ca^{2+} stores (the apoplast and the vacuole) to the cellular Ca^{2+} signaling in non-preceded resolution [87]. The growing number of laboratories that are currently using cameleon in plants will hopefully provide new and interesting findings about the dynamics and regulation of Ca^{2+} signals in plants in the near future.

3. Decoding Ca^{2+} signals in plants

For sensing and decoding the information that is represented in the specific Ca^{2+} signatures plants are equipped with a toolkit of Ca^{2+} sensor proteins. This toolkit is formed by rather complex families of EF hand Ca^{2+} sensor proteins in plants that are represented by the calmodulin (CaM) and calmodulin-like protein (CML) family, the family of Ca^{2+} -dependent protein kinases (CDPK), and the calcineurin B-like protein (CBL) family (Fig. 1). Of course plants also possess Ca^{2+} -binding proteins that do not rely on EF hand mediated Ca^{2+} binding. However, with a few exceptions their function and regulation is not well understood [88]. CaM is highly conserved in all eukaryotes, whereas CML, CDPK and CBL proteins have been identified only in plants and some bikont protists [6,7,89]. Conceptually, plant Ca^{2+} sensor proteins that function as signaling components have been classified into "sensor relays" and "sensor responders" [90]. For example, CDPKs combine a Ca^{2+} sensing function (EF hand motifs) and a responding function (protein kinase activity) within a single protein and have therefore been classified as sensor responders. Consequently, these kinases translate the information

encoded in the Ca^{2+} signatures into phosphorylation events of specific target proteins. In contrast, CaM/CML family members, which have no enzymatic function, alter downstream target activities via Ca^{2+} -dependent protein–protein interactions. Therefore, they represent bona fide sensor relay proteins. CBL proteins also belong to sensor relay proteins due to the lack of any enzymatic activity. However, CBLs specifically interact with a family of protein kinases designated as CBL-interacting protein kinases (CIPKs). Therefore, CBL–CIPK complexes have been considered as bimolecular sensor responders [91]. In the following we will discuss the current knowledge about mechanisms and biological functions of the major Ca^{2+} sensor protein families.

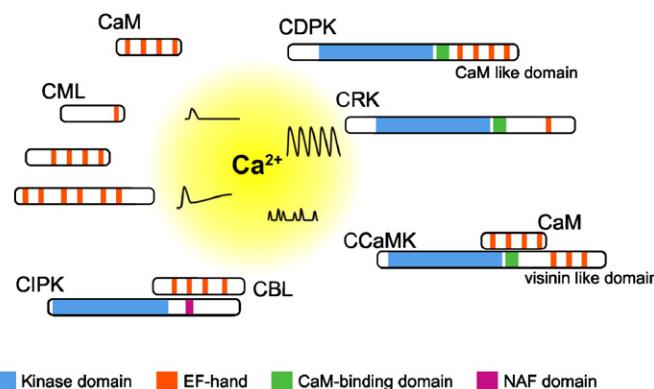


Fig. 1. Ca^{2+} sensor proteins in plants. Representative plant Ca^{2+} sensor proteins are shown and functional domains are highlighted. CaMs, CMLs and CBLs harbor EF hand motifs and regulate target proteins. They do not contain any additional functional domains. CBLs interact and modulate the activity of CIPKs, while CDPKs are directly activated by Ca^{2+} binding to the CaM like domain. CRKs are CDPK related kinases with a degenerated CaM like domain. In contrast to CDPKs, CCaMKs are dual regulated kinases. These proteins bind Ca^{2+} via a visinin like domain, while in addition Ca^{2+} -CaM binds to the regulatory domain of the kinase and mediates further activation. The different Ca^{2+} signatures represented in the center likely mediate a specific activation of these sensors contributing to the decoding of the signal.

4. Calmodulins and calmodulin-like proteins

The evolutionary conserved Ca^{2+} sensor calmodulin is encoded by a small gene family in plants. In *Arabidopsis*, 7 genes encode for 4 calmodulin (CaM) isoforms (CaM1/4; CaM2/3/5; CaM6; CaM7) which differ only in one to five amino acid residues [92,93]. In addition, plants contain several CaM-like (CML) proteins (50 in *Arabidopsis*), which in contrast to the classical CaMs contain variable numbers of functional EF hands (2 to 6) [92,93]. Although most CMLs are cytoplasmic proteins, some CMLs undergo lipid modifications resulting in membrane binding [92,94,95]. Moreover, several CMLs have been identified in different compartments of the cell. A vacuole targeted CML (CML18) was reported to regulate the sodium/proton antiporter NHX1 [96], while a chloroplast targeted CML appears to play a role in assembling Rubisco [97,98]. Furthermore, Tic32, a member of the large protein translocation complex at the inner chloroplast membrane, is a Ca^{2+} /CaM/CML binding protein which is regulated from the stromal side by an unknown CML [99]. Such CML could be CML41, which harbors a potential chloroplast import signal [92]. The number of identified CaM/CML regulated proteins in plants is increasing continuously. These targets belong to different protein and enzyme classes and are targeted to different organelles or are secreted to the apoplast [100–103]. Thereby, CaM/CMLs regulate a wide range of physiological processes like for example cell wall regeneration [104] and the growth of pollen tubes [105,106].

Several types of kinases are regulated by binding of Ca^{2+} /CaM. Some plant species possess dual regulated Ca^{2+} /CaM-dependent kinases (CCaMKs), which are involved in symbiosis of their root system with Rhizobia or mycorrhizal fungi [107,108]. These proteins bind CaM and additionally harbor a Ca^{2+} -binding domain that is composed of three EF hands and related to the protein visinin [109]. Several plasma membrane bound as well as cytoplasmic receptor like kinases are activated upon Ca^{2+} /CaM interaction. One example is CRLK1 from *Arabidopsis*, which functions in plant adaptation to cold-stress [110–113]. Upon Ca^{2+} /CaM binding CRLK1 can interact with and activates the MAP kinase kinase 1, thereby activating a MAP kinase pathway in the cold stress response [113]. Signaling during heat stress partially relies on the function of CaM3 [114,115]. CaM3 activates the CaM-binding protein kinase 3 (CBK3) which then phosphorylates the heat-shock-transcription factor (HSF) HSFA1a. This modulates the binding of the HSF to regulatory promoter elements thereby promoting the expression of heat shock proteins and mediating heat stress responses [116]. Moreover, it was reported that overexpression of the protein phosphatase PP7 also enhanced the expression of heat shock proteins and conferred thermo tolerance [117,118]. Remarkably, PP7 also interacts with CaM3 as well as with HSFA1a [118]. These findings support a model in which PP7 and CBK3 act on the same HSF target, where PP7 removes an inhibitory phosphate group. However, how the simultaneous binding of CaM3 to the kinase and the phosphatase contributes to the regulation of this complex needs to be determined.

Alternatively, transcriptional regulators can also be regulated directly by interaction with CaMs. At least 90 proteins were identified as interacting with CaMs/CMLs and harboring a DNA binding domain [101,119,120]. Among these were several representatives from the bZIP, WRKY and MYB families of transcription factors [101,121–125]. Calmodulin binding transcription activators (CAMTAs) represent a family of transcriptional regulators that all bind to CaMs [119,126,127]. CAMTAs function in diverse biological processes like hormone signaling and in the regulation of cold-regulated gene expression [128,129]. The activation of CAMTA3 by CaMs suppresses the transcription of EDS1 (Enhanced Disease Susceptibility 1), which regulates the synthesis of the phytohormone salicylic acid (SA) an important component in response to wounding and pathogen attack and in acquiring plant immunity [121]. In contrast to this negative regulation by CAMTAs, CaMs are also involved in the positive regulation of SA synthesis. The transcription factor CBP60g induces the expression of Isochorismate Synthase 1 (ICS1), an enzyme involved in SA synthesis [130]. The N-terminal

region of CBP60g from *Arabidopsis* can interact with CaMs and it was shown that CaM binding is required for the induction of SA synthesis [131]. The promoter element to which CBP60g binds was identified and this element is present in several other genes which are induced by pathogens, suggesting that Ca^{2+} /CaM/CBP60 are also involved in the expression of these genes [130]. Another example of transcriptional regulation by CaM/CML proteins is provided by the Ca^{2+} /CaM/CML binding to the nuclear protein IQD1 (IQ-domain 1) that regulates the expression of glucosinolate genes and the production of these metabolites, which are important against pathogen or herbivory attack [132].

A recently uncovered and very interesting example of the interconnection of the plant Ca^{2+} signaling machinery with other major signaling modules is provided by the finding that CaMs bind and activate the MAP kinase 8 (MPK8), which is required for production of reactive oxygen species after wounding. Remarkably, full activation of MPK8 also requires phosphorylation by its upstream MAPK signaling component MPK kinase 3 (MAPKK3). Consequently, MPK8 therefore represents a convergence point of the MAP kinase and Ca^{2+} /Calmodulin signaling pathways [133]. Interestingly, CaMs may in addition also function as repressors of a MAPK pathway, as several MAPK phosphatases were identified as CaM-binding proteins, which activity is increased upon binding of Ca^{2+} /CaM [134,135]. The simultaneous positive and negative regulation of biological responses by Ca^{2+} /CaM/CMLs suggests that these proteins are important for dynamically regulating and thereby fine tuning different response machineries.

While transcriptional regulation is in most cases regulated by CaMs interacting with transcription factors, CaM7 from *Arabidopsis* appears to represent a remarkably exception since it functions as a direct transcriptional regulator. Overexpression of CaM7 resulted in increased expression of light-inducible genes, which regulate the photomorphogenesis of plants. In contrast to the related CaMs2/3/5, which differ in only one amino acid compared to CaM7, only CaM7 was able to directly bind to the Z- and G-box promoter elements of light responsive genes [136]. These promoter elements are similar in sequence to the DRE site, a regulatory promoter element to which the monomeric DREAM protein from animals binds, a Ca^{2+} sensor belonging to the neuronal Ca^{2+} sensor protein family [137,138]. However, at the moment it remains to be addressed if Mg^{2+} and Ca^{2+} binding to CaM7 have similar regulatory functions, as in regulating the DREAM protein [139]. Together all these findings point to a remarkable wide range of mechanisms that CaMs and CMLs employ to fulfill their important roles in regulating a quite diverse range of biological processes.

5. Calcium dependent protein kinases and related proteins

Calcium -dependent protein kinases (CDPKs), also designated as CPKs, are unique Ca^{2+} regulated kinases as they arose by gene fusion of an upstream located serine/threonine kinase with a downstream EF hand Ca^{2+} binding domain which originated from a calmodulin gene [140]. CDPKs are present in plants and in apicomplexa and in general harbor four EF hand domains for Ca^{2+} binding [8,141]. In contrast “CDPK-related kinases” (CRKs) can possess a degenerated Calmodulin domain with non-functional EF hands [142,143]. In contrast to CDPKs from plants, CDPK6 from the apicomplexan species *Toxoplasma gondii* contains additional N-terminal EF hands [141,144].

In all CDPKs the kinase and calmodulin like domain are separated by a junction domain, which physically interacts with the kinase domain and therefore functions as a pseudo-substrate inhibiting the kinase activity towards target substrates. The current model of CDPK regulation supposes that at resting cytoplasmic Ca^{2+} concentrations the C-terminal lobe of the CaM like domain binds Ca^{2+} ions and interacts with the junction domain [8]. Upon an increase in the cytosolic Ca^{2+} concentration, additional Ca^{2+} ions bind to the N-terminal lobe of the Ca^{2+} binding domain resulting in a large conformational change [145,146]. This leads to the displacement of the autoinhibitory domain

from the kinase domain, followed by intra-molecular autophosphorylation resulting in activation of the kinase [145,147]. In contrast, CRKs can be active in absence of Ca^{2+} , but retained the ability and requirement to bind CaM in a Ca^{2+} -dependent manner that leads to full activation of the protein kinase [142,143,148].

In *Arabidopsis* 34 CDPKs and 8 CRKs are encoded in the genome [144]. Most of these proteins are predicted to be modified by N-terminal myristylation and S-acylation within their variable N-terminal domain [144,149]. The lipid modification of rice CPK2 and *Arabidopsis* CPK3 was experimentally confirmed and revealed a crucial role in membrane targeting of these proteins [150,151]. Other CDPKs, like CPK4 from *Arabidopsis* have been found to reside in the cytoplasm and nucleoplasm [152], or were associated with the cytoskeleton [153], the endoplasmatic reticulum [154] or peroxisomes [152]. These findings implicate that CDPKs can regulate diverse targets in various cellular contexts. In addition, membrane binding of CDPKs does not only appear to be important for directing the kinases into the vicinity of target proteins but could further modulate the kinase activity [155–157]. Especially various phospholipids, like phosphatidylinositol and phosphatidic acid, have profound effects on the CDPK protein kinase activity [155,157,158]. One potential binding site for these phospholipids was identified within the N-terminus of CPK1 from *Arabidopsis* [159]. Moreover, this N-terminal domain represents a potential binding site for 14-3-3 proteins [160] and has been found to be phosphorylated in vivo by an upstream kinase in CDPK2 and CDPK3 from tobacco [161]. This phosphorylation occurred only when the proteins were targeted correctly to the membrane. Although the precise consequences of the N-terminal phosphorylation remain to be determined, this phosphorylation is required for the biological function of the kinases [161]. One possible function could be the correct and specific recognition of target proteins, which is mediated by the variable domain [162].

The large family of CDPKs participates in regulating a wide range of physiological functions and developmental processes like root and pollen tube growth [163–167]. Functions of CDPKs have been established in carbon and nitrogen metabolism [168,169], salt and drought stress signaling responses as well as in ion transport processes like regulating potassium or Ca^{2+} homeostasis [170–172]. Remarkably, the function of these Ca^{2+} dependent kinases can counteract the function of other Ca^{2+} sensor proteins. The activity of the Ca^{2+} ATPase ACA2 is inhibited by phosphorylation within the N-terminal regulatory domain that is brought about by CPK1 [173], while activation of the ACA2 depends on binding of CaM [174]. In this way, different Ca^{2+} sensor proteins fine tune the speed of Ca^{2+} extrusion out of the cell, thereby contributing to the generation of a specific Ca^{2+} release signature.

Several CDPKs also fulfill important functions in various aspects of responses to pathogens. Pathogens like *Cladosporium fulvum* activate CDPK2 and 3 in tobacco [175,176] and are required for the adaptive regulation of the transcriptome [177]. Moreover, CDPKs can modulate a MAP kinase pathway that functions in pathogen response [178] and activate NADPH oxidases to promote the production of reactive oxygen species for defense [179]. In addition CPK1 from *Arabidopsis* regulates the production of SA for resistance [180].

However, a single CDPK can also function in diverse biological processes. For example, CPK3 is involved in mediating responses to salt stress [151] but also functions in regulating the rapid stomatal closure in response to the phytohormone ABA. In the latter response CPK3 functions together with the related CPK6 [181] since only *cpk3/cpk6* double knock-out plants were significantly impaired in the activation of the slow(S)-type anion efflux channels in response to ABA or extracellular Ca^{2+} . However, regulation of S-type channels appears not only to require CPK3/CPK6 function but likely also other CPKs. This conclusion is supported by reports that in the heterologous *Xenopus* oocyte system the activation of the slow(S)-type anion efflux channels SLAC1 and SLAH3 can be achieved by CPKs 21 and 23 [182,183].

The interconnection of CDPK signaling with various hormones like ABA and Methyl-jasmonate (MeJA) that impact on the regulation of guard cells appears to be quite complex. In contrast to the stomata closing reaction in response to ABA where CPK3 and CPK6 appear to function synergistically, the stomatal closure in response to MeJA only depends on function of CPK6, but not on the related protein CPK3 [184]. This suggests that only CPK6 is activated by MeJA, although Ca^{2+} transients in guard cells are induced by both ABA and MeJA [184]. Remarkably, other response pathways like the production of reactive oxygen species were still activated by MeJA in *cpk6* mutant plants, while these plants were specifically impaired in activating the slow vacuolar anion channel(s) and therefore failed to close stomata [184].

CDPKs are also involved in additional mechanisms that contribute to guard cell regulation and to the responses to ABA. CPK4 and CPK11, two highly related proteins, as well as CPK32, interact and ABA-dependently phosphorylate ABA-responsive bZIP transcription factors like ABF1 and ABF4 [185,186], which are important to mediate ABA mediated signaling during drought and salt stress [187]. CDPKs also function in signaling and adaptation reactions to other plant hormones. In tobacco, CDPK1 phosphorylates the transcriptional activator Repression of Shoot Growth (RSG) in response to the phytohormone gibberellic acid. The phosphorylation of Ser-114 enables the subsequent binding of 14-3-3 proteins to RSG, which then translocates from the nucleus into the cytosol thereby repressing the function of RSG [188]. Taken together, all these findings support the notion that CDPKs represent important convergence points of signaling pathways in response to different hormones and environmental cues which likely all involve the activation of the CDPKs by modulating the cellular concentration of Ca^{2+} .

6. The CBL–CIPK signaling network

Calcineurin B-like proteins (CBLs) are small Ca^{2+} -binding proteins, which belong to the calmodulin superfamily. They typically harbor four EF hand domains, but it is not clear if all EF hands in every CBL protein are indeed functional [189–191]. CBLs interact with a defined group of SNF related protein kinases, designated as CBL-interacting protein kinases (CIPKs) [192]. The *Arabidopsis* genome encodes 10 CBL proteins and 26 CIPK proteins [190,193]. Green algae species like *Ostreococcus* and *Chlorella* encode one CBL and one CIPK protein each [7,194]. However, CBLs and CIPKs appear to be absent in the green algae *Volvox carterii* and *Chlamydomonas reinhardtii*, suggesting that these algae rely on different Ca^{2+} signaling systems [2,193]. In fact, while the classical IP_3 receptor is also encoded in the genomes of *Volvox* and *Chlamydomonas* it is lacking in other plant organisms, especially in higher plants [5,91]. During the evolution of plants the number of CBL and CIPK genes increased, suggesting that CBLs–CIPKs evolved concurrently with the adaptation and colonization process of plants on the land and with their increasing ability to cope with fluctuating environmental conditions. Remarkably, similar to CDPKs, the occurrence of CBLs and CIPKs is not restricted to the viridiplantae lineage, as putative CBLs and CIPKs were identified in protozoan species such as *Trichomonas vaginalis* and *Naegleria gruberi* [7,193]. These findings raise interesting questions about the origin and the function(s) of these Ca^{2+} -decoding components in non-plant species.

Plant CBLs and the putative protozoan relatives share a common but atypical first EF hand [7]. Crystal structure analysis of CBL2 from *Arabidopsis* revealed that the Ca^{2+} binding region of the EF hand is composed of 14 amino acids, instead of 12 amino acids as in canonical Ca^{2+} -binding loops [195]. Nevertheless, Ca^{2+} binding occurs at the first EF hand, and also on EF hands which are not composed of canonical amino acids normally supposed to be required for Ca^{2+} binding [195,196]. Moreover, the mode of Ca^{2+} binding is further modulated by the interaction with CIPKs. While CBL2 binds four Ca^{2+} ions in complex with a regulatory domain of a CIPK, CBL4

alone binds only two Ca^{2+} ions [196,197]. In contrast, CIPK-complexed CBL4 binds four Ca^{2+} ions [195,198]. The biological consequences of these different Ca^{2+} -binding modes are currently not known and may not necessarily reflect the situation in planta. However, this situation may allow for differential modes of kinase activation and target recognition which is influenced by binding of the CBLs to CIPKs.

The crystal structure studies of the *Arabidopsis* CBL2 and CBL4 proteins consistently revealed that the overall structure of CBLs is composed of two globular domains, each of which contains one EF hand pair, separated by a short linker region between the globular domains [195,198]. Overall, the structural characteristics of CBL proteins are similar to NCS proteins, which are present in animals and fungi [195,199]. Latter proteins harbor an N-terminal hydrophobic crevice that is important for their interaction with effector proteins [199]. A similar mode of interaction was revealed by the crystal structure analysis of CBL2 in complex with the regulatory domain of CIPK14. In the CIPK-unbound state of CBL2, the hydrophobic pocket is intramolecularly blocked, but released upon CIPK14 interaction [195,196]. In addition, in CBL10 the C-terminal tail domain, which closes the pocket, is phosphorylated by the interacting partner CIPK24 at a serine residue, which is conserved in several other CBL proteins from *Arabidopsis* (Hashimoto et al., in revision) [200]. Mimicking phosphorylation of the serine residue enhanced the interaction between Ca^{2+} sensor and kinase partner protein, both in yeast and in *in vitro* interaction studies. The phosphorylation of CBL10 has also physiological relevance, since a non-phosphorylatable version of CBL10 was found to be unable to complement the salt-sensitive phenotype of *cbl10* mutant plants [200].

CIPKs are related to Sucrose-non-fermenting (Snf) protein kinases and AMP-activated kinase (AMPK) from yeast and animals, respectively [144,201]. Plant CIPKs and their related protozoan proteins share a common structural organization. Their N-terminal part contains the kinase domain, while the C-terminal part represents a regulatory region that includes a junction domain, which is required for kinase activity as well as additional functional domains [192,202]. One evolutionary conserved domain is the asparagine–alanine–phenylalanine (NAF)-domain [7,202]. The NAF domain is assumed to have a dual function. While it is required for CBL interaction, it is also implied to function as an auto-inhibitory domain. Binding of CBL proteins releases the kinase domain and thereby switches the kinase into an active enzyme [203,204]. This activation mechanism of CIPKs resembles that of CDPKs and also of CaMKII proteins [8,205]. The activation process of CIPKs is further enhanced by autophosphorylation and by trans-phosphorylation by an unidentified kinase contributing to full kinase activation [203,204]. Two MAP kinases were identified as potential kinases of CIPK by phosphoproteomic screens [206]. However, the position of the phosphorylation sites within the CIPKs modified by the MAP kinases is unknown, as well as if this phosphorylation by MAP kinases is also occurring in planta. A further less well-conserved domain is the protein phosphatase interaction (PPI) domain responsible for interactions with protein phosphatases of the class 2C (PP2Cs), such as ABI1 or AIP [207,208]. Structurally this domain is similar to the kinase-associated domain 1 (KA1) of the KIN2/PAR-1/MARK kinase subfamily [196,197]. Although the functional consequences of the CIPK-PP2C interactions are currently not understood, it appears conceivable that the kinase–phosphatase pairs antagonistically modulate each other's activity and/or reciprocally regulate the phosphorylation status of target proteins [2,207].

Localization studies of all ten *Arabidopsis* CBL proteins revealed that their variable N-terminal extensions determine their sub-cellular targeting. The CBL proteins 1, 4, 5 and 9 harbor N-terminal N-myristoylation as well as S-acylation sites, which mediate plasma membrane targeting [194,209,210]. On the other hand, in CBL2, CBL3, CBL6 and CBL10 the N-terminal extensions, which lack N-myristoylation sites, are required for proper tonoplast targeting [194]. In contrast to CBL proteins, most

CIPKs when expressed as GFP fusion exhibit a cytoplasmic and nucleoplasmic localization [194,211]. However, CBL–CIPK interaction analyses using Bimolecular Fluorescence Complementation (BiFC) revealed that the sub-cellular localization of CIPKs incorporated into distinct CBL–CIPK complexes is determined by the identity of their CBL moiety [194,209,211–213]. This further suggests that the CBL-dependent specific and distinct sub-cellular targeting of CBL–CIPK complexes to different membranes contributes to generating spatial specificity in Ca^{2+} signal decoding [7]. Importantly, preferential complex formation of individual CBLs with defined subsets of CIPKs has been observed in yeast as well as in planta interaction analyses and therefore appears to be one of the mechanisms generating the temporal and spatial specificity of Ca^{2+} signals in plant cells [194,202].

Reverse genetic analyses revealed that CBLs and CIPKs contribute to many different stress responses like for example salt, cold and drought stress [214–218] and to responses to the phytohormone ABA [211,212,219,220]. Moreover, CBL–CIPK complexes modulate a metabolic switch in response to flooding stress in rice [221]. Recent studies further provided important mechanistic insights how CBL–CIPK complexes regulate diverse processes and identified target proteins for CBL–CIPK complexes. Plant potassium homeostasis is regulated by the concerted action of the plasma membrane bound CBLs 1 and 9 and their interacting protein kinase CIPK23 (Fig. 2). Under potassium deprivation they activate the shaker-like potassium inward rectifying channel AKT1 in roots and thereby allow K^+ uptake under K^+ limiting conditions [222,223]. Remarkably, the very same CBL–CIPK complexes are also required in nitrate sensing and transport, mediated by the nitrate-receptor-transporter CHL1/NRT1.1. Especially under low nitrate concentrations, CIPK23 activated by CBL1–CBL9 phosphorylates the nitrate-transporter, which confers a change in affinity of the transporter towards nitrate [224]. Although further studies are needed, it is tempting to speculate that CBL1/9–CIPK23 complexes represent general “nutrient sensing” regulators that may also modulate the uptake of other ions like phosphate. Interestingly, CBLs 1/9 can interact with other CIPKs, and CIPK23 can interact with additional CBLs. However, only this specific combination of Ca^{2+} sensors and kinase is able to mediate AKT1 and NRT1.1 target activation, implicating that both components within the CBL–CIPK complex contribute to generating target specificity [224,225]. Similarly, only CBL4 and its interacting kinase CIPK6 can modulate the activity of the K^+ channel AKT2 [210]. Moreover, this recent study revealed a novel mechanistic principle how target proteins can be regulated by CBL–CIPK complexes. The analyses of AKT2 regulation showed that the Ca^{2+} sensor CBL4 together with the interacting protein kinase CIPK6 modulates the activity and plasma membrane targeting of the K^+ channel AKT2 from *Arabidopsis* by mediating translocation of AKT2 to the plasma membrane [210]. Importantly, an isolated regulatory C-terminal domain of CIPK6 was sufficient for mediating CBL4-dependent and Ca^{2+} -dependent channel translocation from the endoplasmic reticulum membrane to the plasma membrane by a novel targeting pathway that is dependent on dual lipid modification of CBL4 by myristoylation and S-acylation (Fig. 2) [210]. Thereby this work uncovered for the first time in plants a mechanism of ion channel regulation in that a Ca^{2+} sensor modulates K^+ channel activity by conveying a kinase interaction-dependent but phosphorylation-independent translocation to the plasma membrane. Considering the large number of existing CBL–CIPK complexes of which only a few have been characterized in detail it can be safely predicted that further studies will identify many more targets of CBL–CIPK complexes that are involved in diverse cellular processes.

7. Conclusions and perspectives

Ca^{2+} signaling steps are now firmly established as being involved in almost every biological process in plants. During recent years we have witnessed tremendous progress in our knowledge of Ca^{2+}

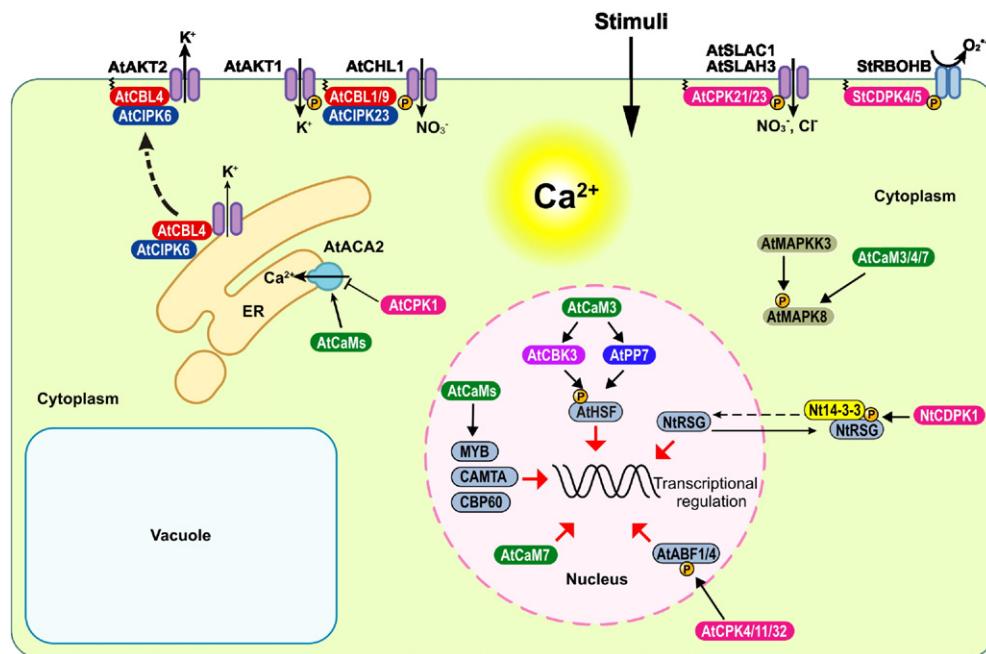


Fig. 2. Ca^{2+} -mediated signal transduction processes in plant cells. Examples of signal transduction processes mediated by CaMs, CDPKs and CBL-CIPK complexes together with their respective target proteins, which were investigated in different plant species (indicated by the acronyms: At, *Arabidopsis*; St, potato; Nt, tobacco). See text for further details. Phosphorylation of target proteins is indicated by the yellow circled P.

signaling processes in plants and especially in our understanding of Ca^{2+} decoding processes. Plants possess an extensive and complex toolkit of Ca^{2+} binding proteins that function as Ca^{2+} sensors for deciphering stimulus-specific Ca^{2+} signatures. CaMs, CMLs, CDPKs and CBL-CIPK complexes form intricate signaling networks for translating Ca^{2+} signatures into downstream responses. Major transduction routes are represented by phosphorylation events of target proteins like ion channels and by transcriptional regulation (Fig. 2). The network-like character of these signaling systems likely represents a mechanistic basis for the flexible but robust information processing that we observe in plants. Our knowledge about biochemical properties and physiological functions of many Ca^{2+} sensor proteins or sensor/kinase complexes has been greatly advanced. Critical for these achievements was the identification of first target proteins for CDPKs and CIPKs. Moreover, our understanding of functional principles governing information processing in such signaling networks has significantly improved and revealed multiple layers of regulation. It has been established that parameters like the specific Ca^{2+} binding affinities, the specific cellular concentration and sub-cellular localization as well as the specific interaction affinities of Ca^{2+} decoders are critical components that contribute to generating specificity in signal-to-response coupling. However, it still remains a main challenge to uncover how exactly defined Ca^{2+} signatures are sensed and translated by all these different Ca^{2+} sensor proteins. In this regard it will be important to systematically determine the differential transcription and protein regulation mechanisms that are responsible for assembling these different signaling modules and pathways on a whole systems level. In addition, it will be most interesting to understand how the distinct Ca^{2+} signaling systems that often function in the same physiological processes in plants are interconnected and cross-regulated. While it is easily appreciable that the interaction-based CaM signaling system provides alternative means for the regulation of a specific biological process than the phosphorylation-based CDPK and CBL-CIPK signaling systems, it is much less obvious why the latter two kinase gene families co-exist. Finally, we are now well positioned to elucidate the convergence points and mechanisms that interconnect Ca^{2+} signaling with other second messengers and hormone regulated signaling systems in plants.

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