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Identification of Phosphomethylethanolamine *N*-Methyltransferase from *Arabidopsis* and Its Role in Choline and Phospholipid Metabolism*

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Three sequential methylations of phosphoethanolamine (PEA) are required for the synthesis of phosphocholine (PCho) in plants. A cDNA encoding an *N*-methyltransferase that catalyzes the last two methylation steps was cloned from *Arabidopsis* by heterologous complementation of a *Saccharomyces cerevisiae cho2, opi3* mutant. The cDNA encodes phosphomethylethanolamine *N*-methyltransferase (PMEAMT), a polypeptide of 475 amino acids that is organized as two tandem methyltransferase domains. PMEAMT shows 87% amino acid identity to a related enzyme, phosphoethanolamine *N*-methyltransferase, an enzyme in plants that catalyzes all three methylations of PEA to PCho. PMEAMT cannot use PEA as a substrate, but assays using phosphomethylethanolamine as a substrate result in both phosphodimethylethanolamine and PCho as products. PMEAMT is inhibited by the reaction products PCho and *S*-adenosyl-L-homocysteine, a property reported for phosphoethanolamine *N*-methyltransferase from various plants. An *Arabidopsis* mutant with a T-DNA insertion associated with locus At1g48600 showed no transcripts encoding PMEAMT. Shotgun lipidomic analyses of leaves of *atpmeamt* and wild-type plants generated phospholipid profiles showing the content of phosphatidylmethylethanolamine to be altered relative to wild type with the content of a 34:3 lipid molecular species 2-fold higher in mutant plants. In *S. cerevisiae*, an increase in PtdMEA in membranes is associated with reduced viability. This raises a question regarding the role of PMEAMT in plants and whether it serves to prevent the accumulation of PtdMEA to potentially deleterious levels.

Choline occurs in plants as free choline, phosphocholine (PCho)[2]; as a component of the integral membrane phospholipid, phosphatidylcholine (PtdCho); and as a precursor for the osmoprotectants choline-*O*-sulfate and glycine betaine (1–3). Along with these important roles in plant metabolism, choline is of interest because of its classification as an essential dietary nutrient for humans (4, 5). Choline biosynthesis is also critical to the viability of *Plasmodium falciparum*, the causative agent of malaria, and the nematode *Caenorhabditis elegans*. As such, the enzymes involved in choline synthesis represent appealing targets for the bioengineering of many traits from more hardy and nutritional crops to the development of drugs with antimalarial and nematicidal properties for use in medicine and agriculture (6–8).

Radiotracer studies provide evidence that choline and hence PtdCho synthesis can proceed along various pathways in plants. Fig. 1*A* shows that synthesis of choline can involve intermediates at the level of phosphobases (P-bases), phosphatidyl bases (Ptd-bases), or a combination of the two routes (9, 10). In each case, the committing step appears to be the *N*-methylation of phosphoethanolamine (PEA) to produce phosphomethylethanolamine (PMEA) (10–12). The production of PMEA is also a key step in choline biosynthesis in *P. falciparum* and *C. elegans* (7, 13).

Relatively little is known about the enzymes involved in choline or PtdCho biosynthesis or factors that regulate their activities. The enzyme phosphoethanolamine *N*-methyltransferase (PEAMT) catalyzes three sequential *N*-methylations of PEA to produce PCho using *S*-adenosyl-L-methionine (AdoMet) as a methyl donor (Fig. 1*A*) (14). Work using castor bean (15), *Lemna*, soybean, and carrot (10) suggests that PEAMT is a rate-limiting enzyme of choline synthesis in plant PtdCho metabolism. Inhibition of *in vitro* PEAMT activity by the reaction products PCho and *S*-adenosyl-L-homocysteine (AdoHcy) offers two possible means by which the activity of this enzyme may be regulated *in vivo* (11, 12, 16).

Evidence that choline content in plants is regulated and probably finite is offered by the results of research using *Arabidopsis* and tobacco, plants that do not naturally oxidize choline for glycine betaine accumulation (2, 3, 17). Nuccio *et al.* (12) engineered transgenic tobacco that expressed the enzymes responsible for converting choline to glycine betaine, but these plants failed to accumulate glycine betaine to levels comparable in accumulating species. These transgenic tobacco plants were then transformed with the gene encoding spinach PEAMT, but

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[2] The abbreviations used are: PCho, phosphocholine; PtdCho, phosphatidylcholine; P-base, phosphobase; Ptd-base, phosphatidyl base; PEA, phosphoethanolamine; PMEA, phosphomethylethanolamine; PtdEA, phosphatidylethanolamine; PtdMEA, phosphatidylmethylethanolamine; PtdDEA, phosphatidyldimethylethanolamine; PDEA, phosphodimethylethanolamine; PEAMT, phosphoethanolamine *N*-methyltransferase; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; PMEAMT, phosphomethylethanolamine *N*-methyltransferase; EA, ethanolamine; MEA, methylethanolamine; PLMT, phospholipid *N*-methyltransferase; SD, synthetic defined.

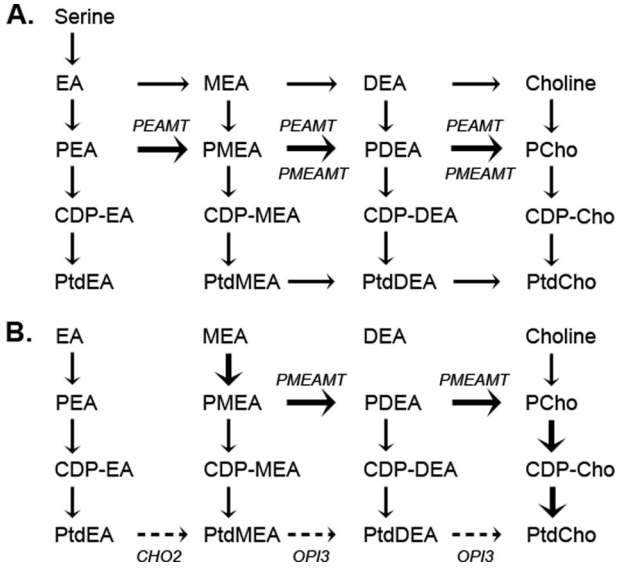

FIGURE 1. **PtdCho synthesis in plants and yeast highlighting the complementation strategy used to identify the gene encoding PMEAMT.** *A*, the methylation of PEA is a committing step in plant PtdCho synthesis with subsequent methylations at the P-base or Ptd-base level. PEAMT catalyzes the methylation of all three P-bases (*heavy arrows*) leading to PCho synthesis, whereas PMEAMT cannot use PEA as a substrate. *B*, yeast synthesizes PtdCho by the Ptd-base route (*dashed arrows*) that is defective in *S. cerevisiae* strain CPBY19. Provision of MEA in the medium allows for rescue of PtdCho production in this strain through a by-pass afforded by *Arabidopsis* PMEAMT.

overexpression of this gene did not lead to expected increases in glycine betaine. This outcome is consistent with PEAMT activity not being a bottleneck for the synthesis of choline for glycine betaine synthesis and led the authors to propose that other factors, including choline transport to chloroplasts, an insufficiency of ethanolamine, and/or inhibition of PEAMT by PCho, may prevent these plants from producing higher levels of glycine betaine.

The NCBI database shows three *Arabidopsis* gene loci to be annotated as associated with PEAMT, namely At3g18000, At1g48600, and At1g73600, with only the product encoded by the first locus having been verified biochemically to encode an enzyme with PEAMT activity (18). An *N*-methyltransferase capable of using PMEA but not PEA was detected in partially purified preparations from spinach leaves (11, 14). This enzyme, designated phosphomethylethanolamine *N*-methyltransferase (PMEAMT), probably also uses phosphodimethylethanolamine (PDEA) as a substrate, but the possibility of a third enzyme showing PDEA specificity cannot be precluded based upon the evidence to date (11, 14). For many dicot plants, PEAMT activity is salt- and light-responsive (14, 19). However, in contrast to PEAMT, PMEAMT activity does not decrease in leaves of plants, including spinach, sugar beet, and canola, following exposure of plants to prolonged dark periods (14, 19).

How PMEAMT contributes toward choline metabolism has been difficult to determine because this enzyme has not been purified to homogeneity; nor has the gene encoding this enzyme been cloned from any plant species. The objective of this study was to clone the gene encoding PMEAMT from *Arabidopsis* and to characterize the contribution of this enzyme toward plant metabolism through analysis of a *pmeamt* T-DNA-tagged mutant. We show that the gene at locus At1g48600 encodes a product with PMEAMT activity that can rescue a choline auxotroph of yeast. This enzyme can use both PMEA and PDEA as substrates for methylation. The *Arabidopsis pmeamt* T-DNA mutant (*atpmeamt*) lacks transcripts associated with *PMEAMT* but has no overt phenotype under any growth conditions used. However, the leaf membrane phospholipid profiles show a greater content of PtdMEA as the 34:3 lipid molecular species in *atpmeamt* plants relative to wild type. Based upon these results, we propose that PMEAMT activity reduces the potential for PMEA incorporation into the polar headgroup of membrane phospholipids.

EXPERIMENTAL PROCEDURES

Media—Minimal synthetic defined (SD) medium was prepared essentially as per Sherman (20) with inositol omitted, 0.1 mM tryptophan (Trp) included, and 2% (w/v) glucose used as the carbon source. When added to SD medium, the ethanolamine (EA), methylethanolamine (MEA), or choline was supplemented at 1 mM.

Cloning PMEAMT by Heterologous Complementation—The *Saccharomyces cerevisiae* yeast mutant CPBY19 (*ura3-52 leu2Δ1 his3Δ200 trpΔ63 opi3*::*HIS3 cho2*::*LEU2*) (18) was transformed using a lithium acetate method (21) with purified plasmid DNA prepared from the *Arabidopsis* (Landsberg *erecta* ecotype) whole seedling cDNA library available in the yeast expression vector pFL61 (ATCC catalog no. 77500) (22). Following transformation, cells were plated on medium lacking uracil but containing 1 mM choline and incubated at 30 °C. The vector pFL61 confers uracil prototrophy, and transformants were subject to selection on SD medium supplemented with 1 mM MEA at 37 °C. Single colonies that were recovered on MEA at 37 °C were tested for growth on medium containing 1 mM EA.

Plasmid DNA was extracted from mutant yeast strains (23) and amplified by PCR with pFL61 vector-specific primers JST46 and JST47 (24). Amplified DNA was subject to DNA sequence analysis, and clones showing heterologous cDNA sequence matches to P-base methyltransferases were subject to further characterization.

Subcloning—cDNAs associated with *Arabidopsis PMEAMT* and *PEAMT* were subcloned into a pET30a expression vector by incorporating N terminus NcoI and C terminus BamHI restriction sites by PCR. The primers for *PMEAMT* were 5′-GCTACTCCATGGAGCATTCTAG-3′ (NcoI) and 5′-ACAGGATCCTTACTTCTTGTCGG-3′ (BamHI), and those for *PEAMT* were 5′-TTCCGCCATGGCTGCATCG-3′ (NcoI) and 5′-GTAGATTTGGATCCGCTTAATTCTTG-3′ (BamHI). PCR-amplified products were ligated into the expression vector, and the resulting plasmids were transformed into *Escherichia coli* BL21 (DE3) cells for expression and DNA sequence analysis.

Protein Expression and Purification—Fifty-ml cultures of SD medium supplemented with 0.1 mM Trp and 1 mM choline were inoculated with cells from a single transformed yeast colony and grown to an A_{600} of 1.0–1.2 at 30 °C. The cells were recovered by centrifugation at 10,000 × *g* for 10 min at 4 °C, and the pellet was suspended in 0.3 ml of 100 mM Hepes-KOH (pH 7.8), 1 mM Na_2EDTA, 5 mM DTT (HED). The yeast cells were dis-

rupted by vortex action with glass beads (18). The supernatant was recovered following centrifugation at 12,000 × *g* for 10 min and used directly for enzyme activity assays or flash frozen in liquid N_2 and stored at −80 °C.

Recombinant His-tagged proteins were recovered using HIS-select® nickel spin columns (H7787, Sigma). Cell-free extract containing His-tagged proteins in 50 mM Hepes-KOH, pH 8.0, 300 mM NaCl, and 1 mM DTT was applied to the affinity gel equilibrated in the same buffer. The column was washed twice with the above buffer, and the bound protein was eluted with 50 mM Hepes-KOH (pH 8.0), 300 mM NaCl, 250 mM imidazole, 1 mM DTT, and 1 mg/ml bovine serum albumin (BSA). After adding Na_2EDTA to a final concentration of 0.25 mM, the eluate was used for enzyme activity measurements.

Enzyme Assays—Enzyme assays were performed using the conditions described by Summers and Weretilnyk (25). Cell-free crude extracts were desalted by centrifugation through Sephadex G-25 medium (Amersham Biosciences) equilibrated with HED buffer. The substrate concentrations for standard assays were 250 μM P-base (PEA, PMEA, or PDEA) and 200 μM AdoMet. K_m values were calculated from Hanes-Wolfe plots showing reaction rates as a function of increasing substrate concentrations. All radioassays represent a minimum of duplicate measurements, and in the case of kinetic determinations, triplicate measurements were used with the entire experiment repeated three times. Product verification by thin layer chromatography (TLC) using ^{14}C-labeled AdoMet was exactly as described by Smith *et al.* (11).

Analysis of Arabidopsis T-DNA Insertion Line—The SALK 006037 line of *Arabidopsis*, which has a T-DNA insertion associated with *At1g48600* (26), was ordered from the Salk Institute Genomic Analysis Laboratory. Plants were grown under a 12-h day/night cycle at 23 °C, and DNA was extracted from leaves of 4-week-old plants according to the protocol of Edwards *et al.* (27). To identify plants homozygous for the T-DNA insertion, the forward (5′-TGTGATGGGAGATTTCAATGG-3′) and reverse (5′-AGAAAACAGTTTGGACTTTTCG-3′) gene-specific primers were used for PCR as well as a left border primer specific to the T-DNA insertion (5′-TGGTTCACGTAGTGGGCCATCG-3′). These primers were used for PCR amplification using plant genomic DNA with an initial 3.5-min denaturation step at 94 °C followed by 30 cycles of 30 s of denaturation at 94 °C, 50 s of annealing at 58 °C, 1 min of extension at 72 °C, and a final extension for 10 min at 72 °C.

RNA was extracted from leaves of plants confirmed to be homozygous for the T-DNA insertion and wild type of the same line using a Qiagen RNeasy extraction kit following the protocol of the supplier (catalog no. 74104). First-strand cDNA synthesis was performed using the gene-specific primers 5′-GATTGGATGGGTCAAGCCAG-3′ (forward) and 5′-GAATAGAGCTGGCTTGTCTTGG-3′ PCR (reverse). A total of 30 cycles were used for RT-PCR, and ubiquitin 10 transcripts were used as a control for template quality (28). PCR conditions used were as described above for gene-specific primers, except the annealing temperature was 67 °C.

Phospholipid Profiling—Lipids were extracted following a modified Bligh and Dyer (29) protocol. Leaf tissue (80–120 mg) was harvested from 4-week-old *Arabidopsis* plants and immediately frozen in liquid N_2. The frozen tissue was ground to a powder in a mortar, and 700 μl of chilled methanol and 58 μl of 1 M NaCl were added. The mortar was rinsed with a second 700-μl aliquot of methanol, and the pooled volumes were shaken at 70 °C for 15 min and then centrifuged at 14,000 × *g* for 3 min at 4 °C. The methanolic supernatant was set aside. The pellet was extracted by adding 750 μl of chloroform and shaking the resuspended pellet at 37 °C for 5 min; the tube was centrifuged, and the resulting supernatant was pooled with the methanolic supernatant. To the pooled supernatants was added 1.4 ml of H_2O; the mixture was vortexed and then centrifuged at room temperature for 15 min at 5000 × *g* for phase separation. Each phase was transferred to a separate vial and dried under a stream of N_2 gas.

Lipidomics analysis was conducted on the chloroform phase using infusion electrospray ionization-mass spectrometry (ESI-MS) in the positive ion mode by the McMaster Regional Centre for Mass Spectrometry according to the method of Basconcillo *et al.* (30). To the lipid (chloroform) phase was added 200 μl of methanol/chloroform (1:1, v/v). Prior to analysis, aliquots were diluted 5-fold with methanol containing 10 mM LiCl. The resulting solutions were infused at a flow rate of 1–2 μl/min into a Waters Quattro Ultima triple quadrupole mass spectrometer equipped with a microelectrospray ionization source and running under MassLynx software. Lithiated lipid adducts were analyzed using neutral loss scans of 147, 161, 175, and 189 mass units, corresponding to the loss of the lithiated polar headgroups of PtdEA, PtdMEA, PtdDEA, and PtdCho, respectively. Typically, 100 spectra were collected and averaged to afford a single spectrum for each lipid analysis. MS/MS analyses were used to determine the identity of the fatty acyl groups in the lipids (30).

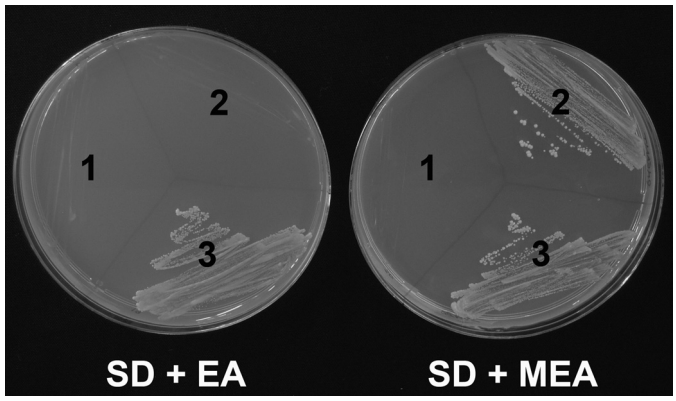

FIGURE 2. **Heterologous complementation of PtdCho synthesis in *S. cerevisiae* CPBY19 by *Arabidopsis* cDNAs encoding P-base methyltransferases.** The CPBY19 (*cho2*, *opi3*) mutant strain was grown on SD medium supplemented with 1 mM EA (*left*) or 1 mM MEA (*right*). Yeast was untransformed (*1*) or transformed with pFL61carrying cDNA encoding either AtPMEAMT (*2*) or AtPEAMT (*3*).

RESULTS

Cloning of PMEAMT—A total of 103 independent yeast isolates were recovered on SD medium containing MEA following transformation of CPBY19 with the pFL61 *Arabidopsis* cDNA library. Of these clones, 100 also grew on medium with EA, suggesting that complementation was associated with more than one plant cDNA (Fig. 2). Using vector-specific primers, 89

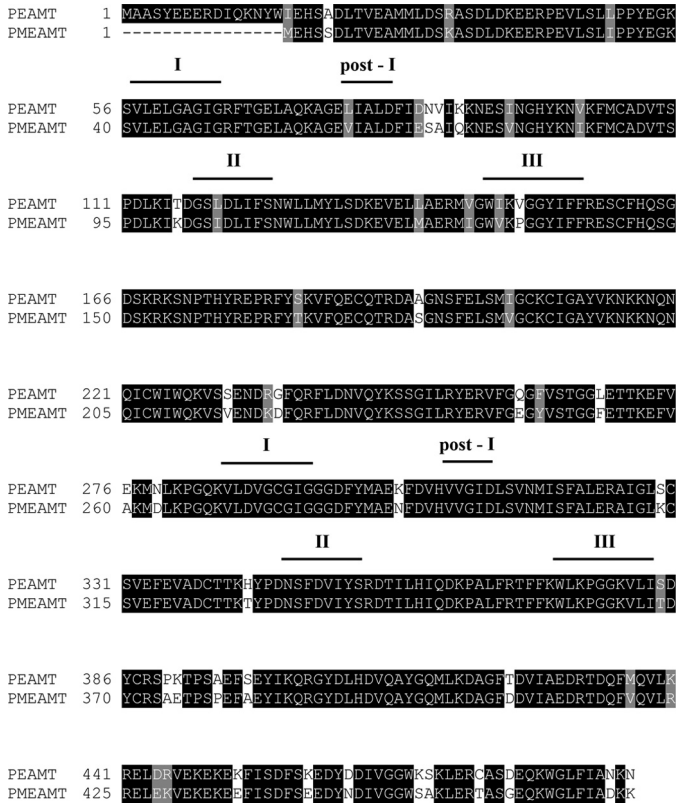

FIGURE 3. **Alignment of deduced amino acid sequences for AtPEAMT and AtPMEAMT.** AdoMet-binding motifs I, post-I, II, and III are indicated by the *horizontal bars*. Amino acids *shaded* in *black* are identical, and conservative substitutions are *shaded* in *gray*.

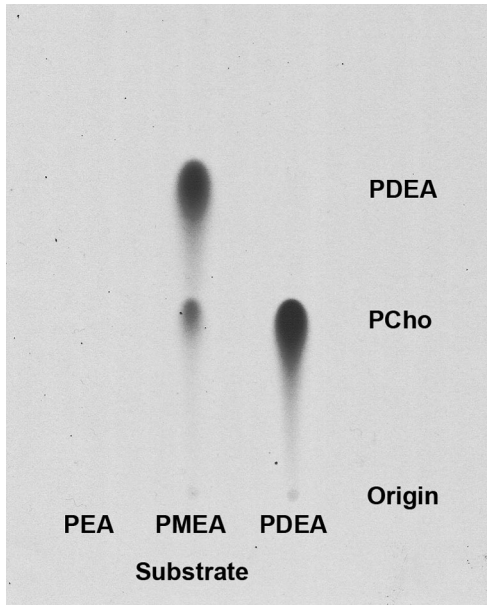

FIGURE 4. **PMEAMT catalyzes the methylation of PMEA to PDEA and PCho.** Autoradiograph of P-base *N*-methyltransferase assay products identified by TLC. Enzyme assay conditions were modified to include [*methyl*-^{14}C]AdoMet, and the assay time was extended to 120 min.

isolates gave a PCR-amplified product of around 2100 bp in length, and three each showed insert lengths of ~2050 and 1900 bp. The cDNAs associated with three random independent clones carrying 2100-bp-long inserts were fully sequenced and shown to be identical to the translated sequence of *PEAMT* (At3g18000) cloned and characterized by Bolognese and McGraw (18) (Fig. 3). Complementation of this yeast mutant by PEAMT is not surprising because this gene product can also catalyze the conversion of PMEA to PCho, as required by our selection strategy (Fig. 1*B*). The translated sequence of the ~1900-bp cDNA insert is shown in Fig. 3, and it corresponds to the *Arabidopsis* gene at locus At1g48600 that is annotated as a putative *PEA N*-methyltransferase 2 (NCBI). Strains containing this insert were able to grow on SD medium supplemented with MEA but not on media containing EA (Fig. 2). The 2050-bp cDNA insert was sequenced and shown to correspond to the gene at locus At4g39800 that is annotated as *myo*-inositol-1-phosphate synthase (NCBI). No clones were found with cDNA inserts corresponding to the third *Arabidopsis* gene encoding the highly similar gene product annotated as a putative PEAMT (NCBI) at locus At1g73600.

Enzyme Activities—Cell-free extracts prepared from yeast were tested for the presence of P-base methyltransferase activity. Extracts were prepared from the untransformed host strain and clones carrying plasmids with cDNA inserts of 2100, 2050, or 1900 bp. All of the strains with a cDNA insert of 2100 bp yielded extracts with methyltransferase activity using PEA, PMEA, and PDEA as substrates, a finding consistent with the substrates used by *Arabidopsis* PEAMT (18). Yeast clones complemented by the plant cDNA inserts of 2050 bp in length were also tested *in vitro* in assays for methyltransferase enzyme activity, but as anticipated, given the identification as *myo*-inositol-1-phosphate synthase, these extracts showed no capacity to methylate PEA, PMEA, or PDEA. The significance of this gene product in supporting growth on SD medium containing MEA was not pursued. The cell-free extracts prepared from yeast clones complemented by the plant cDNA of 1900 bp were shown by *in vitro* assays to have an enzyme capable of methylating PMEA and PDEA but not PEA. This substrate profile is consistent with PMEAMT activity (11, 14), so this gene and its product are referred to in this paper as *AtPMEAMT* and AtPMEAMT, respectively. The reaction products were resolved by TLC, and these results show the substrate PMEA methylated to PDEA and PCho and the substrate PDEA methylated to PCho (Fig. 4). No P-base products were produced when PEA was used as a substrate, consistent with the lack of growth of yeast clones in medium containing EA. The untransformed host CPBY19 was unable to grow on EA- or MEA-containing media (Fig. 2), and cell-free extracts prepared from this strain showed no methyltransferase enzyme activity toward any P-bases tested.

The amino acid sequence predicted by the longest open reading frames of *AtPEAMT* (18) and the *AtPMEAMT* cDNA identified through our complementation strategy shows the amino acid identity to be 87% between these two gene products. *AtPEAMT and AtPMEAMT* both encode proteins with N- and C-terminal AdoMet-binding domains, with each domain possessing three AdoMet-binding motifs (12, 18, 31–33) (Fig. 3). The C-terminal AdoMet-binding motifs are identical between PEAMT and PMEAMT, whereas differences between the post-I, II, and III motifs are present in the N-terminal domains (Fig. 3). Specifically, single amino acid substitutions occur between PEAMT and PMEAMT in SAM-binding motifs post-I

TABLE 1

Inhibition of recombinant AtPMEAMT by PCho and AdoHcy

Methyltransferase activity was assayed using PMEA as a P-base substrate. His-tagged PMEAMT was purified by a nickel affinity matrix with BSA and DTT present. Assays were linear with respect to time ($n = 4$; ± S.E.), and the control activity was 1.8 nmol ml^{-1} min^{-1}.

Addition	Concentration	PMEAMT activity (percentage of control)
	mM	%
PCho	1.0	47 ± 2
	5.0	17 ± 2
Choline	5.0	101 ± 9
AdoHcy	0.01	73 ± 1
	0.20	18 ± 1

and II, and two substitutions are found in motif III. All substitutions within the motifs are conservative with the exception of valine to proline in motif III.

The apparent K_m values of PMEAMT toward PMEA and PDEA as substrates were determined using purified recombinant His-tagged PMEAMT. In this context, it is noteworthy that purification of the recombinant protein by affinity chromatography was found to be problematic and subject to losses in enzyme activity. The inclusion of DTT, BSA, and EDTA prevented the loss of enzyme activity during elution from a nickel ion matrix. In view of this difficulty, apparent K_m values were also calculated for crude, desalted cell-free yeast extracts to determine if exposure to the nickel or the presence of a His tag altered the properties of the enzyme. K_m values for PMEAMT expressed as a purified His tag protein following overexpression in *E. coli* or the non-tagged version in crude, desalted yeast extracts were not statistically different. PMEAMT from either source shows apparent K_m values of 0.16 and 0.03 mM toward PMEA and PDEA, respectively. In a parallel comparison with recombinant *Arabidopsis* PEAMT, we found that the apparent K_m values were 0.32 and 0.14 mM for PMEA and PDEA, respectively. Based upon these determinations, *Arabidopsis* PMEAMT has a 2-fold higher affinity toward PMEA and a 5-fold higher affinity for PDEA compared with PEAMT.

PCho and AdoHcy have been shown to have an inhibitory effect on PCho synthesis through a combination of *in vivo* determinations using cell cultures and by *in vitro* assays of spinach and wheat PEAMT (11, 12, 16, 34, 35). Under standard assay concentrations of PMEA and AdoMet, the inclusion of PCho at 1 and 5 mM final assay concentrations led to a reduction in PMEAMT activity by 53 and 83%, respectively (Table 1). Similarly, PMEAMT activity under the same assay conditions was reduced by 27 and 82% in the presence of 0.01 and 0.2 mM AdoHcy, respectively. The addition of 5 mM choline to the assay had no effect on PMEAMT activity.

Phospholipid Profiling—The T-DNA insertion of the *Arabidopsis* SALK 006037 line is associated with the promoter region of At1g48600. Plants identified as homozygous for the presence of a T-DNA insert were recovered and compared with wild-type plants of the same line. Leaf mRNA used for RT-PCR showed no products associated with *PMEAMT* expression in the mutants homozygous for the T-DNA element (Fig. 5). Although *AtPMEAMT* expression was suppressed in the mutant line, no obvious phenotypic differences in growth or development were observed between wild-type and *atpmeamt* plant lines grown at 23 or 26 °C.

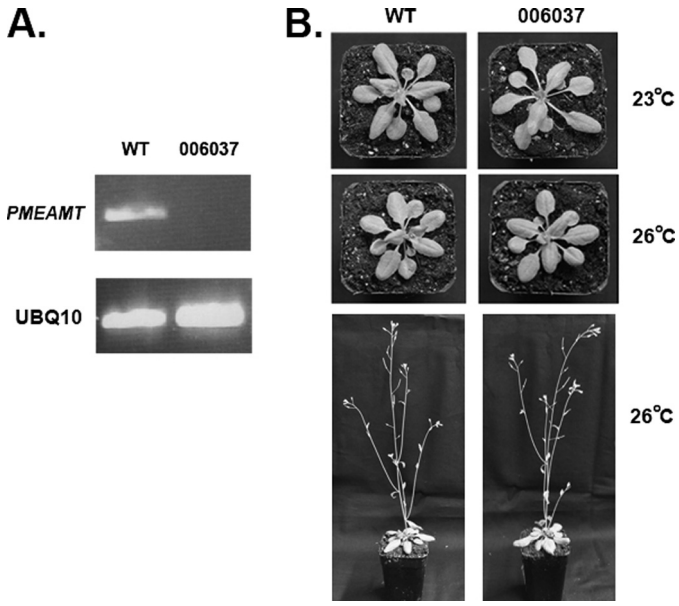

FIGURE 5. **Analysis of *Arabidopsis* SALK 006037 T-DNA insertion line.** *A*, RT-PCR of RNA from wild type (*left*) and T-DNA insertion line SALK 006037. Primers specific for the ubiquitin 10 gene were used as a control. *B*, 4-week-old wild-type and SALK 006037 *Arabidopsis* lines grown at 23 and 26 °C (*top two rows*) under a 12-h photoperiod. Leaves were harvested from plants at this growth stage for RT-PCR analysis. 7-Week-old wild-type and SALK 006037 plants grown at 26 °C are shown in the *bottom row*. When the photoperiod was altered to 8 h light/16 h dark, there were no overt phenotypic differences between the SALK 006037 and wild-type *Arabidopsis* lines.

In view of the possible role of AtPMEAMT in membrane synthesis (Fig. 1*A*), a lipidomics approach was used to compare the phospholipid composition between the wild-type and *atpmeamt* lines. Fig. 6 shows representative phospholipid profiles in positive scan mode, with neutral losses corresponding to the mass of the polar headgroup (30, 36). Across neutral loss profiles, for each additional methyl group added to a given lipid with a specific fatty acid composition, the m/z increases by a factor of 14. As such, neutral losses of 147, 161, 175, and 189 correspond to the lithiated polar headgroups of PtdEA, PtdMEA, PtdDEA, and PtdCho, respectively (30). The ESI-MS/MS profiles for phospholipids extracted from leaves of wild-type *Arabidopsis* plants are on the same scale to show the relative abundance of each Ptd-base species in a given tissue extract. Within each genotype, the relative contributions of PtdCho and PtdEA toward the total leaf phospholipids were equal (data not shown). Also, PtdEA and PtdCho carrying a variety of acyl chains were detected, with the 34:3 and 34:2 lipid molecular species comprising the most abundant classes for these Ptd-bases (Fig. 6). PtdMEA components are frequently near or below the level of detection, and PtdDEA lipid molecular species were not statistically different from the detection limits. Although absolute quantitative estimates for components in profiles can be difficult to make (30, 36), the peak heights associated with PtdMEA are consistent with this species being a minor component of total phospholipids (<2%). However, the 34:3-PtdMEA was significantly different between the genotypes, being, on average, 2.1-fold more abundant among phospholipids of *atpmeamt* plants (Figs. 6*B* and 7). Other than the 34:3-PtdMEA species, we found no significant

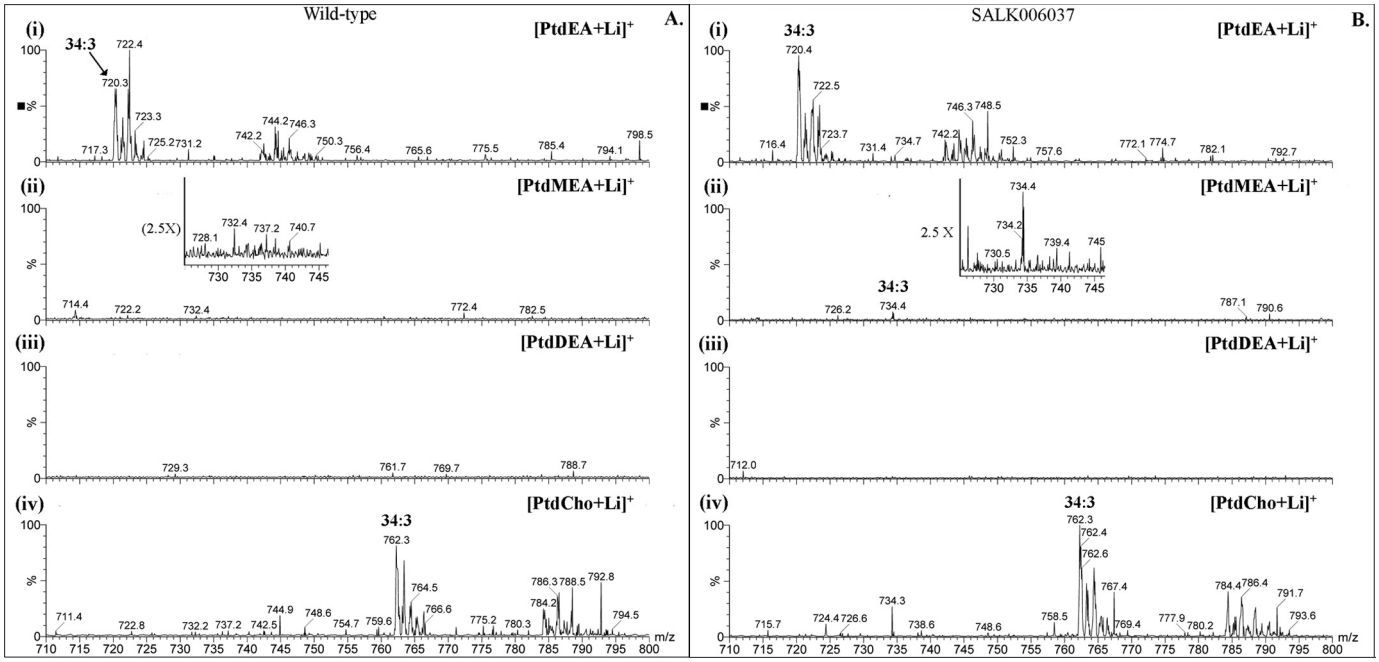

FIGURE 6. **Phospholipid profiles show PtdMEA in leaves of *atpmeamt Arabidopsis* plants.** Positive mode electrospray mass spectra of crude lipid extracts from leaves of wild-type (*A*) and *atpmeamt* (SALK 006037) (*B*) *Arabidopsis* lines. *i*, neutral loss scan of 147 mass units; *ii*, neutral loss scan of 161 mass units; *iii*, neutral loss scan of 175 mass units; *iv*, neutral loss scan of 189 mass units. The *scale inset* (*ii*) has been expanded 2.5-fold to show the presence of a 34:3-PtdMEA peak among the phospholipids of *atpmeamt* (*B*) that was significantly lower in samples from wild-type plants (*A*).

difference with respect to acyl composition of phospholipids and genotype ($p < 0.05$, as determined by Student's *t* test).

DISCUSSION

This study reports on the cloning of the gene encoding a previously uncharacterized P-base *N*-methyltransferase from *Arabidopsis*. This gene was isolated by heterologous complementation of a yeast strain defective in the synthesis of PtdCho by a cDNA associated with the plant gene at locus At1g48600 that encodes a putative PEA *N*-methyltransferase 2 (NCBI). Our selection strategy and *in vitro* assays followed by product identification show that this gene encodes an enzyme capable of methylating the P-base substrates PMEA and PDEA but not PEA and hence is designated AtPMEAMT (Fig. 4).

In many if not all plants, the enzyme PEAMT catalyzes the committing step for choline production (9, 10, 14). In keeping with this important role, it is not surprising that about 97% of the yeast clones rescued by the inclusion of EA or MEA in the selection medium were those complemented by *Arabidopsis* cDNAs encoding AtPEAMT, and only three corresponded to AtPMEAMT. The cDNA used in library construction was obtained from whole seedlings at the two-leaf growth stage (22), and *AtPMEAMT* expression may also be low in this tissue source. A gene at locus At1g73600 also encodes a putative PEAMT showing 85% identity to *Arabidopsis* PEAMT. Despite having screened over 10^6 transformed yeast cells, we recovered no clones complemented by a cDNA associated with this gene. There are two likely explanations for our failure to clone the product associated with this gene. It is possible that full-length transcripts and hence cDNA corresponding to At1g73600 were not present in the library, or the product of this gene does not use PEA or PMEA as substrate. An enzyme using only PDEA as substrate, for example, would not have functionally complemented the yeast phospholipid mutant following our selection strategy. We also did not clone the recently identified *Arabidopsis* phospholipid *N*-methyltransferase (AtPLMT) that is able to methylate PtdMEA and PtdDEA (37). Again, the failure to recover this plant cDNA probably reflects its low abundance or absence in the library. Because there were no differences in growth or lipid profiles between wild-type and PLMT-deficient *Arabidopsis* plants (37), this enzyme may play a more important role in other plant species, such as soybean, where the Ptd-base route for PtdCho synthesis predominates (9, 10, 37). Bolognese and McGraw (18) also noted the lack of complementation by plant PLMT when they used the same strategy to clone *AtPEAMT*. These authors suggested that choline and inositol may regulate AtPLMT, as is the case for this enzyme in yeast (38, 39), and if this were so, AtPLMT expression could be repressed in the seedlings used for the cDNA library because they were grown on medium supplemented with inositol (22).

The existing annotation for the complementing cDNA associated with At1g48600 as a putative PEA *N*-methyltransferase 2 is based upon several factors. Comparison of the ORFs encoded by *AtPMEAMT* and *AtPEAMT* shows the products of these genes to have 87% identity at the level of their amino acid sequences (Fig. 3). This high degree of identity supports an expectation of similar enzymatic activities for their products. Not surprisingly, many of the predicted properties of molecular mass, theoretical pI, and bipartite domain structure with respect to AdoMet binding are also very similar. For example, the longest ORF for AtPMEAMT has a predicted molecular mass of 54,018 Da with a theoretical pI of 5.04, whereas the properties of AtPEAMT are 56,102 Da with a theoretical pI of

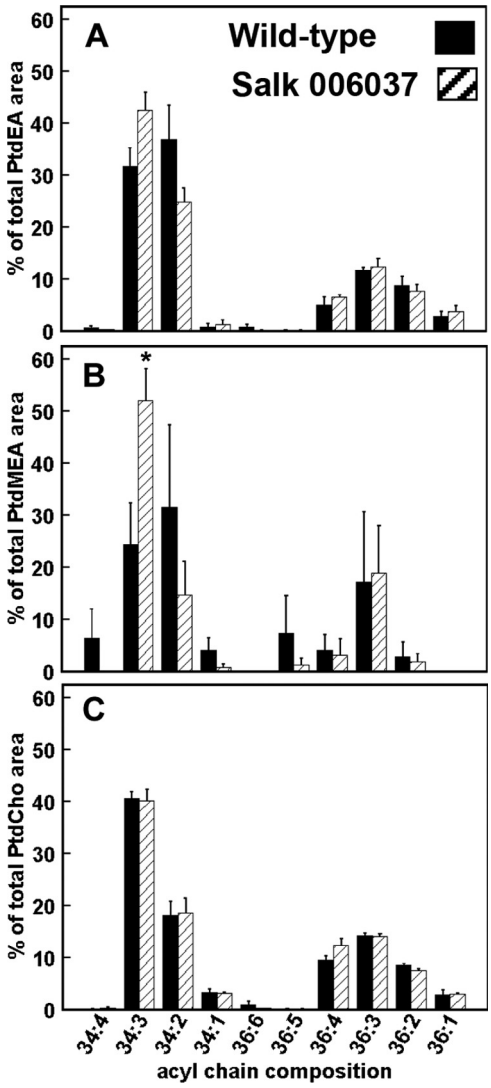

FIGURE 7. **Comparison of lipid molecular species between leaf phospholipids of wild-type and *atpmeamt Arabidopsis*.** PtdEA (*A*), PtdMEA (*B*), and PtdCho (*C*) lipid molecular species (total acyl carbons/total double bonds) are expressed as a percentage of total peak area of their respective Ptd-base as determined by ESI-MS/MS analyses. The *asterisk* indicates a significantly higher level of the 34:3-PtdMEA species for the mutant line relative to wild type as determined by Student's *t* test ($p < 0.05$). $n = 4$ for each genotype. *Error bars*, S.E.

5.39 (18, 40). Neither gene product is predicted to contain a chloroplast or mitochondrion-targeting signal (41, 42) suggesting that both are probably cytosolic, in keeping with the biochemical localization for spinach P-base methyltransferase activities (14). There are also common properties with respect to regulation. Specifically, AdoHcy and PCho have been shown to be inhibitors of spinach, wheat, and corn PEAMT (11, 16, 43), and we show these compounds to be inhibitory to AtPMEAMT activity as well (Table 1). The concentrations tested lie within the estimated physiological range (11, 44, 45) and so are consistent with both PEAMT and PMEAMT being subject to feedback regulation *in planta* by the reaction products AdoHcy or PCho.

To date, there are few comparative studies of P-base methyltransferases from other plants or different organisms, making it difficult to generalize about their features. However, there is evidence that the capacity to methylate PEA and PMEA need not reside on the same enzyme. The enzyme PMT-2 from *C. elegans* only methylates PMEA and PDEA (7), and a second enzyme, PMT-1, methylates PEA to PMEA (46). In contrast to plants, there is apparently no enzyme in *C. elegans* analogous to PEAMT in being able to catalyze the methylation of all three P-base substrates, leading to PCho synthesis (7, 12, 18, 31).

Our estimates of K_m values determined using PMEA and PDEA show that AtPMEAMT has a higher affinity toward PMEA and PDEA than does AtPEAMT. If these enzymes colocalize to the cytosol and are active at the same time, we would expect little opportunity for PMEA to accumulate, and any PDEA generated should be converted by both enzymes to PCho. In this regard, factors that can differentially regulate P-base methyltransferase activity are of interest. For PEAMT, inhibition by PCho and AdoHcy has been proposed as offering an important feedback control to curtail the production of choline when this product is not required (11, 34). In addition to feedback inhibition, PEAMT is light-responsive in many dicot plants (19), including *Arabidopsis* (data not shown). PEAMT shows highest activity when plants are in the light and low to no activity after an extended dark period (14, 19). This temporal regulation should reduce or prevent the production of PMEA in the dark when the continued synthesis of PCho is energetically disadvantageous to the plant. Unregulated operation of this pathway in the dark poses a potentially significant depletion and/or redirection of energy because each molecule of PCho produced requires seven molecules of ATP for EA phosphorylation, AdoMet production, and AdoHcy recycling (48). The reduced production of PMEA by PEAMT in the dark should eventually lead to the depletion of PMEA. This raises a question regarding the role of PMEAMT and whether this enzyme is needed to ensure that PMEA produced by PEAMT is fully converted to PCho.

Plants showing altered expression with respect to *PEAMT* and *PMEAMT* should show perturbed PMEA metabolism and, as such, offer insight into the role for PMEAMT in plants. In this regard, *atpmeamt* plants show clear evidence of altered PtdMEA composition compared with wild-type plants (Figs. 6 and 7). PtdMEA is normally a quantitatively minor and transitory intermediate in PtdCho synthesis (34, 35, 37). Under the growth conditions used, the elevated 34:3-PtdMEA species in lipid fractions of *atpmeamt* was not associated with a deleterious phenotype. Keogh *et al.* (37) also reported that the *atplmt* mutant lacking PLMT showed elevated PtdMEA and PtdDEA content in lipid profiles relative to wild-type, but no other differences were observed with respect to growth or development. In contrast, when a mutant line was grown with suppressed AtPEAMT activity, a variety of aberrant developmental and morphological traits were shown by the plants, including a severe loss of male fertility at 23 or 26 °C (49). Less severe abnormalities were associated with a T-DNA insertion mutant that reduced AtPEAMT activity (50), but even then a distinctive phenotype was noted with respect to diminished root development. We observed no reduced fertility even at 26 °C and no anomalous seedling root development (data not shown). Yeast *opi3* mutants accumulate PtdMEA in their membranes, and this change in phospholipid composition adversely impacts

their growth and viability (38). This deleterious condition is exacerbated when yeast is grown at higher temperatures, and the mutants did not grow at 37 °C. McGraw and Henry (38) suggest that the growth defects associated with this mutation could be related to the transport of required growth factors and/or membrane fluidity. The lack of phenotype for *atpmeamt* and *atplmt* plants may indicate that the PtdMEA content in plant membranes needs to exceed a certain threshold or that exposure to a higher growth temperature is needed before a temperature-responsive phenotype is displayed. The presence of an operational PEAMT that can perform all three activities may be all that is needed to prevent the more deleterious consequences associated with perturbed PCho metabolism.

Radiotracer studies and metabolic modeling almost invariably support the proposal that the flux rates associated with the choline biosynthetic pathways (Fig. 1) are rapid and that pool sizes for the intermediates PMEA and PDEA are small (16, 34, 35, 51). However, under conditions of salt stress in spinach, a salt-responsive increase in PEAMT activity is associated with an increase in radiolabeled PMEA (25). McNeil *et al.* (2) show that overexpression of spinach PEAMT by transgenic tobacco leads to a depletion of EA and PEA and an elevated content of PMEA relative to plants transformed with an empty vector control. This suggests that elevated PEAMT activity can lead to more PMEA produced than can be processed through to PCho. One reason that PMEA content can increase is due to feedback inhibition of PEAMT by PCho. P-base methyltransferases studied to date show varying sensitivity toward PCho. Although the PMT-1 and PMT-2 methyltransferases from *C. elegans* are relatively insensitive to PCho inhibition (7, 46), the methyltransferases from *Plasmodium* with a single AdoMet binding domain are highly sensitive in this regard (13), and bipartite domain PEAMTs from plants show intermediate sensitivity to PCho inhibition (11, 12, 16). The two wheat PEAMTs that have been characterized with respect to this property show that TaPEAMT2 is over twice as sensitive toward PCho as TaPEAMT1 (16). A truncated form of spinach PEAMT (ΔPEAMT) showed a sensitivity toward PCho half that of the recombinant wild-type gene product (12). By way of comparison, AtPMEAMT inhibition at 47% by 1 mM PCho is comparable with that reported for ΔPEAMT from spinach (IC_{50} = 1 mM) and is less sensitive to PCho than the TaPEAMT1 isoform (12, 16). Thus, AtPMEAMT is less sensitive toward inhibition by PCho than plant PEAMTs characterized to date. This feature and the high affinity toward PMEA should allow AtPMEAMT to remove PMEA more efficiently than is possible with AtPEAMT alone. Earlier reports offer support for this action as a proposed role for PMEAMT. The activity associated with PMEA methylation does not decline when spinach plants are put in prolonged dark conditions, whereas PEAMT activity does (11, 14). Thus, although reduced PEAMT would exert a strong level of regulation on PMEA production, continued PMEAMT activity allows for removal of PMEA by a mechanism that is not light-responsive and potentially less inhibited by PCho. As a constitutive housekeeping enzyme, PMEAMT would augment the capacity for plants to convert PMEA to PCho, and less PMEA should reduce the likelihood that this metabolite can be incorporated into the polar headgroups of plant phospholipids. This role for PMEAMT may be particularly advantageous for plants that accumulate glycine betaine under environmental stress, where PEAMT activity is induced and the capacity for PMEA production is much greater (14).

Whether PtdMEA in plant membranes constitutes a deleterious PMEA product is difficult to assess. Few reports document the contribution of PtdMEA or PtdDEA to the lipid composition of plants (34, 35, 37), but the capacity to produce phospholipid membranes containing PtdMEA may be a more general feature of plants. The comparison reported by Keogh *et al.* (37) with respect to the lipid composition between *atplmt* mutant and wild-type *Arabidopsis* shows PtdMEA to be present among the phospholipids found in both lines. Although the distribution of acyl chains showed considerable overlap for the PtdEA, PtdMEA, PtdDEA, and PtdCho phospholipids, only the 34:3 acyl species was absent from the PtdMEA profiles. In contrast, we detected 34:3-PtdMEA in the wild-type and *atpmeamt* mutant (Figs. 6*B* and 7). We cannot explain the difference in acyl species associated with the PtdMEA fractions between our study and the results reported by Keogh *et al.* (37). These authors suggest that the polar headgroup phosphotransferase fails to recognize the 34:3 diacylglycerol in the presence of the intermediate CDP-MEA as a possible explanation for the absence of 34:3 associated with PtdMEA (Fig. 1). Our data would suggest that the CTP-MEA is incorporated by a phosphotransferase that can recognize a 34:3 diacylglycerol, but we cannot preclude the possibility that incorporation of PMEA in *atpmeamt* plants occurs by alternative means. For example, phospholipase D-mediated membrane remodeling could incorporate PMEA after PtdEA or PtdCho synthesis (52). In *atpmeamt* plants, the phosphotransferase enzyme present may have access to unusually high levels of free PMEA that could promote this exchange.

The need for increased environmental stress tolerance in crops has instigated many studies to enhance the capacity of plants to accumulate osmoprotectants like glycine betaine (reviewed in Refs. 47 and 53). Constraints to these goals have included an inability to engineer plants that can furnish sufficient choline for oxidation to glycine betaine (2). One recognized obstacle resides in the innate sensitivity of PEAMT toward PCho. Nuccio *et al.* (12) suggested that a less sensitive recombinant PEAMT that undergoes only the first reaction (PEA → PMEA) coupled to an enzyme like PMEAMT that completes the sequence of methylations might offer a solution to this metabolic challenge. Our results suggest that AtPMEAMT is less inhibited by PCho than spinach PEAMT, and this difference may confer an advantage in attempts to elevate glycine betaine levels in plants that do not normally accumulate this metabolite. However, a potentially more valuable contribution for PMEAMT may be to prevent the accumulation of PtdMEA in plant membranes, an anomaly whose impacts on crop physiology and productivity remains unknown.

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REFERENCES

1. Hanson, A. D., Rathinasabapathi, B., Rivoal, J., Burnet, M., Dillon, M. O., and Gage, D. A. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91,** 306–310
2. McNeil, S. D., Nuccio, M. L., Ziemak, M. J., and Hanson, A. D. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 10001–10005
3. Huang, J., Rozwadowski, K., Bhinu, V. S., Schäfer, U., and Hannoufa, A. (2008) *Plant Physiol. Biochem.* **46,** 647–654
4. Zeisel, S. H., Da Costa, K. A., Franklin, P. D., Alexander, E. A., Lamont, J. T., Sheard, N. F., and Beiser, A. (1991) *FASEB J.* **5,** 2093–2098
5. Blusztajn, J. K. (1998) *Science* **281,** 794–795
6. Chaudhary, K., and Roos, D. S. (2005) *Nat. Biotechnol.* **23,** 1089–1091
7. Palavalli, L. H., Brendza, K. M., Haakenson, W., Cahoon, R. E., McLaird, M., Hicks, L. M., McCarter, J. P., Williams, D. J., Hresko, M. C., and Jez, J. M. (2006) *Biochemistry* **45,** 6056–6065
8. Witola, W. H., El Bissati, K., Pessi, G., Xie, C., Roepe, P. D., and Mamoun, C. B. (2008) *J. Biol. Chem.* **283,** 27636–37643
9. Datko, A. H., and Mudd, S. H. (1988) *Plant Physiol.* **88,** 854–861
10. Datko, A. H., and Mudd, S. H. (1988) *Plant Physiol.* **88,** 1338–1348
11. Smith, D. D., Summers, P. S., and Weretilnyk, E. A. (2000) *Physiol. Plant* **108,** 286–294
12. Nuccio, M. L., Ziemak, M. J., Henry, S. A., Weretilnyk, E. A., and Hanson, A. D. (2000) *J. Biol. Chem.* **275,** 14095–14101
13. Pessi, G., Choi, J. Y., Reynolds, J. M., Voelker, D. R., and Mamoun, C. B. (2005) *J. Biol. Chem.* **280,** 12461–12466
14. Weretilnyk, E. A., Smith, D. D., Wilch, G. A., and Summers, P. S. (1995) *Plant Physiol.* **109,** 1085–1091
15. Moore, T. (1976) *Plant Physiol.* **57,** 382–386
16. Jost, R., Berkowitz, O., Shaw, J., and Masle, J. (2009) *J. Biol. Chem.* **284,** 31962–31971
17. Nuccio, M. L., Russell, B. L., Nolte, K. D., Rathinasabapathi, B., Gage, D. A., and Hanson, A. D. (1998) *Plant J.* **16,** 487–496
18. Bolognese, C. P., and McGraw, P. (2000) *Plant Physiol.* **124,** 1800–1813
19. Lorenzin, D., Webb, C., Summers, P. S., and Weretilnyk, E. A. (2001) *Can. J. Bot.* **79,** 897–904
20. Sherman, F. (2002) *Methods Enzymol.* **350,** 3–41
21. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168
22. Minet, M., Dufour, M. E., and Lacroute, F. (1992) *Plant J.* **2,** 417–422
23. Hoffman, C. S., and Winston, F. (1987) *Gene* **57,** 267–272
24. Stolz, J. (2003) *Yeast* **20,** 221–231
25. Summers, P. S., and Weretilnyk, E. A. (1993) *Plant Physiol.* **103,** 1269–1276
26. José, M., Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W. L., Berry, C. C., and Ecker, J. R. (2003) *Science* **301,** 653–657
27. Edwards, K., Johnstone, C., and Thompson, C. (1991) *Nucleic Acids Res.* **19,** 1349
28. Weigel, D., and Glazebrook, J. (2002) *Arabidopsis: A Laboratory Manual*, p. 176, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
29. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
30. Basconcillo, L. S., Zaheer, R., Finan, T. M., and McCarry, B. E. (2009) *J. Lipid Res.* **50,** 1120–1132
31. Charron, J. B., Breton, G., Danyluk, J., Muzac, I., Ibrahim, R. K., and Sarhan, F. (2002) *Plant Physiol.* **129,** 363–373
32. Kagan, R. M., and Clarke, S. (1994) *Arch. Biochem. Biophys.* **310,** 417–427
33. Joshi, C. P., and Chiang, V. L. (1998) *Plant Mol. Biol.* **37,** 663–674
34. Mudd, S. H., and Datko, A. H. (1989) *Plant Physiol.* **90,** 296–305
35. Mudd, S. H., and Datko, A. H. (1989) *Plant Physiol.* **90,** 306–310
36. Wolf, C., and Quinn, P. J. (2008) *Prog. Lipid Res.* **47,** 15–36
37. Keogh, M. R., Courtney, P. D., Kinney, A. J., and Dewey, R. E. (2009) *J. Biol. Chem.* **284,** 15439–15447
38. McGraw, P., and Henry, S. A. (1989) *Genetics* **122,** 317–330
39. Summers, E. F., Letts, V. A., McGraw, P., and Henry, S. A. (1988) *Genetics* **120,** 909–922
40. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., Bairoch, A. (2005) in *The Proteomics Protocols Handbook* (Walker, J. M., ed) pp. 571–607, Humana Press, Totowa, NJ
41. Emanuelsson, O., Nielsen, H., and von Heijne, G. (1999) *Protein Sci.* **8,** 978–984
42. Claros, M. G., and Vincens, P. (1996) *Eur. J. Biochem.* **241,** 779–786
43. Peel, G. J., Mickelbart, M. V., and Rhodes, D. (2010) *Phytochemistry* **71,** 404–414
44. Bligny, R., Gardestrom, P., Roby, C., and Douce, R. (1990) *J. Biol. Chem.* **265,** 1319–1326
45. Coughlan, S. J., and Wyn Jones, R. G. (1982) *Planta* **154,** 6–17
46. Brendza, K. M., Haakenson, W., Cahoon, R. E., Hicks, L. M., Palavalli, L. H., Chiapelli, B. J., McLaird, M., McCarter, J. P., Williams, D. J., Hresko, M. C., and Jez, J. M. (2007) *Biochem. J.* **404,** 439–448
47. Ashraf, M., and Foolad, M. R. (2007) *Environ. Exp. Bot.* **59,** 206–216
48. Moffatt, B. A., and Weretilnyk, E. A. (2001) *Physiol. Plant.* **113,** 435–442
49. Mou, Z., Wang, X., Fu, Z., Dai, Y., Han, C., Ouyang, J., Bao, F., Hu, Y., and Li, J. (2002) *Plant Cell* **14,** 2031–2043
50. Cruz-Ramírez, A., López-Bucio, J., Ramírez-Pimentel, G., Zurita-Silva, A., Sánchez-Calderon, L., Ramírez-Chávez, E., González-Ortega, E., and Herrera-Estrella, L. (2004) *Plant Cell* **16,** 2020–2034
51. McNeil, S. D., Rhodes, D., Russell, B. L., Nuccio, M. L., Shachar-Hill, Y., and Hanson, A. D. (2000) *Plant Physiol.* **124,** 153–162
52. Wang, X. (2000) *Prog. Lipid Res.* **39,** 109–149
53. Rontein, D., Basset, G., and Hanson, A. D. (2002) *Metabol. Eng.* **4,** 49–56