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Mini-review

Calmodulin and calmodulin-like proteins in plant calcium signaling

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ABSTRACT

Calmodulin (CaM) is a primary calcium sensor in all eukaryotes. It binds calcium and regulates the activity of a wide range of effector proteins in response to calcium signals. The list of CaM targets includes plant-specific proteins whose functions are progressively being elucidated. Plants also possess numerous calmodulin-like proteins (CMLs) that appear to have evolved unique functions. Functional studies of CaM and CMLs in plants highlight the importance of this protein family in the regulation of plant development and stress responses by converting calcium signals into transcriptional responses, protein phosphorylation or metabolic changes. This review summarizes some of the significant progress made by biochemical and genetic studies in identifying the properties and physiological functions of plant CaMs and CMLs. We discuss emerging paradigms in the field and highlight the areas that need further investigation.

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1. Introduction

Calcium (Ca^{2+}) plays a major role in many aspects of plant growth and development including stress responses. In addition to its function as an essential plant nutrient and as a structural element in the plant cell wall, Ca^{2+} is one of the intracellular messengers used to relay information from diverse developmental and environmental stimuli to the cellular machinery responsible for mounting biological responses. A lot of abiotic and biotic factors including light, temperature, salt and osmotic stress, phytohormones and pathogen-derived molecules elicit Ca^{2+} signals that generally occur in the form of transient increases in cytosolic Ca^{2+} concentration. The dynamic changes in free Ca^{2+} level in the cytosol result from a tight control of Ca^{2+} movements, via channels and pumps, between the cytosol where resting Ca^{2+} level is kept low, and neighboring compartments with high Ca^{2+} content such as the vacuole and apoplast. The shape of cytosolic Ca^{2+} signals (the duration, amplitude and frequency) is defined by the nature of the stimulus perceived, and the stimulus-specific pattern of Ca^{2+} pulses, the so-called Ca^{2+} signature, is believed to encode information that specifies the nature of the response [1]. Variations of free Ca^{2+} level also occur in cell organelles including the nucleus, the chloroplasts and the mitochondria [2]. Of particular interest, the nucleus can generate Ca^{2+} signals on its own, and elevations in nuclear free Ca^{2+} level are observed in response to diverse stimuli, indicating a role for Ca^{2+} signals in the regulation of nuclear functions.

As input signals, stimulus-associated Ca^{2+} signatures are recognized and translated into output responses by sensing/responding systems. Most Ca^{2+} sensors bind Ca^{2+} using the EF-hand motif, a helix-loop-helix structure that binds one Ca^{2+} ion. EF-hands often occur in pair forming a domain with positive co-operativity, thereby minimizing the Ca^{2+} signal required to reach protein saturation [3]. Typically, Ca^{2+} binding to a Ca^{2+} sensor induces a conformational change of the protein that either promotes its interaction with downstream effectors or modulates its own catalytic activity. Plants have evolved a superfamily of EF-hand proteins represented by about 250 proteins in rice and *Arabidopsis thaliana*, a well studied model-plant, compared to 132 and 83 EF-hand proteins in *Drosophila melanogaster* and human respectively [4]. Even though many EF-hand proteins remain to be characterized, the extended set of Ca^{2+} -binding proteins in plants appears as a valuable tool kit allowing a versatile use of Ca^{2+} signals in plant biology. Current information on EF-hand proteins that behave as transducers of Ca^{2+} signals in plants comes from calmodulin (CaM) and calmodulin-like proteins (CMLs), calcineurinB-like proteins (CBLs) and Ca^{2+} -dependent protein kinases (CDPKs) [5–7]. CaM, CMLs and CBLs lack an effector domain like the kinase domain of CDPK, and they function as sensor relays via Ca^{2+} -dependent interaction with effector proteins. CBLs specifically interact with a family of protein kinases designated as CBL-interacting protein kinases, whereas CaM and CMLs interact with a wide range of proteins. In contrast to CaM, a universal Ca^{2+} sensor in eukaryotes, CMLs, CBLs and CDPKs are mainly restricted to plants and some protists.

Considerable progress in our understanding of Ca^{2+} signaling systems in plants has been made over the last decade, and critical

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components for generating and processing Ca^{2+} signals have been identified. The integration of Ca^{2+} signaling into networks maps and systems biology is a topic of intensive research that is beginning to shed light on how this ubiquitous messenger mediates stimulus-specific responses. The conceptual framework and the tool kit of Ca^{2+} signaling have been periodically discussed and updated in excellent reviews; therefore, our purpose is to summarize most recent advances in our understanding of Ca^{2+} -decoding processes mediated by the CaM/CML family in plants. Additional information on the topic covered here and other aspects of Ca^{2+} signaling in plants can be found in other recent reviews [8,9].

2. CaM and CML proteins in plants

With the completion of plant genome sequencing projects, the presence of multiple genes predicted to encode identical or highly similar CaM isoforms and diverse CMLs appears to be a typical feature of plant genomes. At the whole genome scale, the CaM/CML family is represented by 7 CaM and 50 CML genes in *Arabidopsis*, 5 CaM and 32 CML genes in rice [10,11]. This expansion of the CaM/CML family is a characteristic of CaMs in plants, and a major challenge is to test experimentally the physiological significance of each of these genes. Accumulating evidence suggests that each of CaM genes have taken distinct and significant functions. The comparison of CaM and CML gene structures and amino acid sequences indicates that CMLs can be broadly grouped into two major groups; those that display >50% sequence identity with typical CaM and contain an intron at the same position as in CaM genes, indicating their close relationship with CaM, and those that exhibit low degrees of identity (<50%) with CaM and various gene structures. In the latter group, CMLs exhibit high structural divergence, suggesting that they have adopted novel functions. Recent data support this assumption and some examples of the divergent expression patterns of CaM/CML genes, the unique biochemical properties and/or subcellular localizations of the gene products are presented below.

2.1. Biochemical properties

CaM is a prototypical Ca^{2+} sensor that interacts with and regulates the activity of a wide range of proteins. It consists of two globular domains, each harboring a pair of EF-hands, connected by a flexible helical region. Ca^{2+} binding to the EF-hands induces the exposure of hydrophobic surfaces that form high affinity binding sites for downstream effectors. Analysis of the mechanisms of CaM/target interaction has revealed that the ability of CaM to accommodate a large array of proteins is due to the flexible structural arrangement between its globular domains and the plasticity of hydrophobic surfaces that form binding interfaces [12]. Plants possess some extremely similar CaM isoforms (3 in *Arabidopsis* and 2 in rice) with minor changes in their primary sequence that determine target specificity [13]. The repertoire of CaM targets in plants includes metabolic enzymes, kinases and phosphatases, transcription factors, channels and pumps, cytoskeletal proteins, proteins of unknown function, and the list is still growing with the use of new tools for interactive proteomic analysis [14,15]. The impressive list of CaM-binding proteins illustrates the versatility of CaM function; however, in many cases, the regulation of target proteins by CaM and the importance of CaM/target interaction *in planta* remain to be demonstrated. Interestingly, a recent study has revealed a novel mechanism of CaM action through a direct interaction of a CaM isoform with DNA. AtCaM7, an *Arabidopsis* CaM isoform, was found to specifically recognize the Z-box and G-box motifs, two similar regulatory elements in light-responsive promoters, whereas AtCaM3, an isoform differing from AtCaM7 by only one amino acid, is

unable to bind these promoters [16]. Chromatin immunoprecipitation experiments confirmed the *in vivo* interaction of AtCaM7 with gene promoters. AtCaM7 was also shown to promote light-induced gene expression, thus supporting the physiological significance of the CaM/DNA interaction. This unexpected finding reveals that CaM can behave as a sequence-specific DNA-binding protein, as observed with other EF-hand proteins like DREAM, a well-characterized Ca^{2+} -dependent transcriptional repressor in animals [17]. This also suggests a direct translation of Ca^{2+} signals by AtCaM7 into the regulation of gene expression; however, the effect of Ca^{2+} on the DNA-binding activity of the CaM isoform has not been investigated. Another report indicates that AtCaM7 interacts *in vitro* with transcription factors [18]. It is therefore tempting to speculate that this CaM isoform may be capable of functioning as a recruiter protein through a dual interaction with DNA and transcription factors. To test this hypothesis, it will be interesting to determine the structure of the CaM/DNA complex, and to examine whether the interaction of AtCaM7 with additional proteins is required for the regulation of light-inducible genes.

Similar to CaM, CMLs are mostly composed of EF-hands and lack other known functional domains. Most CMLs (31/50 in *Arabidopsis* and 20/32 in rice) are predicted to have 4 EF-hands as in CaM, but a majority of them are less than 50% identical to CaM. Variations in the primary sequence of CMLs occur all along the protein, suggesting that they have unique properties in Ca^{2+} binding and target recognition or activation. Despite sequence divergence in the EF-hands, CMLs appear to behave as Ca^{2+} sensors. Several CMLs were shown to display an electrophoretic mobility shift in the presence of Ca^{2+} , a typical feature of a Ca^{2+} sensor [19,20]. A detailed analysis of AtCML42, a protein with only 3 predicted EF-hands indicates that Ca^{2+} affinity is within the range expected for Ca^{2+} sensors, and the protein displays Ca^{2+} -induced exposure of hydrophobic surfaces [21]. Variations in the number and sequence of EF-hands among other CMLs are likely to confer distinct ion-binding properties (ion selectivity and affinity), but this has to be experimentally demonstrated. Diverse proteins including kinases and transcription factors have been identified as CML-binding proteins [18], and the list is expected to grow because only a handful of CMLs have been used so far as bait in screening experiments. Evidence for CML/target interaction in plant cells and specificity in the protein interaction have been reported, suggesting that CMLs can exert unique functions by regulating their own targets [22]. Comparative modeling of the three-dimensional structure of CMLs, definition of the CML-binding domain and structural analysis of CML/target complexes are needed to explain the selective CML/target interaction.

2.2. Expression and localization patterns

There is no doubt that the expression and localization of CaM isoforms and CMLs are critical parameters for defining their functions. The expression of CaM and CML genes is regulated by a variety of stimuli including biotic and abiotic stress, hormones and chemical treatments [23]. Based on microarray experiments, the expression patterns of individual members of the CaM/CML family often differ spatially, temporally and in magnitude in response to the stimuli, suggesting specificity in their roles in signal transduction. CaM and CMLs are also highly regulated throughout normal development. Compilation of transcriptome analyses shows a differential expression of *Arabidopsis* CML genes over the developmental stages, and detailed investigations using promoter-based reporter gene analysis have provided evidence for distinct tissue and cell-type expression profiles among CaM and CML genes [20,24,25].

As plants can establish local Ca^{2+} signatures by restricting Ca^{2+} pulses to a particular compartment of the cell, the subcellular

distribution of Ca^{2+} sensors appears as an additional level of regulation for defining the outcomes of Ca^{2+} signal transduction. It is becoming clear that intracellular trafficking of CaM and CMLs occurs in response to stimulation and generates spatially defined pools of Ca^{2+} sensors. Some examples of the distribution of CaM isoforms, CMLs and downstream targets in plant cells are presented in Fig. 1. CaM has often been reported to display nucleo-cytoplasmic partitioning which is consistent with the fact that the majority of its targets are cytosolic or nuclear proteins. CaM also binds to membrane-associated proteins, and elegant studies have shown the recruitment of cytoplasmic CaM by MLO, a plasma membrane integral protein, upon a pathogen challenge [26]. Like CaM, the majority of CMLs do not contain any particular localization signatures and a nucleo-cytoplasmic distribution of several CMLs has been observed, using fusion proteins with fluorescent tags. CML trafficking like the translocation of AtCML19 (or AtCENTRIN2) from the cytosol to the nucleus in response to UV exposure, has also been reported [27]. A few CMLs contain the consensus sequence for potential myristoylation or prenylation, and experimental evidence for the functionality of these sites has been obtained. PhCaM53, a CML from petunia is associated to the plasma membrane by prenylation of its C-terminal extension, and accumulates in the nucleus when prenylation is inhibited [28]. Interestingly, the prenylation status of the protein and its subcellular location are modulated by light/dark exposure and carbon source availability. Furthermore, the ectopic expression of the wild-type PhCaM53 and a nuclear-localized mutant form in transgenic plants was found to promote distinct phenotypes, indicating that the distribution of the protein influences its roles.

3. Physiological functions of CaM and CMLs

Data on CaM/CML gene expression suggest that they are involved in many developmental and stress-induced signaling

pathways. Accumulating evidence points to specialized functions among members of the CaM/CML family, and the importance of CaM interaction with target proteins *in planta* has been demonstrated. Recent data illustrating CaM and CMLs functions in plant development and stress responses are presented below (see also Fig. 1).

3.1. Roles in plant growth and development

Recent reports document the roles of CaM isoforms and CMLs in various developmental processes including morphogenesis and reproduction. As previously mentioned, AtCaM7 is involved in light-mediated gene expression, and AtCaM7 overexpression in transgenic plants was found to promote photomorphogenic growth [16]. In addition, the ectopic expression of AtCaM7 partially suppresses the phenotype caused by mutations in the *HY5* gene, an essential regulator of photomorphogenesis, indicating that both proteins act to control light-induced seedling development. AtCaM2, another CaM isoform, appears to play a role in pollen germination. A loss-of-function mutation in the *AtCaM2* gene causes a significant reduction in pollen germination [29]. Interestingly, several CaM-binding proteins including ACA9 (an auto-inhibited Ca^{2+} -ATPase), NPG1 (No Pollen Germination) and CNGC18 (a cyclic nucleotide-gated channel) are known to play key roles in pollen germination and pollen tube growth in Arabidopsis [30–32]. It will be interesting to examine if the activities of these targets are differentially regulated by *Arabidopsis* CaM isoforms. CaM also controls plant development through interconnections with plant growth regulators such as brassinosteroids and auxins. CaM interacts with DWARF1 (DWF1) a protein that catalyzes a rate-limiting step in the biosynthesis of brassinosteroids [33]. Mutations in the *DWF1* gene cause a deficiency in brassinosteroid levels that results in a dwarf phenotype. Importantly, this phenotype can be rescued by overexpressing the wild-type protein but not a mutated

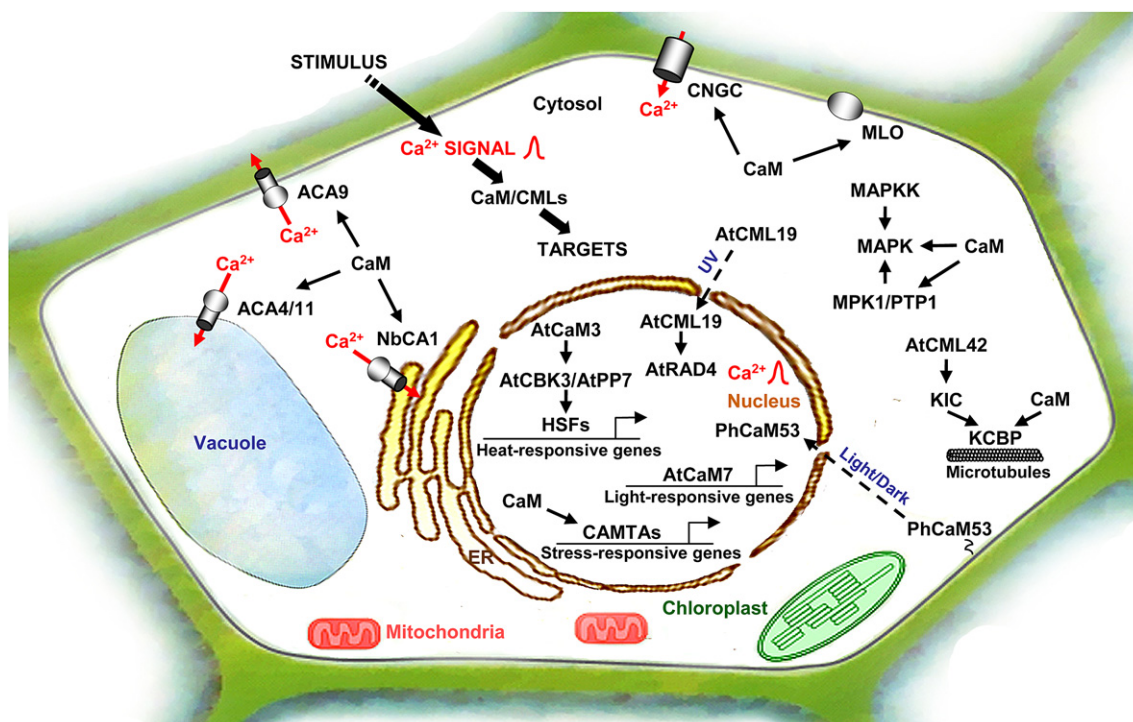


Fig. 1. Examples of the signaling modules made by CaM, CMLs and their target proteins in plant cells. Changes in the location of specific CaM or CMLs in response to stimuli are indicated by a broken arrow. Details are described in the text.

form impaired in CaM binding, thus demonstrating a critical role for Ca^{2+} /CaM complex in DWF1 function. There is evidence supporting a role for CaM in mediating auxin action. Previous studies have shown that the expression of several CaM targets is regulated by auxin [34]. Among these, a CaM-dependent transcriptional activator (CAMTA1) controls the expression of genes known to be associated with auxin signaling, and a recent study indicates that null mutations in the *CAMTA1* gene confer hyper-responsiveness to auxin during seedling development [35].

Mutations in CML genes were also found to cause developmental defects. A knockout mutant in the *AtCML42* gene displays abnormal morphology of trichomes [21]. *AtCML42* specifically interacts with KIC, a kinesin-interacting Ca^{2+} -binding protein, known to regulate trichome morphogenesis. KIC binds Ca^{2+} via a single EF-hand motif and interacts with a kinesin-like protein (KCBP), previously identified as a CaM target [36]. KIC and CaM compete for the same binding site on KCBP and negatively regulate the binding of KCBP to microtubules. However, the inhibitory effect of KIC requires a lower concentration of Ca^{2+} than that required for inhibition by CaM. Genetic studies have indicated that KCBP plays an important role in trichome morphogenesis [37]. Collectively, these data reveal that a network of Ca^{2+} -binding proteins is involved in the regulation of cell morphogenesis by modulating the function of KCBP and microtubule organization (Fig. 1). A role for a CML in reproductive development was recently illustrated by the alteration in the transition to flowering caused by the deregulation of *AtCML24* gene expression in transgenic plants [24]. In conjunction with *AtCML23*, *AtCML24* inhibits the expression of *FLOWERING LOCUS C*, a central repressor of flowering through an as-yet undefined mechanism [38]. Identification of the targets of those CMLs is the next step to clarify their roles in the regulation of flowering.

3.2. Roles in plant responses to environmental stress

Excessive variations in temperature, water availability and soil quality, or attacks by pathogens are common forms of stress that affect plant growth and limit productivity. In order to promote physiological and/or morphological changes and to achieve optimal plant growth and productivity under adverse conditions, environmental factors perceived by plant cells are relayed by Ca^{2+} ions, cyclic nucleotide monophosphates, reactive oxygen species and other small molecules. Changes in cytoplasmic free Ca^{2+} levels have been reported to be one of the earliest cellular responses to various abiotic and biotic stresses, and recent findings have identified CaM and CMLs as key components in stress signal transduction.

3.2.1. Abiotic stress

All organisms respond to elevated temperatures by producing heat-shock proteins (HSPs). Expression of HSPs is regulated by heat-shock transcription factors (HSFs) which play a crucial role in the acquisition of thermotolerance [39]. The activity of HSFs is known to be regulated by reversible phosphorylation via mechanisms involving multiple phosphorylation sites and distinct protein kinases/phosphatases [40]. A recent study has shown that *AtCaM3* mutants exhibit a reduction in thermotolerance which correlates with a decrease in the activity of HSFs and the synthesis of HSPs [41]. Interestingly, knockout mutations in CaM targets including *AtCBK3*, a protein kinase and *AtPP7*, a protein phosphatase impair the thermotolerance as reported for *AtCaM3* [42,43]. Moreover, *AtCBK3* and *AtPP7* are known to interact with HSFs. Thus, genetic dissection of heat-shock signal transduction has led to the identification of a CaM isoform, two downstream targets involved in the reversible modification of HSFs by phosphorylation, as key determinants in the acquisition of thermotolerance, but whether all

these components act in the same signaling pathway needs to be investigated. CaM also functions in cold acclimation through the regulation of cold-responsive gene expression. Exposure to low non-freezing temperatures induces the expression of CBF transcription factors, followed by the expression of the well-characterized CBF regulon which imparts freezing tolerance [44]. Mutation in the *CAMTA3* gene (a CaM-dependent transcriptional activator) was recently reported to reduce the cold-induced accumulation of transcripts for CBF1 and CBF2 [45]. Furthermore, plants carrying a *CAMTA1/CAMTA3* double mutation are impaired in freezing tolerance, indicating an important role for the CAMTA family in cold acclimation.

Several members of the *Arabidopsis* CML family have been found to be highly and/or early induced by abiotic stress factors such as drought, salt and osmotic stress, wounding and irradiation, but in most cases, experimental evidence for the roles of CMLs as regulators of stress tolerance is lacking. *AtCML9* knockout mutants show an enhanced tolerance to drought and salinity stress, and a strong accumulation of branched-chain amino acids [25,46]. Accumulation of those amino acids is known to be induced by abiotic stress in order to provide an alternative carbon source during stress conditions [47]. Several CML9-binding proteins including protein kinases and transcription factors have been identified [18,22], but their roles in stress responses needs to be explored. As already mentioned, *AtCML19* (or *AtCENTRIN2*) translocates from the cytosol to the nucleus in response to UV exposure, and this CML was recently shown to play a role in the repair of cell damage caused by UV irradiation. Plants that are defective in *AtCML19* are hypersensitive to UV-C exposure, exhibiting a reduced efficiency in the repair of DNA damage [48]. The rapid nuclear localization of *AtCML19* upon UV-C exposure and its Ca^{2+} -dependent interaction with *AtRAD4*, a component of the DNA damage recognition complex supports a role for *AtCML19* in an early step of the DNA repair process [27].

3.2.2. Biotic stress

Several studies have shown that the deregulation of CaM expression affects plant immune responses. Down-regulation of *NtCaM13* expression in tobacco results in enhanced susceptibility to virulent bacteria and fungi, whereas overexpression of pepper *CaM1* and *AtCML43* in transgenic plants confers enhanced resistance to pathogens [49–51]. Elevation in cytosolic Ca^{2+} level is an essential signaling event during plant–pathogen interactions and recent studies identified several CaM-regulated proteins as critical regulators of defense responses. For instance, plants lacking a functional Ca^{2+} -conducting cyclic nucleotide-gated channel (CNGC2) have impaired cytosolic Ca^{2+} elevation in response to pathogens, and failed to produce the hypersensitive response (HR), a localized programmed cell death at tissue surrounding the infection site that limits pathogen invasion [52,53]. CNGCs are able to bind CaM at a region overlapping the cyclic nucleotide binding domain, and it has been proposed that CaM negatively regulates CNGCs by reversing channel activation by cyclic nucleotides [54]. CaM-activated Ca^{2+} pumps were also found to play a critical role in the modulation of the HR. Silencing *NbCA1*, an endoplasmic reticulum-localized Ca^{2+} -ATPase in tobacco, alters the cytosolic and nuclear Ca^{2+} signatures and accelerates the HR following exposure to a pathogen [55]. A double mutation of the vacuolar Ca^{2+} -ATPases, *ACA 4* and *11* in *Arabidopsis*, was found to result in a high frequency of HR-like lesions and a more effective defense response to pathogen attack [56]. All together, these findings point to the importance of CaM targets including Ca^{2+} pumps and non-specific cation channels like CNGCs, in the generation of Ca^{2+} signatures, and suggest that CaM contributes to shape pathogen-associated Ca^{2+} signatures by regulating the activity of Ca^{2+} transport systems.

Other studies have revealed connections between Ca^{2+} signals and other signaling molecules including nitric oxide (NO) and salicylic acid via the action of CaM and/or CMLs. In addition to Ca^{2+} rise, generation of NO is an early signaling step which is essential for the development of the HR. As mentioned above, a loss-of-function mutation in CNGC2 impairs not only the pathogen-induced Ca^{2+} signals and the HR, but also NO production [57]. Loss of the HR phenotype in *cngc2* mutants can be rescued by the application of a NO donor, indicating that NO acts downstream of Ca^{2+} signals to initiate the HR. Mutations in CML24 was also found to inhibit pathogen-induced NO generation and the HR [58]. Collectively, this suggests that Ca^{2+} influx into the cytosol promoted by the activation of CNGCs, activates a CML protein which then induces NO synthesis as an additional step in a signaling cascade leading to the HR.

Salicylic acid (SA) is a phenol hormone acting as a primary signal in plant immunity, and a link between CaM and SA-mediated signaling has been proposed. Plants with knockout mutations in *CBP60g*, a pathogen-inducible CaM-binding protein, were found to display a defect in pathogen-induced SA accumulation and an enhanced susceptibility to a bacterial pathogen [59]. Additional analyses indicate that mutations in *CBP60g* that abolish CaM binding, do not restore the SA production and defense response, demonstrating a positive role of CaM in the regulation of SA levels. Importantly, CaM also functions as a negative regulator of SA signaling. Constitutive disease resistance and elevated levels of SA have been observed in *CAMTA3* mutants [60]. *CAMTA3*, a CaM-binding protein, was shown to repress the expression of *EDS1*, a positive regulator of SA production by direct binding to the *EDS1* gene promoter. Collectively, this indicates that CaM targets play antagonistic roles in pathogen-induced production of SA, allowing the Ca^{2+} /CaM complex to promote SA production and SA-dependent defense, but also to prevent over-accumulation of the hormone, potentially leading to developmental defects such as dwarfism and/or cell death.

Like other eukaryotes, plants use mitogen-activated protein kinase (MAPK) cascades to regulate various cellular processes in response to a broad range of biotic and abiotic stress [61]. These cascades promote the transient activation of MAPKs by a dual phosphorylation of Thr and Tyr within the activation loop of the MAPK. Recent studies indicate that MAPKs are not only regulated through phosphorylation by upstream kinases, but also by direct binding of different protein factors. In this respect, the subgroup D MAPKs interact with CaM in a Ca^{2+} -dependent manner, and both CaM and MKK3, a MAPK kinase, are required for the full activation of MAPK8 after wounding [62]. Genetic experiments resulting in the constitutive activation of MAPKs were also found to result in detrimental effects, underlining the importance of a negative regulation of MAPK signaling. MAPK phosphatases (MKPs) are negative regulators of MAPKs, and recent progress in analyzing plant MKP mutants has revealed their important role in fine-tuning MAPK signaling. In particular, the dual-specificity phosphatase MKP1 and the protein tyrosine phosphatase PTP1 negatively regulate defense responses and resistance to a bacterial pathogen by counterbalancing the activation of two MAPKs (MPK3 and MPK6) [63,64]. Interestingly, MKP1 and PTP1 bind CaM, and the phosphatase activity of MKP1 is increased by CaM in a Ca^{2+} -dependent manner [65]. Thus, Ca^{2+} and MAPK signaling pathways appear to be connected through the regulation of plant MAPKs and MKPs by CaM.

Collectively, these data show that CaM/CML-mediated defense signaling pathways form a complex regulatory network through cross-talks with other signaling pathways.

4. Conclusions and perspectives

During the past years, significant progress in our knowledge of the CaM/CML family in plants has been achieved in (i) identifying unique functions among members of this class of Ca^{2+} sensors, (ii)

demonstrating the biological relevance of CaM/target interaction under physiological conditions and (iii) describing the dynamic network built up by CaM-mediated signaling and other pathways. The unprecedented finding of a plant CaM isoform acting as a DNA-binding protein also extends the versatility of this ubiquitous Ca^{2+} transducer. These data provide answers to the central question of how and to what extent a simple ion like Ca^{2+} can specifically control so many processes? It is becoming evident that the large family of CaM/CML proteins in plants provides a powerful tool kit for a flexible processing of the information carried by various Ca^{2+} signatures. Accumulating evidence shows that the Ca^{2+} binding affinity, the dynamic subcellular localization and expression pattern, the specific interaction affinity of individual CaMs and CMLs are critical parameters that contribute to generate specificity in the translation of Ca^{2+} signatures into appropriate responses. However, much remains to be done on CaMs and CMLs in plants because only a few members from this large family have been thoroughly characterized. Investigations into the biochemistry and physiological functions of these Ca^{2+} sensors have to be pursued in order to discriminate specificity from redundancy within the CaM/CML family. This will require investments into biochemical and structural analysis of CMLs in order to understand how Ca^{2+} regulates these proteins. Expanding reverse genetics is needed to explore the physiological functions of these Ca^{2+} sensors. Defining the CML interactome is another major challenge to fully understand how these proteins act to translate Ca^{2+} signals into cellular responses. Recent developments in protein interaction analysis (protein chips, tandem affinity purification and mass spectrometry) should facilitate the identification of downstream targets of CMLs. Because the dynamics in gene expression, protein localization and interaction are essential aspects in defining and tuning CaM/CML-dependent responses, cell biological approaches should be used to visualize spatial and temporal expression profiles of CaM and CML genes, subcellular localization of gene products and protein complex formation. Given that cross-talks between signaling pathways is a mechanism for increasing the specificity of outcomes in different cellular contexts, questions about the interconnections between CaM/CML-dependent processes and other signaling systems should also be addressed.

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