Characterization of a calmodulin-regulated Ca^{2+} -dependent-protein-kinase-related protein kinase, AtCRK1, from *Arabidopsis*

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An AtCRK1 [*Arabidopsis thaliana* CDPK (Ca^{2+} -dependent protein kinase)-related protein kinase 1] has been characterized molecularly and biochemically. AtCRK1 contains the kinase catalytic domain and a CaM (calmodulin)-binding site. Our results demonstrated that AtCRK1 could bind CaM in a Ca^{2+} -dependent manner. This kinase phosphorylated itself and substrates such as histone H1S and syntide-2 in a Ca^{2+} -independent manner and the activity was stimulated by several CaM isoforms through its CaM-binding domain. This domain was localized within a stretch of

39 amino acid residues at positions from 403 to 441 with $K_d = 67$ nM for CaM binding. However, the stimulation amplification of the kinase activity of AtCRK1 by different CaM isoforms was similar.

Key words: *Arabidopsis thaliana*, autophosphorylation, calmodulin, capillary electrophoresis, Ca^{2+} -dependent protein-kinase (CDPK)-related protein kinase (CRK).

INTRODUCTION

Calcium plays an important role as a second messenger in many developmental and physiological processes in both mammals and plants [1–3]. The roles of Ca^{2+} are mediated by a group of Ca^{2+} -binding proteins including CaM (calmodulin) and CDPKs (Ca^{2+} -dependent protein kinases) [4–6]. CaM is ubiquitously expressed and has no enzymic activity of its own. Thus the Ca^{2+} /CaM-mediated signalling pathways are derived from the activities and expression patterns of CaM-binding proteins including CaMKs (Ca^{2+} /CaM-dependent protein kinases) [6–8]. Unlike the mammalian system, little was known about CaMKs in plants [6,8,9]. Until recently, several CBKs (CaM-binding protein kinases) have been reported. These include CB1, a homologue of mammalian CaMKII from apple [10], MCKs [maize (*Zea mays*) homologue of mammalian CaMKs] from maize [11–13], CCaMKs (chimaeric CaMKs) from lily and tobacco [14–18] and CBKs from rice (OsCBK, where Os stands for *Oryza sativa*) and tobacco (NtCBK2, where Nt stands for *Nicotiana tabacum*) [19,20]. Among them, only CCaMK, OsCBK and NtCBK2 have been well characterized molecularly and biochemically. Furthermore, CCaMK has been reported to have a visinin-like domain with three EF hand motifs that are necessary for Ca^{2+} -dependent autophosphorylation and crucial for maximal activation of CCaMK [15,16,18]. The enzymic activity of OsCBK has been indicated to be independent of either Ca^{2+} or CaM [19].

CDPKs, well-documented protein kinases in plants, consist of an N-terminal catalytic domain, a junction domain and a C-terminal CaM-like sequence with four EF hands for Ca^{2+} binding [4]. The activity of CDPKs is modulated by Ca^{2+} rather than by CaM. In contrast with CDPKs, however, CRKs (CDPK-related protein kinases) do not require Ca^{2+} for their activities [21,22]. The C-terminus of members of CRKs from carrot (*Daucus carota*) [21], maize [22] and *Arabidopsis* share sequence similarity with

CaM without typical EF hand motifs for Ca^{2+} binding, but contain apparently degenerate Ca^{2+} -binding sites. No biochemical evidence for CRKs ability to bind CaM is available.

In the present study, we present our work on an AtCRK1 (*Arabidopsis thaliana* CRK1). Whereas the activity of CRK1 for both autophosphorylation and substrate phosphorylation is Ca^{2+} independent as indicated in the CRK of maize [22], this activity is stimulated by several *Arabidopsis* CaM isoforms through their binding to the CaMBD (CaM-binding domain) in a Ca^{2+} -dependent manner. However, the stimulation amplification of the kinase activity of AtCRK1 by different CaM isoforms was similar.

MATERIALS AND METHODS

Screening of a cDNA library and 5'-RACE (5'-rapid amplification of cDNA ends) cloning

The cDNA library (PRL-2) of *Arabidopsis* from Biological Resource Center at Ohio State was screened with the cDNA encoding maize MCK1 [11]. Positive recombinant phages for CRK1 were isolated, and the inserts of pACK1 for AtCRK1 were sequenced [12]. Total RNA of *Arabidopsis* plants was extracted using the TRIzol[®] reagent according to the manufacturer's instructions (Gibco). The 5'-end of the cDNA was obtained by 5'-RACE using 5'-RACE PCR kit (Gibco). The PCR products were cloned into pGEM-T easy vector (Promega) according to the manufacturer's instructions. The plasmids were sequenced for both DNA strands with a DNA sequencing kit (PE Applied Biosystems, Foster City, CA, U.S.A.) and ABI Prism 377 DNA sequencer.

RNA isolation and Northern blot

Total RNA from *Arabidopsis* was isolated with TRIzol[®] as described by the manufacturer (Gibco) and Northern blotting was

Abbreviations used: CaM, calmodulin; CaMBD, CaM-binding domain; CaMK, Ca^{2+} /CaM-dependent protein kinase; CCaMK, chimaeric CaMK; MCK, maize homologue of mammalian CaMK; CBK, CaM-binding protein kinase; NtCBK2, *Nicotiana tabacum* CBK2; OsCBK, *Oryza sativa* CBK; CDPK, Ca^{2+} -dependent protein kinase; CRK, CDPK-related protein kinase; AtCRK, *Arabidopsis thaliana* CRK; AP1, AtCRK1 partial 1; ORF, open reading frame; RACE, rapid amplification of cDNA ends; TBS, Tris-buffered saline; UTR, untranslated region.

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performed as described previously [11]. In brief, a 20 µg sample of total RNA was separated on a 1.5 % (w/v) formaldehyde agarose gel and blotted on to a nylon membrane (NYTRAN®). The membrane was incubated overnight at 65 °C with the α -³²P-labelled probe made from pAtCRK1 and washed under high stringency conditions.

Construction and expression of AtCRK1 and truncated AtCRK1

To define the CaMBD, several constructs were made with plasmid pFastHTb. The cDNAs for full ORF (open reading frame) (AtCRK1) and three truncated forms [AP1 (AtCRK1 partial 1), AP2 and AP3] of AtCRK1 were amplified with four pairs of primers as follows: 5' primer (5'-CGGGATCCATGGGGATCTGTCATGGAAAACCCGTCGAG-3') and 3' primer (5'-CGGGA-TCCCTAAGCTTTTGTAAAGTCCGAG-3') for AtCRK1; 5' primer (5'-CGGGATCCATGGGGATCTGTCATGGAAAACCCGTCGAG-3') and 3' primer (5'-CGGGATCCTGGCCCCAACATGTGAATTG-3') for AP1 of 441 amino acids that contain the N-terminus of AtCRK1 with the tentative CaMBD; 5' primer (5'-CGGGATCCATGGGGATCTGTCATGGAAAACCCGTCGAG-3') and 3' primer (5'-CGGGATCCTTACAAGCTTGTATATGATC-3') for AP2 of 403 amino acids that contain the N-terminus of CRK1 but lacks the tentative CaMBD; 5' primer (5'-CGGGATCCAAAGTGTACATAATGTCAAC-3') and 3' primer (5'-CGGGATCCCTAAGCTTTTGTAAAGTCCGAG-3') for AP3 of 173 amino acids that contain the C-terminus of AtCRK1 including the tentative CaMBD, overlapping 39 amino acid residues with AP1.

*Bam*HI-digested DNA fragments for AtCRK1, AP1, AP2 and AP3 were cloned into *Bam*HI site of plasmid pFastHTb. After sequencing confirmation, the recombinant plasmids were transformed into DH10Bac-competent cells containing the bacmids (baculovirus shuttle vectors) with a mini-att Tn7 target site and helper plasmid. The mini-Tn7 element on the pFastHTb donor plasmid can transpose to the mini-att Tn7 element on the bacmid in the presence of transposition proteins provided by the helper plasmid. Clones containing the recombinant bacmids were identified based on the disruption of the lacZ gene. The Sf9 cells were maintained as monolayers at 27 °C, supplemented with Grace's medium containing 10 % (v/v) fetal bovine serum and transfected by the recombinant bacmids with CELLFECTIN reagent according to the manufacturer's instructions (Gibco). Recombinant virus was harvested after 72 h and identified by PCR with primers described above.

Purification of AtCRK1 and its truncated forms

The Sf9 insect cells were infected with the recombinant virus for 72 h and harvested at room temperature (25 °C). Cells were washed once with Grace's medium, resuspended in 5 ml of lysis buffer [50 mM Tris/HCl, pH 7.5, 10 % (v/v) glycerol, 1 % Nonidet P40 and 0.2 mM PMSF]. The mixture was sonicated for 30 s, followed by centrifugation at 12 000 g for 10 min. The supernatant was applied to a Ni²⁺-nitrilotriacetate resin column pre-equilibrated with buffer A (50 mM potassium phosphate, pH 6.0, 300 mM KCl and 10 % glycerol). After extensive washing with buffer A and then by buffer A containing 25 mM imidazole, AtCRK1 and its truncated forms of proteins were subsequently eluted with buffer A containing 200 mM imidazole. The purified AtCRK1 and its truncated forms of proteins were dialysed in 25 mM Tris/HCl (pH 7.5) for approx. 6 h and used immediately for SDS/PAGE and enzymic analyses. The protein concentration was determined by the quantification of tryptophan [23]. All procedures were performed at 4 °C unless otherwise noted.

Preparation of biotinylated CaM

Arabidopsis CaMs (AtCaM2, AtCaM4, AtCaM7 and AtCaM8) cDNA cloned into the pET5a and pET24d expression vector (gifts from Dr R. Zielinski, Department of Plant Biology, University of Illinois, Urbana, IL, U.S.A.) were introduced into *Escherichia coli* BL21 (DE3) cells and used for CaM purification as described by Lu and Harrington [24] and Zhang et al. [19]. The purified CaM was biotinylated as described in [19]. Briefly, CaM was dialysed overnight at 4 °C against 0.1 M phosphate buffer (pH 7.4). The dialysis residue was supplemented with 1 mM CaCl₂ and incubated with D-biotin- ϵ -amidocaproic acid-*N*-hydroxysuccinimide ester (Sigma) dissolved in *N,N*-dimethylformamide at a final concentration of 1 mM. The incubation was performed for 2 h at 4 °C with constant stirring. The biotinylated CaM was further dialysed in 0.1 M phosphate buffer (pH 7.4) for 48 h at 4 °C and stored in 20 % glycerol at -20 °C until use.

CaM-binding assay

The proteins separated on SDS/polyacrylamide gels were electrophoretically transferred on to PVDF membrane in transfer buffer [20 mM Tris/HCl, pH 8.3, 150 mM glycine and 20 % (v/v) methanol] for 2 h at 75 V using a Bio-Rad Mini-Trans-Blot apparatus as described previously [19]. The membrane was blocked in 2 % (w/v) BSA/TBS (Tris-buffered saline) (50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 50 mM MgCl₂, 0.1 mM CaCl₂ or 2 mM EGTA) and washed three times with TBS for 15 min each. After incubation in TBS containing biotinylated CaM for 3 h at room temperature (25 °C) and then washing with TBS, the membrane was treated with avidin-horseradish peroxidase conjugate (Bio-Rad Laboratories) dissolved in TBS for 1 h. The protein bound to biotinylated CaM was visualized by developing with 4-chloro-1-naphthol and H₂O₂.

CaM affinity measurement

The experiments were performed on a cuvette-based resonant mirror system (IASys) from Affinity Sensors (Thermo Labsystems, Cambridge, U.K.); IASys utilizes a resonant mirror technique and the surface consists of a combination of high and low refractive index layers on a prism and a cuvette system for liquids, allowing real-time observations of the relative amount of molecules bound to the ligand on the sensor surface. Data acquisition was performed at 25 °C under stirring at 100 rev./min. The running buffer used was 25 mM Tris/HCl (pH 7.5), containing 0.1 mM CaCl₂ and 100 mM NaCl. The cuvette was immobilized with purified AtCaM at 25 °C. AtCRK1 with a concentration range 60–400 nM was added to the CaM-loaded cuvette with a defined volume of 50 µl. After each measurement, the sensor surface was regenerated by washing three times with washing buffer (25 mM Tris/HCl, pH 7.5 and 100 mM NaCl) to liberate the molecules from the ligand, and then washed three times with running buffer for the next measurement. The rate constants, K_{diss} and K_{ass} , were respectively read from the slope and the y-intercept in a linear plot of the K_{on} value against the concentration calculated using the Fast-Fit program as described by Kamei et al. [25]. The dissociation constant K_d was then calculated from $K_{\text{diss}}/K_{\text{ass}}$.

Autophosphorylation and substrate-phosphorylation assays of AtCRK1

AtCRK1 autophosphorylation was performed in a 100 µl of reaction mixture containing 25 mM Tris/HCl (pH 7.5), 0.5 mM dithiothreitol, 10 mM magnesium acetate, 100 µM ATP, 10 µCi [γ -³²P]ATP (5000 Ci/mmol) plus 0.1 mM CaCl₂ or 0.1 mM

CaCl₂/1 μ M CaM or 2 mM EGTA at 30 °C for 30 min. For time-course assays, 2 μ g of the kinase protein was used in 100 μ l reaction mixtures. Aliquots for the zero time point were taken immediately after the addition of AtCRK1 to initiate the reaction, and the reactions were terminated by adding 1/5 volume of 5 $\times$ SDS/PAGE sample buffer. All aliquots were separated by SDS/PAGE with 10 % separating gel. After staining with 0.1 % Coomassie Brilliant Blue, the gels were vacuum dried and exposed to X-ray film at – 80 °C. The amount of phosphates transferred to the enzyme was determined by counting the radioactivities of the excised AtCRK1 bands in a liquid-scintillation counter (Beckman LS 6500). The experiments were repeated three times in duplicate.

Substrate phosphorylation by AtCRK1 was performed as described above in addition to 100 μ M histone H1S or 100 μ M syntide-2. For time-course assays with histone H1S as substrate, aliquots for the zero time point were taken immediately after the addition of AtCRK1 to initiate the reaction, and the reaction was terminated by adding 1/5 volume of 5 $\times$ SDS/PAGE sample buffer. Aliquots were separated by SDS/PAGE with 10 % separating gel and after staining with 0.1 % Coomassie Brilliant Blue, the gels were vacuum dried and exposed to X-ray film at – 80 °C. The amount of phosphates transferred to histone H1S was determined by counting the radioactivities of the excised histone H1S bands in a liquid-scintillation counter. The experiments were repeated three times in duplicate.

For time-course assays with syntide-2 as substrate, the experiments with two parallel reactions for different treatments were repeated three times. Aliquots for the zero time point were taken immediately after the addition of AtCRK1 to initiate the reaction and the reaction was terminated by adding 1/5 volume of 5 $\times$ SDS/PAGE sample buffer. Aliquots from one reaction were separated by SDS/PAGE with 10 % separating gel. The amount of phosphates transferred to the kinase was determined by counting the radioactivities of the excised AtCRK1 bands in a liquid-scintillation counter. Aliquots from another reaction were applied to P81 phosphocellulose filters (2 cm $\times$ 2 cm squares; Whatman) for total ³²P incorporation of the kinase and syntide-2. Filters were washed four times for 10 min each in 75 mM phosphoric acid and rinsed in 100 % ethanol and air-dried. ³²P incorporation was determined by liquid-scintillation counting. The amount of phosphates transferred to syntide-2 was determined by subtracting ³²P incorporation of AtCRK1 from total ³²P incorporation of AtCRK1 plus syntide-2.

Assay of AtCRK1 activity

The reaction for AtCRK1 substrate phosphorylation was performed as described above. Aliquots (10 μ l) were removed and applied to P81 phosphocellulose filters (2 cm $\times$ 2 cm squares; Whatman). Filters were washed four times for 10 min each in 75 mM phosphoric acid, rinsed in 100 % ethanol and air-dried. ³²P incorporation was determined by liquid-scintillation counting (Beckman LS 6500).

Phospho-amino acid assays

Both unphosphorylated and autophosphorylated AtCRK1 were hydrolysed in 6 M HCl for 12 h at 110 °C, then dried and dissolved in 20 μ l of 10 mM borate buffer (pH 10.0). The hydrolysed product was mixed with 20 μ l of 1 mM FITC dissolved in acetone containing 0.05 % pyridine (Sigma) and incubated in the dark for 12 h at room temperature. To prepare FITC-tagged standard amino acids and phosphoamino acids, 2 μ l of standard solution containing each amino acid and phospho-amino acid (0.5 mM each) was mixed with 46 μ l of 1 mM FITC, 100 μ l of

20 mM borate buffer (pH 10.0) and 52 μ l of water. The mixture was incubated in the dark for 12 h at room temperature. FITC-tagged amino acids were analysed by capillary electrophoresis as described in [26]. Data were collected by a computer with Spot Advanced software, and further processed with Scion Image and Origin software packages.

RESULTS

Cloning and characterization of AtCRK1 cDNA

An *Arabidopsis* cDNA library was screened using *MCK1* as a probe, resulting in the isolation of a cDNA clone with a 1556 bp insert including 3'-UTR (3'-untranslated region). Additional sequence for the 5'-end of the cDNA was obtained by RACE. This sequence, therefore, has been extended to 2102 bp (GenBank[®] accession numbers AF435448 and AF153351), consistent with the size of the mRNA indicated by Northern-blot analysis (results not shown). The pAtCRK1 contains an ORF of 1731 bp with a start codon ATG at positions 106–108 and a stop codon TGA at positions 1834–1836. This clone was determined to be full-length for the ORF based on the presence of a stop codon TGA in frame at the positions 52–54 in the 5'-UTR (Figure 1).

The deduced amino acid sequence of AtCRK1 consists of 576 amino acids with a calculated molecular mass of 63.4 kDa (Figure 1). It contained all 11 subdomains characteristic of the protein kinase catalytic domain with all the conserved amino acid residues [27,28]. AtCRK1 also shares high sequence identity with CBKs including maize MCK1 (GI number 1839597) (46 %) [11], OsCBK (GenBank[®] accession number AF368282) (72 %) [19] and NtCBK2 (GenBank[®] accession number AF435452) (71 %) [20]; CRKs: carrot DcCRK (GI number 1103386) (71 %) [21] and maize ZmCRK (GI number 1313909) (66 %) [22], whereas it shows low sequence identity with CcAMK such as lily CcAMK (GI number 860675) (19 %) [14] and CDPK such as AtCDPK (GI number 1220099) (32 %) [29].

AtCRK1 did not have typical Ca²⁺-binding domain (EF hand) as shown in CDPKs, but has degeneracy of sequence as an EF-hand motif for potential Ca²⁺ binding [21,22]. Whereas no biochemical evidence for CRKs to bind CaM is available, prediction of secondary structure using the anthwin 45 package suggests that amino acid residues at positions from Leu⁴¹² to Leu⁴²⁹ (LRKSALAA-LAKTLTVPQL) of AtCRK1 might form a basic amphiphilic α -helix, characteristic of CaMBD (Figure 2C) [30].

Characterization of the CaMBD of AtCRK1

Structure prediction of AtCRK1 suggests its possible CaM-binding ability, as indicated above, and this observation was also supported by the fact that AtCRK1 could bind to CaM-Sepharose 4B in the presence of Ca²⁺ and is eluted from it in the absence of calcium (results not shown). The experiments were performed to identify the CaMBD of AtCRK1. For this, the purified AtCRK1 was separated by SDS/PAGE, and then blotted on to PVDF membrane for CaM-binding assay with biotinylated CaM. Our results indicated that AtCRK1 bound to CaM in the presence of Ca²⁺ and lost its binding ability in the presence of EGTA (Figure 2B), demonstrating AtCRK1 as a CaM-binding protein.

To define further its CaMBD, three constructs (pAP1, pAP2 and pAP3) were made to express different truncated forms (AP1, AP2 and AP3) of AtCRK1 for the CaM-binding assay (Figure 2A). Whereas AP2 contains the N-terminus of AtCRK1, AP1 has the same amino acid sequence as pAP2 plus the tentative CaMBD.

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1      TTT CTT GCT TCT CAC ATG AAT CTG TTT CGC AAC CTA ACT TAT TCA TCG GAG TGA TAT CGA
61      TAT TCT TCT CAA ATT TTG AAG CTT TTC GTT TCG AAT TGA CAA TTC ATG GGG ATC TGT CAT
                                         M   G   I   C   H
121     GGA AAA CCC GTC GAG CAA CAA TCA AAA AGC CTC CCA GTT TCC GGC GAA ACT AAT GAA GCT
      G   K   P   V   E   Q   Q   S   K   S   L   P   V   S   G   E   T   N   E   A
181     CCG ACG AAT TCA CAG CCA CCG GCG AAA TCC TCT GGC TTT CCG TTT TAT AGC CCG AGT CCT
      P   T   N   S   Q   P   P   A   K   S   S   G   F   P   F   Y   S   P   S   P
241     GTA CCT AGC TTA TTC AAA TCC TCA CCA TCT GTA TCT TCT AGC GTG AGC TCA ACG CCT CTT
      V   P   S   L   F   K   S   S   P   S   V   S   S   S   V   S   S   T   P   L
301     CGT ATC TTC AAG AGA CCT TTC CCT CCT TCT CCT GCT AAG CAC ATA CGA GCT TTC CTC
      R   I   F   K   R   P   F   P   P   P   S   P   A   K   H   I   R   A   F   L
361     GCG CGT CGC TAT GGC TCT GTC AAG CCT AAT GAG GTT TCG ATT CCC GAA GGT AAA GAA TGT
      A   R   R   Y   G   S   V   K   P   N   E   V   S   I   P   E   G   K   E   C
421     GAG ATC GGT CTC GAT AAG AGT TTT GGC TTT TCA AAA CAG TTT GCT TCA CAC TAT GAG ATT
      E   I   G   L   D   K   S   F   G   F   S   K   Q   F   A   S   H   Y   E   I
481     GAT GGT GAG GTG GGT CGT GGA CAT TTT GGA TAT ACT TGC TCT GCT AAG GGT AAA AAA GGT
      D   G   E   V   G   R   G   H   F   G   Y   T   C   S   A   K   G   K   K   G
541     AGC TTG AAA GGT CAA GAA GTT GCT GTT AAA GTT ATT CCT AAA TCA AAG ATG ACA ATC GCA
      S   L   K   G   Q   E   V   A   V   K   V   I   P   K   S   K   M   T   T   A
601     ATT GCG ATT GAG GAT GTT AGT AGA GAA GTG AAG ATG TTG CGG GCT TTG ACT GGT CAT AAG
      I   A   I   E   D   V   S   R   E   V   K   M   L   R   A   L   T   G   H   K
661     AAC CTA GTC CAG TTC TAT GAC GCG TTT GAA GAT GAT GAA AAT GTT TAT ATT GTC ATG GAA
      N   L   V   Q   F   Y   D   A   F   E   D   D   E   N   V   Y   I   V   M   E
721     TTA TGC AAA GGT GGT GAA CTC TTG GAC AAA ATA CTT CAA AGG GGT GGA AAA TAC TCC GAA
      L   C   K   G   G   E   L   L   D   K   I   L   Q   R   G   G   K   Y   S   E
781     GAT GAT GCT AAA AAA GTT ATG GTT CAG ATT CTC AGT GTT GTG GCT TAC TGT CAT CTA CAA
      D   D   A   K   K   V   M   V   Q   I   L   S   V   V   A   Y   C   H   L   Q
841     GGT GTG GTT CAC CGT GAC CTT AAA CCC GAG AAC TTT CTT TTC TCT ACC AAG GAT GAG ACT
      G   V   V   H   R   D   L   K   P   E   N   F   L   F   S   T   K   D   E   T
901     TCT CCT CTG AAA GCA ATT GAT TTT GGT CTT TCT GAC TAT GTC AAG CCA GAC GAG AGG TTG
      S   P   L   K   A   I   D   F   G   L   S   D   Y   V   K   P   D   E   R   L
961     AAT GAT ATT GTA GGG AGT GCT TAC TAT GTG GCC CCG GAA GTT TTA CAC CGT ACA TAT GGA
      N   D   I   V   G   S   A   Y   Y   V   A   P   E   V   L   H   R   T   Y   G
1021    ACA GAA GCT GAC ATG TGG AGT ATA GGA GTG ATT GCT TAT ATT CTT CTC TGT GGC AGT CGA
      T   E   A   D   M   W   S   I   G   V   I   A   Y   I   L   L   C   G   S   R
1081    CCC TTT TGG GCC CGT ACT GAA TCC GGA ATC TTT CGA GCT GTA CTA AAG GCA GAA CCG AAT
      P   F   W   A   R   T   E   S   G   I   F   R   A   V   L   K   A   E   P   N
1141    TTT GAA GAA GCT CCT TGG CCT TCT CTA TCA CCC GAA GCT GTA GAT TTT GTG AAG AGA TTG
      F   E   E   A   P   W   P   S   L   S   P   E   A   V   D   F   V   K   R   L
1201    CTA AAC AAA GAT TAC CGT AAA AGA CTA ACT GCG GCC CAG GCT TTG TGT CAT CCA TGG CTG
      L   N   K   D   Y   R   K   R   L   T   A   A   Q   A   L   C   H   P   W   L
1261    GTT GGC TCG CAT GAA CTA AAG ATT CCA TCT GAT ATG ATC ATA TAC AAG CTT GTA AAA GTG
      V   G   S   H   E   L   K   I   P   S   D   M   I   I   Y   K   L   V   K   V
1321    TAC ATA ATG TCA ACT TCA TTG AGA AAA TCG GCT TTA GCG GCT CTT GCC AAA ACA TTA ACT
      Y   I   M   S   T   S   L   R   K   S   A   L   A   A   L   A   K   T   L   T
1381    GTA CCG CAG TTA GCG TAT CTT CGG GAG CAA TTC ACA TTG TTG GGG CCA AGC AAA AAT GGA
      V   P   Q   L   A   Y   L   R   E   Q   F   T   L   L   G   P   S   K   N   G
1441    TAT ATT TCG ATG CAG AAT TAC AAA ACA GCA ATC CTA AAG AGT TCA ACA GAT GCT ATG AAG
      Y   I   S   M   Q   N   Y   K   T   A   I   L   K   S   S   T   D   A   M   K
1501    GAT TCT CGA GTG TTT GAT TTT GTT CAC ATG ATA AGT TGT CTT CAA TAC AAG AAA CTA GAC
      D   S   R   V   F   D   F   V   H   M   I   S   C   L   Q   Y   K   K   L   D
1561    TTT GAA GAG TTT TGT GCT TCA GCA TTA AGC GTT TAT CAG CTC GAA GCA ATG GAG ACA TGG
      F   E   E   F   C   A   S   A   L   S   V   Y   Q   L   E   A   M   E   T   W
1621    GAG CAA CAT GCA CGG CGT GCT TAC GAG TTG TTT GAG AAA GAC GGG AAT AGG CCC ATC ATG
      E   Q   H   A   R   R   A   Y   E   L   F   E   K   D   G   N   R   P   I   M
1681    ATT GAA GAA CTT GCA TCG GAA CTC GGA CTA GGG CCA TCA GTG CCG GTG CAC GTT GTT CTT
      I   E   E   L   A   S   E   L   G   L   G   P   S   V   P   V   H   V   V   L
1741    CAG GAT TGG ATA AGA CAT TCT GAT GGC AAA CTT AGT TTC TTA GGG TTT GTC AGA TTG CTA
      Q   D   W   I   R   H   S   D   G   K   L   S   F   L   G   F   V   R   L   L
1801    CAT GGC GTG TCT TCT CGG ACC TTA CAA AAA GCT TAG AGT AAC GCC ATT GTT AGA TGA GAA
      H   G   V   S   S   R   T   L   Q   K   A   *
1861    AAG ATG CAA CTA GGT GGA GTA GAT CTG GTT TCT TCT TTC CTC TCC ACT TCT TGA TAG TTT
1921    TTT TAG TTT TCT TTT TCT ATT CAT GGT TAA GCT TCT GAG TCA AAG ATT CGA TAA TTA TGT
1981    CTG TGA GTG TAT TTG TGT GTG GTG GGA AGG AAA GGT TTA TGA AGA AGA GGA ATG TAC AGA
2041    ATG AGA CAT ATC CGA TTG GTG TTT TTA ATC CAG TGA TGT TTA CGT GTG AAA AAA AAA AAA
2101    AA

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Figure 1 Nucleotide and deduced amino acid sequences of AtCRK1

The nucleotides are numbered on the left. The start codon (ATG) and the stop codon (TAG) are boxed. The stop codon (TGA) in frame in the 5'-UTR is indicated by the grey shaded boxes.

Figure 2 Characterization of the CaMBD of AtCRK1

(A) Diagrams of the truncated forms of AtCRK1. (B) AtCRK1 and its truncated forms (AP1, AP2 and AP3) were separated by SDS/PAGE, blotted on to PVDF membrane and assayed with biotinylated CaM in the presence of either 0.1 mM Ca²⁺ or 2 mM EGTA. (C) Predicted amphiphilic α -helical wheel projection of the CaMBD (LRKSALAALAKTLTPQL) of AtCRK1. The positively charged amino acids are indicated by + and hydrophobic amino acids are boxed.

In addition, AP3, the C-terminus of AtCRK1 including the tentative CaMBD, was also used. When total proteins isolated from the Sf9 insect cells containing pAP1, pAP2 or pAP3 were separated by SDS/PAGE and blotted with biotinylated CaM, the peptides expressed by pAP1 and pAP3 with 51 and 21 kDa respectively, as expected, showed CaM-binding activity in the presence of Ca²⁺, but they lost CaM binding in the absence of Ca²⁺ (Figure 2B), locating the CaMBD into this overlapping region (39 amino acid residues) of these two peptides. Furthermore, when this region was deleted in AP2, CaM-binding ability was lost (Figure 2B). The amino acid sequence within this region shows a typical CaMBD structure (Figure 2C), as suggested in many different CaM-binding proteins [24,30].

To determine the K_d value of CaM binding to AtCRK1, the molecular interaction between AtCRK1 in the mobile phase designated as the ligate and an immobilized CaM referred to as the ligand was determined by IAsys system. AtCRK1 was serially diluted (60–400 nM) for affinity analysis. Changes in the surface concentration were proportional to changes in the refractive index on the surface resulting in changes in the IAsys signal plotted as response units as a function of time. Representative sensorgrams indicated the interaction between CaM and AtCRK1 at different concentrations (Figure 3) and the K_d value was calculated to be 67 nM. K_d has been reported from approx. 10 to 100 nM for CaM binding to many other CaM-binding proteins [31,32].

Figure 3 Sensorgram from IAsys for determination of the K_d value of CaM binding to AtCRK1

AtCRK1 was serially diluted (60–400 nM) and applied to a sensor chip immobilized with CaM.

Autophosphorylation and substrate phosphorylation of AtCRK1

To indicate AtCRK1 experimentally as a protein kinase suggested by database analyses, the autophosphorylation and substrate phosphorylation were performed. Sf9 insect cells were infected with the AtCRK1 recombinant expression virus, and the purified AtCRK1 was used for kinase activity assays.

As shown in Figure 4(A), AtCRK1 phosphorylated itself and histone H1S as substrates in the presence of Ca²⁺. This kinase activity was also assayed in the presence of EGTA, indicating its independence of Ca²⁺ for kinase activation of AtCRK1. Time-course studies showed that purified AtCRK1 could rapidly autophosphorylate and phosphorylate histone H1S and syntide-2 in the presence of either Ca²⁺ or EGTA soon after AtCRK1 was added to the reaction mixture (Figures 4B–4D). Kinetic analysis showed that the V_{max} values were 28.3 and 105.9 nmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹ for histone H1S and syntide-2 respectively as substrates in the presence of either EGTA or Ca²⁺. These results suggested that AtCRK1 has similar kinase properties in the presence of either Ca²⁺ or EGTA. Kinetic parameters of the kinase activity of AtCRK1 were determined from double-reciprocal analysis of data for phosphorylation of various concentrations of histone H1S or syntide-2 in the presence of 100 μ M ATP. The K_m values for histone H1S and syntide-2 was calculated to be approx. 5.9 and 5.2 μ M respectively.

The phospho-amino acid residues of AtCRK1 were also analysed by capillary electrophoresis. In our experiments, 20 amino acids and three phosphoamino acid standards could be separated by capillary electrophoresis (Figure 5A), indicating its application for amino acid identification as reported in [26,33]. When the hydrolysis products of purified AtCRK1, which was not autophosphorylated *in vitro*, were separated by capillary electrophoresis, all amino acids were detected except phospho-amino acids. However, the phosphorylated threonine was identified when the autophosphorylated AtCRK1 was analysed (Figure 5B), demonstrating that AtCRK1 was a threonine protein kinase, consistent with prediction by database analyses of AtCRK1.

Regulation of the kinase activity of AtCRK1 by CaM

As a CaM-binding protein, CaM binding of AtCRK1 may play some roles in the modulation of the kinase activity as

Figure 4 Kinase activity assays of AtCRK1

(A) The purified AtCRK1 was assayed for both autophosphorylation and substrate phosphorylation using histone H1S as substrate in the presence of Ca²⁺ or EGTA. Time-course analyses of AtCRK1 autophosphorylation (B) and substrate phosphorylation with histone H1S (C) or syntide-2 as substrates (D) were performed in the presence of 0.1 mM Ca²⁺ (◆), EGTA (◇), Ca²⁺ plus AtCaM2 (△), AtCaM4 (■), AtCaM7 (▲) or AtCaM8 (□). Results are means from three determinations performed in duplicate. Error bars are $\pm$ S.D.

demonstrated in other CBKs such as CCaMK [14–17], CaMKII [10] and NtCBK2 [9,20]. To test this possibility, four CaMs (CaM2, CaM4, CaM7 and CaM8) were selected as representatives for *Arabidopsis* isoforms based on amino acid homology [34–36]. In the presence of CaM, the maximal activity achieved with CaM was approx. 10-fold of the basal autophosphorylation activity in the presence of Ca²⁺ without CaM (Figures 4B and 6). When substrate phosphorylation activities reached the maximum, activity was approx. 9–10-fold with four AtCaM isoforms (Figures 4C and 6). Kinetic analyses indicated that K_m and V_{max} values for histone H1S are 4.4 μ M and 354.4 nmol $\cdot$ min⁻¹ $\cdot$ (mg of pro-

tein)⁻¹ for CaM2, 4.5 μ M and 358.5 nmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹ for AtCaM4, 4.9 μ M and 349.2 nmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹ for AtCaM7 and 5.3 μ M and 341 nmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹ for AtCaM8. The K_m and V_{max} values for syntide-2 are 3.8 μ M and 768.2 nmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹ for CaM2, 4.0 μ M and 772.3 nmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹ for AtCaM4, 4.6 μ M and 759.1 nmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹ for AtCaM7 and 4.9 μ M and 751 nmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹ for AtCaM8. These results suggested that the kinase activity of AtCRK1 was stimulated by CaMs, but the activity amplification was similar for these four different CaM isoforms.

Figure 5 Phospho-amino acid analyses by capillary electrophoresis

(A) Electropherograms of the hydrolysed unphosphorylated AtCRK1 with FITC labelling. (a) Whole electropherogram; (b) intercepted and enlarged form of (a). (B) Electropherograms of the hydrolysed autophosphorylated AtCRK1 with FITC labelling. (a) Whole electropherogram, (b, c) electropherogram with addition of standard phosphoamino acids for peak identification. (d) Intercepted and enlarged form of electropherogram (a).

This was also suggested by the kinase assay in the presence of low concentration of Ca^{2+} . When the assays were performed with syntide-2 as substrate in the presence of 10, 1 or 0.1 μM CaM, the stimulation of the kinase activity by these CaM isoforms was still found to be similar (results not shown). Furthermore, much higher V_{max} and lower K_m values of AtCRK1 for syntide-2 than histone IIIS also suggested that syntide-2 was better than histone IIIS as the substrate of AtCRK1.

This result was further supported by the similar K_a values for these four CaMs as follows: 30 ± 1.6 , 35 ± 1.2 , 31 ± 0.9 and 38 ± 1.0 nM for AtCaM2, AtCaM4, AtCaM7 and AtCaM8 respectively when AtCRK1 activity was assayed with the addition of increasing amounts of these four CaM isoforms in the presence of 0.1 mM Ca^{2+} (Figure 7).

To explore a possible role of the CaMBD in the regulation of AtCRK1 activity, two truncated constructs (AP1 and AP2) were further used for kinase assays. Both AP1 and AP2 were shown

Figure 6 Assay for CaM-stimulated kinase activity of AtCRK1

Autophosphorylation and substrate phosphorylation using histone IIIS as substrate of AtCRK1 were performed in the presence of Ca^{2+} or Ca^{2+} /CaM and resolved by SDS/PAGE.

Figure 7 Assay for CaM isoforms required for AtCRK1 phosphorylation

Histone IIIS as substrates was phosphorylated in the presence of AtCaM2 (◆), AtCaM4 (▲), AtCaM7 (△) or AtCaM8 (◇) under our standard phosphorylation conditions, except that increasing amounts of AtCaM ($10^{-8.5}$ – $10^{-6.5}$ M) were used at 30 °C for 30 min. Results are averages of three determinations performed in duplicate. Error bars are $\pm$ S.D.

to autophosphorylate and phosphorylate substrates such as histone IIIS in the absence or presence of Ca^{2+} /CaM as full AtCRK1 (Figure 8), suggesting that the C-terminus of AtCRK1 is not absolutely needed for the basal activity of the kinase. Although the kinase activity of AtCRK1 and AP1 can be stimulated by *Arabidopsis* CaM, AP2 was not (Figure 8), indicating that CaMBD is necessary for CaM regulation in the kinase activity.

Figure 8 Assay for CaM-stimulated kinase activity of truncated AtCRK1

Autophosphorylation and substrate phosphorylation using histone IIIS as substrate of AP1 and AP2 were performed in the presence of Ca^{2+} or Ca^{2+} /CaM and resolved by SDS/PAGE.

DISCUSSION

In the present study, a cDNA encoding *Arabidopsis* CRK1 was isolated by using *MCK1* as a probe. AtCRK1 can bind CaM in a Ca^{2+} -dependent manner, whereas this kinase phosphorylated itself and substrate such as histone IIIS in a Ca^{2+} -independent manner and the activity was stimulated by CaM isoforms through its CaMBD.

Several CBKs have previously been identified in plants, and among them CCaMK, OsCBK and NtCBK2 were well characterized molecularly and biochemically. AtCRK1 does not contain any typical EF hand for Ca^{2+} binding. This kinase has Ca^{2+} -independent phosphorylation activity as shown by NtCBK2, OsCBK and ZmCRK. AtCRK1 is a CBK, but shows similar kinase properties in the presence of Ca^{2+} or EGTA. The Ca^{2+} independence of AtCRK1 basal activity is very similar to other kinds of CaM-binding kinases such as CaMK and CCaMK [37,38]. However, this characteristic is different from CDPK, because the activities of most CDPKs are Ca^{2+} dependent.

Our experiments also indicated the stimulation of AtCRK1 autophosphorylation by CaMs. Autophosphorylation has been shown to play a critical role in the regulation of some CaMKs, leading to the activation of the kinases [39]. The presence of phospho-amino acid on the autophosphorylating site is sufficient to disrupt the auto-inhibitory domain, and the kinase retains partial activity (20–80%) even after CaM dissociates from the kinases. For multifunctional CaMK II from rabbit and rat, autophosphorylation exhibits an absolute requirement for Ca^{2+} /CaM and generates a Ca^{2+} /CaM-independent form of the kinase with little loss in the total activity [39,40].

As a CaM-binding protein, the kinase activity of AtCRK1 was demonstrated to be modulated by CaM. It has been documented

that plant cells have multiple divergent CaM isoforms, which allow subtle regulation of Ca^{2+} /CaM-binding proteins. For example, two isoforms of potato CaM, PCaM1 and PCaM7, modulated the activity of CCaMK differently [18]. Whereas the PCaM1 acted as a more efficient inhibitor of autophosphorylation and activator of substrate phosphorylation of tobacco CCaMK than PCaM7, these CaM isoforms did not show this differential regulation of lily CCaMK. To investigate the possible differential regulation of AtCRK1 by different CaM isoforms, AtCaM2, AtCaM4, AtCaM7 and AtCaM8, as representatives of *Arabidopsis* CaM isoforms respectively, were used in our experiments. Whereas all CaM isoforms used in the present study can stimulate the kinase activity of AtCRK1, the kinase activity of AtCRK1 modulated by these different CaM isoforms was found to be similar.

These characteristics are different from OsCBK and NtCBK2. However, it has been reported that NtCBK2 activity is differentially regulated by different CaM isoforms [9,20], the kinase activity of OsCBK is not modulated by CaM [9,19]. These different characteristics of AtCRK1, OsCBK and NtCBK2 for CaM modulation may imply that CaM has many diverse mechanisms to activate its targets and further elucidation of precise roles of each CaM isoforms in regulating CaM target proteins will be needed.

AtCaM8 was also used for AtCRK1 assay. This molecule has been renamed as CAM-like gene (CML8) by McCormack and Braam [36], because it shares only 73% amino acid identity with CaM. In our experiments, this protein, similar to other *Arabidopsis* CaMs, not only has the ability to bind AtCBK1, but also up-regulates the kinase activity, indicating that this molecule can act as a modulator as other CaM isoforms to CaM target proteins, at least to AtCRK1.

In summary, an *Arabidopsis* CRK1-encoded protein kinase was indicated to be different from OsCBK and NtCBK2 in enzymic properties. This kinase showed autophosphorylation and substrate phosphorylation in a Ca^{2+} -independent manner and the activity was stimulated by all CaMs tested. However, the regulation of different CaM isoforms for AtCRK1 activity was similar.

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