Maryam Sarwat · Altaf Ahmad
MZ Abdin *Editors*

# Stress Signaling in Plants: Genomics and Proteomics Perspective, Volume 1

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# Preface

The most important prerequisites imperative for survival of human kind; food, fodder and fuel depend upon the performance of plants. Due to worldwide temperature inversions, the weather conditions have become completely hostile and unpredictable. Thus, there is a growing need of such plants which are better adapted to these adverse climatic conditions. A good understanding of the signalling mechanism within the plant system during these climatic conditions will certainly going to help in raising plants which are better suited for these adverse conditions.

In this book, we have put together both genomics and proteomics approach to further our understanding in this direction. The chapters in this book expand our understanding from bioinformatical approaches to develop the models, as well as proving the ideas up to field conditions. Hence, this book contains comprehensive knowledge of stress signalling useful for graduate students, researchers as well as scientists working in this area.

The ten chapters written by international dignitaries give much weightage to this book.

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# Chapter 1
# $Ca^{2+}$, Calmodulin and Plant-Specific Calmodulin-Binding Proteins: Implications in Abiotic Stress Adaptation

**Prabhjeet Singh and Amardeep Singh Virdi**

## Abbreviations

| | |
|---|---|
| a.a. | Amino acid |
| $Ca^{2+}$ | Calcium |
| CaM | Calmodulin |
| CaMBD | Calmodulin-binding domain |
| CaMBOT | Calmodulin-binding gel overlay technique |
| CaMBP | Calmodulin-binding protein |
| CAMTA | Calmodulin-binding transcription activator |
| CBK | Calmodulin-binding kinase |
| CRCK | Cytoplasmic-localized $Ca^{2+}$-CaM regulated kinase |
| CRLK | Plasma membrane-localized $Ca^{2+}$-CaM regulated kinase |
| HS | Heat stress |
| HSF | Heat shock factor |
| Hsp | Heat shock protein |
| MAPK | Mitogen activated protein kinase |
| MEK | MAPK kinase |
| MEKK | MAPK kinase kinase |
| PCD | Programmed cell death |
| SA | Salicylic acid |

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## Summary

Plants, being sessile, are frequently exposed to different types of abiotic stresses, which may affect their growth and development adversely. Plants adapt to stress conditions by activation of cascade(s) of molecular mechanisms, which result in alterations in gene expression and synthesis of protective proteins/compounds. From the perception of the stimulus to transduction of the signal, followed by an appropriate response, the plants employ a complex network of primary and secondary messenger molecules, of which $Ca^{2+}$ is one of the most well studied. Decoding of spatial and transient changes in $Ca^{2+}$ levels is accomplished by different $Ca^{2+}$-binding proteins (CaBPs), which act as $Ca^{2+}$ sensors. Calmodulin (CaM) is one of the best characterized primary transducer of cytosolic $Ca^{2+}$ changes in all eukaryotes. CaM is an acidic, heat stable and multifunctional protein consisting of two globular domains, each with two $Ca^{2+}$-binding EF-hand motifs. After binding to $Ca^{2+}$, the CaM undergoes a conformational change and binds to diverse range of proteins. The CaM-binding proteins (CaMBPs) have been identified and characterized from different plants and recent studies suggest the involvement of several of the CaMBPs in abiotic stress adaptation. Further, different isoforms of CaM have been reported to modulate the activity of CaMBPs differentially, thus, implying intricate mechanisms of regulation by CaM. Therefore, in the following section, the likely role of $Ca^{2+}$, CaM and plant-specific CaMBPs in abiotic stress adaptation will be discussed.

## Introduction

Plants are frequently exposed to different types of abiotic stresses, thus, affecting their growth and development adversely. Adaptation to stress conditions by plants involves activation of cascade(s) of molecular mechanisms that result in alterations in gene expression and synthesis of protective proteins/compounds (Wang et al. 2003). For conveying the signal in response to stress, the plants employ a complex network of primary and secondary messenger molecules (Shinozaki et al. 2003; Bartels and Sunkar 2005). $Ca^{2+}$ is one of the most well studied secondary messengers involved in signal transduction in eukaryotes (Clapham 2007). The resting cytosolic concentration of $Ca^{2+}$ (100–200 nM) increases up to several folds in response to specific stimulus (Reddy 2001). The intracellular changes in $Ca^{2+}$ are sensed by different $Ca^{2+}$-Binding Proteins (CaBPs) (Reddy 2001; Bouche et al. 2005), which are characterized by the presence of helix-loop-helix motif called EF-hand motif, that typically occur in pairs and facilitate high affinity binding of $Ca^{2+}$ (Gifford et al. 2007). Four broad categories of CaBPs *viz.*, $Ca^{2+}$-dependent protein kinases, calmodulin (CaM), CaM-like proteins and calcineurin B-like proteins have been reported (Bouche et al. 2005 and references therein).

Of the different $Ca^{2+}$ sensors, CaM has been characterized most extensively (Roberts and Harmon 1992; Snedden and Fromm 1998, 2001). The CaM, though primarily a cytosolic protein, is also detected in the mitochondrion, chloroplast,

peroxisome, nucleus, and extracellular matrix (Roberts et al. 1983; Van der Luit et al. 1999), thus, signifying the versatility in its roles. CaM is an acidic protein of approximately 150 amino acid (a.a.) residues. CaM consists of two globular domains connected by a long flexible helix, with each of the globular domains containing two EF-hand motifs that bind to $Ca^{2+}$ cooperatively (Babu et al. 1988). After binding to $Ca^{2+}$, CaM undergoes conformation change, thus, exposing two hydrophobic sites surrounded by negative charges in each of the globular domains, which interact with several target proteins, thereby, regulating their activities (Crivici and Ikura 1995).

Calmodulin is a highly conserved protein in eukaryotes and as compared to animals, which contain only few genes of CaM (e.g. three in humans), there are multiple genes in plants. A total of nine true CaM genes encoding for four different isoforms have been identified in *Arabidopsis thaliana* (McCormack et al. 2005). On the contrary, the wheat genome contains up to 13 genes of CaM and ten cDNAs encoding three different isoforms of CaM have been isolated (Yang et al. 1996). Rice genome has been reported to show the presence of five true CaM genes, which encode two sets of CaM, with three of the genes coding for identical isoforms (Boonburapong and Bauboocha 2007). The different CaM isoforms differ in their ability to regulate target proteins (Lee et al. 1999, 2000), which suggests that multiple CaM isoforms may be enabling the plants to respond differentially to different environmental and developmental signals.

## Detection of CaM-Binding Proteins

Calmodulin targets a vast array of diverse proteins such as metabolic enzymes, transcription factors, kinases, cytoskeletol proteins, chaperones, etc., in plants and animals (Defalco et al. 2010). Since the primary sequence of CaM-binding domains (CaMBDs) among different CaM-binding proteins (CaMBPs) (except within protein families) is not conserved, therefore, the CaMBPs are identified by CaM-binding gel overlay technique (CaMBOT) (O' Day 2003), which employs labelled CaM (Fromm and Chua 1992; Lydan and O' Day 1994; O' Day 2003). This assay identifies the CaMBPs on the basis of protein-protein interactions and provides a crucial beginning point for identifying CaMBPs.

## Molecular Basis of CaM-CaMBPs Interaction

Structural analysis, which has been carried out for some CaMBPs, reveals that two bulky hydrophobic a.a. residues (Phe, Trp, Ile, Leu or Val), situated at specific distance apart, in target proteins play an important role in interaction with CaM (Rhoads and Friedberg 1997). The different motifs, identified to be involved in CaM-binding, are IQ motif, and 1–10, 1–14 and 1–16 motifs, since the key anchor

a.a. residues are 8, 12, and 14 residues apart, respectively (Alexander et al. 1988; Cheney and Mooseker 1992; Osawa et al. 1999). The CaM-binding motifs from different CaMBPs form characteristic basic amphipathic α-helices with several positive residues on one side and hydrophobic residues on the other side (Rhoads and Friedberg 1997). The propensity of a protein to bind to CaM can be analyzed using software (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/browse.html) in which a score of probability ranging from 0 (unlikely) to 9 (very likely) is calculated per a. a. residue (Yap et al. 2000). A stretch of a.a. residues with a score of 7–9 signify the presence of a putative CaM recruitment signal. Although the accuracy of this programme is 80%, it provides a useful tool to determine the CaM-binding property of proteins by *in silico* analysis.

## Role of CaMBPs in Abiotic Stress Response in Plants

The CaMBPs in plants have been implicated in various aspects, such as regulation of ion transport, metabolism, cytoskeleton, protein folding, transcription, protein phosphorylation and dephosphorylation, phospholipid metabolism, disease resistance, cell division, etc. (Reddy et al. 2011). However, the focus of this chapter will be on their role in abiotic stress adaptation since several CaMBPs have been identified which are regulated by different abiotic stress conditions (Table 1.1) (Singh and Virdi 2010).

### *Regulation by CaM of Enzymes Involved in Generation of Reactive Oxygen Species and Programmed Cell Death*

Plants often respond to environmental stresses by producing the reactive oxygen intermediates (ROIs) and their levels in cell are tightly regulated to avoid cellular damage. Exposure to oxidative stress with $H_2O_2$, which results in enhanced $Ca^{2+}$ levels (Lecourieux et al. 2002), also caused an increase in the expression of oxidative stress-responsive genes, including some specific CaMBPs like catalases and superoxide dismutases (SODs) (Gong and Li 1995). Catalases are protective enzymes, which are involved in degradation of $H_2O_2$ to water and oxygen. Regulation of catalases by CaM appears to be plant-specific phenomenon, since animal counterparts of catalases do not show CaM-binding properties and this feature might have evolved in plants due to their sessile nature (Bouche et al. 2005). SODs are another class of ROIs-scavenging enzymes, which show binding to CaM in $Ca^{2+}$-dependent manner (Gong and Li 1995). However, the regulation of SODs by $Ca^{2+}$/CaM in plants needs to be explored further.

**Table 1.1** Abiotic stress modulated calmodulin-binding proteins in plants

| S. no. | Protein | Gene | Source | Stress response | Reference |
|---|---|---|---|---|---|
| 1 | Calmodulin-binding protein kinase | *NtCBK* | *Nicotiana tabacum* | SS, GA | Hua et al. (2004) |
| 2 | $Ca^{2+}$-dependent calmodulin-binding cytoplasmic receptor-like kinase | *CRCK1* | *Arabidopsis thaliana* | SS, CS, $H_2O_2$, ABA | Yang et al. (2004) |
| 3 | Calmodulin-binding receptor-like kinase | *CaMRLK* | *A. thaliana* | CS | Charpenteau et al. (2004) |
| 4 | $Ca^{2+}$/calmodulin-regulated receptor-like kinase | *AtCRLK1* | *A. thaliana* | CS | Yang et al. (2010b) |
| 5 | $Ca^{2+}$-dependent calmodulin-binding recepotor-like kinase | *GsCBRLK* | *Glycine soja* | CS, SS, OS, ABA | Yang et al. (2010a) |
| 6 | Calmodulin-binding protein kinase 3 | *AtCBK3* | *A. thaliana* | HS | Liu et al. (2008) |
| 7 | Mitogen-activated protein kinase phosphatase | *NtMKP1* | *N. tabacum* | SA, wounding | Yamakawa et al. (2004) |
| 8 | Calmodulin-binding Ser/Thr phosphatase | *AtPP7* | *A. thaliana* | HS | Liu et al. (2007) |
| 9 | Calmodulin-binding transcription activator | *CAMTA1*<br>*CAMTA3* | *Brassica napus*<br>*A. thaliana* | CS<br>Plant immunity | Bouche et al. (2002)<br>Du et al. (2009) |
| 10 | Calmodulin-binding transcription factor | *OsCBT* | *Oryza sativa* | Negative regulator of plant defence gene expression | Choi et al. (2005) |
| 11 | MYB2 transcription factor | *AtMYB2* | *A. thaliana* | SS | Yoo et al. (2005) |
| 12 | Calmodulin-binding BTB and TAZ domain protein | *AtBT1-5* | *A. thaliana* | $H_2O_2$, SA | Du and Poovaiah (2004) |
| 13 | Calmodulin-binding protein | *NtCBP4* | *N. tabacum* | Heavy metal tolerance | Arazi et al. (1999) |
| 14 | Heat shock protein 90 | *Hsp90* | *Sorghum bicolor* | HS | Virdi et al. (2009) |
| 15 | Heat shock protein 70 | *Hsp70* | *Zea mays* | HS | Sun et al. (2000) |
| 16 | FK506-binding protein | *FKBP77* | *Triticum aestivum* | HS, DS | Kurek et al. (1999) |
| 17 | Glyoxalase 1 | *GLX1* | *B. juncea* | Heavy metal ($Zn^{2+}$, $Cd^{2+}$) tolerance, OS, SS, ABA | Deswal and Sopory (1991) |

(continued)

**Table 1.1** (continued)

| S. no. | Protein | Gene | Source | Stress response | Reference |
|---|---|---|---|---|---|
| 18 | Soybean $Ca^{2+}$-ATPase 1 | *SCA1* | *Glycine max* | SS, fungal elicitor | Chung et al. (2000) |
| 19 | Glutamate decarboxylase | *GAD* | Petunia *Z. mays* | ABA, MJ, SS, OS, CS, anoxia, mechanical damage | Baum et al. (1993) Zhuang et al. (2010) |
| 20 | Apyrase | Apyrase | *Pisum sativam* *A. thaliana* | Tolerence to xenobiotic compounds | Hsieh et al. (2000) Steinebrunner et al. (2000) |
| 21 | Catalase | Catalase | *A. thaliana* | $H_2O_2$ homeostasis | Yang and Poovaiah (2002) |
| 22 | Calmodulin-binding protein 25 kDa | *AtCAMBP25* | *A. thaliana* | Negative regulator of OS | Perruc et al. (2004) |
| 23 | Superoxide dismutase | SOD | *Z. mays* | $H_2O_2$ homeostasis | Gong and Li (1995) |
| 24 | BCL2-associated athanogene protein (BAG) | *AtBAG6* | *A. thaliana* | PCD, SA, $H_2O_2$, HS | Kang et al. (2006) |

Adapted from Singh and Virdi (2010). Reprinted with permission from Journal of Plant Biology
*ABA* abscisic acid, *CS* cold stress, *DS* drought stress, *GA* gibberellic acid, *HS* heat stress, *MJ* methyl jasmonate, *OS* osmotic stress, *SA* salicylic acid, *SS* salt stress, *PCD* programmed cell death

The production of ROIs under abiotic stress conditions is followed by necrosis and plant cell death (PCD) (Mittler 2002). ROIs act as secondary messengers for execution of cell death during hypersensitivity responses and also play key role in ozone-mediated cell death and PCD (Rao and Davis 1999; Mittler 2002). The BAG (BCL2-associated athanogene) proteins were implicated in antiapoptotic activity (Takayama et al. 1995). Eight genes that encode proteins with the BAG domain have been identified in Arabidopsis genome, with four (AtBAG5, AtBAG6, AtBAG7, and AtBAG8) exhibiting the presence of a CaMBD (IQ motif) close to the conserved BAG domain (Kang et al. 2006). Heterologous expression of AtBAG6, which interacts canonically with different CaM isoforms in $Ca^{2+}$-independent manner, in yeast cells resulted in induced cell death and its expression was enhanced in response to SA, $H_2O_2$ and high temperature stress, all of which are known to be involved in plant PCD processes (Kang et al. 2006). Although the role of CaM-BAG complex in PCD has been well characterized, the precise function of this complex in downstream components *in planta* is yet to be elucidated.

## *Regulation of Stress-Modulated Kinases by CaM*

Kinases constitute an indispensible component of the signal transduction pathways and this is achieved by alteration in autophosphorylation status and/or formation of multi-component complex (Charpenteau et al. 2004). CaM-binding protein kinases (CBKs) have been cloned and characterized from several plant species and elaborately reviewed by Zhang and Lu (2003). The presence of N-terminal CaMBD and C-terminal protein kinase catalytic domain of variable length and sequence is an important feature of plant CBKs. The CBKs from *A. thaliana* (AtCBK1) and tobacco (NtCBK2) bind to CaM in $Ca^{2+}$-dependent manner (Zhang and Lu 2003). The autophosphorylation and substrate phosphorylation activities of these proteins were $Ca^{2+}$-dependent and enhanced by CaM up to 4- to 5-fold. The autophosphorylation activity of CBKs in lily and tobacco was, on the contrary, inhibited in the presence of CaM (Takezawa et al. 1996; Liu et al. 1998). The autophosphorylation activity of maize CBK (ZmCCaMK) was unaffected by CaM (Pandey and Sopory 1998, 2001), though its substrate phosphorylation activity was $Ca^{2+}$-CaM dependent. The autophosphorylation and substrate phosphorylation activities of rice CBK (OsCBK), which showed higher affinity for CaM, were, however, CaM-independent (Zhang et al. 2002). It is, thus, evident that regulation of activity of different CBKs by CaM is differential. The expression of CBKs in plants is reported to be modulated by different stress conditions and, hence, have been implicated in abiotic stress adaptation response (Hua et al. 2004).

Large number of receptor-like serine/threonine kinases (RLKs) are reported in plants and at least 600 RLK homologs have been identified in *Arabidopsis* (Hardie 1999). RLKs are transmembrane proteins, which recognize an extracellular signal that results in autophophorylation on the cytoplasmic kinase domain, thus, leading to transduction of signal (Stone and Walker 1995). About 3/4th of all the RLK homologs known are localized to plasma membrane and rest are cytoplasmic. The presence of cytoplasmic kinase domain, a single membrane spanning domain and an extracellular ligand binding domain, most commonly the leucine rich repeat domain (LRR Domain), are the important features of plasma membrane-localized plant RLKs (Torii 2000; Barre et al. 2002). On the contrary, the cytoplasmic RLKs contain only a kinase domain (Yang et al. 2004). A cytoplasmic-localized $Ca^{2+}$-CaM regulated kinase (CRCK1), isolated from *Arabidopsis*, showed binding to CaM in $Ca^{2+}$-dependent manner and the CaM-binding site was localized around the kinase domain. Both autophosphorylation and substrate phosphorylation activities of CRCK1 were enhanced by CaM. The autophosphorylation activity of CRCK1 was $Mg^{2+}$-dependent and no activity was observed in the presence of $Ca^{2+}$. The CaM-induced enhancement of autophosphorylation and substrate phosphorylation activity of CRCK1 was attributed to direct interaction of the former with a.a. residues 160–183 of this protein. *CRCK1* expression in the seedlings was enhanced at both transcript and protein levels in response to $H_2O_2$, salt, cold, and ABA treatment (Yang et al. 2004), thus, suggesting its role in multiple stress pathways.

The members of RLK family, which are localized to plasma membrane, have also been proposed to play a role in stress response. $Ca^{2+}$-regulated RLK from *Glycine soja (GsCBLRK)* (Yang et al. 2010a) and *Arabidopsis* (*CRLK1*) (Yang et al. 2010b) were demonstrated to act as positive regulators of cold- and salt stress adaptation, respectively. Both GsCBRLK and CRLK1 exhibited binding to CaM, which was $Ca^{2+}$-dependent, and their kinase activity was regulated through $Ca^{2+}$-CaM interaction (Yang et al. 2010a, 2010b). The autophosphorylation activity of another plasma membrane-localized RLK of *A. thaliana* (*AtCaMRLK*), whose binding to CaM is $Ca^{2+}$-dependent, on the contrary, was $Ca^{2+}$-CaM-independent (Charpenteau et al. 2004). The expression of *CRLK1* was observed in roots and leaves. CRLK1 protein levels were enhanced under cold stress (4 °C) without a significant increase in the mRNA level, thus, suggesting that this gene may be regulated at post-transcriptional level. Though *crlk1* mutant knockout plants showed no observable difference as compared to wild type under normal growth conditions, but imposition of cold stress resulted in decrease in root and shoot growth, early signs of senescence and more severe damage due to chilling in the *crlk1* mutants. The *CRLK1*-induced chilling stress tolerance appeared to be through modulation of cold regulated genes viz; *CBF1, RD29A, COR15a,* and *KIN1*, since cold-induced expression of these genes was delayed in *crlk1* mutant plants (Yang et al. 2010b). It, therefore, appears that *CRLK1* is likely to be an important component of cold stress signal transduction pathway in plants.

The role of CaM-binding CBKs in stress tolerance in plants was further emphasized by the cloning of a receptor-like protein kinase, *GsCBRLK,* from a salt tolerant plant, *Glycine soza,* and its over-expression in *A. thaliana*. The *GsCBRLK* binds to CaM in the presence of $Ca^{2+}$. The *GsCBRLK* has been proposed to act as a master regulator of salt stress response (Yang et al. 2010a). The expression of *GsCBRLK* was elevated by different abiotic stress conditions *viz.,* salt-, cold-, and osmotic stress. Constitutive over-expression of *GsCBRLK* in the transgenic *Arabidopsis* plants conferred tolerance, as was evident from enhanced germination, higher root and shoot growth, and increased levels of chlorophyll under salt stress, and in response to ABA treatment.

The differential regulation of phosphorylation activity of the plant CBKs by CaM may be the result of evolutionary divergence resulting from adaptation to different environmental conditions. The stress-inducibility of different CaM-binding kinases in response to diverse stresses implies that these proteins are playing an important role in stress signal transduction pathways and the differential regulation of different kinases by CaM may be enabling the plants to respond in a stimulus-specific manner. Comparative analysis of upstream sequences is, however, required to understand the molecular basis of differential stress-inducibility of the different kinase genes.

## *Regulation of Stress-Modulated Phosphatases by CaM*

Mitogen activated protein kinases (MAPKs) constitute another class of protein kinases that play an important role in signal transduction in eukaryotes. Each MAPK signaling cascade consists of a functionally interlinked pre-kinase module, an MAPK kinase (MEK) and an MAPK kinase kinase (MEKK). MEK carries out activation of MAPKs by phosphorylation of threonine and tyrosine residues with in a conserved TxY motif (Katou et al. 2007). The phosphorylated MAPKs are dephosphorylated by MAPK phosphatases (MKP) thus resulting in inactivation of MAPKs. Modulation of MKP activity, thus, is an important regulatory point in signal transduction in plants.

CaM regulates the activity of plant MKPs, which have been implicated in different abiotic- and biotic stress responses (Ulm et al. 2002). The CaM-binding property appears to be a unique and conserved feature of plant MKPs (Katou et al. 2007). MKPs have been cloned and characterized from tobacco (*NtMKP1*) (Yamakawa et al. 2004), Rice (*OsMKP1*) (Katou et al. 2007), and *Arabidopsis* (*AtMKP1*) (Lee et al. 2008). NtMKP1 and OsMKP1 are orthologs of AtMKP1. Though high similarity is observed in a.a. sequence of NtMKP1 and AtMKP1 but the protein structures, particularly the CaMBDs, are different. The NtMKP1 and OsMKP1 contain a single putative CaMBD. On the contrary, AtMKP1 consists of two different CaMBDs, both of which bind to CaM in $Ca^{2+}$-dependent manner, though the binding affinity of CaMBD2 is higher than CaMBD1 (Lee et al. 2008). The CaMBD2 is absent in NtMKP1 but CaMBD1 of the two correspond with each other. Studies carried out showed that phosphatase activity of AtMKP1 was positively regulated by CaM in a $Ca^{2+}$-dependent manner (Lee et al. 2008). AtMKP1, NtMKP1 and OsMKP1, through their phosphatase activities, were implicated in regulation of wound and defence response in plants. This was supported by the observation that over-expression of *NtMKP1* in transgenic tobacco plants attenuated the kinase activity of several defence-related MAPKs and wound-induced protein kinases (Yamakawa et al. 2004). These studies demonstrated that NtMKP1 may be acting as a negative regulator of MAPKs. The effect of different CaM isoforms on phosphatase activity of MKPs needs to be investigated in order to determine whether the regulation of wound response in plants is mediated through differential expression of different CaM isoforms (Yamakawa et al. 2004). The $Ca^{2+}$-CaM-regulated MKPs, therefore, may provide a critical link between two important signaling pathways in plants i.e., $Ca^{2+}$-signaling and MAPK signaling cascades, which may enable the plants to withstand stressful conditions.

## *Calmodulin-Binding Transcription Factors*

Recent studies suggest that the gene expression at transcriptional level is also regulated by CaM through modulation of activity of transcriptional factors

(Finkler et al. 2007). Various transcription factors, which are involved in cold stress tolerance (Doherty et al. 2009), modulation of plant immune response (Du et al. 2009), auxin signaling (Galon et al. 2010), etc., have been reported to show CaM-binding property. The expression of cold-regulated genes under chilling stress conditions is mediated through an increase in [$Ca^{2+}$] (Minorsky 1989; Knight et al. 1991). Three regulatory genes *viz., CBF1* (C-repeat binding factor), dehydration responsive element binding factor *(DREB1b), CBF2 (DREB1c),* and *CBF3 (DREB1a)* are rapidly expressed (with in 15 min) in response to low temperature. The product of these genes further induce the expression of ~100 genes by binding to their RT/DRE regulating elements in their promoters (Gilmour et al. 1998; Vogel et al. 2005). Recent studies have demonstrated that the CaM-binding transcriptional activator (CAMTA) proteins constitute the molecular link between [$Ca^{2+}$] spike and cold stress-regulated genes in Arabidopsis (Doherty et al. 2009).

Six CAMTA members have been identified in Arabidopsis (Bouche et al. 2002). These proteins carry an IQ domain for CaM-Binding, along with a CG1-1 domain that binds to core sequence VCGLGB (da Costa e Silva 1994; Bouche et al. 2002), which is similar to the conserved motif CM2 sequence [CCGCGT]. The CM2 motif overlaps with ICEr2 (inducer of CBF expression region 1 and 2) in CBF2 and is responsible for cold induction of CBF2. CAMTA proteins, 1, 2, 3 and 5 exhibited binding to CM2 sequence but analysis of T-DNA mutant of *A. thaliana* showed that only *CAMTA3* was responsible for regulating the expression of *CBF1*, *CBF2* and *ZAT12*. CBF3 expression, on the contrary, was not affected in any of the *CAMTA* mutants (Doherty et al. 2009). Studies by Doherty et al. (2009) also demonstrated that as contrary to *camta3* single mutants, *camta1 camta3* double mutants showed significant reduction in cold-induced expression of only *CBF1* but *CBF2* and *ZAT12* (zinc-finger protein) levels were not affected, thus, implying interaction of CAMTA1 and CAMTA2 in regulation of cold-induced gene expression. Analysis of *camta1* and *camta 3* single mutants, and *camta1 camta3* double mutants further revealed the role of *CAMTA1* and *CAMTA2* in cold acclimation of plants but not in the cold tolerance process *per se*. It is likely that both CAMTA1 and CAMTA3 may be required for stabilizing the proteins synthesized during acquisition of chilling tolerance, as was reported for a heat shock-associated protein HSA32 in Arabidopsis (Charng et al. 2006).

The structural homolog of AtCAMTA3 in rice, OsCBT (*Oryzae sativa* Cam-binding transcriptional factor) was also reported to bind to CaM in a $Ca^{2+}$-dependent/independent manner through two distinct types of CaMBDs (Choi et al. 2005). OsCBT was demonstrated to act as a negative regulator in plant defence related gene expression (Koo et al. 2009). CaM is a negative regulator of OsCBT since co-transformation of *OsCBT* and rice CaM genes *(OsCaM)* in *Arabidopsis* resulted in inhibition of transcriptional activation activity of OsCBT. $Ca^{2+}$-CaM, which plays an important role in plant defence signaling (Ali et al. 2003), may be responsible for triggering the plant defence response by inhibiting the OsCBT. The role of *OsCBT1* also need to be investigated in cold stress tolerance since this gene has been proposed as a functional orthologue of *CAMTA3* (Koo et al. 2009), which was implicated in regulation of cold-responsive genes (Doherty et al. 2009). The diverse functions of CAMTA3 and OsCBT1, as observed between *A. thaliana* and rice, signify the versatility in regulation of transcriptional factors by CaM.

A nuclear-localized protein of 25 kDa in Arabidopsis (AtCAMBP25) was also proposed to act as a negative regulator of salt- and osmotic stress tolerance since over-expression of this gene increased the sensitivity of transgenic plants to these stress conditions. On the contrary, suppression of this gene resulted in higher levels of tolerance (Perruc et al. 2004). However, information on the role of AtCaMBP25 as a transcriptional factor is lacking.

MYB proteins constitute another important class of transcriptional regulators in plants. The DNA-binding activity of MYB proteins is also modulated by $Ca^{2+}$-CaM in an isoform-dependent manner (Yoo et al. 2005). The DNA-binding activity of a MYB protein from *Arabidopsis* (AtMYB2) was affected differentially by different *Glycine max* CaM isoforms. Whereas, GmCaM4 enhanced the DNA-binding activity of AtMYB32 in a $Ca^{2+}$-dependent manner, another isoform, GmCaM1, had an inhibitory effect (Yoo et al. 2005). Transgenic Arabidopsis plants, which overexpressed *GmCaM4*, showed higher expression of *AtMYB2* and were more tolerant to salt stress. Higher level of salt stress tolerance, observed in the *GmCaM4* over-expressing plants, was attributed to an increase in expression of dehydration-responsive gene (*RD22*), alcohol dehydrogenase 1 (*ADH1*) and Delta (1)-pyrroline-5-carboxylate synthetase 1 (*P5CS1*), along with elevated levels of proline. On the contrary, over-expression of isoform GmCaM1 had no significant effect on the expression of stress-inducible genes. These observations suggested that salt stress tolerance through AtMYB2 activity is regulated through CaM in an isoform-specific manner. Since different isoforms are regulated differentially by different stimuli (Botella and Arteca 1994; Heo et al. 1999), it may enable the cell to fine tune the response under different environmental conditions.

CaM also regulates the activity of transcriptional factors through mediator proteins. A group of proteins in *Arabidopsis*, designated as AtBT1-5 (*A. thaliana* BTB and TAZ proteins), which bind to CaM in a $Ca^{2+}$- dependent manner, interact with two proteins, *Arabidopsis thaliana* Bromodomain and Extra Terminal domain proteins (AtBET10 and AtBET9), which belong to the family of fsh/ring3 class transcriptional regulators. *In vivo* activation of transcriptional function of AtBET10 ensues after interaction of this protein with AtBT through BTB domain (Du and Poovaiah 2004). The studies carried out till now suggest that some of the responses mediated by messenger molecules like $Ca^{2+}$, SA and $H_2O_2$ are through regulation of expression and modulation of conformation of AtBTs, which in turn facilitate the downstream responses of the cell by activating transcriptional activators such as AtBET10 (Du and Poovaiah 2004).

## *Role of CaM in Regulation of Transport of $Ca^{2+}$, Heavy Metal Ions and Xenobiotic Compounds*

Plasma membrane-localized channel proteins are involved in transport of heavy metals in plants (Arazi et al. 1999). These proteins are characterized by the presence of transmembrane domains and a putative cyclic nucleotide monophosphate

domain that overlaps with a CaM-binding domain located at C-terminus (Köhler et al. 1999). A gene for an 81 kDa plasma membrane-localized CaMBP in *Nicotiana tabacum* (NtCBP4), which showed homology to cyclic nucleotide gated channel protein, CNGC1, of *Arabidopsis,* was cloned (Arazi et al. 1999). Over expression of *NtCBP4* in tobacco resulted in enhanced tolerance to $Ni^{2+}$ but hypersensitivity to $Pb^{2+}$ in the transgenic plants. Tolerance to $Ni^{2+}$ in transgenic plants was due to reduced uptake of this ion and hypersensitivity to $Pb^{+}$ was attributed to increased accumulation of $Pb^{2+}$ in shoots of transgenic plants (Arazi et al. 1999). Deletion of the CaMBD and cyclic nucleotide-binding domains resulted in improved tolerance to $Pb^{2+}$ in transgenic plants, which was primarily the result of decrease in uptake of this metal (Sunkar et al. 2000). $Ca^{2+}$-permeable channels have been identified as a pathway of $Pb^{2+}$ entry into animal and plant cells (Tomsig and Suszkiw 1991; Huang and Cunningham 1996). It is, therefore, likely that transport of $Pb^{2+}$ into the plant cells may be regulated by the CaM through regulation of plasma membrane-localized proteins. This study demonstrates that it may be possible to confer tolerance to heavy metal ions in crop plants by engineering the CaM-binding property of the channel proteins.

Presence of apyrases, which hydrolyse nucleosides di- and tri-phosphates, is an ubiquitous feature of all eukaryotes (Hsieh et al. 2000). Hydrolysis of nucleoside tri- and di-phosphates by apyrases in animals is implicated in neurotransmission (Todorov et al. 1997) and also in preventing thrombosis by inhibition of ADP-induced platelet aggregation (Marcus et al. 1997). The role of apyrases in plants is, however, not very well defined. The activity of animal apyrases has not been reported to be affected by CaM. On the contrary, CaM modulates the activity of plant apyrases (Hsieh et al. 2000), therefore, suggesting the role of CaM in the regulation of these enzymes. An endogenous apyrase from *Pisum sativam* (PsNTP9) was demonstrated to bind to CaM in a $Ca^{2+}$-independent manner (Hsieh et al. 2000) and its activity was reported to be stimulated by $Ca^{2+}$-CaM (Chen and Roux 1986). Transgenic expression of *PsNTP9* in Arabidopsis resulted in enhanced resistance to toxic concentrations of different xenobiotic compounds like cyclohexane, plant growth regulators (Thomas et al. 2000) and different herbicides (Windsor et al. 2003). These studies, thus, supported the role of pea apyrase in multidrug resistance mechanism. Identification and characterization of novel CaM-regulated apyrases from different sources, therefore, may provide versatile tools for exploring strategies for introducing herbicide tolerance in plants.

CaM was also demonstrated to be involved in tolerance to methylglyoxal, a toxic metabolite, which is accomplished through regulation of glyoxalase 1 (Espartero et al. 1995). Glyoxalase I catalyses the conversion of toxic methylglyoxal to a nontoxic metabolite and was reported to be induced by NaCl, mannitol or ABA (Espartero et al. 1995). Glyoxaylase I, isolated from *Brassica juncea* (*BjGly I*), exhibited binding to CaM and its activity was also stimulated by $Ca^{2+}$/CaM (Deswal and Sopory 1991). The *BjGly I* over-expressing transgenic plants showed higher levels of tolerance to salt stress (Veena and Sopory 1999).

The stimulus-induced increase in intracellular $Ca^{2+}$ levels must be restored to basal levels so as to maintain homeostasis. This is achieved by efflux of [$Ca^{2+}$] from