

# LIPID-TRANSFER PROTEINS IN PLANTS

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## ABSTRACT

Lipid-transfer proteins (LTP) are basic, 9-kDa proteins present in high amounts (as much as 4% of the total soluble proteins) in higher plants. LTPs can enhance the *in vitro* transfer of phospholipids between membranes and can bind acyl chains. On the basis of these properties, LTPs were thought to participate in membrane biogenesis and regulation of the intracellular fatty acid pools. However, the isolation of several cDNAs and genes revealed the presence of a signal peptide indicating that LTPs could enter the secretory pathway. They were found to be secreted and located in the cell wall. Thus, novel roles were suggested for plant LTPs: participation in cutin formation, embryogenesis, defense reactions against phytopathogens, symbiosis, and the adaptation of plants to various environmental conditions. The validity of these suggestions needs to be determined, in the hope that they will elucidate the role of this puzzling family of plant proteins.

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## INTRODUCTION

Twenty years after their discovery in plants (51), lipid-transfer proteins (LTPs), defined by their ability to facilitate transfer of phospholipids between membranes *in vitro*, have yet to be assigned a biological role. Their isolation followed a simple idea. Because the enzymes governing the synthesis of phospholipids are not distributed among all membranes (13, 14, 101, 102), membrane biogenesis requires import of newly synthesized phospholipids. Various mechanisms were suggested: flux of vesicles (74), spontaneous movements of phospholipids (52, 123), and involvement of proteins transporting phospholipids (123). The isolation of LTPs from several plants seemed consistent with the latter hypothesis. However, with recent observations showing that LTPs are extracellularly located and are secreted, a possible role for these proteins in intracellular lipid transfer seems unlikely. Other roles in cutin formation or in defense reactions against phytopathogens were recently suggested. This review aims to contribute to the debate existing about this puzzling family of plant proteins.

## BIOCHEMICAL PROPERTIES

### *Assays*

The use of proper assays is of major importance for a correct definition of lipid-transfer proteins. The principle of the assays is to monitor the transfer of labeled lipids from donor to acceptor membranes. Acceptor membranes are either natural membranes such as mitochondria, chloroplasts, plasma membranes, and "microsomes" (endoplasmic rich-fraction) or artificial membranes (liposomes or lipid vesicles) (41, 53, 82). Donor membranes are either natural membranes or liposomes. The lipids to be transferred are either radioactive (118, 125), spin labeled (35, 79, 94), or fluorescent (22, 35, 73). The assays involving radioactive phospholipids are organized as follows: The donor membranes (liposomes) are prepared from  $^3\text{H}$ -phosphatidylcholine (PC) (the phospholipid to be transferred) and  $^{14}\text{C}$ -cholesteryl-oleate (or  $^{14}\text{C}$  trioleoylglycerol) (nontransferable compounds). The acceptors are routinely plant mitochondria. After incubation in the presence of lipid transfer proteins, the mitochondria are collected by centrifugation. The  $^3\text{H}/^{14}\text{C}$  ratio of the lipids recovered in the

mitochondria indicates the extent of the transfer of individual PC molecules from liposomes to mitochondria. The weak  $^{14}\text{C}$  label found in acceptor membranes shows that LTP does not provoke a cosedimentation of donor with acceptor membranes (118).

Fluorescent assays do not require a separation of acceptor and donor membranes. In these assays, donor vesicles consist of self-quenching vesicles of nitrobenzoxadiazol-phosphatidylcholine or of pyrenyl-phosphatidylcholine. When the fluorescent phospholipids leave the donor membranes to reach the acceptor liposomes, the fluorescence increases because of a decrease in the quenching in the donor membrane. This assay allows a continuous monitoring of the lipid transfer (22, 73).

These assays have been used to determine the lipid transfer activity of protein extracts prepared from eukaryotic and prokaryotic cells. Among eukaryotic cells, in addition to plants, mammalian cells, yeasts, and fungi have been studied (28, 92, 108, 123, 124). These assays are important to confirm that a protein belongs to the category of LTPs; one example is the barley LTP, which was initially designated as "putative protease amylase inhibitor" (PAPI) (8, 12, 75). The assays should be carried out with all proteins proposed to be LTPs on the basis of their homology with purified proteins as well as with the products of LTP-like genes.

In all these assays, a bidirectional movement of phospholipids occurs; the lipids of the donor membranes are exchanged with those of the acceptor membranes. For this reason, the proteins involved in this process were first named "phospholipid-exchange proteins" (PLEPs). However, the fact that there is not a true one-for-one exchange of phospholipids led to the name "phospholipid-transfer protein" (PLTP) (52, 123, 124) and then to the generic name "lipid-transfer protein" (LTP), accepted now because of the ability of these proteins to promote the movement of lipids other than phospholipids. The term "non specific lipid-transfer protein" (nsLTP) was also used in reference to the apparent lack of specificity for the various phospholipids.

### *Purification*

The procedures used to purify LTPs from plants are based on LTP biochemical properties (small size—9–10 kDa) and basic character (isoelectric point around 9), which were found to be remarkably similar in all higher plants studied. Plant LTPs are generally purified from soluble proteins through a combination of gel filtration, cation exchange chromatography, and reverse-phase HPLC (20, 26, 27, 53, 72, 77, 110). The lipid transfer proteins are monitored in the purification process by the various transfer assays. Several peaks of lipid transfer activity are often detected, suggesting the existence of various isoforms. The purification of LTPs was helped by their relative abundance in plants [about 4% of the supernatant proteins in maize (40)]. A purification

factor of about 100 is sufficient for obtaining a homogeneous fraction from various seeds or leaves. Another approach has been followed by Pyee & Kolattukudy (89), who extracted wax 9 LTP with a short surface wash of broccoli leaves and then purified this LTP, which represents 90% of the total proteins from the surface wax, with electrophoresis.

LTPs have been purified to homogeneity from various monocotyledonous and dicotyledonous plants (3, 17, 125). Plant LTPs are remarkably stable and keep their activity after months of storage at 4°C. Moreover, after a 5-min incubation at 90°C, the transfer activity of maize LTP is preserved, perhaps because of the presence of several disulfide bridges.

Their molecular mass varies from 9 to 10 kDa as determined either by gel filtration or by SDS-gel electrophoresis and confirmed by amino acid sequence determinations. However, in barley and wheat, shorter (7 kDa) LTPs have been detected (22, 54). Animal cells contain several categories of proteins with molecular mass varying from 11 to 33 kDa and that transfer either specifically PC, or preferentially PI (phosphatidylinositol) (PI-TP) or nonspecific (nsLTP) (123). Yeasts contain 35-kDa proteins transferring preferentially PI (37).

The major part of the lipid transfer activity of protein extracts from plants is associated with basic proteins (95% in the case of spinach leaves) (53). Only the basic LTPs (isoelectric point varying from 8.8 to 10.0, determined by chromatofocalization or isoelectric focusing) have been purified. Acidic proteins, with an apparent molecular mass of 20 to 30 kDa, have been detected in various plants, particularly in castor bean, but neither purified to homogeneity nor sequenced (125).

The availability of polyclonal antibodies prepared against LTPs purified from various plants or fusion proteins (105, 112) has provided tools to confirm, in some cases, the nature of a putative LTP (105), to determine the LTP contents either by ELISA or Western blot (40, 116), or to localize several LTPs immunohistochemically (104, 112). These antibodies also helped in the isolation of cDNAs encoding plant LTPs (109, 115). However, no monoclonal antibody is available for plant LTPs.

### *Specificity for Lipids and Acyl-Binding*

Plant LTPs are able to transfer not only PC from liposomes to mitochondria but also PI and, to lesser extents, PE or PG (phosphatidylglycerol). They are also able to transfer galactolipids but not triacylglycerols (79). The ability of plant LTPs to transfer sterols has not been studied. LTPs from castor bean, spinach, sunflower, and oilseed rape are able to bind acyl-CoA (4, 81, 93, 114). In contrast, animal cells contain fatty acid-binding proteins or acyl-CoA-binding proteins that are unable to transfer phospholipids (44). The binding of fatty acids or acyl-CoA esters by plant LTPs was determined by separation of

the acyl-LTP complex by gel filtration or by temperature-dependent ligand affinity (114). In castor bean, the saturating binding capacities for oleic acid and oleoyl-CoA per mole of LTP were 1:1 (114). In oilseed rape, several isoforms of LTP, separated by cation-exchange chromatography, revealed both ability for transfer of PC and binding of oleoyl-CoA (81). Three of these isoforms have been recently purified and sequenced (80). This ability to bind acyl chains has also been demonstrated with a barley 7-kDa protein that has some features in common with LTPs (16) and with an LTP secreted from carrot embryogenic cultures that was found to be able to bind oleoyl-CoA in a near-equimolar ratio (42, 69).

It can thus be concluded that the same proteins have the dual ability to transfer lipids and to bind acyl chains. Are the same sites involved in these two processes? To answer this question, structural studies of LTPs were performed.

## STRUCTURE AND MODE OF ACTION

### *Amino Acid Sequence*

The complete amino acid sequences have been determined for LTPs purified from various plants (11, 22, 75, 106, 109, 128) and were found to correspond to those deduced from the nucleotide sequences of LTP genes with the exception of the presence of a signal sequence at the amino terminal end of the gene products (Figure 1). These sequences thus correspond to mature LTPs. In the case of barley and rice, LTPs have been primarily called putative amylase-protease inhibitors, because of their homology with a protein isolated from Indian finger millet and because they were initially supposed to belong to this category, although it is probably an LTP (15). Plant LTPs have a total number of amino acids varying from 91 to 95 residues. They lack tryptophan, and they have eight cysteine residues located at conserved positions according to the pattern 2/3-C-8-C-12/15-CC-19-C-1-C-21/23-C-13-C-4/8. The cysteine residues are engaged in four disulfide bridges as determined by tryptic digestion of castor bean LTP (Figure 2) (106).

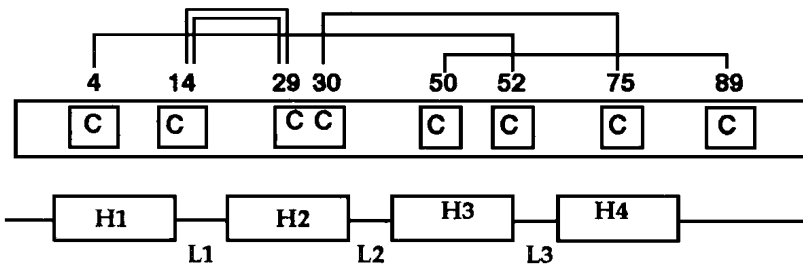
Plant LTPs exhibit strong structural homologies as revealed by sequence alignment and hydrophobic cluster analysis (22, 43, 109). However, no sequence homology was found between LTPs from mammalian and plant LTPs (123). Plant LTPs differ from oleosins, which are hydrophobic proteins associated with oil bodies in seeds (90).

### *Tertiary Structure*

On the basis of the sequences of plant LTPs, a tentative tertiary structure model was proposed that consisted mainly of beta-sheets (65, 109). This conclusion appeared to be wrong in light of the structural studies carried out on various LTPs. Studies of wheat LTP by NMR, infrared, and Raman spectroscopy (22,

			↓			41	
Maize	MARTQQLAVVATAVVALVLLAAATSEAAIS	C	GQV	SASAIAP	C		
Carrot	MGVLRSSFVAMMVMYMLATTPNAEAVLT	C	GQVT	GALAP	C		
Tobacco	MEIAGKIACFVVL	CM	VVAAPCAEAIT	C	GQVTSN	LAP C	
Spinach	MASSAVIKLACAVLLCIVV-AAPYAEAGIT	C	GMV	SSKLAP	C		
Gerbera	MASMVMNVL	CVAVACM	VFSASYADAIS	C	GQVTSGL	VPC	
Barley	MARAQVL-LMAAALV-LMLTAAPRAAVLN	C	GQV	DSKMKP	C		
Broccoli	MAGLMKLA CLIFACMIVAGPITSNAALS	C	GTV	SGYVAP	C		
Rice	MARAQLV LVALVAAA LLAGPHTTM-AAIS	C	GQV	NSAVSP	C		
Castor	MKNVVFSVLLLLSFLFCLANTNEAAVP	C	STV	DMKAAA	C		
Arabido	MLALGLHDCGRSNTSNAALS	C	GSV	NSNLAA	C		
						81	
Maize	ISYARGQSGPSA-G	CC	SGV	RLNNAARTTADRRAA	CNC	LK	
Carrot	LGYLRSQVNPVPLT	CC	NVVR	GLNNAARTTLDKRTA	CGC	LK	
Tobacco	LAYLRN--TGPLGR-	CC	GGV	KALVNSARTTEDRQIA	CTC	LK	
Spinach	IGYKLG---GPLGG	CC	GGIK	ALNAAAATTPDRKTA	CNC	LK	
Gerbera	FGYL--AAGGPVPPA	CC	NGVR	DLNNAAKTTPDRQTA	CGC	LK	
Barley	LTYVOG-GP-GPSGE	CC	NGVR	DLHNQAQSSGDRQTV	CNC	LK	
Broccoli	IGYLAQNAP-AVPTA	CC	SGVT	SLNMMARTTPDRQQA	CRC	LV	
Rice	LSYARGLRP---SAA	CC	SGV	RLNSAASTTADRRTA	CNC	LK	
Castor	VGFATGKDSKP-SQA	CC	TGL	QQLAQTVKTVDDKKAI	CRC	LK	
Arabido	IGYVLQGGVIP--PA	CC	SGV	KNLNSIAKTTTPDRQQA	CNC	IQ	
						120	
Maize	NAAAGVSGLNAGN-AASIPSK	C	GVS	IPYTI	STSD	C	SRVN
Carrot	QTANAVTGLNLNA-AAGLPAR	C	GVN	IPYKIS	PTTD	C	NRVV
Tobacco	SAAGAISGINLGK-AAGLPST	C	GVN	IPYKIS	PSTD	C	SKVO
Spinach	SAANAIKGINYGK-AAGLPGM	C	GVH	IPYAI	SPSTN	C	NAVH
Gerbera	GILANTRINLNN-ANSLPGK	C	GIS	IGYKIT	PNID	C	SKIH
Barley	GIARGIHNLNLN-AASIPSK	C	NVN	VPYTI	SPDID	C	SRIY
Broccoli	GAANALPTINVAR-AAGLPKA	C	GVN	IPYKISK	TTN	C	NSVK
Rice	NVAGSISGLNAGN-AASIPSK	C	GVS	IPYTI	SPSID	C	SS
Castor	ASSKSLGIKDQFLSK--IPAA	C	NIK	VGF	VPVSTNTN	C	ETIH
Arabido	GAARALGSGLNAGRAAGIPKA	C	GVN	IPYKIST	STN	C	KTVR

Figure 1 Comparison of the amino acid sequences of lipid-transfer proteins deduced from cDNAs or genes encoding these proteins in plants. The putative cleavage site of the signal sequence is indicated by an arrow. The cysteine residues are within boxes. The following sequences are presented: maize (109), carrot (105), tobacco (30), spinach (9), *Gerbera hybrida* (58), barley (75), broccoli (wax 9; 88), rice (119), castor bean (nsLTP C, 115), *Arabidopsis thaliana* (111).



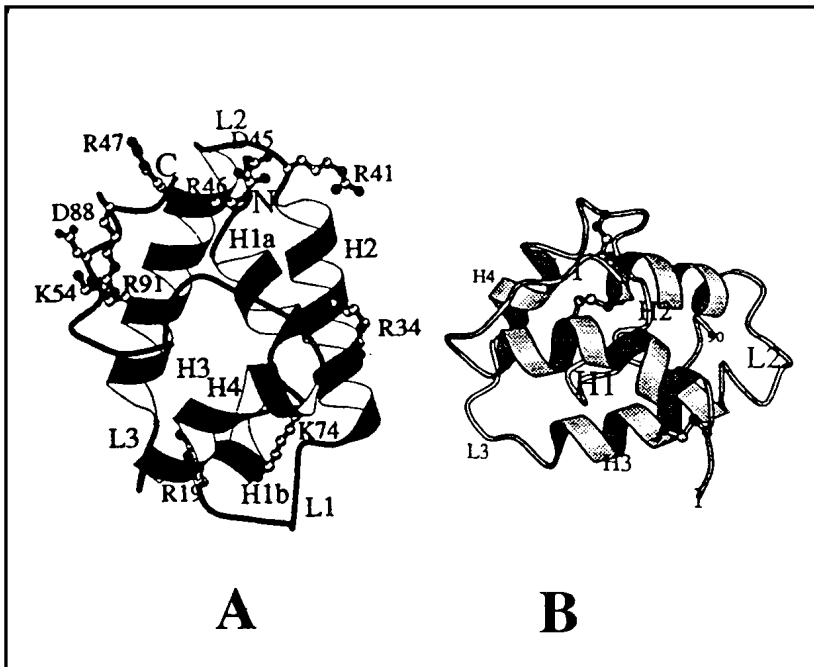
**Figure 2** Structural features of plant lipid-transfer proteins. They appear as 91–95 amino acid polypeptides comprising four  $\alpha$ -helices joined by loops. The eight conserved cysteine residues form four disulfide bridges (97, 106).

98) showed that the protein is organized mainly as helical segments (40% of total structures) connected by disulfide bridges. This helical structure plays a role in the transfer activity. A model of the three-dimensional structure of a wheat LTP was built on the basis of 1H-NMR data (36). The polypeptide backbone appears to be composed of four helices linked by flexible loops.

In order to check these models, crystallographic studies have been carried out. Although wheat (83) and rice (47) LTPs have been crystallized, the first crystal structure has been recently determined on maize LTP either complexed or not complexed with palmitic acid (97). The protein appears composed of a single compact domain with four  $\alpha$ -helices and a long C-terminal domain, which confirms that it is an all- $\alpha$ -type structure, contrary to the initial model proposed for LTPs (Figure 3).

### *Mode of Action*

How do LTPs facilitate movements of phospholipids between membranes? Several years after the detection of their activity, their mode of action is not completely understood. A shuttle mechanism has been proposed for the phosphatidylcholine-specific LTP from mammalian cells, which suggests the formation of a phospholipid-LTP complex that interacts with the membrane and exchanges its bound phospholipid with a phospholipid molecule from the membrane (123). A similar sequence of events has been suggested for plant LTPs (50, 52, 53). However, such a complex has never been isolated with a plant LTP (and also with nonspecific LTPs from mammalian cells), which suggests that the binding is too weak to allow formation of a stable complex (52). In contrast, a strong binding of acyl chains (93) or of lyso-phosphatidylcholine (22) has been noted for several plant LTPs. These binding properties are in agreement with the model suggesting that LTPs contain a hydrophobic cavity that can accept one acyl chain but not a whole phospholipid molecule

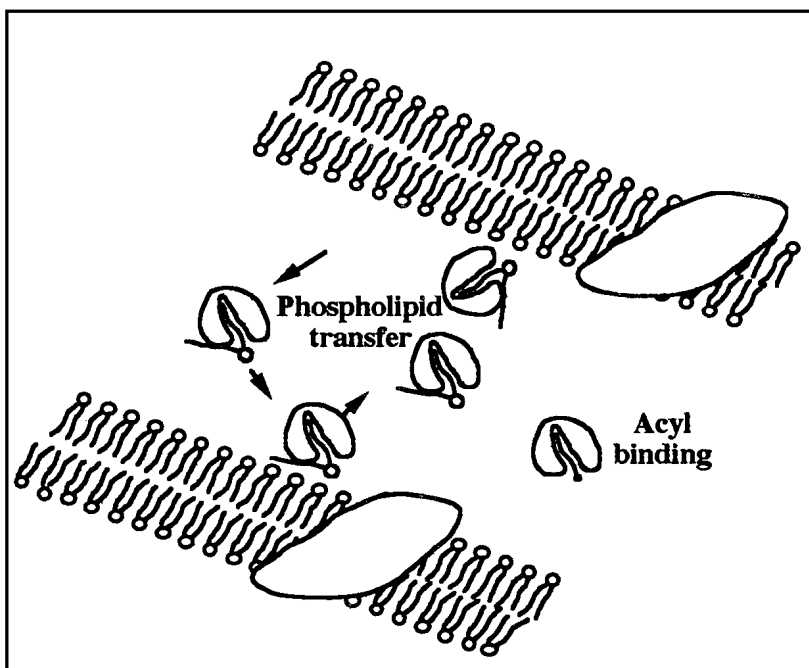


*Figure 3* Ribbon diagrams of plant lipid-transfer proteins: (A) diagram of maize LTP established from crystallographic studies by Shin et al (97); (B) diagram of wheat LTP established from NMR studies by Gincel et al (36). The eight conserved cysteines form four disulfide bridges at the same positions suggested by previous chemical studies of castor bean LTP (107). In the absence of a bound ligand, the protein has a tunnel-like hydrophobic cavity that is large enough to accommodate a long fatty acyl chain. When a complex is formed with the fatty acid, most of the acyl chain is buried inside this hydrophobic cavity (97). One end of the tunnel has a wider mouth and is close to a flexible and polar region, which might play a role in the binding and the subsequent release of lipids. The other end of the tunnel has a narrower opening and nonpolar residues. NMR studies carried out on the same protein have recently yielded similar conclusions (85). This cavity is absent from a hydrophobic protein from soybean exhibiting a four-helices structure (6). It may be interesting to study the structure of modified LTPs after expression of the corresponding genes, normal or mutated, in heterologous organisms, as has been done with wheat and castor bean (24, 66). [Reproduced with permission from *Current Biology* (A) and *European Journal of Biochemistry* (B).]

(97). The two acyl chains of the phospholipid may thus interact differently with the protein, with one more weakly bound than the other, which facilitates the extraction of the phospholipid when the LTP interacts with a membrane surface (Figure 4).

It is not known whether the protein undergoes conformational change when it interacts with lipids or with membranes. The reduction of a wheat LTP by dithiothreitol leads to a decrease of  $\alpha$ -helix proportion (from 40 to 25%).





*Figure 4* Postulated mode of action of plant lipid-transfer proteins. A reversible complex between LTP and phospholipid is formed. A hydrophobic tunnel recently suggested to be able to accommodate only one acyl chain (97) could be involved in this process. The binding and release of phospholipid molecules, facilitated by polar domains situated around the opening of the cavity, leads to their exchange with phospholipids of the membranes. It is also indicated that LTP can bind fatty acids or acyl-CoA esters.

The reduction of maize LTP was also found to inhibit the lipid transfer activity, which confirms the important role of disulfide bridges (39). The fact that the lipid transfer activity can be disturbed when ions are added or when the content of the acceptor liposomes in acidic phospholipids varies (50) indicates that electrostatic interactions between LTP and membranes are involved in the function of the protein. LTP-like proteins present in wheat, barley, pine, and *Petunia* (76, 77, 87) were found to be the substrates, with various phosphorylation sites, of  $\text{Ca}^{2+}$ -dependent protein kinases. Moreover, after labeling of *Petunia* petals with  $^{32}\text{P}$  phosphate, the major labeled protein has an apparent molecular mass close to that of LTPs (77). The physiological significance of these observations remains to be determined. It is worth noting that plants contain calcium-dependent phospholipid-binding proteins called annexins but that their biological role is still unknown (91). The biotech-

nological role of LTP in the stabilization of the beer foam has been recently considered, although the mechanism of this action is unknown (103).

### *Manipulation of Membrane Lipids*

In vitro plant LTPs are able to replace the phospholipids of an acceptor membrane with those of the donor membrane, and vice versa. As a consequence, LTPs were used as tools in order to modify the lipid composition of a membrane and to study the consequences of these changes on its properties. This modification has been done with chloroplast envelope membranes (70), leading to changes in their acyl composition. Plant LTPs have also been used to manipulate the lipid composition of erythrocyte membranes (94) and human platelets (7). The possibility that plant LTPs promote a net transfer—i.e. an increase in the amount of lipids in the acceptor membrane—has not been examined enough, in contrast with animal nsLTP (123).

## GENE EXPRESSION

### *Cloning*

The first cDNA encoding a plant LTP was isolated from a library constructed from mRNAs extracted from maize seedlings (109). The library was screened with polyclonal antibodies prepared against maize LTP. The amino acid sequence, deduced from the cDNA clone, was in agreement with the sequence determined on the purified protein with the exception of an unexpected leader sequence of 27 amino acids. All the cDNAs, further determined for plant LTPs, exhibited the same features. On the basis of the maize cDNA sequence, several homologous cDNAs or genes have been characterized or, in some cases, renamed. This is the case for the cDNA encoding barley LTP, which was published two years before the maize LTP cDNA as a cDNA clone encoding a putative amylase/protease inhibitor (75). Since then, 37 cDNA and gene sequences encoding plant LTPs have been published (Table 1). More than 30 sequences available from projects devoted to partial sequencing of anonymous cDNA clones and expressed sequence tags (EST) from *A. thaliana*, rice, castor bean, maize, tobacco, oilseed rape, *Senecio odoratus*, and *Brassica campestris* have also been deposited in databases. This makes it necessary to clarify the nomenclature of these genes, which are in some cases defined on the basis of a weak sequence homology. It can be easily predicted that the number of LTP gene sequences will considerably increase in the future, owing in part to their general occurrence in plants and to the complexity of this gene family.

The LTP gene family is indeed complex. Several cDNAs encoding LTPs have been characterized in the same plant. This is the case for maize, in which it has been proposed that a mechanism of alternative splicing might exist in

**Table 1** Genes encoding lipid-transfer proteins in higher plants

Source	Gene or cDNA	Accession number <sup>a</sup>	Protein <sup>b</sup>	Ref
<i>Arabidopsis thaliana</i>	LTP1 (cDNA)		110	111
	LTP1 (gene)	S73825	111	111
<i>Brassica napus</i> (oilseed rape)	E2(cDNA)		119(24)	32
<i>Brassica oleracea</i> (broccoli)	wax9A to wax 9D (gene)	L33904 to L33907	117 to 120	88
	<i>Daucus carota</i> (carrot)	EP2 (cDNA)	M64746	120(26) 105
<i>Gerbera hybrida</i>	gltp1 (cDNA)	Z31588	116(25)	58
<i>Gossypium hirsutum</i> (cotton)	GH3 (cDNA)		120	63
<i>Hordeum vulgare</i> (barley)	LTP1 (cDNA)		117(25)	75
	Ltp1 (gene)	X59253, X60292	117(26)	60 100
	LTP2,LTP3, LTP4 (cDNA)	X68654,X68655 X68656	115	71
	blt 4.1 (cDNA)		131	46
	gblt 4.2,gblt4.6, gbl 4.9 (gene)		115 115	122 1
	pKG2316 (cDNA)	Z37114	120	34
	pKG285	Z37115	115	34
	7kDa-LTP2 (cDNA)	X60793	102(35)	54
	TSW12 (cDNA)	X56040	114(23)	113
	<i>Nicotiana tabacum</i> (tobacco)	Ltp1 (cDNA)	D13952	
Ltp1 (gene)		X62395	118(23)	30
<i>Oryza sativa</i> (rice)	Ltp (gene)	Z23271	117	119
<i>Pachyphytum</i>	EPI 12 (cDNA)	L14770	116(23)	18
<i>Pinus taeda</i> (loblolly pine)	pPtlF (cDNA)	U10432	123	55
<i>Ricinus communis</i> (castor bean)	nsLTPC (cDNA)	D11077	106(24)	114
	nsLTPC1 (cDNA)	M86353	118(21)	120
	nsLTPC2 (cDNA)	M86354	118(23)	
<i>Sorghum vulgare</i>	Ltp1,Ltp2 (cDNA)	X71667,X71668	93	84
<i>Spinacia oleracea</i> (Spinach)	PW132 (cDNA)	M58635	118(26)	8
<i>Triticum durum</i> (wheat)	pTd4.90 (cDNA)	X63669	113(23)	23
<i>Vigna unguiculata</i> (cDNA)		X79604	99	59
<i>Zea mays</i> (maize)	9c2 (cDNA)	J04176	120(27)	109
	6B6 (cDNA)	M57249	99	2
<i>Zinnia elegans</i> (cDNA)		U19266	95	127

<sup>a</sup>Other cDNA sequences or EST have been deposited in databases but not yet published. These sequences may be accessed online at URL:<http://ncbi.nlm.nih.gov> (78).

<sup>b</sup>The deduced number of amino acids are indicated as well as the signal peptide (between parentheses) when given by the authors.

the RNA coding for these proteins (3). Several cDNAs encoding LTP-like proteins can be identified in the same plant: castor bean (115, 120), barley (34, 71, 122), and broccoli (88). In *A. thaliana*, a cDNA and the corresponding gene were characterized (111), but several other different sequences are present in the databases.

This complexity was confirmed by Southern blot analysis, which suggested the presence of several LTP genes: at least two in cotton (63), maize (109), *Gerbera hybrida* (58), and tomato (113); three in rice (119); four in broccoli (88); five in *Sorghum* (84); seven in barley (122); and as many as 14 in loblolly pine (55). However, possibly because high stringency hybridization conditions were used, only one gene was detected in carrot (105) and spinach (8). In barley, levels of sequence homology and chromosome location divide the LTP genes into two families, comprising at least seven genes distributed among three chromosomes (3, 5, and 7) (122). Four of these genomic sequences have been determined in barley (60, 100, 122), as well as one additional gene coding for a shorter (7 kDa) LTP that was called *ltp2*, which introduced some confusion with the other LTP genes and which we call 7-kDa-LTP gene (54).

Until now, 14 genomic sequences have been determined for plant LTPs: one in *A. thaliana* (111), tobacco (30), and rice (119); two in *Sorghum* (84); four in broccoli (88); and five in barley (1, 60, 100, 122). Almost all of these genes have an intron placed in the region corresponding to the C-terminus of the protein, generally two codons before the stop codon. The length of the intron varies from one gene to another: 89 bp in rice (119), 114 bp in one of the two *Sorghum* genes (84), 115 bp in *A. thaliana* (111), 133 bp in barley (60, 100), 271 bp in broccoli (88), and as high as 980 bp in tobacco (30). However, no intron has been found in one of the barley genes (*gblt 4.9*) (122) or in one of the *Sorghum* genes (*ltp2*) (84).

### *Organ-Specific and Developmental Expression*

In order to understand the *in vivo* role played by plant LTPs, it is important to determine when and where the LTP genes are expressed. This was done by using Northern blot analyses of mRNA from various tissues, by *in situ* hybridization, and more recently by following the expression for the reporter gene  $\beta$ -glucuronidase-LTP (*GUS*-LTP) constructs in transgenic plants.

The expression patterns reported for the different plant LTP genes were found to be complex and, in large part, temporally and spatially controlled. The studies, generally made by Northern blot analysis and, in one case (88), by reverse transcriptase polymerase chain reaction, led to several unexpected observations. When the vegetative organs were considered, one surprising finding was that no LTP gene transcript was detected in the roots of various plants (9, 30, 34, 63, 71, 88, 113, 120, 122). However, an LTP gene is

expressed—albeit weakly—in rice seedlings (119). Depending on the LTP genes considered, the expression was found to be active in the aerial portions of the plants (leaves, stems, shoot meristems) (9, 30, 31, 63, 71, 88, 111, 119). It was also found that, in a given plant, each LTP gene displays its own peculiar pattern of expression. In barley, one of the two seedling-specific LTP genes is more expressed in leaves than in coleoptiles of seedlings, whereas the reverse is true for the other gene (34). In castor bean, two LTP genes (*ltpC1* and *ltpC2*) have been found exclusively expressed in the cotyledons (120), where another LTP gene (*nsltpD*) is expressed in the axis (115).

Other observations are that the expression of LTP genes is higher in young tissues than in old ones, as observed in tobacco leaves (30), and that the expression of an LTP gene was found to be the highest in the upper part of the tobacco plant and to decline toward the base, which indicates that these LTP genes are expressed in a developmental gradient in this plant. LTP genes were found to be highly expressed early in development in embryo cotyledons and leaf primordia of *A. thaliana* (111) and in somatic embryos of carrot (105).

In flower development, LTP genes are expressed at early stages. Very high expression in inflorescences was found for LTP genes in various plants (29, 30, 56, 88, 105, 111). Highest transcript levels were observed in young developing inflorescences of carrot (105) and *A. thaliana* (111), in the sepals of unopened flowers of tobacco (30), in flower buds of broccoli (88), in microspores of rapeseed (32), and in corolla and carpel of *G. hybrida* (58). The latter genes are strictly specific for the inflorescences, whereas the other LTP genes can be expressed in other organs.

### *Cell-Type Specificity*

A surprising observation, provided by studies using in situ hybridization, is that the LTP gene expression is mainly restricted to defined cell layers, generally situated peripherally. In maize seedlings, for example, the LTP gene transcripts accumulate mainly in the epidermis of the coleoptiles as well as in the leaf veins (104). In carrot (105), the LTP gene is expressed in protoderm cells (which give rise to the epidermis) of somatic and zygotic embryos and in the shoot apical meristem. This cell type-specific expression was also observed in the shoot apical meristem of tobacco, particularly in the epidermis (105). The same pattern was observed in *A. thaliana* in which LTP gene transcripts were detected first in the protoderm cells of the embryo cotyledons, then in the leaf primordia of young seedlings, and at a later stage of development in the epidermal cells of meristem and leaves (111). In the succulent plant, *Pachyphytum*, a cDNA-encoding LTP-like protein has been isolated among epidermis-specific genes (18). In cotton, LTP genes specific for fibers, which are highly elongated trichomes that grow from epidermal cells of ovules,

have been characterized (63). Studies by tissue-print hybridization carried out in barley seedlings also led to the conclusion that the LTP genes are expressed in the epidermis of both leaf and coleoptile (34).

An interesting model in cereal seeds is the aleurone layer, composed of specialized cells rich in lipid bodies and aleurone grains, surrounding the starchy endosperm (48). In barley, the transcripts of *ltp1* (100) or of the 7-kDa LTP genes were found to be specifically accumulated in the aleurone layer. This observation is consistent with studies by Northern blot analysis of RNAs extracted from the aleurone layer of barley seeds (75).

Another example of cell specificity of gene LTP expression is given by floral organs. Koltunow et al (56) found that an LTP gene, with a yet unpublished sequence, was specifically expressed in the tapetum layer of the tobacco anther. In the same plant, transcripts for a gene encoding an LTP showed a restricted distribution to the outermost cell layer in the floral apical meristem at the stage of transition to floral development (29). Another LTP gene, *E2*, was also found to be exclusively expressed in the tapetal cells of *B. napus* anthers (32). LTP gene expression was also detected in the epidermal cells of several floral organs, including *A. thaliana* (111) and *G. hybrida* (58).

It was of interest to extend these observations by the study of transgenic plants containing a fusion of a promoter region of LTP genes to the reporter gene *GUS*. This was carried out in barley using *ltp1* or the 7-kDa LTP genes (54, 100), in *A. thaliana* (111) using the *LTPI* gene, and in *B. oleracea* using the wax 9D gene (88). The data obtained by this approach are remarkably coherent, in some aspects, with the in situ hybridization observations. *GUS* activity was detected in the aleurone layer of barley seeds in the case of two genes (54, 100). When the 7-kDa LTP gene is considered, it was found that the corresponding promoter is able to direct aleurone-specific expression in immature barley grains or in transgenic rice (54). The expression of broccoli LTP promoter-*GUS* constructs was detected in the epidermal cells of various organs of transgenic tobacco (88). In the case of *A. thaliana* (111), the expression of the LTP-*GUS* activity was dependent on the stage of development. In young seedlings, *GUS* activity was detected in the cotyledons and in the hypocotyl. Then, as seedlings matured, *GUS* activity was observed in the shoot meristem, the vascular tissue, in the leaf primordia, and then in the tips of maturing leaves. In adult plants, high *GUS* staining was detected in leaf and stem epidermal cells, guard cells, and flowers. Although no activity was detected in flower buds, high activity was observed in the nectaries, in the stigma, and in pollen grains of opened flowers.

Some of these observations made by the *GUS* approach are not coherent with the in situ hybridization data, probably because of some artifactual expression of this reporter gene: In *A. thaliana*, no clear epidermis specificity of the *GUS* expression was observed in young seedlings, although in some cases

(guard cells, floral nectaries, pollen grains, lateral roots, and stipules) the *GUS* activity was not confirmed by in situ hybridization (111).

### *Localization of LTPs*

LTPs have been purified as soluble proteins after precipitation of cell membranes (50, 51, 124), although they were found to be partly bound to membranes (mitochondria, endoplasmic reticulum) (25). Several unexpected discoveries have changed this conclusion. One finding is that all known plant LTPs are synthesized as precursors with N-terminal extensions having the sequence characteristics of signal peptides. The length of this signal peptide varies from 21 to 27, depending on the LTP gene (Table 1). The longest leader sequence (35 amino acids) was postulated for LTPs from barley (54). As shown in the case of LTP genes from spinach (9) and barley (64, 65) but not in castor bean (126), this signal peptide is able to direct the cotranslational insertion of the polypeptide into the lumen of the endoplasmic reticulum in vitro. It has thus been suggested that LTPs participate in the lipid movements within the lumen of the endoplasmic reticulum (65). This hypothesis is interesting in reason of the presence of plasmodesmata, establishing communications implying membrane continuity and lipid movements between adjacent cells (38, 62). However, because no endoplasmic reticulum retention signal (KDEL) is present at the carboxyterminus of LTPs, they are expected to enter the secretory pathway. Several observations have indeed confirmed that LTPs are secreted proteins: In barley, an LTP first identified as a putative amylase protease inhibitor was found to be secreted into aleurone cell culture medium (75); in carrot, an LTP was detected among extracellular proteins in embryogenic cell cultures (105). A similar observation was made in grapevine because several isoforms of LTP have been purified from the extracellular medium of somatic embryo cultures (20). The extracellular location is consistent with the fact that an LTP from maize is synthesized on membrane-bound polysomes (117).

In addition, immunocytochemical studies carried out at the ultrastructural level using a polyclonal antibody against a fusion protein corresponding to the LTP1 gene of *A. thaliana* clearly indicated that in this plant the LTP was localized to the cell wall (112). In castor bean, a partial localization of LTP in the cell wall was also found (114). Immunocytochemistry at the light microscopy level also indicated some signals corresponding to LTP-immunogold complexes in the cell wall of epidermal cells of maize coleoptiles (104). In broccoli leaves, the wax 9 protein was present mainly in the cell wall of the epidermis and mesophyll tissues as well as in the phloem. The LTP is so abundant in young leaves that it constitutes more than 90% of the protein extracted from these leaves by a brief solvent wash (89). A similar preferential localization of LTP to the cell walls was found in barley, as indicated by

immunological staining of tissue prints (71). This distribution of LTP was confirmed by the fact that these proteins could be extracted by a short dip of intact barley leaves in buffer (71). In conjunction with these data, it is worth noting that a high expression of LTP gene is observed in cotton fibers, particularly in the phase of primary cell wall synthesis (63). Similarly, an LTP gene is specifically expressed during the differentiation of tracheary elements of *Zinnia elegans* in relation to cell wall thickening (127).

It is easy to conclude, from all these observations, that plant LTPs are extracellular proteins. In agreement with this conclusion, no LTP was detected in the stroma of chloroplasts (95). However, it cannot be excluded that some LTP isoforms are addressed to cytosolic compartments. For example, on the basis of immunocytochemical evidence, LTP from castor bean appears to be partly located within the glyoxysomes (114, 126). However, it remains to be explained whether an LTP isoform is truly located in the cytosol and how it is addressed to this compartment in spite of the presence of a signal peptide.

## PROPOSED BIOLOGICAL ROLES

On the basis of their *in vitro* properties of transferring lipids and binding acyl chains, the suggestion that LTPs could be involved in many aspects of cell function where movement of lipids is thought to be important, such as membrane biogenesis and turnover, was a logical one. This hypothesis has been presented in several reviews (2, 50, 52, 124). However, the fact that LTPs are located extracellularly made this hypothesis inconsistent. On the basis of this external location as well as on novel properties discovered in recent years, other roles have been suggested.

### *Cutin Formation and Embryogenesis*

The hypothesis of the group of de Vries (105), which suggests that LTPs are involved in the secretion or deposition of extracellular lipophilic material, including cutin, has been argued convincingly in recent years. According to this theory, LTPs carry acyl monomers necessary for the biosynthesis of cutin. Different facts support this. 1. LTPs are mainly located in the cell wall and are secreted. 2. LTP gene expression and LTP gene products accumulation was detected in high levels in peripheral cell layers, including epidermis. 3. LTPs, particularly in young leaves where cutin deposition is active, are mainly concentrated in the surface wax (88). 4. LTPs are able to bind acyl chains (42, 69).

The "cutin theory" thus seems highly convincing. However, it remains to be validated, either with an antisense approach or by studying eceriferum mutants in *A. thaliana* affected in their epicuticular wax (49). In relation to



this theory, LTPs were also supposed to participate in the deposition of lipophilic material in floral organs: corolla (58), stigma and nectaries (111), and anther (tapetum and pollen), where sporopollenin biosynthesis occurs (32).

The finding that LTP is secreted into the medium of embryogenic cell cultures suggested a new role for LTP in somatic embryogenesis (105). LTP could be involved in the early steps of embryogenesis by participating in the formation of a protecting layer around the young embryo (zygotic or somatic). The high LTP gene expression in young embryos (105, 111) is in agreement with this hypothesis.

### *Defense Reactions Against Pathogens*

The unexpected antibiotic properties of LTP were discovered by screening plant proteins for their ability to inhibit the growth of fungal and bacterial pathogens (72, 110).

Several LTP-like proteins purified from barley leaves or an LTP isolated from maize leaves were shown to inhibit the growth of a bacterial pathogen and a fungus (72). This was confirmed by the isolation of LTP-like proteins from cell-wall preparations from the leaves of *A. thaliana* or spinach (96). A synergistic effect against the fungus occurred when the LTPs were combined with thionins (72). The cloning of three barley antipathogen proteins allowed the study of the induction of the corresponding genes. All LTP mRNA levels were significantly increased when the barley leaves were inoculated with a fungus (71). A similar antifungal activity has been described for a 9-kDa basic protein purified from radish seeds and exhibiting a high sequence homology with LTP in its N-terminal end (110). This protein is able to inhibit in vitro the growth of several fungi. In broccoli, the wax 9 protein was found to inhibit the growth of a fungus (89).

It can be concluded that the antifungal activity of LTPs varies between pathogens and that the pattern of expression of LTP genes in response to infection of barley plants by pathogens is rather complex (33). The idea of a defense-protein shield, suggested by Garcia-Olmedo et al (33), is supported by the fact that LTPs were found, at least for some isoforms, to be accumulated at the tissue surface at much higher concentrations than those required to inhibit many pathogens in vitro. It is interesting to note that an LTP-like protein is secreted into the medium of rice suspension cultures in response to a treatment by salicylic acid known as an elicitor to induce pathogen defense processes (67).

However, we do not yet know how LTPs inhibit the growth of the pathogens. Because of their high isoelectric point, LTPs may act as membrane permeabilizing agents. The fact that LTPs are induced could be due to the fact that cutin biosynthesis is generally stimulated by pathogen infection.

## *Symbiosis*

Another relationship between bacteria and plants is the nodule initiation in legume roots following interaction with symbiotic bacteria such as *Rhizobium*. An early stage of the infection occurs in the root hairs to which *Rhizobium* binds and results in elongation and curling of the root hair that facilitates the penetration of the bacteria. A cDNA, coding for an LTP-like protein, has been isolated from a cDNA library constructed from RNA of *Vigna unguiculata* roots one and four days after inoculation with *Rhizobium* (59). The mRNA levels increased in root hairs after inoculation with *Rhizobium*. Because LTP-like transcripts are absent from differentiated nodules, it was concluded that the LTP gene was transiently expressed during nodule development.

## *Adaptation of Plants to Various Environmental Conditions*

An important domain of research for plant biologists is the study of the ability of plants to modify their metabolism and development in response to changes in the environment, including changes involving temperature, drought, and salt stress. It is of interest to characterize genes involved in acclimation to low or high temperatures or to shortage of water. The search for stress-induced genes has led to the characterization of genes encoding LTP-like proteins. One example is the low-temperature response studied in barley (46, 122). Several genes induced by cold treatment code for LTP-like proteins and are also induced by a treatment of abscisic acid (ABA), which is common with genes induced by low temperature or drought (46). Although all these genes are induced by cold, there are varietal differences in the response of the barley LTP gene family to low temperature. For example, one gene (*blt 4.1*) was upregulated by low temperature in the winter cultivars but not in a spring cultivar (122). In other barley varieties, no induction was observed for the expression of three LTP genes in barley because of cold or other factors (drought, salicylate), and only a moderate increase was observed because of NaCl or ABA.

The isolation of several genomic clones in barley has led to a study of the regulatory elements. A putative ABA-responsive (ABRE) element (GTACGTGG) and a low-temperature responsive element (ACACGTCA) were found in the barley LTP genes (122). Another LTP gene was found to be regulated by auxin (naphthaleneacetic acid) during tracheary element formation in *Z. elegans* (127).

Other LTP genes respond to salt stress or drought stress. In tomato, a gene encoding an LTP-like protein is expressed, specifically in stems, only when plants are treated with NaCl, mannitol, ABA, or high temperature (46). Another gene, only partially homologous to LTP, was also induced by drought in barley

(86). Other genes were found to be expressed in the roots and stems of drought-stressed barley plants (122).

The response of LTP-genes to developmental and environmental signals is thus complex, and additional information is needed to determine the involvement of these proteins in the adaptation of plants to several stresses. However, all these conditions—drought, cold, and salt stresses—are related to desiccation or water stress. If LTPs are involved in cutin deposition, the induction of LTP genes by conditions leading to desiccation seems logical. The promoter regions of the *ltp1* gene from *A. thaliana* contain sequences homologous to putative regulatory elements of genes in the phenylpropanoid biosynthetic pathway and sequence elements that have been found in the promoters of stress-induced genes (111).

## THE SEARCH FOR NOVEL LIPID-TRANSFER PROTEINS

The search for novel LTPs located in the cytoplasm is of major interest. Two categories of proteins have recently been considered.

The first category is acyl-CoA-binding proteins (ACBP). A cDNA encoding this protein was isolated by PCR, using the consensus sequences of ACBP, from a library constructed from mRNAs from oilseed rape (44). This cDNA encodes a protein 92 amino acids in length that is conserved when compared with other ACBP. In spite of its similar size, no homology was found with plant LTPs, including the absence of cysteine residues as well as the lack of signal peptide that indicates that the protein is cytosolic. To confirm this promising finding, functional tests on the product of this ACBP gene are needed. In addition to this gene, several expressed sequence tags, homologous to ACBP, have been detected in *A. thaliana* (45, 78; RS Pakovsky & JB Ohlrogge, unpublished manuscript).

Soluble LTPs worth studying are the specific LTPs, such as the PI-TP transferring preferentially PI and characterized in mammalian cells and in yeasts (37, 123). A growing interest in these proteins started with the discovery that a cytosolic PI-TP, a product of an *sec14* gene in *Saccharomyces cerevisiae*, is essential both for protein transport from a late yeast Golgi compartment and for yeast viability (5, 19). It was then proposed that the SEC14 protein might act as a sensor of Golgi membrane phospholipid composition, which allowed the regulation of PC biosynthesis needed for keeping a PC/PI ratio compatible with the secretory function of Golgi membranes (5, 19, 61, 68, 99). However, in another yeast, *Yarrowia lipolytica*, SEC14P was demonstrated to allow the dimorphic transition from the yeast to the mycelial form that characterizes this species (61). It was also recently suggested that, in mammalian cells, the inositol lipid kinases prefer as substrates the PI molecules bound to PI-TP (21).

It is thus of interest to search for plant genes homologous to *sec14*. This

was recently initiated by following a complementation approach of yeast *sec14* mutant from cDNA from *A. thaliana*, which led to the isolation of cDNA clones homologous to the yeast gene and apparently encoding cytosolic protein, because no signal peptide is present. The properties of the products of these novel genes remain to be characterized (N Jouannic, M Lepetit & V Arondel, unpublished manuscript; R Dewey, unpublished manuscript). Other SEC14 homologous genes have been deposited in databases (78).

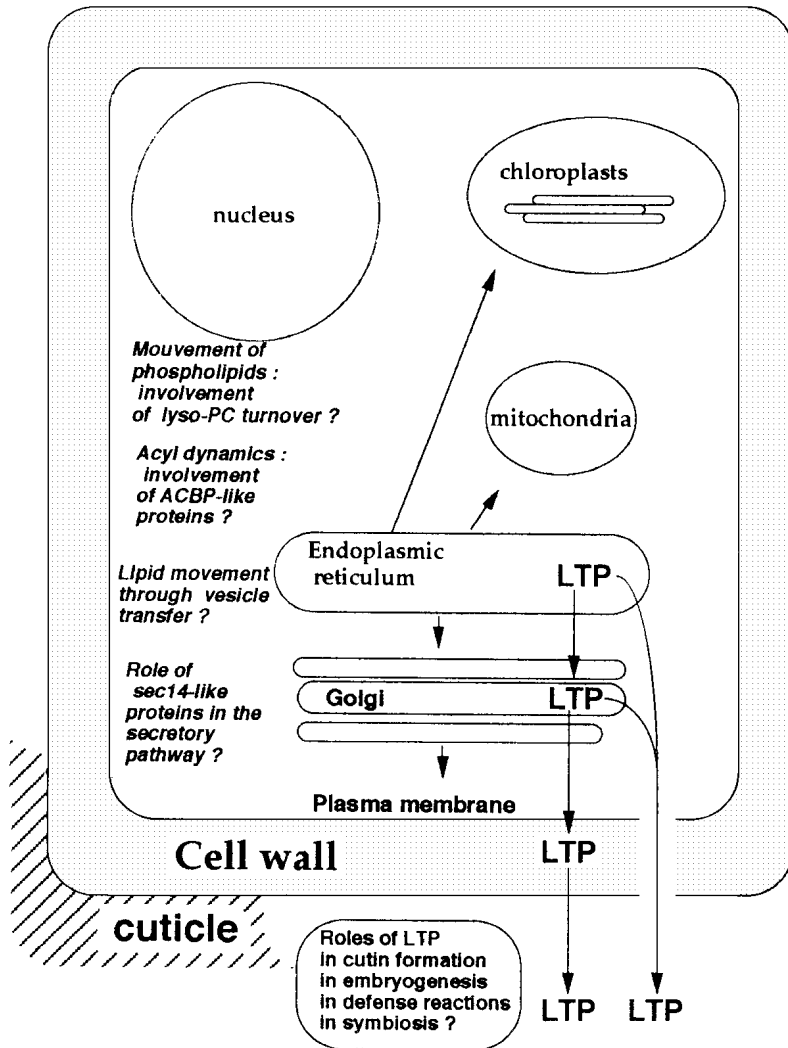
Also a phosphatidylinositol-3-kinase cDNA was identified in *A. thaliana* (121). The protein presents homology in its N-terminal part with a calcium-dependent phospholipid-binding domain, which suggests its involvement in vesicle transport of PI.

## CONCLUDING REMARKS

It is clear that the main question concerning plant LTPs is in regard to their biological role. Although the plant LTPs, by reason of their *in vitro* functional properties, are ideal candidates to play a major role in intracellular lipid dynamics, their external location and their secretion make such a role unlikely (Figure 5). The term "lipid transfer protein" is still valid and, moreover, confirmed by structural studies that showed that these proteins are efficiently built for binding and releasing acyl chains. The problem remaining is to elucidate where lipids are transported *in vivo* by LTPs. The location of all LTP isoforms as well as their functional properties should be studied after their purification or expression of their corresponding cDNAs in heterologous organisms. It would also be worth studying all LTP isoforms of one plant in order to have a complete description of one LTP gene family by choosing, for example, *A. thaliana* or rice as models. In addition, it is worth studying the functional properties of the various isoforms of LTP, including the determination of the structure-function relationship and the elucidation of their mode of action.

In order to determine the biological roles of plant LTPs, several approaches are possible. The antisense strategy might provide a clear answer. The Somerville group has obtained transgenic *A. thaliana* plants in which the amount of the *LTP1* product has been strongly reduced (Thomas & Somerville, unpublished manuscript) (14) by expression of an antisense construct. This decrease in the *LTP1* content has not provoked changes in the morphology of plants—except that the transgenic plants are very late flowering—and no defect in cutin and wax composition. However, the fact that several LTP genes are present in *A. thaliana* makes the antisense approach rather complex.

Another strategy would be to profit from the enormous progress in the genetics of *A. thaliana* (57) by mapping LTP genes. Also, the study of the



*Figure 5* Proposed biological roles of lipid-transfer proteins. LTPs are synthesized as precursor proteins and entered in the secretory pathway after import into the endoplasmic reticulum. They were found to be located in the cell wall (89, 105, 111). They were proposed to be involved in several biological roles, mainly based on this external location, including the formation of protective barriers such as the cuticle. LTP might transport the acyl monomers needed for the synthesis of cutin (42). All things considered, the participation of LTPs in the intracellular lipid flow seems unlikely. Other proteins could be involved in this flow: ACBP in the acyl dynamics (44) and SEC14-like proteins in establishing a PI/PC ratio in Golgi membranes, which allows an efficient secretory process (68). Other pathways could be operative either through lyso-PC turnover or through vesicle transfer (10, 74).

genes involved in mutations in plants might reveal that some of these genes are encoding LTPs.

It can be easily predicted that in the future our knowledge of plant LTPs will increase considerably. I hope that the novel observations that will be made will help to elucidate the role of these enigmatic proteins.

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