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Characterization of Molecular Properties and Expression of Gene *GmPLMT* and Its Effects on the Production of Lipid Metabolites in Soybean and *Arabidopsis thaliana*

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Abstract: Phospholipid N-methyltransferase (PLMT) plays an important role in the synthesis of phosphatidylcholine (PtdCho). The aim of this study was to characterize the molecular properties of *GmPLMT* and the expression of soybean *GmPLMT* and its effects on the production of lipid metabolites. Results showed that *GmPLMT* composed of mainly α -helix was a hydrophobic and transmembrane protein. In soybean leaves, *GmPLMT* was highly expressed during seedling and flowering stages. In transgenic *Arabidopsis thaliana*, the highest and lowest expression levels of *GmPLMT* were detected at flowering and maturity stages, respectively. The total phospholipid contents in soybean grains were decreased from 7.2% (35 days after flowering) to 4.8% (55 days after flowering) and then increased to 7.0% (75 days after flowering). The contents of PtdCho showed a similar pattern to that of total phospholipids. In transgenic *A. thaliana* seeds, the contents of total phospholipids and PtdCho were significantly increased. Significantly positive correlations were revealed between expression of *GmPLMT* and contents of both PtdCho and crude fats, and between the contents of PtdCho and both linoleic acid and linolenic acid, suggesting that increased expression of *GmPLMT* improved the production of lipid metabolites. This study provided solid experimental evidence for further improvement of soybean quality based on *GmPLMT* in the molecular breeding of soybeans.

Keywords: phospholipid compounds; *GmPLMT*; lipid metabolism; soybean; *Arabidopsis*

1. Introduction

Soybean is an important crop for both food and oil, providing a large amount of high-quality nutrients such as proteins and phospholipids. Soybean grains are rich in fats and proteins, and the contents of fatty acids in the grains account for more than 90% of the total oil. Among the fatty acids, two types of unsaturated fatty acids (i.e., linoleic acid and linolenic acid) not synthesized by humans and other mammals are necessary for normal growth and development. The phospholipids are the fundamental materials of life and are important structural components of cell membranes in tissues of both humans and other animals [1,2]. As the indispensable substances for maintaining normal metabolism and the healthy human body, phospholipids are mainly obtained from soybeans. Soybean phospholipids can not only regulate the activities of enzymes related to membrane functions, but also control the information and substances inside and outside the cell membrane. For example, unsaturated fatty acids in soybean phospholipids improve the functions of biofilm [3]. Soybean phospholipids also improve the antioxidant activities and enhance

the nutritional functions of the body [3]. As the main components of phospholipids, both the phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) are important metabolites for plant growth and development, and as the main lipid components of the plant cell membrane, these two types of metabolites account for 40–60% of the total phospholipids [4,5]. PtdCho has also been used as a type of nutritional additive to provide the phospholipids needed in animals to improve the immunity and survival rate of pups [6]. As one of the important sources of plant proteins and edible oil, the content of PtdCho is commonly used as an important indicator to assess the quality of soybeans. Furthermore, as a type of raw natural material, soy lecithin is mainly composed of PtdCho and is widely used in food, medicine, and cosmetic industries [7]. Furthermore, PtdCho is a major membrane phospholipid and a precursor for several major signaling molecules. Therefore, understanding the biosynthesis of PtdCho is important for improving plant growth, nutritional value, and resistance to stress [8].

In recent years, the separation, extraction, and biological functions of phospholipids have been extensively investigated. For example, studies have shown that the yields of lipids and phospholipids using subcritical dimethyl ether are higher than those based on propane and its mixed solvents, while the biological functions of the asymmetric distribution of phospholipids in cell membranes have been well explored [9–11]. However, studies on the functions of the gene encoding phosphatidyl methyltransferase (PLMT) at genetic level are sparsely reported in plants, whereas studies have shown that the PLMTs in animals and yeast catalyze the methylation of PtdEtn to generate PtdCho in vivo [12,13]. Furthermore, fundamental differences have been revealed between the biosynthetic pathways of PtdCho in yeast and plants [14]. The *PLMT* was cloned for the first time in both *Arabidopsis thaliana* and soybean, while studies have further shown that *AtPLMT* is involved in the PtdCho metabolic pathway of *A. thaliana* [15]. Furthermore, as the key enzymes for PtdCho synthesis in *A. thaliana*, two isoforms (i.e., PMT1 and PMT3) of phospho-base N-methyltransferase (PMT) are the primary enzymes for the biosynthesis of phosphocholine, which is involved in the PtdCho biosynthesis and vascular development in *A. thaliana* seedlings [16]. Moreover, it has been reported that the soybean phosphoethanolamine methyltransferase (PEAMT) genes play an important role in PtdCho synthesis [17]. As the biosynthetic precursor of glycine betaine, PtdCho is an osmotic protective agent commonly found in plants to increase the osmotic pressure in the cytoplasm, protect plant cell membranes under stressed conditions, and keep their lipid membranes intact [18–20]. Studies have shown that the genetic engineering technology based on PLMTs can not only increase the contents of PtdCho in transgenic plants, but also improve plant resistance to abiotic stresses [21]. To date, comprehensive studies on the explicit functions of PLMTs in plants are still lacking.

In this study, the molecular characteristics and expression patterns of *GmPLMT* in soybean and transgenic *A. thaliana* were investigated. The correlation between the gene expression of *GmPLMT* and both physiological and biochemical indices related to lipid metabolisms were further analyzed, and the effects of *GmPLMT* on the production of various types of lipid metabolites were also explored. The results showed that transgenic *GmPLMT* gene promoted the growth and development of *A. thaliana*, and the expression of *GmPLMT* regulated the contents of lipid metabolites in soybean grains and the seeds of transgenic *A. thaliana*. This study provided the solid experimental foundation for further studies of the functions of *PLMT* gene in other plants and identified *GmPLMT* as the candidate gene for genetic engineering to further improve the quality of soybean.

2. Materials and Methods

2.1. Plant Materials

The high-oil variety “Jiyu 72” of soybean [*Glycine max* (L.) Merr.] provided by the Jilin Academy of Agricultural Sciences (Changchun, China) was planted in the Teaching and Scientific Research Station of the Jilin Agricultural University in Changchun, China (43.5° N, 125.1° E) in a total of 10 rows each of 5 m in length, row spacing 0.65 m, density

200,000 plants/hm², and repeated three times. The expression levels of *GmPLMT* were measured in soybean leaves at different developmental stages (i.e., seedling, branching, flowering and podding, filling, and maturity stages). Starting from the 35th day after flowering, the pods at the top of soybean plants were collected every five days for a total of nine samples (i.e., T1–T9) to measure the expression levels of *GmPLMT* and the contents of main products generated in lipid metabolism.

Columbia type (Col-0) of *Arabidopsis thaliana* (L.) Heynh. was used as wild type (WT) for genetic transformation of *GmPLMT*. Plants of *A. thaliana* was grown in the artificial climate room of the School of Life Sciences of Jilin Agricultural University with a photoperiod cycle of 12-h light and 12-h dark, a temperature of 22 °C, and a relative humidity of 50%. Flower buds at the top of the stem were treated with *Agrobacterium tumefaciens* EHA105 with transformed *GmPLMT*. The seeds collected were sprayed with 1% glyphosate solution and were repeatedly screened to generate T3 generation. The transgenic plants of *A. thaliana* in T3 generation were sampled in four developmental stages (i.e., four-leaf, bolting, flowering, and maturity stages), with the WT of *A. thaliana* used as the control.

2.2. Molecular Properties of *GmPLMT*

In the previous studies, primers were designed based on the *GmPLMT* sequence (NM_001249156.2) retrieved from the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/>, accessed on 1 January 2018) database to obtain the *GmPLMT* sequence of 627 bp and to investigate its gene expression under salt and alkali stresses [22]. In this study, DNAMAN software was used to comparatively analyze seven protein sequences of PLMT, including *Schizosaccharomyces pombe* (AF004112.1), *A. thaliana* (NP_565246.1), *Glycine max* (NM_001249156.2), *Brassica napus* (XP_013674943.1), *Arachis ipaensis* (XP_016176048.1), *Cannabis sativa* (KAF4387675.1), and *Zea mays* (PWZ27952.1). The molecular properties, functional domains, and tertiary structures of *GmPLMT* were predicted by SMART (<http://smart.embl-heidelberg.de/>, accessed on 1 May 2020). The relative molecular weight and theoretical isoelectric point of *GmPLMT* were calculated by ProtParam [23]. The hydrophobicity of *GmPLMT* was analyzed by ProtScale with the secondary structure predicted by NetSurfP-2.0 at SOPMA [24], the signal peptide predicted by SingaIP [25], and the transmembrane domain predicted by PSIPRED4.0 [26].

2.3. Construction of Plant Expression Vector of *GmPLMT* and Its Genetic Transformation in *Arabidopsis thaliana*

The CDS sequence (504 bp) of *GmPLMT* containing both the HindIII and BamHI restriction sites was PCR-amplified by using the recombinant plasmid of *GmPLMT* as template and primers MQGmPLMT-A (5'-CGCGGATCCATGGGGATTGCGGCGGCTAT-3') and MQGmPLMT-S (5'-CCCAAGCTTTCAATGGAGTGGCTTGGCACG-3') with the PCR conditions as follows: pre-denaturation at 95 °C for 5 min, followed by 35 cycles of "denaturation at 94 °C for 30 s, annealing at 54 °C for 40 s, extension at 72 °C for 2.5 min", and final extension at 72 °C for 15 min. The *GmPLMT* fragment was digested with HindIII and BamHI (Takara Biotechnology Co., Ltd., Dalian, China) and ligated with T4DNA ligase into the plant expression vector pCAMBIA 3301, which was then transformed into *Escherichia coli* DH5 α . The successful transformation was verified by PCR and double restriction endonuclease digestion of HindIII and BamHI. The positive clones were sent to the Shanghai Shengong Bioengineering Co., Ltd. (Shanghai, China) for sequencing. The plant expression vector containing *GmPLMT* gene was successfully constructed and named pCAMBIA3301-*GmPLMT*, and the *E. coli* DH5 α containing the recombinant plasmid was cryopreserved for further experiments. Then, the plant expression vector pCAMBIA3301-*GmPLMT* was transformed into the *Agrobacterium tumefaciens* EHA105 by triparental hybridization method [27], with the successful transformation verified by PCR. During the flowering stage, plants of *A. thaliana* were infected with *Agrobacterium tumefaciens* using the inflorescence infection method [28].

2.4. Detection of Copy Number of *GmPLMT* in Transgenic Plants of *Arabidopsis thaliana* by Southern Blotting

Plants of transgenic *A. thaliana* of T3 generation were selected to detect the copy number of *GmPLMT* by southern blotting [29]. The genomic DNA was digested with EcoRI endonuclease for 2 h with the products (15 µL) run on 1% agarose electrophoresis gel for 4 h. The digested fragments were transferred to the nylon membrane using the downward transfer method [30]. The probes were prepared and hybridized using the Dig High Prime DNA Labeling & Detection Starter Kit according to the manufacturer's instructions (Roche, Basel, Switzerland). The hybridization reaction was set to temperature at 42 °C, with the membrane washed twice with 2× saline sodium citrate (SSC) buffer at 37 °C for 15 min and then twice with 0.5 × SSC buffer at 66 °C for 15 min. BCIP/NBT (Thermo Fisher Scientific Co., Ltd., Shanghai, China) was used to develop the chemical color on the nylon membrane.

2.5. Determination of Gene Expression of *GmPLMT*

The qPCR was carried out to determine the expression of *GmPLMT* using the All-in-One™ qPCR Mix Kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions with the reaction set up at follows: the cDNAs of soybean and *A. thaliana* at different developmental stages were used as templates, primers YGGmPLMT-F (5'-CAGTACGTTGGCAGCATCAT-3') and YGGmPLMT-R (5'-GTGGCTTGGCAGGAGT-AGAT-3') for target gene *GmPLMT*, and primers EIF1B-F (5'-CTGGTTCCTGTGGGATATGG-3') and EIF1B-R (5'-TACTCGTTGCATGGCTCAAC-3') for internal reference gene *EIF1B*. All experiments were repeated with three replicates. The relative expression was calculated based on the average cycle threshold (Ct) value and the $2^{-\Delta\Delta C_t}$ method [31].

2.6. Determination of Physiological and Biochemical Indices Related to the Phospholipid Metabolism

The phospholipid content was determined by the molybdenum blue colorimetric method [32]. Specifically, 1 g of powder sample was added with 20 mL Folch reagent (chloroform:methanol = 2:1) for extraction under ultrasound for 1 h, with the reduced solvent replenished and the supernatant collected. The supernatant (0.1 mL) was first evaporated in a water bath at 40 °C, added with a suppressant (70% perchloric acid, 0.25 mL) until the liquid became colorless, and then after cooling, volumed to 5 mL using a phosphorus chromogenic agent (3 mol/L H₂SO₄:water:2.5% ammonium molybdate:10% ascorbic acid = 1:5:1:1) to determine the absorbance at 700 nm. The total phospholipid content was calculated according to the standard curve generated based on regression equation, and the phospholipid content was obtained by multiplying the phospholipid conversion coefficient 25.

The contents of PtdCho and PtdEtn were determined by the solvent extraction method [33]. Specifically, 1 g of powder sample was added with 10 mL acetone to shake in a water bath for 3 h, extracted with ethanol, and the extract was evaporated and concentrated by rotary evaporator to obtain yellow paste phospholipids. The crude sample was dissolved with ether, filtered, and dried to obtain PtdCho. The insoluble substances extracted by ethanol were repeatedly extracted with petroleum ether five times, with the solution combined, distilled, and concentrated to 10 mL, which was washed and precipitated with an equal amount of ethanol, filtered, and dried to generate PtdEtn shown as yellowish powder. These experiments were repeated with three replicates.

The contents of palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid were determined by gas chromatography [34]. The powder sample (0.1 g) was added with 2 mL sodium methoxide (0.5 mol/L) to esterify for 30 min in a water bath at 30 °C, and extracted twice with 10 mL n-hexane. The relative contents of fatty acid components were determined by Thermo Trace 1300 gas chromatography (Thermo Fisher Scientific Co., Ltd., Shanghai, China) with the chromatographic column of RTX-Wax

(100 m × 0.25 mm × 0.2 µm), the hydrogen flame ion detector (FID), and the temperature set at 300 °C.

The content of crude fats was determined by the Soxhlet extraction method [34]. The powder sample of 1 g was wrapped in filter paper and put into the fat analyzer (SZC-C, Shanghai Fiber Inspection Instrument Co., Ltd., Shanghai, China) for extraction with 60 mL ether at 60 °C for 2 h to make the ether reflux until the ether in the extraction tube was volatilized with no oil stain. After extraction, the crude fats were weighed, and the content of crude fats in the dry weight of the sample was calculated.

Correlation analysis was conducted between the expression of *GmPLMT* and the contents of lipid metabolites in soybean grains at different stages of maturity using SPSS 22 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Bioinformatics Analysis of *GmPLMT* Gene and Its Encoded Protein in Soybean

Comparative analysis of seven PLMT sequences, including *Schizosaccharomyces pombe*, *A. thaliana*, *Glycine max*, *Brassica napus*, *Arachis ipaensis*, *Cannabis sativa*, and *Zea mays*, based on DNAMAN software, showed that the overall similarity of the seven proteins was 59.2%, with a similarity of 81.5% among the six species of plants, indicating that the plant PLMT proteins were relatively conservative in evolution. The soybean protein *GmPLMT* with a molecular formula $C_{956}H_{1363}N_{213}O_{221}S_8$ encoded by *GmPLMT* contained a total of 167 amino acids, with a molecular weight of 19.632 KDa, the theoretical isoelectric point of 8.75, and Phe accounted for the highest proportion (10.2%) and Ala the least proportion (0.6%) among the 20 amino acids. The *GmPLMT* was classified as an unstable protein based on the instability index (II) value of 42.10, with the grand average coefficient of total hydropathicity (GRAVY) of 0.386.

Results of the hydrophobicity analysis of the predicted *GmPLMT* by ProtScale showed that the Val located at position 144 was the most hydrophobic amino acid with a score of 2.411 and the Asp at position 158 was the most hydrophilic with a score of −2.533 (Figure 1). Overall, the amino acids at positions 26–41 and 153–163 showed strong hydrophilicity with the hydrophobic amino acids largely evenly distributed in the entire peptide chain. These results suggested that the *GmPLMT* was a type of hydrophobic protein, which was consistent with the predicted value of GRAVY.

Figure 1. Hydrophobicity and hydrophilicity of soybean *GmPLMT* predicted by ProtScale.

The predicted secondary structure of *GmPLMT* based on NetSurfP-2.0 showed that α -helix accounted for the large portion of the entire protein (68.9%) and β -corner accounted for 29.9%, with no signal peptide identified (Figure 2). A total of four transmembrane regions were identified by PSIPRED4.0 in the amino acid sequence of *GmPLMT*, which was classified as a type of transmembrane protein with its N-terminal and C-terminal located

outside the plasma membrane (Figure 3). These results were consistent with those reported previously based on *S. pombe* [35].

Figure 2. Secondary structure of soybean GmPLMT predicted by NetSurfP-2.

Figure 3. Transmembrane domains (S1–S4) of soybean GmPLMT predicted by PSIPRED4.0.

The three-dimensional structure of the protein encoded by *GmPLMT* predicted based on SMART showed that GmPLMT contained a S-adenosyl-L-methionine (SAM) binding domain which was consistent with the structural properties of methyltransferases (Figure 4), evidently indicating that GmPLMT was a member of the methyl-transferring proteins. Studies have shown that methyltransferases are widely involved in many important cellular processes in plants, including biosynthesis, signal transduction, protein repair, and chromatin regulation [36,37]. Further studies are necessary to identify the explicit biological functions of GmPLMT in plants.

Figure 4. Tertiary structure of soybean GmPLMT shown in XY plan (A) and XZ plan (B) predicted by SMART.

The sequence alignments of seven PLMT proteins (i.e., *Schizosaccharomyces pombe*, soybean, *A. thaliana*, *Brassica napus*, *Arachis ipaensis*, *Cannabis sativa*, and *Zea mays*) were presented in Figure 5. Results showed that the middle portion of GmPLMT was a relatively conserved region, representing a possibly conserved functional domain, i.e. presumably the phospholipid binding site, while the tripeptide AFG and dipeptide ED were the domains of the SAM binding site [15,35]. The PLMTs are involved in the synthesis of PtdCho to transfer a methyl group from SAM to a nucleophilic acceptor, such as oxygen, ultimately generating S-adenosyl-L-homocysteine and a methylated molecule [38]. Results in the current study showed that the amino acid sequence of GmPLMT contained two SAM binding domains, with one composed of 3 amino acids located at positions 77–79 and the other of 2 amino acids located at positions 157 and 158. It has been suggested that all methyltransferases, including those catalyzing the methylation of DNAs, proteins, RNAs, and lipids, contain a tripeptide SAM binding site GXG [39]. Furthermore, studies have revealed tripeptide CFG in the PLMT protein of *S. pombe* [35]. However, the results in the current study identified the tripeptide AFG in six species of plants, including both soybean and *A. thaliana*. These results suggested that the variations in the structural domain of the SAM binding site in plants caused the differentiated functions of PLMTs. These results were consistent with the structural characteristics of methyltransferases [40,41]. Further investigations are necessary to verify these discoveries revealed in the current study.

<i>Schizosaccharomyces pombe</i>	MSLILYPKPTSILYQPFIFKAYFSLNMAIFEINNSFLICAVSIALNPLLWNIAARS	55
<i>Glycine max</i>	...MGIAAAIGLLSPFPFYCLWIWPQWVD.....LCGRG	33
<i>Arabidopsis thaliana</i>	...MGLLAAIGVLLPFPFYWLLWNAQSWVN.....LCGRE	33
<i>Brassica napus</i>	...MGIVAAIGVLLPFPFYWLLWNPQSWVN.....LCGGQ	33
<i>Arachis ipaensis</i>	...MGIFAAVGVLSPFPFYWLLWNPQSWVD.....LCGKD	33
<i>Cannabis sativa</i>	...MGIAAGIGVLLPFPFYWLLWNPQSWVD.....LCGKA	33
<i>Zea mays</i>	MVSMAAAAAVGVLLPFPFYVLLWTHPQRWVD.....LCGRS	37
Consensus	pf	
<i>Schizosaccharomyces pombe</i>	EYNHKTTLTKLANGDSKKACMYLAACIFVAGIVRDLIYQNALKQOFTLGFIMNPLV	110
<i>Glycine max</i>	REARDPSKVMAY....VSFYLKLVQFIS....LFYVSSFHWBPFPFYFWP....	74
<i>Arabidopsis thaliana</i>	R...DPSTVMAR....VSHVLKAAQLLS....LFSVASLSWBPPLYFWP....	71
<i>Brassica napus</i>	K...DPSTVMAR....VSHVLKAAQLLS....LFSVASLSWBPPLYFWP....	71
<i>Arachis ipaensis</i>	R...DPSKVMAL....VAHFLKLIQFIS....LFSVSTLHWBPFPFYFWP....	71
<i>Cannabis sativa</i>	R...DPSKVMAL....VSHFIKLIQFIS....LFSVSTLSWBPPLYFWP....	71
<i>Zea mays</i>	A...DPCRRMAQ....VSHAIKALQLLA....LASVSSFHWBPPLYCPI....	75
Consensus	a l p	
<i>Schizosaccharomyces pombe</i>	QGIAKLIFCFGSVLVLSSMYKGLVGTLYGCDYFCFLPERVSGFPFNVDNDNFM	165
<i>Glycine max</i>	LIAFGQFLNFRVYQLLGETGTYYGVRFCKTIP.WVTEFPFGVIKD..PQ	120
<i>Arabidopsis thaliana</i>	LMAFGQFLNFRVYQLLGEAGTYGVRFCKNIP.WVTEFPFGVIRD..PQ	117
<i>Brassica napus</i>	VMAFGQFLNFRVYQLLGEAGTYGVRFCKNIP.WVTEFPFGVIRD..PQ	117
<i>Arachis ipaensis</i>	LHAFGQFLNFRVYQLLGEAGTYGVRFCKSIP.WVTEFPFGVIKD..PQ	117
<i>Cannabis sativa</i>	LIAFGQFLNFRVYQLLGEAGTYGVRFCKKIP.WVTEFPFGYIQD..PQ	117
<i>Zea mays</i>	LIAVGQFLNFRVYQLLGEAGTYGVRFCKTIP.WVTEFPFGYIKD..PQ	121
Consensus	g l lg gty g lg p v lpf p	
<i>Schizosaccharomyces pombe</i>	YNGSTLCFLSTALRYGKVAGLLLTLEVFVYRIALKFEE.....	204
<i>Glycine max</i>	YVGSIMSILACFSWVPFYIILLWVLGYVFMHVESKEDPSTRAKPLH	167
<i>Arabidopsis thaliana</i>	YVGSIMSLACLSSWVPFYIILLWVLGYVFMHVESKEDPNARAKSIS	164
<i>Brassica napus</i>	YVGSVMSLLACLSSWVPFYIILLWVLGYVFMHVESKEDPSARAKPIS	164
<i>Arachis ipaensis</i>	YVGAIMSLVACLSSWVPFYIILLWVLGYVFMHVESKEDLSTRAKPIH	164
<i>Cannabis sativa</i>	YVGSIMSLACLSSWVPFYIILLWVLGYVFMHVESKEDPNTRANPIS	164
<i>Zea mays</i>	YVGSMLSLVALLCWVPLQYVLLWVLGYVFMHVESKEDSASRAKVIS	168
Consensus	y g l l f k	

Figure 5. Sequence alignments of seven phospholipid methyltransferase (PLMT) proteins including *Schizosaccharomyces pombe* (AF004112.1), *Glycine max* (NM_001249156.2), *Arabidopsis thaliana* (NP_565246.1), *Brassica napus* (XP_013674943.1), *Arachis ipaensis* (XP_016176048.1), *Cannabis sativa* (KAF4387675.1), and *Zea mays* (PWZ27952.1). The conserved amino acids among the PLMTs are indicated by a dark background. The underlined regions represent the predicted phospholipid binding sites. The proposed binding sites (i.e., AFG and ED) for S-adenosyl-L-methionine (SAM) are indicated by two boxes.

3.2. Genetic Transformation of *GmPLMT* in *Arabidopsis thaliana*

3.2.1. Construction of Plant Overexpression Vector of pCAMBIA 3301-*GmPLMT*

The primers with restriction sites of HindIII and BamHI were used to PCR-amplify the *GmPLMT* based on plasmid pCAMBIA3301-*GmPLMT* (Figure 6A). The 504 bp fragment of *GmPLMT* was successfully cloned, ligated with T4 DNA ligase, and transformed into the plant expression vector pCAMBIA3301 to successfully construct the pCAMBIA3301-*GmPLMT* recombinant plasmid, which was verified by PCR and double restriction endonuclease digestion of HindIII and BamHI (Figure 6B).

Figure 6. Construction and verification of recombinant plasmid pCAMBIA3301-*GmPLMT* based on PCR (A) and double restriction endonuclease digestion of HindIII and BamHI (B). Lane M represents DL2000 DNA marker. Lane 1 in (A) indicates *GmPLMT* of 504 bp. Lanes 1–3 in (B) represent the PCR product based on the recombinant plasmid pCAMBIA3301-*GmPLMT* (*GmPLMT* of 504 bp), the bacterial liquid PCR product (*GmPLMT* of 504 bp), and the products of the double restriction endonuclease digestion of plasmid based on HindIII and BamHI (504 bp of *GmPLMT* and 11,319 bp of the plasmid).

The pCAMBIA3301-*GmPLMT* recombinant plasmids were successfully transformed into *Agrobacterium tumefaciens* EHA105 by the triparental hybridization method, as verified by PCR and the sequence of the target gene showing 100% identity as that of the sequence of *GmPLMT* (NM_001249156.2) retrieved from the NCBI database.

3.2.2. Phenotypic Comparison and Southern Blotting Detection of Transgenic *Arabidopsis thaliana* with *GmPLMT*

In this study, the *GmPLMT* gene was overexpressed in *A. thaliana* with the goal to further understand the explicit effects of soybean PLMT on the lipid metabolism. The results of the southern blotting hybridization of *GmPLMT* in transgenic *A. thaliana* showed that *GmPLMT* was integrated into the genome of *A. thaliana* as a single copy (Figure 7). Phenotypic observations were regularly made on the seedlings of WT (control) and transgenic plants of *A. thaliana* in T3 generation, showing evidently phenotypic differentiation (i.e., increased biomass) in the mature transgenic plants of *A. thaliana* in comparison to the WT plants. The transgenic plants of *A. thaliana* were larger, with larger and more leaves than WT plants. In comparison to the WT plants, the number of leaves was increased by 48.6%, the plant height increased by 39.2%, and the root length increased by 20.4% in the transgenic plants. Furthermore, in transgenic plants, the number of pods was increased by 31.8% and the length of pods was increased by 18.1% compared with those of the WT plants. Moreover, the seed size in the transgenic plants was larger than that of the WT plants. These results were consistent with those reported previously, showing that the overexpression of genes led to enhanced growth in plants, increased seed size and yield, and the improved production of metabolites [42,43]. The southern blotting hybridization of *GmPLMT* in transgenic *A. thaliana* revealed a single hybridization signal in each of the

five positive plants with varied locations, a hybridization signal detected in the positive control, and no hybridization signal was detected in the negative controls of WT and H₂O (Figure 7). These results indicated that *GmPLMT* was integrated into the genome of *A. thaliana* as a single copy, with different integration sites in different plants.

Figure 7. Southern blotting hybridization of T3 generation of transgenic *Arabidopsis thaliana* with *GmPLMT*. M, DIG-labeled λ HindIII DNA markers; CK, positive control; WT and H₂O, negative controls, lanes 1–5, five random transgenic plants of *A. thaliana* with *GmPLMT*.

3.3. Effects of *GmPLMT* Gene Expression on the Phospholipid Metabolism in Soybean

3.3.1. Variations of *GmPLMT* Gene Expression in Soybean Leaves during Different Developmental Stages

The results showed that the gene expression of *GmPLMT* in soybean leaves varied significantly at different developmental stages. The expression levels of *GmPLMT* were significantly higher in both seedling and flowering stages than those in other developmental stages, decreasing to the lowest level during the maturity stage (Figure 8). These results revealed that these expression characteristics of *GmPLMT* were consistent with those of plant morphological growth and development, suggesting the regulatory effects of *GmPLMT* on plant growth and development. Flowering and podding periods are the critical growth stages for soybean, while various types of enzymes involved in biosynthetic processes of different substances could adapt to plant growth and development by promoting the expression of genes [44]. It is well known that PLMTs are the main enzymes that synthesize PtdCho in both mammalian cells and yeast [45]. The results revealed in the current study were consistent with those reported previously, showing that the activities of these enzymes have been detected in plants such as soybean, carrot, and olive [46–48].

Figure 8. Relative expression of *GmPLMT* in soybean leaves at different developmental stages. Symbol “*” indicates the significant difference at *p*-value of 0.05 in comparison to the maturity stage.

3.3.2. Variations of *GmPLMT* Gene Expression in Soybean Grains at Different Stages of Maturity

The results revealed significant variations in the gene expression of *GmPLMT* in soybean grains with different stages of maturity (Figure 9). The expression of *GmPLMT* was decreased gradually from 40 to 50 days after flowering, reaching the lowest level at 50 days after flowering, and then increased gradually with the maturity of soybean seeds, reaching the highest level at 70 days after flowering, which was 72% higher than that at 35 days after flowering. At 75 days after flowering, the expression of *GmPLMT* was decreased again. These results indicated that the high expression levels of *GmPLMT* were detected during 55–70 days after flowering, which was the important growth stage for the accumulation of phospholipids during the development of grains, whereas the metabolic activities of plants were decreased after the soybean grains were fully mature, and then the expression of *GmPLMT* declined. These results were similar to those reported previously, showing the temporal and spatial expression patterns of genes involved in lipid metabolism in peony during seed development [49].

Figure 9. Relative expression of *GmPLMT* in soybean grains at different stages of maturity. T1–T9 represent the samples of pods collected every five days starting from the 35th day after flowering. Symbols “*” and “**” indicate significant differences at *p* values of 0.05 and 0.01, respectively, in comparison to T1.

3.3.3. Changes in the Contents of Total Phospholipids, Phosphatidylcholine, and Phosphatidylethanolamine in Soybean Grains at Different Stages of Maturity

The contents of total phospholipids, PtdCho, and PtdEtn in soybean grains at different stages of maturity were presented in Figure 10. The results showed that with the increase of soybean seed maturity, the total phospholipid contents were decreased first and then increased, with the highest content reached at 35 days after flowering and the lowest at 55 days after flowering, and the higher contents detected at 35–40 and 70–75 days after flowering. The contents of PtdCho showed a similar pattern to that of the contents of total phospholipids, i.e., decreased first and then increased. Notably, the contents of PtdEtn were not significantly different at different stages of maturity in the samples of soybean grains collected prior to 75 days after flowering, with the highest content reached at 75 days after flowering. These results revealed the evident effects of the expression of *GmPLMT* on the contents of the intermediate metabolites and the end products of soybean lipid metabolism. These results were consistent with those previously reported, showing that the overexpression of soybean *GmPLDr* gene increased the oil content and fatty acid composition in the seeds of transgenic *A. thaliana* [50].

Figure 10. The contents of total phospholipids, phosphatidylcholine (PtdCho), and phosphatidylethanolamine (PtdEtn) in soybean grains of different stages of maturity. T1–T9 represents the samples of pods collected every 5 days starting from the 35th day after flowering. Symbol “*” indicates the significant difference at p value of 0.05.

3.3.4. Variations in the Contents of Fatty Acids and Crude Fats in Soybean Grains at Different Stages of Maturity

The proportions of several types of fatty acids in the total fatty acids of soybean grains at different stages of maturity were presented in Table 1. The results showed that with the increase of soybean seed maturity, the contents of palmitic acid were increased first and then decreased; they were largely higher at 40–60 days after flowering and then decreased gradually afterward. The contents of both stearic acid and oleic acid showed similar patterns to that of palmitic acid. The contents of linoleic acid were decreased first and then increased, decreasing gradually after 35 days after flowering and reaching the highest levels at 60–70 days after flowering. The contents of linolenic acid showed a similar pattern to that of linoleic acid, an initial decrease followed by an increase, reaching the highest level at 35 days after flowering and showing largely higher levels at 65–75 days after flowering. The contents of crude fats were gradually increased at 40 days after flowering and reached the highest level at 75 days after flowering. Generally, both the lipids and proteins are the main components of soybean grains, while the fatty acids are important raw materials for the synthesis of fats. The results were consistent with those reported previously, showing that the contents of various types of fatty acids and crude fats in the maturity process were correlated with the contents of proteins in soybean seeds [51].

Table 1. The proportion of various fatty acids in the total fatty acids and the contents of crude fats in soybean grains at different stages of maturity.

Sample	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid	Linolenic Acid	Crude Fats
T1	14.9 ± 0.7	4.4 ± 0.2	26.0 ± 1.6	42.3 ± 2.1 *	11.4 ± 0.8 **	10.8 ± 0.4
T2	22.0 ± 1.1 *	7.2 ± 0.4	38.2 ± 3.8	27.5 ± 1.6	3.9 ± 0.2	9.1 ± 0.3
T3	24.4 ± 1.9 *	8.9 ± 0.6	42.5 ± 3.3 *	20.5 ± 2.1	2.4 ± 0.2	9.9 ± 0.6
T4	24.1 ± 1.2 *	9.7 ± 0.7 *	42.4 ± 1.7 *	19.9 ± 0.6	2.3 ± 0.1	10.3 ± 0.6
T5	24.2 ± 1.4 *	10.1 ± 0.9 *	44.3 ± 2.7 *	17.9 ± 1.4	1.9 ± 0.1	10.7 ± 0.5
T6	22.7 ± 1.3 *	9.5 ± 0.8*	44.8 ± 2.2 *	19.7 ± 1.4	1.6 ± 0.1	11.0 ± 0.4
T7	12.5 ± 1.0	5.2 ± 0.2	27.1 ± 0.6	48.3 ± 1.9 *	6.1 ± 0.4	18.1 ± 1.1 *
T8	12.8 ± 1.2	5.6 ± 0.4	23.8 ± 1.4	50.4 ± 2.5 **	6.4 ± 0.5	18.2 ± 0.7 *
T9	10.2 ± 0.6	4.5 ± 0.2	20.6 ± 1.4	54.4 ± 2.2 **	9.2 ± 0.6 *	19.3 ± 0.5 *

T1–T9 represents the samples of pods collected every 5 days starting from the 35th day after flowering. Symbols “*” and “**” indicate significant differences at p values of 0.05 and 0.01, respectively.

3.3.5. Correlation between the Gene Expression of *GmPLMT* and the Contents of Lipid Metabolites in Soybean Seeds at Different Stages of Maturity

The results of the correlation analyses between *GmPLMT* gene expression and the contents of various types of lipid metabolites in soybean seeds at different stages of maturity showed that the expression of *GmPLMT* was significantly positively correlated with the contents of linoleic acid, crude fats, and PtdCho ($r = 0.64, 0.72$, and 0.50 , respectively) and negatively correlated with the contents of palmitic acid and oleic acid ($r = -0.65$ and -0.58 , respectively) (Table 2). Furthermore, the content of PtdCho was positively correlated with that of linoleic acid ($r = 0.74$), extremely significantly correlated with those of linolenic acid and total phospholipid ($r = 0.82$ and 0.93 , respectively), negatively correlated with those of palmitic acid and oleic acid ($r = -0.74$ and -0.76 , respectively), and extremely negatively correlated with that of stearic acid ($r = -0.85$). Fatty acids and ethanolamine/choline are the raw materials for the synthesis of fats and phospholipids in plants, respectively, while diacylglycerol is a commonly shared intermediate product in these synthetic pathways. Results in the current study revealed the significantly positive correlation between the expression of *GmPLMT* and the contents of PtdCho and two types of polyunsaturated fatty acids (i.e., linoleic acid and linolenic acid), indicating that the contents of PtdCho were regulated by gene *GmPLMT* in soybean grains, while gene *GmPLMT* may increase the phospholipid content by promoting the production of PtdCho. Studies have revealed the regulatory effects of different types of fatty acids on the metabolic genes, with the polyunsaturated fatty acids showing the most significant effect [52,53], while the results of the current study were supportive of the speculations proposed and results reported previously [15].

Table 2. Correlation analysis of *GmPLMT* gene expression and contents of various types of lipid metabolites in soybean grains at different stages of maturity.

Correlation Coefficient (r)	Expression of <i>GmPLMT</i>	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid	Linolenic Acid	Crude Fat	Total Phospholipid	PtdCho	PtdEtn
Expression of <i>GmPLMT</i>	1.00									
Palmitic acid	−0.65 *	1.00								
Stearic acid	−0.48	0.93 **	1.00							
Oleic acid	−0.58 *	0.98 **	0.96 **	1.00						
Linoleic acid	0.64 *	−0.99 **	−0.94 **	−0.99 **	1.00					
Linolenic acid	0.30	−0.83 **	−0.93 **	−0.89 **	0.84 **	1.00				
Crude fat	0.72 *	−0.88 **	−0.67 *	−0.81 **	0.87 **	0.50 *	1.00			
Total phospholipid	0.45	−0.75 *	−0.87 **	−0.79 *	0.77 *	0.8 **	0.45	1.00		
PtdCho	0.50 *	−0.74 *	−0.85 **	−0.76 *	0.74 *	0.82 **	0.42	0.93 **	1.00	
PtdEtn	−0.01	−0.62 *	−0.72 *	−0.70 *	0.64 *	0.78 *	0.36	0.75 *	0.58 *	1.00

Symbols “*” and “**” indicate significant differences at p values of 0.05 and 0.01, respectively. PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.

3.4. Effects of *GmPLMT* Gene Expression on the Phospholipid Metabolism in Transgenic *Arabidopsis thaliana*

3.4.1. Variations of *GmPLMT* Gene Expression in Transgenic *Arabidopsis thaliana* at Different Developmental Stages

The biological functions of genes in plants are generally investigated on both the molecular and genetic levels, with the heterologous expression of plant genes as an important technique based on the model plant of *Arabidopsis* [14]. The results of gene expression of *GmPLMT* during different developmental stages of transgenic *A. thaliana* showed that the expression reached the highest level of 2.64 at the flowering stage, followed by 2.26 and 1.66 at the four-leaf and bolting stages, respectively, which were either significantly or extremely higher than that of the maturity stage, showing the lowest expression level, i.e., 38.8% of the expression level at flowering stage in transgenic *A. thaliana* (Figure 11). These results indicated that the gene *GmPLMT* was efficiently expressed in transgenic *A. thaliana*, showing the highest expression level during the flowering stage. In general, plant growth and development are mainly determined by the genes controlling the traits and the environment. Evidently, the *GmPLMT* gene showed consistent expression patterns in both

the transgenic plants of *A. thaliana* and soybeans in different developmental stages, while during the maturity period with the completion of material storage, the enzyme activities were decreased to the lowest level. Therefore, it was speculated that the *GmPLMT* gene plays an important role in plant growth and development. Further studies are needed to verify the discoveries revealed in the current study.

Figure 11. Relative expression of *GmPLMT* in transgenic *Arabidopsis thaliana* at different developmental stages. Symbols “*” and “**” indicate significant differences at *p* values of 0.05 and 0.01, respectively.

3.4.2. Variations of the Physiological and Biochemical Indices Related to Phospholipid Metabolism in Transgenic *Arabidopsis thaliana* Seeds with *GmPLMT*

The variations of the phospholipid metabolic products in transgenic *A. thaliana* seeds with *GmPLMT* are presented in Figure 12. The results showed that the contents of the total phospholipids in transgenic *A. thaliana* were increased significantly to 2.02 times higher than that of WT plants. The contents of PtdCho in transgenic plants accounted for 9.07% and 10.3% of those of the total phospholipids in the WT and the transgenic *A. thaliana*, respectively. Although the content of PtdEtn in transgenic *A. thaliana* was 1.36 times higher than that of WT plants, the difference was not statistically significant. These results showed that overexpression of *GmPLMT* increased the contents of both the total phospholipids and PtdCho in transgenic *A. thaliana*. These results were consistent with those reported previously, showing that PLMTs were involved in the synthesis of PtdCho [54,55].

Figure 12. Contents of total phospholipids, phosphatidylcholine (PtdCho), and phosphatidylethanolamine (PtdEtn) in seeds of wild type (WT) and transgenic plants of *Arabidopsis thaliana* with *GmPLMT*. Symbols “*” and “**” indicate significant differences at *p* values of 0.05 and 0.01, respectively.

3.4.3. Variations of Physiological and Biochemical Indices Related to Lipid Metabolism in Transgenic *Arabidopsis thaliana* Seeds with *GmPLMT*

The contents of crude fats and several types of fatty acids in the total fatty acids in transgenic *A. thaliana* seeds with *GmPLMT* were shown in Figure 13. These results revealed that the contents of both linoleic acid and linolenic acid in the total fatty acids were significantly higher than those of WT plants of *A. thaliana*, increasing by 34.5% and 29.3%, respectively. Although the contents of palmitic acid, stearic acid, and oleic acid were lower than those of WT plants, the differences were not statistically significant. The contents of crude fats in seeds of WT plants of *A. thaliana* were 2.27 times higher than those of transgenic plants ($p < 0.01$). Similarly, previous studies have shown that both seed mass and oil content were increased in transgenic *A. thaliana* by the overexpression of the *wri1*-like gene from *Brassica napus* [56]. These results indicated that the increased expression of *GmPLMT* significantly improved the contents of polyunsaturated fatty acids in the seeds of transgenic plants, whereas the synthesis of crude fats was decreased by the overexpression of *GmPLMT*.

Figure 13. Contents of several types of fatty acids in the total fatty acids and the crude fats in seeds of wild type (WT) and the transgenic plants of *Arabidopsis thaliana* with *GmPLMT*. Symbols “*” and “**” indicate significant differences at p values of 0.05 and 0.01, respectively.

4. Conclusions

Compared with WT plants, the expression levels of *GmPLMT* in transgenic plants of *A. thaliana* were increased, the contents of total phospholipid and PtdCho were increased significantly, the contents of linoleic acid and linolenic acid were significantly up-regulated, and the contents of crude fats were decreased significantly. It was speculated that the heterologous expression of *GmPLMT* in *A. thaliana* altered the dynamic process of lipid metabolism, promoting the synthesis of phospholipids and reducing the fat synthesis, ultimately leading to fatty acid accumulation and significantly increasing the contents of PtdCho and the total phospholipids in transgenic *A. thaliana*. The overexpression of *GmPLMT* increased not only the contents of PtdCho, but also the contents of polyunsaturated fatty acids in plant seeds. These results provide novel experimental evidence to further enhance the understanding of the biosynthetic pathway of PtdCho in legumes. The current study provides the experimental foundation for further exploring the specific pathways of lipid synthesis and its regulation in plants, and identifies the candidate gene *GmPLMT* for future improvement of soybean quality based on genetic engineering and the molecular breeding of soybean.

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