Expression profiles of *PtrLOS2* encoding an enolase required for cold-responsive gene transcription from trifoliate orange

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Abstract

Low expression of osmotically responsive genes 2 (*LOS2*) encodes an enolase (2-phospho-D- glycerate hydrolase, EC 4.2.1.11) that converts 2-phospho-D-glycerate (PGA) to phosphoenolpyruvate (PEP) in the glycolytic pathway in *Arabidopsis*. Meanwhile, it is a transcriptional activator of cold-responsive gene, negatively controlling the expression of *STZ/ZAT10*, a zinc finger transcriptional repressor of cold-responsive gene from *Arabidopsis*. A novel *LOS2* gene, designated *PtrLOS2* (GenBank accession number GQ144341), was isolated from trifoliate orange [*Poncirus trifoliata* (L.) Raf.]. The *PtrLOS2* cDNA is 1 662 bp in length with a 1 338 bp open reading frame (ORF), encoding a deduced 445 amino acid residue protein with a predicted molecular mass of 47.79 kDa and an isoelectric point of 5.54. The deduced protein of the *PtrLOS2* gene shares high identity (over 86 %) with other plant species enolase, which suggests that the *PtrLOS2* probably encodes an enolase. Sequence alignment showed that PtrLOS2 protein has a conserved DNA-binding and a repression domain. Moreover, a conserved start site of alternative translation for the c-myc promoter binding protein (MBP-1) was also found in PtrLOS2 protein. *PtrLOS2* was constitutively expressed in leaves, stems and roots. *PtrLOS2* expression in roots and stems was much higher than that in leaves under normal conditions, however, the expression of *PtrLOS2* was up-regulated in leaves, but down-regulated in roots after cold treatments. The *PtrLOS2* expression in stems was firstly up-regulated and then down-regulated after cold treatments. Meanwhile, after ABA treatment, the expression of PtrLOS2 was up-regulated in leaves but in stems and roots firstly down-regulated followed with up-regulation.

Additional key words: abscisic acid, cold stress, *Poncirus trifoliata*, RACE, RT-PCR.

Introduction

Cold stress is one of the most important environmental factors that limit plant growth and distribution. Most plants from tropical or subtropical regions can increase their freezing tolerance by being exposed to low, nonfreezing temperatures, a process known as cold acclimation (Guy 1990, Thomashow 1999). Plant cold acclimation is associated with various biochemical and physiological changes, such as alterations in lipid composition, leakage of plant metabolites, protein phosphorylation and cytosolic Ca^{2+} influx (Monroy and Dindsa 1995, Reddy and Reddy 2004, Sharma *et al*. 2005, Wang *et al*. 2006). These biochemical and physiological changes stimulate the transcription of hundreds of genes, such as cold regulated (*COR*), low-temperature induced (*LTI*), cold induced (*KIN*), or responsive-to-desiccation (*RD*) (Hajela *et al*. 1990, Kurkela and Borg-Franck 1992, Nordin *et al*. 1993, Thomashow 1999, Viswanathan and Zhu 2002, Kaplan *et al*. 2004). Moreover, a large number of transcription factors which positively or negatively control the expression of cold responsive genes, such as C-repeat binding factor/dehydration-responsive element-binding factor (CBF/DREB), myelocytomatosis (MYC) and myeloblastosis (MYB) family transcription factor, have been identified in plants (Jaglo *et al*. 2001, Gilmour *et al*. 2004, Zhang *et al*. 2004, Chinnusamy *et al*. 2006, Yamaguchi-Shinozaki and Shinozaki 2006, Zhu *et al*. 2007).

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Abbreviations: CBF/DREB - C-repeat binding factor/dehydration-responsive element-binding factor; COR - cold regulated; LTI - low-temperature induced; LOS2 - low expression of osmotically responsive gene 2; MBP-1 - c-myc promoter binding protein; MYB - myeloblastosis; MYC - myelocytomatosis; ORF - open reading frame; PEP - phosphoenolpyruvate; PGA - 2-phospho-D-glycerate; RACE - rapid amplification of cDNA ends; RD - responsive-to-desiccation; RT - reverse transcript; STZ/ZAT10 - salt-tolerance zinc finger ZAT10.
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There are at least two pathways in the stress responsive gene transcriptional system, one ABA dependent and the other ABA independent. Cold responsive genes express largely through the ABA-independent pathway (Thomashow 1999, Shinozaki and Yamaguchi-Shinozaki 2000, Yamaguchi-Shinozaki and Shinozaki 2006). However, ABA dependent pathway also plays little but significant role (Gusta *et al*. 2005) in cold responsive gene transcriptional system. At present, the best understood cold responsive gene regulatory system that belongs to ABA-independent pathway has been identified in *Arabidopsis*. CBF family transcription factors, which play an important role in this system, can bind to the C-repeat/dehydration-responsive element (CRT/DRE) *cis*-elements in the promoters of the cold responsive genes and activate their expression (Yamaguchi- Shinozaki and Shinozaki 1994, Stockinger *et al*. 1997, Liu *et al*. 1998, Thomashow 2001).

Recently, an *Arabidopsis* mutant with substantially reduced cold-induced gene expression, *los2*, was isolated through genetic screen of transgenic *Arabidopsis* with *RD29A-LUC* reporter gene (Lee *et al*. 2002). After cold treatment, *los2* mutation blocks the expression of cold responsive genes, such as *COR15A*, *COR47*, *KIN* and alcohol dehydrogenase (*ADH*). However, *CBF* transcript level is not affected in the *los2* mutation, implying that *LOS2* does not function upstream of the *CBF*. Moreover, the down-regulated expression of cold responsive genes is not significantly detected after ABA, NaCl or PEG treatments, indicating *los2* has a specific defect in cold signaling. The *los2* mutant is chilling sensitive in the light but not in the dark, but the effect of the mutation on cold-responsive gene expression is not dependent on light. These observations suggest that the chilling sensitivity of *los2* plants in the light may be related to metabolic imbalance caused by photosynthesis. Positional cloning reveals that *LOS2* gene encodes an enolase (2-phospho-*D*-glycerate hydrolase, PGA, EC 4.2.1.11) that converts PGA to phosphoenolpyruvate (PEP) in the glycolytic pathway (Lee *et al*. 2002). It is known that salt-tolerance zinc finger ZAT10 (*STZ/ZAT10*) is a negative regulator of cold-responsive genes. *CBF* family genes may play a positive role in inducing *STZ/ZAT10* expression under cold stress because overexpressing CBF3 in *Arabidopsis* increases the expression of *STZ/ZAT10* (Maruyama *et al*. 2004). However, LOS2 protein can bind to the TATA box region of the *c-myc* promoter of *STZ/ZAT10* and represses its expression (Lee *et al*. 2002). Above all, *LOS2* is critical for cold acclimation. It is a positive regulator of cold-responsive genes, which negatively controls the expression of *STZ/ZAT10*.

Citrus is one of the most important economic fruit trees in the world, which is mostly grown in tropic and subtropic regions. Cold stress is a major environmental factor limiting the distribution and productivity of citrus, leading to significant economic losses. Cold resistance breeding is the major method for enhancing the cold resistance of citrus. Traditional breeding methods of citrus have many problems, such as long juvenility periods, polyembryony, inbreeding depression, self- and cross-incompatibility, limiting the producing of cold resistance cultivars. With the rapid development of citrus tissue culture techniques (Tavano *et al*. 2009), genetic engineering techniques become more and more popular in citrus breeding. In order to improve plant cold resistance through genetic engineering, the exact regulatory network of cold-responsive genes must be recognized. Citrus relative species, trifoliate orange (*Poncirus trifoliata*, which can withstand temperatures of -16 °C after being acclimated (Yelenosky 1968), is usually used as rootstocks in cold resistance breeding to produce citrus genotypes with enhancing cold tolerance. In this study, a novel cold regulative gene, *PtrLOS2*, was isolated from trifoliate orange. Furthermore, the expression pattern of *PtrLOS2* after cold and ABA treatments in leaves, stems and roots of trifoliate orange was studied using semi-quantitative RT-PCR.

Materials and methods

Seeds of trifoliate orange [*Poncirus trifoliata* (L.) Raf.] from Huazhong Agriculture University (Wuhan, Hubei province, China) were germinated in soil and grown for 3 months in a greenhouse at 25 °C and about 16-h photo-period with natural irradiance.

Total RNA was extracted from the leaves using *Trizol* reagent (Tiangen, Beijing, China) following the manufacturer's instruction. The first-strand cDNA was synthesized using *ReverTra Ace-α-*^{TM} first strand cDNA synthesis kit according to the manufacturer's instruction (*Toyobo*, Osaka, Japan).

Expressed sequence tags (EST) of Citrus (274), which has high identity with *Arabidopsis LOS2* gene, was selected from the *HarvEST-Citrus* database (University of California, USA) (http://harvest.ucr.edu/) and a consensus sequence was got by sequence alignment program of this database. On the basis of this consensus sequence, a pair of gene special primers (5'-CTCTTGTCGCACTCACA GAC-3') (sense) and (5'-AGCACCGCAACTAGATA CTC-3') (antisense) were designed for gene fragment cloning. RT-PCR was performed using 1 mm^3 of the first-strand cDNA as template in 50 mm^3 volume containing 37.5 mm^3 H_2O, 5 mm^3 5× PCR buffer, 4 mm^3 2.5 mM dNTP, 1 mm^3 10 μM sense and antisense primes, 0.5 mm^3 5 U mm^{-3} r*Taq* DNA polymerase (*TaKaRa*, Daliang, China). The PCR reaction was performed under the following conditions: 1 cycle of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 2 min at 72 °C, 1 cycle of 10 min at 72 °C. The sequencing result shows that the exact length of the amplified cDNA fragment was 1633 bp.

The 3'-RACE ready cDNA was synthesized from 4 μg

of total RNA extracted from trifoliate orange leaves with 3'CDS (5' AAGCAGTGGTATCAACGCAGAGTAC $(T)_{30}$VN3') as primer using *ReverTra Ace-α-*^{TM} first strand cDNA synthesis kit according to the manufacturer's instruction (*Toyobo*). In order to get 3'end of *LOS2* gene from trifoliate orange, 3' RACE were carried out according to *SMART*^{TM} RACE kit following the instructions provided (*Clontech*, Palo Alto, USA). Two nested gene special primers of LOS2-1 (5' CAA ATGTTGGTGATGAAGGCGGT 3') (sense) and LOS2-2 (5' CCGTCAGAATGTCCAAGCAAGC 3') (sense) pointed at 3'end were designed according to the 1633 bp cDNA fragment of *LOS2* gene previously cloned from trifoliate orange. UPM including 0.4 μM UPL (5' CTA ATACGACTCACTATAGGGCAAGCAGTGGTATCA ACGCAGAGT 3') and 2 μM UPS (5' CTAATACGA CTCACTATAGGGC 3') was used as antisense primer of the first round PCR. NUP (5' AAGCAGTGGTAT CAACGCAGAGT 3') was used as antisense primer of the second round PCR. The first round PCR reaction was carried out in a total volume of 50 mm^3 including 32.75 mm^3 H_2O, 5 mm^3 10× *Ex Taq* buffer, 4 mm^3 2.5 mM dNTP mix, 2 mm^3 first strand cDNA template, 5 mm^3 UPM, 1 mm^3 10 μM LOS2-1 and 0.25 mm^3 5 U mm^{-3} *Ex Taq* DNA polymerase. Touch-down PCR temperature program was 1 cycle of 5 min at 94 °C, 5 cycles of 30 s at 94 °C, 2 min at 72 °C; 5 cycles of 30 s at 94 °C, 30 s at 70 °C, 1.5 min at 72 °C; 25 cycles of 30 s at 94 °C, 30 s at 68 °C, 1.5 min at 72 °C, 1 cycle of 10min at 72 °C. Subsequently, PCR product of the first round PCR was 100-fold diluted and used as template in the nested PCR amplification. The second round PCR was performed using the product of the first PCR as template, NUP and LOS2-2 as primers. PCR temperature program was 1 cycle of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 62 °C, 1.5 min at 72 °C, 1 cycle of 10 min at 72 °C. Sequencing results shows that the exact length of 3'end of *LOS2* gene from trifoliate orange was 491 bp.

By assembling the *PtrLOS2* gene fragment and 3' RACE product which have an overlap fragment using *DNAMAN* software, the full-length sequence of *PtrLOS2* was deduced and an entire open reading frame (ORF) was found using ORF finder programme of *NCBI* (www.ncbi.nlm.nih.gov). To confirm the *PtrLOS2* cDNA sequence, sense prime (5' CTCTTGTCGCACTCAC AGAC 3') and antisense primer (5' AGCACCGCAAC TAGATACTC 3') located in 5'-UTR and 3'-UTR respectively, were designed for RT-PCR using first strand cDNA as template. PCR reaction was carried out in a total volume of 50 mm^{-3} including 36.75 mm^{-3} H_2O, 5 mm^{-3} *Ex Taq* buffer, 4 mm^{-3} 2.5 mM dNTP, 1 mm^{-3} 10 μM sense and antisense primers, 2 mm^{-3} first strand cDNA and 0.25 mm^{-3} 5 U mm^{-3} *Ex Taq* DNA polymerase. PCR temperature program was 1 cycle of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 1.5 min at 72 °C, 1 cycle of 10 min at 72 °C.

BLAST program in National Center for Biotechnology Information Server (http://www.ncbi.nlm.nih.gov/) was used to find the identity of PtrLOS2 with other plant and animal species. *Clustal W* program in *Jalview 2.3* version was used for multiple protein sequence alignment. For phylogenetic and multiple protein sequence alignment analysis, other plant and animal enolase protein sequences were obtained from the database online (http://www.ncbi.nlm.nih.gov/), including *Arabidopsis thaliana* LOS2 (*A.thaliana*, GenBank accession number NP_181192), *Ricinus communis* enolase (*R. communis*, CAA82232), *Populus trichocarpa* enolase (*P. trichocarpa*, EEE71910), *Vitis vinifera* enolase (*V. vinifera*, XP_002267091), *Zea mays* enolase (*Z. mays*, AAD04187), *Oryza sativa* enolase (*O. sativa*, ABB46862), *Brassica rapa subsp. chinensis* enolase (*B. chinensis*, ABD92697), *Lupinus luteus* enolase (*L. luteus*, CAB75428), *Rattus norvegicus* enolase (*R. norvegicus*, AAH81847). The phylogenetic tree of LOS2 proteins was constructed using the Neighbor-Joining algorithm of *MEGA 4* version and 1000 bootstrap replicates were performed for the phylogenetic tree construction. Theoretical isoelectric point and mass values for the protein was predicted using *ExPASy ProtParam* tool (http://www.expasy.org/prosite/).

For the expression analysis, trifoliate orange seedlings were cultivated in soil at a greenhouse for 3 months at above mentioned conditions. For the cold treatments, the plants were placed in a 4 °C refrigerator for 0, 15, 30 min, 1, 2, 4, 8, 16, 24, 48 and 72 h, respectively. For the ABA treatments, the seedlings were removed from the soil and placed in Hoagland solution contained 0.1 mM ABA for the same periods. The materials were frozen immediately in liquid nitrogen and stored at -70 °C. Total RNA was extracted using *Trizol* reagent. The first-strand cDNA was synthesized with 4 μg total RNA in 20 mm^{-3} reaction volume using *ReverTra Ace-α-*^{TM} first strand cDNA synthesis kit.

Semi-quantitative RT-PCR was carried out to investigate the expression of *PtrLOS2* in different tissues including leaves, roots and stems of trifoliate orange. *LOS2* gene fragments of trifoliate orange were amplified using specific sense primer (5' TGAACTAAGAG ACGGAGGCT 3') and antisense primer (5' CTTGCTTGG ACATTCTGACG 3'). The quantity of each template was adjusted to an equal level by amplification of 18S RNA gene from trifoliate orange (GenBank accession number AF206997) using the sense primer (5′ TCTGCCCGT TGCTCTGATGAT 3′) and antisense (5′ CCTTGGAT GTGGTAGCCGTTT 3′). The PCR condition was as follows: 5 min pre-amplification at 94 °C, 25 cycles of 30 s at 94 °C, 40 s at 60 °C, 1 min and 30 s at 72 °C, a final extension of 10 min at 72 °C. The products were subjected to electrophoresis on 1 % agarose gel.

Results

Isolation and sequence analysis of *PtrLOS2*: Based on the RT-PCR and RACE strategy, a novel gene designated as *PtrLOS2* (GenBank accession number GQ144341) was isolated from trifoliate orange. The *PtrLOS2* cDNA is 1662 bp in length with a 1338 bp ORF, encoding a deduced 445 amino acid residue protein with a predicted molecular mass of 47.79 kDa and an isoelectric point of 5.54.

PtrLOS2 sequence homology was verified by database searching at the National Center for Biotechnology Information server using *BLAST* algorithm. The results revealed that PtrLOS2 protein shares high identity with other species enolase, such as *A. thaliana* (88 %), *R. communis* (93 %), *P. trichocarpa* (92 %), *V. vinifera* (91 %), *Z. mays* (88 %), *O. sativa* (88 %),

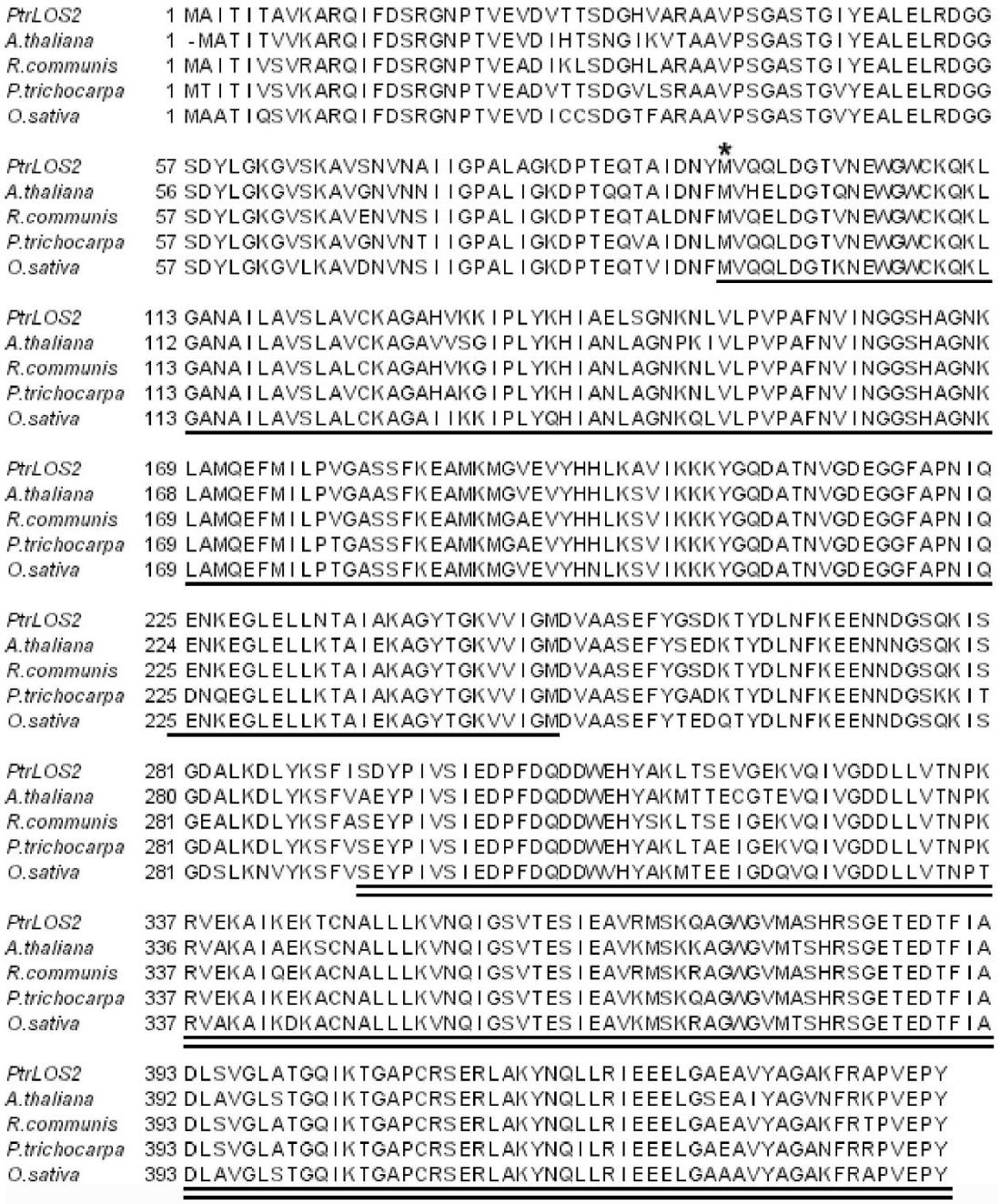

Fig. 1. Multiple amino acid sequence alignment among the PtrLOS2 protein with the corresponding protein sequences of other plants. The amino acid sequence is displayed in one-letter code under the coding sequence. The DNA-binding domain is underlined, and the transcriptional repression domain is double-underlined. The start site of alternative translation for the c-myc promoter binding protein (MBP-1) is indicated by *asterics*.

B. *chinensis* (89 %), *L*. *luteus* (86 %), *R. norvegicus* (69 %). It is well known that *Arabidopsis LOS2* gene encodes an enolase (Lee *et al*. 2002). To confirm the enolase activity of PtrLOS2 protein, five known plants enolase proteins were used for alignment. Multiple sequence alignment was performed using the *ClustalW* of *Jalview 2.3* version. Alignment analysis revealed that the whole amino acid sequence of the PtrLOS2 protein was highly conserved compared with other species enolases (Fig. 1). There are two functional domains including the DNA-binding domain and the transcriptional repression domain found in PtrLOS2 protein sequence and other enolases (Fig. 1).

To investigate the evolutionary relationship between PtrLOS2 and other plants species enolase, a phylogenetic tree was constructed using Neighbor-Joining algorithm of *MEGA 4* version. The *R. norvegicus* enolase protein (animal protein) was defined as the outgroup for purposes of tree-rooting. The phylogenetic analysis revealed that the enolase family can be divided into two groups. The first group includes only one animal protein of *R. norvegicus*, whereas the other plants compose the second group. The second group is subdivided into four subgroups. The first subgroup is composed of PtrLOS2, *R. communis*, *P. trichocarpa*, the second subgroup is composed of *V*. *vinifera*, *Z. mays* and *O*. *sativa*, the third subgroup is composed of *A*. *thaliana* and *B*. *chinensis*, the fourth subgroups is composed of *L*. *luteus* solely (Fig. 2).

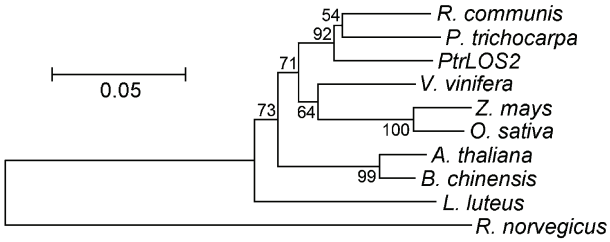

Fig. 2 . Phylogenetic tree of the PtrLOS2 and enolase proteins sequences from different plant and animal species. Construction of the phylogenetic tree was performed with Neighbor-Joining algorithm of *MEGA 4* version. Numbers at each node represent bootstrap values for 1000 replicates.

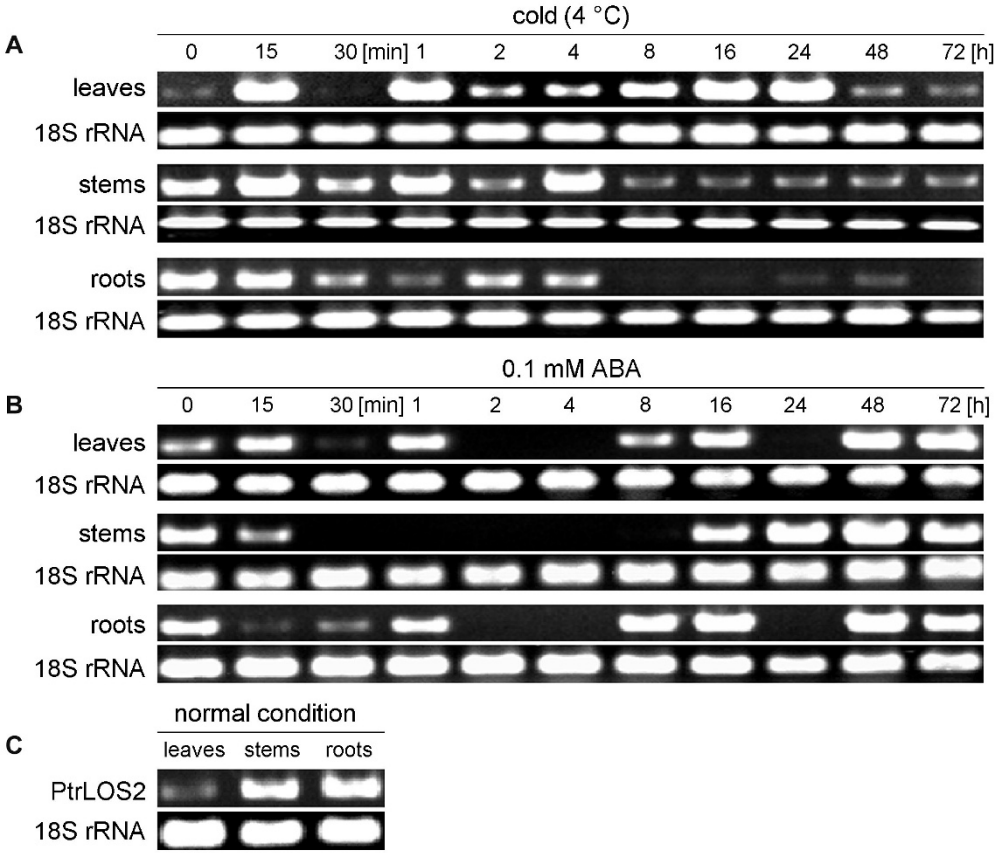

Fig. 3. Expression of *PtrLOS2* gene in different tissues. Trifoliate orange *18S rRNA* was amplified as a control to ensure the equal amounts of templates were added to each RT-PCR reaction. Cycle numbers of RT-PCR amplification were 25 for each gene. *A* - Expression patterns of the *PtrLOS2* gene in leaves, stems and roots of trifoliate orange induced by cold treatment (4 °C). Total RNA was extracted at different times after cold treatment. *B* - Expression patterns of the *PtrLOS2* gene in leaves, stems and roots induced by ABA treatment (0.1 mM). Total RNA was extracted at different times after ABA treatment. *C* - Expression patterns of the *PtrLOS2* gene in leaves, stems and roots under normal condition.

Expression patterns of *PtrLOS2* gene after cold and ABA treatments: Semi-quantitative RT-PCR was used to identify the expression pattern of the *PtrLOS2* gene in different tissues of trifoliate orange after cold and ABA treatments. In leaves, low level of *PtrLOS2* expression was detected under normal condition. At 15 min after cold treatment, *PtrLOS2* expression increased rapidly and transitorily, but declined to almost undetectable at 30 min after cold treatment and recovered quickly at 1 h. However, *PtrLOS2* expression level declined again at 2 h after cold treatment and then recovered gradually until 24 h, at which the expression reached the maximum level (Fig. 3*A*). Meanwhile, the *PtrLOS2* expression pattern in leaves before 16 h after ABA treatments was similar to that after cold treatments. At 24 h after ABA treatment, *PtrLOS2* expression declined to undetectable level and then increased to maximum from 48 h to 72 h after ABA treatments (Fig. 3*B*). Reversely, the maximum of *PtrLOS2* expression appeared at 24 h and declined strongly from 48 to 72 h after cold treatment (Fig. 3*A*).

In stems, high level of *PtrLOS2* expression was detected from normal condition to 4 h after cold treatments. Subsequently, the *PtrLOS2* expression declined to the minimum level from 8 h to 72 h after cold treatments (Fig. 3*A*). Meanwhile, *PtrLOS2* expression declined slightly at 15 min after ABA treatment. Subsequently, the *PtrLOS2* expression level declined to undetectable from 30 min to 8 h. From 16 h to 72 h, the *PtrLOS2* expression recovered to high level. The maximum level of *PtrLOS2* expression appeared at 48 h after ABA treatment (Fig. 3*B*).

In roots, *PtrLOS2* expression was detected under normal condition and declined slightly from 30 min to 4 h after cold treatments. Finally, the *PtrLOS2* expression declined sharply to almost undetected from 8 h to 72 h after cold treatments (Fig. 3*A*). Meanwhile, at 15 min after ABA treatment, the *PtrLOS2* expression declined to almost undetectable and then increased gradually from 30 min to 1 h. From 1 h to 72 h after ABA treatments, *PtrLOS2* expression pattern in roots was similar to that in leaves. The maximum level of *PtrLOS2* expression in roots appeared at 48 h after ABA treatment (Fig. 3*B*).

Expression of *PtrLOS2* in different tissues of trifoliate orange under normal condition: The different expression of *PtrLOS2* in different tissues under normal conditions was also examined in trifoliate orange by semi-quantitative RT-PCR. The results showed that the *PtrLOS2* expression in roots and stems is much higher than in leaves (Fig. 3*C*).

Discussion

In this study, a novel *LOS2* gene, designated *PtrLOS2*, was cloned from leaves of trifoliate orange. The deduced protein of the *PtrLOS2* gene shares high identity (over 86 %) with other plant species enolase, which suggested that the PtrLOS2 protein belongs to the enolase family. Enolase (2-phospho-*D*-glycerate hydrolase, EC 4.2.1.11) has been found in both plants and animals (Feo *et al*. 2000, Subramanian *et al*. 2000). Alignment analysis showed that PtrLOS2 protein has a conserved DNA-binding and repression domains. Moreover, a conserved start site of alternative translation for the *c-myc* promoter binding protein (MBP-1) was also found in PtrLOS2 protein (Fig. 1). MBP-1 is a 37 - 38 kDa protein that binds to the c-myc P2 TATA motif and represses *c-myc* gene expression in human cell (Ray and Miller 1991). Interestingly, in human cells, part of the enolase has been identified as MBP-1 since the two genes share 97 % similarity at the cDNA level and both genes have been mapped to the same region of human chromosome 1 (Giallongo *et al*. 1986). *Arabidopsis LOS2* gene, which encodes an enolase, can bind to the *cis*-element of the human *c-myc* gene promoter. Moreover, *Arabidopsis LOS2* can also bind to the promoter of *STZ/ZAT10*, a zinc finger transcriptional repressor of cold responsive genes, and repress the expression of *STZ/ZAT10*. Therefore, *Arabidopsis LOS2* positively regulated cold responsive gene expression and plays an important role in enhancing cold resistance (Lee *et al*. 2002). Because PtrLOS2 protein shares high identity (over 86 %) with *Arabidopsis* LOS2 and other plant species enolase, *LOS2* gene must be highly conserved during evolution and further demonstrating their critical roles in cold resistance in plants.

As mentioned above, *PtrLOS2* probably encodes an enolase which plays an important role in glycolytic pathway. Because glycolytic activity is expected to be higher in nonphotosynthetic tissues and the expression of enolase gene in roots is much higher than in leaves of *Arabidopsis* and tomato (Van der Straeten *et al*. 1991). We investigated whether this difference expression of *PtrLOS2* gene in different tissues was also observed in trifoliate orange. The results showed that *PtrLOS2* expression in roots and stems was much higher than that in leaves under normal condition (Fig. 3*C*).

The expression of *LOS2* gene was constitutively detected in *Arabidopsis* and slightly up-regulated in response to cold treatment (Lee *et al*. 2002). In this paper, *PtrLOS2* expression was detected under normal condition in leaves, stems and roots, suggesting that *PtrLOS2* is also a constitutive expression gene (Fig. 3*C*). In general, after cold treatment, the *PtrLOS2* expression was up-regulated in leaves. However, unlike *Arabidopsis LOS2*, the *PtrLOS2* expression level in leaves increased quickly and transitorily at 15 min after cold treatment and declined to almost undetectable level at 30 min after cold treatment. This rapid and transient reduction of *PtrLOS2* expression in response to cold treatments was also detected in the stems. Moreover, the *PtrLOS2* expression showed recurrent fluctuation changes in response to cold treatments in leaves, stems and roots. To our surprise, the *PtrLOS2* expression was down-regulated in roots (Fig. 3*A*).

Because *PtrLOS2* expression is much higher in roots than in leaves at normal condition, perhaps the high expression of PtrLOS2 will activate the expression of some negative regulator to limit further *PtrLOS2* expression. *Arabidopsis LOS2* gene is a negative regulator of *STZ/ZAT10* (Lee *et al*. 2002). Because PtrLOS2 protein shares high identity (over 86 %) with *Arabidopsis* LOS2, it is also deduced to have negative regulator activity. As a negative regulator, the decline expression of *PtrLOS2* may be necessary for the accumulation of its downstream genes.

Abscisic acid (ABA) acts as a signaling molecule mediating responses to a lot of abiotic stresses including cold stress (Leung and Giraudat 1998, Thomashow 1999, Xiong *et al*. 2002). ABA accumulation in plants can lead to a number of physiological changes including changes in membrane structure, modulation of polyamines and soluble sugars, as well as changes of specific gene expression (Lee *et al*. 1997). There is much overlap in the expression pattern of stress genes after cold, drought, salt, or ABA treatment (Leung and Giraudat 1998, Thomashow 1999, Shinozaki and Yamaguchi-Shinozaki 2000, Finkelstein *et al*. 2002). In order to confirm whether the *PtrLOS2* expression is affected by ABA, we examined the expression pattern of *PtrLOS2* after ABA treatments. Generally, the expression of *PtrLOS2* is up-regulated in leaves after ABA treatments. However, the *PtrLOS2* expression pattern in roots and stems is down-regulated followed by up-regulation after ABA treatment (Fig. 3*B*). The *PtrLOS2* expression also shows fluctuation changes in response to ABA treatment in leaves, stems and roots (Fig. 3*B*), suggesting the *PtrLOS2* expression is regulated by ABA and confirm the negative regulator activity of *PtrLOS2*.

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