Calmodulin, calmodulin-related proteins and plant responses to the environment
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In any organism, one of the fundamental properties of defense mechanisms is that they must be inducible in response to the external threat. This ensures that resources are not wasted. Thus, plant cells have evolved to perceive different signals from their surroundings, to integrate them and to respond by modulating various functions via signal transduction pathways. Recently, tremendous progress has been made in understanding the role of Ca\(^{2+}\) as a second messenger in plants. This has become possible in several ways: by employing innovative types of technology to measure, in real time, changes in the concentration of Ca\(^{2+}\) in cells\(^1,2\); by using novel approaches to introduce signaling components and reporter genes into single plant cells\(^3\); and by using methods for the isolation of downstream cellular targets of calmodulin\(^4\). These studies suggest that Ca\(^{2+}\) has a vital role in mediating plant responses to external stimuli of both abiotic origin (e.g. light, cold, heat, movement, hypoxia and drought) and biotic origin (e.g. phytohormones, pathogens and interactions with symbionts). Thus, Ca\(^{2+}\) triggers a myriad of cellular processes that influence growth, development and physiology, which allow plants to adapt to the changing environment.

Ca\(^{2+}\)-dependent modulation of cellular processes occurs via intracellular Ca\(^{2+}\)-binding proteins, of which calmodulin is one of the best characterized. It has no catalytic activity of its own, but Ca\(^{2+}\)-bound calmodulin (Ca\(^{2+}\)-calmodulin) activates numerous target proteins involved in a variety of cellular processes (Fig. 1). Recent studies have revealed that plants possess unique calmodulin-related proteins, the functions of which are still unknown. This review focuses on recent developments in studying calmodulin and calmodulin-related proteins, their downstream effectors and their role in regulating responses to external signals.

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**Fig. 1. Ca\(^{2+}\)-bound-calmodulin-mediated signal transduction in plants.** Biotic and abiotic signals are perceived by receptors, resulting, in some cases, in transient changes in Ca\(^{2+}\) concentrations in the cytosol and/or organelles (e.g. nucleus). Increases in free Ca\(^{2+}\) concentrations originating from either extracellular pools or intracellular stores are capable of binding to Ca\(^{2+}\)-modulated proteins including calmodulin and calmodulin-related proteins. Structural modulations of these proteins enable them to interact with numerous cellular targets that control a multitude of cellular functions, such as metabolism, ion balance, the cytoskeleton and protein modifications. In addition, Ca\(^{2+}\) and calmodulin might also regulate the expression of genes by complex signalling cascades or by direct binding to transcription factors. Rapid changes in cellular functions result from direct interactions of calmodulin and calmodulin-related proteins with their targets (within seconds to minutes). Slower responses require gene transcription, RNA processing and protein synthesis (variable times from minutes to days). These calmodulin-mediated processes, together with cellular changes triggered by other signaling pathways, constitute the response of the plant to the external signals. Broken arrows denote Ca\(^{2+}\) fluxes from extracellular or intracellular stores, and question marks signify unknown signal transduction intermediates.
Most proteins that function as intracellular transducers of Ca\(^{2+}\) signals contain a common structural motif, the ‘EF hand’, which is a helix-loop-helix structure that binds a single Ca\(^{2+}\) ion. These motifs typically occur in closely linked pairs, interacting through antiparallel \(\beta\)-sheets. This arrangement is the basis for cooperativity in Ca\(^{2+}\) binding. The superfamily of EF-hand proteins is divided into several classes based on differences in number and organization of EF-hand pairs, amino acid sequences within or outside the motifs, affinity to Ca\(^{2+}\) and/or selectivity and affinity to target proteins. Calmodulin is an acidic EF-hand protein present in all eukaryotes. The calmodulin prototype is composed of 148 amino acids arranged in two globular domains connected with a long flexible helix. Each globular domain contains a pair of intimately linked EF hands (Fig. 2a).

One of the intriguing properties of calmodulin is that it can bind and activate numerous target proteins that share very little amino acid sequence similarity in their binding sites. Thus, the positioning of these binding domains must be empirically determined. However, the majority of known target sites for calmodulin are composed of a stretch of 12–30 contiguous amino acids with positively charged amphiphilic characteristics and a propensity to form an \(\alpha\)-helix upon binding to calmodulin (Fig. 2b). This affords a tremendous potential for variability in primary sequence (i.e., target diversity). In addition, calmodulin-binding domains, or closely juxtaposed regions, often also function as autoinhibitory or pseudosubstrate domains, maintaining the target in an inactive state in the absence of a Ca\(^{2+}\) signal. Recent X-ray diffraction and NMR studies of calmodulin have provided a model for the structural basis of calmodulin-target interactions. The binding of Ca\(^{2+}\) to calmodulin (affinity in the range of 10\(^{-7}\) to 10\(^{-3}\) M) exposes two hydrophobic surfaces surrounded by negative charges, one in each globular domain. Ca\(^{2+}\)-calmodulin can then bind to its targets (affinity in the nanomolar range), mainly by hydrophobic interactions with long hydrophobic side chains in the target sites. Electrostatic interactions contribute to the stability of the calmodulin-target complex. In solution, the two globular domains wrap around the target, forming an almost globular structure (Fig. 2b). However, functions of calmodulin in the Ca\(^{2+}\)-free state have also been described. For example, a mutant yeast calmodulin lacking the ability to bind calcium can still complement a mutant yeast strain lacking its calmodulin gene. Subsequent to this finding, calcium-independent calmodulin binding to the yeast spindle-pole body, required for chromosome segregation, was characterized. Some target proteins bind calmodulin with higher affinity in the absence of Ca\(^{2+}\) than in its presence (e.g., neurocalmodulin). Other proteins, such as phosphorylase kinase, can form stable complexes with calmodulin such that the calmodulin does not dissociate even at low Ca\(^{2+}\) concentrations, but activation of the target protein is still Ca\(^{2+}\) dependent. These findings raise the possibility that Ca\(^{2+}\)-free calmodulin functions also occur in plants.
Although the physiological significance of the multiple types of calmodulin-related proteins in plants is unclear, recent studies have revealed that plant calmodulin-related proteins differ in their ability to activate known calmodulin-regulated enzymes in vitro. Soybean SCaM-4, which differs at 32 amino acid residues from SCaM-1 (Fig. 3), was unable to activate pea NAD kinase, but was able, like SCaM-1, to activate the mammalian enzyme 3′,5′ cyclic nucleotide phosphodiesterase. Similarly, differential activation of NAD kinase by different calmodulins from Arabidopsis has also been reported. Thus, there might be target specificity for different members of the calmodulin superfamily. In addition, at least one other protein as well as other genes encoding various calmodulin-related proteins, but the emerging picture of stimulus a cell perceives and in magnitude, depending upon the type of signal, might be distinct. A striking example is the induced expression of a group of calmodulin-related genes in response to physiological signals (e.g. touch, dark, heat and light) and chemical (e.g. auxin and NaCl) stimuli. The induced expression of at least some of these calmodulin-related genes is mediated by a rise in cytosolic Ca²⁺ in response to the external stimulus. The dynamic modulation of calmodulin gene expression in plants is also reflected in gene-specific, developmentally regulated, organ-, tissue- and cell-specific expression patterns. These observations imply that the intracellular Ca²⁺-sensing machinery is continuously changing, probably reflecting a more general phenomenon of tuning of the plant’s signal transduction network, the significance of which might be sensitization or desensitization to external signals, preparation for a longer-term response, or other functions.

Dynamic expression of calmodulin-related genes

Many plant species have been shown to possess calmodulin multi-gene families composed of several genes encoding an identical protein as well as other genes encoding various calmodulin-related proteins. For example, in wheat, at least seven genes encode the conserved calmodulin TaCaM-1; at least two other genes encode TaCaM-4, which differs from TaCaM-1 in just two conserved amino acid substitutions; and at least one other gene encodes TaCaM-III, a novel calmodulin-related protein lacking the first Ca²⁺-binding site.

| Human CaM | MADLTERGQ13345KKLPIQ8791DGSQTV109515 |
| Arabidopsis CaM-2 | DD-S-D-D |
| Petunia CaM1 | K-E-N-V |
| Wheat CaM-1 | M-P-D-L |
| Soybean SCaM-1 | D-S-D-S-D |
| Soybean SCaM-4 | I-V-Y-D-D |

Fig. 3. Comparison of amino acid sequences of selected plant calmodulins with human calmodulin (CLP, 'calmodulin-like protein'). The amino acid sequences of some phylogenetically conserved calmodulins are aligned with respect to their four Ca²⁺ binding sites (asterisks). Amino acids are numbered (right) from the start methionine codon of the human calmodulin cDNA. GenBank accession numbers: human calmodulin, M19311; human CLP, X13461; Arabidopsis CaM-2, M38380; Petunia CaM1, M80832; Petunia CaM81, M18036; soybean SCaM-1, U46242; soybean SCaM-4, L01433; soybean CaM72, M80832; soybean CaM81, M18036; wheat CaM1-1, U46242; soybean SCaM-1, L01433; soybean SCaM-4, L01433.
light-mediated gene activation and chloroplast development. Genes involved in anthocyanin biosynthesis (e.g. the gene encoding chalcone synthase) can be inducted in the aurea mutant by microinjection of cGMP, whereas Ca$^{2+}$-calmodulin microinjection leads to the induction of genes associated with the development of the photosynthetic complex (e.g. CAB genes). Moreover, these signaling molecules operate in a reciprocally repressive manner: Ca$^{2+}$-calmodulin microinjection inhibits anthocyanin production, whereas cGMP microinjection inhibits CAB expression. In addition, UV-B light, but not UV-A light, appears to modulate chalcone synthase gene expression through a calmodulin-mediated pathway. Similarly, microinjection of calmodulin into the zygotes of fucoid algae enhanced photopolarization of the zygotic axis. In general, however, the targets calmodulin that mediate these responses remain unknown.

**The diversity of plant calmodulin-binding proteins**

In animals, over 25 calmodulin targets have been identified, including kinases, receptors, ion channels and G-proteins. In plants, relatively few calmodulin-regulated proteins have been isolated, even though calmodulin has been implicated in a range of cellular processes as diverse as responses to pathogens, gravitropism, and pollen viability. These plants have significantly elevated Ca$^{2+}$-calmodulin-dependent and -independent pathways for light-mediated gene activation and chloroplast development. Genes involved in anthocyanin biosynthesis (e.g. the gene encoding chalcone synthase) can be induced in the aurea mutant by microinjection of cGMP, whereas Ca$^{2+}$-calmodulin microinjection leads to the induction of genes associated with the development of the photosynthetic complex (e.g. CAB genes). Moreover, these signaling molecules operate in a reciprocally repressive manner: Ca$^{2+}$-calmodulin microinjection inhibits anthocyanin production, whereas cGMP microinjection inhibits CAB expression. In addition, UV-B light, but not UV-A light, appears to modulate chalcone synthase gene expression through a calmodulin-mediated pathway. Similarly, microinjection of calmodulin into the zygotes of fucoid algae enhanced photopolarization of the zygotic axis. In general, however, the targets calmodulin that mediate these responses remain unknown.

**Fig. 4. Schematic structural presentation of selected plant calmodulin-related proteins.** Loops denote high-affinity Ca$^{2+}$-binding sites. C-terminal extensions, denoted by filled boxes, of Petunia CaM53 and rice CaM61 contain a large number of positively charged amino acids. Each of these two proteins ends with a Ca$^{2+}$-calmodulin-dependent and -independent pathways for light-mediated gene activation and chloroplast development. Genes involved in anthocyanin biosynthesis (e.g. the gene encoding chalcone synthase) can be induced in the aurea mutant by microinjection of cGMP, whereas Ca$^{2+}$-calmodulin microinjection leads to the induction of genes associated with the development of the photosynthetic complex (e.g. CAB genes). Moreover, these signaling molecules operate in a reciprocally repressive manner: Ca$^{2+}$-calmodulin microinjection inhibits anthocyanin production, whereas cGMP microinjection inhibits CAB expression. In addition, UV-B light, but not UV-A light, appears to modulate chalcone synthase gene expression through a calmodulin-mediated pathway. Similarly, microinjection of calmodulin into the zygotes of fucoid algae enhanced photopolarization of the zygotic axis. In general, however, the targets calmodulin that mediate these responses remain unknown.

**Table 1.** The multiplicity of calmodulin-related proteins and the diversity of calmodulin targets imply that a broad spectrum of processes is probably modulated by these proteins in plants. Indeed, manipulation of calmodulin and calmodulin targets, by microinjection studies and transgenic analysis, can significantly alter the developmental profile of plant organelles, cells and tissues. A particularly interesting example of the involvement of calmodulin in plant development comes from studies of photomorphogenesis. Microinjection studies of calmodulin into a phytochrome-deficient aurea mutant of tomato have allowed the identification of Ca$^{2+}$-calmodulin-dependent and -independent pathways for light-mediated gene activation and chloroplast development. Genes involved in anthocyanin biosynthesis (e.g. the gene encoding chalcone synthase) can be induced in the aurea mutant by microinjection of cGMP, whereas Ca$^{2+}$-calmodulin microinjection leads to the induction of genes associated with the development of the photosynthetic complex (e.g. CAB genes). Moreover, these signaling molecules operate in a reciprocally repressive manner: Ca$^{2+}$-calmodulin microinjection inhibits anthocyanin production, whereas cGMP microinjection inhibits CAB expression. In addition, UV-B light, but not UV-A light, appears to modulate chalcone synthase gene expression through a calmodulin-mediated pathway. Similarly, microinjection of calmodulin into the zygotes of fucoid algae enhanced photopolarization of the zygotic axis. In general, however, the targets calmodulin that mediate these responses remain unknown.

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Future perspectives

The existence of multigene families of calmodulin and calmodulin-related proteins in plants and the growing list of known targets of calmodulin suggest a complex Ca\(^{2+}\)-mediated regulatory network controlling development and responses to the environment. Future studies should aim to dissect the specific roles of members of the calmodulin superfamily, their subcellular distribution and the physiological relevance of their interaction with target proteins. Different approaches include affinity purification, mapping of calmodulin-binding domains, assessment of protein-protein interactions in vitro, functional analysis using reverse genetics and expression of calmodulin isoforms. The critical role of domain I, the helix-loop-helix calcium-binding proteins, in the regulation of development and responses to the environment, has been recognized by several groups. However, the mechanism by which these proteins interact with calmodulin and calmodulin targets involved in these processes remain uncharacterized. Thus, future characterization of calmodulin target proteins is essential. Continued integrated studies in model systems, such as Arabidopsis, should help us to understand the mechanisms of Ca\(^{2+}\)-mediated signal transduction pathways.

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Table 1. Calmodulin-binding proteins in plants

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Protein</th>
<th>Features</th>
<th>Refs</th>
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</thead>
<tbody>
<tr>
<td>Metabolism</td>
<td>Glutamate decarboxylase* (GAD)</td>
<td>Catalyzes conversion of L-glutamate to y-aminobutyric acid; activated by Ca(^{2+})-bound calmodulin (Ca(^{2+})-calmodulin) and many stresses; forms a multivalent complex, tightly associated with calmodulin.</td>
<td>29, 31 and 32</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>NAD kinase (gene not cloned)</td>
<td>Catalyzes conversion of NAD to NADP. Activated by Ca(^{2+})-bound calmodulin; might be involved in oxidative burst.</td>
<td>28</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>Apyrase (nucleoside triphosphatase)</td>
<td>Subcellular localization is light-regulated; ectopic expression affects metabolism and growth in Arabidopsis.</td>
<td>33, and S.J. Roux, pers. commun.</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>Ca(^{2+})-dependent kinase (Ca(^{2+})-calmodulin-dependent kinase (CCaMK))</td>
<td>Has a visinin-like (three ‘EF hands’) Ca(^{2+})-binding domain and a separate Ca(^{2+})-calmodulin-binding domain, which is autoinhibitory.</td>
<td>34</td>
</tr>
<tr>
<td>Kinase homologue (MCK1)</td>
<td>Homologous to known Ca(^{2+})-calmodulin-regulated kinases from other eukaryotes; isolated from maize roots; might be involved in gravitropism.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ion transport</td>
<td>Ca(^{2+})-ATPases (BCA1 and ACA2)</td>
<td>Endomembrane Ca(^{2+}) pumps; calmodulin-binding domain autoinhibitory; localized near the N-terminus; endomembrane localization is unique to plants.</td>
<td>35 and 36</td>
</tr>
<tr>
<td>Cytoskeleton function</td>
<td>Elongation factor-1 (EF-1)</td>
<td>Motor domain protein; calmodulin modulates association with tubulin; highly expressed during mitosis; might be involved in acentriolar spindle and phagoplast formation; involved in rice development.</td>
<td>37</td>
</tr>
<tr>
<td>Cytoskeleton function</td>
<td>Kinesin-like* (KCPB)</td>
<td>Similar to class V myosins; contains IQ repeats with putative calmodulin-binding characteristics.</td>
<td>38</td>
</tr>
<tr>
<td>DNA binding</td>
<td>Basic leucine zipper protein (TEA3)</td>
<td>Transcription factor; interacts in vitro with a DNA element in the promoter of the Cam-3 Arabidopsis calmodulin gene.</td>
<td>39</td>
</tr>
</tbody>
</table>

*Note that other unidentified plant calmodulin-binding proteins have been cloned. *Calmodulin-regulated forms of these proteins are unique to plants.

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