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Full Length Research Paper

Molecular cloning and characterization of *P5CS* gene from *Jatropha curcas* L.

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Δ1-Pyrroline-5-carboxylate synthetase, *(P5CS)* is a bifunctional enzyme (EC 2.7.2.11/1.2.1.41) that catalyses the first two steps of glutamate pathway in proline biosynthesis in plant. The *JcP5CS* was cloned from the leaves of *Jatropha curcas L*. The lengthen of the cDNA of *JcP5CS* was 2675 bp, containing a 2148 bp open reading frame, a 117 bp 5'-untranslated region and a 410 bp 3'-untranslated region. The open reading fragment (ORF) encoded a 715 amino acid polypeptide with the molecular weight of 77.54 kDa and the pI value of 6.11. *JcP5CS* was composed of N-terminal Glutamate-5-kinase (G5K, ProB) and C-terminal glutamic-γ-semialdehyde dehydrogenase (GSA-DH, ProA) domains. The conserved Glu-5-kinase, GSA-DH domains, conserved leucine zipper and the putative ATP and NAD(P)H-binding sites was also found. The *JcP5CS* protein was successfully expressed in *Escherichia coli* and showed high enzymatic activities. The real-time quantitative PCR results showed that the *JcP5CS* was induced by drought and salt stress, but not cold stress.

Key words: Δ1-Pyrroline-5-carboxylate synthetase, proline, *Jatropha curcas L.*, expression, molecular cloning, real-time quantitative polymerase chain reaction (RT-PCR).

INTRODUCTION

Drought and high salinity are the most important environmental factors that cause osmotic stress and impact negatively on plant growth and crop productivity (Boyer, 1982; Chen et al., 2009; Matsui et al., 2008). During osmotic stress, many plants accumulate osmolytes such as proline, glycine, betaine, mannitol and trehalose to maintain a stable intracellular environment (Wang et al., 2007; Zhu, 2002). Proline is one of the most accumulated osmolytes found in plants with salinity and water deficit conditions (Delauney and Verma, 1993; Jime´nez-Bremont et al., 2006; Tripathi et al., 2007; Takano et al., 2009). It has many functions during osmotic stress, osmoprotectant, protein stabilizer, hydroxyl radical scavenger and stabilizer of cell membranes by interacting with phospholipids and carbon and nitrogen reserve after stress relief (Kavi et al., 2005).

In plants, there are two pathways of proline biosynthesis; either from glutamate or ornithine. The glutamate pathway is primarily for proline biosynthesis, especially under osmotic stress conditions (Delauney and Verma, 1993; Hu et al., 1992). There are four steps in the process of proline biosynthesis in plants and the whole process is catalyzed by two enzymes, *P5CS* and *P5CR* (Delauney and Verma, 1990; Willett and Burton, 2002). *P5CS* is a bifunctional enzyme (EC 2.7.2.11/1.2.1.41) that catalyses the first two steps of glutamate pathway in proline biosynthesis in plants. It is a rate-limiting enzyme in the glutamate pathway and exhibits both γ-glutamyl kinase and glutamic-γ-semialdehyde dehydrogenase activities (Delauney and Verma, 1993; Yoshiba et al., 1995). *P5CS* was cloned from many high plants, for example, *Phaseolus vulgaris* (Chen et al., 2009), *Medicago truncatula* (Armengaud et al., 2004),

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Abbreviations: P5CS, Δ 1-Pyrroline-5-carboxylate synthetase; **PCR,** polymerase chain reaction; **ORF,** open reading fragment; **G5K,** glutamate-5-kinase; **GSA-DH,** glutamic-γ-semialdehyde dehydrogenase.

Lycopersicon esculentum (Fujita et al., 1998), *Arabidopsis thaliana* (Strizhov et al., 1997) etc.

Jatropha curcas L. (*Euphorbiaceae*) is a bush/small tree, which thrives in many areas of the tropics and sub-tropics in Africa, Asia and America (Keith, 2000). In recent years, there is growing concern about *J. curcas L.*, because its seed can be used as raw material for biodiesels. In addition to this, it can be used to prevent and/or control erosion, reclaim land and produce feedstuff, soap, cosmetics, pesticides, and anti-cancer and anti-HIV medicines (Dalziel, 1955; Liu et al., 2009; Muanza et al., 1995).

MATERIALS AND METHODS

Plant materials and stress treatments

Roots, stems, leaves, flowers, fruits and seeds of *J. curcas L.* were collected from Panzhihua City, Sichuan Province and quickly frozen in liquid nitrogen and stored at -70°C. Seeds of *J. curcas L.* were germinated in plates filled with soil and irrigated daily with a half-strength Hoagland solution. *J. curcas L.* grew under 12 h light/12 h dark condition at 25°C. 25 days later, the uniform seedlings were transplanted into watertight culture containers filled with half-strength Hoagland solution. After 5 days, some plants were subjected to drought by adding PEG to the half-strength Hoagland solution until 50% (w/v), some were subjected to salt stress by adding NaCl until 500mM, while the plants were exposed to 4°C. After stress treatments, leaves were collected and quickly frozen in liquid nitrogen, and stored at -70°C.

Isolation of total RNA and synthesizing first strand cDNA

Total RNA was extracted from young leaves of *J. curcas L.*, with plant RNA isolation reagent (Tiangen Biotech, Beijing, China). Then, the first strand cDNA was sythesized using oligo $(dT)_{18}$ (Table 1). The middle fragment primers *JcP5CSM-1*, *JcP5CSM-2* and *JcP5CSM-3* (Table 1), were designed based on *Ricinus communis P5CS* sequence (GenBank accession number: XM 002524184). The nucleotide sequences of the 3′ and 5′ ends of *JcP5CS* were amplified by the rapid amplification of cDNA ends (RACE) method (Sambrook and Russell, 2001), using three universal primers (oligo $(dT)_{18}$, AP_1, and AP_2) and four gene specific primers (*JcP5CS3'-1*, *JcP5CS5'-T*, *JcP5CS5'-1* and *JcP5CS5'-2*) (Table 1). *JcP5CSO-1* and *JcP5CSO-2* (Table 1) were used to amplify the ORF of *JcP5CS* gene. All resulting products were sub-cloned into pMD18-T vectors (Takara, Dalian, China) and sequenced (Invitrogen, Shanghai, China).

Sequence and phylogenetic analysis

The sequence homology and the deduced amino acid sequence comparisons were carried out using BLAST at NCBI (http://www.ncbi.nlm.nih.gov/blast). The theoretical isoelectric point (pI) and molecular weight (Mw) of proteins were computed by DNAMAN (version5.2.2). Multiple alignments of amino acid sequences were carried out with the sequence analysis software DNAMAN (version5.2.2) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was constructed with MEGA program (version4.0) based on amino acid sequences alignment.

Expression of *JcP5CS* in *Escherichia coli*

The coding region of *JcP5CS* was amplified by PCR with the primer *JcP5CS* E-1 containing the BamHI site and three protection bases, and the primer *JcP5CS* E-2 containing the HindIII site and three protection bases (Table 1). The pMAL-c2E vector and the PCR products were double digested by BamHI and HindIII, after the purification of the digested products by Gel Extraction Mini Kit (Tiangen Biotech, Beijing). Then the ligation was done to obtain the expression plasmids pMAL-*JcP5CS*. The recombinant plasmid pMAL-*JcP5CS* and the empty vector pMAL-c2E were transformed into *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) cells carrying pMAL-*JcP5CS* and empty vector pMAL-c2E was grown at 37°C and 220 rpm, in LB (Luria-Bertani) media supplemented with 50g/mL kanamycin. Isopropylthiogalactoside was added to cell cultures (OD_{600} = 0.6 to 0.8) to a final concentration of 1 mmol/L and grown at 37°C for 4 h. The cells of 1 ml culture were harvested by centrifuging at 5,000 rpm for 5 min, dissolved in 100 µL 1 × SDS sample buffer and boiled at 100°C for 10 min. Overexpressed proteins were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of *JcP5CS* activity from transfected cells

The transformants with plasmid pMAL-*JcP5CS* were grown at 37°C and 220 rpm in LB media supplemented with 50 g/ml kanamycin. Isopropylthiogalactoside was added to cell cultures (OD_{600} = 0.6 to 0.8) to a final concentration of 1 mmol/L and grown at 37°C for 4 h. The cells were harvested and washed twice with ice-cold phosphate buffer saline. They were then re-suspended in lysis buffer (50mM Hepes, pH7.5, 10% glycerol, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol, and 1mM phenylmethanesulphonyl fluoride) and sonicated five times for 30 s. The cell lysate was then centrifuged at 12,000 rpm for 10 min at 4°C to remove unbroken cells. Protein concentrations of the supernatant were measured by the Bradford method. *P5CS* activity was measured according to the method described by Garcia-Rios M. et al. (1997)(Garcia-Rios M. et al.,1997). The *P5CS* assay was carried out in 100 mM Tris Cl (pH 7.2), 25 mM $MgCl_2$, 75 mM Na-glutamate, 5 mM ATP, 0.4 mM NADPH and 10 to 50 µg crude proteins at 30°C for 20 min. The NADPH was measured at 340 nm. One unit of the enzyme activity was defined as the decrease in absorption at 340 nm.

Real-time quantitative PCR and free proline determination

Expression of *JcP5CS* mRNA was analyzed quantitatively using SYBR Green II real time PCR (Takara, Dalian, China) with Bio-Rad IQ5 detection system according to the manufacturer's instructions. The primer pair (Jc18s1 and Jc18s2) (Table 1) was used to amplify the *J. curcas L.* 18S ribosomal RNA(18SrRNA) (Gene Bank accession no.AY823528) that was used as an internal standard. The *P5CS*-specic PCR primers was Rq-F and Rq-R (Table1). The thermocycling program was 40 cycles of 95°C for 15 s and 60°C for 1 min with an initial cycle of 95°C for 10 min. After the PCR, a dissociation curve (melting curve) was constructed from the range of 55 to 95°C to ensure that primer-dimers and other nonspecific products had been eliminated. Data were collected and processed, including baselines subtraction and threshold definition, with iQ5-Cycler software (Biorad, Milano, Italy).

Also, free proline was quantified according to the method of Bates et al. (1973). Fresh leaf material (0.5 g) was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and filtered through filter paper. Half milliliter of the filtrate was mixed with 1 ml each of acid-ninhydrin and glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100°C. The reaction mixture was extracted with 4 ml toluene and the chromophore containing

Table 1. The primers used in *JcP5CS* gene clone, prokaryotic expression and Real-time quantitative PCR.

Primer	Sequence (5′→3′)	Purpose
Oligo(dT)18	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)18	Adaptor
AP1	GTCAACGATACGCTACGTAACG	Anchor
AP2	TACGTAACGGCATGACAGTG	
JcP5CSM-1	CTTGATGGGAAAGCATGTGC	Middle fragmengt primers
JcP5C M-2	CCTCGAGCATGAATCCTACTT	
JcP5CSM-1	CTTGATGGGAAAGCATGTGC	
JcP5CSM-3	GTGTACAAGAAGTGTTTCCATAGC	
JcP5C 3′-1	CCTGTTCTGGGTCATGCTGA	3′-RACE primers
JcP5CS5′-T	CCCTCTTCCCACCCTGGACTTATCACC	
JcP5CS5′-1	GCTTTCCTGGTACTGACCGCATC	5′-RACE primers
JcP5CS5′-2	AATCACTATCGGTCACAAGGAGC	
JcP5CSO-1	ATGAATGACGGCGTGGATCG	The ORF primers
JcP5CSO-2	TTATGTTACATCCTTGTGGGT	
Rq-F	GGGCAAGCAAGGAGATGAAC	Real-time quantitative PCR
Rq-R	TCCAACCTCAGCACCTAAGC	
Jc18s1	AGAAACGGCTACCACATC	
Jc18s2	CCAAGGTCCAACTACGAG	
JcP5CS E-1	CGCGGATCCATGAATGACGGCGTGGATCG	expression in *E. coli*
JcP5CS E-2	CCCAAGCTTTTATGTTACATCCTTGTGGGT	

toluene was aspirated, cooled to room temperature, and the absorbance was measured at 520 nm with a UV visible spectrophotometer. Appropriate proline standards were included for the calculation of proline in the samples.

RESULTS

Cloning of full length cDNA of *JcP5CS*

A 1389 bp fragment was amplified form young leaves of *J. curcas L.* by middle fragment primers. The nucleotide sequence showed a high homology with many known *P5CS* genes, especially the *Ricinus communis*. The 3′ and 5′ RACE primers were designed based on the fragments. The 3′ and 5′ fragments were amplified by RACE and sequenced. We obtained a 2675 bp long full-length cDNA sequence of *P5CS* from *J. curcas L.* (GenBank accession no. GU358610) by comparing and aligning the sequences of the 3′ and 5′ RACE products.

Sequence and phylogenetic analysis

The nucleotide sequence of the cDNA was 2675 bp, containing a 2148 bp open reading frame, a 117 bp 5′-untranslated region and a 410 bp 3′-untranslated region. The ORF encoded a 715 amino acid polypeptide with the molecular weight of 77.54 kDa and the pI value of 6.11. The gene was named *JcP5CS* (*J. curcas P5CS*). Multiple sequence alignments showed that *JcP5CS* shared high homology in amino acid sequence, with *Ricinus communis P5CS* (83.75%), *Phaseolus vulgar P5CS* (78.49%), *Arabidopsis thaliana P5CS1* (75.36%), *A. thaliana P5CS2* (76.75%), *Oryza sativa P5CS* (73.74%) and *Vigna aconitifolia P5CS* (75.80%). A comparison of the amino acid sequence of *JcP5CS* with the *P5CS* of the other plants by BLAST showed *JcP5CS* was composed of N-terminal Glutamate-5-kinase (G5K, ProB) and C-terminal glutamic-γ-semialdehyde dehydrogenase (GSA-DH, ProA) domains. The conserved Glu-5-kinase, GSA-DH domains, conserved leucine zipper and the putative ATP and NAD(P)H-binding sites (Figure 1) were also found. The conserved proline feedback inhibition amino acid residue was found in *JcP5CS* (Phe, at position 129). A Phylogenetic tree of *JcP5CS* with several plants *P5CS* was built based on their amino acid sequence by MP (Maximum parsimony) method of cluster analysis using MEGA program, computing 1,000 permutations. Phylogenetic relationships of *JcP5CS* with several species *P5CS* is shown in Figure 2.

Recombinant protein expression in *E. coli* and *JcP5CS* activity assay

After induction with IPTG for 4 h, the clones with recombinant plasmids of pMAL-*JcP5CS* were harvested. Whole cell lysate, soluble cell extracts and cell pellets were analyzed by 12% SDS-PAGE. The recombinant protein pMAL-*JcP5CS* with a molecular weight of about

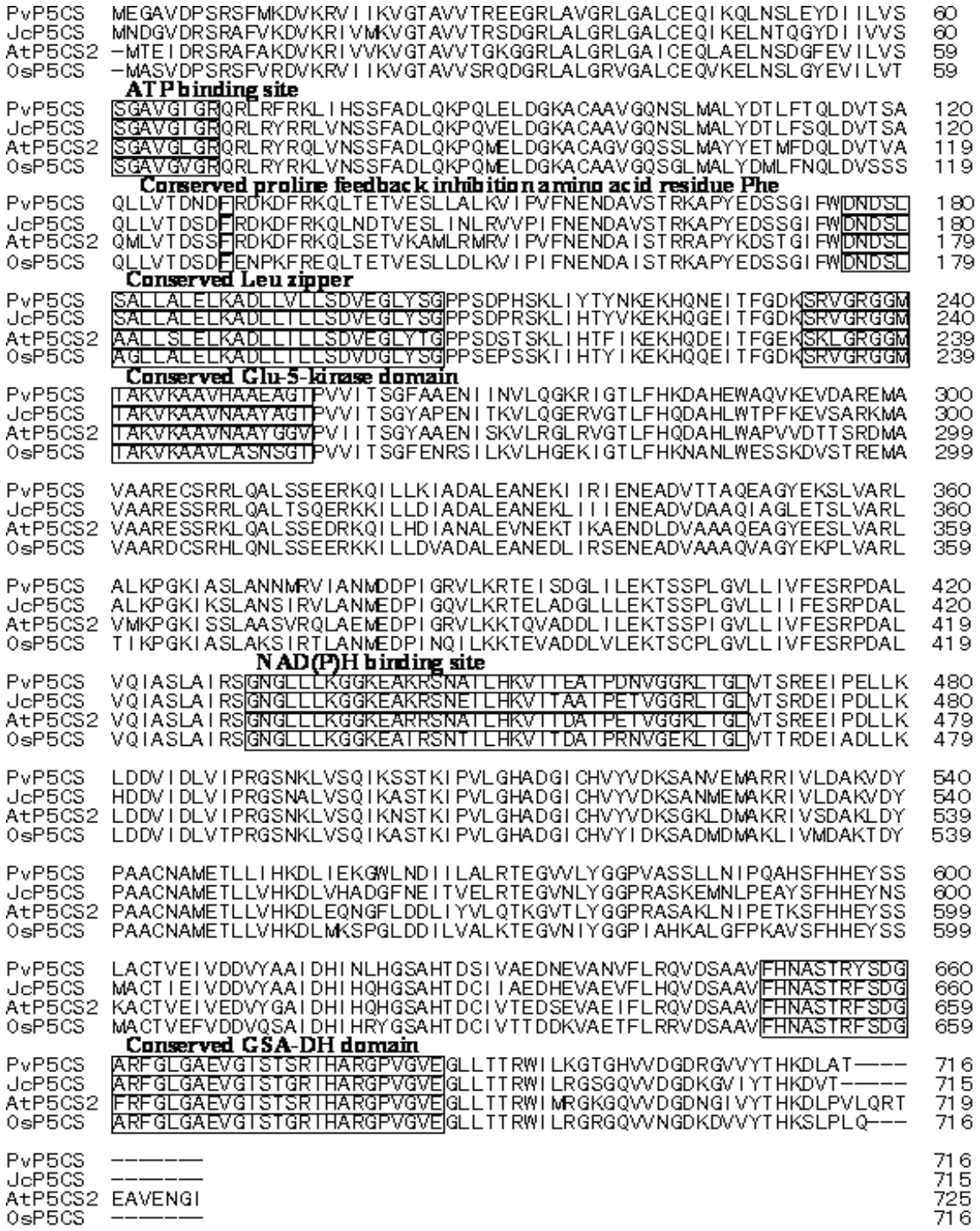

Figure 1. Amino acid sequence alignment of *PvP5CS* (accession no. ABY61079), *JcP5CS* (accession no. GU358610), *AtP5CS2* (accession no. NP191120) and *OsP5CS* (accession no. BAA19916).The conserved Gk, GSA-DH domains, putative ATP and NAD(P)H-binding sites, conserved leu zipper and the conserved proline feedback inhibition amino acid are boxed.

119kD was observed in total protein extracts, soluble cell extracts and cell pellets (Figure 3, lane 4 to 6). The recombinant protein pMAL-*JcP5C*S molecular weight was higher than the molecular weight of *JcP5CS* (77.54 kD) because the recombinant protein contained maltose-binding protein (42.3 kD).

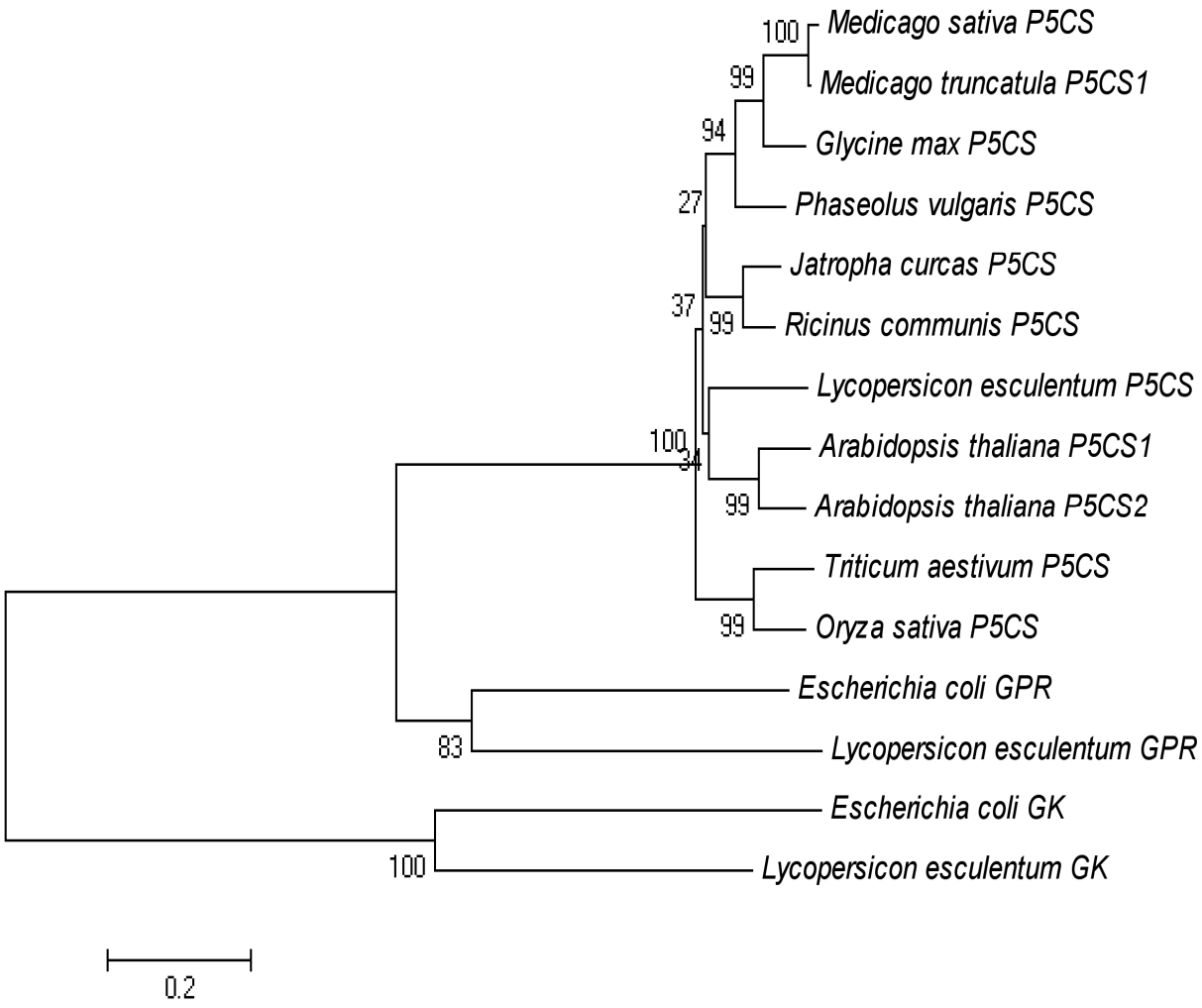

Figure 2. The phylogenetic tree of *JcP5CS* (*J. curcas P5CS*) was built from amino acid sequences. GeneBank. accession numbers for these sequences are as follows: *Medicago sativa P5CS* (CAA67069), *Medicago truncatula P5CS* (CAC82184), *Glycine max P5CS* (AAR86688), *Phaseolus vulgaris P5CS* (ABY61079), *Ricinus communis P5CS* (XP002524230), *Lycopersicon esculentum P5CS* (AAB67875), *Arabidopsis thaliana P5CS1* (NP181510), *Arabidopsis thaliana P5CS2* (NP191120), *Triticum aestivum P5CS* (AAX35536), *Oryza sativa P5CS* (BAA19916), *Escherichia coli GPR* (CAA25364), *Lycopersicon esculentum GPR* (AAB67877), *Escherichia coli GK* (CAA25363) and *Lycopersicon esculentum GK* (AAB67876). The reliability of the tree is measured by bootstrap analysis with 1,000 replicates.

The *JcP5CS* activity assay was measured as Garcia-Rios M described. Protein concentrations of the soluble part were measured by the Bradford method. The soluble part of *E. coli* with recombinant plasmids pMAL-*JcP5CS* after 4 h post-induction with 1.0 mM IPTG was set as the control. The *JcP5CS* activity was 95.87 U/mg.

Expression patterns of *JcP5CS* in response to drought, salt and cold stresses

The expression of *JcP5CS* and proline levels were measured as previously described, in drought, salt and cold stress treated *J. curcas* leaves at different time points (Figure 4). Drought stress treatment caused rapid introduction of *JcP5CS* expression up to 6.1 fold at 2 h after treatment, followed with a reduction to 4 fold at 4 h. Then, *JcP5CS* expression sharply reduced to1.2 fold at 6 h, followed by an increase to 1.5 fold at 9 h. It maintained at low level from 12 to 36 h. After that it reduced to 0.24 fold at 48 h. During the drought stress treatment process, proline level increased from 1.0 fold at 2 h to 4.6 fold at 12 h, followed by a reduction from 24 h and then an increase to 4.2-fold again at 48 h. Salt stress treatment caused rapid introduction of *JcP5CS* expression up to 6.5 fold at 2 h after treatment, followed with a reduction to 2.8 fold at 4 h. Then, *JcP5CS* expression sharp reduced to1.1 fold at 6 h and maintained at low level from 9 to 24 h. After that it reduced to 0.12 fold at 36 h and 0.08 fold at 48 h. During the drought stress treatment process,

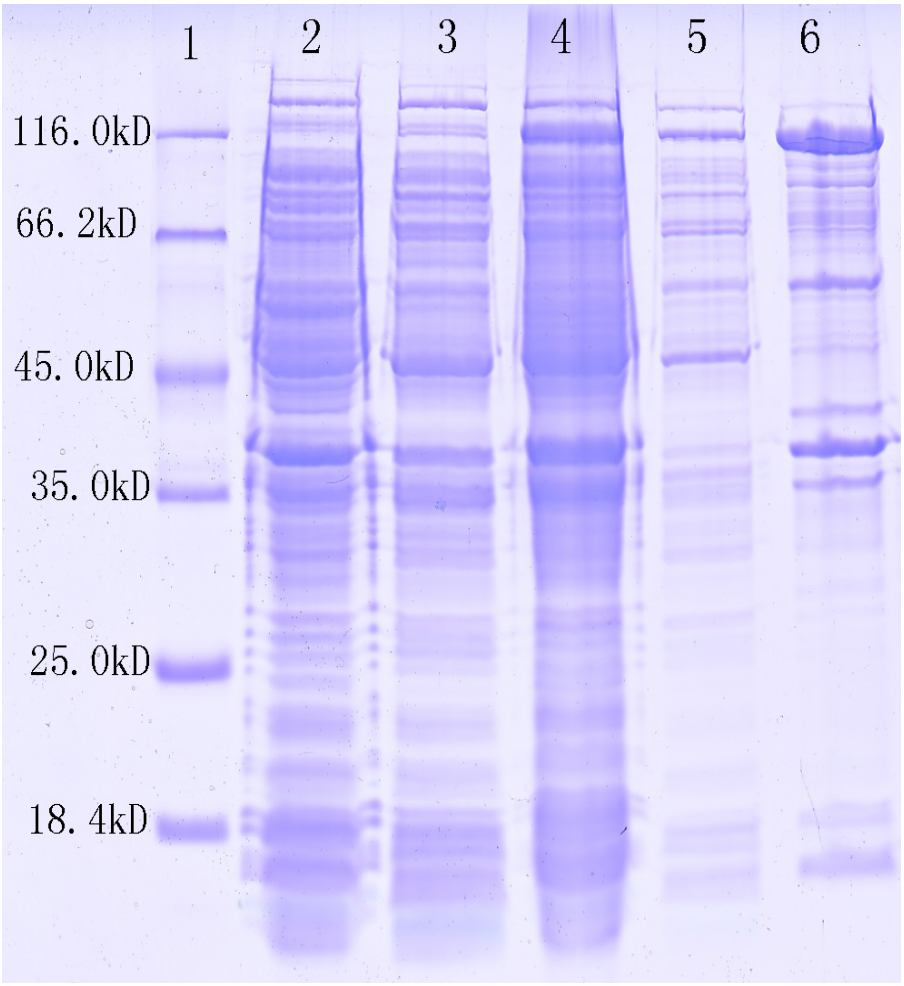

Figure 3. Expression of *JcP5CS* in *Escherichia coli*. Lane 1, Protein marker; lane 2, total protein extracts from BL21 *E. coli* cells containing the empty vector pMAL-c2E after 4 h post-induction with 1.0 mM IPTG; lane 3, total protein extracts from non-induced BL21 *E. coli* cells containing the plasmid pMAL-*JcP5CS*; lane 4, total protein extracts from BL21 *E. coli* cells containing the plasmid pMAL-*JcP5CS* after 4 h post-induction with 1.0 mM IPTG; lanes 5 and 6, soluble and pellet part of *E. coli* with recombinant plasmids pMAL-*JcP5CS* after 4 h post-induction with 1.0 mM IPTG.

proline steadily accumulated from 1.0 fold at 2 h to 13.1 fold at 24 h, followed by a reduction from 24 h. More also, cold stress treatment caused introduction of *JcP5CS* expression up to 1.25 fold at 2 h after treatment, followed with a rapid reduction to 0.41 fold at 4 h. It maintained a very low level from 4 to 48 h. During the cold stress treatment process, proline steadily accumulated from 0.75-fold at 2 h to 6.7-fold at 24 h, followed by a reduction from 24 h.

DISCUSSION

In *E. coli*, conversion of glutamate to Δ1–pyrroline-5-carboxylate is mediated by two enzymes, γ-glutamyl kinase (GK, EC 2.7.2.11) and glutamic-γ-semialdehyde dehydrogenase (GSA-DH, EC 1.2.1.41). These two enzymes are encoded by proB and proA, respectively (Delauney and Verma, 1993; Deutch et al., 1984). In higher plants the process is mediated by *P5CS*, which exhibited both GK and GSA-DH activities. *VaP5CS* (*V. aconitifolia P5CS*) was the first cloned gene that encoded the *P5CS* enzyme (Hu et al., 1992). *PvP5CS, AtP5CS, OsP5CS* and *RcP5CS* are also encoding a bifunctional enzyme like *VaP5CS*. However, in tomato, the *tomPRO1* locus is unusual, because it contains two open reading frames encoding GK and GSA-DH, respectively, thus resembling prokaryotic polycistronic operons (Garcia-Rios M. et al.,1997).

We succeeded in cloning the full length *P5CS* cDNA from *J. curcas L.* by RACE. Multiple sequence alignments showed that *JcP5CS* shared high homology in amino

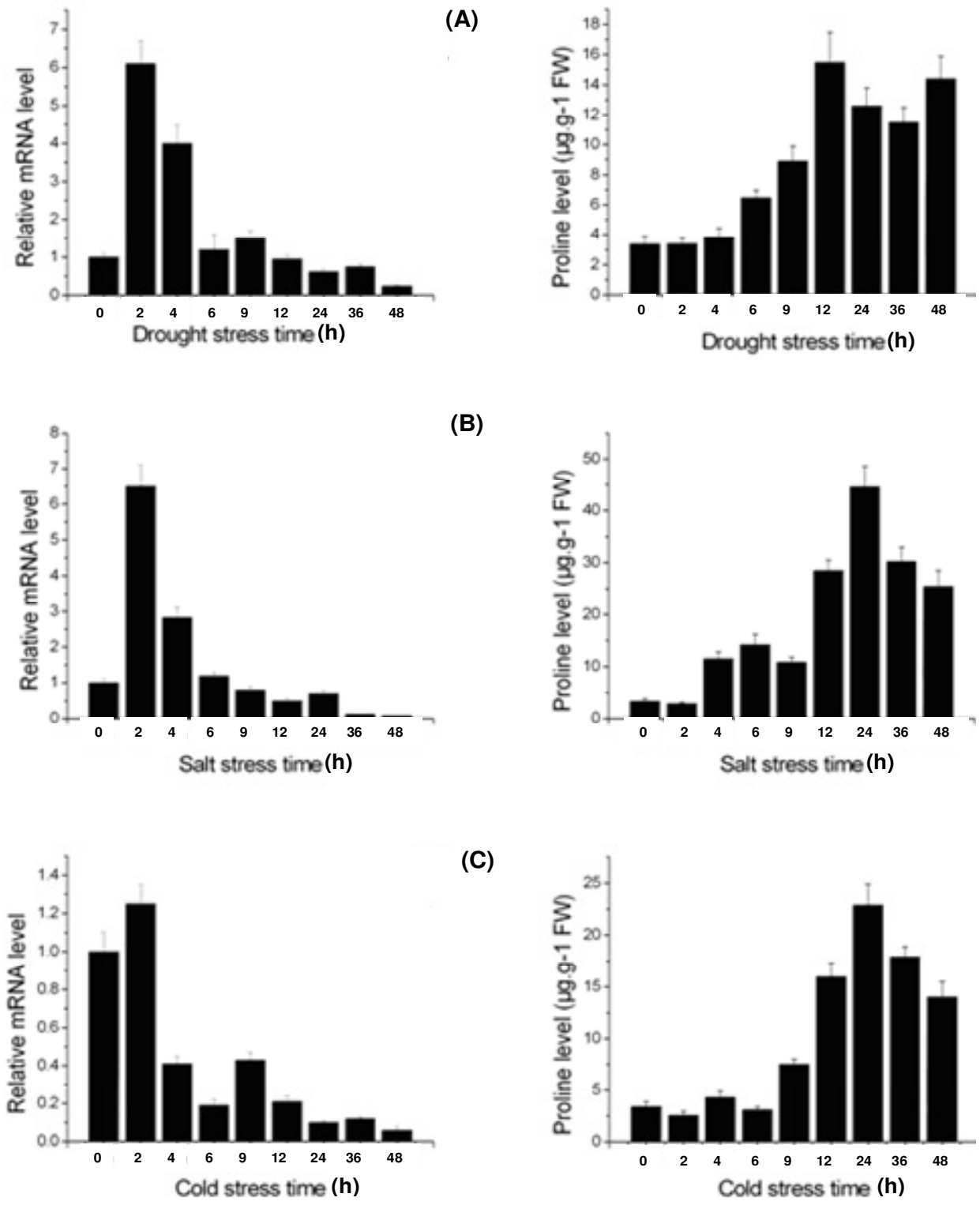

Figure 4. Expression of *JcP5CS* and proline levels in different stress treatments. (A) Represents drought stress treatments; (B) represents salt stress treatments. (C) represents cold stress treatments. Data are presented as the mean ± SD (n = 3).

acid sequence, with *R. communis P5CS* (83.75%), *P. vulgar P5CS* (78.49%), *A. thaliana P5CS1* (75.36%), *A. thaliana P5CS2* (76.75%), *O. sativa P5CS* (73.74%) and *V. aconitifolia P5CS* (75.80%). Sequence analysis showed *JcP5CS* had a complete ORF and encoded a bifunctional enzyme *P5CS*. As shown in Figure 3, the result

indicated that the *JcP5CS* gene could express in *E. coli* cells. Crude protein extract was used for the enzyme activity assay and results showed that the *JcP5CS* activity was 95.87 U/mg. It indicated that we obtained the functional *P5CS* enzyme. In higher plant, *tomPRO2* which encode the *P5CS* in *Lycopersicon esculentum,* was expressed in *E. coli* cells (Fujita et al., 1998), but this enzyme was not obtained in a soluble form. Va*P5CS* was expressed and exhibited high level of GK activity (Hu et al., 1992).

There have been many researches about *P5CS* expression pattern in higher plants. The level of *VaP5CS* transcripts in *V. aconitifolia* leaves and root is increased by treatment with 200 mM NaCl (Hu et al., 1992). The expression of *OsP5CS* was induced by salt, dehydration, cold and exogenous ABA treatments, but not by heat treatment (Igarashi et al., 1997). *PvP5CS* expression was increased by the treatment of drought, salt and cold stress (Chen et al., 2009). RT-PCR analysis showed that the transcription of *MsP5CS1* and *MsP5CS2* were enhanced in roots exposed to NaCl (Ginzberg et al., 1998). The expression of *AtP5CS1* increased by 5 to 6 fold and the *AtP5CS2* increased 2 fold in drought stress treatment (Strizhov et al., 1997). The expression of *AtP5CS1* also increased in *Arabidopsis* when subjected to drought, NaCl and ABA treatments (Yoshiba et al., 1995).

Our results show that *JcP5CS* is induced by drought and salt stress, but not by cold stress. The *JcP5CS* expression accumulated at the early time of drought and salt stress treatments, but the proline accumulated at the later time of osmotic stress treatments. Proline biosynthesis is regulated by end-product inhibition of GK in bacteria (Hayzer and Leisinger, 1980). The wild type *E. coli* GK showed 50% inhibition at 0.2 mM proline, while a mutant form of the enzyme is about 200 fold less sensitive to end-product inhibition. The mutation from aspartic acid to asparagine is responsible for the resistance to end-product inhibition of GK. The amino acid site is also found in *VaP5CS* (128, Asp) (Hu et al., 1992).

There is a different opinion that the conserved Phe residue (129) in *VaP5CS* was involved in feedback inhibition instead of Asp residue (128) (Hong et al., 2000). Sequence analysis showed the *JcP5CS* both had Asp residue (128) and Phe residue (129). It therefore implies *JcP5CS* enzyme might be inhibited by proline to regulate the biosynthesis.

In conclusion, we succeeded in obtaining the *P5CS* from *J. curcas L.* and performed osmotic expression analysis and functional analysis. To further understand the function of this gene, more researches based on these primary results are needed.

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