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Review Article

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Calmodulin in Plant Responses to Abiotic Stresses and Signalling

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ABSTRACT

Abiotic stresses are considered as major limiting factors of crop yields and cause billions of dollars of losses annually around the world. It is hoped that understanding at the molecular level how plants respond to adverse conditions and adapt to a changing environmental conditions may help in developing plants that can efficiently cope with multiple abiotic stresses. Abbreviation for (CaM) is calcium-modulated protein, a calcium-binding messenger protein expressed in all eukaryotic cells. It is an intracellular target of the secondary messenger Ca^{2+} *and the binding of* Ca^{2+} *is required for the activation of Calmodulin. Once bound to* Ca^{2+}*, Calmodulin participate in calcium signal transduction pathway by altering its interactions with various target proteins such as kinases or phosphatase. Calmodulin is a ubiquitously present and highly conserved calcium sensor throughout the eukaryotes. Plants have evolved a complex network of calmodulin and calmodulin-binding target proteins that serve to play an important role in mediating stress-signaling pathways. Many of the target proteins of CaM directly or indirectly regulates plant responses to environmental stresses and bind proteins include transcription factors, ion channels, and metabolic enzymes that assist plant to effectively cope with environmental stress and pathogens. Extensive research over the past decade has been focused on understanding the function of calmodulin in biotic and abiotic stress response. How CaM/CMLs are involved in regulating plant responses to abiotic stresses are emphasized in this review. Future finding in* Ca^{2+}*/CaM-mediated signaling will improve our understanding of how plants respond to environmental stresses, also provide the knowledge base to improve stress-tolerance of crops. Basic information about stimulus-induced* Ca^{2+} *signal and overview of* Ca^{2+} *signal perception and transduction are briefly discussed in the beginning of this review.*

Key words: *CaM- Calcium-modulated protein, CML- CaM-like protein EF-hand, Secondary Messenger, Signal Transduction, Ion Channels, Environmental Stress, Signal Perception.*

INTRODUCTION

The sessile nature of plants necessitates their adaptation to continuously changing and often unfavorable environmental conditions. These include many abiotic stresses that arise from an excess or deficit of water, temperature, and light in the physical environment[5,30,47].

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Classic agricultural technologies such as irrigation, applications of fertilizer, insecticides, fungicides, and chemical phytoprotectants have helped to improve crop yield, but the effects are limited, costs are high and the impacts on the ecosystems and human health are undesirable and dangerous. Plants showed extraordinary plasticity in many of their growth and developmental processes in response to changes in their environment. Elucidation of mechanisms by which plants recognize and respond to various stresses is of great interest to agriculturist not only to elucidate basic principles in cellular signaling mechanisms but also to knowledge to generate plants that can be grown under adverse environmental conditions. Understanding how plants adopt and respond to various environmental stresses provides the necessary framework to create new crop varieties which may fit better into the stress environments, and has become one of the most important tasks for plant scientists around the world. Here, we focus primarily on the role of Ca2+- and Ca2+/calmodulin (CaM)-regulated gene expression in stress signaling. For those aspects of calcium signaling in plants that are not covered, referred into some recent reviews[19,6,8,10,21]. High Ca^{2+} concentrations can be toxic to cellular energy metabolism, cytosolic Ca^{2+} level in unstimulated cells is maintained at a submicromolar concentration by removing Ca^{2+} ion from the cytosol to either the apoplast or the lumen of intracellular organelles such as the vacuole or the endoplasmic reticulum. Transient elevations in the cytosolic Ca^{2+} concentrations occur via an increased Ca^{2+} influx and a rapid return to the basal level by Ca^{2+} efflux in response to a variety of stimuli including hormones, light, gravity, abiotic stress factors or their interactions with pathogens and symbionts. These small Ca^{2+} pulses are briefly available to act as cellular signals and exert changes in cellular functions. Hypothesized that a myriad of stimuli uses Ca^{2+} as an intracellular intermediate, a central question on Ca^{2+} signaling is how this signal carrier conveys information from a perceived stimulus and controls the specificity of cellular responses. In recent years, significant progress have been made in elucidating the patterns of Ca^{2+} signals and the relays that convert these messages into cellular responses.

Structure: Calmodulin is a small, highly conserved protein that is 148 amino acids long (16.7 KDa) and first discovered by Ebashi and Kodama[9] who demonstrated calcium requirement of calcium-binding protein troponin C in skeletal muscle. Calmodulin protein has two approximately symmetrical globular domains each containing a pair of EF-hand motifs (the N- and C-domain) separated by a flexible linker region for a total of four Ca^{2+} binding sites. Each EF-hand motif allows calmodulin to sense intracellular calcium levels by binding one Ca^{2+} ion. Calcium ion binding regions are found in the following positions in the sequence of amino acids are 21-32, 57-68, 94-105 and 130-141; each region that calcium binds to is exactly 12 amino acids long. These regions are located between two alpha helices in the EF-hand motifs, the first two regions (21-32 and 57-68) are on one side of the linker region the other two (94-105 and 130-141) are on the other side. Survey of genomes of different organisms revealed a greater diversity in sequence of CaM displayed by plants unlike animals that have only few genes encoding calmodulin[56].

Mechanism: Four calcium ions are bound by calmodulin via its four EF hand motifs. EF hands supply an electronegative_environment for ion coordination. After calcium binding, hydrophobic methyl groups from methionine residues become exposed on the protein via conformational change. Using X-Ray and NMR studies, revealed that the conformational changes occurred in the alpha-helices of the EF motif, which changes the binding affinity for target proteins. When the alpha helices are perpendicular to one another, the Calmodulin is in an open conformation giving it a higher affinity for target proteins Moreover specifically, this conformational change presents hydrophobic surfaces, which can in turn bind to Basic Amphiphilic Helices (BAA helices) on the target protein. These helices contain complementary hydrophobic region. The flexibility of calmodulin's hinged region allows the molecule to wrap around its target. This property allows it to tightly bind to a wide range of different target proteins. The C-domain of calmodulin has a higher affinity for calcium than does the N-domain.

Changes in cellular ca^{2+} levels in response to abiotic and biotic stress signals as a secondary messenger: Secondary messengers are intracellular signaling molecules released by the cell to trigger physiological modification such as proliferation, differentiation, migration, survival and apoptosis. Secondary messengers are therefore one of the initiating components of intracellular signal transduction cascades. Examples of second messenger molecules include cyclic AMP, cyclic GMP, inositol trisphosphate, diacylglycerol, and calcium. The cell releases multiple secondary messenger in response to exposure to extracellular signaling molecules—the **first messengers**. Secondary messenger systems can be synthesized and activated by enzymes, for example, the cyclases that synthesize cyclic nucleotides, or by opening of ion channels to allow influx of metal ions, for example Ca^{2+} signaling. These small molecules are bind and activate protein kinases, ion channels and other proteins, thus continuing the signaling cascade.

In response to a variety of stimuli, such as hormones, gravity, light, and abiotic and biotic stresses, plants generate a “Ca^{2+} spike,” a transient increase in intracellular calcium level. Given that a plethora of stimuli can generate a “Ca^{2+} spike,” specificity in signaling is achieved by downstream transducers of Ca^{2+} signal, the “EF-hand”-containing proteins called “Ca^{2+} sensors” or Ca^{2+}-binding proteins[4]. Presence of a large repertoire of Ca^{2+}-transducing proteins in plants points to the existence of a wide variety of cellular responses regulated by calcium. Signal-induced Ca2+ changes in plant nuclei have been reported[46,28] but not studied as extensively as signal-induced [Ca2+]cyt. Thus, nuclei have the potential to generate a Ca2+ signature[51,23,27,28]. In vitro studies with plant nuclei indicate that Ca2+ does not pass through nuclear pores passively and requires energy in term of ATP (Nicotera et al., 1989; Pauly et al., 2000). However, an in vivo study with animal cells indicates that Ca2+ can freely diffuse through nuclear pores at very low concentrations but not more than 300 nM, indicating that [Ca2+]cyt change may influence [Ca2+]nuc levels under certain condition but not others. Plant nuclei are also capable of generating Ca2+ changes that are not dependent on [Ca2+]cyt changes, suggesting that [Ca2+]nuc and [Ca2+]cyt levels can be regulated independently[34,28]. The mechanisms and the channels involved in signal-induced changes in [Ca2+]nuc have not been identified[27,28]. Therefore, the regulation of transcription by Ca2+ in plants may occur through processes controlled in the cytosol and in the nucleus or by a combination of both. For instance, studies on stress gene regulation in tobacco (Nicotiana tabacum) showed that wind-induced expression of one CaM isoform is regulated by a Ca2+-signaling pathway in the nucleus, while expression of a cold shock–induced isoform is regulated by a pathway in the cytoplasm[46]. Although the effect of individual stresses on cellular Ca2+ levels has been extensively studied, the effect of combinations of stresses that plants are subjected to normally has not been investigated in any detail. To understand the effects of multiple stresses, it will be necessary to investigate the type of Ca2+ signatures elicited by a combination of stresses. Calcium signatures elicited by a combination of stresses are likely to be different from those evoked by individual stresses.

CALMODULIN AND CROSSTALK BETWEEN DIFFERENT STRESSES

Drought Stress: drought stress is the major environmental stress which continuously experienced by plants, and both impose osmotic stress on plant cells. Osmotic stress induces a series of responses at the molecular and cellular levels and a primary event is an increase in the cytosolic Ca^{2+} concentration and subsequent transduction of Ca^{2+} signals that promotes cellular responses in an effort to mitigate potential damages[51]. MYB2 is reported to interact with CaM in a Ca^{2+}-dependent manner and regulate dehydration responsive genes[1,58]. Another similar CML protein AtCML9 was found to be involved in the osmotic stress tolerance through ABA-dependent pathways[26]. *AtCML9* was induced by abiotic stress and ABA, knock-out mutant *atcml9* showed a hypersensitive response to ABA during seed germination and seedling growth stages, and exhibited enhanced tolerance to salt and dehydration stresses. Furthermore, expression of several stress gene and ABA-dependent genes including *RAB18*, *RD29A*, and *RD20* was altered in *atcml9*. As the most abundant vacuolar Na^+-proton exchanger in *Arabidopsis*, Na^+/H^+ exchanger 1 (AtNHX1) regulates various cellular activities such as maintaining pH, ion homeostasis, and protein trafficking. Yamaguchi et al.[54] found that AtCaM15 (also called AtCML18) is localized in the vacuolar lumen and interacts with the C-terminus of AtNHX1. The cross talk between AtCaM15 and AtNHX1 is affected by both Ca^{2+} presence and pH, and the binding of AtCaM15 to AtNHX1 alters the Na^+/K^+ selectivity of the exchanger by decreasing its Na^+/H^+ exchange speed. The interaction between AtCaM15 and AtNHX1 suggests the presence of Ca^{2+}-pH-dependent signaling components in the vacuole, which are involved in mediating plant responses to salt stress.

Oxidative stress and ROS: Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2), and hydroxyl radical (·OH) are usually produced in various physiological processes and serve as a class of second messengers[45,2]. While controlled production of ROS is essential to signal appropriate actions to protect plants from various environmental stresses, excessive accumulation of ROS causes damages to plant cells. Oxidative stress is defined as disruption of the cellular redox balance, which could be triggered by a wide range of biotic and abiotic stimuli[37]. Because of its long half-life and excellent permeability, H_2O_2 is broadly accepted as the major form of ROS in plant cells. It is known that H_2O_2 trigger the increases in cytosolic Ca^{2+} by activating the Ca^{2+}-permeable channels[36,37]. On the other hand, H_2O_2 production during oxidative burst is also dependent on continuous Ca^{2+} influx, which activates not only the NADPH oxidase, an EF-hand containing enzyme on the plasma membrane[50,] but also the CaM-binding NAD kinase (NADK), which supplies NADP cofactor for ROS production through NADPH oxidase[16,17,43].

Heat Stress: Prolonged high temperature is usually lethal to all organisms; fluctuations in temperature above optimal level, usually called heat shock (HS), impose major stress affecting plant growth and productivity. Almost all organisms including plants synthesize HS proteins (HSPs), a class of chaperons to assure normal function of various client proteins under adversely high temperature conditions. It was observed that HS induced a quick and strong elevation in cytosolic Ca^{2+} in tobacco[13]. Expression of CaM protein in the maize coleoptiles was found to be induced during heat stress and was affected by Ca^{2+} level, suggesting that Ca^{2+} and CaM may be involved in the acquisition of HS-induced thermotolerance[14]. Liu et al.[25] observed an increase in intracellular Ca^{2+} within one min after wheat was subjected to 37^{oC} HS. Expression level of CaM mRNA and protein was both induced during HS in the presence of Ca^{2+} and expression of *HSP26* and *HSP70* was stimulated by exogenous application of Ca^{2+}. HS-induced expression of *CaM* was 10 min earlier than that of *HSP*s, and both were suspended by pharmacological reagents which interfere with Ca^{2+} signaling. These results showed that Ca^{2+} and CaM are directly involved in HS regulation[25]. The Ca^{2+}/CaM signaling system was also proposed that the induction of *HSP* genes in *Arabidopsis*[24]. Using molecular and genetic tools, Zhang et al. (2009) found that *Arabidopsis* AtCaM3 was involved in the Ca^{2+}/CaM-mediated HS signal mediated pathway. *atcam3* knockout mutant showed a decrease in thermotolerance after 50 min of incubation at 45°C. The compromised thermotolerance of *atcam3* mutant could be rescued by functional complementation with 35S promoter driven AtCaM3, and over expression of *AtCaM3* in wild-type (WT) background increased thermo tolerance of the transgenic plants. Furthermore, the DNA-binding activity of HS transcription factors and the expression of tested HS genes at both mRNA and protein levels were shown to be down-regulated in *atcam3* null mutant and up-regulated in its over expressing mutant upon HS treatment[59]. A role for CaM protein in HS signaling was also reported in rice[49,48]. HS was reported to induce biphasic cytosolic Ca^{2+} transients, and this feature was found to be reflected in the HS-induced expression of *OsCaM1-1*. OsCaM1-1 was observed to localize to the nucleus and over expression of *OsCaM1-1* in *Arabidopsis* resulted in enhanced thermotolerance which coincided with elevated expression of HS-responsive *AtCBK3, AtPP7, AtHSF*, and *AtHSP* at a non-inducing temperature. Nitric oxide (NO) signaling molecules level is found to be elevated by high temperature stress[15] and exogenous application of NO donor provides effective protection to plants under heat stress[44,40]. However, for a long time it was unknown how NO is involved in protecting plants from damage by HS. Recently, *Arabidopsis* CaM3 was reported that it act as a downstream factor of NO signaling in activation of HS transcription factors, accumulation of HSPs and establishment of thermo tolerance[53]. In addition, early studies from heat-stressed maize seedling suggested that ROS homeostasis and the entire antioxidant system including catalase, superoxide dismutase (SOD) and ascorbate peroxidase, could be regulated by Ca^{2+} influx and intracellular CaM[14]. Later plant Catalases, catalyzing ROS to water and oxygen was found to bind CaM in a Ca^{2+}-dependent manner[57]. The activity of the *Arabidopsis* CAT3 is stimulated by Ca^{2+}/CaM combine rather than Ca^{2+} or CaM alone, but catalases from other organisms such as *Aspergillus niger*, human and bovine, do not interact with CaM[57]. Peroxidase enzyme is a type of CBP, isolated from *Euphorbia latex*, activated by Ca^{2+}/CaM[29,31]. Evidence suggested that another class of ROS-scavenging enzyme SOD regulated by CaM in maize, although the specific *SOD* gene has not been cloned[13]. The critical role of Ca^{2+}/CaM in balancing ROS actions was further supported by the observation that the oxidative damage caused by heat stress in *Arabidopsis* seedlings is exacerbated by pretreatment with CaM inhibitors[22].

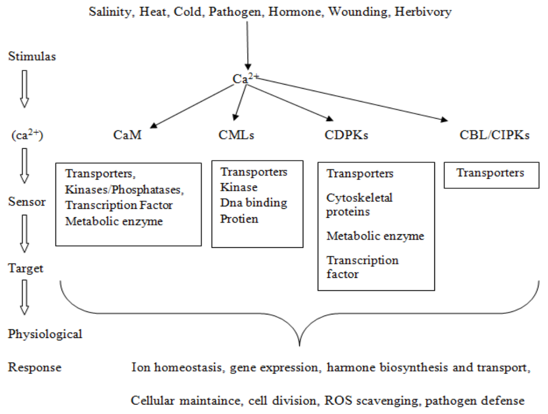
Cold Stress: Ca^{2+} has been identified as a vital second messenger coupling to cold stress (Knight et al., 1991; Dodd et al., 2010). In addition to downstream effectors of Ca^{2+}/CaM-mediated signaling , CaM-regulated receptor-like kinases CRLK1, which is mainly localized in the plasma membrane, was found to be involved in cold tolerance[56]. CBPs are also reported to be involved in plant responses to cold stress. Ca^{2+}/CaM-mediate receptor-like kinase CRLK1, which is mainly localized in the plasma membrane, was found to be involved in cold tolerance (Yang et al., 2010b). CRLK1 carries two CaM-binding sites, N- and C-termini with differ affinities for Ca^{2+}/CaM of 25 and 160 nM, respectively[56]. *crlk1* knockout mutant plants grow and behave similar to WT plants under control conditions, but are more sensitive to chilling and freezing stress than WT plants[56]. In addition, cold response genes *CBF1*, *RD29A*, *COR15a* and *KIN1* showed delayed in responses to cold in *crlk1* mutant , suggesting a putative role of CRLK1 in regulating cold tolerance .Transgenic studies on *Arabidopsis* by over expressing *CaM3* showed the decreased levels of *COR* (cold regulated) transcripts, suggesting that CaM may function as a negative regulator of cold-induced gene expression (Townley and Knight, 2002). Genes encoding CMLs, such as *AtCML24/TCH2* and *OsMSR2* (*O. sativa* Multi-Stress-Responsive gene2, a novel CML gene), were also induced by cold treatment and thus, likely to involve in the transducing cold-induced Ca^{2+} signals[35,7,52].

MEKK1, which is a member of the MAP kinase kinase kinase family, was shown to interact with CRLK1 both in vitro and in vivo[55]. Knockout mutation of CRLK1 abolished that cold-triggered MAP kinase activities and modify cold-induced expression of genes involved in MAP kinase signaling[55]. Therefore, Ca^{2+}/CaM-regulated CRLK1 may modulate cold acclimation through MAP kinase cascade in plants. Other CaM-binding kinases are also reported to be involved in cold acclimation. The expression of *PsCCaMK* in pea (*Pisum sativa*) roots was found to be up-regulated by low temperature or salinity stress[33] and the activity of the Ca^{2+}/CaM-dependent NADK was also found to be increased by cold shock[39].

Heavy Metal Stress: Elevated concentration of both essential (Cu and Zn) and non-essential (Cd, Hg, Pb, and Ni) heavy metals in the soil can cause toxicity and impair plant growth. It was showed that Ca^{2+}/CaM is involved in responses to Cd^{2+} toxicity during the early phases of seed germination in radish (*Raphanus sativus* L.)[38]. Ca^{2+} added in the medium could partially reduce the Cd^{2+}-toxicity in the germinating embryo, and this coincides with the decreased Cd^{2+} uptake. An equilibrium dialysis study reported that Cd^{2+} interfare with Ca^{2+} for CaM-binding, hence Cd^{2+} reduce the binding of Ca^{2+}/CaM to its target site. In tobacco (*N. tabacum*) cyclic nucleotide gated ion channel (CNGC) called NtCBP4 was identified to be a CBP through protein–protein interaction-based library screening, and shown to be localized to plasma membrane. Transgenic tobacco plants with elevated level of *NtCBP4* displayed tolerance to Ni^{2+} and hypersensitivity to Pb^{2+}, and consistently showed decreased Ni^{2+} and increased Pb^{2+} accumulation, suggesting that NtCBP4 is involved in heavy metal uptake across the plant plasma membrane[3]. However, transgenic plants mediates a truncated version of NtCBP4 lacking the C-terminal stretch covering the CaMBD and part of the putative cyclic nucleotide-binding domain showed improved tolerance to Pb^{2+} and lower accumulation of Pb^{2+}, and loss-of-function mutation of AtCNGC1, a homolog of NtCBP4 in *Arabidopsis*, also resulted in Pb^{2+} tolerance. These results revealed that CaM-binding is required for the normal function of both AtCNGC1 and NtCBP4 for the transport of heavy metals[41].

EFFECT OF CELLULAR CA2+ CHANGES ON GENE EXPRESSION AND SINALLING

The transduction of a Ca^{2+} signaling can be showed 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 undergoes post-translational modifications 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 a. 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.



CONCLUSION

Number of studies have been conducted on shed light on CaM-modulated target proteins expanding understanding of CaM function in both abiotic stress response. Development of both physiological and biochemical method by different research groups has facilitated identification of novel proteins regulated by CaM in response to different stimulus. However, mechanistic details of how CaM is regulating the activity of such target proteins within the cell in cross talk with other pathways are just beginning to emerge. Work over the last decade has provided us with the knowledge of many novel roles played by calcium signaling and calmodulin in vast majority of abiotic and biotic stress pathways. Future studies try to discover many more such targets of calmodulin and their different role in abiotic stress-signaling pathway would be a major research focus that would also provide a platform for utilizing this basic knowledge in creation of various stress-resistant crop varieties.

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