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Lipid transfer in plants

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Summary. Plant cells contain cytosolic proteins, called lipid transfer proteins (LTP), which are able to facilitate in vitro intermembrane transfer of phospholipids. Proteins of this kind from three plants, purified to homogeneity, have several properties in common: molecular mass around 9 kDa, high isoelectric point, lack of specificity for phospholipids, and binding ability for fatty acids. The comparison of their amino acid sequences revealed striking homologies and conserved domains which are probably involved in their function as LTPs. These proteins could play a major role in membrane biogenesis by conveying phospholipids from their site of biosynthesis to membranes unable to form these lipids. Immunochemical methods were used to establish an in vivo correlation between membrane biogenesis and the level of LTP or the amount of LTP synthesized in vitro from mRNAs. The recent isolation of a full-length cDNA allows novel approaches to studying the participation of LTPs in the biogenesis of plant cell membranes.
Key words. Lipids; phospholipids; galactolipids; fatty acids; transfer; binding; membranes; mitochondria; chloroplasts; biogenesis.

Introduction

Lipid dynamics play a major role in membrane biogenesis. The need for intracellular lipid movements, demonstrated for several living cells, is based on the observation that the enzymes involved in the biosynthesis of some lipids are localized in specific membranes and compartments, although the lipids synthesized are distributed among all the membranes of the cells. This is also true for plant cells which contain, in addition to organelles similar to those of other eucaryotic cells, some characteristic ones, like plastids. Studies on plant lipid metabolism [18,19] have concluded that such intracellular translocation of lipids must occur. For example, phosphatidylcholine (PC), which is the major phospholipid of the envelope membranes of chloroplasts as well as of mitochondrial membranes, is not synthesized by these membranes; in contrast, the endoplasmic reticulum contains all the enzymes needed for the de novo synthesis of this lipid [18,19]. The same observation has been made for other phospholipids, like phosphatidylethanolamine (PE) or phosphatidylinositol (PI). Moreover, movements of lipids within organelles are needed since, for example, galactolipids which accumulate in thylacoid membranes of chloroplasts are synthesized in the envelope; an intrachloroplastidial transfer of these lipids from the envelope to the thylacoids therefore seems to be necessary [12] (fig. 1).

In addition to this need for membrane biogenesis, intracellular movements of lipids are strongly suggested by the cooperative pathway assumed to occur between the endoplasmic reticulum and the plastid for the biosynthesis of galactolipids containing polyunsaturated fatty acids. This pathway involves an exportation of acyl chains (mainly in the form of oleyl-CoA), synthesized within the plastid, towards the endoplasmic reticulum. The desaturation reaction which takes place in this membrane leads to the formation of diunsaturated acyl chains which are transferred to the plastid as phosphatidylcholine molecules; the final desaturation step, which occurs within the plastid, allows the synthesis of galactolipids containing triunsaturated fatty acids. In this rather complicated pathway, the movements of lipids between the endoplasmic reticulum and the plastid play a major role (fig. 1).

What are the mechanisms of these intracellular fluxes of lipids? A first possibility is a spontaneous movement of lipids as free molecules. However, the weak solubility of phospholipids in the aqueous phase leads to the movement of these molecules being very slow [21,50]. A second mechanism is the transfer of membrane vesicles from the endoplasmic reticulum to other membranes through the Golgi membranes, according to the membrane flux theory [28]. An argument in favor of this hypothesis in plant

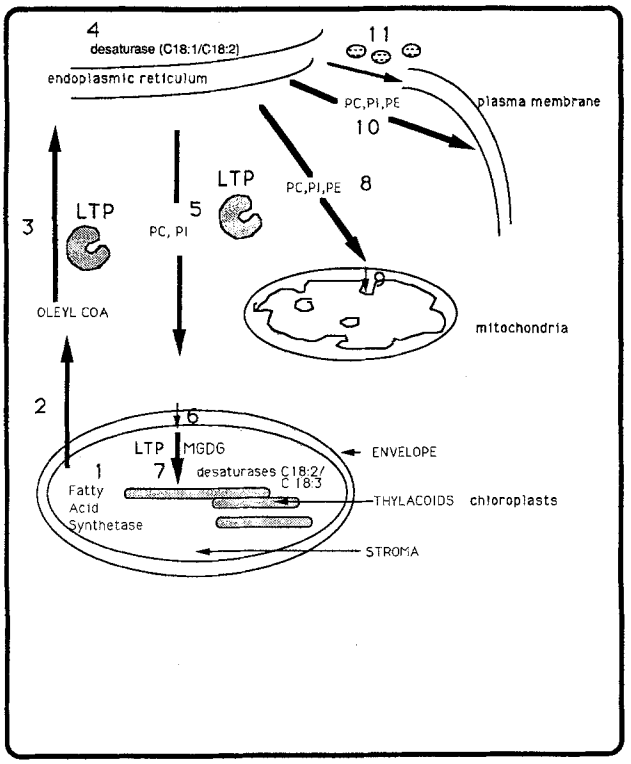

Figure 1. Involvement of lipid transfer proteins (LTP) in membrane biogenesis in plant cells. LTPs are suggested to participate in the biogenesis of the various membranes by transferring various phospholipids (phosphatidylcholine, PC; phosphatidylinositol, PI; phosphatidylethanolamine, PE) towards mitochondria (8), plasma membrane (10), chloroplasts (5); in this case, a transfer of PE is unlikely since PE is absent from chloroplast membranes. Other membranes (peroxisomes, proplastids) can be involved in these transfers. Intermembrane transfers occur between outer and inner membranes of chloroplasts (6) or mitochondria (9). LTPs can also participate in the cooperative pathway between the endoplasmic reticulum and chloroplasts for the synthesis of triunsaturated fatty acids. According to this pathway fatty acids, synthesized by the fatty acid synthetase (1) located within the stroma of chloroplasts, are desaturated and exported (2) from chloroplasts as oleyl-CoA molecules. LTPs, acting as fatty-acid binding proteins, can regulate the intracellular level of oleyl-CoA (3). Oleyl-CoA (C18:1) is esterified to PC and then desaturated to linoleic acid (C18:2) (4). The transfer of PC from endoplasmic reticulum to chloroplasts, where a final desaturation step occurs (5), leads to the biosynthesis of galactolipids (monogalactosyldiacylglycerol, MGDG) containing triunsaturated fatty acids (C18:3). An intrachloroplastidial transfer of this galactolipid (7) from the envelope to thylacoids can be mediated by LTP located within stroma.

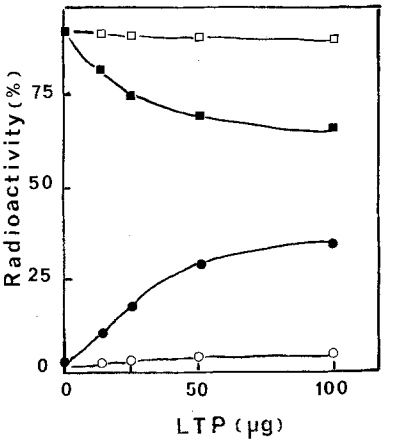

Figure 2. Transfer of phosphatidylcholine from liposomes to chloroplast envelope vesicles in the presence of lipid transfer protein from spinach leaves. Liposomes made from ^{3}H phosphatidylcholine and ^{14}C-cholesteryl-oleate were incubated at 30 °C for 30 min with envelope vesicles isolated from spinach leaf chloroplasts (corresponding to 0.37 mg of protein) in the presence of increasing amounts of spinach leaf LTP. The ^{3}H radioactivity in the envelope pellet collected by centrifugation (●) and in the supernatant (■), and the ^{14}C radioactivity in the envelope pellet (○) and in the supernatant (□), are expressed as percent of the initial radioactivity of liposomes. This experiment indicates that spinach LTP facilitates a transfer of ^{3}H-phosphatidylcholine from liposomes to the chloroplast envelope. Data are from Miquel et al.[26].

cells was given by the demonstration of an in vivo intracellular movement of very long chain fatty acids through various membrane compartments, namely plasma membrane, Golgi and endoplasmic reticulum[27]. A third mechanism – which will be mainly discussed in this review – implies the participation of water-soluble transport proteins, able to facilitate the movement of phospholipid molecules between membranes. The discovery of proteins of this category in plant cells, designed as 'lipid transfer proteins' (LTP) has provided a strong argument in favor of this protein-mediated transport of lipids.

Assay and purification of lipid transfer proteins

Assay

The lipid transfer assay is different from the classical enzymatic assays since it is based on the use of two categories of membranes, one containing a radioactive lipid. These two membranes are incubated with the active transfer protein and then separated. The appearance and increase of radioactive lipid in the initially unlabelled membrane indicates the rate of the transfer of this lipid. The two membranes widely used are liposomes and mitochondria. In a typical assay liposomes, prepared by ultrasonication of a mixture of lipids, are incubated with non-radioactive mitochondria. Liposomes usually contain, in addtition to non-radioactive lipids, ^{3}H-phosphatidylcholine – the lipid to be transferred – and ^{14}C-cholesteryloleate, which is a non transferrable lipid. The determination of the $^{3}H/^{14}C$ ratio in the mitochondria collected after incubation indicates the rate of transfer of ^{3}H-phosphatidylcholine from liposomes to mitochondria. The ^{14}C label recovered determines the level of cross-contamination (generally low) of mitochondria by liposomes. In this assay, the lipid composition of the 'donor' membranes – liposomes – can be easily modified by replacing labeled phosphatidylcholine by other phospholipids or galactolipids whereas the 'receptor' membranes – mitochondria – can be replaced by other plant membranes (chloroplasts[26], plasma membranes, etc.) (fig. 2). Other assays, which are less frequently used, are based on the incubation of multilamellar and unilamellar vesicles, separable by centrifugation, or liposomes containing glycolipids, separable by concanavalin A precipitation. As-

says based on the incubation of liposomes containing spin-labelled lipids were used to follow, by ESR spectroscopy, the movement of various phospholipids or of monogalactosyldiacylglycerol, the major galactolipid of chloroplast thylacoids[31].

Purification of lipid transfer proteins

After the demonstration, in 1975, that the cytosolic extracts from potato tubers contained proteins facilitating the movement of phospholipids between membranes[20], the first purifications to homogeneity of lipid transfer proteins from plants were achieved from maize seedlings[9,10], spinach leaves[24,25] and castor bean seedlings[49]. The purification steps performed on proteins precipitated from cytosolic extracts involved gel filtration and anion-exchange chromatography and, finally, separation on a carboxymethylsepharose column. The elution of this cation exchanger by a phosphate gradient gave several fractions corresponding to isoforms of basic LTPs. The final purification of the major isoforms was achieved either by gel filtration (maize[10], spinach[24]) or by using hydroxyapatite (castor bean)[49]. With these purification protocols, pure LTPs, exhibiting a single band corresponding to a molecular mass of 9 kDa after SDS-electrophoresis, were obtained in a good yield. As an example, 60 mg of pure LTP was prepared from 2 kg of maize seeds. By following similar methods, fractions enriched in LTP have been isolated from various seeds; sunflower, barley and wheat (Arondel, Guerbette and Kader, unpublished). Interestingly, a major 9 kDa band was found with all these proteins. This is also the case for a barley protein, initially described as a putative amylase/protease inhibitor, which was recently identified as lipid transfer protein[6].

Biochemical properties

The availability of considerable amounts of pure LTPs from the three plants studied so far – maize, spinach and castor bean – has allowed studies of their properties and, recently, the determination of their complete amino acid sequences.

Molecular mass. The molecular masses determined from their sequences are remarkably similar for the three proteins. They vary from 8832 to 9313 Da, which is in good agreement with the apparent molecular mass of 9 kDa found in SDS-gel electrophoresis (table). These values are close to those established for non-specific lipid transfer proteins (nsL-TP) from animal cells (11.2 kDa for rat hepatoma; 12.5 kDa for nsL-TP from rat liver, identical to sterol carrier protein 2; 14.5 kDa for bovine liver nsL-TP)[50]. Higher values have been determined for proteins specific for PC (24.68 kDa for PC-TP from bovine liver) or PI (32.3 kDa for bovine liver PI-TP)[50]. Higher molecular masses have been found for LTPs from yeasts (35 kDa[8] and 33.4[5] kDa).

Biochemical properties of lipid transfer proteins from higher plants

Source	Protein	Molecular mass (Da)	Isoelectric point	Specificity	Reference
Maize	LTP	9054	8.8 (9.0[a])	PC, PE, PI	9, 10
Spinach	LTP	8832	9.0 (9.4[a])	PC, PE, PI, PG	24, 43
Castor bean	LTP	9313	10.5 (8.6[a])	PC, PE, PI, PG	49, 43
Oat	FABP	8700	8.4 (4.8[b])	Oleic acid Linoleic acid Oleyl CoA	36

[a] Calculated from the amino acid sequence; [b] lipidated protein; PG = phosphatidylglycerol.

Isoelectric point. All the three plant LTPs are basic, as indicated by their binding properties to cationic columns at neutral pH. The values of isoelectric points are 8.8 and 9 for maize and spinach proteins, respectively[10,24], while an isoelectric point of 10.5 was found for the castor bean LTP[49]. These values are consistent with the data calculated from the amino acid sequence. In addition to these basic LTPs, acidic transfer proteins have also been detected in castor bean seedlings and in spinach leaf[22,24]. The other LTPs isolated from animal cells or microorganisms differ in their isoelectric points, which range from 4.6 for yeast[8] to 9.75 for bovine liver nsL-TP[50].

Specificity. A common property of the plant LTPs is their broad specificity for phospholipids since PC, PI and to a lesser extent PE are all transferred. In addition, spinach and castor bean proteins transfer phosphatidylglycerol. It is interesting to note that the spinach protein is also able to transfer monogalactosyldiacylglycerol[31]. Phospholipid transfer proteins from animal tissues are classified into three categories according to their specificity; specific for PC (PC-TP) or PI (PI-TP), or non-specific, able to transfer a wide variety of phospholipids as well as sterols. Interestingly, PI is the lipid preferentially transferred by LTPs from yeasts[8,9] and fungi[17]. PG, PC and PE are transferred by a LTP from photosynthetic bacteria[40].

Fatty acid binding properties of LTPs

A surprising finding concerning spinach LTP was its ability to bind oleic and linoleic acids as well as oleyl-CoA[37]. This confers on this protein the bifunctional character of transferring lipids and binding fatty acids. The same is true for maize protein (Douady and Kader, unpublished). These observations have to be correlated with the isolation of a protein able to bind fatty acids from oat seedlings[36]. This protein has a molecular mass of 8.7 kDa, close to that of LTPs, as well as a basic character (an isoelectric point of 8.4; this is, however, reduced to 4.8 when the protein is lipidated). From all these observations, it can be suggested that in a plant tissue the same protein is involved in transferring phospholipids and binding fatty acids. In animal cells, these two pro-

cesses are associated with two different categories of proteins, phospholipid transfer proteins [50] and fatty-acid binding proteins (FABPs) [1,14]. However, in rat liver cells the properties of transferring phospholipids and sterols are shared by the same protein, called nSL-TP [3] or sterol-carrier protein 2 [32].

Structure and mode of action

The comparison of the amino acid composition of the LTPs purified from three plants (maize, spinach and castor bean) indicated several common features, like the total number of residues (varying from 91 to 93) or the number of cysteine residues (6–8, with two disulfide bridges in the case of castor bean protein) [4,41–43]. By aligning the amino acid sequences domains of high homologies were found, especially in the central core of the proteins, which is a hydrophilic region surrounded by two hydrophobic domains. The N-terminal end, which could be involved in LTP-membrane interactions, is hydrophobic. It is to be noted that the positions of cysteinyl residues and of charged amino acids are remarkably conserved. In addition to these three sequences, the sequence established for a barley protein which was identified as an LTP [6] presents a 50% homology with the maize protein [2] (fig. 3).

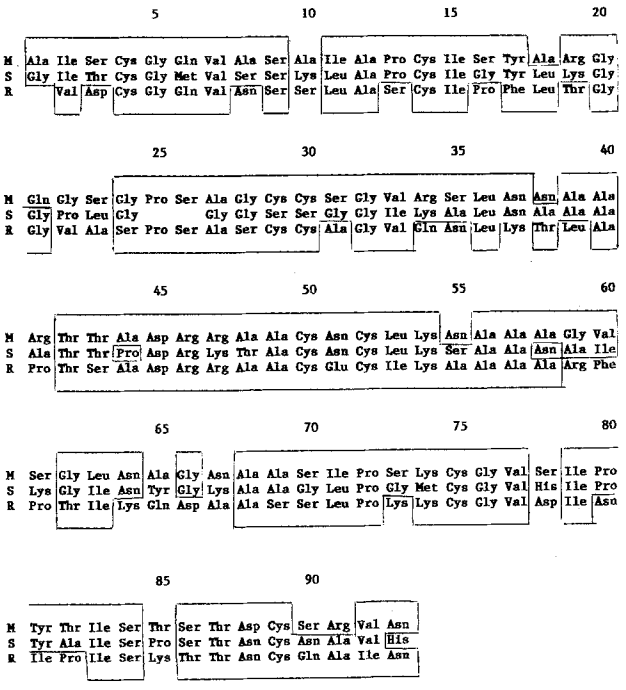

Figure 3. Comparison of the amino acid sequences of plant LTPs. The amino acid sequences of maize seed (M) [43], spinach leaf (S) [25] and castor bean seed (R) [41] are presented. Data are from Tchang et al. [43]. It should be noted that there is a very low number of aromatic amino acids (no tryptophan, no phenylalanine, and only two tyrosines), and histidine, methionine and glutamic acid are absent. A high homology of the three sequences is observed, as well as the presence of positively charged residues in the central part of the molecule and the conserved positions of cysteinyl residues.

Knowledge about the structure of LTPs has shed some light on their mode of action. The ability of plant LTPs to transfer phospholipids suggests that, as has been shown for phospholipid transfer proteins from animal cells, a reversible complex is formed between the LTP and a phospholipid molecule. This complex can react with membranes and exchanges its phospholipid with those of the membranes. This leads to a transfer of phospholipids between membranes which gives an overall exchange process, since bidirectional transfer occurs. This scheme has been clearly described for bovine liver PC-TP [50]. In the case of LON-specific transfer proteins from animal cells, and of plant LTPs, such complexes between the transfer protein and the transported lipid have not been clearly demonstrated by studies on the binding of radiolabelled phospholipids. However, recent experiments established that rat liver nsL-TP can bind a fluorescent lipid containing a short-chain fatty acid [30]. This successful demonstration, which has been confirmed with maize LTP (Guerbette and Kader, unpublished), could be explained by the fact that there is a competition between LTP and the membrane for binding the lipid molecule; this competition is in favor of LTP when the length of the acyl chain of the bound lipid decreases. These experiments strongly suggest that, like PC-TP, plant LTPs and animal nsL-TP act as lipid-carriers. Further experiments are needed to establish the sites of binding of phospholipids as well as of fatty acids on the LTPs/FABPs from plants.

According to the mode of action suggested for PC-TP as well as for the other transfer proteins, a transient complex consisting of bound lipid, LTP and membrane is formed. Such a process implies an interaction with the surface of the membrane, and one can predict that the surface charges control the activity of the transfer protein. It has indeed been found, that the activity of several plant LTPs is inhibited by the increase in the ionic strength of the medium while the introduction of positive charges into the membrane inactivates plant LTPs. In contrast, the introduction of negative charges at the surface of the membranes (for example, by adding PI into pure PC liposomes) enhances the lipid transfer mediated by maize LTP (Guerbette and Kader, unpublished). Similar observations have previously been published with animal proteins [50]. All these observations suggest a possible control in vivo – for instance by charges provided by the membrane proteins – of the activity of plant LTPs. This could lead to the formation of membranes with different lipid compositions in spite of the non-specific character of the LTPs. In connection with this point, one important question is whether LTP catalyzes an exchange or a net transfer process. Since LTPs facilitate a bidirectional transfer of lipids, they act as lipid-exchange mediators, which explains why they were originally designated as phospholipid-exchange proteins [22,23,50,51]. However, a precise study of the lipid pools involved in the process revealed that this exchange is not strictly a

one-for-one process. Depending on the relative importance of the lipid pools, a net transfer has been observed, leading to an increase in the amount of lipids present in one of the two membranes. This has been clearly shown with chloroplasts[26]. Animal nsL-TP (or PC-TP under some precise conditions) also catalyze a net transfer process[50,51].

Physiological functions of lipid transfer proteins

It is clear that, since LTPs are able to transfer phospholipids between membranes in vitro, it could be assumed that in vivo they convey phospholipids like PC, which are synthesized in the endoplasmic reticulum, towards mitochondria or chloroplasts which are unable to form this lipid. According to this hypothesis, LTPs could play an important role in membrane biogenesis as well as in membrane renewal. However, several points are still obscure. If LTPs are assumed to interact with the outer surface of the outer membranes of mitochondria or chloroplasts, how are lipids transported to the inner layer of the inner membranes? Careful studies of the outer membrane of yeast mitochondria have shown that this membrane has a lipid composition distinct from that of the inner membrane[39]. In addition to transmembrane movements of lipids, an intermembrane movement is needed. Another question concerns the intrachloroplastidial transfer of galactolipids. In addition to possible transfer of membrane vesicles[7], the isolation of a 28 kDa protein from the chloroplast stroma, which is able to transfer galactolipids, provides an argument in favor of a protein-mediated process[31]. The origin of the lipid components of the plasma membrane is still a matter of debate. First experiments have indicated that this type of membrane could be involved in protein-catalyzed transfer of phospholipids. However, intracellular transfer of membrane vesicles through the pathway endoplasmic reticulum-Golgi-plasma membrane is also possible[27,28]. In addition to these functions, LTPs could play a major role in the cooperative pathway which needs a transfer of phosphatidylcholine containing diunsaturated acyl chains towards chloroplasts, where the final desaturation and synthesis steps occur, leading to the formation of galactolipids esterified with triunsaturated fatty acids. In vitro transfer of lipids from liposomes or microsomes to chloroplasts has also been observed[13,26,33,34]. LTPs can also play another role in this cooperative process by interacting with oleyl-CoA molecules exported from the plastids where they are synthesized. Since they bind oleyl-CoA, LTPs/FABPs, similarly to FABPs from animal cells[1], can control the intracellular level of fatty acids or oleyl-CoA.

The fact that plant LTPs actively transfer PI is of high interest in the light of recent observations providing evidence for the functioning of the cycle of polyphosphoinositides in plant cells[38]. In response to various stimuli, like hormone signals, phosphorylation and degradation of PI occur, leading to the formation of second messengers, such as inositol trisphosphate. The replacement of the hydrolyzed PI molecules could be mediated by LTPs. Van Paridon et al.[47] suggested the same hypothesis for animal cells.

Immunochemical characterization of LTPs

In order to provide arguments in favor of the participation of LTPs in membrane biogenesis, it could be interesting to establish a correlation between this biogenesis and the level and activity of LTPs in plant cells. The preparation of specific antibodies directed against maize[15] and castor bean[45] LTPs has allowed the use of immunochemical methods like immunoblots and ELISA to determine the levels of LTPs. The immunoblotting technique showed that the amounts of LTPs increased in castor bean seedlings in parallel with the increase in lipid transfer activities[45].

Quantitative data were given by the ELISA method developed for maize LTP. It was found that an increase in the amount of LTP occurs in the aerial parts of the seedling, where active biogenesis of membranes is observed, whereas no major change was observed in the endosperm, which is not tissue where membranes are actively formed[16]. These observations strongly support the hypothesis of the participation of LTPs in membrane formation. Immunochemical methods have provided additional evidence. One study was concerned with the amount of LTP in maize cell; LTP was found to represent up to 4% of the proteins precipitated with ammonium sulfate from a seedling homogenate. This result confirms that LTPs are abundant proteins, at least in seedlings[15]. Immunoblots performed with purified membranes also showed that LTPs, although mainly cytosolic, can be partly bound to intracellular membranes[11].

Molecular biology

In order to provide more direct correlation between membrane biogenesis and the function of LTPs, it is of great interest to study the biosynthesis of LTPs and the expression of the genes coding for these proteins. The in vitro synthesis of LTP was studied with poly(A+)RNA isolated from maize[43,48] or castor bean[45] seedlings. After translation in a rabbit reticulocyte lysate in the presence of ^{35}S cysteine, the newly-synthesized proteins were immunoprecipitated with specific antibodies. Polypeptides with a molecular mass 3 kDa higher than that of the mature proteins (9 kDa) were detected both with maize[48] and castor bean[45] mRNAs. This suggests that these LTPs are synthesized as precursors. Interestingly, in rat liver, a high-molecular mass polypeptide is detected by an antibody against nsL-TP[46], whereas rat adrenal nsL-TP is synthesized in vitro as a precursor with a molecular mass 3 kDa higher than the mature protein[44]. The recent isolation and characterization of a full-length

cDNA clone corresponding to maize LTP has provided new tools for the study of the expression of genes coding for this protein[43]. The amino acid sequence deduced from the nucleotide sequence of this cDNA (822 base pairs) is identical to the sequence determined for the purified maize protein. Interestingly, an extra-peptide of 27 amino acids, having the characteristics of a signal peptide, is present at the N-terminal end. This is the first demonstration that a signal peptide is involved in the synthesis of a plant LTP. Interestingly, signal peptides of 26 amino acids have been detected in the cDNAs corresponding to spinach LTP (Somerville, unpublished) and to barley protein[29]. The physiological significance of this signal peptide is not clear at the moment. However, this observation is consistent with the previous observations that maize and castor bean LTPs are synthesized as precursors.

Lipid transfer proteins as probes for membrane studies

The high activity of plant LTPs, their stability (they can be stored for months at +4 °C or at −80 °C in the presence of 50 % glycerol), and their wide specificity for phospholipids, facilitate their use as probes for membrane studies as well as for modification of the lipid composition of membranes. The basis of the latter procedure is to incubate membranes with liposomes of a given composition in the presence of LTP. The partial replacement of membrane lipids by liposomal lipids changes the initial composition of the membrane. As an example, a change of the lipid composition of chloroplast envelopes was observed when these membranes were incubated with spinach LTP, and liposomes made from a phospholipid present in low proportions in the chloroplast membrane[26]. LTPs can also be used as probes since they only interact with the outer surface of the membranes. Interesting information about transmembrane movements of lipids or asymmetry of lipid pools can be provided by the use of plant LTPs. Furthermore, plant LTPs are active with membranes from animal origin, e.g. rat liver mitochondria (Guerbette and Kader, unpublished) or erythrocytes (Devaux and Kader, unpublished).

Concluding remarks and prospects

The recent progress made in the field of plant LTPs opens novel perspectives for studying their participation in membrane assembly and renewal. Although it is very likely that these proteins are involved in membrane biogenesis, direct in vivo evidence is still lacking. The approaches of molecular biology can provide such evidence, since it is now possible to follow the expression of the genes coding for LTP carefully, by using the cDNA now available for maize LTP as a probe. The variation of the levels of LTP mRNAs in membrane-forming cells, and the study of the localization of these mRNAs within tissues by in situ hybridization, will be some of the topics for future investigations. By performing experiments on site-directed mutagenesis, the domains of LTPs essential for their activity will be explored. At a later stage the methods of transformation of plants, which are already well developed for genes of other systems, could give novel and decisive information about the physiological roles of LTPs in plant cells.

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