

Full Length Research Paper

Differential expression of ozone-induced gene during exposures to salt stress in *Polygonum sibiricum* Laxm leaves, stem and underground stem

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The response of plants to environmental stresses is characterized by a number of physiological and biochemical changes that ultimately result from the selective increase or decrease in the biosynthesis of a large number of distinct proteins. In this report, we describe the characterization of an ozone-induced transcript, *PcOZI-1*, which has been identified from *Polygonum sibiricum* Laxm by the rapid amplification of cDNA ends method (RACE). *PcOZI-1* mRNA in untreated plants was detected at low levels in underground stem, leaves and at higher levels in stem. *PcOZI-1* mRNA accumulation was transiently induced in stem 7-fold within the first 8 h of 3% NaHCO₃ treatment. *PcOZI-1* mRNA accumulation was also induced 22-fold in underground stem after 72 h. Sequence analysis of *PcOZI-1* revealed that it encodes a 8.45 kDa basic protein that contains a putative signal peptide. Our results suggest that *PcOZI-1* represents a novel stress-related protein that accumulates in response to the production of active oxygen species.

Key words: *Polygonum sibiricum* Laxm, *PcOZI-1*, rapid amplification of cDNA ends, gene expression.

INTRODUCTION

Abiotic stresses, such as water deficit, increased soil salinity and extreme temperature, can limit plant growth and productivity (Yancey et al., 1982). Therefore, plants have developed various strategies for coping with unfavorable conditions. One of the response of plants to environmental stresses such as salt stress is characterized by a number of physiological and biochemical changes that ultimately result from the selective increase or decrease

in the biosynthesis of a large number of distinct proteins (Moons et al., 1995; Akihiro et al., 2002; Pitzschke et al., 2006). These changes in protein patterns are due at least in part to changes in the transcriptional activity of the corresponding genes. Ozone-induced proteins are supposed to be one of the proteins to be involved in coping with unfavorable conditions for plant. Ozone-induced gene (OZI) was first reported by Yogesh et al., (1995). They found that the accumulation of a number of OZI mRNAs induced during a hypersensitive response was also induced by ozone. Until now, OZI has been cloned in two species of plants including *Arabidopsis thaliana* and *Zea mays*. Before we have constructed a cDNA library of *Polygonum sibiricum* Laxm mixed leaves, some salt stress induced gene was selected from EST library of *P. sibiricum* Laxm including the *PcOZI-1*. We presume that the *PcOZI-1* has a tight connection with salt stress (Liu et al., 2008, 2009). In this report, we present the molecular and functional characterization of the *PcOZI-1*. Further-

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Abbreviations: OZI, Ozone-induced gene; EST, expressed sequence tag; cDNA, complementary DNA; RACE, Rapid amplification of cDNA ends; *PcOZI-1*, *Polygonum sibiricum* Laxm ozone-induced gene transcript; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; ORF, open reading frame; SDS, sodium dodecyl sulfate; UTR, 5'-untranslated region.

more, we demonstrated here that *PcOZI-1*, which was expressed in the leaves, stem and underground stem, responds to various salt stresses. To our knowledge, this is the first experimental evidence that *PcOZI-1* have different express modes in leaves, stem and underground stem under the salt stress.

MATERIALS AND METHODS

Plant

P. sibiricum Laxm obtained from saline-alkali fields in Zhaodong, Heilongjiang and grown in phytotron at 24°C. The samples were treated at different stages using 3% NaHCO₃. A total of 150 *P. sibiricum* Laxm (plant high 10-15 cm) were allotted into 5 treatments randomly which was 0 h (blank) 8 h, 24 h, 48 h and 72 h (Liu et al., 2008). Each treatment consists of 6 replicates with 5 *P. sibiricum* Laxm each. After harvesting, all samples were immediately maintained in liquid nitrogen and kept at -80°C until they were used for isolating the RNA.

RNA isolation from *P. sibiricum* Laxm and reverse transcription (RT)

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

Obtaining 3' and/or 5' regions by RACE

To isolate the complete 5' and 3' regions of this gene, the rapid amplification of cDNA ends (RACE) method was used. First-strand cDNA synthesis was performed using SmartTM RACE cDNA amplification kit (Clontech). We have previously obtained the *PcOZI-1* 3' EST sequences from the *P. sibiricum* Laxm cDNA library constructed. According to the EST, two specific primers were designed on the basis of the *PcOZI-1* 3'UTR for 5'-RACE. *PcOZI* N:3'-GTAACCTTAGTAGAAAACAACC-5', *PcOZI*:3'-GCACTACTAAA GTTTCAAATT-5'. The 5' 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 were designed to amplify the ORF, the primer: *OZI-A*:5'-GCCAACGGCAATAAACAA-3', *OZI-S*: 5'-GGGGCGAGTAACCCTA AA-3', which contained ATG, the start codon sites and the primer 3' which contained TAA, the stop codon sites.

Subcloning

The PCR fragments were subjected to electrophoresis on 0.8% agarose gel for length differences and amplified cDNA fragments were cloned into the pGEM-T Easy vector following the instructions provided (Promega, Madison, WI, USA). 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 *PcOZI-1* gene sequence was analysed and compared using the BLASTP and ORF search programs with GeneBank

database search. The multiple sequence alignment of *PcOZI-1* gene was created by using the Clustal W analysis program. Predicting signal-peptide site by Signal P3.0. ProtParam (<http://au.expasy.org/tools/protparam.html>) compute the OZI protein MW and pI.

Quantification of *PcOZI-1* gene expression by real-time PCR

Total RNAs were isolated by SDS method from different tissues including stem, underground stem and leaf at different handling stages inducing by 3% NaHCO₃, which is 0 h (blank), 8 h, 24 h, 48 h and 72 h. The residue of DNA were removed by DNase I digesting at 37°C for 30 min. Four (4) microgram of the total RNA were used in each lane and electrophoresed in a 0.8% agarose gel, at 100 V 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 six as the primers according to reaction conditions recommended by the manufacturer's instructions. The cDNA was used for the assay of quantitative real-time PCR. The SYBR Green I real-time PCR assay was carried out in an Option- \square Sequence Detection System (MJ Research, USA). The amplifications were performed in a 96-well plate in a 25 μ l reaction volume containing 12.5 μ l of 2 \times SYBR Green Master Mix (TARAKA), 2.5 μ l (each) OZI-F and OZI-R primers (10 mM), 1 μ l of template and 9 μ l of DEPC-water. The thermal profile for SYBR Green real-time PCR was 95°C for 2 min, followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. In a 96-well plate, each sample was conducted in triple. DEPC-water for the replacement of template was used as negative control. The relative expression was calculated as 2^{- $\Delta\Delta$ Ct}, Ct: cycle threshold (Giulietti et al., 2001; Livak et al., 2001).

RESULTS

cDNA cloning of *PcOZI-1*

In order to isolate cDNA encoding from *P. sibiricum* Laxm, PCR reactions were performed using degenerated 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, assigned as *PcOZI-1*, the full-length *PcOZI-1* cDNA fragment of *P. sibiricum* Laxm was obtained by overlapping two cDNA fragments. The full-length *PcOZI-1* cDNA comprised of 514 bp, containing 103 bp in the 5'-untranslated region (UTR), 240 bp in the open reading frame (ORF) and 171 bp in 3'-UTR without poly (A) tail (Figure 1). The ORF encodes a polypeptide of 79 amino acids. The calculated molecular mass of the mature protein (79 amino acids) is 8.45 kDa with an estimated pI of 9.52. Ozone-induced signatures from 4 to 75 were observed (Figure 2).

PcOZI-1 expression in different tissues

Expression of *PcOZI-1* in each organ of *P. sibiricum* Laxm is shown in Figure 5. In a RT-PCR study, specific primers (*PcOZI-1-F*:5-AACCTTTGCTCTGGCTTATGT-3 and *PcOZI-1-R*: 5-GATGATGAAATTCTGGCGACT-3) were used to amplify a 187 bp fragment with cDNA from leaf,

2	GCA CTG GTT CTC TTC AAA AGA TAA GCA GCG CCG AGT AAC CCT AAA	46
47	CAA TAC GCA TCC ATC GTC TCC CCC AAA TCA CAC TCG CCA ACC TTT	91
92	CTC AGG TTG AGT ATG GCA ACC AAA TAC ATT ATT GGT TCT GTT GTT	136
1	M A T K Y I I G S V V	11
137	GGA ACC TTT GCT CTG GCT TAT GTT TTG GAT ACT ACC ATT TCA GAC	181
12	G T F A L A Y V L D T T I S D	26
182	AAG AAG ATA TTT GGA GGT ACT ACC CCT CAC ACA GTT TCC AAT CGC	226
27	K K I F G G T T P H T V S N R	41
227	GAA TGG TGG GAA GAG ACC GAC AAG AAG TTC CAA GCC TGG CCT CGT	271
42	E W W E E T D K K F Q A W P R	56
272	ACC GCT GGA CCT CCG GTT GTA ATG AAC CCA ATA AGT CGC CAG AAT	316
57	T A G P P V V M N P I S R Q N	71
317	TTC ATC ATC AAG TCA ACT GAC TCT TGA AGG TTT TAA GAG CTA GAT	361
72	F I I K S T D S *	79
362	TTT TTT TCT CCT TGA AAT ATA CTG ATT GTT GAA GTT ACT GCT TGA	406
407	TAT TTC ATT GTT GTT TAT TGC CGT TGG CCC ATG TTT ATG CTT CAT	451
452	ATT ATT GCG TTT TGA ACT CTA TTA TGT ACG TGT CTG CAC ACA TTG	496
497	AAT CAT CTT TTG TTG GAA	514

Figure 1. Nucleotide and deduce amino acid sequences of *PcOZI-1* cDNA from *P. sibiricum* Laxm. The PCR products of *PcOZI-1* cDNA were sequenced. The full length was 514 bp, 5'untranslated region of 103 bp, 3'untranslated region of 171 bp and an open reading frame (ORF) encoding 79 amino acid (240 bp).

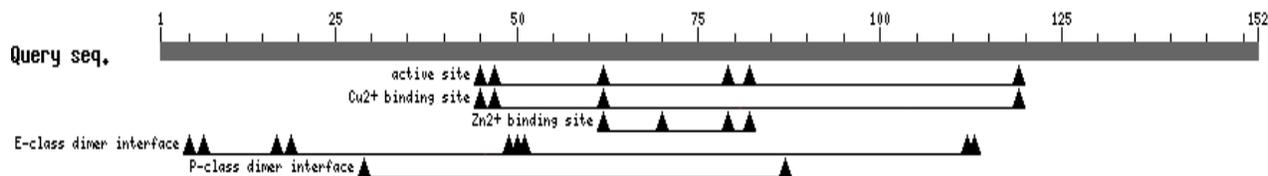


Figure 2. Search for the conserved domains in deduced sequence of amino acid of *PcOZI-1* gene.

stem and underground stem organ using 18S as a positive control. The RT-PCR showed that the *PcOZI-1* was detected in leaf, stem and underground stem. In underground stem, the contents of *P. sibiricum* Laxm *PcOZI-1* is significantly higher than 0 h after 72 h (Figure 5) under the saline-stress. In stem, the contents of *P. sibiricum* Laxm *PcOZI-1* is significantly higher than 0 h after 8 h (Figure 5). That is, in underground stem and stem they were up-regulated during salt stress, whereas in leaf organ, the *PcOZI-1* transcripts were fluctuated after salt stress during 72 h.

DISCUSSION

Differential display was used to identify a novel stress-

induced gene, named *PcOZI-1*. In this work, the full length of a *PcOZI-1* gene was isolated from *P. sibiricum* Laxm by the rapid amplification of cDNA ends method. Analysis of nucleotide sequence revealed that the *PcOZI-1* gene cDNA clone consists of 514 bp, containing 103 bp in the 5' untranslated region, 240 bp in the open reading frame (ORF) that encodes a protein of 79 amino acids and 171 bp in 3' untranslated region. Sequence analysis indicated that the protein, like most of others plant OZI, include the conserve domain from the 4th amino acids to the 75th amino acids (Figures 3 and 4).

Stress-induced genes are sometimes expressed at specific times during normal development and often exhibit some level of tissue specificity in their expression (Liu et al., 2008, 2009). We therefore analyzed the expression pattern of *PcOZI-1* by real-time quantitative PCR. Results

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Arabidopsis thaliana -----MSRATYIIIGALAGSAVVAYVCDKVISDDKFLGGT
Triticum aestivum -----MA-GKYIVAGLVGSCVISYACDYIVSQKKIFGGT
Zea mays -----MT-AGYIVGSLVGSFAIAYLCDTFISDKKAFGGG
Sorghum bicolor -----MT-AGYIVGSLVGSFAIAYLCDTFVSDKKAFGGG
Ricinus communis -----MA-TKYIISALAGSFAIAYVCDQLISDKKIFGGT
Populus trichocarpa -----MS-TKYIVSALVGSFAIAYVCDYVVDKKIFGGT
Polygonum sibiricum Laxm -----MA-TKYIIGSVVGTALAYULDTTISDKKIFGGT
Oryza sativa GDVQFHYPHFQGFIVSRAINQDMA-TKYIVGSVTASFAFAYVCGVYFADKKVLGGT

* * : : : * * * : : : * * * * : : * * * : : : : :
Arabidopsis thaliana PGTIITNKEWGAATEERLQAWPRVAGPPVVMNPISRQNFIVKSRPE-----
Triticum aestivum PGTIVSDKEWLKATEQRFQAWPRVAGPPVIMNPISRQNFIVKDLNP-----
Zea mays PKTVSEKEWQQATDTKFKQAWPRTAGPPVVMNPISRQNFIVKSTE-----
Sorghum bicolor PKTVSEKEWQQATDTKFKQAWPRTAGPPVVMNPISRQNFIVKSTE-----
Ricinus communis PHTVANKDWWEEETDKKFQAWPRTAGPPVVMNPISRQNFIVKPRE-----
Populus trichocarpa PRTVSNKEWWEETDKKFQAWPRTGGPPVVMNPITRQNFIVKSQDS-----
Polygonum sibiricum Laxm PHTUSNREWWEETDKKFQAWPRTAGPPVVMNPISRQNFIIKSTDS-----
Oryza sativa PRTVADKEWGGKVTTEKLDAWPRVAGKPVAMNPVTRQNYVLVKKKKASGSKKASEP

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Figure 3. Full length cDNA sequences of genes were searched using the BLAST algorithm against NR Database from the National Center for Biotechnology Information (NCBI). GenBank accession codes of the corresponding nucleotide sequences are: *A. thaliana* 69% (NP_191995), *Triticum aestivum* 57% (ADB92599), *Z. mays* 78% (NP_001151357), *Sorghum bicolor* 77% (XP_002436372), *Ricinus communis* 72% (XP_002519491), *Populus trichocarpa* 71% (XP_002308950) and *Oryza sativa* 67% (AAT12488).

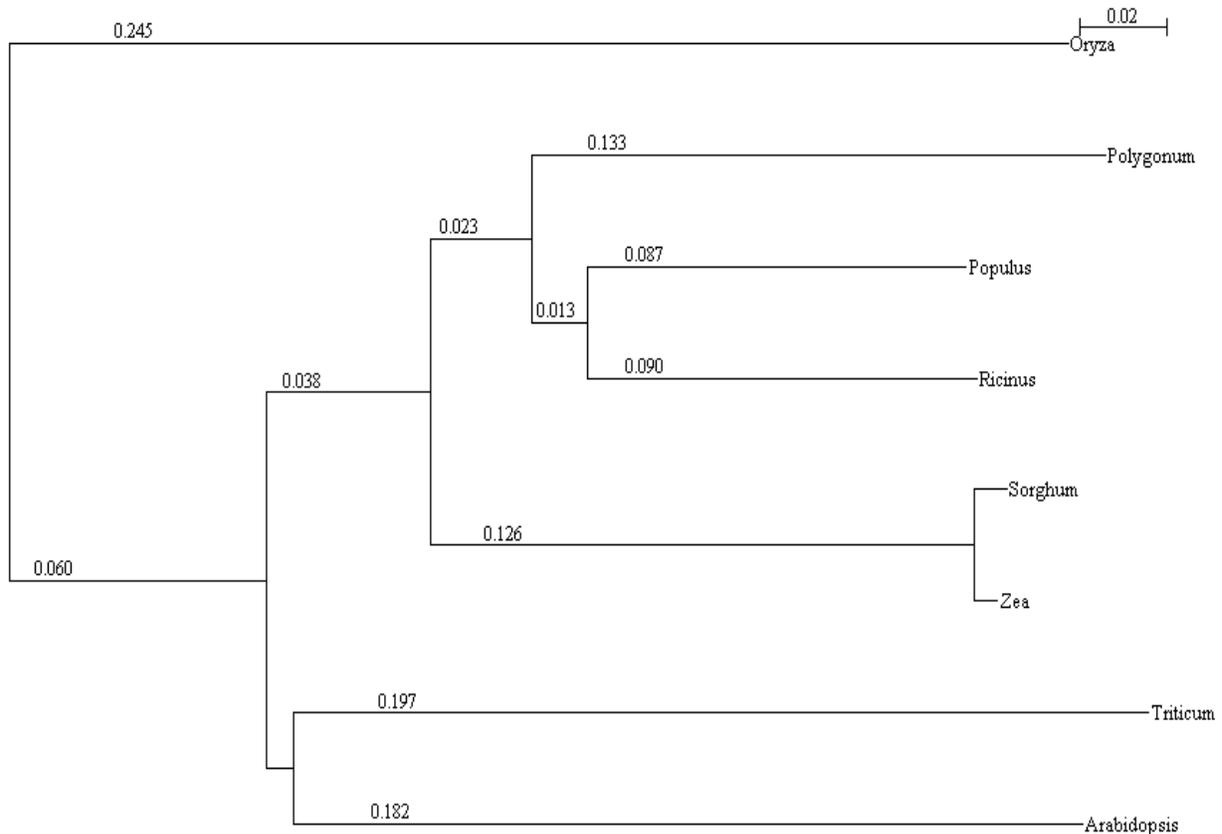


Figure 4. The phylogenetic tree of PcOZI-1 from plants and animals. The tree was based on the amino acid alignment as in Figure 3. The homologous sequences were selected from the BLAST search results as the input for phylogenetic analysis. The phylogenetic analysis was conducted using Treeview software. It showed a higher conservation of the PcOZI-1 with the *Populus* and *Ricinus*.

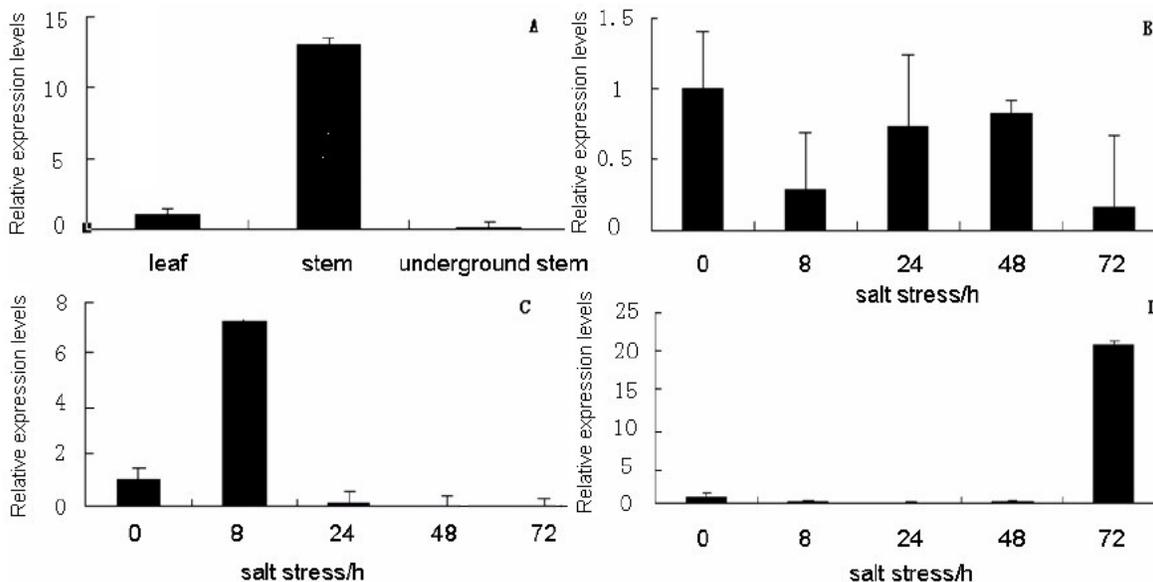


Figure 5. The change of *PcOZI-1* after 3%NaHCO₃ exposure in leaf, stem, underground stem organ. Total RNA was prepared using SDS reagent from the independently 3% NaHCO₃ stress *Polygonum sibiricum* Laxm at 0, 8, 24, 48, 72 h. After digested with DNase I to eliminate the genome contamination, the cDNA was synthesized using the oligo d(T) primer and random 6 primer. Real-time PCR was performed with the DNA Engine Opticon-II sequence detection system. SYBR green Real-time PCR mix (TaKaRa) was used for PCR. (A) The expression of *PcOZI-1* gene in leaf, stem, underground stem organ without stress comparison. (B) The levels of *PcOZI-1* mRNA in leaf tissues. (C) The levels of *PcOZI-1* mRNA in stem tissues. (D) The levels of *PcOZI-1* mRNA in underground stem tissues.

demonstrate that *PcOZI-1* transcripts are differentially expressed at organ specific level during induced by salt stress, but the specific factors underlying the regulatory mechanism have not been clearly understood. Future studies are required to clarify the mechanism by involved in *PcOZI-1* changes in plant physiology and metabolism and may help us gain a better understanding of the molecular genetic basis of the salt damaging effects of *PcOZI-1* on plants.

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