Calcium Signaling through Protein Kinases. The Arabidopsis Calcium-Dependent Protein Kinase Gene Family[1]

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In plants, numerous Ca^{2+}-stimulated protein kinase activities occur through calcium-dependent protein kinases (CDPKs). These novel calcium sensors are likely to be crucial mediators of responses to diverse endogenous and environmental cues. However, the precise biological function(s) of most CDPKs remains elusive. The Arabidopsis genome is predicted to encode 34 different CDPKs. In this *Update*, we analyze the Arabidopsis CDPK gene family and review the expression, regulation, and possible functions of plant CDPKs. By combining emerging cellular and genomic technologies with genetic and biochemical approaches, the characterization of Arabidopsis CDPKs provides a valuable opportunity to understand the plant calcium-signaling network.

Calcium is a ubiquitous second messenger in eukaryotic signal transduction cascades. In plants, intracellular Ca^{2+} levels are modulated in response to various signals, including hormones, light, mechanical disturbances, abiotic stress, and pathogen elicitors (Sanders et al., 1999; Evans et al., 2001; Rudd and Franklin-Tong, 2001). How response specificity is regulated during Ca^{2+}-mediated signal transduction is an important biological issue. It appears that different stimuli elicit specific calcium signatures, generated by altering the kinetics, magnitude, and cellular source of the influx (Malhó et al., 1998; Allen et al., 2000, 2001; Evans et al., 2001; Rudd and Franklin-Tong, 2001). Unlike most other ions, calcium does not freely diffuse within cells (Trewavas, 1999). Plants have multiple calcium stores, including the apoplast, vacuole, nuclear envelope, endoplasmic reticulum (ER), chloroplasts, and mitochondria. Therefore, each stimulus can elicit a characteristic Ca^{2+} wave by specifically altering the activities of various differentially localized Ca^{2+} channels, H^+/Ca^{2+} antiporters, and Ca^{2+}- and H^+-ATPases (Bush, 1995; Thuleau et al., 1998; Allen et al., 2000; Hwang et al., 2000; Harper, 2001). Different calcium sensors recognize specific calcium signatures and transduce them into downstream effects, including altered protein phosphorylation and gene expression patterns (Sanders et al., 1999; Rudd and Franklin-Tong, 2001).

In plants, there are several known classes of Ca^{2+}-binding sensory proteins, including calmodulins, calcium-dependent protein kinases (CDPKs), and calcineurin B-like proteins. Among them, CDPKs are the best characterized and are of particular interest. They represent a novel class of Ca^{2+} sensors, having both protein kinase and calmodulin-like domains in a single polypeptide. As a result, CDPKs directly bind calcium, and their calcium-stimulated kinase activities are independent of calmodulins, unlike calcium/calmodulin-dependent protein kinases (CaMKs; Roberts and Harmon, 1992). Currently, most of the known calcium-stimulated protein kinase activities in plants are associated with CDPKs. A genome-wide analysis of Arabidopsis CDPKs provides an overview of the diversity of this large multigene family and should facilitate the elucidation of their functions. It appears likely that gene duplication and subsequent evolution generated CDPKs with both redundant and distinct functions. Furthermore, the functional specificity of individual CDPKs may be determined by regulations at both transcriptional and posttranslational levels, as well as targeted subcellular compartmentalization, calcium and lipid sensitivity, and substrate recognition.

THE ARABIDOPSIS CDPK GENE FAMILY

The first calcium-dependent, calmodulin-independent protein kinase activities were reported in pea (*Pisum sativum*) extracts 20 years ago (Hetherington and Trewavas, 1982). Since their initial purification and characterization from soybean (*Glycine max*; Harmon et al., 1987), CDPKs have been identified throughout the plant kingdom from green algae to angiosperms (Hrabak, 2000; Harmon et al., 2001).

[1] This work was supported by the National Science Foundation (grant nos. MCB9985881 to S.-H.C. and J.S. and DBI007692 to J.S. and predoctoral fellowship to M.R.W.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.005645.

Other than plants, CDPKs are found only in some protozoans, and are notably absent from the sequenced eukaryotic genomes of yeast (*Saccharomyces cerevisiae*), nematodes (Harmon et al., 2000), fruitflies (*Drosophila melanogaster*; Adams et al., 2000), and humans (*Homo sapiens*; International Human Genome Sequencing Consortium, 2001; Venter et al., 2001). Analysis of the genome sequence of Arabidopsis indicates the presence of 34 CDPK genes (The Arabidopsis Genome Initiative, 2000). Information available from limited genomic sequencing, as well as several extensive expressed sequence tag (EST) projects, also indicate the presence of multigene families of CDPKs in other plants, including soybean, tomato (*Lycopersicon esculentum*), rice (*Oryza sativa*), and maize (*Zea mays*; Harmon et al., 2001).

Domain Structure

Four distinct domains typify CDPK family members: an N-terminal variable domain, a protein kinase domain, an autoinhibitory domain, and a calmodulin-like domain (Fig. 1). Based on phylogenetic analysis, it is thought that the CDPK gene family arose through the fusion of a CaMK and a calmodulin (Harper et al., 1991; Suen and Choi, 1991; Harmon et al., 2000; Zhang and Choi, 2001). This unique molecular structure allows the direct activation of CDPKs by Ca^{2+}. Unlike the analogous mammalian protein, the multisubunit CaMKII, CDPKs function as monomers (Roberts and Harmon, 1992).

Alignments of the predicted amino acid sequences of all 34 Arabidopsis CDPKs reveal a high conservation of the kinase (44%–95% identity and 60%–98% similarity), autoinhibitory (23%–100% identity and 42%–100% similarity) and calmodulin-like (27%–97% identity and 50%–98% similarity) domains, whereas the N-terminal variable domain shows little sequence similarity. (The alignments and pair-wise comparisons of the amino acid sequences of all 34 Arabidopsis CDPKs can be viewed online at http://xanadu.mgh.harvard.edu/sheenweb/index.htm by selecting "Arabidopsis CDPKs.") The kinase domain (264–273 amino acids long) contains all 12 of the highly conserved subdomains of typical eukaryotic Ser/Thr protein kinases (Hanks and Hunter, 1995). Nearly 100% identity is found in the region of the active site among all 34 Arabidopsis CDPKs. Using casein, histone IIIS, or syntide as substrates, heterologously expressed CDPKs have been shown to possess Ca^{2+}-stimulated protein kinase activity in vitro (e.g. Harper et al., 1993; Urao et al., 1994; Lee et al., 1998; Yoon et al., 1999). An absolutely conserved Lys residue located within subdomain II is thought to be the ATP-binding site, and mutagenesis of this residue abolishes the catalytic activities (Sheen, 1996).

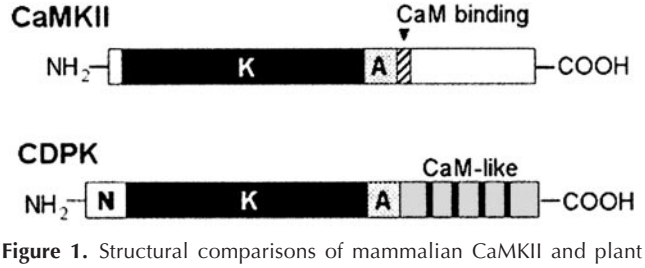

Figure 1. Structural comparisons of mammalian CaMKII and plant CDPKs. The kinase domain of CDPKs is up to 44% identical (65% similar) to that of mouse (*Mus musculus*) CaMKII alpha (accession no. S04365) and 43% identical (65% similar) to that of mouse CaMKII beta (accession no. NP_031621). N, Amino-terminal variable domain; K, kinase domain; A, autoinhibitory domain; CaM, calmodulin. The four bars within the CaM-like domain represent the EF hand Ca^{2+}-binding sites.

The autoinhibitory domain is a basic amino acid region (31 amino acids long) that functions as a pseudosubstrate (Harmon et al., 1994). Sixteen of the 34 Arabidopsis CDPKs contain a potential autophosphorylation site (Basic-X-X-S/T, where X is any residue) in the autoinhibitory domain (Harmon et al., 1994). Whether these CDPKs autophosphorylate at this site has yet to be determined. Although autophosphorylation at the analogous site of CaMKII results in a constitutively active enzyme that is no longer dependent upon Ca^{2+}/calmodulin for activation (Schulman and Lou, 1989), it has not been established that an analogous phosphorylation plays a role in the activity of plant CDPKs (see "Regulation by Phosphorylation and Dephosphorylation" below).

The calmodulin-like domain (94–147 amino acids long) contains Ca^{2+}-binding EF hands allowing the protein to function as a Ca^{2+} sensor. Each EF hand consists of a loop of 13 amino acid residues flanked by two α-helices. A single Ca^{2+} molecule is bound to each EF hand via the loop domain (Zhang and Yuan, 1998). The number of EF hands differs depending on the isoform. Most Arabidopsis CDPKs contain four EF hands, whereas a few of them have one, two, or three (Table I). The most conserved EF hand sequences are those of the hands in positions 1 and 2 and the least conserved is that for position 4. The positions where the EF hands are absent also vary. These differences in numbers and positions of EF hands likely yield variations in the allosteric properties of Ca^{2+} binding and the activation threshold. Sequential deletion of the EF hands demonstrates that the number of EF hands may be important for determining calcium regulation of CDPK activity (Hong et al., 1996). In addition, site-directed mutagenesis of a highly conserved Glu residue in each EF hand shows that the closer the EF hand is to the autoinhibitory domain, the greater its effect on the Ca^{2+} regulation of CDPK activity (Zhao et al., 1994).

The mechanism by which CDPK activity is regulated is largely controlled through interactions between the kinase, autoinhibitory, and calmodulin-like domains. Under the basal condition of low free Ca^{2+}, the autoinhibitory domain is bound by the kinase domain, keeping substrate phosphorylation activity low. Upon binding Ca^{2+} via the EF hand

Table I. *Characteristics of Arabidopsis CDPKs*

Name (Previous Name)[a]	AGI ID[b]	Genomic Locus[c]	Expression[d]	M_r	V.D.[e]	EF[f]	N-Term. a.a.[g]	N-Myrist.[h]	Subcel. Dist.[i]	Ref[j]
				kD						
CPK1(AK1)	At5g04870	MUK11.19	3 ESTs	68.2	149	4	M**G**NTCVGP	Yes		1
CPK2	At3g10660	F13M14.5	4 ESTs	72.2	185	4	M**G**NACVGP	Yes	ER	2
CPK3(6)	At4g23650	F9D16.120	9 ESTs	59.3	77	4	M**G**HRHSKS	No	–	2,3
CPK4	At4g09570	T25P22.10	2 ESTs	56.4	24	4	MEKPNPRR	No	–	2
CPK5	At4g35310	F23E12130	7 ESTs	62.1	96	4	M**G**NSCRGS	No	ME	2
CPK6(3)	At2g17290	F5J6.13	4 ESTs	61.6	84	4	M**G**NSCRGS	No	ME	2
CPK7	At5g12480	T2L20	Yes	60.3	58	3 (1)	M**G**NCCGNP	Yes	–	2
CPK8(19)	At5g19450	F7K24.200	6 ESTs	59.9	56	3 (1)	M**G**NCCASP	Yes	–	2,3
CPK9	At3g20410	MQC12.17	8 ESTs	60.4	90	4	M**G**NCFAKN	Yes	–	2
CPK10(1)	At1g18890	F6A14.1	2 ESTs	61.4	62	3 (3)	M**G**NCNACV	Yes	–	4
CPK11(2)	At1g35670	F15O4.8	Yes	55.9	25	4	METKPNPR	No	–	4
CPK12(9)	At5g23580	MQM1.15	Yes	55.4	21	4	MANKPRTR	No	–	3
CPK13	At3g51850	ORF10	R16737	56.5	53	2 (2,3)	M**G**NCCRSP	No	–	–
CPK14	At2g41860	T6D20.24	?	60.1	53	3 (1)	M**G**NCCGTA	Yes	–	–
CPK15	At4g21940	T8O5.150	Yes	62.6	101	4	M**G**CFSSKH	Yes	–	6
CPK16	At2g17890	T13L16.9	1 EST	64.8	107	4	M**G**LCFSSA	Yes	–	6
CPK17	At5g12180	MXC9.14	R11445	58.5	72	4	M**G**NCCSHG	Yes	–	–
CPK18	At4g36070	T19K4.200	?	60.5	70	4	M**G**LCFSSP	Yes	–	–
CPK19	At1g61950	F8K4.26	?	62.4	97	3 (3)	M**G**CLCINL	No	–	–
CPK20	At2g38910	T7F6.8	Yes	64.7	133	4	M**G**NTCVGP	Yes	–	7
CPK21	At4g04720	T4B21.13	5 ESTs	59.9	79	4	M**G**CFSSKH	Yes	–	6
CPK22	At4g04710	T4B21.12	3 ESTs	64.7	35	4	M**G**NCCGSK	Yes	–	–
CPK23	At4g04740	T4B21.15	R15960	58.6	68	3 (1)	M**G**CFSSKH	Yes	–	–
CPK24	At2g31500	T9H9.2	RAFL19-07-H23	66.2	65	4	M**G**SCVSSP	Yes	–	–
CPK25	At2g35890	F11F19.20	Yes	58.9	131	1 (2,3,4)	M**G**NVCVHM	Yes	–	5
CPK26	At4g38230	F20D10.350	Yes	54.3	23	4	MKHSGGNQ	No	–	5
CPK27	At4g04700	T4B21.11	Yes	54.9	27	4	M**G**CFSSKE	Yes	–	5
CPK28	At5g66210	K2A18.29	Ceres18901	59.0	61	4	M**G**VCFSAI	Yes	–	–
CPK29	At1g76040	T4O12.25	1 EST	63.8	111	4	MLQNQHKT	No	–	–
CPK30(1a)	At1g74740	F25A4.29	2 ESTs	64.2	58	4	M**G**NCIACV	Yes	–	–
CPK31	At4g04695	T19J18.7	?	54.7	27	4	M**G**CYSSKN	Yes	–	–
CPK32	At3g57530	T8H10.130	7 ESTs	63.4	62	3 (1)	M**G**NCCGTA	Yes	–	–
CPK33	At1g50700	F17J6.22	Yes	58.6	72	4	M**G**NCLAKK	Yes	–	5
CPK34	At5g19360	F7K24.110	Yes	58.2	67	4	M**G**NCCSHG	Yes	–	5

[a] Nos. in parentheses indicate names given to previously published Arabidopsis CDPKs. 6, CDPK6; 3, CDPK3, 19, CDPK19; 1, CDPK1; 2, CDPK2; 9, CDPK9; 1a, CDPK1a. [b] Systematic designation given to gene by Arabidopsis Genome Initiative. [c] Designation of gene locus on annotated bacterial artificial chromosome. [d] Gene expression status is based on cloned cDNA (see reference) or presence of cognated EST or full-length cDNA. The cDNA clone for CPK24 is from Riken (www.gsc.riken.gp.jp). [e] Nos. of amino acids in variable domain. [f] No. of EF hands predicted by PROSITE program (Hoffmann et al., 1999) and by sequence alignment. Nos. in parentheses indicate the position in which EF hand is absent. [g] The first eight amino acids at N terminus of the CDPKs. [h] The prediction of myristoylation motif is based on PROSITE program with considerations drawn from Towler et al. (1988); Ellard-Ivey et al. (1999); Martin and Busconi (2000); Lu and Hrabak (2002); and Raíces et al. (2001). [i] Subcellular distribution. ME, Membrane. [j] 1, Harper et al. (1993); 2, Hrabak et al. (1996); 3, Hong et al. (1996); 4, Urao et al. (1994); 5, M.R. Willmann, S.-H. Cheng, and J. Sheen, unpublished data; 6, Harmon et al. (2001); 7, Thuemmler et al. (1995).

motifs, CDPKs undergo conformational changes that release the pseudosubstrate from the catalytic site, activating the protein (Harmon et al., 1994; Harper et al., 1994). This model is supported by the observation that deletion of only the calmodulin-like domain generates an inactive enzyme that cannot be activated by Ca^{2+}, whereas deletion of both the autoinhibitory and calmodulin-like domains creates a constitutively active, Ca^{2+}-insensitive enzyme (Harper et al., 1994; Sheen, 1996).

Little is known about the function of the N-terminal variable domain. It has been proposed that this region contains subcellular targeting information (Schaller and Sussman, 1988; Harper et al., 1994; Hrabak et al., 1996). CDPKs are reported to associate with various membranes (Ellard-Ivey et al., 1999; Martin and Busconi, 2000; Lu et al., 2001; Lu and Hrabak, 2002). However, none of the 34 Arabidopsis CDPKs are predicted to be integral membrane proteins. The N-terminal leader sequence of CDPKs is variable not only in amino acid sequence, but also in length, ranging from 25 (AtCPK11) to nearly 200 (AtCPK2) amino acids in Arabidopsis (Table I). Despite this variability within the N-terminal domain, most Arabidopsis CDPKs have a Gly residue at the second position. When placed in a proper context,

this N-terminal Gly residue can be modified by covalent attachment of myristic acid (a C 14:0 fatty acid; Towler et al., 1988). In many systems, *N*-myristoylation promotes protein-membrane and protein-protein interactions (Johnson et al., 1994). Mutation of the N-terminal Gly abolishes such a lipid modification and thus prevents membrane association (Martin and Busconi, 2000). Twenty-four of the Arabidopsis CDPKs are predicted to have *N*-myristoylation motifs for membrane association (Table I). Among them, however, only AtCPK2 has been shown experimentally to be myristoylated at the N-terminal Gly residue, and the first 10 amino acids are critical for localization to the ER membrane (Lu and Hrabak, 2002; Table I). This lipid modification also has been shown to occur in CDPKs from four other species, and similarly to Arabidopsis, two of these proteins have been found to be membrane associated (Farmer and Choi, 1995; Ellard-Ivey et al., 1999; Martin and Busconi, 2000; Raíces et al., 2001). AtCPK 5 and 6 are not predicted to be myristoylated (Table I), but are partially associated with membranes nevertheless (Lu et al., 2001), suggesting that other mechanisms (e.g. glycosylation) may account for their subcellular localization.

The addition of a myristic acid residue is not always sufficient for membrane attachment. Often, a second lipid modification, such as palmitoylation (addition of palmitate, a C 16:0 fatty acid), is necessary to stabilize the interaction with the membrane. All 24 AtCPKs predicted to have a myristoylation consensus sequence also have at least one Cys residue at position 3, 4, or 5 (Table I), a potential palmitoylation site (Milligan et al., 1995). Recently, both myristoylation at the N-terminal Gly and palmitoylation at the Cys residues in positions 4 and 5 have been shown experimentally to occur in the membrane-bound rice OsCPK2 (Martin and Busconi, 2000). When myristoylation of OsCPK2 is abolished by mutating the N-terminal Gly, the protein can no longer be palmitoylated either, indicating that myristoylation may be a prerequisite for palmitoylation. Whether Arabidopsis CDPKs also have such a prerequisite, however, remains to be determined.

Sequence Homology and Chromosomal Distribution

All 34 Arabidopsis CDPKs are highly homologous to each other. Pair-wise analyses with the full protein sequences indicate that the overall identities and similarities are 39% to 95% and 56% to 96%, respectively. High identities are found between AtCPK 4 and 11 (95%), AtCPK 17 and 34 (93%), AtCPK 7 and 8 (90%), AtCPK 10 and 30 (86%), AtCPK 9 and 33 (85%), AtCPK 1 and 2 (81%), and AtCPK 21 and 23 (81%), and among AtCPK 5, 6, and 26 (85%–88%). Because both AtCPK 10 and 30 specifically activate a stress pathway (Sheen, 1996), such high homologies may indicate similar functions. AtCPK16, 18, and 28 are the most divergent CDPKs, as indicated by their relatively low average pair-wise identity/similarity values (45% and 64%, respectively).

To examine protein relationships of Arabidopsis CDPKs further, an unrooted tree was constructed from alignments of the full CDPK sequences (Fig. 2). Based upon sequence homology, the CDPKs of Arabidopsis cluster into four subgroups (I–IV). Subgroup IV is the least complex, with three members, and subgroup II is the most complex, with 13 members. This pattern of grouping was also found when the tree was constructed based on the sequences of the kinase domain only (data not shown; Harmon et al., 2001). Subgroups I through III are closer in sequence identity to each other than to subgroup IV. It is not known whether such a pattern of clustering reflects any functional differences between the subgroups.

The 34 Arabidopsis CDPKs are distributed among all five chromosomes (Fig. 3). Chromosome IV has the most CDPKs (11), whereas chromosome III has the least (4). The only region that contains no CDPKs is the short arm of chromosome II. Interestingly, one gene cluster on the short arm of chromosome IV contains five genes (AtCPK 21, 22, 23, 27, and 31), all within subgroup IV. They are organized in tandem in the same transcriptional orientation (Fig. 3), and their amino acid sequences are very homologous (61%–82% identity and 74%–89% similarity). Furthermore, sequence homology also exists in the N-terminal variable domain in this gene cluster (21%–78% identity and 22%–85% similarity). These results suggest that they arose relatively recently by gene duplication and that they may have similar or overlapping functions.

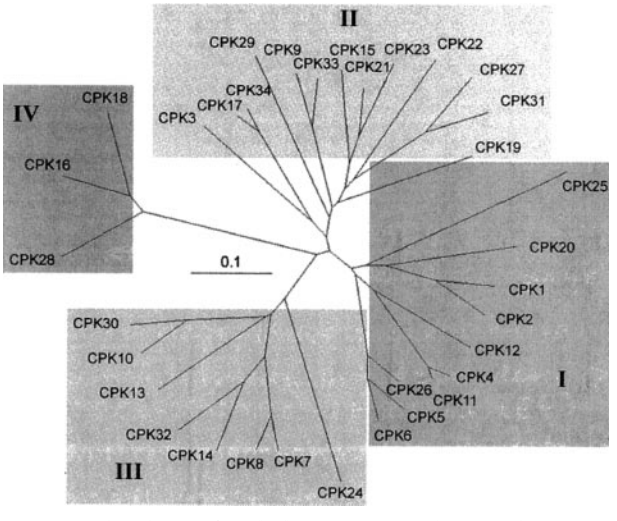

Figure 2. Relatedness of Arabidopsis CDPKs. The complete protein sequences of the AtCPKs were aligned and analyzed by the Treeview 1.6.5 program (http:/taxonomy.zoology.gla.ac.uk/rod/rod.html). The unrooted distance tree reveals the presence of four distinct, branched subgroups (I–IV). The branch lengths are proportional to divergence, with the scale of "0.1" representing 10% change.

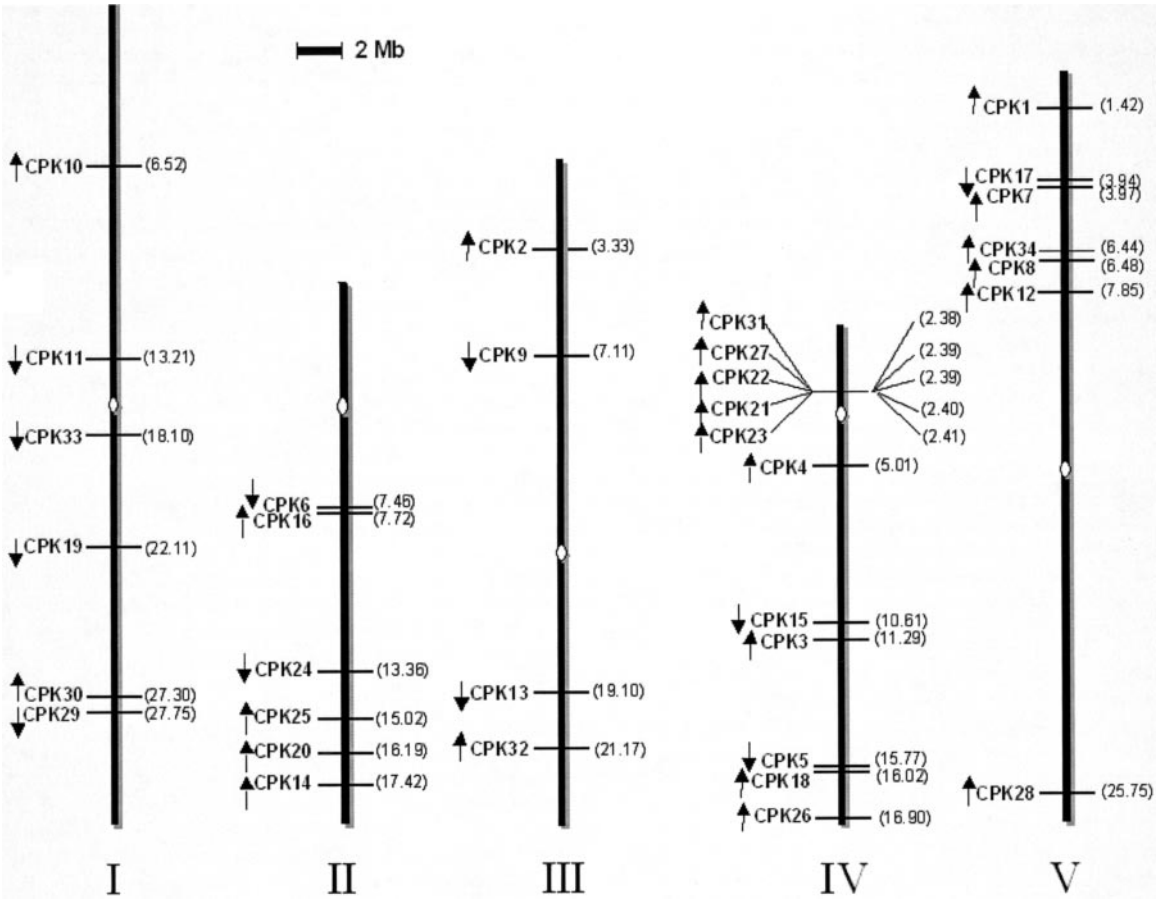

Figure 3. Genomic distribution of CDPKs on the Arabidopsis chromosomes. Ovals on the chromosomes indicate the location of the centromeres. The arrows next to the gene names show the direction of transcription. The numbers in parentheses designate the position of the first exon of each CPK gene in megabases (Mb). The chromosome numbers are indicated by Roman numerals.

Expression and Subcellular Distribution

Although 34 CDPK genes are predicted in the completed Arabidopsis genome, the transcription of all of these genes has not been demonstrated (Table I). Full-length cDNAs have been obtained for AtCPKs 1 through 12 (Harper et al., 1993; Urao et al., 1994; Hong et al., 1996; Hrabak et al., 1996); for AtCPKs 15, 16, and 21 (Harmon et al., 2001); and AtCPKs 13, 17, and 23 (http://signal.salk.edu). Twelve of the remaining 16 CDPKs either have corresponding EST clones or have been cloned by the Sheen laboratory (Table I). Whether the AtCPK 14, 18, 19, and 31 genes are transcribed is still unknown. Therefore, there are at least 30 distinct CDPKs expressed in Arabidopsis.

The subcellular localization of only three Arabidopsis CDPKs has been studied. All three of these proteins are associated with membrane systems, with AtCPK2 localized to the ER (Table I; Lu et al., 2001, 2002). In other species, CDPKs have been found associated with both soluble and membrane fractions, nuclei, chromatin, and the cytoskeleton (e.g. Putnam-Evans et al., 1989; Schaller et al., 1992; Martin and Busconi, 2000; Patharkar and Cushman, 2000). The plasma membrane localization of some CDPKs is highly significant because many critical calcium-signaling events are initiated by fluxes across the plasma membrane (Malhó et al., 1998; Rudd and Franklin-Tong, 2001).

REGULATION OF CDPK ACTIVITY

Regulation by Phosphorylation and Dephosphorylation

In addition to Ca^{2+}, reversible phosphorylation also may regulate CDPK kinase activity. Autophosphorylation has been observed in both native and recombinant CDPKs (Saha and Singh, 1995; Chaudhuri et al., 1999; Harmon et al., 2000). However, the role of autophosphorylation in the activities of CDPKs is unclear. For example, in vitro autophosphorylation activates a groundnut (*Arachis hypogea*) CDPK but inhibits one in winged bean (*Psophocarpus tetragonolobus*; Saha and Singh, 1995; Chaudhuri et al., 1999). Furthermore, autophosphorylation shows no effect on the Ca^{2+} dependence of groundnut and soybean CDPK activities (Chaudhuri et al., 1999; Harmon et al., 2000). CDPK activation may be mod-

ulated by other protein kinases as well. For example, full activation of a tobacco (*Nicotiana tabacum*) CDPK (NtCDPK2) requires both calcium and phosphorylation. Because the phosphorylation event is insensitive to the calmodulin and CDPK antagonist W7 in vitro, and in vitro autophosphorylation cannot substitute for the in vivo phosphorylation-dependent activation, autophosphorylation likely is not responsible for this activation (Romeis et al., 2000, 2001). Therefore, NtCDPK2 is probably activated through direct phosphorylation by an upstream protein kinase. How this phosphorylation event affects CDPK activation is still unknown.

Dephosphorylation is as important as phosphorylation in controlling signaling pathways. A soluble phospho-Ser phosphatase from winged bean shoots dephosphorylates an inactivated, autophosphorylated winged bean CDPK1 (WbCDPK1) in vitro (Ganguly and Singh, 1999). It is thought that this action releases an inhibitory effect of autophosphorylation and suggests the existence of a regulatory feedback loop. These findings indicate an intricate interplay between protein kinases and phosphatases in regulating some CDPK activities.

Regulation by Phospholipids

Biochemical analysis has revealed that in the presence of Ca^{2+}, specific phospholipids can enhance in vitro substrate phosphorylation by CDPKs from oat (*Avena sativa*), Arabidopsis (AtCPK1), and carrot (*Daucus carota*; DcCPK1) by 2 to 30 times above that observed with Ca^{2+} alone (Schaller et al., 1992; Harper et al., 1993; Binder et al., 1994; Farmer and Choi, 1999). Interestingly, the phospholipids regulating kinase activity are not the same for each of the CDPKs studied, which may provide an added layer of CDPK specificity (Farmer and Choi, 1999). At least two mechanisms for phospholipid activation of CDPKs likely exist. Although both phosphatidylinositol and lyso-phosphatidylcholine increase substrate phosphorylation by AtCPK1, only the prior enhances CDPK autophosphorylation and partially relieves inhibition by poly-Lys (Binder et al., 1994). Sequence analysis has revealed a putative phosphatidylinositol binding site in the N terminus of AtCPK1 (Binder et al., 1994). Because this phospholipid still activates a truncated AtCPK1 protein lacking this site, however, this cannot be the only phosphatidylinositol-binding site in the protein.

It remains unknown whether phospholipids will be important in regulating CDPK activities in vivo. Because of the specificity of the phospholipids stimulating each CDPK, the effects will likely have physiological relevance. Some of these phospholipids do act as second messengers in plant signal transduction, and, therefore, might elicit their effects in part through CDPKs. In addition, the activation of DcCPK1 by phosphatidyl-Ser, which is known to activate protein kinase C (PKC) in animal cells, suggests CDPKs may be responsible for some PKC-like responses in plant cells (Farmer and Choi, 1999). Whether there is a plant PKC homolog is still unknown. This observation also may help explain reports of kinases seemingly having properties of both CDPKs and PKC (Abo-el-Saad and Wu, 1995; Van der Hoeven et al., 1996; Lino et al., 1998).

Regulation by 14-3-3 Proteins

Three different 14-3-3 isoforms have been demonstrated to specifically bind and activate AtCPK1 in vitro in the presence of Ca^{2+} (Camoni et al., 1998b). Calcium may be needed in part to induce autophosphorylation of the CDPK, because 14-3-3 proteins typically regulate the activities of many enzymes by binding specific phosphorylated residues. Although the specific sites of autophosphorylation and 14-3-3 protein interaction for AtCPK1 are unknown, AtCPK1 does contain one site within the N terminus similar to the most common 14-3-3 consensus binding site, R-S/T-X-S-X-P, where the underlined Ser is phosphorylated (Camoni et al., 1998b). Using The Arabidopsis Information Resource Patmatch to search the AtCPKs for similar sites revealed that only AtCPK24 (amino acids 28–33) and AtCPK28 (amino acids 40–45) also possess such putative 14-3-3 binding sites. The putative binding sites for all three proteins are located in the N-terminal variable domain. Whether 14-3-3 proteins bind these sites remains to be determined, but these results nonetheless suggest that 14-3-3 proteins may be regulators of only a subset of CDPKs in Arabidopsis.

PHYSIOLOGICAL FUNCTIONS

Although CDPKs have been implicated biochemically to act as key regulators of many signaling pathways, very little is known about which particular CDPK acts as the calcium sensor in each case. The absence of specific inhibitors for CDPKs (there are general inhibitors), the lack of dominant negative constructs, and the possibility of functional redundancy have made it difficult to assign functions to individual CDPKs. Nevertheless, significant progress has been made in our understanding of the physiological roles of CDPKs in Arabidopsis and other plant species.

Hormones

Changes in the cytosolic Ca^{2+} concentration are known to occur during hormone signaling (Bethke et al., 1995; Bush, 1995), and CDPKs may act as the calcium sensors modulating these responses. For example, hormone-induced increases in CDPK activity have been demonstrated in gibberellin-treated rice seeds (Abo-el-Saad and Wu, 1995), and in brassinolide-

treated rice green lamina (Yang and Komatsu, 2001). Significantly, CDPKs have also been shown to activate hormone-responsive genes in vivo. Expression of a constitutively active AtCPK10 or AtCPK30 directly activates a barley (*Hordeum vulgare*) promoter induced by abscisic acid (ABA) in the absence of stress signals (Sheen, 1996). CDPKs also are regulated at the transcriptional level by hormones. GA, ABA, and cytokinin all induce the expression of *NtCDPK1* mRNA in detached tobacco leaves (Yoon et al., 1999), whereas indole-3-acetic acid stimulates the expression of specific CDPK genes in mung bean (*Vigna radiata*) cuttings (Botella et al., 1996) and in cultured alfalfa (*Medicago sativa*) cells (Davletova et al., 2001).

Growth and Development

A wide variety of growth and developmental processes in plants are regulated by Ca^{2+} fluxes (Evans et al., 2001; Hepler et al., 2001). One such process involving CDPKs is pollen tube growth. In maize, the expression of a pollen tube-specific CDPK gene is restricted to the late stages of pollen development (Estruch et al., 1994). The addition of a calmodulin antagonist, CDPK inhibitor, or antisense oligo-nucleotides directed against the maize CDPK mRNA impair pollen germination and tube growth. Further, it has been shown that localized increases in cytosolic Ca^{2+} concentration leading to pollen tube reorientation in *Agapanthus umbellatus* also increase CDPK activities (Moutinho et al., 1998). The ability of a pollen tube CDPK from *Nicotiana alata* to phosphorylate a style self-incompatibility RNase suggests a role for CDPKs in self-incompatibility regulation, as well (Kunz et al., 1996).

CDPKs may also regulate other developmental processes. In the early stages of potato (*Solanum tuberosum*) tuberization, the spatial and temporal accumulation of *StCDPK* mRNA correlates with the increased activity of the protein (MacIntosh et al., 1996; Raíces et al., 2001). In addition, CDPKs may modulate nodulation because two nodule-specific proteins, nodulin-26 (a voltage-sensitive ion channel) and nodulin-100 (a Suc synthase [SuSy]), are phosphorylated by CDPKs in vitro (Weaver and Roberts, 1992; Zhang and Chollet, 1997). Other processes likely involving CDPKs include embryogenesis, seed development and germination in sandalwood (*Santalum album*; Anil et al., 2000), and sexual organ development in liverwort (*Conocephalum conicum*; Nishiyama et al., 1999).

Guard Cells and Stomatal Movements

Stimulus-triggered calcium oscillations regulate the guard cell ion fluxes necessary for driving stomatal movements (McAinsh et al., 1997; Assmann and Wang, 2001; Schroeder et al., 2001a, 2001b). During ABA-induced stomatal closure, calcium enhances K^+ efflux in part via the direct inhibition of inward-rectifying K^+ channels localized within the plasma membrane (McAinsh et al., 1997; Schroeder et al., 2001a, 2001b). A *Vicia faba* CDPK from guard cells is capable of phosphorylating the Arabidopsis guard cell voltage-gated K^+ influx channel KAT1 in vitro in a calcium-dependent manner. This phosphorylation requires the integration of KAT1 into a membrane, suggesting that this regulation is likely to occur in vivo (Li et al., 1998). Co-injecting *Xenopus laevis* oocytes with mRNAs of *KAT1* and a soybean CDPK inhibits K^+ currents typically seen when injecting *KAT1* transcripts alone (Berkowitz et al., 2000). These results suggest that stimulus-induced calcium fluxes in guard cells may activate a CDPK that phosphorylates an inward-rectifying K^+-channel, down-regulating its activity and resulting in a net efflux of K^+ from the cell and stomatal closure.

Actually, Ca^{2+} influxes are implicated in hormone and blue light-induced stomatal opening, as well (McAinsh et al., 1997; Schroeder et al., 2001a), and CDPKs may also be important calcium sensors in these cases (Pei et al., 1996). During stomatal opening, anion uptake into vacuoles is necessary to balance K^+ uptake. A vacuolar chloride channel in *V. faba* guard cells is highly activated by AtCPK1 in the presence of calcium. AtCPK1 also induces malate uptake into *V. faba* guard cell vacuoles and Cl^- uptake into red beet (*Beta vulgaris*) root vacuoles (Pei et al., 1996). These data suggest that CDPKs may be important in the general regulation of vacuolar anion uptake in plant cells. It is not known if AtCPK1 is directly phosphorylating the ion channels or acts on an intermediate protein.

Carbon and Nitrogen Metabolism

In response to various environmental and growth and developmental changes, plants must modify aspects of carbon and nitrogen metabolism. Two key enzymes of carbon metabolism modulated by CDPKs are SuSy and Suc-phosphate synthase (SPS), which catalyze Suc cleavage and Suc synthesis, respectively (Huber and Huber, 1996). In vitro experiments with SuSy from several plant species have shown that the primary CDPK phosphorylation site is an N-terminally located Ser between residues 11 and 15 (Huber et al., 1996; Nakai et al., 1998; Zhang et al., 1999; Loog et al., 2000). Phosphorylation of this site appears to be CDPK specific because the residue is not phosphorylated by a heterologous SuSy protein kinase (mammalian protein kinase A) (Zhang et al., 1999; Loog et al., 2000). In many species, including Arabidopsis, this Ser and the surrounding amino acids required for CDPK phosphorylation are highly conserved, suggesting such phosphorylation of SuSy may be important for the regulation of Suc metabolism (Huber et al., 1996; Zhang et al., 1999). It has been shown that this phosphorylation reduces membrane associ-

ation, increasing the amount of cytosolic SuSy (Winter et al., 1997; Zhang et al., 1999). The physiological significance of this phosphorylation-driven change in subcellular localization is not clear, but may be important for directing the use of the cleavage products by specific biosynthetic pathways (Zhang et al., 1999).

Dark inactivation of spinach (*Spinacia oleracea*) leaf SPS is dependent upon phosphorylation of Ser-158 (McMichael et al., 1993, 1995a, 1995b; Huang et al., 2001). Ser-158 is phosphorylated by a 45-kD CDPK (PK_I) and a 150-kD multiple subunit SNF1-related protein kinase (SnRK1 or PK_{III}) in vitro (McMichael et al., 1995a, 1995b; Huang and Huber, 2001). However, a Pro at position P-4, as is observed in many dicots besides spinach, greatly reduces the ability of CDPK, but not SnRK1, to phosphorylate a synthetic peptide designed from this site. This suggests a more important role for calcium signaling in the phosphorylation-based inactivation of SPS in spinach and many monocots than in most dicots (Huang and Huber, 2001). CDPKs can phosphorylate the homologous site in maize SPS, which lacks a Pro at this site (Huber et al., 1995). The Arabidopsis SPS possesses this Pro, likely preventing phosphorylation by CDPKs in vivo (Huang and Huber, 2001).

Interestingly, it appears that there is an overlap in the specific kinases able to phosphorylate SPS and a rate-limiting enzyme of nitrogen metabolism-nitrate reductase (NR)-in vitro, indicating a possible central role for CDPKs in the coordination of carbon and nitrogen metabolism in planta. Similar to the phosphorylation of Ser-158 in SPS, phosphorylation of Ser-543 in NR takes place in the dark and results in inactivation (Kaiser and Huber, 2001; MacKintosh and Meek, 2001). However, such inhibition of NR activity additionally requires the binding of 14-3-3 proteins to the phosphorylated residue (Douglas et al., 1995; Bachmann et al., 1996). The CDPK PK_I phosphorylates both SPS and NR (McMichael et al., 1995a; Bachmann et al., 1996; Douglas et al., 1997, 1998). NR also appears to be inactivated through phosphorylation of the same site by an additional CDPK (PK_{II}) that has little effect on SPS (McMichael et al., 1995a; Bachmann et al., 1995, 1996). Importantly, these different protein kinases may allow for the activities of SPS and NR to be coordinately or separately regulated, depending upon the needs of the cell. Thus, carbon and nitrogen metabolism can be similarly or differentially modulated.

Little is known about the molecular identity of the CDPKs regulating metabolism and how they are activated in vivo. Suc is known to induce cytosolic calcium influxes (Furuichi et al., 2001) and to increase the protein expression and autophosphorylation of a plasma membrane-associated CDPK from tobacco leaves (Iwata et al., 1998). Because SuSy is important for Suc cleavage in sink locations, Suc induction of the CDPK acting on SuSy might take place in this case. Dark-induced CDPK inactivation of SPS and NR may be explained in part by a reported increase in cytosolic calcium in the absence of photosynthesis (Miller and Sanders, 1987; Johnson et al., 1995). In all cases, a direct demonstration of CDPK activation and substrate phosphorylation in vivo will be necessary to fully demonstrate physiological relevance. In addition, the relative importance of CDPKs and calcium-independent protein kinases, such as SnRK1 for SPS, in phosphorylating such enzymes could be explored further.

Abiotic Stress

Many stress signals, such as wounding, cold, high salinity, and drought, are known to elicit fluctuations in cytosolic Ca^{2+} levels, as well as changes in protein phosphorylation (Bush, 1995; Trewavas, 1999; Knight and Knight, 2001). Several lines of evidence suggest that CDPKs mediate abiotic stress signaling pathways. Transcriptional activation of many different CDPKs by a variety of abiotic stresses has been demonstrated in tissues from diverse species (Urao et al., 1994; Monroy and Dhindsa, 1995; Botella et al., 1996; Yoon et al., 1999; Patharkar and Cushman, 2000; Saijo et al., 2000; Chico et al., 2002). The enzymatic activities of CDPKs also increase in response to these stresses. For example, cold treatments enhance activity of a membrane-bound rice CDPK (Martin and Busconi, 2001). In addition, CDPKs have been shown to elicit abiotic stress responses. Using a protoplast transient expression system, specific CDPKs (AtCPK10 and 30 but not AtCPK1 or AtCPK11) have been demonstrated to activate a stress and ABA-inducible promoter. This result shows the connection of particular CDPKs to specific signaling pathways in vivo (Sheen, 1996). Furthermore, overexpression of a rice CDPK (OsCDPK7) in vascular bundles confers cold, salt, and drought tolerance in transgenic rice (Saijo et al., 2000, 2001). These results demonstrate the usefulness of engineering CDPKs to enhance abiotic stress tolerance in crops.

Pathogen Defense

Extensive studies in various plant/pathogen systems have demonstrated that a cytosolic calcium influx is a crucial early step for the activation of pathogen-induced signal transduction cascades (Xu and Heath, 1998; Blume et al., 2000; Fellbrich et al., 2000; Grant et al., 2000; Nürnberger and Scheel, 2001; Rudd and Franklin-Tong, 2001). Pathogen response pathways are often activated by the interaction between a pathogen-encoded elicitor (such as the *Cladosporium fulvum* Avr9 peptide) and a corresponding plant-encoded receptor (such as the tomato Cf-9 resistance protein). Recently, a CDPK activated in vivo after a Cf-9/Avr9 gene-for-gene interaction has been identified in *Cf-9* transgenic tobacco, suggesting that

CDPKs are important calcium sensors in inducible defense responses (Romeis et al., 2000, 2001). The transition from a nonelicited to an elicited form increases enzyme activity by 10- to 200-fold (Romeis et al., 2000). Silencing of tobacco *NtCDPK2* and *NtCDPK3* (encoding the CDPKs likely responsible), using a viral-induced gene silencing system, has shown that CDPKs are essential for mediating the Cf-9-/Avr9-induced hypersensitive response in planta (Romeis et al., 2001). Significantly, similar results are also observed in the Cf-4/Avr4 gene-for-gene interaction, indicating a more general role for CDPKs in elicitor signaling events. It will be of particular interest to examine whether the Arabidopsis orthologs of NtCDPK2 and NtCDPK3, AtCPK2 and AtCPK1, also are involved in pathogen defense.

The specific function of CDPKs in early defense responses is not yet understood, but, because calcium fluxes and CDPK activation are upstream events of defense signaling (Blume et al., 2000; Fellbrich et al., 2000; Grant et al., 2000; Romeis et al., 2000), they likely regulate other early processes. One of these steps could be the formation of reactive oxygen species by NADPH oxidase, which is both calcium and phosphorylation dependent (Mehdy, 1994; Grant et al., 2000). Although NADPH oxidase may be directly regulated by calcium (Keller et al., 1998), studies have shown that calmodulin/CDPK antagonists can inhibit the phosphorylation and membrane translocation of putative cytoplasmic complex components and block reactive oxygen species formation (Xing et al., 1997; Romeis et al.; 2000). It has been suggested that CDPKs may mediate this indirect calcium regulation (Mehdy, 1994; Xing et al., 1997; Romeis et al., 2000). Recent data revealing that ectopic expression of AtCPK1 can enhance NADPH oxidase activity in a cell-free system and in tomato protoplasts (Xing et al., 2001) support these studies.

CDPKs activated by pathogen elicitors may also initiate other early ion fluxes. For instance, CDPKs may be responsible for changes in H^+ fluxes resulting from the calcium- and phosphorylation-dependent regulation of plasma membrane proton ATPases (PM H^+-ATPases; Schaller and Sussman, 1988; Schaller et al., 1992; Camoni et al., 1998a; Lino et al., 1998; De Nisi et al., 1999; Schaller and Oeckling, 1999). Depending on the elicitor, elicitor treatment may increase the net H^+ influx through negative regulation of PM H^+-ATPases or enhance the net H^+ efflux through positive regulation of these pumps (Blumwald et al., 1998). Several studies have shown that purified CDPKs can phosphorylate PM H^+-ATPases in vitro (Schaller et al., 1992; Harmon et al., 1996; Camoni et al., 1998a). The most detailed report shows that a CDPK from maize roots phosphorylates a PM H^+-ATPase in the C terminus (Camoni et al., 1998a), phosphorylation of which is thought to be important for 14-3-3 protein-mediated activation of the protein (Morsomme and Boutry, 2000). Further biochemical analysis will be necessary to identify the precise phosphorylation sites and to determine how these phospho-modifications affect the ATPase activity because CDPK phosphorylation of different sites may yield positive or negative regulation.

CDPK SUBSTRATES

Although CDPKs have been shown to phosphorylate a large number of protein substrates in vitro (Table II), limited information is available regarding substrate specificity and phosphorylation sites in vivo. Although CDPKs are highly homologous, an examination of in vitro substrate phosphorylation by spinach and soybean CDPKs suggests that CDPKs will exhibit substrate specificity differences in vivo (Bachmann et al., 1996; Lee et al., 1998). The use of synthetic peptides has facilitated delineation of the potential phosphorylation motifs recognized by some CDPKs. Most of these studies have been conducted with spinach leaf CDPKs PK_I and PK_{II}, which both appear to recognize two different general phosphorylation motifs (McMichael et al., 1995b; Bachmann et al., 1996; Huang and Huber, 2001; Huang et al., 2001). The first minimal consensus phosphorylation site is φ_{-5}-X_{-4}-Basic$_{-3}$-X_{-2}-X_{-1}-S, where S is the phosphorylated Ser, X is any residue, and φ is a hydrophobic residue (McMichael et al., 1995b; Bachmann et al., 1996; Huang and Huber, 2001). Substituting a Thr for the Ser makes the synthetic peptide a poor substrate for PK_{II} but not PK_I (Bachmann et al., 1996). Further studies of PK_I reveal that basic residues at P − 6 and P + 5 are not required but enhance phosphorylation (Huang and Huber, 2001). Therefore, maximal phosphorylation by PK_I is attained with the motif Basic$_{-6}$-φ_{-5}-X_{-4}-Basic$_{-3}$-X_{-2}-X_{-1}-S-X_{+1}-X_{+2}-X_{+3}-φ_{+4}-Basic$_{+5}$. This motif is similar to the site delineated for cauliflower (*Brassica oleracea*) floret PK_I and maize CDPK-1, except that the maize protein also requires a basic residue at P+2 (Toroser and Huber, 1998; Loog et al., 2000). This recognition site also is similar to that recognized by mammalian PKC (Loog et al., 2000).

Making peptides with various Ala substitutions, a second substrate phosphorylation motif for PK_I and PK_{II} has been identified as Basic$_{-9}$-Basic$_{-8}$-X_{-7}-Basic$_{-6}$-φ_{-5}-X_{-4}-X_{-3}-X_{-2}-X_{-1}-S-X_{+1}-Basic$_{+2}$ (Huang et al., 2001). Using this motif, several new potential CDPK substrates have been identified, including the spinach plasma membrane aquaporin PM28A, ACC synthase, a splicing factor, the RNA polymerase β-chain, a protein kinase, and two disease resistance protein homologs (Huang et al., 2001). Both PK_I and PK_{II} phosphorylate a synthetic peptide based on the phosphorylation site of PM28A (Huang et al., 2001). Furthermore, PM28A is regulated by calcium-dependent phosphorylation of this site (Johansson et al., 1998), suggesting the physiological relevance of the prediction. These results support

Table II. *Known potential CDPK substrates*[a]

Substrate	Site[b]	Substrate Source (Species)[d]	CDPK Species (CDPK)[e]	Potential Effect[f]	Ref[g]
Metabolism					
Nitrate reductase	SGPTLKRTA<u>S</u>TPFMN	Leaves (spinach)	Spinach (PK_I, PK_{II}); AtCPK3	A	1–3
Phospho*enol*pyruvate carboxylase	–	Root nodules	Soybean	–	4
Phospho*enol*pyruvate carboxylase	APGPGEKHH<u>S</u>IDAQL	Leaves	Maize	–	5
PSII D1	–	Peptide	*Spirodela* sp.	B	6
Suc-phosphate synthase	TKGRMRRIS<u>S</u>VEMMD	Leaves	Spinach (PK_I)	A	1,7
Suc synthase	ATDRLTRVH<u>S</u>LRERL	Rec (mung bean)	Soybean (nodule)	C	8
Suc synthase 2	GDRVLSRLH<u>S</u>VRERI	Leaves	Maize	A,D	9,10
Suc synthase (nod 100)	ATDRLTRVH<u>S</u>LRERL	Root nodules	Soybean	D	11
Stress-related proteins					
PAL	VSXVXKRXL<u>T</u>TXXXX	Cell culture, rec (poplar [*Populus* × *generosa*])	French bean (*Phaseolus vulgaris*); AtCPK1	E	12,13
Pseudoresponse regulator	–	Leaves	Ice plant (*Mesembryanthemum crystallinum* Mc-CDPK1)	–	14
Antifungal proteins					
Carboxypeptidase inhibitor	AWFCQACWN<u>S</u>ARTCG	Tuber (potato)	Wheat (embryo)	–	15
γ-Thionins	UKLCQRP<u>S</u>GTWSG[c]	Seeds (yellow mustard [*Sinapis alba*])	Wheat (embryo)	–	16
Lipid transfer proteins	VXSXXXPCX<u>S/T</u>YXXGX	Seeds (wheat [*Triticum aestivum*] and barley)	Wheat (embryo)	–	17
Napins	QGQQLQQVI<u>S</u>RIYQT[c]	Seeds (kohlrabi [*Brassica napus* var. rapifera])	Wheat (embryo)	–	18
Protease inhibitor	SDMRLNSCH<u>S</u>ACKSC	(Soybean)	Wheat (embryo)	–	19
Ion/water transport					
Aquaglyceroporin (nod 26)	PLSEITKSA<u>S</u>FLKGR	Root nodules	Soybean	F	20,21
Aquaglyceroporin (LIMP2)	PLRFITKNV<u>S</u>FLKGI	Peptide	*Lotus japonicus*	–	22
Aquaporin PM28A	RAAAIKALG<u>S</u>FRSN	Peptide	Spinach (PK_I, PK_{II})	–	23
Ca^{2+}-ATPase ACA2	RRFRFTANL<u>S</u>KRYEA	Rec	Arabidopsis (AtCPK1)	G	24
PM H^+-ATPase	–	Roots	Oat, maize	–	25,26
Potassium channel KAT1	–	Guard cells	*V. faba*	G	27,28
Other					
Actin-depolymerizing factor	MANAR<u>S</u>GVAVN	Cell cultures (maize)	French bean	G	29
CDPK-related kinase	RKAALRAL<u>S</u>KTLTV	Peptide	Carrot (DcCPK1)	–	30
Myosin light chain	–	–	*Chara*	–	31
PI 4-kinase activator	–	Cell cultures	Carrot	B	32
Proteasome regulatory sub.	–	Peptide	Tobacco (NtCDPK1)	–	33
Self-incompatibility	–	Style	*N. alata* (pollen tube)	–	34
RNases					
Ser acetyltransferase	–	Cell cultures	Soybean	H	35

[a] Potential substrates included are those shown to be phosphorylated by a CDPK (rather than CaMK). [b] Site or motif phosphorylated by the CDPK with the modified residue underlined. [c] The site for only one protein of this class is shown. [d] Substrate species is in parentheses if different from that of the CDPK. rec, Recombinant source. [e] CDPK plant species, with the name or tissue of the CDPK, if different from the substrate tissue, in parentheses. [f] A, Inactivation; B, activation; C, higher substrate affinity; D, localization; E, lower V_{max}; F, higher voltage sensitivity; G, inhibition; H, reduced feedback inhibition. [g] 1, McMichael et al. (1995a); 2, Douglas et al. (1997); 3, Douglas et al. (1998); 4, Zhang and Chollet (1997); 5, Ogawa et al. (1998); 6, Swegle et al. (2001); 7, McMichael et al. (1995b); 8, Nakai et al. (1998); 9, Huber et al. (1996); 10, Winter et al. (1997); 11, Zhang et al. (1999); 12, Allwood et al. (1999); 13, Cheng et al. (2001); 14, Patharkar and Cushman (2000); 15, Neumann et al. (1996c); 16, Neumann et al. (1996a); 17, Neumann et al. (1993); 18, Neumann et al. (1996b); 19, Neumann et al. (1994); 20, Weaver and Roberts (1992); 21, Lee et al. (1995); 22, Guenther and Roberts (2000); 23, Huang et al. (2001); 24, Hwang et al. (2000); 25, Harmon et al. (1996); 26, Camoni et al. (1998a); 27, Li et al. (1998); 28, Berkowitz et al. (2000); 29, Allwood et al. (2001); 30, Farmer and Choi (1999); 31, McCurdy and Harmon (1992); 32, Yang and Boss (1994); 33, Lee et al. (2001); 34, Kunz et al. (1996); 35, Liu et al. (2001).

the usefulness of predicting CDPK substrates using these motifs. Interestingly, the second motif is not recognized by the spinach SNF1-related protein kinase SnRK1 (PK_{III}), which has been shown to phosphorylate some of the same substrates as PK_I and PK_{II} (McMichael et al., 1995a; Douglas et al., 1997; Huang and Huber, 2001). Therefore, using the first phosphorylation motif may allow for phosphoregulation by both CDPKs and SNF1-related protein kinases in vivo, whereas the second motif

Table III. *Insertional mutants of Arabidopsis CDPKs*[a]

Name	Insertional Mutagen	Clone Name(s)[b]	Hit[c]	Source[d]	Ref[e]
CPK1	T-DNA	SALK_010530, 007698	Exon	A	–
	T-DNA	SALK_007911, 007917, 007918, 007919	Intron	A	–
	T-DNA	Garlic_346_B04	Promoter	B	–
CPK2	T-DNA	SALK_014870	300-3′	A	–
	T-DNA	SALK_036166	Exon	A	–
	T-DNA	Garlic_1288_C10	Exon	B	–
CPK3	T-DNA	Garlic_234_B06	300-5′	B	–
CPK4	T-DNA	SALK_000685	Intron	A	–
	T-DNA	–	?	D	1
CPK6	T-DNA	SALK_034106, 033392, 033739	300-5′	A	–
	T-DNA	Garlic_690_H07	Exon	B	–
	T-DNA	–	?	D	1
CPK7	T-DNA	SALK_035601	Exon	A	–
CPK8	T-DNA	SALK_036581	Exon	A	–
CPK9	T-DNA	SALK_034324	300-5′	A	–
	T-DNA	–	?	D	1,2
CPK10	T-DNA	SALK_015994	300-3′	A	–
	T-DNA	SALK_032021	Intron	A	–
	T-DNA	SALK_003484	Exon	A	–
	T-DNA	–	?	D	1,2
CPK11	T-DNA	SALK_007814	Intron	A	–
	T-DNA	–	?	D	1,2
CPK12	T-DNA	Garlic_839_H10	Intron	B	–
CPK15	T-DNA	Garlic_1284_H09	Promoter	B	–
	Transposon	GT1712	300-5′	C	–
CPK16	T-DNA	SALK_052257	Intron	A	–
CPK17	T-DNA	SALK_001818	300-3′	A	–
	T-DNA	SALK_057146	Intron	A	–
CPK19	T-DNA	SALK_001691, 001685	Exon	A	–
	T-DNA	Garlic_1215_F07	Exon	B	–
	Transposon	ET1931	Exon	C	–
CPK20	T-DNA	SALK_045192	300-3′	A	–
	T-DNA	SALK_044320	Intron	A	–
	T-DNA	Garlic_377_G02	Promoter	B	–
CPK21	T-DNA	SALK_029412	Exon	A	–
	T-DNA	SALK_043765	300-5′	A	–
CPK22	Transposon	ET9246	Exon	C	–
	Transposon	ET6769	Intron	C	–
CPK23	T-DNA	SALK_007958	Intron	A	–
	T-DNA	Garlic_754_E09	Intron	B	–
CPK24	T-DNA	SALK_015986	Exon	A	–
	T-DNA	SALK_035333	300-3′	A	–
CPK27	T-DNA	Garlic_61_A09	300-5′	B	–
CPK29	T-DNA	SALK_013734	300-3′	A	–
CPK30	T-DNA	SALK_042171	300-5′	A	–
	T-DNA	Garlic_337_G01	Promoter	B	–
CPK32	Transposon	ET5772	Exon	C	–
CPK33	T-DNA	Garlic_26_C12	Exon	B	–

[a] The insertional mutants listed are all Columbia ecotype. This list was completed March 13, 2002. A regularly updated list is found at http://xanadu.mgh.harvard.edu/sheenweb/index.htm under "Arabidopsis CDPKs." [b] The criteria used to determine insertional mutants from Torrey Mesa Research Institute/Syngenta and Genetrap after BLAST search are an E value less than 1e-04 and a score greater than 460. [c] 30D-3′(5′) indicates that insertion is within 300 nucleotides in the 3′(5′) of the open reading frame, whereas insertion between 300 and 1000 nucleotides in the 5′ of the open reading frame is listed as an insertion in the promoter. [d] A, Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu); B, Torrey Mesa Research Institute/Syngenta (http://www.tmri.org); C, Genetrap (Cold Spring Harbor Laboratory; http://genetrap.cshl.org); D, Wisconsin Arabidopsis Knockout Facility (http://www.biotech.wisc.edu). [e] 1, Satterlee and Sussman (1998); 2, Krysan et al. (1996).

may allow for sole regulation by CDPKs (Huang et al., 2001).

CDPK SPECIFICITY

To carry out the myriad of Ca^{2+}-dependent cellular processes, plants have acquired a multitude of CDPK genes during evolution. How different CDPKs translate the information encoded in the "calcium signatures" to specifically affect metabolism and gene expression is mostly unknown. Available evidence indicates that functional specialization of individual CDPKs can occur through different types of regulation. For example, detailed analysis of three soybean CDPK isoforms has shown conclusively that each one

possesses unique Ca^{2+}-binding properties, presumably allowing different thresholds of Ca^{2+} activation (Lee et al., 1998). In addition, modulations by reversible phosphorylation (winged bean WbCDPK1; Ganguly and Singh, 1999), different phospholipids (carrot DcCPK1; Farmer and Choi, 1999), and 14-3-3 proteins (AtCPK1; Camoni et al., 1998b) further contribute to CDPK specificities. Targeted subcellular localization, such as to the plasma membrane (rice OsCPK2; Martin and Busconi, 2000), ER (AtCPK2; Lu and Hrabak, 2002), and nuclei (ice plant McCDPK1; Patharkar and Cushman, 2000), allows interactions with different substrates (Table II). Variations in phosphorylation motif recognition or substrate phosphorylation may occur as well (Bachmann et al., 1995, 1996; McMichael et al., 1995a; Lee et al., 1998). Specific roles of CDPKs also are determined by stimulus-specific (e.g. wound-induced tomato LeCDPK1; Chico et al., 2002) and spatiotemporal regulation of expression (maize pollen-specific CDPK; Estruch et al., 1994). Thus, plants may use a combination of various strategies to functionally specialize individual CDPKs, as evidenced by two sandalwood CDPKs isoforms that differ in tissue-specific distribution, subcellular localization, and enzyme kinetics and properties (Anil et al., 2001).

PERSPECTIVES

As we enter the post-sequencing era of Arabidopsis, understanding the physiological roles of the array of Arabidopsis CDPKs poses a new challenge. A combination of biochemical, molecular, cellular, and genetic approaches will be required to elucidate the function(s) of each individual CDPK. The spatial and temporal expression patterns of CDPKs can be determined by using promoter/reporter (e.g. β-glucuronidase or green fluorescence protein; Jefferson et al., 1987; Chiu et al., 1996) fusions in transgenic plants and in situ hybridization with isoform-specific probes. The expression of epitope-tagged CDPKs in a transient expression system or transgenic plants can provide information regarding subcellular localization (Chiu et al., 1996; Hwang and Sheen, 2001). New CDPK-interacting proteins and substrates can be identified using bacterial expression, phage display, or yeast two-hybrid techniques (Patharkar and Cushman, 2000; Shinohara et al., 2000). Substrate specificity can be determined by performing protein kinase assays with epitope-tagged CDPKs expressed in protoplasts (Cheng et al., 2001). This approach could be further explored on a genomic scale, using immobilized substrates (proteins or peptides) on microarray surfaces (Zhu et al., 2000). The previously delineated phosphorylation motifs and potential substrates (Table II) could be used as a starting point. Finally, gene disruption and silencing techniques, such as insertional mutagenesis, RNA interference, and virus-induced gene silencing, can be used to study altered phenotypes (Krysan et al., 1996; Waterhouse et al., 1998; Romeis et al., 2001). Sequencing of publicly and privately generated insertion mutants of Arabidopsis has already identified a number of putative CDPK mutants, as listed in Table III, with others rapidly becoming available. Due to possible redundancy in CDPK functions (Sheen, 1996), the simultaneous inactivation of highly homologous CDPKs with similar expression patterns, cellular localization, and substrate specificity may be necessary to reveal a mutant phenotype and to identify physiological functions. This integrated approach, in combination with bioinformatics, should shed light on the role of CDPKs in the complex web of signaling networks that regulate cellular metabolism, growth and development, and responses to the environment. Because specific CDPKs act as positive regulators to selectively activate a stress pathway (Sheen, 1996) and to enhance drought/salt tolerance (Saijo et al., 2000), increasing our understanding of the specific role of CDPKs in many aspects of plant biology will prove invaluable for many future biotechnology applications.

ACKNOWLEDGMENT

We thank Dr. Brandon D. Moore for critical reading of the manuscript.

Received March 15, 2002; accepted March 17, 2002.

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