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Functional Characterization of Phospholipid *N*-Methyltransferases from *Arabidopsis* and Soybean*[S]

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Matthew R. Keogh[‡], Polly D. Courtney[§], Anthony J. Kinney[¶], and Ralph E. Dewey[§1]

From the Departments of [§]Crop Science and [‡]Plant Biology, North Carolina State University, Raleigh, North Carolina 27695-8009 and [¶]Crop Genetics Research, DuPont Experimental Station, Wilmington, Delaware 19880-0353

Phospholipid *N*-methyltransferase (PLMT) enzymes catalyze the *S*-adenosylmethionine-dependent methylation of ethanolamine-containing phospholipids to produce the abundant membrane lipid phosphatidylcholine (PtdCho). In mammals and yeast, PLMT activities are required for the *de novo* synthesis of the choline headgroup found in PtdCho. PLMT enzyme activities have also been reported in plants, yet their roles in PtdCho biosynthesis are less clear because most plants can produce the choline headgroup entirely via soluble substrates, initiated by the methylation of free ethanolamine-phosphate. To gain further insights into the function of PLMT enzymes in plants, we isolated *PLMT* cDNAs from *Arabidopsis* and soybean (*Glycine max*) based upon primary amino acid sequence homology to the rat PLMT, phosphatidylethanolamine *N*-methyltransferase. Using a heterologous yeast expression system, it was shown that plant PLMTs methylate phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine but cannot utilize phosphatidylethanolamine as a substrate. Identification of an *Arabidopsis* line containing a knock-out dissociator transposon insertion within the single copy *AtPLMT* gene allowed us to investigate the consequences of loss of PLMT function. Although the accumulation of the PLMT substrates phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine was considerably elevated in the *atplmt* knock-out line, PtdCho levels remained normal, and no obvious differences were observed in plant morphology or development under standard growth conditions. However, because the metabolic routes through which PtdCho is synthesized in plants vary greatly among differing species, it is predicted that the degree with which PtdCho synthesis is dependent upon PLMT activities will also vary widely throughout the plant kingdom.

Phosphatidylcholine (PtdCho)[2] is the most abundant phospholipid in most non-plastid membranes of eukaryotes. PtdCho biosynthesis has been studied intensively in plants not only because of its importance as a structural membrane lipid, but also because of its role as a precursor to important lipid-based signaling molecules, such as phosphatidic acid, and phospholipase A_2-derived free fatty acids (1). The choline headgroup of PtdCho serves multiple functions as well. In addition to being an essential human nutrient (2), in many plant species choline can be oxidized to produce the potent osmoprotectant glycine betaine (3, 4).

For over 2 decades it has been apparent that there are fundamental differences between the manner in which PtdCho is produced in plants *versus* how it is synthesized in mammals and fungi. In the latter two systems, PtdCho can be formed through two distinct pathways as follows: (*a*) the "nucleotide pathway" in which free choline is incorporated in PtdCho using CDP-choline as an intermediate, and (*b*) the "methylation pathway" whereby PtdCho is produced directly from phosphatidylethanolamine (PtdEtn) via three sequential methylation reactions using *S*-adenosylmethionine (AdoMet) as the methyl donor (5, 6). In contrast, PtdCho biosynthesis in plants occurs through a branched pathway that utilizes components of both the nucleotide and methylation pathways (7). The greatest distinction between the contrasting mechanisms of PtdCho biosynthesis can be attributed to the presence of plant enzymes that are capable of converting ethanolamine headgroups to choline at the phospho-base level, activities that are absent in mammals and yeast. Conversely, mammals and fungi possess methylation enzymes that act directly on PtdEtn, a reaction that cannot be detected in most plant systems investigated (reviewed in Ref. 7).

A diagram of the most widely accepted model of phosphoamino alcohol biosynthesis in plants is shown in Fig. 1. Similar to animals and yeast, free choline can be directly incorporated into PtdCho via nucleotide pathway enzymes in plants. In the absence of choline, however, the methylation of Etn-phosphate represents the first committed step in PtdCho biosynthesis. The resulting monomethylethanolamine-phosphate (MMEtn-P) metabolite can be further methylated at the phospho-base level to produce Cho-P. Alternatively, MMEtn-P can be incorporated into phosphatidylmonomethylethanolamine (PtdMMEtn) via the cytidylyltransferase and amino alcohol phosphotransferase activities of the nucleotide pathway and then methylated at the phosphatidyl-base level to

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) FJ858262.

[1] To whom correspondence should be addressed: North Carolina State University, Campus Box 8009, Raleigh, NC 27695-8009. Fax: 919-515-7959; E-mail: ralph_dewey@ncsu.edu.

[2] The abbreviations used are: PtdCho, phosphatidylcholine; PLMT, phospholipid *N*-methyltransferase; PtdMMEtn, phosphatidylmonomethylethanolamine; PtdDMEtn, phosphatidyldimethylethanolamine; PtdEtn, phosphatidylethanolamine; MMEtn-P, monomethylethanolamine-phosphate; PEAMT, phosphoethanolamine *N*-methyltransferase; PEMT, phosphatidylethanolamine *N*-methyltransferase; AdoMet, *S*-adenosylmethionine; ESI/MS/MS, electrospray ionization/tandem mass spectrometry; dH_2O, distilled H_2O; MES, 4-morpholineethanesulfonic acid; Ds, dissociator.

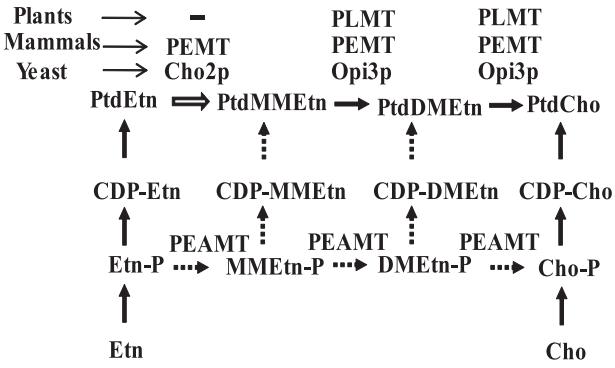

FIGURE 1. **Phosphatidylcholine biosynthetic pathways.** Steps common to plants, mammals, and yeast are indicated by *black arrows*. *Dashed arrows* indicate pathways specific to plants. Methylation of PtdEtn, which occurs in mammals and yeast, is indicated by on *open arrow*. Enzymes catalyzing phosphoamino alcohol methylation reactions in plants, mammals, and yeast are indicated.

complete the synthesis of PtdCho (Fig. 1). The extent with which PtdCho is formed by the flow of metabolites through phospho-bases as opposed to phosphatidyl-bases varies greatly among different plant species. In most higher plants, it is likely that the methylation of the phosphoamino alcohol headgroups involves the flow of metabolites through both branches of the pathway, as has been shown in species such as barley, carrot, and tobacco (3, 8, 9). Nevertheless, examples have also been reported where only one of the branches appears to be utilized. In *Lemna paucicostata*, for example, the methylation steps in PtdCho biosynthesis were shown to occur almost exclusively at the phospho-base level (10). At the other end of the spectrum is soybean, where all methylations beyond the initial formation of MMEtn-P were reported to occur on phosphatidyl-bases (8, 11). The tremendous variability observed among plants with regard to PtdCho formation is also exemplified by a study conducted by Williams and Harwood (12) where it was shown that the predominant route of PtdCho synthesis in olive culture cells involved the first two methylation reactions taking place at the phospho-base level (producing dimethylethanolamine phosphate) and the final methylation occurring on a phosphatidyldimethylethanolamine (PtdDMEtn) substrate.

Our understanding on the mechanisms by which plants synthesize PtdCho and regulate its accumulation has been further enhanced as the genes encoding the various steps of the phosphoamino alcohol pathway have been isolated and characterized. For example, molecular characterizations led to the conclusion that all of the amino alcohol phosphotransferase reactions depicted in Fig. 1 can be mediated by the product of a single gene (designated *AAPT1*) that displays a broad substrate specificity (13, 14). Similarly, it was the isolation of the phosphoethanolamine methyltransferase (PEAMT) genes from *Arabidopsis* and spinach that led to the discovery that all three phospho-base methylation reactions could be catalyzed by a single enzyme (15, 16). Inhibition of PEAMT gene function in *Arabidopsis* through T-DNA insertion or co-suppression revealed unexpected associations between the phosphoamino alcohol pathway and root development, salt hypersensitivity, and male sterility (17, 18).

Although most of the reactions depicted in Fig. 1 have been characterized at the molecular genetic level, conspicuously absent is information on the plant genes/enzymes responsible for the methylation reactions conducted at the phosphatidylbase level. In contrast, these reactions are among the most well characterized in animals and yeast, catalyzed by enzymes commonly referred to as phospholipid *N*-methyltransferases (PLMTs). In mammals, the 18-kDa integral membrane protein phosphatidylethanolamine *N*-methyltransferase (PEMT) is a PLMT that is expressed primarily in the liver (19). PEMT catalyzes all three of the methylation reactions needed to convert PtdEtn to PtdCho. Yeast uses two distinct PLMT enzymes to catalyze the three methylation reactions as follows: Cho2p/Pem1p that mediates the direct methylation of PtdEtn to produce PtdMMEtn (20, 21), and Opi3p/Pem2p, an enzyme homologous to the mammalian PEMT, that primarily catalyzes the methylation of PtdMMEtn to PtdDMEtn and PtdDMEtn to PtdCho, the final two steps of the methylation pathway (20, 22). PLMT activities are critical in both of these systems. Mice possessing *pemt* knock-out mutations are completely dependent on dietary choline for survival, and they display abnormal levels of choline metabolites within the liver and develop hepatic steatosis even when fed diets supplemented with choline (23). Yeast lacking PLMT activities (*cho2*/*opi3* double mutants) are obligate choline auxotrophs, unable to synthesize PtdCho *de novo* in the absence of exogenous choline.

To gain a greater understanding of the specific function of PLMT reactions in higher plants, and their contribution toward PtdCho biosynthesis, we cloned and characterized PLMT homologs from *Arabidopsis* and soybean. By expressing the candidate cDNAs in yeast, we were able to confirm that they encoded functional PLMT activities as well as to establish their substrate specificities. We also identified a mutant *Arabidopsis* line containing a knock-out allele in the single copy *PLMT* gene found in the *Arabidopsis* genome, allowing us to characterize the consequences of loss of gene function in this model species.

EXPERIMENTAL PROCEDURES

Yeast Growth and Strains—Yeast were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) (w/v) media for complex media or yeast nitrogen base without amino acids for defined media (supplemented as needed with amino acids). Yeast strain CDS100 was grown on minimal media with 1 mM choline unless otherwise stated. Yeast strains used included CTY182 (*MAT***a**, *ura3-52, his3-200, lys2-801*), CTY410 (*MAT***a** *his3-200, leu2*Δ*9, cho2*::*LEU2*), CTY411 (*MATα, ade2-101, his3-200, leu2, trp1, can1, opi3*::*URA3*), and CSD100 (*his3-200, opi3*::*URA3, cho2*::*LEU2*). Strain CSD100 was obtained by crossing strains CTY410 and CTY411 using the procedure described by Guthrie and Fink (24).

Cloning of Plant PLMTs for Expression in Yeast—To place the plant *PLMT* cDNAs under the regulatory control of a strong constitutive yeast promoter, HindIII restriction sites were initially engineered into both the 5′- and 3′-untranslated regions of the *AtPLMT* and *GmPLMT* cDNAs via PCR. Subsequent digestion with HindIII enabled the insertion of the plant cDNAs between the promoter and terminator regions of the yeast *ADH1* gene in yeast expression vector pDB20 (25). A BamHI digest of the resulting plasmids yielded a 3-kb band containing the yeast *ADH* promoter/plant *PLMT*/*ADH* termi-

nator (partial digests were required for *AtPLMT* to avoid an internal BamHI site), which was gel-extracted and ligated into BamHI-digested yeast expression vector pRS313 (26). pRS313 contains a *HIS3* selectable marker that facilitated the subsequent transformation of the expression constructs into strains CTY411 (Δ*his3-200*) and CSD100 (Δ*his3-200*). Yeast transformation was conducted using the PEG/lithium acetate transformation protocol described by Gietz and Schiestl (27).

Phospholipid N-Methyltransferase Assays—Yeast cultures were grown overnight to stationary phase, and microsomes were isolated as described by Tang *et al.* (28). *In vitro* methylation assays were performed following the protocol of Kodaki and Yamashita (20). Reactions included 50 μg of microsomal protein, 40 mM Tris-HCl, pH 8.8, 3 mM $MgSO_4$, and 4 μCi of [*methyl*-^{3}H]AdoMet (0.72 μM) (60 Ci/mmol; American Radiolabeled Chemicals Inc.). A total reaction volume of 400 μl was achieved by the addition of dH_2O. Samples were incubated at 30 °C for 15 min and terminated by the addition of 400 μl of 2:1 chloroform/methanol. The bottom phase was transferred to a new tube, and the remaining aqueous phase was extracted again using 400 μl of 2:1 chloroform/methanol. The pooled organic phases were washed using dH_2O (40% final volume), and the extracted organic phase was evaporated to dryness and resuspended in 100 μl of 2:1 chloroform/methanol. Reaction products were spotted on silica gel 60 plates (Whatman) and resolved using chloroform/methanol/acetic acid (65:35:5, v/v/v). Labeled compounds corresponding to PtdMMEtn, PtdDMEtn, and PtdCho were identified by co-migration with authentic standards (Avanti Polar Lipids, Alabaster, AL) in adjacent lanes and quantitated using scintillation spectroscopy.

Analysis of Arabidopsis Ds-transposon Insertion Line GT9768—All plants used in this study were grown under 16-h/8-h light/dark cycle at 22 °C. Genomic DNA was extracted from young leaves according to Edwards *et al.* (29). Plants were genotyped by PCR using the following three primers: forward (5′-CGTAATAAGTCCCAGCTTCACCTAACA-3′) and reverse (5′-AAACAGAACTTTAAGGAGCGATTGC-3′ corresponding to genomic *AtPLMT* sequences flanking the predicted insertion site, and a primer specific to the Ds element (5′-GAAACGGTCGGGAAACTAGCTCTAC-3′). The PCR conditions were as follows: 94 °C incubation for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1.5 min and a final 7-min extension at 72 °C. For transcript analysis of line GT9763, total RNA was isolated with TRIzol™ reagent (Invitrogen) according to the manufacturer's protocol. First strand cDNA was synthesized using the First Strand synthesis kit (Invitrogen). The primers used to determine *AtPLMT* expression were 5′-CATCGACGGTAATGGCACGTGTTT-3′ (forward) and 5′-ACCCAAGAGGCATGCCAATAGA-3′ (reverse). To amplify transcripts corresponding to the *actin-8* gene control, 5′-CTTTCCGGTTACAGCGTTTG-3′ and 5′-GAAACGCGGATTAGTGCCT-3′ were used as forward and reverse primers, respectively. The PCR conditions were as follows: 94 °C incubation for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final 7-min extension at 72 °C.

DNA and RNA Gel Analyses—Genomic DNA was isolated from young leaf tissue according to Murray and Thompson (30). Total RNA was extracted using the TRIzol™ reagent as described above. Nucleic acid blot hybridizations were conducted using either the protocols described by Sambrook and Russell (31) or using the Perfect-Hyb Plus hybridization buffer according to the manufacturer's protocol (Sigma). ^{32}P-Labeled hybridization probes were generated using the Random Prime Labeling kit according the method provided by the manufacturer (Roche Diagnostics). After overnight incubation with labeled probe, blots were washed as follows: two washes with 2× SSPE 0.1% SDS (1× SSPE = 150 mM NaCl, 10 mM $NaHPO_4$, 1 mM EDTA, pH 7.4) at room temperature for 15 min, two washes using 1× SSPE 0.1% SDS at 65 °C for 15 min, two washes with 0.5× SSPE 0.1% SDS at 65 °C for 15 min, and a final wash using 0.1× SSPE 0.1% SDS at 65 °C for 10 min. Autoradiograms were generated by exposing the washed blots to Kodak Biomax XAR film.

[^{14}C]Formate Radiolabeling of Arabidopsis Seedlings—The protocol for labeling whole *Arabidopsis* plantlets with [^{14}C]formate was adapted from Hanson and Wyse (32). *Arabidopsis* seeds were germinated on 1% phytoagar plates with Murashige and Skoog (MS) media and 1% sucrose. Eight to 10 days after germination, plantlets were transferred aseptically to 1 ml of liquid medium containing MS salts, 1% sucrose, and 10 mM MES, pH 5.6. After equilibration in the liquid medium by growth for 24 h on a rotary shaker (120 rpm) using a 16-h light/8-h dark cycle, 20 μCi of [^{14}C]formate (56 mCi/mmol; Sigma) was added to the 1-ml volumes, and the seedlings were shaken in the dark for up to 24 h. Labeling was terminated by the addition of 5% trichloroacetic acid (final concentration), followed by a 20-min incubation on ice. Seedlings were rinsed with dH_2O, then ground with a micropestal in 200 μl of extraction solvent (dH_2O/ethanol/diethyl ether/pyridine at 15:15:5:1, v/v), followed by incubation at 65 °C for 20 min. One ml of chloroform/methanol (2:1, v/v) was added to each sample, vortexed thoroughly, and incubated overnight at 4 °C. To promote phase separation, 400 μl of dH_2O was added, vortexed, and centrifuged at 2 min at 13,000 × g. The bottom phase was extracted, dried under a vacuum, and resuspended in 50 μl of chloroform/methanol (2:1, v/v). Twenty five microliters of each extract were separated on a LK5D silica gel 60- Å TLC plate and developed in a tank containing chloroform/methanol/glacial acetic acid (65:35:5, v/v/v). Regions corresponding to unlabeled standards (visualized with iodine vapor) were scraped from the plate, and radioactivity was quantified by liquid scintillation spectroscopy.

Lipidomics Analysis—Arabidopsis seedlings were grown on 1% phytoagar plates containing MS salts and 1% sucrose. Phospholipids were extracted from 10-day-old plants as described previously (33). Plant tissue remaining after lipid extraction was placed in a drying oven for 18 h at 80 °C and then weighed to allow for normalization according to dry weight. Lipidomics analysis was conducted using ESI/MS/MS by the Kansas State Lipidomics Research group.

RESULTS

Identification of PLMT Homologs from Arabidopsis and Soybean—Using the rat PEMT protein and yeast Opi3p as a query sequences, tblastn searches of the expressed sequence tag

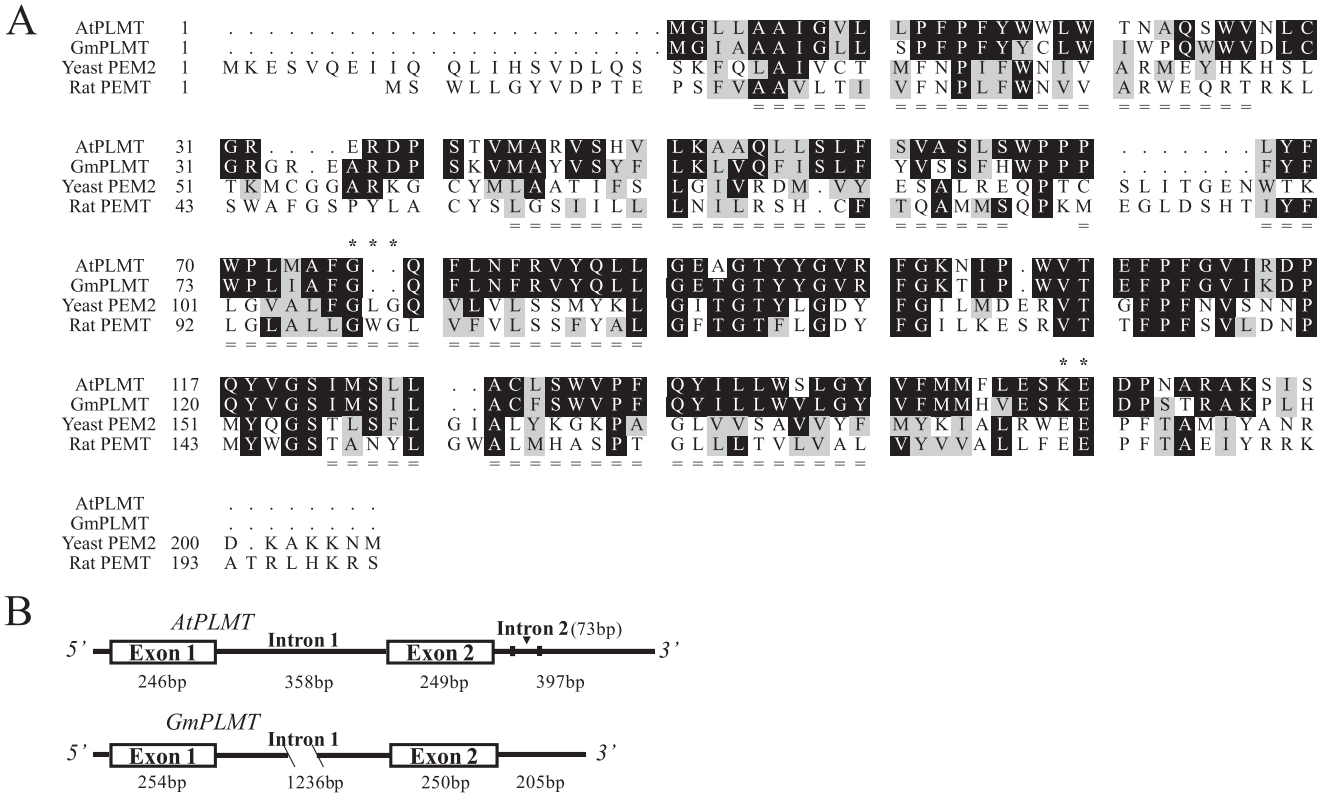

FIGURE 2. **Predicted protein sequence and structure of *Arabidopsis* and soybean *PLMT* genes.** *A,* amino acid alignment of PLMT proteins from *Arabidopsis* (GenBank™ accession number BT043493), soybean (GenBank™ accession number FJ858262), yeast, and rat. Residues shown to function in AdoMet binding in the rat PEMT are indicated by *asterisks*. Amino acids identical to those found in either plant species are *shaded black*; conservative substitutions are *shaded gray*. Predicted transmembrane domains for the plant PLMTs are indicated by *dotted lines*. *B,* gene structures for the *Arabidopsis* and soybean *PLMTs*. The 73-bp intron located in the 3′-flanking region of *AtPLMT* is excised in ~50% of *AtPLMT* transcripts.

(EST) data bases of GenBank™ revealed an *Arabidopsis* EST whose predicted protein product shared homology with the rat and yeast sequences. No *Arabidopsis* sequences with homology to the yeast Cho2p sequence were observed. A partial cDNA lacking part of its 5′ sequence was obtained from the Arabidopsis Biological Resource Center, and a full-length clone was obtained using 5′-rapid amplification of cDNA ends PCR. A homologous sequence from soybean was obtained by screening a soybean cDNA library using the *Arabidopsis* cDNA as a probe. As shown in Fig. 2, the predicted *Arabidopsis* and soybean proteins are 164 and 167 amino acids in size, respectively. The plant proteins share 74% sequence identity to each other, but only about 25% identity to either the yeast Opi3p or rat PEMT enzymes. The presence of three predicted membrane-spanning domains suggests that the plant proteins function as integral membrane proteins.

Analysis of the complete *Arabidopsis* genome sequence showed that the *PLMT*-like sequence exists as a single copy within the genome (GenBank™ accession number At1g80860). Investigation of the soybean genome revealed two distinct chromosomal segments possessing *PLMT*-like genes (data not shown). Chromosome 8 contains the gene that corresponds to the soybean cDNA isolated from the library screen. The other soybean *PLMT*-like sequence (found on chromosome 7) is likely to be a pseudogene, as described below. DNA blotting results were consistent with the interpretation that soybean possesses two loci with homology to this sequence (supplemental Fig. 1).

The genomic structures of the *Arabidopsis* and soybean (chromosome 8) *PLMT*-like genes are shown in Fig. 2*B*. Both genes contain a single intron midway between two protein-encoding exons. Comparison of *Arabidopsis* EST sequences with the At1g80860 genomic sequence reveals that two transcript variants originate from this gene. A 73-bp intron within the 3′-untranslated region of the gene is excised in about half of the *Arabidopsis* ESTs corresponding to At1g80860 (data not shown). Comparison of the soybean *PLMT*-like gene on chromosome 8 with that on chromosome 7, together with their corresponding ESTs, showed that a series of small deletions and a splice junction mutation in the intron results in incomplete, frame-shifted transcripts from the latter gene that would not be likely to produce a functional protein (data not shown). It is therefore probable that the gene residing on chromosome 8 is the only viable copy within the soybean genome.

Biochemical Function and Substrate Specificity—Because of the low primary sequence identity shared between the rat and yeast PLMT protein sequences and the predicted products of the *Arabidopsis* and soybean *PLMT*-like sequences (~25%), it was incumbent to test whether the putative plant proteins were indeed functional homologs of the animal and yeast enzymes. According to the most widely accepted model of PtdCho biosynthesis in plants (Fig. 1), PtdMMEthn and PtdDMEtn would be the most likely substrates for a plant PLMT enzyme. Because of the integral membrane nature of PLMTs, we took advantage of mutant yeast strains to test the function and substrate spec-

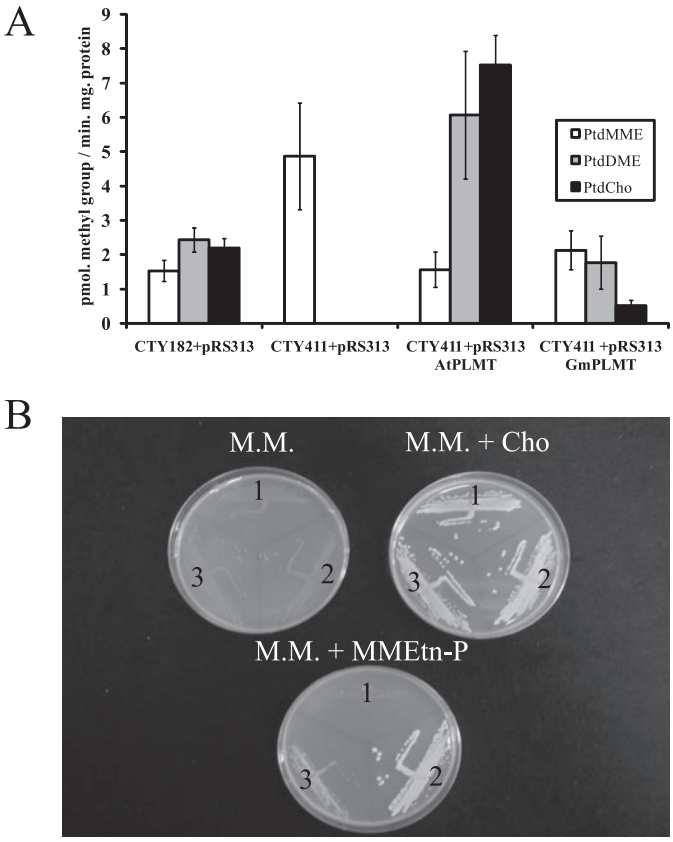

FIGURE 3. **Plant PLMTs methylate PtdMMEtn and PtdDMEtn.** *A, in vitro* phospholipid *N*-methylation assay utilizing microsomes from wild-type yeast strain CTY182 and *opi3* mutant strain CTY411 expressing *AtPLMT*, *GmPLMT*, and vector control. *Error bars* represent S.E. ($n = 3$). *B,* strain CSD100 (*opi3*/*cho2*) grown on minimal media (*M.M.*) alone or supplemented with 1 mM Cho or 1 mM MMEtn-P. CSD100 was transformed with the pRS313 vector control (*1*), *GmPLMT* (*2*), and *AtPLMT* (*3*).

ificities of the candidate plant PLMTs. Yeast strains possessing an *opi3* knock-out allele display negligible PtdMMEtn and PtdDMEtn methylation activities and have been shown to accumulate high levels of the Cho2p product PtdMMEtn (22). Full-length cDNAs of both the *Arabidopsis* and soybean *PLMT*-like sequences were cloned into a yeast transformation vector and introduced into the *opi3* knock-out strain CTY411. Microsomal preparations were incubated with [^{3}H]AdoMet and assayed for their ability to methylate the endogenous phospholipids within this membrane fraction.

The results of the *in vitro* methylation assays are shown in Fig. 3. All possible PtdEtn derivatives (PtdMMEtn, PtdDMEtn, and PtdCho) were readily observed using microsomal membranes from a wild-type control yeast strain (CTY182). As expected, PtdMMEtn was the only product observed from microsomes of CTY411 transformed with the control vector alone. In CTY411 cells expressing either the *Arabidopsis* or soybean cDNAs, however, radiolabeled PtdDMEtn and PtdCho species were observed in addition to PtdMMEtn (Fig. 3*A*), demonstrating that the plant enzymes were capable of catalyzing the final two steps in the methylation pathway of PtdCho synthesis in yeast. Because of their abilities to *N*-methylate phosphatidylamino alcohol substrates *in vitro*, the *Arabidopsis* and soybean genes were designated *AtPLMT* and *GmPLMT*, respectively. Although both plant enzymes gave the same reaction products, under our assay conditions, microsomal fractions recovered from yeast expressing *AtPLMT* consistently displayed greater activity than those observed from yeast expressing *GmPLMT*.

Classical biochemical studies have suggested that plants are fundamentally different from animals or yeast by not being able to produce PtdCho directly via methylation of PtdEtn (7, 8, 34). This concept is further supported by the fact that previous efforts using *Arabidopsis* and spinach cDNA libraries to complement yeast strains defective in the PtdCho methylation pathway only recovered genes encoding enzymes capable of methylating phospho-base substrates (15, 16). If the hypothesis is true that plant cells cannot synthesize PtdCho directly from PtdEtn, then plant PLMT enzymes, unlike their animal and yeast counterparts, should be incapable of utilizing PtdEtn as a substrate. We tested this by determining whether the *Arabidopsis* or soybean *PLMT* genes could complement a yeast strain with knock-out mutations in both the *cho2* and *opi3* loci. In yeast, choline auxotrophy is not mediated by the *cho2* mutation alone because the Opi3p enzyme is capable of a catalyzing a low level of PtdEtn methylation (35). Yeast possessing *cho2*/*opi3* double mutations, however, have an absolute requirement for exogenous choline. Yeast strain CDS100 (*cho2*/*opi3*) was generated through the mating of CTY410 (*cho2*) with CTY411 (*opi3*). *AtPLMT* and *GmPLMT* cDNAs were cloned downstream of the strong constitutive alcohol dehydrogenase promoter of yeast and transformed into CDS100.

As shown in Fig. 3*B*, the expression of plant *PLMT*s failed to support the growth of the *cho2*/*opi3* mutant on minimal media lacking choline. The inability to complement choline auxotrophy in CDS100 suggests that neither AtPLMT nor GmPLMT can serve as PtdEtn *N*-methyltransferases. To verify that the plant enzymes are functionally expressed in CDS100, each strain was grown on minimal media supplemented with MMEtn-P, a metabolite that can be incorporated into PtdMMEtn via enzymes of the nucleotide pathway. Although CDS100 transformed with the empty vector control remained inviable on this media, expression of both plant *PLMT* genes restored growth (Fig. 3*B*). Consistent with the results obtained from the *in vitro* enzyme assays, expression of *AtPLMT* gave a stronger growth restoration phenotype than *GmPLMT*. Microsomal membrane preparations of strain CDS100 expressing the plant PLMTs (grown in minimal media supplemented with choline) were also tested in the *in vitro* methylation assay described above. No traces of radiolabeled PtdMMEtn were detected using this assay (data not shown). Cumulatively, the expression studies in yeast support the claim that plant PLMT enzymes are able to carry out the final two steps of the traditional PtdEtn to PtdCho methylation pathway, but they cannot catalyze the initial methylation of PtdEtn.

Expression of AtPLMT—Examination of the Affymetrix-based expression profiling data base AtGenExpress suggests that *AtPLMT* (At1g80860) is expressed at relatively similar levels in most plant tissues, except fully mature or senescent tissue where a modest decrease in transcript accumulation is observed (data not shown). Furthermore, *AtPLMT* expression levels appear to be largely unaffected by exposure to either biotic or abiotic stresses. In *Saccharomyces cerevisiae*, PLMT

activities are regulated at the level of transcription in response to soluble phospholipid precursors (35, 36). Specifically, *CHO2* and *OPI3* mRNA accumulation is repressed in media containing inositol and/or choline. To address whether *AtPLMT* expression is regulated in a similar manner, *Arabidopsis* seedlings were grown on MS media supplemented with varying levels of choline or inositol. RNA blot analysis revealed no obvious effect on *AtPLMT* transcript levels in response to either compound (supplemental Fig. 2). Consistent with the observation of two classes of *At1g80860*-derived ESTs (the 3′-untranslated region intron either spliced or unspliced as shown in Fig. 2*B*), *AtPLMT* transcripts appear on RNA blots as a near equimolar doublet.

Characterization of AtPLMT Function in Planta—In animals and yeast, PLMTs serve critical functions, representing the sole route for *de novo* choline synthesis in these systems. The existence of the well characterized phospho-base pathway for *de novo* choline synthesis in plants, however, raises interesting questions regarding the necessity of the PLMT enzymes in plants. To explore this issue, a reverse genetic approach was taken to ascertain the consequences of the loss of *AtPLMT* gene function in *Arabidopsis*. A Ds-transposon insertion mutant line, GT9768, was identified in the Cold Spring Harbor Genetrapper Collection (37). DNA sequence analysis revealed that the Ds element was inserted at the first nucleotide of the exon1-intron1 junction (supplemental Fig. 3*A*). PCR-based genotype analysis of numerous GT9768 progeny showed that the line was homozygous for the insertion allele (data not shown). To assess the effect of the insertion event on *AtPLMT* expression, total RNA was isolated from wild-type *Arabidopsis* and the GT9768 line and analyzed by reverse transcriptase-PCR. Using forward and reverse primers corresponding to exon 1 and exon 2, respectively, *AtPLMT*-specific amplification products were readily detected in wild-type RNA preparations but could not be observed using GT9768 RNA (supplemental Fig. 3*B*). The failure to detect *AtPLMT* transcripts in GT9768 suggests that the Ds element created a null mutation. The insertion mutation in GT9768 at the *AtPLMT* locus was also confirmed by Southern blot analysis (supplemental Fig. 3*C*).

When grown under standard growth conditions, either in soil or on MS nutrient plates lacking choline, *atplmt* conferred no obvious morphological or developmental phenotype (data not shown). To gain insights into the effect of the mutant *atplmt* allele at the cellular level, experiments were conducted to compare the synthesis and accumulation of phospholipids with methylated amino alcohol headgroups (PtdMMEtn, PtdDMEtn, and PtdCho) in wild-type *versus* GT9768 plants. Initially, young *Arabidopsis* seedlings were incubated in the presence of [^{14}C]formate for up to 24 h. Formate is rapidly incorporated into the methyl donor group of AdoMet via the 1-carbon pathway (3). Labeling was carried out in the dark to minimize the incorporation of labeled carbon that had been oxidized to CO_2, which could then be integrated into other carbon moieties of phospholipids through photosynthetic respiration (9). As shown in Fig. 4*A*, ^{14}C counts incorporated into PtdMMEtn were significantly greater in the mutant line at all time points assayed. The differential was particularly dramatic at the 2-h time point, where ^{14}C counts in PtdMMEtn exceeded that found in PtdCho in the mutant *Arabidopsis* plants. [^{14}C]PtdDMEtn levels in this study were too low to accurately measure above background.

To establish whether the lack of AtPLMT activity results in an increase in the steady-state levels of PtdMMEtn and PtdDMEtn in GT9768 plants, whole plant lipid extracts were analyzed using ESI/MS/MS at the Kansas State Lipidomics Research Center. PtdMMEtn and PtdDMEtn levels were increased by ~9- and 3.5-fold, respectively, in *atplmt* mutant plants compared with wild-type *Arabidopsis* (Fig. 4*B*). No significant change, however, was observed in the steady-state pool of PtdCho (Fig. 4*C*). These observations suggest that production of PtdCho strictly through the phospho-base pathway and/or modulation of optimal PtdCho levels via phospholipase activities can compensate for deficiencies in PtdCho synthesis mediated by the PLMT enzyme. Similar to PtdCho, no differences were observed in any of the other major phospholipids or galactolipids in GT9768 plants (Fig. 4, *C* and *D*).

Fatty Acid Composition of Phosphatidylamino Alcohols—In animal cells it has been shown that PtdCho synthesized through the nucleotide pathway is not necessarily functionally equivalent with PtdCho produced via the methylation pathway. Differences in acyl chain composition between the two sources of PtdCho is believed to be one of the main causes for this lack of redundancy (38, 39). In yeast as well, substrate selectivity of the Cho1p and Opi3p enzymes results in the formation of PtdCho pools whose acyl composition differs from that derived through the enzymes of the nucleotide pathway (40). To determine whether the flux of metabolites through the PLMT enzyme has the potential of yielding PtdCho species that differ from that produced strictly through the phospho-base/nucleotide route (Fig. 1), the fatty acid composition of the individual phosphatidylamino alcohols was quantified in both GT9768 and wild-type *Arabidopsis* by ESI/MS/MS. No differences in PtdCho fatty acid profiles were observed between mutant and wild-type plants (Fig. 5). Although it cannot be concluded from this result that AtPLMT lacks substrate specificity based on acyl chain composition, it is clear that AtPLMT is not solely responsible for producing any specific PtdCho species, as has been shown for PEMT in the animal model. Similarly, there were no differences observed in the fatty acid compositions of PtdEtn, PtdMMEtn, and PtdDMEtn (Fig. 5) or any of the other major phospholipid and galactolipid species (data not shown) between wild-type and *atplmt Arabidopsis* plants. Even though no differences were observed in the fatty acid compositions of the phosphatidylamino alcohols in mutant *versus* wild-type plants, one notable observation from this analysis was the lack of 34:3 fatty acid species in PtdMMEtn and PtdDMEtn. Although 34:3 fatty acids (presumably composed of 16:0 + 18:3) account for ~15% of the total PtdEtn and PtdCho species in both genotypes, this acyl combination is nearly undetectable in PtdMMEtn and represents less than 3% of PtdDMEtn (Fig. 5).

DISCUSSION

This study represents the first characterization of genes encoding the PLMT branch of the PtdCho biosynthetic pathway in higher plants. Similar to the rat PEMT and yeast Opi3p proteins, the predicted soybean and *Arabidopsis* PLMT

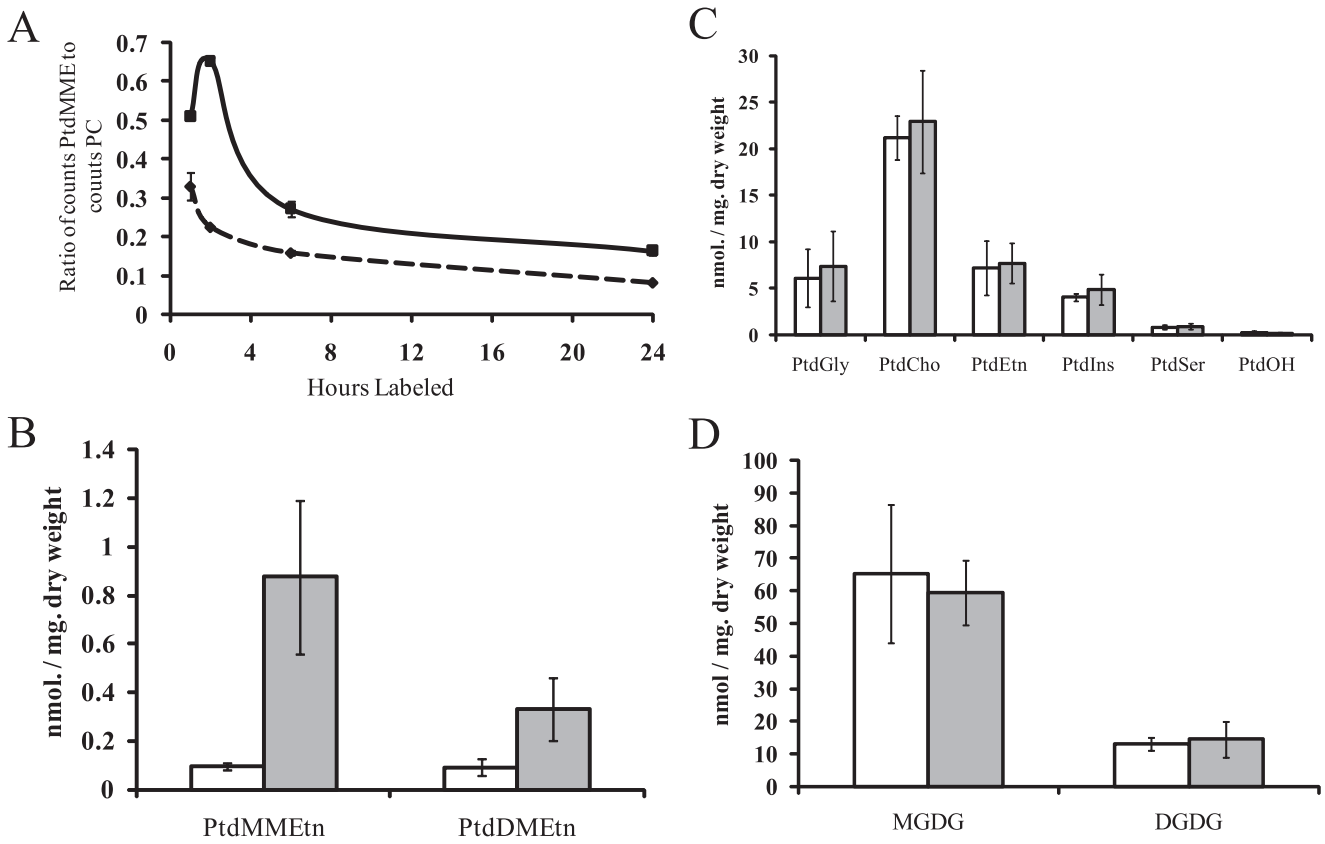

FIGURE 4. ***Arabidopsis atplmt* knock-out mutants accumulate elevated levels of PtdMMEtn and PtdDMEtn.** *A,* time course labeling of intact 8–10-day-old plantlets with [^{14}C]sodium formate. Data presented show the mean ± S.E. of six biological replications using wild-type *Arabidopsis* (*dashed line*) and *atplmt* mutant line GT9768 (*solid line*). *B–D,* steady-state quantification of PtdMMEtn and PtdDMEtn (*B*), major phospholipids (*C*), and galactolipids (*D*) using ESI-MS/MS. Wild-type *Arabidopsis* is represented by *white bars* and GT9768 by *gray bars. Error bars* represent the S.E. of five biological replications. For PtdMMEtn and PtdDMEtn, differences were found to be significant as determined by Student's *t* test ($p < 0.05$).

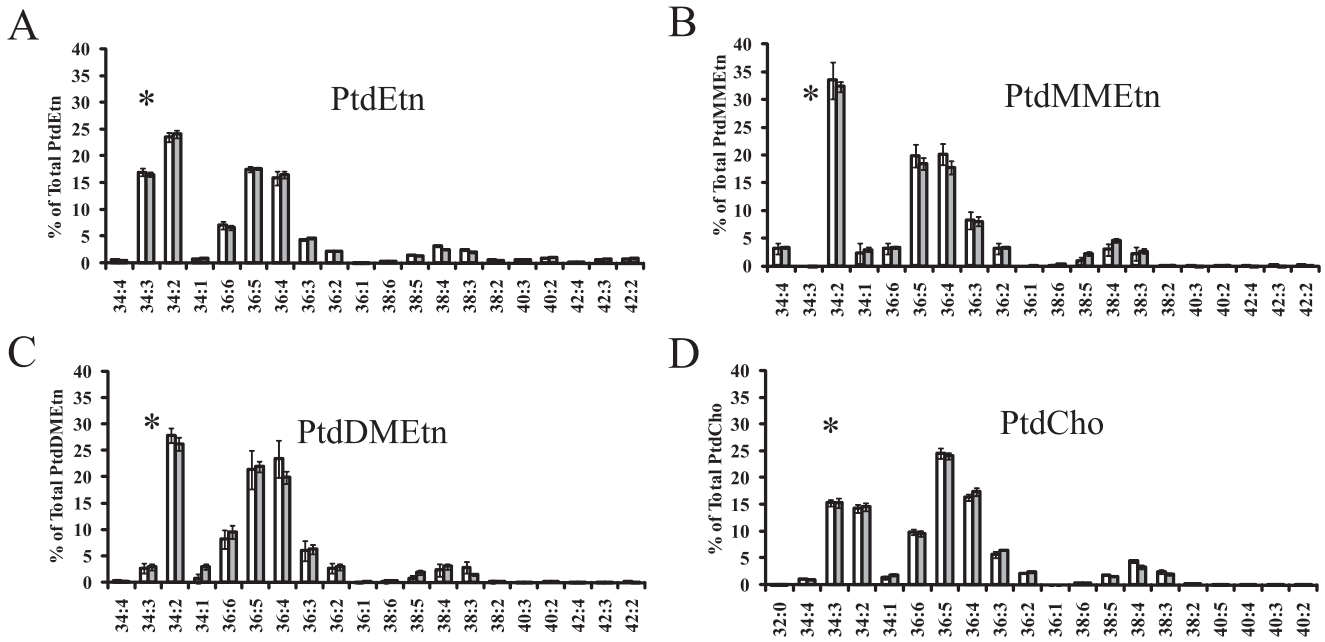

FIGURE 5. **ESI-MS/MS derived data showing distribution of acyl chains among PtdEtn, PtdMMEtn, PtdDMEtn, and PtdCho in wild-type (*white bars*) and GT9768 (*atplmt*) *Arabidopsis* plants.** The 34:3 acyl species found to be disproportionately lower in PtdMMEtn and PtdDMEtn are highlighted with an *asterisk*. Wild-type *Arabidopsis* is represented by *white bars* and GT9768 by *gray bars*.

enzymes are small, displaying calculated molecular masses of 18.8 and 19.6 kDa, respectively. The observation of multiple predicted membrane-spanning domains is consistent with the localization of PLMT activities within microsomal membrane fractions of disrupted plant cells (11, 34). Despite catalyzing similar reactions, the *Arabidopsis* and soybean PLMT enzymes

share only ~25% primary amino acid sequence identity with their mammalian and yeast counterparts. Particularly intriguing is the nature of the AdoMet-binding sites in the plant enzymes. Because PLMTs in general do not contain AdoMet-binding motifs that are typical of most non-DNA AdoMet-dependent methyltransferases, Shields *et al.* (41) conducted a study to define the sites involved in AdoMet binding for human PEMT. Two AdoMet-binding sites were identified in human PEMT, a G*X*G motif (*X* = any amino acid) located in the middle of the protein, and an EE motif near the C terminus (Fig. 2). Both enzymatic activity and AdoMet binding in the human PEMT were completely abolished when the second glycine residue in the G*X*G motif or the first glutamic acid of the EE sequence was replaced with conservative amino acid substitutions. The soybean and *Arabidopsis* enzymes, however, lack these critical residues. A small deletion in the plant PLMTs (relative to the rat and yeast sequences) occurs at the residues corresponding to the *X*G of the G*X*G motif, and a nonconservative lysine residue is found in place of the first, presumably invariant, glutamate in the EE motif (Fig. 2). These observations suggest that plant PLMTs utilize different motifs to bind and orient the AdoMet substrate. Furthermore, the observation that plant PLMTs exclude PtdEtn as a substrate, in contrast to the mammalian enzyme that readily methylates PtdEtn (as well as yeast Opi3p to a lesser extent), also suggests that there are significant differences in the tertiary structures of plant *versus* animal and yeast PLMTs.

The inability of plant PLMTs to utilize PtdEtn as a substrate coupled with several classical biochemical studies collectively suggests that the direct methylation of PtdEtn does not occur in plants (7, 8, 34). This means that the *de novo* synthesis of a choline moiety must originate via the methylation at the phospho-base level by the PEAMT enzyme. MMEtn-P and DMEtn-P can serve as substrates for the nucleotide pathway, which results in the synthesis of PtdMMEtn or PtdDMEtn lipid intermediates. Wang and Moore (42) showed that the cholinephosphate cytidylyltransferase of castor bean has a similar level of activity using either MMEtn-P or Cho-P as a substrate. Therefore, for most plants species it appears that the flow of metabolites through phospho-base *versus* phosphatidyl-base intermediates is dictated by the relative efficiencies of the cytidylyltransferase enzyme(s) with the PEAMT enzymes as they compete for common MMEtn-P and DMEtn-P substrates.

The elimination of *AtPLMT* gene function in *Arabidopsis* did not lead to any obvious perturbations in normal plant growth and development, but it did result in increased PtdMMEtn and PtdDMEtn accumulation, lipid species that are typically found in only trace amounts in membrane fractions. The 9- and 3.5-fold increases in PtdMMEtn and PtdDMEtn concentrations, respectively, in *atplmt* plants elevated the accumulation of these species to levels similar to that observed for phosphatidylserine (Fig. 4). In contrast, the steady-state levels of the end product of the PLMT reaction, PtdCho, remained unchanged. Given that the phospho-base route alone appears to be sufficient for supplying adequate concentrations of PtdCho within *atplmt* mutant plants (Fig. 4), it is worth speculating on why PLMT function has been maintained during *Arabidopsis* evolution. Perhaps the most reasonable explanation would be that PLMT functions to optimally channel metabolites of the PtdCho pathway to the desired end product, and thus minimize the accumulation of the PtdMMEtn and PtdDMEtn intermediates. Although the enhanced accumulation of these minor phosphatidylamino alcohols appears to be benign when *atplmt* plants are grown in an ideal environment, they may be deleterious during growth at suboptimal conditions, or when the plant is exposed to biotic or abiotic stresses. Subjecting *atplmt* plants to an array of stress conditions would be a high priority for future endeavors to elucidate gene function on whole plant physiology.

Because of the great variability observed among different plant species with regard to the methylation of amino alcohol intermediates in the PtdCho pathway, the conclusions derived from our study of the *Arabidopsis atplmt* mutant may not necessarily reflect the relative importance of this gene in other higher plants. In soybean, for example, *in vivo* labeling studies conducted both in cell cultures and leaf disks, and *in vitro* enzyme assays using cell fractions, provided clear evidence that the final two methylation reactions occur exclusively at the phosphatidyl-base level in this species (8, 11). Therefore, the soybean *GmPLMT* gene would be predicted to be essential for PtdCho synthesis, and its inactivation would likely result in a lethal phenotype. In contrast, although the disruption of PLMT activity in *L. paucicostata* would likely yield a phenotype similar to that observed in *Arabidopsis*, minimal metabolite flux was shown to occur through phosphatidyl-base intermediates in this system, despite the fact that PLMT activities were readily measured *in vitro* (8, 11).

Although no differences were observed in the fatty acid profiles of the phosphatidylamino alcohol lipids from wild-type *versus atplmt Arabidopsis* plants, the lack of 34:3 species within PtdMMEtn and PtdDMEtn of both genotypes was notable, particularly in light of the fact that this combination is very prevalent in PtdEtn and PtdCho (Fig. 5). Because of the virtual absence of unsaturated 16 acyl chain species within all phospholipids of *Arabidopsis* except phosphatidylglycerol (43), it is reasonable to assume that the 34:3 species detected in this analysis were composed of 16:0 plus 18:3. The near absence of this fatty acid combination in PtdMMEtn and its great reduction in PtdDMEtn could be explained by the exclusion of the corresponding diacylglycerol species by the amino alcohol phosphotransferase enzyme when presented with a CDP-MMEtn or CDP-DMEtn substrate. Alternatively, cellular pools of 16:0/18:3 diacylglycerol may be inherently low, and the abundance of this fatty acid combination in PtdCho and PtdEtn could be a reflection of 16:0/18:2 PtdCho and PtdEtn species serving as good substrates for the *FAD3*-encoded ω-3 desaturase and the corresponding species of PtdMMEtn and PtdDMEtn representing poor substrates for this enzyme. Finally, the paucity of 16:0/18:3 PtdMMEtn and PtdDMEtn could be explained by an enhanced susceptibility of these species to phospholipase degradation, or even through an acyl-editing mechanism as recently proposed by Bates *et al.* (44).

In conclusion, through the heterologous expression of candidate cDNAs in yeast, we have demonstrated that the *AtPLMT* and *GmPLMT* genes identified in this study encode PLMT enzymes involved in PtdCho biosynthesis. The enhanced accu-

mulation of PtdMMEtn and PtdDMEtn intermediates in an *Arabidopsis atplmt* mutant line confirmed that the *AtPLMT* gene product functions within the PtdCho pathway *in vivo*. The identification and characterization of the genes encoding PLMT activity in plants not only enhance our understanding of the PtdCho pathway *per se* but also provide an important tool for further defining the great variability observed among different plants species regarding the specific route by which PtdCho is synthesized within the plant cell and how this process is regulated.

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