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Spatial and Temporal Expression of a Maize Lipid Transfer Protein Gene

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We studied the temporal and spatial pattern of lipid transfer protein (LTP) gene expression, as well as the localization of this protein, in maize. Using an LTP gene, we observed an accumulation of LTP mRNA in embryos and endosperms during seed maturation. LTP gene expression was also investigated in young seedlings. After germination, the level of LTP mRNA in the coleoptile increased, with a maximum at 7 days, whereas LTP mRNA levels were low in the scutellum and negligible in roots. The high levels of LTP mRNA found in coleoptiles and embryos were confirmed by in situ hybridization. Moreover, LTP gene expression appeared to be localized in the external cellular layers and around the leaf veins. Using immunogold methods, we also observed that LTP was distributed heterogeneously in the different cells of coleoptiles and leaves. The highest concentrations of LTP were found in the outer epidermis of the coleoptiles as well as in the leaf veins. Together, our observations indicate that LTP gene expression is not only organ specific and time specific but also cell specific.

INTRODUCTION

A group of plant proteins called lipid transfer proteins (LTPs) have been shown to facilitate in vitro transfer of lipids between membranes (for reviews, see Kader, 1990; Arondel and Kader, 1990). LTPs have been purified as water-soluble proteins from various plants (Kader, 1990). They exhibit similar properties: (1) a high pI (9 to 10), (2) a molecular mass of approximately 9 kD (90 to 93 amino acids), (3) high sequence homologies including the presence of 8 cysteine residues at conserved locations, and (4) a broad specificity for lipids (Kader et al., 1984; Watanabe and Yamada, 1986; Kader, 1990). cDNAs encoding LTPs have been isolated and characterized from maize seedlings (Tchang et al., 1988), spinach leaves (Bernhard et al., 1991), and barley (Mundy and Rogers, 1986; Breu et al., 1989). Because of their ability to transfer lipids in vitro, LTPs are assumed to play a role in membrane biogenesis by mediating the transport of lipids from their sites of biosynthesis, typically the endoplasmic reticulum (ER), to other membranes (Kader, 1990). However, the in vivo physiological functions of LTPs have not been demonstrated. To contribute to the demonstration of a role in lipid transfer in vivo, we have studied the pattern of gene expression and the cellular localization of maize LTP. In addition to a full-length cDNA coding for maize LTP, a specific polyclonal antibody is available (Grosbois et al., 1987).

In this paper, we present an analysis of LTP gene expression during maize seed maturation and germination. The LTP gene expression pattern was investigated at different developmental stages and in different tissues. We also used in situ hybridization and immunogold methods to show that LTP mRNAs were preferentially located in the outer cellular layers of the organs where LTP genes are actively expressed.

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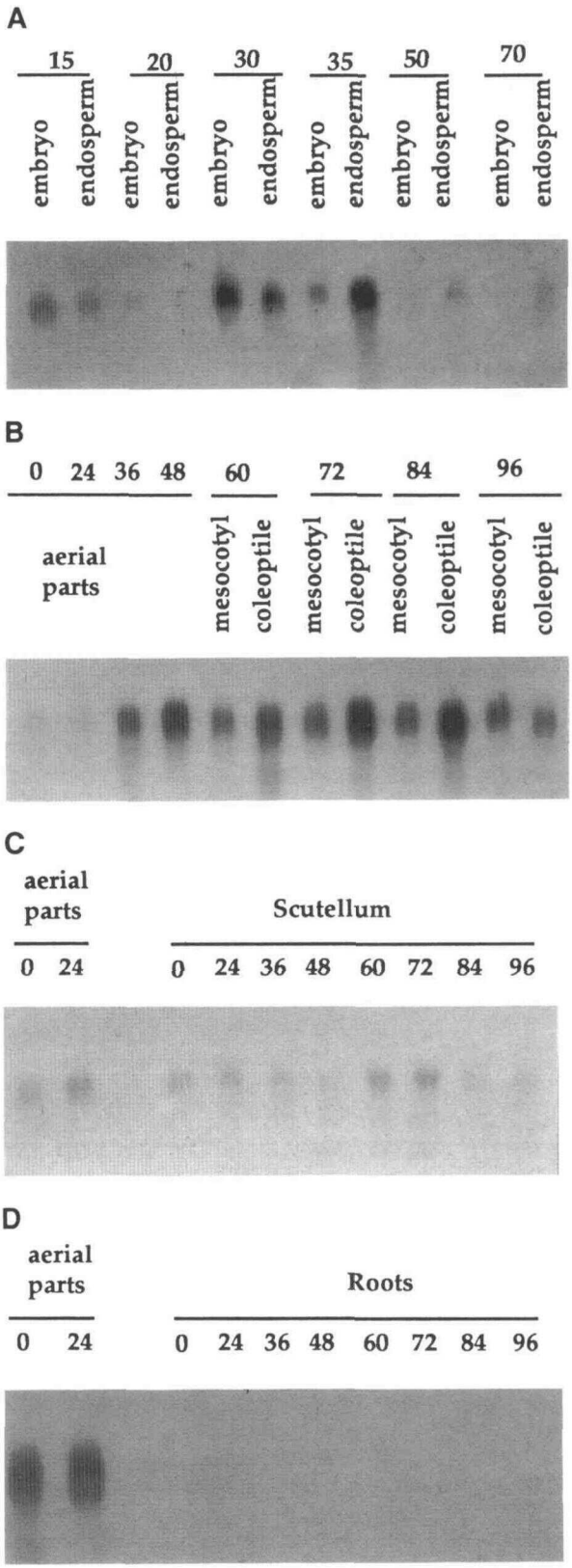


RESULTS

LTP mRNAs Accumulate during Seed Maturation

The accumulation of LTP transcripts in maize seeds was examined by RNA gel blot analysis. Equal amounts of total RNAs from the following two samples were analyzed: (1) isolated embryos (scutellum and embryonic axis), and (2) endosperms (with aleurone tissues, without periderm). Blots were hybridized with the 9C2 cDNA probe, which corresponds to the maize LTP gene (Tchang et al., 1988). As shown in Figure 1A, for each developmental stage and each tissue, the probe hybridized with an abundant 0.9-kb mRNA. If the exposure time was extended, a second mRNA (0.7 kb) was detected at 35 days after pollination (DAP) (data not shown).

Figure 1A shows that the 0.9-kb mRNA level increased in endosperms late in embryogenesis and reached a maximum at 35 DAP. The 0.9-kb mRNA level then quickly decreased. By the dry seed stage (70 DAP), the 0.9-kb mRNA level was only one-tenth of the level observed at 35 DAP, revealed by densitometric scanning (data not shown). In embryos, the 0.9-kb LTP transcript was present at low levels at 15 and 20 DAP. The 0.9-kb mRNA level then increased during the early developmental stages to peak at 30 DAP. However, the maximum level of the 0.9-kb mRNA in embryos was only about one-third of that observed at 35 DAP in endosperms. At the dry kernel stage, the amount of 0.9-kb RNA in embryos was less than one-tenth of the maximum level obtained for endosperm.

Figure 1. Accumulation of LTP mRNA during Maturation and Germination of Maize Seeds.

Total RNA (10 μg) was isolated from various parts of developing seeds or seedlings, fractionated on denaturating agarose gels, transferred to nylon membranes, and hybridized with labeled gene LTP probe, as outlined in Methods. The signals correspond to the 0.9-kb LTP mRNA.

(A) Levels of LTP mRNA in developing seeds. Total RNA was isolated from embryos and endosperms of developing seeds at 15 to 70 DAP.

(B) Levels of LTP mRNA in seedlings. Total RNA was isolated from aerial parts of seedlings 0 to 48 hr after germination and from mesocotyl or coleoptile 60 to 96 hr after germination.

(C) Levels of LTP mRNA in seedlings. Total RNA was isolated from scutellum of seedlings 0 to 96 hr after germination. For comparison, RNAs from aerial parts were analyzed.

(D) Levels of LTP mRNA in seedlings. Total RNA was isolated from roots of seedlings 0 to 96 hr after germination. For comparison, RNAs from aerial parts were analyzed.

Film exposure times were 10 hr for **(A)**, 24 hr for **(B)**, 6 days for **(C)**, and 3 days for **(D)**.

LTP mRNAs Accumulate during Seed Germination

LTP gene expression was also investigated in young seedlings after germination. We detected an LTP mRNA (0.9 kb) in coleoptile and mesocotyl (Figure 1B) and in scutellum (Figure 1C) but not in roots (Figure 1D).

Figure 1B shows that, during the first 24 hr after germination, the abundance of the 0.9-kb mRNA in aerial parts increased steadily to reach a maximum at 84 hr after germination. In mesocotyl, the 0.9-kb mRNA level remained constant and low from the 60th hour (about half of the maximal value obtained for coleoptile according to densitometric scanning). In the scutellum (Figure 1C), the amount of 0.9-kb mRNA remained constant and very low in comparison with the levels attained in aerial parts (about one-sixth of the maximum value obtained for coleoptile).

LTP Is Located Mainly in the Outer Epidermis of the Coleoptile

The low magnification view of a section of maize coleoptile in Figure 2A shows the organization of this tissue. Surrounded by an uniseriate outer epidermis with sparsely distributed stomata, the coleoptile appears to consist mainly of large parenchymal cells, which are highly vacuolated and contain few amyloplasts. The vascular system is represented by two vascular strands placed in opposite positions. They contain well-developed phloem-conducting and xylem-conducting elements and small parenchymal cells. As shown in Figures 2A and 2C, an inner epidermis is close to the leaf rolls. Figure 2C also shows numerous leaf veins formed by a few small cells, surrounded by larger cells that constitute the bundle sheath and by parenchymal cells.

Peroxidase-antiperoxidase and immunogold methods, used to visualize LTP, gave identical staining patterns (Figures 2A and 2D). The antibody used in these studies is specific for maize LTP, as indicated by the protein gel blot of Figure 3A: the antibody stains a band in protein extracts from coleoptile, mesocotyl, and scutellum but not root. Lipid transfer activity was also detected in coleoptile, mesocotyl, and scutellum but not in roots (Figure 3B).

Figures 2A and 2B show that nearly all coleoptile and leaf cells were stained by the antibody, but the intensity varied. More intense staining was observed in the outer epidermal cells of the coleoptile (Figures 2A, 2B, and 2D). In contrast, a weak staining was observed in the parenchymal cells, in the inner epidermis of the coleoptile, and in the leaf epidermis (Figures 2A, 2B, 2C, and 2D). In Figures 2B and 2C, the vascular strands of the coleoptile and leaves appeared more heavily stained than the surrounding parenchymal cells. When observed at a higher magnification, as shown in Figure 2D, the staining appeared to be associated with the cytoplasm. The background, as indicated by the control experiments in Figure 2E, was very low. Very low or no immunostaining was observed when immune serum was preincubated with its corresponding antigen (Figure 2E) or in control sections in which treatment with primary antibody was omitted from the immunostaining protocol or preimmune serum was used (data not shown).

LTP mRNA Is Located Mainly in the Outer Cellular Layers

Sections of the same tissue samples as those used for light microscopy immunocytochemistry were hybridized in situ with a biotinylated LTP gene probe. Hybridization was visualized with immunogold silver staining, and sections were examined by epipolarization microscopy. LTP mRNAs were located in the same cells as LTP protein, as shown in Figure 4A (compare with Figure 2B). Staining was more intense in the outer epidermal layer of the coleoptile than in the inner epidermis (compare Figures 4A and 4B). The two vascular coleoptile strands and the leaf minor veins showed more staining than their surrounding parenchymal cells, as shown by Figures 4A and 4B. Pretreatment of the sections by RNase A showed that labeling was specific (Figure 4C).

Similar results were obtained when sections of immature maize embryos were hybridized in situ with a ^{35}S-labeled riboprobe. As shown in Figures 5 and 6, hybridization was observed in the outer and inner epidermis of the coleoptile in the axis. The inner epidermis was more heavily labeled than with the biotinylated probe (see Figures 5A and 5B). As shown in Figure 5C, hybridization in the axis disappears below the scutellar node. In the scutellum, the hybridization is restricted to the single layer of secretory epidermal cells located in the abaxial part, as observed in Figures 5A and 6C. The pattern is maintained through early embryogenesis, at least between 12 (Figure 6A), 15 (Figure 6B), and 20 DAP (Figure 5C).

DISCUSSION

LTP Gene Expression Is Temporally and Spatially Regulated

We followed the changes in the amount of LTP mRNA during seed maturation and germination and localized LTP mRNAs within the tissues. Our results indicate that the LTP gene is specifically expressed in certain tissues and cells and that it is regulated during maize development.

Our results are consistent with previous observations showing that the amount of LTP, determined by ELISA and immunoblotting methods, varies during maturation and germination of maize (Grosbois et al., 1989) and castor

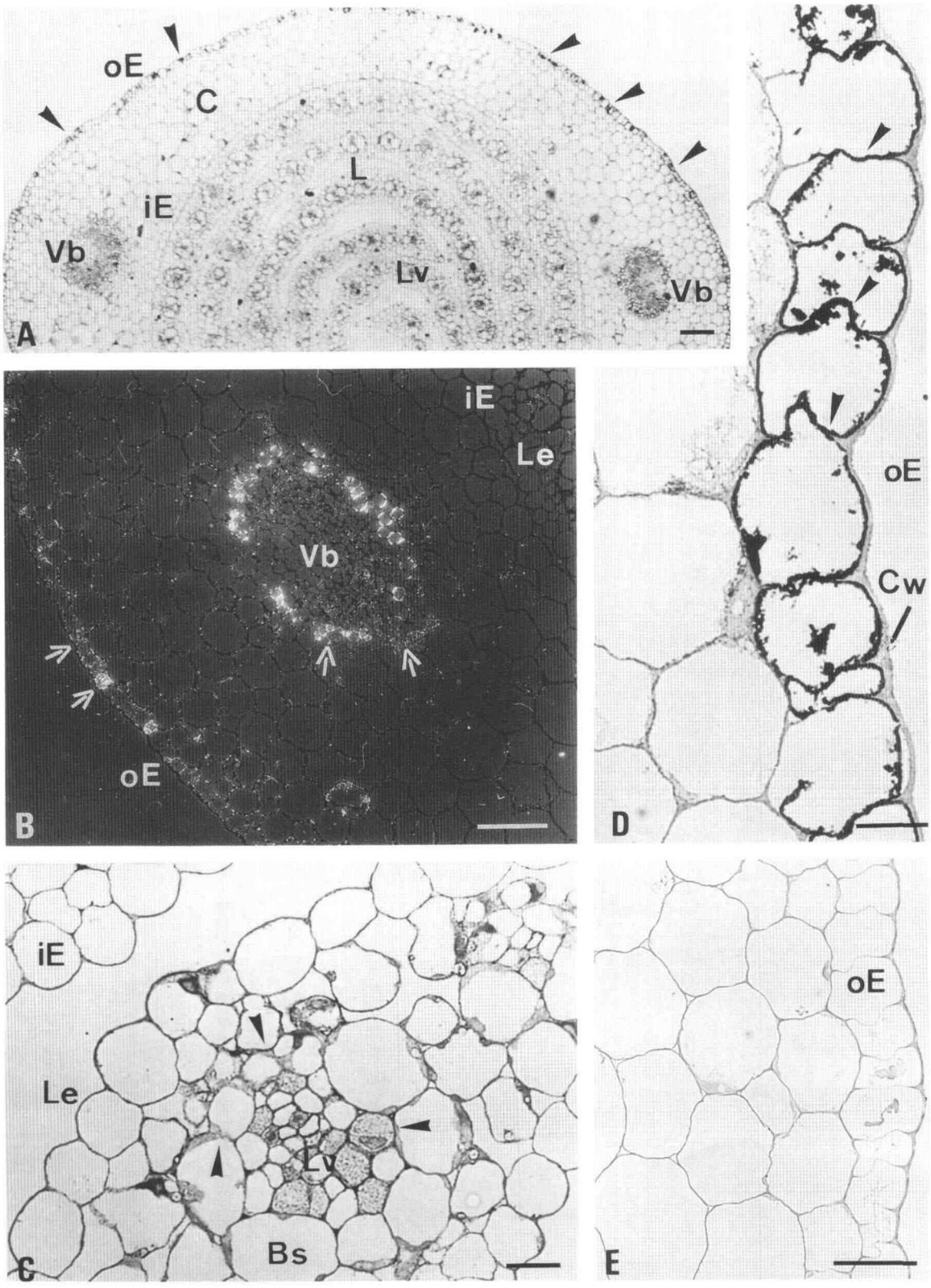


Figure 2. Immunolocalization of LTP in Maize Coleoptile and Its Enclosed Leaves.

bean (Tsuboi et al., 1989). These authors observed the same kinetics for LTP accumulation and enhancement of phospholipid transfer activity. Therefore, there is a good correlation between LTP mRNA and the corresponding protein.

The spatial and temporal pattern of LTP gene expression during seed maturation suggests that the protein participates in two aspects of lipid metabolism. One is storage lipid formation, which is restricted to the scutellum and the aleurone layer. The storage lipids are packaged into lipid bodies as a triacylglycerol core surrounded by a layer of phospholipids and proteins (Vance and Huang, 1988). These lipid bodies probably derive from ER, and their formation requires an acyl-CoA transfer from plastids (Huang et al., 1987). By their ability to bind fatty acids (Kader, 1985), LTP could be involved in storage lipid formation. Second, the formation of new membranes requires active lipid metabolism. The embryo is the center of numerous divisions. The membrane proliferation is especially important at the 30 to 35 DAP developmental stage and affects the ER (Millerd, 1975). It is interesting to note that the developmental time course of LTP mRNA in the embryo was the same as that observed for L_3 protein (Vance and Huang, 1988) and acyl-carrier protein (Hannapel and Ohlrogge, 1988), which are two important proteins involved in lipid metabolism.

The same results have been obtained with zein mRNAs, which are the major storage proteins in maize. However, these proteins are found only in the endosperm (Sanchez-Martinez et al., 1986), whereas LTP is located in endosperm and embryo. Moreover, zein gene expression ceases as the seed matures and the endosperm dies. In contrast, LTP gene expression continues at a low level throughout the first day of germination. This level, which is higher in endosperm than in embryo, suggests that the mRNA encoding LTP is stable.

The presence of a high LTP mRNA level in aerial portions can be correlated with active membrane synthesis. However, a surprising result is the absence of mRNA encoding LTP in roots, although an active membrane biogenesis takes place in this tissue. This absence of LTP synthesis has been observed in barley aerial parts (Mundy and Rogers, 1986). One explanation could be the existence of LTP isoforms specifically expressed in root tissues, as suggested by the fact that, in addition to the major 0.9-kb mRNA, another minor one (0.7 kb) was detected in our experiments. However, it should be noted that it is difficult to distinguish the activity of the various isoforms because our transfer assays detect all LTPs present in the extracts. Nevertheless, the apparent absence of LTP in roots introduces some doubt about an essential participation of LTP in the intracellular lipid transfer necessary for membrane biogenesis.

One of the most interesting observations of our work is the fact that LTP mRNA was preferentially located at the periphery of the organs studied, embryos or coleoptiles. This is to be related to recent observations (Koltunow et al., 1990), indicating that among anther-specific mRNAs, some are coding for LTP, as deduced from sequence homologies. Interestingly, these LTP mRNAs are specifically expressed in the tapetal cells.

LTP Is Heterogeneously Distributed within Organs

One of the main findings of the present work is the demonstration that the level of LTP protein, as well as the LTP gene expression, differed in the various cells and organs. The most noteworthy feature revealed by the present study concerns the high LTP gene expression and the high level of immunoreactive sites in the outer epidermal layer in the coleoptile. These observations suggest a specific function of LTP in this layer. It is known that auxin and its corresponding putative receptor play a role in the growth of coleoptile and are present in the epidermis (Klämbt, 1990). The study of the relation between auxin and LTP distribution in the epidermis is of potential interest.

Figure 2. (continued).

Transverse sections were labeled with affinity-purified rabbit anti-LTP IgG, followed by peroxidase-antiperoxidase staining **(A)** and **(E)** or goat anti-rabbit IgG coupled to 5-nm colloidal gold staining, further enhanced with silver intensification reagent **(B)** to **(D)**. They were viewed by light microscopy using bright-field **(A)** and **(C)** to **(E)** or epipolarization optics **(B)**. Arrows indicate the location of LTP in some zones chosen as typical examples.

(A) General view of the coleoptile and leaves. Black stainings represent cells with LTP. C, coleoptile; iE, inner epidermis of the coleoptile; L, leaves; Lv, leaf veins; oE, outer epidermis of the coleoptile; Vb, vascular bundle of the coleoptile. Bar = 100 μm.

(B) Higher magnification view of the coleoptile. White grains represent silver/gold grains of the LTP signal. Le, leaf epidermis. Bar = 50 μm.

(C) Positive immunolabeling of the leaf vein; inner epidermal cells of the coleoptile and leaf epidermis are weakly stained. Bs, bundle sheath. Bar = 20 μm.

(D) Outer epidermis of the coleoptile with strong cytosolic labeling. Note that the cell wall is not labeled. Cw, cell wall. Bar = 20 μm.

(E) Specificity control through the outer epidermis of the coleoptile. The section was immunostained with preabsorbed anti-LTP antibody with purified LTP before its application. Bar = 20 μm.

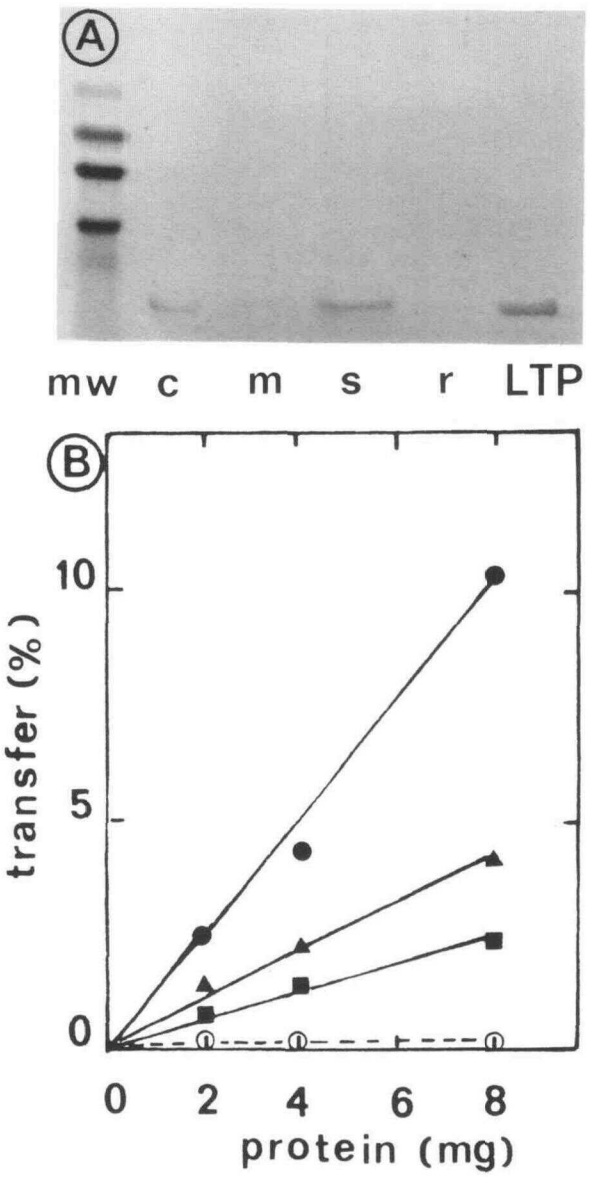


Figure 3. Immunodetection and Activity of Lipid Transfer Protein in Extracts Prepared from Coleoptiles and Leaves of Maize Seedlings.

(A) Immunoblots of proteins extracted from coleoptiles (lane c), mesocotyl (lane m), scutellum (lane s), and roots (lane r) excised from 200 seedlings. These proteins were separated by SDS-PAGE (240 μg per lane), electroblotted to nitrocellulose, and probed with anti-maize LTP antibody. In addition, prestained SDS-PAGE standards (Bio-Rad) were run in lane mw as a control for transfer efficiency. The apparent molecular masses of the major bands detected as standards were, in increasing order: 18.5, 27.5, and 32.5 kD. A major band was detected in the protein extracts from coleoptiles and scutellum, and weakly in mesocotyl proteins. No band was observed with root proteins. Purified maize LTP (3 μg) was deposited as a control (lane LTP).

(B) The protein extracts were assayed for lipid transfer activity. The assays were performed by following the transfer of ^{3}H-phosphatidylcholine from liposomes to mitochondria, as described in Methods. The transfer activity, expressed as percent of the initial liposomal radioactivity, was detected in coleoptile (●), mesocotyl (■), scutellum (▲), and root (○) extracts.

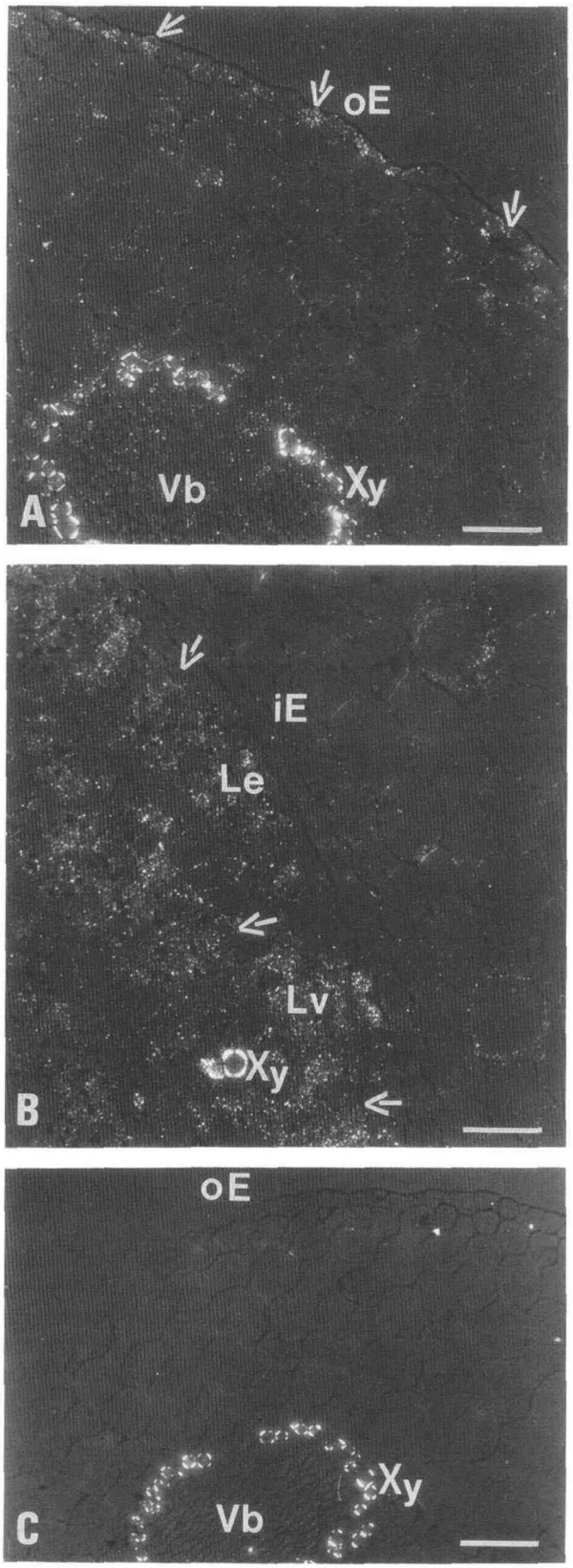
The localization of LTP protein and LTP gene expression in the epidermis should be considered in the context of evidence that LTP is secreted. The sequence of a full-length cDNA encoding maize LTP indicates the presence of a leader peptide of 27 residues (Tchang et al., 1988). This peptide has characteristics of signal peptides and may allow membrane transit. Vergnolle et al. (1988) showed that maize LTP was synthesized as a precursor form. In connection with this observation, Bernhard et al. (1991) found that spinach LTP is synthesized by a secretion process involving a crossing of the ER membrane. If LTP crosses the membrane, this suggests that LTP can be partly secreted outside the cell. It is worth remembering that a barley protein, recognized to be an LTP, was shown to be secreted from aleurone cells (Breu et al., 1989). It is possible that various isoforms of LTP are either cytosolic or secreted. However, the secretion from maize cells remains to be demonstrated, and factors acting on this process have to be determined. The finding that LTP may be secreted raises again the question of the in vivo involvement of these proteins in the intracellular lipid flux. Interestingly, immunolocalization experiments made in maize coleoptile suggested that LTP (or isoforms of LTP) could have a multiple localization, being cytosolic and membrane bound (L. Sossountzov, unpublished results).

It is clear that further studies are required to assess the full role of LTP in the development of plant organs. The present findings provide important clues for studying the regulation of gene expression of LTP in relation with plant morphogenesis.

METHODS

Biological Materials

For RNA gel blot analysis, seeds of W 64A +/+, a pure inbred line of maize (*Zea mays*), grown in the greenhouse, were harvested at different developmental stages and stored at −80°C. For each stage, embryos (scutellum and embryonic axis) and endosperms (with aleurone tissues, leaving the periderm) were carefully dissected by hand in the presence of liquid nitrogen and separately stored at −80°C. In germination studies, dry seeds were grown on moist vermiculite in the dark at 30°C. For each stage of germination, the seedlings were then divided into scutellum, mesocotyls, roots, and coleoptiles in liquid nitrogen and



stored at −80°C. For immunocytochemical and in situ hybridization observations, etiolated coleoptiles and enclosed leaf rolls 4 to 5 cm long were harvested from the same sets of seedlings.

RNA Preparation and Gel Blot Analysis

In maturation studies, total cellular RNAs were isolated from both tissues at 4°C, as previously described by Laroche-Raynal et al. (1984). Extraction of RNAs from germinated seedlings was carried out at 65°C according to Tchang et al. (1988). Ribosomal RNAs (25S and 18S) were used as internal controls to check the RNA recovery.

Total cellular RNAs (10 μg/stage) were analyzed on a 1.5% agarose gel as described by Maniatis et al. (1982). The membrane hybridization was performed according to Tchang et al. (1988) with the probe corresponding to the PstI-PstI largest fragment of 9C2 cDNA. The filters were exposed at −80°C to Kodak X-Omat film with intensifying screen for various periods.

The relative amounts of LTP mRNAs were normalized by densitometric scanning to 25S mRNAs present in the same samples as detected by hybridization with rDNA cDNA (Gerlach and Bedbrook, 1979). Film densitometry was performed with a Shimadzu CS-930 densitometer, and the maximum value obtained was taken as 100%.

Production and Characterization of Anti-LTP Antibody

Antibodies raised in rabbits against purified LTP from maize seedlings were characterized as previously described (Douady et al., 1986; Grosbois et al., 1987). IgGs were purified by CM-Affi-Gel blue chromatography from anti-LTP or preimmune sera. These antibodies were checked by protein gel blot on protein extracts as indicated before (Grosbois et al., 1989). The protein extracts were performed on coleoptiles and leaves taken from 200 seedlings germinated for 4 days at 30°C in the dark, according to Grosbois et al. (1989).

Lipid Transfer Activity

The activity of the protein extracts was measured by following the transfer of phosphatidylcholine between liposomes and mito-

Figure 4. Localization of mRNAs in the Coleoptile and Enclosed Leaves.

Semithin transverse sections from Spurr-embedded samples, 1 μm thick, were hybridized with cDNA biotinylated probe, followed by rabbit anti-biotin IgG and goat anti-rabbit IgG coupled to 5-nm gold particles. Photographs were taken with epipolarization optics: white grains represent cDNA/RNA hybrids.

(A) In the outer part of the coleoptile, the hybridization signal is mainly observed over the outer epidermis.

(B) The hybridization signal is also observed in the inner part of the coleoptile and in rolled leaf.

(C) As a control, the section was treated by RNase before the application of the probe. A few white grains represent background level. Xylem vessels (Xy) appear highly autofluorescent. Bar = 100 μm. Other abbreviations are as in Figure 2.

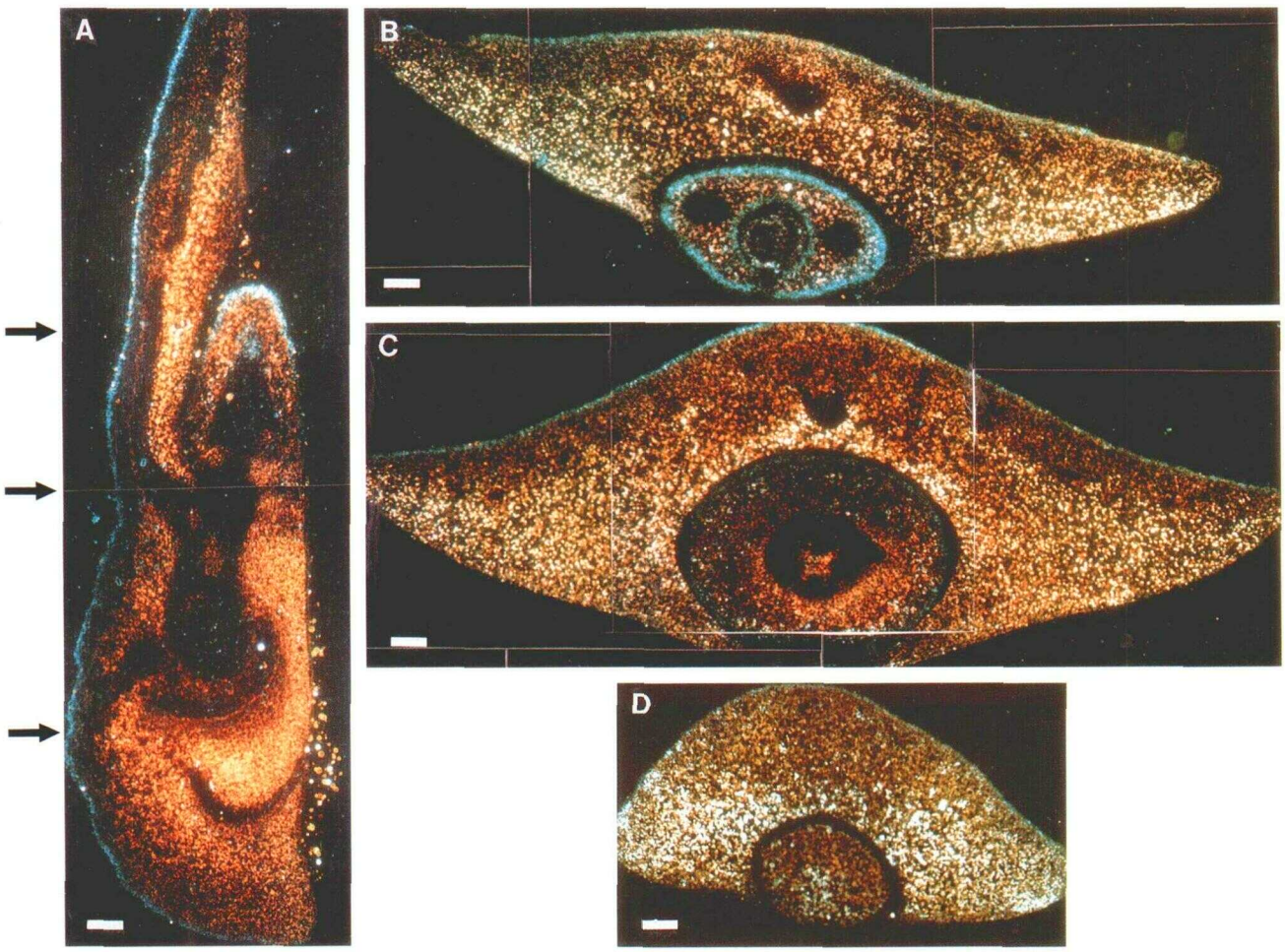


Figure 5. Maize LTP mRNA Localization in Sections from Embryos at 17 and 20 DAP.

Longitudinal **(A)** and transverse **(B)** to **(D)** sections of maize embryos at 17 and 20 DAP were hybridized with a partial cDNA LTP riboprobe and examined under dark field. The hybridization with LTP mRNA was observed as blue areas. Bars = 100 μm.
(A) Longitudinal section of a 17 DAP embryo. The approximate location of transverse sections is indicated by arrows in the left side of the plate.
(B) Transverse section of a 20 DAP embryo through the upper coleoptile.
(C) Transverse section of a 20 DAP embryo through the scutellar node.
(D) Transverse section of a 20 DAP embryo through the coleorhyza .

chondria (Grosbois et al., 1989). Liposomes containing ^{3}H-phosphatidylcholine (260 nmol, 740 Bq) as a lipid to be transferred and 1-^{14}C-cholesteryl oleate (1 nmol; 740 Bq) as a nontransferable tracer were incubated at 30°C for 30 min with maize mitochondria (2 mg protein) in the presence of protein extract. The ^{3}H label recovered in mitochondria, collected by centrifugation, indicated the extent of transfer of phosphatidylcholine (as percent of the initial radioactivity of lipids), whereas the ^{14}C label (usually low, around 1%) allowed the determination of the contamination of mitochondria by intact liposomes.

Tissue Processing for Immunocytochemistry and in Situ Hybridization

The sliced coleoptiles were fixed for 6 hr at 4°C in a solution containing 4% paraformaldehyde and 0.5% glutaraldehyde in a 0.05 M sodium phosphate buffer, pH 7. The tissues were rinsed in cold buffer containing 0.2% glycine for 30 min and left overnight at 4°C in buffer containing 3% sucrose. Tissues were then conventionally dehydrated with ethanol and infiltrated in Spurr's resin.

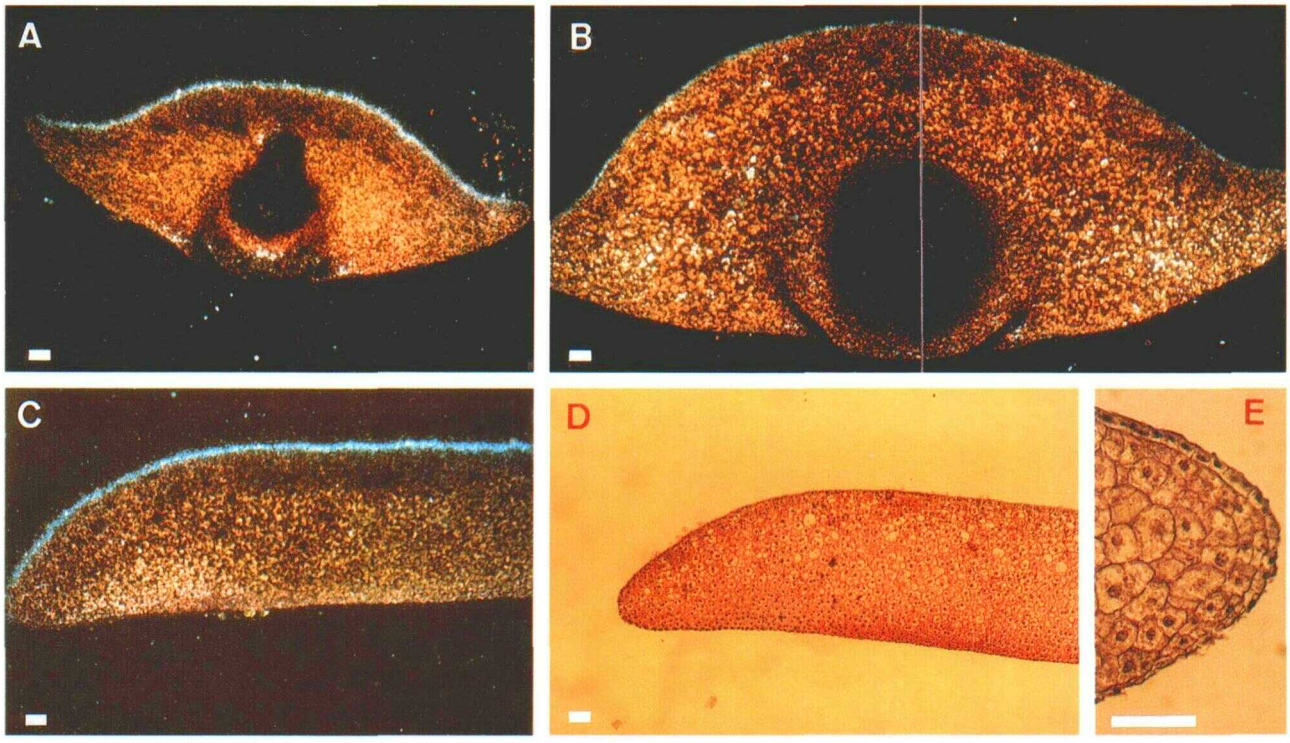


Figure 6. Evolution of the Maize LTP mRNA Localization in Sections from Embryos at Different DAP.

Transverse or longitudinal sections of maize embryos at various DAP were hybridized with a partial cDNA LTP riboprobe **(A)** to **(C)**. The morphology of the scutellum is presented in **(D)** and **(E)**. Bars = 100 μm.
(A) Transverse section of a maize embryo 13 DAP through the scutellar node.
(B) Transverse section of a maize embryo 15 DAP through the radicle.
(C) Longitudinal section of the lateral scutellum from a 17 DAP embryo.
(D) A section similar to that of **(C)** stained with hematoxylin to observe the morphology of the scutellum.
(E) A detail of a transverse section of the lateral scutellum stained with hematoxylin at greater magnification.

The samples were soaked overnight. Polymerization was done at 60°C for 14 hr. Low-temperature UV polymerization of Lowicryl was initiated at −25°C. The specimens were then brought to room temperature and further hardened for 48 hr under UV illumination.

For ^{35}S in situ hybridization, embryos were collected from hand-dissected kernels at 13, 15, 17, and 20 DAP and immediately submerged in fixing solution. The whole procedure of in situ hybridization was performed essentially according to Langdale et al. (1988).

Tissue Processing for Light Microscopy

Semithin sections, 1 μm thick, were cut from Spurr-embedded samples. The resin was first etched by bromine vapors for 1 min according to Yensen (1968). After four rinses with absolute acetone, they were hydrated by passing them through ethanol, 0.1 M, phosphate-buffered saline, pH 7.4, containing 0.1% Triton X-100 (PBS-T), and then aqueous 5% H_2O_2 solution for 15 min.

For immunocytochemistry, after rinsing with PBS-T, the sections were treated by 100 μL of PBS containing 0.2% Triton X-100 and Tween 20, 0.2% carrageenan gelatin (Type IV, Sigma), and goat IgG, 1 mg mL^{-1} (Jackson Immunochemical Research Products, Baltimore, MD) (PBS-C) for 30 min. Without washing, sections were incubated with 100 μL of anti-LTP IgG diluted 1/100 in PBS-C and placed in humidified chambers at room temperature overnight in the dark. After washing with PBS-T, the antigen-antibody complex was revealed according to the method (Sternberger et al., 1970) using peroxidase-antiperoxidase complex (Nordic, The Netherlands) or to the immunogold silver staining method (IGSS, Holgate et al., 1983) using goat anti-rabbit IgG labeled with 5-nm gold particles (Auroprobe LM GAR G5, Janssen, Belgium).

For in situ hybridization of Spurr sections, biotinylation of the probe corresponding to the PstI-PstI largest fragment of 9C2 cDNA maize clone (Tchang et al., 1988) was performed as described by Singer and Ward (1982) using a biotinyl-dUTP derivative (Bio-11-UTP, Enzo Biochemical, NY).

For ^{35}S in situ hybridization, a 270-bp PstI fragment of the 5′ coding region of the LTP gene (Tchang et al., 1988) was cloned in a pBluescript SK+ vector (Stratagene) and used as template for synthesis of sense and antisense riboprobes. Transcripts from T3 and T7 promoters were produced following the instructions of the manufacturers, using ^{35}S-CTP (37 TBq $mmol^{-1}$, Amersham) as a radioactive precursor. Final concentration of the probes was 0.1 $\mu g \cdot mL^{-1}$, and each slide was hybridized with about 0.5 kBq of label. Hybridization was then performed as previously described (Langdale et al., 1988). The slides were exposed for 10 days using Kodak nitro blue tetrazolium emulsion and stained after developing with fast green 0.5% (w/v) in 95% ethanol. The photographs were taken using a Zeiss automated camera on a Zeiss microscope.

Immunocytochemical Controls

For immunocytochemistry, specificity of labeling was assessed by the following control tests: (1) incubation with antibodies against LTP previously treated with an excess of its corresponding antigen: 100 μg of the pure LTP was added to 1 mL of the diluted antiserum for 16 hr at 4°C before use on sections (preabsorbed antibodies); (2) substitution of the LTP antibodies by preimmune rabbit IgG diluted at the same concentration; (3) incubation with goat anti-rabbit gold antibodies, the primary antibodies being omitted.

For in situ hybridization, sections were treated with RNase A at 200 μg/mL for 1 hr at 37°C before the application of the biotinylated 9C2 cDNA probe.

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