NARRATIVE STUDENT REVIEW

The Role of Calmodulin and Related Proteins in Plant Cell Function: An Ever-Thickening Plot

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Abstract Stimuli-induced fluctuations in intracellular free calcium (Ca^{2+}) serve as secondary messenger signals that regulate diverse biochemical processes in eukaryotic cells, such as developmental transitions and responses to biotic and abiotic stresses. Stimuli-specific Ca^{2+} signals are manifested as spatially and temporally defined differential Ca^{2+} signatures that are sensed, decoded, and transduced to elicit distal responses via an array of Ca^{2+} binding proteins (CBPs) that function as intracellular Ca^{2+} sensors. Calmodulin (CaM), the most important eukaryotic CBP, senses and responds to fluctuations in intracellular Ca^{2+} levels by binding to this ubiquitous second messenger, and transducing given Ca^{2+} signatures that differentially activate distal effector (target) proteins regulating a broad range of biochemical responses. Ca^{2+}/CaM targets include an increasing number of proteins whose functions continue to be elucidated. Hundreds of reports have highlighted the importance of CaM, and other CBPs, in the transduction of Ca^{2+}-mediated signals involved in transcriptional regulation, protein phosphorylation/dephosphorylation, and metabolic shifts. Other Ca^{2+}-binding proteins are known to play significant functional roles in plant cells as well. This review is primarily focused on the role of CaM in some key plant processes, and discusses recent advances in understanding the pivotal role of CaM in an ever-increasing number of plant cell functions and biochemical responses. We also discuss recent work highlighting the emerging importance of CaM in nuclear and organellar signaling.

Keywords Calcium · Calmodulin · Intracellular signaling · Plant defense · Secondary messenger

Introduction

The free calcium ion (Ca^{2+}) emerged very early during evolution as a critically important soluble secondary messenger, and became a fundamental intracellular signaling component in an array of developmental and physiological processes and responses to biotic and abiotic stresses [139]. Ca^{2+} signals originate from several sources in eukaryotic cells, including the extracellular space and release from intracellular stores. Eukaryotic cells have evolved several membrane-bound channel and pump systems that ensure Ca^{2+} levels in the cytosol are maintained at significantly lower levels (up to 10,000-fold lower) than the extracellular environment and within various organelles, such as the endoplasmic reticulum (ER). These dramatic concentration gradients provide potent force potentials that can be exploited for modulating various cellular processes in response to dynamic changes in Ca^{2+} concentrations across membranes. Major Ca^{2+} storage compartments in plants include the vacuole, ER, and the apoplast. More recently other organelles such as the chloroplast, mitochondria, and

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nucleus have also been implicated for their role in Ca^{2+} signaling [32, 90, 146, 162]. Most current research on Ca^{2+} signaling in plants is focused on characterizing cytosolic Ca^{2+} flux parameters and the molecules that sense, decode, and transduce these intracellular Ca^{2+} signals [56, 80, 90, 96, 140].

When intracellular Ca^{2+} concentrations increase above threshold levels, potentially cytotoxic calcium salts such as $Ca_3(PO_4)_2$ can precipitate and trigger apoptotic cell death and disrupt cellular processes [9, 99, 101, 183]. Therefore, active removal of Ca^{2+} from the cytosol appears to have been an evolutionary prerequisite for cells to develop life-sustaining processes. This would be beneficial since removal of Ca^{2+} from the cell would minimize, or prevent, random chelation of negatively charged molecules. Notably, the ability to minimize and regulate intracellular Ca^{2+} levels would also allow a minimal concentration of soluble phosphate ion to persist in the cytosol and establish conditions that would favor subsequent selection and development of phosphate-based metabolic processes. In plant cells, cytosolic levels of Ca^{2+} are actively maintained in the range of ca. 100–200 nM, whereas the cell wall space, vacuole, and ER compartments typically have levels between 1 and 10 mM [30, 43, 56, 93, 120, 171].

A multitude of stimuli including hormones, light, gravity, biotic and abiotic stresses, and defense responses to pathogens or trauma can activate rapid, transient spikes in intracellular Ca^{2+} levels. A critical determinant of a cell's response specificity to a given stimulus is the Ca^{2+} influx signature characterized by its duration, amplitude, frequency, and location. The other critical determinant is the presence, or absence, of specific Ca^{2+}-binding (sensor) proteins that are differentially activated when bound to Ca^{2+}. These activated Ca^{2+}-binding proteins decipher and transduce given Ca^{2+} signatures (i.e., differential stimuli) to specific physiological responses by interacting with, and differentially regulating, various downstream effector molecules, such as kinases, transcription factors, and lipases. However, it remains unclear how the cell selectively tailors distal intracellular responses to subtle qualitative changes in proximal Ca^{2+} influx signatures such as influx spike frequency, amplitude, and duration. In its role as a classic second messenger molecule, Ca^{2+} relays signals from specific cell surface receptors (primary messengers) to various intracellular target molecules that, in turn, directly mediate changes in diverse cellular activities, developmental processes, and stress or defense-related responses. Major challenges remain in elucidating how the cell deciphers exquisitely subtle variations in stimuli-specific Ca^{2+} signatures and precisely integrates corresponding distal cellular responses.

Calcium-Binding Proteins

Eukaryotes have evolved a large array of Ca^{2+}-binding proteins (CBPs) that effectively buffer intracellular Ca^{2+} levels to maintain internal Ca^{2+} homeostasis, and serve as sensors that govern distal Ca^{2+}-dependent cellular responses. Hundreds of proteins involved in calcium signaling networks have been identified in plants, and the number of downstream targets regulated by these calcium sensor proteins continues to increase. Many of these CBPs play crucial cellular roles by differentially modulating a range of cellular activities in response to specific Ca^{2+} influx signatures. CBPs have been under increasing scrutiny in recent years as they have been found to play roles in an expanding range of plant cell functions and processes.

Interaction of a given CBP with Ca^{2+} results in the formation of biologically active Ca^{2+}/CBP complex. These active complexes, in turn, initiate biological responses by either altering the inherent activity of the specific CBP directly, or by interacting with other effector molecules such as enzymes, transcription factors, cytoskeletal components, or even DNA to regulate cellular activities or initiate signaling cascades, which amplify the primary signal. Collectively, CBPs form a cellular network of integrated stimulus–response feedback loops that regulate the relative response levels, and resulting distal cellular effects, of the calcium signal. Over the past few years, an increasing number of CBPs has been identified and characterized, and it is clear the extent of their functional diversity will continue to expand as progress in understanding the regulatory parameters and complex interplay of Ca^{2+}-mediated plant signaling pathways advances.

CBPs can be divided into a handful of major sub-groups, which include calmodulin (CaM), CaM-like proteins (CML), calcineurin B-like proteins (CBLs) and their interacting kinases (CIPKs) [11, 12, 111, 155], calcium-dependent protein kinases (CDPKs) [7, 26, 95, 188], and various protein kinase signaling networks in plant innate immunity [168]. In plant genomes, these sub-groups of Ca^{2+} sensors exist as multi-gene families that form sophisticated signaling networks that integrate the information processing controlling diverse cellular processes. CaM is highly conserved, found in all eukaryotes, and is the most important of all known CBPs. However, in contrast to CaM, the CMLs, CBLs, and CDPKs are found primarily only in plants. The focus of this review is CaM, and thus CMLs and other Ca^{2+}-binding proteins will not be discussed in great detail here. More in-depth discussion on calcium signaling and related CBPs can be found in the following reviews [12, 56, 80, 137], and an entire issue of Plant Physiology dedicated to calcium signaling (October 2013, vol. 163).

Over the past several years an increasing number of plant species have been subjected to whole genome sequencing, analysis, and profiling. Sequencing data to date has revealed the existence of multiple genes that encode identical or highly homologous CaM isoforms (97–99 % identity), and further indicated that a diverse assortment of CMLs is a common feature of higher plant, but not animal, genomes [47, 199]. All eukaryotic CBPs, including CaM, possess one or more discrete amino acid domains called E–F hands that can bind Ca^{2+}. In a genome-wide analysis of *Arabidopsis thaliana,* Day et al. [47] identified 250 genes encoding proteins that contained at least one predicted EF-hand motif. Seven of these genes encoded four CaM isoforms sharing 97–99 % amino acid identity with each other, and a high level of sequence identity with CaM isoforms found in vertebrates and insects. Based on these two criteria these four isoforms are considered to be typical CaM proteins, as compared to CMLs or other CBPs.

Eukaryotic cells have developed a multitude of ways to exploit intracellular Ca^{2+} gradients to regulate cellular processes. Ca^{2+} acts as a diffusible second messenger by relaying information from initial stimuli to activate cellular responses, and its critical role in eukaryotic intracellular signaling is well established. CaM has evolved to be the primary transducer of Ca^{2+} signals in eukaryotic cells. Structurally, CaM consists of a single polypeptide, typically 148 amino acids long, that has no known inherent biological activity until binding Ca^{2+}. Each CaM protein contains two globular Ca^{2+}-binding domains, and each of these domains has two EF-hand motifs capable of binding one Ca^{2+} ion each. Therefore, each CaM protein can bind up to four Ca^{2+} ions.

The EF hand is a helix–loop–helix structure consisting of about 40 amino acids that is one of the most common structural motifs found in plant and animal genomes—an observation that is consistent with Ca^{2+} being a versatile and ubiquitous messenger for dynamic regulation of cellular signaling pathways. EF-hand domains are often found in single or multiple pairs, giving rise to various structural variations in proteins that contain EF-hand motifs. These structural variations confer functional diversity to these proteins that provides the cell with the capacity to integrate and fine-tune target molecule binding, and subsequent distal cellular responses, to a broad continuum of differential Ca^{2+} influx signatures. In animal genomes, for example, over 1,000 different genes have been identified from their unique EF-hand sequence motifs [33, 81].

In both plants and animals, there is a notable lack of understanding on how the integral EF-hand domains in CaM and other CBPs impart differential specificity to these

Ca^{2+} sensors, and what subtle structural shifts enable these proteins to distinguish among the numerous known target proteins within the cells they regulate. The presence of EF-hand motifs in all classes of CBPs is consistent with Ca^{2+} being a global intracellular regulator.

CaM itself is a monomeric peptide containing two symmetrical, globular Ca^{2+}-binding domains. Each domain contains two canonical EF-hand motifs that are separated by a flexible alpha helix linker sequence. The flexibility of this linker sequence between the EF-hand motifs is a defining structural feature for CaM function in that it allows the Ca^{2+}-activated CaM peptide to wrap around and form a stable complex with the target molecule being regulated. Binding of calcium to the two helix–loop–helix Ca^{2+}-binding motifs in each of the globular domains induces conformational changes that expose a methionine-rich hydrophobic patch on the surface of each domain of the protein, which promotes binding to specific peptide sequences present in specifically bound target proteins [191]. Using a structural systems approach Velayev et al. [179] concluded that specificity and selectivity of CaM target regulation likely depends upon two key factors: (1) variations in target-specific Ca^{2+} dissociation and cooperatively effected association constants, and (2) variations in the threshold number of Ca^{2+} ions required to bind CaM for effective target activation. Binding of Ca^{2+} to EF-hand domains induces a conformational change in CaM that is transmitted to its target proteins to, typically, catalyze enzymatic reactions.

CMLs are characterized by possessing two to six predicted EF-hand motifs, lacking any other known functional domain, and having at least 15 % amino acid homology with CaMs. The CaM/CML gene family in *Arabidopsis* consists of seven CaM and fifty CML genes [121, 137], while the rice genome comprises of five CaM and thirty-two CML genes [24]. Although overlapping and redundant functional roles are frequently observed for some CaM isoforms, it is known that different CaM gene variants can exhibit distinct, yet often subtle, patterns of temporal or spatial regulation and differentially affect various biological functions.

Comparisons between CaM and CML genes, and amino acid sequences, indicated that CMLs can be broadly classified into two major groups. One group of CMLs displays significant sequence identity (>50 %) with CaM and contains an intron at the same position found in CaM genes, indicating close evolutionary relationship with CaM. In contrast, the second CML group displays low homology (<50 %) with CaM, and exhibits high structural divergence suggesting novel functions for the various isoforms [80, 121, 137, 140].

Calcium Signal Transduction by CaM

The transduction of a Ca^{2+} signal can be viewed as a two-step process involving an initial activation of CaM (or any CBP) by the ionic signal, followed by binding to, and modulation of, a specific target protein. Since Ca^{2+} signatures result from the coordinated action between Ca^{2+} influx and efflux pathways, how Ca^{2+}-permeable channels and transporters are regulated during calcium signaling processes, including plant–pathogen interactions, must be considered. CaM found in plants and animals can bind up to four Ca^{2+} ions. In animals, CaM also undergoes post-translational modifications such as phosphorylation, acetylation, methylation, and proteolytic cleavage, each of which can potentially modulate its activity. Although post-translational modification of CaM has yet to be carefully investigated in animals, it is likely that similar kinds of modifications occur to plant CaMs as well to endow cells with another strategy for fine-tuning the regulatory effects of CaM on cellular processes. For example, recent work by Banerjee et al. [11] examining CaM N-methyltransferase (CaM KMT) activity confirmed that the methylation status of CaM plays a role in CaM-mediated signaling. In *Arabidopsis* plants overexpressing, partially expressing, or knocked out with regard to CaM KMT, the authors found differential, discrete spatial- and tissue-specific patterns of CaM KMT expression in these transgenic plant lines. Moreover, microarray analysis revealed numerous putative target proteins having specificity for methylated CaM. Differential methylation of CaM thus adds another strategy for expanding the target protein repertoire mediated by Ca^{2+}/CaM signaling, and fine-tuning their differential activity.

CaM Target Molecules in Plants

CaM interacts with a wide range of downstream target molecules, mainly proteins that mediate diverse cell processes. The list of CaM-binding proteins continues to expand and includes transcription factors, kinases and phosphatases, ion channels, membrane transporters, and metabolic regulators. A comprehensive list of known plant CaM-binding proteins can be found in Poovaiah et al. [140].

Several important target proteins known to bind CaM and mediate key plant cell processes will be discussed.

Mitogen-Activated Protein Kinases

Mitogen-activated protein kinases (MAPKs) are evolutionarily conserved proteins that function as key signal transducers of external stimuli in plants, animals, and fungi. MAPK cascades are known to regulate processes involved in plant growth and development, and cellular responses to biotic and abiotic stresses [27, 46, 63, 65, 145, 147, 154, 165]. The lesser understood MAPK phosphatases (MKPs) also play key roles in regulating biotic and abiotic processes through their ability to deactivate MAPK signaling cascades by altering activation levels and kinetics of MAP kinases [75, 104, 131]. After screening an *Arabidopsis* cDNA library, Lee et al. [104] identified an MKP (AtMKP1) and subsequently demonstrated that it binds CaM in a Ca^{2+}-dependent manner. Moreover, CaM binding was confirmed to enhance the phosphatase activity of AtMKP1 in a Ca^{2+}-dependent manner. Recent work shows specific interaction among individual MAPKs and cognate MKPs in plant responses to UV light stress [75], and regulatory integration of these antagonistic activities with other defense signals (JA, SA, and ET) in plant immune responses [131]. Unraveling the discrete regulatory circuits governed by given MAPK-MKP pairs will be an ongoing challenge.

Ca^{2+} is known to regulate and activate MAPKs and cyclin dependent protein kinases, or CDPKs [6, 26, 69, 107, 138, 168, 188]. A growing body of evidence implicates CaM in the activation of specific MAPK variants. For example, *Arabidopsis* MPK8 is activated through mechanical wounding and requires direct binding of CaMs in a Ca^{2+}-dependent manner [166], and other reports have described CaM-mediated activation of specific MAPKs [104, 107]. Moreover, CaM signaling through the MAPK pathway and elevations in cytosolic Ca^{2+} are hallmarks of general plant stress responses [104, 136, 147, 160, 165, 166, 188, 189]. The functional coordination of Ca^{2+} and MAPK interaction may occur through the formation of unique CaM-MAPK phosphatase multi-protein complexes [83, 144, 166].

Deciphering the complex cross-talk among MAPKs and various other signaling messengers such as nitric oxide (NO), reaction oxygen species (ROS), jasmonic acid (JA), salicylic acid (SA), and ethylene is an intensely active area of research, but will be discussed here in limited context only where CaM involvement has been clearly established. More exhaustive coverage of these areas can be found in recent reviews, including Boudsocq and Sheen [26], Liese and Romeis [108], Meng and Zhang [122], Sanchez-Barrena et al. [155], Danquah et al. [46], Romeis and Herde [151], and Smékalová et al. [161].

WRKY Transcription Factors

WRKY transcription factors are a large superfamily of transcriptional regulators unique to plants that are involved in signal transduction networks that govern an increasing number of physiological processes including responses to

biotic and abiotic stresses [34, 153, 172], systemic acquired resistance (SAR), and the hypersensitive response (HR) [66, 82]. WRKY proteins can act as repressors, as well as activators, and often affect seemingly unrelated cellular processes. Most known WRKY transcription factors studied have been implicated in SA and/or JA signaling pathways, and several WRKY family members have been shown to interact with CaM [10, 11, 37, 134, 152]. Using a CaM probe to screen an *Arabidopsis* cDNA expression library, Park et al. [134] isolated positive clones encoding AtWRKY7, and demonstrated Ca^{2+}-dependent CaM binding in gel mobility shift assays and competition assays using a Ca^{2+}/CaM-dependent enzyme. The AtWRKY7 protein contains a short amino acid stretch called the C-motif (VAVNSFKKVISLLGRSR) that functioned as the CaM-binding domain [134]. Chi et al. [37] noted that similar C-motif domains have been found in at least 10 other *Arabidopsis* WRKY proteins also bound by CaM.

A genome-wide analysis of CaM genes from three Solanaceous species (tomato, tobacco, and potato) by Zhao et al. [199] reported that upstream sequences from CaM genes carry a variety of potential regulatory motifs, including binding sites for transcription factors that are regulated by hormones such as abscisic acid (ABA), gibberellin (GA), auxin, JA, and ethylene. The sequence patterns of these upstream elements differed significantly among the tomato CaM genes suggesting differential regulation potential. For example, one tomato CaM gene promoter contained several sequence elements that could potentially respond to all five noted hormones, while the other tomato CaM family members completely lacked elements responsive to one or more of these hormones. Notably, all tomato CaM genes contained upstream elements having multiple W-box elements, which are the cis-acting regions specifically recognized by WRKY transcription factors. These results strongly suggest that all tomato CaM genes are regulated, at least to some degree, by WRKY transcription factors.

IQD Proteins

IQD proteins (also referred to as IQ67-domain proteins) are a large group of plant-specific CaM/CML-target molecules that share a unique common domain comprised of multiple CaM retention motifs in tandem orientation. Genetic studies in *Arabidopsis* and tomato have revealed roles for IQD proteins in plant defense responses and plant development, but specific functions for most IQD proteins have not been determined. A recent genome-wide comparative screen of *Arabidopsis* and rice revealed the presence of 33 and 29 IQD proteins, respectively [3]. All 33 predicted *Arabidopsis* IQD proteins share a unique conserved domain of 67 amino acids that is characterized by a specific arrangement of multiple CaM recruitment domains, in tandem orientation, referred to as IQ motifs. Therefore, all IQD family members are potential CaM targets.

A specific IQD isoform, IQD1 (also known as IQ67 domain 1), contains several CaM-binding motifs as well as a putative nuclear localization signal. Glucosinolates are a class of secondary metabolites, primarily characterized in *Brassicaceae* species, that have important functions in human nutrition and plant defense against microbes and herbivory [1, 15, 84, 109, 186]. Levy et al. [106] screened *Arabidopsis thaliana* T-DNA activation-tagged lines and identified a high-glucosinolate mutant caused by overexpression of IQD1. Overexpression had increased levels of glucosinolates, as well as reduced herbivory, whereas loss-of-function iqd1 mutants had reduced glucosinolate levels. The authors proposed that IQD1 integrates intracellular Ca^{2+} signals to fine-tune glucosinolate accumulation in response to biotic challenges. Burstenbinder et al. [29] showed that IQD1 binds to multiple *Arabidopsis* CaM and CML proteins in yeast two-hybrid interaction assays, and in vitro. Green florescent protein (GFP)-tagged IQD1 proteins were found to localize to the microtubules, nucleus, and nucleolus in transiently and stably transformed plant tissues. Since IQD1 harbors several nuclear localization signals and localizes to the nucleus, it is likely that IQD1 regulates gene expression by interacting with DNA.

Burstenbinder et al. [29] and Abel et al. [4] suggested that IQD1 and related proteins provide Ca^{2+}/CaM-regulated scaffolds for facilitating cellular transport of specific cargo along microtubular tracks via kinesin motor proteins. IQD1 has also been shown to interact in vitro with single-stranded nucleic acids, suggesting it and related IQD family members, facilitates cellular RNA localization as a means of fine-tuning gene expression and protein sorting [4].

Plant Cyclic Nucleotide-Gated Ion Channels

Plant cyclic nucleotide-gated ion channels (CNGCs) function as non-selective cation channels and play key roles in development, ion homeostasis, thermotolerance, and defense responses by providing a pathway for Ca^{2+} and K^+ movement across the plasma membrane [2, 40, 59, 88, 112, 113, 123, 180, 197]. CNGCs are found in the plasma membranes of plant, animal, and (recently) prokaryotic cells. These ancient cation channels are activated by cyclic nucleotides that bind at specific channel sites that partially overlap the CaM-binding sites they also possess [197]. Binding of CaM results in the inactivation of the CNGCs by interfering with the binding of cyclic nucleotides via competitive inhibition. The negative action of CaM on CNGC activity therefore provides an intriguing negative feedback system that allows Ca^{2+} itself to restrict its own influx into plant cells, or across intracellular membranes [112, 180].

During plant immune responses, elevations of CNGC-dependent Ca^{2+} activate a signaling cascade that results in the accumulation of defense-related molecules, such as H_2O_2 and NO, and the induction of defense gene expression [180]. Fischer et al. [61] showed that a specific CNGC (CNCG20) from *Arabidopsis thaliana* binds CaM in a Ca^{2+}-dependent manner and also interacts with all known AtCaM isoforms, but not with the CaM-like proteins CML8 and CML9. GFP-localization studies by the same authors further showed that this interaction occurred at the plasma membrane.

Although IQ domains are known to be conserved among plant CNGCs [197], the CaM-binding site within CNGC20 was identified as an isoleucine glutamine (IQ) domain, which had not been reported previously for any plant CNGCs. It was also revealed that the binding sites for cyclic nucleotides and CaM within CNGC20 are sequentially arranged, rather than overlapping [61]. This particular structural difference is also unique for CNGC20, compared to corresponding binding sites found in other known plant CNGCs. The presence of alternative binding domains in CNGC20 expands the regulatory potential of the cell for controlling Ca^{2+}-dependent channel activity, and clearly indicates that ligand-mediated regulation of plant CNGC activity is more complex than previously known for this functionally diverse gene family.

The role Ca^{2+}-ATPases in cellular Ca^{2+} efflux mechanisms is well known, and in recent years it has become clear that these ion pumps also play key roles in sensing intracellular calcium fluctuations and transducing distal signals by activating specific target molecules to modulate corresponding metabolic pathways [23, 110]. Ca^{2+}-activated CaM plays vital roles in numerous stress tolerance responses, and the presence of a unique CaM-binding site in type IIB Ca^{2+}-ATPases indicates their potential role in mediating biotic and abiotic stress tolerances [23, 64].

Autoinhibited Ca^{2+}-ATPases (ACA) belong to a subgroup of CaM-regulated Ca^{2+}-ATPases that contain an N-terminal CaM-binding site and an auto-inhibitory domain. CaM has been shown to stimulate the activity of these particular Ca^{2+} pumps by binding to this domain and preventing their auto-inhibition. Control of Ca^{2+} transport systems involving both CNGCs and ACAs can therefore be regulated by CaM [61, 72].

Calmodulin and Salicylic Acid

Transient changes in intracellular calcium levels are crucial early signaling events in the activation of plant–pathogen interactions that initiate local defense and systemic acquired resistance, or SAR [103]. Salicylic acid (SA) is well known to be a key signal molecule in plant resistance,

yet its precise functional role has yet to be clearly defined [66, 67, 73, 126, 127].

Doares et al. [55] showed that SA specifically inhibits JA- and systemin-mediated activations of proteinase inhibitor genes via the octadecanoid pathway, which is induced by wound trauma such as herbivory. Bergey et al. [20] purified and obtained partial amino acid sequence information to identify numerous proteins, including CaM, that were differentially regulated in transgenic tomato plants overexpressing the wound response peptide systemin, a potent activator of the octadecanoid (or JA) signaling pathway. This was the first report implicating CaM induction with the plant wound response and octadecanoid signaling. Since then an expanding body of work has established a central role for CaM in mediating interplay among numerous defense and stress-related responses [18, 21, 22, 36, 142, 173].

Although CaM and various CMLs have been clearly linked to SA-mediated responses to development, stress, and defense response, the nature of this interaction is just beginning to be unraveled [17, 42, 89, 173, 176, 178, 198].

A Ca^{2+}/CaM-Regulated Transcription Factor Family

A family of Ca^{2+}/CaM-binding transcription factors generally referred to as signal responsive/CaM transcription activators (SR/CAMTAs) is known to play important roles in fruit ripening and response to myriad abiotic and biotic stresses [18, 21, 25, 60, 68, 78, 142, 194]. Using differential display, Zegzouti et al. [196] first identified a large class of cDNA clones from tomato that were differentially regulated by ethylene. This group of cDNAs were referred to as ethylene-regulated (ER) sequences, one of which was ER66. The following year Yang and Poovaiah [192] isolated and characterized a tobacco ER66 homolog after screening a tobacco anther cDNA library with 35S-labeled CaM. The authors referred to this homolog as an early ethylene-responsive up-regulated gene (NtER1), and this report was the first to link Ca^{2+}/CaM signaling to ethylene activity. Reddy et al. [148] followed closely by identifying an ER66 homolog, and several related hypothetical sequences, in *Arabidopsis*. Yang and Poovaiah [193] subsequently characterized six NtER1-related proteins in *Arabidopsis*, and called these homologs *Arabidopsis thaliana* Signal Response genes (AtSRs). SR/CAMTA is the more general name for this family of CaM-binding transcription factors, and homologs of this family have since been found in all plant and animal species surveyed to date. All SR/CAMTA family members share a similar structural organization with a novel type of sequence-specific DNA-binding domain (designated CG-1) that directly binds DNA to activate transcription, or interact with other transcription factors to function as a co-activator.

Du et al. [57] reported that plants with loss-of-function mutant alleles for atsr1 constitutively expressed genes associated with SAR, resulting in elevated SA levels and enhanced disease resistance. Moreover, the wild-type AtSR1 protein product was shown to be regulated by Ca^{2+}/CaM, and to reduce SA levels via transcriptional repression of the gene encoding enhanced disease susceptibility 1 (EDS1), a protein previously shown to promote SA biosynthesis [185]. In work by Qui et al. [142] SR/CAMTA homogues in *Arabidopsis* (AtSRs or AtCAMTAs) responded differentially to wounding, and atsr1 mutants were more susceptible to herbivore attack than wild-type plants, and that complementation of the atsr1 mutant plants by overexpressing wild-type AtSR1 protein restored resistance to herbivore attack [142]. These authors further reported that the elevated levels of SA in atsr1 mutant plants suppressed both basal and induced biosynthesis of jasmonates, and concluded that Ca^{2+}/CaM regulates the plant wound response by modulating, or coupling, JA-SA cross-talk through AtSR1.

AtSR1 also functions as a novel regulator of glucosinolate metabolism and subsequent herbivory tolerance in *Arabidopsis* [100]. Recently, Zhang et al. [198] provided some clarification on this system by identifying and characterizing an AtSR1 interaction protein 1 (called SR1IP1) that turns out to be an important component of ubiquitin ligase that is associated with AtSR1 turnover. The authors showed that SR1IP1 is a loss-of-function mutant that was more susceptible to bacterial pathogens, and that overexpression of SR1IP1 conferred enhanced resistance, indicating that SR1IP1 acts as a positive regulator of plant defense. SR1IP1 contains the structural features of a substrate adaptor in E3 ubiquitin ligase, and was shown to function as a substrate adaptor that recruits AtSR1 for ubiquitination and subsequent degradation when plants are challenged with pathogens. Therefore, SR1IP1 positively regulates plant immunity by effectively removing the immune repressor AtSR1 [198]. A host of other transcription factors are known to interact with CaM [57, 87, 90, 149, 184, 189], thus ensuring a future of continuing discovery and deeper understanding of the complex circuitry involved in Ca^{2+}/CaM signaling.

The complex, and typically antagonistic, interplay between JA and SA signaling in plant development and defense has been an area of intense investigative interest for several years, and will continue to provide challenges and rich intellectual rewards in coming years [52, 73, 178, 184]. As progress in this area continues to advance, differential regulation of CaM family isoforms will undoubtedly be confirmed as a common strategy for fine-tuning the crosstalk that coordinates the sophisticated interplay among these fundamental signaling pathways. Excellent recent reviews covering this area can be found in Reddy et al. [149], Weng

et al. [184], Denancé et al. [51], Derksen et al. [52], Gimenez-Ibanez and Solano [73], and González et al. [74].

CaM in Plant Defense and Stress Responses

All animal and plant cells are continually threatened by invading microorganisms throughout their life spans. Although plants lack versatile and mobile sentinel cells such as macrophages and neutrophils found in animal innate immune systems, plant cells possess their own very effective innate immune systems that perceive and respond to invading pathogens and wound trauma. Like animals, plant innate immune response activation is triggered by the recognition of foreign (non-self) structural components called pathogen (or microbe) associated molecular patterns (PAMPs or MAMPs), which are essential, evolutionarily conserved components of pathogenic microbes. The recognition of PAMPs by pattern recognition receptor (PRRs) proteins localized in the host plasma membrane, and some intracellular membranes, leads to the activation of innate defense responses, referred to as PAMP-triggered immunity. Some excellent recent reports and reviews on this topic include [14, 35, 48, 62, 124, 127, 130, 158, 164, 169]. In addition, a recent book dedicated to innate pathogen recognition signals in plants is available.

All known plant PRRs identified to date are receptor-like kinases/proteins (RLK/Ps). Most plant viruses have RNA genomes that form inherent double-stranded RNA (dsRNA) structures that are recognized as PAMPs by plant cells, which subsequently activate defensive RNAi (RNA interference) responses as a fundamental antiviral defense strategy [53, 54].

In classic host-pathogen warfare style, selection pressure has lead to the result that most plant viruses now encode RNA silencing suppressor (RSS) proteins that can neutralize plant antiviral RNAi defense strategies. Many known RSSs bind dsRNAs, which can be functionally regarded as viral PAMPs. Tadamura et al. [164] recently identified a tobacco CaM-like protein, called rgs-CaM, as a putative PRR that is capable of binding to diverse viral RSSs by interacting with their dsRNA-binding domains and subsequently targeting the viral RSSs for autophagic degradation. Rapid induction of Rgs-CaM expression (within 1 h) was observed at damaged sites following wounding of tobacco leaves. Plant virus entry into cells is typically associated with some kind of wound damage, therefore the rapid wound-induced expression of rgs-CaM suggests an early counter-attack strategy that would be initiated before an effective viral infection could be established. Known CaM and CML proteins transduce calcium signals by binding endogenous target molecules, whereas rgs-CaM appears unique in that it not only binds to

exogenous targets but also functions as an antiviral PRR. Further evidence supporting rgs-CaM as a valid plant PRR was described by Nakahara et al. [129] who overexpressed rgs-CaM in tobacco and demonstrated increased resistance against viruses, and reduced resistance in plants in which rgs-CaM was repressed by RNAi.

Perception of, and interaction with, a pathogen can also result in the initiation of HR, which involves rapid programmed cell death (PCD) around the pathogen-infected site and prevents the spread of pathogen within the plant beyond the initial infection site. Production of primary signaling molecules such as NO and ROS are known to be key signaling events during the PCD process [13, 16, 44, 50, 76, 157, 181, 187]. Sphingolipids are essential components of all eukaryotic cell membranes that are known to play roles in plant defense and stress signaling [118, 125, 132, 143]. Synthesis of sphingolipids increases both cytosolic and nuclear levels of Ca^{2+} in plant cells [99, 135, 156], and recent work shows that sphingolipid metabolites are involved in the activation of calcium-dependent cell death [20, 99, 170].

Harding et al. [79] overexpressed an endogenous dominant acting CaM mutant (VU-3) in tobacco cells, which resulted in elevated production of ROS. The authors also showed that VU-3 CaM differs from endogenous plant CaM in that it cannot be methylated post-translationally, and as a consequence directly hyperactivates CaM-dependent NAD kinase resulting in increased ROS production. This report was the first to provide evidence suggesting that CaM-activated NAD kinase potentiates ROS production in plants by altering NAD(H)/NADP(H) homeostasis.

Similar to animal cells, plant cells also utilize Ca^{2+} signaling as an essential early signaling event in response to pathogen perception. Transient elevations in cytosolic Ca^{2+} levels are a pivotal event in signaling pathways that trigger plant responses to a wide range of biotic stresses, including innate immune responses [36, 58, 137, 140, 149, 159], and it is well documented that Ca^{2+}, CaM, CMLs, and NO work together to mediate specific responses to pathogenic microorganisms and MAMPs [41, 78, 86, 114, 180].

NO is a small redox-active gas that has become established as a central regulator of growth, development, and abiotic and biotic stress responses in plants. The primary molecular mechanism for initiating NO bioactivity is through S-nitrosylation, which involves the covalent attachment of NO to a protein cysteine thiol to form an S-nitrosothiol entity called SNO [195]. Abundant evidence supports NO as a key messenger involved in mediating Ca^{2+} signaling in plants, and in recent years it has become increasingly clear that CaM and CML Ca^{2+} sensor proteins play pivotal roles in NO production and plant defense signaling [86, 112, 113, 116, 175], as well as an ever-growing number of plant processes including developmental transitions, autophagy, and exposure to environmental stresses [75, 91, 102, 149, 175, 184, 189, 190, 194].

Arabidopsis lines expressing mutant forms of different CML genes exhibited elevated levels of NO and were responsible for FLOWERING LOCUS C transcript accumulation [114, 174]. One of these CML gene products (CML24) was shown to interact directly with an autophagy related (ATG) gene to mediate progression of autophagy [36].

Arabidopsis showed that pathogen-induced Ca^{2+} results in CaM and/or CML activation of NOS, and that CaM antagonists prevent NO production and subsequent activation of the plant HR [114]. Overexpression of an animal CaM-dependent mammalian neuronal NOS (nNOS) in tobacco plants led to the spontaneous formation of lesions in leaves, the accumulation of high levels of SA and H_2O_2, and the up-regulation of an array of SA-, JA-, and ethylene-related genes [42]. These transgenic tobacco plants also exhibited enhanced resistance to a broad range of pathogens including bacteria, fungi, and viruses. The authors proposed the likely existence of a sophisticated regulatory hierarchy involving NO in SA-, JA-, and/or ethylene (ET)-dependent pathways that lead to disease resistance. Jeandroz et al. [86] showed that cytosolic Ca^{2+} fluxes initiated changes in NO production and provided the first evidence that CaM may be regulated at the post-translational level by NO through S-nitrosylation. Besides playing a critical role in plant immune signaling, several recent reports show that NO can mediate differential post-translational modifications (PTM) of various target proteins, and thus may have a much more expansive role in plant physiological processes [8, 70, 182]. Overall, these reports indicate that specific CML gene products transduce calcium signals that specifically regulate NO production to mediate plant cell processes.

Perception of pathogen signals by plants results in cyclic nucleotide production and the activation of CNGCs, which provide a conduit for Ca^{2+} movement across the plasma membrane and subsequent transient increases in cytosolic Ca^{2+} levels. CNGCs have been implicated in numerous signaling pathways [113, 115, 123, 180, 197]. Work by Ma [112] and Walker and Berkowitz [180] indicates that the recognition of pathogens results in cyclic nucleotide production and the activation of CNGCs, which leads to the production of pivotal signaling molecules such as NO. CaM and other CBPs are also involved in Ca^{2+} signaling processes that mediate the synthesis of NO during plant-pathogen signaling [113, 141].

An increasing body of evidence points to the dynamic integration of CaM/CMLs, CNCGs, and NO, a fundamental signaling triad regulating a broad range of plant defense and stress responses [86, 112, 113, 123, 180].

CaM in Biotic and Abiotic Stress Tolerances in Transgenic Plants

Ca^{2+} influx is one of the earliest events to occur following biotic or abiotic stress in plant cells [71, 94, 200], and Ca^{2+} influx has also been directly correlated with the activation of numerous endogenous defense responses including the induction of defense-related genes, and hypersensitive cell death [28, 77, 92, 105, 167, 184]. Since the Ca^{2+} signal is necessary for the initiation of plant defense responses, transgenic plants that overexpress one of a number of the divergent CaM isoforms have been evaluated for their role in plant disease-resistance responses. Transgenic tobacco plants overexpressing the soybean CaM isoform 4 or soybean CaM isoform 5 gene were tolerant to *Phytophthora parasitica* var. *nicotianae* and *Pseudomonas syringae* pv. *tabaci*. In addition, these transgenic plants exhibited increased resistance to the tobacco mosaic virus (TMV) by developing TMV-induced HR lesions [82]. Overexpression of a synthetic gene-derived CaM (VU-3 CaM) in tobacco also resulted in enhanced levels of active oxygen species (AOS) in plants [79]. Salt stress-tolerant transgenic plants have been developed by overexpressing a calcineurin, a Ca^{2+}/CaM-dependent protein phosphatase [133].

In addition to an important role in biotic stress, CaMs have been observed to modulate abiotic stress in transgenic plants. Overexpression of the *Nicotiana tabacum* CaM-binding protein (NtCBP4) was observed to regulate plant tolerance to heavy metal and confer Ni^+ tolerance and Pb^{2+} hypersensitivity [5].

Trihelix-GT factors comprise a family of plant-specific transcription factors characterized by their binding specificity for GT-elements located in the promoters of many plant genes [128]. Xi et al. [189] identified a new member of the GT transcription factor family in *Arabidopsis* (AtGT2L) as a Ca^{2+}/CaM-binding protein, and demonstrated it was specifically targeted to the nucleus and possessed transcriptional activation and DNA-binding capability. The authors further showed that AtGT2L was induced by cold and salt (NaCl) stress, as well as abscisic acid, and when overexpressed in transgenic plants enhanced the expression of known cold- and salt-inducible marker genes.

CaM Function in Cellular Organelles

Ca^{2+}/CaM-mediated regulation of a multitude of cytosolic processes is well established, and there are increasing numbers of reports demonstrating CaM and CML presence in various organelles such as chloroplasts, mitochondria, peroxisomes, and the nucleus [31, 39, 117, 163, 177]. In addition, processes such as protein import into chloroplasts and mitochondria have been shown to be governed by CaM regulation [85, 97, 119]. Jarrett et al. [85] reported that CaM entered the chloroplast and played a role in photosynthesis, and more recently chloroplast protein import has been shown to be influenced by calcium and CaM [38, 39, 163]. Using CaM-affinity chromatography and mass spectrometry, Dell'Aglio et al. [49] identified over 200 candidate CaM-binding proteins from *Arabidopsis* and spinach chloroplast sub-fractions. In vitro CaM-binding assays confirmed a subset of these proteins to be valid CaM-binding proteins. These results indicate there may be a much larger population of plastid targeted CaM-binding proteins than previously thought, and suggests CaM may be entering plastids to mediate various functions. These findings indicate that chloroplast protein import is integrated into the Ca^{2+}-signaling circuit of the cell, and thus adds an additional, separate regulatory level for the chloroplast to define its protein complement and integrate its metabolic needs with those of the surrounding cell and tissue.

Peroxisomes are generally thought to be the primary source of NO in plants, mainly because NO and the enzyme responsible for the biosynthesis of NO, L-arginine-dependent NO synthase (NOS), are found in these oxidative organelles. Using calcium channel blockers and a CaM antagonist, Corpas et al. [45] showed that the import of the NOS protein into *Arabidopsis* peroxisomes is a Ca^{2+}/CaM-dependent process.

Signaling cross-talk among different organelles is an important aspect of cellular Ca^{2+} signaling that is gaining increasing interest with regard to dynamic intracellular reorganization in response to stimuli, the localization of Ca^{2+} effectors, Ca^{2+}-dependent translocation, and post-translational modifications of proteins. Kuhn et al. [97] showed that the import of nuclear-encoded mitochondrial proteins into the mitochondria of pea plants is influenced by calcium and CaM, and that Ca^{2+}-loaded CaM enters the nucleus, ostensibly to interact with transcription factors and regulate specific suites of genes [32, 90, 146].

In 1999 Rodríquez-Concepcíon et al. [150] showed that a previously unknown CaM variant (CaM53) from petunia is post-translationally isoprenylated at its C-terminal end. Using GFP fusion constructs the authors showed that inhibition of isoprenoid biosynthesis resulted in targeting of the CaM53 protein to protoplast nuclei, indicating that isoprenylation mediates the subcellular localization of this unusual CaM variant. In leaves exposed to light for several days, CaM53 was found to localize to the plasma membrane, whereas this CaM variant accumulated in the nucleus when leaves were maintained in the dark during the same period. However, nuclear translocation was blocked when leaves were exposed to dark on a medium supplemented with sucrose. Recombinant CaM53 was

shown to activate the enzyme glutamate decarboxylase, a known plant CaM-dependent enzyme, and the authors used genetic complementation to demonstrate that an intact CaM53 gene was able to rescue a yeast mutant (cmd1Δ) defective in this CaM gene variant. The later result confirmed that this nuclear-targeted CaM isoform (CaM53) is both functionally active, and biologically relevant. Nuclear localization of CaM has also been reported by Kushwaha et al. [98] who identified four CaM isoforms in *Arabidopsis* and demonstrated that one of these isoforms (CAM7) directly interacted with promoters of several light-inducible genes to promote photomorphogenesis. Overexpression of CAM7 resulted in hyperphotomorphogenic growth and increased expression of the light-inducible genes.

As efforts to understand the seemingly infinite versatility of Ca^{2+}/CaM signaling in plant cell processes advance, it appears certain that CaM will continue to be revealed as a fundamental global regulator and facilitator of intracellular signaling cross-talk, organelle function, and gene regulatory cascades in plant cells.

Summary Comments

As work to unravel and understand the exquisitely complicated signaling networks that control plant processes forges ahead, an increasingly prominent regulatory role for CaM and CMLs will emerge. The evolutionary conservation and persistence of CaM, and related CMLs, as key Ca^{2+} sensors support their role as regulatory allies that modulate the expression and activity of other Ca^{2+}/CaM-dependent effector proteins, such as those noted. The complexity and functional ubiquity of the Ca^{2+} signal in regulating diverse plant growth, development, and defense responses presents major challenges and opportunities for investigators in coming years to elucidate specific biological functions for each of these highly conserved CaM gene family members. Transduction of the Ca^{2+} signal by CaM (or any CBP) can be viewed as a two-step process involving initial activation of CaM by Ca^{2+} ion binding, followed by direct interaction with, and consequent modulation of, a target protein. In this scenario, challenging questions whose answers have remained stubbornly elusive to date include (i) How do seemingly minor differences in amino acid sequences among highly conserved CaM isoforms dictate differential downstream responses to given Ca^{2+} signatures(?) and (ii) How do different CaM isoforms (or other EF-hand proteins) interact with, and differentially modulate, their respective target proteins(?).

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