

Characterization of an Oxidative Stress Inducible Nonspecific Lipid Transfer Protein Coding cDNA and its Promoter from Drought Tolerant Plant *Prosopis juliflora*

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Abstract Abiotic stress is the principal cause of crop failure worldwide. *Prosopis juliflora* is a hardy plant reported to be tolerant to drought, salinity, extremes of soil pH, and heavy metal stress. Here, we report the characterization of a cDNA clone for a putative nonspecific lipid transfer protein (Pj LTP1) found abundantly in a drought stressed leaf cDNA library of *P. juliflora* and its promoter. A multiple sequence analysis of Pj LTP1 with well-established and officially designated nonspecific lipid transfer proteins allergens revealed high similarity at the amino acid level. Northern analysis of Pj LTP1 in *P. juliflora* leaves under oxidative stress revealed steady upregulation at the time points analyzed. A 929-bp fragment was isolated from the 5' end of Pj LTP1, and transient reporter gene expression studies revealed it to be a functional promoter. Several cis-acting elements previously reported to function in stress response were found in this promoter. The possible reasons for changes in gene expression during stress in relation to the host plant's stress tolerance mechanisms are discussed.

Keywords *Prosopis juliflora* · nsLTP · Promoter · Allergen

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Introduction

Plant growth and productivity are adversely affected by various abiotic and biotic stress factors. Abiotic stress is the principal cause of crop failure worldwide, causing a dip in average yields for most major crops by more than 50% (Boyer 1982; Bray et al. 2000). The development of crop plants able to grow in these adverse conditions assumes importance in this emerging global context. Despite the wealth of information on abiotic stress and stress tolerance in plants, many aspects still remain unclear. One of the effective ways of analyzing a stress response is to use model organisms, chosen either for their relative amenability for study for their tolerance to the stress in question.

Prosopis juliflora, a leguminous phreatophytic perennial deciduous thorny small tree (Burkart and Simpson 1977), is now the dominant woody plant found in about 45 million hectares of grazing lands worldwide from sea level to 1,500 m. It grows in very hot and dry regions with temperatures as high as 48°C and annual precipitation of 150–750 mm (Drake 1993; Geilfus 1994). The roots penetrate to great depths in the soil, and the tree can grow in a variety of areas including saline, alkaline, sandy, and rocky soils. *P. juliflora* can withstand high leaf-to-air vapor pressure deficit by reducing metabolic activity and by effective adjustments in the partitioning of electron flow between assimilation and non-assimilation processes, which in turn imposes a strong limitation on the potential carbon gain (Shirke and Pathre 2004).

Plant LTPs are small basic cytoplasmic proteins defined by their ability to facilitate the transfer of phospholipids between membranes in vitro (Kader 1996; Kader et al. 1984). On the basis of molecular mass and disulfide bond assignments, nonspecific lipid transfer proteins (nsLTPs)

are classified into two major subfamilies: nsLTP1 (*9 kDa) and nsLTP2 (*7 kDa; Wang et al. 2007). nsLTP1 family composes small basic peptides of 90 to 94 amino acids, arranged in a three-dimensional structure formed by four α -helixes with a long carboxy-terminal tail, generally devoid of defined secondary structure, except by the presence of one turn of 310 helix (Shin et al. 1995; Lee et al. 1998). The most striking structural characteristic is an internal hydrophobic cavity in the peptide structure, whose volume is variable depending on the loaded molecule. Through this flexible cavity, LTP1 are capable of reversibly binding and transporting of hydrophobic molecules such as phospholipids, acyl CoA, and fatty acids of different sizes (Carvalho and Gomes 2007). Plant LTPs have been proposed to be involved in cutin biosynthesis or surface wax formation, embryogenesis and development (Kader 1996; Soufleri et al. 1996), pollen adherence to the stigma during pollen elongation (Park et al. 2000), adaptations of plants to environmental changes (Dunn et al. 1998; Kader 1996), β -oxidation (Tsuboi et al. 1992), plant defense signaling (Buhot et al. 2001; Blein et al. 2002; Maldonado et al. 2002), and plant defense against phytopathogens (Carvalho and Gomes 2007).

In this paper, we report the isolation and characterization of a cDNA clone for a putative nsLTP coding cDNA and its promoter sequence from *P. juliflora*. This cDNA clone was obtained from a cDNA library created from drought-stressed leaf tissue of 2-month-old *P. juliflora* plants and happened to be one of the most abundant EST clones in this stress-induced transcripts enriched cDNA library (George et al. 2007). The present study was undertaken as a part of the overall program of identifying the stress tolerance mechanisms in *P. juliflora*.

Materials and Methods

Isolation of the cDNA Clone and In Silico Analysis

A cDNA library was created from the leaf tissue of drought-stressed 2-month-old *P. juliflora* seedlings (George et al. 2007). Among the 1,750 random ESTs that were sequenced from this library, those showing maximum similarity to nsLTP in the BLASTX analysis (NCBI, Altschul et al. 1990) formed the second largest category (12 clones). Upon CAP3 analysis (Huang and Madan 1999), these ESTs could be assembled into two contigs, with seven and five clones each. Representative clones from each contig (Pj124 and Pj408, later renamed Pj LTP1 and Pj LTP2, respectively) were sequenced completely (ABI prism 310, Applied Biosystems).

Open reading frames (ORFs) were identified in these cDNAs using ORF finder at NCBI (www.ncbi.nlm.nih.gov),

and the longest ORF with a predicted start and stop codon was identified as the putative coding region. Protein-targeting predictions were done using the Target P 1.1 Server (Emanuelsson et al. 2000; Nielsen 1997). Molecular mass predictions were made using Prot Param (Gasteiger et al. 2005). HMMTOP (Tusnady and Simon 1998, 2001) was used for making putative transmembrane predictions. Multiple sequence alignments were done using the ClustalW program at NPS (Network Protein Sequence analysis). The conserved domains present in the ORFs were identified using the NCBI Conserved Domain Database (CDD; Bauer et al. 2007).

RNA Isolation and Northern Analysis of Pj LTP1

Seeds of *P. juliflora* (Swartz) DC used in this study were collected from Kudankulam, a semiarid region in coastal South Tamil Nadu (India). The seeds were scarified with a blade, treated with 30% H_2SO_4 for 15 min, washed thoroughly and grown in a mixture of soil and sand (1:1) in the greenhouse under a 12-h light/12-h dark photoperiod (illuminated from 0600 to 1800 hours) with daily watering. Two-month-old *P. juliflora* seedlings were then removed from soil, and the roots were washed thoroughly and allowed to acclimate in half strength MS (Murashige and Skoog 1962) solution for 48 h before stress treatment. For oxidative stress, the acclimatized plants were kept in half strength MS containing 90 mM H_2O_2 , and leaf tissue was frozen at 12, 24, and 48 h after stress application. Total RNA from *P. juliflora* leaf tissue was isolated according to Chomczynski and Sacchi (1987). The expression patterns of *Pj LTP1* in *P. juliflora* leaf tissue under H_2O_2 stress at different time points were studied using Northern analysis. Full-length cDNA amplified using UTR-specific primers Pj LTP1 5' UTR F and Pj LTP1 3' UTR R (Table 1) was used as the probe (polymerase chain reaction (PCR) conditions—1 min preamplification at 94°C, 30 PCR cycles of 30 s at 94°C, 40 s at 60°C, 1 min and 30 s at 72°C, and a final extension of 10 min at 72°C). The northern experiments were carried out as described in George et al. (2007).

Isolation of the 5' Upstream Region of Pj LTP1

For isolation of *P. juliflora* genomic DNA, leaves were ground in liquid nitrogen and resuspended in CTAB buffer (2% CTAB, 100 mM Tris-Cl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 1% PVP, 0.2% β -ME). The tubes were incubated at 60°C for 30 min. The sample was extracted once with an equal volume of $CHCl_3$ /IAA and centrifuged at 12,000 $\times g$ for 10 min. To the aqueous phase, 0.5 volume of 5 M NaCl and 0.4 volume of isopropyl alcohol was added and centrifuged at 12,000 $\times g$ for 10 min. The genomic DNA pellet was rinsed in wash buffer (10 mM Ammonium

Table 1 List of primers used in the study

Primer name	Sequence (5'–3')
Pj LTP1 5' UTR F	GTGTAGCCGCAGCATCTGTC
Pj LTP1 3' UTR R	GGCACAAGGGAGAAGCCAT
Pj LTP1 GSP1	CTTCTGCAGGTAAGCGATGCATGG
Pj LTP1 GSP2	GTGCAACCACCAGGGCCACGC
Pj LTP1 GSP3	GAGACCAGCCATTGTTGGGAGC
AD1	NGTCGASWGANAWGAA
AD2	TGWGNAGSANCASAGA
AD3	WGTGNAGWANCANAGA
AD4	STTGNTASTNCTNTGC
Pj LTP1 PRM F	CTGCCAACTCAATTCTCCCATTC

acetate; 76% ethanol), centrifuged at 12,000 rpm for 10 min, air-dried, and resuspended in 10 mM ammonium acetate, 0.25 mM EDTA (pH 8.0). Sequence corresponding to the 5' upstream region of Pj LTP1 was amplified from genomic DNA using thermal asymmetric interlaced PCR (TAIL-PCR) with modifications according to Sessions et al. (2002). Three nested gene-specific reverse primers (Pj LTP1 GSP1–3) and one of four arbitrary degenerate (AD1–4) primers were used in successive rounds of TAIL-PCR cycling. The primary PCR product was diluted 40-fold and used in the secondary reaction, while the latter was diluted 10-fold for the tertiary reaction. The products of the primary, secondary, and tertiary reactions were analyzed on a 0.8% agarose gel. Fragments exhibiting a difference in size consistent with nested gene-specific primer positions were cloned using InsTA Clone PCR cloning kit according to the manufacturer's instructions (MBI Fermentas) and sequenced completely. The AD primers as well as the gene specific reverse primers used are listed in Table 1. The specific products obtained were gel-eluted sequence. The putative cis-acting DNA elements in the isolated 5' regions were identified using the PLACE database (Higo et al. 1999).

Promoter Functional Analysis by Transient Reporter Gene Expression

The Pj LTP1 putative promoter was reamplified from *P. juliflora* genomic DNA using the primers Pj LTP1 PRM F and Pj LTP1 GSP3 (Table 1) and cloned in T/A cloning vector (InsTA Clone PCR cloning kit—MBI Fermentas) according to the manufacturer's instructions. After confirming the sequence, Pj LTP1 putative promoter was released from the T/A cloning vector by restriction digestion with *EcoRI* and *BamHI* and cloned in the promoter fusion vector pCambia 1391z in the *EcoRI* and *BamHI* sites with *Uida* (GUS) as the reporter gene

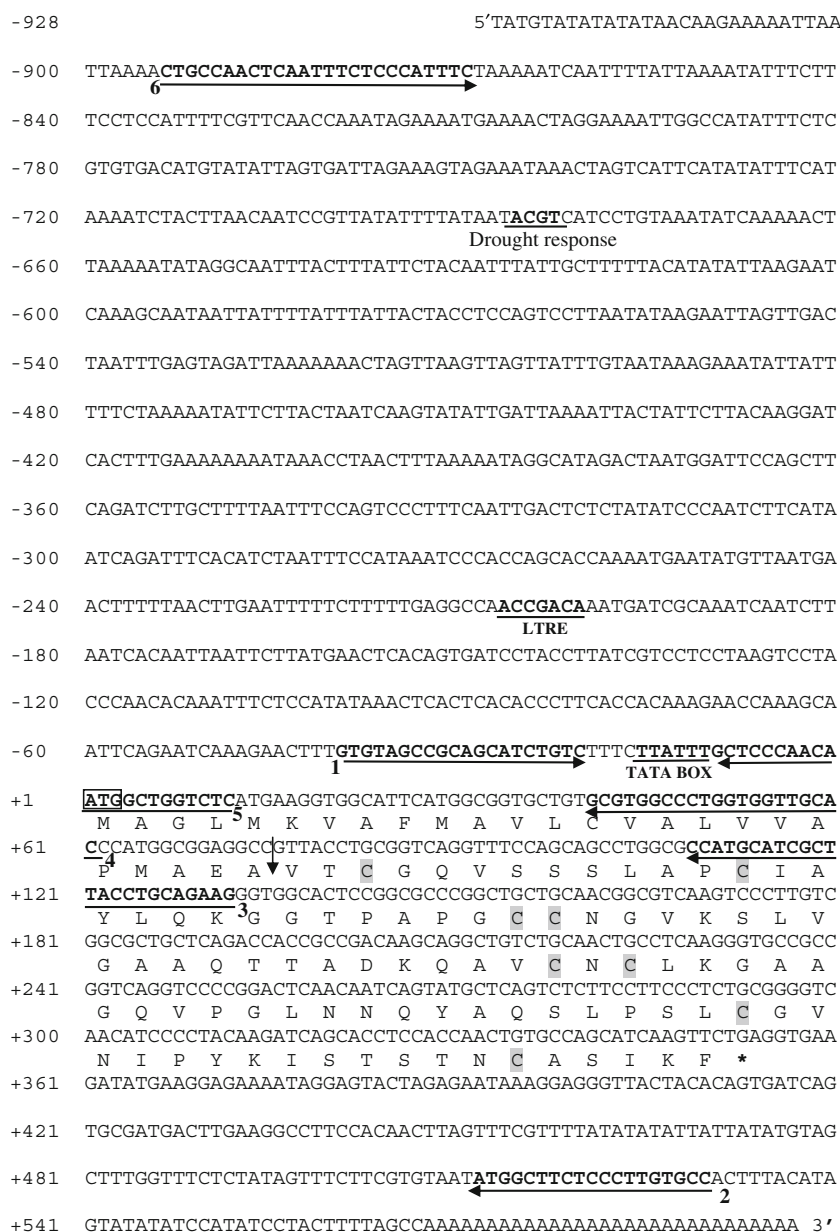
(pPRM-1391z). pPRM-1391z was transformed in to *Agrobacterium* strain LBA4404 by freeze-thaw method. Recombinant *Agrobacterium* colonies were selected on YEP medium supplemented with rifampicin (10 mg/ml) and Kanamycin (50 mg/ml) followed by colony PCR with Pj LTP1 PRMF and Pj LTP1 GSP1 primers. A single positive *Agrobacterium* colony was resuspended in 100 µl of sterile-distilled water and then spread-plated on rif⁺, kan⁺ YEP. After 2 days of incubation in dark at 28°C, the lawn of culture was scraped off carefully and resuspended in half strength MS medium (30) to obtain an optical density of 1–2 at A595. Acetosyringone (100 µM) was added to the suspension and gently mixed for about 10 min. Green house grown tobacco cv. Petit Havana leaves were infiltrated in the epidermal region with this culture using a syringe and needle. After 2 days, GUS staining was carried out in infiltrated leaf regions according to (31). The leaf pieces were mounted in water and examined under the Nikon Optiphot-2 phase contrast microscope using ×10, ×20, and ×40 objectives and photographed with a Nikon CoolPix 4300.

Results

The full-length cDNA of Pj LTP1 has a total length of 668 bp, with an ORF of 354 bp (Fig. 1) while Pj LTP2 is 677 bp in length and also with an ORF of 354 bp. Domain search at the NCBI's CDD showed the presence of pfam domain pfam00234 in both Pj LTP1 and Pj LTP2. This family consists of trypsin-alpha amylase inhibitors, seed storage proteins, and lipid transfer proteins from plants (Rico et al. 1996). During contig analysis, however, they were found to cluster in two different contigs. ClustalW analysis of Pj LTP1 and Pj LTP2 revealed that 70.09% amino acids are identical, 17.09% are strongly similar, 5.98% are weakly similar, and only 6.84% are different (figure not shown). Both Pj LTPs lack tryptophan and contain the eight cysteine residues that are conserved in LTPs (Kader 1996). A signal peptide cleavage site is predicted to occur between amino acids 25 and 26. After the cleavage of the signal peptide, the mature protein sizes are expected to be 9.2 kDa (Pj LTP1) and 9.5 kDa (Pj LTP2), respectively. Based on the molecular mass criteria, both Pj LTPs could be type I LTPs (Kader 1996). Further, both Pj LTPs lack the ER retention signal H/KDEL and might enter the secretory pathway as predicted for other cloned plant LTPs (Kader 1996).

Pj LTP1 was analyzed in detail. Upon BLASTX analysis, it showed maximum similarity to lipid transfer protein-like protein from *Retama raetam* (Genbank ID: AAL32039). A multiple-sequence analysis of Pj LTP1 with first 100 hits from the BLASTX search (ESM 1) revealed

Fig. 1 Nucleotide, protein, and promoter sequence of Pj LTP1. The A nucleotide of the ATG translation initiation codon (boxed) is assigned as position 1, and the nucleotide positions upstream of position 1 are given negative number. The deduced amino acid sequence is shown below the nucleotide sequence. The signal peptide cleavage site is marked by an arrow. An asterisk marks the stop codon. The conserved cysteine residues in the protein sequence are shaded gray. Important cis-acting elements in the promoter are underlined and named. Primer sequences are indicated in bold letters underlined by arrows, followed by their number. Forward arrows indicate sequence of forward primers; while reverse arrows indicate reverse complement of the actual primer sequence. Numbering is / Pj LTP1 5' UTR F, 2 Pj LTP1 5' UTR R, 3 Pj LTP1 GSP1, 4 Pj LTP1 GSP2, 5 Pj LTP1 GSP3, and 6 Pj LTP1 PRM F



that the eight cysteine residues involved in the formation of disulfide bonds are not absolutely conserved among them as previously reported (Da Silva et al. 2005). In two of the analyzed proteins (Genbank ID: Q42762 and Q43129, nsLTPs from *Gossypium hirsutum*), the first conserved cysteine residue (C28 as per Pj LTP1 numbering) was replaced by weakly similar serine. The seventh conserved cysteine residue (C98 as per Pj LTP1 numbering) was replaced by “unknown or other,” X, in one of the analyzed proteins (Genbank ID: AAY83344 from *Fragaria x ananassa*). In addition to these, G₅₅, K₇₇, P₉₅, Y₁₀₄, I₁₀₆, and S₁₀₇ were also found to be conserved among the analyzed proteins.

The BLASTX results showed that many of the initial hits were putative allergens (e.g., AAR22488, Q9M5X7 (Mal d 3, *Malus x domestica*), Q9M5X6 (Pyr c, *Pyrus communis*), Q9M5X8 (Pru av 3, *Prunus avium*), and ABG54494 (Rub i 3, *Rubus idaeus*)). A multiple-sequence analysis of Pj LTP1 mature protein sequence with these proteins and peach Pru p 3 (*Prunus persica*), a well-established and officially designated allergen from *P. persica* (CAB96876) revealed 46.7% identity in amino acids. Amino acids (19.57%) were strongly similar, and 17.39% were weakly similar. Only 16.3% were different (Fig. 2). Pj LTP1 shares 58.70% amino acid identity with peach Pru p 3 (which is more than the identity between Pru p 3 and Lyc e 3 (53%)),

Genbank ID	Amino acid sequence	% Id
CAB96876	ITCGQVSSSLAPCIPYVRGGGAVPPACCNIRNVNNLARTTPDRQAACNCLKQLSASVPGVNPNNAAALPGKCGVSIPIYKISASTNCATVK-	87.91 %
Q9M5X8	LTCGQVSSNLAPCIAYVRGGGAVPPACCNIRNVNNLAKTTADRQTACNCLKQLSASVPGVNPNNAAALPGKCGVNPVYKISPSTNCATVK-	81.32 %
AAR22488	ITCGQVTSSLAPCIGYVRSGGAVPPACCNIRNTINGLARTTADRQTACNCLKLAGSISGVNPNNAAALPGKCGVNPVYKISTSTNCATVK-	81.32 %
Q9M5X7	ITCGQVTSSLAPCIGYVRSGGAVPPACCNIRNTINGLARTTADRQTACNCLKLAGSISGVNPNNAAALPGKCGVNPVYKISTSTNCATVK-	78.02 %
Q9M5X6	ITCSQVSANLAPCINIVRSGGAVPPACCNIRKTINGLAKTTTPDRQAACNCLKLAGSVSGVNPNGNAESLPGKCGVNPVYKISTSTNCATVK-	69.23 %
ABG54494	ITCGQVTQNVAPCFNYVKNNGGAVPAACCNVRLNSQAATTADRQTCNCLKNAAGSIPGLNPSLAAGLPGKCGVSPVYKISTSTNCATVK-	58.70 %
PjLTP1	VTCGQVSSSLAPCIAYLQKGGTPAPGCCNGVSKLVGAQTTADKQAVCNCLKGAAGQVPGLNQYQSLPSLCGVNIPYKISTSTNCASIKF	
	:**.**: .:***: **: **: ..*****:..: . *:*.*:* ***** :...:.*: * .**.*:*****.*****:.*	

Fig. 2 Multiple sequence alignment of Pj LTP1 mature protein sequence with peach Pru p 3 and other allergens. Regions corresponding to the IgE-epitopes located in peach Pru p 3 are marked in *gray*, and percentages of sequence identity (% Id) with respect to this allergen are shown. Asterisks indicate identical amino

acids, *colon* indicate strongly similar (16.81%), and *full stop* indicate weakly similar (10.92%) amino acids. Accession nos. CAB96876 (Pru p 3 from *P. persica*), AAR22488, Q9M5X7 (Mal d 3), Q9M5X6 (Pyr c), Q9M5X8 (Pru av 3), ABG54494 (Rub i 3)

another well-established and officially designated allergen from *Lycopersicon esculentum*.

Northern analysis revealed that the transcript level of Pj LTP1 in leaves increased rapidly at 12 hours after PEG stress and decreased thereafter until 48 h. However, the transcript levels were still higher than control (unstressed) plants (George et al. 2007). With 90 mM H₂O₂ stress, the transcript level increased steadily with maximum expression at 48 h (Fig. 3a).

Isolation of the 5' Upstream Region of Pj LTP1, In Silico and Functional Analysis of the Promoter

The TAIL-PCR products of Pj LTP1 were separated on an agarose gel. The amplified fragments indicated by arrows in

Fig. 3b (F1, F2, F3, and F4—roughly 750 bp, 1 Kb, 500 bp, and 1.2 Kb, respectively) were cloned and sequenced. Of these, F1, F2, and F3 were found to have sequence overlaps with the Pj LTP1 cDNA sequence at their 3' ends and were, thus, confirmed to be specific amplification products. Using this sequence information, a 969-bp fragment was reamplified from *Prosopis* genomic DNA using primers Pj LTP1 PRM F and Pj LTP1 GSP3 (Fig. 1).

Several putative cis-acting DNA elements were identified in the 5' upstream sequence of Pj LTP1 using the PLACE database. The first ATG of the longest ORF was considered as the putative translation start site and numbered +1. The nearest TATA box was found at -15. Several CAATBOX1 (CAAT) were found at positions -885, -862, -707, -648, -631, -595, -330, -311, -187,

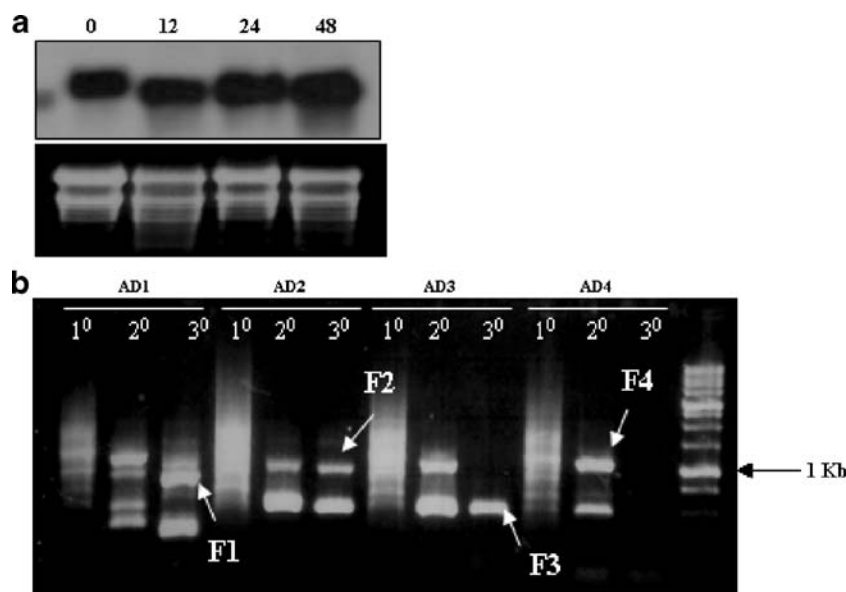


Fig. 3 **a** Northern analysis of Pj LTP1 under 90 mM H₂O₂. Samples were collected at 0, 12, 24, and 48 h of stress application. *Upper panel* shows transcript level of Pj LTP1 in *P. juliflora* leaves under 90 mM H₂O₂ stress. *Lower panel* shows ethidium bromide stained gel prior to transfer. **b** TAIL-PCR amplification products of Pj LTP1. Primary secondary and tertiary TAIL-PCR reactions using the degenerate

primers AD1, AD2, AD3, and AD4 and Pj LTP1-specific nested reverse primers are shown. The fragments F1, F2, F3, and F4 indicated by arrows are cloned and sequenced. F1, F2, and F3 were found to have sequence overlap with the 5' end of the Pj LTP1 cDNA and thus confirmed to be the specific amplification products

–175, and –61. The 5' upstream region of Pj LTP1 included many stress regulatory elements such as dehydration-responsive elements, pathogen- and salinity-responsive elements, etc. A low-temperature-responsive element (LTRE), containing the CCGAC core motif was found in the 5' upstream region of Pj LTP1 (–207). An ACGT motif, reported to be an element involved in early response to drought, was found at position –686. A putative plant Myb- (MACCWAMC) consensus sequence was found at –403, –210, –147, and –124. Three Myc consensus sequence CANNTG were found at –330, –202, and –27. Several elements involved in tissue and developmental stage-specific expression were found in the putative promoter of Pj LTP1 including YACT that is required for mesophyll-specific expression (–420, –714, –643, –576, –464, –438, and –93), AAAG, required for binding of Dof proteins associated with expression of multiple genes involved in carbon metabolism (–755, –599, –494, –74, –65, and –49), AGAAA, responsible for pollen specific gene expression (–911, –816, –750, –757 and –492), and ATATT, which drives root-specific expression (–849, –789, –769, –729, –698, –611, –488, –471, and –452). Three putative cis-acting elements, TTGAC, TGACT, and TGACY, known to bind to WRKY proteins and involved in salicylic acid (SA), sugar, and wound signaling, respectively, were also found in the 5' upstream region of Pj LTP1. A possible regulatory element required for circadian regulation (CAANNNNATC) was found at –192. Five GT1 elements (GRWAAW) found in the promoter of several light regulated genes were found at –910, –815, –801, –800, and –414 positions. No abscisic acid (ABA)-responsive element (ABRE, G-box, coupling element (CE)) could be identified in the 5' upstream region of Pj LTP1.

Intense GUS staining was detected in tobacco leaves transiently expressing the Pj LTP1 promoter: *Uid A* construct (pPRM-1391z). This indicated that the isolated Pj LTP1 promoter was a functional promoter in tobacco.

Discussion

Among LTPs, two major multigenic families have been identified: type I LTP (9 kDa, LTP1) and type II LTP (7 kDa, LTP2). They share several structural features, the most striking being a consensus motif of eight cysteine residues that are engaged in four disulfide bridges with identical connectivity (Kader 1996). In both types I and II LTPs, these disulfide bonds stabilize an α -helix fold in which a hydrophobic cavity or tunnel can bind different types of lipids and hydrophobic molecules except sterols (Douliez et al. 2000; Marion et al. 2004). Pj LTP1 and Pj LTP2 are both putative type I LTPs showing 70.09% identical amino acids.

Pj LTP1 upon BLASTX analysis showed maximum similarity to lipid transfer protein-like protein from *R. raetam* (white weeping broom), a highly drought-tolerant plant of *Fabaceae*. The eight cysteine residues involved in the formation of disulfide bonds are not absolutely conserved in nsLTPs as previously thought. The significance of replacement of cysteine with other amino acids and the conservation of a different set of amino acids (G₅₅, K₇₇, P₉₅, Y₁₀₄, I₁₀₆, S₁₀₇ as per Pj LTP1 numbering) in the 100 proteins analyzed in relation to protein overall folding and function need to be analyzed.

The multiple sequence analysis of Pj LTP1 with previously reported nsLTP allergens revealed a high degree of similarity at the amino acid level. The identification of the 10–13 kDa IgE-binding components from peach and apple as members of the nsLTP family was independently published in 1999 by Spanish and Italian groups (Sanchez-Monge et al. 1999; Pastorello et al. 1999a, b). A major allergen of around 9 kDa was isolated from each species and designated Pru p 3 (peach, *P. persica*) and Mal d 3 (apple), according to the guidelines of the IUIS Allergen Nomenclature Subcommittee. Since then, the works of several clinical and biochemical research groups have confirmed and extended the role of nsLTPs in allergic reactions to plant foods and even pollens (Salcedo et al. 2007). Thus, allergenic members of the nsLTP family with amino acid sequence identity with peach Pru p 3 varying between 92–47% have been identified and at least partially characterized in other *Rosaceae* fruits (apricot, plum, etc.), grape, citrus fruits, tomato, vegetables (asparagus, lettuce, etc.), nuts (hazelnut, walnut, etc.), and maize (Salcedo et al. 2007). The considerable similarity at the amino acid level between Pj LTP1 and Pru p 3 points towards possible allergenic properties for this protein. Further experiments are needed to confirm this.

Many abiotic stresses such as drought, salt, cadmium stress, etc., are known to cause oxidative stress (Ezaki et al. 2000; Richards et al. 1998; Jithesh et al. 2006). In addition, increased antioxidant potential has been shown to improve tolerance to many abiotic stresses, including drought and salt (Van Camp et al. 1996; Borsani et al. 2001). George et al. (2007) reported upregulation of Pj LTP1 in *P. juliflora* leaves under conditions of drought stress (PEG 25%). The transcript level of Pj LTP1 in leaves was shown to increase rapidly at 12 h after PEG stress, which began to decrease thereafter until 48 h but was still higher than control (unstressed) plants. The initial rapid increase in transcript level of Pj LTP1 suggests that this gene may have a role in early drought stress. Under 90 mM H₂O₂ stress, the transcript level increased steadily with maximum expression at 48 h. This indicates a possible role for Pj LTP1 in reactive oxygen species removal. It could be possible that a common response mechanism perceives both the stresses

and induces expression of Pj LTP1 under drought and H₂O₂ stress conditions.

TAIL PCR reaction carried out using different combinations of AD primers with Pj LTP1 gene-specific primers revealed that all the three PCR products in the tertiary reaction (fragments F1, F2, and F3) had sequence similarity with the cDNA sequence at their 3' ends and were, thus, confirmed to be the specific amplification products.

Several putative cis-acting DNA elements were identified in the upstream sequence and 5' UTR of Pj LTP1 including the TATABOX and CAATBOX. Putative LTREs containing the CCGAC core motif were found in the 5' upstream region of Pj LTP1. Gene responses to cold appear to be mainly regulated by an ABA-independent pathway (Shinozaki and Yamaguchi-Shinozaki 2000). In *Arabidopsis*, LTREs, containing the CCGAC core motif, have been found in many cold- and drought-responsive genes (Baker et al. 1994; Jiang et al. 1996; Shinozaki and Yamaguchi-Shinozaki 1996). In barley, a similar motif, CCGAAA, has been shown to confer low temperature responsiveness to the blt4.9 gene promoter (Dunn et al. 1998), although no transcription factor binding to this motif has been identified to date. Thus, the motif ACCGACA in the 5' upstream sequence of Pj LTP1 might contribute to cold responsiveness. An ACGT motif, reported to be an element involved in early response to drought and shown to be the cis element that modulates the level of expression of some genes in rice seed (Washida et al. 1999) was identified. Several putative plant Myb (MACCWAMC) and Myc (CANNTG) consensus sequence were also found at the 5' upstream region of Pj LTP1. MYB and MYC recognition sites have been identified in stress-responsive plant genes such as those in the ABA-mediated dehydration-responsive expression of the rd22 promoter gene from *Arabidopsis* (Abe et al. 1997). Several elements involved in tissue and developmental stage-specific expression were found the 5' UTR of Pj LTP1 including a cis-acting element, YACT, required for mesophyll-specific gene expression (Gowik et al. 2004), core site AAAG required for binding of Dof proteins associated with expression of multiple genes involved in carbon metabolism (Yanagisawa 2000), AGAAA, one of two co-dependent regulatory elements responsible for pollen specific activation of tomato lat52 gene (Filichkin et al. 2004) and a cis-acting element, ATATT found in the *Agrobacterium rhizogenes* rol D promoter which drives root-specific expression. Three cis-acting elements, TTGAC, TGAAT, and TGACY, binding to WRKY DNA binding proteins and involved in SA, sugar, and wound signaling, respectively, were also found in the 5' upstream region of Pj LTP1. (Eulgem et al. 2000; Nishitani et al. 2004; Sun et al. 2003). A possible regulatory element required for circadian regulation (CAANNNNATC), five GT1 elements (GRWAAW; found in the promoter of several

light-regulated genes) were also present. Putative cis-acting elements such as the ABRE, G-box, or coupling element which are required for ABA-induced gene expression were not found in the 5' upstream region of Pj LTP1.

The Pj LTP1 promoter was found to be functional in tobacco as revealed by transient reporter gene expression. Further characterization of this promoter would throw light into the possible functions of this gene. The results of our studies could be used as a starting point for generation of crop plants tolerant to abiotic stresses.

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