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Osmotic Stress-Inducible Expression of a Lipid Transfer Protein Gene in Poplar

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Abstract - We have cloned an LTP gene (*PoLTP1*) from poplar (*Populus alba* × *P. tremula* var. *glandulosa*) suspension cells and examined changes in its expression levels in response to various stresses and ABA treatment. The full-length *PoLTP1* cDNA clone encodes a polypeptide of 116 amino acids with typical characteristics of LTPs, notably a conserved arrangement of cysteine residues. Southern blot analysis indicate that two or three copies of the *PoLTP1* are present in the genome of the investigated hybrid poplar. In addition, northern analysis of samples from soil-grown plants indicate that *PoLTP1* is tissue-specifically expressed in the leaves and flowers. The gene is significantly up-regulated by treatment with mannitol, NaCl and ABA, but not by either cold or wounding. These results indicate that *PoLTP1* is involved in osmotic stress responses in poplar plants and suspension cells.

Key words - ABA, Drought, Lipid transfer protein, Poplar

Introduction

Plants exchange metabolites, ions, and signals with various biotic and abiotic components of their external environment through their cell membranes. Many cell wall proteins are involved in these exchange processes, including lipid transfer proteins (LTPs), which have been shown to have wide substrate specificities and to be involved in transporting a variety of both phospholipids and glycolipids (Kader, 1996). The LTPs are low molecular weight, basic, soluble proteins with high contents of proline, glycine, alanine and serine. Other notable features of the proteins include transit peptide sequences targeting the cell wall or extracellular surfaces, and the formation of four disulfide bridges (Castro *et al.*, 2003).

Two major classes of LTPs have been identified: large and small, designated LTP1s and LTP2s, with molecular weights of: 9-10 kDa and ca. 7 kDa, respectively (Douliez *et al.*, 2000). LTP1s have been shown to have four helices, a long C-terminal, and a hydrophobic cavity to which both phospholipids and fatty acids bind (Kader, 1997). These structural features have prompted many workers to speculate that LTP1s are involved in the biosynthesis and transportation of membrane components to the cell membrane, and thus function as cell membrane-binding enzymes that use lipids as substrates (Arondel and Kader, 1990). Recently, LTP1s have been shown to contribute to drought tolerance of plants since secretion of these proteins to external surfaces of cells results in the accumulation of a wax layer on them, thereby reducing water conductivity (Cameron *et al.*, 2006). In addition, LTP1s are known to be involved in embryogenesis in plants (Thoma *et al.*, 1993), defenses against pathogens (Kim *et al.*, 2006) and nitrogen symbiosis (Krause *et al.*, 1994).

Trees are long-lived and thus subject to diverse environmental stresses, one of the most serious being drought, which is capable of severely reducing yields of stands, or even destroying large areas of forests in extreme cases (Seki *et al.*, 2003; Gonzalez-Martinez *et al.*, 2007). Therefore, it is important to understand drought tolerance mechanisms and to identify ways to improve it in forest trees (Gonzalez-Martinez *et al.*, 2007). Poplars are ideal model tree systems for studying drought tolerance at the molecular level for the following reasons: 1) they are easy to manipulate vegetatively, as either cuttings or cultures of cells or tissues, 2) transformation protocols are readily available, and 3) the entire genome sequence of one poplar species (*Populus trichocarpa*) and large amounts of genetic information regarding other species are publicly available (Jansson and Douglas, 2007). In the present study, we cloned LTP1 cDNA from poplar (*Populus alba* × *P. tremula* var. *glandulosa*) cell suspensions and attempted to characterize its expression pattern in relation to drought stress.

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Materials and Methods

Plant materials and growth conditions

Poplar cell suspensions were maintained by subculturing them biweekly according to Choi *et al.* (2001). Briefly, 0.4g (fresh weight) of cells were transferred every two weeks to 100ml portions of liquid MS medium containing 1mg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid), 0.1mg l^{-1} NAA (1-naphthalene acetic acid) and 0.01mg l^{-1} BAP (6-benzylaminopurine) in 250ml flasks. The suspensions were maintained at 120rpm on a gyratory shaker in a culture room at 22 ± 1℃ under 20μmol m^2s^{-1} cool, white fluorescent light. For tissue-specific expression analysis, leaves, stems and roots were harvested from 1-year-old poplar plants growing in a nursery, and flowers were harvested from approximately 25-year-old plants.

Construction of cDNA library and isolation of lipid transfer protein gene

Cell suspensions were harvested 8 days after subculturing, messenger RNA was extracted and double-strand cDNAs were synthesized using reverse transcriptase following the procedures described by Lee *et al.* (2005). The ZAPII vector (Stratagene, La Jolla, CA) was used to trap the synthesized cDNAs to construct a cDNA library. For expressed sequence tag analysis, the cDNA library was randomly excised *in vivo* using the ExAssist helper phage and the plasmid DNAs were then isolated and 5'-single pass sequences were determined. Clones with high levels of homology to known plant lipid transfer proteins were then sought by BLASTX searches of the public databases. The cDNA clones thus identified were then sequenced and analyzed by the Vector NTI program (Invitrogen, Maryland, USA).

Genomic DNA isolation and southern blot analysis

Genomic DNA was extracted from leaves of 1-year-old poplar plants growing in a nursery using a MagExtractor-Plant Genome kit (Toyobo, Osaka, Japan), and 10μg portions of the genomic DNA were digested with the restriction enzymes *Bam*HI, *Eco*RI, *Hind*III or *Xba*I overnight. The DNA was then electrophoretically separated on 1% agarose gels, transferred to Hybond-XL nylon membrane (Amersham-Pharmacia, Piscataway, NJ) by the capillary transfer method and probed with full-length *PoLTP1* cDNA, using the labeling, hybridization and washing protocols described by Lee *et al.* (2005).

Stress treatments and northern blot analyses

To impose drought-mimicking osmotic stress, four-day-old cell suspensions were supplemented with 250mM mannitol or 150mM NaCl. To assess their responses to the drought-associated hormone ABA, 25μM of ABA was added to replicate suspensions, and to assess the effects of cold stress suspension cells were incubated in Erlenmeyer flasks at ca. 2℃ in ice on a gyratory shaker. In each case replicate flasks (n = 3) were harvested after 2h and 10h, then the harvested cells were immediately frozen in liquid nitrogen and stored at -70℃ until analysis. In addition, to evaluate the effects of wounding, ca. 1cm cuts were made along the edges of leaves of 1-year-old poplar plants, and sets of 10 wounded leaves were harvested 2h and 10h later. Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, Ohio), then 10μg portions of the RNA were run on 1.2% formaldehyde agarose gel and transferred to Hybond-XL nylon membrane. Labeling of the probe, hybridization and membrane washing were done as described by Lee *et al.* (2005).

Results and Discussion

Structural features of *PoLTP1*

Based on homology to known plant LTP1s, a cDNA clone was selected from the cDNA library derived from poplar suspension cells. The whole sequence of the selected clone was determined and then analyzed by homology search with a BLASTX program. The selected clone showed high homology to *Arabidopsis* LTP1, with a blast score of 117 and was therefore named *PoLTP1* (Poplar Lipid Transfer Protein1). The 658-bp cDNA clone *PoLTP1* contains a 345-bp open reading frame beginning with the start codon (ATG) at nucleotide (nt) position 68 and ending with the stop codon (TGA) at nt position 415 (Fig. 1). The protein encoded by this ORF, PoLTP1, has an expected molecular mass and pI of 11.8 kDa and 9.14, respectively, and it contains 116 amino acids, a high proportion of which (15%) are alanines. The next most abundant amino acids are cysteine, glycine, leucine, asparagine and valine; each of which accounts for ca. 9% of its total complement. PoLTP1 also contains signal peptides, including 24 amino acid residues required for cell wall or extracellular transport. Furthermore, PoLTP1 contains the eight cysteine residues involved in the formation of the four disulfide bridges (Fig. 1) that are known to be conserved in all plant LTPs (Heinemann *et al.,* 1996). The C-terminal domain of the PoLTP1 protein also appears to contain both an AAI-LTSS domain,

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  1   CTTCTCCTTCACTCTCAAACACTCGAGAAAAAAAAGGTTCTTCCAAGCCTTCCCTAACCT
 61   ATAAAACATGGCTGGTGCTAAGTCGGTCTGTGTTCTCTTGCTGTGCATGGTGCTCTCCGC
             M  A  G  A  K  S  V  C  V  L  L  L  C  M  V  L  S  A
121   CCCCATGTTGAACGTTCAAGCCTTGACATGTGGTGTTGTGGCCAGTAACTTGGCACCGTG
       P  M  L  N  V  E  A  L  T  C  G  V  V  A  S  N  L  A  P  C
181   CATCGGCTACCTAAGAAATGGCGGGAAAGTTCCTGACCCTTGCTGCAAAGGAGTTGGGGC
       I  G  Y  L  R  N  G  G  K  V  P  D  P  C  C  K  G  V  G  A
241   TCTCAACAATGCTGCTAAAACAACTAAAGATCGCCCGAGATGCTTGTAATTGCATTAAAAC
       L  N  N  A  A  K  T  T  K  D  R  R  D  A  C  N  C  I  K  T
301   AACCGCCACTCAAATTGGTGGGGTTAATGCTGCCAACGCGGCGGCTCTCCCTGGTTTCTG
       T  A  T  Q  I  G  G  V  N  A  A  N  A  A  A  L  P  G  F  C
361   CCGTGTTAATATTCCGTACAAGATCAGCACATCCACCAACTGCGCCAGCATTAAGTGAGG
       R  V  N  I  P  Y  K  I  S  T  S  T  N  C  A  S  I  K  *
421   GGAACAAAATCGAGCATCATCCATCGGATTGCACCGTGATCATAATAAAGTAGGTGTTTG
481   CCTCTGTTGATGGGTCTCCACTAGGGCGAGTCCCACACTCAGTCTTGGGTCGTTGTCTCT
541   TTTTCTTTTTAATTTAATGAATATCATGCGTCGTTTCATGTATCTTTGGGCGTTATGCAA
601   TATGTTAAGAAAATAAATTTCTCCTAAAACCAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide and deduced amino acid sequences of the cDNA encoding PoLTP1 (GenBank Accession No. EF613275). The conserved AAI-LTSS domain is indicated in bold, cysteine residues involved in disulfide bridges in gray boxes, and the N-terminal signal peptide sequences in italics.

a highly conserved domain of storage proteins in barley, rye and wheat (Kreis *et al.,* 1985), and a Leu-Pro-Xaa-Xaa-Cys-Xaa-Haa sequence (Fig. 1). The latter is found in LTP1 proteins and various other proteins, including seed storage proteins (Kader, 1996) and trypsin-alpha amylase inhibitors (Reeck and Hedgcoth, 1985), which are involved in the cleavage of alpha-glucoside bonds of glycogen and starch.

Southern blot analysis

To determine the copy number of *PoLTP1* genes in the genome of the hybrid poplar used in this study, Southern blot analysis was performed using the full-length *PoLTP1* cDNA as a probe (Fig. 2). All restriction digests gave 3 to 4 bands, including 1-2 strong positive signals. Since plant *LTP1* genes contain an intron at their C-terminal region (Kadar, 1996), we consider it highly likely that poplars carry a small family of *LTP1* genes with 2-3 members. Our findings that poplar has a low *PoLTP1* copy number are consistent with low numbers previously reported for cotton (Ma *et al.,* 1995) and rice (Vignols *et al.,* 1994).

Tissue-specific expression of *PoLTP1*

To examine the tissue-specificity of *PoLTP1* gene expression, northern blot analysis was performed with total RNAs extracted from suspension cell cultures and from leaves, stems, roots and flowers of soil-grown plants (Fig. 3). The results showed that while the gene is highly expressed in leaves and flowers, it is only weakly expressed in cell suspensions. No expression signals were detected from samples of roots or stem tissues. The results are consistent with patterns reported for other plant *LTP1*s, except that rice root

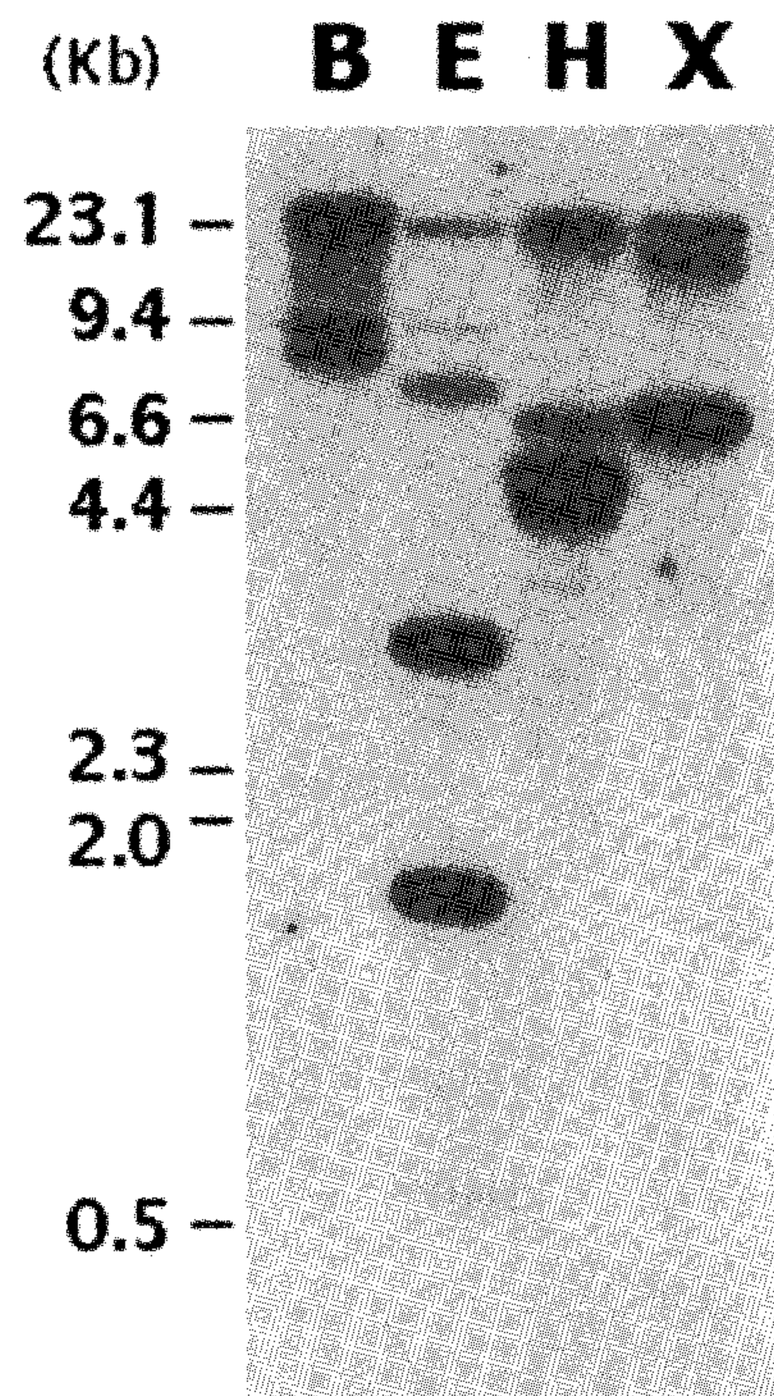

Fig. 2. Genomic Southern blot analysis of *PoLTP1*. Portions of genomic DNA (10㎍) digested with *Bam*HI (B), *Eco*RI (E), *Hind*III (H) and *Xba*I (X) were electrophoretically fractionated in a 1.0% agarose gel, transferred to a nylon membrane and hybridized with ^{32}P-labeled full-length *PoLTP1* cDNA.

Fig. 3. Tissue-specific expression of *PoLTP1*. Total RNA was extracted from leaf (L), stem (S), root (R), flower (F) tissues, and cell suspension culture (SC), then probed with full-length *PoLTP1* cDNA. Ethidium bromide-stained ribosomal RNA (rRNA) served as a loading control.

tissues have been found to weakly express an *LTP1* gene (Kadar, 1996). Floral expression of the *LTP1* gene has been observed in tobacco (Fleming *et al.*, 1992) and Arabidopsis (Thoma *et al.*, 1993).

Induction of *PoLTP1* gene expression by stress

LTPs have been recently reported to contribute to drought tolerance in plants by participating in the accumulation and modification of cuticular wax layers after stomatal closure (Cameron *et al.*, 2006). Therefore, to examine whether or not drought stress induced the *PoLTP1* gene, northern blot analysis was performed with total RNAs extracted from suspension cells after various stress treatments, including treatments with mannitol, salt, cold and ABA (Fig. 4). The results showed that *PoLTP1* was highly expressed in suspension cells after 10h treatment with mannitol or salt. Similar up-regulation of *PoLTP1* was also observed in the cells after 10h treatment with the plant hormone ABA, which has been strongly implicated in drought tolerance in plants (Yang *et al.*, 2005). It is well known that many genes up-regulated by osmotic

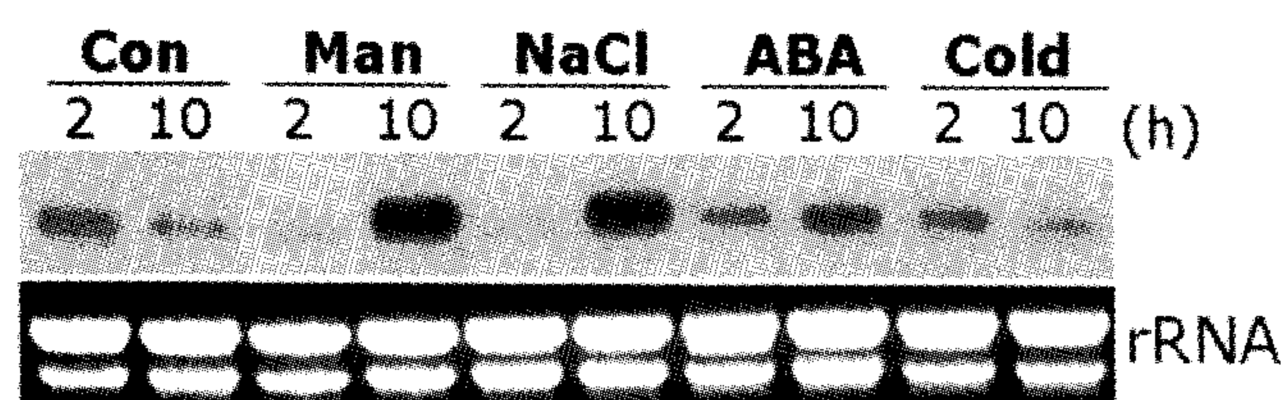

Fig. 4. *PoLTP1* expression in suspension cells in response to treatments with mannitol (Man, 250mM), NaCl (150mM), ABA (25μM) and cold (2℃) for 2 and 10h. Untreated control cells (Con) were incubated for the same durations. Ethidium bromide-stained ribosomal RNA (rRNA) served as a loading control.

stresses, such as those imposed by salt or mannitol treatments, are also induced by ABA treatment via the ABA-dependent pathway (Seki *et al.*, 2003). In contrast, no changes in expression level were detected in cold-treated cells.

We also analyzed the *PoLTP1* expression pattern in response to wounding injuries applied to the leaves. The expression level appeared to fall shortly after the injuries, but returned to levels observed in untreated tissues within 24h (Fig. 5). The reason why *PoLTP1* mRNA levels did not increase following wound injuries is not known. However, the lack of an observed rise is consistent with the results obtained from our suspension culture cells, in which *PoLTP1* expression levels remained low although such cells are exposed to another type of physical stress; agitation (Cazale *et al.*, 1998). These findings suggest that the *PoLTP1* gene product is not involved in responses to physical stresses such as wounding.

The genomes of members of various plant taxa have all been found to contain 2-5 copies of *LTP1* genes, which may have different functions in different tissues, and respond differently to different stresses including drought, cold, and pathogen infection. For instance, in tree tobacco (*Nicotiana glauca* L. Graham), the *NgLTP* gene has been found to be induced by drought treatment, but not the *NgLTP2* gene, which has been shown to be induced by wounding injury (Cameron *et al.*, 2006). In addition, Dunn *et al.* (1991) demonstrated that *LTP* expression can be induced by cold and water stress treatments in cereals, and Hughes *et al.* (1992) showed that *LTP* gene expression can be induced not only by cold and water stresses, but also by ABA. In contrast, Pearce *et al.* (1996) found that three different copies of *LTP*s induced by cold treatments were not induced by either water stress or nutrient deficiency. Other noteworthy agents that can induce the expression of *LTP* genes include microbial pathogens (Molina *et al.*, 1996) and cadmium (Hollenbach *et al.*, 1997). Thus, various published studies indicate that different copies of *LTP* genes are involved in different defense mechanisms and have different functions in different

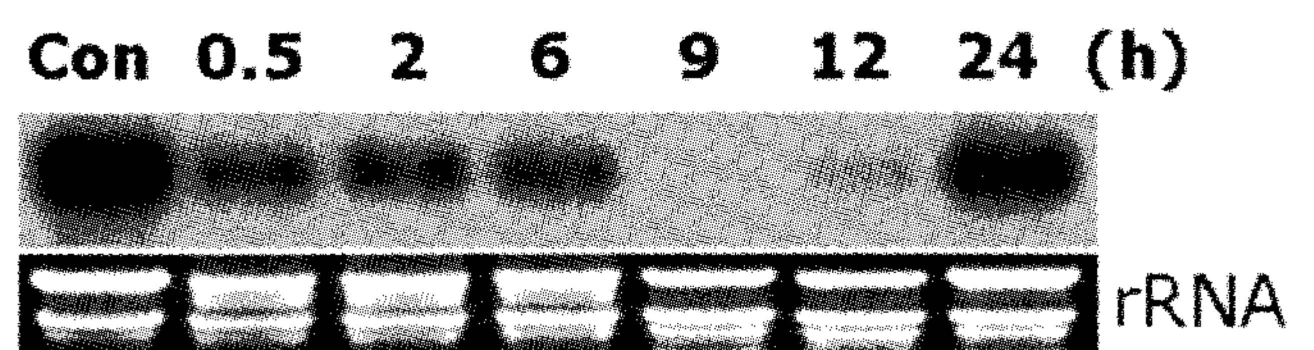

Fig. 5. *PoLTP1* expression in leaf tissue in response to wounding. Ethidium bromide-stained ribosomal RNA (rRNA) served as a loading control.

tissues. However, little is still known about the functions of plant *LTP*.

The *PoLTP1* gene isolated in the present study appeared to be up-regulated by mannitol, salt and ABA, but did not respond to cold or wounding injury. Therefore, the *PoLTP1* product might be involved in cuticular wax accumulation and confer drought tolerance. We believe that the information acquired regarding *PoLTP1* in this and ongoing studies will provide useful contributions to knowledge of the functions and regulation of plant *LTPs*, especially *LTPs* in trees, for which relatively little is known compared to their counterparts in annual crops and herbaceous plants.

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