thematic review series

Thematic Review Series: Lipid Transfer Proteins

Plant lipid transfer proteins: are we finally closing in on the roles of these enigmatic proteins?

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Abstract The nonspecific lipid transfer proteins (LTPs) are small compact proteins folded around a tunnel-like hydrophobic cavity, making them suitable for lipid binding and transport. LTPs are encoded by large gene families in all land plants, but they have not been identified in algae or any other organisms. Thus, LTPs are considered key proteins for plant survival on and colonization of land. LTPs are abundantly expressed in most plant tissues, both above and below ground. They are usually localized to extracellular spaces outside the plasma membrane. Although the in vivo functions of LTPs remain unclear, accumulating evidence suggests a role for LTPs in the transfer and deposition of monomers required for assembly of the waterproof lipid barriers, such as cutin and cuticular wax, suberin, and sporopollenin, formed on many plant surfaces. Some LTPs may be involved in other processes, such as signaling during pathogen attacks. Here, we present the current status of LTP research with a focus on the role of these proteins in lipid barrier deposition and cell expansion. We suggest that LTPs facilitate extracellular transfer of barrier materials and adhesion between barriers and extracellular materials. A growing body of research may uncover the true role of LTPs in plants.—Edqvist, J., K. Blomqvist, J. Nieuwland, and T. A. Salminen. **Plant lipid transfer proteins: are we finally closing in on the roles of these enigmatic proteins?** ***J. Lipid Res.*** **2018.** 59: **1374–1382.**

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OVERVIEW OF PLANT NONSPECIFIC LIPID TRANSFER PROTEINS

The plant nonspecific lipid transfer proteins (LTPs) are abundant, secreted, soluble, cysteine (Cys)-rich, and small proteins with a molecular size usually below 10 kDa (1, 2). In the LTPs, four conserved disulfide bridges, formed by an eight-Cys motif (8CM) with the general form C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C, stabilize the folding of four or five α-helices into a very compact 3D structure (3, 4) (**Fig. 1**). The folding of the helices results in a central hydrophobic cleft suitable for the binding of hydrophobic ligands, such as fatty acids and other lipids (**Fig. 2**). The compact structure renders the LTPs very insensitive to heat and denaturing agents (5, 6). LTPs are expressed in all investigated land plants, but have not been detected in any other organisms (7). LTPs are encoded by large gene families in seed plants (2, 8–11). In bryophytes and ferns, the gene families are significantly smaller (7, 12). LTPs are classified in five major types (LTP1, LTP2, LTPc, LTPd, and LTPg) and five minor types (LTPe, LTPf, LTPh, LTPj, and LTPk) (7). The classification is based on the spacing between the Cys residues in the 8CM, the polypeptide sequence identity, and the position of evolutionarily conserved introns. The classification also reflects posttranslational modifications, e.g., LTPs with a GPI-anchor belong to LTPg. LTPd and LTPg are encoded in all land plants, which suggests that these were possibly the first LTP types that evolved in land plants. The most well-studied LTP types in flowering plants, LTP1 and LTP2, probably evolved later because these are not found in liverworts, mosses, or other non-seed plants (7). The LTPs are translated with an N-terminal signal peptide that has a potential to localize the protein to the apoplastic space (1).

Several in vitro binding experiments show that LTPs bind both saturated and unsaturated fatty acyl chains,

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Abbreviations: 8CM, eight-Cys motif; DPMG, 1,2-dimyristoyl phosphatidylglycerol; H1–H4, helixes 1–4; LMPC, lyso-myristoyl-phosphatidylcholine; LTP, lipid transfer protein; PDB ID, Protein Data Bank identification number; PGB_2, prostaglandin B2.

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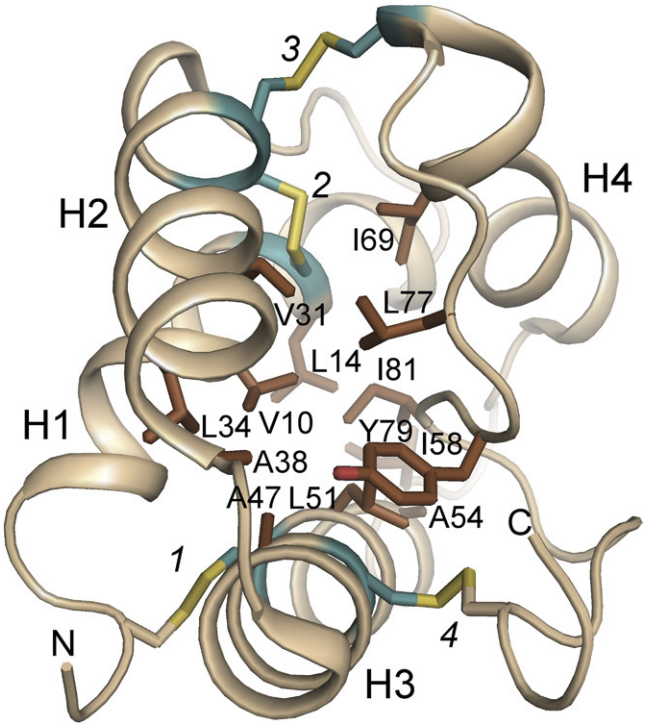

Fig. 1. The 3D fold of wheat LTP1 [renamed TaLTP1.1 in (2)] structure [PDB ID 1GH1 (3)]. The four helices are stabilized by four disulfide bridges (green sticks) of which the first and fourth bond connect the N- and C-terminal parts to H3, respectively. The second and third bond link H2 to H1 and H4. The hydrophobic central cavity of the LTP1 structure is formed by residues (brown sticks) from each helix and the unstructured C-terminal part.

presented in various molecules, such as in LPC, PG, acyl-CoA, or as free fatty acids (13–17). Furthermore, many LTPs can fit two fatty acyl chains in the cavity (18–21). Some LTPs are reported to bind to hydroxylated acyl chains (12, 16, 19). The rice LTP2, OsLTP2.3, binds to dehydroergosterol (22), which is, to our knowledge, the only report of a LTP binding to a sterol. The dissociation constants (K_d) for LTP-ligand interactions are usually in the micromolar range, suggesting that the LTPs are involved in low-affinity interactions. The preferred ligands are, in most cases, fatty acyl-chains with 14 to 18 carbons (19, 23). One of rather few LTPs shown in vitro to bind very-long-chain-fatty acids is Arabidopsis AtLTP1.4, which, in a lipid-protein-overlay assay, could bind to fatty acids from C22 to C27 (24). There is still rather poor knowledge of the in vivo binding patterns for LTPs. A phytosphingosine bound camptothecin derivative was associated with purified peach LTP Pru p 3 and could hence represent an in vivo ligand (25). Nevertheless, the biological relevance of this observation is at present unclear.

The biological role of the LTPs remains rather obscure. However, there are data accumulating suggesting that these proteins are required for the deposition and function of wax and lipid-based polymers, such as suberin, sporopollenin, and the cuticle that forms waterproof barriers on plant surfaces (24, 26–32). Additionally, some LTPs with cell wall-loosening activities could be important for plant growth (33). The roles of LTPs in lipid barrier deposition and cell wall loosening will be the primary focus of this review. A more encyclopedic review on plant LTPs was published rather recently (2). Nevertheless, it is also important to point out here that LTPs are suggested to be involved in many other processes in plants, such as signaling (34, 35), defense against biotic hazards (36–40), and tolerance to abiotic stresses, just to mention a few examples (12, 41–45).

LTP: A MAJOR PLANT FOOD ALLERGEN

The plant LTPs are the most frequent cause of food allergy in adults living in the Mediterranean area (46). Curiously, such LTP-sensitized allergies are rare in Northern Europe and the United States. Exposure to birch pollen

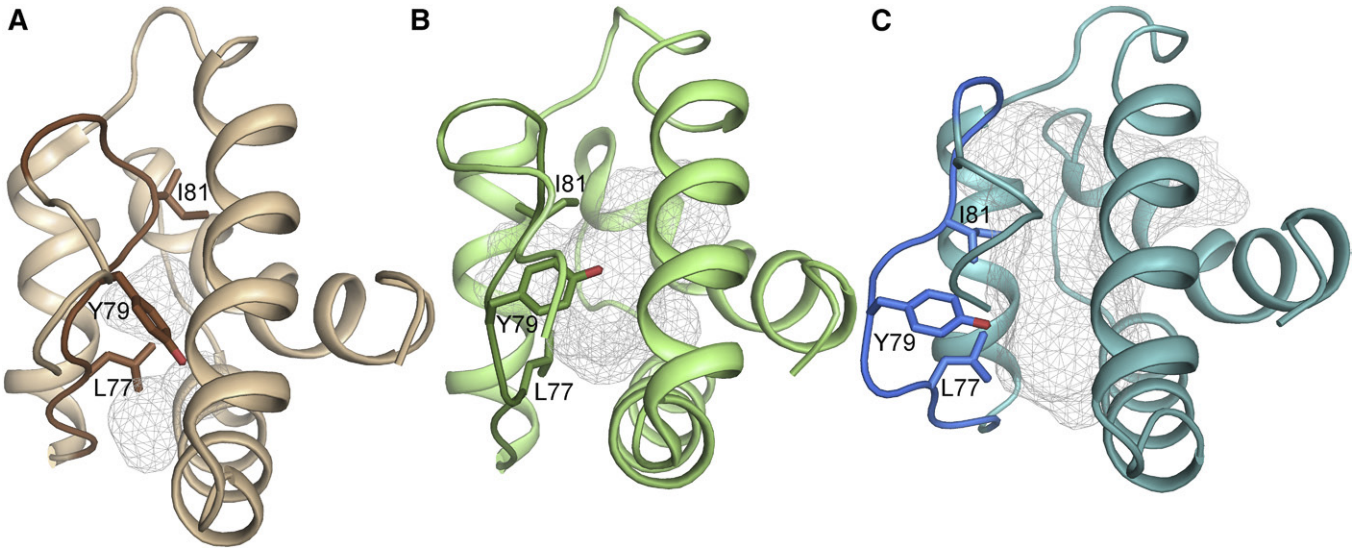

Fig. 2. The size and shape of the ligand binding cavity of wheat LTP1 [renamed TaLTP1.1 in (2)] without a ligand [PDB ID 1GH1 (3)] (A), complexed with PGB_2 [PDB ID 1CZ2 (83)] (B) and complexed with two LMPC molecules [PDB ID 2BWO (15)] (C). The size of the cavity (shown as white wireframe) is adjusted by the size of the bound ligand by the movement of the C-terminal residues shown as sticks.

may counteract the sensitivity to LTPs. This could explain the geographical distribution of the LTP-sensitized allergies (47). The peach LTP, Pru p 3, is the molecule that dominates the immune response to LTPs and is considered to be a marker for severe systemic reactions to plant-derived food. However, because LTPs are abundantly expressed in most plants, LTP-sensitized patients may show reactions against a large number of plant foods. The role of LTPs in allergic reactions is only briefly mentioned here. We recommend reading other reviews for an introduction and update on these interesting and clinically relevant aspects of plant LTPs (47–50).

LTPs IN LIPID BARRIER POLYMER DEPOSITION

When plants conquered land more than 500 million years ago (51), they developed specialized tissues to survive the harsh climate outside the water. They developed new cell components, like lignin, which gave sturdier cell walls and waxes, and hydrophobic lipid-based polyesters, like suberin, cutin, and sporopollenin, that form waterproof barriers to protect the plants from abiotic and biotic stresses, such as water loss, radiation, pathogens, and herbivores. Suberin is a heteropolymer with polymeric aliphatic carbon chains associated with aromatic compounds. Long-chain oxygenated fatty acids provide the core of the suberin polyester. Suberin is usually found in seed coats and in periderms in roots and on stems, where it accumulates in layers between the plasma membrane and the cell wall. The suberin layers contribute to the control of diffusion of water and solutes across seeds, roots, stems, and other tissues (52). Cutin is the structural polymer of the epidermal cuticle, the waterproof layer covering the exterior surface of the cell wall of primary aerial organs. Cutin is a polyester of C16 and C18 hydroxy fatty acids and glycerol, which in the cuticle is interspersed with and covered by waxes (53–55). Sporopollenin is another very complex lipid-based biopolymer derived mainly from saturated precursors, such as long-chain fatty acids and long aliphatic chains. Sporopollenin is found in the exine layer of spores and pollen walls. The exine protects spores and pollen in harsh environments and serves as a barrier against various physical and chemical factors and biological pathogens (56). The sporopollenin is chemically inert due to insolubility in both aqueous and organic solvents.

More and more details of the synthesis of the cuticle, suberin, and sporopollenin polymers are elucidated (57). Basically, the synthesis of the lipid barriers requires de novo synthesis of precursors, which occurs mainly in the endoplasmic reticulum. This is followed by transfer of the precursors to extracellular spaces through the plasma membrane. This process is likely facilitated by ABC- or ABCG-transporters located in the plasma membrane (58–61). When the monomeric precursors have reached the extracellular apoplastic environment, they need to diffuse or transfer to the site for polymer assembly (62).

It has been suggested that LTPs are important for the last step described above, thus, in facilitating the movement of lipid polymer and wax components to the sites of polymer accumulation on the extracellular side of the plasma membrane (26, 56, 62). This could involve passage of hydrophobic waxes or polymer precursors through the hydrophilic cell wall for cuticle accumulation, across the locule for pollen exine assembly, or in the apoplast for suberin deposition. From a mechanistic perspective, it is still rather unclear how the LTPs aid in the extracellular transport of the building blocks for lipid polymers. It is possible that the ABC transporters deliver the polymer building blocks to LTPgs, which are attached to the apoplastic side of the plasma membrane through their GPI-anchor. The cargo may then be transferred from an LTPg to an LTP of another type that may diffuse freely in the relevant apoplastic environment. Proposed models for LTPs in the deposition of barrier lipids in different tissues are illustrated in **Fig. 3**.

Although the details regarding the role of LTPs in lipid polymer biosynthesis remain somewhat mysterious, there are more and more data accumulating to support that the LTPs are involved and also important for this process. For instance, when gene expression data of LTPgs from rice and Arabidopsis were investigated for coexpression patterns, the LTPgs could be arranged in three coexpressed LTPg clusters (63). For the first cluster (I), expression was observed in aerial parts of the plant. The second cluster (II) was the only one with expression in roots, while expression of the third cluster (III) was restricted to reproductive tissues. Thus, LTPs in seed plants, based on expression patterns, can be classified into root LTPs, green LTPs, and reproductive LTPs. Further, gene ontology analyses of genes coexpressed with the three Arabidopsis LTPg-clusters showed an enrichment of genes involved with cuticular wax accumulation for cluster I, an enrichment of genes involved with suberin synthesis or deposition for cluster II, and, for cluster III, an enrichment for genes acting in sporopollenin accumulation. Hence, the coexpression patterns suggest that the LTPgs in the three clusters are involved in the assembly of the cuticle, suberin, and sporopollenin, respectively (63).

This division of LTPs in separate functional clusters is also supported from the analysis of knockdown, knockout, and overexpressing plant lines. In Arabidopsis, the knockdown of At*LTPg1* results in reduced wax load on stem surfaces (26), while in *Atltpg1* and *Atltpg2* knockout single mutants there is a 4–20% reduction in stems and siliques of the C29 alkane (nonacosane) component of cuticular wax (27). In the *Atltpg1Atltpg2* double-mutant, there are even stronger reductions of nonacosane and less total wax load in the stems and siliques (29). Curiously, overexpression of the *Brassica rapa BrLTPd1* gene in *Brassica napus* causes reduced wax deposition on leaves and morphological changes of leaves and flowers (63). Possibly, the overexpression of *BrLTPd1* may lead to disordered secretion of wax, which is lost from the surface, or to the inhibition of other LTPs involved in wax deposition (63).

In rice, silencing of *OsC6* [later renamed to *OsLTPg25* (2)] results in reduced pollen fertility. In the silenced plants, the anthers follow normal development until young

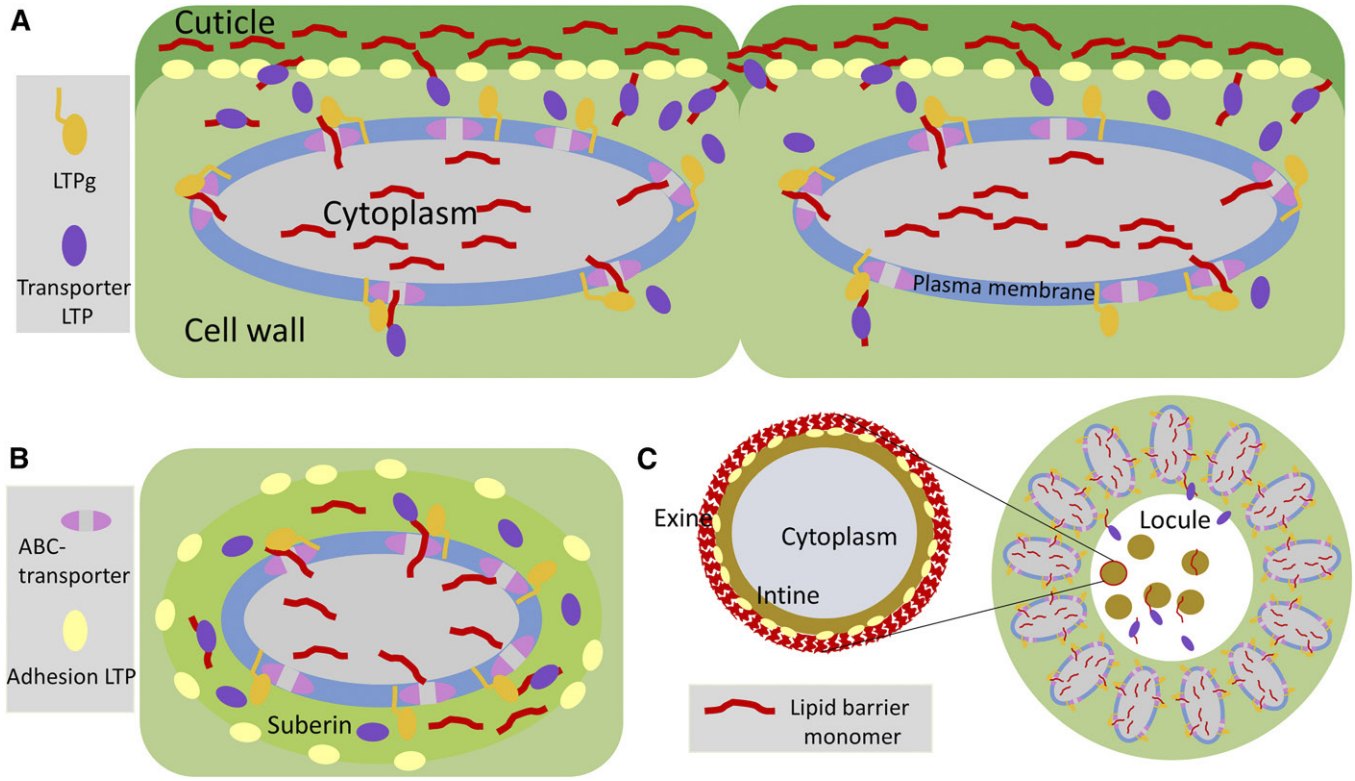

Fig. 3. Putative roles for different LTPs during lipid barrier assembly in green tissues (A), roots (B), and pollen (C). Three different roles for LTPs in lipid barrier assembly are indicated. LTPg (orange) is attached to the plasma membrane through its GPI-anchor and dock the lipid barrier monomers when they leave the plasma membrane-localized ABC transporters. The Transporter LTPs (purple) facilitate the transfer of the lipid barrier monomers from the LTPg to the site of barrier deposition. The Adhesion LTPs (yellow) have a structural role adhering the hydrophobic barrier to the hydrophilic cell wall (A, B) or intine (C).

microspores are prematurely released from the tetrad (28). The *OsLTPg25*-silenced lines also show microspores and pollen walls with irregular shapes and structures. Further, ectopic expression of OsLTPg25 results in granule-like droplets on the inner surface of the tapetal cells. These obtained phenotypes suggest that OsLTPg25s are facilitating the transport of exine precursors in the locule from the tapetal cells to the pollen grains (28).

In plants, crown gall tumors develop after infection of virulent *Agrobacterium tumefaciens* strains. These tumors are covered with a suberin-containing periderm that protects against water loss and pathogen infections. In Arabidopsis, the LTP, *AtLTP1.4*, is expressed in the crown gall tumors (24). In an *Atltp1.4* T-DNA mutant, the suberin composition in crown galls is altered, as there is a decrease in C18 alkanoic acids, as well as an increase in C18-C24 2-hydroxy alkanoic acids. The results indicate that AtLTP1.4 enhances the extracellular delivery in the apoplast of fatty acyl precursors for suberin deposition. Interestingly, although *AtLTP1.4* is not normally expressed in epidermis, ectopic expression of the gene in epidermis resulted in altered wax composition in the cuticular wax (24).

Artemisin is a highly oxygenated sesquiterpene that is used for malaria treatment. Artemisin is produced by the plant, *Artemisia annua*, on the glandular trichomes on leaves and ovules. It is formed nonenzymatically from the precursors, artemisinic acid and dihydroartemisinic acid (64). When artemisin was transiently produced in *Nicotiana benthamina*, it was found that the simultaneous expression of genes required for artemisin biosynthesis together with an *A. annua* LTP and the *A. annua* ABC-transporter, pleiotropic drug resistance (PDR), resulted in a higher yield of artemisinic acid and dihydroartemisinic acid in the *N. benthamina* apoplast (65). The *A. annua* LTP seems to be able to retain artemisin precursors in the apoplast, thereby preventing reflux back to the cell. The mechanism behind the retaining effect is unknown, although the authors speculated that the LTPs may deposit the artemisin precursors on the outer side of the cell wall, and once deposited, reflux would be prevented (65). Possibly, such a retention mechanism that improves the yield for export of precursors from the cell, as suggested for artimisin biosynthesis, could explain the requirement for LTPs in lipid polymer deposition.

We suggest a classification of the LTPs involved in extracellular lipid polymer precursor transfer and secretion as Transporter LTPs (Fig. 3). This classification is relevant, as there are also data that suggest that some other LTPs rather act by stabilizing the adhesion between lipid polymer barriers and cell walls, such as between the cuticle and the cell wall in green tissues, between the exine and intine in pollen, or between suberin and the cell wall in the seed coat. We classify these LTPs as Adhesion LTPs (Fig. 3). Intriguingly, such a role as an Adhesion LTP was suggested for Arabidopsis AtLTP1.4 [previously named AtLTP2, but renamed to emphasize that it is of type LTP1 (2)] (32). An

Atltp1.4-mutant has increased cuticle permeability and structural defects at the cell wall-cuticle interphase. It was therefore proposed that AtLTP1.4 could play a major structural role by maintaining the integrity of the adhesion between the mainly hydrophobic cuticle and the underlying hydrophilic cell wall (32).

An adhesive role for LTPs is also supported from the investigation of the Arabidopsis AtLTPc1, AtLTPc2, and AtLTPc3. These three LTPs all have an expression pattern restricted to the tapetum of developing anthers (66). Double RNAi silencing of AtLTPc1 and AtLTPc3 did not cause any abnormalities regarding pollen morphology or fertility. However, in the silenced plants, the intine underneath the exine is impaired and uncharacteristically separated from the exine and the microspore plasma membrane (66). Further, several Arabidopsis *Atltpg* single mutants have deformed or collapsed pollen grains (31). The seeds of these *Atltpg* mutants are unable to restrict salt uptake and show other abnormal phenotypes, such as the protrusion of seed hairs or a shrunken and irregular appearance. Lipid analysis of the seed coats from *Atltpg4-1*, *Atltpg4-2*, *Atltpg6-1*, and *Atltpg6-2* revealed a large decrease in the ω-hydroxy fatty acid, 24-hydroxytetracosanoic acid (C24ωOH), and an increase in unsubstituted C20:0, C22:0, and C24:0 fatty acids (31). These ω-hydroxy fatty acids are all important constituents of the barrier polymer, suberin (67). We speculate that the phenotypes shown for the *Atltpg*-mutants could result from defects in adhesion between the suberin layer and the cell wall.

Hence, there are rather different roles suggested for LTPs in the assembly and biosynthesis of the lipid polymer barriers. The Transporter LTPs may facilitate extracellular transport or diffusion of the hydrophobic polymer monomers and waxes and the Adhesion LTPs stabilize the interaction between lipid polymer barriers and cell walls, such as between the cuticle and the cell wall in green tissues, between the exine and intine in pollen, or between suberin and the cell wall in the seed coat. These different hypotheses on the function of LTPs are not necessarily contradicting each other. In the light of the variety of different LTP-types and the large number of members in the LTP family, it is possible that distinct members of the LTP family could participate as either a Transporter LTP or an Adhesion LTP. It is also possible that both functions in transfer and adhesion could be fulfilled by singular LTPs, as has already been suggested for AtLTP1.4 (24, 32). Further studies will hopefully reveal whether particular LTPs are involved in specific processes during lipid polymer deposition.

LTPs MAY FACILITATE CELL EXPANSION AND PLANT GROWTH

As described above, LTPs seem to have an important role in the deposition and adhesion of wax and lipid barrier polymers. However, there are also many other functions that have been linked to LTPs, such as signaling and tolerance to abiotic and biotic stress (2). Underpinning the plethora of proposed physiological functions are the more specific biochemical activities of LTPs. Nieuwland et al. (33) described a novel function of LTP as cell wall loosening proteins. In plant cells, growth by increase of volume is achieved by turgor-driven expansion, which is limited by the extensibility of the cell wall [for a recent review see (68)]. The cell wall is a complex matrix consisting mainly of polysaccharide chains, but also of proteins. Cellulose microfibrils provide the load-bearing structure and this cellulose network is tethered together by branched polysaccharides. The structural scaffold provided by cellulose can be found throughout the plant kingdom, but the cross-linking tethers vary (69). In dicotyledonous plants, the matrix in which cellulose is embedded consists of essentially xyloglucans and pectins. Although, historically, xyloglucan was thought to be the main cross-link involved in cell wall strength and extensibility, it has become clear that the pectin matrix also plays a critical role (70–73). Nevertheless, as turgor pressure remains constant, the expansion of the cell depends on the rigidity of the cell wall. Upon growth, cells actively decrease the rigidity by cell wall loosening, which causes the wall polymers to slide along each other. The expansin protein family was shown to have cell wall loosening activity, but it is important to note that cell wall loosening by expansins is not achieved by conventional enzymatic activity (74).

As a β-expansin was expressed in the tobacco stigma, it was not a surprise to find that cell wall loosening activity was detected in stigma exudate. However, after purification of the protein with wall loosening activity, it was identified as a LTP (33). Although expansins and LTPs do not share sequence similarity, it is possible that they have the same effect on the cell wall matrix. The availability of the hydrophobic cavity of this tobacco LTP was shown to be essential for cell wall loosening in vitro and the activity could be shown on an artificial matrix of cell wall material from bacterial cellulose and xyloglucan, indicating that it acts on the interface between those polysaccharides, similarly again to expansins (75). Further analysis and numerical simulation of cellulose-xyloglucan network extension with or without LTPs revealed that this system shows slow glassy dynamics that depend on the weakening of xyloglucan-cellulose interactions (76). Besides the initial discovery of LTPs as cell wall loosening proteins, no further work directly on cell wall dynamics has since been published. However, indirect data does support a role of LTPs in plant growth. Proteomic and transcriptomic experiments have revealed LTPs spatially and temporally associated with plant growth. For example, in a genome-wide transcriptome analysis of growth in *Populus trichocarpa*, LTP transcripts correlated with stem development (77). In maize, transcripts of elongating internodes were compared with non-elongating internodes and LTPs were found to be expressed preferentially in the elongating internodes (78). In an overexpression experiment of BELLRINGER, a homeodomain transcription factor involved in development and growth, only the expression of genes associated with the cell wall were changed, including several LTPs (79). Furthermore, the transcriptional regulator, MED/PFT1, in

Arabidopsis was shown to regulate cell wall gene expression, including two LTPs (80). In a meta-analysis of cell wall proteomics data, LTPs were found to be a significant part of the protein population (81).

Although there is an overlap between LTP expression and plant growth, which fits with a function of cell wall loosening, it could also fit the more traditional function of cuticle deposition. However, the cuticle does not represent the majority of the organs used in cell wall proteomics approaches in Arabidopsis (81), although this is not true for other studies [to exemplify (82)]. Taken together and based on gene expression and phenotypic analyses, it is clear that LTPs are associated with plant growth. It is possible that LTPs could function in growth through both cell wall loosening and lipid deposition. It remains unclear whether different LTPs have different or identical biochemical functions at the same time. Further experiments focusing on dissecting the two biochemical functions would be required to answer that question.

STRUCTURAL PLASTICITY OF LTPs

The 3D structure of a protein is a key to unlock the secrets and reveal their biological functions. In the case of the LTPs, the first 3D structures of LTPs were presented in the early 1990s; still, we are not entirely confident about their activities. Nevertheless, a large number of reports describing the structural or ligand-binding properties of LTPs were published thereafter [recently reviewed in (2)]. To exemplify, in wheat LTP1 TaLTP1.1, four helices are linked together by flexible loops (3). The helices are packed against the unstructured C-terminal part, which is stabilized by a hydrogen bond to helix (H)3 (Fig. 1). Four disulfide bridges are formed by the eight Cys in the 8CM to stabilize the fold of the protein. Both the N-terminal end of H1 and the C-terminal part are linked to H3 by disulfide bridges (labeled 1 and 4 in Fig. 1), respectively. The position of H2 is stabilized by two disulfide bonds; one of them links the N-terminal part of H2 to the C-terminal part of H1 and the other one links H2 to H4 (bridges 2 and 3 in Fig. 1). The central hydrophobic cleft is formed by the residues from H1 (Val10, Leu14), H2 (Val31, Leu34, Ala38), H3 (Ala47, Leu51, Ala54), loop H3-H4 (Ile58), H4 (Ile69), and from the C-terminal part (Leu77, Tyr79 and Ile81) (Fig. 1).

The crystal structure of a TaLTP1.1:lyso-myristoylphosphatidylcholine (LMPC) complex (15) [Protein Data Bank identification number (PDB ID) 2BWO] showed that TaLTP1.1 can accommodate two molecules of LMPC (Fig. 2) positioned head to tail. The aliphatic chains are positioned inside the cavity, while the polar head groups are directed toward the solvent areas at each end of the tunnel. One of the bound LMPC ligands (in site 1) contacts wheat TaLTP1.1 via hydrophobic interactions and through a hydrogen bond with the side chain hydroxyl of Tyr79, whereas the other LMPC (in site 2) is only involved in a few hydrophobic interactions. In the solution structure of a TaLTP1.1:prostaglandin B2 (PGB_2) complex, the C-terminal part, which in the unliganded form makes contact with the H4 helix, moves outward (3, 83) (PDB ID 1CZ2). The interaction induces a 100° rotation around the Cβ-Cγ bond of the Tyr79 ring. This rotation facilitates the formation of a hydrogen bond between the carboxyl group of the ligand and the hydroxyl group of Tyr79 (Fig. 2). In addition, several hydrophobic residues lining the internal cavity are pushed away by the ligand, which results in the volume of the cavity increasing from 300 ± 50 $Å^3$ in the unliganded protein to 786 ± 43 $Å^3$ in the TaLTP1.1:PGB_2 complex (83). Also, in the TaLTP1.1:1,2-dimyristoyl phosphatidylglycerol (DMPG) complex, as assayed with ^1H NMR and fluorescence spectroscopy, both acyl chains are accommodated into the hydrophobic cavity. The only structural alteration induced by DMPG is seen in the C-terminal part of the structure where the aromatic ring of Tyr79 is moved outwards into the solvent, which excludes formation of hydrogen bonds between DMPG and TaLTP1.1. The volume of the cavity was estimated to increase to 750 ± 250 $Å^3$ when occupied by the two acyl chains.

The swelling of the cavity is the result from conformational changes in the unstructured C-terminal end of the protein. In particular, Tyr79 located close to the opening of the cavity is a key residue that may act as a gate keeper controlling the shape, size, and binding capacity of the hydrophobic cavity. Curiously, some ligands, as determined for DMPG, cause a shift in the orientation that moves the aromatic ring of Tyr79 outwards to the solvent to exclude the formation of hydrogen bonds between Tyr79 and the ligand, while other ligands, as demonstrated for PGB_2, seem to adjust the orientation of Tyr79 to allow for the formation of bonds to the ligand. Nevertheless, the properties of the ligand clearly influence the 3D structure of and around the cavity in the LTP. Similarly, in the maize LTP1 ZmLTP1.6, Tyr81 is often displaced from the cavity upon binding of a ligand to the protein (4, 16), as well as Tyr79 in rice OsLTP1.18 (17) and Tyr79 in NtLTP1.1 from *Nicotiana tabacum* (84), just to mention some additional examples.

SUMMARY

The research on plant LTPs has now formed a solid ground with a wealth of information available regarding 3D structures, lipid binding, and expression patterns. More and more results are also accumulating from phenotypic investigations of knock-out or knock-down plants. When the current LTP research is reviewed and summarized, a role of many LTPs in the deposition lipid-based surface barriers seems more and more likely. However, this is not excluding that some LTPs also could have other biological functions to fulfill in plants, such as in signaling or pathogen defense. Still, we cannot precisely describe the function and activity of the LTPs in lipid barrier formation. Here, we have suggested that LTPs are facilitating the extracellular transfer of barrier materials and/or are involved in enhancing the adhesion between the lipid-based barriers and other extracellular materials, such as the cell wall or the intine layer in pollen grains (Fig. 3). Nevertheless,

we need to learn more about the details surrounding the mechanisms of the LTP activities to truly pinpoint their different roles in the plants.

Thus, it is certainly a good time to advance with more exciting experiments on these intriguing and fascinating plant proteins. There are many challenges involved in LTP research, such as the complex gene families that potentially result in a high degree of gene redundancy, low sequence conservation, low specificity of the ligand:LTP interactions, and the complex nature of the lipid polymer synthesis. Anyway, with systematic approaches, it will definitely be possible to significantly advance our knowledge in a few years from now. Mosses and liverworts are emerging as models for studies on the assembly, function, and evolution of the plant cuticle (31, 85, 86). Because the number of LTP genes is much lower in bryophytes compared with vascular plants (2, 7), suggesting less redundancy, it may be useful to apply bryophyte model systems to investigate the function of LTPs in lipid barrier biosynthesis. Another way forward could be to use sophisticated microscopy to trace the movements of LTPs in living cells. There is also a need for identifying in vivo interaction partners, such as lipids, carbohydrates, or proteins. Anyway, we look forward to entering a very productive and exciting period for LTP research.

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