Calcium/calmodulin-mediated signal network in plants

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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 microdomains 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

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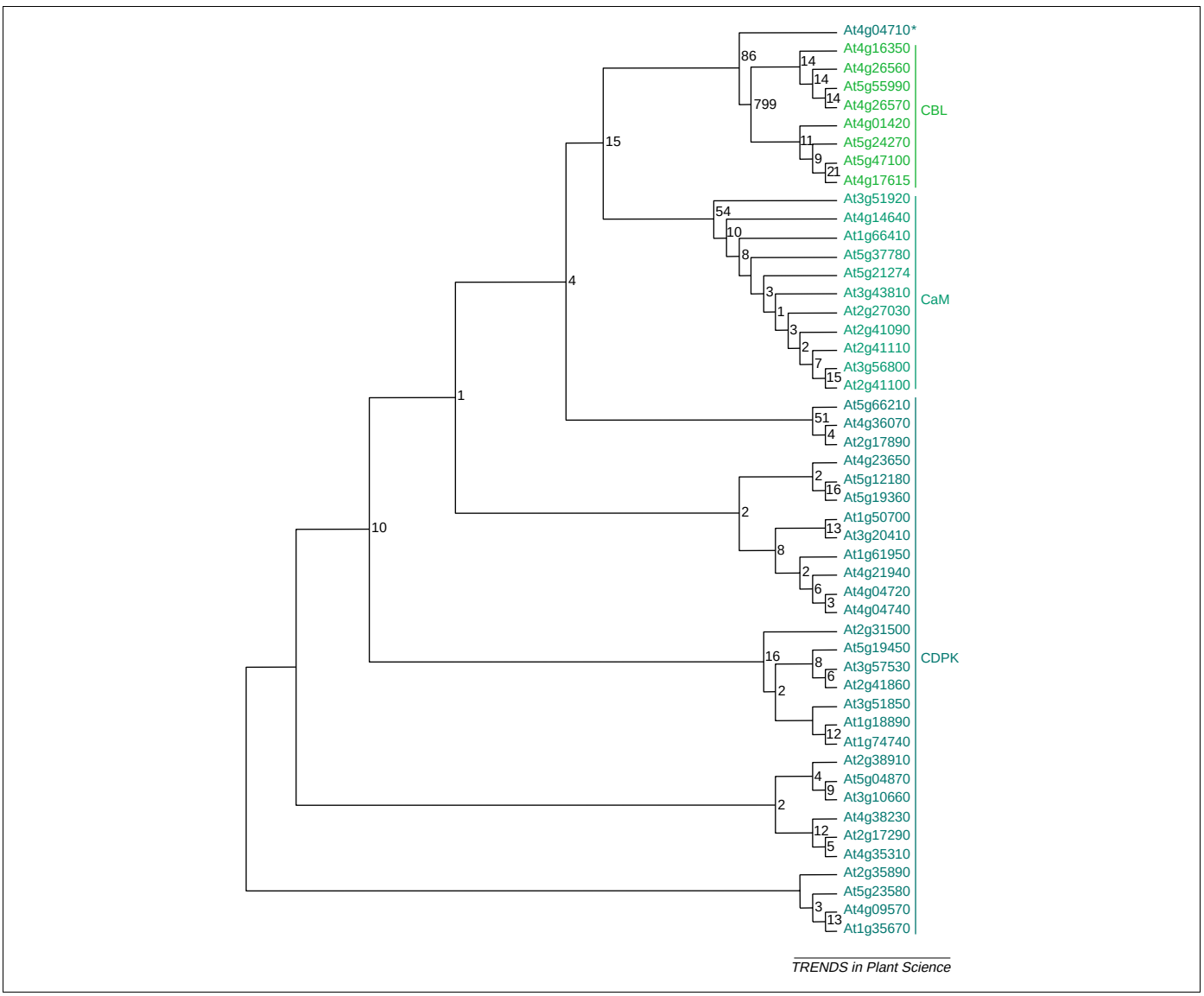

Figure 1. Phylogenetic tree showing the relationship between three *Arabidopsis* calcium sensor families: calmodulin (CaM), calcium-dependent protein kinase (CDPK) and calcineurin-B-like protein (CBL). The complete protein sequences were aligned and analyzed in GCG 10 and Treeview 1.6.5 program (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). 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 At4 g04710 (indicated by *) is CDPK.

exhibit extreme sequence variability, reflecting the flexibility of CaM regulation [9,18]. The CaM-binding domains are usually stretches of 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 adenylyl 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.

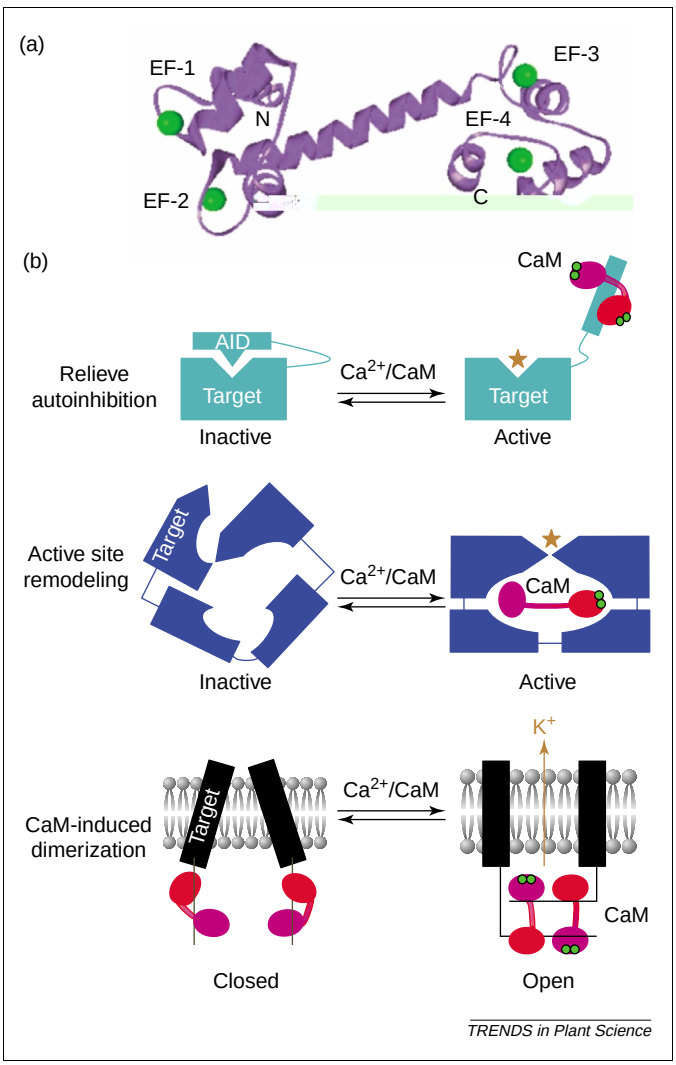

Figure 2. Calmodulin structure and its activation mechanisms. **(a)** Crystal structure of Ca^{2+}-bound calmodulin. Calcium ions are indicated by green balls. Four EF-hands are indicated. **(b)** Protein activation mechanisms by calmodulin. Relieving autoinhibition: Calmodulin binding to the target induces a conformational rearrangement that displaces the autoinhibitory domain (AID) and allows for full enzyme activity. Active site remodeling: upon Ca^{2+}/CaM binding, a helical domain of oedema factor undergoes a rotation away from the catalytic core, which stabilizes a disordered loop and leads to enzyme activation. Calmodulin-induced dimerization: two CaM molecules interact with two K^+ channel domains of a potassium channel 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 and direct coupling between changes in intracellular Ca^{2+} concentrations and altered membrane potential. Calcium ions are depicted as green circles and active sites as stars. (b) is adapted from Ref. [18].

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 proteins. 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 pathogens, 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].

Different 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 Ca^{2+}. 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 Ca^{2+}/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 1 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