

Update section

Short communication

Structure and expression of the barley lipid transfer protein gene *Ltp1*

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Abstract

We have characterized a gene (*Ltp1*) encoding a barley lipid transfer protein. Northern blot analysis showed that *Ltp1* mRNA accumulates specifically in the aleurone layer of developing and germinating seeds. Southern blot analysis indicated that LTP1 protein is encoded by a single gene in barley. Sequence analysis of *Ltp1* showed that it contains an open reading frame of 351 bp interrupted by a single intron of 133 bp. Transient expression assays indicated that 702 bp of the 5' upstream region of *Ltp1* is sufficient to direct aleurone-specific expression during late seed development and early germination.

Several years ago we reported the sequence of a cDNA encoding a barley protein with amino acid sequence homology to inhibitors of amylases and proteases [11]. The protein was therefore referred to as PAPI for Probable Amylase/Protease Inhibitor. Immunoprecipitation experiments showed that this protein accumulates specifically in the aleurone layer during late seed development and early germination. Recently, the protein was found to be homologous to lipid transfer proteins [3], and was also shown to transfer phospholipids between membranes *in vitro* [5]. The gene encoding this protein has therefore been named *Ltp1* (Lipid transfer protein).

We are studying gene regulation in barley seeds,

focusing on genes whose expression is modulated by phytohormones [12], as well as genes that show developmental or tissue-specific expression patterns [9]. Northern hybridizations showed that *Ltp1* mRNA accumulates specifically in the aleurone layer during late seed development and early germination (Fig. 1A). In contrast, *Ltp1* mRNA was not detectable in leaves and roots of mature plants, in coleoptiles or seedling shoots, or in the embryo or starchy endosperm of developing and germinating seeds. We therefore undertook to clone *Ltp1* as a model for studying determinants of aleurone-specific gene expression.

Southern blots of genomic DNA digested with restriction enzymes that do not cut within the

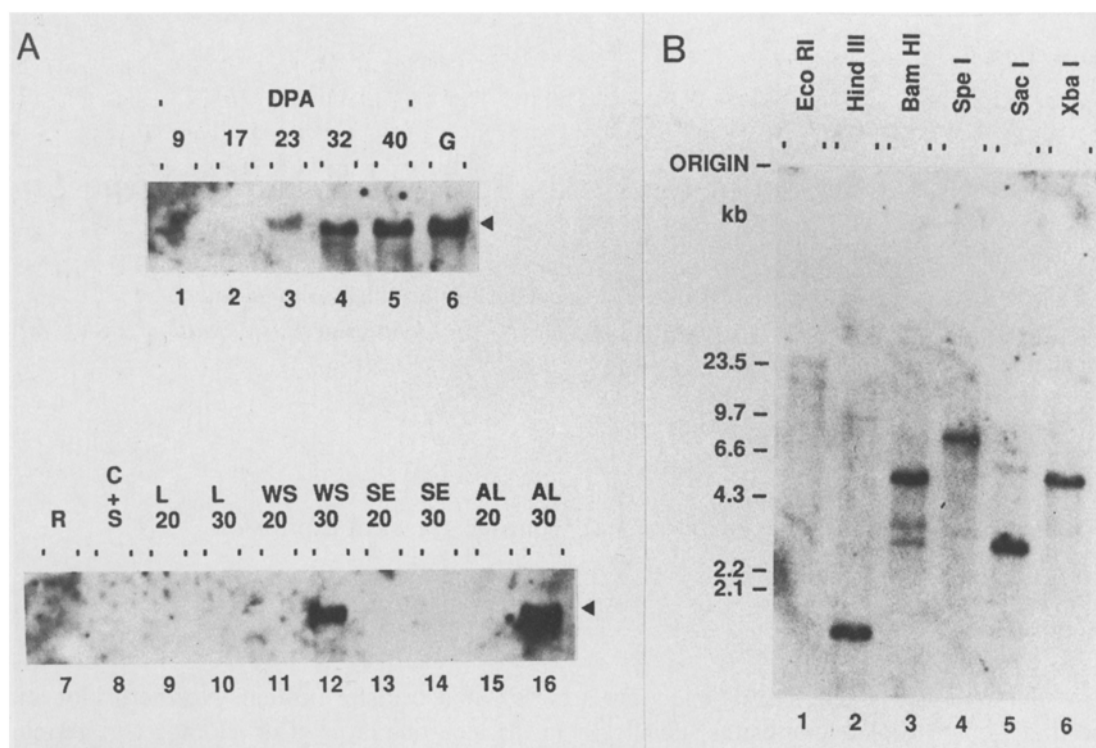


Fig. 1. A. Steady-state *Ltp1* mRNA levels in seeds, leaves, roots and shoots of barley (cv. Piggy). Northern blot analysis of *Ltp1* mRNA from developing (9–40 DPA, lanes 1–5), and germinating (lane 6) seeds, from roots (lane 7), and shoots/coleoptiles (lane 8) of mature seeds germinated for 6 days, and from leaves (lanes 9, 10), whole developing seeds (lanes 11, 12), isolated developing starchy endosperm (lanes 13, 14), and isolated aleurone layers (lanes 15, 16) from plants harvested 20 and 30 DPA, respectively. The northern blot using the ^{32}P -labelled cDNA probe was performed as described previously [9]. The arrow shows the single transcript of approximately 650 bases. B. Southern blot analysis of *Ltp1* DNA. 10 μg DNA was digested with *Eco* RI (lane 1), *Hind* III (lane 2), *Bam* HI (lane 3), *Spe* I (lane 4), *Sac* I (lane 5) and *Xba* I (lane 6). Southern blotting was performed as described previously [9]. *Hind* III-digested λ phage DNA was used as size marker.

Ltp1 sequence and probed with the full-length cDNA [11] showed one strongly hybridizing band, indicating that there is a single *Ltp1* gene in barley (Fig. 1B). In addition, the blot showed one or two faint bands, suggesting that barley contains one or two other genes with weak homology to *Ltp1*.

A genomic fragment containing *Ltp1* was isolated from a library of *Spe* I-digested barley (*Hordeum vulgare* L. cv. Piggy) genomic DNA ligated to the *Spe* I site of λ ZAP II (Stratagene). 7×10^5 plaque-forming units plated on *Escherichia coli* ER1647 (McrA⁻ and McrB⁻, New England Biolabs) were screened with the full-length, ^{32}P -labelled cDNA probe [11]. Excision of pBlue-script SK⁻ plasmid from lambda ZAP II was performed according to the λ ZAP II cloning kit protocol (Stratagene). Primers (17-mers) derived

from the cDNA and genomic sequences were used to sequence double-stranded plasmid DNA in both directions by the dideoxy chain termination method with Sequenase (United States Biochemicals).

The sequence of the *Ltp1* gene is shown in Fig. 2. The coding region is identical to that of the original cDNA [11]. Primer extension (data not shown) determined the transcript start site (Fig. 2, position + 1), and showed that the original cDNA is full-length.

To determine whether the apparent aleurone-specific expression of the gene is at least in part due to transcriptional control, we fused *Ltp1* 5' upstream sequences to the bacterial β -glucuronidase (GUS) reporter gene. This *Ltp1*/GUS gene contained a 769 bp fragment (Fig. 2; -702 to + 67) of *Ltp1* obtained by PCR amplification

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GTCCCAACTCATGAGCATCACGGAATGGCATGAGTTGAAATATAACTACATTGCTCAAA -1621
GCAACAAAAGCACATTAGAATCTTGAGCATTGAGATAAGAGTTTTCTCATGCTCTAAA -1561
TATATATTTTGAGAACTCCTTTGAGGAGAAAATCCATATTTACAATTGTTGTAATTT -1501
GAGTCCATGATCCTAAAGAGATTAGCATGCGAATTACCCAAACATCAAAATTTGTGCCA -1441
TTGAAACTAAGAGTGTAGAGAACTCTAATCCCTAGTTGACATACTTACTCTCTAGGTT -1381
GTGAAACCTAATAATGAGAGATCTAGCTCTAATACCAATTGAGAGGATGTGGATGTCGCC -1321
TAGAGGGGCGGTGAATAGCGCTTTAAAATAATTACGGTTTAGGCTCGAACAAATGTGGA -1261
ATAAACTAACCTTTTATTGTCAAGCGCAAAACCTAAACCAACTAGGCTCACCTATGTG -1201
CACCAACAACCTTATGATAAGCAAGATAAAAAAATAAGTGATGGCAGAAATATAACAAAG -1141
AAACAATATGGCTATCACAAGTGAAGTGCATAAGTAAACAGCTCGGGTAAGGGACAACC -1081
GAGCCATGCGGAGACGACGATGTATCCTCAAGTTCACACACTTGGCGATGCTAATCTCCG -1021
TTTGAAGCAGTGTGGAGGCACAATCGTCCCCAAGAGCCACTAAGGCCACCGTAATCTCC -961
TCACGCCCTCGCACAACTGAAGATGTTGTGATTCCACTAAGGGACCCCTGAGGGCAGTCA -901
CTGAACCCGTATAAACATGGTTGAACAACTCCACGACTTAATTGGAGACTCCCAACAA -841
CACCAAGCACTTATCATAACGAATATGGCTTCGAGGTAACTCAAACTGCTCGGGCA -781
ATTTTACAACTTAATTGAAGACCTCGACGCTTGGCTGGAGCTTACACTATAATGATTG -721
AGCTCCAAGGGCATCACCAAGCTTCTATGACGCCAAAACATCCAGAAAGATATGTACTA -661
GGATACCAAGCACCCAGAGATAAAGCGGAGGAAGTATAATATAAGGCCCTGTTTATAACA -601
AAGTAGTAAAAAACTAAAGTATTAATAAATGTCAGTAATTTACGTGTAGATAGAAAAATA -541
CCATGGTTTAAATATAATATATTTTTCAGTATTACAAATGTAGAGAACTGTTTGAT -481
TACGCCACATATTACTGCAGTTTAGATCGAGCAAGTACACGGGAAGAGATAACGACGTC -421
CCACCCCTTCTTTTCGCCCTTCTCTGTTTAAAAAGAGGTCTGGGGTTAGTTTTCCTAA -361
TACTGCAGTTTAAAAATCACAAATCTTAGAGGCAACCAACACCTCATGTAAATAAAAC -301
TATGATAATCTCCAAAACGAGTATTCTAAAAATACTACAAAAATCTTTGTTATCAAA -241
CAGGGCCTAAGGAGTTAAAAAAATTTAGCCGTAAGTACGAGCTCGGCGAGGCACGAGC -181
TAGCAGTCATCAACACTTGATGGTTGGCAAGGCGAGTCGACGTGTCGCGGGGCTCGGCC -121
TGAGCGGAGATACAACTGTTCTCCAGTAACCCCGTCGATTTGGCCCGCGACTAAAGC -61
ATCCAGGCATCTCTCGCTCGAACCCCTATTTAAGCCCTCCATCTCCCAACATTCTCC -1
ACACCTCCACGAGTGTCTCATCTAGCTAGTACGTTGTACTGTAGCTACAGATTAAAG 60
AGTGATCATGGCCCGCGCTCAGGTAAGTCTGCTAGGCGCGCGCTTGGTGTGATGCTCAC 120
M A R A Q V L L M A A A L V L M L T
GGCGGCCCGCGCGCTCGCGTGGCCCTCAACTGCGGCCAGGTTGACAGCAAGATGAAAC 180
A A P R A A V A L N C G Q V D S K M K P
TTGCTGACCTACGTTTCAAGGCGGCGCGCGCGCTCGCGGAATGCTGCAACGCGCTCAG 240
C L T Y V Q G G P G P S G E C C N G V R
GGATCTCCATAACAGGCGCAATCTCGGGCGACCGCAACCGTTTCAACTGCTGAA 300
D L H N Q A Q S S G D R Q T F V C N C L K
GGGATCGCTCGCGGCATCCCAATCTCAACCTCAACACGCGCGCAGCATCCCTCCAA 360
G I A R G I H N L N L N N A A S I P S K
GTGCAATGTCAACGTCCTCCATACCATCAGCCCGGACATCGACTGCTCCAGTgattaaa 420
C N V N V P Y T I S P D I D C S R
tttactcatccagagtgaatctttaaaagaactatatttacgaacggagtgagtat 480
ataggaaacattcatccacgtaaaatttggatatttaacattaacacgcatgattgacct 540
gcagGATTACTGAGCGACGATCCGTCAGCTGGTGTCTCAGCTCATCGATCCACGTGGAG 600
I Y *
CTGAAGCGCGCAGCCTCTGTCCCTATGTAGTATGGCTACCAGTTATGCCGAGTTTATGCT 660
GAATAAGAACTCTCTCTGTACTCCTTTGGAGGAGATCAGTATCTATGTACGTGAGAGTT 720
GAGAGTTTGTACCATCGGCACTCCAGTGTATTGGACTATATGCATACACCTCTCTTG 780
TGCTCAGTGTGTAACCTGTCTCTCTGTTTCTCACGTTTCGCTCATATAAATTTAC 840
TTATGTGCTCTAGGATCGTAGTACAGTATCATATATATACCTCTATGAATTAGTTTAC 900
CGTAGACCGTATGTTTCTGAATCTGGATGAAAATTACGGATTCAAGCGTGGCTCCCGCA 960
TATAATAAGCTTGCTTACGGATTCAAGCGTGGCTCAGCGGGCTCAGTAGATGATGAGGAT 1020
ACTCGCTGCTGCATCTCTACATCCCGCTCATGAGCTGAGCTGAGCCGGGTCTCCCCCG 1080
CTCCGGCCCGCTGGCCACCCCGCGCGCGCACCTCAACAGCCTTCATGACGAGCGGCC 1140
CGCGAGCAAGATCTGTTGGCTCTCCCTGTGCGTGTAGAGAAACCGCA 1192

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Fig. 2. Structure of *Ltp1*. The 351 bp open reading frame is interrupted by a 133 bp intron (+ 412 to + 544). The transcript start site is at position + 1, putative CAAT and TATA boxes at -107 and -34. A putative poly (dA) site (+ 785) has been noted earlier [11].

of the plasmid insert DNA according to the GeneAmp PCR Reagent Kit (Perkin Elmer Cetus). A CaMV 35S/GUS reporter gene which exhibits strong expression in numerous plant tissues was used as a control [2]. Developing barley caryopses were harvested and prepared by partially separating the pericarp/testa, aleurone and starchy endosperm boundaries. Aleurone layers were also prepared from mature, de-embryonized seeds imbibed for 3 days at 15 °C [11]. Delivery of DNA into these tissues was performed by the biolistic process using plasmid DNA gold-coated microprojectiles, and GUS activity was assayed histochemically [7, 8].

The results of a typical GUS transient expression assay of seeds harvested 25 days after anthesis (DPA) are shown in Fig. 3. While the 35S/GUS gene was expressed in several developing

seed tissues (Fig. 3A), expression of the *Ltp1*/GUS gene was confined to the developing aleurone layer (Fig. 3B). The *Ltp1*/GUS gene was expressed to the same level in aleurones of mature seeds germinated for 3 days, while expression was much lower in aleurones of seeds harvested at 19 DPA, and expression was not detectable in seeds harvested at 15 DPA (results not shown). These results are in agreement with those obtained by northern blot analysis. We have also seen a similar pattern of aleurone-specific GUS expression using a construct containing 5' upstream sequences of a gene encoding a 26 kDa chitinase (*Chi26*; R. Leah, unpublished results). Previous work has shown that *Chi26* mRNA, like *Ltp1* mRNA, accumulates to high levels in aleurone tissue during late development and early germination [9]. We conclude that sequences be-

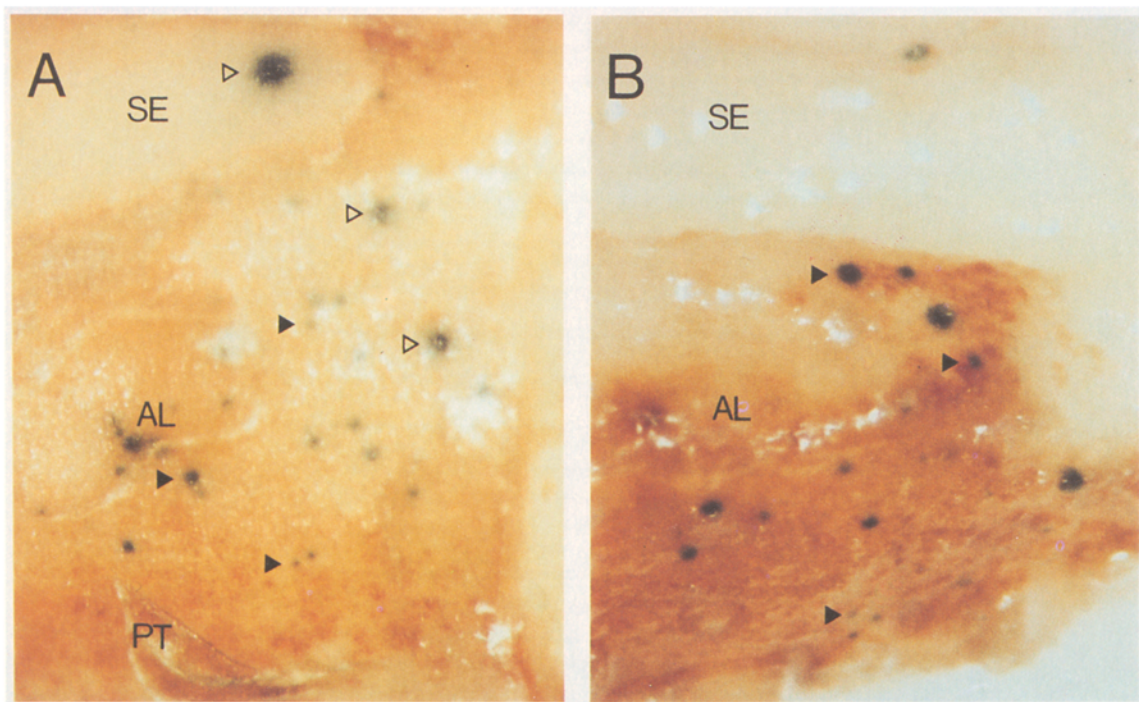


Fig. 3. Histochemical visualization of GUS activity in 25 DPA developing barley seed tissues. A. Expression promoted by CaMV 35S 5' upstream sequences (–941 to +8). Appearance of blue spots represents GUS expression. Partial dissection separated the pericarp/testa (PT), aleurone (AL), and starchy endosperm (SE) tissues. The biolistic process is often able to deliver DNA through the pericarp/testa layer to aleurone cells (filled arrowheads) or deeper to starchy endosperm cells (open arrowheads). 35S upstream sequences promote GUS expression in aleurone and starchy endosperm cells, as well as in cells of the testa/pericarp and embryo (not shown). B. Expression promoted by *Ltp1* 5' upstream sequences (–702 to +67). This *Ltp1* upstream fragment only promotes GUS expression in aleurone cells. This was further verified by cryosectioning the seed tissues and microscopic localization of GUS activity.

tween -702 and +67 of *Ltp1* contain DNA elements that specifically modulate its transcription in aleurone cells.

Work on the *in vivo* functions of plant lipid transfer proteins indicates their involvement in membrane biogenesis [4]. It therefore seems reasonable that *Ltp1* is highly expressed in aleurone cells which develop an extensive membrane system [6]. However, LTP1 is synthesized as a pre-protein and vectorially discharged into the lumen of the ER, where it forms 4 disulfide bonds [5,10,11]. It has therefore been proposed that the LTP1 type molecules constitute translocators of phospholipids in the lumen of the endomembrane system, while the cytosolic lipid flow is effected by proteins corresponding to the yeast 32kD SEC14 phospholipid transfer protein and mammalian counterparts [10]. Recently, it was shown that maize LTP isoforms, which may have different *in vivo* functions, arise by alternative splicing of a single gene [1]. Interestingly, the barley *Ltp1* intron occurs close to the 3' end of the LTP1-coding region in the same position as that inferred from comparison of the maize cDNA sequences. However, the amino acid sequences encoded by the possible open reading frames present in the barley *Ltp1* intron do not appear to be related to the C-terminal extension of the maize minor isoform. Further studies are required to elucidate the *in vivo* function of the LTP1 protein and whether alternative splicing of *Ltp1* gives rise to LTP isoforms.

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