Cellular localization and biochemical characterization of a novel calcium-dependent protein kinase from tobacco

Yun WANG[¶], Mei ZHANG[¶], Ke KE, Ying Tang LU[*]

Key Lab of MOE for Plant Developmental Biology, College of Life Sciences, Wuhan University, Wuhan 430072, China

ABSTRACT

By screening tobacco cDNA library with *MCK1* as a probe, we isolated a cDNA clone *NtCPK5* (accession number AY971376), which encodes a typical calcium-dependent protein kinase. Sequence analyses indicated that NtCPK5 is related to both CPKs and CRKs superfamilies and has all of the three conserved domains of CPKs. The biochemical activity of NtCPK5 was calcium-dependent. NtCPK5 had V_{max} and K_m of 526 nmol/min/mg and 210 µg/ml respectively with calf thymus histone (fraction III, abbreviated to histone IIIs) as substrate. For substrate syntide-2, NtCPK5 showed a higher V_{max} of 2008 nmol/min/mg and a lower K_m of 30 µM. The $K_{0.5}$ of calcium activation was 0.04 µM or 0.06 µM for histone IIIs or syntide-2 respectively. The putative myristoylation and palmitoylation consensus sequence of NtCPK5 suggests that it could be a membrane-anchoring protein. Indeed, our transient expression experiments with wild type and mutant forms of NtCPK5/GFP fusion proteins showed that NtCPK5 was localized to the plasma membrane of onion epidermal cells and that the localization required the N-terminal acylation sites of NtCPK5/GFP. Taking together, our data have demonstrated the biochemical characteristics of a novel protein NtCPK5 and its subcellular localization as a membrane-anchoring protein.

Keywords: calcium-dependent protein kinase, phosphorylation, GFP, subcellular localization.

INTRODUCTION

Calcium (Ca^{2+}) as a ubiquitous signal molecule plays vital roles in plant response to various stimuli, including light, environmental stresses, pathogen attack, and hormones by fluctuation of cytosolic Ca^{2+} concentration in all living cells [1]. In plants, different calcium signal was transduced through several calcium-binding proteins such as calmodulin (CaM), calcium-dependent protein kinase (CDPK or CPK), calcineurin B-like protein (CBL) and chimeric Ca^{2+}/CaM-dependent protein kinase (CCaMK) into downstream responses, including altered protein phos-

phorylation and gene expression patterns [2-4]. CPKs are a group of well-characterized protein kinases and have been found in many plants including Arabidopsis, soybean, rice, tobacco and potato [5]. CPKs contain three conserved domains: kinase catalytic domain, junction domain and calmodulin-like domain. Junction domain can serve as a pseudosubstrate and block the catalytic pocket of kinase domain in the absence of calcium. Upon the calcium binding to EF-hands, junction domain (the autoinhibitory region) is released from the kinase domain to activate CPKs [6].

Except of the three highly conserved domains, CPKs also have an N-terminal variable region, which often contains N-terminal acylation (including myristoylation and palmitoylation) sites required for the subcellular localization of certain CPKs [7-10]. Myristoylation often occurs at Gly residue at position 2 and may or may not sufficient for membrane anchoring. Palmitoylation occurred at Cys residue at position 4 as a supplement can promote and stabilize the targeting of CPKs to plasma membrane [7-9]. In Arabidopsis, there are 34 CPK isoforms, among them, only five AtCPKs lack both Gly2 and Cys4. Different N-terminal acylation from variable N-terminal sequences produces isoform-specific subcellular localization of AtCPKs, sug-

gesting that this family of protein kinases may be involved in multiple signaling pathways due to differential distribution in cells [10].

In this paper, we reported the cloning of *NtCPK5*, a new *CPK* isoform from tobacco plants and the biochemical characterization of NtCPK5 activity. NtCPK5 shares high sequence homology to NtCPK4. However their variable N-terminal regions are diverse. Our experiments showed that these two tobacco NtCPK isoforms have different subcellular distribution and that the two amino acids Gly2 and Cys4 of NtCPK5 are crucial for its membrane localization.

MATERIALS AND METHODS
Isolation of the cDNA encoding *NtCPK5*

A tobacco cDNA library was constructed from mRNAs isolated from leaves of *Nicotiana tabaccum* cv. W38 using ZAPcDNA Synthesis Kit, following the manufacturer's instructions (Invitrogene). The library was then screened using maize *MCK1* cDNA [11] as a probe, and a positive plasmid containing *NtCPK5*, named p*NtCPK5* was isolated and sequenced.

Construction of plasmids NtCPK5 and truncated NtCPK5 P1 and P2

To identify the kinase properties of NtCPK5, several constructs were made in plasmid pFastHTb following the protocol described previously [12, 13]. Using p*NtCPK5* as template, the cDNAs for the full open reading frame (NtCPK5) and two truncated forms P1 (residues 1-372) and P2 (residues 1-408) of NtCPK5 were amplified and purified. P1 contains the N-terminal 372 amino acids without the C-terminal calmodulin like domain and junction domain of NtCPK5 while P2 contains the N-terminal 408 amino acids without the C-terminal calmodulin like domain of NtCPK5 only. The PCR products were digested with *Hin*dIII and cloned into the *Hin*dIII site of plasmid pFastBacHTb. The orientation of the insertion was checked by restriction digestion.

The recombinant plasmids were sequenced and transformed into DH10Bac competent cells containing the bacmids with a mini-att Tn7 target site and helper plasmid. The mini Tn7 element on the pFastBacHTb 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* gene. The sf-9 cells were maintained as mono-layer 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 (Invitrogen). Recombinant virus were harvested after 72 h and identified by PCR, then was used in following assay or saved at –80°C.

Purification of recombinant proteins

The NtCPK5 protein expression in Insect sf-9 cells and protein purification were performed as described previously [14, 15]. In briefly, sf-9 cells were harvested at room temperature after infected by the recombinant virus for 72 h. 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 12000 g for 10 min. The supernatant was applied to a Ni-NTA (Ni^{2+}-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, recombinant proteins were subsequently eluted with buffer A containing 100 mM imidazole. The eluted recombinant proteins were dialyzed against 25 mM Tris-HCl, pH 7.5 for approximately 6 h, and then used for SDS-PAGE and enzymatic analyses. Protein concentration was determined by the method of Bradford using BSA as a standard. All procedures were performed at 4°C, unless stated otherwise.

Kinase assays

Autophosphorylation and substrate phosphorylation assays were carried out in kinase buffer containing 25 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 50 μM ATP, 10 μCi [γ-^{32}P] ATP (5000 Ci/mM), 0.1 mM $CaCl_2$ or 2 mM EGTA at 30°C for 30 min, with histone IIIs as substrate (1 mg/ml). The reactions were initiated by addition of 100 ng NtCPK5, P1 or P2, terminated by adding 1/5 volume 5 × Laemmli Sample Buffer, and analyzed by 10% SDS-PAGE. After staining with 0.1% Coomassie Brilliant Blue, the gels were vacuum-dried and exposed to x-ray film at –80 °C with a screen.

The kinase activities were measured under different pH (pH 6-9.5) and Mg^{2+} concentration (0-25 mM), or different reaction times (0-60 min). Aliquots (50 μl) were terminated by adding a 1/5 volume of 5 × Laemmli Sample Buffer, and analyzed by SDS-PAGE. After staining with 0.1% Coomassie Brilliant Blue, the substrate bands (histone IIIs) were collected and the ^{32}P incorporation was determined by liquid scintillation counting (Beckman LS 6500). Enzyme assays were also performed in the presence of different concentrations of Ca^{2+} (10 nM-60 μM with histone IIIs or syntide-2 as substrates), histone IIIs (0.01-1 mg/ml with 0.1 mM $CaCl_2$) and syntide-2 (1-150 μM with 0.1mM $CaCl_2$). For Ca^{2+}-dependent curves, free Ca^{2+} levels were set using Ca^{2+}/EGTA buffer as described by Bers [16]. When using syntide-2 as substrate, the reaction mixture was pipetted onto squares of 2×2 cm phosphocellulose paper (type P81, Whatman) and then the paper was immediately immersed into 75 mM phosphoric acid, followed by five times wash 5 min each in 75 mM phosphoric acid and one time wash in acetone, and then dried and counted by liquid scintillation.

Construction of GFP fusion proteins

DNA fragments containing ORFs for both NtCPK5 and NtCPK4 were obtained by PCR with forward primers (GGA TCC ATG GGC AGC TGT TTT TCT AGC TCC and GGA TCC ATG GGT AAT AAC TGT TTT TCT AGC) and reverse primers (GGA TCC CAA AGC TAC ATT TCT CCG TGA ATC and GGA TCC ACT TTT CCG AGA GCC TCT AAC AG). The full length cDNAs of NtCPK5 with mutated myristoylation site (Gly2Ala) or mutated palmitoylation site (Cys4Ala) or mutated both myristoylation and palmitoylation sites (Gly2Ala/Cys4Ala) were PCR generated by *in vitro* mutagenesis with site-mutated forward primers (GGA TCC ATG GCC AGC TGT TTT TCT AGC TCC, or GGA TCC ATG GGC AGC GCC TTT TCT AGC TCC, or GGA TCC ATG GCC AGC GCC TTT TCT AGC TCC) respectively together with reverse primer (GGA TCC CAA AGC TAC ATT TCT CCG TGA ATC). All of these PCR products were cloned in pGM T-vector, digested and further cloned into the pUC/GFP made by ligating 35S promoter

from pBIm [17] and GFP from pBI101-GFP [17] into pUC18 in frame to produce C-terminal GFP tagged fusion protein. After sequence confirmation, these vectors were bombarded into the epidermal cells of onion.

Transient expression of NtCPK4/GFP, NtCPK5/GFP and three mutation forms of NtCPK5/GFP

The experiments were performed as previously described by Scott [18]. The inner epidermal peels of onion (*Allium cepa*) buld (2×2 cm) were riped and placed on agar plates containing 1 × Murashige andSkoog (MS) salts, 30g/L sucrose and 3% agar, pH 5.7. Peels were bombarded within 1 h after transferred to agar plates. The five constructs (including NtCPK4/GFP, NtCPK5/GFP, Gly2Ala NtCPK5/GFP, Cys4Ala NtCPK5/GFP, and Gly2Ala/Cys4Ala NtCPK5/GFP) were delivered into onion epidermal cells with a biolistic PDS-1000/HeTM (BioRad) particle gun with 1100 psi rupture discs under a vacuum of 28 in Hg [18]. Three bombardments were performed for each construction. Onion epidermal cells were allowed to recover for about 20 h at 22ºC in continual light before they were analyzed by confocal laser scanning microscopy at 488 nm (Leica, TCS-SP).

RESULTS

Molecular cloning of NtCPK5

To clone a maize *MCK1* ortholog in tobacco, a tobacco cDNA library was screened and a cDNA clone, named *NtCPK5*, was isolated. The *NtCPK5* cDNA (accession number AY971376) is 2368 bp long with an open reading frame (ORF) of 1704 bp encoding 567 amino acids with a molecular weight of 64 kDa (Fig. 1). NtCPK5 belongs to CPKs superfamily [5, 6], comprising an N-terminal variable region, a kinase domain, a junction domain, and a C-terminal calmodulin-like domain with four EF-hand motifs implicated in Ca^{2+} binding. The kinase domain (266 amino acids) of NtCPK5 contains all of the 11 conserved subdomains of Ser/Thr protein kinases including a putative ATP-binding site in the N-terminal region and a Ser/Thr protein kinase active site in the center of the kinase domain. By searching ScanProsite in PlantsP database [19], seven putative N-myristoylation sites are found in NtCPK5 as shown in Fig. 1 with underlines. The N-terminal putative myristoylation site (MGxxxSxx, PlantsP database) is conserved within plant CPKs superfamily [7-10], which may be required for the subcellular localization of NtCPK5.

The amino acid sequence of NtCPK5 was aligned with those of other plant CPKs and CRKs by using DNAstar alignment program (Fig. 2A and 2B). The results show that NtCPK5 is highly identical to other CPKs. It shares 87.1% and 89.4% amino acid sequence identity to that of NtCPK4 (lab data) and of StCPK1 [20] respectively. Except of NtCPK4 and StCPK1, other CPKs including NtCDPK1 (accession number AF072908, 42.6% identity) [21] and NtCDPK2 (accession number AJ344154, 40.6% identity) [22] in tobacco, LeCPK1 in tomato (accession number AJ308296, 42.3% identity) [8], and CpCPK1 in
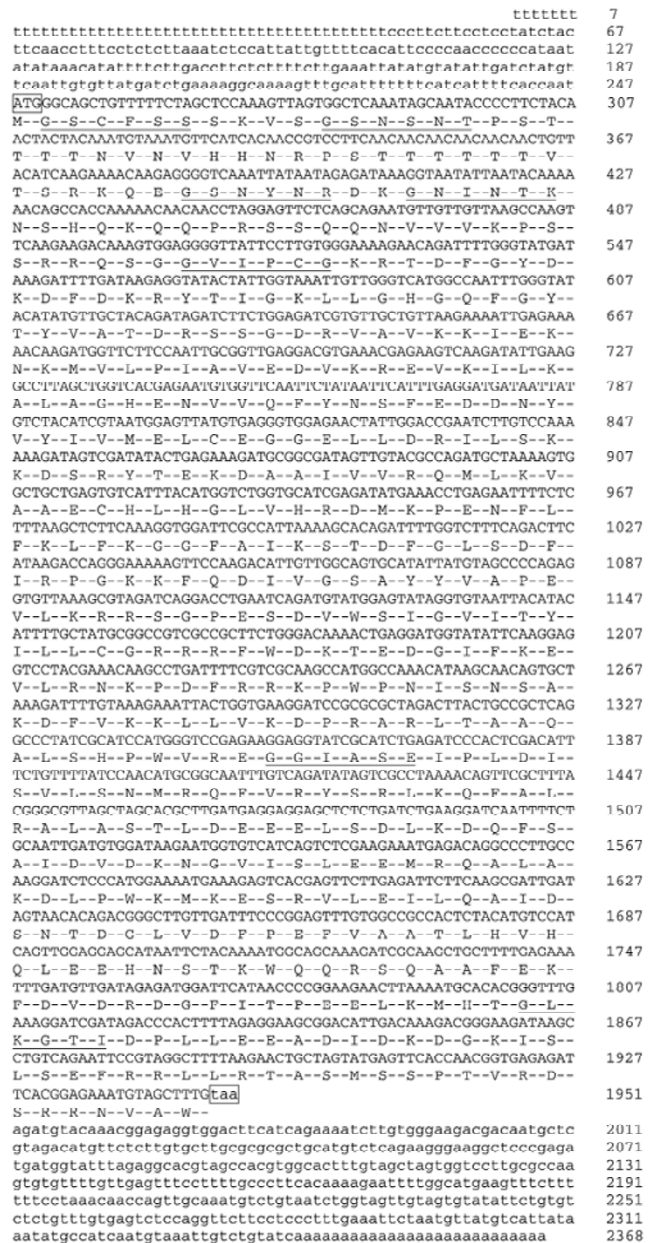

Fig. 1 Nucleotide and deduced amino acid sequences of NtCPK5. The nucleotides are numbered on the right. The start codon (ATG) and the stop codon (taa) in frame are boxed respectively. The underlined sequences are putative myristoylation consensus predicted by ScanProsite in PlantsP.

zucchini (accession number U90262, 41.5% identity) [23] also share sequence homology to NtCPK5. Phylogenic tree analyses indicate that NtCPK5 subfamily including NtCPK4 and StCPK1 is more related to CRK than to CPK. The sequence identity between NtCPK5 and CRK proteins such as maize MCK1 (accession number 1839597) [11] and

ZmCRK (accession number D84507), carrot DcCRK (accession number X83869) [24], and *Arabidopsis* AtCRK1 (accession number AF435448) [13] are 43.3%, 43.3%, 44.3%, and 44.3% respectively. NtCPK5 may be thus considered as an evolutionary link between CPKs and CRKs (Fig. 2B).

A putative myristoylation site (MGxxxSxx, PlantsP database) at the beginning of the NCPK5 coding region, which is also present in other plant CPKs [7-10], was presumed involving in the subcellular localization of NtCPK5.

Analysis of the kinase activity of NtCPK5 protein

To characterize the biochemical activity of NtCPK5 as a calcium-dependent protein kinase, the full length as well as two truncated proteins of NtCPK5 was tagged with HT and the recombinant proteins were expressed in sf-9 cells (Fig. 3A). The proteins were purified, checked on an SDS-PAGE (Fig. 3B), and used for enzymatic analyses.

The full-length NtCPK5 has autophosphorylation activity. The phosphorylation occurred in the presence of 0.1 mM Ca^{2+} (Fig. 3C). Addition of EGTA greatly reduced the autophosphorylation of NtCPK5. NtCPK5 is an active kinase towards histone IIIs in the presence of Ca^{2+}. Similar to its autophosphorylation activity, the presence of EGTA decreased the kinase activity of NtCPK5, similar to other known CPKs [21].

To examine the possible role of the putative junction domain and calmodulin-like domain in regulation of the kinase activity of NtCPK5, two truncated forms of NtCPK5, P1 (residues 1–372) and P2 (residues 1–408) were used in the kinase assay. P1 was expressed and purified as the full-length NtCPK5 protein (Fig. 3B). It con-

tains the N-terminal region and the kinase domain, but lacking the junction domain and the calmodulin-like domain. It has the full autophosphorylation and kinase activities (Fig. 3D). P1's activity was however much lower than NtCPK5's, but similar to NtCDPK1 (date not shown). Differing from the full-length NtCPK5, the phosphorylation activity of P1 does not respond to Ca^{2+} by showing similar activities in the presence of both $CaCl_2$ and EGTA,
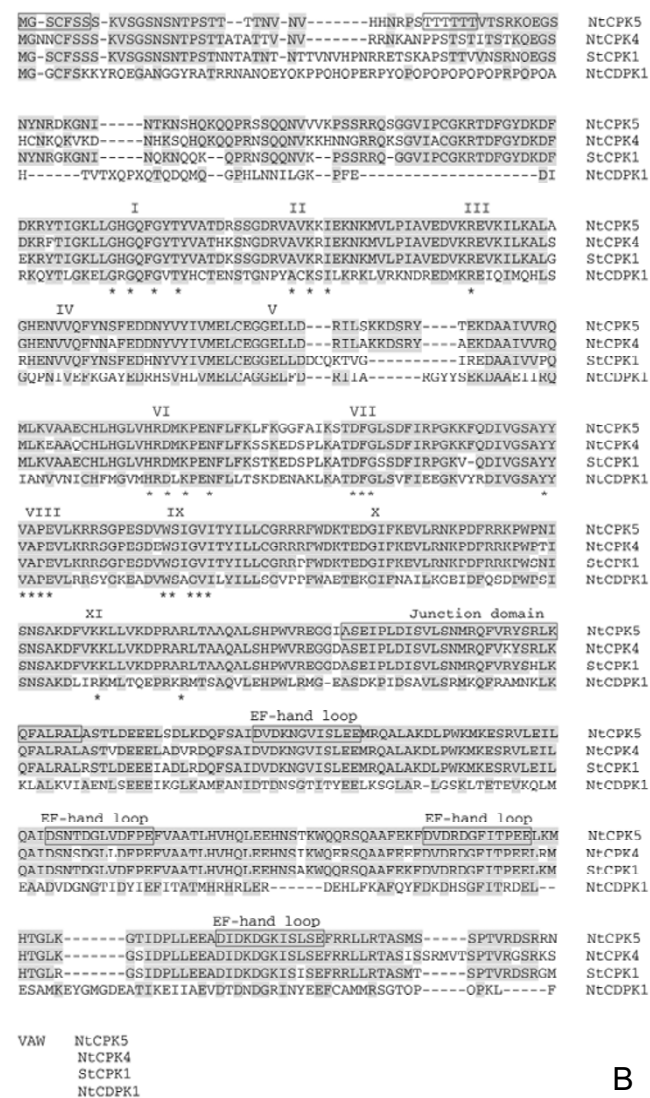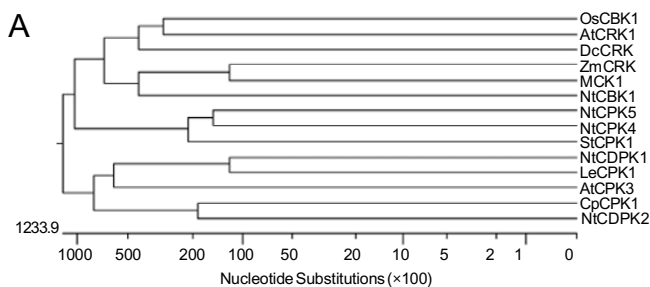

Fig. 2 Alignment of the predicted amino acid sequence of NtCPK5 with other plant protein kinases. **(A)** Phylogenic tree of NtCPK5 and other protein kinases. **(B)** Alignment of the predicted amino acid sequence of NtCPK5 with NtCPK4, StCPK1 and NtCDPK1. Identities between these kinases are indicated by shade squares. Gaps were indicated by dashes (-). The eleven subdomains in kinase domain are indicated. Asterisks (*) indicate residues that are conserved in all Ser/Thr kinases. The N-terminal presumed myristoylation site and continuous six threonines, junction domain and C-terminal four EF-hand loops are marked with a box. Genebank accession number: OsCBK1 (AF368282), AtCRK1 (AF435448), DcCRK (X83869), ZmCRK (D84507), MCK1 (1839597), NtCBK1 (AF435450), NtCPK5 (AY971376), NtCPK4 (AF435451), StCPK1 (AF030879), NtCDPK1 (AF072908), LeCPK1 (AJ308296), AtCPK3 (AL035394), CpCPK1 (U90262), NtCDPK2 (AJ344154).
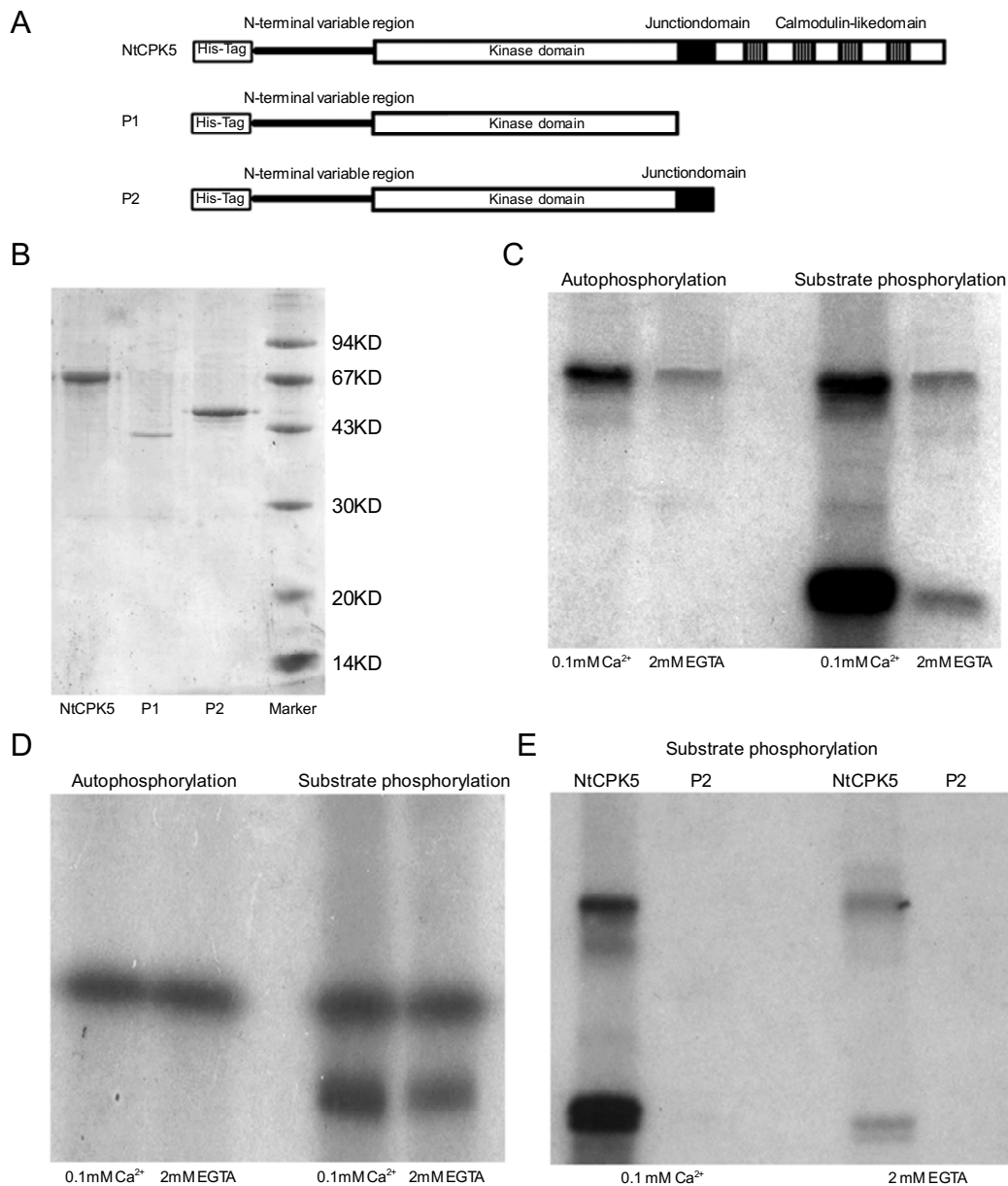
Fig. 3 Analyses of kinase activity of the recombinant NtCPK5 and two truncated proteins P1 and P2. **(A)** Diagrams of full-length and truncated forms of NtCPK5, P1 and P2. **(B)** Expression of the recombinant NtCPK5, P1 and P2 from sf-9 cells. After SDS-PAGE, the purified recombinant proteins were visualized with Coomassie brilliant blue staining. Molecular size markers are indicated on the right. **(C)** Autophosphorylation and substrate phosphorylation (1 mg/ml histone IIIs) of recombinant NtCPK5. **(D)** Autophosphorylation and substrate phosphorylation (1 mg/ml histone IIIs) of P1. **(E)** Substrate phosphorylation (1 mg/ml histone IIIs) of recombinant NtCPK5 and P2. 100 ng of the purified recombinant proteins were subjected to kinase reactions in the presence of 0.1 mM Ca^{2+} or 2 mM EGTA (for autophosphorylation), or plus 1 mg/ml histone IIIs (for substrate phosphorylation), and electrophoresed on SDS-PAGE gels. The gel was vacuum-dried and exposed to x-ray film at – 80°C.

suggesting the regulatory role of its C-terminal calmodulin-like domain in calcium signaling pathway (Fig. 3D). Surprisingly, P2, which is P1 plus the junction domain does not show detectable kinase activity regardless of the presence or absence of Ca^{2+} (Fig. 3E), suggesting that the junction domain may be a negative regulatory domain of the kinase. This result is supporting the model of CPKs activation that the binding of Ca^{2+} can trigger conformational change of the kinase to release an auto-inhibitor from the active site [25]. In the absence of the calcium-binding

domain, access to the active site is permanently blocked by the pseudosubstrate auto-inhibitor rendering the enzyme inactive [6].

Characterization of the kinetic properties of NtCPK5

To investigate the optimum conditions for NtCPK5's enzymatic activity, a range of Mg^{2+} concentration (0 - 25 mM) and of pH (6 - 9.5) was tested. Using histone IIIs as substrates, the optimum concentration of Mg^{2+} for NtCPK5 activity was 10 mM (Fig. 4A). The kinase activity of NtCPK5 was reduced to 51% of the maximum when Mg^{2+} concentration was used at 20 mM. Maximum activity of NtCPK5 was observed at pH 8.0. An acidic condition of pH 6.0 and a basic condition of pH 9.5 reduced the kinase activity to 34% and 60% respectively. The pH-oriented activity of NtCPK5 was similar to that observed for OsCBK in rice [12]. NtCPK5 can phosphorylate histone IIIs rapidly and reached about 60% of the maximal activity within 10 min in the presence of 0.1 mM $CaCl_2$ (Fig. 4C). In the presence of 2 mM EGTA, the enzyme remained the minimum activity (Fig. 4C).

To analyze the effect of Ca^{2+} on NtCPK5's activity, a range of Ca^{2+} concentration was used for the activity assay (Fig. 4D). The kinase activity of NtCPK5 on histone IIIs was stimulated by increasing amount of Ca^{2+} with one-half maximal activation ($K_{0.5}$) 0.04 μM, while on syntide-2 with one-half maximal activation of 0.06 μM. Thus NtCPK5 behaves similarly to other plant CPKs in terms of their Ca^{2+}-dependent and stimulating activity [26], probably via their highly conserved Ca^{2+}-binding domain.

The kinase activities of NtCPK5 on histone IIIs or syntide-2 were further analyzed by using different concentrations of the substrates and fitted to Lineweaver-Burk curve (Fig. 5). For histone IIIs, K_m value of 210 μg/ml and V_{max} of 526 nmol/min/mg were observed (Fig. 5A). For syntide-2, a K_m value of 30 μM and V_{max} value of 2008 nmol/min/mg were observed (Fig. 5B). A fixed ATP concentration (50 μM) was used in the reaction.

Subcellular localization of NtCPK5

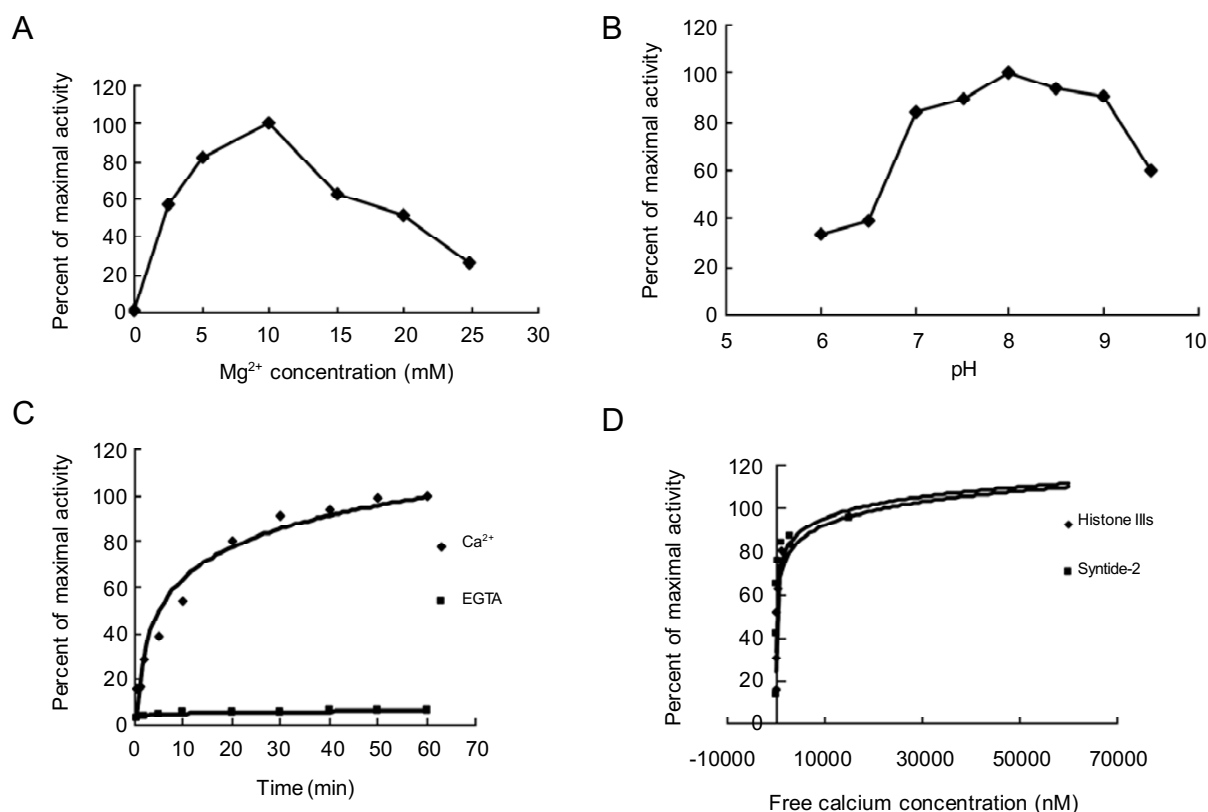The N-terminal variable region has been shown essential for the subcellular localization of many CPKs [7-10].

Fig. 4 Analysis of the kinetic properties of NtCPK5. **(A)** Effects of Mg^{2+} and **(B)** pH concentration on NtCPK5 activity. **(C)** Time course of phosphorylation of histone IIIs by NtCPK5 in the presence of 0.1 mM Ca^{2+} [◆] or 2 mM EGTA [■]. **(D)** Dependence on Ca^{2+} concentration for protein kinase activity of NtCPK5. The enzyme activity was assayed with a histone IIIs [◆] or syntide-2 [■] as substrate in the presence of various concentrations of calcium ions.
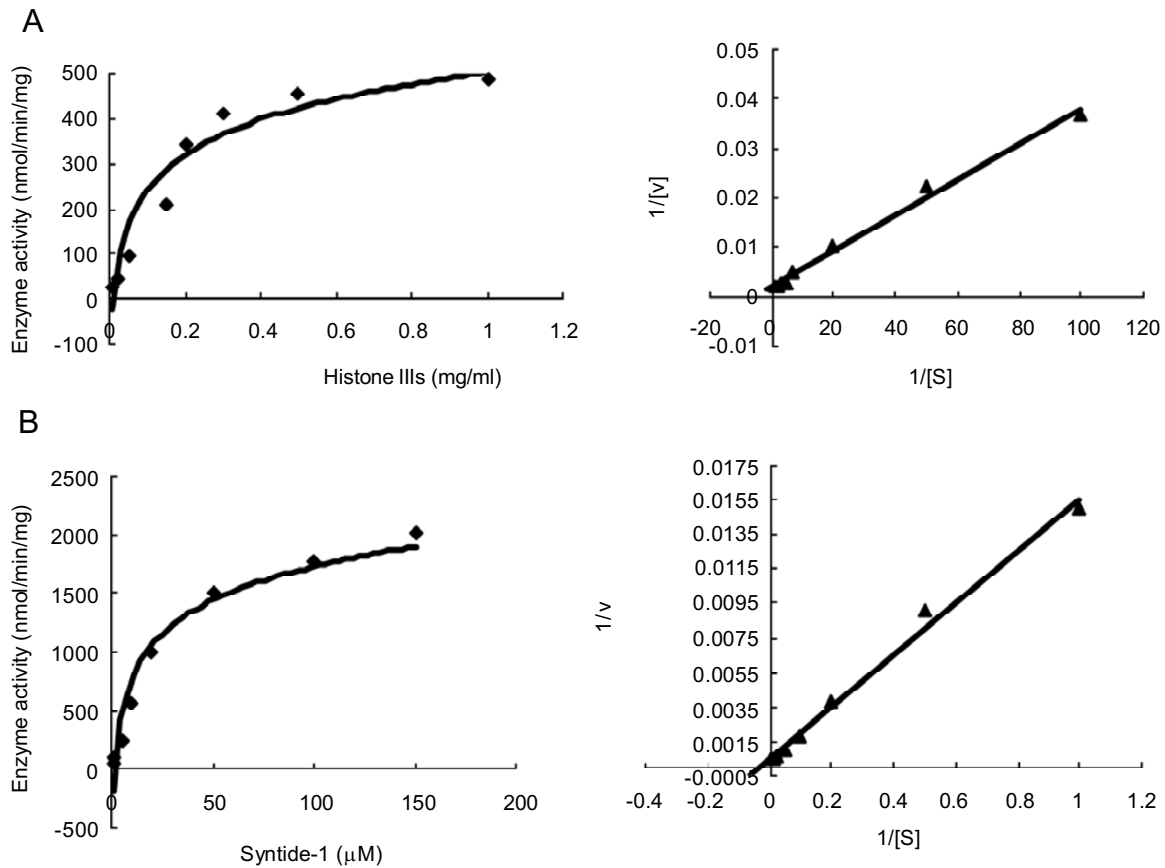
A

B

Fig. 5 Determination of K_m and V_{max} values for NtCPK5 using different concentration of substrate. The protein kinase activity was assayed in the presence of various concentrations of histone IIIs **(A)** and syntide-2 **(B)** as substrate. The right shows a Lineweaver–Burk plot for the same values.

The N-terminal acylation is critical for membrane-anchor of CPKs. NtCPK4 and NtCPK5 are high homologous in tobacco but their N-termini are obviously diverse. NtCPK5 but not NtCPK4 has putative N-terminal myristoylation site by ScanProsite searching of PlantsP database. The Cys at the 4th position from N-terminus as a presumed palmitoylation site in NtCPK5 is replaced by Ala. It is likely that these two kinases have different cellular localizations. In order to analyze the role of CPKs' N-termini in subcellular distribution of these two CPKs, GFP fusion proteins of NtCPK4/GFP and NtCPK5/GFP and three mutant proteins of NtCPK5/GFP with mutated acylation sites were generated and transiently expressed in onion epidermal cells by bombardment.

Confocal microscopy showed that NtCPK5/GFP was efficiently targeted to the cell periphery, suggesting the plasma membrane localization of NtCPK5/GFP (Fig. 6A). NtCPK4/GFP was distributed throughout the cell including the cytoplasm and the nucleus (Fig. 6B), indistinguish-

able from that of GFP (Fig. 6F). Because NtCPK5 had putative N-terminal acylation sites which are missing in NtCPK4, we assume that these sites played vital roles in differential subcellular localizations between NtCPK5 and NtCPK4. To test this hypothesis, three mutant proteins were made by site-directed mutagenesis as described in Material and Methods. To summarize, mutations of Gly2Ala or Cys4Ala or a double mutant of Gly2Ala/ Cys4Ala were made, fused to GFP and transiently expressed in onion epidermal cells (Fig. 6C-E). Analysis by confocal microscopy showed that all these mutation forms lose their plasma membrane localization, instead a distribution throughout the cells was observed, suggesting the importance of both N-terminal myristoylation and palmitoylation sites for plasma membrane targeting of NtCPK5 in onion cells. N-myristoylation or palmitoylation alone is not sufficient for NtCPK5 membrane localization and both amino acids Gly2 and Cys4 are required for the plasma membrane localization of NtCPK5.
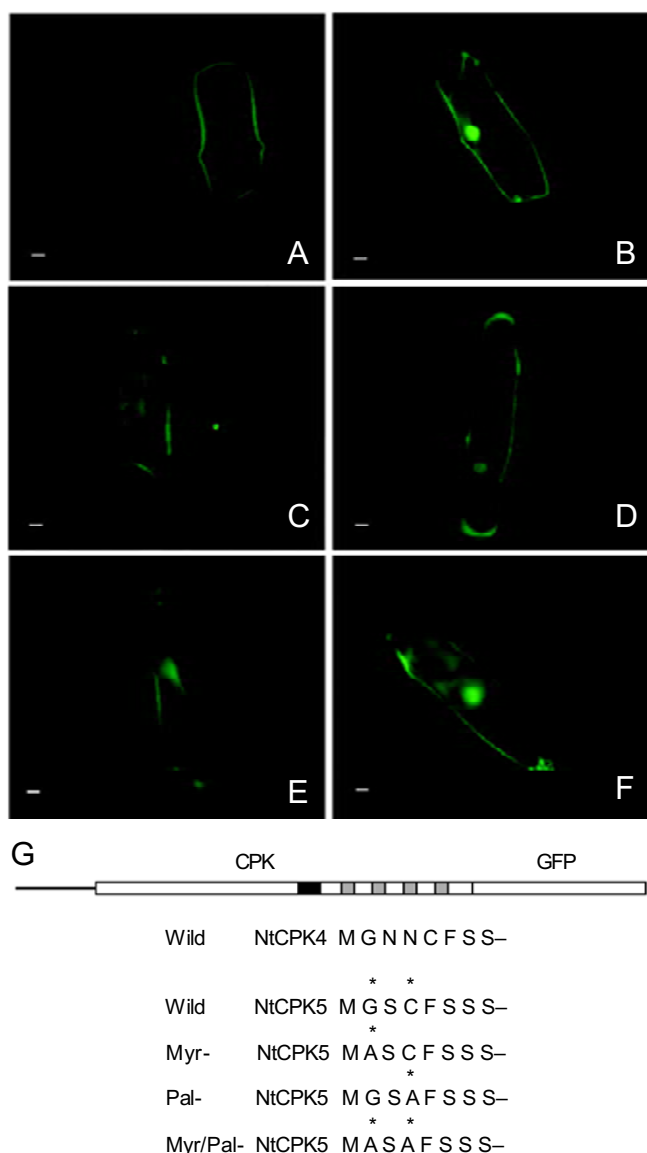
Fig. 6 Subcellular localization of NtCPK5/GFP and NtCPK4/GFP fusion proteins. Wild-type NtCPK5 and N-terminal myristoylation and palmitoylation mutants were transiently expressed as C-terminal GFP-fusion proteins in onion epidermal cells. **(A)** Localization of wild NtCPK5/GFP fusion protein. **(B)** Localization of wild NtCPK4/GFP fusion protein. **(C)** Localization of mutated myristoylation site Gly2Ala NtCPK5/GFP fusion protein. **(D)** Localization of mutated palmitoylation site Cys4Ala NtCPK5/GFP fusion protein. **(E)** Localization of mutated myristoylation and palmitoylation sites Gly2Ala/Cys4Ala NtCPK5/GFP fusion protein. **(F)** Control. Soluble localization of GFP protein. **(G)** Diagrams of wild NtCPK4/GFP, NtCPK5/GFP and mutant NtCPK5/GFP. The length of the bars corresponds to 10 µm.

DISCUSSION

In this study, a tobacco cDNA designated as *NtCPK5*

was cloned and characterized. Analysis of amino acid sequence indicates that it could be a calcium-dependent protein kinase, since it contains all the features of Ca^{2+}-dependent protein kinases, including conserved kinase domain, junction domain and CaM-like domain. Searching the PlantsP database with NtCPK5 amino acid sequence [19], it was found of a protein kinase catalytic domain (pfscan), four EF-hand binding Ca^{2+} motives (ScanProsite), a protein kinase ATP-binding region signature (ScanProsite), a Ser/Thr protein kinase active-site signature (ScanProsite) and seven potential N-myristoylation sites (ScanProsite). Compared to other CPKs, NtCPK5 showed high homology to that of NtCPK4 and potato StCPK1 [20], especially to that of NtCPK4. They differ in N-terminal variable regions of the N-termini acylation sites and the six continuous threonines from 34th to 39th in NtCPK5. The N-termini acylation sites have been shown required for membrane-anchoring of many CPKs [7-10], while the role of the six threonines stretch in NtCPK5's N-terminal variable region is still unknown.

CPK-related protein kinases (CRKs) have both kinase domain and junction domain closely related to those of CDPKs, while their C-terminal domains have degenerated EF-hands [6]. Biochemical analyses showed that the activation of CRKs does not require calcium [13, 24, 27]. Phylogenetic analyses indicated that there was a common evolutionary origin for plant CPKs and CRKs [6]. An evolutionary link between *CPKs* and *CRKs*, named *StCPK1*, has been cloned in potato plants [20]. StCPK1 shares more similarity to CRKs than to CPKs, although sequence analyses indicate that it contains conserved EF-hands at the C-terminus. NtCPK5 showed high homology to StCPK1 (89.4%, identity), NtCPK5 could thus be considered as an evolutionary link between CPKs and CRKs similar to StCPK1.

To characterize the biochemical activity of NtCPK5 as a calcium-dependent protein kinase, both full-length and truncated NtCPK5 were used for kinase assays. Substrate phosphorylation by full-length NtCPK5 can be greatly stimulated by Ca^{2+}. The $K_{0.5}$ of calcium-stimulating NtCPK5's activity is different for different substrates such as histone IIIs and syntide-2 used. All these dada suggest that NtCPK5 is a new member of the CPK subfamily in tobacco.

Previous reports have shown that many CPKs were modified by N-acylation including myristoylation and palmitoylation and targeted to membrane [7-10]. The N-myristoylation site was a conserved N-terminal sequence of 7-10 aa long signature as MGxxxS/T (K) [28]. In our experiments, NtCPK5/GFP was targeted to the plasma membrane, while NtCPK4/GFP was distributed within the whole cell. This could due to the putative N-terminal acy-

lation sites in NtCPK5. When the N-acylation sites (Gly2Ala and Cys4Ala) were mutated, NtCPK5/GFP lost plasma membrane localization.

In summary, a new CPK isoform NtCPK5 from tobacco was cloned and characterized. NtCPK5 behaviors like a typical calcium-dependent protein kinase. NtCPK5 localized to the plasma membrane in onion cells, while NtCPK4, a homologue of NtCPK5 was distributed evenly within the cell, suggesting that the N-terminal sequence of the two NtCPKs plays vital roles in their subcellular localization. An understanding of the role of acylation in NtCPK5 activity needs further analysis of the physiological function of NtCPK5 and its mutant proteins in plants.

REFERENCES

1   Reddy ASN. Calcium: silver bullet in signaling. Plant Sci 2001; **160:**381-404.
2   Cheng SH, Willmann MR, Chen H, Sheen J. Calcium Signaling through Protein Kinases. The *Arabidopsis* Calcium-dependent Protein Kinase Gene Family. Plant Physiol 2003; **129:**469-85.
3   Zhang L, Lu YT. Calmodulin-binding protein kinase in plants. Trends Plant Sci 2003; **8:**123-7.
4   Rudd JJ, Franklin-Tong VE. Unravelling responsespecificity in Ca^{2+} signaling pathways in plant cells. New Phytol 2001; **151:**7-33.
5   Hrabak EM. Calcium-dependent protein kinases and their relatives. Advances in botanical sciences, plant protein kinases. New York: Academic Press 2000; **32:**185-223.
6   Harmon AC, Gribskov M, Harper JF. CDPKs: a kinase for every Ca^{2+} signal? Trends Plant Sci 2000; **5:**154-9.
7   Martin ML, Busconi L. Membrane localization of a rice calcium-dependent protein kinase (CDPK) is mediated by myristoylation and palmitoylation. Plant J 2000; **24:**429-35.
8   Rutschmann F, Stalder U, Piotrowski M, Oecking C, Schaller A. LeCPK1, a calcium-dependent protein kinase from tomato plasma membrane targeting and biochemical characterization. Plant Physiol 2002; **129:**156-68.
9   Raíces M, Gargantini PR, Chinchilla D, *et al*. Regulation of CDPK isoforms during tuber development. Plant Mol Biol 2003; **52:**1011-24.
10 Dammann C, Ichida A, Hong B, *et al*. Subcellular targeting of nine Calcium-dependent protein kinase isoforms from Arabidopsis. Plant Physiol 2003; **132:**1840-8.

11 Lu YT, Hidaka H, Feldman LJ. Characterization of a calcium/calmodulin-dependent protein kinase homolog from maize roots showing light- regulated gravitropism. Planta 1996; **199:**18-24.
12 Zhang L, Liu BF, Liang S, Jones RL, Lu YT. Molecular and biochemical characterization of a calcium/calmodulin-binding protein kinase from rice. Biochem J 2002; **368:**145-57.
13 Wang Y, Liang S, Xie QG, Lu YT. Characterization of a calmodulin-regulated Ca^{2+}-dependent-protein-kinase-related protein kinase AtCRK1 from Arabidopsis. Biochem J 2004; **383:** 73-81.
14 Hua W, Liang S, Lu YT. A tobacco (*Nicotiana tabaccum*) calmodulin-binding protein kinase, NtCBK2, is regulated differentially by calmodulin isoforms. Biochem J 2003; **376:**291-302.
15 Ma L, Liang S, Jones RL, Lu YT. Characterization of a novel calcium/calmodulin-dependent protein kinase from tobacco. Plant Physiol 2004; **135:**1280-93.
16 Bers DM, Patton CW, Nuccitelli R. A Practical Guide to Preparation of Ca^{2+} Buffers. Methods Cell Biol 1994; **40:**3-29.
17 Hua W, Zhang L, Liang S, Jones RL, Lu YT. A Tobacco Calcium/Calmodulin-binding Protein Kinase Functions as a Negative Regulator of Flowering. J Biol Chem 2004; **279:**31483-94.
18 Scott A, Wyatt S, Tsou PL, Robertson D, Allen NS. Model system for plant cell biology: GFP imaging in living onion epidermal cells. Biotechniques 1999; **26:**1125-32.
19 Gribskov M, Fana F, Harper J, *et al*. PlantsP: a functional genomics database for plant phosphorylation. Nucleic Acids Res 2001; **29:**111-3.
20 Lakatos L, Hutvagner G, Banfalvi Z. Potato protein kinase StCPK1: a putative evolutionary link between CDPKs and CRKs. Biochim Biophys Acta 1998; **1442:**101-8.
21 Yoon GM, Cho HS, Ha HJ, Liu JR, Lee Pai HS. Characterization of NtCDPK1, a calcium-dependent protein kinase gene in *Nicotiana tabacum*, and the activity of its encoded protein. Plant Mol Biol 1999; **39:**991-1001.
22 Romeis T, Ludwig AA, Martin R, Jones JDG. Calcium- dependent protein kinases play an essential role in a plant defence response. EMBO J 2001; **20:**5556-67.
23 Mary EI, Hopkins RB, White TJ, Lomax TL. Cloning, expression and N-terminal myristoylation of CpCPK1, a calcium-dependent protein kinase from zucchini (*Cucurbita pepo L.*). Plant Mol Biol 1999; **39:**199-208.
24 Furumoto T, Ogawa N, Hata S, Izui K. Plant calcium-dependent protein kinase-related kinases (CRKs) do not require calcium for their activities. FEBS Lett 1996; **396:**147-51.
25 Harper JF, Sussman MR, Schaller GE, *et al*. A calciumdependent protein kinase with a regulatory domain similar to calmodulin. Science 1991; **252:**951-4.
26 Chehab EW, Patharka OR, Hegeman AD, Taybi T, Cushman JC. Autophosphorylation and subcellular localization dynamics of a salt- and water deficit-induced Calcium-dependent protein kinase from ice plant. Plant Physiol 2004; **135:**1430-46.
27 Du W, Wang Y, Liang S, Lu YT. Biochemical and expression analysis of an Arabidopsis Calcium-dependent protein kinase-related kinase. Plant Sci 2005; **168:**1181-92.
28 Thompsona Jr GA, Okuyamab H. Lipid-linked proteins of plants. Prog Lipid Res 2000; **39:**19-39.