Journal of Plant Physiology **163** (2006) 1083—1093

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JOURNAL OF PLANT PHYSIOLOGY
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# Heterologous expression and biochemical characterization of two calcium-dependent protein kinase isoforms CaCPK1 and CaCPK2 from chickpea

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Received 10 February 2006; accepted 11 April 2006

**KEYWORDS**
Biochemical and kinetic properties;
Calcium-dependent protein kinase isoforms;
Chickpea (*Cicer arietinum*);
cDNA cloning;
Recombinant expression

## Summary

In plants, calcium-dependent protein kinases (CPKs) constitute a unique family of enzymes consisting of a protein kinase catalytic domain fused to carboxy-terminal autoregulatory and calmodulin-like domains. We isolated two cDNAs encoding calcium-dependent protein kinase isoforms (CaCPK1 and CaCPK2) from chickpea. Both isoforms were expressed as fusion proteins in *Escherichia coli*. Biochemical analyses have identified CaCPK1 and CaCPK2 as $Ca^{2+}$-dependent protein kinases since both enzymes phosphorylated themselves and histone III-S as substrate only in the presence of $Ca^{2+}$. The kinase activity of the recombinant enzymes was calmodulin independent and sensitive to CaM antagonists W7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide] and calmidazoilum. Phosphoamino acid analysis revealed that the isoforms transferred the $\gamma$-phosphate of ATP only to serine residues of histone III-S and their autophosphorylation occurred on serine and threonine residues. These two isoforms showed considerable variations with respect to their biochemical and kinetic properties including $Ca^{2+}$ sensitivities. The recombinant CaCPK1 has a pH and temperature optimum of pH 6.8–8.6 and 35–42 °C, respectively, whereas CaCPK2 has a pH and temperature optimum of pH 7.2–9 and 35–42 °C, respectively. Taken together, our results suggest that CaCPK1 and CaCPK2 are functional serine/threonine kinases and may play different roles in $Ca^{2+}$-mediated signaling in chickpea plants.


*Abbreviations:* CaM, calmodulin; CCaMK, calcium and calmodulin-dependent protein kinase; CaCPK, *Cicer arietinum* CPK; CPK or CDPK, calcium-dependent protein kinase; CRK, CPK-related kinase; IPTG, isopropyl-$\beta$-D-thiogalactoside; Ni-NTA, $Ni^{2+}$-nitrilotriacetate; OsCPK, *Oryza sativa* CPK; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; W5, *N*-(6-aminohexyl)-1-naphthalene sulphonamide; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide

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doi:10.1016/j.jplph.2006.04.005

## Introduction

Protein kinases regulate diverse cellular activities by post-translational phosphorylation of enzymatic and non-enzymatic target proteins (Cohen, 1985; Hunter, 1987). Phosphorylation may facilitate an increase or decrease in enzymatic activity or it may alter the interaction of the target proteins with other regulatory proteins. Several physiological processes, such as cellular metabolism, cell division, growth and differentiation, are regulated by reversible phosphorylation of numerous cellular proteins in response to an array of diverse external stimuli. Four major classes of $Ca^{2+}$-regulated protein kinases have been characterized in plants: calcium-dependent protein kinases (CPKs), CPK-related kinases (CRKs), calmodulin-dependent protein kinases (CaMKs) and calcium and calmodulin-dependent protein kinases (CCaMKs). Of these four classes, the predominant forms of $Ca^{2+}$-regulated protein kinases in plants are CPKs, which are identified as Ser/Thr protein kinase family. They have been implicated as key elements in signaling processes. The CPKs have acquired CaM independence due to the presence of internal high affinity $Ca^{2+}$-binding sites (Roberts and Harmon, 1992). They have a variable N-terminal domain, a catalytic domain, an autoinhibitory region, and a calmodulin – like domain (Harper et al., 1991; Suen and Choi, 1991; Huang et al., 1996; Cheng et al., 2002; Hrabak et al., 2003). From an evolutionary standpoint, it has been suggested that the genes encoding CPKs have evolved by the fusion of a gene encoding the catalytic/autoinhibitory domain of a $Ca^{2+}$/CaM-dependent protein kinase and a gene encoding a CaM-like protein (Harmon et al., 2000; Hrabak, 2000).

Many CPKs have been cloned and characterized from a wide variety of plant species including soybean (Harper et al., 1991), *Arabidopsis* (Hrabak et al., 2003), maize (Estruch et al., 1994; Wang et al., 2001), rice (Kawasaki et al., 1993; Breviario et al., 1995), mungbean (Botella et al., 1996), potato (Raíces et al., 2001), strawberry (Llop-Tous et al., 2002), cucumber (Kumar et al., 2004), zucchini (Ellard-Ivey et al., 1999) and tobacco (Yoon et al., 1999; Zhang et al., 2005). There has been considerable progress in plant CPK studies form identifying the new isoforms and cloning corresponding genes to clarify their specific roles in signal transduction cascades involving plant growth and development, and various stress responses. In *Arabidopsis* (Cheng et al., 2002; Hrabak et al., 2003) and in rice (Asano et al., 2005) it has been found that there are 34 and 29 CPK isoforms, respectively. Different CPK isoforms in a given plant species may be distinct in their expression patterns and physiological functions.

Since differences in biochemical properties, $Ca^{2+}$ sensitivity and substrate specificity could contribute specific roles for the CPK isoforms, we undertook characterization of these two recombinant chickpea CPK isoforms. The overexpressed fusion CaCPK1 and CaCPK2 proteins have permitted the study of their biochemical and kinetic properties. We characterized in detail the $Ca^{2+}$-binding and kinetic properties of the recombinant proteins under physiological conditions. We show that substrate phosphorylation and autophosphorylation of the recombinant CaCPK1 and CaCPK2 are $Ca^{2+}$ dependent and the two isoforms differ in kinetic and $Ca^{2+}$-binding properties. These observations suggest that these two isoforms may play different roles in the transduction of calcium signaling.

## Materials and methods

### Isolation of CaCPK1 and CaCPK2 cDNAs

A cDNA library of chickpea (*Cicer arietinum* L. cv. *Kabuli*) stem constructed in a Uni-ZAP XR vector was a gift from Tom W. Okita (Washington State University). For library screening, a rice CPK cDNA, OsCPK2 (Breviario et al., 1995) was randomly labeled with [$\alpha$-$^{32}$P] dCTP using random primer labeling kit (Fermentas, Germany) and used as a probe to screen membrane lifts of $2 \times 10^5$ plaques grown in *E. coli* XL-1 Blue MRF cells. Hybridization and washing were performed as described previously (Kumar et al., 2004). Of the five plaques affording positive signals, two of them were purified through two additional cycles of hybridization. The purified $\lambda$ZAP II clones were in vivo excised as pBluescript SK (−) phagemids and transformed into *E. coli* SOLR cells (Stratagene). The plasmids were sequenced and the two cDNAs were compared at both the nucleic acid and amino acid levels by using PILEUP and GAP programs (Devereux et al., 1984) and found to be partial lacking 5′ ends. To obtain 5′ terminal sequence of the two truncated clones, 5′ RACE was performed by using the FirstChoice™ RLM-RACE kit (Ambion, USA) according to the manufactures instructions with sequence specific reverse primers. The 5′ RACE products were cloned into pTZ57R TA cloning vector (Fermentas, Germany) and sequenced.

### Heterologous expression and purification of recombinant chickpea CaCPK1 and CaCPK2

The coding regions of CaCPK1 and CaCPK2 were RT-PCR amplified using total RNA isolated from