Biochemical characterization of a calcium-sensitive protein kinase
LeCPK2 from tomato

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Received 30 December 2010; revised 06 May 2011

LeCPK2 (GenBank GQ205414), a versatile calcium-dependent protein kinase (CDPK or CPK) gene was isolated from tomato in our previous study. In this study, the biochemical properties of LeCPK2 were further investigated. To examine the role of the C-terminal calmodulin-like domain (CLD) of LeCPK2 with respect to Ca2+ activation, the kinase activities of recombinant full-length and truncated LeCPK2 were measured by Kinase-Glo® Luminescent kinase assay (Promega). The results showed that LeCPK2 activity was Ca2+-dependent and the C-terminal CLD of 161 residues was essential for the activation of LeCPK2. The activity of LeCPK2 was sharply stimulated by Ca2+ with K0.5 (concentration of Ca2+ for half-maximal activity) of 48.8 and 45.5 nM with substrate histone IIIs and syntide 2, respectively. The optimal concentration of Mg2+ for LeCPK2 activity was 20 and 10 mM for substrate histone IIIs and syntide 2, respectively. The Km value of LeCPK2 towards histone IIIs and syntide 2 was 44.9 µg/ml and 89.52 µM, respectively. The determination of biochemical properties of LeCPK2 would provide some clues on how its activity was regulated in vivo.

Keywords: Calcium-dependent protein kinase, LeCPK2, Kinase-Glo® Luminescent Kinase Assay, Tomato

Calcium (Ca2+) is a ubiquitous signal molecule that plays vital role in plant responses to various stimuli, including light, abiotic stresses, pathogen attack, and hormones1. In cells, the fluctuation of cytosolic Ca2+ concentration induces specific calcium signals which can be recognized and transduced into downstream responses, including altered protein phosphorylation and gene expression patterns through several calcium-binding proteins, such as calmodulin (CaM), calcineurin B-like protein (CBL), and calcium-dependent protein kinases (CDPK or CPK)2,3. Among them, CDPKs represent a novel class of calcium sensors having both protein kinase and calmodulin-like domains (CLDs) in a single polypeptide. As a result, kinase activities of CDPKs are directly stimulated by Ca2+, unlike calcium/calmodulin-dependent protein kinases (CCaMs) which respond to Ca2+ through CaM2.

CDPKs comprise an N-terminal variable region and three conserved domains: kinase catalytic domain, auto-inhibitory domain (junction domain, JD) and C-terminal CLD2,4,5. CDPKs activation can self-regulate through the JD and CLD. For parasite Plasmodium falciparum PfCDPK4, the N-terminus of its JD serves as a pseudo-substrate, blocking the activity and the C-terminus may interact with the CLD to regulate the kinase activation6. Recently, the regulation of JD and CLD on the CDPKs activation has been further depicted through solving the structures of auto-inhibited and activated CDPKs from parasite Apicomplexan. In inactivated state, the JD and subsequent N-terminal segment of CLD form a long helix to inhibit the kinase activity, but in activated form, the helix unwinds partially and is separated into a JD and a CLD helix, resulting in a striking conformational change which is distinct from that occurs in calmodulins7.

CDPKs exist as a multi-gene family in the plants. Through genome-based scanning, 34 and 31 CDPK

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Abbreviations: CaM, calmodulin; CBL, calcineurin B-like protein; CCaMs, calcium/calmodulin-dependent proteins; CDPK or CPK, calcium-dependent protein kinase; CLD, calmodulin-like domain; ET, ethylene; IPTG, isopropyl thio-β-D-galactoside; JD, junction domain; MeJA, methyl jasmonate; RLU, relative light units; SA, salicylic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
genes have been identified in *Arabidopsis* and rice, respectively. Tobacco, wheat and corn also have multiple CDPK isoforms. The wide distribution of CDPK isoforms in different plant species implicates their vital roles in plants. CDPKs have shown wide functions, in response to various internal developmental signals and environmental stimuli, such as hormones, wounding, salt, drought, cold, heat, and attacks. In a given species, the specific functions of each CDPK isoform may be related to its biochemical properties, such as calcium binding and activation. Three CDPK isoforms from soybean have shown different calcium binding activities, though they exhibit high homology in amino acid sequence. Thus, it is essential to investigate the biochemical properties of individual CDPK and compare them with that of other CDPK isoforms, in order to assess specific functions of a CDPK in a calcium signal transduction.

Earlier, we have isolated a novel CDPK gene *LeCPK2* (GenBank GQ205414), which to date is the third CDPK gene observed in the tomato besides *LeCDPK1* and *LeCPK1*. *LeCPK2* has been found to be involved in flower development and response to heat, wounding, and three phytohormones, ethylene (ET), methyl jasmonate (MeJA) and salicylic acid (SA). In this study, we have investigated the Ca\(^{2+}\) activation and enzymatic kinetic properties of recombinant *LeCPK2*, in order to understand the roles of this versatile calcium-dependent protein kinase.

**Materials and Methods**

**Construction of expression plasmids of full-length and truncated *LeCPK2***

To understand the regulation of the C-terminal CLD of *LeCPK2* on kinase activation, cDNAs encoding full-length and truncated *LeCPK2* lacking the CLD of 161 residues were amplified and cloned into expression vector pET-30a through restriction sites *Sac* I and *Xho* I. The forward primer 5'-ATCGAGCTCATGGGTAGTTGTTTTTCAAGCT-3' used was the same for both wide-type *LeCPK2* (1,700 bp) and its truncated mutant (1,200 bp). The reverse primer for wide-type *LeCPK2* was 5'-ATTCTCGAGTTACATTCCCCGTGAATCTCT-3' and for mutant was 5'-AACCTCGAGTTACATTCCCCGTGAATCTCT-3'. After washing with ddH\(_2\)O, cell pellets were resuspended in ice-cold lysis buffer (25 mM Tris-HCl pH 7.5, 500 mM NaCl, 1% Triton X-100, 40 mM imidazole) and incubated on ice for 10 min, followed by two freeze-thaw cycles to enhance lysis.

After centrifugation at 12,000 g for 30 min at 4°C, supernatant was applied to a Ni-NTA (Ni\(^{2+}\)-nitrilotriacetate) resin column, pre-equilibrated with buffer A (25 mM Tris-HCl, pH 7.5, 500 mM NaCl, 40 mM imidazole, and 10% glycerol). Following extensive washing with buffer A, recombinant proteins were subsequently eluted with buffer A containing 300 mM imidazole. The purified proteins were desalted and exchanged into storage buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10% glycerol) using centrifugal filter device MicroconYM-30 (Millipore) and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and enzymatic assays. Protein concentration was determined by the method of Bradford using BSA as a standard.

**Kinase assays**

Kinase reaction was carried out in kinase buffer containing 25 mM Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 10 \(\mu\)M ATP, 0.1 mM CaCl\(_2\) or 2 mM EGTA at 30°C, with histone IIIs as substrate (1 mg/ml). Reaction was initiated by addition of 100 ng purified protein. At each time points (0-50 min), 20 \(\mu\)l of reaction mixture was pipetted off and heated at boiling water for 1 min to terminate the reaction. Kinase activity was measured using Kinase-Glo\textsuperscript{®} Luminescent kinase assay kit (Promega, Madison, WI), which quantifies the amount of ATP remaining in solution, following a kinase reaction by a luminescence-producing reaction.

Briefly, solutions from each time points were mixed with an equal volume of Kinase-Glo\textsuperscript{®} Reagent (20 \(\mu\)l) in a 96-well white microplate and incubated for 20 min at room temperature. Following the reaction, the microplate was recorded for luminescence value (relative light units, RLU) on a GloMax\textsuperscript{®} Multi-detection system (Promega). Kinase activities were also measured under different Mg\(^{2+}\) concentrations (5-25 mM) and free Ca\(^{2+}\) concentrations (0-300 nM), which were set using Ca\(^{2+}\)/EGTA buffer as described by Bers at 30°C for
30 min using histone IIIs (1 mg/ml) and syntide 2 (100 µM) as substrate, respectively. For determination of the \( K_m \) values of LeCPK2, different concentrations of histone IIIs (0-500 µg/ml) and syntide 2 (0-200 µM) were used in the reactions.

**Data analysis and curve fitting**

Each kinase assay was performed repeatedly at least three times and the RLU value for each sample (well) was also read three times at intervals of 1 min. The activity-time and activity-Mg\(^{2+}\) concentration curves were fitted through Microsoft® Excel. Curve fitting and the determination of the Ca\(^{2+}\) \( K_{0.5} \) (concentration required for the half-maximal activity) value of LeCPK2 were performed using software GraphPad® Prism dose-response-stimulation (variable slope) model. Curve fitting and the calculation of \( K_m \) values were carried out using GraphPad® Prism enzyme kinetics Michaelis-Menten model. Since the recorded RLU value was positively correlated with the amount of ATP present, and thus inversely correlated with the amount of kinase activity due to ATP consumed by the enzyme in kinase reaction, the decrease of RLU value against control (background) for each sample was calculated and used as the representation of kinase activity in the Ca\(^{2+}\) \( K_{0.5} \) and \( K_m \) curve fittings.

**Results**

**Expression and purification of recombinant proteins**

Based on the sequence alignment with some known CDPKs, the C-terminal of 161 residues of LeCPK2 was determined to be the CLD (Fig. 1A), so in the truncated LeCPK2, this segment was removed to examine its role in Ca\(^{2+}\)-induced kinase activation. Recombinant full-length and truncated LeCPK2 were expressed as soluble proteins and purified with predicted molecular masses of 69.8 and 51.6 kDa, respectively (Fig. 1B). For *in vitro* expression of LeCPK2, given a few rare codon clusters existing in LeCPK2 sequence, *E. coli* strain Rosetta 2 (DE3) was recruited for the expression of recombinant LeCPK2 in place of BL21 (DE3), with which no recombinant target protein was induced with IPTG (data not shown here). *E. coli* strain Rosetta 2 (DE3) may supply tRNAs for seven rare codons, rarely used in *E.coli* on a compatible chloramphenicol-resistant plasmid and was thus designed to enhance the expression of eukaryotic proteins in *E. coli*.

**Analysis of calcium-dependent activity of LeCPK2**

To assess the role of C-terminal CLD in the activation of LeCPK2, full-length and truncated LeCPK2 were generated and subjected to the activity assays in the presence of Ca\(^{2+}\) or EGTA (a Ca\(^{2+}\)-specific chelating agent, which removes free Ca\(^{2+}\) from reaction solution). For wild-type LeCPK2, RLU values decreased persistently throughout the experimental time, indicating that ATP was continuously consumed in the reaction solution in the presence of Ca\(^{2+}\), while the insignificant variation in RLU values in EGTA solution suggested no kinase reaction or only weak reaction in the absence of Ca\(^{2+}\) (Fig. 2A).

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**Fig. 1—Amino acid sequence and *in vitro* expression of full-length and truncated LeCPK2** [A: Amino acid sequence of full-length LeCPK2. LeCPK2 comprises N-terminal variable domain, kinase catalytic domain (box), junction domain (highlighted) and C-terminal calmodulin-like domain of 161 aa (underline); and B: SDS-PAGE analysis of expression and purification of full-length and truncated LeCPK2. Lane Mr; a protein standard; lane 1, expression of un-induced control; lanes 2 and 3, expression of induced truncated and full-length LeCPK2, respectively; and lanes 4 and 5, purified proteins of truncated and full-length LeCPK2, respectively (indicated with arrows)]
For mutant LeCPK2, the approximately constant luminescent values during reaction time from 0 to 40 min indicated that the truncated protein had no kinase activity, regardless of the presence of Ca^{2+} (Fig. 2B). Therefore, it is reasonable to propose that kinase activity of LeCPK2 is Ca^{2+}-dependent and is regulated by the essential C-terminal CLD of 161 residues, which support the mechanism by which Ca^{2+} binds to the EF-hands of CLD to release the auto-inhibitory motif from the active site and triggers the CDPK activity. In our previous study, we have shown that the deduced amino acid sequence of LeCPK2 shares high homology with other CDPK proteins and comprises the typical CDPK domains. Here, the calcium-dependent and calcium-regulative LeCPK2 activity further confirmed that LeCPK2 is a member of tomato CDPK family.

Kinetic properties of LeCPK2

A kinase reaction is Mg^{2+}-dependent. For LeCPK2, the optimal Mg^{2+} concentration was 10 and 20 mM using syntide 2 and histone III as substrate (Fig. 3A) respectively, which suggested that the effect of Mg^{2+} on the activity of LeCPK2 was substrate-dependent.
Different concentrations of Ca\(^{2+}\) were set to investigate the stimulation of LeCPK2 activity by the Ca\(^{2+}\). Result showed that catalytic activity of LeCPK2 was sharply stimulated by Ca\(^{2+}\) using either histone IIIs or syntide 2 (Fig. 3B). The concentration of Ca\(^{2+}\) required for the half-maximal activity (\(K_{0.5}\)) of LeCPK2 was 48.8 and 45.5 nM with histone IIIs and syntide 2, respectively. LeCPK2 activity towards different substrates was also analyzed using different substrate concentrations. Result indicated that the \(K_m\) value of LeCPK2 was 44.9 µg/ml and 89.52 µM using histone IIIs and syntide 2, respectively (Fig. 4A and B).

**Discussion**

Traditional kinase activity assays are based on the monitoring of radion-labelled products generated by the phosphoryl transfer of \([\gamma-3^P]\)ATP in the kinase reaction. These methods are sensitive and reliable, but they are labor-intensive and involve use of radiation. In this study, Kinase-Glo\textsuperscript{TM} Luminescent kinase assay (Promega), a non-radioactive, homogeneous, ATP quantitative kit was recruited to assay the activity and kinetic properties of LeCPK2. ATP is used as a universal kinase substrate and produces reliable, sensitive and reproducible results. The reliability of determining kinase parameters by the ATP quantitation through luminescence signal could be validated by the utilization of ADP-Glo\textsuperscript{TM} assay (Promega) in the determination of \(K_m\) values for the lipid kinases. In our study, data from the luminescence records were homogeneous and logical in replicate tests, which thus provided a new choice for the determination of kinase parameters, such as \(K_m\) values.

As indicated, the activity of LeCPK2 was Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-sensitive. The \(K_{0.5}\) value of Ca\(^{2+}\) for LeCPK2 was lower than that for most of the reported CDPKs, such as soybean CDPK isoforms \(\beta\) and \(\gamma\) (\(K_{0.5} = 0.4\) and 1.0 µM, respectively)\textsuperscript{24}, McCPK1 (\(K_{0.5} = 0.15\) µM)\textsuperscript{25} and PfCPK1 (\(K_{0.5} = 15\) µM)\textsuperscript{26} and similar to that for soybean CDPK isoform \(\alpha\) (\(K_{0.5} = 0.06\) µM)\textsuperscript{24} and NtCPK5 (\(K_{0.5} = 0.04\) and 0.06 µM for histone IIIs and for syntide 2, respectively)\textsuperscript{27}.

The Ca\(^{2+}\) \(K_{0.5}\) values for different CDPK isoforms in a given species vary significantly. The value for individual soybean CDPK isoforms \(\alpha\), \(\beta\) and \(\gamma\) differs by over two orders of magnitude from 0.06 µM (\(\alpha\)) to 0.4 µM (\(\beta\)) and to 1.0 µM (\(\gamma\)) with syntide 2 as substrate\textsuperscript{24}. Also, the sensitivity of CDPKs to Ca\(^{2+}\) can be influenced by the type of protein substrate. In the absence of any substrate, CDPKs binds Ca\(^{2+}\) with a \(K_d\) of 50 mM. However, in the presence of substrates, Ca\(^{2+}\) sensitivity can increase ten-fold or more\textsuperscript{3}. In plants, CDPKs exist as multiple isoforms and this difference in sensitivity to Ca\(^{2+}\) suggests that each CDPK isoform responds to a specific set of calcium signals which differ in frequency of oscillation, magnitude and duration, depending on the stimulus\textsuperscript{3}.

Taken together, the study indicated that activity of LeCPK2 was highly sensitive to Ca\(^{2+}\) and possibly regulated \textit{in vivo} by integrated factors, including Ca\(^{2+}\), Mg\(^{2+}\), and specific substrates rather than only by the fluctuation of Ca\(^{2+}\) concentration.

**Acknowledgements**

This work was supported by Chinese National Non-profit Institute Research Grant of CATAS-ITBB.

**References**