



Non-specific Lipid Transfer Proteins in Legumes and Their Participation During Root-Nodule Symbiosis

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In Memoriam:

This paper is dedicated to the
memory of Dr. Tomás Ruiz-Argüeso.

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Non-specific lipid transfer proteins (LTPs) constitute a large protein family in plants characterized by having a tunnel-like hydrophobic cavity, which allows them to transfer different lipid molecules. LTPs have been studied in various model plants including those of agronomic interest. Recent studies have demonstrated that LTPs play key functions in both biotic and abiotic stress. In plants-pathogen interaction, they act as either positive or negative regulators of defense responses. However, little is known about the roles of LTPs in symbiotic interactions, especially in root nodule symbiosis. Here, we performed a broad genome analysis of LTP family members in legumes and other important model plants, focusing on their possible roles in legume-rhizobium symbiosis. *In silico* analysis showed that legumes contain large LTP families, with at least 70 LTP members clustered into four clades. Although the structures of LTP genes and proteins are conserved among species, differences were observed between clades from different species. LTPs are widely expressed in different plant tissues. In general, genes of the *LTP1* and *LTP2* classes are highly expressed in shoot and reproductive tissues in all analyzed species. Furthermore, genes of the different classes are also expressed in roots inoculated with rhizobia and nodules of legumes. RT-qPCR expression profile analysis of seven *PvLTP* genes in common bean (*Phaseolus vulgaris*) revealed that these genes are differentially expressed during the early and late stages of nodulation and they are genetically regulated by *PvRbohA*. These findings provide insight into the putative roles of LTP family members in legume-rhizobium symbiosis and their possible interactions with RBOH-dependent ROS production.

Keywords: expression profile, legumes, lipid transfer proteins, nodule symbiosis, phylogenetic analysis

INTRODUCTION

Lipid transfer proteins (LTPs) constitute a large family of plant proteins that bind to different lipids in a non-specific manner. As LTPs can transfer a wide range of lipid molecules, such as phospholipids, galactolipids, and free fatty acids, they are also known as non-specific LTPs (nsLTPs) (Wei and Zhong, 2014; Edqvist et al., 2018). LTPs have a low molecular weight and contain four or five α -helices connected by four disulfide bonds. These boundaries are formed by the conserved eight-cysteine motif (8CM) with the general structure C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C, where X represents any amino acid (Shin et al., 1995; Boutrot et al., 2008; Lascombe et al., 2008). LTPs are also characterized by the presence of a hydrophobic cavity that allows them to bind to lipid molecules (Lascombe et al., 2008).

LTP family members have been identified in various angiosperms, including rice (*Oryza sativa* L.), *Arabidopsis thaliana* (L.) Heynh, wheat (*Triticum aestivum* L.) (Boutrot et al., 2008), barley (*Hordeum vulgare* L.) (Zhang et al., 2019), maize (*Zea mays* L.) (Wei and Zhong, 2014), and potato (*Solanum tuberosum* L.) (Li et al., 2019). Also in non-vascular plants, such as *Marchantia polymorpha* L., *Physcomitrium* (*Physcomitrella*) *patens* (Hedw.) Bruch and Schimp., *Selaginella moellendorffii* Hieron, the fern *Adiantum capillus-veneris* L., and the conifer *inus taeda* L. (Edstam et al., 2011).

Plant LTPs are classified into 2 subgroups based on their structural organization: LTP1s, with a molecular weight of 9–10 kDa and ~90–95 amino acids; and LTP2s, with a molecular weight of 6–7 kDa and 65–70 amino acids (Finkina et al., 2016). LTPs have also been characterized based on the presence and positions of introns, the presence of a glycosylphosphatidylinositol (GPI) modification site, the Cys spacing in the 8CM, and the similarity of their amino acid sequences. Based on these features, LTPs are grouped into 10 subtypes: LTP1, LTP2, LTPc, LTPd, LTPe, LTPf, LTPg, LTPh, LTPj, and LTPk (Edstam et al., 2011).

LTPs participate in lipid barriers synthesis, facilitating the transportation of lipid polymers in the apoplastic space, as well as in the loosening of the cell wall (Edqvist et al., 2018). Under *in vitro* conditions, AsE246, an LTP from Chinese milk vetch (*Astragalus sinicus* L.), was shown to bind to different fatty acids, mainly those with chains 16–18 carbons in length. Under the same conditions, AsE246 also binds to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), monogalactosyldiacylglycerol (MGDG), and to digalactosyldiacylglycerol (DGDG) (Lei et al., 2014).

Genetic approaches have revealed LTPs functions in plant responses to pathogens. For instance, the *Arabidopsis* LTP3 gene transcript accumulated in response to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 and its overexpression enhanced the susceptibility to this pathogen (Gao et al., 2016). Transcript accumulation of the LTP4 from *Nicotiana tabacum* L. (*NtLTP4*) also increased in response to wounding and infection with *Ralstonia solanacearum* (Xu et al., 2018). Moreover, salicylic acid (SA), a hormone that induces the plant systemic acquired resistance (SAR) of plants against biotrophic bacterial pathogens (Zhao, 2015), enhanced *NtLTP4* transcription (Xu et al., 2018). Xu et al. (2018) also found that jasmonic acid (JA), a hormonal regulator of defense responses to wounding and pathogen attack (Halim et al., 2006), increased *NtLTP4* transcription.

The *Arabidopsis* nsLTP-related gene *DRN1* (disease related nonspecific lipid transfer protein) is suppressed in response to Pst DC3000 and *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm* ES4326). Interestingly, when plants were challenged with bacteria with a defective type three secretion system (TTSS), *DRN1* was not suppressed. Additionally, *drn1* mutants showed less resistance to both bacterial (Pst DC3000 and Psm ES4326) and fungal pathogens (*Botrytis cinerea*). Since *DRN1* expression was also abrogated in SA, JA, and ethylene (ET)-mediated resistance, it is probably regulated by a pathway independent of these

hormones. In this context, it was shown that *DRN1* expression is apparently regulated by superoxide radicals (Dhar et al., 2020).

In root nodule symbiosis, LTP functions have also been reported. For instance, AsE246 was shown to bind DGDG from Chinese milk vetch nodule extract; interestingly, AsE246 knockdown reduced lipid abundance (PC, PE, PI, and DGDG) in the nodules. AsE246 knockdown also reduced number of nodules and infection threads (IT) and impaired the development of symbiosomes. Conversely, AsE246 overexpression increased the number of nodules and IT (Lei et al., 2014). Recently, AsE246 was observed to interact with the high temperature protein G (HtpG) of *Mesorhizobium huakuii* in infected nodule cells of *A. sinicus* and in symbiosome membranes (Zhou et al., 2019). Furthermore, downregulation of the LTP nodulin 5 from *Medicago truncatula* Gaertn. (MtN5) caused an increase in the curling of the root hair, but a reduction of invaded nodule primordia. On the contrary, MtN5 overexpression favored both root hair curling and invaded nodule primordia, also, the overexpression of MtN5 increased the content of lipids, MGDG, DGDG, PE and glucosyl ceramides, in infected roots (Santi et al., 2017).

Since LTPs bind to and transfer a variety of lipids, they are thought to play important roles in membrane remodeling. Both membrane remodeling and the formation of new membranes are key processes for the development of nodules during legume-rhizobium symbiosis. During this symbiotic interaction, rhizobium (soil bacteria capable of fixing atmospheric nitrogen) are internalized into nodule cells via a mechanism similar to endocytosis that encapsulates the bacteria within the plant membrane (Oldroyd and Downie, 2008; Roy et al., 2020). Inside the nodules, bacteria differentiate into bacteroids, which are surrounded by a membrane of plant origin. In these compartments, known as symbiosomes, bacteroids fix atmospheric nitrogen, and the plant-derived membrane acts as a symbiotic interface, allowing nutrient exchange (Oldroyd and Downie, 2008; Bapaume and Reinhardt, 2012).

Some legume species are widely exploited as crops, serving as a source of nutrients for human and animal consumption. The ability of legumes to develop symbiosis with nitrogen-fixing bacteria makes them suitable crops for the development of sustainable agriculture. However, little is known about the roles of LTPs in legume-rhizobium symbiosis. Therefore, in the current study, we examined the possible roles of these proteins in this process. We identified LTP family members in several model plant species, including seven species of legumes, using the criteria proposed by Edstam et al. (2011). To expand our knowledge of the evolutionary relationships of the LTPs, we constructed a phylogenetic tree based on the amino acid sequences of LTPs from legumes, cereals, and non-seed plants. Analysis of the gene structures of LTPs and their expression profiles in different organs of legumes and other species was performed based on several databases. Several LTPs are expressed in the roots and nodules of legumes. RT-qPCR assays showed that seven LTPs genes of common bean (*Phaseolus vulgaris* L.) were differentially expressed in roots at different stages of nodule development and that some of these genes were regulated by downregulation of the NADPH oxidase *PvRbohA*. Therefore, we hypothesize that LTPs could regulate legume-rhizobium

symbiosis under the regulation of ROS production, dependent on RBOHs.

MATERIALS AND METHODS

Identification of LTP Family Members

To identify *LTP* genes in various plants (15 species, **Supplementary Table 1**), several databases were searched. LTPs from 12 species were identified in the *Phytozome 12.1.6* database (<https://phytozome.jgi.doe.gov>) (Goodstein et al., 2012). LTPs from *Lotus japonicus* L. were identified by searching *LotusBase* (<https://lotus.au.dk>) (Mun et al., 2016). The *LIS: Legume Information System* database (<https://legumeinfo.org>) (Dash et al., 2016) was used to identify *LTPs* in the genomes of *Pisum sativum* L. and *Lupinus albus*. *A. thaliana* LTP amino acid sequences were used as queries in all BLASTP searches.

Gene/Protein Sequence Analysis, Chromosomal Localization, and Synteny Analysis of LTPs in Legume Species and *A. thaliana*

Exon-intron gene structure information was obtained from the corresponding databases (**Supplementary Table 2**). Gene structure mapping was carried out using Gene Structure Display Server 2.0 (http://gsds.gao-lab.org/Gsds_about.php). The conserved motifs and domains in the full-length amino acid sequences of all LTPs of legume species and *A. thaliana* were identified using the Multiple Expression motifs for Motif Elicitation version 5.2.0 tool (MEME) (Bailey et al., 2009). Chromosome localization was carried out with PhenoGram Plot (<http://visualization.ritchielab.org/phenograms/plot>) (Wolfe et al., 2013).

Synteny analysis was performed using the amino acid sequences of LTPs of *L. japonicus*, *Glycine max* L., *P. vulgaris*, *M. truncatula*, and *A. thaliana*. In all cases, a BLASTP file in m8 format and a simplified gff file were used as inputs for the MCScanx Collinearity Scanner Toolkit (<http://chibba.pgml.uga.edu/mcscan2/>) (Wang et al., 2012).

In silico Analysis of LTP Expression in *P. vulgaris*, *G. max*, *M. truncatula*, *L. japonicus*, and *A. thaliana*

The expression profiles of *P. vulgaris* LTPs were obtained from the Common Bean Gene Expression Atlas, PvGEA (<https://plantgrn.noble.org/PvGEA/>) (O'Rourke et al., 2014). The *LTP* expression profiles of *G. max*, *A. thaliana*, *S. moellendorffii*, *Sorghum bicolor* (L.) Moench, *O. sativa*, and *Z. mays* were retrieved from Bio-Analytic Resource for Plant Biology (BAR) (Schmid et al., 2005; Libault et al., 2010). The expression profiles of *M. truncatula* LTPs were downloaded from the *M. truncatula* Gene Expression Atlas (MtGEA) (Benedito et al., 2008; He et al., 2009). The expression profiles of *L. japonicus* LTPs were retrieved from LotusBASE (<https://lotus.au.dk/>) (Mun et al., 2016), and the expression profiles of *P. patens* LTPs were retrieved from PEATmoss (<https://peatmoss.online.uni-marburg.de/>) (Fernandez-Pozo et al., 2020). Heat maps of the transcript levels of the genes were constructed

using the heatmap.2 function of the gplot package (Warnes et al., 2015) with R Project.

Phylogenetic Analysis of LTPs

All protein sequences were aligned with the MUSCLE algorithm and manually edited using MEGA version X (Kumar et al., 2018) to remove misaligned sequences. The phylogenetic tree was constructed with the IQ-TREE algorithm 1.6.12 (Nguyen et al., 2014) using the maximum-likelihood method based on a VT+R9 substitution model with 1,000 bootstraps.

Wild-Type Plant Growth and Generation of Composite Plants

Common bean seeds (*P. vulgaris* cv. Negro Jamapa) were surface sterilized as described by Estrada-Navarrete et al. (2007) and incubated in a germination chamber for 2 days at 28°C in the dark. At 2 days postgermination (dpg), the seedlings were placed in pots with sterile vermiculite and inoculated with *Rhizobium tropici* (CIAT 899) at an OD₆₀₀ of 0.05 or they were not inoculated (control plants). All plants were watered three times per week with Fahraeus medium; control plants were treated with KNO₃ (10 mM) to avoid rhizobial contamination. At 7, 14, 21, and 30 days post-inoculation (dpi), the roots were harvested, frozen in liquid nitrogen, and stored at -75°C until RNA extraction.

To generate common bean composite plants (wild-type plants with transgenic hairy roots), 2 dpg seedlings were infected with *Agrobacterium rhizogenes* K599 strains carrying the *PvRbohA-RNAi* (Arthikala et al., 2017) and *PvRbohB-RNAi* (Montiel et al., 2012) constructs. As a control, composite plants carrying an inert RNAi vector were used (see details in Montiel et al., 2012); these plants are referred to as pTdT-SAC hereafter. All composite plants were generated as described by Estrada-Navarrete et al. (2007) with some modifications (**Supplementary Material**: protocol 1). Three to five days after removing the primary root, hairy roots of composite plants were observed under an epifluorescence microscope to confirm the presence of the reporter gene (*RFP*) and to remove non-transformed roots. Composite plants were potted in sterile vermiculite and inoculated with *R. tropici* (CIAT 899) as previously described. Hairy roots were harvested at 24 h after inoculation, frozen in liquid nitrogen, and stored at -75°C until RNA extraction.

RNA Extraction and qPCR Assays

Total RNA was extracted from frozen root tissue using TRIzol Reagent (Ambion[®], Life Technologies[™], USA) following the manufacturer's instructions. RNA integrity and concentration were verified by agarose gel electrophoresis (1%) and spectrometry (NanoDrop 2000/200c, Thermo Scientific, Waltham, MA, USA), respectively. DNA contamination was removed by incubation with RNase-free DNase (10 U/μl, Roche) at 37°C for 30 min. Complementary DNA (cDNA) was synthesized from 200 ng/μl of DNA-free RNA using RevertAid Reverse Transcriptase (200 U/μl, Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) assays were performed

TABLE 1 | Number of LTP genes identified in the analyzed species.

Species	LTP identified	LTP classes					
		LTP1	LTP2	LTPc	LTPd	LTPe	LTPg
<i>C. reinhardtii</i>	1	–	–	–	1	–	–
<i>M. polymorpha</i>	13	–	–	–	13	–	–
<i>P. patens</i>	28	–	–	–	20	–	8
<i>S. moellendorffii</i>	23	–	–	–	13	–	10
<i>Z. mays</i>	68	10	9	2	17	–	30
<i>S. bicolor</i>	63	10	13	1	17	–	22
<i>O. sativa</i>	73	19	13	3	12	–	26
<i>A. thaliana</i>	82	14	17	3	15	2	30
<i>P. vulgaris</i>	77	37	5	1	10	1	23
<i>G. max</i>	120	29	8	2	31	2	48
<i>M. truncatula</i>	95	45	6	1	15	1	27
<i>T. pratense</i>	85	39	5	–	18	1	22
<i>L. japonicus</i>	72	16	3	4	16	–	33
<i>L. albus</i>	87	36	9	1	11	2	28
<i>P. sativum</i>	73	32	3	–	16	1	21
	960						

using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, Waltham, MA, USA) on a RT-qPCR system (QuantStudio 5; Applied Biosystems, Waltham, MA, USA) as follows: 95°C for 10 min, 30 cycles of 95°C for 15 s, and 60°C for 60 s. The primer sequences used in the RT-qPCR assays are listed in **Supplementary Table 1**.

Relative transcript levels were calculated by the $2^{-\Delta\Delta CT}$ method using the transcript profile of the *elongation factor 1 α* (*EF1 α*) gene for normalization. The samples for the RT-qPCR assays were derived from three independent plants, with three biological replicates and three technical repeats performed per sample.

Statistical Analysis

The non-parametric Mann-Whitney test was performed using the *wilcox.test* function from the statistics package in R Project.

RESULTS

Phylogenetic Analysis Indicates That LTPs Are an Ancient Protein Family

Previous studies aimed at identifying the members of the LTP family have focused on only a few model species, such as *A. thaliana* and a few cereal crops (Edstam et al., 2011; Salminen et al., 2016). To analyze the potential roles of LTP subfamily members in legume-rhizobium symbiosis, we identified these proteins in the most recent versions of the genomes of 14 plants and a green alga from various databases (**Supplementary Table 1**). We searched for LTP homologs in each species, followed by domain analysis of homologous proteins to verify the presence of the characteristic lipid transfer protein domain of the LTP family. We identified 960 LTPs in the 15 species analyzed (**Table 1**, **Supplementary Table 3**).

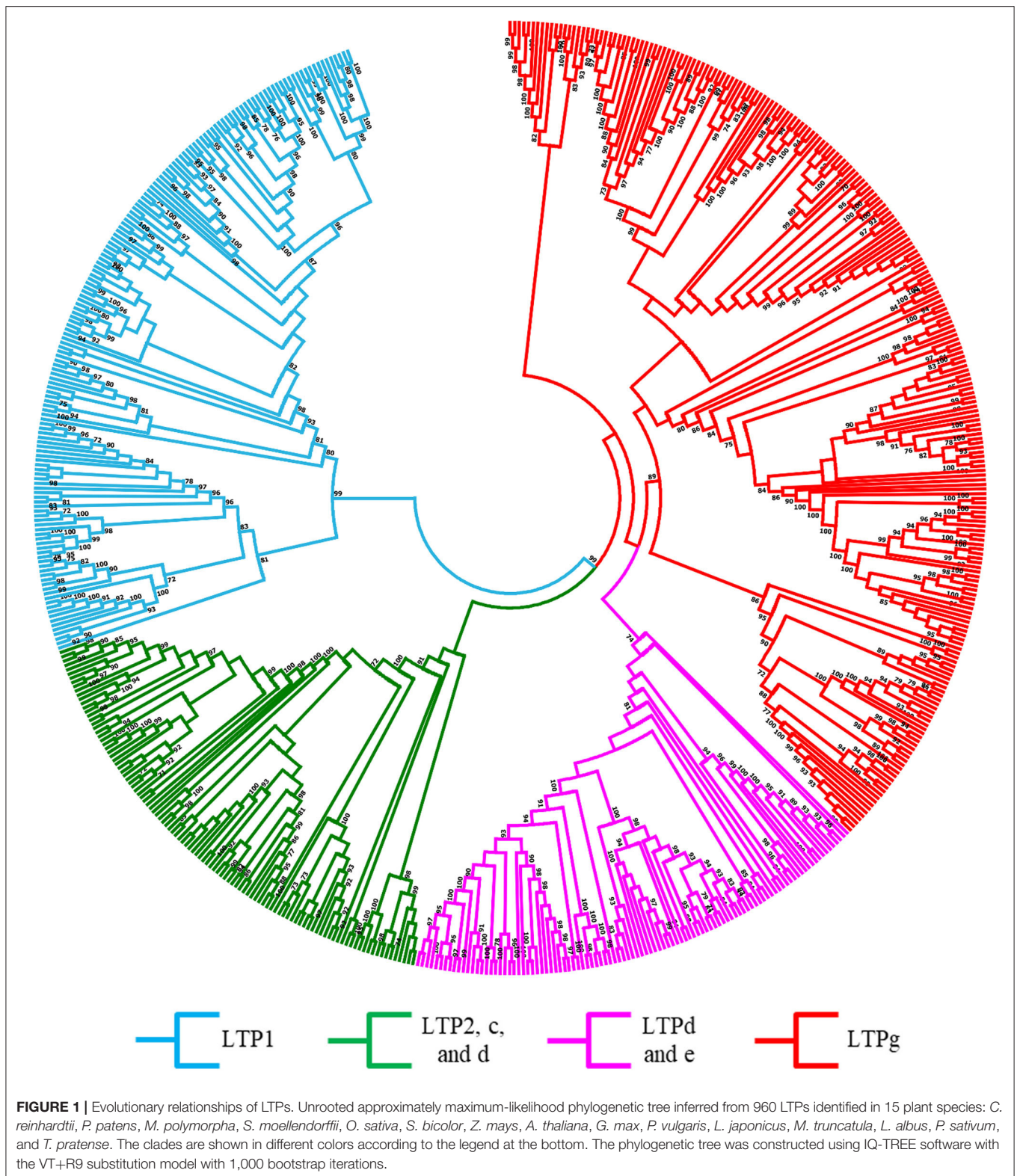
Remarkably, the *Chlamydomonas reinhardtii* P. A. Dang. genome had one significant hit related to the LTP family, making it the first reported LTP in a chlorophyte species. In addition, we identified at least 13 LTPs encoded in each terrestrial plant genome, with fewer homologous LTPs present in the analyzed marchantiophyta, bryophyte, and lycophyte species (**Supplementary Table 3**).

A robust phylogenetic analysis of the amino acid sequences of these 960 LTPs divided them into 4 major clades (**Figure 1**, **Supplementary Figure 1**). Following the guidelines proposed by Edstam et al. (2011), the clades were named according to the content of the characteristic 8CM motif C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C (**Figure 1**, **Supplementary Table 3**). One of these clades, consisting exclusively of the LTP1 class, was identified in all angiosperm species analyzed in this study (**Figure 1**, **Supplementary Figure 1**, light blue clade). Additionally, another clade consisting exclusively of the LTPg class was identified in all bryophyte and angiosperm species studied (**Figure 1**, **Supplementary Figure 1**, red clade). By contrast, two clades contained a mixture of classes, including one with classes LTP2, LTPc, and LTPd (**Figure 1**, **Supplementary Figure 1**, green clade) and the other with classes LTPd and LTPe (**Figure 1**, **Supplementary Figure 1**, lilac clade). At least one member of the LTPd class was present in all species analyzed. A few previously classified LTPs were reassigned based on a phylogenetic analysis and on the arrangement of 8CM motifs determined using the latest available versions of their genomes. These results suggest that LTPs are an ancient protein family that appeared before the first terrestrial plant; it was represented in the chlorophyte species by the LTPd class.

Features of LTPs Are Variable Among Plant Species

Our phylogenetic analysis showed that LTPs are a large family of conserved proteins in the phylum Plantae. However, some differences were detected in the physicochemical characteristics of LTPs in legumes vs. the other species. The angiosperm species contained more LTPs than the ancient lineages, suggesting that a major duplication event occurred during evolution. Although the total number of LTPs identified in legumes was similar to that in *A. thaliana* and cereals, the number of members in different classes varied. Our sequence analysis showed that LTPs in the legumes studied had a reduced number of members of the LTP2 class, with an average of 6 members, whereas *A. thaliana* and the cereal species had an average of 13 members (**Table 1**). By contrast, the LTP1 class was larger in legumes than in the other species, with an average of 33 members in the legume species studied and only an average of 13 members in the other angiosperm species (**Table 1**). These differential characteristics in the legumes could be related to their specific functions in this plant family, likely in root nodule symbiosis.

The 960 LTPs have variable amino acid length and molecular weight (MW), ranging from 55 to 793 amino acids and 8.8–20.8 kDa (**Supplementary Table 4**), respectively. In all species studied, the MW was closely related to the LTP class, with the



highest MW in the LTPg class and the lowest in the LTPc class (Supplementary Figure 2A). Similarly, the isoelectric point (IP) showed some differences between LTP classes, ranging from

3.7 to 11.1 (Supplementary Table 4, Supplementary Figure 1B). The LTPc class showed more acidic IP values, while the remaining LTP classes tended to show basic IP values. As expected, the LTPd

identified in the alga *C. reinhardtii* showed a slightly basic IP of 7.56, confirming this categorization of IP values by LTP class and the trend toward a basic IP for the LTPd class.

LTPs are characterized by the presence of a lipid transfer domain and an 8CM arrangement along the sequence (Figure 2B). To characterize these conserved motifs and examine their relationship to legume nodule symbiosis, we analyzed the LTP sequences of the legumes and *A. thaliana* using MEME software (Figure 2). In the 691 sequences examined, 15 different motifs with diverse cysteine arrangements were identified throughout the sequences (Figures 2A,C). The motifs were mostly class specific for all species analyzed, showing clear differential patterns between the LTP1 and LTP2 classes and the remaining classes. Classes LTP1 and LTPg showed five specific motifs, while classes LTPc and LTPd shared two or three motifs in some genes. The motif located in the LTP2 class (motif number two) was the longest and one of the most conserved motifs. Surprisingly, we identified a specific motif in the LTP1 class in legumes that form indeterminate nodules, suggesting it might play a specific role in this type of symbiotic nodule (Figures 2A,C). Our findings indicate that the physicochemical characteristics of LTPs in plants vary between classes, but also between species, suggesting specific functions for each species.

Synteny of LTP Genes in Legumes and *A. thaliana* Showed a Duplicated Gene Family

To analyze the distribution of LTP genes in the genomes of the legumes and *A. thaliana*, we performed *in silico* mapping of the gene loci using PhenoGram Plot. The LTP gene family is distributed among all *A. thaliana* and legume chromosomes (Supplementary Figure 2). The LTP loci are clustered in a particular manner in some legume chromosomes by LTP class, which was not observed in *A. thaliana*. In *P. vulgaris*, 51% of the LTP1 genes are located on chromosome 9, while 40% of LTP1 genes are located on chromosome 7 in *M. truncatula*. Similarly, 50% of the LTPd genes are located on chromosome 18 in *G. max*, and 50% of LTPg genes are clustered on chromosomes 1 and 5 in *L. japonicus*.

We explored the evolutionary pathways of the LTP genes in the four legumes and *A. thaliana* individually and between species using MCScanx software. Individual syntenic analysis showed that *A. thaliana*, *P. vulgaris*, *L. japonicus*, and *M. truncatula* have a low percentage of LTP collinear genes in their genomes (10–17%), whereas *G. max* contains 52% of these genes (Supplementary Table 4, Supplementary Figure 3). These collinear genes represent less than half of the percentage of genetic synteny in the overall genomes of *A. thaliana* and *P. vulgaris*, which range from 27 to 29%. By contrast, in *L. japonicus* and *M. truncatula*, these collinear genes represent 1- or 2-fold that of their overall genomes, i.e., 5–13% (Supplementary Figure 4).

Syntenic analysis between species revealed 112 collinear LTP genes between *P. vulgaris* and *G. max*; 46 between *P. vulgaris* and *L. japonicus*; 54 between *P. vulgaris* and *M. truncatula*; 87 between *L. japonicus* and *G. max*; 93 between *G. max* and *M. truncatula*; and 49 between *L. japonicus* and

M. truncatula (Figures 3A–C,E–G, Supplementary Table 4). Within these collinear genes, the LTPg class represents the highest percentage of syntenic genes (58%), followed by the LTP1 and LTPd classes (17 and 14%, respectively). Analysis of the collinearity between the LTPs of these four legumes against *A. thaliana* revealed 124 legume genes that are syntenic to those of the Brassicaceae. Of these 124 syntenic genes, 29 belong to *P. vulgaris*, 21 to *L. japonicus*, 52 to *G. max*, and 22 to *M. truncatula* (Figure 3D, Supplementary Table 5). *G. max* has the highest number of syntenic genes, and *M. truncatula* has the lowest number compared with *A. thaliana*, which are mainly represented by the LTPg class in all instances. These results indicate that LTP genes were subjected to various duplication events throughout the evolution of legumes.

Conserved Exon-Intron Arrangement of LTP Genes in Legumes and *A. thaliana*

To analyze the functional roles of LTP genes, we assessed the exon-intron organization of the LTP genes in *P. vulgaris*, *G. max*, *L. japonicus*, *M. truncatula*, *T. pratense*, and *A. thaliana* using Gene Structure Display Server 2.0 software. The positions of the introns are variable between all species analyzed and the LTP classes, but the number of introns showed little variation. In *A. thaliana*, four LTP genes lack introns (*AtLTP2.8*, *AtLTP2.11*, *AtLTPe.2*, and *AtLTPg.18*); the remaining genes of the LTP1, LTPc, and LTPd classes have one to three introns, with more genes containing three introns; genes of the LTP2 class have one or two introns; *AtLTPe.1* has one intron, while LTPd genes have two to four introns, most with four introns (Figure 4A).

Among legumes, *M. truncatula* and *P. vulgaris* have the highest number of LTP genes without introns (20 and 11, respectively), followed by *G. max* and *L. japonicus* (6 and 1, respectively); all these genes are distributed among LTP1, LTPc, LTPd, LTPe, and LTPg (Figures 4B–F). Legumes that form determinate nodules have one to three introns in the LTP1 class (Figures 4B–D), while genes belonging to this class from legumes that form indeterminate nodules have up four introns (Figures 4E,F). Additionally, the classes LTP2, LTPd, and LTPg exhibit a homogeneous intron distribution, with two, one to three, and one to five introns, respectively, in almost all legumes. Finally, genes in the LTPc and LTPe classes contain one to three introns, depending on the legume species. Although some conservation of introns was observed among the six plants analyzed, more introns are present in LTP1 and LTPg genes in the legumes than in *A. thaliana*. However, more similarities between this non-legume dicot and legumes that form determinate vs. indeterminate nodules were observed.

Analysis of the Expression Profiles of LTP Genes

To analyze the expression profiles of the LTP genes in various plant organs, we retrieved expression data for ancient species, cereals, legumes, and *A. thaliana*, available in different gene expression atlases. Comparison of expression data between plant species and between LTP classes was performed. The results are presented in the following sections.

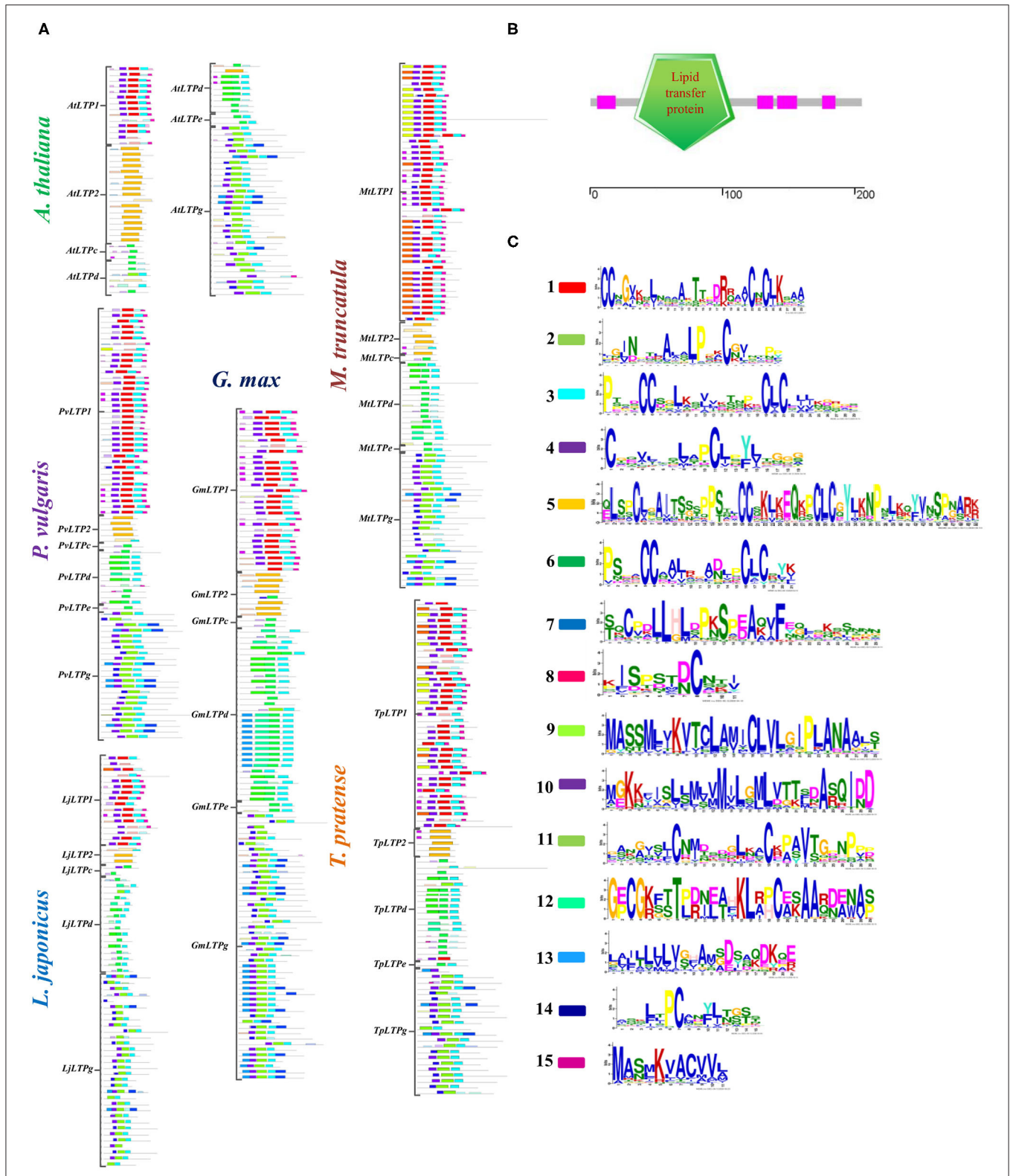
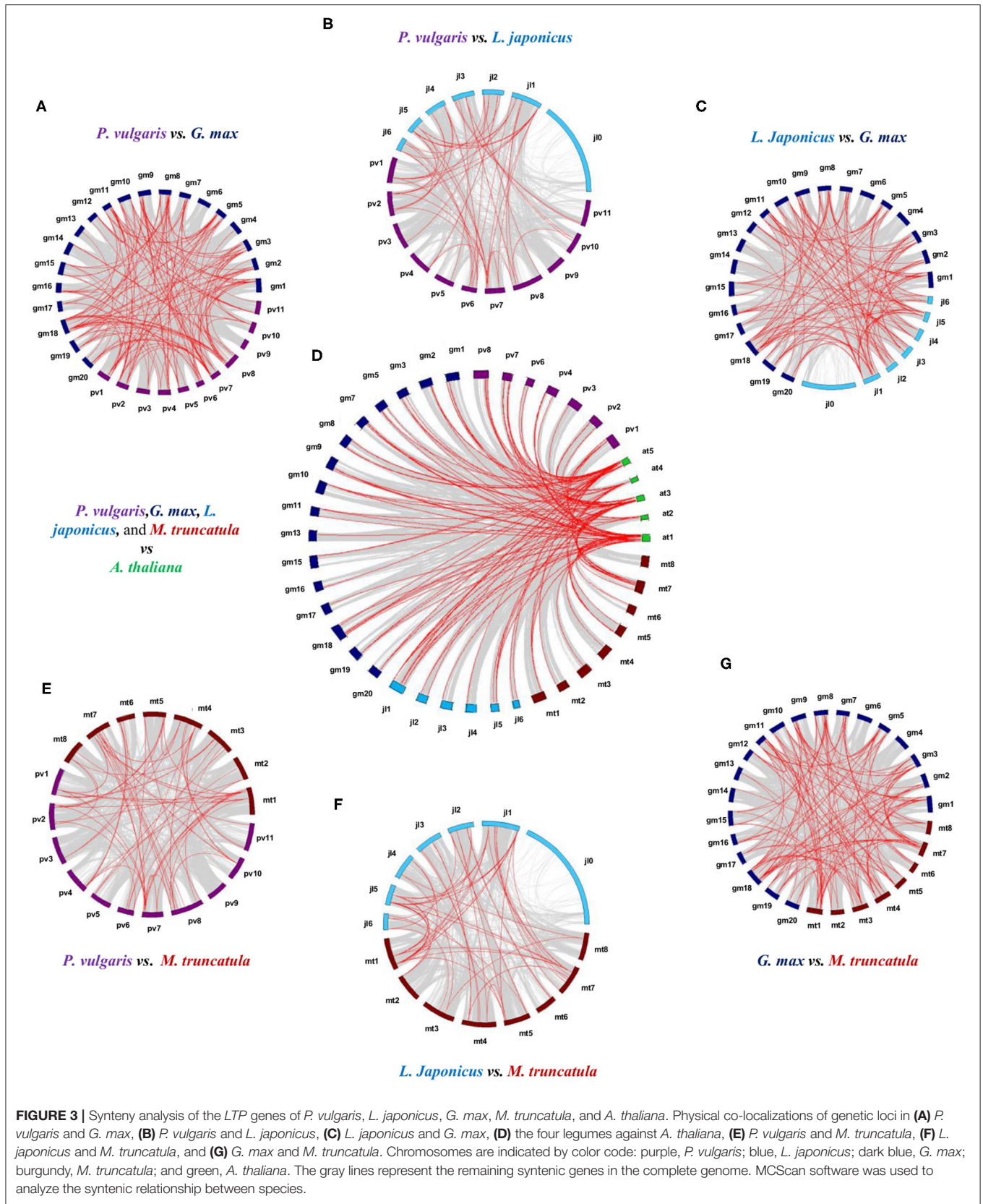
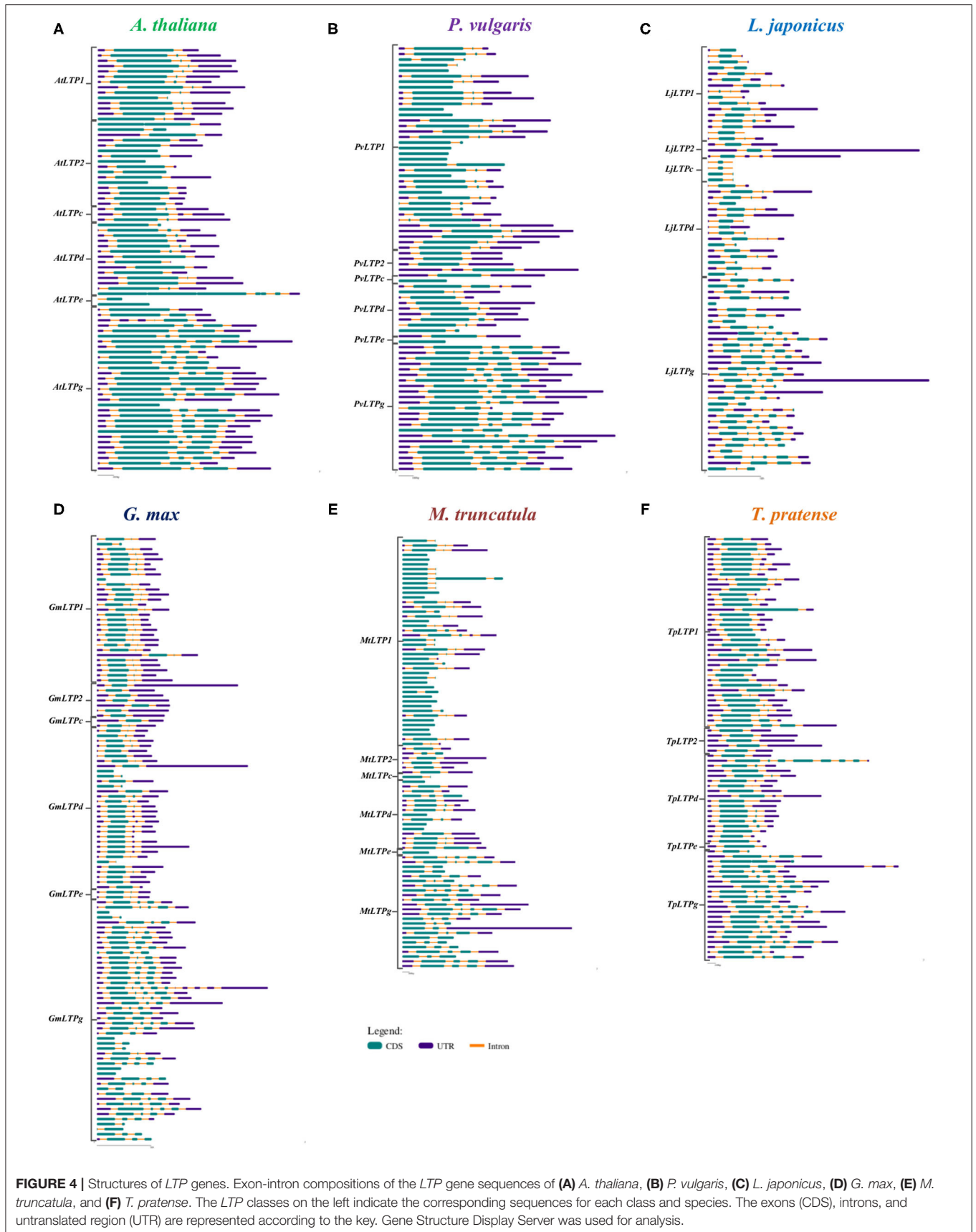


FIGURE 2 | Conserved functional motifs in LTPs. **(A)** Schematic representation of the motifs identified in LTP amino acid sequences from *P. vulgaris*, *G. max*, *L. japonicus*, *M. truncatula*, *T. pratense*, and *A. thaliana*. Significantly overrepresented motifs are shown by bars in their predicted positions. **(B)** Characteristic lipid transfer domains in LTP sequences. **(C)** Logo of the identified overrepresented motifs; the color code is the same as in **(A)**. The LTP classes on the left indicate the corresponding sequences for each class and species. MEME software was used to identify significantly overrepresented motifs.





Ancient Plants

Expression data for *P. patens* (a bryophyte) and *S. moellendorffii* (a lycophyte) were retrieved from the BAR for Plant Biology database. These species only possess the *LTPd* and *LTPg* classes, which exhibit differential expression patterns between species (**Supplementary Figures 5A,B**). In *P. patens*, the *LTPd* class (*LTPd15*, *LTPd17*, and *LTPd18*) is highly expressed in whole gametophores, gametophores without rhizoids, and green sporophytes (**Supplementary Figure 5A**). Genes in the *LTPg* class are expressed 90% lower than *LTPd* genes, but are expressed in germinating spores and protonema. In *S. moellendorffii*, the *LTPd* class (*LTPd.8* and *LTPd.9*) is mainly expressed in roots and rhizophores, while the *LTPg* class (*LTPg.9*) is 99% higher expressed in strobili than in other tissues (**Supplementary Figure 5B**). These data suggest that the *LTPd* class is more strongly associated with aboveground tissue in bryophytes and belowground tissue in lycophytes, but members of the *LTPg* class are more closely involved in reproductive organ activity in both species. However, additional comparative analyses with ancient species are needed to test this hypothesis.

Monocotyledons

To analyze the expression patterns of *LTP* family genes in monocot species, we also retrieved expression data for the cereal species, *S. bicolor*, *O. sativa*, and *Z. mays* from the BAR for Plant Biology database. The *LTP* genes are mainly expressed in the shoot systems (basically leaves, stems, flowers, and seeds) of the three monocot species, with *LTP1* genes being the most highly represented class (**Supplementary Figures 5C–E**). In *S. bicolor*, the most highly expressed gene is *SbLTP1.9* in the vegetative meristem, followed by *SbLTP1.3* in shoots (**Supplementary Figure 5C**). In *O. sativa*, the most highly expressed gene is *OsLTP1.4* in stems, followed by *OsLTP2.4* in stems (**Supplementary Figure 5D**). The most highly expressed gene in *Z. mays* is *ZmLTP1.8* in young seeds (**Supplementary Figure 5E**). In these cereal species, the *LTP* genes were expressed 70% lower in belowground vs. aboveground tissue, where genes in classes *LTP1* and *LTPd* are mainly expressed (**Supplementary Figures 5C–E**). *SbLTPd.10* and *ZmLTPd.10* show the highest expression levels in *S. bicolor* and *Z. mays*, respectively, whereas 11 *OsLTP1* genes and *OsLTP2.3* show the highest expression levels in *O. sativa*. These data suggest that genes belonging to the *LTP1* class could play various roles in shoot development and reproduction in cereals.

Non-legume Dicotyledons

LTP family proteins have been studied more extensively in *A. thaliana* than in any other species. In the present study, *A. thaliana* was included as a model non-legume dicotyledon to compare the expression profiles of *LTPs* in legumes against dicotyledons. We retrieved expression data for *A. thaliana* from the BAR for Plant Biology database. In this dicot species, *AtLTP* genes are expressed at their highest levels in siliques and flowers, followed by leaves and seeds (**Figure 5A**). The *AtLTP1*, *AtLTP2*, and *AtLTPd* classes are the most highly represented classes in these organs, whereas genes in the *LTPg* class are expressed 67% less. Despite the generally lower expression levels of *AtLTP* genes

in roots compared to siliques and flowers, *AtLTP2.5*, *AtLTP2.11*, *AtLTPd.4*, and *AtLTPg.19* are more highly expressed in roots than in these organs. These results suggest that members of the *LTP* family could play an important role in the reproductive process of this dicot species, as observed in the monocot species.

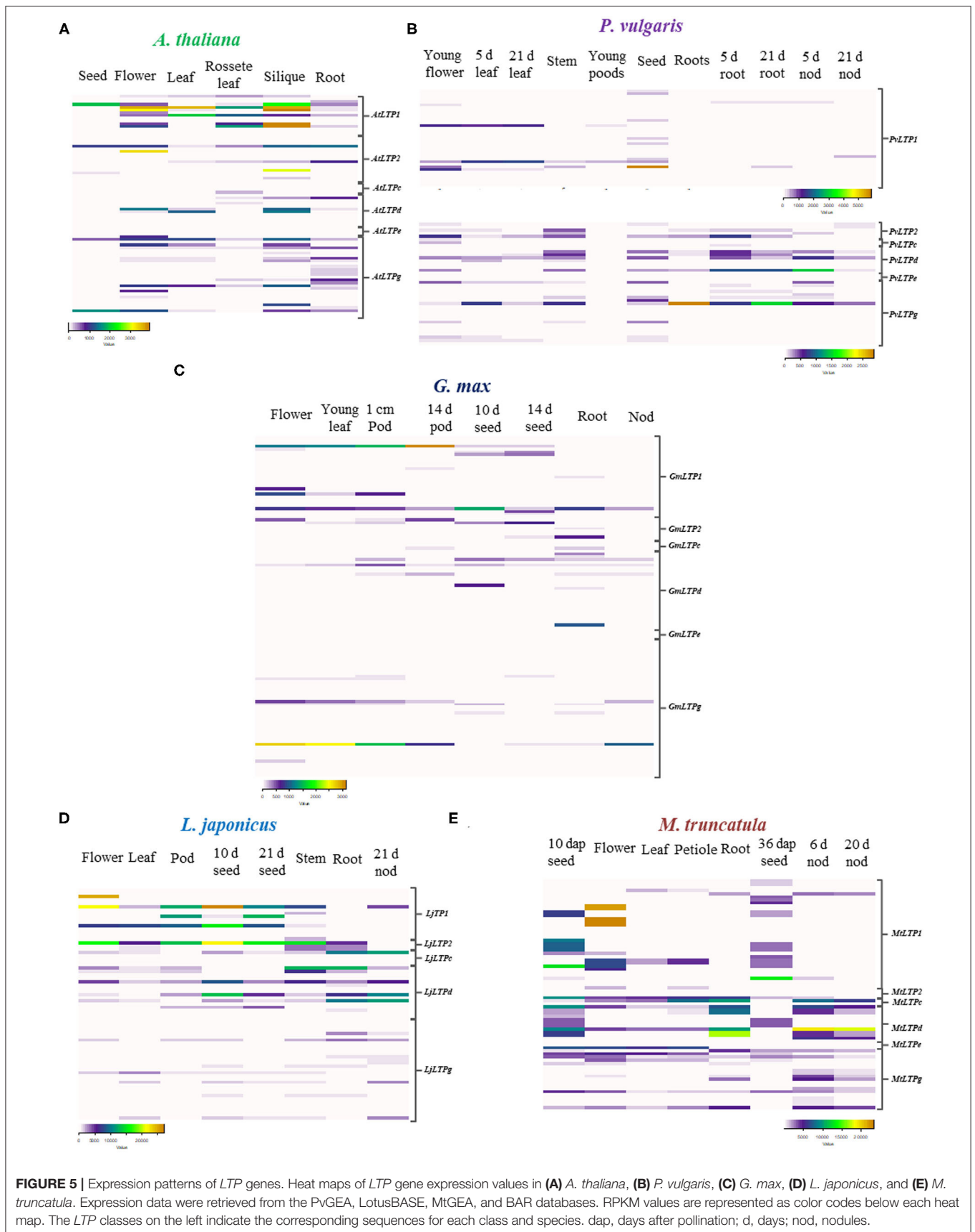
Legumes

To analyze the possible roles of *LTP* family members in root nodule symbiosis, we evaluated the expression patterns of these genes in the following legumes: *P. vulgaris*, *G. max*, *L. japonicus*, and *M. truncatula*. We observed both similarities and differences in the expression patterns of *LTP* genes in these legumes compared to non-legumes species. Genes in classes *LTP1* and *LTP2* are highly expressed in leaves, flowers, and seeds at different stages of development (**Figures 5B–E**). Although some *LTP* genes are highly expressed in flowers and seeds in all four legumes, the different classes of *LTPs* are also highly expressed in roots and nodules of the legumes (**Figures 5B–E**).

In *P. vulgaris*, *PvLTPg.11* is the most highly expressed gene in roots inoculated with rhizobia and in young nodules, followed by *PvLTPd.4*, *PvLTPd.6*, and *PvLTPd.10* (**Figure 5B**), whereas in *G. max*, *GmLTPd.1* and *GmLTP1.1* are the most expressed genes in roots (**Figure 5C**). *LjLTPd.1*, *LjLTPd.2*, *LjLTPd.3*, and *LjLTPc.1* are primarily expressed in roots and mature nodules of *L. japonicus* (**Figure 5D**). In *M. truncatula*, which forms indeterminate nodules, *MtLTPd.1*, *MtLTPd.2*, and *MtLTP2.1* show high expression levels in young and mature nodules and roots (**Figure 5E**). Among legumes, the *LTP1* and *LTPd* classes are the most commonly represented classes in roots and nodules, showing a differential expression pattern during early and late stages of nodulation.

These data suggest that some *LTP* classes are active in legume roots, roots inoculated with rhizobia, and nodules. To validate the expression patterns of *LTP* genes in the legumes analyzed *in silico*, we evaluated the expression profiles of seven *P. vulgaris* genes during the early and late stages of nodulation by RT-qPCR. We selected *LTP* genes that are potentially expressed under nodulation conditions in this legume that might also be expressed in the three other legumes examined. Also, we analyzed the expression profile of these genes in a home transcriptomic data of *P. vulgaris* roots inoculated and non-inoculated at 7 dpi with *R. tropici* (Fonseca-García et al., 2019), here they showed the highest fold changes with the highest read counts of the gene family in this legume (**Supplementary Table 6**). These seven genes (*PvLTP1.6*, *PvLTP2.3*, *PvLTP2.5*, *PvLTPd.4*, *PvLTPd.6*, *PvLTPd.10*, and *PvLTPg20*) presented two different expression profiles during nodulation in *P. vulgaris* (**Figure 6**).

Two genes were downregulated during the late stages of nodulation: *PvLTP1.6* was significantly suppressed at 14, 21, and 30 dpi, while *PvLTP2.3* was clearly downregulated at 14 and 21 dpi (**Figures 6A–C**). By contrast, 4 genes were upregulated between the early and late stages of nodulation: *PvLTPd.6*, *PvLTPd.10*, and *PvLTPg20* were upregulated at 7, 14, and 21 dpi, and *PvLTPg20* was also upregulated at 30 dpi (**Figures 6E–G**). Similarly, *PvLTPd.4* was upregulated at 7 and 14 dpi, but, intriguingly, it was downregulated at 30 dpi (**Figure 6D**). These results indicate that these seven *LTP* genes are differentially



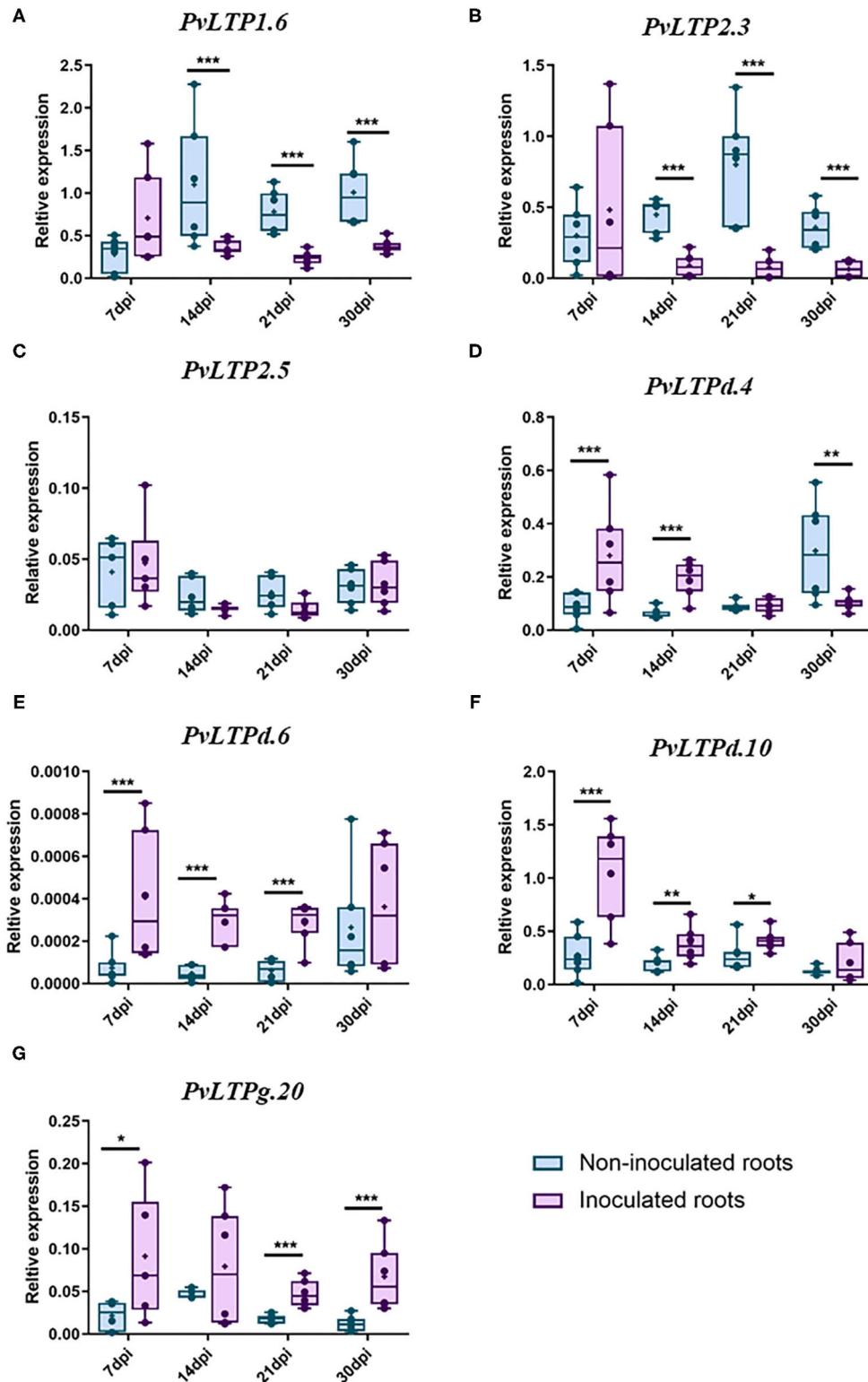


FIGURE 6 | RT-qPCR analysis of selected *P. vulgaris* LTP genes. The relative expression profiles of seven LTP genes from wild-type *P. vulgaris* roots inoculated or non-inoculated with *R. tropici*: (A) *PvLTP1.6*, (B) *PvLTP2.3*, (C) *PvLTP2.5*, (D) *PvLTPd.4*, (E) *PvLTPd.6*, (F) *PvLTPd.10*, and (G) *PvLTPg.20*. The transcript abundance of the selected genes was evaluated by RT-qPCR and normalized according to the expression of the *elongation factor 1 α* (*EF1 α*) gene. The blue bars represent non-inoculated roots, and the purple bars represent roots inoculated with *R. tropici*. The borders of the boxes represent the first quartile to the third quartile, the horizontal line within the box represents the median, and the whiskers represent the smallest and the largest outlier point of the data set ($n = 6$). A non-parametric Mann-Whitney test was performed to evaluate significant differences, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

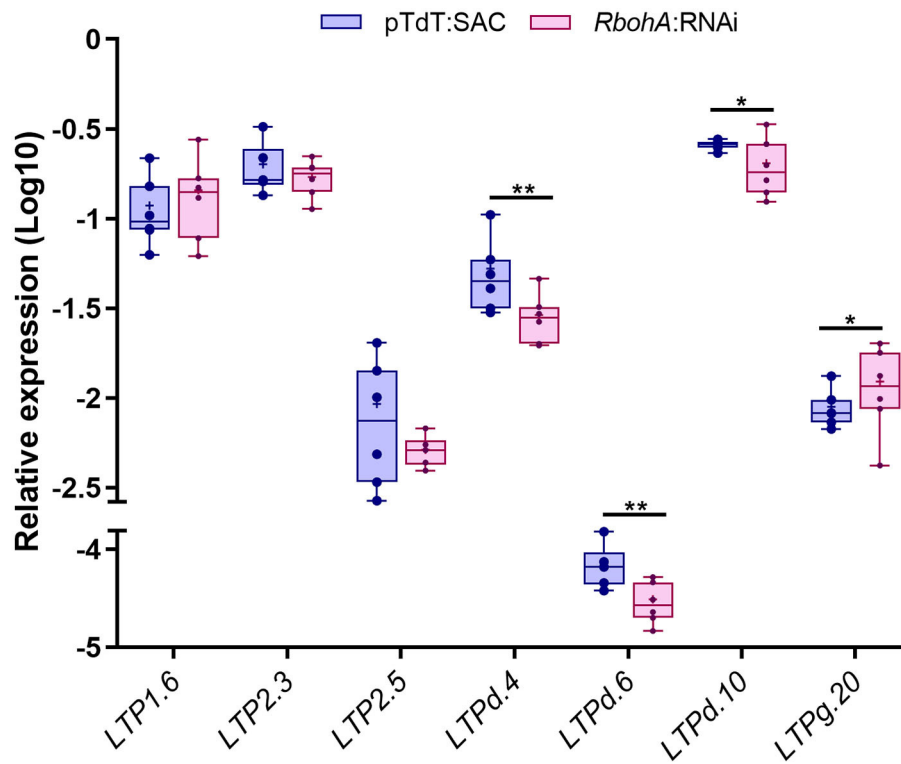


FIGURE 7 | RT-qPCR analysis of selected *P. vulgaris* LTP genes in *RbohA:RNAi* transgenic roots. Relative expression profiles of the seven LTP genes in control (pTdt:SAC) and *RbohA:RNAi* transgenic roots of *P. vulgaris* inoculated with *R. tropici*. The transcript levels were normalized according to the expression of the *elongation factor 1 α* (*EF1 α*) gene. The blue bars represent control (pTdt:SAC) transgenic roots, and the purple bars represent *RbohA:RNAi* transgenic roots inoculated with *R. tropici*. The borders of the boxes represent the first quartile to the third quartile, the horizontal line within the box represents the median, and the whiskers represent the smallest and the largest outlier point of the data set ($n = 6$). A non-parametric Mann-Whitney test was performed to evaluate significant differences, * $p \leq 0.05$, ** $p \leq 0.01$.

expressed during different stages of nodulation in common bean, suggesting that they are fine-tuning regulated in root nodule symbiosis.

Previously, our group demonstrated that ROS play an important role in common bean nodulation through RBOHs activity (Cárdenas et al., 2008; Montiel et al., 2012; Arthikala et al., 2017). To investigate the possible roles of these seven *PvLTP* genes in nodulation in more detail, we evaluated their transcript accumulation in transgenic roots expressing an RNA interference construct targeting *PvRbohA* following inoculation with *R. tropici*. *PvRbohA* encodes a NADPH oxidase required for the early stages of root nodule development in common bean (Arthikala et al., 2017). All three *LTPd* genes were downregulated with 23–54% less in the inoculated roots carrying the *RbohA:RNAi* construct at 1 dpi than in control roots, whereas *LTPg.20* was upregulated with 38% more under the same conditions (Figure 7). Furthermore, the *LTP2.5* gene presented a downregulation tendency with 44% less in the *RbohA:RNAi* roots than control, but *LTP1.6* and *LTP2.3* were expressed at similar levels in silenced and control roots. These results indicate that five of the seven genes analyzed could be regulated by ROS, produced by *RbohA* during the early stages of rhizobial symbiosis.

DISCUSSION

Legumes are valuable crops that serve as sources of food and animal feed throughout the world. These plants interact with soil bacteria called rhizobia, which through a molecular dialog and a coordinated cellular reorganization in the plant cortex, form a new organ, the nodule. In the nodules, rhizobia differentiate into bacteroids and are enclosed to form symbiosomes, in which atmospheric dinitrogen is transformed into ammonia, a source of nitrogen that is assimilable by the plant (Roy et al., 2020). In plants, *LTP* genes comprise an abundant family of proteins and perform various functions in their life cycle (Edqvist et al., 2018). This gene family has been identified in some important plant species such as, *A. thaliana*, rice, wheat (Boutrot et al., 2008), maize (Wei and Zhong, 2014), barley (Zhang et al., 2019) and potato (Li et al., 2019). However, little is known about *LTPs* in legumes and their role in the root-nodule symbiosis. In this study, we performed a genome identification of *LTPs* family in various legumes species and other important plant model, moreover, we analyzed the putative roles of *LTPs* in rhizobial symbiosis.

Through a robust phylogenetic analysis, we identified 960 *LTP* genes in 15 different plant species, which were grouped into 6 classes (*LTP1*, *LTP2*, *LTPc*, *LTPd*, *LTPe*, and *LTPg*), according to

their phylogenetic features and the characteristic motif 8CM. We used the latest versions of the genomes of all species studied, allowing us to use the complete sequences of the putative *LTP* genes instead of the ESTs used in previous studies (Edstam et al., 2011); consequently, some previously reported *LTP* genes were reassigned in the current study. In addition, 30 genes showed incomplete 8CMs with at least 1 missing C; however, these sequences were considered to be *LTPs* because they all possess characteristic lipid transfer domains. This modification occurred in the 15 species studied and in the 6 classes of *LTPs*, suggesting that this feature was conserved during evolution. Notably, we identified the first reported *LTP* gene (*LTPd*) in an algae species, *C. reinhardtii*, indicating that *LTPs* are an ancient family of genes that has been present since the division of Chlorophyta, before the first land plant appeared.

Our phylogenetic analysis clustered the 960 *LTPs* into 4 clades (**Figure 1**, **Supplementary Figure 1**). Ancient species here studied, *P. patens* (Rensing et al., 2020) and *S. moellendorffii* (Ferrari et al., 2020), only contained classes *LTPd* and *LTPg*, suggesting that these two classes are the oldest in this gene family. On the contrary, the *LTPe* class was present only in dicot species, indicating that the class is specific to these angiosperms and that these genes could be the youngest members of the *LTP* family (**Table 1**). Additionally, we detected notable differences in the number of *LTP1* and *LTP2* genes among monocotyledonous and dicotyledonous species. In particular, legumes had fewer *LTP2* genes and more *LTP1* genes than the other species (**Table 1**), which contained a specific motif in legumes that form indeterminate nodules (**Figures 2A,C**). These results suggest that these classes of genes exhibit specific gene duplication, as well as structural modifications in legumes that could have specific functions in these plants, which has also been suggested in other plant systems (Moore and Purugganan, 2005; Conant and Wolfe, 2008; Flagel and Wendel, 2009).

Genome duplication is a primary source of genetic novelty, providing increased functionality in angiosperms and has played a fundamental role in their evolution (Davies et al., 2004). Many reports have documented duplication events of *LTP* genes in various species, such as *A. thaliana*, rice, sorghum (Salminen et al., 2016), potato (Li et al., 2019), maize (Wei and Zhong, 2014), and wheat (Kouidri et al., 2018; Odintsova et al., 2019). Gene duplication and functional redundancy allow *LTP* gene sequences to gather mutations, that result in increased divergence and thus the expansion and evolution of the gene family (Conant and Wolfe, 2008). Our synteny analysis of *LTP* genes revealed a conserved homology between some genes in the four legumes and *A. thaliana*. We observed a low percentage of collinear *LTP* genes in the individual genomes of *A. thaliana*, *P. vulgaris*, *L. japonicus*, and *M. truncatula*, while more than half of the *LTP* genes in *G. max* showed collinearity (**Supplementary Table 4**, **Supplementary Figure 3**). This could be due to *G. max* is an allotetraploid species that originated from hybridization events involving two different diploid progenitors (Cannon and Shoemaker, 2012). Also, we observed a higher percentage of collinear genes between *G. max* and the other legumes than within *A. thaliana*, *P. vulgaris*, *L. japonicus*, and *M. truncatula*. The *LTP* class with the most syntenic genes was *LTPg*, which was

generally observed in all species tested with an extended number of members. It could be because *LTPg* class regulate the export of cuticular wax and suberin (Kim et al., 2012; Lee and Suh, 2018), important processes for plant development.

Gene function has been associated with different structural characteristics such as exon-intron composition, which bear the marks of the evolution of a gene family (Moore and Purugganan, 2005; Flagel and Wendel, 2009). According to our data, the *LTP* family formed before the differentiation of terrestrial plants and generated new classes during their evolution. The dicotyledonous species analyzed have a variable arrangement of exon-introns among the *LTP* classes, and legume genes have more introns than *A. thaliana* genes (**Figure 4**). Also, legumes have more genes without introns in all *LTP* classes compared to *A. thaliana*. *M. truncatula* has the highest number of genes without introns (22, including 17 in the *LTP1* class), followed by *P. vulgaris* (10 genes) and *A. thaliana* (only 5 genes). A compact genetic structure could be advantageous to plants responding to stimuli by allowing rapid gene expression (Jeffares et al., 2008). However, the expression patterns of intron less *LTP* genes in legumes were similar to those in *A. thaliana* (**Figure 5**).

Even though many potential *LTP* genes have been identified in some plant species using bioinformatics approaches, the functions of only a few *LTPs* have been revealed. However, some reports suggest that the mechanism of action of *LTPs* is related to the transfer and deposition of the monomers required for the assembly of the waterproof lipid barriers (Charvolin et al., 1999; Han et al., 2001; Cheng et al., 2004). Root nodule symbiosis involve two complex processes that are simultaneously coordinated, rhizobial infection and nodule development, where the number of dividing cells increases dramatically, requiring membrane biosynthesis by the plant (Roy et al., 2020). Also, for the formation of symbiosomes the synthesis of the peribacteroidal membrane to enclose the bacteroids is required (Verma, 1992). The mentioned ability of *LTPs* to bind to and transfer a wide variety of lipids suggests that these proteins participate in membrane remodeling during root nodule symbiosis. In fact, the *LTP* AsE246 from Chinese milk vetch binds DGDG from nodule extract and its knockdown impairs symbiosomes development (Lei et al., 2014). In addition, *in silico* analysis of the expression profiles showed that many *LTP* genes, especially *LTPd* genes, are active in legume roots and nodules (**Figure 5**).

RT-qPCR assays confirmed that genes belonging to the *PvLTPd* and *PvLTPg* classes are highly expressed in *P. vulgaris* roots inoculated with rhizobia during the early and late stages of nodulation (**Figures 6D–G**). In addition, genes of the *PvLTP1* and *PvLTP2* classes are repressed during late stages of nodulation in this legume (**Figures 6A–C**). Indeed, a novel *LTP* gene in Chinese milk vetch might be involved in the establishment of rhizobial infection and the transport of plant-synthesized lipids to the symbiosome membrane (Lei et al., 2014). Another report suggests that an *AtLTP1* gene is involved in regulating the ethylene response and signaling in *A. thaliana* (Wang et al., 2016), importantly, this phytohormone is a well-known negative regulator of nodulation. Our results suggest that genes of the *PvLTPd* and *PvLTPg* classes could

mediate positively root nodule symbiosis, transporting plant-synthesized lipids. Whereas, members of the *PvLTP1* and *PvLTP2* classes might inhibit this process interacting with negative regulators.

ROS function as early signals in root hairs during the establishment of root nodule symbiosis (Montiel et al., 2016). In root hair cells of *P. vulgaris*, a rapid, transient ROS burst was detected 15–30 s after treatment with Nod factors (Cárdenas et al., 2008). In plants, ROS are mainly produced by the activity of the NADPH oxidase enzymes RBOHs, which have been implicated in effective rhizobial infection and in promoting nodule organogenesis in *P. vulgaris* (Montiel et al., 2012; Arthikala et al., 2014) and *M. truncatula* (Marino et al., 2011). In the current study, we provided evidence that the expression of *LTPd* and *LTPg* class genes is regulated by the downregulation of a *PvRBOH* (*PvRBOHA*) in *P. vulgaris* roots during the early stages of nodulation. Our findings strongly suggest that *LTP* genes could play different roles during nodulation in *P. vulgaris*, and possibly interacting with RBOH-dependent ROS production since the early stages. ROS as early signals in this symbiotic process could activate the synthesis of lipids in the plasma membrane necessary for rhizobia infection and nodule organogenesis, however further functional analysis is needed to confirm this interaction.

CONCLUSIONS

In this study, we performed a comprehensive genome analysis of the LTP family in legumes and other important model plants. We identified 960 LTPs in 14 plant species and 1 green alga, which were grouped into 4 clades according to the LTP class. We report the first *LTP* gene identified in a chlorophyte species, *C. reinhardtii*, indicating that the LTP family appeared before the first terrestrial plant. Sequence analysis showed that the gene and protein structures of the LTP family members are highly conserved among species, but clear differences were observed between the LTP classes. Expression profiling analyses revealed that *PvLTP* genes are differentially expressed during the early and late stages of nodulation and that they might be regulated by RBOH-dependent ROS production. These findings shed light on the possible roles of LTP family members in the legume-rhizobium symbiosis, but functional analyses are necessary to

increase our knowledge and, as a good start, *PvLTP* genes could be good candidates.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Phytozome 12.1.6 database <https://phytozome.jgi.doe.gov>; LotusBase <https://lotus.au.dk>; LIS: Legume Information System database <https://legumeinfo.org>.

AUTHOR CONTRIBUTIONS

CQ and CF-G conceived the study. CF-G and JS-M analyzed the data. RP and CF-G collected some of the plant tissues and performed RNA extraction and purification, cDNA synthesis, and RT-qPCR assays. CQ, CF-G, and RP drafted, edited, and revised the manuscript, which was approved by all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fagro.2021.660100/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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