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Expression of three members of the calcium-dependent protein kinase gene family in *Arabidopsis thaliana*

Yan Hong[1,7,*], Makoto Takano[2,3], Chun-Ming Liu[1,4], Alexander Gasch[2,5], Mee-Len Chye[1,6] and Nam-Hai Chua[2]

[1] *Institute of Molecular and Cell Biology, National University of Singapore, Republic of Singapore, 0511;* [2] *Laboratory of Plant Molecular Biology, The Rockefeller University, New York, NY 10021, USA; Present addresses:* [3] *Laboratory of Molecular Genetics, National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, Japan;* [4] *John Innes Institute, Norwich, UK;* [5] *BIOTECON, Gesellschaft für Biotechnologische Entwicklung and Consulting MBH, Gustav-Meyer-Allec 25, 13355 Berlin, Germany;* [6] *Department of Botany, University of Hong Kong, Pokfulam Road, Hong Kong;* [7] *Institute of Molecular Agrobiology, National University of Singapore, 59A, The Fleming, Science Park Drive, Singapore 118240, Republic of Singapore (* author for correspondence)*

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Abstract

Calcium-dependent protein kinases (CDPKs) belong to a unique family of enzymes containing a single polypeptide chain with a kinase domain at the amino terminus and a putative calcium-binding EF hands structure at the carboxyl terminus. From *Arabidopsis thaliana*, we have cloned three distinct cDNA sequences encoding CDPKs, which were designated as *atcdpk6*, *atcdpk9* and *atcdpk19*. The full-length cDNA sequences for *atcdpk6*, *atcdpk9* and *atcdpk19* encode proteins with a molecular weight of 59343, 55376 and 59947, respectively. Recombinant atCDPK6 and atCDPK9 proteins were fully active as kinases whose activities were induced by Ca^{2+}. Biochemical studies suggested the presence of an autoinhibitory domain in the junction between the kinase domain and the EF hands structure. Serial deletion of the four EF hands of atCDPK6 demonstrated that the integrity of the four EF hands was crucial to the Ca^{2+} response. All the three *atcdpk* genes were ubiquitously expressed in the plant as demonstrated by RNA gel blot experiments. Comparison of the genomic sequences suggested that the three *cdpk* genes have evolved differently. Using antibodies against atCDPK6 and atCDPK9 for immunohistochemical experiments, CDPKs were found to be expressed in specific cell types in a temporally and developmentally regulated manner.

[1] The cDNA and genomic sequences for atCDPK6 (U20633, U20625), atCDPK9 (U20388, U20626) and atCDPK19 (U20624, U20627) were submitted to GenBank.

Introduction

Ca^{2+} is involved in various physiological and biochemical responses of plants, elicited by a number of environmental factors, such as light and cold stress [10, 15]. Although the precise mechanism is still not known, it is generally thought that Ca^{2+} acts through intracellular protein mediators. One such mediator is calmodulin (CaM) which is also present in plants. CaM has a high binding affinity for calcium and the resulting Ca^{2+}/CaM complex is able to activate a number of downstream target enzymes. In animal cells, the target enzymes include CaM kinases, whose activity depends critically on CaM. The cloning of a plant cDNA encoding a protein with homology to CaM kinase II has recently been reported [22], although the encoded protein was not sufficiently characterized to allow a definitive identification.

In contrast to animal cells, plant cells contain a group of kinases, designated as calcium-dependent protein kinases (CDPKs), which are dependent only on calcium and does not require CaM for activation [8]. Molecular cloning of kinases revealed that they are made up of an N-terminal kinase domain fused to a C-terminal CaM-like domain [11]. The presence of the latter presumably allows the enzyme to be directly responsive to intracellular changes in calcium, without the need of CaM mediation. In addition to plants, CDPKs have also been found in *Paramecium tetraurelia* [6] and *Plasmodium falciparum* [24].

cDNA clones encoding CDPKs have been isolated from a number of higher plants [11, 12, 14, 19, 20, 21] and Southern blot hybridization experiments showed that the enzyme is encoded by a multi-gene family [12]. In *Arabidopsis*, at least 3 different cDNAs for CDPK have been characterized [12, 20, 21]. The encoded proteins share homology in the kinase and the CaM-like domains but are different in other sequences, particularly at the N-terminus. As far as we know, no genomic sequence for any plant CDPK has yet been reported.

In this paper, we report the isolation and characterization of three new members of the *Arabidopsis thaliana cdpk* gene family. Comparison of their genome sequences indicates that these genes are likely to have evolved differently. Using polyclonal antibodies to the CDPKs we demonstrated that this family of enzymes is expressed in specific cell types in a temporally and developmentally regulated manner. Finally, our mutagenesis experiments showed that all of the four EF hands structures are needed for calcium response.

Materials and methods

Plant material

Arabidopsis thaliana (ecotype Columbia) plants were grown in a growth chamber under a 12 h light/12 h dark cycle at 20 °C.

Isolation of cDNA clones for atCDPKs

From a cDNA library constructed from poly(A) RNA isolated from young ovules of *Arabidopsis thaliana*, a 1.0 kb fragment was isolated by differential screening, cloned into pBluescript vector (Stratagene) and sequenced. Sequence analysis revealed homology to the soybean calcium-dependent protein kinase (CDPK) [11]. This clone (*atcdpk6*) was used to screen an *Arabidopsis* λZAPII cDNA library (ecotype C24). Five positive clones were excised from λZAPII and cloned into pBluescript vectors. The nucleotide sequence of double stranded DNA was determined by a Sequenase dideoxy sequencing kit (United States Biochemical) using oligonucleotide primers.

During our screening for full-length cDNA clone as mentioned above, several weakly positive clones were obtained. Some of these clones were later sequenced and found to encode another CDPK, designated *atCDPK19*.

In another approach, we used the polymerase chain reaction technique to obtain cDNA clones encoding kinases from *Arabidopsis*. Two degenerate oligonucleotides corresponding to the amino acid sequences, KI(A/T)DFG and DVW(T/V/M)FGV were synthesized and used for PCR. The

amplified fragments were cloned into pBluescript (Stratagene) and their sequences determined (USB). A 160 bp fragment with sequence homology to the soybean CDPK cDNA was random-labelled and used to screen a λZAPII cDNA library of *A. thaliana*. Fourteen independent clones were obtained from 5×10^5 phage screened and all were found to encode the same CDPK, which was designated atCDPK9.

Sequence analysis

DNA and amino acid sequence analyses were performed using various programs of GCG (Genetics Computer Group, Wisconsin) sequence analysis package.

RNA gel blot hybridization

Total RNA was isolated from root, stem, leaf, and flower of *A. thaliana* using RNaid resin of BIO101. Poly(A) RNA was isolated with the Promega PolyATract mRNA Isolation System following the manufacturer's instructions. Poly(A) RNA samples (3 μg) from different plants parts were electrophoresed through a formaldehyde-containing gel and transferred to a Duralon-UV membrane (Stratagene). The RNA gel blot hybridization was carried out according to Sambrook *et al.* [17]. The same filter was hybridized sequentially to random-prime-labelled *Eco*RI-*Eco*RI fragments of cDNA clones of *atcdpk6*, *atcdpk19* and *atcdpk19*.

Southern blot hybridization

Genomic DNA of *A. thaliana* was isolated according to Dellaporta *et al.* [4]. DNA (5 μg) was digested with restriction enzymes, fractionated on an agarose gel and transferred to Hybond N membrane (Amersham). cDNA probes used for RNA gel blot hybridizations were used. Southern blot hybridizations were performed as described by Sambrook *et al.* [17].

Expression of atCDPK6 and atCDPK9 in E. coli

Sequences flanking the translation initiation codon *atcdpk9* were altered to create a *Nde*I site and the 1527 bp *Nde*I fragment was ligated into the *Nde*I site of pET3b (Novagene), *Escherichia coli* strain BL21(DE3)pLysE was used to express the recombinant atCDPK9 protein. Cells were grown at 30 °C to an OD_{600} of 0.7 and then induced with 0.5 mM IPTG. After 3 h of induction, cells were harvested, lysed and contrifuged for 10 min at 5000 × **g**. The pellet was resuspended in 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5 M NaCl and 1% Triton X-100 and incubated for 1 h at room temperature. After centrifugation, the pellet, consisting mainly of inclusion bodies, was solubilized with 6 M guanidium hydrochloride. Refolding and renaturation of the denatured atCDPK9 was carried out by the rapid dilution method of Berndt and Cohen [2]. The enzyme was concentrated and further purified by adsorption to a Mono Q column (equilibrated in 10 mM Tris-HCl pH8.0, 0.25 mM mercaptoethanol and 0.02% Tween 20) and elution with a NaCl gradient (0.04–0.8 M) in the same buffer.

Full-length *atcdpk6* cDNA encoding amino acids 1 to 529 was cloned by amplifying a cDNA clone using Vent polymerase (New England Biolabs) and primers with *Bam*HI sites at the 5′ ends. Partial *atcdpk6* cDNA encoding amino acids 1 to 412 with only one EF hand (EF1) and that encoding amino acids 1 to 490 with three EF hands (EF3) were cloned by amplifying cDNA clone using appropriate primers with *Bam*HI sites at the 5′ ends. A partial *atcdpk6* cDNA encoding amino acids 1 to 390 without any EF hand (EF0) and that encoding amino acids 1 to 447 with two EF hands (EF2) were cloned by amplifying cDNA sequence using primers with *Nde*I sites at the 5′ ends. Polymerase chain reactions were performed according to the manufacturer's instructions. PCR products were purified from agarose gel, digested with the appropriate restriction enzymes, and cloned into pET16b (Novagene). After sequence verification, the appropriate clones were used to transform *E. coli* strain BL12(DE3) by electroporation. *E. coli* cells were grown at

30 °C to OD_{600} 0.6–1.0 and protein expression was induced by the addition of 1 mM IPTG for 2–3 hr at 30 °C. Cells were harvested, broken by sonicaton and the recombinant proteins were purified using His Bind affinity resin (Novagene) according to the manufacturer's instructions. The proteins were dialyzed against PBS (10 mM sodium phosphate buffer pH 7.4, 0.15 M NaCl) and the cencentration determined according to Bradford [3].

Kinase activity assay

Protein kinase activities were measured according to Harper *et al.* [12]. The reaction mix (25 μl) contained 0.1 μg of purified protein in 20 mM Tris-HCl, pH 7.4, and 6 mM $MgCl_2$, with 25 μg of histone III-S (Boehringer Manheim) as a substrate. Control reaction contained an equivalent amount of bovine serum albumin (BSA). Control levels of phosphorylation were determined by chelating free Ca^{2+} with 1 mM EGTA. To test the effect of Ca^{2+}, the reaction mix without EGTA was supplemented with 1 mM $CaCl_2$. Reaction was initiated by the addition of 5 μCi γ-^{32}P-ATP (100 μM final ATP concentration) and the mixture was incubated for 20 min at room temperature. Samples were spotted onto Whatman 3MM filters followed by immersion in ice-cold 10% (w/v) trichloroacetic acid with 10 mM sodium pyrophosphate. Filters were washed with the above solution three times for 15 min each, followed by 15 min in 95% ethanol. Radioactivity on the dried filters was determined by scintillation counting. Each reaction was carried out in triplicate and the average value was used. Phosphorylation of substrates was also monitored by electrophoresis of samples on SDS polyacryamide gel followed by autoradiography.

To compare the kinase activities of various atCDPK6 deletion mutants, a fixed amount of purified recombinant proteins was fractionated on SDS-PAGE and the protein profile was scanned by a Visage Image System (Millipore). The percentage of atCDPK6 or its derivatives in the protein samples was estimated and the value used for the calculation of specific activity.

To investigate their effects on the atCDPK6 activity, inhibitors or calmodulin antagonists, including staurosporin, calmodozolium, trifluoperizine, KN252a and KN62 (all from CalBiochem) were added into the reaction system at various concentrations of atCDPK6. The Ki's of different inhibitors were calculated according to the Dixon's graphic method [5]. The effects of four oligopeptides covering 293–376 amino acids of atCDPK6 (conjunction sequence between kinase domain and EF hands structure) were also investigated. The sequences are as follows:
pep1, 346–365: PLDNAVLSRMKQFRAMNKLK
pep2, 293–327: GQLDFSADPWPALSDGAKDLVRKMLKYDPKDRLTA
pep3, 318–347: KYDPKDRLTAAEVLNHPWIREDGEASDKPL
pep4, 354–376: RMKQFRMNKLKKMALDVIAENL

Preparation of antibodies against atCDPKs

Purified atCDPK6 and atCDPK9 proteins were used to immunize rabbits using a standard protocol. Purified antigens were immobilized onto CNBr-activated Sepharose 4B (Pharmacia) and antibodies were purified by affinity chromatography with an antigen-Sepharose 4B column followed by a protein A-Sepharose column [7].

Western blot analysis of protein extracts

Different organs of *A. thaliana* plant were freshly harvested and grounded in a mortar and a pestle on ice with phosphate-buffered saline containing 1 mM PMSF (Sigma), 10 μg/ml leupeptin (Sigma) and 100 KI unit/ml Aprotinin (Sigma). Extracts were clarified by spinning in a microcentrifuge at full speed for 10 min at 4 °C. Protein concentration in the supernatant was determined by the Bradford method [3]. Equal amounts of protein were mixed with Laemmli's sample buffer and boiled for 2 min before being separated on 10% SDS-polyacryamide gels according to

Laemmli [13]. Proteins in gels were transferred onto a nitrocellulose membrane (S & S BA85). Membranes were blocked with a PBST solution containing 3% non-fat milk in phosphate buffered saline with 0.05% Tween-20 for 1 h at room temperature before incubation with affinity purified anti-atCDPK6 or anti-atCDPK9 (all were diluted with PBST to 1 μg/ml). Alkaline phosphotase conjugated anti-rabbit IgG (Promega, diluted 1:1000 with PBST) was used as the second antibody. Reactions were vizualized with 33 μg/ml nitroblue tetrazolium and 16.5 μg/ml of 5′-bromo-4-chloro-3-indolyl phosphate (all from Promega) in 10 mM Tris-HCl, 100 mM NaCl and 5 mM $MgCl_2$ pH 9.5.

Immunohistochemical localization of atCDPK

Different plants organs of *A. thaliana* were harvested and immediately fixed in modified FAA (50% ethanol, 6% acetic acid, 5% formaldehyde and 5% glycerol) by applying vacuum 2 min every 30 min for 2 h. The materials were dehydrated through a series of ethanol solutions (50%, 70%, 95%, 100%) for 1.5 h each, and then embedded in paraffin. The paraffin blocks were sectioned in an ultramicrotome (Reichert-Jung 2050) and 7 μm sections were transferred to polylysine-coated glass slides. Slides dried overnight were hydrated by a series of ethanol solutions (100%, 90%, 50%) and washed with PBS twice for 5 min each. Endogenous peroxidase activity was blocked by treatment with 3% H_2O_2 in PBS. The sections were treated with PBS and 0.1% saponin (Sigma) for 5 min before incubation with PBS containing 0.1 saponin, 1% BSA and 2% goat serum for 1 h at room temperature, then treated with affinity-purified anti-atCDPK6 or anti-atCDPK9 IgG at 4 °C overnight. The antisera were diluted by PBS containing 0.1% saponin, 1% BSA and 2% goat serum to 5 μg/ml. IgG from preimmune sera used as control always gave negative results. After several washes with PBS with 0.1% saponin, the sections were treated with horseradish peroxidase-conjugated anti-rabbit IgG (Promega, 1:500 diluted with PBS with 0.1% saponin and 1% BSA) as the second antibody. The sections were washed with PBS several times and the signals were developed in GPN solution (2.5% nickel ammonium sulphate, 0.05% diaminobenzidine tetrahydrochloride, 0.2% glucose, 0.04% ammonium chloride and 0.001% glucose oxidase, all from Sigma) for 20 min.

Results

Three cDNA sequences encode distinct calcium-dependent protein kinases

In our attempt to search for genes that are specifically expressed during embryogenesis, we isolated five cDNA clones that showed sequence homology to the soybean CDPK [11]. All of them contained the same open reading frame but they differed slightly from each other in length at the 5′ and 3′ ends. The longest clone was 1.76 kb long and had an open reading frame of 529 amino acids, encoding a protein (*M*r 59343) with a kinase domain at the N-terminus and a structure with four EF hands at the C-terminus. We designated this gene as *atcdpk6* and the encoded protein atCDPK6. Some weakly hybridizing cDNA clones were also sequenced and found to encode another CDPK, designated as atCDPK19. The longest *atcdpk19* clone contained 1.8 kb encoding a long open reading frame, but with no stop codon upstream of the first methionine. The corresponding genomic clone was later isolated and found to contain another in-frame methionine located 55 amino acids upstream of this methionine with several translation stop codons further upstream of it. Considering that the cDNA clone was 300 to 400 bp smaller than the mRNA band on Northern blot, we assumed that the first methionine of the genomic clone was used for translation. RNase protection assays localized the transcriptional initiation site 32 bp upstream of the ATG for this methionine (data not shown). The encoded polypeptide has 533 amino acid residues with an estimated molecular weight of 59947, which we designated atCDPK19.

To obtain DNA probes for genes encoding

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    AK1   (1-50)     MGNTCVGPSR NGFLQSVSAA MWRPRDGDDS ASMSNGDIAS EAVSGELRSR

atCDPK19 (1-7)                                                    MGNCCAS
    AK1   (51-100)  LSDEVQNKPP EQVTMPKPGT DVETKDREIR TESKPETLEE ISLESKPETK
atCDPK6  (1-28)                          MGHRHSKS KS*DPPPSSS S*SSGNVVHH

atCDPK19 (8-57)     PGSETGSKKG KPKIKSNPFY SEAYTTNGSG TGFKLSVLKD PTGHDISLMY
atCDPKa  (1-11)                                        MSN Q*..Q**DK*
atCDPK9  (1-22)                             MA*KPR *RW....VLP YKTKNVEDN*
atCDPKb  (1-26)                             METKPN PRRPSNTVLP YQTPRLRDH*
    AK1   (101-150) QETKSET*PE SKPDPPAKPK KPKHMKRV*S A*LRTESVLQ RKTENFKEF*
atCDPK3  (1-24)                             MRR..N VDNQSYYVLG HKTPN*RDL*
atCDPK6  (29-128)   VKPAGERRGS SGSGTVGSSG SGTGGSRSTT STQQNGRILG RPMEEVRRT*


atCDPK19 (58-107)   DLGREVGRGE FGITYLCTDI KTGEKYACKS ISKKKLRTAV DIEDVRREVE
atCDPKa  (12-61)    I****L**** *********R E*H*AL**** ***R****** *********A
atCDPK9  (23-72)    F**QVL*Q*Q **T*F***HK Q**Q*L**** *P*R**LCQE *YD**L**IQ
atCDPKb  (27-76)    L**KKL*Q*Q **T*****EK S*SAN***** *P*R**VCRE *Y***W**IQ
    AK1   (151-200) S***KL*Q*Q **T*F**VEK T**KEF**** *A*R**L*DE *V******IQ
atCDPK3  (25-74)    T*S*KL*Q*Q **T******* A**VD***** ***R**ISKE *V******IQ
atCDPK6  (79-128)   EF***L***Q **V***V*HK E*KQQV**** *PTRR*VHKD *********Q
                          a

atCDPK19 (108-157)  IMKHMPRHPN IVSLKDAFED DDAVHIVMEL CEGGELFDRI VARGHYTERA
atCDPKa  (62-111)   **STL*E*** V*K**ASY** NEN**L**** ********** **********
atCDPK9  (73-122)   **H*LSEY** V*RIES*Y** TKN**L**** ********** *K****S**E
atCDPKb  (77-126)   **H*LSE*** V*RI*GTY** SVF******V ********** *SK***S**E
    AK1   (201-250) **H*LAG*** VI*I*G*Y** VV***L***C *A******** IQ*******K
atCDPK3  (75-124)   **H*LAG*K* **TI*G*Y** PLY****I** *A******** IH****S**K
atCDPK6  (129-178)  **H*LSG*R* **D**G*Y** RHS*NLI*** ********** ISK*L*S***


atCDPK19 (158-207)  AAAVMKTILE VVQICHKHGV MHRDLKPENF LFANKKETSA LKAIDFGLSV
atCDPKa  (112-161)  ****AR**A* **MM**SN** ********** *******N*P **********
atCDPK9  (123-172)  **KLI***VG **EA**SL** V********** **SSSD*DAS ***T******
atCDPKb  (127-176)  *VKLI***LG **EA**SL** ********** **DSP*DDAK **ST******
    AK1   (251-300) **ELTR**VG **EA**SL** ********** **VS*H*D*L **T******M
atCDPK3  (125-174)  **ELT*I*VG **EP**SL** ********** *LV**DDDFS **********
atCDPK6  (179-228)  **DLCRQMVM **HS**SM** ********** **LS*D*N*P ***T******
          •                                 b

atCDPK19 (208-257)  FFKPGEGFNE IVGSPYYMAP EVLRRNYGPE VDIWSAGVIL YILLCGVPPF
atCDPKa  (162-211)  *****DK*T* ********** ***K*D***G **V******I ******A***
atCDPK9  (173-222)  *CT***A*S* L***A**V** ***HKH**** C*V******* ******F***
atCDPKb  (177-226)  *Y***QYLYD V******V** ***KKC**** I*V******* ****S*****
    AK1   (301-350) ****DDV*TD V******V** ****KR**** A*V******V ****S*****
atCDPK3  (175-224)  *****QI*KD V******V** ***LKH**** A*V*T***** ****S*****
atCDPK6  (229-278)  *****DK*KD L***A**V** ***K****** A********* ****S*****


atCDPK19 (258-307)  WAETEQGVAQ AIIRSVIDFK RDPWPRVSET AKDLVRKMLE PDPKKRLSAA
atCDPKa  (212-261)  ********L **L*G*L*** *****QI**S **S**KQ**D ***T***T*Q
atCDPK9  (223-272)  ***S*I*IFR K*LQGKL**E IN***SI**S ****IK**** SN*****T*H
atCDPKb  (227-276)  *****S*IFR Q*LQGKL*** S****TI**A ****IY**** RS*******H
    AK1   (351-400) *******IFE QVLHGDL**S S****SI**S *********V R******T*H
atCDPK3  (225-274)  ****Q**IFD *VLKGY***D T****VI*DS ****I****C SS*SE**T*H
atCDPK6  (278-328)  *G*N*T*IFD **LQGQL**S A****AL*DG *********K Y***D**T**


atCDPK19 (308-357)  QVLEHSWIQN AKKAPNVSLG ETVKARLKQF SVMNKLKKRA LRVIAEHLSV
atCDPKa  (262-311)  ***A*P**** *******P** DI*RS***** *M**RF**KV *********I
atCDPK9  (273-322)  ***C*P**VD D*V**DKP*D CA*VS***NG *A******M* ******R**E
atCDPKb  (277-326)  EA*C*P**VD EQA**DKP*D PA*LS***** *Q***I**M* ******R**E
    AK1   (401-450) ***C*P*V*V DGV**DKP*D SA*LS*M*** *A***F**M* ******S**E
atCDPK3  (275-324)  E**R*P**CE NGV**ARA*D PA*LS***** *A******M* *K****S**E
atCDPK6  (329-378)  E**N*P**RE DGE*SDKP*D NA*LS*M*** RA******M* *K****N**E


atCDPK19 (358-407)  EEVAGIKEAF EMMDSKKTGK INLEELKFGL HKLGQQQIPD TDLQILMEAA
atCDPKa  (312-360)  Q**EV**NM* SL**DD*D** *TYP***A** Q*V*.S*LGE PEIKM***V*
atCDPK9  (323-371)  **IG*L**L* K*I*TD*S*T *TF****D*M RRV*.SELME SEI*E*LR**
atCDPKb  (327-375)  **IG*L**L* K*I*TDNS*T *TF****A** KRV*.SELME SEIKS**D**
    AK1   (451-500)  **I**L**M* N*I*AD*S*Q *TF****A** KRV*.ANLKE SEILD**Q**
atCDPK3  (325-373)  **I**LRAM* *A**TDNS*A *TFD***A** R*Y*.STLES *EIHD**D**
atCDPK6  (379-427)  **II*L**M* KSL*TDNN*I VT****RT** P***.SK*SE AEIRQ*****
                              T
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atCDPK19  (408-457) DVDGDGTLNY GEFVAVSVHL KKMANDEHLH KAFSFFDQNQ SDYIEIEELR
atCDPKa   (361-410) ****N*F*D* ******II** Q*IE***LFK L**M***KDG *T***LD***
atCDPK9   (372-421) ***ES**ID* ***L*ATI** N*LERE*N*V A******KDA *G**T****Q
atCDPKb   (376-425) *I*NS**ID* ***L*ATL*M N**ERE*I*V A***D**KDG *G**T*D**Q
    AK1   (501-550) ***NS**ID* K**I*ATL** N*IERED**F A**TY**KDG *G**TPD**Q
atCDPK3   (374-423) ***NS**ID* S**I*ATI** N*LERE***V S**QY**KDG *G**T*D**Q
atCDPK6   (428-477) *M**D*SID* L**ISATM*M N*IERED**Y T**Q***NDN *G**TM***E
                        II                                  III

atCDPK19  (458-507) EALNDEVDTN SEEVVAAIMQ DVDTDKDGRI SYEEFAAMMK AGTDWRKASR
atCDPKa   (411-460) ***A**LGEP DAS*LSD**R E********* N*D**VT*** **********
atCDPK9   (422-469) Q*W.K*FGI* DSN.LDEMIK *I*Q*N**Q* D*G**V***R K*NGTGGGIG
atCDPKb   (426-471) S*C.T*FGLC DTP.LDDMIK EI*L*N**K* DFS**T***R K*DGVGRS..
    AK1   (551-597) Q*C.E*FGVE DVR.IEEL*R ***Q*N**** D*N**V***Q K*SITG.GPV
atCDPK3   (424-468) QSC.I*HGMT DVF.LED*IK E**Q*N**** D****V***Q K*N..A.GVG
atCDPK6   (478-526) L*M.KKYNMG DDKSIKE*IA E*****R**K* N****V**** K*NPELVPN*
                                                 IV

atCDPK19  (508-533) QYSRERFNSL SLKLMREGSL QLEGEN
atCDPKa   (461-483) *Y*****K** *IN**KD*** H*HDALTGQT VPV
atCDPK9   (470-490) RRTMRNSLNF GTT*PD*SMN V
atCDPKb   (472-495) .RTMMKNLNF NIA...DAFG VDGEKSDD
    AK1   (598-611) KMGL*KSF*I A*K*
atCDPK3   (469-483) RRTMKNSLNI *MRDV
atCDPK6   (527-529) RRM
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Fig. 1. Amino acid alignment of CDPKs from *Arabidopsis thaliana*. AK1: CDPK1 from *Arabidopsis* by Harper *et al.* [12]. atCDPKa and atCDPKb from *Arabidopsis* by Urao *et al.* [21] and atCDPK3 from *Arabidopsis thaliana* by Urao *et al.* [20]. atCDPK6, 9, and 19 are described in this paper. atCDPK19 is a composite sequence with the N-terminal most sequence being derived from the cognate genomic clone. Stars denote sequence identity to that of atCDPK19 and gaps represented by dots are introduced to maximise alignment. a, b are signature domains of serine/threonine kinases and I, II, III and IV are calcium binding EF hands.

protein kinase in *A. thaliana*, we carried out PCR amplification of a cDNA library of *A. thaliana* with degenerate oligonucleotides. One of these fragments was found to be very similar in sequence to the soybean CDPK cDNA. Using this fragment as a probe, a 1.8 kb cDNA clone was isolated from a λZAPII cDNA library of *A. thaliana*. This cDNA encodes a protein (atCDPK9) of 490 amino acid residues with a molecular weight of 55 376. There was an in-frame translation stop codon upstream of the first ATG.

The protein sequences of atCDPK6, atCDPK9 and atCDPK19 all show homology to the soybean CDPK [11], the carrot CDPK [19] and AK1 from *Arabidopsis* [12]. Recently, Urao *et al.* [20, 21] identified three additional *Arabidopsis* cDNA sequences encoding three novel CDPKs. Figure 1 shows the amino acid sequence alignment of all the CDPKs from *Arabidopsis thaliana*. All encoded proteins share a common structure: the amino terminus contains sequences typical of catalytic domains of serine/threonine protein kinases and the carboxyl terminus contains four putative EF-hand Ca^{2+}-binding motifs. Important residues for a helix-loop-helix structure are conserved in each EF-hand motif. The atCDPKs differ from each other mostly in the extreme N and C termini although there are also some differences in the EF-hand structures.

Recombinant atCDPK6 and 9 are Ca^{2+}-dependent protein kinase

Recombinant atCDPK9 was insoluble in E. coli, and was purified from guanidium HCl extracts of inclusion bodies using Mono-Q ion-exchange chromatography. atCDPK6 was more soluble although the expressed protein had extra heterologous 21 amino acids at the N-terminus, among which were 10 consecutive histidines. The polyhistidine tag was used for affinity purification with His.Bind resins. After elution, the purified protein contained 80–90% of a major band of the expected size, as determined by SDS-PAGE.

Using histone III-S as a substrate, both atCDPK6 and atCDPK9 were found to be fully functional calcium-dependent protein kinases.

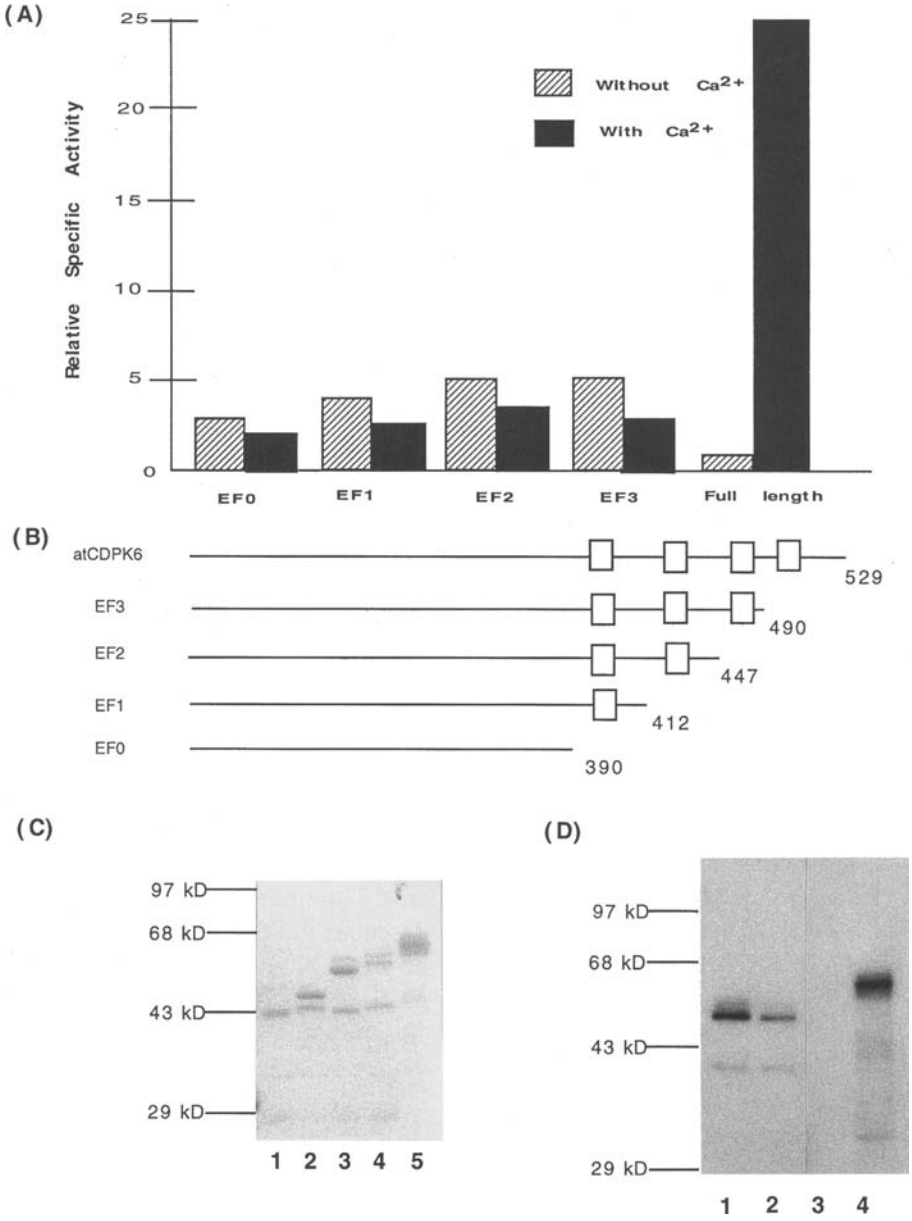


Fig. 2. Kinase activity of full-length atCDPK6 and deletion mutants. A. A graphic representation of kinase activities of different deletion mutants of atCDPK6 in the presence (solid black bar) or absence (striped bar) of Ca^{2+}. The basal kinase activity of the full-length atCDPK6 was taken as one unit of kinase activity. B. A schematic representation of the structures of different deletion mutants of atCDPK6. Open boxes represent EF hands structure. The full-length atCDPK6 contains 529 amino acids. The deletion mutants EF3, EF2, EF1 and EF0 contain 490, 447, 412 and 390 amino acids respectively. C. Protein prifiles of the purified atCDPK6 and derivatives. 5μg each of the purified full-length atCDPK6 and derivatives were separated on 10% SDS-PAGE and stained with Coomassie brilliant blue. Lane 1, atCDPK6 with no EF hand; lane 2, atCDPK6 with 1 EF hand; lane 3, atCDPK6 with 2 EF hands; lane 4, atCDPK6 with 3 EF hands; lane 5, full-length atCDPK6. Molecular size markers are given on the left. D. Comparison of autophosphorylation of full-length atCDPK6 (lanes 3 and 4) with that of EF2 (lanes 1 and 2) in the presence (lanes 2 and 4) and absence of Ca^{2+} (lanes 1 and 3). Molecular size markers are given on the left.

There was very little or no kinase activity in the absence of Ca^{2+}; however, in the presence of Ca^{2+}, kinase activity was stimulated. atCDPK6 was a active kinase with specific activity of 18 nmol phosphate/mg^{-1} min^{-1}. Its activity was stimulated about 25 times by Ca^{2+}. atCDPK9 was less active as compared to atCDPK6. It had a specific activity of 1–2 nmol phosphate Hmg^{-1} min^{-1} and induction by Ca^{2+} was only about 3–5 times. Besides phosphorylating substrates, both atCDPKs also underwent autophosphorylation which was also Ca^{2+}-dependent.

All the 4 EF hands of CDPK are needed for Ca^{2+} response

To investigate the importance of the EF hand structure to the atCDPK kinase activity as well as the Ca^{2+} response, we constructed cDNA derivatives encoding proteins with different numbers of EF hands (0, 1, 2, 3). Full-length atCDPK6 and four deletion derivatives were assayed for kinase activity as well as Ca^{2+} dependence. The protein profiles in Fig. 2C show that the major bands for atCDPK6 and derivatives were of the correct sizes: 59.3 kDa for atCDPK6, 54.7 kDa for atCDPK6(EF3), 49.6 kDa for atCDPK(EF2), 45.7 kDa for atCDPK6(EF1), 43.6 kDa for atCDPK(EF0).

Figure 2A shows that the full length atCDPK6 protein, in the presence of 1 mM EGTA and depleted of Ca^{2+}, had a basal level of kinase activity. Upon addition of Ca^{2+}, the activity increased about 25 times over the basal level. In the absence of Ca^{2+}, the atCDPK6 deletion derivatives showed kinase activity 2.9–5.3 times of the basal level of the full-length protein. Derivatives with more EF hands appeared to be more active. In contrast to the full-length atCDPK6, kinase activities of the deletion mutants were not stimulated by Ca^{2+} but instead were slightly inhibited. This result demonstrates that all the four EF hands are needed to mediate the Ca^{2+} response.

Biochemical similarities of atCDPK6 to calmodulin-dependent kinase

As mentioned earlier, CDPK contains a serine/threonine kinase domain at the N-terminus and a calmodulin-like structure at the C-terminus. We tested the effects of kinase inhibitors as well as calmodulin antagonists on atCDPK6 activity.

Table 1 shows staurosporin, a general serine/threonine kinase inhibitor, was a very effective inhibitor of atCDPK6 with a Ki of 135 nM. Two other kinase inhibitors, KN252a and KN62, were also effective although higher concentrations were required. Of the two calmodulin antagonists tested, both inhibited atCDPK6 activity, suggesting that the CaM-like domain is important for kinase activity.

Studies on mammalian Ca^{2+}/calmodulin-dependent protein kinase II [18] suggested that the substrate binding site in the catalytic core is normally blocked by an autoinhibitory domain thus rendering the enzyme inactive. Binding of Ca^{2+}/calmodulin disrupts the interaction between the autoinhibitory and the catalytic domain, thereby activating the kinase and allowing autophosphorylation of the kinase as well as phosphorylation of exogenous substrates. To see whether atCDPK6 might contain a similar autoinhibitory domain, four oligopeptides covering the junction sequence (amino acids 293 to 376) between the kinase and the calmodulin-like domains were synthesized and assayed for possible inhibitory activity, only one oligopeptide with the sequence RMKQFRAMNKLKKMALDVIAENL (amino acids 354–376 of atCDPK6) was found to be effective (85% inhibition at 100 μM), suggesting that it may constitute a part of the

Table 1. Inhibition of atCDPK6 activity.

Inhibitors	K_i (μM)
Staurosporin	0.135
KN252a	0.609
KN62	21.5
Trifluoperazine	38
calmodazolium	4.2

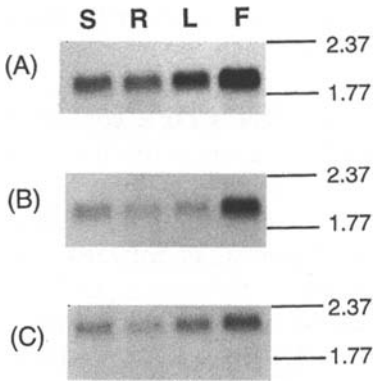


Fig. 3. Distribution of *atcdpk* mRNAs in various plant organs. About 3 μg of poly(A) RNA of root (R), stem (S), leaf (L) and flower (F) were loaded in each lane. The same filter was hybridized sequentially with labelled cDNAs encoding atCDPK6 (A), atCDPK9 (B) and atCDPK19 (C). Low- and high-molecular-weight RNAs (Gibco-BRL) were used as markers. Numbers are in kb.

autoinhibitory domain. This result confirms the finding of Harmon *et al.* [9] that the junction domain in the soybean CDPK can function as an autoinhibitory domain.

The atCDPKs are ubiquitously expressed in the plant

To study the expression pattern of the atCDPKs, Poly(A) RNA isolated from different plant organs isolated were analyzed by RNA gel blot hybridization using as probes cDNAs of *atcdpk6*, *atcdpk9* and *atcdpk19*. These probes did not cross hybridize with each other and thus were gene-specific. Fig. 3 shows that all the three *atcdpk* genes are ubiquitously expressed. The transcript size of *atcdpk6* (1.8 kb) and *atcdpk9* (1.9 kb) corresponded to the size of their respective cDNA clone isolated. The transcipt size of *atcdpk19* was about 2.2 kb, 0.3 to 0.4 kb longer than that of the corresponding cDNA, indicating the latter was not full-length.

atcdpk6, atcdpk9 *and* atcdpk19 *are divergent in evolution*

Southern blot hybridizations show only one hybridizing band for *atcdpk6* and *atcdpk9*, suggesting that they exist as single-copy genes (Fig. 4). On the other hand, the hybridization pattern for

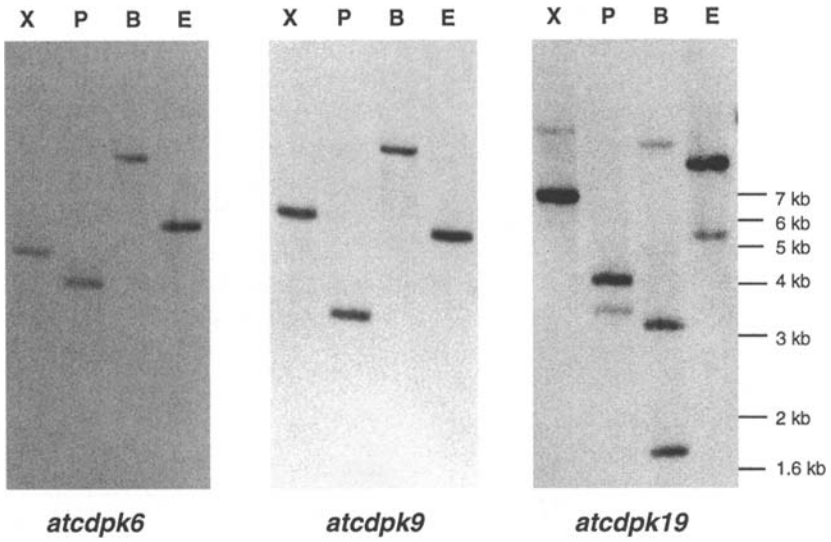


Fig. 4. Southern blot hybridization. Genomic DNA from *Arabidopsis thaliana* was digested by *Xba*I (X), *Pst*I (P), *Bam*HI (B), or *Eco*RI (E). Digested DNA samples (10 μg) were subjected to electrophoresis on an agarose gel, transferred to a Hybond N membrane and probed with random-primer-labelled cDNAs encoding atCDPK6, atCDPK9 and atCDPK19. A 1 kb DNA ladder (Gibco-BRL) was used as molecular size markers.

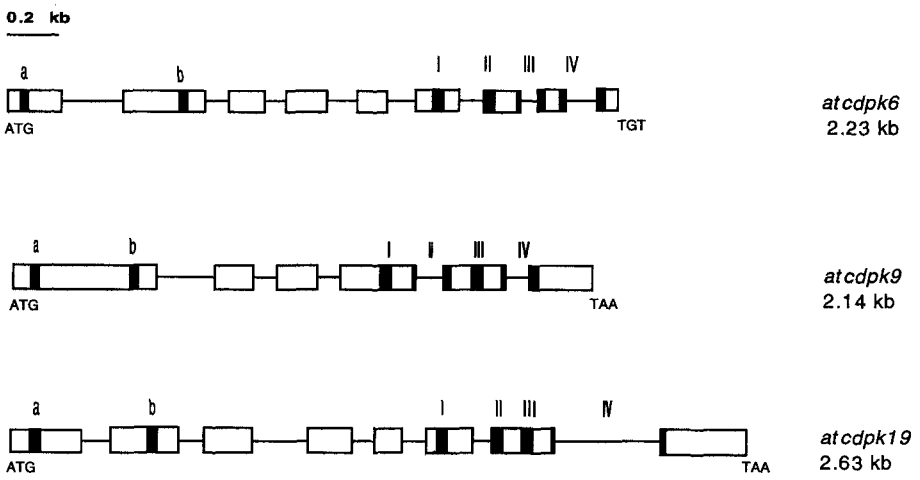


Fig. 5. Structures of the three *atcdpk* genes. Structures of the 3 *atcdpk* genes are represented in schematic forms. Exons are shown as boxes whereas introns are shown as lines. a and b are DNA sequences endoding signature domains of serine/threonine kinases and I, II, III and IV encode Ca^{2+}-binding EF hands. The start codon ATG and the stop codons TGT, TAA are indicated.

atcdpk19 was more complex, suggesting that it is encoded by two related genes.

The same *atcdpk* probes used for RNA gel and Southern blot hybridizations were used to screen an *Arabidopsis* genomic library (Stratagene). Five clones encoding atCDPK6, 4 clones encoding atCDPK9 and 4 clones encoding atCDPK19 were mapped by restriction enzyme digestion followed by Southern blot hybridization. The clones for each atCDPK shared the same coding sequence by differed from each other in size. One genomic clone each for atCDPK6 (4.9 kb), atCDPK9 (4.4 kb), atCDPK19 (3.3 kb) were fully sequenced. Figure 5 shows the physical maps for the coding sequences of the three *atcdpk* genes. The *atcdpk6* genomic clone contained the entire encoding sequence for atCDPK6 and about 1.6 kb of 5′-upstream sequence, the *atcdpk9* genomic clone contained the whole coding sequence for atCDPK9 and about 1.1 kb of 5′-upstream sequence, and the *atcdpk19* genomic clone contained the whole encoding sequence for atCDPK19 with about 100 bp 5′-upstream sequence. The overall sequence identity among the three genomic clones was only 48–55%. Moreover, the organizations of the three genes are also very different. There are eight introns in the *atcdpk6* gene, ranging in size from 69 to 244 bp. The first intron is the longest. In comparison, there are only five introns in the *atcdpk9* gene, with different locations from those in the *atcdpk6* gene. The introns of *atcdpk9* vary in size from 83 bp to 233 bp. In the *atcdpk6* gene, there are introns separating the coding sequences for EF hands III and IV whereas in the *atcdpk9* gene, there are introns separating coding sequences for EF hands II and IV. In the *atcdpk19* gene, there

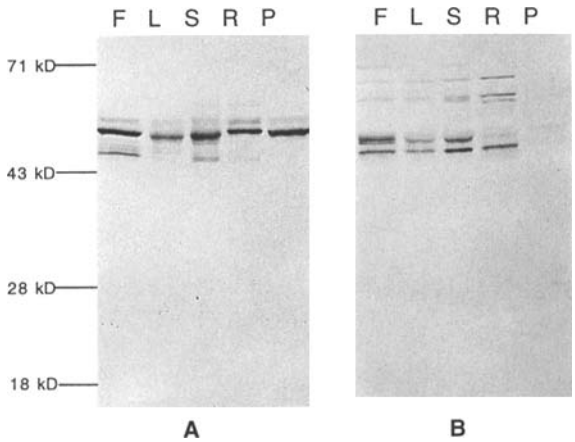
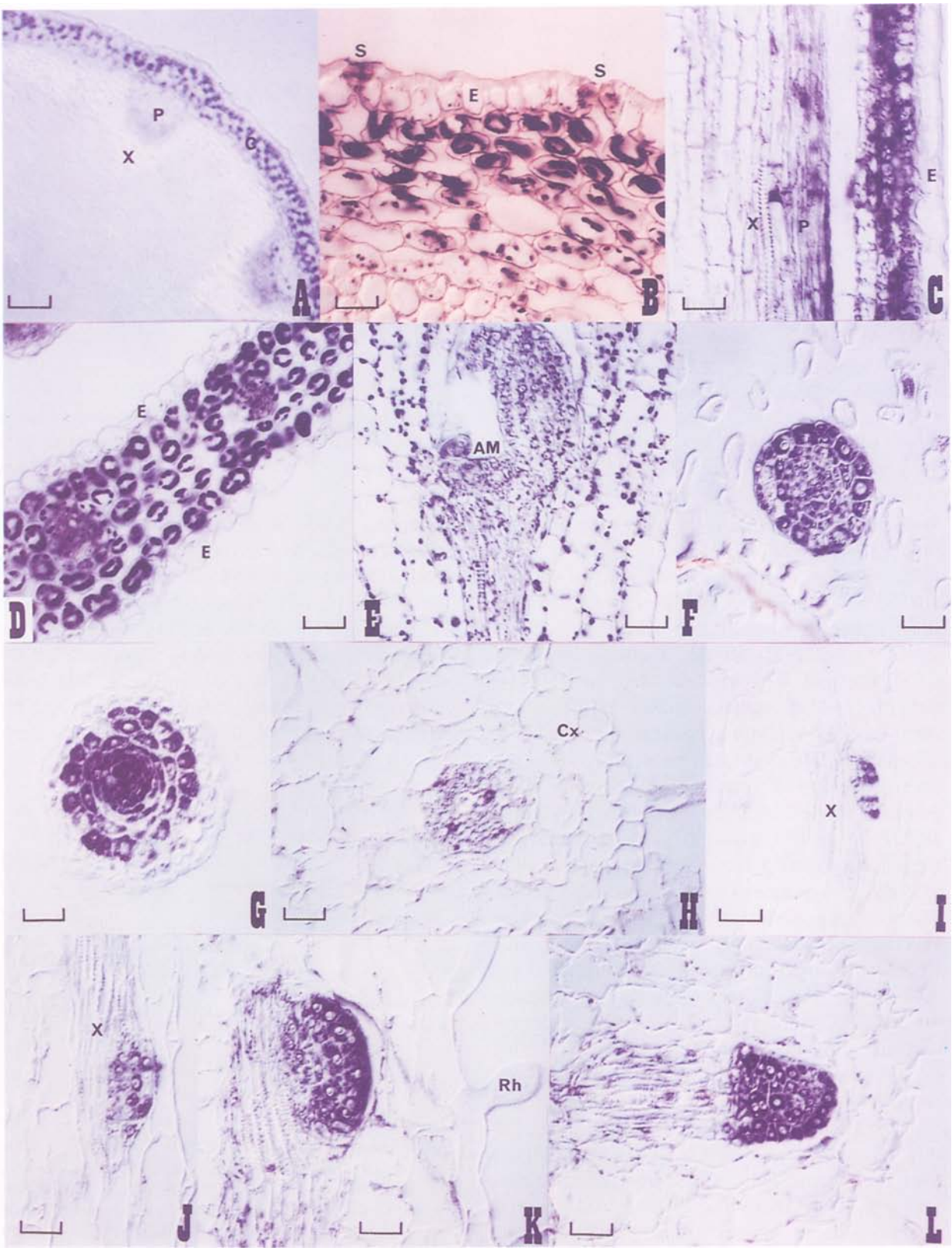


Fig. 6. Western blot analysis of protein extracts from different organs of *Arabidopsis thaliana*. Each lane contained 20 μg of protein extracts. F, flower; L, leaf; S, stem; R, root; P, pod. A. Membrane was probed with affinity purified anti-atCDPK6. B. Membrane was probed with affinity purified anti-atCDPK9.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are 7 introns ranging in size from 72 to 406 bp (the longest is located at the 3′ end) and the EF hand IV is interrupted by an intron. The exon/intron junction sequences of *atcdpk6*, *atcdpk9* and *atcdpk19* conform to the GT/AG rule of plant genes. All these data indicate that the three *atcdpk* genes are divergent in evolution.

Cell-type distribution of atCDPKs

Purified, recombinant atCDPK6 and atCDPK9 were used to raise antibodies in rabbits. Specific antibodies were purified by antigen affinity chromatography. Anti-atCDPK6 IgGs could recognize atCDPK9 and vice versa and both specific IgG preparations also reacted with *E. coli* expressed atCDPK19. The lack of specificity of these IgGs to any individual atCDPK is understandable considering that all the three atCDPKs have similar functional domains and the antisera are polyclonal. Figure 6 shows that the affinity-purified IgGs cross reacted with several bands in different *Arabidopsis* organs. These cross-reacting bands ranged in mass 43–75 kDa, which are likely to be different atCDPKs or their proteolytic products. Because there were no positive bands below 43 kDa, we concluded that calmodulins (less than 40 kDa in size) were not recognized by these IgGs. Although the affinity purified IgGs could not discriminate between different atCDPK isoforms, there was some subtle difference in the pattern obtained with anti-atCDPK6 and that with anti-atCDPK9. Possibly, this might reflect the presence of different amounts of isoform-specific IgG or proteolysis artifact.

Affinity-purified IgGs for atCDPK6 and atCDPK9 were used for cellular immuno-localizations of atCDPKs. The results obtained with anti atCDPK6 were quite similar to those obtained with anti-atCDPK9. Preimmune IgG from the same rabbit, which was used as a negative control, consistently gave no staining. Figure 7 and 8 show immunolocalization results obtained with anti-atCDPK9. Because of the cross-reactivity of these two antibodies and the similarity of the immunostaining results, the results we present here likely reflect the expression of most, if not all, of atCDPKs in the plant.

Figs. 7 and 8 show that chloroplast-containing parenchyma cells in leaves (mesophyll, Fig. 7, D), stem (collenchyma, Fig. 7A–C), sepal (Fig. 8B, H) and silique (Fig. 8L–2N) were all stained by the antibodies, indicating expression of CDPK in these cells. By contrast, other parenchyma cells without chloroplasts, like those in the pith (Fig. 7H) were not stained. Among the cells in the epidermis only the guard cells (Fig. 7B) were stained. Although it is clear that all tissues containing chloroplasts show positive staining, we could not determine whether the CDPK(s) are localized in the cytoplasm, associated with the chloroplast envelope membranes or compartmentalised within the chloroplasts. Recently, we have detected CDPK activity in both chloroplast stromal and thylakoid membrane fractions (data not shown), suggesting that one or more CDPK could be imported into chloroplasts after cytoplasmic synthesis.

In addition to chloroplast-containing tissues, a number of other tissues were also stained positive for CDPK. These include tapetum cells (Fig. 8E and F), nucellus (Fig. 8J), phloem (Fig. 7A and C), sigmatic or papillae cells (Fig. 8C) and endosperm (Fig. 8L). CDPK expression was detected in suspensor cells of embryos at the globular to torpedo shaped stage (Fig. 8L, M), although not at earlier stages (Fig. 8K). Only the cotyle-

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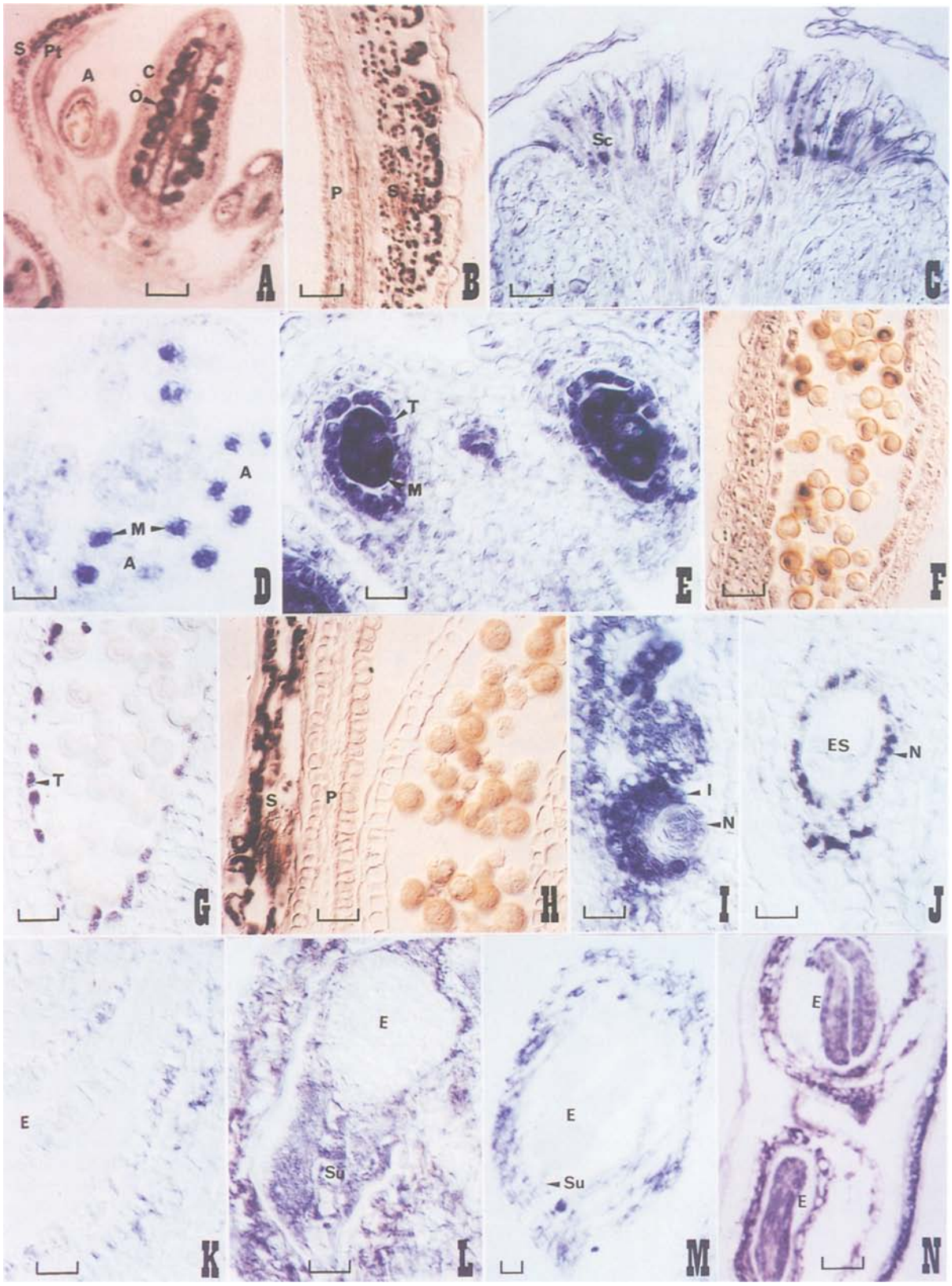
Fig. 7. Immunolocalization of atCDPKs in cells of vegetative organs. A. Transverse section of a stem. B. Transverse section of a stem at a higher magnification. C. Longitudinal section of a stem. D. Transverse section of a leaf. E. Apical meristem of a 4-day old seedling. F. Transverse section of a root tip (meristem region). G. Transverse section of a root tip. H. Transverse section of root tip (root hair region). I–L: Longitudinal section of roots, showing different stages of lateral root development. A: bar = 100 μm; B to L: bar = 25 μm. AM, apical meristem; C, collenchyma; Cx, cortex; E, epidermis; P, phloem; Rh, root hair; S, stomata; X, xylem.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donary-staged embryos (Fig. 8N) were strongly stained by the antibody, whereas globular to torpedo-staged embryos were negative (Fig. 8K–M).

CDPK was present in shoot primordium including apical meristem and leaf primordium (Fig. 7E), floral buds (data not shown), root meristem (Fig. 7F–H) and meristem for producing lateral root (Fig. 7I–L). Although CDPK was detected in microsporocyte mother cells (Fig. 8D and E), the protein level declined during pollen maturation (Fig. 8H).

Discussion

In this paper we describe the cDNA and genomic sequences encoding 3 new *Arabidopsis* CDPKs, designated as atCDPK6, 9 and 19, which are different from the 4 CDPK sequences (AK1, atCDPK1, atCDPKb, and atCDPK3) reported previously [12, 20, 21]. A comparison of the 7 *Arabidopsis* CDPK sequences revealed low sequence identify. Using atCDPK19 as a reference, amino acid sequence identities with atCDPK6, atCDPK9, AK1, atCDPKa, atCDPKb and atCDPK3 are 52%, 51.9%, 52.3%, 67%, 51.8% and 54.8%, respectively. Given this low level of sequence identity it is therefore not surprising that the *cdpk* genes do not cross-hybridize to one another in genomic Southern blot hybridization expriments (Fig. 4).

We also present here for the first time nucleotide sequences of three *A. thaliana cdpk* genes, *atcdpk6, 9* and *19*. In addition to the low sequence homology, it is striking that these 3 genes differ in the number and the size of their introns and exons, as well as the relative locations of the introns. For example, *atcdpk9* contains 5 introns whereas *atcdpk19* has 7 introns and *atcdpk6* had 8 introns. The differences in the genomic structures are particulary evident when the sequences encoding the CaM-like domain are compared. The exon encoding EF hand II in *atcdpk9* but not *atcdpk6* and *atcdpk19* is interrupted by an intron whereas the exon encoding EF hand III in *atcdpk6* but not *atcdpk9* and *atcdpk19* is interrupted (Fig. 5). Based on our results it is unlikely that the *cdpk* genes were derived from a common ancestor by gene duplication events. The results are more consistent with a devergent evolution of the 3 *atcdpk* and with the hypothesis that introns are added later on in evolution.

RNA gel blot analysis revealed that the 3 *atcdpk* genes are expressed at a low level in all tissues examined (Fig. 3).

CDPK belongs to a class of calcium-modulated proteins, including calmodulin, troponin C, myosin light chain, etc., that contain 2 or 4 copies of a calcium-binding structure called EF hand. It is thought that the presence of multiple copies of EF hand in a protein may enhance its affinity for calcium. Each EF hand consists of a loop of 12 amino acid residues, which is flanked by two alpha-helixes. The refinement of the crystal structure of CaM [1] has delineated the crucial role of the conserved glutamic acid at position 12. Recently Zhao *et al.* [25] mutated the conserved glutamic acid residue within the EF hands of *Plasmodium falciparum* CDPK in order to understand the role of the individual calcium-binding site. They found that, for a given EF hand, mutation of the conserved glumatic acid residue to either lysine or glutamine eliminates the calcium-binding activity of the particular EF hand. Although the kinase activity was also affected by the mutation it continued to be calcium-dependent. By contrast, we found that the deletion of just the C-terminal EF hand from atCDPK6 rendered the kinase activity constitutive, although the mutant activity is reduced to about 20% of the calcium-stimulated activity of the wild-type enzyme

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Fig. 8. Immunolocalization of atCDPKs in cells of reproductive organs. A. Longitudinal section of a flower. B. Transverse section of sepal and petal. C. Longitudinal section of a stigma. D–H: Different stages of pollen development. I–N: Different stages of embryo development. A and N: bar = 100 μm. B to M: bar = 25 μm. A, anther; E, embryo; Es, embryo sac; I, integument; M, microsporocyte; N, nucellus; O, ovule; Pt, P, petal; S, sepal; Sc, stigmatic cell; Su, suspensor; T, tapetum.

(Fig. 2). Our result indicates the integrity of all the four EF-hand structures are important for the calcium activation.

Biochemical studies on atCDPK6 were conducted in an attempt to differentiate CDPK from other kinases and from CaM. We found that the kinase inhibitors staurosporin, KN252a and KN62 could all inhibit atCDPK6 activity, suggesting the kinase domains of CDPK are very much similar to those in other serine/threonine kinases. In fact, atCDPK6 was more sensitive to staurosporin (with K_i of 135 nM) than many other kinases. atCDPK6 is also sensitive to the two calmodulin antagonists calmodozolium and trifluoperizine, indicating the Ca^{2+}-binding motifs which are similar to those of calmodulin are also needed for activities. The inhibition of atCDPK6 kinase activity by the synthetic peptide, pep4, confirmed the finding by Harmon *et al.* [9] regarding the presence of an autoinhibitory domain in CDPK. This conclusion was further supported by our finding that the autoinhibitory domain of the Ca^{2+}/calmodulin-dependent protein kinase II (CaM Kinase II), the inhibitory peptide of PKC and the inhibitory peptide of PKA (data not shown) can all inhibit atCDPK6 activity. Together, these results suggest a model silimar to that of CaM Kinase II: in the absence of Ca^{2+}, the autoinhibitory domain blocks the substrate binding and renders the kinase inactive whereas the conformation change upon binding of Ca^{2+} releases the inhibition.

However, experiments on the serial deletion of EF hands structures of atCDPK6 suggested that CDPK may not be simply constituted from a fusion of a kinase with a calmodulin. atCDPK6 mutants with less than four EF hands were constitutively active and not responsive to Ca^{2+}. The mutant activities were 11.6%–21.4% that of wild type in the presence of Ca^{2+}. Even though the putative autoinhibitory domain remained intact in these mutants, it failed to inhibit the kinase activity fully. Our interpretation is that the interdependence of kinase domain with CaM-like domain was established during evolution and the right conformation for normal function requires the contribution of the two domains. It will be interesting to check if the CaM-like domain alone could function as a calmodulin.

The presence of 7, and possibly more, isoforms of CDPK in *A. thaliana* raises the question of their physiological functions. Since the CDPKs differ in their N-terminal sequences it is possible that they are differentially localized within the cell and might even possess different substrate specificities. As a first step toward elucidating the function of the CDPKs, we determined the tissue and cell type distribution of this group of enzymes by immuno-localization. Because polyclonal antibodies were used in these experiments it is very likely that they reacted with all CDPK isoforms. Nevertheless, we found that CDPKs are differentially expressed in cells of *A. thaliana* with specific temporal and spatial patterns. The enrichment of this group of enzymes in chloroplast-containing parenchyma cells, stigmatic (papillae) cell, phloem, tapetal cells, and meristematic cells, etc. (Fig. 7 and 8) suggest a role of these enzymes in these cell types. However, it is clear that the elucidation of the cellular functions of different CDPK isoforms depends critically on the identification of their *in vivo* substrates. In this connection, we note that Weaver *et al.* [23] have reported that nodulin-26, a nodule-specific membrane protein, is a substrate of a soybean CDPK. Future work should be directed toward identification of downstream targets of this group of kinases.

Acknowledgements

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