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Cloning and functional characterization of NtCPK4, a new tobacco calcium-dependent protein kinase☆

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Abstract

A cDNA clone, encoding calcium (Ca^{2+})-dependent protein kinase (CDPK or CPK), was isolated from tobacco (*Nicotiana tabacum*). The full-length cDNA of 2360 bp contains an open reading frame for *NtCPK4* consisting of 572 amino acid residues. Sequence alignment indicated that NtCPK4 shared high similarities with other CPKs and some CPK-related protein kinases (CRKs). Biochemical analyses showed that NtCPK4 phosphorylated itself and calf thymus histones fraction III-S (histone III-S) in a calcium-dependent manner, and the $K_{0.5}$ of calcium activation was 0.29 μM or 0.25 μM with histone III-S or syntide-2 as substrates, respectively. The V_{max} and K_m were 588 nmol min^{-1} mg^{-1} and 176 μg ml^{-1}, respectively, when histone III-S was used as substrate, while they were 2415 nmol min^{-1} mg^{-1} and 58 μM, respectively, with syntide-2 as substrate. In addition, the phosphorylation of NtCPK4 occurred on threonine residue, as shown by capillary electrophoresis analyses. All of these data demonstrated that NtCPK4 was a serine/threonine protein kinase. *NtCPK4* as a low copy gene was expressed in all tested organs including the root, leaf, stem, and flower of tobacco, while its expression was temporally and spatially modulated in both productive and vegetative tissues during tobacco growth and development. *NtCPK4* expression was also increased in response to the treatment of gibberellin or NaCl. Our study suggested that NtCPK4 might play vital roles in plant development and responses to environmental stimuli.

Keywords: Calcium-dependent protein kinase; Phosphorylation; Gene expression; *Nicotiana tabacum*

1. Introduction

Calcium (Ca^{2+}) as a second messenger is a ubiquitous intracellular signaling molecule that mediates many growth and developmental processes in plants as well as their responses to external stimuli [1]. The role of calcium is mediated by a group of calcium-binding proteins including calcium-dependent protein kinase (CPK or CDPK), calmodulin (CaM), and calmodulin-binding protein kinase (CBK) [2]. Since the CPK activities were first evidenced in pea shoot membranes [3] and CPK cDNA from *Arabidopsis* [4], many subsequent CPKs have been characterized from other plants. The analysis of the *Arabidopsis* genome indicates that there is a large CPK multigene family of 34 CPK members [5]. So far, CPKs have been shown to share a similar primary structure consisting of three domains: the kinase domain, followed by an autoinhibitory region (or junction domain), and a C-terminal calmodulin-like domain (or calcium-binding domain). The autoinhibitory region contains a pseudo-substrate site that, in the absence of Ca^{2+}, binds to the catalytic center, resulting in an inactive state of the kinase. However, the binding of Ca^{2+} to the calmodulin-like domain can induce a conformational shift for the release the pseudo-substrate domain from the

Abbreviations: CPK or CDPK, Ca^{2+}-dependent protein kinase; CRK, CPK-related kinase; CaM, calmodulin; CBK, calmodulin-binding protein kinase; CaMK, Ca^{2+}/CaM-dependent protein kinase; CCaMK, chimeric Ca^{2+}/CaM-dependent protein kinase; MCK, maize homologue of mammalian CaMK; histone III-S, calf thymus histones fraction III-S; Ni-NTA, Ni^{2+}-nitrilotriacetate; FITC, fluorescein isothiocyanate

☆ The NtCPK4 nucleotide sequence data reported in this paper have been deposited in the GenBank database with accession number AF435451.

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active site and kinase activation [4,6,7]. Besides these three conservative domains, all CPKs have an N-terminal variable region, which often contains N-terminal acylation (including myristoylation and palmitoylation) sites for the subcellular localizations of CPKs [8–10]. The first reported tobacco CPK, NtCDPK1, is a membrane-associated protein, and its expression is stimulated by several phytohormones, Ca^{2+}, wounding, fungal elicitors, chitosan, and NaCl [11]. Based on the interaction of this CPK and NtRpn3, a regulatory subunit of 26S proteasome, NtCDPK1 and NtRpn3 are proposed to interact in a common signal transduction pathway possibly for the regulation of cell division, differentiation, and cell death in tobacco [12]. Two other tobacco CPKs (NtCDPK2 and NtCDPK3) are suggested to be involved in plant defense response [13,14]. So far, increasing evidence has been provided for the involvement of CPKs in biotic and abiotic stress signaling including drought, salt, injury, cold, light, and hormones [15–18].

In our investigation, a tobacco cDNA encoding NtCPK4 was isolated and characterized molecularly and biochemically. The activity of NtCPK4 was dependent upon free calcium ion, and the phosphorylation was occurred at the threonine site, indicating that NtCPK4 is a serine/threonine calcium-dependent protein kinase. The expression of *NtCPK4* was regulated spatially and temporally during plant growth and development and in plant response to the gibberellin and high salt treatment. NtCPK4 as a new member of the tobacco CPK superfamily could be proposed to play roles in plant development and environmental responses.

2. Materials and methods

2.1. Isolation of the cDNA encoding NtCPK4

The tobacco cDNA library was constructed with mRNA isolated from leaves of tobacco (*Nicotiana tabacum*) W38, then was screened with plasmid p550 containing a maize cDNA encoding MCK1 [19]. Positive recombinant phages for NtCPK4 were isolated and excised in vitro into recombinant pBluescript SK (−). Candidate plasmid p*NtCPK4* was sequenced.

2.2. RNA isolation and Northern blot

Total RNAs from different tobacco tissues (root, stem, flower and leaf; 60-day-old adult plants) or under different conditions (30-day-old seedlings) were isolated with Trizol reagent, and Northern blotting was carried out as described previously [20]. Briefly, 20 μg total RNA was separated on a 1.2% formaldehyde agarose gel and then blotted onto Hybond N^+ nylon membranes (Amersham). The mRNA for *NtCPK4* was detected by a [^{32}P]-labeled probe made with the 3′-untranslational region of *NtCPK4*.

The expression of *NtCPK4* was also examined under various conditions. For hormone treatments, tobacco plants were cultured in solutions of 100 μM of auxin (IAA, 2, 4-D) or 100 μM gibberellin for different times with control plants in water. For NaCl treatment, the young tobacco plants were incubated with 200 mM NaCl solution for different periods. For cold and heat treatments, the plants were placed at 4 °C and 42 °C for different periods.

2.3. Genomic DNA isolation and Southern blot

Genomic DNA was extracted from tobacco leaves using the CTAB method [21,22], and 10 μg genomic DNA was digested with the restriction enzymes *Bam*HI and *Eco*RV (New England Biolabs, Beverly, MA), which cut only once in the *NtCPK4* cDNA coding region, separated on 1.0% agarose gels, and blotted under denaturing conditions onto Hybond N^+ nylon membrane (Amersham). Membranes were hybridized overnight at 65 °C with [^{32}P]-labeled *NtCPK4* probes generated by random-primer labeling using a random-primer labeling kit (Promega). The blots were washed twice at 65 °C for 10 min each in 2× SSC/0.1% SDS and subsequently washed twice in 0.2× SSC/0.1% SDS for 10 min at 65 °C before exposure to X-film at −80 °C.

2.4. Construction of recombinant virus and purification of NtCPK4 and truncated NtCPK4 T1-411

The experiments were performed as previously described [23,24]. Two cDNA fragments for the full-length NtCPK4 and the truncated form NtCPK4 T1-411 containing the variable amino-terminal, kinase domain, and autoinhibitory region (residues 1–411) were amplified. Then the PCR products were digested with *Eco*RI and subsequently cloned into the *Eco*RI site of plasmid pFastBacHta to generate the recombinant proteins fused to the C-terminus of six-histidine (His-tag). The recombinant plasmids were transformed into DH10Bac competent cells containing the bacmid with a mini-att Tn7 target site and helper plasmid, and the mini Tn7 element on the pFastBacHTa donor plasmid transposed 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 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 the manufacturer's instructions (Invitrogen). Recombinant virus were harvested after 72 h and identified by PCR with the primers described above.

The insect cells sf-9 were harvested at room temperature after being infected with the recombinant virus for 72 h. Cells were washed once with Grace's medium and resuspended in 5 ml lysis buffer (50 mM Tris–HCl, pH 7.5, 10% glycerol, 1% Nonidet P-40, and 0.2 mM phenyl-