A tobacco (*Nicotiana tabaccum*) calmodulin-binding protein kinase, NtCBK2, is regulated differentially by calmodulin isoforms

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A calcium (Ca^{2+})/calmodulin (CaM)-binding protein kinase (CBK) from tobacco (*Nicotiana tabaccum*), NtCBK2, has been characterized molecularly and biochemically. NtCBK2 has all 11 conserved subdomains of the kinase-catalytic domain and a CaM-binding site as shown by other kinases, including Ca^{2+} -dependent protein kinase and chimaeric Ca^{2+} /CaM-dependent protein kinases. However, this kinase does not contain an EF-hand motif for Ca^{2+} binding, and its activity was not regulated by Ca^{2+} . Whereas NtCBK2 phosphorylated both itself and other substrates, such as histone H1S and syntide-2, in a Ca^{2+} /CaM-independent manner, as also shown by OsCBK, a CaM-binding protein kinase from rice (*Oryza sativa*), the kinase activity of NtCBK2 was greatly stimulated by Ca^{2+} /CaM, whereas that of OsCBK was not. By molecular dissection analyses, the CaM-binding domain of NtCBK2 has been localized in a stretch of 30 amino acid residues at residue positions 431–460 as a 1-5-10 protein motif. Three

tobacco CaM isoforms (NtCaM1, NtCaM3 and NtCaM13) used in the present study have been shown to bind to NtCBK2, but with different dissociation constants (K_d s), as follows: NtCaM1, 55.7 nM; NtCaM3, 25.4 nM; and NtCaM13, 19.8 nM, indicating that NtCBK2 has a higher affinity for NtCaM3 and NtCaM13 than for NtCaM1. The enzymic activity of NtCBK2 was also modulated differently by various CaM isoforms. Whereas the phosphorylation activity of NtCBK2 was shown by assay to be enhanced only ≈ 2 –3-fold by the presence of NtCaM1, the activity could be amplified up to 8–9-fold by NtCaM3 or 10–11-fold by NtCaM13, suggesting that NtCaM3 and NtCaM13 are better activators than NtCaM1 for NtCBK2.

Key words: calmodulin-binding protein kinase (CBK), capillary electrophoresis, dissociation constant, phosphorylation, tobacco.

INTRODUCTION

Ca^{2+} has been reported to be involved in a wide variety of cellular and physiological processes in all life forms [1–4]. In plants, Ca^{2+} -mediated processes are regulated by its targets, including CDPKs (Ca^{2+} -dependent protein kinases) and CaM (calmodulin) [5,6]. CDPK, as a well-known Ca^{2+} target protein, consists of an N-terminal catalytic domain and a C-terminal CaM-like sequence, having EF-hand motifs for Ca^{2+} binding [7], and it has been shown to have physiological and developmental roles in plants [8–10]. CaM, another well-identified Ca^{2+} target protein, has no enzymic activity by itself: it has important roles in regulating its binding proteins, including CaMKs (Ca^{2+} /CaM-dependent protein kinases) [11–13].

In yeast and mammals, CaMKs have been shown to be a key component of CaM-mediated signal transduction, and there has been a dramatic disclosure of information on biochemical and functional aspects of these proteins [11,14,15]. However, little was known about CaMKs in plants [13,16,17]. Recently, several kinds of CBKs (CaM-binding protein kinases) in plants have been isolated, including CB1, a homologue of mammalian CaMKII from apple [18,19], MCKs (maize homologues of mammalian CaMK) from maize [20,21], CCaMKs (chimaeric CaMKs) from lily [22], tobacco [23] and maize [24], and CBK from rice (OsCBK; 'Os' derived from *Oryza sativa*) [25]. Among them, only CCaMK and OsCBK have been well characterized

biochemically. Whereas CCaMK has been reported to have a visinin-like domain with three EF-hand motifs that are both necessary for Ca^{2+} -dependent autophosphorylation and crucial for maximal activation of CCaMK [23,26,27], the enzymic activity of OsCBK has been shown to be independent of either Ca^{2+} or CaM [25].

In plants, numerous CaM isoforms are expressed from a small multigene family within a single organism. At least five expressed CaM isoforms are present in soya bean [28], eight CaM isoforms are produced in *Arabidopsis* [29] and potato [30], and thirteen CaMs are found in tobacco [31]. Studies have shown that different CaM isoforms from *Arabidopsis*, potato and soya bean regulate Ca^{2+} /CaM-binding proteins, including CCaMK, differently [23,32,33]. The biological consequences of the expression of CaM isoforms that exhibit differential regulation of target enzymes could provide a primary mechanism for altering the Ca^{2+} signal.

Here, we report molecular and biochemical characterization of a tobacco CBK (NtCBK2; 'Nt' derived from *Nicotiana tabaccum*) that is structurally different from CCaMK, and has different enzymic properties from OsCBK. NtCBK2 binds to CaM in a Ca^{2+} -dependent manner and phosphorylates itself at serine residues. The kinase-activity assays indicate that this kinase has enzymic activity in the absence of Ca^{2+} and CaM. However, this activity can be stimulated by tobacco CaMs. Whereas NtCaM3 and NtCaM13, two isoforms of tobacco CaM, amplify the

Abbreviations used: CaM, calmodulin; CaMK, Ca^{2+} /CaM-dependent protein kinase; CBK, CaM-binding protein kinase; CCaMK, chimaeric CaMK; CDPK, Ca^{2+} -dependent protein kinase; CRK, CDPK-related kinase; DTT, dithiothreitol; MCK, maize homologue of mammalian CaMK; Ni-NTA, Ni^{2+} -nitrilotriacetate; NtCBK2, *Nicotiana tabaccum* CBK2; OsCBK, *Oryza sativa* CBK; TBS, Tris-buffered saline.

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enzymic activity of NtCBK2 up to 11-fold, the isoform NtCaM1 stimulates it only ≈ 2 –3-fold, indicating its diverse regulation by various CaM isoforms.

MATERIALS AND METHODS

Screening of a tobacco cDNA library

The mRNAs were isolated from leaves of *N. tabaccum* cv. W38, and were used for cDNA library construction with the ZAP-cDNA Synthesis kit, following the manufacturer's instructions (Stratagene). The cDNA library was screened with plasmid p550 containing a maize cDNA encoding MCK1 [20]. Positive recombinant phage for NtCBK2 were isolated and excised into recombinant pBluescript SK⁻ *in vivo*. The candidate plasmid pNtCBK2 was then sequenced.

RNA isolation and Northern blotting

Total RNA from tobacco was isolated with TRIZOL[®], as described by the manufacturer (Gibco), and Northern blotting was carried out as described previously. [20]. In brief, 20 μ g of total RNA was separated on a 1.5 % formaldehyde/agarose gel and blotted on to a nylon membrane (NYTRAN[®]). The membrane was incubated with the α -³²P-labelled probe made from pNtCBK2 at 65 °C overnight, and washed under high-stringency conditions.

Construction of NtCBK2 and truncated NtCBK2

To define the CaM-binding domain, several constructs were made with plasmid pFastHTb (see Figure 6A). The cDNAs for the full open reading frame (NtCBK2) and three truncated forms (T1-460, T1-424 and T431-602) of NtCBK2 were amplified with four pairs of primers, as follows: 5' primer CGGGATCC-ATGGGTGCTTGCACTTCAAAACC and 3' primer CGGGA-TCTCATTTGAACCTTTTGTAAATTGAGCGGC for NtCBK2; 5' primer CGGGATCCATGGGTGCTTGCACTTCAAAACC and 3' primer CGGGATCCCTGCTCCTTCAGATAAAATA for T1-460; 5' primer CGGGATCCATGGGTGCTTGCACTTCAAAACC and 3' primer CGGGATCCCTATCAAAATATCCAAAG-GCTCCT for T1-424; and 5' primer CGGGATCCCTACAT-GAGGTCCTCTGCTCTTAGA and 3' primer CGGGATCCT-CATTGAACCTTTTGTAAATTGAGCGGC for T431-602. Whereas T1-460 contains the N-terminus of NtCBK2, including the kinase catalytic domain and a tentative CaM-binding domain, T1-424 has the same N-terminal amino acid sequence as T1-460, but lacks the tentative CaM-binding domain. The third truncated form, T431-602, contains the C-terminus of NtCBK2 with the tentative CaM-binding domain. *Bam*HI-digested DNA fragments for NtCBK2, T1-460, T1-424 and T431-602 were cloned into the *Bam*HI site of plasmid pFastHTb. After confirmation of their sequences by sequencing, the recombinant plasmids were transformed into DH10Bac-competent cells containing the bacmids 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 recombinant bacmids were identified on the basis of disruption of the *lacZ* gene. The Sf9 cells were maintained as monolayers at 27 °C in Grace's medium containing 10 % (v/v) fetal bovine serum, and were transfected by the recombinant bacmids with Cellfectin reagent, according to manufacturer's instruction (Gibco). Recombinant virus was harvested after 72 h and identified by PCR, using the primers described above.

Purification of NtCBK2 proteins

Insect Sf9 cells were infected by the recombinant virus for 72 h, and harvested at room temperature (≈ 25 °C). The 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 P40/0.2 mM PMSF], and then homogenized by sonication for 30 s, followed by centrifugation at 12 000 *g* for 10 min. The supernatant was applied to a Ni-NTA (Ni²⁺-nitrilotriacetate) resin column pre-equilibrated with buffer A [50 mM potassium phosphate (pH 6.0)/300 mM KCl/10 % glycerol]. After extensively washing with buffer A and buffer A containing 25 mM imidazole, NtCBK2 was subsequently eluted with buffer A containing 200 mM imidazole. The eluted NtCBK2 was dialysed in 25 mM Tris/HCl, pH 7.5, for approx. 6 h, supplemented with CaCl₂ at a final concentration of 0.5 mM, and loaded on to a CaM-Sepharose 4B (Amersham Pharmacia Biotech) column pre-equilibrated with buffer B [25 mM Tris/HCl (pH 7.5)/2 mM CaCl₂]. After washing with buffer B plus 200 mM NaCl, elution of NtCBK2 was performed with buffer C [25 mM Tris/HCl (pH 7.5)/2 mM EGTA]. Purified NtCBK2 was dialysed in 25 mM Tris/HCl, pH 7.5, for 6 h, and either used immediately or stored at -80 °C prior to SDS/PAGE and enzymic analysis. The protein concentration was determined by the quantification of tryptophan in NtCBK2 [34]. All procedures were performed at 4 °C, unless stated otherwise.

Autophosphorylation and dephosphorylation of NtCBK2

Autophosphorylation assays were performed in 100 μ l of reaction mixture, containing 25 mM Tris/HCl, pH 7.5, 0.5 mM DTT (dithiothreitol), 10 mM magnesium acetate, 100 μ M ATP and 10 μ Ci of [γ -³²P]ATP (5000 Ci/mmol) plus 0.5 mM CaCl₂ or 0.5 mM CaCl₂/1 μ M CaM or 2 mM EGTA, at 30 °C for 30 min. For time-course assays, 15 μ g of NtCBK2 was used in 100 μ l of reaction mixture. Aliquots for the zero time point were taken immediately after the addition of NtCBK2 to initiate the reaction, and the reactions were terminated by adding a one-fifth 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 NtCBK2 bands in a liquid scintillation counter (Beckman LS 6500). The experiments were repeated three times in duplicate.

Dephosphorylation of NtCBK2 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 MnSO₄ at 30 °C for 10 min. The dephosphorylated NtCBK2 was purified further with CaM-Sepharose 4B.

Substrate phosphorylation assay

Substrate phosphorylation assays were performed in 100 μ l of reaction mixture containing 25 mM Tris/HCl, pH 7.5, 0.5 mM DTT, 10 mM magnesium acetate, 100 μ M ATP, 10 μ Ci of [γ -³²P]ATP (5000 Ci/mmol), 50 μ M histone H1S or 50 μ M syntide-2 plus 0.5 mM CaCl₂ or 0.5 mM CaCl₂/1 μ M CaM or 2 mM EGTA with 15 μ g of NtCBK2 at 30 °C for 30 min.

For time-course assays with histone H1S as the substrate, aliquots for the zero time point were taken immediately after the addition of NtCBK2 to initiate the reaction, and the reaction was terminated by adding a one-fifth 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 the 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 NtCBK2 to initiate the reaction, and the reaction was terminated by adding a one-fifth volume of $5 \times$ SDS/PAGE sample buffer. Aliquots from one reaction were separated by SDS/PAGE with a 10 % separating gel. The amount of phosphates transferred to the kinase was determined by counting the radioactivities of the excised NtCBK2 bands in a liquid scintillation counter. Aliquots from another reaction were applied to P81 phosphocellulose filters ($2\text{ cm} \times 2\text{ cm}$ squares; Whatman) for total [^{32}P]P_i incorporation of the kinase and syntide-2. Filters were washed four times (10 min each wash) in 75 mM phosphoric acid, rinsed in 100 % ethanol and then air-dried. [^{32}P]P_i incorporation was determined by liquid scintillation counting. The amount of phosphates transferred to substrate syntide-2 was determined by subtracting [^{32}P]P_i incorporation of NtCBK2 from the total [^{32}P]P_i incorporation of NtCBK2 plus syntide-2.

Activation assay of NtCBK2 by NtCaM isoforms

The reaction for NtCBK2 substrate phosphorylation was performed as described above. Aliquots ($10\text{ }\mu\text{l}$) were removed and applied to P81 phosphocellulose filters ($2\text{ cm} \times 2\text{ cm}$ squares; Whatman). Filters were washed four times (10 min each wash) in 75 mM phosphoric acid, rinsed in 100 % ethanol and air-dried. [^{32}P]P_i incorporation was determined by liquid scintillation counting (Beckman LS 6500). The experiments were repeated three times in duplicate.

Preparation of ^{35}S -labelled and biotinylated CaM

Tobacco CaM cDNAs (isoforms NtCaM1, NtCaM3 and NtCaM13) cloned into the pET15 expression vector (a gift from Dr Y. Ohashi, National Institute of Agrobiological Sciences, Japan) [31] were used to prepare ^{35}S -labelled CaM, as described previously [35,36]. Bovine CaM was biotinylated as described by Billingsley et al. [37]. Briefly, bovine CaM was dialysed overnight at 4°C against 0.1 M phosphate buffer, pH 7.4. The bovine CaM was then supplemented with 1 mM CaCl_2 and incubated with D-biotinoyl- ϵ -amidocaproic acid-*N*-hydroxysuccinimide ester (Sigma) dissolved in *N,N*-dimethyl formamide at a final concentration of 1 mM. The incubation was performed for 2 h at 4°C with constant stirring. The biotinylated CaM was dialysed further in 0.1 M phosphate buffer, pH 7.4, for 48 h at 4°C , measured with BSA as a standard and stored in 20 % (v/v) glycerol at -20°C . The amounts of ^{35}S -labelled NtCaMs were measured from 0.5×10^6 c.p.m./ μg of material, as described previously [36].

CaM binding assay

NtCBK2 and its truncated forms were separated by SDS/PAGE and blotted on to a PVDF membrane. For the biotinylated CaM binding assay, the membrane was blocked in 2 % BSA/Tris-

buffered saline (TBS; composition: 50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 50 mM MgCl_2 plus 0.5 mM CaCl_2 or 2 mM EGTA) and washed three times with TBS (15 min each wash). After incubation in TBS containing biotinylated CaM for 3 h at room temperature and then washing with TBS, the membrane was treated with avidin-horseradish peroxidase 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 .

CaM binding affinity assay for NtCBK2

CaM binding affinity assays were carried out as described previously [26]. Briefly, purified NtCBK2 protein (3 pmol) separated by SDS/PAGE was electrophoretically transferred on to nitrocellulose filters and incubated with different concentrations of ^{35}S -labelled CaM with 1 mM CaCl_2 overnight. After washing with solution containing 25 mM Tris/HCl, pH 7.5, 50 mM NaCl and 1 mM CaCl_2 , the radioactivities of the bound CaM on each filter and the free CaM in the incubation buffer collected before washing were measured using a liquid scintillation counter (Beckman LS 6500). Average background counts were subtracted from the counts in NtCBK2 protein samples when calculating the specific binding. The dissociation constants (K_d s) were calculated using SPSS Enzyme Kinetics (SPSS Inc.). Data points are expressed as means of the results from three independent assays in duplicate.

Phospho amino acid assays

Both NtCBK2 and autophosphorylated NtCBK2 were hydrolysed in 6 M HCl for 12 h at 110°C , then dried and dissolved in $20\text{ }\mu\text{l}$ of 10 mM borate buffer, pH 10.0. The hydrolysed product was mixed with $20\text{ }\mu\text{l}$ 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 phospho amino acids, $2\text{ }\mu\text{l}$ of standard solution containing each amino acid and phospho amino acid (0.5 mM each) was mixed with $46\text{ }\mu\text{l}$ of 1 mM FITC, $100\text{ }\mu\text{l}$ of 20 mM borate buffer, pH 10.0, and $52\text{ }\mu\text{l}$ 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, as described previously [38]. Data were collected on a computer with Spot Advanced software, and processed further with Scion Image and Origin software packages.

RESULTS

Cloning and characterization of NtCBK2 cDNA

A tobacco cDNA library was screened with maize *MCK1* as a probe, resulting in the isolation of a cDNA clone (designated pNtCBK2) with a 2444 bp insert (GenBank® accession no. AF435452). The pNtCBK2 contains an open reading frame of 1809 bp, with a start codon ATG at positions 229–231 and a stop codon TGA at positions 2035–2037 (Figure 1). This clone was determined to be full length for the open reading frame, as judged on the basis of the presence of a stop codon TAG in-frame at the positions 223–225 in the 5'-untranslated region (Figure 1). Northern blot analysis revealed a single band of approx. 2.5 kb (results not shown), consistent with the cDNA size.

The deduced NtCBK2 from pNtCBK2 consists of 602 amino acid residues with a calculated molecular mass of 66 kDa. To

Figure 1 Nucleotide and deduced amino acid sequences of NtCBK2

The nucleotides are numbered on the right. The start codon (ATG) and the stop codon (TGA) are shown in non-shaded boxes. The stop codon (TAG) in-frame in the 5'-untranslated region is shown in a grey-shaded box.

search for a putative function of NtCBK2, a database comparison was conducted to identify similarity between the NtCBK2 sequence and other genes with known functions (Figure 2). NtCBK2 contains all eleven subdomains, characteristic of the protein kinase catalytic domain, as well as the conserved CaMK amino acid residues [39] (Figure 2). NtCBK2 shares a high sequence identity with CBKs and CDPKs, including rat CaMKII α -subunit (GenBank® accession no. J02942; 21 %) [39], maize MCK1 (GenBank® accession no. 1839597; 59 %) [20], OsCBK (GenBank® accession no. AF368282; 66 %) [25], lily CCaMK (GenBank® accession no. 860675; 23 %) [22], carrot CRK (CDPK-related kinase) (GenBank® accession no. 1103386; 78 %) [40] and soya-bean CDPK (GenBank® accession no. P28583; 34 %) [41] (Figure 2). The NtCBK2 sequence was also analysed using programs found on the ExPASy Proteomics tools. Using MotifScan, we found that, unlike CDPKs and CCaMKs, NtCBK2 does not possess Ca^{2+} -binding EF-hands at its C-terminus (Figure 2). Prediction of secondary structure using the Anthwin 45 package suggests that amino acid residues at positions 439–456 (KAALRALSKTLTVDELFFY) might be a basic amphiphilic α -helix for CaM binding [42]. These structural features implied that NtCBK2 could be a CBK.

Autophosphorylation and substrate phosphorylation of NtCBK2

To analyse NtCBK2's enzymic properties as a protein kinase, NtCBK2 was purified by Ni-NTA and CaM-affinity chromatography from Sf9 insect cells infected with the NtCBK2 recombinant expression virus, and assayed by SDS/PAGE as a single 68 kDa polypeptide (Figure 3), consistent with the predicted molecular mass of the fusion protein. This purified NtCBK2 was then assayed for the kinase autophosphorylation and substrate phosphorylation.

Considering the high level of similarity between NtCBK2 and CDPK/CBK, Ca^{2+} and EGTA were used in the kinase assays of NtCBK2. As shown in Figure 3, NtCBK2 phosphorylated itself and histone H1S as substrates in the presence of Ca^{2+} . This kinase activity was also assayed in the presence of EGTA (Figure 3), demonstrating that the kinase activation of NtCBK2 is independent of Ca^{2+} . Time-course studies showed that purified NtCBK2 could rapidly autophosphorylate and phosphorylate histone H1S and syntide-2 soon after NtCBK2 was added to the reaction mixture (Figure 4). Kinetic analysis showed that the V_{max} values were 0.35 nmol/min per mg of protein for histone H1S and 11 nmol/min per mg of protein for syntide-2 in the presence

1	MGACTSKPSNFSVDDITVAGD-GAIFPVKSGPSNDDVNSHQTKNDE-----PSVGKKSP	NtCBK2
1	MGQCYGKARDASSRADHDADPSGAGSVAPPSPPLPANGAPLPATPRRHKSGSTTFVHHHQA	MCK1
1	MGLCHGK-SAAVLEPTVEEEEGATRVAEAAAAPAKPASPAPSAAAAAAKPGTPKQHK--	OsCBK
1	MGICVSKPSP-EPDLNHHSTI--PVNDTSLPPQDNSIPPKDIAIPAQDNNKEP-GKKSP	carrot CRK
0	-----	rat CaMKII
0	-----	lily CCaMK
0	-----	soybean CDPK
55	FFPFYSPSPAHYLFSSKKSPATNA-----SSNSTPMRF-FKRPFPPSPAKHIRSLARRHG	NtCBK2
61	ATPGAAAWPSPYPAGGASPLPAGVSPSPARSTPRRF-FKRPFPPSPAKHIKATLAKRLG	MCK1
58	F-PFYLPSPLPASSYKSPA-NS-----SVASTPARGGFKRPFPPSPAKHIRALLARRHG	OsCBK
57	FLPFYSPSPAHYLFSSKKSPAVGSPAAGSSNSTPKRL-F--PFPPSPAKHIKAAWARRHG	carrot CRK
0	-----	rat CaMKII
1	-----MSRHE	lily CCaMK
0	-----	soybean CDPK
110	TVKPNEAISIEGNESEVGDGGGAG-----LDKSFSGFSKNFVNKYEMGEE	NtCBK2
120	GGKPKEGTIPEEGGAGAGAGAGAGAAVGAADSAEADRPLDKTFGFAKNFGAKYDLGKE	MCK1
112	SVKPNEAISIEGNESEVGDGGGAG-----LDKSGFSGFSKNFVNKYELGRE	OsCBK
114	SVKPNEAISIEGNESEVGDGGGAG-----LDKSGFSGFSKNFVNKYELGRE	carrot CRK
1	-----MATITCTR-FTEEVVFGKT	rat CaMKII
6	SRKLSDDYEVVDVLGKGGSVVRRGISKSRGKNNDVAIKTLRRY-GYTLPGAQRSQPGQR	lily CCaMK
1	-----MAAKSSSSSTTTNVVTLKAAWVLPQRTQNIREV--YEVGRK	soybean CDPK
154	VGRGHFGYTCKAKFKKGEVVKQEVAVKVIKSKMTAIAIEDVRREVKILRALTGHNHLV	NtCBK2
180	VGRGHFGHTCSAVVKKGEHKGHTVAVKIIISKAKMTAISIEDVRREVKILKALSGHDNLV	MCK1
150	VGRGHFGYTCAATCKKGELKGODVAVKVIKSKMTAIAIEDVRREVRLSSLAGHSNLV	OsCBK
154	VGRGHFGYTCKAKFKKGEVVKQEVAVKVIKSKMTAIAIEDVRREVKILRALTGHNHLV	carrot CRK
19	LKGAFSVVRRVCVKV---LAGQYAAKIINTKLSA-RDHQKLEREARICRLK-HPNIV	rat CaMKII
65	-GKSPLG-----MPTLKQVSVDALLTNEILVMRR---IVEDVSPHPNVI	lily CCaMK
40	LQQGQFGTTTFECTRRASG--GKF-ACKSIPKRKLLCKEDYEDVWREIQIMHHLSEHANVV	soybean CDPK
214	KFYDAYEDPNNVYIVMELCEGGELLDRIILSRGGKYTEDDAKSVMIQILKVVAFCHLQGVV	NtCBK2
240	RFYDACEALNVYIVMELCEGGELLDRIILATGGRYTEEDAKAIIVQILSVVAFCHLQGVV	MCK1
210	QFYDAYEDENYIVMELCKGGELLDRIILARGGKYSEEDAKVVMRQILSVASFCHLQGVV	OsCBK
214	QFYDAFEDHTNVYIVMELCEGGELLDRIILSRGGKYTEDDAKAVMIQILNVVAFCHLQGVV	carrot CRK
74	RLHDSISEEGHHYILFDLVTGGELFEDIVARE-YSEADASHCIQQILEAVLHCHQMGGV	rat CaMKII
106	HLHDVYEDANGVHLVLELCGGELFDRIIV-DDRYSEEAEEVQVQIASGLAALHKSTII	lily CCaMK
97	RIEGTYEDSTAVHLMELCEGGELFDRIIVQKG-HYSERQAARLIKTIVEVVEACHSLGVM	soybean CDPK
274	HRDLKPENFLFTSKDENAQIKAIIDFGLSDFVKPDERLNDIVGSAY-YVAPEVLHR-SYST	NtCBK2
300	HRDLKPENFLFTTRDESAPMKLIDFGLSDFTRPDERLNDIVGSAY-YVAPEVLHR-SYSM	MCK1
270	HRDLKPENFLFSSKDENSAMKVIDFGLSDFVKPDERLNDIVGSAY-YVAPEVLHR-SYGT	OsCBK
274	HRDLKPENFLFSSKDENSAMKVIDFGLSDFVKPDERLNDIVGSAY-YVAPEVLHR-SYST	carrot CRK
133	HRDLKPENLLLASLKLGAIVKLADFGLAIEVEGEQQAWFGAGTGYLSPEVLKDPYK	rat CaMKII
165	HRDLKPENCLFLNQEKSTLKLIMDFGLSSVEDFTDPIVALFGSID-YVSPEALSQRQVSS	lily CCaMK
156	HRDLKPENFLFDITIDAKLKATDFGLSVFYKPGESFCDDVVGSPY-YVAPEVL-RKLYGP	soybean CDPK
332	EADVWSIGVIAYILLCGSRPFWARTESGIFRAVLKADPSFDEQPWPTLSSEAKDFVKRL	NtCBK2
358	EADIWSIGVITYIILLCGSRPFWARTESGIFRSVLRADPNFDDSPWPSVSAEAKDFVKRL	MCK1
328	EADMWSIGVIVYIILLCGSRPFWARTESGIFRAVLKADPSFEEAPWPTLSAEAKDFVRRLL	OsCBK
332	EADVWSIGVISYIILLCGSRPFWARTESGIFRAVLKANLSFDEPPWPSVSEAKDFVKRL	carrot CRK
198	PVDLWACGVILYIILLVGYPFWDEDQHRLYQQIKAGAYDFPSPEWDTVTPEAKDLINKML	rat CaMKII
224	ASDMWSLGVILYIILLSGCPPFHAPSREKQORILAGDFSFEHTWKTITSSAKDLISSLL	lily CCaMK
214	ESDVWSAGVILYIILLSGVPPFAESEPQIFRQILLGKLDHFSEPWPSISDSAKDLIRKML	soybean CDPK

Figure 2 For legend see next page.

392	NKDPKRMTAAQALGHPWIKNSHNMEE-PLDILIFKLMKAYMRSSALRKAALRALSKTLT	NtCBK2
418	NKDYRKRMTAVQALTHPWLRD-EQRQI-PLDILIFRLVKQYLRTPLKRLALKALSKALS	MCK1
388	NKDYRKRMTAAQALCHPWIRGTEEVKL-PLDMIIYRLMAYISSSLRRAALRALAKTLT	OsCBK
382	NKDPKRMTAAQALCHSWIKNSNDIKF-PLDILVFKLKVMYMRSSPLRKAALRALSKTLT	carrot CRK
253	TINPSKRITAAEALKHPWISHRSTVASCMHRQETVDCLKFNARRKIKGAILTTMLATRN	rat CaMKII
284	SVDPYKRPTANDLLKHPWVIGDSAKQELIEPE-VVSRLRSFNARRKLRAAAIASVLSKV	lily CCaMK
274	DQNPKTRLTAHEVLRHPWIVDDNIAPDKPLDSAVLSRLKQFSAMNKLKFMALRVIAERIS	soybean CDPK
451	VDELFFYLKEQFALLEPNKNGTISFMNIKTALMKHATDAMKEARMHDFLASLNALQYRMD	NtCBK2
476	EDELLYLRLQFKLLEPRD-GFVSLDNFRTALTRYSTDAMRESRVLEFQHAEPLAYRKMD	MCK1
447	TQDIYYLREQFELIGPNKSDLITLQNLKTALMKNSTNAMKDSRVVDFVNTISNIQYRKLD	OsCBK
451	VDELFFYLKEQFVLLLEPTKNGTISLENIKQALMRNSTDAMKDSRVLDLLVSLNALQYRMD	carrot CRK
313	FSGGKSGGNKNDGVKESSESTNTTIEDETKVRKQEIIVTEQLIEAISNGDF-ESYTK	rat CaMKII
343	LLRTKKLKNLLGSHDMKSEELNLAHFKRICANGDNATLPEFEEVLKAMKMNSLIPLAP	lily CCaMK
334	EEEIGGLKELFKMIDTDNSGTITFDELKDGKRVGSELMESEIKDLMDAADIDKSGTIDY	soybean CDPK
511	FEFCAAALSVMHLEALDRWEQHARCAYEIFEKEGNRA-----IMTEELASELG	NtCBK2
535	FEFCAAALISPYQLEALERWEEIAGTAFQHFEQEGNRV-----ISVEELAQLN	MCK1
507	FEFSAALISVYQMEGLETWEQHARQAYEFFDKEGNRP-----IVIDEASGLG	OsCBK
511	FEFCAAALSVMHLEALDRWEQHARCAYDLFEKDNRA-----IMTEELASELG	carrot CRK
372	MCDPGMTAFEPALGNLVEGLDFHRFYFENLWSRNSKPVHTTILNPHIH-MGDESACIAY	rat CaMKII
408	RVFDLFDNNRDGTIDMREILCGLSNLRNSQGDDALQLCFQMYDADRSGCISKEELASMR	lily CCaMK
394	GEFIAATVHLNKLREENLVSAFSYFDKDGSGYITLDEIQACKDFGLDDIHIDDMKEI	soybean CDPK
560	LSPSVPVHAVLHDWLRHTDGKLSFLGFAKLLHGVSSRSITKVO	NtCBK2
584	LAPT--HYSIVQDWIRKSDGKLNFLGFTKFLHGVITIRGSNTRRH	MCK1
556	LGPSVPLHVVLQDWIRHPDGKLSFLGFMKLLHGVSSRTIPKT	OsCBK
560	LGPSIPVHAVLHDWLRHTDGKLSFLGYVVLHGVSTRATAKAO	carrot CRK
432	IRITQYLDAGGIP--R-TAQSEETR-----VWHRRDGKWOI	rat CaMKII
463	ALPEDCVPADITEPGKLDIEFDQMDANSBGVVTDFEFAAMQRDSSLQDGVVLSLRTI	lily CCaMK
464	DQDNDGQIDYGEFAAMMRKNGGIGRRTMRKTLNLRDALGLVDNGSNQVIEGYFK	soybean CDPK

Figure 2 Alignment of NtCBK2 with the other calcium-signal-system protein kinases

Identities between NtCBK2 and other kinases (maize MCK1, GenBank[®] accession no. 1839597; OsCBK, accession no. AF368282; carrot CRK, accession no. 1103386; rat brain CaMK-II subunit, accession no. J02942; lily CCaMK, accession no. 860675; and soya-bean CDPK, accession no. P28583) are indicated by shaded boxes. The putative CaM-binding domain of NtCBK2 (residues 439–456) is shown underlined.

Figure 3 Phosphorylation assays of NtCBK2 in the presence of Ca^{2+} or EGTA

The purified NtCBK2 was either silver stained or assayed for both autophosphorylation and substrate phosphorylation using histone H1S as substrate in the presence of Ca^{2+} or EGTA. NtCBK2 and reaction products were resolved by SDS/PAGE. The positions of molecular-mass markers are shown on the left.

of either EGTA or Ca^{2+} . These results suggested that NtCBK2 showed similar kinase properties in the presence of Ca^{2+} or EGTA. Kinetic parameters of the kinase activity of NtCBK2 were determined from double-reciprocal analysis of data for phosphorylation of various concentrations of histone H1S or syntide-2 in the presence of 100 μM ATP. The K_m for histone H1S was calculated to be approx. 1.81 μM ; that for syntide-2 was 1.02 μM .

To test the possibility that NtCBK2 had become phosphorylated in Sf9 cells and, therefore, that it had been activated *in vivo* and had enzymic activity *in vitro*, regardless of the presence or absence of Ca^{2+} , the purified NtCBK2 was autophosphorylated (labelled with [^{32}P]P_i) *in vitro* and then dephosphorylated with the catalytic subunit of the α -isoform of protein phosphatase 1 [43]. [^{32}P]P_i, added after the autophosphorylation, could be removed from NtCBK2 by treatment with the α -isoform of the protein phosphatase 1 catalytic subunit (Figure 5A, lane 2); this dephosphorylated NtCBK2 was used in subsequent experiments for autophosphorylation and in substrate phosphorylation assays. Our experiments showed that the dephosphorylated NtCBK2 still had kinase activity in a Ca^{2+} -independent manner (Figure 5B), suggesting that the activation of the kinase in the absence of Ca^{2+} was not due to the phosphorylation of the purified protein *in vivo*.

Figure 4 Time-course analyses of NtCBK2 phosphorylation

Time courses (s) of NtCBK2 autophosphorylation (A) and substrate phosphorylation with histone IIIS (B) or syntide-2 (C). Substrates in the presence of EGTA (■), Ca²⁺ (●), Ca²⁺/NtCaM1 (▲), Ca²⁺/NtCaM3 (▼), or Ca²⁺/NtCaM13 (◆) are shown.

Characterization of the CaM-binding domain of NtCBK2 and its role in the regulation of the kinase activity

As shown by database comparison studies, NtCBK2 contains a tentative CaM-binding domain that may bind to CaM. This notion was also supported by the purification procedures of NtCBK2, in which NtCBK2 was eluted from CaM-Sepharose 4B only in the absence of Ca²⁺.

To localize the CaM-binding domain experimentally, four constructs were made to express full NtCBK2 and the three truncated variants of NtCBK2, T1-424, T1-460 and T431-602 (Figure 6A). Whereas T1-460 contained the N-terminal catalytic domain of NtCBK2, including a tentative CaM-binding domain (KAALRALSKTLTVDEL^{FY}) at its C-terminus, T1-424 had the same amino acid sequence as T1-460, but lacked the amino acid residues 425–460 predicted for CaM binding at its C-terminus. T431-602 was the C-terminus of NtCBK2 lacking the kinase catalytic domain, but having the 30 amino acid residues at its N-terminus overlapping with the C-terminus of T1-460. These constructs, as recombinant expression viruses, were used to infect Sf9 insect cells, and proteins isolated from the Sf9 cells by

Ni-NTA were used for Western blot analysis with biotinylated bovine CaM. The results indicated that NtCBK2, T1-460 and T431-602 bound to CaM in a Ca²⁺-dependent manner, whereas T1-424 lost its ability to bind to CaM (Figures 6B and 6C), demonstrating a CaM-binding domain within the C-terminus of T1-460. On the basis of the positions of conserved hydrophobic residues, this CaM-binding domain [amino acid residues 445–454; **LSKTLTVDEL** (the hydrophobic residues highlighted in bold)] can be fitted to a 1-5-10 motif [44].

Three of these four constructs were used further for kinase assays in order to explore a possible role of the CaM-binding domain in the regulation of NtCBK2 activity. Both T1-424 and T1-460 were shown to autophosphorylate and phosphorylate substrates such as histone IIIS in the absence or presence of Ca²⁺/CaM, as found for the full NtCBK2 (Figure 7), suggesting that the C-terminus of NtCBK2 is not absolutely needed for the basal activity of the kinase. However, whereas the kinase activity of NtCBK2 and T1-460 can be stimulated by tobacco CaM3, T1-424 was not (Figure 7), indicating that the CaM-binding domain is necessary for CaM regulation of the kinase activity.

Figure 5 Kinase assays of the dephosphorylated NtCBK2

(A) The purified NtCBK2 that was autophosphorylated *in vitro* was analysed by SDS/PAGE either directly (lane 1) or after dephosphorylation (lane 2). The dephosphorylated NtCBK2 was assayed for both autophosphorylation and substrate phosphorylation with histone III S as the substrate in the presence of either Ca^{2+} or EGTA, and the reactions were resolved by SDS/PAGE (B). The positions of molecular-mass markers are shown on the left.

Figure 6 Identification of the CaM-binding domain of NtCBK2

NtCBK2 (0.75 μg) and its truncated forms (T1-424, T1-460 and T431-602, 0.5 μg each) were separated by SDS/PAGE, blotted on to PVDF membrane and assayed with biotinylated CaM in the presence of either 0.5 mM Ca^{2+} (B) or 2 mM EGTA (C). Diagrams of the truncated forms of NtCBK2 are shown in (A).

Differential regulation of the kinase activity of NtCBK2 by various CaM isoforms

It has been reported that diverse CaM bind proteins have different binding affinities with CaM isoforms, resulting in the subtle regulation by different concentrations of either CaM or CaM binding proteins [45,46]. To explore whether NtCBK2 binds to various CaM isoforms with different affinities or not, ^{35}S -labelled

NtCaM isoforms (NtCaM1, NtCaM3 and NtCaM13) were used, and the binding of NtCaM isoforms to NtCBK2 was measured directly by counting for radioactivity of the CaM–NtCBK2 complex. NtCBK2 (3 pmol) was immobilized on membranes, and then incubated with increasing concentrations of ^{35}S -labelled NtCaM isoforms until the binding was saturated. The K_d values of NtCaMs for NtCBK2 were estimated to be 55.7 ± 3.0 nM for NtCaM1, 25.4 ± 1.5 nM for NtCaM3 and 19.8 ± 0.8 nM for NtCaM13, on the basis of the saturation curve of ^{35}S CaM binding to NtCBK2 and by fitting the data using SPSS Enzyme Kinetics (SPSS Inc.) (Figure 8). The binding of CaMs to NtCBK2 was completely blocked in the presence of EGTA.

In order to investigate whether these CaMs regulate the kinase activity of NtCBK2 differentially or not, the K_a value of NtCBK2 activity was determined by assay, with the addition of increasing amounts of these three CaMs in the presence of 0.5 mM Ca^{2+} (Figure 9A). Our experiments indicated that when histone III S was employed as a substrate of NtCBK2 in the presence of CaM isoforms, NtCaM1, NtCaM3 and NtCaM13 half-maximally activated NtCBK2, with K_a values of 67 ± 2.1 nM, 25 ± 1.4 nM and 15.8 ± 0.5 nM respectively. If syntide-2 was used as the substrate instead of histone III S, NtCaM1, NtCaM3 and NtCaM13 half-maximally activated NtCBK2 with K_a values of 56.2 ± 1.9 nM, 31.6 ± 1.0 nM and 20.1 ± 0.3 nM respectively (Figure 9B). Furthermore, in the presence of Ca^{2+} without CaM, the enzyme had basal autophosphorylation activity. The maximal activity achieved with Ca^{2+} /NtCaM3 and Ca^{2+} /NtCaM13 was, in both cases, approx. 10-fold higher than that of the basal autophosphorylation activity, whereas the maximal activity achieved with Ca^{2+} /NtCaM1 was 3-fold higher (Figure 4A). In addition, substrate phosphorylation activities with histone III S and syntide-2 as substrates were also analysed. When phosphorylation activities using histone III S as the substrate in all reactions reached the maximum, the activity was shown to be amplified up to ≈ 9 -fold with NtCaM3 or ≈ 10 -fold with NtCaM13 as activators, while the phosphorylation activity was enhanced only ≈ 2 -fold in the presence of NtCaM1 (Figure 4B). Whereas the kinase had a $V_{\max}$ value of 0.35 nmol/min per mg of protein when assayed in the presence of EGTA or Ca^{2+} without CaM, the $V_{\max}$ was increased to 1.2, 2.9 and 3.3 nmol/min per mg of protein by the presence of NtCaM-1, -3 and -13 respectively. Alternatively, when syntide-2 was employed as the substrate, the activity was stimulated 3-fold, 8-fold and 11-fold, respectively, by NtCaM-1, -3 and -13 (Figure 4C): $V_{\max}$ values in the presence of NtCaM-1, -3 and -13 were 36, 184 and 227 nmol/min/per mg of protein respectively, whereas $V_{\max}$ was only 11 nmol/min per mg of protein in the presence of EGTA or Ca^{2+} without CaM. Thus NtCaM3 and NtCaM13 are better activators than NtCaM1 when histone III S and syntide-2 are used as substrates. These results suggest a diverse regulation of the kinase by different CaM isoforms. Furthermore, much higher $V_{\max}$ values and lower K_m s of NtCBK2 with syntide-2 compared with histone III S also suggested that syntide-2 was better than histone III S as a substrate of NtCBK2.

Phospho amino acid analysis

To assay for the phospho amino acid residues, capillary electrophoresis analysis of the purified NtCBK2 was performed. Capillary electrophoresis has been reported as a successful tool to identify amino acid residues [38,47]. In our experiments, 20 amino acids and three phospho amino acid standards could be separated efficiently by capillary electrophoresis (Figures 10A and 10C), demonstrating its applicability for amino acid identification. When the hydrolysis products of purified NtCBK2 that was not

Figure 7 Phosphorylation assays of NtCBK2

Autophosphorylation and substrate phosphorylation assays using histone III_S as the substrate of NtCBK2, T1-460 and T1-424 were performed in the presence of Ca^{2+} or Ca^{2+} /NtCaM3, and resolved by SDS/PAGE. The positions of molecular-mass markers are shown on the left.

Figure 8 Assay to determine the K_d value of NtCBK2 to CaMs

The saturation curve of ^{35}S -labelled CaM binding to the purified NtCBK2 was generated as follows. Purified NtCBK2 protein (3 pmol) was transferred on to a nitrocellulose filter and incubated with different amounts of ^{35}S -labelled CaM. After washing in buffer without ^{35}S -CaM, radioactivity of the filter was measured by using a liquid scintillation counter. The amount of bound CaM at each point was represented as a percentage of the maximal binding. (A) NtCaM1; (B) NtCaM3; (C) NtCaM13.

Figure 9 Assay for CaM isoforms required for NtCBK2 phosphorylation

Histone H1S (A) or syntide-2 (B) as the substrate was phosphorylated with NtCBK2 in the presence of NtCaM1 (■), NtCaM3 (●) or NtCaM13 (▲) under our standard phosphorylation conditions, except that increasing amounts of NtCaM ($10^{-8.5}$ – $10^{-6.5}$ M) were used at 30 °C for 30 min. The results are averages of three determinations performed in duplicate.

autophosphorylated *in vitro* were separated by capillary electrophoresis, all amino acids were detected, except the phospho amino acids (Figure 10A), indicating that the purified NtCBK2 used in the kinase activity assays was not phosphorylated. When autophosphorylated NtCBK2 was assayed with capillary electrophoresis, a phosphorylated serine residue was detected (Figure 10B and 10D) and identified by addition of standard phosphorylated serine (Figure 10E), indicating that NtCBK2 was a serine protein kinase, as inferred from the database analysis.

DISCUSSION

In the present study, a cDNA encoding tobacco NtCBK2 was isolated by using *MCK1* as a probe. NtCBK2 shares high similarity with other CaMKs in terms of structure, and was

biochemically characterized as a CBK. Although this protein kinase can be fully activated for its enzymic activity by the presence of Ca^{2+} /CaM, it has autophosphorylation and substrate phosphorylation activity even in the absence of Ca^{2+} .

Several CBKs have been identified previously in plants and, among them, CCaMK and OsCBK have been well characterized, both on the molecular level and biochemically. CCaMK has a Ca^{2+} -binding, visinin-like domain with three EF-hand motifs. However, NtCBK2 as shown in the present study does not contain any EF-hand for Ca^{2+} binding. This kinase has Ca^{2+} -independent phosphorylation activity, as also shown by OsCBK. OsCBK has a high-binding affinity to CaM, which is unnecessary for the full activation of this kinase [25]. This is different from NtCBK2, in which the kinase activity can be stimulated up to 10-fold by CaM. This difference could be due to the differential use of CaM isoforms. Various CaM isoforms can act on the activation of CBK differentially, as indicated by our data with NtCBK2. Whether other plant isoforms modulate OsCBK activity or not remains to be determined. We also note that NtCBK2 shares high similarity with CRKs, which are CDPK-related protein kinases with degenerated EF hands in plants. Whereas NtCBK2 is regulated by CaMs, biochemical evidence for CRKs as Ca^{2+} /CaM-binding proteins is unavailable [40,48]. Considering the high similarity between these two kinases, it may be useful to investigate whether CRK binds to CaM experimentally.

In our experiments, NtCBK2 has been shown to have kinase activity in the presence of either Ca^{2+} or EGTA, suggesting its Ca^{2+} -independent enzymic activity. However, an alternative explanation for this could be that the purified NtCBK2 from Sf9 insect cells was constitutively activated via its phosphorylation *in vivo*, either by itself or another kinase. To address this possibility, NtCBK2 was dephosphorylated by a phosphatase, and then this dephosphorylated NtCBK2 was used to assay kinase activity. The results revealed that the dephosphorylated NtCBK2 still retained its kinase activity in a Ca^{2+} -independent fashion, indicating that the Ca^{2+} -independent activity of the kinase did not result from the phosphorylation of the purified NtCBK2 *in vivo*. This conclusion was supported further by the data from capillary electrophoresis analyses of phospho amino acid residues of NtCBK2. When amino acid residues of the phosphorylated NtCBK2 were separated by capillary electrophoresis, a phosphorylated serine plus all unphosphorylated amino acid residues were identified, demonstrating the usefulness of this technique as documented by others [38,47]. However, none of the phosphorylated residues were detected with purified NtCBK2 that was not phosphorylated *in vitro*. These results indicate that the purified NtCBK2 used in these kinase activity assays was not phosphorylated, and that the Ca^{2+} -independence of NtCBK2 was not because of the enzyme being phosphorylated *in vivo*. Thus we conclude that NtCBK2 has some basal enzymic activity that is independent from Ca^{2+} /CaM.

As a CaM-binding protein, the kinase activity of NtCBK2 was demonstrated to be modulated by CaM. It has been documented that plant cells have multiple divergent CaM isoforms that allow subtle regulation of Ca^{2+} /CaM-binding proteins. For example, two isoforms of potato CaM, PCaM1 and PCaM6, modulated the activity of CCaMK differently [23]. Whereas PCaM1 acted as a more efficient inhibitor of autophosphorylation and activator of substrate phosphorylation of tobacco CCaMK than did PCaM6, these CaM isoforms did not show this differential regulation with respect to lily CCaMK. In tobacco, 13 NtCaMs have been identified and divided into three groups on the basis of deduced amino acid sequences [31], as follows: NtCaM1 and 2 as group I, with high similarity to PCaM1; NtCaMs 3–12 as group II, similar to the ordinary-type plant CaMs, such as soya-bean

Figure 10 Phospho amino acid analysis

(A) Electropherograms of the hydrolysed, unphosphorylated NtCBK2 with FITC labelling. (B) Electropherograms of the hydrolysed phosphorylated NtCBK2 with FITC labelling. (C) Intercepted and enlarged form of electropherograms of FITC-labelled standard phospho amino acids (p-Ser, p-Thr and p-Tyr, where the 'p' denotes 'phospho'). (D) Intercepted and enlarged form of electropherograms of the hydrolysed phosphorylated NtCBK2. (E) Intercepted and enlarged form of electropherograms of the hydrolysed phosphorylated NtCBK2, with additional phosphorylated serine for peak identification.

SCaM1; and NtCaM13 as group III, a homologue of SCaM4. On the basis of the interaction between SCaM isoforms and their targets, CaM target enzymes could be categorized into three different types, as follows: 1, enzymes activated equally by both SCaM1 and SCaM4 (e.g. phosphodiesterase, plant Ca^{2+} -ATPase); 2, enzymes activated only by SCaM1 (e.g. plant NAD kinase); and 3, enzymes activated only by SCaM4 (e.g. nitric oxide synthase) [28,33,49,50]. Specific binding sequences for SCaM1 and SCaM4 have been identified as a 1-5-10 motif or a 1-8-14 motif respectively [51]. It has been suggested that NtCaM3 and NtCaM13 seem to resemble SCaM1 and SCaM4, which activated only NAD kinase or nitric-oxide synthase respectively, whereas NtCaM1 appears to be a unique plant CaM isoform, via which both enzymes can be activated [31]. To investigate the possible differential regulation of NtCBK2 by the different CaM isoforms, NtCaM1, NtCaM3 and NtCaM13, as representatives of the three respective groups of tobacco CaM isoforms, were used in our experiments. Based on the classification of tobacco CaMs, an interaction between NtCBK2 and NtCaM1/3 can be predicted, because the CaM-binding domain of NtCBK2 has been classified as a 1-5-10 motif, and thus these CaM isoforms could modulate the kinase activity of NtCBK2, whereas NtCaM13 could not. However, our experimental results indicated that, whereas both NtCaM1 and NtCaM3 stimulated the enzymic activity of the NtCBK2 as predicted above, NtCaM13 could also amplify the kinase activity as efficiently as NtCaM3. These results are contrary to the prediction above. It may imply that CaM has many more ways to activate its targets than we can envisage at present, and further elucidation of precise roles for each CaM isoform in regulating CaM target proteins is needed.

In summary, a CBK gene for NtCBK2 was cloned from tobacco, and the encoded protein kinase is different from CCaMK and OsCBK, in terms of both structural and enzymic properties. This kinase showed Ca^{2+} -independent and Ca^{2+} /CaM-stimulating enzymic activity. Various CaM isoforms not only have different binding affinity for NtCBK2, but also regulate the kinase activity differently.

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REFERENCES

- Hepler, P. K. and Wayne, R. O. (1985) Calcium and plant development. *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* **36**, 397–439
- Reddy, A. S. N. (2001) Calcium: silver bullet in signaling. *Plant Sci.* **160**, 381–404
- Sanders, D., Pelloux, J., Brownlee, C. and Harper, J. F. (2002) Calcium at the crossroads of signaling. *Plant Cell (suppl.)* **14**, S401–S417
- Carafoli, E. (2003) The calcium-signalling saga: tap water and protein crystals. *Nat. Rev. Mol. Cell Biol.* **4**, 326–332
- Chin, D. and Means, A. R. (2000) Calmodulin: a prototypical calcium sensor. *Trends Cell Biol.* **10**, 322–328
- Harmon, A. C., Gribskov, M. and Harper, J. F. (2000) CDPKs – a kinase for every Ca^{2+} signal? *Trends Plant Sci.* **5**, 154–159
- Harmon, A. C., Gribskov, M., Gubrium, E. and Harper, J. F. (2001) The CDPK superfamily of protein kinases. *New Phytol.* **151**, 175–183
- Cheng, S. H., Willmann, M. R., Chen, H. C. and Sheen, J. (2002) Calcium signaling through protein kinase. The *Arabidopsis* calcium-dependent protein kinase gene family. *Plant Physiol.* **129**, 469–485
- Huang, J. F., Teyton, L. and Harper, J. F. (1996) Activation of a Ca^{2+} -dependent protein kinase involves intramolecular binding of a calmodulin-like regulatory domain. *Biochemistry* **35**, 13222–13230
- Lee, J. and Rudd, J. J. (2002) Calcium-dependent protein kinases: versatile plant signalling components necessary for pathogen defence. *Trends Plant Sci.* **7**, 97–98
- Braun, A. P. and Schulman, H. (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. *Annu. Rev. Physiol.* **57**, 417–445
- Snedden, W. A. and Fromm, H. (1998) Calmodulin, calmodulin-related proteins and plant responses to the environment. *Trends Plant Sci.* **3**, 299–304
- Reddy, V. S., Ali, G. S. and Reddy, A. S. (2002) Genes encoding calmodulin-binding proteins in the *Arabidopsis* genome. *J. Biol. Chem.* **277**, 9840–9852
- Soderling, T. R. and Stull, J. T. (2001) Structure and regulation of calcium/calmodulin-dependent protein kinases. *Chem. Rev.* **101**, 2341–2352
- Vetter, S. W. and Leclerc, E. (2003) Novel aspects of calmodulin target recognition and activation. *Eur. J. Biochem.* **270**, 404–414

- 16 Snedden, W. A. and Fromm, H. (2001) Calmodulin as a versatile calcium signal transducer in plant. *New Phytol.* **151**, 35–66
- 17 Zhang, L. and Lu, Y.-T. (2003) Calmodulin-binding protein kinases in plants. *Trends Plant Sci.* **8**, 123–127
- 18 Watillon, B., Kettmann, R., Boxus, P. and Burny, A. (1993) A calcium/calmodulin-binding serine/threonine protein kinase homologous to the mammalian type II calcium/calmodulin-dependent protein kinase is expressed in plant cells. *Plant Physiol.* **101**, 1381–1384
- 19 Watillon, B., Kettmann, R., Boxus, P. and Burny, A. (1995) Structure of a calmodulin-binding protein kinase gene from apple. *Plant Physiol.* **108**, 847–848
- 20 Lu, Y.-T., Hidaka, H. and Feldman, L. J. (1996) Characterization of a calcium/calmodulin-dependent protein kinase homolog from maize roots showing light-regulated gravitropism. *Planta* **199**, 18–24
- 21 Wang, L., Liang, S. P. and Lu, Y.-T. (2001) Characterization, physical location, and expression of the genes encoding calcium/calmodulin-dependent protein kinases in maize (*Zea mays* L.). *Planta* **213**, 556–564
- 22 Patil, S., Takezawa, D. and Poovaiah, B. W. (1995) Chimeric plant calcium/calmodulin-dependent protein kinase gene with a neural visinin-like calcium-binding domain. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4897–4901
- 23 Liu, Z., Xia, M. and Poovaiah, B. W. (1998) Chimeric calcium/calmodulin-dependent protein kinase in tobacco: differential regulation by calmodulin isoforms. *Plant Mol. Biol.* **38**, 889–897
- 24 Pandey, S. and Sopory, S. K. (1998) Biochemical evidence for a calmodulin-stimulated calcium-dependent protein kinase in maize. *Eur. J. Biochem.* **255**, 718–726
- 25 Zhang, L., Liu, B. F., Liang, S. P., Jones, R. L. and Lu, Y.-T. (2002) Molecular and biochemical characterization of a calcium/calmodulin-binding protein kinase from rice. *Biochem. J.* **368**, 145–157
- 26 Takezawa, D., Ramachandiran, S., Paranjape, V. and Poovaiah, B. W. (1996) Dual regulation of a chimeric plant serine/threonine kinase by calcium and calcium/calmodulin. *J. Biol. Chem.* **271**, 8126–8132
- 27 Ramachandiran, S., Takezawa, D., Wang, W. and Poovaiah, B. W. (1997) Functional domains of plant chimeric calcium/calmodulin-dependent protein kinase: regulation by autoinhibitory and visinin-like domains. *J. Biochem. (Tokyo)* **121**, 984–990
- 28 Lee, S. H., Kim, J. C., Lee, M. S., Heo, W. D., Seo, H. Y., Yoon, H. W., Hong, J. C., Lee, S. Y., Bahk, J. D., Hwang, I. and Cho, M. J. (1995) Identification of a novel divergent calmodulin isoform from soybean which has differential ability to activate calmodulin dependent enzymes. *J. Biol. Chem.* **270**, 21806–21812
- 29 Zielinski, R. E. (1998) Calmodulin and calmodulin-binding proteins in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 697–725
- 30 Takezawa, D., Liu, Z. H., An, G. and Poovaiah, B. W. (1995) Calmodulin gene family in potato: developmental and touch-induced expression of the mRNA encoding a novel isoform. *Plant Mol. Biol.* **27**, 693–703
- 31 Yamakawa, H., Mitsuhashi, I., Ito, N., Seo, S., Kamada, H. and Ohashi, Y. (2001) Transcriptionally and post-transcriptionally regulated response of 13 calmodulin genes to tobacco mosaic virus-induced cell death and wounding in tobacco plant. *Eur. J. Biochem.* **268**, 3916–3929
- 32 Liao, B., Gawienowski, M. C. and Zielinski, R. E. (1996) Differential stimulation of NAD kinase and binding of peptide substrates by wild-type and mutant plant calmodulin isoforms. *Arch. Biochem. Biophys.* **327**, 53–60
- 33 Lee, S. H., Johnson, J. D., Walsh, M. P., Van Lierop, J. E., Sutherland, C., Xu, A., Snedden, W. A., Kosk-Kosicka, D., Fromm, H., Narayanan, N. and Cho, M. J. (2000) Differential regulation of Ca^{2+} /calmodulin-dependent enzymes by plant calmodulin isoforms and free Ca^{2+} concentration. *Biochem. J.* **350**, 299–306
- 34 Aitken. A. and Learmonth, M. (2002) Part I: quantitation of proteins. In *Protein Protocols Handbook*, 2nd edn (Walker, J. M., ed.), pp. 41–44, Humana Press, Totowa, NJ
- 35 Lu, Y.-T. and Harrington, H. M. (1994) Isolation of tobacco cDNA clones encoding calmodulin-binding proteins and characterization of a known calmodulin-binding domain. *Plant Physiol. Biochem.* **32**, 413–422
- 36 Fromm, H. and Chua, N.-H. (1992) Cloning of plant cDNAs encoding calmodulin-binding proteins using ^{35}S -labeled recombinant calmodulin as a probe. *Plant Mol. Biol. Rep.* **10**, 199–206
- 37 Billingsley, M. L., Pennypacker, K. R., Hoover, C. G., Brigati, D. J. and Kincaid, R. L. (1985) A rapid and sensitive method for detection and quantification of calcineurin and calmodulin-binding proteins using biotinylated calmodulin. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7585–7589
- 38 Liu, B.-F., Zhang, L. and Lu, Y.-T. (2001) Characterization of a novel protein kinase in rice cells by capillary electrophoresis. *J. Chromatogr. A* **918**, 401–409
- 39 Lin, C. R., Kapiloff, M. S., Durgerian, S., Tatemoto, K., Russo, A. F., Hanson, P., Schulman, H. and Rosenfeld, M. G. (1987) Molecular cloning of a brain-specific calcium/calmodulin-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5962–5966
- 40 Lindzen, E. and Choi, J. H. (1995) A carrot cDNA encoding an atypical protein kinase homologous to plant calcium-dependent protein kinase. *Plant Mol. Biol.* **28**, 785–797
- 41 Harper, J. F., Sussman, M. R., Schaller, G. E., Putnam-Evans, C., Charbonneau, H. and Harmon, A. C. (1991) A calcium-dependent protein kinase with a regulatory domain similar to calmodulin. *Science* **252**, 951–954
- 42 O'Neil, K. T. and DeGrado, W. F. (1990) How calmodulin binds its targets: sequence independent recognition of amphiphilic α -helices. *Trends Biol. Sci.* **15**, 59–64
- 43 Zhang, A. J., Bai, G., Deans-Zirattu, S., Browner, M. F. and Lee, E. Y. (1992) Expression of the catalytic subunit of phosphorylase phosphatase (protein phosphatase-1) in *Escherichia coli*. *J. Biol. Chem.* **267**, 1484–1490
- 44 Rhoads, A. R. and Friedberg, F. (1997) Sequence motifs for calmodulin recognition. *FASEB J.* **11**, 331–340
- 45 Sikela, J. M. and Hahn, W. E. (1987) Screening an expression library with a ligand probe: isolation and sequence of a cDNA corresponding to a brain calmodulin-binding protein. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3038–3042
- 46 Reddy, V. S., Safadi, F., Zielinski, R. E. and Reddy, A. S. N. (1999) Interaction of a kinesin-like protein with calmodulin isoforms from *Arabidopsis*. *J. Biol. Chem.* **274**, 31727–31733
- 47 Crowder, M. W., Numan, A., Haddadian, F., Weitzel, M. A. and Danielson, N. D. (1999) Capillary electrophoresis of phosphoamino acids with indirect phosphometric detection. *Anal. Chim. Acta* **384**, 127–133
- 48 Furumoto, T., Ogawa, N., Hata, S. and Izui, K. (1996) Plant calcium-dependent protein kinase-related kinase (CRKs) do not require calcium for their activities. *FEBS Lett.* **396**, 147–151
- 49 Kondo, R., Tikunova, S. B., Cho, M. J. and Johnson, J. D. (1999) A point mutation in a plant calmodulin is responsible for its inhibition of nitric-oxide synthase. *J. Biol. Chem.* **274**, 36213–36218
- 50 Chung, W. S., Lee, S. H., Kim, J. C., Heo, W. D., Kim, M. C., Park, C. Y., Park, H. C., Lim, C. O., Kim, W. B., Harper, J. F. and Cho, M. J. (2000) Identification of a calmodulin-regulated soybean Ca^{2+} -ATPase (SCA1) that is located in the plasma membrane. *Plant Cell* **12**, 1393–1407
- 51 Choi, J. Y., Lee, S. H., Park, C. Y., Heo, W. D., Kim, J. C., Kim, M. C., Chung, W. S., Moon, B. C., Cheong, Y. H., Kim, C. Y. et al. (2002) Identification of calmodulin isoform-specific binding peptides from a phage-displayed random 22-mer peptide library. *J. Biol. Chem.* **277**, 21630–21638

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