

# Recent Advances in Calcium/Calmodulin-Mediated Signaling with an Emphasis on Plant-Microbe Interactions<sup>1</sup>

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During calcium ( $\text{Ca}^{2+}$ ) signaling, decoding the stimulus-response coupling involves a set of  $\text{Ca}^{2+}$  sensor proteins or  $\text{Ca}^{2+}$ -binding proteins (DeFalco et al., 2010a; Kudla et al., 2010). These proteins usually possess one or more classical helix-loop-helix elongation factor (EF) hand motifs. Three major types of  $\text{Ca}^{2+}$ -sensor proteins in plants are calmodulin (CaM)/CaM-like proteins, calcium-dependent protein kinases (CDPKs), and calcineurin B-like proteins. As compared with animals, plant genomes encode more diversified  $\text{Ca}^{2+}$  sensors; with the exception of canonic CaM, all other types of  $\text{Ca}^{2+}$  sensors (CaM-like proteins, CDPKs, and calcineurin B-like proteins) are plant specific. The large population and unique structural composition of  $\text{Ca}^{2+}$ -binding proteins and the diversity of the target proteins regulated by the  $\text{Ca}^{2+}$  sensors reflect the complexity of  $\text{Ca}^{2+}$  signaling, which helps plants adapt to the changing environment. This update will be limited primarily to discussions on CaM and CaM-binding proteins and the recent advances in  $\text{Ca}^{2+}$ /CaM-mediated signaling.

CaM is a conserved  $\text{Ca}^{2+}$ -binding protein found in all eukaryotes. The discovery of CaM can be traced back to the 1970s. An activator of cyclic nucleotide phosphodiesterase was shown to be involved in the regulation of cAMP concentration, which was stimulated by  $\text{Ca}^{2+}$  (Kakiuchi and Yamazaki, 1970; Cheung, 1971). The activator was found to bind  $\text{Ca}^{2+}$  and was eventually named “calmodulin,” an abbreviation of  $\text{Ca}^{2+}$ -modulated protein. Since its discovery over 40 years ago, CaM has been regarded as a model  $\text{Ca}^{2+}$ -binding protein and has been subjected to intensive studies in

biochemistry, cell biology, and molecular biology because of its importance in almost all aspects of cellular regulation (Poovaiah and Reddy, 1987, 1993; Bouche et al., 2005; DeFalco et al., 2010a; Du et al., 2011; Reddy et al., 2011b). Disruption or depletion of the single copy of the CaM gene in yeast (*Saccharomyces cerevisiae*) results in a recessive lethal mutation (Davis et al., 1986), suggesting that CaM has a critical role in eukaryotic cells.

The structure of CaM has been well studied, and the prototype of CaM found in all eukaryotes has 149 amino acids with two globular domains, each containing two EF hands connected by a long flexible helix (Meador et al., 1993; Zhang et al., 1995; Yun et al., 2004; Ishida et al., 2009). As more and more genomes are sequenced, it is becoming clear that CaM belongs to a small gene family in plants. In the model plant *Arabidopsis thaliana*, seven CaM genes encode for four highly conserved isoforms (CaM1/4, CaM2/3/5, CaM6, and CaM7) that differ in only one to five amino acid residues. Loss-of-function mutations of individual CaMs indicate that the different CaMs may have overlapping yet different functions. For example, a loss of function in *Arabidopsis AtCaM2* affects pollen germination (Landoni et al., 2010). Phenotypic analysis showed that in normal growth conditions, *atcam2-2* plants were indistinguishable from the wild type, while genetic analysis showed a reduced transmission of the *atcam2-2* allele through the male gametophyte, and in vitro pollen germination revealed a reduced level of germination in comparison with the wild type. However, the *atcam3* knockout mutant showed a clear reduction in thermotolerance after heat treatment at 45°C for 50 min (Zhang et al., 2009). Overexpression of *AtCaM3* in either the *atcam3* knockout or wild-type background significantly rescued or increased the thermotolerance, respectively. Further analysis of individual CaM mutants under different stress conditions should reveal more on the functional significance of individual CaM genes.

## STRATEGIES TO IDENTIFY CaM-BINDING PROTEINS

CaM has no inherent catalytic activity, but its activity is reflected in modulating the function of the target proteins by physically interacting with them

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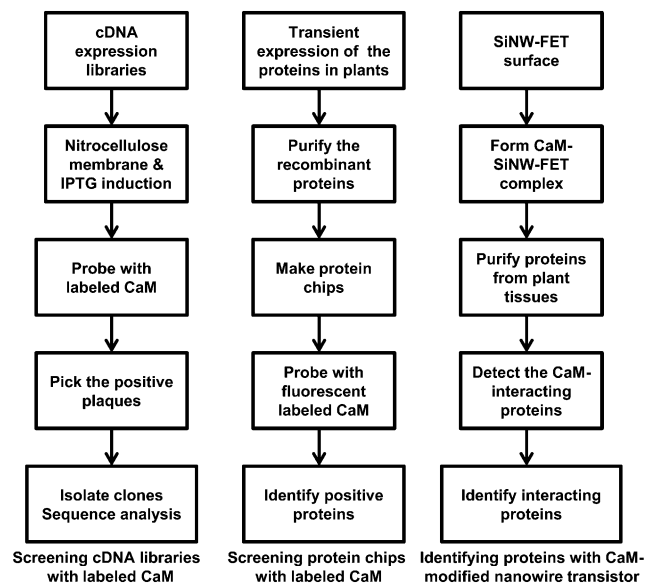
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(Rhoads and Friedberg, 1997; Hoeflich and Ikura, 2002). The CaM-binding domain (CaMBD)/motifs in the target proteins are not conserved. However, the target peptides usually form a basic amphipathic  $\alpha$ -helix, which contains hydrophobic residues on one side and basic residues on the other side. The hydrophobic portion of the target peptide is often held in the hydrophobic pocket of CaM to anchor the target peptide, and the acidic clusters of CaM then interact with the basic portion of the target peptide. The remarkable flexibility of the central linker and the exceptionally large numbers of Met residues in the hydrophobic pocket give CaM conformational plasticity to adjust to a variety of target peptides (Ikura and Ames, 2006). However, the variation in the primary structure of CaMBDs makes it very difficult to identify CaM targets just based on the amino acid sequences of proteins.

Yeast hybrid systems, based on the reconstitution of functional transcription factors, are the most commonly used approaches to isolate interacting proteins. However, these approaches are not effective for obtaining CaM-binding proteins, possibly because CaM does not undergo  $\text{Ca}^{2+}$ -induced conformational changes in yeast cells under normal conditions. Coimmunoprecipitation and purification with a CaM-Sepharose column are also not very effective. So far, the majority of CaM-binding proteins in plants have been identified by screening complementary DNA (cDNA) expression libraries with radiolabeled or biotinylated CaM. The most useful probe is  $^{35}\text{S}$ -labeled CaM (Fromm and Chua, 1992; Reddy et al., 1993; Yang and Poovaiah, 2000b), and a general scheme for this strategy is shown in Figure 1. The cDNA expression libraries are grown on plates until the plaques appear. The isopropyl  $\beta$ -D-1-thiogalactopyranoside-absorbed nitrocellulose membranes are overlaid onto the plaques to induce the expression of protein. Then, those membranes are incubated with the labeled CaM. The positive plaques are picked up and verified.

Screening cDNA libraries is easy and straightforward. However, many false-positive clones can be picked up, and researchers could miss the real targets because eukaryotic proteins expressed in bacteria are often misfolded. Popescu et al. (2007) developed a protein microarray approach in which proteins expressed in plants were purified and used to make protein chips. The initial cost for preparing the protein chips with a large collection is high, because each protein needs to be expressed and purified from plants. However, once the system is established, this could be used as a high-throughput approach to find the targets for different CaM isoforms in an entire genome (Fig. 1). So far, over 80 plant CaM-target proteins have been characterized using these approaches. However, it is believed that there are many more putative CaM-target proteins yet to be discovered. The current estimate of CaM targets in the *Arabidopsis* genome is about 500 (T. Yang and B.W. Poovaiah, unpublished data). These target proteins are involved in almost all aspects of plant growth and development as well as in responses to abiotic and biotic stresses. Table I summarizes the characterized CaM-binding proteins and their CaM-binding motifs.



**Figure 1.** Illustration of the three main approaches used to identify CaM-binding proteins. IPTG, Isopropyl  $\beta$ -D-1-thiogalactopyranoside; SiNW-FET, silicon nanowire field-effect transistor.

Recently, an mRNA display technique and a CaM-modified nanowire transistor method have been developed for isolating CaM-binding proteins in humans (Shen et al., 2005; Lin et al., 2010). The mRNA display technique to identify  $\text{Ca}^{2+}$ /CaM-binding proteins is to screen the mRNA-displayed proteome libraries with biotinylated CaM. Covalent fusions between an mRNA and the peptide or protein that it encodes can be generated by in vitro translation of synthetic mRNAs that carry puromycin. The mRNA display provides a powerful means for reading and amplifying a protein sequence after it has been selected from large libraries (Shen et al., 2005). The CaM-modified nanowire transistor (biosensor) approach is to use a highly sensitive and reusable silicon nanowire field-effect transistor for the detection of protein-protein interactions (Fig. 1). The reusable device is made possible by the reversible association of glutathione S-transferase-tagged CaM with a glutathione-modified transistor (Lin et al., 2010). The minimum concentration of  $\text{Ca}^{2+}$  required to activate CaM is  $1 \mu\text{M}$ , and this sensitive nanowire transistor can serve as a high-throughput biosensor and substitute for immunoprecipitation methods used in the identification of interacting proteins. These approaches will be useful for the further identification and characterization of true plant CaM-target proteins, especially those proteins triggered by different stimuli.

#### Ca<sup>2+</sup>/CaM-MEDIATED REGULATION OF PROTEIN PHOSPHORYLATION

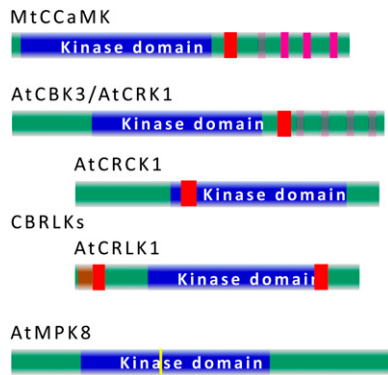
Ca<sup>2+</sup>/CaM-stimulated phosphorylation in plants was first observed in the 1980s (Veluthambi and Poovaiah, 1984), long before the cloning of the first CaM-binding kinase from apple (*Malus domestica*; Watillon et al.,

**Table 1.** Plant CaM-binding proteins with defined CaMBDs

CaM-Binding Proteins	References
Protein phosphorylation/dephosphorylation	
Chimeric Ca <sup>2+</sup> /CaM-dependent protein kinase (CCaMK)	Patil et al. (1995); Takezawa et al. (1996)
Diacylglycerol kinase (LeCBDGK)	Snedden and Blumwald (2000)
NAD kinase (NAD2)	Turner et al. (2004)
Cytoplasmic receptor-like kinase (CRCK1)	Yang et al. (2004)
<i>Nicotiana tabacum</i> Ca <sup>2+</sup> /calmodulin-dependent protein kinase 1 (NtCaMK1)	Ma et al. (2004)
PP2C-like phosphatase	Takezawa (2003)
NPK phosphatase (NtMKP1)	Yamakawa et al. (2004); Ishida et al. (2009)
Receptor-like kinase (CRLK1)	Yang et al. (2010)
Nuclear proteins/transcription regulators	
SRs/CaMTAs	Yang and Poovaiah (2002a); Du et al. (2009)
AtBT	Du and Poovaiah (2004)
Auxin-induced protein ZmSAUR1	Yang and Poovaiah (2000)
IQD1	Levy et al. (2005)
AtMYB2	Yoo et al. (2005)
CBP60g	Wang et al. (2009)
AtGT2L	Xi et al. (2012)
Nuclear protein PCBP	Reddy et al. (2002)
WRKY group IId AtWRKY7	Park et al. (2005)
ASYMMETRIC LEAVES1 (AS1)	Han et al. (2012)
Metabolism	
Glu decarboxylase	Baum et al. (1993, 1996)
Catalase	Yang and Poovaiah (2002b)
FAD-dependent oxidoreductase, DWF1	Du and Poovaiah (2005)
Membrane proteins/ion channels/transporters	
Disease resistance gene MLO	Kim et al. (2002a, 2002b)
Cyclic nucleotide-gated cation channels (CNCG)	Arazi et al. (1999); Kohler et al. (1999); Ali et al. (2007)
Vacuolar Ca <sup>2+</sup> -ATPase	Malmstrom et al. (2000)
Endoplasmic reticulum Ca <sup>2+</sup> -ATPase	Hong et al. (1999)
Plasma membrane Ca <sup>2+</sup> -ATPase**	Chung et al. (2000)
AAA family CIP111	Zielinski (2002)
AAA+-ATPase AFG1L1	Bussemer et al. (2009)
Apyrase	Hsieh et al. (2000); Steinebrunner et al. (2003)
Others	
Kinesin-like protein	Reddy et al. (1996); Wang et al. (1996)
Pollen-specific protein (MPCBP, NPG1)	Safadi et al. (2000)
Chaperonin10	Yang and Poovaiah (2000c)
DRL1	Nelissen et al. (2003)
NADPH-dependent dehydrogenase Tic32	Chigri et al. (2006)
Ubiquitin-specific protease6 (AtUBP6)	Moon et al. (2005)

1993). Plant CaM-binding kinases can be classified into four distinct subgroups based on their structural features (Fig. 2). The first subgroup is the CaM-binding protein kinases similar to mammalian CaMK, such as apple calmodulin-binding peptide 1, *Nicotiana tabacum* Ca<sup>2+</sup>/calmodulin-dependent protein kinase, maize Ca<sup>2+</sup>/calmodulin-dependent protein kinase, and *Arabidopsis* calmodulin binding protein kinase 1 (AtCBK1) to AtCBK3. These kinases are sometimes called CDPK-related kinases (CRKs, also called CBKs) because they carry a kinase domain in the N terminus and degenerated, nonfunctional EF hands in their C terminus (Zhang et al., 2002; Zhang and Lu, 2003; Hua et al., 2004; Wang et al., 2004). The second subgroup is Ca<sup>2+</sup> and calcium/calmodulin-dependent protein kinases (CCaMKs), found in most of the higher plants (Patil et al., 1995). CCaMK is a plant-specific protein kinase that carries a Ser/Thr kinase domain in the N-terminal

portion, a CaM-binding autoinhibitory domain, and a visinin-like domain (VLD) with three distinct Ca<sup>2+</sup>-binding EF hands in the C terminus. The third subgroup belongs to receptor-like kinases (RLKs); hence, they are called CaM-binding receptor-like kinases. Plant genomes carry a relatively large RLK family that shares homology with animal receptor kinases, with an extracellular domain, a transmembrane domain, and a kinase domain. Some RLKs from plants contain only a kinase domain and thus are named receptor-like cytoplasmic kinases. Some receptor-like cytoplasmic kinases, such as CRCK1 and homologs (Yang et al., 2004), and RLKs, such as SRK, CaM-binding receptor-like kinase, AtCaMRLK, AtCRLK1, and BRI1, are CaM-binding proteins (Vanoosthuyse et al., 2003; Charpentreau et al., 2004; Kim et al., 2009a; DeFalco et al., 2010b; Yang et al., 2010; Oh et al., 2012). Last but not least, some of the mitogen-activated protein kinases (MAPKs), specifically members of



**Figure 2.** Schematic presentation of the domain structure of  $\text{Ca}^{2+}$ /CaM-regulated protein kinases. One example of each class of CaM-regulated kinases, MtCCaMK (UniProt Q6RET7), AtCBK3/AtCRK1 (UniProt O80673), AtCRCK1 (UniProt Q9FIL7), AtCRLK1 (UniProt Q9FIU5), and AtMPK8 (UniProt Q9LM33), is presented. The kinase domain is in blue, CaMBD is in red, functional EF hands are in bright purple and degenerated EF hands are in light purple, the transmembrane domain is in light brown, and the yellow bar in MAPK8 represents the conserved TDY motif recognized and phosphorylated by MAPK kinases. CaMBDs in the MAPK D subgroup, including MPK8, are not currently resolved.

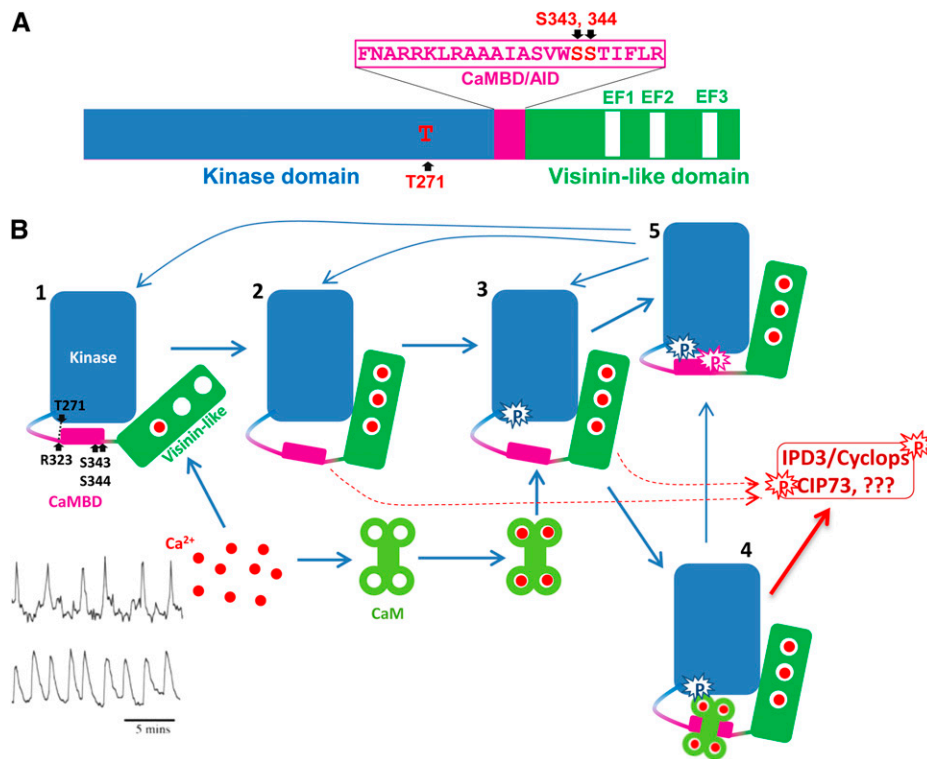
subgroup D of MAPK in Arabidopsis and rice (*Oryza sativa*), were reported to be regulated by  $\text{Ca}^{2+}$ /CaM (Ding et al., 2009; Takahashi et al., 2011). Structural features of CaM-regulated protein kinases are summarized in Figure 2. Although the regulation of some of these kinases by CaM remains to be confirmed, accumulated evidence indicates that  $\text{Ca}^{2+}$ -mediated signals could regulate a broad range of physiological activities related to plant growth, development, and responses to environmental stimuli (Zhang et al., 2002; Vanoosthuysen et al., 2003; Charpentreau et al., 2004; Hua et al., 2004; Liu et al., 2008; Kim et al., 2009a; Yang et al., 2010; Oh et al., 2012). Among all these CaM-regulated kinases, CCaMK has been widely studied because of its role in symbioses, developmental processes, and stress responses.

## ROLE OF $\text{Ca}^{2+}$ /CaM-REGULATED KINASE IN PLANT-MICROBE INTERACTIONS

$\text{Ca}^{2+}$  spiking in the nucleus and perinuclear region of root hair cells has been documented as one of the earliest cellular responses after the perception of symbionts by host plants (Ehrhardt et al., 1996; Wais et al., 2000; Walker et al., 2000; Kosuta et al., 2008). Accumulating results have revealed a common symbiotic pathway composed of eight components, SYMRK/DMI2, POLLUX/DMI1, CASTOR, NENA, NUP85, NUP133, CCaMK, and CYCLOPS/IPD3, which are all required for the normal establishment of both root nodulation symbiosis (RNS) and arbuscular mycorrhizal symbiosis (AMS; Singh and Parniske, 2012; Oldroyd, 2013). CCaMK, which carries structural features enabling it to interact with  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ /CaM, acts as a decoder

of the encrypted  $\text{Ca}^{2+}$  signal (Levy et al., 2004; Mitra et al., 2004). An activation mechanism of CCaMK is proposed in the next section of this update (Fig. 3). CCaMK was first identified and cloned in lily (*Lilium* spp.), and biochemical studies on lily CCaMK showed that its kinase activities are regulated by both  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ /CaM (Patil et al., 1995; Takezawa et al., 1996). CCaMK alone is inactive, with little or no kinase activity. Its autophosphorylation, predominantly at the Thr-271 residue, is significantly stimulated when  $\text{Ca}^{2+}$  levels increase (Takezawa et al., 1996). Autophosphorylation at this position drastically increases its affinity for CaM, a phenomenon called CaM trapping (Sathyanarayanan et al., 2000), and also increases its substrate phosphorylation activity (Takezawa et al., 1996). The kinase activities of CCaMK in leguminous plants are closely related to its function in supporting the establishment of RNS and AMS. Site mutations T271A of MtCCaMK and T265I of LjCCaMK resulted in deregulated and constitutive substrate phosphorylation activity and produced spontaneous nodulation when used for the complementation of *ccamk* null mutations in both *Medicago truncatula* and *Lotus japonicus* (Ramachandiran et al., 1997; Gleason et al., 2006; Tirichine et al., 2006). A recent study on *L. japonicus* CCaMK indicated that Thr-265 in its native state interacts with other amino acids along with the positively charged Arg-317 in the CaMBD/autoinhibitory domain (AID) region, and this interaction is disrupted when Thr-265 is mutated to Ala or Ile (Shimoda et al., 2012). Together with the aforementioned biochemical data, this suggests that the native Thr-265 (Thr-271 in *M. truncatula*) is critical in maintaining an ideal intramolecular interaction between the autoinhibitory domain and the kinase domain that keeps CCaMK inactive. Changing the structure at this position through autophosphorylation provides a regulatory mechanism for the substrate phosphorylation capacity of CCaMK and the associated physiological function in supporting plant-microbe symbioses.

Very recently, Ser-337 in LjCCaMK (corresponding to Ser-343 in MtCCaMK) and Ser-344 in MtCCaMK (corresponding to Ser-338 in LjCCaMK) in the CaMBD were reported to be novel autophosphorylation sites in CCaMK (Liao et al., 2012). Phosphorylation at Ser-337 of LjCCaMK and Ser-344 of MtCCaMK both negatively regulate their interaction with CaM and their kinase activities. Furthermore, the phosphomimicking mutation of Ser-337 of LjCCaMK and Ser-344 of MtCCaMK failed to complement null mutants of CCaMK in both RNS and AMS. These results indicate that autophosphorylation at Ser-337 of LjCCaMK and Ser-344 of MtCCaMK has negative regulatory functions in LjCCaMK and MtCCaMK at both the biochemical and physiological levels. Interestingly, the negative regulation through the autophosphorylation of Ser-337 of LjCCaMK is required for the proper progression and establishment of both RNS and AMS (Liao et al., 2012; Routray et al., 2013), but autophosphorylation at Ser-344 acts solely as a negative control to switch off the activated



**Figure 3.** A, Schematic diagram of MtCCaMK showing the domain structure and autophosphorylation sites. B, Activation of CcCaMK by  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}/\text{CaM}$ . State 1, CcCaMK most likely with  $\text{Ca}^{2+}$  loaded in the first EF hand. CcCaMK is inactive in this state. State 2, CcCaMK loaded with  $\text{Ca}^{2+}$  in its three EF hands in the VLD. Because of the  $\text{Ca}^{2+}$ -induced changes in the tertiary structure of CcCaMK, the kinase is active and Thr-271 is ready for autophosphorylation. State 3, Thr-271 is autophosphorylated and CcCaMK shows a dramatic increase in its affinity for CaM, a phenomenon called CaM trapping. State 4,  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}/\text{CaM}$ -loaded CcCaMK. The kinase is fully active and should be able to phosphorylate substrates as well as CcCaMK itself. As long as the CcCaMK is bound by  $\text{Ca}^{2+}/\text{CaM}$ , it should remain active. State 5, Ser-343 and/or Ser-344 are phosphorylated, CcCaMK can no longer interact with CaM, and its kinase activity is also turned off, whether the  $\text{Ca}^{2+}$  concentration is high enough to load in its EF hands. Mycorrhizal and Nod factor-induced  $\text{Ca}^{2+}$  spikings (bottom left corner: mycorrhizal is at top and Nod factor is at bottom) are adapted from Kosuta et al. (2008).

MtCCaMK (Routray et al., 2013), implying a complex regulation of CcCaMK through autophosphorylation.

The well-characterized CaMKII, the closest homolog of CcCaMK in animals, also has several autophosphorylation sites in its autoinhibitory/CaMBD, and its activities are delicately regulated through differential autophosphorylation at different sites (Hanson and Schulman, 1992). Similar to CcCaMK, phosphorylation of CaMKII at its autophosphorylation sites in the CaMBD also interferes with its interaction with CaM and negatively regulates its kinase activity (Colbran and Soderling, 1990; Hanson and Schulman, 1992). A high sequence homology of 79% between CaMKII and CcCaMK around the CaM/autoinhibitory region (Colbran and Soderling, 1990; Patil et al., 1995), the presence of autophosphorylation sites in both of the kinases, and their similar impact on CaM-binding properties indicate that CcCaMK could be inactivated in a similar way to CaMKII, and this hypothesis has now been confirmed (Liao et al., 2012; Routray et al., 2013). Although CcCaMK is very similar to CaMKII, especially in its kinase domain and CaMBD/AID region, CcCaMK is drastically different

from its cousin in the C terminus and acquired the ability to receive  $\text{Ca}^{2+}$  signals using both the CaMBD and the  $\text{Ca}^{2+}$ -binding VLD. Hence, it is not surprising to see that its mode of activation is different from that of CaMKII.

Although CcCaMK was reported to be a major regulator of plant-microbe symbioses, evidence also suggests that CcCaMK is involved in other aspects of plant life. Tobacco (*Nicotiana tabacum*) CcCaMK was indicated to play a role in controlling the development of anther (Poovaiah et al., 1999), and pea (*Pisum sativum*) CcCaMK was up-regulated in roots in response to low temperature and salt treatment (Pandey et al., 2002). Recent results showed that CcCaMK from maize (*Zea mays*; ZmCCaMK) and rice (OsCCaMK) both play important roles in abscisic acid-induced antioxidant protection (Ma et al., 2012; Shi et al., 2012). Together, these results suggest that CcCaMK acts as a multifunctional regulatory protein in plants.

#### PROPOSED ACTIVATION MECHANISM OF CcCaMK

Based on published data, we propose an activation mechanism of CcCaMK (Fig. 3). The first EF hand of

CCaMK has a very high affinity for  $\text{Ca}^{2+}$  (Swainsbury et al., 2012). Hence, in the resting condition, it is likely that CCaMK is loaded with  $\text{Ca}^{2+}$  in its EF1 site, and at least one of the other two sites must be unloaded to keep CCaMK responsive to  $\text{Ca}^{2+}$  spiking. It could also be postulated that in the resting condition (state 1), the CaMBD/AID interacts with the kinase activity center and keeps the kinase at an inactive or low-activity status (Takezawa et al., 1996). The hydrogen bond-based interaction between Thr-271 and Arg-323 is predicted to be critical for maintaining this intramolecular interaction to seal the kinase activity of CCaMK under this condition (Shimoda et al., 2012). Alteration in the status of Thr-271 or changes in the  $\text{Ca}^{2+}$ -loading status of VLD could break this inhibition of the kinase activity.  $\text{Ca}^{2+}$  binding to the three EF hands in the VLD induces a change in the tertiary structure of CCaMK, activates its kinase activity, and also makes the Thr-271 accessible for autophosphorylation (state 2, transient and active, may phosphorylate substrates; Sathyanarayanan et al., 2000, 2001; Gleason et al., 2006; Swainsbury et al., 2012). Thr-271 was reported to be the preferred and likely the first autophosphorylated site of CCaMK (Sathyanarayanan et al., 2001; Routray et al., 2013), and autophosphorylation at this site induces an increase in its affinity for CaM by about 200-fold, a phenomenon called CaM trapping (state 3, active and may phosphorylate substrate, transient, and CaM trapping; Sathyanarayanan et al., 2001; Gleason et al., 2006; Tirichine et al., 2006). Once the CCaMK reached state 3, CaM in the vicinity was attracted to CCaMK, even though the  $\text{Ca}^{2+}$  concentration could have already decreased to a level lower than that in the stage of  $\text{Ca}^{2+}$  loading to EF hands in the VLD. Once bound by CaM in the CaMBD/AID region, CCaMK is fully activated and can phosphorylate substrates such as IPD3/Cyclops, CIP73, and itself (state 4, CCaMK fully loaded with  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ /CaM and highly active; Messinese et al., 2007; Yano et al., 2008; Kang et al., 2011). In addition, CaM binding could protect Ser-343 and Ser-344 from being phosphorylated by CCaMK, which is supported by the *in vitro* phosphorylation assays that demonstrated that CaM binding decreases the autophosphorylation level of CCaMK (Takezawa et al., 1996).

Recent results showed that, if Ser-343 and/or Ser-344 are autophosphorylated, CCaMK will no longer interact with  $\text{Ca}^{2+}$ /CaM and its kinase activity will be turned off (Liao et al., 2012; Routray et al., 2013). Hence, another impact of  $\text{Ca}^{2+}$ /CaM binding to CCaMK could be to maintain its activity for a prolonged period even after  $\text{Ca}^{2+}$  decreases to some extent. Since the interaction between  $\text{Ca}^{2+}$ /CaM and CCaMK is dynamic, the fully activated CCaMK will still have a chance to be autophosphorylated at Ser-343 and Ser-344 in its CaMBD; this will cap the CCaMK from further CaM binding (a phenomenon called CaM capping) and also inactivate itself (state 5; Liao et al., 2012; Routray et al., 2013). This inactivation can be postponed by decreasing  $\text{Ca}^{2+}$  concentration. If CCaMK is regulated to state 3 and there is no CaM available in the vicinity of CCaMK,

the Thr-271-phosphorylated active kinase could immediately phosphorylate the unprotected Ser-343 and Ser-344 in its CaMBD and inactivate the kinase activity rapidly (state 5). This was observed to occur *in vitro* within 30 s (Sathyanarayanan and Poovaiah, 2002). Hence, the phosphorylation of Thr-271 acts as a “binary logic switch” in the decision-making process; it could quickly activate or quickly turn off the kinase activity of CCaMK depending on whether CaM is available once the Thr-271 is phosphorylated. Logically, this inactivation via the autophosphorylation of Ser-343 and/or Ser-344 in the CaMBD could be mitigated or avoided by decreases in  $\text{Ca}^{2+}$  concentration; this could be the reason why this kinase is activated *in vivo* by  $\text{Ca}^{2+}$  spiking (Oldroyd and Downie, 2004). After losing the phosphate groups at Thr-271, Ser-343, and/or Ser-344, the CCaMK at state 5 could be changed back to state 1, 2, or 3. Different from CaMKs in animals, which need to form a dodecamer to read the oscillative  $\text{Ca}^{2+}$  signals (Hudmon and Schulman, 2002), the dually regulated CCaMK, which receives  $\text{Ca}^{2+}$  signals from both VLD and CaMBD, could sense and respond to oscillative  $\text{Ca}^{2+}$  signals in a monomer format. Recent empirical data obtained from truncated versions of CCaMK also support that CCaMK may not form multimers in the presence or absence of  $\text{Ca}^{2+}$  (Swainsbury et al., 2012).

#### **$\text{Ca}^{2+}$ /CaM-MEDIATED REGULATION OF TRANSCRIPTIONAL CONTROL IN PLANTS**

Transcriptional control is an end result of many signal transduction pathways, including  $\text{Ca}^{2+}$ /CaM-mediated signaling. CaM interacts with a variety of DNA-binding proteins/transcription factors. Over 90 CaM-binding proteins (CBPs) are DNA-binding proteins that fit into several families of known transcription factors, including CAMTAs (also known as AtSRs), WRKY IID, bZIP, MYB, Trihelix, NAC, CBP60, MADS, and GRAS (Reddy et al., 2011a), and the CaMBDs in some of the newly identified CaM-binding transcription factors remain to be determined (Popescu et al., 2007). In this section, we will focus only on recent developments in the direct regulation of  $\text{Ca}^{2+}$ /CaM on transcriptional machinery through the actions of CAMTAs/SRs.

#### **The Best Characterized CaM-Regulated Transcription Factors: CAMTAs**

NtER1 was the first member of the CAMTA family reported to be a CaM-binding protein (Yang and Poovaiah, 2000a). Follow-up studies showed that CAMTAs belong to a conserved transcription factor family that exists in all the examined multicellular eukaryotes (Reddy et al., 2000; Bouche et al., 2002; Yang and Poovaiah, 2002a; Finkler et al., 2007). The expression of CAMTAs is developmentally regulated (Yang et al., 2012) and responds to different abiotic and biotic signals (Yang and Poovaiah, 2002a; Yang et al., 2013). Target cis-elements for this family are (A/C/G)



CGCG(T/C/G) (Yang and Poovaiah 2002a) and (A/C)CGTGT (Choi et al., 2005; Doherty et al., 2009; Du et al., 2009; Kim et al., 2009b; Galon et al., 2010b). All members of the CAMTA family carry a CG-1 DNA-binding domain in the N terminus, followed by a TIG domain, ankyrin repeats, a  $\text{Ca}^{2+}$ -dependent CaMBD, and tandem repeats of the IQ motif that interact with CaM in a  $\text{Ca}^{2+}$ -independent manner (Bouche et al., 2002; Yang and Poovaiah, 2002a; Finkler et al., 2007; Du et al., 2009). The functions of AtSRs/CaMTAs were found to be dependent on their interaction with  $\text{Ca}^{2+}$ /CaM (Choi et al., 2005; Du et al., 2009).

Loss-of-function mutants of AtSR1/CAMTA3 were reported to have pleiotropic, temperature-dependent, constitutive disease-resistant phenotypes, including compromised growth, spontaneous leaf chlorosis with autonomous lesions, constitutive expression of pathogenesis-related genes, and elevated resistance against both virulent and avirulent strains of *Pseudomonas syringae* pv *tomato* DC3000 (Galon et al., 2008; Du et al., 2009). These phenotypes were correlated with higher levels of endogenous salicylic acid (SA), demonstrating that AtSR1/CAMTA3 is a negative regulator of SA-mediated defense responses (Du et al., 2009). It was also shown that AtSR1/CAMTA3 interacts with a CGCG box motif in the -1-kb promoter region of EDS1 both in vivo and in vitro and suppresses the transcription of EDS1, a critical player in the SA activation loop and toll interleukin 1 receptor-nucleotide binding domain (NB)-leucine rich repeat (LRR)-type R gene-mediated defense in Arabidopsis (Du et al., 2009). Recently, AtSR1/CAMTA3 was also shown to negatively regulate ethylene-mediated senescence by recognizing a CGCG box in the promoters of EIN3 and disease resistance by interacting with a CGCG box in the promoters of NDR1, a key signaling component required for coiled-coil-NB-LRR-type R gene-mediated plant immunity (Nie et al., 2012). Similar to the function of CAMTA3, OsCBT, a CAMTA member from rice, also plays a negative role in regulating rice defense against both the bacterial pathogen *Xanthomonas oryzae* pv *oryzae* and the rice blast fungus *Magnaporthe grisea* (Koo et al., 2009). Very recently, CAMTA3 was shown to play a critical role in plant defense against insect herbivory (Laluk et al., 2012; Qiu et al., 2012). *atsr1/camta3* null mutants are more vulnerable to herbivore attack, and CaM binding was shown to be required for AtSR1/CAMTA3 in maintaining normal levels of plant resistance to herbivore attack. In addition, it was observed that elevated SA levels in *atsr1* mutant plants have a negative impact on both basal and induced biosynthesis of jasmonates, a critical hormone-mediated wounding response in plants (Qiu et al., 2012). Furthermore, compared with the wild type, the *atsr1* mutant accumulates less of the insect repellent metabolite glucosinolate, and this coincides with the altered expression of several genes involved in glucosinolate metabolism, such as MYB51, AtST5 $\alpha$ , and IQD1 (Laluk et al., 2012). In a different line of research, CAMTA3 was shown to recognize the conserved

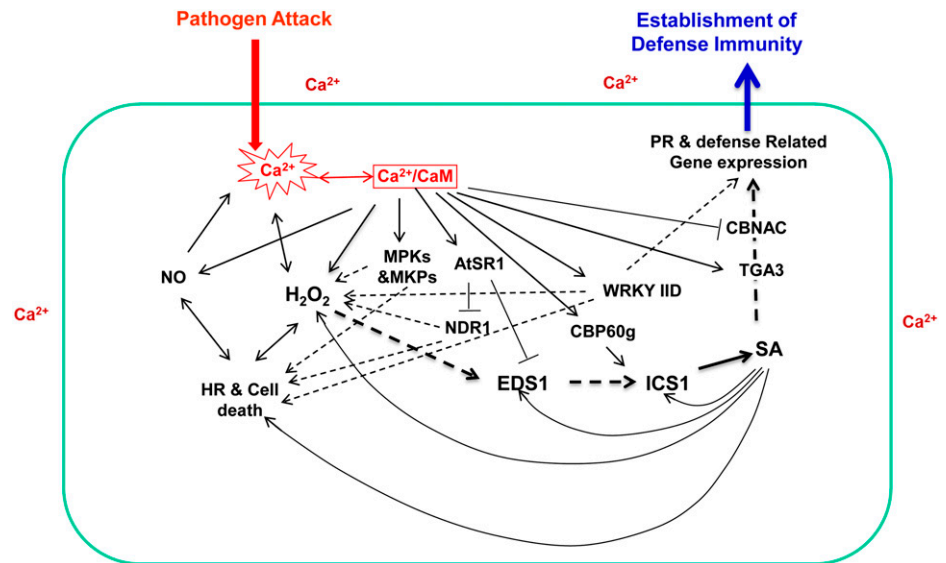
DNA motif 2 (CM2, CCGCGT) in the promoter of CBF2, a critical transcription factor required for Arabidopsis cold acclimation and the subsequent establishment of freezing tolerance as well as the regulation of cold-induced gene expression (Doherty et al., 2009). Very recently, results showed that CAMTA1 and CAMTA2 share some functional redundancy with CAMTA3 in suppressing SA biosynthesis and the quick induction of CBF1, CBF2, and CBF3 transcription (Kim et al., 2013). AtCAMTA1 was reported to be an auxin-responsive gene, and hypocotyl elongation of AtCAMTA1 knockout or repression lines is hyperresponsive to exogenous application of auxin, indicating that CAMTA1 could regulate plant growth through the action of auxin (Galon et al., 2010a).

### Recent Breakthrough in $\text{Ca}^{2+}$ /CaM-Mediated Transcriptional Control of Plant Immunity

$\text{Ca}^{2+}$ /CaM-mediated signaling has been documented to be involved in almost every aspect of a plant's life, including plant growth and development, as well as plant responses to biotic and abiotic stresses (Du and Poovaiah, 2005; Lecourieux et al., 2006; Yang et al., 2007; DeFalco et al., 2010a; Du et al., 2011). In the past several years, mechanisms by which  $\text{Ca}^{2+}$ /CaM regulate plant defenses against pathogenic microbes have been revealed at an impressively rapid pace. Figure 4 summarizes the accumulated information of how  $\text{Ca}^{2+}$ /CaM regulates the functions of target proteins involved in plant responses to pathogen attack. Rapid increases in intracellular  $\text{Ca}^{2+}$  concentration, oxidative burst, nitric oxide (NO) production, hypersensitive responses and the associated cell death, accumulation of SA, induced expression of pathogenesis-related genes, and the establishment of local and systemic resistances are common defense responses that occur at different stages during the establishment of immune responses after plants are challenged with pathogens (Nimchuk et al., 2003; Lecourieux et al., 2006; Fu and Dong, 2013). These changes are presented in Figure 4 in a rough temporal order. The key defense-related events/signaling components, such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), NO, and SA, are well known to induce the production of each other and, hence, are positioned directly or indirectly in various feed-forward amplification loops and act as positive regulators of hypersensitive and defense responses (Lecourieux et al., 2006; Ali et al., 2007; Fu and Dong, 2013). Accumulated data revealed that  $\text{Ca}^{2+}$ /CaM-mediated regulations oversee and coordinate the progression of the entire plant immune system.

Properly controlled production of NO with the involvement of  $\text{Ca}^{2+}$ , CaM, and NO synthase after the perception of lipopolysaccharides or avirulent pathogen and the balanced action of NO and  $\text{H}_2\text{O}_2$  contribute to the appropriate progression of the hypersensitive reaction and the establishment of plant defenses (Ali et al., 2007). On the other hand, NO has also been reported to be a powerful stimulator of intracellular  $\text{Ca}^{2+}$  in plants,

**Figure 4.** Multiple controls of plant immunity by  $\text{Ca}^{2+}/\text{CaM}$ . Empirically confirmed regulations are presented with solid lines, regulations not directly confirmed are presented with dashed lines, arrowheads indicate positive control, and T-heads indicate negative control. HR, Hypersensitive response; PR, pathogenesis related.



providing a path of feedback to the  $\text{Ca}^{2+}$  signaling system (Besson-Bard et al., 2008). While well known as a regulator of endogenous  $\text{Ca}^{2+}$  levels,  $\text{H}_2\text{O}_2$  production during the oxidative burst requires  $\text{Ca}^{2+}$  influx, which activates the plasma membrane-localized NADPH oxidase (Keller et al., 1998). Furthermore,  $\text{Ca}^{2+}/\text{CaM}$  has been proposed to increase  $\text{H}_2\text{O}_2$  generation through  $\text{Ca}^{2+}/\text{CaM}$ -dependent NAD kinase, which affects the concentration of available NADPH during the assembly and activation of NADPH oxidase (Harding et al., 1997; Turner et al., 2004). Moreover,  $\text{Ca}^{2+}/\text{CaM}$  binds to plant catalase and enhances its activity (Yang and Poovaiah, 2002b). Recently,  $\text{Ca}^{2+}/\text{CaM}$  was also reported to regulate  $\text{H}_2\text{O}_2$ -mediated defense responses by regulating the MAPK cascade through the action of MAPKs and MAPK phosphatases (Lee et al., 2008; Bartels et al., 2009; Takahashi et al., 2011).

SA has been generally accepted as a defense hormone that controls plant defense against biotrophic pathogens (Nimchuk et al., 2003; Fu and Dong, 2013). The SA activation pathway is under extensive regulation by  $\text{Ca}^{2+}/\text{CaM}$ -mediated signaling. Transcription of the SA biosynthesis gene *ICS1/SID2* is controlled by the  $\text{Ca}^{2+}/\text{CaM}$ -binding transcription factor CBP60g (Wang et al., 2009, 2011; Zhang et al., 2010), providing a venue for the  $\text{Ca}^{2+}$  signal to activate the production of SA. The transcription of two critical genes, *EDS1* and *NDR1*, required for the activation of both toll interleukin 1 receptor-NB-LRR and coiled-coil-NB-LRR gene-activated immunities, is negatively controlled by  $\text{CaM}$ -regulated AtSR1/CAMTA3, enabling tight control over the synthesis of SA and providing an effective approach to avoid the misactivation of effector-triggered immunity as well as pathogen-associated molecular pattern-triggered immunity, since SA is critical for both (Nimchuk et al., 2003; Fu and Dong, 2013). In addition,  $\text{Ca}^{2+}/\text{CaM}$  could also provide both positive and negative controls through WRKY7, WRKY11, WRKY17, and

WRKY53 (Park et al., 2005; Journot-Catalino et al., 2006; Kim et al., 2006; Murray et al., 2007; Popescu et al., 2007), although their direct downstream target genes and their regulation by  $\text{CaM}$  remain unknown.

Defense-related gene expression after the accumulation of SA also seems to be regulated by  $\text{Ca}^{2+}/\text{CaM}$ -mediated signaling.  $\text{CaM}$  binding to TGA3 enhances its interaction with the target promoter (Szymanski et al., 1996); furthermore, TGA3 interacts with NPR1, a critical transcription cofactor involved in SA perception and the expression of a broad spectrum of defense-related genes (Fu and Dong, 2013), providing a possible option to regulate the output of defensive reactions. Furthermore,  $\text{CaM}$  could also repress the expression of pathogenesis-related genes through the action of the transcription factor CBNAC (Kim et al., 2012). It is very interesting to see that  $\text{Ca}^{2+}/\text{CaM}$  can exert a well-balanced control even at the final stage of defense responses after the induced accumulation of SA.

Defense comes with a price; mutant plants with misactivation or constitutive activation of defense responses usually suffer significantly in their growth and development or can even die (Heil and Baldwin, 2002). The deployment of multiple positive and negative regulatory controls on different progression stages during the establishment of plant immunity demonstrates the critical importance of balancing these immune responses. As summarized in this update,  $\text{Ca}^{2+}$ , the universal messenger in eukaryotes, including plants, could act as a competent conductor in orchestrating these powerful physiological activities, which could protect plants from pathogen attack or cause them to commit "suicide."

## CONCLUSION

Since the discovery of  $\text{CaM}$  over 40 years ago, and especially during the last decade, tremendous progress



has been made in the isolation and characterization of Ca<sup>2+</sup>/CaM and its target proteins. A complex Ca<sup>2+</sup>/CaM-regulated network is beginning to emerge, but it is far from complete. While accepting the unique roles of Ca<sup>2+</sup> as a messenger, we are also realizing the complexities and the challenges of Ca<sup>2+</sup>/CaM-mediated signaling and its cross talk with different signal transduction pathways in plants. As more genomes are sequenced and more high-throughput tools are developed, the significance and the scope of this network are being realized at an unprecedented rate. However, there are several issues that still need to be resolved. First, we believe that many CaM-binding proteins remain to be identified, not to mention the target proteins of CaM-like proteins. Hence, the identification of novel CaM-binding proteins will still be one of the most important tasks for plant scientists. Classic approaches to identify targets of CaM through protein-protein interaction-based expression library screening may still be used, but improved approaches, such as the CaM-modified nanowire transistor method, are highly desirable. Second, the functional significance of these target proteins in terms of biochemical, molecular, and physiological activities needs to be adequately studied. Third, we need to determine how Ca<sup>2+</sup>/CaM and its interactors respond to upstream signals and how they regulate various downstream signal transduction pathways. For example, scientists need to determine whether and how Ca<sup>2+</sup>/CaM helps CcMK to initiate RNS or AMS based on the subtle differences in Ca<sup>2+</sup> spiking induced by Nod or Myc factors. Fourth, we need to understand the significance and complexity of Ca<sup>2+</sup>/CaM-mediated regulations, their roles as both positive and negative regulators to balance a particular signaling pathway, and how these regulatory roles are coordinated. To cite a specific example: when, where, and how Ca<sup>2+</sup>/CaM helps plants to produce a positive or negative regulation of the SA-mediated immune response to ensure appropriate protection against a potential pathogen and avoid the negative consequences of overreaction during defense. Progress in these areas will significantly improve our understanding of Ca<sup>2+</sup>/CaM-mediated signaling in plants.

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