

CDPKs – a kinase for every Ca^{2+} signal?

Alice C. Harmon, Michael Gribskov and Jeffrey F. Harper

Numerous stimuli can alter the Ca^{2+} concentration in the cytoplasm, a factor common to many physiological responses in plant and animal cells. Calcium-binding proteins decode information contained in the temporal and spatial patterns of these Ca^{2+} signals and bring about changes in metabolism and gene expression. In addition to calmodulin, a calcium-binding protein found in all eukaryotes, plants contain a large family of calcium-binding regulatory protein kinases. Evidence is accumulating that these protein kinases participate in numerous aspects of plant growth and development.

Four types of protein kinases constitute the calcium-dependent protein kinase or calmodulin-like domain protein kinase (CDPK) superfamily. These kinases differ in whether they are regulated by binding Ca^{2+} (CDPKs), Ca^{2+} /calmodulin [calmodulin-dependent protein kinases (CaMKs)], a combination of both [calcium and calmodulin-dependent protein kinases (CCaMKs)], or neither [CDPK-related protein kinases (CRKs)].

The abundant calcium-stimulated protein kinase activity found in plant extracts is associated with CDPKs. These enzymes contain three functional domains¹⁻⁴: catalytic, autoinhibitory and calcium-binding (Fig. 1). The calcium-binding domain of the archetypal CDPK is similar to calmodulin in sequence (~40% identity) and contains four EF-hand calcium-binding motifs. In addition to plants, CDPKs are found in protozoans such as paramecium and *Plasmodium falciparum* (the causative agent of malaria). Notably, CDPKs are absent from the completed genome sequence of yeast (*Saccharomyces cerevisiae*) and of nematode

(*Caenorhabditis elegans*). Thus, it is tempting to speculate that CDPKs might be present in plants and protozoans only.

CCaMKs are rarer than CDPKs, and might be expressed in a few plant tissues only⁵. Like CDPKs, they contain a calcium-binding domain⁶ (Fig. 1), but this domain contains only three EF-hands and is more similar to visinin (another EF-hand protein) than to calmodulin. The autoinhibitory domain contains a binding site for calmodulin, and calmodulin stimulates the activity of these kinases.

A third type of calcium-regulated protein kinases, the CaMKs, is well characterized from animals and yeast, but only one putative representative is known in plants⁷. The plant CaMK is more similar in sequence to CCaMKs than to animal CaMKs, having an identical calmodulin-binding site, but lacking the C-terminal domain containing EF-hands (Fig. 1). The biochemical properties of this enzyme have not been characterized fully.

The fourth type of protein kinase in the superfamily is the CDPK-related protein kinases (CRKs). They have catalytic domains closely related to those of CDPKs, and their C-terminal domains have some sequence similarity to calmodulin (20% identity), but their EF-hands are poorly conserved. Representative members of this group appear to be unresponsive to calcium⁸⁻¹⁰. It is not known how these protein kinases are regulated or what their physiological roles are. Another type of CRK was reported recently: phosphoenolpyruvate carboxylase kinase has a catalytic domain related to those of CRKs, but no C-terminal domain. This protein kinase phosphorylates and regulates phosphoenolpyruvate carboxylase *in vivo* and is regulated at the level of transcription¹¹.

In phylogenetic analyses (Fig. 2), the clustering of the plant CDPKs and CRKs away from the non-plant CaMKs and the SNF1-like kinases suggests a single common origin for plant CDPK and CRK genes. However, an important evolutionary question remains unresolved. Did different branches of the superfamily have a common origin or did the fusion of genes encoding a protein kinase and a calcium-binding domain occur more than once in evolution?

Based on the analysis of the currently available (~70% complete) genomic sequence from *Arabidopsis*, we estimate that there will be a total of 40 CDPKs and seven CRKs. An *Arabidopsis* CCaMK sequence has not yet been identified, but southern blot analysis suggests that there is a single gene¹². Proliferation of family members might be related to expression of some of these genes in specific tissues, physiological conditions or developmental stages (reviewed in Ref. 13). Also there might be specialization of cellular roles, which might be related to differences in substrate specificity, subcellular location and calcium sensitivity.

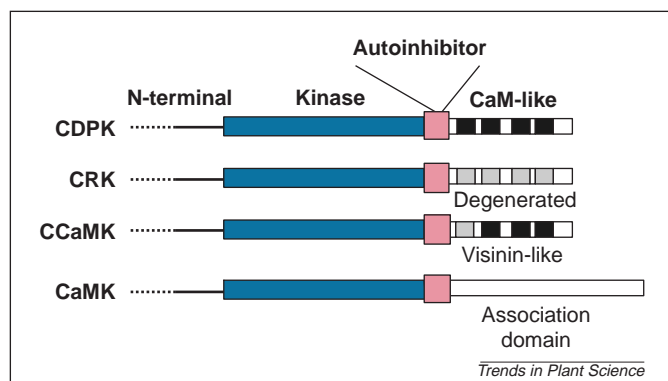


Fig. 1. Domain structure of calcium-dependent protein kinase or calmodulin-like domain protein kinases (CDPKs) and three related protein kinases. The N-terminal domain is highly variable in length and sequence. An autoinhibitor is predicted in the region immediately following the kinase domain. A distinguishing feature of CDPKs, CDPK-related protein kinases (CRKs) and calcium and calmodulin-dependent protein kinases (CCaMKs) is the number of functional EF-hands in a C-terminal regulatory domain: EF-hands that can bind calcium are denoted by black boxes, whereas degenerated EF-hands are denoted by gray boxes. In a conventional CDPK the regulatory domain has four EF-hands and an overall sequence similarity to calmodulin (CaM). CaMK, calmodulin-dependent protein kinase.

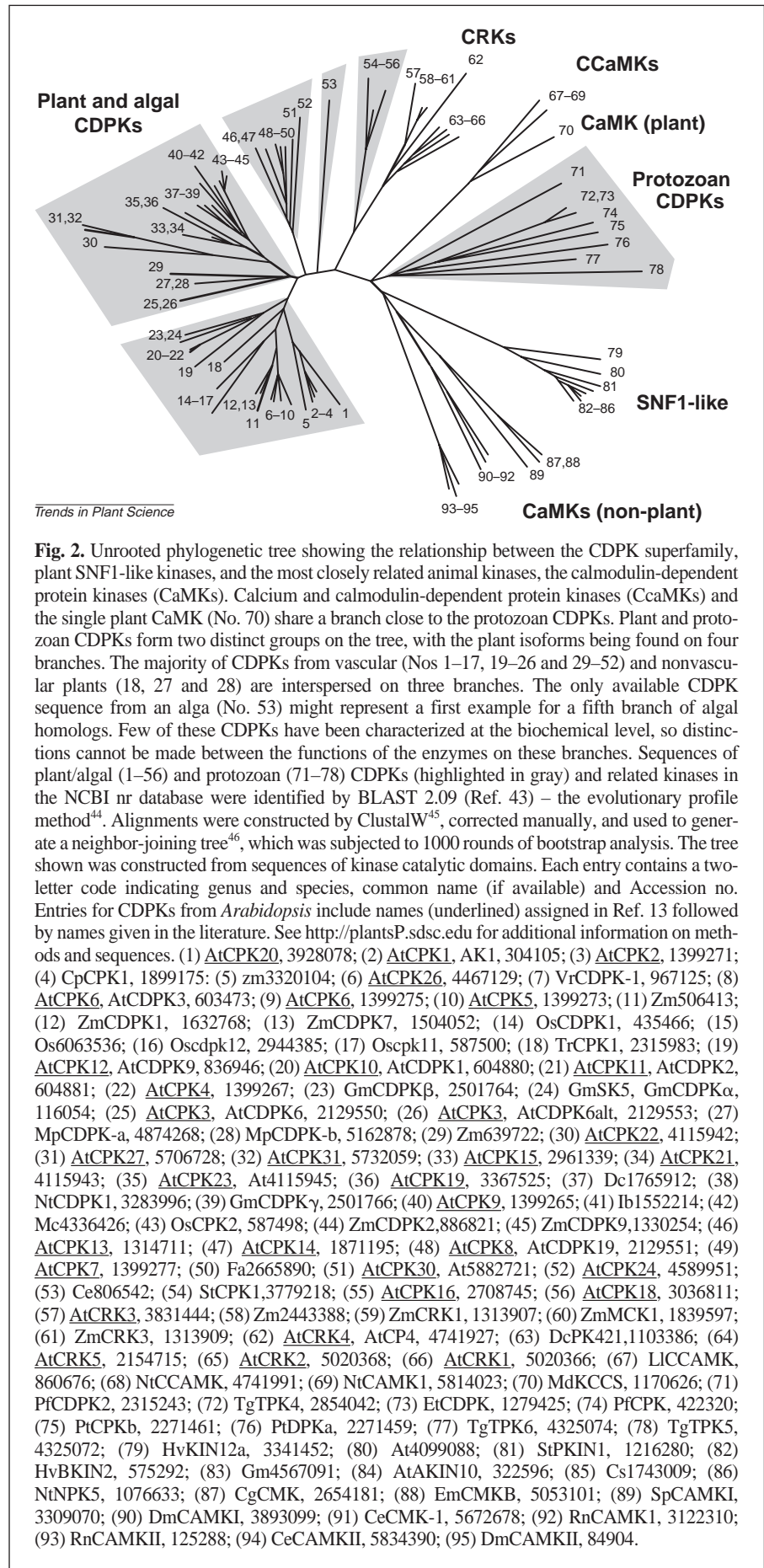
Activity and regulation

Regulation of CDPKs by Ca²⁺

How calcium regulates CDPKs has been the subject of studies with recombinant soybean (*Glycine max*) CDPK α and *Arabidopsis* CPK1 (Refs 1–4,14). CDPKs are kept in a low basal state of activity by an autoinhibitor located in a junction domain that connects the kinase to its C-terminal calmodulin-like domain. A peptide sequence from the junction inhibits the activity of wild-type enzymes and of a constitutively active mutant, in a competitive fashion with respect to the peptide substrate, suggesting that the autoinhibitory sequence functions through a pseudosubstrate mechanism³, analogous to that proposed for a typical CaMK from animals.

The simplest model for the activation of CDPK by Ca²⁺ is provided by analogy to the mechanism for stimulation of animal CaMKs. For CaMKs, calcium promotes a bimolecular binding of calmodulin to a region immediately downstream of an autoinhibitory sequence. This binding event somehow disrupts the autoinhibitor and results in a 'release of inhibition'. The distinction for a CDPK is that this 'release of inhibition' involves intramolecular binding with its calmodulin-like domain (Fig. 3). One line of evidence supporting a close analogy to a CaMK is the observation that the activity of a truncated CDPK (Δ C), in which the calmodulin-like domain is deleted, can be partially stimulated by either calmodulin or an isolated calmodulin-like domain, with half maximal activation at ~3 μ M for both activators^{1,2}. Although this indicates that a CDPK can be reconstituted as a bimolecular interaction with calmodulin (i.e. like a CaMK), it is possible that the natural mechanism of intramolecular activation (i.e. the whole) is distinct from its reconstitution as two separate fragments (i.e. the sum of the parts). An important challenge is to understand the structural basis for activation of CDPK (and CaMKs), and to determine whether the presence of a tethered calmodulin-like domain endows CDPKs with unique biochemical and physiological properties.

One reason for the multiplicity of CDPKs in a given plant species might be related to the specialization of different isoforms with respect to calcium binding and activation. Dose–response curves (Fig. 4) for three CDPK isoforms from soybean show that they are responsive to different ranges of calcium concentrations¹⁵. The concentration of calcium required for half-maximal activity ($K_{0.5}$) for CDPKs α , β and γ varies over two orders of magnitude when using the synthetic peptide substrate syn-*tide*-2. Other CDPKs, such as *P. falciparum*



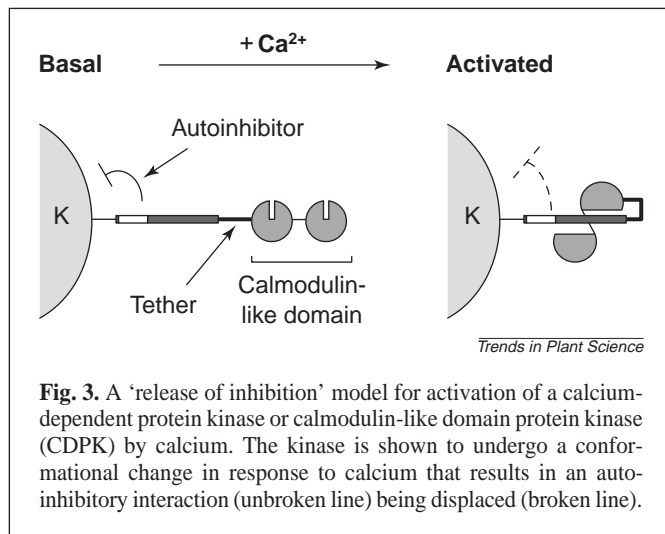


Fig. 3. A 'release of inhibition' model for activation of a calcium-dependent protein kinase or calmodulin-like domain protein kinase (CDPK) by calcium. The kinase is shown to undergo a conformational change in response to calcium that results in an autoinhibitory interaction (unbroken line) being displaced (broken line).

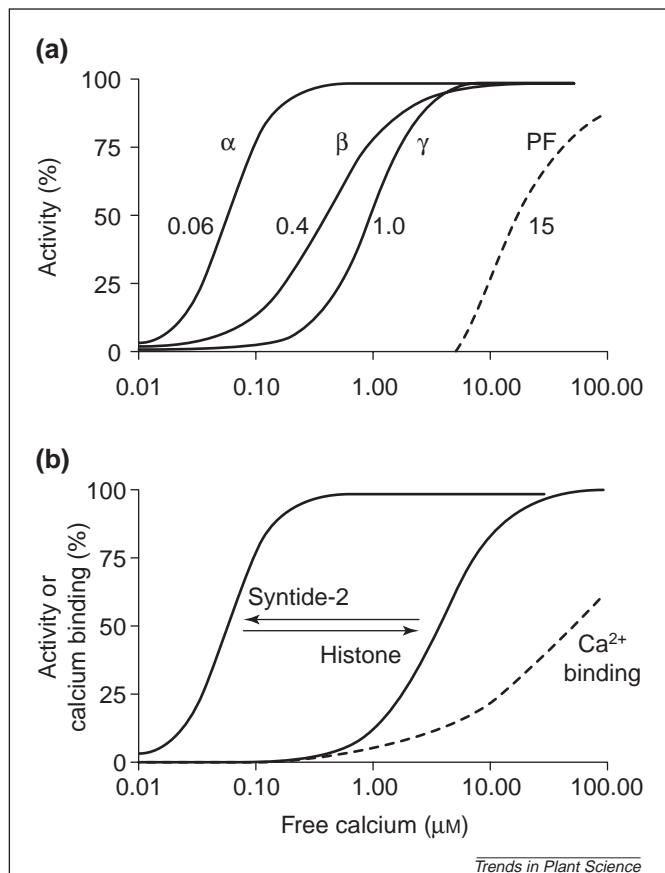


Fig. 4. Sensitivity of calcium-dependent protein kinase or calmodulin-like domain protein kinase (CDPK) isoforms to Ca^{2+} . (a) Isoform-specific thresholds for Ca^{2+} -activation showing the Ca^{2+} dose-response curves for phosphorylation of syntide-2 by soybean CDPKs α , β and γ (data from Ref. 15), and phosphorylation of casein by *Plasmodium falciparum* CDPK1 (data from Ref. 16). To emphasize differences in calcium sensitivity, the maximal activity for each enzyme was set to 100%. The $K_{0.5}$ for each CDPK is indicated. (b) Substrate-dependent thresholds for Ca^{2+} -activation, showing the influence of substrate on the Ca^{2+} sensitivity of soybean CDPK α (data from Ref. 15). In the absence of any substrates, CDPK α binds Ca^{2+} (broken line) with a K_d of 50 μM . In activity assays, the $K_{0.5}$ s were 0.4 and 4.0 μM syntide-2 and histone IIS, respectively.

PfCPK1 (Ref. 16), require concentrations of calcium that are another order of magnitude higher than those observed for the soybean enzymes.

Calcium-binding properties have been experimentally determined for only a few CDPKs, and it is difficult to predict from sequence information what the calcium-binding properties of each isoform will be. Some CDPKs appear to have defects in one or more of their EF hands that would affect their calcium-binding properties¹⁷. Studies with PfCPK1 (Ref. 16), in which each EF hand was disabled by the mutation of a critical glutamate residue, showed that for the enzyme to be stimulated by calcium only the first EF-hand must be functional. Therefore, CDPKs with defects in EF hands can still be regulated by calcium.

Another nuance in the regulation of CDPKs is that their sensitivity to calcium can be influenced by the type of protein substrate (Fig. 4). In the absence of any substrates, CDPK α binds Ca^{2+} with a K_d of 50 μM . However, in the presence of substrates, calcium sensitivity can increase tenfold or more (Fig. 4).

These differences in sensitivity to calcium might mean that each isoform of CDPK responds to a specific set of calcium signals, which differ in frequency of oscillation, magnitude and duration depending on the stimulus (reviewed in Refs 18,19). The difficult question of how to test *in vivo* for differential activities of specific isoforms remains unanswered.

Regulation by calmodulin

CCaMK binds both calcium ions and Ca^{2+} /calmodulin²⁰. Ca^{2+} stimulates autophosphorylation, but not phosphorylation of the *in vitro* substrate histone IIS. By contrast Ca^{2+} /calmodulin stimulates histone IIS phosphorylation, but inhibits autophosphorylation. Activation is proposed to occur through the binding of Ca^{2+} /calmodulin to a site in the autoinhibitory domain, similar in position and sequence to the intramolecular binding site for the calmodulin-like domain in CDPKs.

The isolated autoinhibitory domains of soybean CDPK α and *Arabidopsis* AtCPK1 bind calmodulin^{2,21}, but these enzymes are not greatly stimulated by calmodulin. Because the calmodulin-binding sequence in these CDPKs interacts intramolecularly with the calmodulin-like domain in the holoenzyme, it is probably unavailable for binding calmodulin. Carrot CRK is also unaffected by the addition of calmodulin (J. Choi, pers. commun.) in spite of the presence of a potential calmodulin-binding site in its putative autoinhibitory domain. Nevertheless, the question of whether calmodulin might regulate some isoforms is still open, as apo-calmodulin can bind to the variable amino-terminal domain of AtCPK1 (Ref. 1). Whether this binding site might have a role in docking the kinase into a protein complex or in modifying a more subtle feature of regulation is not known.

Regulation by myristoylation and lipids

The question of regulation by lipids is an important one because this could represent a possible point of crosstalk between signaling pathways, or a means of targeting activity to specific locations by reversible membrane association. CDPKs are found in several subcellular locations including membranes. The structural basis for membrane-association is not known because CDPKs have neither predicted membrane spanning regions nor C2 domains, which are responsible for the interaction of proteins such as protein kinase C with lipids and Ca^{2+} . However, it is possible that myristoylation, which is known to affect the membrane localization of several proteins, might underlie membrane-association. Many CDPKs have predicted myristoylation sites at their N-termini^{13,17}. A carrot CDPK can be myristoylated during co-expression with appropriate modifying enzymes in *E.coli*²², and a zucchini (*Cucurbita pepo*) CDPK

has been found to be myristoylated *in vivo*²³. Because CDPK activity is found in the cytosol as well as in membranes, the question arises as to whether their association with membranes might be regulated by a Ca²⁺-dependent myristoyl switch, in a manner similar to that of recoverin²⁴.

The activity of some CDPK isoforms is stimulated by phospholipids in addition to activation by calcium. Activation of AtCPK1 (Ref. 21) and carrot (*Daucus carota*) DcCPK1 (Ref. 10) by phospholipids such as phosphatidylserine and phosphatidylinositol is synergistic. However, because these lipids are not considered to be signaling molecules, it is arguable whether they serve as regulators or as structural components of membrane-associated CDPKs. One possible scenario is that certain CDPKs have low activity when located in the cytosol, but are activated upon translocation to the membrane.

It is possible that some CDPKs are regulated *in vivo* by signaling molecules derived from lipids, but only a few have been tested. Recombinant DcCPK1 is stimulated by phosphatidic acid¹⁰, a component in phospholipase D signaling pathways. It is not stimulated by diacylglycerol, which is produced by the cleavage of phosphatidylinositol bisphosphate by phospholipase C and is a regulator of protein kinase C in animals. It will be interesting to see if CDPKs are subject to cross-regulation by components of other pathways such as jasmonic acid or brassinosteroids.

Regulation by phosphorylation

Both native and recombinant CDPKs exhibit intramolecular autophosphorylation, but the sites of autophosphorylation have not been identified and there is no consensus as to the role of autophosphorylation. Many CDPK isoforms contain a potential autophosphorylation site (Lys-Gln-Phe-Ser) in their auto-inhibitory domains. Because autophosphorylation of CaMKII at a similar site yields an active enzyme that no longer requires Ca²⁺/calmodulin, it is tempting to speculate that these CDPKs are potentially activated by a similar mechanism. However, available evidence does not support this possibility. Soybean CDPK α does not phosphorylate peptides matching the sequence of the auto-inhibitory domain³, and autophosphorylation does not affect the calcium requirement of either groundnut (*Arachis hypogaea*)²⁵ or soybean CDPK α (B.C. Yoo and A.C. Harmon, unpublished). In addition, the activity of a CDPK purified from spinach (*Spinacia oleracea*) was not altered by incubation in conditions that favor phosphorylation or by treatment with phosphatase²⁶. In the case of CCaMK, autophosphorylation of the lily (*Lilium*) isoform stimulates activity fivefold, but it still requires Ca²⁺/calmodulin²⁰.

However, other reports have shown that autophosphorylation affects the activity of some CDPKs. Autophosphorylation of groundnut CDPK is required for its activity, but it occurs at low concentrations of Ca²⁺ and might not have a regulatory role *in vivo*²⁵. By contrast, autophosphorylation of CDPK purified from winged bean (*Psophocarpus tetragonolobus*) is inhibitory²⁷. Thus, clear evidence showing phosphorylation-dependent activation of a CDPK has not yet emerged.

The 14-3-3 connection

The 14-3-3 proteins serve as both regulatory and docking proteins (reviewed in Ref. 28). Several CDPKs bind 14-3-3 proteins, and the activity of at least one is stimulated through binding 14-3-3 (Refs 29–31). The 14-3-3s also bind to sites in proteins, such as nitrate reductase, that have been phosphorylated by CDPK (Ref. 28). Several other proteins including sucrose-phosphate synthase, trehalose-6-phosphate synthase, glutamine synthetases and LIM17 bind 14-3-3 proteins in a phosphorylation-dependent manner³⁰. It has been proposed that 14-3-3s might dock enzymes

together that are involved in two consecutive metabolic steps³². Roles for 14-3-3s in the activation and targeting of CDPKs need to be explored further.

Substrates and physiological roles

Insight into the physiological roles of CDPKs has come from identification of substrates and from experiments using constitutively active CDPKs to activate a pathway in the absence of a calcium signal³³. For example, it has been shown that expression of a constitutively active version of isoform AtCPK10 [but not AtCPK1 or AtCPK11 (numbering as in Ref. 17), or four protein kinases from another family] led to the expression of a stress-, Ca²⁺- and ABA- responsive reporter gene. This is the first stimulus-response pathway shown to be activated by a specific isoform. It will be interesting to see what protein(s) in this pathway are phosphorylated by this CDPK.

Biochemical approaches have identified a variety of CDPK substrates that suggest potential regulatory roles in gene expression, metabolism and signaling pathway components, traffic of ions and water across membranes, and the dynamics of the cytoskeleton (Fig. 5). More information on these substrates can be found at the protein kinase and phosphatase Web site (Box 1), and in a recent review¹³. Here, we highlight two substrates for which there is strong experimental support for regulation by CDPK.

Sucrose phosphate synthase (SPS) is a key enzyme in the sucrose synthesis pathway, and nitrate reductase is the rate-limiting enzyme in the assimilation of nitrogen from nitrate (reviewed in Refs 34,35). Both enzymes are phosphorylated in the dark, resulting in their inhibition. SPS is directly inhibited by phosphorylation of Ser153, and nitrate reductase is inhibited by a two-step mechanism involving phosphorylation of Ser543 and binding of a 14-3-3 protein to the phosphorylated site (reviewed in Ref. 28). Inhibition of these enzymes in the dark when carbon fixation is not occurring diminishes the partitioning of carbon skeletons into exported sucrose and amino acids, and conserves glucose and fructose for use in other pathways in leaf cells, such as glycolysis or starch synthesis. Evidence that supports this *in vivo* function includes co-purification of a CDPK that has been identified as a homolog of AtCPK3 (Ref. 26), which phosphorylates both of these enzymes at the regulatory sites^{26,36–38}. These observations raise the possibility that a single CDPK can coordinately regulate both activities.

The hypothesis that CDPK down-regulates nitrate reductase and SPS in response to the dark is consistent with the observation that cytoplasmic calcium concentrations are higher at night than during the day³⁹. However, it should be noted that a calcium-independent kinase with properties of SNF1-related kinases also phosphorylates Ser153 of SPS and Ser543 of nitrate reductase^{38,40,41}. Thus, it is possible that both types of kinase down-regulate SPS and nitrate reductase *in vivo*, but in response to a separate signaling pathway.

A CDPK might also be involved in activating SPS by phosphorylation at a site (Ser424) distinct from the inhibitory site⁴². This phospho-dependent activation occurs in response to hypo-osmotic stress and presumably increases cytosolic sucrose and thereby decreases the water potential of the cell to help retain water. These intriguing results raise the possibility that two different CDPK pathways that are differentially activated by separate stimuli oppositely regulate SPS activity.

The future

In the past ten years we have progressed from identifying the first member of a new family of calcium-dependent protein kinases to the understanding that:

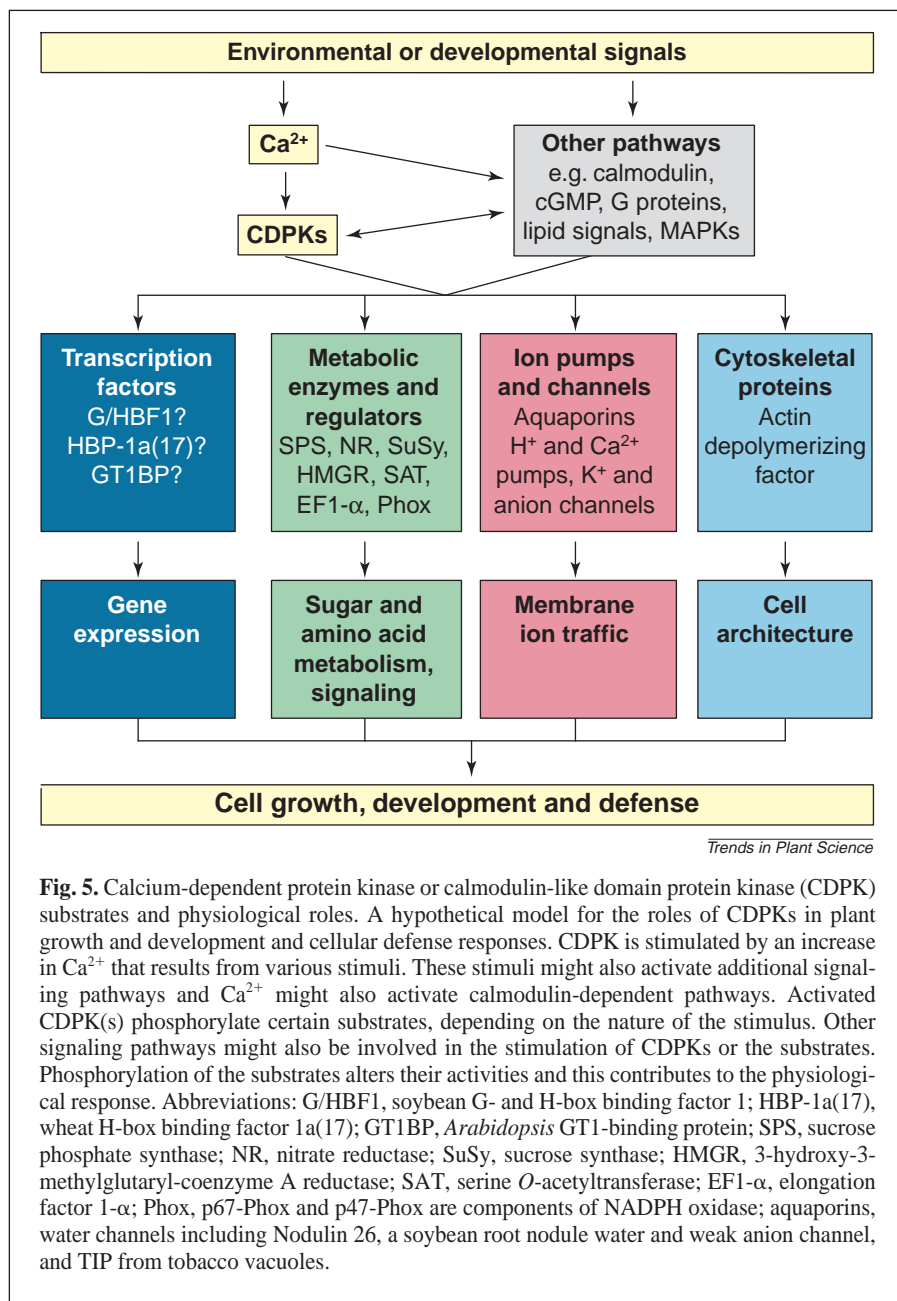


Fig. 5. Calcium-dependent protein kinase or calmodulin-like domain protein kinase (CDPK) substrates and physiological roles. A hypothetical model for the roles of CDPKs in plant growth and development and cellular defense responses. CDPK is stimulated by an increase in Ca^{2+} that results from various stimuli. These stimuli might also activate additional signaling pathways and Ca^{2+} might also activate calmodulin-dependent pathways. Activated CDPK(s) phosphorylate certain substrates, depending on the nature of the stimulus. Other signaling pathways might also be involved in the stimulation of CDPKs or the substrates. Phosphorylation of the substrates alters their activities and this contributes to the physiological response. Abbreviations: G/HBF1, soybean G- and H-box binding factor 1; HBP-1a(17), wheat H-box binding factor 1a(17); GT1BP, *Arabidopsis* GT1-binding protein; SPS, sucrose phosphate synthase; NR, nitrate reductase; SuSy, sucrose synthase; HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; SAT, serine *O*-acetyltransferase; EF1- α , elongation factor 1- α ; Phox, p67-Phox and p47-Phox are components of NADPH oxidase; aquaporins, water channels including Nodulin 26, a soybean root nodule water and weak anion channel, and TIP from tobacco vacuoles.

- Plants have many CDPK isoforms (>40 in *Arabidopsis*), which are predicted to have kinase activities directly activated by calcium and potentially modified by cross-talk with other signaling systems.
- CDPKs are multifunctional, with individual isoforms providing specific pathways to control transcription, metabolic enzymes, membrane transport and cell structure.
- Plants also have at least three types of CDPK-related kinases, with some members regulated by calmodulin and some totally unresponsive to calcium signals.

A major challenge for the future is to create an integrated picture of how members of this kinase family are used in plant development and physiology. To this end, we offer the following basic strategy for investigating every isoform in a model plant such as *Arabidopsis*.

- Use biochemistry to define isoform-specific calcium activation thresholds and substrate specificities.
- Use cell biology tools to delineate subcellular locations.

Box 1. Plant protein kinase and phosphatase Web site

This Web site (<http://plantsP.sdsc.edu>) focuses on *Arabidopsis* genes encoding protein kinases and phosphatases. When completed, this site will have an annotated database of all families of protein kinases and phosphatases, and will announce the availability of protein kinase and phosphatase mutants as they are identified and made available to the research community.

Information on the CDPK family can be found at <http://plantsP.sdsc.edu/cdpk>, including fully annotated figures from this review, expanded information on phylogenetic trees and sequence alignments, and a categorized list of literature related to all the CDPKs that have been found in plant and protist species to date.

- Use genetics to identify biological functions, as indicated by the phenotypes that result from the disruption of genes or the expression of de-regulated mutants (e.g. calcium independent).
- Use bioinformatics to provide a picture of how CDPK signaling pathways are integrated into the dynamic interactions of all signaling pathways in a cell.

CDPKs represent a potential gold mine of opportunity for biotechnology applications. Given the involvement of calcium signals in so many aspects of plant biology, including biotic and abiotic stress responses, the different CDPK pathways provide many potential points of intervention to suppress or activate a specific response. Structure–function studies on CDPKs have provided an important paradigm by showing how a CDPK can be converted into an active, calcium-independent kinase to be used as a dominant, positive transgene¹. This approach has provided a precedent by establishing a role for one CDPK isoform in selectively activating a

cold, dark and osmotic stress response pathway in the absence of other calcium signaling pathways³³.

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*Alice C. Harmon is at the Program in Plant Molecular and Cellular Biology, Dept of Botany, University of Florida, PO Box 118526, Gainesville, FL 32611-8526, USA; Michael Gribskov is at the San Diego Supercomputer Center, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0537, USA (tel +1 858 534 8312; fax +1 858 822 0873; e-mail gribskov@sdscc.edu); Jeffrey F. Harper is at the Dept of Cell Biology, The Scripps Research Institute, 10550 N. Torrey Pines Rd, La Jolla, CA 92037, USA (tel +1 858 784 2862; fax +1 858 784 9840; e-mail harper@scripps.edu).

*Author for correspondence (tel +1 352 392 3217; fax +1 352 392 3993; e-mail harmon@botany.ufl.edu).