Calcium/calmodulin-mediated signal network in plants

Tianbao Yang and B.W. Poovaiah

Center for Integrated Biotechnology and Department of Horticulture, Washington State University, Pullman, WA 99164-6414, USA

Various extracellular stimuli elicit specific calcium signatures that can be recognized by different calcium sensors. Calmodulin, the predominant calcium receptor, is one of the best-characterized calcium sensors in eukaryotes. In recent years, completion of the Arabidopsis genome project and advances in functional genomics have helped to identify and characterize numerous calmodulin-binding proteins in plants. There are some similarities in Ca\(^{2+}\)/calmodulin-mediated signaling in plants and animals. However, plants possess multiple calmodulin genes and many calmodulin target proteins, including unique protein kinases and transcription factors. Some of these proteins are likely to act as ‘hubs’ during calcium signal transduction. Hence, a better understanding of the function of these calmodulin target proteins should help in deciphering the Ca\(^{2+}\)/calmodulin-mediated signal network and its role in plant growth, development and response to environmental stimuli.

Calcium, a universal second messenger, acts as a mediator of stimulus-response coupling in the regulation of diverse cellular functions [1–3]. In general, in the absence of a stimulus, cytosolic Ca\(^{2+}\) concentration in plant cells is maintained at a concentration of \(~100\) nM. Levels of Ca\(^{2+}\) in extracellular and intracellular stores are in the mM range [2,4]. In response to a variety of stimuli, including light, gravity, abiotic and biotic stresses and hormones, the cytosolic Ca\(^{2+}\) concentration in plants is rapidly elevated via an increased Ca\(^{2+}\) influx, and then quickly returns to the basal level by Ca\(^{2+}\) efflux – this produces a Ca\(^{2+}\) spike [5–7]. In recent years, significant progress has been made in measuring in vivo free calcium changes in plant cells using calcium-imaging techniques. Free Ca\(^{2+}\) changes have been observed not only in the cytosol but also more recently in the nucleus as well [8]. Even within subcellular compartments, spatially and temporally regulated micro-domains of different Ca\(^{2+}\) oscillations are likely to exist.

Transient Ca\(^{2+}\) elevations are sensed by several Ca\(^{2+}\) sensors or Ca\(^{2+}\)-binding proteins, which usually contain the ‘EF-hand’ motif(s), a helix–loop–helix structure [7,9,10]. In Arabidopsis, it is estimated that there are \(~250\) putative EF-hand proteins [11]. To date, three major classes of EF-hand Ca\(^{2+}\) sensors have been characterized in plants, based on the number and organization of EF-hands and on the similarity of the amino acid sequences. These classes are calmodulin (CaM) [7,9,12], calcium-dependent protein kinase (CDPK) [13,14] and calcineurin B-like protein (CBL) [15]. CaM is one of the most conserved Ca\(^{2+}\)-binding proteins in eukaryotes. Like the counterpart in animals, a typical plant CaM has four EF hands [7,9]. CBL is a new family of plant-specific Ca\(^{2+}\) sensors similar to the regulatory B-subunit of calcineurin in animals [15]. Each CBL usually contains three EF hands. Unlike CaM and CBL, which act only as calcium sensors, CDPK contains a kinase domain and a CaM-like domain (four EF-hands) in a single protein and acts as a Ca\(^{2+}\) sensor and as an effector [13,14]. CDPK is found in certain protozoans in addition to plants, but not in other animals. In Arabidopsis, each of the three classes of Ca\(^{2+}\) sensors is encoded by a multi-gene family: 11 CaMs, 9 CBLs and 34 CDPKs [11,13–15]. To examine the relationships of these Ca\(^{2+}\) sensors further, a phylogenetic tree was constructed based on their amino acid sequence homology (Figure 1). These Ca\(^{2+}\) sensors each form three clusters, and CaM has a closer relationship with CDPK than with CBL. This close relationship between CaM and CDPK supports the hypothesis that the ancestral CDPK gene originated from a fusion of protein kinase and CaM [16]. Because of space limitations, this review will focus only on recent advances in the characterization of CaM and its target proteins.

Calmodulin, a conserved Ca\(^{2+}\) receptor with a flexible conformation

CaM is a small acidic protein present in all eukaryotes that have been tested [7,9,17]. The prototype of vertebrate CaM has 148 amino acids with pI 3.92. Alignment of CaM amino acid sequences indicates a high degree of conservation between animals and plants (\(>70\%\) identity). The four EF-hand Ca\(^{2+}\)-binding domains occur in pairs embedded within two separate globular regions in the N- and C-terminal regions separated by a flexible central helix that forms the hallmark dumbbell-shaped structure [17] (Figure 2a). The ability of CaM to regulate the target enzyme activity appears to reside in hydrophobic regions that are exposed in the conformational change that occurs upon the binding of Ca\(^{2+}\) to CaM [18]. However, electrostatic interaction can also play a role in CaM-binding. For example, the major force for CaM-binding to basic-helix–loop–helix DNA-binding proteins is electrostatic interaction [19].

In contrast to CaM, whose sequence shows exceptional conservation, the CaM-binding domains of target proteins
CaM-binding domains usually stretch 16–35 residues, formed by basic amphiphilic helices. These CaM recruitment motifs currently fall into three groups: two related motifs for Ca\(^{2+}\)-dependent binding, termed 1-5-10 and 1-8-14, which are distinguished by their spacing of bulky hydrophobic and basic amino acids, and the third motif, denoted as the ‘IQ motif’, which binds CaM in a Ca\(^{2+}\)-independent manner [18]. However, CaM-binding domains do not always have these features.

Currently, three CaM-activation mechanisms have been observed in animal systems [18] (Figure 2b). The first activation mechanism is relieving autoinhibition: the CaM binding site is adjacent to or within an autoinhibitory domain of the enzyme such as the CaM kinases. CaM binding to the target induces a conformational rearrangement that displaces the pseudosubstrate inhibitory domain and allows full enzyme activity [20]. The second activation mechanism is active site remodeling: in the case of activation of anthrax adenyl cyclase (oedema factor), four discrete regions of the oedema factor form a surface that recognizes an extended conformation of CaM. Upon CaM binding, a helical domain of the oedema factor undergoes a 30° rotation away from the catalytic core, which stabilizes a disordered loop and leads to enzyme activation [21]. The third activation mechanism is CaM-induced dimerization: two CaM molecules tightly interact with two K\(^{+}\) channel domains of the Ca\(^{2+}\)-activated potassium channels upon Ca\(^{2+}\)-binding. The C-terminal EF hands mediate tethering to the channel and the N-terminal EF hands are responsible for Ca\(^{2+}\)-induced dimerization leading to channel gating [22]. Recently, it has been reported that a single CaM molecule interacts with two peptides derived from the C-terminal CaM-binding domain of petunia glutamate decarboxylase (GAD) [23]. This provides evidence of the conformational flexibility of plant CaMs.

Figure 1. Phylogenetic tree showing the relationship between three Arabidopsis calcium sensor families: calmodulin (CaM), calcium-dependent protein kinase (CDPK) and calcineurin-B-like protein (CBL). The complete protein sequences were aligned and analyzed in GCG 10 and Treeview 1.6.5 program (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). The bootstrap values are shown near the internal nodes. CaM, a highly conserved calcium-binding protein in both animals and plants, has a closer relationship with plant-specific CDPKs than CBLs do. Note that At4g04710 (indicated by *) is CDPK.
Plant calmodulin: a multigene family with functional redundancy?

There are only a few CaM genes encoding for one or a few isoforms in animals. By contrast, each plant possesses a repertoire of multiple CaM and CaM-like genes that encode for several CaM isoforms [7,9]. Note that there are no criteria for classifying CaM and CaM-like genes. Here, we refer to those proteins with four EF-hands, ~148 amino acids and >50% identity to vertebrate CaM as CaM, and refer to others as CaM-like proteins. As indicated earlier, Arabidopsis has 11 CaM genes encoding for at least seven isoforms. In addition, there is also an extended family of CaM isoforms and CaM-like proteins in plants. Some of these proteins have varied EF-hands, from three (wheat CaM-III) [24,25] to six (Arabidopsis TCH3) [26]. The reasons that multiple CaM genes encode for the same or similar proteins in plants are not fully understood. Although the possibility of gene redundancy still cannot be ruled out, accumulating evidence suggests that each of those CaM genes could have functional significance.

Differential expression

In all plants examined, CaM genes, even genes encoding the same isoform, are differentially expressed in response to numerous external stimuli such as touch, heat shock, cold, light and pathogen, and to hormones such as auxin [7,9,27–29]. CaMs are also differentially expressed in different stages, as well as in different tissue and cell types [9,30,31].

Differential subcellular localization

Generally, CaM is a cytosolic protein. However, CaM has been found in the nucleus [32], in the peroxisome [33], and even in the extracellular matrix [34]. The necessity of multiple subcellular locales is understandable because the CaM-target proteins are present in different subcellular locations. However, the manner in which CaM targets these organelles is not clear. One possibility is that it is regulated by post-translational modification. A CaM-like protein (petunia CaM53) has been shown to associate with the plasma membrane when the protein is prenylated. If the prenylation is inhibited, it is localized predominantly in the nucleus [35,36].

Differential activation of targeting proteins

Distinct plant CaM isoforms differ in their ability to bind and activate known CaM-regulated enzymes in vitro [37–39]. It is not clear whether this is the case in vivo. In addition, the various CaMs and CaM-like proteins might have differential affinities to Ca2+. This could be the situation for those CaMs containing a varied number of EF-hand motifs, or those that contain mutations in the EF-hand motifs. The transgenic approach has also been used to define the function of CaM in vivo. It has been observed that different CaM genes are involved in disease resistance [40] and cold stress [27]. However, to define their functional significance, it is necessary to carry out loss-of-function and gain-of-function studies for each CaM gene in one plant species such as Arabidopsis.

Number of CaM target proteins identified is booming

CaM has no catalytic activity of its own. Its regulatory activities are manifested by its ability to modulate a certain set of enzymes. Thus, it is crucial to isolate and to characterize the CaM-binding proteins to understand the role of the Ca2+/CaM-mediated network. In the past decade, molecular approaches such as protein–protein interaction screening have provided a powerful tool to isolate CaM-binding proteins. Identification of novel CaM-binding proteins has also been accelerated as a result of the Arabidopsis genome project. To date, ~50 CaM-binding proteins in plants have been identified, and the total number is expected to increase [41]. Table I lists some plant CaM-binding proteins with their dedicated CaM-binding domains. Other proteins that exhibit CaM-binding without the identification of CaM-binding domains, such

Figure 2. Calmodulin structure and its activation mechanisms. (a) Crystal structure of Ca2+-bound calmodulin. Calcium ions are indicated by green balls. Four EF-hands are indicated. (b) Protein activation mechanisms by calmodulin. Relieving autoinhibition: Calmodulin binding to the target induces a conformational rearrangement that displaces the autoinhibitory domain (AID) and allows for full enzyme activity. Active site remodeling: two CaM molecules interact with two K+ channel domains of a potassium channel upon Ca2+-binding. The C-terminal EF hands mediate tethering to the channel and the N-terminal EF hands are responsible for Ca2+-induced dimerization leading to channel gating and direct coupling between changes in intracellular Ca2+ concentrations and altered membrane potential. Calcium ions are depicted as green circles and active sites as stars. (b) is adapted from Ref. [18].
as NAD kinase, EF-1α and FKBP73 [9,12,42], are not included in Table 1. Table 1 shows that CaM has a diverse set of target proteins that are involved in a variety of processes, including ion transport [43,44], gene regulation [45,46], cytokinesis organization [47], defense tolerance [7,9]. These CaM-binding proteins can be classified into three groups. The first group is plant-specific CaM-binding proteins, such as auxin-responsive SAURs [49] and pollen-specific MPCBP [50]. The second group is composed of proteins that are similar to animal homologs but have additional CaM-binding domains, such as GAD [9], kinesin [7] and catalase [33]. The third group is composed of proteins that are similar to the CaM homologs in animals, such as the CGCG protein [46]. Interestingly, plants have a greater number and more diversified CaM-binding proteins than animals do. Many CaM-binding proteins are plant-specific, suggesting that Ca²⁺/CaM-mediated signal networks are distinctly different in plants as compared with those in animals. Here we highlight several CaM-binding proteins that have been defined recently.

### Protein kinases

CaM kinases are the best-characterized CaM-binding proteins in animals and include a kinase cascade in which all members are regulated by Ca²⁺ and CaM [18]. By contrast, plants have a large family of CDPKs that contains a CaM domain directly fused to the kinase domain. However, not much is known about plant CaM kinase. Several plant homologs of CaM kinase II have been cloned from apple, maize and rice [51]. CaM is able to bind to those recombinant proteins, but no effect on the auto-phosphorylation or substrate phosphorylation has been observed. However, a structurally unique Ca²⁺/CaM-regulated protein kinase (CCaMK) has been reported in plants. In addition to having a kinase domain and a CaM-binding domain, CCaMK has an additional visinin-like domain. However, not much is known about plant CaM kinase. Several plant homologs of CaM kinase II have been cloned from apple, maize and rice [51]. CaM is able to bind to those recombinant proteins, but no effect on the auto-phosphorylation or substrate phosphorylation has been observed. However, a structurally unique Ca²⁺/CaM-regulated protein kinase (CCaMK) has been reported in plants. In addition to having a kinase domain and a CaM-binding domain, CCaMK has an additional visinin-like domain.

### Transcription factors and nuclear proteins

It has been known for some time that CaM can regulate transcription activators indirectly through CaM kinases and the phosphatase calcineurin in animals [18]. Certain transcription factors in the bHLH family in mammals have been shown to bind to CaM, thus inhibiting their DNA-binding properties by interacting with the DNA-binding

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**Table 1. Plant calmodulin-binding proteins with defined calmodulin-binding domains**

<table>
<thead>
<tr>
<th>Protein</th>
<th>CaM binding domain (CaMBD)</th>
<th>Arabidopsis</th>
<th>Non-plant system</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate decarboxylase (GAD)</td>
<td>VHKTDSEVOLMETAVKKFVEEKKKTNRVC (Petunia)</td>
<td>5</td>
<td>P, No CaMBD</td>
<td>[9]</td>
</tr>
<tr>
<td>Arylase</td>
<td>VKKSDIKORDITGWKKFDRKKTSGC (Arabidopsis GAD1)</td>
<td>2</td>
<td>P</td>
<td>[69,71]</td>
</tr>
<tr>
<td>Catalase</td>
<td>FKNCNKTRALKNLNY (pea)</td>
<td>2</td>
<td>P, No CaMBD</td>
<td>[33]</td>
</tr>
<tr>
<td>Auxin-induced protein ZmSAUR1</td>
<td>NKKRIVRLQQQLWWKVLATVTPSA (maize)</td>
<td>&gt;30</td>
<td>NP</td>
<td>[49]</td>
</tr>
<tr>
<td>DNA-binding protein CGCG</td>
<td>IWSVGILEKVLWRKSGSRLGFK (Arabidopsis AtSR1)</td>
<td>6</td>
<td>P</td>
<td>[45,46,56,57]</td>
</tr>
<tr>
<td>Nuclear protein PCBP</td>
<td>SKLKKLILRSKALEKARKF (potato)</td>
<td>3</td>
<td>NP</td>
<td>[50]</td>
</tr>
<tr>
<td>Disease resistance gene MLO</td>
<td>MKALMNWRRKAMEKVKVR (rice OSMlo)</td>
<td>1</td>
<td>P</td>
<td>[48,70]</td>
</tr>
<tr>
<td>Cyclic nucleotide gated cation channels (CNGC)</td>
<td>FRLHSKQLRHTFRFYSGQWRTW (tobacco NtCBP4)</td>
<td>20</td>
<td>P</td>
<td>[43,64–67]</td>
</tr>
<tr>
<td>Vacuolar Ca²⁺-ATPase</td>
<td>RQWRSSSVSVKRNARFRFMISNL (Brassica)</td>
<td>14</td>
<td>NP</td>
<td>[61]</td>
</tr>
<tr>
<td>Endoplasmic reticulum Ca²⁺-ATPase</td>
<td>LEKWRNLCGVVKPNNRFRTANL (Arabidopsis AtACA2)</td>
<td>1</td>
<td>NP</td>
<td>[62]</td>
</tr>
<tr>
<td>Plasma membrane Ca²⁺-ATPase</td>
<td>VLQRWRRLGIVKNPFFFREF (soybean SCA1)</td>
<td>P</td>
<td></td>
<td>[63]</td>
</tr>
<tr>
<td>AAA family CIP111</td>
<td>RERTQFKRLAILVSKA (soybean SCA1)</td>
<td>1</td>
<td>P, No CaMBD</td>
<td>[72]</td>
</tr>
<tr>
<td>Kinesin-like protein</td>
<td>LWTPLKSVAMLFRRHIA (Arabidopsis)</td>
<td>1</td>
<td>NP</td>
<td>[7,9]</td>
</tr>
<tr>
<td>Chimeric Ca²⁺/CaM-dependent protein kinase (CCaMK)</td>
<td>ISSKEMVRKLKLAVYWKEOAGKK (Arabidopsis)</td>
<td>1</td>
<td>NP</td>
<td>[52–55]</td>
</tr>
<tr>
<td>Diacylglycerol kinase I (LeCBDGK)</td>
<td>SFNARLKRAAAIASVLS (lilly)</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pollen-specific protein TCP60</td>
<td>KRQRSHGRKPRPLLWRLNLLAFRLERH (tomato)</td>
<td>3</td>
<td>P, No CaMBD</td>
<td>[76]</td>
</tr>
<tr>
<td>Heat shock repressed protein TCP60</td>
<td>VSKGWRLLALILSAAQRF (maize)</td>
<td>3</td>
<td>NP</td>
<td>[50]</td>
</tr>
<tr>
<td>Chaperonin 10</td>
<td>GWLKKAAARMWGGFVRKKA (tobacco)</td>
<td>6</td>
<td>NP</td>
<td>[9]</td>
</tr>
<tr>
<td></td>
<td>LYSKYAGNDFKGKDGSNIVIALRADVMAILS (Arabidopsis)</td>
<td>1</td>
<td>NP</td>
<td>[9]</td>
</tr>
</tbody>
</table>

*NP, not present in non-plant systems; P, present in non-plant systems; P, No CaMBD: present but no calmodulin-binding domain in non-plant systems.

*ND, Not defined.
domain [19]. In plants, TGA3, a member of a family of basic leucine zipper transcription factors, exhibits Ca\textsuperscript{2+}/CaM enhanced-binding activities to the C/G box in vitro. The CaM-binding to TGA3 enhances the binding of TGA3 to the promoter [12]. However, the CaM-binding property of TGA3 is not well defined. Recently, the novel CGCG family of DNA-binding proteins has been reported in plants [45,46,56,57]. CGCG proteins are also present in animals and are referred to as one of the ancient architectures. The CGCG family shares the structural features of a CGCG-DNA-binding domain in the N-terminus, a CaM-binding domain in the C-terminus, and ankyrin repeats in the central portion. In Arabidopsis, a CGCG protein (AtSR1) exhibits special DNA-binding activity to a novel CGCG-box [45]. A transcription activation domain has also been identified for one AtSR1 homolog [46]. Furthermore, six Arabidopsis CGCG genes are induced by a variety of environmental and hormonal signals [45]. Thus, CGCG genes might play an important role in plant stress tolerance.

In addition, a nuclear CaM-binding protein (PCBP) has been isolated from potato tubers [58]. PCBP is a plant-specific protein containing several strong PEST motifs. Proteins containing PEST regions are thought to be degraded rapidly. Recently, it has been reported that CaM binds to DRL, a homolog of yeast TOT4 (also known as KTI12) protein. TOT4/KTI12 associates with Elongator, a complex binding to the RNA polymerase II transcription elongator complex. DRL has a function in meristem activity and organ growth in plants [59].

**Ion transporters, channels and membrane proteins**

In animal systems, CaM regulates both fluxes of transient Ca\textsuperscript{2+}: Ca\textsuperscript{2+} influx by Ca\textsuperscript{2+} channel and Ca\textsuperscript{2+} efflux by calcium transporters [44]. Recent findings have revealed that plant cells have many CaM-regulated Ca\textsuperscript{2+} pumps in multiple subcellular locations [60–63]. In Arabidopsis, there are at least three CaM-regulated Ca\textsuperscript{2+}-ATPases, ACA2 in the endoplasmic reticulum, ACA4 in the vacuolar membrane, and ACA8 in the plasma membrane. By contrast, in mammals, only plasma membrane (type II) Ca\textsuperscript{2+}-ATPase is a CaM-binding protein [44,60]. This suggests that plants have a more-complex regulatory mechanism for Ca\textsuperscript{2+} efflux via Ca\textsuperscript{2+}/CaM-regulated Ca\textsuperscript{2+}-ATPases than mammals do.

A family of CaM-binding cyclic nucleotide-gated non-selective cation channel proteins (CNGC) has been reported from barley, tobacco and Arabidopsis [9]. Animal CNGCs are permeable for monovalent cations and Ca\textsuperscript{2+}, whereas plant homologs are also permeable for K\textsuperscript{+} and Ca\textsuperscript{2+} [64]. The CaM-binding site in CNGC overlaps with a putative cNMP-binding domain, suggesting that Ca\textsuperscript{2+}/CaM signaling and cyclic nucleotide signaling interact [65,66]. Overexpression of NtCBP4, a tobacco CNGC, confers tolerance to Ni\textsuperscript{2+} uptake and hyposensitivity to Pb\textsuperscript{2+} uptake [67]. Mutating AtCNGC2 abolishes the
hypermethylation response upon pathogen infection, suggesting that AtCNCG2 is involved in apoptosis [68].

There are other CaM-binding membrane proteins, such as apyrase [69] and MLO [70]. Apyrases hydrolyze nucleoside tri- and diphosphates and are involved in xenobiotic resistance. Only one of two Arabidopsis apyrases is a CaM-binding protein. Overexpression of CaM-binding pea apyrase in Arabidopsis leads to increased scavenging of extracellular phosphate. Disruption of two apyrases inhibits pollen germination in Arabidopsis [71]. In addition, CaM binds to MLO, a powdery mildew disease resistance protein. MLO is a plasma membrane protein reminiscent of G-coupled receptor proteins in animals. In barley, MLO is known to function as a negative regulator of broad-spectrum disease resistance and leaf cell death. Loss of CaM binding halves the ability of MLO to regulate defense negatively against powdery mildew in vivo [48]. In addition, Arabidopsis has a CaM-binding ATP-like protein CIP111 [72]. However, its function is still not clear.

Cross-talk with other signaling pathways
Recent studies suggest that changes in intracellular redox and calcium homeostasis are unifying consequences of biotic and abiotic stresses. Reactive oxygen species, such as H$_2$O$_2$ and O$_2^*$ are important components of the stress signal transduction cascade [73]. One recent finding is that Ca$^{2+}$/CaM binds to and activates plant catalases, but does not have any effect on catalases from other sources [33]. Catalase, which is involved in the degradation of H$_2$O$_2$ into water and oxygen, is the major H$_2$O$_2$-scavenging enzyme in all aerobic organisms. Taken together with other studies, it is suggested that Ca$^{2+}$ has dual functions in regulating H$_2$O$_2$ homoeostasis that in turn influence plant redox signaling: (i) positive regulation – generating H$_2$O$_2$ by directly activating NADPH oxidase that has affinity to Ca$^{2+}$/CaM, and indirectly producing more NADPH via Ca$^{2+}$/CaM-regulated NAD kinase [74,75]; (ii) negative regulation – reducing H$_2$O$_2$ by stimulating catalase activity through Ca$^{2+}$/CaM modulation [33].

Diacylglycerol kinases (DGKs) catalyse the phosphorylation of diacylglycerol to yield phosphatidic acid (PA). PA, a putative signal molecule, has been shown to accumulate rapidly in plants in response to stimuli. Two tomato DGKs are derived from the same gene via alternative splicing. Interestingly, one DGK (LeCBDGK) has a 29 residue C-terminal extension that represents a CaM-binding domain. LeCBDGK is found in association with membranes and in soluble cell extracts, but its membrane association is Ca$^{2+}$/CaM-dependent [76].

Progress has also been made in understanding the interaction of Ca$^{2+}$/CaM-mediated signaling with hormone actions by identifying ZmSAUR1 [49] and NtER1 [56] as CaM-binding proteins. SAURs are a family of small auxin up RNAs that are rapidly induced by auxin (within minutes) and localized to tissues that are targets of auxin-induced cell elongation. NtER1 is rapidly responsive to ethylene (within minutes) and highly expressed in senescing tissues [56]. The six genes of the Arabidopsis NtER1 homolog (CGCG genes) are rapidly and differentially induced by the hormones ethylene and abscisic acid, as well as other stress signals and signal molecules [45]. Therefore, these genes belong to a group of early signal-responsive genes. It is believed that, in response to certain signals, early-responsive genes play a prominent role in regulating late-responsive genes [77].

Conclusions and outlook
In recent years, calcium signaling has received a great deal of attention because of the realization that it is involved in many aspects of plant biology, including biotic and abiotic stress responses. A decade ago, there was a perception that Ca$^{2+}$/CaM-mediated signaling in plants and animals might be similar because CaM is a remarkably conserved Ca$^{2+}$ sensor. This is true in that the concentration of the messenger, Ca$^{2+}$, changes in response to stimuli, and it is also true that CaM shares a similar structure in both plants and animals. However, it is becoming obvious that plants have a larger repertoire of CaM genes that encode for multiple isoforms, as well as extremely diversified CaM-target proteins, many of which are plant specific. This suggests that there are aspects of Ca$^{2+}$/CaM-mediated signaling that are unique to plants. This is understandable because plants are sessile organisms and must therefore adapt to a changing environment to survive. Figure 3 summarizes a complex Ca$^{2+}$/CaM-mediated signal network that affects many aspects of plant growth, development and responses to environmental changes. However, our understanding of this complex network is still in its infancy. Much is yet unknown about the functional significance of CaM and CaM-targeting proteins. Some of these questions include: what are the expression profiles for each of these genes in the tissue, cell type, developmental stages and their responses to external stimuli? What are the subcellular localizations of each of these proteins? What are the CaM target proteins and the downstream-regulated proteins? How does the Ca$^{2+}$/CaM-mediated network interact with other signal transduction pathways? How does each CaM perceive and decode the varied Ca$^{2+}$ signatures generated from various signals and ultimately lead to a physiological response? In the next decade, a concerted multi-disciplinary approach involving functional genomics, proteomics, metabolites, bioinformatics and imaging techniques should help in our understanding of the Ca$^{2+}$/CaM-mediated plant signal network.

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