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Full Length Research Paper

Differential expression of cysteine protease inhibitor (CPI) gene of *Polygonum sibiricum* Laxm. leaves, stems and rhizomes in response to $NaHCO_3$

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A cDNA clone which encodes a cysteine protease inhibitor gene, named *PsCPI*, has been identified in *Polygonum sibiricum* Laxm. by the rapid amplification of cDNA ends method (RACE). Analysis of the nucleotide sequence reveals that the *PsCPI* gene cDNA clone consists of 437 bp, containing 38 bp in the 5' untranslated region, 336 bp in the open reading frame (ORF) encoding 111 amino acids and 63 bp in 3' untranslated region. The gene accession nucleotide sequence number in GenBank is GU562861. Expression analysis by real-time quantitative PCR reveals that the *PsCPI* gene is expressed in leaves, stems and rhizomes. The result shows that *PsCPI* gene expression can be induced by 3% $NaHCO_3$ in the three earlier mentioned tissues, but there are different expression modes in leaves, stems and rhizomes under the salinity-alkalinity stress.

Keywords: Polygonum sibiricum Laxm., Polygonum sibiricum Laxm cysteine protease inhibitor, rapid amplification of cDNA ends, real-time polymerase chain reaction, gene expression.

INTRODUCTION

Natural protease inhibitor (PI) is a kind of small molecule protein which has inhibitory activity to proteolytic enzyme and is widely distributed in all organisms (Knight et al., 1994; Sangadala et al., 1994). At present, three kinds of PIs have been found in plants, which are, serine protease inhibitors, metal protease inhibitors and cysteine protease inhibitors (CPIs) (Xu et al., 2008). CPI, also called thiol protease inhibitor, is a kind of natural protease inhibitor, and exists in animals, plants and microorganisms. Plant CPIs, homologous to animal CPIs (Brown and Dziegielewska, 1997), have been characterized in several plants, including *Oryza* (Echao et al., 1996; Hao et al., 1999), soybean (Miguel et al., 1996), chestnut (Monica et al., 1998), *Arabidopsis thaliana* (Beatrice et al., 2003), cowpea (Xue and Liu, 2003), kiwi (Rassam and Laing, 2004), strawberry (Manuel et al., 2005) and so on. Most CPIs have QxVxG(Gln-Xaa-Val-Xaa-Gly) in the central areas of protein sequence(Li and Gai, 2010). However, in some plants, the CPI does not have the conserved sequence, but still have a high CPI activity, like pineapple stem bromelain inhibitors that lack conserved sequence (Hatano et al., 1996, 1998; Sawano et al., 2005). This conserved sequence is an important site combined with cysteine protease, so that, CPIs can inhibition cysteine proteases. Previous research suggested that CPI plays an important role in plants subjected to biotic and abiotic stress (Clarence et al., 2000), and researchers found that plants experience programmed cell death (PCD) when they are exposed to salt stress (Maki et al., 2000; Gyung-Hye et al., 2002; Jian-you et al., 2007; Jiusheng et al., 2006;). Recently,

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Abbreviations: CPI, Cysteine protease inhibitor; **EST,** expressed sequence tag; **cDNA,** complementary DNA; **RACE,** rapid amplification of cDNA ends; **PsCPI,** *Polygonum sibiricum* Laxm cysteine protease inhibitor; **PCR,** polymerase chain reaction; **ORF,** open reading frame; **UTR,** 5'-untranslated region; **PCD,** programmed cell death.

Table 1. Oligo nucleotide primers used in the experiment.

Primer name	Primer sequence (5'-3')	Remark
A1	CATTGAGAGATAAGACTGGTTGTTG	PsCPI 5' RACE
S1	GAACGGTCGTGGTAACTGGTAATGG	PsCPI 3' RACE
PsCPI-S1	CATAATAGAGCTAGCTCTTCT	full length cloning
PsCPI-A1	ATTGAACGGTCGTGGTAACTG	full length cloning
PsCPI-S2	CATGTACTACATCACTCTGGAGGC	PsCPI real-time PCR
PsCPI-A2	ACTGGTAATGGTAAGAAATTGACA	PsCPI real-time PCR
18S-S	GTATGGTCGCAAGGTGAAAC	18S rRNA real-time PCR
18S-A	TTAGCAGGCTGAGGTCTCGT	18S rRNA real-time PCR

Mazal (1999) revealed that CPI and PCD have direct connection, and PCD can be regulated by activity poised between the cysteine proteases and the cysteine protease inhibitors in plant (Mazal et al., 1999).

In this study, the *PsCPI* gene with the complete coding cDNA sequence was cloned from the leaves and bioinformatics analysis was conducted. The expression pattern of the gene in different organ after 3% $NaHCO_3$ stress was detected using real time quantitative PCR. These results may further reveal the relationship between CPI and PCD induced by salt stress.

MATERIALS AND METHODS

Polygonum sibricum Laxm. (height 10 to 15 cm) were collected from salinity-alkalinity fields (pH = 8.68) in Zhaodong, Heilongjiang. The rhizomes of *P. sibiricum* Laxm. were in a mixture of humus and sand (volume ratio 3:1) in greenhouse for one month. Then, the samples were treated at different stages using 3% $NaHCO_3$. A total of 150 plants (plant height 15 to 20 cm) were divided into five treatments randomly: 0, 8, 24, 48 and 72 h (Shouhai et al., 2010). Each treatment consisted of six replicates with five plants of *P. sibricum* Laxm. each. After harvesting, all samples were immediately preserved in liquid nitrogen and kept at -80°C until they were used for isolating the RNA.

Total RNA extraction

Total RNA was extracted using a phenol sodium dodecyl sulfate extraction/LiCl precipitation procedure (Davis et al., 1991).

Obtaining 3' and 5' regions by RACE

The rapid amplification of cDNA ends (RACE) method was used to isolate the complete 5' and 3' regions of this gene. First-strand cDNA synthesis was performed using Smart™ RACE cDNA amplification kit (Clontech). Previously, we obtained the CPI EST sequences from the *P. sibiricum* Laxm. cDNA library (Liu et al., 2008). According to the EST, two specific primers A1 and S1 (Table 1) were designed on the basis of the CPI 3' UTR for 5'-RACE. The fragment PCR was carried according to the manufacturer's instructions (Clontech Kit). Next, the fragments were linked, transformed and sequenced. At last, the full-length cDNA was obtained by linking two fragments. A pair of specific primers PsCPI-S1 and PsCPI-A1 (Table 1) was designed to amplify the ORF of PsCPI.

Subcloning

The PCR fragments were subjected to electrophoresis on 0.8% agarose gel for length differentiation, and amplified cDNA fragments were cloned into the pGEM-T Easy vector following the instructions provided (Promega, Madison, WI, U.S.). Recombinant bacteria were identified by blue/white screening and confirmed by PCR. Plasmids containing the insert were purified (Promega minipreps) and used as a template for DNA sequencing.

Nucleotide sequence analysis

The fragments were linked by the soft Bio-Edit CAP contig assembly program. The *PsCPI* gene sequence was analyzed and compared using the BLAST P and ORF search programs with GenBank database search. The multiple sequence alignment of *PsCPI* gene was created by using the Clustal Wanalysis program, Predicting signal-peptide site by Signal P3.0, Predicting phosphorylation site by KinasePhos 2.0. ProtParam (http://au.expasy.org/tools/protparam. html), the CPI protein molecular weight (MW) and isoelectric point (pI) were computed by ProtParam (*ProtParam Tool.*), and amino acid composition was analyzed. The hydrophilicity/hydrophobicity was analyzed by ExPASy ProtScale (http://www.expasy.org/cgi-bin /protscale.pl) with Kyteh Doolittle method.

Quantification of PsCPI gene expression by RT-PCR

Total RNAs were extracted by SDS method from different tissues including stem, rhizome and leaf at different handling stages induced by 3% $NaHCO_3$, and treated as mentioned earlier. The residue of DNA was removed by DNase I digesting at 37°C for 30 min. 4 µg of the total RNA were used in each lane and electrophoresed in a 1.0% agarose gel, at 100 V/12 cm for 15 min. First-strand cDNA synthesis was performed using M-MLV reverse transcriptase (TaKaRa Biotechnology (Dalian) Co., Ltd. Japan) to transcribe poly (A)+ RNA with oligo-d(T)18 and random 6 as the primers, using reaction conditions recommended by the manufacturer. The cDNA was used for the assay of quantitative real-time PCR. The SYBR Green I real-time RT-PCR assay was carried out in an Option-IISequence Detection System (MJ Research, U. S.). The amplifications were performed in a 96-well plate in a 25 µl reaction volume containing 12.5 µl of 2×SYBR Green Master Mix (TARAKA), 2.5 µl (each) PsCPI-S2 and PsCPI-A2 primers (10 mM), 1 µl of template, and 9 µl of DEPC-water. The thermal profile for SYBR Green RT-PCR was 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 55°C for 30 s. In a 96-well plate, each RT-PCR was repeated three times. DEPC-water for the replacement of template was used as negative control. The

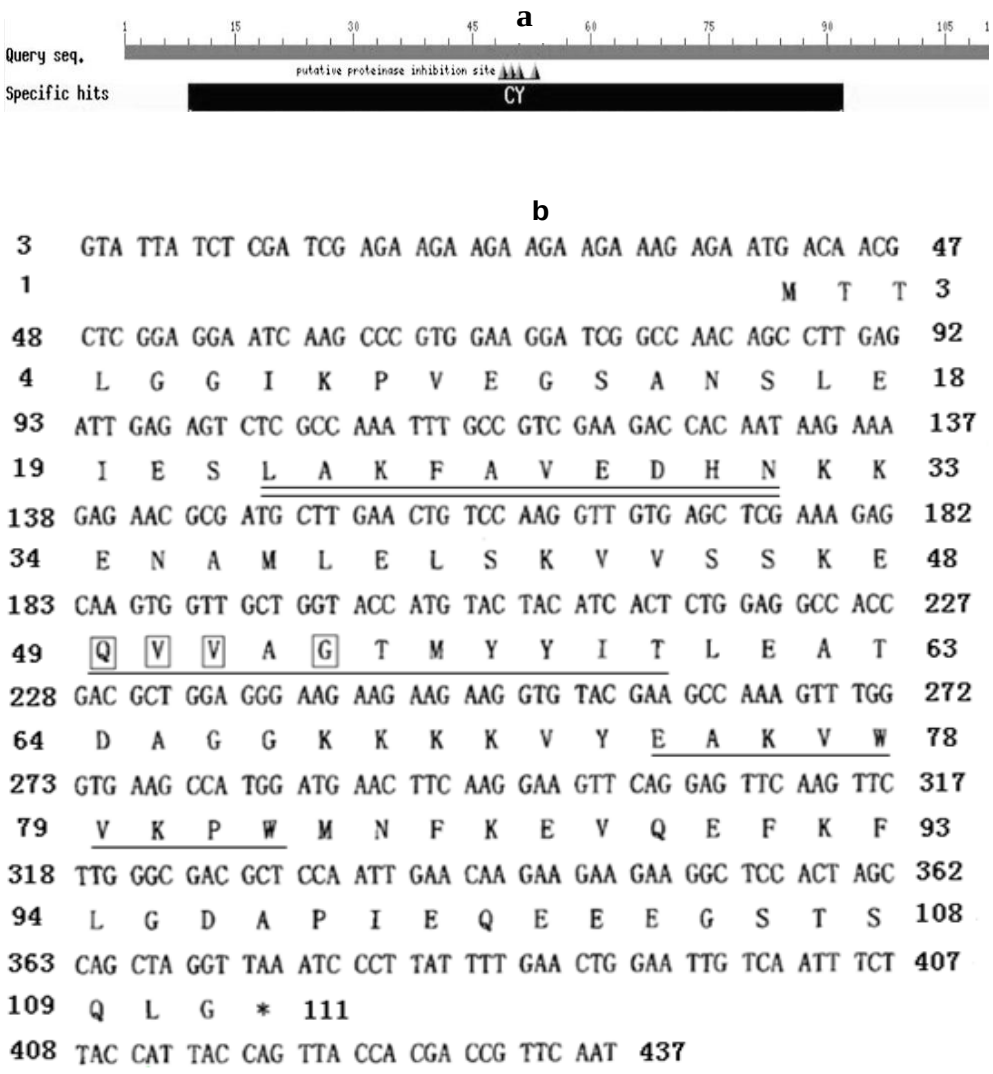

Figure 1. Nucleotide and deduced amino acid sequences of *PsCPI* cDNA from *P. sibiricum* Laxm. The PCR products of *PsCPI* cDNA were sequenced. Rectangle indicates proteinase inhibition site and conservative domain; *Indicates the stop codon; single underline indicates hairpin loop; double underline indicates alpha helix.

relative expression was calculated as $2^{-\Delta\Delta Ct}$; Ct: cycle threshold (Livak et al., 2001).

Statistical analysis

A multiple comparison (Duncan's test) was conducted to compare significant differences in *PsCPI* expression between leaves, stems and rhizomes, using the SPSS software. A significant level of p = 0.05 was chosen.

RESULTS

cDNA cloning, sequencing and bioinformatics analysis of PsCPI

In order to isolate cDNA encoding for cysteine protease inhibitor, PCR reactions were performed using specific primers and total cDNA of *P. sibiricum* Laxm leaf. Products of amplification were cloned and sequenced. Computer analysis, using the BLAST algorithm, confirmed that the selected sequence corresponded to a cysteine protease inhibitor. The full-length cysteine protease inhibitor cDNA fragment of *P. sibiricum* Laxm. was obtained by overlapping two cDNA fragments. The cloned cDNA of *PsCPI* gene comprised of 437 bp, containing 38 bp in 5'-untranslated region (UTR), 336 bp in the open reading frame (ORF) and 63 bp in 3'-UTR without poly (A) tail (Figure 1b).

The ORF encodes polypeptide of 111 amino acids. The calculated molecular mass of the mature protein (111 amino acids) was 12.3 KDa, with an estimated pI of 5.08. The protein had no signal peptide, and phosphorylation.

Table 2. Amino acid composition analysis on CPI of *P. sibiricum Laxm*.

Amino acid	Percentage (%)	Amino acid	Percentage (%)
A	8.1	M	3.6
D	2.7	N	3.6
E	13.5	P	2.7
F	3.6	Q	3.6
G	8.1	S	7.2
H	0.9	T	5.4
I	3.6	V	9.0
K	12.6	W	1.8
L	7.2	Y	2.7

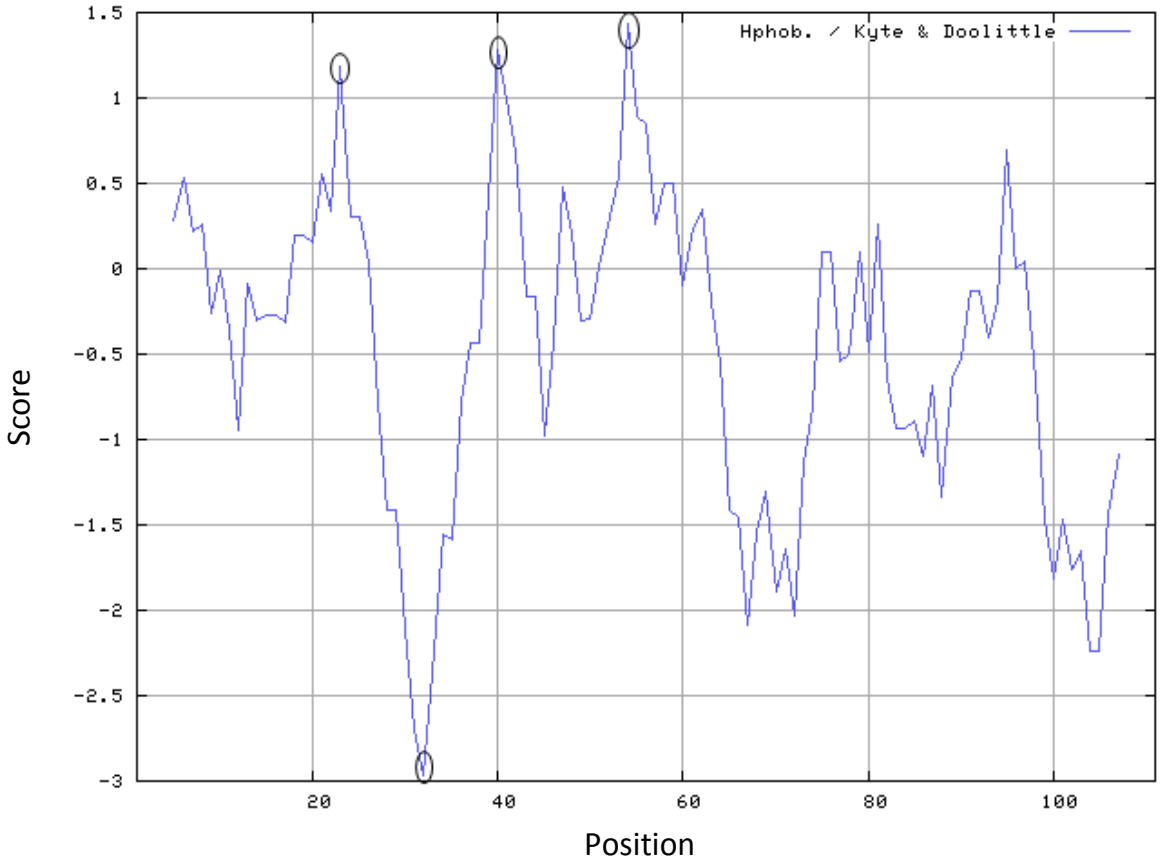

Figure 2. The hydrophilic/hydrophobic result of CPI protein from *P. sibiricum* Laxm. Rectangle indicates hydrophobic region, circles indicate hydrophilic regions.

The protein has four proteinase inhibition sites. The best matching domain is CY, from 9 to 92, according to NCBI BlastP (http://blast.ncbi. nlm. nih.gov) (Figure 1a). In the 111 amino acids, E, K, V, A, S and L accounted for the highest, followed by T, F, I, M, N, Q, H and W which was the least (Table 2). There were several obvious hydrophilic regions and one obvious hydrophobic region in PsCPI protein (Figure 2). The cDNA sequence and deduced amino acid sequence has been submitted to the NCBI GenBank as accession number GU562861.

Homology comparison of PsCPI

The comparison of the ORFs with other known CPIs indicates that the P. sibiricum Laxm CPI shows homology: identities = 87/108 (81%) with Rumex obtusifolius; identities = 78/101 (71%) with Spinacia

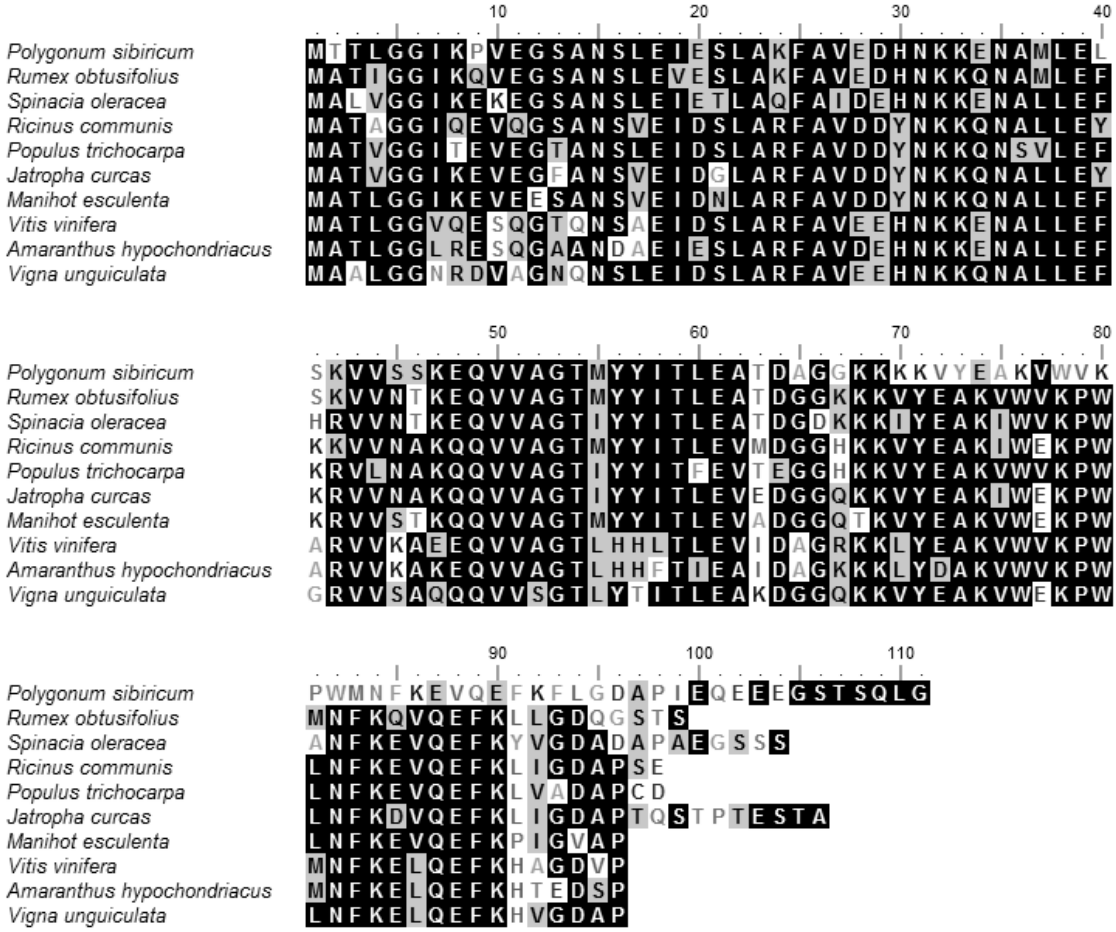

Figure 3. Alignment of the deduced amino acid sequences of *PsCPI* and the known CPI from GenBank. Shaded regions show identical amino acids. Identities are compared with other plants mentioned earlier.

oleracea; identities = 71/100 (71%) with Ricinus communis: identities = 67/100 (67%) with Populus trichocarpa; identities = 69/108 (64%) with Jatropha curcas; identities = 70/98 (72%) with Manihot esculenta; identities = 65/98 (67%) with Vitis vinifera; identities = 65/98 (67%) with Amaranthus hypochondriacus; identities = 66/98 (68%) with Vigna unguiculata and so on. PsCPI was genetically distant from other kinds of CPI (Figure 3). A phylogenetic tree based on evolutionary distances was constructed from amino acid sequence using the njplotWIN95 program (Figure 4). All the bioinformatics analysis results suggest that *P. sibiricum* Laxm. CPI should be a plant cysteine protease inhibitor.

Tissue expression of PsCPI

CPI expressed in each organ of *P. sibiricum* Laxm. is shown in Figure 5. In a RT-PCR study, specific primers PsCPI-S2 and PsCPI-A2 were used to amplify a 209 bp fragment with cDNA from leaf, stem and rhizome organ using 18S as a positive control. The RT-PCR showed that the *PsCPI* was detected in leaf, stem and rhizome. In normal condition, the expression of *PsCPI* in stems was the highest, and the lowest was in the rhizomes (Figure 5a). When treated with 3% $NaHCO_3$, the expression situations were variable amongst different organs. In leaves, the increase of the *PsCPI* mRNA expression level reached its peak in 48 h after 3% $NaHCO_3$ stress, then suddenly disappeared (Figure 5b). In stems, the increase of the *PsCPI* mRNA expression reached its peak in 48 h after salinity-alkalinity stress, and suddenly decreased (Figure 5c). In rhizome, the contents of *P. sibiricum* Laxm CPI gradually increased; it peaked in 72 h after 3% $NaHCO_3$ stress (Figure 5d).

DISCUSSION

We isolated the full length of a *PsCPI* gene from *P.*

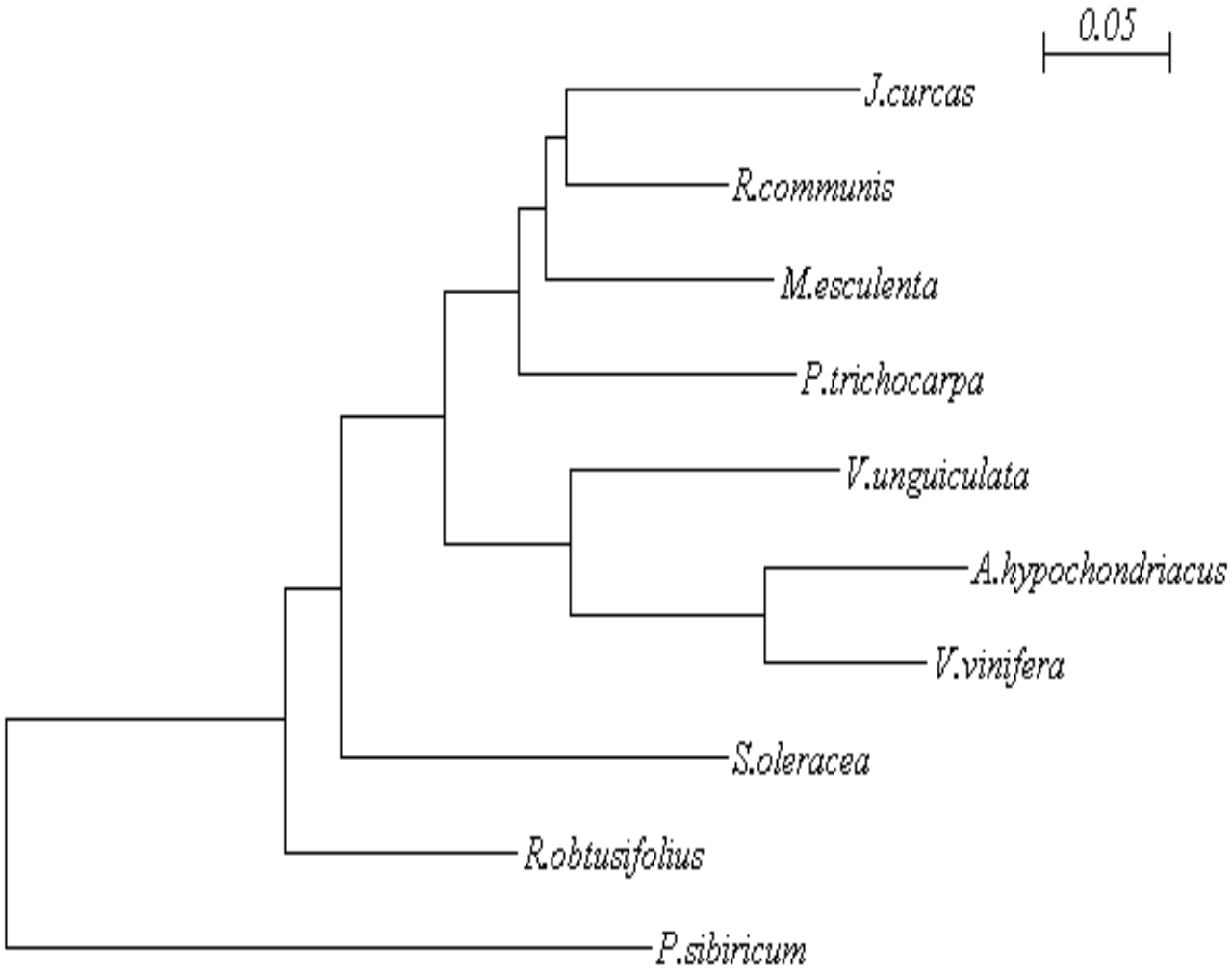

Figure 4. The phylogenetic tree of *PsCPI* from plants. ClustalW was used to establish the phylogenetic tree, and the result are displayed using Treeview software.

sibiricum Laxm. by the rapid amplification of cDNA ends method and 336 bp in the open reading frame (ORF) encoding 111 amino acids. The CPI protein was 12.3 KDa, the pI was 5.08, and it had obvious hydrophilic regions and hydrophobic region. *PsCPI* was compared with other plant species, the homology of nucleotide was high, but it was an independent branch on phylogenetic tree, which would probably be as a result of its special salinization-tolerance. Margis (1998) aligned CPIs amino acid sequences from 32 species of plants, discovering three conserved regions; N-terminal LAK(R)FAVDEHN sequences formed alpha-helix, middle QVVAGTMYYIT sequences formed hairpin loop between the second beta-bridge and the third beta-bridge, C-terminal EAKVWVKPW sequences also formed hairpin loop between the fourth beta-bridge and the fifth beta-bridge. This particular structure plays an important role in maintaining the stability of the protein (Margis et al., 1998). The two hairpin loops which formed the hydrophobic interaction constituted a wedge structure, and the wedge structure was complementary to the active site of target enzyme (Martin et al., 1995; Auerswald et al., 1996). Similarly, *PsCPI* amino acid sequences have the three earlier-mentioned special conserved sequences, so we inferred that the special structure has the same function as the earlier-mentioned. Expression of *PsCPI* showed different trends with the stress time; the phenomenon suggest that *PsCPI* is a salt stress-related gene. Salt stress can induce PCD (Lin et al., 2006; Gyung-Hye et al., 2002; Maki et al., 2000; Jianyou et al., 2007), and PCD could be blocked by CPI (Minami et al., 1995). In our experiment, when stress time reached 72 h, *PsCPI* expression quantity dropped rapidly in leaves and stems. So we presumed that 3% $NaHCO_3$ stressed *P. sibiricum* Laxm., and caused PCD, at stress time of 72 h, leaves and stems had PCD, at about stress time of 48 h,

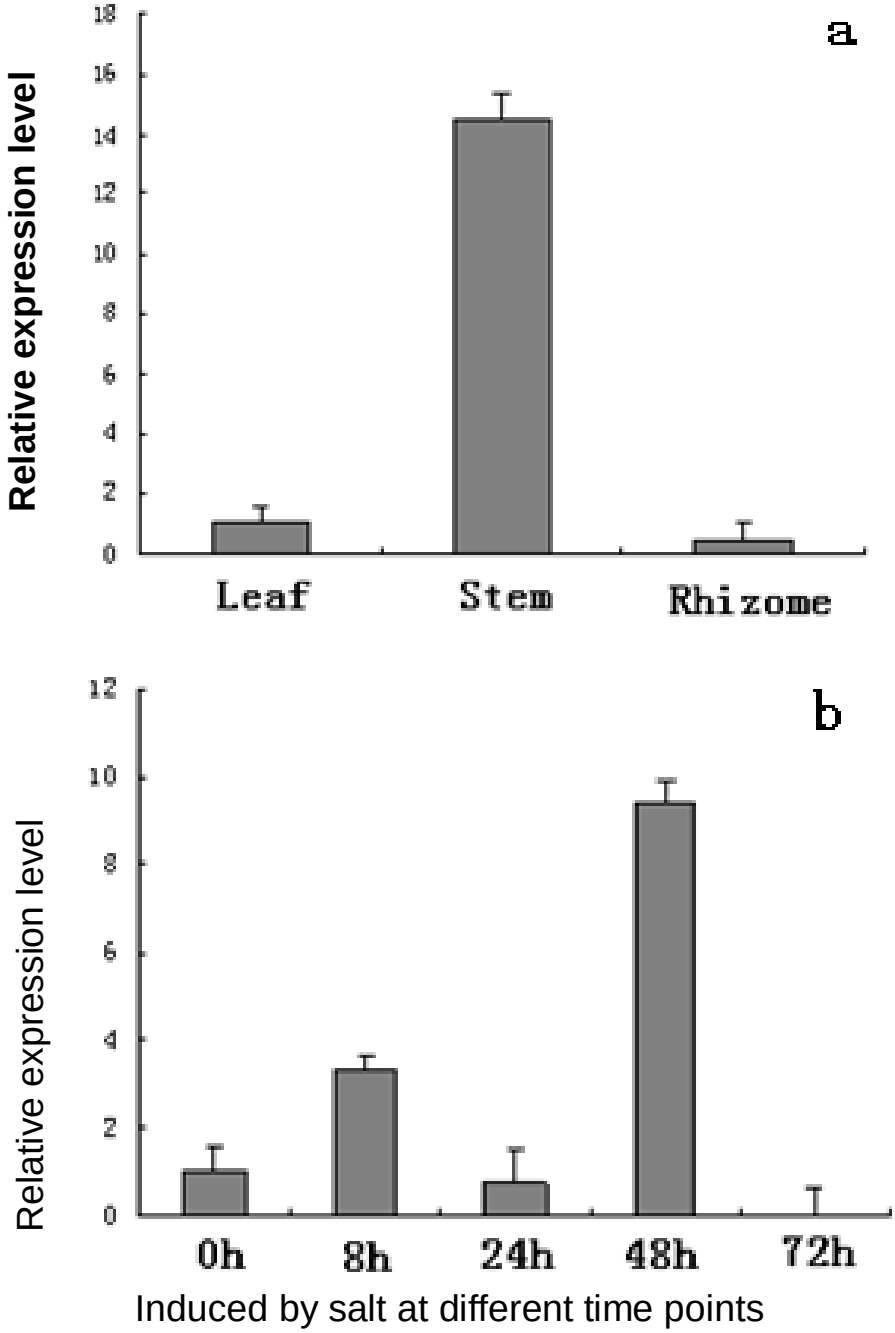

Figure 5. The expression pattern of *PsCPI* after 3% $NaHCO_3$ exposure in leaf, stem, rhizome organ. (a) The expression of *PsCPI* gene in leaf, stem, rhizome organ without stress comparison; (b) the levels of *PsCPI* mRNA in leaf tissues; (c)the levels of *PsCPI* mRNA in stem tissues; (d) the levels of *PsCPI* mRNA in rhizome tissues. A multiple comparisons test was conducted to compare significant differences in *PsCPI* expression between leaves, stems and rhizomes using the SPSS software. A significant level of p = 0.05 was chosen.

the activity of *PsCPI* enzyme was the strongest, while the rhizomes as the direct sensor to salt stress showed that expression of *PsCPI* had been up-regulated. We show that the ability of CPI resistant to salt stress was stronger in rhizome.

Obviously, the different expression models of *PsCPI* in

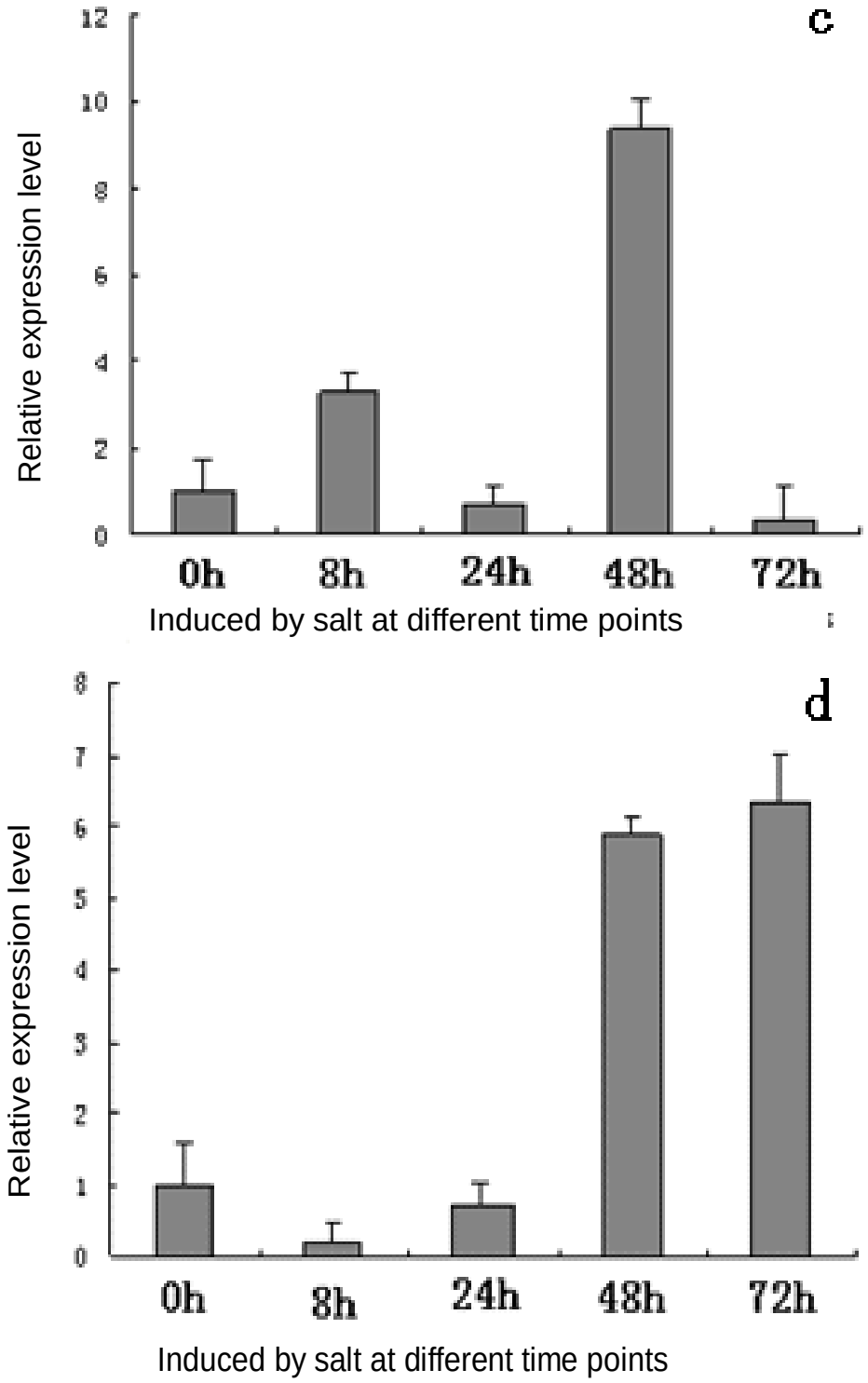

Figure 5. Contd.

leaves, stems and rhizomes indicate that there are different tolerant mechanisms in these parts. However, the tolerance mechanism is unclear. In our next work, we will study the relationship between *PsCPI* and PCD. Our results may provide the basis for future investigations of *PsCPI* roles in *P.* sibiricum Laxm. resistance to salinity-

alkalinity stress.

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