Molecular and biochemical characterization of a calcium/calmodulin-binding protein kinase from rice

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A Ca^{2+}/calmodulin (CaM)-binding protein kinase from rice (*Oryza sativa*), OsCBK, has been characterized that lacks Ca^{2+}-binding EF hands and has Ca^{2+}/CaM-independent autophosphorylation and substrate-phosphorylation activity. OsCBK has all 11 subdomains of a kinase catalytic domain and a putative CaM-binding domain, and shares high identity with Ca^{2+}-dependent-protein-kinase ('CDPK')-related protein kinases in plants. OsCBK bound CaM in a Ca^{2+}-dependent manner as previously reported for Ca^{2+}/calmodulin-dependent protein kinases in animals, but autophosphorylation and phosphorylation of histone IIIs were Ca^{2+}/CaM-independent. Surface plasmon resonance analysis showed that OsCBK specifically bound CaM with high affinity ($K_D = 30$ nM). Capillary electrophoresis showed that phosphorylation of OsCBK occurred on serine and threonine residues. These data show that OsCBK is a serine/threonine protein kinase that binds Ca^{2+}/CaM, but whose enzymic activity is independent of Ca^{2+}/CaM. *In situ* hybridization showed that OsCBK is expressed in reproductive and vegetative tissues of rice and shows temporal and spatial changes during plant growth and development. OsCBK is highly expressed in zones of cell division and it is particularly abundant in sporogenous cells of the anther at meiosis.

Key words: autophosphorylation, calcium-dependent protein kinase (CDPK), capillary electrophoresis (CE), *in situ* hybridization, surface plasmon resonance (SPR).

INTRODUCTION

The involvement of Ca^{2+} as a second messenger in a wide variety of cellular and physiological processes is well documented [1–5]. It is believed that changes in cytosolic Ca^{2+} are sensed by a group of Ca^{2+}-binding proteins including calmodulin (CaM) and Ca^{2+}-dependent protein kinases (CDPKs) [6,7].

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 [8]. Thus, the activity of CDPKs is modulated by Ca^{2+} rather than CaM. In addition to CDPKs, CDPK-related protein kinases (CRKs) have been reported [9,10]. In contrast to the CDPKs, however, CRKs do not require Ca^{2+} for their activities. The C-termini of members of CRKs from carrots [9], maize [10] and *Arabidopsis* (GenBank® accession number 7446421) share sequence similarity with CaM (20 % identity) without typical EF-hand motifs for Ca^{2+} binding, but contain apparently degenerate Ca^{2+}-binding sites. No biochemical evidence for CRK's ability to bind CaM is available.

CaM plays roles in regulating CaM-binding proteins. Several types of CaM-binding kinase genes have been cloned in plants: CB1 from apple [11], MCK from maize [12,13], and chimaeric Ca^{2+}/calmodulin-dependent protein kinases (CCaMKs) from lily [14], tobacco [15] and maize [16,17]. Furthermore, while CB1 and MCK lack Ca^{2+}-binding EF hands, CCaMKs are characterized by a neural visinin-like Ca^{2+}-binding domain in addition to a CaM-binding domain [14,15]. So far, only CCaMKs have been reported to have kinase activity that is regulated by Ca^{2+}/CaM [16,18].

We report here on the molecular and biochemical characterization of a novel Ca^{2+}/CaM-binding protein kinase of rice, *Oryza sativa* (OsCBK). The results indicate that the expression of OsCBK is regulated spatially and temporally. We show that OsCBK differs from other Ca^{2+}/CaM-dependent protein kinases (CaMKs), CDPKs and CRKs from animals or plants, displaying Ca^{2+}/CaM-independent kinase activity while binding CaM with high affinity in a Ca^{2+}-dependent manner.

MATERIALS AND METHODS

Rice cDNA library screening

The cDNA library was constructed with leaf mRNA isolated from rice (*O. sativa*) IR62266 and a λZAP cDNA Synthesis kit following the manufacturer's instructions (Stratagene), and then screened with plasmid p550 containing a maize cDNA encoding MCK1 [12]. Positive recombinant phages for OsCBK were isolated and excised *in vivo* into recombinant pBluescript SK(−). Candidate plasmid pOsCBK was sequenced.

RNA isolation and RNA analyses

Total RNA from rice leaves was isolated with TRIzol® as described by the manufacturer (Gibco), and Northern blotting was carried out as described previously [12]. Briefly, a 20 μg sample of total RNA was loaded and separated on a 1.5 %

Abbreviations used: CaM, calmodulin; CaMK, Ca^{2+}/CaM-dependent protein kinase; CCaMK, chimaeric CaMK; CDPK, Ca^{2+}-dependent protein kinase; CRK, CDPK-related protein kinase; DIG, digoxigenin; DTT, dithiothreitol; HRP, horseradish peroxidase; Ni-NTA, Ni^{2+}-nitrilotriacetate; ORF, open reading frame; OsCBK, *Oryza sativa* Ca^{2+}/CaM-binding protein kinase; SPR, surface plasmon resonance.

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The nucleotide sequence data reported here have been submitted to the GenBank® Nucleotide Sequence Database under the accession number AF368282.

formaldehyde/agarose gel. The mRNA for OsCBK was detected by α-^{32}P-labelled pOsCBK probe.

Construction of recombinant virus

A 5′ primer (5′-CGGGATCCATGGGTCTCTGCCATGGCAAG-3′) and a 3′ primer (5′-CGGGATCCTCAGGTTTTTGGGATGGTACGC-3′) were used to amplify the full open reading frame (ORF) of pOsCBK cDNA for OsCBK. A 5′ primer (5′-CGGGATCCATGGGTCTCTGCCATGGCAAG-3′) and a 3′ primer (5′-CGGGATCCAGTACCACGAATCCATGGATG-3′) were used for truncated OsCBK (R1-408). The PCR products were digested with *Bam*HI and subsequently cloned into the *Bam*HI site of plasmid pFastBac HTb. After the sequences were confirmed, the recombinant plasmids were transformed into DH10BAC competent cells containing the bacmid with a mini-att Tn7 target site and helper plasmid. The mini-att Tn7 element on the pFastBac HTb 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 recombinant bacmid were identified based on the disruption of the *lacZ* α gene.

Sf-9 cells were maintained as monolayers at 27 °C in 10 % fetal bovine serum supplement with Grace's medium and transfected with the recombinant bacmid with CELLFECTIN reagent according to manufacturer's instructions (Gibco). Recombinant virus was harvested after 72 h and identified by PCR with the primers described above.

Purification of OsCBK protein

The Sf9 insect cells were infected with the recombinant virus for 72 h and harvested at room temperature. Cells were washed once with Grace's medium, re-suspended in 5 ml of lysis buffer (50 mM Tris/HCl, pH 7.5, 10 % glycerol, 1 % Nonidet P-40 and 0.2 mM PMSF). The mixture was sonicated for 30 s, followed by centrifugation at 12000 ***g*** for 10 min. The supernatant was applied to a Ni^{2+}-nitrilotriacetate (Ni-NTA) 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 followed by buffer A containing 25 mM imidazole, OsCBK was subsequently eluted with buffer A containing 200 mM imidazole. OsCBK was dialysed against 25 mM Tris/HCl, pH 7.5, containing 2 mM $CaCl_2$ for 6 h and loaded on to a CaM–Sepharose 4B column (Amersham Biosciences) pre-equilibrated with buffer B (25 mM Tris/HCl, pH 7.5, and 2 mM $CaCl_2$). After washing with buffer B plus 200 mM NaCl, OsCBK was eluted with buffer C (25 mM Tris/HCl, pH 7.5, and 2 mM EGTA). Purified OsCBK was used immediately for SDS/PAGE and enzymic analyses. Protein concentration was determined by the method of Bradford [19] using BSA as a standard. All procedures were performed at 4 °C unless otherwise noted.

Protein blotting

An antibody against MCK1 was prepared according to the method of Yang et al. [20]. OsCBK (2 μg) separated on 10 % SDS/polyacrylamide gels was electrophoretically transferred to PVDF membrane in transfer buffer (20 mM Tris/HCl, pH 8.3, 150 mM glycine and 20 % methanol) for 120 min at 75 V using a Bio-Rad Mini-Trans-Blot apparatus. After blocking with Tris/saline (10 mM Tris/HCl, pH 7.4, and 150 mM NaCl) containing 5 % (w/v) non-fat dry milk for 1 h, the membrane was treated with the antibody against MCK1 (diluted with 1 % BSA in Tris/saline) for 1 h, followed by washing with Tris/saline. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody for 1 h followed by extensive washing with Tris/saline. HRP was detected with 4-chloro-1-naphthol and H_2O_2.

Construction and expression of truncated OsCBK in *Escherichia coli*

To define the CaM-binding domain, several truncated constructs were made with pET32a expression vector and PCR fragments (see Figure 5A, below). R1-455 (5′ primer, 5′-CGGGATCCATGGGTCTCTGCCATGGCAAG-3′; 3′ primer, 5′-CGGGATCCCTGTTCTCTTAGGTAATAGAT-3′) contains the N-terminus of OsCBK including the kinase catalytic domain and the tentative CaM-binding domain. R1-408 (5′ primer, 5′-CGGGATCCATGGGTCTCTGCCATGGCAAG-3′; 3′ primer, 5′-CGGGATCCAGTACCACGAATCCATGGATG-3′) has the same amino acid sequence as R1-455 but lacks the tentative CaM-binding domain. R418-597 (5′ primer, 5′-CGGGATCCATAATTTATAGGCTTATGAGG-3′; 3′ primer, 5′-CGGGATCCTCAGGTTTTTGGGATGGTACGC-3′) contains the C-terminus of OsCBK including the tentative CaM-binding domain. All inserts in the clones were sequenced. The clones were introduced into *E. coli* BL21 (DE3) and incubated in LB medium at 37 °C. Isopropyl β-D-thiogalactoside was then added to the cultures at a final concentration of 1 mM, and the cells were further incubated for 4–6 h. *E. coli* cells were collected, re-suspended in 2 × loading sample buffer. After boiling for 5 min and centrifugation at 12000 ***g*** for 5 min the supernatants were analysed by SDS/PAGE (12 % gels).

Expression and purification of rice CaM

Rice CaM (GenBank® accession number D29693; a gift from Dr Uchimiya Hirofumi, University of Tokyo, Tokyo, Japan) was sequenced (accession number AF441190) and used as a template for PCR with the 5′ primer (5′-CATGCCATGGCGGATCAGCTCACCGACGAG-3′) and 3′ primer (5′-CATGCCATGGTCACTTGGCCATCATGACCT-3′). The *Nco*I-digested PCR product was cloned into the *Nco*I site of the pET14b expression vector. After sequencing confirmation, the recombinant plasmid was introduced into *E. coli* BL21 (DE3) cells and used for CaM purification as described by Gopalakrishna and Anderson [21] and Lu and Harrington [22] with some modification. Briefly, the *E. coli* cells were grown to A_{600} of 0.6 and induced by 1 mM isopropyl β-D-thiogalactoside for 6 h in 500 ml of LB medium containing 100 μg/ml ampicillin. Cells were harvested and then washed in 50 mM Tris/HCl (pH 7.5). Then cells were re-suspended in lysis buffer [50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol (DTT), 0.1 mM $CaCl_2$ and 200 μg/ml lysozyme], followed by incubating the suspension on ice for 30 min. After brief sonication to shear the bacterial DNA, the mixture was heated to 90 °C for about 3 min, cooled down to less than 10 °C within a few minutes and centrifuged at 27000 ***g*** for 30 min at 4 °C. After adjusting the $CaCl_2$ concentration to 5 mM, the supernatant enriched in CaM protein was loaded on to a phenyl-Sepharose CL-4B column pre-equilibrated with 50 mM Tris/HCl, pH 7.5, 0.1 mM $CaCl_2$ and 0.5 mM DTT. The column was then washed with the same buffer containing 500 mM NaCl. The CaM protein was eluted with 50 mM Tris/HCl, pH 7.5, 5 mM EGTA and 0.5 mM DTT. The eluted CaM was dialysed in water and then stored at −80 °C until use.

CaM-binding assay

CaM was biotinylated as described by Billingsley et al. [23]. Briefly, CaM was dialysed overnight at 4 °C against 0.1 M

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cctcctcctcctcctctctctcctccttcctccgattcacacacacacacactcctcgccg   60
tcttgtttcttcttcgtctgggtggttgatccatccatccagagattgcctgtgcgagga   120
agggagcgggcgagtggcggatttgttttttggtttgttttttgagtttgttttttattggtg   180
gtatcctctcctcctcctgtgcgagggagcagctggtggattgttccgttccagctccgttt   240
cttgtcttgatttggaatccgggacatttttccgcgccccgtttctggatttccggtacc   300
ccccctttcttggtggaagtgggaaggggagggggaaggatttttttggtggggaattcga   360
gggggcgaggtgatgaactcttgcctctttgaccagccttgatttttcctggtgattttgc   420
ttggaatttctcggattttgtgggggttctttgatccgggctccATGGGTCTCTGCCAT   480
                                                M  G  L  C  H

GGCAAGTCGGCGGCGGTGCTGGAGCCAACGGTGGAGGAGGAGGAGGAGGGGGCGACGCGC   540
G  K  S  A  A  V  L  E  P  T  V  E  E  E  E  E  G  A  T  R

GTAGCCGAGGCCGCGGCGGCGCCGGCGAAGCCAGCGTCGCCTGCGCCTTCGGCGGCGGCG   600
V  A  E  A  A  A  A  P  A  K  P  A  S  P  A  P  S  A  A  A

GCGGCGGCGAAGCCCGGGACGCCGAAGCAGCACAAGTTCCCGTTCTACCTGCCGAGCCCG   660
A  A  A  K  P  G  T  P  K  Q  H  K  F  P  F  Y  L  P  S  P

CTCCCGGCGTCGAGCTACAAGGGCTCGCCGGCGAACTCGAGCGTGGCGTCGACGCCCGCG   720
L  P  A  S  S  Y  K  G  S  P  A  N  S  S  V  A  S  T  P  A

CGCGGCGGGGTTCAAGCGGCCGTTCCCGCCGCCGTCGCCGGCGAAGCACATCCGCGCGCTC   780
R  G  G  F  K  R  P  F  P  P  P  S  P  A  K  H  I  R  A  L

CTGGCGCGGCGCCACGGCTCGGTGAAGCCGAACGAGGCGTCCATCCCGGAGTCCGGCGAG   840
L  A  R  R  H  G  S  V  K  P  N  E  A  S  I  P  E  S  G  E

CCCGGCGTGGCGCTGGACAAGGGCTTCGGCTTCTCCAGGCACTTCGCCGCCAAGTACGAG   900
P  G  V  A  L  D  K  G  F  G  F  S  R  H  F  A  A  K  Y  E

CTCGGCCGCGAGGTTGGCCGCCGCCACTTCGGCTACACCTGCGCCCGCCACCTGCAAGAAG   960
L  G  R  E  V  G  R  G  H  F  G  Y  T  C  A  A  T  C  K  K

GGCGAGCTCAAGGGCGACGACGTCGCGGTCAAGGTCATCCCCAAGGCCAAGATGACTACT   1020
G  E  L  K  G  D  D  V  A  V  K  V  I  P  K  A  K  M  T  T

GCTATTGCCATTGAAGATGTCAGGAGAGAAGTCAGAATATTGAGTTCTTTGGCAGGGCAC   1080
A  I  A  I  E  D  V  R  R  E  V  R  I  L  S  S  L  A  G  H

AGCAACTTAGTGCAGTTTTATGATGCTTATGAGGATGAAGAAAATGTATATATAGTTATG   1140
S  N  L  V  Q  F  Y  D  A  Y  E  D  E  E  N  V  Y  I  V  M

GAGTTATGCAAAGGAGGTGAACTGCTGGACAGGATTTTGGCTAGAGGTGGAAAATATTCT   1200
E  L  C  K  G  G  E  L  L  D  R  I  L  A  R  G  G  K  Y  S

GAGGAAGATGCAAAGGTTGTTATGCGCCAAATCCTAAGTGTTGCTTCATTTTGTCATCTT   1260
E  E  D  A  K  V  V  M  R  Q  I  L  S  V  A  S  F  C  H  L

CAGGGTGTTGTTCATCGGGATCTGAAACCAGAGAATTTCCTTTTCTCGTCAAAGGATGAG   1320
Q  G  V  V  H  R  D  L  K  P  E  N  F  L  F  S  S  K  D  E

AACTCTGCCATGAAGGTCATAGACTTCGGTTTGTCTGACTTTGTAAAGCCAGATGAGAGG   1380
N  S  A  M  K  V  I  D  F  G  L  S  D  F  V  K  P  D  E  R

CTTAATGACATTGTCGGAAGTGCATATTATGAAGCTCCTGAAGTGCTCCATCGATCTTAT   1440
L  N  D  I  V  G  S  A  Y  Y  V  A  P  E  V  L  H  R  S  Y

GGCACTGAGGCAGATATGTGGAGCATTGGAGTAATAGTGTATATTTTGCTTTGTGGCAGC   1500
G  T  E  A  D  M  W  S  I  G  V  I  V  Y  I  L  L  C  G  S

 CGTCCTTTCTGGGCACGTACTGAGTCAGGAATATTCCGAGCTGTGCTGAAAGCAGACCCT   1560
R  P  F  W  A  R  T  E  S  G  I  F  R  A  V  L  K  A  D  P

AGTTTTGAGGAAGCCCCATGGCCAACCCTCTCTGCTGAAGCAAAAGACTTTGTAAGGAGG   1620
S  F  E  E  A  P  W  P  T  L  S  A  E  A  K  D  F  V  R  R

CTGCTCAATAAAGATTACCGCAAGAGGATGACTGCTGCACAAgccctctgtcatccatgG   1680
L  L  N  K  D  Y  R  K  R  M  T  A  A  Q  A  L  C  H  P  W

ATTCGTGGTACTGAAGAAGTGAAGCTTCCTTTGGACATGATAATTTATAGGCTTATGAGG   1740
I  R  G  T  E  E  V  K  L  P  L  D  M  I  I  Y  R  L  M  R

GCTTACATAAGTTCATCTTCTTTACGGAGAGCTGCATTGAGGGCCCTTGCCAAGACATTG   1800
A  Y  I  S  S  S  S  L  R  R  A  A  L  R  A  L  A  K  T  L

ACAACAGATCAAATCTATTACCTAAGAGAACAGTTTGAATTGATAGGCCCAAACAAGAGC   1860
T  T  D  Q  I  Y  Y  L  R  E  Q  F  E  L  I  G  P  N  K  S

GATCTTATTACTTTGCAAAATTTGAAGACGGCCTTGATGAAGAACTCCACAAATGCAATG   1920
D  L  I  T  L  Q  N  L  K  T  A  L  M  K  N  S  T  N  A  M

AAGGATTCTAGGGTGGTTGACTTTGTTAACACAATATCCAATATTCAGTACAGAAAGTTG   1980
K  D  S  R  V  V  D  F  V  N  T  I  S  N  I  Q  Y  R  K  L

GATTTTGAGGAGTTTTCTGCTGCTGCCATCAGTGTTTACCAGATGGAAGGTTTGGAAACC   2040
D  F  E  E  F  S  A  A  A  I  S  V  Y  Q  M  E  G  L  E  T

TGGGAACAACATGCCCGGCAAGCGTACGAGTTCTTTGATAAGGAGGGTAACCGACCAATT   2100
W  E  Q  H  A  R  Q  A  Y  E  F  F  D  K  E  G  N  R  P  I

GTGATCGACGAGCTCGCATCGGGACTTGGATTGGGTCCTTCGGTTCCCCTACATGTTGTT   2160
V  I  D  E  L  A  S  G  L  G  L  G  P  S  V  P  L  H  V  V

CTCCAAGATTGGATCAGACATCCTGATGGAAAACTCAGCTTCCTTGGGTTCATGAAACTA   2220
L  Q  D  W  I  R  H  P  D  G  K  L  S  F  L  G  F  M  K  L

TTGCATGGAGTTTCTTCGCGTACCATCCCAAAAACCTGAgataataacttattttcttac   2280
L  H  G  V  S  S  R  T  I  P  K  T  *

atcaaccaacaaaagtgtgatgactaaactaatctctggctgtctggagaagcggcatgg   2340
aaccagtacaaagattaacaaagctgtcctagtgtaacaagtgaAaaagctctcctgtca   2400
ggcccgcgaatttcgcctctctctttaccagatgattgatgttgcgtttgatatcaggttt   2460
gtttagaggtggaatgtacagatgagtttaggaaacagcaaaaaTgaggtgctaagaagt   2520
gaaacatggtttaatttcgattggtgtaactgtgtgcgtttgatCttcgccgatctgtga   2580
atgtcgtgattggactgaaattgagaagtgaggtggtatcagtaccatattcgtagatat   2640
ttcttgagttttttttcttattgaaatttactcgtgtttatttgcaatagtttggctca   2700
ttggctgtacatcgatgtgatgaaaacgatatcaatttttttttgcataaacaataTcag   2760
tatggttaggaatttttataaaaaaaaaaaaaaaaaaaa
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Figure 1 Nucleotide and deduced amino acid sequences of OsCBK from rice

The nucleotides are numbered on the right. The start codon (ATG) and the stop codon (TGA) are boxed.

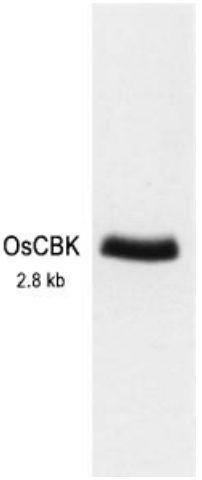

Figure 2 Northern blot for OsCBK in rice leaves

Total RNA (20 μg) from leaves was subjected to formaldehyde/agarose gel electrophoresis and blotted on to nitrocellulose. The blot was hybridized to ^{32}P-labelled DNA made from pOsCBK and autoradiographed at −80 °C.

phosphate buffer (pH 7.4). The dialysate was supplemented with 1 mM $CaCl_2$ and incubated with D-biotinoyl-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 about 48 h at 4 °C and stored in 20 % glycerol at −20 °C.

The purified OsCBK or total proteins isolated from *E. coli* cells were separated by SDS/PAGE and blotted on to PVDF membrane as described above. The membrane was blocked in 2 % BSA/Tris-buffered saline (TBS; 50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 0.5 mM $CaCl_2$ and 50 mM $MgCl_2$) and washed three times with TBS for 15 min each. After incubation in TBS containing biotinylated CaM for 3 h at room temperature and then washing with TBS, the membrane was treated with avidin-HRP conjugate (Bio-Rad) dissolved in TBS for 1 h. Protein bound to biotinylated CaM was visualized by colour development with 4-chloro-1-naphthol and H_2O_2.

Affinity measurement with surface plasmon resonance (SPR)

Experiments were performed on a BIAcore 2000 instrument (Pharmacia Biosensor) at 25 °C. The running buffer used was 25 mM Tris/HCl, pH 7.5, containing 2 mM $CaCl_2$ and 100 mM NaCl. The biotinylated CaM was captured on sensor chip SA-5 containing immobilized streptavidin on its surface. OsCBK with a concentration range of 45–600 nm was injected as the analyte on to the CaM-loaded surface with a flow rate of 30 μl/min. For each experimental cycle, a defined concentration of OsCBK (30 μl) was injected. During the dissociation phase, only buffer was passed over the surface.

Association and dissociation rate constants were calculated by non-linear fitting of the primary sensorgram data using

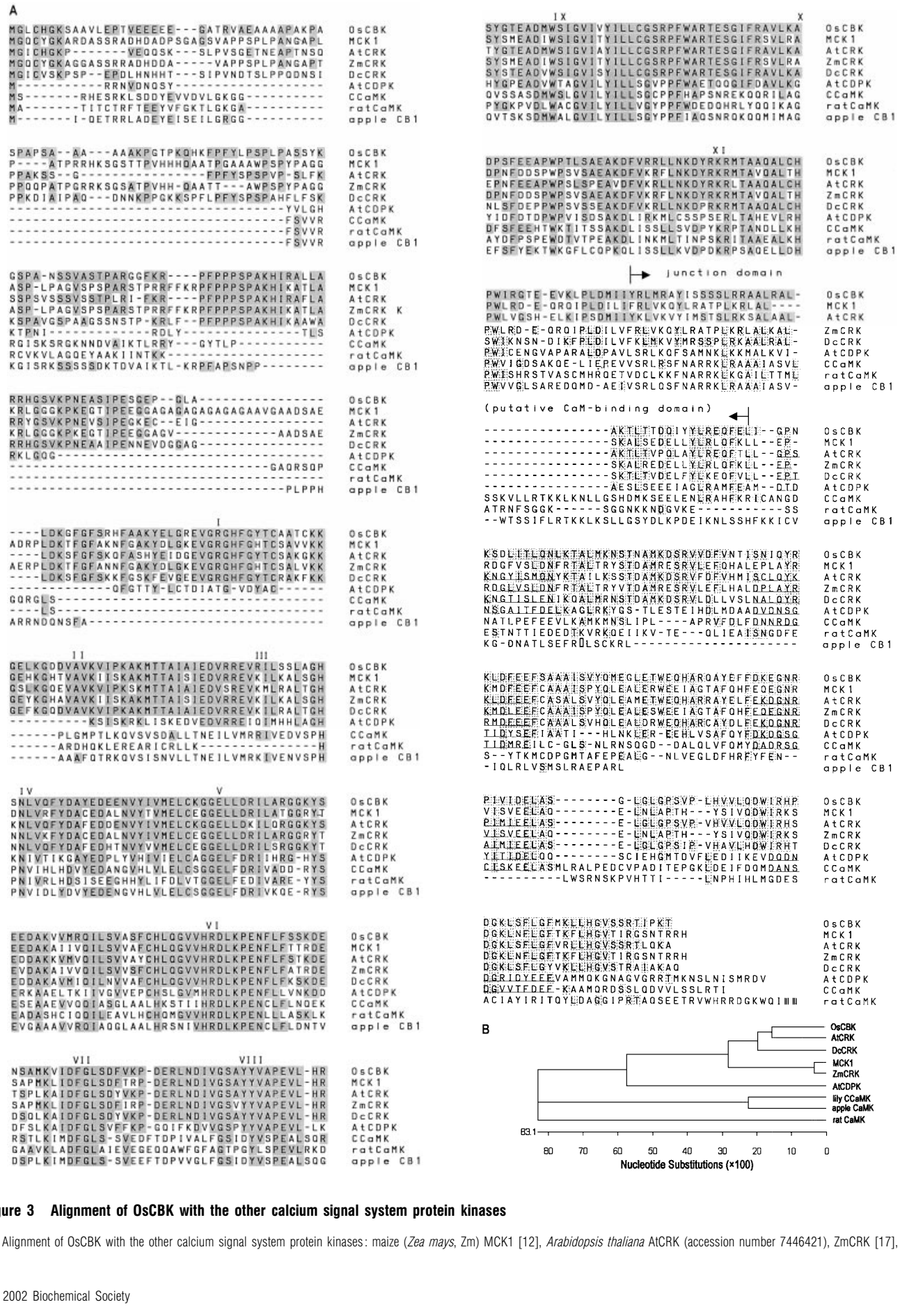

Figure 3 Alignment of OsCBK with the other calcium signal system protein kinases

(**A**) Alignment of OsCBK with the other calcium signal system protein kinases: maize (*Zea mays*, Zm) MCK1 [12], *Arabidopsis thaliana* AtCRK (accession number 7446421), ZmCRK [17], carrot

BIAEVALUATION 3.0 software (Biacore). The dissociation rate constant was derived using the following equation:

$$R_t = R_{t0} \cdot e^{-k\,off(t-t0)} \quad (1)$$

where R_t is the response at time t, R_{t0} is the amplitude of the initial response and the k_{off} is the dissociation constant. The association rate constant can then be derived using eqn (2):

$$R_t = k_{on} C R_{max}[1-e^{-(k\,on C + k\,off)t}]/(K_{on} C + k_{off}) \quad (2)$$

where R_{max} is the maximum response, C is the concentration of the ligand in solution and k_{on} is the association rate constant. The steady-state dissociation constants (K_D) were calculated by:

$$K_D = k_{off}/k_{on} \quad (3)$$

Autophosphorylation and dephosphorylation assays

OsCBK autophosphorylation was carried out in a 100 μl reaction mixture containing 25 mM Tris/HCl, pH 7.5, 0.5 mM DTT, 10 mM magnesium acetate, 100 μM ATP, 10 μCi [γ-^{32}P]ATP (5000 Ci/mM) and either 1 μM CaM/1 mM $CaCl_2$ or 2 mM EGTA at 30 °C for 15 s or 30 min. The reactions were initiated by addition of OsCBK, terminated by adding a one-fifth vol. of 5 × SDS sample buffer, and analysed by SDS/PAGE (10 % gels). After staining with 0.1 % Coomassie Brilliant Blue, the gel was vacuum-dried and exposed to X-ray film at −70 °C.

For OsCBK dephosphorylation, autophosphorylated OsCBK was purified with CaM–Sepharose. Dephosphorylation was catalysed by the protein phosphatase-1 catalytic subunit α-isoform (Sigma) in a mixture containing 20 mM Tris/HCl, pH 7.0, 2 mM DTT and 1 mM $MnSO_4$ at 30 °C for 10 min. The dephosphorylated OsCBK was further purified with CaM–Sepharose 4B.

Substrate-phosphorylation assay

Substrate phosphorylation by OsCBK was performed in a 100 μl reaction mixture containing 25 mM Tris/HCl, pH 7.5, 0.5 mM DTT, 10 mM magnesium acetate, 100 μM ATP, 10 μCi [γ-^{32}P]ATP (5000 Ci/mM), 1 mg/ml histone IIIs and either 1 μM CaM/1 mM $CaCl_2$ or 2 mM EGTA at 30 °C for 15 s or 30 min. The reaction was initiated by the addition of the OsCBK, terminated by adding a one-fifth vol. of 5 × SDS sample buffer, and analysed by SDS/PAGE as described above.

Assay of OsCBK activity

The reaction for OsCBK substrate phosphorylation was performed as described above. Aliquots (10 μl) were removed and applied to P81 phosphocellulose filters (2 cm × 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. ^{32}P incorporation was determined by liquid scintillation counting (Beckman LS 6500).

Phosphoamino acid assays

OsCBK and autophosphorylated OsCBK were hydrolysed in 6 M HCl for 12 h at 110 °C, then dried and dissolved in 20 μl of 10 mM borate buffer (pH 10.0). The hydrolysed product was mixed with 20 μl 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 μl of standard solution containing each amino acid and phosphoamino acid (0.5 mM each) was mixed with 46 μl of 1 mM FITC, 100 μl of 20 mM borate buffer (pH 10.0) and 52 μl of H_2O. The mixture was incubated in the dark for 12 h at room temperature.

FITC-tagged amino acids were analysed by capillary electrophoresis [24,25]. Data were collected by a computer with Spot Advanced software, and further processed with Scion Image and Origin software packages.

RNA *in situ* hybridization and detection

Vegetative and reproductive tissues of rice were fixed in 4 % paraformaldehyde, dehydrated in an ethanol series, cleared with xylene and embedded in paraffin as described by Drews [26]. Paraffin-embedded tissues were cut into 8 μm-thick sections, which were attached to glass microscope slides coated with polylysine hydrobromide. Digoxigenin (DIG)-labelled antisense and sense RNA probes were transcribed from either *Sac*I- or *Nco*I-digested pOsCBK using either T7 (antisense) or T3 (sense) RNA polymerase. The template for the antisense probe was the 3′ untranslated region and the template for the sense probe was the 5′ untranslated region. *In situ* hybridization analyses with paraffin-embedded tissue sections were performed as described by Drews [26]. Hybridization was carried out at 55 °C overnight. Slides were washed twice in 2 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) at room temperature, then in 1 × SSC and 0.1 × SSC at 57 °C, each for 15 min. DIG-labelled RNA was detected using an anti-DIG alkaline phosphatase conjugate according to the manufacturer's instructions (Boehringer Mannheim). The development of Nitro Blue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) was carried out as described by Block and Debrouwer [27]. Briefly, polyvinyl alcohol was added to the NBT-BCIP mixture. After enzyme-catalysed colour reaction, an insoluble blue precipitate was observed. Slides were visualized with an Olympus BX60 microscope, photographed and images captured on 100 ASA colour negative film.

RESULTS

Cloning and characterization of OsCBK cDNA

To isolate the rice homologue of maize MCK1, a rice cDNA library was screened with the MCK1 probe, resulting in the isolation of a cDNA clone (designated pOsCBK) having a 2800 bp insert. pOsCBK contains an ORF of 1794 bp with a start codon at nucleotide position 466–468 bp and a stop codon at nucleotide position 2257–2259 bp. This clone was determined to be a full-length ORF based on the presence of an in-frame stop codon at nucleotide position 412–414 bp in the 5′ untranslated region (Figure 1). Northern Blot analyses revealed a single band of about 2.8 kb (Figure 2), consistent with the predicted size of the cDNA.

DcCRK [10], AtCDPK [9], lily CCaMK [14], rat brain CaMK II α-subunit [30] and apple CaMK [11]. Identities between OsCBK and other kinases are indicated by shaded squares. The subdomains identified by Hanks et al. [28] in the catalytic domain are indicated by roman numerals. The calcium-binding domains (EF hands) and the non-canonical EF hands in CRKs are underlined. (**B**) Phylogenetic tree showing the relationship between OsCBK and the closest kinases.

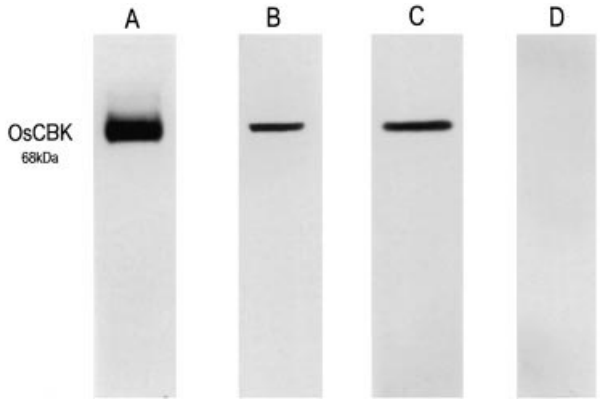

Figure 4 Characterization of purified OsCBK

(**A**) Silver staining of purified OsCBK. (**B**) Protein blotting with MCK1 antibody. (**C**) and (**D**) Biotinylated CaM-binding assay in the presence of 1 mM Ca^{2+} (**C**) or 2 mM EGTA (**D**).

The deduced amino acid sequence of OsCBK consists of 597 residues with a calculated molecular mass of 65.7 kDa. OsCBK contains all 11 subdomains characteristic of the protein kinase catalytic domain [28] (Figure 3A). While OsCBK shares low identities with plant CDPKs [34.4 % with *Arabidopsis thaliana* (At) CDPK, accession number 1220099] [29], CCaMKs (22.1 % with lily CCaMK, accession number 860675) [14] and CaMKs (22.2 % with CaMK II α-subunit, accession number J02942; 25.1 % with apple CB1, accession number 1170626) [11,30], the highest identities are found between OsCBK and CRKs isolated from *Arabidopsis* (AtCRK, 71.9 % identity, accession number 7446421), carrot (DcCRK, 65.2 % identity, accession number 1103386) [9] and maize (*Zea mays*, ZmCRK, 56.4 % identity, accession number 1313909) [10] (Figure 3A). The phylogenetic tree based on the amino acid sequences of the entire polypeptide also shows that the OsCBK is more closely related to CRKs than to the other protein kinases (Figure 3B). However, while OsCBK lacks the Ca^{2+}-binding domain as CDPKs and CCaMKs do (Figure 3A), OsCBK is also different from CRKs because its C-terminus shares only about 16–18 % identity with CaM and does not have non-canonical EF-hand motifs as CRKs do (Figure 3A) [10]. These structural features suggest that OsCBK may be a novel protein kinase with a CaM-binding domain and without EF-hand motifs, and high homology with CRKs. OsCBK also has high identity with MCK1 (55.1 % identity, accession number 1839597) [12]. The high identity between OsCBK and MCK1 was further confirmed by showing that OsCBK was recognized by antibody against MCK1 (Figure 4, lane B).

Characterization of a tentative CaM-binding domain of OsCBK

OsCBK has the ability to bind CaM. This was shown by the following observations. (i) OsCBK was eluted from a CaM–Sepharose 4B column only in the absence of Ca^{2+}. (ii) OsCBK bound CaM in the presence of Ca^{2+} (Figure 4, lane C) and lost its binding ability in the absence of Ca^{2+} (Figure 4, lane D). (iii) Further evidence came from the analysis of truncated OsCBK. Three expression constructs, R1-455, R1-408 and R418-597, were made to express truncated OsCBKs (Figure 5A). R1-455 contained the OsCBK catalytic domain and a tentative CaM-binding domain (SSSLRRAALRALAKTLT) at its C-terminus, and R1-408 lacked residues 408–455 predicted to be involved in CaM binding at its C-terminus. R418-597 was the C-terminus of OsCBK lacking the catalytic domain, but having the 38 amino acid residues (IIYRLMRAYISSSSLRRAALRALAKTLTTD-QIYYLREQ) at its N-terminal overlapping with R1-455. These three constructs were introduced into *E. coli* BL21 (DE3) and total proteins isolated from the *E. coli* cells were used for Western blot analyses with biotinylated CaM. The results indicated that R1-455 and R418-597 bound to CaM in a Ca^{2+}-dependent manner while R1-408 lost its binding ability to CaM once these 38 amino acids were truncated (Figure 5B), showing a CaM-binding domain within this region. The analysis with the biological software Anthewin suggested amino acid residues SSSLRRAALRALAKTLT to be a basic amphiphilic α-helix, a classic structure of the CaM-binding domain (Figure 5C). Finally, the kinetic rate constant of OsCBK to CaM was determined to be 30 nM by affinity measurement with SPR, indicating its high affinity to CaM. The molecular interaction between an immobilized component, referred to as the ligand (CaM), and a molecule in the mobile phase, designated as the analyte (OsCBK), was determined by SPR studied in the BIAcore instrument. Changes in surface concentration are proportional to changes in the refractive index on the surface resulting in changes in the SPR signal plotted as response units as a function of time [31]. The purified rice CaM (Figure 6A) was biotinylated and immobilized to the sensor chip SA-5 covered with avidin. The association and dissociation of OsCBK were determined on a surface containing 750 response units of CaM (Figure 6B). OsCBK was serially diluted (600, 300, 120, 60 and 45 nM) and used for SPR analyses. Representative sensorgrams in Figure 6(C) demonstrated the interaction between CaM and OsCBK in different concentrations and the analyses showed that OsCBK specifically bound CaM with high affinity ($K_D = 30$ nM).

Autophosphorylation and substrate phosphorylation of OsCBK

To indicate OsCBK as a protein kinase, the autophosphorylation and substrate phosphorylation of OsCBK were analysed. To perform these experiments, Sf9 insect cells were infected with the OsCBK recombinant expression virus. OsCBK was purified by Ni-NTA and CaM-affinity chromatography, and assayed by SDS/PAGE as a single 68 kDa polypeptide, consistent with the predicted molecular mass of the fusion protein (Figure 4, lane A). Purified OsCBK was used for kinase activity assays.

OsCBK was shown to autophosphorylate and phosphorylate substrates such as histone IIIs (Figure 7). However, unlike CaMKs, CCaMKs and CDPKs, OsCBK carried out autophosphorylation and substrate phosphorylation in both the presence and absence of Ca^{2+}/CaM despite whether the reaction time was 30 min or 15 s (see Figure 7A; the top panel was for 30 min and the bottom panel was for 15 s), just like CRKs in plants [9,10]. Time course analyses showed that purified OsCBK rapidly phoshorylated histone IIIs in the presence of Ca^{2+} or EGTA, soon after OsCBK was added to the reaction mixture at 30 °C (Figure 7B). The results showed that Ca^{2+}/CaM binding to OsCBK does not take part in the kinase activity.

Autophosphorylation plays pivotal roles in activation of CaMK II in animals [32,33]. A time course for the phosphorylation of histone IIIs in the presence of 1 μM ATP is shown in Figure 7(E). The observed lag during substrate conversion suggested that autophosphorylation of OsCBK is required for the full activation of the enzyme towards exogenous substrate.

To rule out the possibility that OsCBK had become phosphorylated in Sf9 cells, the purified OsCBK was autophosphorylated (labelled by ^{32}P) *in vitro*, and dephosphorylated with the catalytic subunit of the α-isoform of protein phosphatase-1

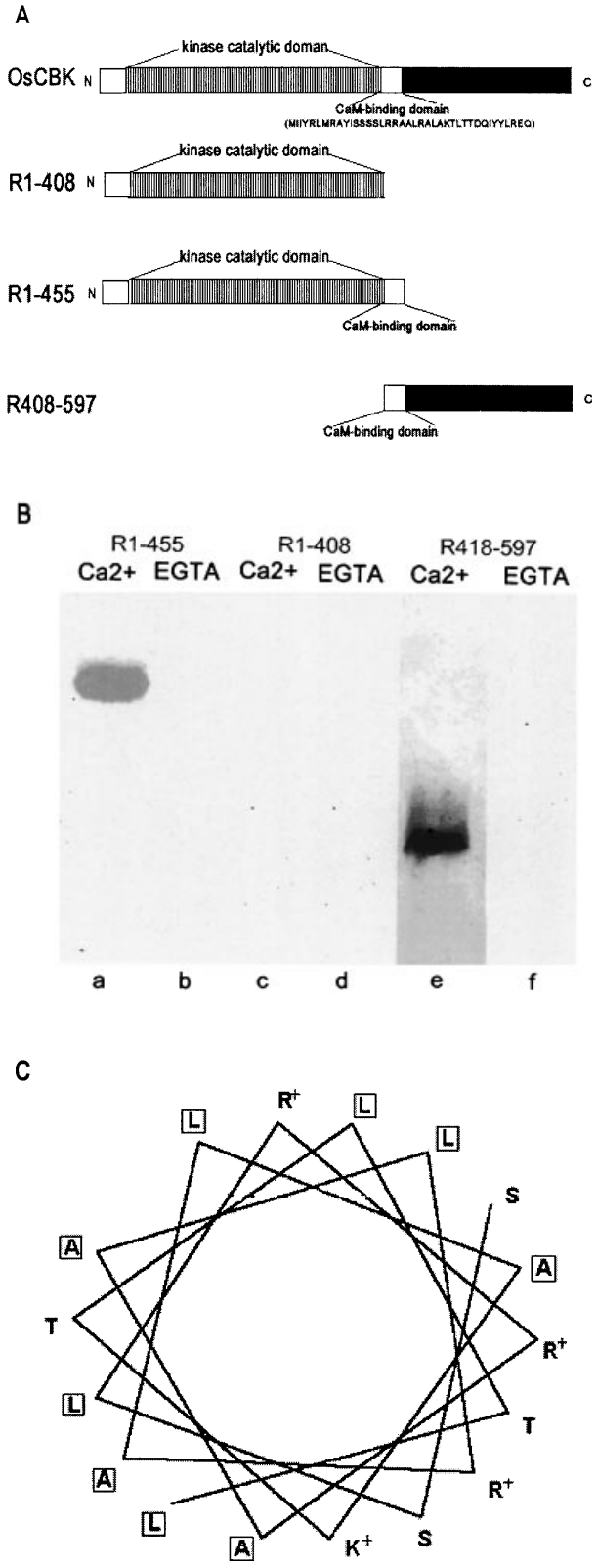

Figure 5 Identification of the CaM-binding domain of OsCBK

(**A**) Diagrams of the truncated forms of OsCBK. (**B**) Biotinylated CaM-binding assays. Lanes a and b, assays were done with R1-455 in the presence of Ca^{2+} (lane a) or EGTA (lane b); lanes c and d, assays were done with R1-408 in the presence of Ca^{2+} (lane c) or EGTA (lane d); lanes e and f, assays were done with R418-597 in the presence of Ca^{2+} (lane e) or EGTA (lane f). (**C**) Helical wheel projection of the CaM-binding sequence of OsCBK. Hydrophobic amino acid residues are boxed. Basic amino acid residues are marked with +.

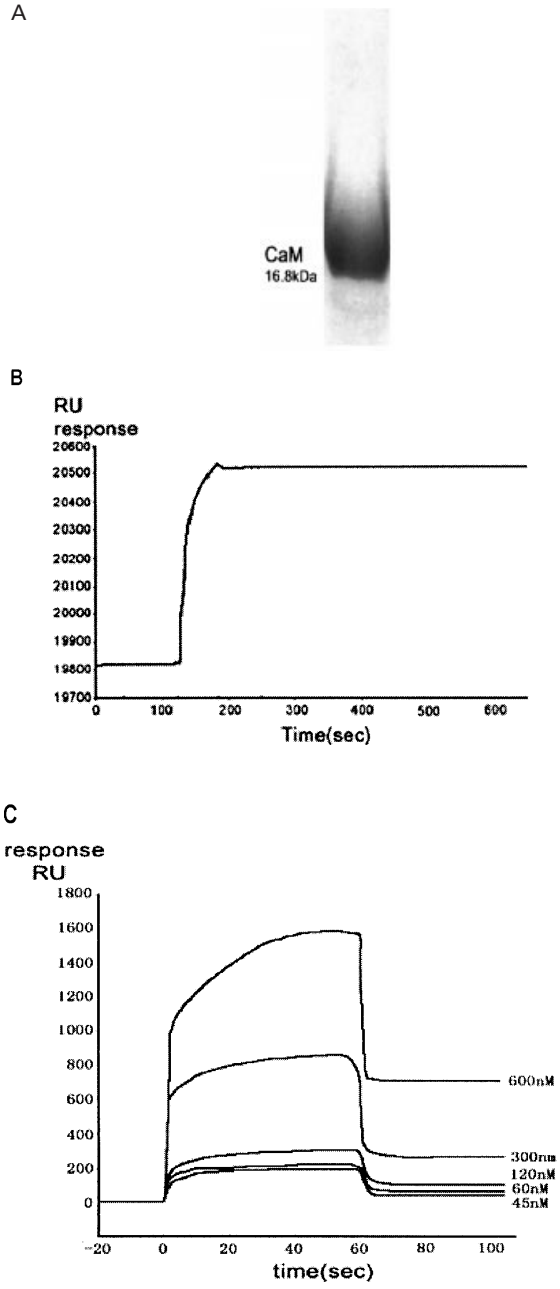

Figure 6 SPR assays for binding affinity of OsCBK to CaM

(**A**) Rice CaM purified from *E. coli*. (**B**) Biotinylated CaM was immobilized on to the sensor chip covered with avidin. (**C**) The sensorgram from SPR used for determination of the affinity constant. OsCBK was serially diluted (600, 300, 120, 60 and 45 nM) and applied to a sensor chip immobilized with CaM. RU, response units.

[34]. The results showed that ^{32}P can be removed from OsCBK (Figures 8A and 8B), and we used this phosphatase to dephosphorylate purified OsCBK for further experimentation. The Ca^{2+}/CaM-independence of OsCBK kinase activity was confirmed by showing that the dephosphorylated OsCBK could phosphorylate both itself and the substrate in the absence of Ca^{2+}/CaM (Figure 8C).

Capillary electrophoresis was also used to confirm that purified OsCBK lacks phosphoamino acids. Capillary electrophoresis has been used successfully for the identification of phosphoamino

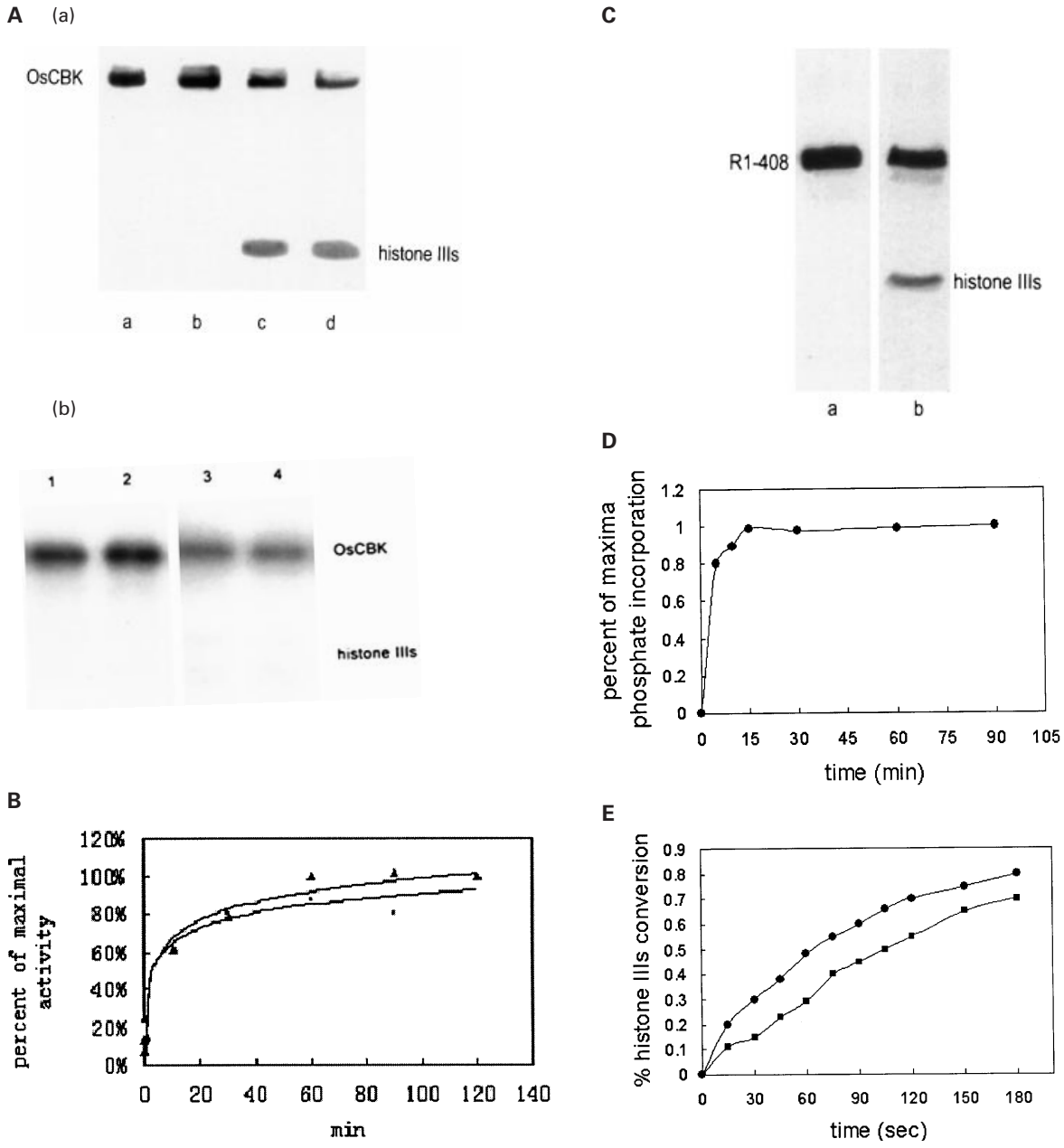

Figure 7 Phosphorylation assays

(**A**) OsCBK autophosphorylation and substrate phosphorylation using histone IIIs as substrate at 30 min (top panel) or 15 s (bottom panel). Lane a/1, autophosphorylation of OsCBK in the presence of Ca^{2+}/CaM; lane b/2, autophosphorylation of OsCBK in the presence of 2 mM EGTA; lane c/3, substrate phosphorylation of OsCBK in the presence of Ca^{2+}/CaM; lane d/4, substrate phosphorylation of OsCBK in the presence of 2 mM EGTA. (**B**) Time course of phosphorylation of histone IIIs by OsCBK in the presence of Ca^{2+}/CaM (●) or EGTA (▲). The data are means from three determinations done in duplicate. (**C**) R1-408 autophosphorylation and substrate phosphorylation using histone IIIs as substrate. (**D**) Time course of phosphorylation of histone IIIs by R1-408 in the absence of Ca^{2+}/CaM. (**E**) Effect of autophosphorylation on the time course of histone IIIs phosphorylation. Phosphorylation of histone IIIs by autophosphorylation (●) or control kinase (■) was carried out as described for the standard kinase assay; the concentration of ATP was under 1 μM.

acid [25,35]. When the hydrolysis products of purified OsCBK that was not autophosphorylated *in vitro* were separated and analysed by capillary electrophoresis all amino acids were detected except phosphoamino acids (Figure 9A). These results presented further evidence that the purified OsCBK used in kinase activity assays was not phosphorylated. Capillary electrophoresis analyses of autophosphorylated OsCBK showed that serine and threonine residues were phosphorylated (Figure 9B), indicating that OsCBK was a serine/threonine protein kinase, as also suggested by database analysis.

In order to define the possible roles of the CaM-binding domain for the kinase activity of OsCBK, R1-408 was also expressed in Sf9 cells. After being purified from cells through a Ni-NTA resin column, R1-408 activity was analysed. R1-408 was shown to autophosphorylate and phosphorylate substrates such as histone IIIs in the absence of Ca^{2+}/CaM (Figure 7C).

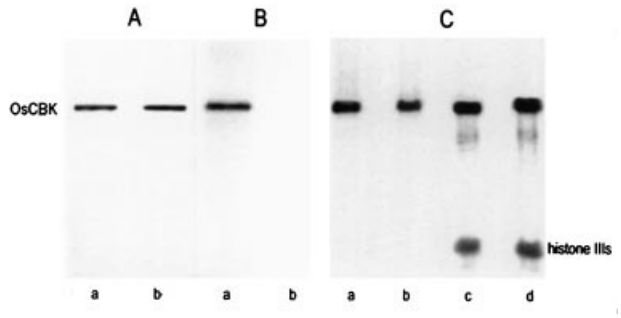

Figure 8 Kinase assays of the dephosphorylated OsCBK

(**A**) Silver staining of underphosphorylated (lane a) or dephosphorylated OsCBK (lane b) that was autophosphorylated. (**B**) Same as (**A**) but autoradiography. (**C**) Autophosphorylation and substrate-phosphorylation assays of the dephosphorylated OsCBK. Lane a, autophosporylation in the presence of Ca^{2+}/CaM; lane b, autophosphorylation in the presence of 2 mM EGTA; lane c, substrate phosphorylation in the presence of Ca^{2+}/CaM; lane d, substrate phosphorylation in the presence of 2 mM EGTA.

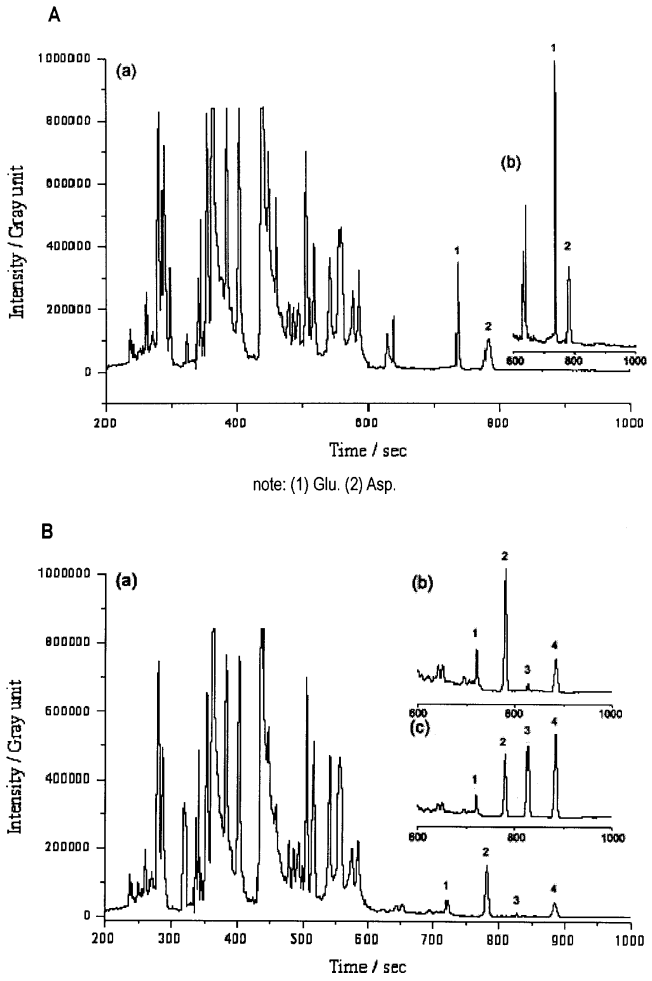

Figure 9 Phosphoamino acid analysed by capillary electrophoresis

(**A**) Electropherograms of the hydrolysed unphosphorylated OsCBK with FITC-labelling. Panel a, whole electropherogram; panel b, intercepted and enlarged form of panel a. (**B**) Electropherograms of the hydrolysed autophosphorylated OsCBK with FITC labelling. Panel a, whole electropherogram; panel b, intercepted and enlarged form of electropherogram a; panel c, electropherogram with addition of standard phosphoamino acids for peak identification.

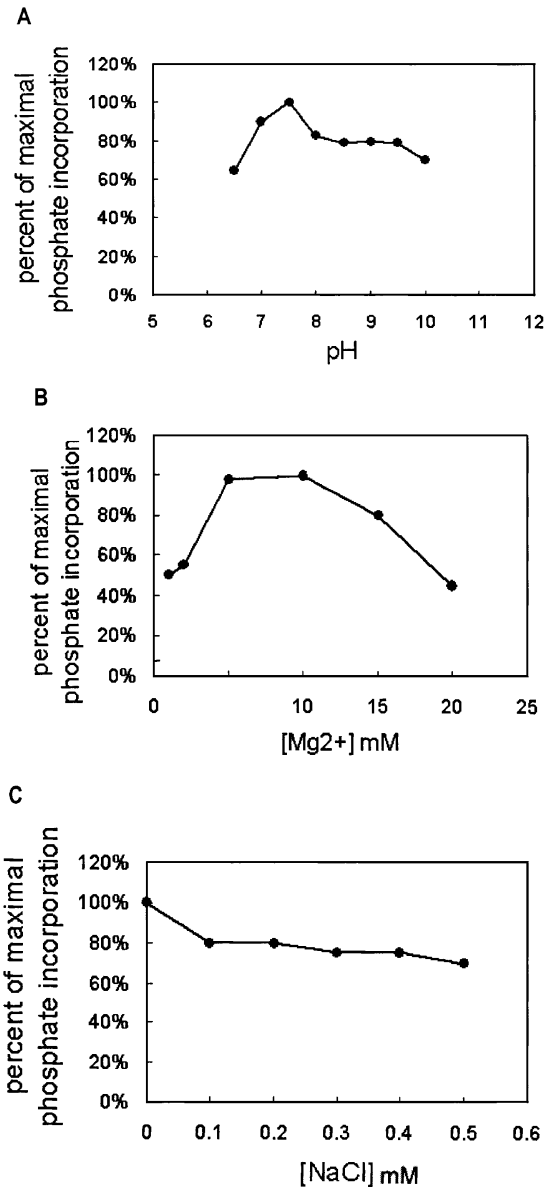

Figure 10 Effects of pH, Mg^{2+} and salt concentration on OsCBK activity

(**A**) pH. (**B**) Mg^{2+}. (**C**) NaCl. The data are means from three determinations done in duplicate.

Time-course analyses showed that R1-408 rapidly phoshorylated histone IIIs in the absence of Ca^{2+}/CaM, soon after R1-408 was added to the reaction mixture at 30 °C (Figure 7D). The similar characteristics of R1-408 with the full protein kinase OsCBK in autophosphorylation and substrate-phosphorylation assays further implied that the C-terminus of OsCBK including the CaM-binding domain might not be involved in the regulation of kinase activity of OsCBK.

Kinetic parameters of the kinase activity of OsCBK were determined from double-reciprocal analyses of data after phosphorylation of various concentrations of histone IIIs in the presence of 20 μM ATP. The results were the average of three determinations done in duplicate. The K_m for histone IIIs was found to be 50 ± 16 μg/ml when Ca^{2+}/CaM was added. On the other hand, the K_m was 43 ± 14 μg/ml in the absence of

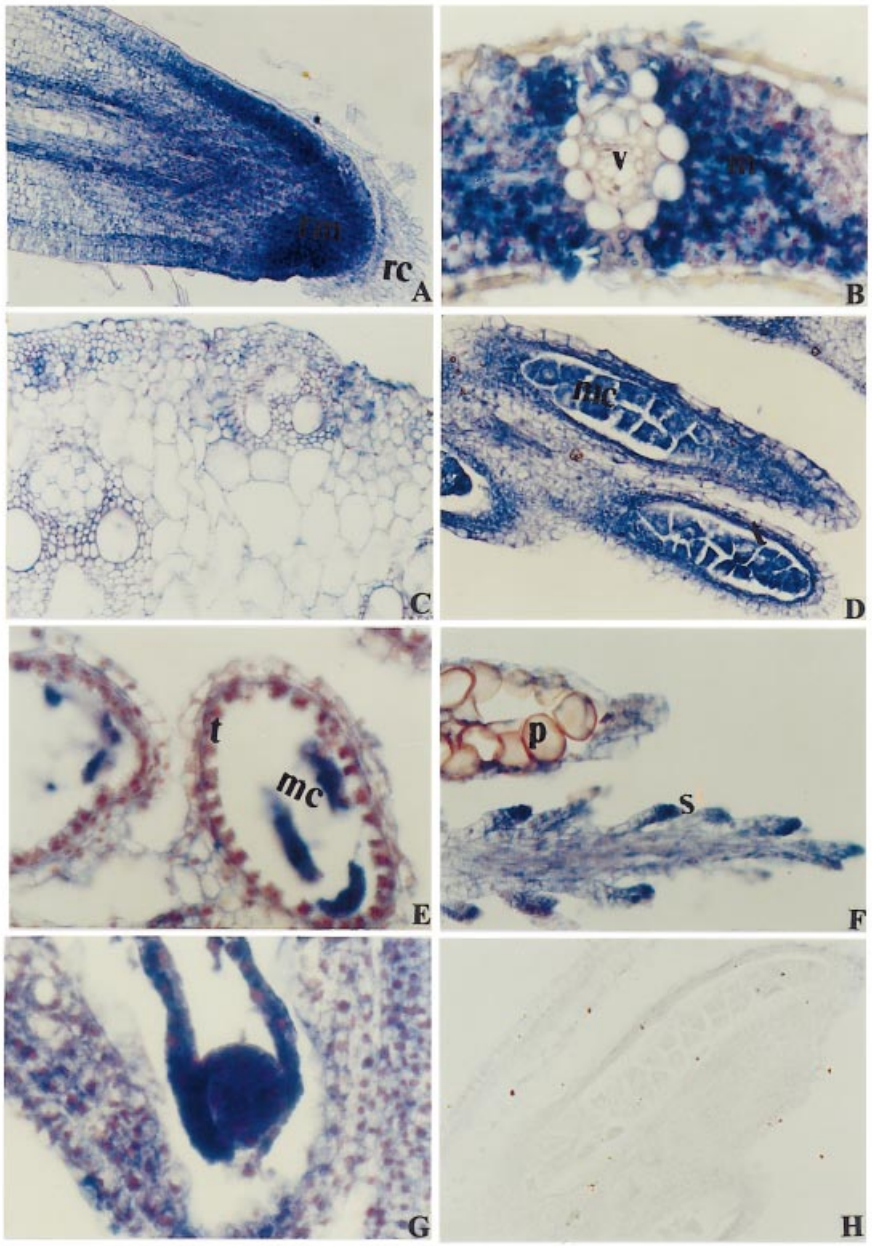

Figure 11 ***In situ*** **hybridizations showing the expression pattern of OsCBK in rice tissues**

(**A**) Root; (**B**) leaf; (**C**) stem; (**D**) sporogenous cell stage; (**E**) meiosis; (**F**) stigma and mature pollen cell; (**G**) proembryo; (**H**) control, sporogenous cell stage. Expression is seen as blue coloration under bright field. Magnification: ×96 in **A**; ×368 in **B**; ×96 in **C**; ×368 in **D**; ×184 in **E**; ×368 in **F**; ×368 in **G**; ×184 in **H**. rc, root cap; rm, root meristem; v, vascular bundle; mc, microspore cell; t, tapetum; p, pollen; s, stigma; m, mesophyll.

Ca^{2+}/CaM. This result indicated that Ca^{2+}/CaM had little effect on OsCBK activity when histone IIIs were used as substrates.

Effect of pH, salt and Mg^{2+} on OsCBK activity

The activities of several histone kinases from animal and plant sources have been shown to exhibit high-pH optima [36,37]. With histone IIIs as substrates, OsCBK showed a typical bell-shaped pH-dependence profile in the range of pH 6.0–10.0. Maximum activity was observed at pH 7.5 and this was reduced to 70–75% of maximum at pH 6.0 and 10.0 (Figure 10A). This pH-activity curve for OsCBK is similar to that for soya bean CDPK [38], but different from ground nut CDPKs. As for ground nut CDPKs, the activity increased exponentially from pH 5.5 to 9.0 and levelled off at pH 9.0–10.2 when MLCpep (the phosphate-accepting domain of smooth muscle myosin light

chain) was used as the kinase substrate [39]. Mg^{2+} forms a complex with ATP and this complex appears to be the actual protein kinase substrate [40]. In our assay, the optimum concentration of Mg^{2+} for OsCBK activity was found to be 5–10 mM (Figure 10B). OsCBK retained activity in various concentrations of NaCl, and 70% of maximum activity was retained in a concentration of NaCl as high as 0.5 M (Figure 10C). Such stability in the presence of a high concentration of salt suggests that the OsCBK–histone III interaction is not simply ionic in nature as reported for ground nut CDPK [39].

Localization of OsCBK expression

In situ hybridization was used to localize the cells and tissues of rice in which OsCBK is expressed. Using a DIG-labelled antisense RNA probe made from the OsCBK 3′ untranslated region, we showed that OsCBK was expressed ubiquitously during rice growth and development, but that the level of expression was regulated both temporally and spatially (Figure 11). Sense RNA of OsCBK was used as a control (Figure 11H). OsCBK was highly expressed in roots and was particularly abundant in the zone of cell division, but the mRNA was notably absent from the root cap (Figure 11A). OsCBK was expressed in leaf mesophyll cells but its expression was not detected around the vascular bundle or in epidermal cells (Figure 11B). Little OsCBK mRNA was detected in the tissues of mature rice stems (Figure 11C).

The expression of the OsCBK gene was regulated temporally and spatially during flower development. At the early stages of anther development, OsCBK was expressed in sporogenous cells and in the tapetum (Figure 11D). OsCBK expression decreased in the tapetum, but large amounts of OsCBK mRNA accumulated in the sporogenous cells at meiosis (Figure 11E). At the mature pollen stage, OsCBK mRNA was below the level of detection in mature pollen and the anther wall (Figure 11F). OsCBK was relatively strongly expressed in the stigma, especially at the stigmatic surface (Figure 11F). Following fertilization, OsCBK mRNA was abundantly accumulated in the proembryo (Figure 11G).

DISCUSSION

We have isolated and characterized a novel CaM-binding protein kinase from rice that lacks Ca^{2+}-binding EF hands and has Ca^{2+}/CaM-independent autophosphorylation and substrate-phosphorylation activity, while it has high affinity for CaM. Our results indicate that OsCBK represents a new class of CaM-binding protein kinase in plants.

A cDNA encoding OsCBK was isolated from a rice cDNA library using maize CaMK (MCK1) as a probe and characterized as a Ca^{2+}/CaM-binding protein kinase. Whereas OsCBK exhibits full kinase activity in the absence of Ca^{2+}/CaM, it retains the ability to bind Ca^{2+}/CaM with high affinity. It has been reported that *Z. mays* CRKs also do need calcium for their activities [10]. However, no biochemical evidence for their CaM-binding ability is available.

OsCBK was identified as a CaM-binding protein kinase based on the following observations. (i) OsCBK bound to CaM–Sepharose 4B only in the presence of Ca^{2+}. (ii) OsCBK bound biotinylated CaM in the presence of Ca^{2+} and lost its binding ability in the absence of Ca^{2+}. (iii) Further evidence came from analysis of the CaM-binding domain of OsCBK. Only truncated forms of OsCBK, R1-455 and R418-597, containing the predicted CaM-binding domain showed CaM-binding ability. (iv) Finally, the kinetic rate constant of OsCBK to CaM was determined to be about 30 nM by affinity measurement with SPR, indicating its high affinity to CaM.

It has been well-documented that the Ca^{2+}/CaM complex modulates the activities of CaMKs via its binding to CaMKs in mammalian systems [41]. However, the Ca^{2+}/CaM complex does not regulate the activity of OsCBK even though it binds to OsCBK. A possible explanation for the Ca^{2+}/CaM binding but Ca^{2+}/CaM -independent activity of OsCBK is that recombinant protein purified from Sf9 cells is in the form of a Ca^{2+}/CaM-kinase complex. The complex retains full activity regardless of Ca^{2+} concentration as long as CaM remains in the complex [42]. The explanation is unlikely in our experiments, because the purified OsCBK was eluted from a CaM–Sepharose affinity column with EGTA, indicating that OsCBK interacts with CaM–Sepharose and must be free of CaM. Furthermore, no CaM was found when the purified OsCBK was resolved by SDS/PAGE and visualized by silver and Coomassie staining.

An alternative explanation could be that the purified OsCBK from Sf9 cells was autophosphorylated or was phosphorylated by a protein kinase *in vivo*, and thus the phosphorylated OsCBK showed kinase activity in a Ca^{2+}/CaM-independent fashion. Autophosphorylation has been shown to play a critical role in the regulation of some CaMKs, leading to activation of the kinases [43]. The presence of phosphoamino acid on the autophosphorylation site is sufficient to disrupt the auto-inhibitory domain, and the kinase retains partial activity (20–80%), even after CaM dissociates from the kinases [3]. 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 total activity [43,44]. Is it possible that OsCBK, when expressed in Sf9 cells, was phosphorylated *in vivo*, resulting in its Ca^{2+}/CaM-independent activity? To address this possibility, OsCBK was dephosphorylated and the dephosphorylated OsCBK used to assay kinase activity. The dephosphorylated OsCBK retained its kinase activity in the absence of Ca^{2+}/CaM, suggesting that the Ca^{2+}/CaM-independence of OsCBK was not because the enzyme was phosphorylated *in vivo*. It could not be ruled out entirely that protein phosphatase did not remove all phosphates from the purified OsCBK and that a few remaining phosphoamino acids conferred OsCBK activity. To test this possibility, the purified OsCBK was separated by capillary electrophoresis, and amino acids/phosphoamino acids were identified. The results showed that OsCBK from Sf9 cells lacked phosphoamino acids, indicating that the kinase activity of OsCBK was indeed Ca^{2+}/CaM-independent. Thus we conclude that OsCBK is a CaM-binding protein kinase, but its enzymic activity is independent of Ca^{2+}/CaM.

It has been reported that the autoinhibitory domain of soya bean truncated CDPKα without the CaM-like domain binds CaM, but the holoenzyme is not greatly stimulated by CaM. This is because the CaM-binding sequence in these CDPKs interacts intra-molecularly with the CaM-like domain in the holoenzyme, probably resulting in CaM binding being unavailable [45]. In OsCBK, its C-terminus has only about 16–18% identity with CaM without the conserved EF hand for Ca^{2+} binding, and its putative CaM-binding domain binds CaM in the holoenzyme in a Ca^{2+}-dependent manner. The fact that CaM is able to bind to OsCBK, but is unable to activate OsCBK, suggests that the simple binding is not related to activation of OsCBK. OsCBK may represent an evolutionary link between CRK/CDPKs and CaMKs.

Although the potential regulatory roles of CaM binding to OsCBK are not understood, it is possible that CaM binding exhibits an accessory form of regulation, in which CaM binding promotes phosphorylation of the protein by other kinases [5]. Another hypothetical role could be that CaM binding brings about a conformational change in OsCBK that alters its cellular location or substrate specificity [5]. Whether this binding site might have a role in docking the kinase into a protein complex or in modifying more subtle features of regulation is not known. These hypotheses need to be further investigated experimentally.

We localized the expression of OsCBK and showed that it was expressed temporally and spatially during plant growth and development. OsCBK mRNA accumulated in the tissues/cells that are required for rapid growth and metabolic activity, including the growing region of the root, sporogenous cells in the anther, the stigma and the developing proembryo. However, OsCBK mRNA was not detectable in mature pollen and the anther epidermis. These results imply that OsCBK may be related to cell metabolism and the cell cycle.

In summary, our results indicated that a temporally and spatially regulated kinase gene, OsCBK, was cloned from rice. The encoded protein showed Ca^{2+}/CaM-independent kinase activity, even though it was a Ca^{2+}/CaM-binding protein. OsCBK is not a CaM-dependent protein kinase or a Ca^{2+}-dependent protein kinase, and also is not a CDPK-related protein kinase, and also is not a CRK in which CaM binding is not shown experimentally, but a novel Ca^{2+}/CaM-binding protein kinase in plants. These findings lead us to consider a new subtle regulatory role for CaM in various plant protein-phosphorylation events.

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