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Lipid-transfer proteins: a puzzling family of plant proteins

Jean-Claude Kader

Lipid-transfer proteins are small, basic proteins, and have been purified from various plant sources. They are able to transfer lipids between membranes *in vitro* and, on the basis of this, were initially thought to participate in the intracellular flux of lipids during membrane synthesis. However, the finding that these proteins are located in the cell wall and can be secreted has led to the suggestion that they are not required for intracellular lipid transport. Instead, they may be involved in cutin biosynthesis, surface wax formation, pathogen-defence reactions, or the adaptation of plants to environmental changes.

Lipids have to move from their sites of biosynthesis, mainly in the endoplasmic reticulum, to other cellular organelles, such as mitochondria and chloroplasts¹. The search for lipid carrier proteins led to the discovery of lipid-transfer proteins (LTPs) in a variety of monocotyledons and dicotyledons. These proteins are characterized by their ability to transfer phospholipids between membranes and to bind fatty acids *in vitro*. Recent nuclear magnetic resonance (NMR) and X-ray diffraction studies have confirmed that, structurally, LTPs are well suited to this task. However, none of these observations prove involvement in the intracellular flux of lipids. Moreover, in the light of recent observations on the expression of several LTP genes, the proposed cytoplasmic role for LTPs now appears unlikely and other novel roles, taking into account the extracellular location of the proteins, have been proposed.

Assay, purification and basic properties

The determination of LTP activity is a complex process involving two types of membranes (donor and acceptor). The donor membranes are usually liposomes prepared by ultrasonication using a radioactive phospholipid. Acceptor membranes are from mitochondria or any other subcellular fraction that can be separated from liposomes by centrifugation. When the donor and acceptor membranes are incubated in the presence of LTP and then separated, radioactive phospholipid is detected in the acceptor membrane, indicating transfer from the donor membranes. In the absence of LTP, no transfer is observed – the spontaneous movement of phospholipids is very slow.

Alternatively, liposomes used as donor membranes can be prepared from fluorescent lipids, and fluorescence quenching used to measure LTP activity in all extracts. These assays can be performed either with crude extracts or with purified LTPs.

LTPs have been purified from various sources of plant tissue (e.g. leaves and seedlings). They are 9–10 kDa in size, and have isoelectric points ranging between 8.8 and 10, depending on the plant source^{2,3}. They are also relatively abundant – on the basis of enzyme-linked immunosorbent assays (ELISAs) and immunoblotting, it has been calculated that LTPs constitute 4% of the soluble proteins extracted from maize seedlings⁴. The LTPs have a broad specificity for phospholipids and are able to transfer phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) between various membranes. Galactolipids [e.g. monogalactosyl-diacylglycerol, digalactosyl-diacylglycerol and a sulfolipid (sulphoquinovosyl-diacylglycerol)], but not triacylglycerols, are also transferred⁵.

Structure

The primary amino acid structures have been determined for the LTPs from maize, castor bean, wheat and rice, and the primary structures of several other LTPs have been deduced from their cDNA sequences (Fig. 1). The proteins consist of 91–95 amino acid residues, differing in sequence but containing eight strictly conserved cysteines forming four disulfide bridges^{3,5}. Within a given species, great divergence can be observed between the

sequences of different LTPs. For example, in castor bean seeds, the degree of sequence identity of the LTPs varies from 34 to 97% (Ref. 5). Despite such divergence in homology, all the deduced proteins retain features characteristic of the LTPs.

The three-dimensional structure of LTPs has been determined by both X-ray crystallography⁶ and NMR (Ref. 7). Both approaches, performed with maize seed LTP (93 amino acids), led to a similar structural model in which the protein is predicted to have four α -helices and a C-terminal tail, with the structure stabilized by the four disulfide bridges (Fig. 2). There also appears to be an internal hydrophobic cavity running through the molecule. NMR and fluorescence studies have shown that LTPs form complexes in aqueous solution with lyso-phosphatidylcholine^{3,7}. A model for a lipid-protein complex has been proposed in which the aliphatic chain of the phospholipid is inserted in the internal cavity, and the polar head interacts with the charged side chains within⁷; according to NMR studies, the cavity is large enough to accommodate a whole phospholipid molecule, but X-ray analysis suggests that only one acyl chain can be inserted⁶.

How do lipid-transfer proteins work?

It has been suggested that LTPs may act as carriers capable of extracting one lipid molecule from a membrane and transferring it to another (Fig. 3). This implies the formation of a reversible lipid-protein complex in agreement with the structural analyses performed with lysophospholipid-protein complexes. However, it has so far proved impossible to isolate stable complexes with diacylphospholipids. A similar mechanism has been suggested for proteins purified from animal cells and yeasts, with specific transfer of PC and PI (Refs 8 and 9). Yet, despite their apparently similar mode of action, no sequence homology has been found between LTPs from animal, plant and yeast sources.

As indicated by their three-dimensional structure, LTPs are able to bind acyl chains. This observation is in agreement with the results of ligand-binding experiments with radiolabeled fatty acids or acyl-CoA esters. This property is of interest in the light of the role suggested for LTPs in transporting acyl monomers for cutin biosynthesis^{3,10}.

When and where are lipid-transfer protein genes expressed?

Genes and cDNAs encoding LTPs have been isolated from various plant species. As a general rule, several copies of LTP genes are present per genome [e.g. tomato¹¹, castor bean^{5,12}, rice¹³, *Sorghum*¹⁴, barley¹⁵, *Pinus taeda* (loblolly pine)¹⁶, cotton¹⁷, broccoli¹⁸, *Gerbera hybrida*¹⁹ and rape²⁰]. In *Arabidopsis*, in addition to a gene already isolated and characterized²¹, more than 100 expressed

	10	20	30	40
Maize	AISC	GGV	ASAIAP	CISYARGQSG-PS-AGCCSGVRS
Spinach	GITC	GMVSSKLAP	CIGYLKG-G---	PLGGCCGGIKALNAAATTPDRKTA
Castor bean	-VDC	GGVNSSLAS	CIPFLTGA	VAS-PS-ASCCAGVQNLKTLAPTSADRRAA
Wheat	-IDC	GHVDSLVRP	CLSYVQG-GPG-PS-GQCCDGVKNLHNQARSQSDRQSA	
Rice		CGQVNSAVGP	CLTYARG-GAG-PSAA-CCSGVRS	LKAAASTTADRRTA
Barley	-LNC	GQVDSKMKP	CLTYVQG-GPG-PS-GECCNGVRDLHNQAQSSGDRQTV	
Rape	HITC	GTVTSTMTQ	CISYLTNGGPL-PSS--CCVAVKSLNQMAQTTPDRRQV	
Carrot	VLTC	GGVTGALAP	CLGYLRSQVNV-PVPLTCCNVVRGLNNAARTTLDKRTA	
<i>Arabidopsis</i>	LMSC	GTVNGNLAC	CIAYL-TRG-A-PLTQCCNGVTNLKNMASTTPDRQQA	
	50	60	70	80
Maize	CNCLKNA	AAGVS-GLNAGNAASIPSKCGVSI	PYTISTSTDCSRVN---	
Spinach	CNCLKSA	ANAIAK-GINYGKAAGLPGMCGVHIP	YAIISPSTNCNAVH---	
Castor bean	CECIKAAA	ARFP-TIKQDAASSLPKKGVDINIP	ISKTTNCQAIN---	
Wheat	CNCLKGI	ARGIH-NLNEDNARSIPPKCGVNL	PYTISLNDICSRV----	
Rice	CNCLKNA	ARGIK-GLNAGNAASIPSKCGVSV	PYTISASIDICSRVS---	
Barley	CNCLKGI	ARGIH-NLNLNNAASIPSKCNVNV	PYTISPDIICSRIV---	
Rape	CECLKS	SAGKEIK-GLNIDLVAALPTTCGVSL	SYPIGFNTNCDSISIAV	
Carrot	CGCLKQ	TANAVT-GLNLNAAAGLPARGVNI	PKISPTTDCNRVV---	
<i>Arabidopsis</i>	CRCLQSA	AKAVGPGLN	TARAAGLPACKVNIPYKISASTNCNTVR---	

Fig. 1. Primary structure of lipid-transfer proteins isolated from plants: maize³, spinach³, castor bean⁵, wheat³⁵, rice¹³, barley¹⁵, rape seed²², carrot¹⁰ and *Arabidopsis*²⁶. The alignment of the amino acid sequences was carried out using the Clustal method³⁶. Dashes represent regions where the sequence has been expanded to allow optimal sequence alignment. The sequences from maize, castor bean, wheat and rice were determined by analyzing purified proteins, and the other sequences were deduced from cDNA clones. The eight conserved cysteine residues are highlighted.

sequence tags (ESTs) encoding LTP-like proteins have been identified. These EST sequences have been assembled by building contigs (contiguous segments) into tentative consensus sequences (TIGR database, accessible on the World Wide Web at <http://www.tigr.org>). Twenty-two different groups of ESTs have been found (11 assemblies of ESTs and 11 individual ESTs that do not group into assemblies). It remains to be determined whether these sequences correspond to functional LTP genes.

The expression pattern of LTPs is complex, characterized by strong developmental and tissue specificity with distinct patterns of expression for the different genes (Table 1). For example, a strong cell specificity, mainly in epidermal cells, has been observed in both maize seedlings⁴ and carrot embryos¹⁰. However, in rape seedlings, an LTP gene was found to be expressed in the entire cotyledon²⁰ (Fig. 4). In contrast, another LTP gene is specifically expressed in the tapetal cell layer in rape seed²² and *Lilium henryi*²³.

Recent studies on embryogenesis were based on the position-specific expression of an LTP gene from *Arabidopsis* (*AtLTP1*). This gene is highly expressed in embryo protoderm, which is the precursor of the plant epidermis. The expression of the gene was studied in several *Arabidopsis* mutant embryos (*knolle*, *keule* and *gnom*) in which the mutation affects the formation of the apical-basal and radial axes²⁴. Using *in situ* hybridization, as well as by following *AtLTP1* promoter- β -glucuronidase transgene expression, it was observed that pattern formation in the

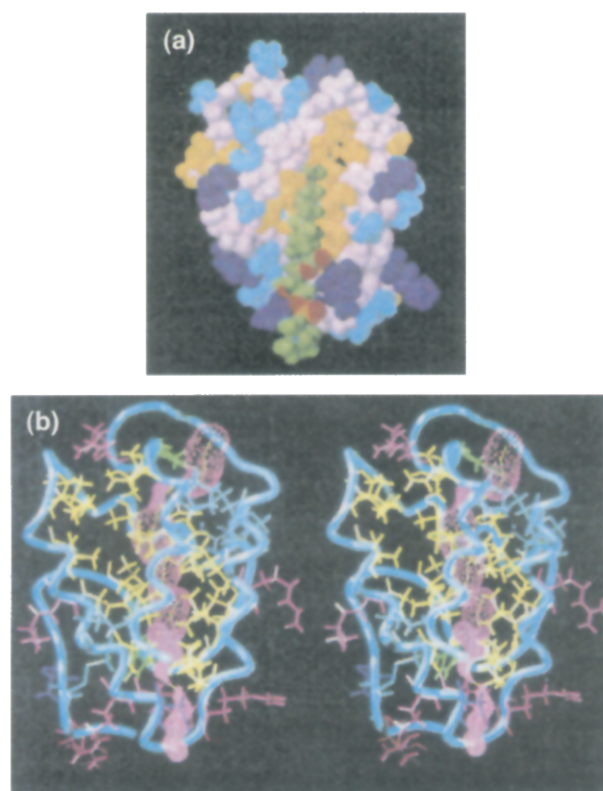


Fig. 2. Structural model of the lysophospholipid-lipid-transfer protein (LTP) complex. This model has been established by analyzing the complex formed by an LTP from maize seeds and lyso-palmitoyl-phosphatidylcholine using nuclear magnetic resonance spectroscopy. Part (a) shows a space-filling model of the complex: green, lipid; red, phosphate and carbonyl groups; pink, protein backbone; orange, hydrophobic side-chains; blue, neutral polar residues; purple, basic residues. Part (b) shows a stereo view of the pore through the protein: cyan, protein backbone; yellow, hydrophobic residues lining the cavity; pink, basic residues; pink circles, surface of the cavity; green, Tyr17 and Tyr81; deep purple, cysteine residues. These pictures are available as 'kinemages' (dynamic structures) on the World Wide Web (<http://www.prosci.uci.edu>). Reproduced, with permission, from Ref. 7.

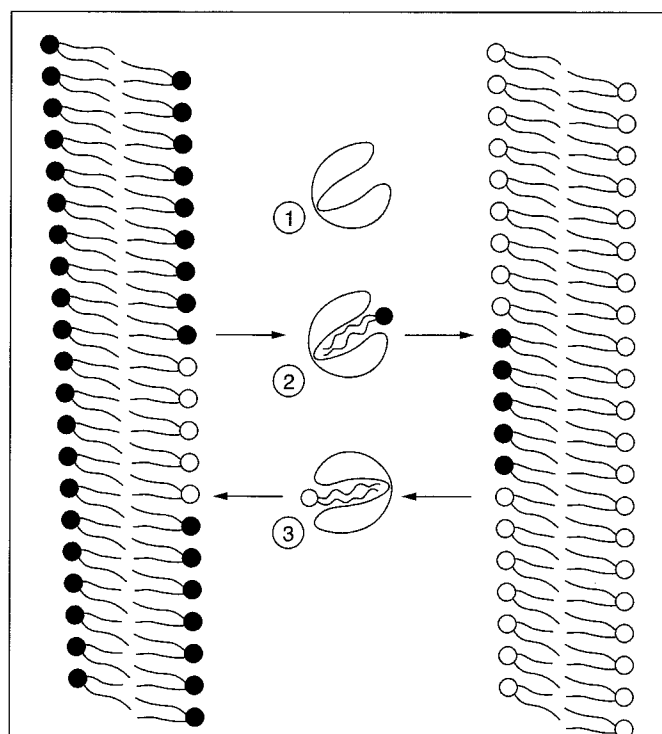


Fig. 3. Postulated mode of action of lipid-transfer proteins. The proteins (1) form a reversible complex with phospholipids (2 and 3) allowing the exchange of phospholipids between membranes.

embryo is reflected in the position-specific expression of this LTP gene²⁴. It was also found that *AtLTP1* is expressed in the outer cell layer of *Arabidopsis* raspberry embryos, which are morphologically arrested at the first stage (globular) of their formation²⁵. Together, these observations suggest that cell differentiation is uncoupled from morphogenesis during embryo development. The studies also show the utility of LTP genes as tissue-specific markers in mutant embryos.

The cellular location of LTPs has also been found to be complex. Although immunolocalization studies on rape seeds indicated a uniform distribution²⁰, LTP appears to be concentrated in the peripheral cell layers of maize seedlings⁴. It has also been reported that in *Arabidopsis*²⁶, castor bean⁵ and broccoli²⁷ LTPs are mainly (or exclusively) located in the cell wall. These findings are consistent with the secretion of LTPs from embryogenic cell cultures of carrot¹⁰ or from developing castor bean fruits²⁷. The location can be explained by the presence of a potential signal peptide, deduced from all the sequences of LTP genes, able to drive the LTPs into

Table 1. Expression patterns of lipid-transfer protein genes

Gene	Species	Cell specificity/induction	Refs
<i>EP2</i>	Carrot	Epidermal cells	21
<i>AtLTP1</i>	<i>Arabidopsis</i>	Epidermal cells	20, 24, 25
<i>9C2</i>	Maize	Epidermal cells	4
<i>glp1</i>	<i>Gerbera hybrida</i>	Epidermal cells	18
<i>wax9</i>	Broccoli	Epidermal cells	27
<i>Ltp</i>	Barley	Epidermal cells	31
<i>E2</i>	Rape	Tapetal cells	22
<i>LHM</i>	<i>Lilium henryi</i>	Tapetal cells	23
<i>GH3</i>	Cotton	Fibre cells	16
<i>TSW12</i>	Tomato	Salt-induced	10
<i>gblt4</i>	Barley	Low temperature-induced	14
<i>gblt4</i>	Barley	Abscisic acid-induced	14
<i>Ltp2-4</i>	Barley	Abscisic acid-induced	31
<i>Ltp2-4</i>	Barley	Fungal pathogen-induced	31

the lumen of the endoplasmic reticulum⁸. However, in endosperm tissues of castor bean fruits, LTPs were shown by immunohistochemical approaches to be translocated to the vacuoles by dense vesicles²⁸.

Multiple roles for multiple genes

The extracellular location of LTPs led to the hypothesis that they are active in processes requiring the transport of lipids for the cell wall. It has been suggested that LTPs participate in the biosynthesis of the cutin layer and the surface wax by carrying acyl monomers^{10,29} (Fig. 5). The fact that LTP is a major protein in the surface wax of broccoli leaves is in agreement with this hypothesis²⁷. The relationship established between LTP secretion and somatic embryogenesis can be explained by the potential involvement of these proteins in the formation of a protective layer around the young embryo^{10,30}. Alternatively, LTPs could be involved in the deposition of lipophilic material in floral organs^{19,21,22}.

According to recent studies, LTPs may also be active as plant-defence proteins³¹. The purification of plant proteins that inhibit the growth of bacterial and fungal pathogens has led to the discovery that these proteins possess some homology with LTPs^{31,32}. LTPs isolated from maize, spinach, radish, broccoli and *Arabidopsis* were shown to be active against several pathogens with various degrees of specificity^{27,31,32}. Moreover, several LTP genes in barley are upregulated in response to the infection by various strains of fungal pathogens³¹. However, it is not known if this inhibiting activity is dependent on the ability of the LTP to transfer lipids. An onion-seed protein with homology to LTPs exhibits a strong antimicrobial activity, but is unable to transfer lipids, and both maize and wheat LTPs show low antipathogen activity³². The antifungal property of LTPs might be linked to their interaction, as cationic proteins, with fungal membranes.

Several observations indicate that the expression of LTP genes can be induced by environmental factors, such as extreme temperatures and salt or drought stresses. For example, an LTP gene is specifically expressed in the stems of tomato plants treated with NaCl, mannitol or abscisic acid (ABA) (Ref. 11). In barley, several LTP genes that contain a putative ABA-responsive element and a low temperature-responsive element are induced by low temperatures¹⁵. The relationship between the putative protective role of LTPs and their differential expression in various stress conditions needs to be determined.

Although the regulation of LTP gene expression is unknown, the promoter region of an LTP gene from *Arabidopsis* contains sequences homologous to sequence elements that have been found in the promoters of stress-induced genes²¹. Although LTPs have been reported to be

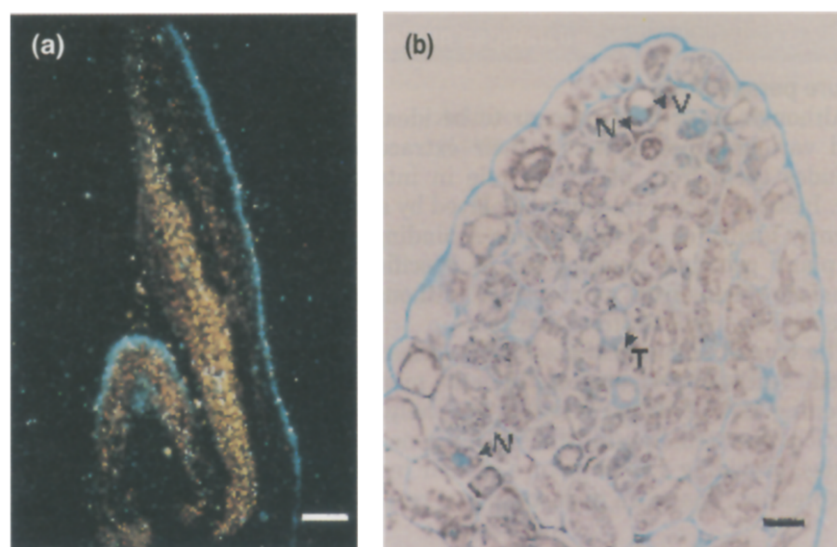


Fig. 4. Expression pattern of lipid-transfer protein (LTP) genes determined by *in situ* hybridization. Part (a) (dark-field image) shows the location of the LTP expression in a longitudinal section of maize embryo (17 days after pollination) stained with fast green (yellowish background). The blue colour, corresponding to LTP mRNA, is produced by hybridization of tissue sections with a 35S-labeled LTP gene probe from maize (see Ref. 4). The LTP mRNA is observed mainly in the outer cell layer of the scutellum (right) and in the outer epidermal cell layers of the coleoptile (left). Part (b) (bright-field image) shows the location of the LTP expression in the cotyledons of rape seeds using an antisense probe (oligonucleotide specific to an LTP and labeled with 5'-bromo-2'-deoxyuridine) (see Ref. 20). The dark zones, corresponding to LTP mRNA, are seen in the entire cotyledon. The section was subsequently stained with fast green, giving the blue-coloured structures. Abbreviations: N, nucleus; V, vacuole; T, tracheid. Bars represent 100 µm (part a) and 10 µm (part b). Reproduced, with permission, from Ref. 4 (part a) and Ref. 20 (part b).

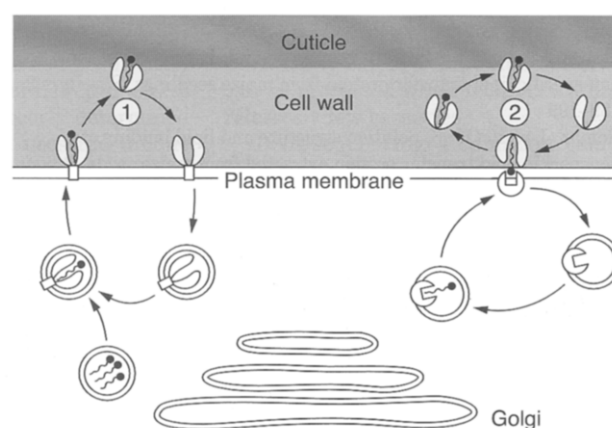


Fig. 5. Model for the postulated role of lipid-transfer proteins (LTPs) in the formation of cutin. According to the hypothesis put forward by Hendricks *et al.* (Ref. 29), LTPs function as shuttles for cutin monomers from their site of synthesis towards the cuticle. Two possible processes are presented: LTPs are taken up by receptor-mediated endocytosis and loaded by the fusion of endocytotic vesicles with cutin monomer-containing vesicles derived from the Golgi apparatus; LTPs remain outside the cell and are loaded by a membrane-bound cutin monomer carrier. Reproduced, with permission, from Ref. 29.

substrates for kinases *in vitro*, there is no evidence that LTPs are phosphorylated *in vivo*³³.

Future perspectives

Although plant LTPs appear to be ideally designed to bind various types of lipids, their extracellular location excludes them from playing a role in intracellular lipid flux. Instead, such a role may be played by another class of recently identified acyl-Coenzyme-A-binding proteins³⁴, or by plant proteins homologous to specific phospholipid transfer proteins from animal cells⁸ (although as yet these have not been isolated from plants).

The true *in vivo* role of plant LTPs remains to be established. Antisense strategies, genetic mapping and the screening of *Arabidopsis* mutants may help to clarify their function. A systematic study of all LTP genes within a plant species, providing a clear picture of their regulated pattern of expression, is also an important next research step.

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