GENETICS AND GENOMICS

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Isolation and characterization of lipid transfer protein (LTP) genes from a wheat-rye translocation line

Received: 31 July 2001 / Revised: 7 November 2001 / Accepted: 12 November 2001 / Published online: 24 January 2002 © Springer-Verlag 2002

Abstract Two genes (*TaLTP1* and *TaLTP2*) encoding lipid transfer proteins (LTPs) were isolated from a cDNA library constructed from leaf tissue harvested from 4-week-old seedlings of a wheat-rye near-isogenic line (NIL) involving a translocation of rye chromosome 2RL with wheat 2BS. The spatial and temporal patterns of expression of *TaLTP1* and *TaLTP2* were examined by Northern blot analysis and in situ hybridization. Both TaLTP1 and TaLTP2 contained a 270-bp open reading frame and encoded a putative LTP precursor molecule of 90 amino acids. Expression of the two LTPs was detected in leaves, stems, and crowns of the NILs but not in the roots. The expression levels of *TaLTP1* and *TaLTP2* remained constant in response to cold and ABA treatments over a period of 24 h but increased 3 days after the initiation of drought stress. An in situ hybridization study indicated that TaLTP1 was expressed in the cells within the vascular bundles of leaves and in the tissue layers between the vascular bundles in the crowns of the control and drought-treated plants. Expression of *TaLTP1* in the tissue layers between the vascular bundles was higher in the drought-treated plants than in the control plants. The results suggested that high levels of expression of TaLTPs in the tissue layers between the vascular bundles might play a role in the drought tolerance response of the wheat crown.

The nucleotide sequence data reported appear in the GenBank, EMBL, and DDBJ Nucleotide Sequence Database under the accession numbers AF302788 (*TaLTP1*) and AF334185 (*TaLTP2*).

Communicated by I. S. Chung

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Keywords Lipid transfer protein · Abiotic stress · Northern blot · In situ hybridization · Wheat

Abbreviations *ABA*: Abscisic acid · *ESTs*: Expressed sequence tags · *LTP*: Lipid transfer protein · *NILs*: Near-isogenic lines · *ORF*: Open reading frame · *STS*: Sequence-tagged site · *TaLTP*: *Triticum aestivum* lipid transfer protein

Introduction

Lipid transfer protein genes have been isolated from numerous plants, including *Arabidopsis thaliana* (Thoma et al. 1994; Arondel et al. 2000), *Zea mays* (Sossountzov et al. 1991), *Hordeum vulgare* (Gausing 1994; Molina et al. 1996; Pearce et al. 1998; Chen and Foolad 1999), *Oryza sativa* (Vignols et al. 1997), and *Sorghum vulgare* (Pelèse-Siebenbourg et al. 1994). Plant LTPs have been shown to enhance the in vitro transfer of phospholipids between membranes and to bind acyl chains (for review see Kader 1996). Most of the plant LTPs reported are basic 9-kDa proteins with eight conserved cysteine residues. In addition, the deduced amino acid sequences of the cDNAs of LTPs isolated from plants possess putative N-terminal signal peptides and lack trytophan residues. LTPs are generally members of a small multigene family.

The expression of LTPs can be induced by abiotic and/or biotic stresses such as cold, water deficit, NaCl, heavy metal treatment, or pathogen invasion (Torres-Schumann et al. 1992; Molina et al. 1996; Colmenero-Flores et al. 1997; Hollenbach et al. 1997; Pearce et al. 1998). The expression pattern of each gene within a family is typically affected differentially by plant growth stage, anatomy, and biotic and abiotic stress (Molina et al. 1996; Vignols et al. 1997; Arondel et al. 2000). cDNAs of LTPs of hexaploid wheat (*Triticum aestivum* L., 2n=42) have not yet been reported.

Numerous wheat-rye translocation lines have been developed because they possess broad adaptability, high productivity, and a resistance to diseases and insects (for review see Friebe et al. 1996). Rye chromosome arm 2RL from Chaupon rye (Secale cereale L.) was found to carry a gene or gene complex that conditioned antibiosis to biotype L of Hessian fly [Mayetiola destructor (Say)] larvae (Friebe et al. 1990). The hard red winter wheat germplasm Hamlet (PI549276) possesses 2RL chromatin in the form of a 2BS/2RL translocation and carries the resistance gene (H21) of biotype L of Hessian fly (Sears et al. 1992). Seo et al. (1997) developed NILs for H21 by backcross introgression – $BC_3F_{3:4}$ (Coker 797 × 4/Hamlet). These two NILs were identified as being different by the presence or absence of H21 located on 2RL. A molecular marker associated with the H21 Hessian fly resistance gene was ultimately identified using these NILs and bulked segregant analysis (Seo et al. 1997). More recently, STS markers have been developed to assist in identifying wheat-rye translocations possessing 2RL (Seo et al. 2001).

Jang et al. (1999) described the development of expressed sequence tags from a cDNA library of an NIL carrying 2RL in order to construct a profile of genes unique to rye chromosome arm 2RL. A single EST from this cDNA library showed high homology with LTPs isolated from other plants. However, this clone did not show any homology with wheat genes previously registered in DNA data base. We report here the isolation of two LTP genes from the wheat-rye 2BS/2RL translocation line and the characterization of these genes by Northern blot analysis and in situ hybridization.

Materials and methods

Plant materials

NILs were developed by backcross introgression and repeated selection for 2RL (Coker 797×4 /Hamlet) (Seo et al. 1997). An NIL carrying 2RL was identified and verified by the presence of the resistance gene H21 of Hessian fly. NILs were grown for 4 weeks at 25°/18°C (day/night) in a glasshouse. Plant tissues were harvested from the leaves, stems, crowns, and roots. For the cold treatment, 4-week-old plants of the NIL carrying 2RL were transferred to a growth chamber (constant 4°C) and incubated for 24 h under a 12/12-h (day/night) photoperiod. For the ABA treatment, 4-weekold leaves of the NIL carrying 2RL were sprayed with a 100 μM solution of ABA containing 0.05% (v/v) Tween 20 and placed in a growth chamber at 25°/18°C (day/night) for 24 h on a 12/12-h (day/night) photoperiod. Tissues from the cold- and ABA-treated plants were harvested at 0, 6, 12, 18, and 24 h after treatment initiation and were immediately frozen in liquid nitrogen. All tissue samples for RNA extraction were stored at -80°C. For the drought treatment, several plants of the NIL carrying 2RL were grown for 4 weeks under a normal watering regime and subsequently exposed to drought stress (no further applications of water). Plants were harvested at 1, 2, 3, 4, and 5 days after initiation of the drought treatment.

Isolation of LTP family genes

A cDNA library was constructed from leaves taken from a 4-week-old NIL (2BS/2RL) using the Uni-ZAP XR vector (Stratagene) as previously described by Jang et al. (1999). About 2×10^6 plaques from this cDNA library were transferred to nylon membrane (MSI). The LTP clone reported by Jang et al. (1999) was la-

beled using digoxygenin (Dig) and a random primer labeling kit (Boehringer Mannheim, Indianapolis, Ind.) and subsequently used as a probe. Membranes were hybridized for 18 h at 65° C with the Dig-labeled LTP probe. The hybridization solution contained $5\times$ SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 1% blocking reagent. After hybridization, the membranes were washed twice with $2\times$ SSC and 0.1% SDS at room temperature, followed by three washes of 15 min each with 0.1× SSC, 0.1% SDS at room temperature. Hybridization was detected using a Dig Luminescent Detection kit (Boehringer Mannheim).

DNA sequence analysis

Positive phages were transferred to pBluescript plasmids by in vivo excision (Stratagene) and cloned in SOLR cells (Stratagene). Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen, Valencia, Calif.). cDNAs were amplified using the Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, Calif.) according to the manufacturer's instructions. Sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Perkin Elmer). DNA sequencing data were analyzed using the internet network program (http://www.expasy.ch). Comparisons of deduced amino acids were performed with nonredundant entries in the database at the National Center for Biotechnology Information (NCBI) using the BLASTP program.

RNA isolation and Northern blot analysis

Total RNA was isolated from individual tissues using Trizol according to commercial protocols (Gibco/BRL, Gaithersburg, Md.). Total RNAs (20 µg) were fractionated on a 1% formaldehyde agarose gel and transferred to nylon membrane (MSI). cDNAs to be used as probes were excised using the restrictions enzymes *Eco*RI and *XhoI* and labeled with α -[³²P]-dATP using the Random Primed DNA Labeling kit (Boehringer Mannheim). The blots were prehybridized for 2 h and then hybridized for 18 h at 60°C in 1 mM EDTA, 7% SDS, 0.25 M disodium phosphate pH 7.2, and 5% dextran sulfate. Following hybridization, the membranes were washed twice for 30 min in 2× SSC, 1% SDS at room temperature and twice in 0.1× SSC, 1% SDS for 10 min at room temperature or until the background radioactivity was near zero.

In situ hybridization

In situ hybridization was carried out as described by Lee et al. (2000). Tissue sections were vacuum-infiltrated for 10 min with a fixing solution containing 30 mM sodium phosphate, 130 mM sodium chloride, 4% paraformaldehyde, and 0.1% Triton X-100, and then incubated for 2 h at room temperature. The tissues were subsequently washed three times in PBS buffer (30 mM sodium phosphate, 130 mM sodium chloride, pH 7.1), dehydrated in a graded ethanol and xylene series, and embedded in paraplast.

The digoxigenin-labeled TaLTP1 probe was synthesized using the Dig High Prime kit (Boehringer Mannheim). The tissues (10 µm) were attached to poly-L-lysine-coated glass slides. After removal of the paraplast with xylene, the tissues were rehydrated through the ethanol series. The sections were then treated with 1% BSA in 10 mM Tris-HCl (pH 8.0), incubated in proteinase K solution (5 mg/ml proteinase K in 50 mM EDTA, 100 mM Tris-HCl pH 8.0) at 37°C, and treated in 100 mM triethanolamine containing 0.25% acetic anhydride. The sections were prehybridized for 1 h and then hybridized for 18 h at 42°C in 50% formamide, 4× SSC, 0.5% blocking reagent, and 150 µg/ml tRNA. After hybridization, the tissues were washed twice with 50% formamide and 4× SSC, and twice with 4× SSC, at 42°C. The hybridization signal was detected using the Digoxigenin Detection kit with nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), according to the manufacturer's protocols (Boehringer Mannheim).

TaLTP1

GC	TAA	TTA	ccc	ACC	CTC	TCC	CTG	CGA	acc	CTC	CAC	CAC	AAC	TAC	AGC	TAA	CTC	GOT	CGAG
AT	GGC	TCG	CAC	TGC	AGC	AAC	AAA	GCT	CGT	GCT	GGT	CGC	CCT	GGT	GGC	GGC	AAT	GCT	CCTO
н	A	R	т	A	A	т	ĸ	L	v	L	v	A	L	v	A	A	м	L	L
GT	AGC	CTC	CGA	CGC	GGC	GAT	CTC	CTG	CGG	TCA	GGT	GAA	CTC	TGC	CCT	GGC	CTC	CTG	CGTO
v	A	s	D	A	A	I	s	C	G	Q	v	N	s	A	L	A	s	C	v
TO	CTA	CGC	AAA	AGG	CAG	CGG	GGC	CAG	CCC	GCC	TGG	GGC	CTG	CTG	TTC	CGG	AGT	TAG	GAG
s	Y	A	ĸ	G	s	G	A	s	P	P	G	A	C	C	s	G	v	R	R
TT	GGC	CGG	CTT	AGC	GCG	GAG	CAC	CGC	CGA	CAA	GCA	AGC	GGC	ATG	CAG	GTG	CAT	CAA	GAG
L	A	G	L	A	R	s	т	A	D	ĸ	Q	A	A	d	R	C	I	ĸ	s
GC	TGC	CGG	TGG	GCT	CAA	ccc	CGG	AAA	GGC	TGC	AAG	TAT	CCC	CTC	CAA	GTG	CGG	CGT	CAG
A	A	G	G	L	N	P	G	ĸ	A	A	s	I	P	s	ĸ	d	G	v	s
AT	CCC	CTA	CTC	CAT	CAG	CGC	ATC	CGT	GGA	CTG	CTC	TAA	GAT	CCA	CTG	ATC	GAT	TAC	TTG
I	P	Y	s	I	s	A	s	v	D	R	s	ĸ	I	н					
CG	CCA	TCA	TCG	CCG	ATT	ATA	GCT	CCA	aca	ATA	GAT	GCC	GAG	TAC	GTT	GAG	GTC	ACA	CACI
CA	CAT	ATA	TAT	ATA	TAT	ATA	TAC	ACA	TAT	ACA	TGA	ATA	AAT	GCT	CTG	ATA	TGA	TCT	CCAT
GT	GAG	AGA	CAG	GAG	TAC	GTA	CGT	CGA	GCC	AGC	TCT	GCA	TAG	CCG	acc	ACT	GTT	GTA	TCT
TT	TTG	TAT	CAG	CGT	TTG	ATT	GTT	CGT	TCA	CTC	CCT	TTG	AGC	AGA	GAC	GGG	AGT	TTG	TTGT
AC	TTT	GTA	CCA	TGT	GCA	CTC	ATG	GTA	TTA	TAA	ATT	AAT	CTA	GCT	CTA	TTC	AGT	TAA	AAA
AA	777	AAA		222		6													
	20																		
LTF	2																		
LTR	2	TCT	AGC	TAT	ccc	ACC	CTC	TCT	CCG	TGA	GCT	AGC	CCA	CCA	CCA	CAA	GGT	CCA	CAA
GC	22	TCT	AGC	TAT	CCC	ACC	TGG	TCT	GCA	TGA	GCT	AGC	CCA	CCA	CCA	CAA	GGT TGG	CCA	CAAC
	GC AT M GT V TC S TT L GC A AT I CG CA GT TT ACA	GCTAA ATGGC M A GTAGC V A GTAGC V A TCCTA S Y TTGGC L A GCTGC A A ATCCC I P CGCCA CACAT GTGAG TTTTG ACTTT AAAAA	Ο CTAATTA ATGGCTCC W A S R TTCCTACGC S Y A TTGGCCGG L A G GCTGGCCGG A A G GCTGCCCGTA I P Y CGCCATCAT GCGACATCA GCGC	GCTANTIACCC ARGGCTCCGAA M & R T GTAGCCTCCGAA V & S D TCCTACGCAA S Y & K TTGGCCGGCG L & G G A & G G A TCCCCTACTC I P Y S CGCCATCATCC CGCCATCATCAC CGCCATCATCAC CGCCATCATCAC ARAAAAAAAA	GCTANTIACCACC ARGGCTCGCACTGC W A R T A GTAGCCTCGACGG V A S D A TCCTACGCAAAAGG S Y A K G TTGGCCGGCTAGG L A G L A GCTGCCGGTGGGGT A A G G L ATCCCCTACTCCAT I P Y S I CGCCATCATCGCCG CACATATATATAT GTGAGAGACAGGAG TTTTGTACCACGT AAAAAAAAAA	GCTANTIACCACCCTT ATGGCTCGCACTGCAGC M & R T & A GTAGCCTCGCACGGGG V & S D & A TCCTACGCAAAAGGCAG S Y & A K G S TTGGCCGGTGGGGCTCAA C & A G L & R GCTGCCGGTGGGGGTCAA A & G G L N ATCCCCTACTCCATCAG I P Y S I S CGCCATCATCGCCGAT CGCCATCATCGCCGAT CGCCATCATCGCGGATAC TTTTGTACACATGTGCA AAAAAAAAAA	GCTANTIACCACCCTTCC ARGGCTCGAACGCAGCAG M & R T & A T TATAGCTCCGACGCGGCGAT V A S D A A I TCCTACGCAAAGGCAGCGG S Y A K G S G TTGGCCGGCTGAGCGCGGGG L A G L A R S GCTGCCGGTGGGGCTCAACCC A A G G L R P ATCCCTACTCCATCAGCGC I P Y S I S A CGCCATCATCCATCAGCATATAT CGCACATCATCAGCGTTTGAT ACTTTGTACCACGTTGTTAT TTTTGTACCACGTTGTACA	GCTAATTACCACCCTTCCCTC ATGGCTCGACACAAAAAAAAAA	GCTANTIACCACCCTTCCCTGCGA ATGGCTCGCACTGCGGCGATCTTCCTG W A R T A A T K L GTAGCCTCGACGGGGGATCTCTG V A S D A A I S \bigcirc TCCTACGCAAAGGCAGGGGGGCGA S Y A K G S G A S TTGGCCGGCTGAGCGCGGGGGGCA C A G L A R S T A GCTGCCGGTGGGGCTCAACCCGGAA A A G G L R P G K ATCCCCTACTCCATCAGGCACACCCG I P Y S I S A S V CGCCATCATCGCCGATATAGCTCCA CACATATATATATATATATACACAC GTGAGAGACAGGAGTACGTACGTCGA TTTTGTACACGTTGTGCACTCATGGTA ANANANANANANANA	GCTANTIACCACCCTTCCCTTGCAGCC ArgGCTCGCACTGCAGCACAAGGCTGT M A R T A A T K L V GTAGCCTCGACGCGGGATCTCTCGGG C C T A B A T S \square TCCTACGCAAAGGCAGCGGGGCCAGCCC S Y A K G S G A S P TTGGCCGGCTGAGCGCGGAGGCCCGGC A A G L A R S T A D GCTGCCGTGGGGGTCAACCCGGGAAGGC A A G G L R P G K A ATCCCCTACTCCATCAGCGCACCCGTGGA I P Y S I S A S V D CGCCATCATCCCATCAGGTATAGCTCCAGCG CACATATATATATATATATACACATT TGGAGAGCAGGAGTACGTCCAGCG CTTTGTACCATGTGCACTCATGGTATA ANAAAAAAAAAAAAAAA	GCTAATTACCACCCTTCCCTGCGAGCCGCC ArGGCTCGCACTGCGGCGATCTCCTGCGGTGCT M A R T A A T K L V L GTAGCCTCCGACGCGGGATCTCCTGCGGTGC V A S D A A I S \square G Q TCCTACGCAAAAGGCAGCGGGGGCCACACCCCCC S Y A K G S G A S P P TTGGCCGGCTGAGCGCGGGAGCACCGCCGACAA L A G L A R S T A D K GCTGCCGTGGGGGTCCAACCGGGAAAGCCTGC A A G G L R P G K A A ATCCCCTACTCCATCAGGCCACCCCCGGGATGGCACC I P Y S I S A S V D \square CGCCATCATCGCCGATATGTCCAGCGACA GCGACATCATCGCGATATGTCCAGCGACA GCGACATCATCGACGTAGGCACCCCTTTGTATCAGCGTTGGCCACTCACCCAGGCTAGCCACCTCAGGCTATTATAA AAAAAAAAAA	GCTAATTACCCACCCTTCCCTTCCAGGCCCTCCAG ARGGCTCGCACTGCGGCGACAACAAGGCTCGTGCTGGT W A R T A A T K L V L V TAGCCTCCGACGCGGGACTCCTCCGGCGGCGGG V A S D A A I S \square G Q V TCCTACGCAAAGGCAGCGGGGGCCAGCCCCCCGG S Y A K G S G A S P P G TTGGCCGGCTGAGCGCGGGGGCAGCCCCCCGGGAAGGC L A G L A R S T A D K Q GCTGCCGGTGGGGCTCAACCCGGGAAAGCCTCCAG A A G G L R P G K A S ATCCCCTACTCCATCAGGCCACCCCGGGAAGGCTCCAGC I P Y S I S A S V D \square S GCGCCATCATCCCATCAGGCTACCCCGGGAAGAGC GCGCATCATCGCCGATATATATATATACACATTACACTG GTGAGAGACAGGAGTACGTCCGTGGGCCACCTCT TTTTGTACCATGTGCCACCCATGGGTATTATAAATT AAAAAAAAAA	GCTAATTACCCACCCTTCCCTGCAGCCCTCCCACCAC ARGGCTCGCACTGCGGCGACACAAGCCCGTGCTGGGTGG M A R T A A T K L V L V A GTAGCCTCCGACGCGGGACTCCTCCGGCGGCTGGGTGGG V A S D A A I S \square G Q V N TCCTACGCAAAGGCAGCGGGGGCCAGCCCCCCGGGGG S Y A K G S G A S P P G A TTGGCCGGCTGAGCGCGGGGGCAGCCCCCCGGGAGGGCCGGCGGGGGGGCCAACCCGGGAAAGCCGGGAAGAC L A G L A R S T A D K Q A GCTGCCGGTGGGGCTCAACCCGGGAAAGCCTCCAA A G G L N P G K A A S I A C CGCCATCCCCCCAGCAACCCCCGGGACAGCCTCCTAA I P Y S I S A S V D \square S K CGCCATCATCCCCATCAGCTCCCAGGACTAGCCTCCAA TTTGGACGACGAGAGACGTCCGACGACTCGCCA TTTTGTACACGTTGTGCAGCCACCCCTCT ACTTTGTACCATGTGCACCCCTGGGACTGCCCTTTG	GCTAATTACCCACCCTTCCCTTGCAGCCCTCCACCACAAC ARGGCTCGCACTGCAGCACAAAGCTCGTGCTGGTCGGCCCT W A R T A A T K L V L V A L GTAGCCTCCGACGCGGGACTCCTGCGGCGCGGGCAGGTGAACTC V A S D A A I S \bigcirc Q V N S TCCTACGCAAAAGGCAGCGGGGGCCAGCCCGCCGGGGGCGCGGGGGCCTAGCGCGGGAGGCCCGGCGAACAGCAGCGCC S Y A K G S G A S P P G A \bigcirc TTGGCCGGCGTGGGCCAGCGCGGGAAAGCTGCCAAGTATCCC A A G G L A R S T A D K Q A A GCTGCCGTGCGGCTCAACCCGGGAAAGCCTGCAAGTATCCC A A G G L N P G K A A S I P ATCCCCTACTCCATCAAGCAACCCCGGGACAAGCTGCCTAAGAT I P Y S I S A S V D \bigcirc S K I CGCCATCATCCCATCAAGCTACCCCGGGACAAGCTGCCGAG CACATATATATATATATATACACATATACATGAAGATGCCGA TTTTGTACACGTTGTGCAGCCAGCTCGCCGCCTGCATGAT TTTGTACACGTGTGGCCACTCGCCGCCAGCTCTGCATGA ACTTTGTACCATGTGCCACCCATGGTATTATAAATTAATCTA AAAAAAAAAA	GCTAATTACCCACCCTTCCCTTCCAGGCCGTCCACCACAACTAC ATGGCTCGCACTGCGGCGACACAAGCCCGTGGTGGGCCGGGCGGG	GCTAATTACCCACCCTTCCCTTCCAGGCCTTCCACCACAACTACACC ARGGCTCGCACTGCAGCACAAAGCTCGTGCGGGCCGGCCTGGTGGGC M & R T & A T K L V L V A L V A GTAGCCTCCGACGCGGGACTCCTCGGGGCAGGTGAACTCTCCCCT V A S D A A I S \bigcirc Q V N S A L TCCTACGCAAAAGGCAGCGGGGGCCAGCCGCCGGGGGCCTGCTGTC S Y A K G S G A S P P G A \bigcirc C TTGGCCGGCTGAGCGCGGGGACAAGCAGCAAGCAGCGGCATACAG L A G L A R S T A D K Q A A \bigcirc R GCTGCCGGTGGGGCTCAACCGGGGAAAGCTCCAAGATCCCCCTCCAA A A G G L N P G K A A S I P S K ATCCCCATCCCATCAAGCAACCACCGTGGACTGCTCTAAGATCCACCG I P Y S I S A S V D \bigcirc S K I H * CGCCACTACTCCATCAAGCTACCACGGACAAGATACCCGACGATATATAT	GCTAATTACCCACCCTTCCCTTCCAGCCCTCCACCACAACTACACCAAA ATGGCTCGCACTGCAGCACAAAGCTCGTGCGGGCGCCCGGGGGGGG	GCTAATTACCCACCCTTCCCTTCCAGCCCTCCACCACAACTACACCTAACACC ARGGCTCGCACTGCAGCACAAAGCTCGTGCGGGCCCGGGGCGGCAAT W A R T A A T K L V L V A L V A A M GTAGCCTCCGACGCGGGACACTCCTGCGGGCACGCTGGGCGCCTCCGCTGCTCCCGCGGCCT V A S D A A I S \bigcirc Q V N S A L A S TCCTACGCAAAAGGCAGCGGGGGCCAGCCGCCGCGGGCGCTGCCGCTGCCGCGGAGGC S Y A K G S G A S P P G A \bigcirc S G V TTGGCCGGCGGGGGGCCAGCCGCCGACAAGCAGCGGCATCCAGGTGCGG L A G L A R S T A D K Q A A \bigcirc R \bigcirc I S G V TGGCCGGCGGGGGGCCAACCCGCCGGGAAAGCCTCCCGCTGACGGCGGGAGGCCCCCCCGGGAAGCCCCCCCC	GCTAATTACCCACCCTTCCCTTGCAGCCGTCCACCACAACTACACGCAATGCT ARGGCTCGCACTGCAGCACAAAGCTCGTGCGGGCCAGCTGGTGGCGGCAATGCT M & R T & A T K L V L V A L V A A M L GTAGCCTCCGACGCGGGACTCCTGCGGTGAGCTGACTCTCCCCGGGGCTCGTG V A S D A A I S \bigcirc Q Q V N S A L A S \bigcirc TCCTACGCAAAAGGCAGCGGGGCCAGCCGCCGCCGGCGCTGCTGTCCGGAGTTAG S Y A K G S G A S P P G A \bigcirc S C V R TTGGCCGGCGGGGGGCCAGCCGCCGACAAGCAGCGGCATCAAGGTGCACTA L A G L A R S T A D K Q A A \bigcirc R \bigcirc I K GCTGCCGGTGGGGCTCAACCGGGGAAAGGCTGCCAGATCCCCCCCAGTGCGGCT A A G G L R P G K A A S I P S K \bigcirc G V R ATCCCCTACCCCATCAAGCCACCGCCGACTAGATCCCACTGATCGAGTAC I P Y S I S A S V D \bigcirc S K I H * CGCCACTACTCCCATCAAGTTCCCAGGATAGCTCGAGTACGTGAGGTCCAC CACATATATATATATATATACACATATACATGAATAACTCTGGACGGAC

							м	A	R	т	A	A	т	ĸ	L	v	L	v	A	L
121	GGT	GGC	GGC	AAT	GAT	CCT	CGC	AGC	CTC	CGA	CGC	GGC	CAT	CAG	CTG	CGG	CCA	GGT	GAG	CTC
	v	۸	A	м	I	L	A	A	s	D	A	л	I	s	e	G	Q	v	s	s
181	TOC	CTT	GAC	CCC	CTG	CGI	CGC	CTA	TGC	GAA	GGG	CAG	CGG	GAC	CAG	CCC	GTC	TGG	GGC	CTG
	a	L	т	P	d	v	A	¥	A	ĸ	G	s	G	т	s	P	s	G	A	C
241	CTG	CAG	COG	CGT	CAG	GAA	ATT	GOC	CGG	CTT	AGC	GCG	GAG	CAC	CGC	CGA	CAA	GCA	AGC	TAC
	c	s	G	v	R	ĸ	L	A	G	L,	A	R	s	т	A	D	ĸ	Q	A	T
301	ATG	CAA	GGT	GCC	TCA	AGA	GTG	TTG	CCG	GAG	GGC	TCA	ACC	CCA	ACA	AGG	CCG	CAG	GCA	TTC
	c	R	P	L	ĸ	s	v	A	G	G	L	N	P	N	к	A	A	G	I	P
361	CCT	CCA	GTG	CGG	CGT	CAG	CGT	CCC	CTA	CAC	GAT	CAG	CGC	ATC	CGT	GGA	CTG	CTC	TAA	GAT
	5	ĸ	e	G	v	s	v	P	Y	т	Ι	s	А	s	v	D	C	s	ĸ	I
421	CCA	CTG	ATC	GAT	TTC	TGG	CCG	CCA	TCA	TCG	CCG	ATT	ATA	GTT	CCA	GCG	ATT	GAT	GCT	GAG
	н	*																		
481	TAC	GTT	GAG	GTC	TCA	CAC	ACA	CAT	ATA	TAC	ATA	TAC	ATG	AAT	AAA	TGC	TCT	GAT	ATG	ATC
541	TCC	ATG	TGA	GAG	ATA	GGA	GTA	CGT	ACA	TCG	AGC	CAG	CTT	TGC	ATA	GCT	GGC	CAC	TGT	TGT
601	ATC	TAT	TTT	GTA	TCA	GCG	TTT	GAT	TGT	TCG	TTC	ACT	CCC	TTT	GAG	CTA	GAC	ACG	GGA	GTT
661	TGT	TGT	ACT	TTG	TAC	GAT	GTA	CGC	TTA	TCA	TAT	AGG	ATT	ATA	AAT	TAA	TCT	222		AAA
721	AAA	AAA	AAA	A																

Fig. 1 Nucleotide sequences and derived amino acid sequences of *TaLTP1* and *TaLTP2*. *Asterisks* indicate the transcription stop sites. The *underlined* sections represent the putative polyadenylation signal sequences. Cysteine residues are enclosed in *boxes* and are located in the conserved regions identified throughout all of the plant LTPs

Results

Isolation and characterization of two LTP cDNAs

In the previous article (Jang et al. 1999), we reported the isolation of a novel LTP EST from a wheat-rye translocation line carrying 2RL. This LTP cDNA clone was designated as TaLTP1 (Triticum aestivum Lipid Transfer Protein 1). Twenty-eight positive plaques were identified among the 2×10^6 plaques of the cDNA library that was constructed from 4-week-old leaves of an NIL carrying 2RL. Of these 28 clones, eight were selected at random for sequencing. Four clones were ultimately identified as TaLTP1 based on a homology search. Other positive cDNA clones that were not identified as TaLTP1 were designated as <u>Triticum aestivum Lipid Transfer Protein 2</u> (TaLPT2). Both TaLTP1 and TaLTP2 contained a 270-bp ORF that encoded a putative LTP precursor of 90 amino acids. This ORF also contained a signal peptide of 25 amino acids (Fig. 1). The molecular masses of TaLTP1 and TaLTP2 were calculated to be 8.7 kDa and 8.8 kDa, respectively (excluding the signal peptide), with an estimated isoelectric focusing point of 9.3. The polyadenylation signal sequences (AATAAA) of TaLTP1 and

TaLTP1	1	MARTAATKLVLVALVAAMLLVASDAAISCGOVNSALASCV	40
TaLTP2	1	MARTAATKLVLVALVAAMILAASDAAISCGOVSSALTPCV	40
NSLTP_CW18	1	MARTAATKLALVALVAAMLLVAADAAITCGOVSSALGPCA	40
NL41 HORVU	1	MARAAASOLVLVALVAAMLLVAADAALSCGOVSSALSPCT	40
NLTP_MAIZE	1	MARTOOLAVVATAVVALVLLAAAT-SEAAISCGOVASAIAPCI	42
NLT2_SORBI	1	MARSMKLAVAIAVVAAAAAVVLAATTSEAAVTCGQVSSAIGPCL	44
TaLTP1	41	SYAKGSGASPPGACCSGVRRLAGLARSTADKQAACRCIKSAA	82
TaLTP2	41	AYAKGSGTSPSGACCSGVRKLAGLARSTADKQATCRCLKSVA	82
NELTP CW18	41	AYAKGSGTSPSAGCCSGVKRLAGLARSTADKQATCRCLKSVA	82
MEAL HODING	41	SYARGNGAKPPAACCSGVKRLAGAAQSTADKQAACKCIKSAA	82
NL41 HORVU	43	SYARGOGSGPSAGCCSGVRSLNNAARTTADRRAACNCLKNAAAG	86
NLT2_SORBI	45	SYARQQGSGPSAGCCSGVRSLNSAARTTADRRAACNCLKNAARG	88
TaLTP1	83	-GGLNPGKAASIPSKCGVSIPYSISASVDCSKIH	115
TaLTP2	84	-GGLNPNKAAGIPSKCGVSVPYTISASVDCSKIH	115
NsLTP CW18	84	-GAYNAGRAAGIPSRCGVSVPYTISASVDCSKIH	115
NL41 HORVU	84	-GGLNAGKAAGIPSMCGVSVPYAISASVDCSKIR	115
NLTP MAIZE	87	VSGLNAGNAASIPSKCGVSIPYTISTSTDCSRVN	120
NLT2 SORBI	89	IRGLNVGKAASIPSKCGVSIPYTISTSTDCSRVS	122

Fig. 2 Comparison of the derived amino acid sequences from wheat (*Triticum aestivum*) *TaLTP1* and *TaLTP2* with other plant LTPs including: NsLTP_CW18 (nonspecific lipid-transfer protein Cw-18) of barley (S45370), NL41_HORVU (nonspecific lipid-transfer protein 4.1) of barley (Q43767), NLTP_MAIZE (nonspecific lipid-transfer protein precursor) of maize (P19656), and NLT2_SORBI (nonspecific lipid-transfer protein 2 precursor) of sorghum (Q43194). The *arrow* indicates the putative signal peptide cleavage site



Fig. 3 Northern blot hybridization of *TaLTP1* and *TaLTP2* genes in different tissues. Total RNA (20 μ g per sample) of four tissues from 4-week-old plants of the NIL carrying 2RL and a nontranslocated NIL (Coker 797) was fractionated on a 1% denaturing agarose gel. *L* leaf, *S* stem, *C* crown, *R* root

TaLTP2 were located 192 bp and 187 bp, respectively, upstream of the poly(A) tail.

A comparison of the deduced amino acid sequences of *TaLTP1* and *TaLTP2* with other plant LTPs is presented in Fig. 2. Both *TaLTP1* and *TaLTP2* contained eight cysteine residues that are a conserved structural characteristic of plant LTPs. Neither LTP contained tryptophan, glutamate, or phenylalanine. The similarity between *TaLTP1* and *TaLTP2* (including the signal peptide) was 88% (101/115) identity based on their deduced amino acid sequences. When *TaLTP1* and *TaLTP2* were compared with other plant nonspecific LTPs, such as those from barley, maize, and sorghum, identities ranged from 57% to 80% and from 56% to 85%, respectively.

Tissue specificity of *TaLTP1* and *TaLTP2* expression

Northern blot hybridization indicated that both *TaLTP1* and *TaLTP2* were expressed in the leaves, stems, and crowns of the NILs but not in the roots (Fig. 3). The expression of both *TaLTP1* and *TaLTP2* was highest in the stems of an NIL carrying 2RL. In contrast, *TaLTP1* and *TaLTP2* mRNAs were more abundant in the leaves of the nontranslocated plants.





Fig. 4 Northern blot hybridization of *TaLTP1* and *TaLTP2* genes from leaves of the NIL carrying 2RL that was treated with cold and ABA. Total RNA (20 µg per sample) of leaves from 4-weekold plants was fractionated on a 1% denaturing agarose gel. *Left* Leaf material was harvested from the plant incubated at 4°C for 6, 12, 18, and 24 h. *Right* Leaf material was harvested from the plant sprayed with a 100 µM solution of ABA with 0.05% (v/v) Tween 20 for 6, 12, 18, and 24 h. *C* Control, *h* hours

Fig. 5 Northern blot hybridization of *TaLTP1* and *TaLTP2* genes from leaves of the NIL carrying 2RL that underwent drought treatment. Total RNA (20 μ g per sample) of leaves from 4-week-old plants was fractionated on a 1% denaturing agarose gel. The leaf material was harvested from plants that underwent drought treatments of 1, 2, 3, 4, and 5 days. *C* Control, *d* days

Fig. 6A-F In situ hybridization of TaLTP1 mRNA in various wheat tissues of control and drought-treated plants. A blue-purple color indicates a positive signal. A, B Transverse section of vascular bundles of control plant leaves: A positive signal (hybridized with TaLTP1) (arrow stain in cells within a vascular bundle), **B** negative signal (hybridized without TaLTP1). C Transverse section of root cells of droughttreated plant. D Transverse section of crown cells of control plant. E, F Transverse section of crown cells of drought-treated plant: E positive signal (hybridized with TaLTP1), F negative signal (hybridized without TaLTP1). Arrow (**D**, **E**) Stain in cells within a vascular bundle and in the tissue layers between the vascular bundles. C Cortex, En endodermis, P phloem, Vs vascular bundle, X xylem. Bar 100 µm



TaLTP1 and TaLTP2 expression following abiotic stresses

Northern blot analysis (Fig. 4) indicated that both *TaLTP1* and *TaLTP2* were expressed throughout the cold treatment in the NIL carrying 2RL. A similar pattern of gene expression was detected in the control plants. Treatment of plants with exogenous ABA did not affect the expression of LTPs over a period of 24 h in either the control plants or in the NIL carrying 2RL.

The drought treatment had a pronounced effect on the expression of both LTPs (Fig. 5). The expression of *TaLTP1* in the NIL carrying 2RL was highest 3 days after the initiation of the drought treatment, at which time visually detectable symptoms of drought stress were evident and leaf water potential values were decreasing (data not shown). Expression levels then remained near constant through day 5. In contrast, the expression of *TaLTP2* mRNAs in the NIL carrying 2RL increased during the 3 days following the drought treatment initiation and then continued to increase through day 5.

In situ hybridization of the TaLTP1 gene

In order to examine the cell specificity of the expression of *TaLTP1* in response to drought, we performed in situ hybridization using the leaves, crowns, and roots of both control and drought-treated plants (Fig. 6). The spatial patterns of expression of *TaLTP1* mRNA were similar in both the control and drought-treated plants. In the leaves, *TaLTP1* mRNA was detected in cells within the vascular bundles of both the control and the drought-stressed plants. *TaLTP1* mRNA was also detected in the tissue layers between the vascular bundles in the crowns of both the control and drought-treated plants. However, expression of *TaLTP1* in the tissue layers appeared to be greater in the drought-treated plants than in the control plants. Expression of *TaLTP1* was not detected in the roots.

Discussion

We have described the isolation of two LTPs (*TaLTP1* and *TaLTP2*) from a cDNA library constructed from 4-week-old leaves of a wheat-rye translocation line (2BS/2RL). *TaLTP1* and *TaLTP2* have many features in common with other plant *LTPs*. For example, both genes code for proteins that each contain eight cysteine residues and no tryptophan residues and have a molecular weight of about 9 kDa with an isoelectric focusing point of 9.3. Both contain signal peptide sequences that are identical to those of LTP genes present in other plants.

In plants, nonspecific LTPs occur as small multigene families. However, individual LTPs are often differentially expressed in response to environmental variables, stages of growth, and tissue types (Kader 1996; Vignols et al. 1997; Arondel et al. 2000). The spatial patterns of expression of *TaLTP1* and *TaLTP2* suggest tissue-specific differences. *TaLTP1* and *TaLTP2* were expressed in leaf tissues of both NILs. However, the highest levels of expression of both *TaLTP1* and *TaLTP2* occurred in the stems of the NIL carrying 2RL. In contrast, the highest levels of expression of *TaLTP1* and *TaLTP2* occurred in the leaves of the nontranslocated NIL. These apparent tissue-specific differences with respect to expression level may, or may not, be associated with the presence or absence of the 2RL chromosome. Additional study is required to more fully elucidate the possible role of 2RL in the expression of these LTP genes. *TaLTP1* and *TaLTP2* were expressed in the aerial portions (leaves, stems, and crowns) of the plants but not in the roots. Similar findings have been reported elsewhere (Torres-Schumann et al. 1992; Gausing 1994).

In earlier studies the transcripts of the LTP genes of barley reached high steady-state levels 7 days after the initiation of a low-temperature treatment (Dunn et al. 1994) and the promoters of the LTP genes were also induced by low temperature (Molina et al. 1996). We found that *TaLTP1* and *TaLTP2* were continuously expressed both in control plants and in cold-treated plants over a period of 24 h.

Drought-induced plant stress is a common occurrence in agriculture. Abiotic stress typically results in the expression of numerous stress-related genes and in the accumulation of ABA. ABA accumulation is believed to provide plants with a tolerance to drought, high salinity, and cold (for review see Chandler and Robertson 1994). The exogenous application of ABA can result in the expression of some, but not all, genes that typically are induced in response to water stress and cold (Shinozaki and Yamaguchi-Shinozaki 1997). Nakashima et al. (1997) reported a drought-inducible gene that did not respond to either cold stress or the application of ABA. Vignols et al. (1997) reported that the expression of rice LTP genes was stimulated by exposure to salt but not by treatment with ABA. In the present study, the continued expression of TaLTP1 and TaLTP2 following exogenous application of ABA over a period of 24 h suggests either that the expression of both TaLTP1 and TaLTP2 was independent of an ABA pathway, or that the two genes responded very slowly to ABA exposure.

Plant LTPs have also been shown to be inducible in response to water deficit. Plant et al. (1991) reported that expression of the le6 gene, which shows homology with the LTP of maize, was induced by drought or ABA treatment. An LTP from tomato (TSW12) was detected following treatment of a plant with NaCl, mannitol, or ABA (Torres-Schumann et al. 1992). The LTP from Phaseolus vulgaris was induced by water deficit or ABA treatment (Colmenero-Flores et al. 1997). In our case, visually detectable symptoms of water deficit were evident 3 days after the initiation of the water-stress treatment. At this time, the plants also exhibited lower water potential values than the control plants (data not shown). Increased transcripts of TaLTP1 and TaLTP2 mRNAs were observed 3 days after the initiation of the drought treatment. This coincided with the initiation of visually detectable symptoms of water deficit. The relationship between water deficit and the expression pattern of the LTP genes suggested that the LTP genes were over-expressed in the leaves in response to the drought treatment.

The localization of expression of LTP genes has been previously investigated using in situ hybridization. In maize seedlings, the highest expression of LTP occurred in the outer epidermis of the coleoptiles and in the veins (Sossountzov et al. 1991). Thoma et al. (1994) reported that LTP1, isolated from Arabidopsis, was active in the protoderm cells of the embryo, vascular tissues, lignified tips of the cotyledons, shoot meristem, and stipules. The expression of TaLTP1 mRNAs in the vascular bundles of leaves and crowns may indicate that LTP gene products play a role in the secretion of lipophilic substances in the cell walls of secretory tissues (Thoma et al. 1994). Pearce et al. (1998) reported that blt101 was strongly expressed in the vascular-transition zone of the cold-treated crown and suggested that it might be involved in bestowing tolerance to cellular dehydration caused by extracellular freezing. We observed that TaLTP1 was expressed in the cells within the vascular bundles of leaves and in the tissue layers between the vascular bundles, in the crowns of both control and drought-stressed plants. The similar patterns of expression of *TaLTP1* in nonstressed and stressed plants suggest that the function of nonstressrelated and stress-related expression of LTPs might be similar (Pearce et al. 1998). However, TaLTP1 was also strongly expressed in the tissue layers between the vascular bundles in the crowns of the drought-treated plants. As noted previously, these results suggest that the tissue layers between the vascular bundles were damaged by dehydration and that the expression of TaLTP genes in these tissues might be expected to mediate drought tolerance.

Acknowledgements The authors thank Dr. R.A. Graybosch (USDA-ARS, University of Nebraska-Lincoln, Neb.) and Dr. R.L. Jarret (USDA-ARS, University of Georgia, Griffin, Ga.) for thoughtful comments and critical review of the manuscript. We especially thank Dr. B.K. Hwang and Mr. S.C. Lee (Department of Agricultural biology, Korea University) for their assistance with the in situ hybridization.

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