

Molecular Cloning and Characterization of a Novel Calcium-dependent Protein Kinase Gene *liCPK2* Responsive to Polyploidy from Tetraploid *Isatis indigotica*

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Received 29 March 2006, Accepted 7 June 2006

A novel calcium-dependent protein kinase gene (designated as *liCPK2*) was cloned from tetraploid *Isatis indigotica*. The full-length cDNA of *liCPK2* was 2585 bp long with an open reading frame (ORF) of 1878 bp encoding a polypeptide of 625 amino acid residues. The predicted *liCPK2* polypeptide included three domains: a kinase domain, a junction domain (or autoinhibitory region), and a C-terminal calmodulin-like domain (or calcium-binding domain), which presented a typical structure of plant CDPKs. Further analysis of *liCPK2* genomic DNA revealed that it contained 7 exons, 6 introns and the length of most exons was highly conserved. Semi-quantitative RT-PCR revealed that the expression of *liCPK2* in root, stem and leaf were much higher in tetraploid sample than that in diploid progenitor. Further expression analysis revealed that gibberellin (GA₃), NaCl and cold treatments could up-regulate the *liCPK2* transcription. All our findings suggest that *liCPK2* might participate in the cold, high salinity and GA₃ responsive pathways.

Keywords: Defense/stress, *Isatis indigotica*, *liCPK2*, Polyploidy, RACE

Introduction

Calcium is a ubiquitous signal molecule and changes in cytosolic Ca²⁺ concentration are involved in plant responses to various stimuli, including light, environmental stresses, pathogen attack, and hormones (Poovaiah and Reddy, 1993; Bush, 1995). Calcium acts through various intracellular mediators, including calmodulin (CaM) and Ca²⁺-regulated kinases (Sopory and Munshi, 1998). Plants contain a novel group of kinases, designated as Ca²⁺-dependent protein kinases (CDPKs; EC 2.7.1.37), which are found only in plants, green algae, and protozoa (Hrabak, 2003). So far, CDPKs have been shown to share a similar primary structure consisting of three domains: a kinase domain, a junction domain (or autoinhibitory region), and a C-terminal calmodulin-like domain (or calcium-binding domain). The kinase domain contains all 11 of the highly conserved subdomains of typical eukaryotic Ser/Thr protein kinases (Hanks *et al.*, 1988). The autoinhibitory region contains a pseudo-substrate site that, in the absence of Ca²⁺, binds to the catalytic center, resulting in an inactive state of the kinase. However, the binding of Ca²⁺ to the calmodulin-like domain can induce a conformational shift for the release the pseudo-substrate domain from the active site and kinase activation (Harper *et al.*, 1991; Harmon *et al.*, 1994; Harmon *et al.*, 2000). Besides these three conservative domains, all CDPKs have an N-terminal variable region, which often contains N-terminal acylation (including myristoylation and palmitoylation) sites for the subcellular localizations of CDPKs (Martin and Busconi, 2000; Rutschmann *et al.*, 2002; Dammann *et al.*, 2003).

The first isolation of a cDNA encoding a CDPK was from soybean (Harper *et al.*, 1991). Since then, genes encoding CDPKs from a diverse spectrum of plants including *Arabidopsis*, maize, rice, and mung bean have been cloned and, in all cases, they are encoded by a multigene family (Kawasaki *et al.*, 1993; Estruch *et al.*, 1994; Urao *et al.*, 1994; Breviario *et al.*, 1995; Botella *et al.*, 1996; Hong *et al.*, 1996;

Database Accession No: DQ458915

Abbreviations: CPK: calcium-dependent protein kinase, CTAB: Cetyl trimethyl ammonium bromide, ORF: open reading frame, PCR: Polymerase chain reaction, RACE: rapid amplification of cDNA ends, RT-PCR: reverse transcriptase-polymerase chain reaction.

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Hrabak *et al.*, 1996). This large number of genes suggests that individual isoforms have different functions and participate in multiple, distinct signaling pathways (Romeis *et al.*, 2001). Increasing evidence shows that CDPKs are involved in environmental stress and hormone signaling. Drought, salt, cold, light, and hormones can influence the expression or activity of various CDPKs in plants (Sopory and Munshi, 1998). Increased CDPK activity and Ca^{2+} -dependent protein phosphorylation were observed in rice seedlings exposed to cold (Li and Komatsu, 2000; Komatsu *et al.*, 2001) and hormone treatment (Kawasaki *et al.*, 1993; Karibe and Komatsu, 1997; Yang and Komatsu, 2000).

Polyploidy is recognized as a common phenomenon in the evolution of plants. It is estimated that 50 to 80% of angiosperms are polyploids, including many of our most important crop plants with the hallmarks of autopolyploidy (e.g. alfalfa and potato) or allopolyploidy (e.g. wheat, oat, cotton, coffee and canola) (Wendel, 2000). Polyploids often show novel phenotypes that are not present in their diploid progenitors or exceed the range of the contributing species (Levin, 1983; Ramsey and Schemske, 2002). Some of these traits, such as increased drought tolerance, apomixis (asexual seed production), pest resistance, flowering time, organ size and biomass, could allow polyploids to enter new niches or enhance their chances of being selected for use in agriculture. Many insights have emerged from recent explorations using laboratory generated or synthetic polyploids (Madlung *et al.*, 2002; Han *et al.*, 2003; Liu and Wendel, 2003; Pires *et al.*, 2004; Adams and Wendel, 2005). Analysis of the most recent genome duplication event in Arabidopsis revealed that some classes of genes, such as those involved in transcription and signal transduction, had been preferentially retained and their transcription profiles were variant. (Blanc and Wolfe, 2004).

Isatis indigotica Fort., the same family Cruciferae with *Arabidopsis thaliana*, is a prevalent Chinese medicinal herb. After selection for five years, tetraploid *I. indigotica* ($2n = 28$) with better yield and enhanced resistance had been obtained, compared with diploid progenitor ($2n = 14$) (Qiao *et al.*, 1989). Whether the polyploidisation has effect on genes involved in signal transduction in *I. indigotica* is unknown. In the present study, a CDPK gene from tetraploid *I. indigotica* (*IiCPK2*) was cloned and characterized. The expression profiling analyses of *IiCPK2* under stress conditions and phytohormone treatment suggest that *IiCPK* may be involved in environmental stress and hormone signaling. Higher expression level and changed transcriptional profile in tetraploid *I. indigotica* compared with diploid progenitor suggests that *IiCPK* probably participates in polyploidy evolution of *I. indigotica*.

Materials and Methods

Plant materials. Seeds of tetraploid and diploid *I. indigotica* were kindly provided by professor C.Z. Qiao of School of Pharmacy, Second Military Medical University, Shanghai, China. Seeds were

pretreated with 75% alcohol for 1 min, washed 3 times with distilled water, followed by the treatment of 0.1% HgCl_2 for 5 min and by 4 rinses with sterile distilled water. The sterilized seeds were then incubated between several layers of sterilized wet filter paper under 30°C for germination. Germinated seeds were sowed and cultured in small plastic flowerpots naturally. The seedlings were all grown at 25°C under 14 h light/10 h dark photoperiod cycles for two months until various treatments and RNA, DNA isolation.

Various treatments. The leaves from two-month-old tetraploid *I. indigotica* seedlings were sprayed with solution of 100 μM GA_3 , 100 μM Absciscic Acid (ABA) and 250 mM NaCl respectively followed by RNA isolation. Another set of control plants were similarly treated with distilled water. For cold treatment, the seedlings were first grown at 28°C for 4 d, transferred from 28°C to 4°C and grown for 8 h, 16 h and 32 h respectively, and then returned to 28°C for 32 h.

RNA and DNA isolation. Total RNAs of different tissues including root, stem, leaf from two-month-old tetraploid and diploid *I. indigotica* were extracted using TRIzol Reagent (GIBCO BRL) according to the manufacturer's instruction (Jaakola *et al.*, 2001) as well as total RNAs of tetraploid plant with various treatments. The genomic DNAs of tetraploid and diploid plant were isolated using a CTAB-based method (Reichards, 1995). The quality and concentration of RNA and DNA samples were examined by EB-stained agarose gel electrophoresis and spectrophotometer analysis.

Molecular cloning of the *IiCPK2* Full-length cDNA. Molecular cloning of *IiCPK2* from tetraploid *I. indigotica* was carried out by Rapid amplification of cDNA ends (RACE) method using a SMART™ RACE cDNA Amplification Kit (Clontech).

For 3'RACE of *IiCPK2*, about 100 ng of total RNA was reverse transcribed with 3'-CDS primer (5'-AAGCAGTGGTATCAACGCA GAGTAC(T)₃₀VN-3', N = A, C, G, or T; V = A, G, or C; Clontech USA) by BD PowerScript Reverse Transcriptase (Clontech). Universal Primer A Mix (UPM, 5'-CTAATACGACTCACTATAGGGCAAG CAGTGGTATCAACGCAGAGT-3', Clontech), Nested Universal Primer A (NUP, 5'-AAGCAGTGGTATCAACGCAGAGT-3', Clontech), gene-specific primers CPK3'GSP1 (5'-CCATTGTTGG GGTGTAGAGGCTTGC-3', as 3' RACE first amplification primer) and CPK3'GSP2 (5'-GCATCGAGACCTCAAGCCCCGAG AATT-3', as 3' RACE nest amplification primer) were used. The PCR was conducted in accordance with the protocol provided by the manufacture (Clontech). The nested amplified PCR product was purified and cloned into PMD18-T vector (TaKaRa) and then sequenced.

For 5' RACE of *IiCPK2*, about 100 ng of total RNA was reverse transcribed with 5'-CDS primer (5'-(T)₂₅VN-3', N = A, C, G, or T; V = A, G, or C; Clontech USA) and SMART™ II A Oligonucleotide (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3', Clontech USA). Universal Primer A Mix (UPM), Nested Universal Primer A (NUP) (Clontech), gene-specific primers CPK5'GSP1 (5'-TCTGG AGCCACGCCGTCGATTGTAC-3', as 5' RACE first amplification primer) and CPK5'GSP2 (5'-GCTACATAATACGGGCTACCAAC AAC-3', as 5' RACE nest amplification primer) were used. The PCR was conducted in accordance with the protocol provided by

the manufacture (Clontech). The nested amplified PCR product was purified and cloned into PMD18-T vector (TaKaRa) and then sequenced. By aligning and assembling the products of 3' and 5' RACE, the full-length *IiCPK2* from tetraploid *I. indigotica* was deduced and subsequently amplified by proof-reading PCR amplification with primers CPKF-S (5'-ATTAGACTGTTTGTGAGC GTTTTGTGA-3') and CPKF-AS (5'-ACTCTTGCTTTGGCTTTA GTTTTGTGA-3'). The PCR procedure was conducted under the following conditions: 5 min at 94°C, 5 cycles (30 s at 94°C, 30 s at 70°C, 3 min at 72°C), 30 cycles (30 s at 94°C, 30 s at 58°C, 3 min at 72°C) and 10 min at 72°C. The amplified PCR product was purified and cloned into PMD18-T vector (TaKaRa) and then sequenced. To examine whether the sequence of *IiCPK2* in tetraploid plant was the same as that in diploid plant, the full-length *IiCPK2* from diploid plant was amplified by RT-PCR (One-step RT-PCR Kit TaKaRa) using primers CPKF-S and CPKF-AS mentioned above. Aliquot of 2 µg total RNA (1 µg/µl) of diploid plant was reversely transcribed at 50°C for 30 min and denatured at 94°C for 2 min, followed by 35 cycles of amplification (94°C for 35 s, 56°C for 35 s, 72°C for 3 min). The amplified PCR product was purified and cloned into PMD18-T vector (TaKaRa) and then sequenced.

Amplification of genomic sequence corresponding to the *IiCPK2* Full-length cDNA. In order to detect whether there exist introns within the *IiCPK2*, PCR amplification was carried out using the same reaction system as for the cloning of the full-length cDNA except that the template was substituted by 1.5 µg of total genomic DNA of tetraploid plant and the extension time at 72°C in the amplification cycles was prolonged up to 3.5 min.

Sequence analyses of *IiCPK2*. Sequence alignments, ORF translation and molecular mass calculation of the predicted protein were carried out on Vector NTI Suite 8. Genbank BLASTs were carried out on NCBI (<http://www.ncbi.nlm.nih.gov/>), while bioinformatic analysis of the predicted *IiCPK2* protein was carried out on website of ExPASy Molecular Biology Server (<http://www.expasy.org>). Homology-based structural modeling was performed by Swiss-Model, and WebLab Viewer Lite 4.0 was used to display 3-D structures.

Expression profile of *IiCPK2* in different tissues and stress, phytohormone assays. Semi-quantitative RT-PCR was used to investigate the expression profiling of *IiCPK2* in different tissues of tetraploid and diploid *I. indigotica* and under various stresses and phytohormone treatments. A mock treatment was carried out with H₂O as a control to exclude any other factors inducing *IiCPK2* gene expression that might result from the spraying. Total RNA was extracted separately from different tissues (leaves, roots, and stems) and under different treatments. After establishing agreement between the OD values of the RNAs, aliquot of 1 µg total RNA (1 µg/µl) was used as the template in one-step RT-PCR analysis using One-step RT-PCR Kit (TaKaRa) with CPK-RT-S (5'-GTTCC TCCATTCTGGGCTGAATCCGA-3') and CPK-RT-AS (5'-TCTG ACCGCTTTTGTCTGGCATCTATC-3') as primers. The template was reversely transcribed at 50°C for 30 min and denatured at 94°C for 2 min, followed by 35 cycles of amplification (94°C for 35 s, 50°C for 35 s, 72°C for 2 min). The RT-PCR reaction for the house-keeping gene (18S gene) using specific primers 18SF (5'-

ATGATAACTCGACGGATCGC-3') and 18SR (5'-CTTGGATGT GGTAGCCGTTT-3') was performed as described above as the control except for 20 cycles. The densities of the target bands were measured with a WEALTEC Dolphin-DOC ultraviolet analyzer (WEALTEC). Each sample was assayed in triplicate.

Results and Discussion

Molecular cloning of the *IiCPK2* full-length cDNA. Using the RACE method, cDNA ends of 1233 bp and 1496 bp were amplified by 3' RACE and 5' RACE respectively. 3' and 5' ends were assembled with Vector NTI Suite 8.0 and the deduced full-length *IiCPK2* cDNA was subsequently amplified by proof-reading PCR amplification with primers mentioned above. The full-length cDNA of *IiCPK2* was 2585 bp, consisting of a 495-bp 5' untranslated region, a 212-bp 3' untranslated region, and a 1878-bp ORF encoding a 625-amino-acid protein (Fig. 1). The sequence of *IiCPK2* in tetraploid plant was the same as that in diploid plant.

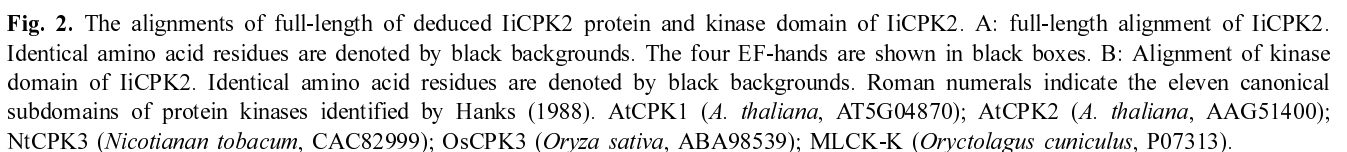
Characterization of *IiCPK2* protein. The predicted *IiCPK2* protein had a calculated molecular mass of 69.84 kDa and a theoretical pI at 5.43. Protein-protein BLAST showed that on the amino acid level *IiCPK2* protein shared high homology with CDPKs from other plant species. As shown in Fig. 1, the predicted *IiCPK2* polypeptide included a kinase domain, a junction domain, and a C-terminal calcium-binding domain, which presented a typical structure of plant CDPKs. Besides these three conservative domains, *IiCPK2* had an N-terminal variable region. By using Plant-Specific Myristoylation Predictor in PlantsP database (Gribskov *et al.*, 2001) (<http://plantsp.genomics.purdue.edu/plantsp/html/myrist.html>), putative N-myristoylation site was found in *IiCPK2* as shown in Fig. 1, which might be required for the subcellular localization of *IiCPK2*.

Through Clustal W/X, full-length Alignment result showed that *IiCPK2* protein shared 90% identity to AtCPK1 from *Arabidopsis*, 80% identity to AtCPK2 from *Arabidopsis*, 73% identity to NtCPK3 from tobacco and 67% identity to OsCPK3 from rice respectively (Fig. 2A). In C-terminal calcium-binding domain, four conserved Ca²⁺-binding EF-hands were identified by ScanProsite (<http://au.expasy.org/tools/scanprosite/>) (Fig. 2A). As shown in Figure 2B, the kinase domain of *IiCPK2* contained 11-signature protein kinase subdomains characteristic of the serine/threonine protein kinase family (Hanks *et al.*, 1988).

Secondary structure analysis of *IiCPK2* protein was performed by SOMPA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). The result showed that *IiCPK2* consisted of 258 α -helices, 45 β -turns joined by 80 extended strands and 242 random coils (Fig. 3). The secondary structure of *IiCPK2* had high similarity in kinase domain and Ca²⁺-binding domain, while the N-terminal variable region was not (Fig. 3). Subsequently, molecular homologous modeling of kinase domain and Ca²⁺-binding domain of

1 ggggggtagtgotattaaactatctcttcttatttttctcctca
 46 ttttcccccacctaattcatcttcccatctttttctactctccagatctctgtttttttttttgtttccttgccotgggggtga
 136 tttctactctctgggttggttattgcatctctctctttttgggttgccttcacttcacaaattttccagtaaccttttgataattctg
 226 ctccagataacctctgtctgttttcccttcaattttttgtctgcctatatacccaagcagggtttatcagctgtctgtgtacaaa
 316 ttgaaagccaattatgggttgatccttgaacttcgaggatttgatgttgcgatgtttagcatagaaggatttgactttcatggcatt
 406 aggaatgagattagactgttttgagcgttttgatcagatcttagatgctattttotggaaagagattaagacacggtttagggtattgg
 496 **atgggtaatactt**gtgttggaaccaagcagaatgggttcttgcacatccgtttcggctgcaatgtggcggcctcgggatgcagatgattcg
 M G N T C V G P S R N G F L Q S V S A A M W R P R D A D D S
 586 gtttctcagagcaatggagatactgcaagtgaagctgtttcaggagagcttcgatctccatcatctgatcaggtccagaataaacccct
 V S Q S N G D T A S E A V S G E L R S P S S D Q V Q N K P P
 676 gagcagctcacaatgccaaagccagggaactaacatcgagataaaggcgaaatccgatgtagagattoagcctgaaatcaagctggaacg
 E Q L T M P K P G T N I E I K A K S D V E I Q P E I K L E T
 766 caagaggtgacaaaacccggagtcacaagccggagacaaagtcagagactaagcctgagacaaagtcggagactaagccggagacaaagtc
 Q E V T K P E S K P E T K S E T K P E T K S E T K P E T K S
 856 gagactaagccggagacaaaagctgatactccggcctaagcctaagaaacctaaacacatgaagagagtgctcagtgaggtcttaggact
 E T K P E T K A D T P A K P K K P K H M K R V S A G L R T
 946 gactgtgtgtgcagaggaactgaaaactttaaggattctattccttgggaaggaaacttggaacaggcgaatttgggaacgactttt
 E S V L Q R K T E N F K E F Y S L G R K L G Q Q G Q F G T T F
 1036 ctatgcgtgagaagacttcagggaaggagtttgccgtcgaactctattgctaagaggaaactgttgaccgatgaggacgtggaagatgtg
 L C V E K T S G K E F A C K S I A K R K L L T D E D V E D V
 1126 agaagggaattcagattatgcacacttggctggcaccgaatgcatatccattaaaggagcttatgaggatgtttotggcgggtgac
 R R E I Q I M H H L A G H P N V I S I K G A Y E D V L A V H
 1216 ottgttatggagtggtgacaggtggcgagctttttgacagaataattcaacgggtcactacacagagaggaaagctgtgagctcact
 L V M E C C A G G E L F D R I I Q R G H Y T E R K A A E L T
 1306 agaaccatagtcgggttgtagagctgtgctattctcttgggttatgcatcgagaccccaagcccgagaattttctgtttgctagtaaa
 R T I V G V V E A C H S L G V M H R D L K P E N F L F V S K
 1396 caccgaagattcactcttgaagacgattgattttggactctctatgttctttaagccagatgatgttttacagatgttgttgtagcccg
 H E D S L L K T I D F G L S M F F K P D D V F T D V V G S P
 1486 tattatgtagctccggaagttcttgcagaacattatggctcgaagctgatgtctggagtgctggagtgattgtatacattttgttaagt
 Y Y V A P E V L R K H Y G P E A D V W S A G V I V Y I L L S
 1576 ggagttctccattctgggtggaatccgaacaggtattttgcagaagctccatggtgatcttgacttttccatccgatccctggcgg
 G V P P F W A E S E Q G I F E Q V L H G D L D F S S D P W F
 1666 agcatatctgaaagtgcacaaagacttagtgaggaaaatgcttggctgggacccaagagaagactaactgcacacaaagattattgtcat
 S I S E S A K D L V R K M L V R D P K R R L T A H Q V L C H
 1756 ccattgggtacaaatgcacggcgtggctccagacaaacctatagattctgtgttctgagccgatgaagcaattttctgcaatgaacaaag
 P W V Q I D G V A P D K P L D S A V L S R M K Q F S A M N K
 1846 ttcaagaaatggctccttagagctcattgtctgagagcttatctgaagaggaaatagccggcttgaaagaaatgtttatgatagatgcc
 F K K M A L R V I A E S L S E E E I A G L K E M F N M I D A
 1936 gacaaaagggtcagataactttcgaagaactgaagcaggactaaaacgagtagggcgcaatctcaagagtcagagattctcgacttg
 D K S G Q I T F E E L K A G L K R V G A N L K E S E I L D L
 2026 atgcaagctgctgatgtggacaacagtggaacaatagactacaaagagttcatagcagcaacattgcatttaacaagatagagagagaa
 M Q A A D V D N S G T I D Y K E F I A A T L H L N K I E R E
 2116 gaccatttgtttgcagcctttacatactttgacaaagatgggagcggttatatcaacccggacagagcttcaacaagcttgtgaggagttt
 D H L F A A F T Y F D K D G S G Y I T P D E L Q Q A C E E F
 2206 ggagttgaggatgtccgcatagaagaatgatgctgtgatgttgatcaagacaatgacggggaatagattataacagagtttggcgatg
 G V E D V R I E E M M R D V D Q D N D G R I D Y N E F V A M
 2296 atgcagaaggagcatcacaggagttccagtgagatgggactagagaaaagctttagcattgtctttaaactc *tagt*ttcaattcttt
 M Q K G S I T G V P V K M G L E K S F S I A L K L *
 2386 taagtacaaaactaaagccaaagacaagagttcctctgatgatataatgattgtgaagacaaatagcacagccaagattctcttttcg
 2476 attctcaattctttgaatgagttttgtctgttttgaaacttggtttgttgcactgttagttgttgagcatataacaaggactttgt
 2566 caaaaaaaaaaaaaaaaa

Fig. 1. The full-length cDNA sequence and the deduced amino acid sequence of *liCPK2*. The start codon (atg) is in bold and the stop codon (tag) is in italics. The putative N-myristoylation site (A₁-A₂₄) is boxed with white background, the kinase domain (A₁₆₅-A₄₂₃) is shown in the grey background, the junction domain (A₄₂₄-A₄₆₅) is underlined. The calcium-binding domain (A₄₆₆-A₆₀₇) is boxed with grey background.



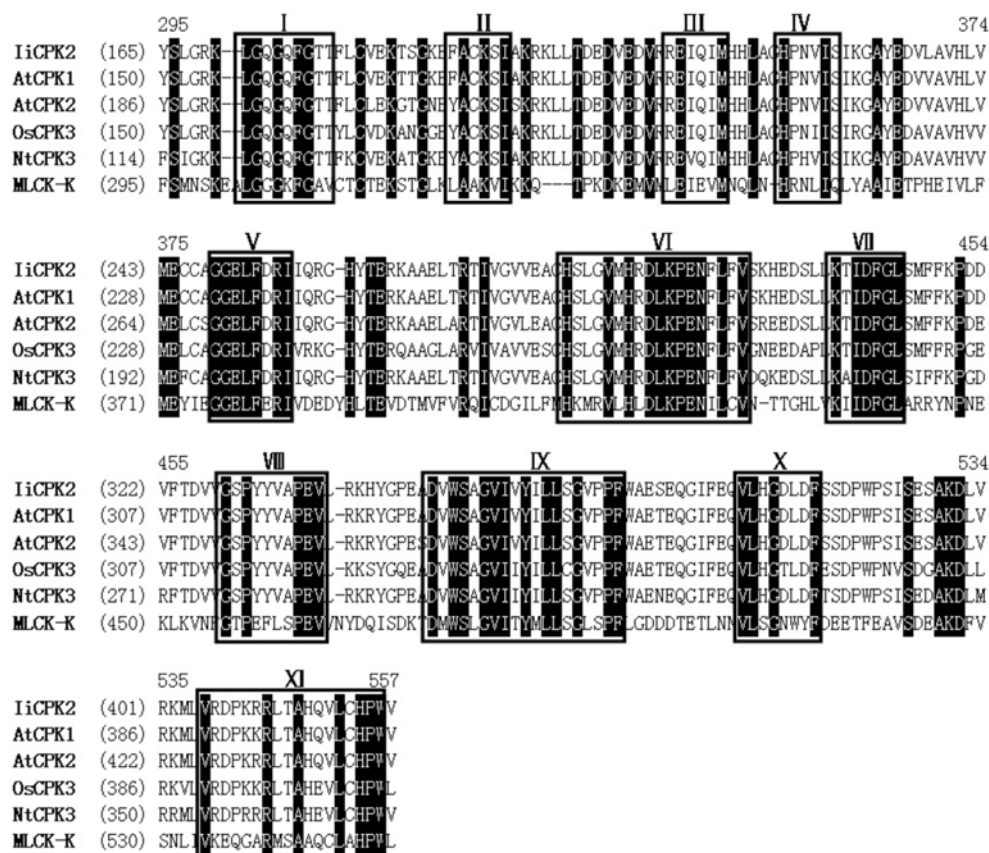


Fig. 2. Continued.

IiCPK2 was performed by Swiss Model Server (<http://swissmodel.expasy.org/>) respectively. The result indicated that folding mode and spatial configuration of two domains of IiCPK2 shared high similarity with AtCPK1 from Arabidopsis (Fig. 4). Polypeptide of four EF-hands formed four loop structures in different directions which might be favourable for binding calcium (Fig. 4).

Genomic DNA analysis of *IiCPK2* gene. Genomic Sequence Corresponding to the *IiCPK2* Full-length cDNA was 3638 bp (GenBank Accession No: DQ458916). The alignment result of *IiCPK2* cDNA sequence and the genomic sequence indicated that the *IiCPK2* gene contained 7 exons and 6 introns (Fig. 5). The intron-exon junctions obeyed the GT/AG rule (Shapiro and Senapathy, 1987) (Fig. 5). Comparison of

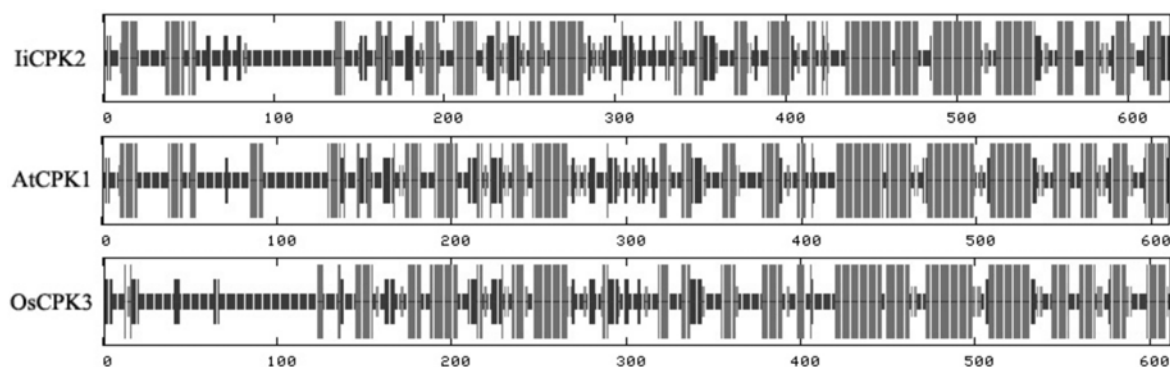


Fig. 3. The secondary structure of the deduced IiCPK2 protein. AtCPK1 (the highest identity); OsCPK3 (the lowest identity). Alpha helix, extended strand, beta turn and random coil are denoted by the longest, the second longest, the second shortest and the shortest bars respectively.

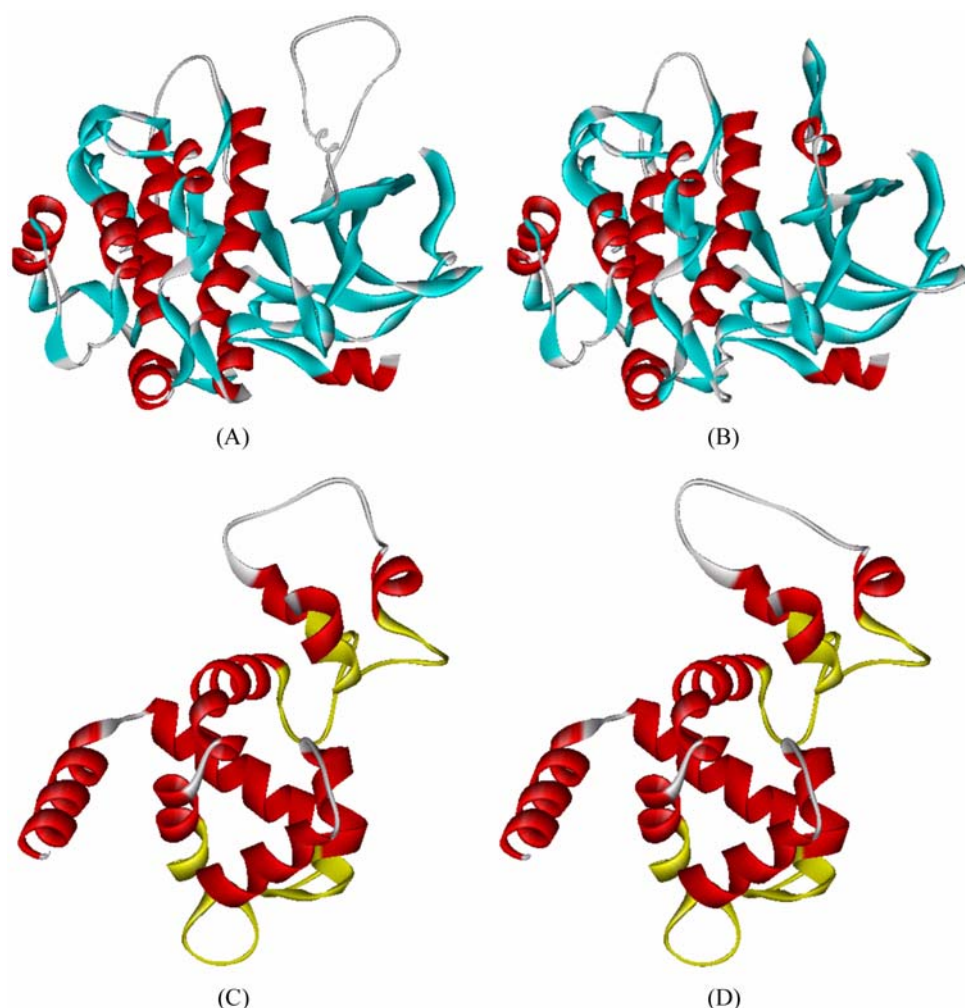


Fig. 4. Three-dimensional structures of the deduced *IiCPK2* kinase domain and calcium-binding domain. A: kinase domain of *IiCPK2*, B: kinase domain of *AtCPK1*, C: calcium-binding domain of *IiCPK2*, D: calcium-binding domain of *AtCPK1*. α -helices are indicated in red and β -sheets are indicated in blue. Turns and loops are indicated by gray lines. Four EF-hands in C and D are labeled by yellow.

the genomic sequence with the cDNA revealed that the 5' portion of exon1 encoded the 5'-untranslated region (UTR); the protein-coding region extended from 3' portion of exon 1 through exon 7; exon7 encoded part of the *IiCPK2* protein and the entire 3'-UTR. Further sequence analysis revealed that the length of exons of *IiCPK2* was high conserved compared with genomic sequence from *Arabidopsis* (*AtCPK1* and *AtCPK2*) and Rice (*OsCPK3*) except for exon 1 (Fig. 5), which differed somewhat in sequence and length. The probable reason was that this part of the protein contained N-terminal variable region and had a slightly different function in *I. indigotica*.

Effects of applied stress and phytohormones on *IiCPK2* transcription. CDPKs were suggested to be associated with various stress tolerance and hormones in different plant species (Sopory and Munshi, 1998). To clarify the possible role of *IiCPK2* in tetraploid *I. indigotica*, the expression of the

IiCPK2 in response to NaCl, low-temperature, exogenous ABA and GA₃ was analyzed by semi-quantitative RT-PCR. When treated with 250 mM NaCl and 100 μ M GA₃ by spraying, the expression of *IiCPK2* was induced and reached the highest 16 h after the treatment, and then decreased gradually thereafter (Fig. 6). Under cold treatment, the expression of *IiCPK2* was up-regulated and reached the highest 16 h after the treatment. However, ABA treatment had no significant effect on the expression of *IiCPK2* (Fig. 6).

The results verified that the expression of *IiCPK2* might be relevant to the stress tolerance including low-temperature and salt tolerance. The relationship between low-temperature, salt and GA₃ regulated gene expression was not understood, but accumulating evidence suggested that independent signal-transduction pathways may operate in response to different environmental signals (Nordin *et al.*, 1991; Nordin *et al.*, 1993; Yamaguchi-Shinozaki and Shinozaki, 1994; Zhu, 2002; Kim *et al.*, 2003). Previous evidence suggested that CDPKs

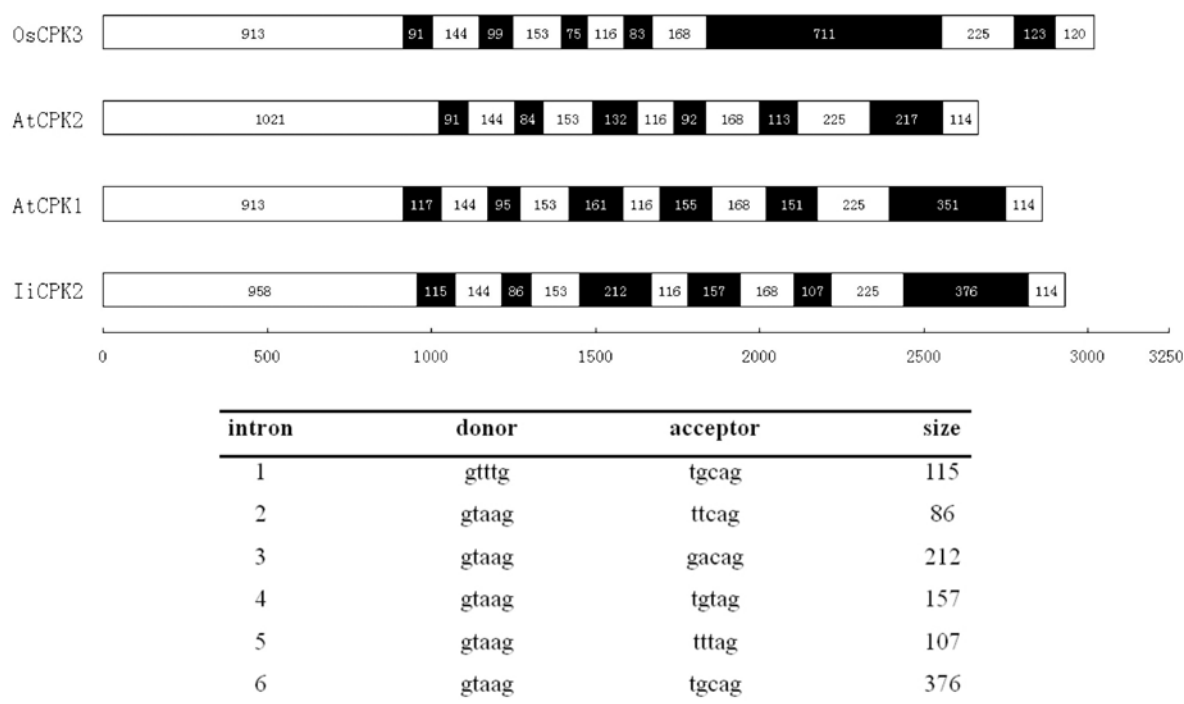


Fig. 5. Genomic organization of the *LiCPK2* and other CDPK genes (corresponding to coding region). In this schematic diagram, the exons are represented as boxes without background and the introns filled with grey background.

may be involved in certain plant hormone responses. For example, the activity of a rice seed membrane CDPK was increased by GA₃ (Abo-El-Saad and Wu, 1995). Furthermore, a GA₃-induced increase in Ca²⁺ influx had been observed in barley aleurone protoplasts (Gilroy and Jones, 1992), providing a possible mechanism to link the GA₃ response with activation of CDPKs. In this study, *LiCDPK2* was up-regulated in response to GA₃ treatment (Fig. 6). The results suggest that *LiCDPK2* might act in a GA₃-mediated signaling.

Dehydration stresses including drought, low-temperature and high salinity adversely affect the growth of plants and the productivity of crops (Rabbani *et al.*, 2003; Shen *et al.*, 2004). Both ABA-independent and ABA-dependent signal transduction pathways link dehydration and gene expression (Guerrero *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992; Yamaguchi-Shinozaki and Shinozaki 1993). Low-temperature inducible genes had been shown to be regulated by three separate signal pathways, one of which is ABA-independent (Nordin *et al.*, 1991; Gilmour and Thomashow, 1991). In the present study, low-temperature and high salinity stress up-regulated the expression of *LiCPK2*, while ABA had no significant effect on the expression of *LiCPK2* (Fig. 6). These results suggest that *LiCPK2* gene induced expression should occur through ABA-independent pathway.

An alternative explanation for the involvement of *LiCPK2* in cold stress, high salinity stress and GA signaling was crosstalk between these pathways. Signaling pathways have to be regarded as complex networks (Zhao *et al.*, 2005). These signal networks are characterized by multiple points of

convergence and divergence that enable integration of signaling pathways at different levels and provide the molecular basis for appropriate downstream responses. For example, *Nicotiana benthamiana* plants with reduced levels of NtCDPK show severe abnormalities in cell morphology, spontaneous necrotic lesions, and increased expression of marker genes for the plant defense response (Lee *et al.*, 2003). These results suggest that plant cell development and defense responses could be interconnected at the level of NtCDPK1. Similarly, cold and high salinity stress responsiveness and GA₃ dependent signal transduction may be promoted through distinct signaling pathways that crosstalk at the level of *LiCPK2*.

Divergence of transcription profiles of *LiCPK2* in different tissues between diploid and tetraploid *I. indigotica*. It was suggested that some classes of genes, such as those involved in transcription and signal transduction, had been preferentially retained and their transcription profiles were variant after genome duplication event in Arabidopsis. (Blanc and Wolfe, 2004). To determine whether the expression profile of *LiCPK2* was influenced by genome duplication, total RNA was extracted from different tissues (roots, stems and leaves) of diploid and tetraploid *I. indigotica* and used for semi-quantitative RT-PCR. It was found that the *LiCPK2* was constitutively expressed in all the tested tissues including root, stem and leaf in both tetraploid and diploid plant (Fig. 7). As expected, the expression level of *LiCPK2* in root, stem and leaf from tetraploid *I. indigotica* was much higher than that of diploid samples (Fig. 7). Furthermore, the result was interesting

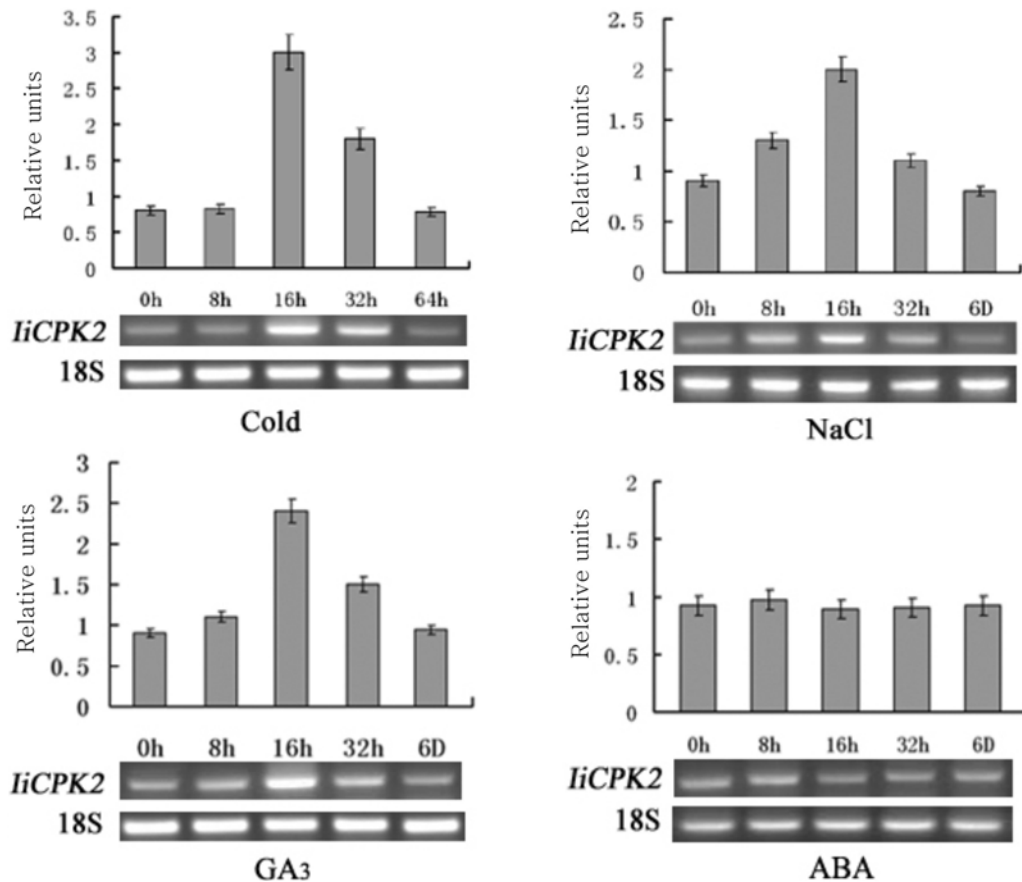


Fig. 6. Effects of applied stress and phytohormones on *IiCPK2* transcript levels. Data represents the mean values \pm SE of three replicates.

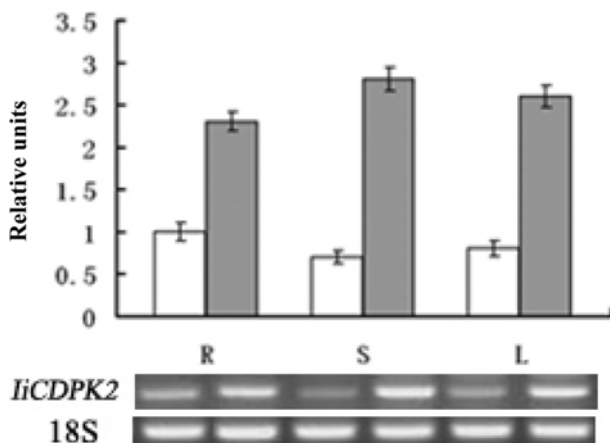


Fig. 7. Expression profiling of *IiCPK2* in different tissues of tetraploid and diploid *I. indigotica*. R: root; S: stem; L: leaf. White columns indicate *IiCPK2* expression of diploid plant; Grey columns indicate *IiCPK2* expression of tetraploid plant. Data represents the mean values \pm SE of three replicates.

that the highest expression of *IiCPK2* in diploid plant was found in root, followed by in leaf, and stem, while the expression of *IiCPK2* in tetraploid plant was almost the same

in these tissues (Fig. 7). These results suggested that transcription profiles of *IiCPK2* might be influenced by genome duplication.

One of the intriguing findings is the level of *IiCPK2* expression in the tetraploid *I. indigotica* is more than twice of that found in the diploid plant, especially in stems. The probable explanation for this phenomenon is altered regulatory networks in tetraploid *I. indigotica* after genome duplication. The expression of most genes is dependent on networks of regulators, such as transcription factors, that are organized into hierarchies (Guo and Birchler, 1994; Birchler *et al.*, 2001). The numbers of regulators in diploid networks is high, but in polyploids they can be expanded several fold. The functioning of regulatory networks in polyploids will depend, in part, on how effectively regulators encoded by the contributing genomes interact. Polyploidy could alter the functioning of these networks by potentially increasing the number of different interacting regulators through an increase in number of alleles (Osborn *et al.*, 2003). Similarly, regulatory network functioning might be severely altered in tetraploid *I. indigotica* derived from diploids. Therefore, the overall effects on the expression of genes in tetraploid *I. indigotica* at the ends of regulatory cascades might be significant.

In conclusion, we have cloned a new plant CDPK gene

from tetraploid *I. indigotica* and named it *IiCPK2*. Although the details of *IiCPK2* function are still unclear, its sequence characterizations and induced expression responsive to NaCl, low-temperature and GA₃ suggest that *IiCPK2* might participate in the cold, high salinity and hormone response pathways. Higher expression and changed transcription profiles of *IiCPK2* in tetraploid plant suggest that polyploidisation might have effect on *IiCPK2* expression, which also might partially result in increased adaptable flexibility to environmental cold and high salinity conditions for tetraploid *I. indigotica* compared with diploid plants. In succession, the cloning of *IiCPK2* enables us to test whether the *IiCPK2* gene could increase the tolerance and recovery from cold and high salinity stress of the whole transgenic plants by transferring it into model plants in the future.

Acknowledgments This research was financially supported by the National Natural Science Foundation of China (30371746).

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