Cloning and Functional Identification of Phosphoethanolamine Methyltransferase in Soybean (Glycine max)

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Phosphoethanolamine methyltransferase (PEAMT), a kind of S-adenosylmethionine-dependent methyltransferases, plays an essential role in many biological processes of plants, such as cell metabolism, stress response, and signal transduction. It is the key rate-limiting enzyme that catalyzes the three-step methylation of ethanolamine-phosphate (P-EA) to phosphocholine (P-Cho). To understand the unique function of PEAMT in soybean (Glycine max) lipid synthesis, we cloned two phosphoethanolamine methyltransferase genes GmPEAMT1 and GmPEAMT2, and performed functional identification. Both GmPEAMT1 and GmPEAMT2 contain two methyltransferase domains. GmPEAMT1 has the closest relationship with MtPEAMT2, and GmPEAMT2 has the closest relationship with CcPEAMT. GmPEAMT1 and GmPEAMT2 are located in the nucleus and endoplasmic reticulum. There are many light response elements and plant hormone response elements in the promoters of GmPEAMT1 and GmPEAMT2, indicating that they may be involved in plant stress response. The yeast cho2 opi3 mutant, co-expressing Arabidopsis thaliana phospholipid methyltransferase (PLMT) and GmPEAMT1 or GmPEAMT2, can restore normal growth, indicating that GmPEAMTs can catalyze the methylation of phosphoethanolamine to phosphate monomethylethanolamine. The heterologous expression of GmPEAMT1 and GmPEAMT2 can partially restore the short root phenotype of the Arabidopsis thaliana peamt1 mutant, suggesting GmPEAMTs have similar but different functions to AtPEAMT1.

Keywords: phosphoethanolamine methyltransferase, phosphatidylcholine, lecithin, Glycine max, phospholipid metabolism

INTRODUCTION

Phosphatidylcholine (PC) is a type of cell membrane phospholipid (Bolognese and McGraw, 2000), which plays a vital role in various eukaryotic organisms. PC is necessary for the rapid proliferation of malaria parasites in human red blood cells, so its synthesis pathway has become a potent target for malaria chemotherapy (Pessi et al., 2004). PC can also be used as a special nutritional additive to supplement the phospholipids needed in the animals to improve the immunity and survival rate of pups (Zhang et al., 2006). As an essential membrane structure lipid, PC can repair biological membrane damage caused by free radical production and improve antioxidant activity in plants.
PC can be hydrolyzed to produce phosphatidic acid (PA) and choline (Cho) by phosphatase D (Bolognesi and McGraw, 2000). As a second messenger in plant cells, PA plays an important role in the signal transduction pathways related to plant stress response (Hong et al., 2010). In spinach (Spinacia oleracea L.), sugar beet (Beta vulgaris L.), barley (Hordeum vulgare L.), and other plants, PC is the synthetic precursor of glycine betaine (GB) (McNeil et al., 2001). GB is a kind of osmotic protection agent that exists universally in plants. It can increase the osmotic pressure in the cytoplasm, stabilize protein complexes, protect plant cell membranes under stress conditions, and maintain their lipid membrane integrity (Sakamoto and Murata, 2000; Sakamoto et al., 2000).

In mammals and fungi, there are two main pathways for the synthesis of PC: (1) The nucleotide pathway, also known as the “Kennedy pathway” (Kennedy and Lehninger, 1949) or the cytidine diphosphate-choline (CDP-Chol) pathway, which is highly conserved in eukaryotes. Free choline is phosphorylated to form P-Chol by choline kinase, and then P-Chol is converted into CDP-Chol through cytidine triphosphate: phosphocholine cytidylyl transferase (CTP). Then it is converted into PC by cytidine diphosphate-choline: 1,2-diacylglycerol choline phosphotransferase (CEPT). (2) The methylation pathway, with S-adenosylmethionine (SAM) as the methyl donor, phosphatidylethanolamine (Ptd-EA) is converted to PC through three-step continuous methylation catalyzed by phospholipid methyltransferase (PLMT) (Carman and Henry, 1989).

The synthesis pathway of PC in plants is relatively complex, requiring the joint contribution of the nucleotide pathway and the methylation pathway (Hitz et al., 1981). Similar to mammals and yeast, free choline can also be finally converted into PC through the nucleotide pathway in plants. However, when there is no free choline in plants, or when the Kennedy pathway is blocked, the methylation of phosphoethanolamine (ethanolamine-phosphate, P-EA) acts as the first step in the process of PC biosynthesis. Under the catalysis of plant-specific phosphoethanolamine methyltransferase (PEAMT), phosphoethanolamine (ethanolamine-phosphate, P-EA) is converted into phosphorylcholine (P-Chol) via two intermediate products, monomethylethanolamine-phosphate (P-MMEA) and dimethylethanolamine-phosphate (P-DMEA). And, then, it merges into the nucleotide pathway to form PC finally. Alternatively, P-MMEA and P-DMEA can also produce phosphatidylmonomethylethanolamine (Ptd-MMEA) and phosphatidyltrimethylethanolamine (Ptd-DMEA), respectively, through the nucleotide pathway, and then PC can be produced by PLMT at the phosphatidyl level (Lykidis, 2007). It is worth noting that PLMT that has been discovered in plants so far can only use Ptd-MMEA or Ptd-DMEA as a catalytic substrate (Keogh et al., 2009). Therefore, even though there are considerable differences in phosphate-level methylation and phosphatidyl-level methylation among different plants, the methylation of P-EA catalyzed by PEAMT to produce P-MMEA is the only entrance for PC synthesis (Chen et al., 2018a). Given the critical role of PEAMT in the PC synthesis, it has been studied in plants such as Arabidopsis thaliana (Bolognesi and McGraw, 2000), wheat (Charron et al., 2002), and corn (Zea mays L.) (Wu et al., 2007).

Phosphatidylethanolamine is essential to improve the quality of soybean (Glycine max), which is an important source of plant protein and edible oil. In addition, soybean lecithin, mainly composed of PC, has been widely used as a natural raw material in the food, medical, and cosmetics industries (Gu et al., 2001). The PC synthesis pathway of soybean is slightly different from other plants, mainly in the methylation reactions at the phosphate level (Datko and Mudd, 1988a), suggesting a special function of GmPEAMTs. Since PEAMTs have not been identified from soybean or other legumes, we isolated the coding sequences and promoters of PEAMTs from soybean and conducted a systematic study on them. The findings of this study make us better understand the unique role of GmPEAMTs in the synthesis of PC. It is also of guiding significance for promoting the molecular breeding process of legumes.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Arabidopsis thaliana T-DNA mutant peamt1 (SALK_036291) was obtained from ABRC. Arabidopsis thaliana ecotype Col-0 and peamt1 mutant were grown under 16-h light/8-h dark conditions at 24°C/20°C. A ½/MS solid medium was used for plant culture. Glycine max (cv. “Williams82”) was cultivated in soil at 24°C with 12-h light/12-h dark.

### Identification of GmPEAMT and Its Promoter

Potential GmPEAMT sequences of soybean were identified, using BLASTN at the Phytozome v12.1 website based on Arabidopsis thaliana PEAMT1 (At3g18000). The results were filtered with a score value ≥100 and an E-value ≤1e-10. The filtered genes were further analyzed for the potential domains, using SMART (Letunic and Bork, 2018). The promoter region and the coding sequences of GmPEAMT were amplified by PCR (primers are listed in Supplementary Table 1), cloned into the pMD19-T vector (TaKaRa, Japan) and sequenced (Tsingke, China).

### Sequence and Phylogenetic Analysis

Genome structure was analyzed by online program GSDS. Structural domains were predicted through SMART. The sequences of other plant PEAMTs were obtained from NCBI, using BLASTP. Multiple alignments were conducted with ClustalX 2.0 and viewed with GeneDoc. The transmembrane domains were predicted by TMHMM. Phylogenetic analyses were conducted in MEGA X. The evolutionary history was inferred, using the neighbor-joining method.

1. http://www.arabidopsis.org/abrc/
2. https://phytozome.jgi.doe.gov
3. http://smart.embl-heidelberg.de/
4. http://gsds.cbi.pku.edu.cn/
5. http://www.cbs.dtu.dk/services/TMHMM/
The bootstrap set value was 1,000 replicates. The possible 
 cis-acting elements in the promoters were predicted by 
 PlantCARE (Lescot et al., 2002) and visualized with TBtools 
 (Chen et al., 2020).

**RESULTS**

**Identification of GmPEAMT1 and GmPEAMT2**

Through filtering and domain analysis, two candidate genes 
 named “GmPEAMT1” (Glyma05g33790) and “GmPEAMT2” 
 (Glyma07g11580) were determined. Both GmPEAMT1 and 
 GmPEAMT2 contain 11 exons and 10 introns (Supplementary 
 Figure 1). GmPEAMT1 encodes a predicted protein of 488 
 amino acids with a calculated molecular mass of 56.01 kDa, and 
 GmPEAMT2 encodes a predicted protein of 531 amino acids 
 with a calculated molecular mass of 60.74 kDa. Their isoelectric 
 points are both less than 7. GmPEAMT1 and GmPEAMT2 
 contain a methyltransferase (MT) domain at the N-terminal 
 and the C-terminal, respectively (Figure 1A). Each domain 
 comprises four SAM-binding motifs (I, p-I, II, and III). It 
 is found that the arrangement of motif I in GmPEAMT1 
 and GmPEAMT2 meets the standard requirement proposed 
 by Clawson (Clawson et al., 1990). The alignment analysis 
 shows that both MT1 and MT2 domains of GmPEAMTs 
 are homologous to the respective domains in the PEAMTs of 
 plants, nematodes, and plasmodium. Most phosphorylation sites 
 and catalytic sites found in nematodes and plasmodium PEAMT (Lee 
 and Jez, 2014) are conserved in soybean (Figures 1B,C). Neither 
 GmPEAMT1 nor GmPEAMT2 has a transmembrane domain 
 (Supplementary Figure 2).

To explore the phylogenetic relationship of GmPEAMT1 
 and GmPEAMT2 with other plant PEAMTs, we used the 
 neighbor-joining method of MEGA to construct a phylogenetic 
 tree of plant PEAMTs (Figure 2). These plant PEAMTs are 
 separated into two clades, monocotyledon, and dicotyledon. 
 Both GmPEAMT1 and GmPEAMT2 are clustered together 
 with other legumes. GmPEAMT1 is closely related to 
 Medicago truncatula MtPEAMT2, and GmPEAMT2 shows high similarity 
 with Cajanus cajan CcPEAMT, suggesting that they may have 
 similar functions.

**Identification of GmPEAMT1 and GmPEAMT2 Promoters**

The putative promoters of GmPEAMT1 and GmPEAMT2, 
 which mainly cover different classes of regulatory motifs, 
 were isolated and sequenced. The possible cis-acting elements in 
 the promoters of GmPEAMT1 and GmPEAMT2 were predicted by 
 PlantCARE (Figure 3). Light response elements are found to 
 be the most abundant cis-acting elements in the regions of 
 GmPEAMT1 and GmPEAMT2 promoters. In addition, there is 
 a methyl jasmonate response element in the promoter region 
 of GmPEAMT1. Many plant hormone response elements are 
 also distributed in the promoter region of GmPEAMT2, such as 
 abscisic acid response elements, salicylic acid response elements, 
 and gibberellin response elements.

**Tissue Expression Analysis of GmPEAMT1 and GmPEAMT2**

To examine the expression patterns of GmPEAMT1 and 
 GmPEAMT2, the relative expression level of GmPEAMT1s in
FIGURE 1 | Multiple amino acid sequence alignments of the two domains of PEAMTs. (A) A schematic diagram of plant PEAMTs. Methyltransferase domains MT1 and MT2 are shown in black and gray, respectively. (B,C) Multiple alignments of MT1 (B) and MT2 (C) domains. Dashes represent gaps introduced to improve the alignment. Identical and similar amino acids are shown in black and gray, respectively. The putative phosphorylation sites and catalytic sites deduced from the structural studies (Lee and Jez, 2014) are indicated by triangles and diamonds, respectively. The four SAM-binding motifs (I, p-I, II, and III) are indicated. The alignments of MT1 and MT2 domains were conducted with ClustalX 2.0 and viewed with GeneDoc. GmPEAMT1, Glycine max PEAMT1 (NP_001348858.1); GmPEAMT2, Glycine max PEAMT2 (XP_025984992); MtPEAMT1, Medicago truncatula PEAMT1 (XP_003619836.1); MtPEAMT2, Medicago truncatula PEAMT2 (XP_003631124); AtPEAMT, Arabidopsis thaliana PEAMT (XP_003631124); OsPEAMT, Oryza sativa PEAMT (XP_015622327.1); CaPEAMT, Chlamydomonas applanata PEAMT (LC228965-1); CePEAMT1, Caenorhabditis elegans PEAMT1 (NP_494991.1); CePEAMT2, Caenorhabditis elegans PEAMT2 (NP_504248.1); PfPEAMT, Plasmodium falciparum PEAMT (XP_001350151.1).
different tissues during the vegetative and reproductive stages was obtained by qRT-PCR (Figure 4). Transcripts of both GmPEAMT1 and GmPEAMT2 can be detected in all tissues, but their expression patterns are different. GmPEAMT1 has high expression levels in roots, leaves, flowers, and seeds, but low expression levels in stems and fruits. GmPEAMT2 is most...
Subcellular Localization of GmPEAMT1 and GmPEAMT2

To determine the proteins localization in plant cells, the GmPEAMT-GFP fusion expression vector was successfully transformed into *N. benthamiana* leaves by *Agrobacterium tumefaciens*. The green fluorescent signals of GmPEAMT1-GFP fusion proteins partly coincided with the red fluorescent signals of nucleus marker proteins (Figure 5A). Notably, expression of GmPEAMT1-GFP led to the formation of punctate fluorescent signals with a radial network, particularly around the signals of nucleus marker proteins. Further research found that the radial network signals of GmPEAMT1-GFP overlapped with the red fluorescent signals of the endoplasmic reticulum marker proteins (Figure 5B). The location of GmPEAMT2 was consistent with GmPEAMT1 (Figures 5A, B). The signals of the empty plasmid pFGC5941-GFP were distributed throughout the whole cell (Figures 5A, B). The results reveal that GmPEAMT1 and GmPEAMT2 localize in the nucleus and endoplasmic reticulum.

Functional Analysis of GmPEAMT1 and GmPEAMT2 in Yeast

Yeast cells lack PEAMT activity, and the synthesis of PC in *vivo* relies on PLMT1/CHO2 and PLMT2/OPI3 to catalyze the three-step methylation of Ptd-EA to PC (Figure 6C). The deletion of *CHO2* and *OPI3* in yeast will completely hinder the biosynthesis of PC, resulting in a temperature-sensitive growth defect at 37°C, which is rescued by exogenous supplementation of choline (Kodaki and Yamashita, 1987, 1989; Keogh et al., 2009). To examine whether GmPEAMT1 and GmPEAMT2 encode a functional methyltransferase to produce P-Cho, we took advantage of the *cho2 opi3* mutant and observed its growth when expressing *GmPEAMT1* or *GmPEAMT2* under the control of GAL1 inducible promoters. The yeast *cho2 opi3* mutant transformed into empty vectors was used as a negative control, and the yeast *cho2 opi3* mutant transformed into *AtPEAMT1* was used as a positive control, as *AtPEAMT1* expression could fully rescue *cho2 opi3* mutant growth in the absence of exogenous choline (Figure 6D; Bolognese and McGraw, 2000). As shown in Figure 6A, the yeast *cho2 opi3* mutant expressing GmPEAMT1 or GmPEAMT2 alone could not restore normal growth in the absence of exogenous choline. These results suggest that GmPEAMT1 and GmPEAMT2 cannot continuously catalyze the three-step methylation of P-EA to P-Cho. At least one of the three steps has defects.

To further determine the catalytic abilities of GmPEAMT at the phosphate level, we co-expressed *AtPLMT* and *GmPEAMT* in the yeast *cho2 opi3* mutant. *AtPLMT* has a similar function to *OPI3*, which can catalyze the two-step methylation from Ptd-MMEA to PC via Ptd-DMEA (Figure 6D; Keogh et al., 2009). As shown in Figure 6B, the yeast *cho2 opi3* mutant co-expressing GmPEAMT1 and *AtPLMT* could restore normal growth without exogenous additives. In addition, co-expressing of GmPEAMT2 and *AtPLMT* could partly restore the growth of *cho2 opi3* cells. These results indicate that GmPEAMT1 and GmPEAMT2 can at least catalyze the methylation from P-EA to P-MMEA (Figure 6D).

Functional Verification of GmPEAMT1 and GmPEAMT2 in Arabidopsis

To further investigate the biochemical functions of GmPEAMT1 and GmPEAMT2 in planta, we individually expressed *GmPEAMT1* and *GmPEAMT2* in the Arabidopsis *peamt1* mutant, which exhibits a distinctive short-root phenotype (Cruz-Ramírez et al., 2004). As shown in Figure 7A, *peamt1* roots were significantly shorter than wild-type roots, as expected. The root length of the *peamt1* mutant expressing *GmPEAMT1* is longer significantly than *peamt1* (Figure 7C), indicating that the heterologous expression of *GmPEAMT1* in the *peamt1* mutant can partially restore normal root development but

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**FIGURE 4** Relative expression of GmPEAMTs in different tissues. Relative transcript abundance of GmPEAMT1 (A) and GmPEAMT2 (B) in vegetative and reproductive organs was quantified by qRT-PCR and normalized to the abundance of *GmTUBLIN*. Expression analyses were relative to the expression amounts of the GmPEAMT1 and GmPEAMT2 in the leaf sample. The error bars represent the standard error of three replicates. Each replicate corresponded to the three individual plants.
cannot revert to the wild-type level. The peamt1 mutants expressing GmPEAMT2 have a similar phenotype to the peamt1 mutants, expressing GmPEAMT1 (Figures 7B,D). These results demonstrate that GmPEAMT1 and GmPEAMT2 are partly homologous to AtPEAMT1 in planta, and there is some functional redundancy between GmPEAMT1 and GmPEAMT2.
FIGURE 6 | Functional complementation of GmPEAMTs in Saccharomyces cerevisiae. (A,B) Heterologous complementation of PCho-dependent growth of the S. cerevisiae cho2 opi3 mutant. Yeast mutant strain cho2 opi3 harboring empty vectors (negative controls), Arabidopsis PEAMT1 (positive controls), GmPEAMT1, GmPEAMT2, AtPLMT, GmPEAMT1+AtPLMT, and GmPEAMT2+AtPLMT were grown on agar supplemented with minimal media and 2% (w/v) galactose, and 0-mM ethanolamine (CK) or 1-mM ethanolamine (EA) or 1-mM monomethylethanolamine (MEA) or 1-mM choline (CHO). These transformants were grown at 37°C for 3 days. Different transformants and additives are indicated at the left and the top of the images, respectively (C,D). The currently proposed PC biosynthesis pathway in S. cerevisiae and plants, respectively (Keogh et al., 2009; Liu et al., 2018). The methyltransferases involved in the synthesis of PC in S. cerevisiae, Arabidopsis, and soybeans are shown in blue, green, and red. Ptd-EA, phosphatidylethanolamine; Ptd-MMEA, phosphatidylmonomethylethanolamine; Ptd-DMEA, phosphatidyldimethylethanolamine; Ptd-CHO, phosphatidylcholine; CDP-EA, cytidine diphosphate-ethanolamine; CDP-CHO, cytidine diphosphate-choline; P-EA, ethanolamine-phosphate; P-MMEA, monomethylethanolamine-phosphate; P-DMEA, dimethylethanolamine-phosphate; P-CHO, phosphate-choline; EA, ethanolamine; Cho, choline; PEAMT, phosphoethanolamine methyltransferase; and PLMT, phospholipid methyltransferase.

DISCUSSION

Possible Functional Differences Between GmPEAMTs and Other Plants PEAMTs

Phosphatidylcholine is an essential component of plant cell membranes (Meijer and Munnik, 2003). The PC synthesis pathways of different plants are distinct (Datko and Mudd, 1988b; Williams and Harwood, 1994; Lykidis, 2007). This difference is mainly reflected in the methylation at the level of phosphate and phosphatidylmediated by PEAMT and PLMT, respectively (Keogh et al., 2009; Chen et al., 2018a). In this study, the coding sequences and promoters of PEAMTs were successfully isolated from soybeans. GmPEAMT1 and GmPEAMT2 are very similar to PEAMTs of other plants, such as Arabidopsis (Bolognese and McGraw, 2000) and spinach (Nuccio et al., 2000) in terms of protein size and an isoelectric point. Protein domain analysis shows that the MT1 and MT2 domain sequences of plant PEAMT are highly conserved, especially the catalytic residues and phosphorylation sites, which found in some nematodes or plasmodia (Lee and Jez, 2014), are also present in GmPEAMT1 and GmPEAMT2, indicating that GmPEAMT1 and GmPEAMT2 may have similar biological functions to other plant PEAMTs.

GmPEAMT1 and GmPEAMT2 were predicted to localize in the cytoplasm based on the protein properties, such as hydrophilicity, the lack of transmembrane structure, and the lack of organelle-targeting signal sequences. The reported plant PEAMTs are all located in the cytoplasm (Bolognese and McGraw, 2000; Smith et al., 2000). However, the results of subcellular localization show that GmPEAMT1 and GmPEAMT2 are located in the nucleus and endoplasmic reticulum, which indicates that GmPEAMT1 and GmPEAMT2 may have different properties from other plant PEAMTs. The phenomenon of non-unique subcellular localization is common in plants. The localization of some transporters, transcription factors or photoreceptor proteins will change with external signals. For example, it is reported that the localization of phosphate transporter PHT1 is affected by phosphorus content. When phosphorus is sufficient, PHT1 is phosphorylated and localized in the endoplasmic reticulum, while, when phosphorus is deficient,
PHT1 is dephosphorylated and carried to the cell membrane by PHF1 (Ham et al., 2018). Therefore, the non-uniqueness of GmPEAMTs position may have a certain relationship with its response to external signals, which requires further experimental verification.

The Potential of GmPEAMT1 and GmPEAMT2 to Participate in Plant Stress Response

When responding to various abiotic stresses, plants will induce the expression of specific genes through their signal transduction mechanisms, thereby reducing the harm of stress to plants, and this induced expression is related closely to the cis-acting elements in the upstream promoter region of the gene and the corresponding transcription factors (Liu et al., 2014). The prediction of the cis-acting elements in the promoters of GmPEAMT1 and GmPEAMT2 shows that a large number of light response elements, some plant hormone response elements, and stress response elements are distributed in the promoter region. The promotors of corn (Gou, 2017), Suaeda liaotungensis (Xie, 2013), and Salicornia (Ma, 2008) PEAMTs also contain these elements. PEAMTs of many dicotyledonous plants have light-responsive characteristics. For example, when measuring the phosphoethanolamine methyltransferase activity of spinach, it was found that its activity was highest at the end of the photoperiod and lowest at the end of the dark period. When the plant was exposed to the light again, the activity would be restored (Weretilnyk et al., 1995). Nuccio found that salt treatment improved the expression level of PEAMT in spinach, and then increased the production of choline to promote the accumulation of betaine (Nuccio et al., 2000). Low-temperature treatment and salt treatment will lead to the upregulation of wheat PEAMT and corresponding protein expression. The corresponding increase in enzyme activity may be related to the accumulation of betaine in wheat (Charron et al., 2002). These findings are consistent with the results in this study. It is preliminarily inferred that GmPEAMT1 and GmPEAMT2 are both plant stress-related genes, and their promoters may have the characteristics of responding to abiotic stress.

The Specificity of GmPEAMT1 and GmPEAMT2 Function

Through the application of yeast heterologous complementation, some plant PEAMTs have been biochemically verified, such as...
spinech (Nuccio et al., 2000) and Arabidopsis (Bolognese and McGraw, 2000; Chen et al., 2018a,b). Deletion of CH02 and OP13 involved in the methylation reaction of PC biosynthesis at the phosphatidyl level is lethal unless choline is exogenously provided (Kodaki and Yamashita, 1987; Summers et al., 1988; Preitschopf et al., 1993; Keogh et al., 2009). ApLMT is homologous to OP13, which can catalyze the transformation of Ptd-MMEA→Ptd-DMEA→PC (Keogh et al., 2009). The independent expression of GmPEAMT1 or GmPEAMT2 in the yeast cho2 opi3 mutant cannot compensate for its growth defect. In the absence of exogenous choline, co-expression of AtPlmt and GmPEAMT1 or GmPEAMT2 enabled the cho2 opi3 mutant to grow. These results indicate that GmPEAMTs can at least catalyze the first step of methylation at the phosphate level to convert P-EA to P-MMEA. Then P-MMEA is converted into Ptd-MMEA through the nucleotide pathway, and finally, PC is formed under the catalysis of AtPlmt in cho2 opi3 cells. In soybeans, it has been proved that only the first step methylation (P-EA→P-MMEA) conducts at the phosphate level by the metabolic analysis (Datko and Mudd, 1988a). Therefore, it is deduced that GmPEAMT1 and GmPEAMT2 can only catalyze the methylation of PEA to P-MMEA.

Studies have shown that the MT1 domain of PEAMT is responsible for catalyzing the initial methylation reaction from P-EA to P-MMEA; the MT2 domain catalyzes the two-step subsequent methylation reaction from P-MMEA to P-Cho via P-DMEA (BeGora et al., 2010; Lee and Jez, 2013, 2014, 2017). Although GmPEAMT1 and GmPEAMT2 have the MT2 domain, they cannot catalyze the last two steps of methylation. The loss of catalytic activity may result from mutations in some key positions of the MT2 domain. However, studies have also shown that there is no absolute correspondence between structural domains and catalytic functions. For example, Caenorhabditis elegans PEAMT1 can only methylate P-EA to P-MMEA, while the second enzyme PEAMT2 can methylate P-MMEA and P-DMEA, but they both have two domains. However, a single protein has only one methyltransferase domain to function, and the other does not have catalytic activity (Lee and Jez, 2013). What is more, Plasmodium falciparum PEAMT only has one domain to catalyze a continuous three-step methylation reaction (Reynolds et al., 2008).

In animals and yeast, the three-step methylation reaction on the phosphatidyl group is the only way for these organisms to de novo synthesis PC, and PLMT is the key enzyme that functions in this pathway (Keogh et al., 2009). However, the phosphorylation pathway for de novo synthesis of PC in plants starts with the methylation of P-EA by PEAMT (McNeil et al., 2001). The subsequent two-step methylation may occur at the phosphate level or the phosphatidyl level, depending on the species (Lykidis, 2007). For example, in Lemna and carrot (Daucus carota L.), the sequential methylation of P-EA and the sequential methylation of Ptd-MMEA are carried out simultaneously (Datko and Mudd, 1988b). Olive (Olea europaea L.) is more extraordinary than the first two-step methylation occurs at the phosphate level, and the last step occurs at the phosphatidyl level (Williams and Harwood, 1994). These results all illustrate the considerable differences in the PC synthesis pathway between different plants and the diversity of PEAMT functions. The differences in the role and necessity status of PEAMT among plants are also worthy of our in-depth exploration.

**Possible Reasons for the Difference in the PEAMT Function Between Arabidopsis and Soybeans**

In order to better understand the role of PEAMT in plants, we analyzed the biological function of GmPEAMT1 and GmPEAMT2 through the genetic transformation in Arabidopsis. Similar to PEAMTs in most plants, AtPEAMT1 has two methyltransferase domains, MT1 and MT2, which can catalyze the three-step methylation reaction of P-EA to P-Cho at the phosphate level (Bolognese and McGraw, 2000). When AtPEAMT1 was mutated by a T-DNA insertion, the root growth of the mutant plants was greatly restricted (Mou et al., 2002; Cruz-Ramirez et al., 2004). There were severe defects in the meristem and elongation zone, such as the abnormal morphology of the epidermal cell. In addition, the inhibition of PC synthesis also induces the death of root epidermal cells (Cruz-Ramirez et al., 2004). In this study, GmPEAMT1 and GmPEAMT2 were driven by the 35S promoter and transferred into the Arabidopsis peamt1 mutant. The overexpression of GmPEAMT1 and GmPEAMT2 in the peamt1 mutant partially restored the root growth of the mutant, indicating that GmPEAMT1 and GmPEAMT2 are similar to AtPEAMT1, which play an essential role in maintaining the normal growth and development of the root.

The introduction of GmPEAMT1 and GmPEAMT2 did not restore the root length of the mutant to the level of wild type, which indicates that, in the biosynthesis of PC, GmPEAMT1, and GmPEAMT2 may have similar but not identical biochemical functions to AtPEAMT1. This may result from the difference of substrate catalytic ability between soybeans and Arabidopsis. Yeast complementation verification results show that GmPEAMT1 and GmPEAMT2 can catalyze the methylation of PEA to P-MMEA. In contrast, other plant PEAMTs represented by Arabidopsis can catalyze the triple methylation of PEA to P-Cho (Chen et al., 2018b). The difference in tissue expression patterns and the subcellular localization between GmPEAMT1 and GmPEAMT2 may also result in the different functions between soybeans and Arabidopsis.

It is also worth noting that the overexpression of GmPEAMT1 and GmPEAMT2 in the peamt1 mutant has similar phenotypes, indicating that GmPEAMT1 and GmPEAMT2 have a certain degree of functional redundancy in maintaining the normal growth and development of the root. This kind of genetic redundancy is widespread in organisms. It is a coping strategy to reduce the impact of unfavorable external environmental factors on their survival, which gradually formed in the evolution process of organisms adapting to the environment. Through the genetic redundancy, phenotypic changes caused by a knockout or loss of function of a gene can be reduced (Li et al., 2018).
Given the difference in tissue expression patterns of GmPEAMT1 and GmPEAMT2, whether they have different functions in other processes of plant growth and development remains to be explored. Elucidating the synergistic but unequal functions of GmPEAMT1 and GmPEAMT2 in soybean PC synthesis, and the contribution of GmPEAMT-mediated phosphate level methylation to PC synthesis will be important future effort.

In summary, the identification and functional analysis of PEAMTs in soybeans enhanced our understanding of the synthesis pathway of phosphatidylcholine in legumes. It provides valuable materials for further exploration of the specific pathways of lipid synthesis between different plants and the massive variability in the regulation of this process. At the same time, it is also of great significance for improving stress resistance and soybean quality.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.612158/full#supplementary-material

AUTHOR CONTRIBUTIONS

YC and YS conceived the study. XW contributed to the cultivation of test materials. WC performed the qRT-PCR. QY performed the subcellular localization experiment. XJ carried out the functional verification experiments in Arabidopsis and yeast, analyzed the data, and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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