Plant Physiol. (1994) 105: 35–45

Tissue-Specific Expression of a Gene Encoding a Cell Wall-Localized Lipid Transfer Protein from *Arabidopsis*[1]

Sharon Thoma, Ursula Hecht, Arina Kippers, Jose Botella, Sacco De Vries, and Chris Somerville[2]*

Michigan State University-Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824 (S.T., U.H., J.B., C.S.); and Department of Molecular Biology, Agricultural University of Wageningen, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands (A.K., S.D.V.)

Nonspecific lipid transfer proteins (LTPs) from plants are characterized by their ability to stimulate phospholipid transfer between membranes in vitro. However, because these proteins are generally located outside of the plasma membrane, it is unlikely that they have a similar role in vivo. As a step toward identifying the function of these proteins, one of several LTP genes from *Arabidopsis* has been cloned and the expression pattern of the gene has been examined by analysis of the tissue specificity of β-glucuronidase (GUS) activity in transgenic plants containing LTP promoter-GUS fusions and by in situ mRNA localization. The LTP1 promoter was active early in development in protoderm cells of embryos, vascular tissues, lignified tips of cotyledons, shoot meristem, and stipules. In adult plants, the gene was expressed in epidermal cells of young leaves and the stem. In flowers, expression was observed in the epidermis of all developing inflorescence and flower organ primordia, the epidermis of the siliques and the outer ovule wall, the stigma, petal tips, and floral nectaries of mature flowers, and the petal/sepal abscission zone of mature siliques. The presence of GUS activity in guard cells, lateral roots, pollen grains, leaf vascular tissue, and internal cells of stipules and nectaries was not confirmed by in situ hybridizations, supporting previous observations that suggest that the reporter gene is subject to artifactual expression. These results are consistent with a role for the LTP1 gene product in some aspect of secretion or deposition of lipophilic substances in the cell walls of expanding epidermal cells and certain secretory tissues. The LTP1 promoter region contained sequences homologous to putative regulatory elements of genes in the phenylpropanoid biosynthetic pathway, suggesting that the expression of the LTP1 gene may be regulated by the same or similar mechanisms as genes in the phenylpropanoid pathway.

LTPs are small, soluble, basic proteins that are characterized by an ability to catalyze the transfer or exchange of lipids between membranes in vitro (reviewed in Arondel and Kader, 1990). The plant proteins of this class have a broad specificity for the type of lipid they will transfer in vitro, and LTPs from spinach and castor bean also bind fatty acids (Rickers et al., 1985; Yamada et al., 1990). On the basis of this in vitro activity, there has been sustained interest in the possibility that LTPs may be involved in intracellular lipid transport.

All known plant LTPs are synthesized with N-terminal extensions that have the sequence characteristics of signal peptides. In those cases where it has been tested, these signal sequences direct the cotranslational insertion of the polypeptide into the lumen of the ER in vitro (Bernhard et al., 1991; Madrid, 1991). It has also been shown that a maize LTP is synthesized on membrane-bound polysomes (Tchang et al., 1988). Since all known LTPs lack the ER retention signal H/KDEL, they would be expected to enter the secretory pathway. Indeed, Mundy and Rogers (1986) reported that a barley aleurone protein, which was subsequently identified as an LTP (Breu et al., 1989), was secreted into aleurone cell culture medium. Similarly, Sterk et al. (1991) demonstrated that a carrot LTP was secreted by embryogenic cell cultures. Finally, an *Arabidopsis* LTP was localized to the cell wall by immunocytochemical labeling at the ultrastructural level (Thoma et al., 1993). Using immunocytochemical light microscopy, Sossountzov et al. (1991) have shown that a maize LTP is localized to the periphery of epidermal cells in maize coleoptiles and have suggested that the labeling might be associated with the cytoplasmic face of the plasma membrane. However, since light microscopy does not usually have the ability to resolve the cell wall and plasma membrane, this must be considered speculative. Thus, we believe that the weight of evidence indicates that most LTPs are cell wall proteins. A notable exception is the report that in castor bean cotyledons LTPs are located in microbody-like organelles (Tsuboi et al., 1992). The mechanism of this atypical localization has not been reported.

With the mounting evidence that most plant LTPs investigated are extracellular, a possible role for these proteins in intracellular lipid transfer seems unlikely. This raises the question as to the biological role of these proteins. One important observation, in this respect, is that LTPs are not found in all cell types. A maize LTP was shown by immu-

[1] This work was supported in part by grants from the U.S. Department of Agriculture/National Science Foundation/Department of Energy Plant Science Center Program, the U.S. Department of Energy (DE-FG02-94ER20133), and the European Community Program BRIDGE (BIOT-CT90-0177).

[2] Present address: Carnegie Institution of Washington, Department of Plant Biology, 290 Panama Street, Stanford, CA 94305.

* Corresponding author; fax 1–415–325–6857.

Abbreviations: EST, expressed sequence tag (i.e. a partially sequenced anonymous cDNA clone); GUS, β-glucuronidase; LTP, lipid transfer protein; SSC, 10 mM sodium citrate, 150 mM NaCl.

nocytochemical methods to be present mainly in epidermal cells and vascular strands of coleoptiles (Sossountzov et al., 1991). A carrot LTP gene was shown by in situ hybridization to be expressed in the protoderm cells of embryos, the epidermal cells of leaf primordia and all flower organs, and epidermal tissues of developing seeds (Sterk et al., 1991). A tobacco LTP was highly expressed in leaf epidermal cells and in the shoot apical meristem (Fleming et al., 1992). Another tobacco gene, with significant homology to LTPs, was expressed predominantly in tapetal cells (Koltunow et al., 1990). In spinach (Bernhard et al., 1991), maize (Sossountzov et al., 1991), carrot (Sterk et al., 1991), and tobacco (Fleming et al., 1992), LTPs were shown by northern blot analysis to be expressed in the aerial portions of the plant, with little or no expression in root tissue. In *Arabidopsis* (Thoma et al., 1993), western analysis showed the protein to be present predominantly in the aerial tissues of the plant. In consideration of the known in vitro activity, the tissue specificity of gene expression, and the extracytoplasmic location of LTPs, Sterk et al. (1991) speculated that these proteins may play a role in the transport of cutin monomers through the extracellular matrix to sites of cutin formation.

Because of the potential utility of applying a genetic approach to the analysis of LTP function, we have investigated the expression of an LTP gene in *Arabidopsis* by the analysis of transgenic plants that contain an LTP gene promoter fused to the GUS reporter gene and by in situ mRNA hybridization. The results of these studies confirm and extend previous investigations by other methods and provide a basis for the interpretation of the effects of genetic alterations of LTP expression in *Arabidopsis* (Thoma, 1992).

MATERIALS AND METHODS

Plant Material

The Rschew ecotype of *Arabidopsis thaliana* was used for all experiments because of the high efficiency of transformation. Plants were grown under continuous fluorescent illumination (100–150 μmol m^{-2} s^{-1} photon flux density) at 23°C on a mixture of perlite:vermiculite:sphagnum (1:1:1) irrigated with mineral nutrients.

Transgenic seeds were surface sterilized and germinated on mineral salts medium containing kanamycin (50 μg/mL). Kanamycin-resistant seedlings were transferred to potting medium and grown as described above. Plants used for analysis of GUS expression during seedling development were grown on agar-solidified mineral salts medium in 100 × 20 mm Petri dishes under the same light and temperature conditions described above.

Genetic Materials

The λGT10 cDNA library was obtained from Nigel Crawford (University of California, San Diego, CA). The λGEM11 library was constructed from a *Sau*3A partial digest of Columbia wild-type DNA. The following genetic materials are available from the *Arabidopsis* Biological Resource Center at Ohio State University (Columbus, OH). Plasmid pJ5–3 contains a cDNA of an *Arabidopsis* LTP (GenBank accession number M80566). Plasmid pUH1 contains a genomic LTP1 clone (GenBank accession number M80567). Plasmid pWB2 contains a cDNA clone of a spinach LTP cDNA (Bernhard et al., 1991). The four homozygous transgenic lines LTP-GUS 1A, 1B, 1C, and 1D contain the 1150-bp LTP1 promoter fused to GUS.

Sequence Analysis and Retrieval

Sequence comparisons were performed using the electronic mail BLAST servers of the National Library of Medicine. Information on analysis and retrieval of EST sequences can be obtained by sending the word HELP by electronic mail to est_report@ncbi.nlm.nih.gov and blast@ncbi.nlm.nih.gov.

Isolation of LTP Clones

A cDNA clone of an *Arabidopsis* LTP was isolated by probing a cDNA library constructed from leaf and shoot mRNA in λGT10 with a cDNA clone for a spinach LTP (Bernhard et al., 1991). Filters were prehybridized for 4 h, and hybridized for 16 h, at 42°C in a solution of 5× Denhardt's solution, 5× SSC, 0.1% SDS, and 0.5 μg/μL sonicated herring DNA. The filters were washed (for 15 min each time) at room temperature in 4× SSC, then in 2× SSC, and finally at 42°C in 2× SSC. The insert from the longest clone was excised with *Eco*RI and cloned into the *Eco*RI site of pBluescript to produce plasmid pJ5–3.

A genomic clone for an *Arabidopsis* LTP was isolated from a genomic library in λGEM11 by probing nitrocellulose plaque lifts with the insert from pJ5–3. The filters were prehybridized for 15 min at 65°C in a solution containing 200 mM NaCl, 20 mM sodium phosphate (pH 7.7), 2 mM EDTA, 1% SDS, 0.5% dry milk powder, 10% dextran sulfate and hybridized for 8 h in the same solution. The filters were washed three times for 10 min at 65°C in a solution containing 2× SSC, 0.1% SDS and once in a solution containing 0.5× SSC, 0.5% SDS under the same conditions. A 1.9-kb *Cla*I fragment containing the complete coding sequence for the *Arabidopsis* LTP1 gene and approximately 1 kb of the 5′ upstream region was subcloned into the corresponding site of pBluescript KS to produce plasmid pUH1 and the sequence was determined completely on both strands manually by the dideoxy chain-termination method.

Nucleic Acid Manipulations

Unless specified otherwise, nucleic acid manipulations were carried out as described by Sambrook et al. (1989). DNA was extracted from leaves of *Arabidopsis* as described by Rogers and Bendich (1988). For Southern analysis, DNA was restricted, resolved by electrophoresis through 0.8% agarose (1.7 μg/lane), and transferred to a nylon membrane (Hybond N^+, Amersham). The membrane was prehybridized for 3 h at 65°C in a solution containing 4× SET (3 M NaCl, 0.6 M Tris-HCl, pH 7.4, 40 mM EDTA), 0.1% $Na_4P_2O_7$, 0.2% SDS, 0.1% heparin. Hybridization was carried out for 12 h at 65°C in a solution containing 4× SET, 0.1% $Na_4P_2O_7$, 0.2% SDS, 10% dextran sulfate, and an *Arabidopsis* LTP gene fragment labeled with [α-^{32}P]dCTP by random priming. The membranes were washed for 15 min each time, first at 65°C in

2× SSC, 0.1% SDS, then at 65°C in 1× SSC, 0.1% SDS, and finally at 65°C in 0.1× SSC, 0.1% SDS.

Construction of Promoter-Fusion Constructs

Synthetic oligonucleotides homologous to portions of the 5′ untranslated regions of the genomic clone were used as primers to amplify an 1150-bp promoter fragment by PCR. The downstream primer (U19), which introduced a unique *Hin*dIII site, was ACCAAAGGATCCATATTGATCTCTTA-GGTA. The upstream primer (U20), which introduced a unique *Bam*HI site, was ACCAAAAAGCTTAATCTCAAA-ACCAAAGTC. The amplified product was digested with *Bam*HI and *Hin*dIII and subcloned into the corresponding sites of the binary Ti plasmid pBI101 (Clontech, Palo Alto, CA), which contains a promoterless GUS gene (Jefferson et al., 1987).

Production of Transgenic Plants

The promoter-GUS fusion constructs were transformed into *Agrobacterium tumefaciens* C58 (pGV3850) by electroporation and selected for resistance to kanamycin (50 μg/mL). *Arabidopsis* was transformed as described by Valvekens et al. (1988). Transgenic plants that expressed GUS activity were identified by enzyme assay. Leaves from transgenic plants were homogenized in 50 mM $NaPO_4$ (pH 7.0), 1 mM EDTA, 0.1% Triton X-100, 0.7% β-mercaptoethanol, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide and incubated at 37°C. The samples were checked visually for appearance of blue color.

Histochemical Localization of GUS Activity

Tissue sections were placed in 100 mM $NaPO_4$ (pH 7.0), 1 mM spermidine, vacuum infiltrated for 15 min, then incubated at 37°C in 0.5 M KeFCN, 0.01% Triton X-100, 50 mM $NaPO_4$ (pH 7.0), 10 mM β-mercaptoethanol, 1 mM EDTA, 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, until a blue color appeared (usually after 5 h). Following incubation with the substrate, dehydration and embedding were carried out essentially as described by De Block and Debrouwer (1992), except that the activator concentration in the embedding step was decreased from 0.8 to 0.6%. Eight-micrometer sections were cut with a dry glass knife, and sections were flattened on a drop of water. Sections were viewed using bright-field microscopy on a Zeiss Axiophot microscope.

In Situ mRNA Hybridizations

In situ mRNA hybridizations were carried out essentially as described by Cox and Goldberg (1988). To facilitate handling, seedlings were placed in 1% low-melting agarose at 40°C prior to fixation and embedding. ^{35}S-labeled antisense and sense (control) RNA probes were transcribed from pJ5–3 using either the T7 (sense) or the T3 (antisense) promoter. Hybridization was carried out for 16 h at 42°C in a solution containing 0.3 M NaCl, 50% (v/v) formamide. Sections were then washed in 4× SSC at 37°C, treated with 50 μg mL^{-1} RNAse A in 0.5 M NaCl at 37°C, and finally washed in 2× SSC at room temperature. Slides were coated with Kodak NTB2 nuclear emulsion, exposed for 1 to 3 weeks at 4°C, and developed with Kodak D19 developer. Sections were stained with toluidine blue and photographed with a Nikon Optiphot-2 microscope equipped with dark-field and epipolarization optics.

RESULTS

An *Arabidopsis* LTP cDNA Clone

Several dozen LTP cDNA clones were isolated by probing an *Arabidopsis* cDNA library with a spinach LTP cDNA clone. All of the clones cross-hybridized with similar intensity at high stringency (i.e. 68°C in 5× SSC) and, therefore, appeared to represent the products of a single gene that we designated LTP1. The cDNA from the clone with the longest insert was subcloned to produce plasmid pJ5–3 and the nucleotide sequence of the 590-bp insert was determined. The deduced amino acid sequence of the open reading frame encoded a polypeptide of 110 amino acid residues that exhibited high homology to the spinach LTP (Fig. 1), the most intensively studied plant LTP at the biochemical level (Kader et al., 1984; Arondel and Kader, 1990). The deduced amino acid sequence of the *Arabidopsis* cDNA contained 10 fewer amino acid residues than the spinach protein at the amino terminus, raising the possibility that the cDNA clone was not full length. However, the corresponding genomic clone lacked an in-frame ATG codon in the 60 bp of upstream sequence (Fig. 2). There was very low homology between the spinach and *Arabidopsis* proteins in the region of the signal peptide. However, the sequence of the putative mature peptide was 54% identical to the spinach LTP sequence, and this increased to 68% when conservative amino acid substitutions were considered. The *Arabidopsis* LTP1 contained the eight conserved Cys residues that are characteristic of this family of proteins. Thus, the *Arabidopsis* LTP1 gene product has a typical primary structure for this class of proteins.

Characterization of a Genomic Clone

A genomic clone was isolated by using the LTP1 cDNA clone as a hybridization probe. A 1948-bp fragment, which included 1237 bp upstream from the putative translation

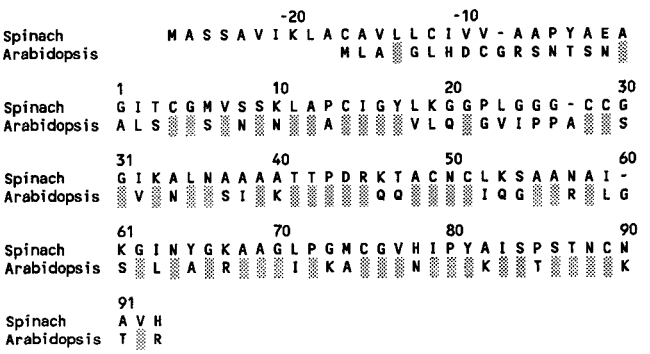

Figure 1. Comparison of the deduced amino acid sequences of the *Arabidopsis* LTP1 gene product and a spinach LTP. The spinach sequence was from Bernhard et al. (1991). Shaded boxes represent identical amino acids, and gaps are indicated by hyphens (-).

```
CTGATACTACGTGATTACAGTTGGGAAGTATCAAGTAAACAACATTTTGTTTTTGTTTGA   60

TATCGGGAATCTCAAAACCAAAGTCCACACTAGTTTTTGGACTATATAATGATAAAAGTC  120
      →U20
AGATATCTACTAATACTAGTTGATCAGTATATTCGAAAACATGACTTTCCAAATGTAAGT  180

TATTTACTTTTTTTTTGCTATTATAATTAAGATCAATAAAAATGTCTAAGTTTTAAATCT  240

TTATCATTATATCCAAACAATCATAATCTTATTGTTAATCTCTCATCAACACACAGTTTT  300

TAAAATAAATTAATTACCCTTTGCATGATACCGAAGAGAAACGAATTCGTTCAAATAATT  360

TTATAACAGGAAATAAAATAGATAACCGAAATAAACGATAGAATGATTTCTTAGTACTAA  420

CTCTTAACAACAGTTTTATTTAAATGACTTTTGTAAAAAAAACAAAGTTAACTTATACAC  480

GTACACGTGTCGAAAATATTATTGACAATGGATAGCATGATTCTTATTAGAGTCATGTAA  540

AAGATAAACACATGCAAATATATATATGAATAATATGTTGTTAAGATAAACTAGACGATT  600

AGAATATATAGCACATCTATAGTTTGTAAAATAACTATTTCTCAACTAGACTTAAGTCTT  660

CGAAATACATAAATAAACAAAACTATAAAAATTCAGAAAAAAACATGAGAGTACGTTAGT  720

AAAATGTATTTTTTTGGTAAAATAATCACTTTTCATCAGGTCTTTTGTAAAGCAGTTTTC  780

ATGTTAGATAAACGAGATTTTAATTTTTTTTAAAAAAAGAAGTAAACTAACTATGTTCCT  840
Box 1
ATCTACACACCTATAATTTTGAACAATTACAAAACAACAATGAAATGCAAAGAAGACGTA  900

GGGCACTGTCACACTACAATACGATTAATAAATGTATTTTGGTCGAATTAATAACTTTCC  960

ATACGATAAAGTTGAATTAACATGTCAAACAAAAGAGATGAGTGGTCCTATACATAGTTA 1020
                                    Box 2
GGAATTAGGAACCTCTAAATTAAATGAGTACAACCACCAACTACTCCTTCCCTCTATAAT 1080
                                Box 1
CTATCGCATTCACACCACATAACATATACGTACCTACTCTATATAACACTCACTCCCCAA 1140
                    *                   Box 3
ACTCTCTTCATCATCCATCACTACACACATCTCCTATTGCAAACGAACATAAAACACTAC 1200

CTAAGAGATCAATATGGCTGGAGTGATGAAGTTGGCATGCTTGCTCTTGGCCTGCATGAT 1260
          →U19                    M  L  A  L  G  L  H  D          8

TGTGGCCGGTCCAATACATCGAACGCTGCGCTAAGCTGTGGCTCAGTTAACAGCTCCAAC 1320
C  G  R  S  N  T  S  N  A  A  L  S  C  G  S  V  A  S  S  N     28

TTGGCAGCGTGCATTGGCTACGTGCTCCAAGGTGGTGTCATTCCCCCAGCGTGTTGCTCC 1380
L  A  A  C  I  G  Y  V  L  Q  G  G  V  I  P  P  A  C  C  S     48

GGCGTTAAAAACCTCAACAGCATAGCCAAGACGACCCCAGACCGTCAGCAAGCTTGCAAT 1440
G  V  K  N  L  N  S  I  A  K  T  T  P  D  R  Q  Q  A  C  N     68

TGCATTCAAGGTGCCGCTAGAGCCTTAGGCTCTGGTCTCAACGCTGGCCGTGCAGCTGGA 1500
C  I  Q  G  A  A  R  A  L  G  S  G  L  N  A  G  R  A  A  G     88

ATTCCTAAGGCATGTGGAGTCAATATTCCTTACAAAATCAGCACCAGCACCAACTGCAAA 1560
I  P  K  A  C  G  V  N  I  P  Y  K  I  S  T  S  T  N  C  K    108

ACgtatgttaatctctctcactctctcagatattaagctataatttgtttcttttgggtt 1620
T                                                              109

aaatattcgaaaggttatattaacggtgttatattaatttgtatatatgtggttgcagCG 1680

TGAGGTGATGAGCTAGCAACGGTGAGATGATGCTACTACCGGAAGTTTCGAATCCTTATT 1740
V  R                                                           111

ATATAATGGATGAGATTAATATTAAATAAGATGTTCGAATGGTTTGTTTTTAGAGTTTTT 1800

AATTTCTTGTCTTTTTCTATTGTGGTGTTCTTGTTATATGGGTTTGTCTGTACTATGTTC 1860

GCAGGCAACAACGTTATATGAAATTTCAGAGTACTTGAAGTTTAAGTTTTTCTATATCTT 1920

ACTTATTAAATCTTGCATTAGTTTAATCGAT
```

Figure 2. Sequence of the LTP1 genomic region. The deduced amino acid sequence is shown below the nucleotide sequence. The putative signal peptide is underlined. The intron is shown in lowercase letters. A putative TATA box is indicated with a double underline, and an asterisk (*) above the sequence marks the putative transcription start site. Sequence elements corresponding to phenylpropanoid promoters are indicated by shaded and numbered boxes. The extent of the region used for the LTP1 promoter-GUS fusion is marked below the sequence with arrows.

initiation codon and 266 bp of the 3′ untranslated region, was sequenced (Fig. 2). Comparison of the nucleotide sequences of the cDNA and genomic clones indicated that the coding region of the genomic clone was interrupted by a 115-bp intron at the same location as in tobacco and barley (Linnestad et al., 1991; Fleming et al., 1992). A putative TATA box, with 75% homology to the consensus TATA sequence T(G/C)TATA(T/A)A, was found 111 bp from the first in-frame ATG codon (Fig. 2). Comparison of the sequence of the 5′ region of the LTP1 gene to known transcription start sites of plant genes (Joshi, 1987) revealed a probable transcription initiation site at nucleotide 1157 (Fig. 2). This is based on the observation that in 85% of genes examined, transcription initiates at an A residue in the context CTCATCA. This sequence closely resembles the sequence TCCATCA, which is found at positions 1154 to 1160 in the LTP1 gene, 79 bp upstream of the putative ATG.

The 5′ untranslated region of the LTP gene showed no overall sequence homology to any other sequence in GenBank Release 70, but contained several small regions that are conserved among promoters of phenylpropanoid biosynthetic genes (Cramer et al., 1989; Lois et al., 1989; Ohl et al., 1990). Two elements (Boxes 1 and 2; Figs. 2 and 3) are homologous to sequences in bean, parsley, and *Arabidopsis* Phe ammonia-lyase promoters. In parsley, these sequence elements have been shown to display elicitor-inducible and light-inducible footprints in vivo. A 9-bp AC-rich element (Box 3; Figs. 2 and 3), which is found in the 5′ untranslated regions of an *Arabidopsis* Phe ammonia-lyase gene and a bean chalcone synthase gene, was also observed in this region of the LTP1 gene. In addition, another sequence element, which has been found in the promoters of several stress-

```
BOX 1    Arabidopsis LTP1    -314  ACACACCTATAA
         Arabidopsis LTP1     -52  ACGTACCTACTC
         Parsley PAL         -119  TCTCACCTACCC
         Bean PAL             -89  ACCCACCTACCA
         Bean PAL            +113  ACTCACCTACCC
         Arabidopsis PAL     -435  ACACACCTACTC
         Arabidopsis PAL     -359  TCTCACCAACCG
         Arabidopsis PAL      -64  GCTTACCTACCA
                  CONSENSUS        (A/T)CTCACCTACC(A/C)

BOX 2    Arabidopsis LTP1    -110  ACAACCACCAAC
         Parsley PAL         -208  CCAACAAACCCC
         Bean PAL            -157  TCCACCAACCCC
         Arabidopsis PAL    -1631  CCAACACACCAC
         Arabidopsis PAL     -109  TCAACCAACTCC
                  CONSENSUS        (C/T)CAAC(A/C)AACC(A/C)C

BOX 3    Arabidopsis LTP1     +26  AACGAACAT
         Bean CHS             +23  AACCAACAA
         Arabidopsis PAL      +23  AACCAACAA
                  CONSENSUS        AACCAACAT
```

Figure 3. Comparison of putative regulatory sequences in the LTP1 gene and in genes of the general phenylpropanoid pathway. Putative regulatory sequences, designated as box 1, 2, and 3, from bean, parsley, and *Arabidopsis* Phe ammonia-lyase (PAL) and bean chalcone synthase (CHS) were aligned with the sequences found in the LTP1 clone. The positions of the sequences are given relative to the proposed transcription start sites (i.e. nucleotide 1157 in Fig. 1 for the LTP1 gene).

induced genes (Goldsbrough et al., 1993), has been found in the LTP1 promoter region (Figs. 2 and 3).

Evidence for Multiple LTP Genes

To assess the number of LTP genes in *Arabidopsis*, genomic DNA was digested with a variety of restriction enzymes and hybridized with the labeled *Arabidopsis* LTP1 cDNA. There are no cleavage sites for *Bam*HI, *Kpn*I, or *Xba*I within the DNA sequence of the genomic clone. Thus, one band would be expected on a Southern blot if there were one gene. Since there are cleavage sites for *Eco*RV, *Hin*dIII, and *Pvu*II near the middle of the LTP1 coding sequence, two bands would be expected if there were only one gene. Under stringent hybridization and washing conditions, two to four bands of different intensities were observed (Fig. 4), indicating that there are at least two LTP genes in *Arabidopsis*. The existence of additional LTP genes was confirmed by an analysis of sequence information, which is available from projects devoted to partial sequencing of anonymous cDNA clones or ESTs. Comparison of the genomic sequence with the sequences of ESTs in the dbEST data base resulted in the identification of three *Arabidopsis* ESTs with high homology to LTP1 (accession numbers 14215, 14037, and 14299). These EST sequences had average overall sequence homologies to LTP1 of approximately 80% at the DNA level but were essentially identical to each other (i.e. since ESTs are derived by single-pass sequencing, they may have a significant number of errors that prevents a critical analysis of perfect identity), indicating that they are the products of a second gene that we designate LTP2 for convenience. A fourth EST (accession number 21488) with 65% homology was also present in the dbEST data base and may represent a more distantly related third LTP gene.

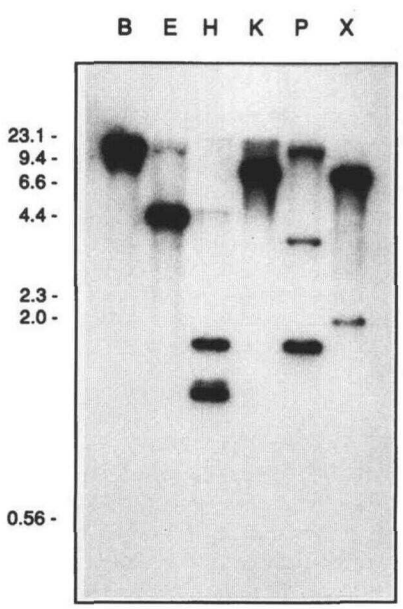

Figure 4. Southern blot of *Arabidopsis* genomic DNA probed with an *Arabidopsis* LTP1 cDNA. DNA was digested with *Bam*HI (B), *Eco*RV (E), *Hin*dIII (H), *Kpn*I (K), *Pvu*II (P), and *Xba*I (X). Size markers on the left are in kb.

Cell-Specific Accumulation of LTP mRNA

Antisense transcripts of the LTP1 gene were used as probes for in situ mRNA hybridizations on tissue sections from various developmental stages. The earliest stage at which LTP1 transcripts were detected was in the protoderm cells of the embryo cotyledon (Fig. 5, A and B). In 1-week-old seedlings, transcripts were most abundant in the leaf primordia (Fig. 5, C and D). At 3 weeks, LTP transcripts were abundant in the meristem and the primary and secondary leaves. In these tissues, hybridization was mainly to the epidermal cells (Fig. 5, G and H). There was no significant accumulation of mRNA in stomata or in root tissues, including lateral root primordia (Fig. 5, E–H).

Analysis of LTP expression in floral tissues indicated that LTP is expressed in very young flower buds (stages 2–4, Smyth et al., 1990), mainly in the epidermal cells of the inflorescence meristem (Fig. 6, A–D). In longitudinal sections of the gynoecium of a stage-11 flower, LTP expression was evident in the epidermis of the stigma, style, and ovary (Fig. 6, E and F) and in the epidermal cells of ovules (Fig. 6, O and P). Transverse sections through the upper part of a stage-12 flower showed LTP expression in many epidermal cells of most organs, but most intensely in the epidermal layer of the petals and the ovary (Fig. 6, G and H). LTP mRNA was very abundant in the stigma of stage-15 flowers (Fig. 6, K and L) and in the epidermal cells of nectaries from stage-15 flowers (Fig. 6, M and N). Accumulation of LTP mRNA in epidermal cells of floral stem tissues was evident in longitudinal sections (Fig. 6, Q and R) and cross-sections (Fig. 6S). High levels of LTP mRNA accumulation were also evident in the sepal/petal abscission zone at the base of siliques (Fig. 6, T and U). LTP mRNA was not detected in pollen of stage-15 flowers (Fig. 6, I and J).

Expression of LTP-GUS Promoter Fusions in Transgenic Plants

To exploit the advantages of using GUS-promoter fusions for LTP localization studies, the 1149-bp region of the LTP1 gene from nucleotide 68 to 1217 (Fig. 2) was placed upstream of a promoterless GUS gene and transformed into *Arabidopsis*. The 3′ end of this fragment corresponds to the 5′ end of the cDNA clone. Control plants containing GUS under transcriptional control of the cauliflower mosaic virus 35S promoter were also generated. From among 15 independent transformants, four LTP-GUS fusion lines designated LTP-GUS 1A, 1B, 1C, and 1D were chosen for further study. As is commonly observed in GUS-promoter fusion experiments, there was variation in the relative levels of GUS expression in different transformed lines. Line LTP-GUS 1A had a high relative level of GUS expression, LTP-GUS 1D had a low level of expression, and LTP-GUS 1B and 1C had intermediate expression levels. These differences were probably due to positional effects from insertion of the gene construct in various sites of the genome. To obviate concern about the influence of position effects on the pattern of expression, all observations were made on sections from the four transgenic lines. All four lines exhibited qualitatively the same pattern of expression of GUS activity.

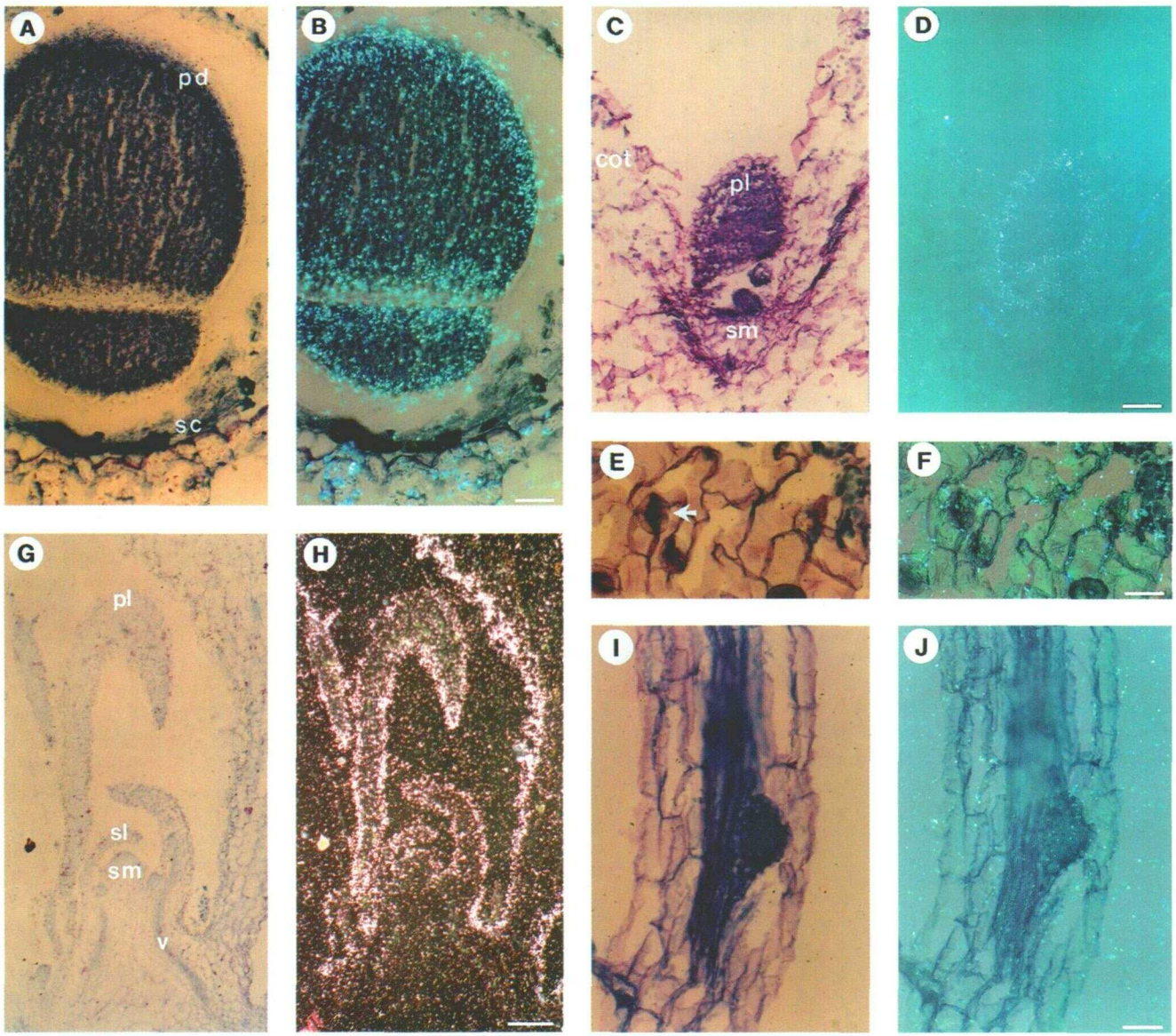

Figure 5. Localization of LTP1 mRNA in embryos, seedlings, leaf, and stem tissue by in situ hybridization. Tissue sections are presented as bright-field images (A, C, E, G, I). Silver grains are visible as bright white dots in dark-field images (H) and as blue dots in epipolarization images (B, D, F, J). A and B, Embryo cotyledons; pd, protoderm; sc, seed coat. Bar = 20 μm. C and D, Shoot meristem of a 1-week-old seedling; cot, cotyledon; pl, primary leaf; sm, shoot meristem. Bar = 40 μm. E and F, Parallel section of the leaf epidermis of a 4-week-old plant. The arrow indicates a guard cell. Bar = 20 μm. G and H, Shoot meristem of a 3-week-old plant; pl, primary leaf; sl, secondary leaf; sm, shoot meristem; v, vascular tissue. Bar = 100 μm. I and J, Lateral root primordium of a 2-week-old plant. Bar = 20 μm.

In young seedlings that had recently emerged from their seed coats (3 d after imbibition), GUS staining was strong in the hypocotyl region adjacent to the root and was also evident at lower levels in the cotyledons (Fig. 7A). By 5 d the cotyledons were weakly stained, with more intense staining in the vascular tissue (Fig. 7B). Intense GUS staining was also seen in the shoot meristem, in the primary leaves, and in the stipules (Fig. 7, B–D). As the seedling developed, GUS staining in the cotyledon decreased and eventually was confined to the tip of the cotyledon (Fig. 7, D and F). Similarly, as the primary leaves matured, GUS staining also became localized to the tips (Fig. 7E). Analysis of leaf tissue at 2, 3, and 4 weeks showed that GUS staining was seen in vascular tissue and leaf and stem epidermal cells (Fig. 7G), in guard cells (Fig. 7K), and in hydathodes (Thoma, 1992). Light GUS staining was observed in cortical cells (Fig. 7G), confirming previous observations (Thoma, 1992). The highest level of GUS activity was always obtained from tissues near the top of the stem, with little or no color formation near the base of the plant (Thoma, 1992).

No GUS activity was observed in stage-6 or younger flower buds (Thoma, 1992), but as the flower developed, GUS

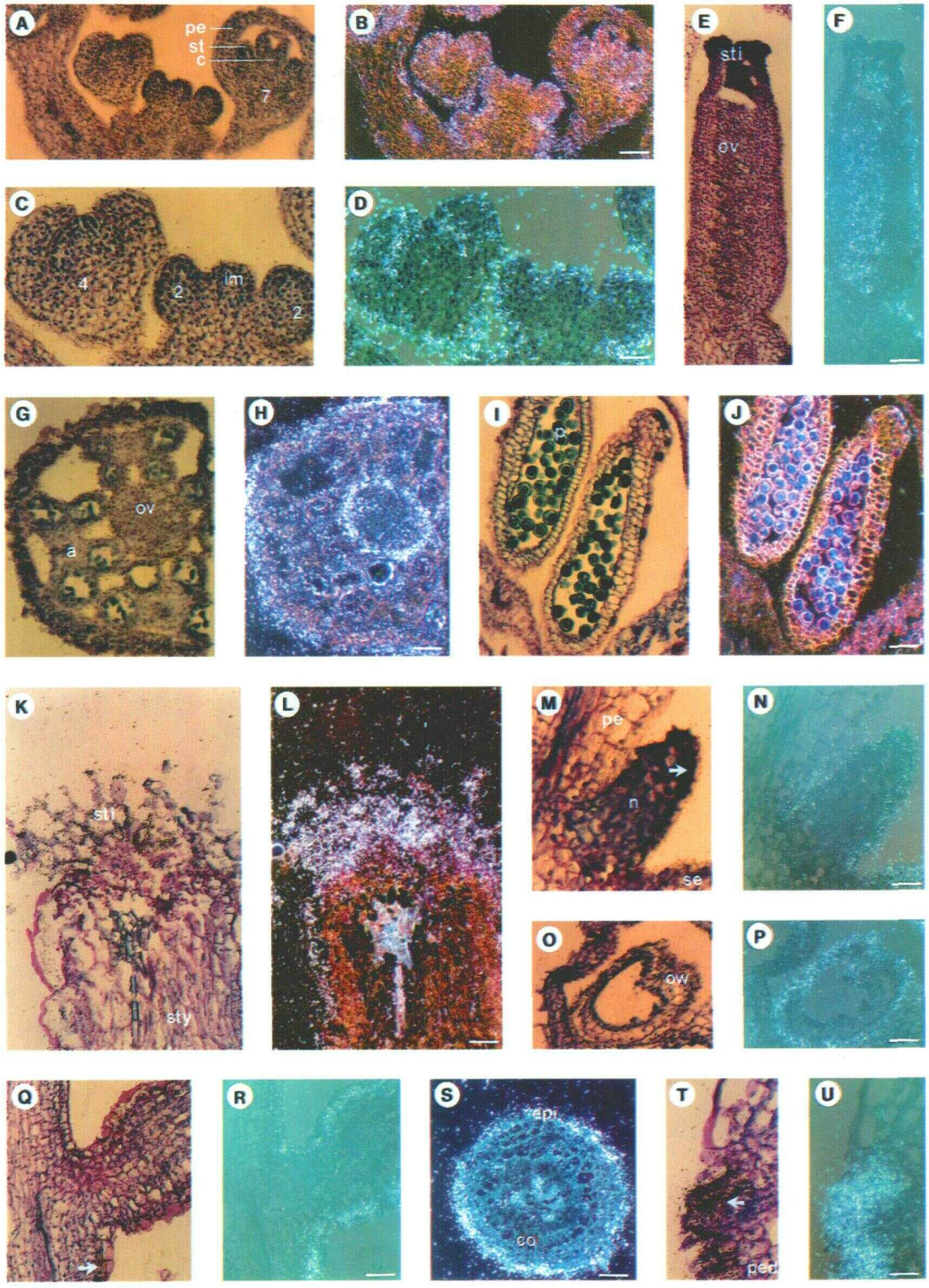

Figure 6. Localization of LTP1 in flowers by in situ hybridization. Tissue sections are presented as bright-field images (A, C, E, G, I, K, M, O, Q, T). Silver grains are visible as bright white dots in dark-field images (B, H, J, L, S) and as blue dots in epipolarization images (D, F, N, P, R, U). A and B, Inflorescence meristem and stage-2 to -7 flower buds; c, gynoecium exposed of 2 carpels; pe, petal; st, stamen; 7, stage-7 flower bud. Bar = 40 μm. C and D, Stage-2 to -7 flower buds; im, inflorescence meristem; 2, stage-2 flower bud; 4, stage-4 flower bud. Bar = 20 μm. E and F, Gynoecium and ovules of stage-11 flower; ov, ovary; sti, stigma. Bar = 40 μm. G and H, Cross-section of the upper part of a stage-12 flower; a, anther; ov, ovary. Bar = 40 μm. I and J, Pollen of stage-15 flower; p, pollen. Bar = 40 μm. K and L, Stigma of a stage-15 flower; sti, stigma; sty, style. Bar = 40 μm. M and N, Nectary of a stage-15 flower; n, nectary; pe, petal; se, sepal. The arrow indicates the epidermal cells. Bar = 20 μm. O and P, Ovule; ow, ovule wall. Bar = 40 μm. Q and R, Longitudinal section of the top of the stem near the flowers. The arrow indicates the epidermal cells. Bar = 40 μm. S, Cross-section of the top of the stem near the flowers; co, cortical cells; epi, epidermis. T and U, Base of a silique. The arrow indicates the sepal/petal abscission zone; ped, pedicel. Bar = 40 μm.

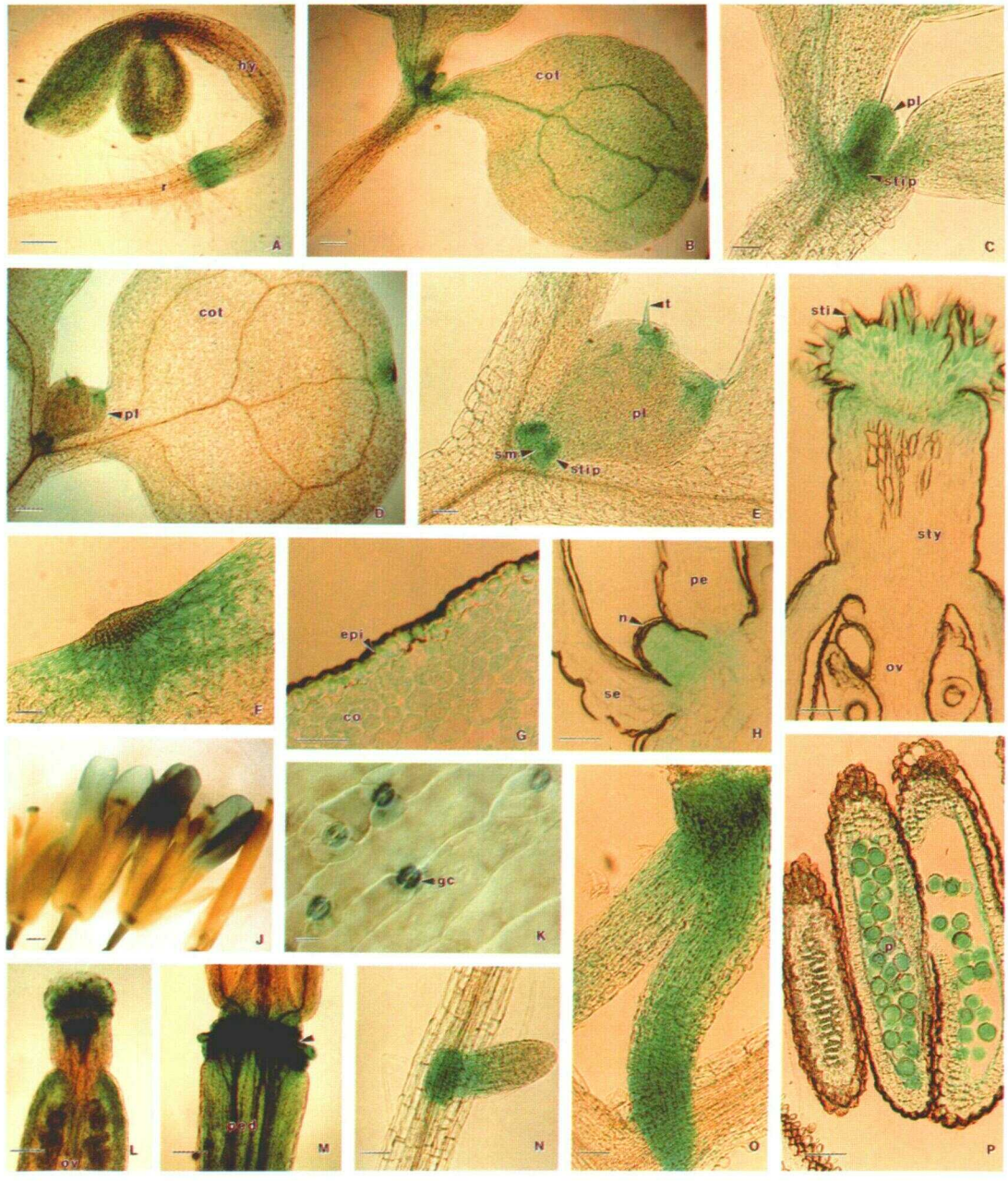

Figure 7. GUS activity in transgenic plants containing LTP1-GUS promoter fusions. A, Three-day-old seedling; cot, cotyledon; hy, hypocotyl. Bar = 250 μm. B, Five-day-old seedling; cot, cotyledon. Bar = 200 μm. C, Shoot meristem, stipules, and primary leaves of a 5-d-old seedling; pl, primary leaf; stip, stipules. Bar = 100 μm. D, Six-day-old seedling. The labeling in the cotyledon is confined to the tip; cot, cotyledon; pl, primary leaf. Bar = 200 μm. E, Shoot meristem, stipules, and primary leaves of a 6-d-old seedling; cot, cotyledon; pl, primary leaf; sm, shoot meristem; stip, stipule; t, trichome. Bar = 100 μm. F, GUS activity near lignified tissue at tip of cotyledon. Bar = 200 μm. G, Cross-section through stem of 6-week-old plant. Section taken from top of stem, near flowers; co, cortical cells; epi, epidermis. Bar = 200 μm. H, Longitudinal section through the base of a stage-15 flower; n, nectary; pe, petal; se, sepal. Bar = 50 μm. I, Longitudinal section through the stigma and style of a stage-15 flower; ov, ovary; sti, stigma; sty, style. Bar = 50 μm. J, Whole mount of flower. Bar = 250 μm. K, Whole mount of 4-week-old, fully expanded rosette leaf; gc, guard cell. Bar = 10 μm. L, Whole mount of distal end of silique; ov, ovary; sti, stigma. Bar = 100 μm. M, Whole mount of proximal end of silique; ped, pedicel. The arrow points to the sepal abscission zone. Bar = 100 μm. N, Lateral root forming on 5-d-old seedling. Bar = 100 μm. O, Lateral root of 14-d-old seedling. Bar = 50 μm. P, Longitudinal section through the anther of a stage-15 flower; p, pollen. Bar = 50 μm.

activity appeared. In the newly opened flowers (stages 11 and 12), weak staining was observed in the stigma but not in the pollen or in the floral nectaries (Thoma, 1992). In the mature flower (stage 15), intense staining was observed in the nectaries (Fig. 7H), in the stigma (Fig. 7I), and in pollen grains (Fig. 7P). Weak GUS activity was observed in the ovary walls (Fig. 7P). Whole mounts of flowers showed GUS staining of petals (Fig. 7J) and the sepal and petal abscission zone (Fig. 7, L and M).

GUS activity was not observed in emerging roots, but as the roots developed, GUS staining was seen at the base of lateral roots (Fig. 7N). Staining was also occasionally observed at the tip of lateral roots as they developed (Fig. 7O). GUS activity was occasionally observed in trichomes and their associated basal cells (Fig. 7E). However, since this was not always observed, it may reflect induction of GUS by mechanical damage to the trichomes or basal cells during sample preparation.

DISCUSSION

LTPs were originally isolated based on their ability to transfer lipids between membranes in vitro. However, a role for *Arabidopsis* LTPs in intracellular lipid transfer now seems unlikely in view of the observations indicating that they are secretory proteins (Bernhard et al., 1991; Madrid, 1991), which, in the cases of barley, carrot, and *Arabidopsis*, have been shown to be located outside the plasma membrane in the cell wall (Mundy and Rogers, 1986; Sterk et al., 1991; Thoma et al., 1993). This raises the question of what biological role LTPs play.

One approach to this question is to determine when and where the LTP genes are expressed. Previous studies of this question in other species have utilized immunohistochemical methods, northern blots of mRNA from various tissues, and in situ hybridization. We have extended this analysis by the use of GUS-LTP promoter constructs in transgenic *Arabidopsis* plants combined with in situ hybridizations. The use of promoter fusions obviates possible concern about cross-hybridization of a nucleic acid probe, or an antibody, with the products from different genes in a multigene family. As a first step, cDNA and genomic clones for an *Arabidopsis* LTP were characterized. The *Arabidopsis* LTP1 gene product is highly homologous to the LTPs isolated from other plants. Analysis of Southern blots probed with the LTP1 cDNA indicate that *Arabidopsis* has at least two closely related LTP genes. Three partial cDNA sequences for a highly homologous second gene (LTP2) are present in the dbEST data base. The fact that LTP2 cDNAs were repetitively isolated during anonymous sequencing of cDNAs from siliques and etiolated seedlings, whereas LTP1 cDNAs were not, may suggest that it is more highly expressed than LTP1 in these tissues. A fourth EST with significant but lower homology to LTP1 may represent the product of a third gene in *Arabidopsis*. LTP isoforms have also been found in castor bean (Tsuboi et al., 1991), barley (Mundy and Rogers, 1986; Linnestad et al., 1991), tobacco (Koltunow et al., 1990; Fleming et al., 1992), and wheat (Dieryck et al., 1992), and Southern analysis suggests the presence of several LTP genes in maize (Tchang et al., 1989) and tomato (Torres-Schumann et al., 1992).

Examination of the pattern of GUS activity in conjunction with the results from in situ hybridizations indicated that the LTP1 gene is expressed in specific cell and tissue types. Employing in situ hybridization, the earliest stage at which LTP expression was detected was in embryos of heart-stage cotyledons, where the expression was confined to the protoderm cells, which give rise to the epidermis. Similar results were obtained with carrot (Sterk et al., 1991), although in carrot, LTP expression was detected much earlier in embryo development. In newly emerged seedlings, GUS activity was observed in the cotyledons and in the hypocotyl near the root. As the cotyledons expanded, GUS activity became most prominent in the highly lignified area at the tip of the cotyledons. As the seedlings matured, GUS activity became localized to the shoot meristem, the stipules, the vascular tissue, and the emerging leaf primordia. No clear epidermis-specific GUS expression was observed, possibly due to the use of whole mounts for the analysis. As the primary leaves matured, GUS activity became localized to the tips. In contrast, in situ mRNA hybridization showed an intense signal in the epidermal cells of entire primary leaves of older seedlings. This discrepancy could be due to hybridization of the probe to another member of the LTP gene family. The failure to detect significant nonepidermal expression of LTP genes by in situ hybridization is more difficult to explain. Given the high level of homology between individual members of this gene family, at least some signal should have been detected. Therefore, it is unlikely that the GUS staining seen in cortical cells is the result of LTP1 promoter activity.

In mature plants, expression of the GUS reporter was evident in leaf and stem epidermal cells, guard cells, and flowers. The expression of LTP1 in epidermal cells and the higher level of expression in guard cells is consistent with the results of immunocytochemical studies that showed a similar pattern of protein localization (Thoma et al., 1993). The expression of the gene in stem epidermal cells was confirmed by the in situ studies, but these analyses showed no specific hybridization in guard cells. It is possible that the LTP1 transcripts are short-lived in leaf epidermal and guard cells, whereas GUS may be very stable in these cells. GUS activity in the stem was much more prominent at the top portion of this structure, near the flowers, than at the bottom of the stem. A similar spatial expression pattern was seen for a tobacco LTP (Fleming et al., 1992), where it was also observed that transcript levels were higher in young tissues at the top of the plant than in older organs. GUS activity was also present at low levels in stem cortical cells. This expression pattern is consistent with immunocytochemical localization (Thoma et al., 1993).

An LTP was expressed at a very early stage in flower development. In situ analysis showed that a signal is present as early as the formation of flower primordia (stage 2; Smyth et al., 1990) and in the inflorescence meristem. However, no GUS staining was seen in young flower buds or the inflorescence meristem, indicating that the LTP1 promoter is not active in these tissues. This discrepancy could be due to the fact that there are multiple LTP genes in *Arabidopsis*, and in situ analyses detect all homologous genes. It is also possible that additional control elements exist, either upstream of the LTP1 promoter region used or in the 3′ region of the gene.

At stage 7, very little LTP message remained in the petal epidermis, but occasionally a strong signal in the abaxial epidermis of the upper parts of stage-12 petals was observed. This was also evident by GUS staining in slightly later flower stages. GUS staining was observed on the stigma surface in flowers where the stigmatic papillae have appeared and the petals are level with long stamens (stages 11 and 12). By stage 15, when the stigma extends above the anthers, staining is observed in the stigma surface, the floral nectaries, and developing pollen grains. GUS staining in the stigma was confirmed by in situ studies. In the floral nectaries, in contrast, the in situ hybridization showed only a detectable signal restricted to the epidermis. High levels of GUS activity in the abscission zone of the pedicel was also paralleled by strong in situ mRNA hybridization signals.

No specific in situ mRNA hybridization signal was evident in pollen grains, in lateral roots, or in the internal cells of stipules, but high levels of GUS activity were observed in these tissues. As stated before, these discrepancies could be due to transient expression of GUS coupled with high stability of the enzyme activity. Thus, if the tissue used for the GUS assays was at a slightly different stage than that used for the in situ analysis, this would explain the observed results. It is also possible that the GUS staining of pollen grains, vascular tissue, and lateral roots is an artifact. It has recently been observed that although a pathogenesis-related protein (PR-1a)-GUS promoter fusion specifies expression in pollen grains, the same promoter fused to other reporter genes does not show this effect (Uknes et al., 1993), indicating that the pollen expression is due to sequence elements in the GUS coding region. A similar discrepancy has also been observed recently by Drews et al. (1992). The possibility of artifacts when using the GUS reporter gene to detect tissue- and cell-type-specific expression highlights the necessity of either performing in situ mRNA hybridization analysis to confirm the results or using several different reporter genes for promoter fusion constructs. This is especially true in situations where the gene under study is part of a multigene family, the members of which exhibit highly complex temporal and spatial regulation.

Although the LTP1 gene is clearly under tissue-specific developmental regulation, there have been several recent reports in which the search for stress-induced genes has led to the isolation of genes encoding LTPs. A barley gene, *blt4*, is induced in shoot meristems by cold-temperature treatment, by drought stress, and by ABA application, and its deduced amino acid sequence shows a high similarity to that of maize LTP (Hughes et al., 1992). A tomato LTP has been shown to be greatly induced in stems by NaCl, mannitol treatment, and ABA treatment (Torres-Schumann et al., 1992). Another tomato gene, *le16*, which has approximately 50% homology to the N terminus of a maize LTP, is induced by drought stress and ABA treatment (Plant et al., 1991). In addition, a radish seed protein that shows sequence homology to LTPs has been shown to exhibit in vitro antifungal activity (Terras et al., 1992). Also, LTPs from barley and maize leaves were shown to be inhibitors of the growth of bacterial and fungal pathogens in vitro (Molina et al., 1993). The existence in the LTP1 5′ region of promoter elements that have previously been implicated in transcriptional control of the phenylpropanoid pathway (Cramer et al., 1989; Lois et al., 1989; Ohl et al., 1990) raises the possibility that the LTP1 gene may be regulated by the same or similar mechanisms. Indeed, the expression of LTP1 in the sepal/petal abscission zone is similar to that seen with an *Arabidopsis* Phe ammonia-lyase promoter (Ohl et al., 1990). In addition, a 10-bp sequence motif, designated box 1 in this study and found in the 5′ nontranscribed region of many stress-induced genes (Goldsbrough et al., 1993), is present twice in this region of the LTP1 gene.

All things considered, the expression pattern observed for LTP1 as detected by in situ hybridization is consistent with a proposed role in secretion or deposition of extracellular lipophilic materials (Sterk et al., 1991). The elevated expression in young tissues suggests a role that is diminished in fully expanded tissues. This observation, in conjunction with the localization in epidermal cell walls, is generally consistent with a role in assembly or deposition of cell wall or cuticular structural material as previously suggested (Sterk et al., 1991). The carrot LTP was recently shown to have biochemical properties similar to those of other plant nonspecific LTPs (i.e. it is able to transfer phospholipids between donor and acceptor membranes in vitro, and it is able to bind fatty acids) (Hendriks et al., 1993; Meijer et al., 1993). Such a role would also be consistent with the high level of LTP expression in the petal and sepal abscission zone, where it may be expected that additional structural materials are deposited to seal off the ruptured tissue. The high level of expression in the lignified zone at the tip of cotyledons and in vascular elements is also consistent with this general function. However, an additional aspect seems necessary to explain how expression in stipules, nectaries, and stigmas relates to this pattern of expression. One possible connection is that all of these tissues may secrete structurally related materials and that the LTP is somehow involved in the secretory process or subsequent reactions of the secreted materials. In view of the fact that LTP has the ability to transfer lipids, it seems reasonable to propose that the secreted materials are lipophilic substances. Indeed, stigma cells produce a secretion that contains lipids and phenolic compounds (Martin and Brewbaker, 1971). Unfortunately, very little is known about stipules or nectaries, so it is not possible to argue for or against a particular function for LTP based on expression in this tissue. The fact that the LTP protein is detectable in the mature leaf epidermis (Thoma et al., 1993), whereas expression of the LTP1 gene is not readily detected by GUS-promoter fusions or in situ hybridization, suggests that the proteins are stable in the cell wall.

In conclusion, the available evidence indicates that the LTP1 promoter specifies a complex developmental pattern of expression in transgenic plants and also contains elements involved in wound-induced expression of other genes. Although the pattern of expression is suggestive of a possible biological role, additional studies will be required to establish an in vivo function.

ACKNOWLEDGMENT

We are grateful to Nigel Crawford for the gift of the λgt10 cDNA library.

Received September 2, 1993; accepted January 18, 1994.

LITERATURE CITED

Arondel V, Kader J-C (1990) Lipid transfer in plants. Experientia **46:** 579–585

Bernhard WR, Thoma S, Botella J, Somerville CR (1991) Isolation of a cDNA clone for spinach lipid transfer protein. Evidence that the protein is synthesized by the secretory pathway. Plant Physiol **95:** 164–170

Breu V, Guerbette F, Kader J-C, Gamini Kannangara C, Svensson B, von Wettstein-Knowles P (1989) A 10 kD barley basic protein transfers phosphatidylcholine from liposomes to mitochondria. Carlsberg Res Commun **54:** 81–84

Cox GN, Goldberg RB (1988) Analysis of plant gene expression. *In* CH Shaw, ed, Plant Molecular Biology: A Practical Approach. IRL Press, Oxford, UK, pp 1–34

Cramer CL, Edwards K, Dron M, Liang X, Dildine SL, Bolwell GP, Dixon RA, Lamb CJ, Schuch W (1989) Phenylalanine ammonia-lyase gene organization and structure. Plant Mol Biol **12:** 367–383

De Block M, Debrouwer D (1992) In situ enzyme histochemistry on plastic-embedded plant material. The development of an artefact-free β-glucuronidase assay. Plant J **2:** 261–266

Dieryck W, Gautier M-F, Lullien V, Joudrier P (1992) Nucleotide sequence of a cDNA encoding a lipid transfer protein from wheat (*Triticum durum* Desf.). Plant Mol Biol **19:** 707–709

Drews GN, Beals TP, Bui AQ, Goldberg RB (1992) Regional and cell-specific gene expression patterns during petal development. Plant Cell **4:** 1383–1404

Fleming AJ, Mandel T, Hofman S, Sterk P, de Vries SC, Kuhlemeier C (1992) Expression pattern of a tobacco lipid transfer protein gene within the shoot apex. Plant J **2:** 855–862

Goldsbrough AP, Albrect H, Stratford R (1993) Salicylic acid-inducible binding of tobacco nuclear protein to a 10-bp sequence which is highly conserved amongst stress-inducible genes. Plant J **3:** 563–571

Hendriks T, Meijer EA, Thoma S, Kader JC, de Vries SC (1994) The carrot extracellular lipid transfer protein EP2: quantitative aspects with respect to its probable role in cutin synthesis. *In* G Coruzzi, P Puidgomenech, eds, Molecular-Genetic Analysis of Plant Metabolism and Development. NATO-ASI Seminars. Plenum, New York (in press)

Hughes MA, Dunn MA, Pearce RS, White AJ, Zhang L (1992) An abscisic-acid responsive, low-temperature inducible barley gene has homology with a maize phospholipid transfer protein. Plant Cell Environ **15:** 861–865

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive, versatile gene fusion marker in higher plants. EMBO J **6:** 3901–3907

Joshi CP (1987) An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucleic Acids Res **15:** 6643–6653

Kader J-C, Julienne M, Vergnolle C (1984) Purification and characterization of a spinach-leaf protein capable of transferring phospholipids from liposomes to mitochondria or chloroplasts. Eur J Biochem **139:** 411–416

Koltunow AM, Truettner J, Cox KH, Wallroth M, Goldberg RB (1990) Different temporal and spatial gene expression patterns occur during anther development. Plant Cell **2:** 1201–1224

Linnestad C, Lönneborg A, Kalla R, Olsen OA (1991) Promoter of a lipid transfer protein gene expressed in barley aleurone cells contains similar myb and myc recognition sites as the maize Bz-McC allele. Plant Physiol **97:** 841–843

Lois R, Dietrich A, Hahlbrock K, Schulz W (1989) A phenylalanine ammonia-lyase gene from parsley: structure, regulation, and identification of elicitor and light-responsive *cis*-acting elements. EMBO J **8:** 1641–1648

Madrid S (1991) The barley lipid transfer protein is targeted into the lumen of the endoplasmic reticulum. Plant Physiol Biochem **29:** 695–703

Martin FW, Brewbaker JL (1971) The nature of stigmatic exudate, its role in pollen germination. *In* J Heslop-Harrison, ed, Pollen Development and Physiology. Butterworth, London, pp 262–272

Meijer EA, de Vries SC, Sterk P, Gadella Jr DWJ, Wirtz KWA, Hendriks T (1993) Characterization of the nsLTP EP2 from carrot (*Dacus carota* L.). Mol Cell Biochem **123:** 159–166

Molina A, Segura A, Garcia-Olmeda F (1993) Lipid transfer proteins (nsLTPs) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens. FEBS Lett **316:** 119–122

Mundy J, Rogers JC (1986) Selective expression of a probable amylase/protease inhibitor in barley aleurone cells: comparison to the barley amylase/subtilisin inhibitor. Planta **196:** 51–63

Ohl S, Hedrick S, Chory J, Lamb CJ (1990) Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. Plant Cell **2:** 837–848

Plant AL, Cohen A, Moses MS, Bray EA (1991) Nucleotide sequence and spatial expression pattern of a drought- and abscisic acid-induced gene of tomato. Plant Physiol **97:** 900–966

Rickers J, Spener F, Kader J-C (1985) A phospholipid transfer protein that binds long-chain fatty acids. FEBS Lett **180:** 29–32

Rogers SO, Bendich AJ (1988) Extraction of DNA from plant tissues. Plant Mol Biol Manual **A6:** 1–10

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Smyth DR, Bowman JL, Meyerowitz EM (1990) Early flower development in *Arabidopsis*. Plant Cell **2:** 755–767

Sossountzov L, Ruiz-Avila L, Vignois F, Jolliot A, Arondel V, Tchang F, Grosbois M, Guerbette F, Migniac E, Delseny M, Puigdomenech P, Kader J-C (1991) Spatial and temporal expression of a maize lipid transfer protein gene. Plant Cell **3:** 923–933

Sterk P, Booij H, Schellekens GA, Van Kammen A, De Vries SC (1991) Cell-specific expression of the carrot EP2 lipid transfer protein. Plant Cell **3:** 907–921

Tchang F, This P, Stiefel V, Arondel V, Morch M-D, Pages M, Puigdonmench P, Grellet F, Delsney M, Bouillon P, Huet J-C, Guerbette F, Beauvais-Cante F, Duranton H, Kader J-C (1988) Phospholipid transfer protein: full length cDNA and amino acid sequence in maize. J Biol Chem **263:** 16849–16855

Terras FRG, Goderis IJ, van Leuven F, Vanderleyden J, Cammue BPA, Broekaert WF (1992) In vitro fungal activity of a radish (*Raphanus sativus* L.) seed protein homologous to nonspecific lipid transfer proteins. Plant Physiol **100:** 1055–1058

Thoma SL (1992) Molecular, cellular characterization of non-specific lipid transfer proteins from spinach and *Arabidopsis*. PhD Thesis, Michigan State University, East Lansing

Thoma SL, Kaneko Y, Somerville C (1993) An *Arabidopsis* lipid transfer protein is a cell wall protein. Plant J **3:** 427–437

Torres-Schumann S, Godoy JA, Pintor-Toro JA (1992) A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. Plant Mol Biol **18:** 749–757

Tsuboi S, Suga T, Takishima K, Mamiya G, Matsui K, Ozeki Y, Yamada M (1991) Organ-specific occurrence, expression of the isoforms of nonspecific lipid transfer protein in castor bean seedlings, and molecular cloning of a full-length cDNA for a cotyledon-specific isoform. J Biochem **110:** 823–831

Tsuboi S, Suga T, Takishima K, Mamiya G, Matsui K, Ozeki Y, Yamada M (1992) Non specific lipid transfer protein in castor bean cotyledon cells. Subcellular localization and a possible role in β-oxidation. J Biochem **111:** 600–608

Uknes S, Dincher S, Friedrich L, Negrotto D, Williams S, Thompson-Taylor H, Potter S, Ward E, Ryals J (1993) Regulation of pathogenesis-related protein-1a gene expression in tobacco. Plant Cell **5:** 159–169

Valvekens D, Van Montagu M, Van Lijsebettens M (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci USA **85:** 5536–5540

Yamada M, Tsuboi S, Osafune T, Suga T, Takishima K (1990) Multifunctional properties of non-specific lipid transfer proteins from higher plant. *In* PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure, Function, and Utilization. Portland Press, London, pp 278–280