

Cell-Specific Expression of Genes of the Lipid Transfer Protein Family from *Arabidopsis thaliana*

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We have characterized three cDNAs from a gene family encoding lipid transfer proteins, LTP, from *Arabidopsis thaliana* (Wassilewskaja). In addition to the already characterized *Ltp1*, our analysis includes *Ltp2* and *Ltp3*, two sequences previously known as expressed sequence tags (EST) only. The deduced amino acid sequences of the three cDNAs share 56 to 57% identity and show unique tissue- and cell-specific expression. Genes *Ltp1* and *Ltp2* are located within approximately 1.4 kb of each other in tandem orientation. RNA hybridizations showed that all three LTP are expressed in flowering meristems, flowers and developing seeds. *Ltp1* is expressed in leaves in addition. *Ltp3*, though not *Ltp2*, is also expressed in a short segment of the stem close to the flowering meristem. In contrast to the epidermis-specific *Ltp1*, both *Ltp2* and *Ltp3* are not restricted to the epidermis, but are also expressed in sub-epidermal layers of the organs in which they are found. In the upper stem segment, *Ltp3* is predominantly cortical. It appears that the expression of these three cDNAs is sufficient to account for the formation of LTP in all meristematic and expanding cells of the aboveground plant. Evolutionary analysis allows the conclusion that each *Ltp* belongs to a different sub-family of genes. Additionally, parsimony analysis provides evidence that several copies of *Ltp* genes already existed in ancestors of the *Brassicaceae* family.

Key words: *Arabidopsis thaliana* — Cell-specificity — Gene family — Lipid transfer protein — LTP.

Lipid transfer proteins, LTPs, are a class of small proteins, approximately 9 kDa, which are capable of transferring phospholipids between membranes in vitro (Arondel and Kader 1990, Kader 1996). Based on biochemical data, the biological function originally assigned to these proteins was the transfer of lipids between intracellular membranes. Gene expression data, however, indicated that *Ltp* transcripts and LTPs are predominantly localized to epidermis cells. Their implied role is in the secretion of lipids into the cell wall space and, for exam-

ple, lipid deposition into cuticular waxes (Sterk et al. 1991, Thoma et al. 1993, 1994, Kader 1996). The presence of a signal peptide sequence for traffic through the endomembrane system and isolation of LTP from cell walls supports this view (Kader 1996).

LTPs have been reported from the epidermis of several plant organs, including leaves, stems and flowers (Kader 1996). Among the extracellular proteins of the epidermis, LTP are abundant. A non-specific LTP, WAX9, may constitute 90% of all wax-associated proteins (Pyee et al. 1994). High *Ltp* mRNA abundance characterizes cells of the epidermis in a succulent plant *Pachyphytum* (Clark et al. 1992, Clark and Bohnert 1993) and also in cells of the corolla of *Gerbera* sp. which possess a well-developed cuticle (Kotilainen et al. 1994). Also supporting a role in secretion is the presence of LTP in stigmata (Thoma et al. 1994), in which epidermal and sub-epidermal layers produce lipid-containing compounds which are deposited over epidermal walls (Esau 1977). Participation in secretory processes could also explain the presence of LTP transcripts in styles and nectaries. In nectaries, papillate epidermal cells fulfill a secretory function, while the transmitting tissue in stylae provides the glandular lining of the canal.

Small families of genes encode LTPs (Skriver et al. 1992, Kader 1996). The presence of multiple members of the LTP family may reflect plasticity of the plant genome, a consequence of gene duplications and subsequent sequence variation, while those residues crucial to function are conserved (Dean et al. 1989). Equally likely, however, is that individual LTP may carry out specific functions. Such a view has become more plausible recently, since the extreme genetic complexity of several proteins for basic, ubiquitous functions has become known. Prime examples are the gene families encoding plasma membrane ATPases, with at least 12 genes in *Arabidopsis* (Sussman 1994), or the 23 genes encoding (putative) water channel proteins in *Arabidopsis* (Chrispeels and Agre 1994, Weig et al. 1997). Thus, a detailed characterization of distinct LTP protein family members may shed light on their participation in the overall process of secretion and deposition of lipids and reveal whether individual LTP have specific functions.

We have characterized the gene structure of two and the expression of three members of the *Ltp* gene family in

Abbreviation: LTP, lipid transfer protein; CHS, chalcone synthase; PAL, phenylalanine ammonia lyase.

Arabidopsis with emphasis on cell-specificity of expression, providing a picture of their expression in space and time. It is possible that there are yet other sub-families of LTPs which may have diverged enough to escape detection by hybridization, and which are expressed during different stages of development. The *Arabidopsis Ltps* described here are all active during embryo formation and largely specific for meristematic cells of the flowering stage. The most abundant transcript of this family, *Ltp1*, is epidermis-specific and, in addition to its expression in flower structures and embryos, is also highly expressed in leaves. *Ltp2* and *Ltp3* are not expressed in leaves, but in flowers and in developing embryos, and their cell specificity is different from that of *Ltp1*. *Ltp2* and *Ltp3* are not only expressed in the epidermis but also in cell layers internal to the flower and embryo organs.

Materials and Methods

Clone isolation and DNA sequence analysis—Clones of genomic DNA corresponding to LTP1 and LTP2 genes were isolated from a genomic library λ GEM12 (provided by Dr. K. Feldmann, Univ. Arizona) after hybridization with a radioactively-labeled *Ltp1* partial cDNA clone, obtained by PCR using the primer 5'-GGGAATTCTGT/CATT/C/AGGNTAT/CC/TTI-3'. This primer is complementary to the conserved amino acid sequence CIGYL (amino acid 14–18) in LTP. *Ltp3* cDNA (EST 21488) was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH). DNA sequences were obtained by dideoxysequencing of overlapping deletion clones in pBluescript II KS+ (Stratagene, La Jolla, CA). Sequences were analyzed using programs of the Genetics Computer Group (Madison, WI) (Devereux et al. 1985, Higgins and Sharp 1989).

DNA and RNA blot analysis—*Arabidopsis thaliana* ecotype Wassilewskija (WS), were grown in a controlled environment chamber at 22°C under a 12-h photoperiod. Genomic DNA and total RNA were copurified from plant tissues using the method of Gustincich et al. (1991). Genomic DNA was digested with restriction endonucleases and DNA fragments were electrophoretically separated in 0.9% agarose gels. Ten micrograms of LiCl-purified total RNA was resolved on formaldehyde-agarose gels. Either DNA or RNA gels were then transferred to nitrocellulose and hybridized with ^{32} P-labeled probes derived from each of the three LTP cDNAs. Following the hybridizations, blots were washed at room temperature with $2 \times$ SSC, then with $0.1 \times$ SSC at 42°C, dried and exposed to x-ray film (X-Omat, Kodak). The presence of RNA in preparations from various plant organs was monitored by hybridization with an actin probe. *Arabidopsis* actin DNA was generated by PCR using selected primers: upstream 5'-GGIACTGGAATGGTIAAGG-3' and downstream 5'-GIGATCTCCTTGCTCATACG-3'.

Tissue preparation and in situ hybridization—Plant tissues were fixed at room temperature in 2% glutaraldehyde/50 mM KPO4 pH 7.0, dehydrated in ethanol and tertiary butyl alcohol, and embedded in paraffin. Paraffin blocks were sectioned at 8 μ m thickness. Preparation of digoxigenin-labeled sense and antisense riboprobes, and hybridization steps were performed as described by Yamada et al. (1995). About 20 ng of sense or antisense riboprobe was applied to each slide. In order to eliminate

nonspecific signals, slides were blocked in 4% nonspecific sheep serum and 0.3% Triton-X100 in buffer containing 100 mM Tris-HCl, pH 7.5 and 150 mM NaCl, overnight instead of 30 minutes. The sections were then incubated with the (1:1,000) diluted alkaline phosphatase-conjugated anti-digoxigenin Fab-fragment.

PAUP analysis—*A. thaliana* sequences were compared with sequences in the EMBL and SwissProt databases. BLASTx analysis (Altschul et al. 1990) was used to limit the sequences to those most closely related. Those sequences were subsequently considered in the evolutionary analysis. Parsimony analysis was implemented with PAUP 3.1 (Swofford 1993). The search used heuristic algorithm with TBR branch-swapping and ten replicates of random sequence additions. The sequence from gymnosperm Loblolly pine (*Pinus taeda* L.) was used as an outgroup. There were four equally parsimonious trees each requiring 200 steps. A consensus tree is presented here. A bootstrap analysis of 200 replicates was performed on the data set to determine the significance of the branching patterns.

Results and Discussion

There are at least three genes in the family encoding LTPs in *Arabidopsis*. Thoma et al. (1994) reported sequence and expression pattern for one of the *Arabidopsis Ltp* genes (*Ltp1*; accession: M80567) and indicated the presence of at least two additional genes, *Ltp2* (EST accession numbers: 14215, 14037 and 14299) and *Ltp3* (EST 21488). There appears to be a fourth distinct *Ltp* cDNA (T45302) (Newman et al. 1994), which might be closely related to LTP1 (Vignols et al. 1997). In this report we present a detailed characterization of patterns of expression of two cDNAs, *Ltp2* (N37745) and *Ltp3* (T04673) (Newman et al. 1994) in comparison with *Ltp1* cDNA. We also describe the gene (AF057357) corresponding to the *Ltp2* cDNA.

LTP comparisons—Figure 1 presents the deduced amino acid sequences of the three *Arabidopsis* LTPs. All three sequences share the characteristics of functionally analyzed LTPs: A signal peptide sequence (25 amino acids in LTP1 and LTP2, and 23 in LTP3), eight conserved cysteine residues, a valine residue at the 7th position of the

	.1	*	50.
Ltp1	MAGVMK <u>L</u> AC LLLACMIVAG PITSNAALSC GSVNSNLAC IGVVLQGGVI		
Ltp2	MAGVMK <u>L</u> AC MVLACMIVAG PITTANALMSC GTVNGNLACG IAYLITRGAPL		
Ltp3	MAFALRFFITC LVLITVCIVAS SNTVDAALSC GTVAGSLAPC ATYLSKGGLV		
	= = === = = == = = = = = =		
	.51		100.
Ltp1	PPACCSGVKN LNSTAKITPD RQQAQNCIQG AARALGSGLN AGRAAGIPKA		
Ltp2	TQCCNGVIN LKMASTITPD RQQAQRCIQS AAKAVGRGLN TARAAGIPSA		
Ltp3	PPSCCAGVKT LNSTAKITPD RQQAQRCIQS TAKSIGSGLN PSLASGLPGK		
	== == = = ===== = = == = = =		
	.101		
Ltp1	CGVNISYKIS TSTNCKIVR	118 amino acids	
Ltp2	CKVNIPYKIS ASTNCKIVR	118 amino acids	
Ltp3	CGVSIPYPIS MSTNCKQH	118 amino acids	
	= = = = = =====		

Fig. 1 Deduced amino acid sequences of three *Arabidopsis* LTPs. Identical amino acids in all sequences are underlined. The processing site in the amino terminal region of the precursor protein is indicated by an asterisk.

mature peptide, and two charged residues (aspartic acid in position 44 and arginine 45) located centrally in the mature protein (Kader 1996). The presence of a signal peptide in the *Arabidopsis* LTPs has been demonstrated by Segura et al. (1993), who determined the N-terminal sequence of two LTPs isolated from *Arabidopsis* leaves (ecotype Columbia). One invariant region, residues 41–47, TTPDRQQ, is present in *B. napus* (Soufleri et al. 1996) and *B. oleracea* (Pyee and Kolattukudy 1995) and seems to be characteristic for the *Brassicaceae*. A functional role has been ascribed to the two charged residues, aspartic acid and arginine, in this region. They are thought to interact with the phosphate group of a phospholipid to be bound for transport (Tchang et al. 1988).

The comparison of amino acid sequences for the mature proteins showed 60% (LTP1 to LTP3) and 63% (LTP1 to LTP2) identity, and the respective similarities were 82% and 80%. These identity values for LTP1, LTP2 and LTP3 are lower than the range of identities reported for LTP family members within the *Brassicaceae*, 68–79% (Pyee and Kolattukudy 1995), and may argue for the existence of different sub-families. Similarly, three of the LTPs identified in *B. napus* showed very high sequence identities of 85–92% (Soufleri et al. 1996). However, when these transcripts were compared to yet another *B. napus* LTP the tapetum-specific E2, they showed significantly lower identity, less than 50%. In addition, the N-terminal amino acid sequences of two *Arabidopsis* (ecotype Columbia) LTP are different from the deduced protein sequences presented here (Segura et al. 1993). Together, we take the significant variations between these sequences as evidence that there should be other, more widely divergent genes and sub-families encoding LTPs in *Arabidopsis* and evidently also in other plants (Vignols et al. 1997).

Characterization of the *Ltp2* gene—Screening of genomic libraries with *Ltp* cDNAs as probes resulted in the identification of genomic clones corresponding to two genes, *Ltp2*, and the previously characterized *Ltp1* (Thoma et al. 1994). Both genes were contained on the same lambda clone (8.0 Kb), a situation reminiscent of *Ltp* gene organization in *B. oleracea* (Pyee and Kolattukudy 1995). Two *EcoRI* subclones were analyzed in detail and the nucleotide sequence of 3.5 kb was determined. One region, 1,607 bp, was located on two *EcoRI* fragments and included the sequence of *Ltp1*. The coding region with the intron, 473 nucleotides, was preceded by a 5' sequence including the promoter of 870 bp, and followed by a 3' untranslated region of 257 bp. The sequence was identical to that reported by Thoma et al. (1994), although their work had been performed with DNA from a different ecotype (Rschew). A third subclone, 1,937 bp in size, included 898 bp upstream of *Ltp2*, 465 bp of the *Ltp2* coding region with an 111 bp intron, and 600 bp of the 3' untranslated portion of the gene. Sequence analysis showed that EST

#14299 contained the amino terminal 79 amino acids of LTP2, and EST #14215 included 68 amino acids of the carboxyterminal portion of the protein, with an overlap of 29 amino acids. Alignment of the overlapping sequences permitted positioning *Ltp1* and *Ltp2* in tandem orientation, with an intervening region of 1,465 kb. Part of the intervening region separating *Ltp2* and *Ltp1* has previously been deposited into the database (accession number M80567). A similar distance of 1.3 kb has been reported to separate two LTP genes, *Wax9 D* and *Wax9 C*, in *B. oleracea* (Pyee and Kolattukudy 1995). Interestingly, although physically close, they show closer identity to a non-linked members of the LTP family. The amino acid identity of gene C to A was 76%, and gene D to B was 79%. The positions of the introns in *Arabidopsis thaliana* and *B. oleracea* are identical, as each intron is inserted two codons amino terminal from the stop codon. There were, however, differences in the sizes of the introns in two species. Introns in *Arabidopsis* were 116 bp (*Ltp1*) and 111 bp (*Ltp2*), whereas in *B. oleracea* intron sizes were 242 bp, 272, 166 and 271 bp, for genes A, B, C, and D, respectively.

The arrangement of genes deduced from the DNA sequencing data was subsequently confirmed by Southern analysis using the sequences of the three cDNAs as probes (Fig. 2). The DNA hybridization indicated that each of the cDNAs generated a unique hybridization pattern, with the exception of a single *NsiI*-generated DNA fragment (panel 1 and 2; lane N; 5 kb), which includes the entire coding sequences for both *Ltp1* and *Ltp2*. With *EcoRI*, *Ltp1* hybridizes to three DNA fragments (6.3 and 1.15 kb) and to a fragment of 8.3 kb on which also *Ltp2* is located (panels 1 and 2, lane E) in its entirety. Also, *Ltp1* hybridized to two *EcoRV* DNA fragments, while *Ltp2* is located on only one of these (panels 1 and 2, lane R).

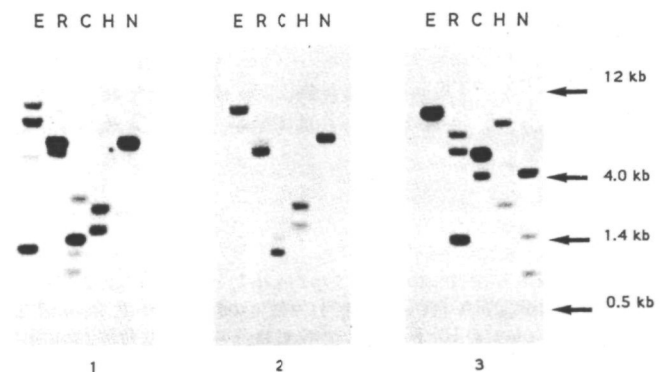


Fig. 2 Southern-hybridization of three *Arabidopsis* LTP cDNAs to total DNA. Ten μ g of total DNA was digested with the restriction endonucleases *EcoRI* (E), *EcoRV* (R), *HincII* (C), *HindIII* (H), and *NsiI* (N). Positions of fragment sizes are indicated. Each panel was hybridized with a different cDNA probe: panel 1, *Ltp1*; panel 2, *Ltp2*; panel 3, *Ltp3*.

Similarly, the fragment patterns generated by *Hinc*II (lane C), and *Hind*III (lane H) produced hybridization patterns that distinguished *Ltp1* and 2 clearly, and the hybridization of the *Ltp3* probe had no band in common, although the *Eco*RI pattern identifies *Ltp3* in a DNA fragment that is similar in size to the 8.3 kb fragment to which *Ltp1/2* hybridize. Figure 2 shows hybridizations to a few very minor bands, which are not explained by the sequenced DNA fragments. We suggest that these might represent incomplete digestion of the DNA, but it is possible that some of these bands might also indicate the presence of additional *Ltp*-like genes in the *Arabidopsis* genome. The hybridization results, with very low cross-hybridization between *Ltp1* and *Ltp2*, also demonstrated that the three cDNAs could be used as gene-specific probes.

Spatial and developmental *Ltp* expression—Analysis of total RNA indicated that only *Ltp1* was expressed in vegetative and in floral tissues (Fig. 3). *Ltp2* and *Ltp3* mRNAs were not detected in young or mature leaves, but were expressed in immature and mature flowers. In addition, *Ltp2* and *Ltp3* transcripts were also identified in the stem immediately below the flowers. The presence of the transcripts was controlled by a parallel hybridization of the same RNA-blot to an actin probe from *Arabidopsis* (see methods). All lanes were loaded with the same amount of RNA as judged by visual inspection of the rRNA bands. Although not completely uniform, actin mRNA amounts were comparable within particular tissues. For example, relatively low amounts of actin transcripts were present in RNA isolated from leaves (lanes 1–4) and the amount was comparable for all three *Ltp* transcripts tested. The *Ltp1* message was abundant and produced a clear signal, while the two other mRNAs were either absent (*Ltp3*) or barely detectable (*Ltp2*). The *Ltp1* expression data presented here agree with those reported by Thoma et al. (1994).

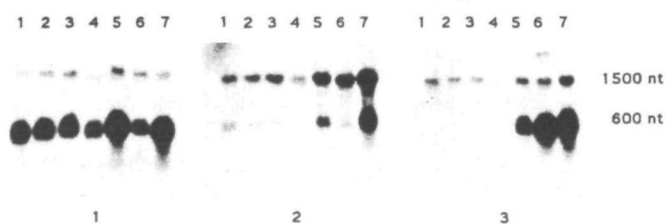


Fig. 3 RNA blot analysis of *Ltp1*, *Ltp2*, and *Ltp3* in different organs. Total RNA ($10 \mu\text{g lane}^{-1}$) was used. Lanes 1, 2, and 3, leaves from plants, 10, 20, 30 d of age; lane 4, leaves from mature plants at bolting stage; lane 5, RNA from stems and flower buds; lane 6, RNA from stems; lane 7, RNA from flowers. Each panel was hybridized with a different cDNA probe: panel 1, *Ltp1*; panel 2, *Ltp2*; panel 3, *Ltp3*. The blots were simultaneously hybridized with actin probe (approximately 1,500 nucleotides) and *Ltp* probes (approximately 600 nucleotides). The hybridization intensity of the actin probe varies between lanes, because actin varies in abundance in different tissues.

The pattern of expression of four *Ltp* genes in *B. oleracea* (Pyee and Kolattukudy 1995) has been documented by analyzing the presence of PCR-generated products in various plant organs. All genes were expressed in leaves and flower buds. Genes A, B, and D were also expressed in stems and mature, open flowers. In *B. napus* (Soufleri et al. 1996), three *Ltp* cDNAs were found to be expressed only in germinating seedlings. Transcripts were detected in cotyledon and hypocotyl but not in roots of 1 to 5 d old seedlings. No expression was found in seeds, mature leaves, stems, or in flowers. These different patterns of organ-specific expression reflect most likely the complexity of the *Ltp* gene family. It is possible that each of the studies looked at different transcripts in sets of subfamilies or, in the case of *B. napus*, analyzed three members of the same subfamily.

Detailed, cellular level localization of the *Ltp2* and *Ltp3* transcripts which has been missing up to now was tested by in situ hybridizations. RNA blot analyses indicated that both transcripts were expressed mainly in floral organs, both in buds and mature flowers, and in developing seeds. Figure 4 illustrates the results. Both transcripts were already detected in floral meristems, stages 2 to 7 (Fig. 4, panel A). Strong hybridization signals were detected in petal primordia, and weaker signals were observed in sepal primordia. The transcripts persisted throughout flower development and were still detectable in ovules following fertilization during stage 15 of flower development (Fig. 4; panel C). Both the *Ltp2* and *Ltp3* transcripts showed identical signal strength in the same flower organs at similar stages for example, strong hybridization signals in the gynoecium and ovules of the flower at a young developmental stage. The mRNAs of all three genes were detectable in anthers but not in pollen. Hybridization signals were also present in the young stigma, stage 11 (not shown), however, the mRNAs were no longer detectable in stigmatic tissue after pollination. The disappearance of *Ltp* mRNAs from stigma tissue may correlate with the state of receptiveness of the stigma. In the stigmata, both the epidermal and sub-epidermal layers secrete a fluid mainly consisting of lipids and phenolic compounds (Esau 1977). The lipids, likely similar to components of the waxy cuticle, may slow down evaporation of the secretion. *Ltp2* and *Ltp3* transcripts were abundant in the nectaries. The secreting cells of the nectaries resemble other epidermal cells, but they are devoid of a cuticle. The only difference in the expression of *Ltp2* and *Ltp3* was the presence of *Ltp3* in the epidermis and the outer cortical layers of the stem in a region subtending the flower (Fig. 4, panel G). This region of the stem showed also a signal with *Ltp1* RNA-probes (Thoma et al. 1994). There was no significant hybridization signal detected in cells of vascular tissues of any of the organs examined. The data on *Ltp2* and *Ltp3* indicate that their expression is consistent with the proposed

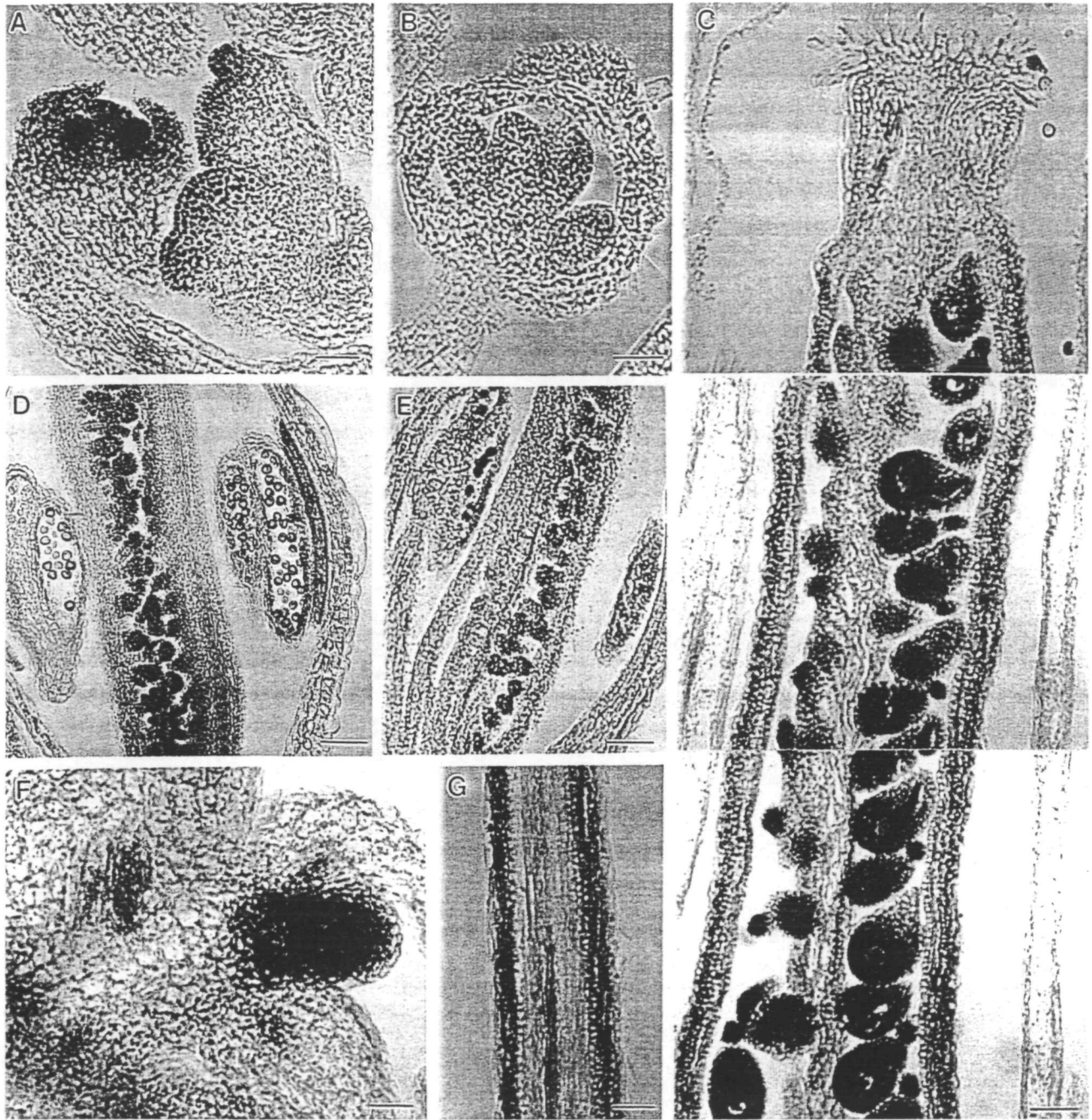


Fig. 4 Cytological detection of *Ltp* transcripts by in situ hybridizations. Transverse sections of various stages of *Arabidopsis* flowers were hybridized with digoxigenin-labeled antisense-RNA (panels A, C, D, F and G) or sense-RNA (panels B and E) of either *Ltp2* or *Ltp3*. (A) *Ltp1* in flower meristems (antisense-RNA). *Ltp2* and *Ltp3* gave identical signals. (B) Sense-RNA with all *Ltp* produced no signal. (C) Strong *Ltp2* and *Ltp3* signals with antisense-RNA in developing seeds. *Ltp* transcripts were also present in the gynoecium; the signal was absent in the stigma at this stage, but was detected in stigmata at earlier stages of development (similar to the signal shown in (D)). (D) *Ltp* transcripts (similar pattern with all three *Ltp* probes) are present in the gynoecium and ovules of this stage of flower development and signals are present in filaments and the pollen sacks, but are absent in pollen. (E) Sense-RNA hybridization produced no signal in gynoecium and stamen. (F) *Ltp* transcripts in nectaries of flowers late in development. (G) *Ltp3* transcript is present in sub-epidermal cells of the stem section immediately below the flowers. This result was the only discernable difference between *Ltp2* (not expressed in stems) and *Ltp3* (abundant in stems), respectively. Bars represent 50 μm (in B, D, E, F) and 100 μm (A, C, G).

role of LTP in the deposition and/or secretion of lipophilic substances. It also illustrates similarities in the overall expression of the three *Ltps* in flowers. Only *Ltp1* is also expressed in leaves, documenting that the in-situ hybridization signals observed are gene-specific.

There exists, however, a pronounced difference in the cell layers in which the three *Ltps* are expressed. *Ltp1* mRNA was restricted to the single layer of epidermis cells in tissues in which it is expressed (Thoma et al. 1994), whereas *Ltp2* and *Ltp3* were expressed in sub-epidermal cell layers as well. A comparative study of elements controlling the expression of *Ltp1* with *Ltp2* and/or *Ltp3* should lead to an identification of sequence elements responsible for the epidermis-specificity. The alignment of over 800 bp of the non-transcribed regions located upstream of the *Ltp1* and *Ltp2* genes revealed a relatively low sequence conservation. There was higher sequence identity within 200 bp immediately upstream of the coding regions. Detailed analysis of the *Ltp1* promoter sequence (Thoma et al. 1994) indicated the presence of several known expression-controlling motifs. A search for these elements within the *Ltp2* promoter region led to identification of at least two of these conserved sequences. One was a sequence termed Box 3 which was present in the promoter regions of *Ltp2*, bean chalcone synthase (CHS) and *Arabidopsis* phenylalanine ammonia lyase (PAL) (Thoma et al. 1994). Both CHS and PAL are known to be pathogen-induced proteins (Lois et al. 1989, Feinbaum and Ausubel 1988). Elevated expression of three barley LTP mRNAs upon pathogen induction has been previously reported (Molina and Garcia-Olmedo 1993). The second motif, a 10 bp sequence TCATCTTCTT was found in 30 different plant genes which are inducible by various types of stress, most of which also show cell-specific and/or developmental expression (Goldsbrough et al. 1993). This motif, present at position -568 bp in the *Ltp2* non-transcribed region, agrees in seven out of ten residues, whereas all but one of the sequences compared differ only in two sites from the consensus sequence. Also in most cases, this sequence was present repeatedly within the same gene including the 5' proximal, exons and 3' untranslated sequences (Goldsbrough et al. 1993). The *Ltp2* promoter region contained also sequences found to be conserved among the four *B. oleracea* genes (Pyee and Kolattukudy 1995). Particularly high similarity exists around the TATA-box and sequences downstream, termed box IV.

From the data it is conceivable that each of the studies analyzing genes of the *Ltp* family has focussed on different members, and that, in fact, several sub-families of sequences exist. The different organ-, tissue- and cell-specific expression patterns that have been reported seem to indicate that this is the case. Members of different sub-families might be divergent to an extent to be undetectable by heterologous hybridizations.

Evolutionary analysis of plant LTPs—Vignols et al. (1997) indicated two ancestral genes as it is reflected in two distinct phylogenetic groups among LTP sequences. Further duplications followed by plant speciation created complex relationships among LTP genes. Here, we analyzed evolutionary relationship of the *A. thaliana* *Ltp2* gene and closely related sequences, revealed by BLAST. The primary goal was to test the relationships among proteins sharing tissue- or organ-specific expression. The PAUP analysis presented here is similar to one provided by Vignols et al. (1997). The sequences examined tended to group according to the botanical classification and not according to tissue- or organ-specificity. However, the ab-

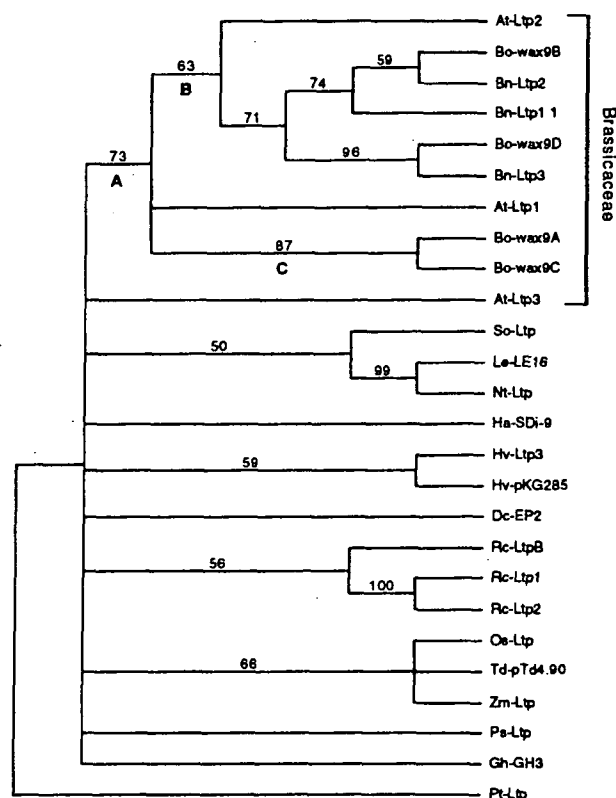


Fig. 5 Evolutionary relationship of the *Arabidopsis thaliana* *Ltp* genes. Proteins showing the closest relationship to the LTP2 sequence were subjected to the parsimony analysis PAUP as described in Methods. Each protein sequence presented here is identified by the species abbreviation and by the EMBL accession number. At-Ltp2 (AF057357; N37745), Bo-wax9B (L33905), Bn-Ltp2 (U22174), Bn-Ltp1 (U22105), Bo-wax9D (L33907), Bn-Ltp3 (U22175), At-Ltp1 (M80567), Bo-wax9A (L33904), Bo-wax9C (L33906), At-Ltp3 (T04673), So-Ltp (P10976), Ha-SDi-9 (X92648), Hv-Ltp3 (Cw19) (X68656), Hv-pKG285 (Z37115), Dc-EP2 (M64746), Le-Ltp (U81996), Tob-Ltp (S29227), Rc-Ltp-B (P10974), Rc-Ltp1 (M86353), Rc-Ltp2 (M86354), Os-Ltp (Z23271), Td-pTd4.90 (S22528), Zm-Ltp (S45635), Ps-Ltp (L14770), Gh-GH3 (S78173), Pt-Ltp (U10432). Sequences from the Brassicaceae family are indicated. Three of the branches were labeled A, B and C, see text for description.

sence of statistics and the lack of detail given about the construction methods make it difficult to assess this analysis. Our analysis provides a closer look at the evolutionary relationships of the already characterized members of the *Brassicaceae* family (Fig. 5). With the exception of *At-Ltp3* all *Brassicaceae* sequences are monophyletic (branch A). Among the *Brassicaceae* sequences there are at least two distinct *Ltp* sequences represented by the B- and C-branches. *At-Ltp1* may represent a third clade for which additional sequences from other species have not yet been found. The structure in clade A implies that the ancestor of the *Brassicaceae* family already possessed multiple copies of *Ltp*. This explains why *Ltp* sequences from a particular species are not monophyletic. For example *Bo-wax 9D* is more closely related to *Bn-Ltp3* than it is to the other sequences from *B. oleracea*. *At-Ltp2* clearly falls into clade B, while *At-Ltp1* appears to be a distinct lineage not yet identified in other Brassicas (Fig. 5). *At-Ltp3* is unrelated to any of the *Brassica Ltp* sequences. Clearly, the parsimony analysis, with which we anchored the *Arabidopsis Ltp* sub-family, suggests that the family is likely more diverse than what is represented in the databases. This is confirmed by the relationships of the other *Ltp* sequences included in this analysis. Not surprising is that the rice, wheat and maize sequences are monophyletic. However, the barley sequences are distinct from the three other monocots, suggesting an early gene duplication event, which preceded speciation in the Gramineae as it had been observed before (Vignols et al. 1997). The sequences from the other dicots are scattered throughout the tree indicating that the evolution of the *Ltp* genes has been complex. Phylogenetic analysis and the diversity of expression patterns suggest the existence of multiple additional copies in each genome. Their functions remain to be studied.

The localization of three *Ltp* from *Arabidopsis* transcripts indicates overlapping expression patterns in the flower organs and distinct differences in their cellular localization. The patterns in cells of the L1 as well as L2/3 layers and in oocytes, and developing embryos and seeds suggest functions—which remain to be studied with different techniques—that are compatible with cuticle formation, excretion in nectaries and the intracellular and possibly intercellular transfer of lipids.

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