Phosphocholine (pCho) is a precursor for phosphatidylcholine and osmoprotectants in plants. In plants, de novo synthesis of pCho relies on the phosphobase methylation pathway. Phosphoethanolamine methyltransferase (PMT) catalyzes the triple methylation of phosphoethanolamine (pEA) to pCho. The plant PMTs are di-domain methyltransferases that divide the methylation of pEA in one domain from subsequent methylations in the second domain. To understand the molecular basis of this architecture, we examined the biochemical properties of three Arabidopsis thaliana PMTs (AtPMT1–3) and determined the X-ray crystal structures of AtPMT1 and AtPMT2. Although each isoform synthesizes pCho from pEA, their physiological roles differ with AtPMT1 essential for normal growth and salt tolerance, whereas AtPMT2 and AtPMT3 overlap functionally. The structures of AtPMT1 and AtPMT2 reveal unique features in each methyltransferase domain, including active sites that use different chemical mechanisms for phosphobase methylation. These structures also show how rearrangements in both the active sites and the di-domain linker form catalytically competent active sites and provide insight on the evolution of the PMTs in plants, nematodes, and apicomplexans. Connecting conformational changes with catalysis in modular enzymes, like the PMT, provides new insights on interdomain communication in biosynthetic systems.

Conformational changes in the di-domain structure of Arabidopsis phosphoethanolamine methyltransferase leads to active-site formation

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This article was selected as one of our Editors’ Picks. This article contains supplemental Figs. S1–S5 and Table S1. The atomic coordinates and structure factors (codes 5WP4 and 5WP5) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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2 The abbreviations used are: pCho, phosphatidylcholine; PMT, phosphoethanolamine methyltransferase; pEA, phosphoethanolamine; SAM, S-adenosylmethionine; PtdCho, phosphatidylcholine; pMME, phospho-monomethylethanolamine; pDME, phosphodimethylethanolamine; PDB, Protein Data Bank; SAH, S-adenosylhomocysteine; ITC, isothermal titration calorimetry; r.m.s.d., root mean squared deviation; qRT, quantitative RT; F, forward; R, reverse; IPTG, isopropyl 1-thio-β-D-galactopyranoside.

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by functional complementation of a yeast mutant (13). Subsequent work demonstrated that silencing of *atpmt1* resulted in temperature-sensitive male sterility and salt hypersensitivity and that the *Arabidopsis xipotl1* mutant, which disrupts *atpmt1*, is characterized by a short primary root, increased lateral root formation, and short epidermal cells (24, 25). Limited analysis of AtPMT2 suggests that this protein may not catalyze the methylation of pEA, but only the conversion of pMME to pCho (26). No phenotypic or functional information is available on AtPMT3, and no detailed biochemical examination of either AtPMT1 or AtPMT3 has been described.

The plant PMTs are important for the synthesis of PtdCho; however, the biochemical roles of the three PMT isoforms in the phosphobase methylation pathway of *Arabidopsis* remain to be fully examined. In addition, the structural basis for the di-domain architecture of the type I PMT from plants that separate enzymatic steps of the pathway (i.e. pEA to pMME in MT1 and pMME to pDME and pDME to pCho in MT2) is unclear. Here, we present the biochemical analysis of AtPMT1–3, which indicates that although each isoform catalyzes all the steps in the phosphobase methylation pathway, *atpmt1* is the only isoform with a growth phenotype and hypersensitivity to salinity in T-DNA knock-out plants. This appears linked to expression differences of *atpmt1* versus *atpmt2* and *atpmt3*. The X-ray crystal structures of AtPMT1 in complex with S-adenosylhomocysteine (SAH) and pCho and of AtPMT2 in complex with SAH at 1.34- and 1.50-Å resolution, respectively, provide details that differentiate the MT1 and MT2 domains both structurally and mechanistically. These structures also reveal the conformational flexibility in the di-domain architecture and provide insight on the molecular evolution of the different types of PMT in various organisms.

### Results

#### Biochemical analysis of *Arabidopsis PMT*

To examine the biochemical activities of the three *Arabidopsis* PMT isoforms, the coding region of each enzyme was PCR-amplified from a cDNA library with the obtained sequences matching those reported in GenBank™. AtPMT1 (491 amino acids; 56.1 kDa) shares 89 and 80% amino acid sequence identity with AtPMT2 (491 amino acids; 56.1 kDa) and AtPMT3 (504 amino acids; 57.8 kDa), respectively. AtPMT2 and AtPMT3 are 80% identical. The AtPMT1s share 33–36% sequence identity with the nematode PMT and 42% identity with the *Plasmodium* enzyme, but this is localized entirely to the MT2 domain of the plant enzymes (supplemental Fig. S1). The three AtPMT1s were expressed in *Escherichia coli* as N-terminal His-tagged proteins and purified using Ni²⁺-affinity and size-exclusion chromatographies. Each isoform migrated with a molecular mass of ∼55 kDa on SDS-PAGE (supplemental Fig. S2), and eluted from the size-exclusion column as a monomer (∼50–60 kDa) (supplemental Fig. S2D), which was similar to the spinach and nematode enzymes (8, 10, 11, 17, 20).

Steady-state kinetic parameters for AtPMT1–3 were determined using pEA, pMME, or pDME and SAH as substrates (Table 1). The *k_cat* and *K_m* values for pEA and SAH were similar to those reported for the spinach, wheat, nematode, and *Plasmodium* enzymes (8–11, 20, 21). Comparison of *k_cat/K_m* values indicates that AtPMT1 displays a modest 3-fold preference for pEA versus either pMME or pDME. Both AtPMT2 and AtPMT3 also prefer pEA with a 1.5–2-fold higher catalytic efficiency compared with pMME and pDME.

Multiple studies suggest that the plant PMT contain two methyltransferase domains defined by SAM-binding sequence.

### Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Parameter</th>
<th>AtPMT1</th>
<th>AtPMT2</th>
<th>AtPMT3</th>
</tr>
</thead>
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<tr>
<td>pEA</td>
<td><em>k_cat</em> (min⁻¹)</td>
<td>12.3 ± 0.6</td>
<td>16.1 ± 2.5</td>
<td>5.2 ± 1.0</td>
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<td></td>
<td><em>K_m</em> (µM)</td>
<td>156 ± 24</td>
<td>408 ± 143</td>
<td>284 ± 96</td>
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<tr>
<td></td>
<td><em>k_cat/K_m</em> (M⁻¹ s⁻¹)</td>
<td>1.314</td>
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<td>305</td>
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<tr>
<td>pMME</td>
<td><em>k_cat</em> (min⁻¹)</td>
<td>8.2 ± 0.4</td>
<td>12.7 ± 0.6</td>
<td>2.5 ± 0.3</td>
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<tr>
<td></td>
<td><em>K_m</em> (µM)</td>
<td>278 ± 38</td>
<td>577 ± 72</td>
<td>201 ± 59</td>
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<tr>
<td></td>
<td><em>k_cat/K_m</em> (M⁻¹ s⁻¹)</td>
<td>489</td>
<td>367</td>
<td>209</td>
</tr>
<tr>
<td>pDME</td>
<td><em>k_cat</em> (min⁻¹)</td>
<td>13.1 ± 1.4</td>
<td>16.0 ± 1.0</td>
<td>2.4 ± 0.2</td>
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<tr>
<td></td>
<td><em>K_m</em> (µM)</td>
<td>507 ± 115</td>
<td>653 ± 98</td>
<td>249 ± 69</td>
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<td></td>
<td><em>k_cat/K_m</em> (M⁻¹ s⁻¹)</td>
<td>433</td>
<td>408</td>
<td>161</td>
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<tr>
<td>SAM (pEA)</td>
<td><em>k_cat</em> (min⁻¹)</td>
<td>7.8 ± 0.4</td>
<td>16.0 ± 0.7</td>
<td>3.7 ± 0.2</td>
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<td><em>K_m</em> (µM)</td>
<td>89.7 ± 11.8</td>
<td>308 ± 33</td>
<td>69.1 ± 9.6</td>
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<td><em>k_cat/K_m</em> (M⁻¹ s⁻¹)</td>
<td>1,453</td>
<td>866</td>
<td>897</td>
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<tr>
<td>SAM (pDME)</td>
<td><em>k_cat</em> (min⁻¹)</td>
<td>9.0 ± 0.9</td>
<td>12.8 ± 0.3</td>
<td>1.5 ± 0.1</td>
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<tr>
<td></td>
<td><em>K_m</em> (µM)</td>
<td>215 ± 41</td>
<td>212 ± 13</td>
<td>92.8 ± 7.5</td>
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<tr>
<td></td>
<td><em>k_cat/K_m</em> (M⁻¹ s⁻¹)</td>
<td>767</td>
<td>1,006</td>
<td>266</td>
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</tbody>
</table>

Figure 1. Plant phosphatidylcholine biosynthesis and the di-domain architecture of the type I PMT from plants. A, phosphatidylcholine biosynthesis. The de novo choline or Kennedy pathway (gray arrow), the Bremer-Greenberg pathway (white arrow), and the phosphobase methylation pathway (black arrow) are shown. Metabolite names consist of a prefix (p, phospho; CDP-, cytidine 5’-diphosphate; or Ptd, phosphatidyl) and a core name (EA, ethanolamine; MME, monomethyllethanolamine; DME, dimethyllethanolamine; Cho, choline). B, reactions and domain structure of the type I PMT from plants. Methylation of phosphoethanolamine (pEA) to phosphoethanolamine (pMME) is catalyzed by the first methyltransferase domain (MT1) and the methylation reactions converting pMME to phosphocholine (pCho) catalyzed by the second methyltransferase domain (MT2).
motifs (8, 13, 14). Isothermal titration calorimetry (ITC) was used to evaluate the stoichiometry and energetics of ligand binding to AtPMT2. The other two isoforms were not stable enough during the time of experiment for analysis. Titration of AtPMT2 with SAM, pEA, and pCho did not yield sufficient heat to accurately determine binding constants. Addition of SAH to AtPMT2 (supplemental Fig. S3) showed a 2:1 ligand/protein stoichiometry consistent with the predicted di-domain structure. The $K_d$ value for SAH (4.6 ± 0.4 μM) and the binding energy ($\Delta G = -7.19 \pm 0.20$ kcal mol$^{-1}$), which was largely driven by the enthalpic contribution ($\Delta H = -10.8 \pm 0.2$ kcal mol$^{-1}$; $\Delta S = 3.6$ kcal mol$^{-1}$), were similar to those reported for the nematode and Plasmodium PMT (15–17).

In planta analysis of Arabidopsis PMT

 Examination of the role of each AtPMT isoform in Arabidopsis has been limited to the xipotl1 mutant phenotype of atpmt1, which suggests a key role for this enzyme in root development (24, 25), and a report of a change in one phospholipid form in atpmt2 knock-out lines (26). For a comparison of the role of each isoform in planta, T-DNA insertion mutants for each atpmt were obtained with the knock-out confirmed by reverse transcript PCR (Fig. 2, A and B). As described previously (24, 25), the atpmt1 mutant displayed a short root phenotype under normal growth conditions and hypersensitivity, as determined by root length, under salt-stress conditions. Comparison of all three lines showed the reported phenotype with atpmt1 and no phenotypic differences with either atpmt2 or atpmt3 under normal conditions (Fig. 2C). Under salt stress, atpmt1 also showed the hypersensitive short primary root phenotype with the atpmt2 and atpmt3 lines comparable with wild type (Fig. 2D). Earlier work suggested that NaCl treatment induces PMT expression (8). Comparison of isoform expression changes in Arabidopsis seedlings treated with either 0 or 50 mm NaCl indicates that expression of atpmt1 increased 2-fold relative to untreated seedlings with unchanged atpmt2 expression and a 2-fold reduction in atpmt3 transcript (Fig. 2E). At the metabolic level, previous studies describe a modest 10% decrease in PtdCho in roots and leaves of the atpmt1/xipotl1 mutant (25) and only a 2-fold decrease for one polar lipid (34:3) species in the atpmt2 knock-outs (26). We compared the polar lipid profiles of wild-type and atpmt3 knock-out plants, which indicate no significant differences (supplemental Fig. S4). As our results suggest a singular role for AtPMT1 versus functional redundancy of AtPMT2 and AtPMT3, the tissue-specific expression for each isoform was examined by qRT-PCR using gene-specific primers (Fig. 2F). In roots, atpmt1 and atpmt2 are more highly expressed than atpmt3, but in leaves the expression of atpmt2 and atpmt3 predominates. The differences in expression of atpmt1 in root tissue compared with the higher transcript levels of atpmt2 and atpmt3 in leaf tissues, along with

Figure 2. Comparison of atpmt T-DNA knock-out mutants and tissue-specific expression of AtPMT isoforms. A, a schematic of the genomic structures of atpmt1, atpmt2, and atpmt3 with T-DNA insertion sites indicated. Boxes represent exons, and the lines represent introns. B, RT-PCR analysis of homozygous mutant plants using AtPMT isoform-specific primers. β-Tubulin (TUB) was used as a control. C, effect of T-DNA knock-out on root length. Wild-type, atpmt1, atpmt2, and atpmt3 Arabidopsis thaliana seedlings were grown for 10 days on half-strength MS agar plates and photographed. D, effect of T-DNA knock-out on salt tolerance. As in C, seedlings were grown for 10 days on half-strength MS agar plates ± 50 mm NaCl, and then primary root length was determined. Error bars represent the standard error of the mean (n = 25). E, effect of NaCl treatment on atpmt1–3 expression. Wild-type A. thaliana seedlings were grown as in D. Total RNA was extracted from 7-day-old seedlings for qRT-PCR amplifications. Relative expression values for atpmt1 (black), atpmt2 (white), and atpmt3 (gray) in NaCl-untreated and -treated seedlings were normalized against tub gene expression. Error bars are the standard error of the mean (n = 3). F, tissue-specific expression of AtPMT isoforms. Total RNA was extracted from 7-day-old seedlings and 1-month-old plant tissues. The qRT-PCR amplifications were performed in triplicate independently for each target gene and the data averaged. The relative expression values for atpmt1 (black), atpmt2 (white), and atpmt3 (gray) in different tissues were normalized against act7 gene expression. Error bars represent the standard error of the mean (n = 6).
induction of atpmt1 expression by NaCl, likely explain the effects of the atpmt1/xipotl1 mutant under salt stress versus the phenotype of the knock-out lines for the other two isofoms.

**X-ray crystal structure of AtPMT1: Overall di-domain structure**

To understand the molecular basis for the distinct methyltransferase activities of the PMT from plants, the 1.34-Å resolution X-ray crystal structure of AtPMT1 in complex with SAH and pCho was determined by molecular replacement (Table 2). The overall structure of AtPMT1 reveals the N-terminal MT1 and C-terminal MT2 domains connected by a linker between β1g and α9 (Fig. 3A). The MT1 domain, which is responsible for conversion of pEA to pMME, contains a 7-stranded β-sheet (β1α–β1g) and multiple α-helices (α1–α8). The MT2 domain, which catalyzes the methylation of pMME to pDME and pCho, retains the core β-sheet structure (β2α–β2g) but with 11 α-helices (α9–α19) arranged differently compared with MT1 (Fig. 3, B and C, left). Clear electron density for SAH and pCho in the active site of each domain was observed (supplemental Fig. S5, A and B). In each domain, SAH binds along the central β-sheet; however, the regions around the phospho-base-binding site comprise structurally different arrangements of α-helices and loops that cap the active site of each domain. For example, the β1α–α7 loop caps the MT1 active site, whereas two α-helices (α18 and α19) serve a similar role in the MT2 active site. Thus, the MT1 and MT2 domains are structurally distinct in terms of both overall three-dimensional fold and local active-site architecture.

The structure of AtPMT1 reveals the conservation of the overall fold of each MT domain with the respective catalytic domains from the type III PMT of the parasitic nematode H. contortus (17) but also key differences with the vestigial non-catalytic N-terminal domain of HcPMT1. The MT1 domain of AtPMT1 is similar to the N-terminal catalytic domain of HcPMT1 (1.2 Å root mean squared deviation (r.m.s.d.) for 224 Cα atoms; Z = 31.9; 34% identity) but differs from the vestigial non-catalytic N-terminal domain of HcPMT2 (2.4 Å r.m.s.d. for 192 Cα atoms; Z = 20.0; 14% identity). The loss of regions corresponding to α1, α2, α5, and β1c (Fig. 3B), along with sequence variations (supplemental Fig. S1), yields a vestigial MT1 domain in HcPMT2. The MT2 domain of AtPMT1 shares a common fold (1.0 Å2 r.m.s.d. for 255 of Cα atoms; Z = 40.5; 42% identity) with the type II PMT from P. falciparum (15, 16) and the C-terminal domain of HcPMT2 (1.1 Å2 r.m.s.d. for 257 Cα atoms; Z = 40.3; 41% identity). It should be noted that a DALI search using the MT2 domain of AtPMT1 does not identify any portion of the nematode PMT1 as similar in fold. Comparison of the AtPMT1 MT2 domain and the vestigial non-catalytic C-terminal domain of HcPMT1 highlights key structural differences, including shortening of the α18 and α19 helices compared with the plant enzyme and a longer β2α–α12 loop, which occludes the SAM/SAH-binding site (Fig. 3C). These changes lead to loss of the MT2 domain activity in HcPMT1.

**X-ray crystal structure of AtPMT2: Changes in di-domain structure and formation of the MT2 active site**

The reported structures of the nematode PMT (17) and the structure of AtPMT1 (Fig. 3A) show the N- and C-terminal domains oriented adjacent to each other; however, the linker region connecting the domains could be conformationally flexible. During initial crystallization efforts, AtPMT2 (89% amino acid identity to AtPMT1) was used to increase the odds of obtaining crystals of a plant PMT. Fortuitously, the X-ray crystal structure of AtPMT2 reveals how changes in di-domain structure are linked to MT2 active-site formation. The 1.50-Å resolution structure of AtPMT2 in complex with SAH was solved by molecular replacement (Table 2) with clear electron density for an SAH molecule observed in each active site (supplemental Fig. S5, C and D). In contrast to previously reported PMT structures, AtPMT2 crystallized in a domain-swapped form (Fig. 4A). In the asymmetric unit, two AtPMT2 molecules pack with the MT1 and MT2 domains in a crossed orientation, instead of a side-by-side arrangement. An overlay of the MT2 domains of AtPMT1 and AtPMT2 emphasizes the global movement of the MT1 domain and shows how a structural transition of an α-helix positions one domain relative to the other (Fig. 4B).

In the AtPMT2 structure, residues from Gly-236–Gly-259 form the α9-helix that extends between the two domains (Fig.
Structure of Arabidopsis PMT

The changes in di-domain orientation and conformational changes in the linker helix affect access to the MT2 domain active site. In the AtPMT2:SAH complex structure, the extended conformation of α9 leaves the MT2 domain active site open for potential access of substrates (Fig. 5A). In comparison, the AtPMT1:SAH:pCho complex structure suggests that breaking the linker helix into two segments allows for movement of α9 and α10 into the MT2 active site for substrate binding (Fig. 5B). Residues from the core regions of the MT2 domain provide multiple interactions with SAH (Fig. 5C). The amino acid group of SAH binds in a pocket formed by Val-263, Ser-264, and Arg-355. A bidentate interaction between the ribose hydroxyl groups of SAH and Asp-312 anchors the ligand in the active site. Leu-241, Leu-313, Ser-314, Cys-339, and Ile-361 provide a largely apolar binding site for the adenineosine ring of SAH with Asp-388 forming a hydrogen bond to the nitrogen of the adenine ring. These positions display the sulfur group, which would be methylated in SAM, toward the phosphobase-binding site of the active site.

Within the MT2 domain active site of AtPMT1, extensive protein–ligand contacts are involved in phosphobase binding (Fig. 5D). Hydrogen bonds from Gln-245, Tyr-254, Tyr-386, Tyr-400, and Tyr-406 and charge–charge interactions from Arg-404 and Lys-472 lock the pCho phosphate group into the active site to orient the choline group toward SAH. Two residues from the α9–α10 loop form key contacts with pCho, and the amide side-chain nitrogen of Gln-245 forms a hydrogen bond with the bridging oxygen of pCho. As noted above, the structural transition from a single linker helix (i.e. α9) to two helices (α9 and α10) brings Tyr-246 into the active site (Fig. 4). In the functional active site, the hydroxyl group of Tyr-246 is positioned between the amine of pCho (3.9 Å) and the sulfur of SAH (3.8 Å). The Nε of His-360 interacts with the hydroxyl group of the tyrosine (2.8 Å), which would allow this residue to serve as a general base to activate Tyr-246. Previous studies of PfPMT and HcPMT2 suggest a critical role for these residues as a catalytic dyad during the methylation of pMME to pDME and pDME to pCho (15–17).

Structure of the MT1 domain active site

The N-terminal residues of α9 (Gly-236–Asn-243) retain an α-helical structure and swing toward the active site of the MT2 domain; however, Val-244–Ser-248 adopt a loop conformation that places Gln-245 and Tyr-254 in proximity with the bound SAH and pCho in the AtPMT1-active site. The remaining stretch of α9, including Ser-249–Tyr-254, undergoes minor side-chain movement between the two structures to become α10 in the AtPMT1 structure.

Figure 3. Overall three-dimensional structure of AtPMT1. A, a di-domain structure of AtPMT1. The MT1 and MT2 domains are indicated in the ribbon diagram. The schematic represents α-helices as gold cylinders and β-strands as blue arrows with numbering as shown. SAH and pCho are represented as space-filling molecules. The β1e–α7 loop that caps the active site of the MT1 domain is indicated. B, comparison of the secondary structure features of the MT1 domain of AtPMT1 (left) and the non-catalytic vestigial N-terminal domain of HcPMT2 (right). Coloring of the MT1 domain is as in A with the α9-helix of the AtPMT1 MT2 domain shown for reference. The schematic of the vestigial domain shows α-helices as rose cylinders and β-strands as green arrows. C, comparison of the secondary structure features of the MT2 domain of AtPMT1 (left) and the non-catalytic vestigial C-terminal domain of HcPMT2 (right). Coloring is as in A and B. The AtPMT1 MT2 domain α9 is shown for reference. The position of the insertion loop in the vestigial domain of HcPMT1 that occludes the SAM/SAH-binding site is indicated.
binding. There is also a corresponding shift of His-162–Ser-171 in the MT1 domain active site, binding of SAH/SAM to the active site of HcPMT1 (17).

**Mutagenesis of the catalytic tyrosines in the MT1 and MT2 domains of AtPMT2**

To test the possible roles of Tyr-131 in MT1 and Tyr-246 in MT2 during the methylation reactions catalyzed by the plant PMT, each tyrosine was mutated to alanine and phenylalanine in AtPMT2 with the resulting mutant proteins expressed and purified as described for the wild-type enzyme. Enzymatic assays with either pEA or pDME as substrates indicate that these two residues are key for function (Fig. 7). The AtPMT2 Y131A and Y131F mutants exhibited decreased activity with pEA with less than 10% specific activity retained but were comparable with wild type and pDME. The AtPMT2 Y246A and Y246F mutants retained wild-type activity with pEA but were nearly inactive with pDME. This suggests that the plant PMT use similar residues as the nematode PMT for catalysis.

**Discussion**

Synthesis of pCho in plants provides the headgroup for the predominant membrane phospholipid PtdCho and precursors for synthesis of the key osmoprotectants (1–3). Unlike animals, which can obtain choline from dietary sources, plants use the phosphobase methylation pathway to convert pEA into pCho (Fig. 1A) (5–8). The PMT that catalyze the triple methylation of pEA to pCho are the committed enzymes in this process (8). In plants, the PMT are structurally distinct from the nematode and *Plasmodium* enzymes (12). The di-domain organization of the bi-functional plant PMT divides the first methylation reaction from subsequent steps in the pathway (Fig. 1B). Here, our work reveals the molecular basis for distinct function and suggests how conformational transitions lead to catalytically competent active sites in each methyltransferase domain.

Initial cloning of the PMT from *A. thaliana*, *S. oleracea* (spinach), *T. aestivum* (wheat), *B. napus* (canola), and *Z. mays* (corn) identified homologs (supplemental Fig. S1), but only some were biochemically examined (8, 13, 14, 21–23). Of the three PMT isoforms in *Arabidopsis*, AtPMT1/XIOPTL is the best studied with *in planta* experiments showing that a loss of activity leads to temperature-sensitive male sterility, salt hypersensitivity, and altered root development (13, 24, 25). One report examining AtPMT2 suggests that this enzyme lacks the ability to convert pEA to pMME (i.e. the first step in the phosphobase methylation pathway) and that its gene knock-out alters levels of one phospholipid form (26). No information is available on AtPMT3, and the substrate preferences of the AtPMT have not been systematically examined.

Biochemical assays of the AtPMT demonstrate that each isoform catalyzes all three reactions in the phosphobase methylating system.
tion pathway (Table 1). This result is consistent with the proposed di-domain structure of these enzymes (8). In contrast to previous work on AtPMT2, which reported that the enzyme does not methylate pEA (26), our data indicate that it accepts all three phosphobase substrates. In addition, the 2:1 stoichiometry of SAH binding to AtPMT2 (supplemental Fig. S3) and the 1.50-Å resolution X-ray crystal structure of the protein (Table 2; Fig. 4) reveal a structurally intact MT1 domain. The previous study (26) described a clone lacking the first 16 amino acids of full-length AtPMT2 (At1g48600). Deletion of the N-terminal residues would eliminate the α9 linker helix of the MT1 domain, which removes key structural features and protein–ligand contacts needed for substrate binding (Fig. 6). This explains the lack of activity with pEA for the N-terminally truncated protein.
Although the AtPMT isoforms share common biochemical properties, their physiological roles appear to be different and likely depend on specific expression patterns. The Arabidopsis xipotl1 phenotype associated with loss of atpmt1 expression suggests a key role for this enzyme in root development and pCho synthesis during salt stress (24, 25). No clear phenotype was reported for atpmt2 knockout lines in Arabidopsis (26).

Comparing the T-DNA knockout lines of each isoform (Fig. 2, A and B) confirmed the previously described atpmt1/xipotl1 mutant effects when grown under either normal or salt-stress conditions (25), whereas the atpmt2 and atpmt3 knockout lines displayed no unusual growth phenotypes under either condition (Fig. 2, C and D). In addition, only atpmt1 expression increased under salt stress (Fig. 2E). Earlier studies report that the Arabidopsis atpmt1 knockout lines reduced PtdCho levels by 10% in roots and leaves (25) and that loss of atpmt2 decreased the level of one polar lipid (34:3) species by 2-fold (26). Likewise, comparison of the polar lipid profiles of wild-type Arabidopsis plants with the atpmt3 knockout line shows no significant changes in phospholipid content of the T-DNA insertion line (supplemental Fig. S4). These results suggest functional redundancy for the AtPMT2 and AtPMT3 isoforms and that the phenotype associated with the atpmt1 knockout may result from tissue-specific expression differences (Fig. 2F).

Elevated expression of atpmt1 in root compared with other tissues and the higher transcript levels of atpmt2 and atpmt3 in leaf may contribute to the differential effects of the atpmt1/xipotl1 mutant versus the other two isoforms. In the root, AtPMT1 is clearly key for normal growth and tolerance against salt stress (24, 25), whereas the other isoforms may have overlapping functions primarily in leaves. In Arabidopsis, the three PMT provide a route to pCho for production of PtdCho; however, production of downstream metabolites of the phosphobase methylation pathway may also contribute to phenotypic differences. For example, hydrolysis of PtdCho into choline releases phosphatidic acid, which is a lipid second messenger in stress signaling (21, 27). In addition, oxidation of choline yields the osmoprotectant glycine betaine (3). Thus, the roles of the PMT in plants may be broader than just pCho and PtdCho synthesis, and the resulting metabolic complexity in different tissues under normal growth and stress-response conditions requires additional effort to dissect their contributions.

At the molecular level, the three-dimensional structures of AtPMT1 and AtPMT2 provide new insights on the di-domain organization of these proteins (Fig. 3). The X-ray crystal structures of AtPMT1 and AtPMT2 show how the type I PMT retain an evolutionarily conserved core structure that forms the SAM/SAH-binding site in each methyltransferase domain, but they also show structural differences in the phosphobase-binding sites that lead to distinct MT1 and MT2 domain activities, which rely on two different sets of catalytic residues. As reported previously, key residues in the active sites of each domain are highly conserved across the PMT from a variety of organisms (12). Ultimately, the three types of PMT use different structural organizations for the same metabolic pathway (Fig. 8).

Based on sequence comparisons, the bi-functional di-domain architecture of the AtPMT is conserved across eudicots, monocots, conifers, mosses, and green algae, as the residues of each active site are highly conserved in their respective domain (Fig. 8). Interestingly, bioinformatic comparisons suggest that plant-like type I PMT may also be found in a variety of marine organisms, including oomycetes (water molds), tunicates, segmented seaworms, bivalves, gastropods, crustaceans, lancelets, bony fishes, frogs, toads, sea urchins, and anemones; however, the biochemical function and metabolic contribution of the PMT-like enzymes in these organisms remain to be determined. In the apicomplexans, which evolved from a plastid-containing ancestor, type II PMT perform all three methylation reactions in a single domain (Fig. 8). Structural and biochemical analysis of the P. falciparum PMT indicates that its active site contains substritions to allow for methylation of pEA and that these residue changes are not found in the MT2 domains of the plant and nematode enzymes (15–18). It appears that the loss of the MT1 domain and alteration of the MT2 domain active site led to a multifunctional single domain PMT of the apicomplexans (12, 19). In contrast to the simplification of PMT architecture observed in the apicomplexans, the evolutionary path to the two mono-functional di-domain proteins found in nematodes appears to have involved gene duplication and structural alterations that yielded vestigial non-functional domains (Fig. 8) (10, 11, 20). Although the domain organization of each type of PMT varies, the overall structure within each domain is highly conserved across a range of evolutionarily distant and diverse organisms.

The published structures of the type II apicomplexan and type III nematode PMT show active sites that enclose reactants, suggesting that catalysis requires conformational changes (15–19). As described here, crystallization differences between AtPMT1 and AtPMT2 yielded structures that reveal the local and global conformational changes associated with each step in the phosphobase methylation pathway (Figs. 4–6). We propose an overall reaction sequence that takes into account the structural flexibility of the PMT.
The methylation of pEA to pMME occurs in the MT1 domain of the plant PMT. The open MT1 active site observed in the AtPMT2 structure allows sufficient contacts for SAM/SAH binding, which along with parts of a disordered N-terminal region and orientation of the β1e–α7 loop (Ser-159–Tyr-176) away from the active site provide access for phosphobase binding (Fig. 6A, green). Previous studies of C. elegans PMT1 and wheat PMT show that these enzymes use a random sequential kinetic mechanism in which either pEA or SAM binds first, followed by the other substrate (11, 21). Based on the structure of the AtPMT1-SAHPCho complex (Fig. 6, A–C), we suggest that binding of both substrates leads to structural rearrangements that form a functional active site and that those movements are linked to local dynamic changes that stabilize residues of α1–α2 and reposition the β1e–α7 loop to enclose the site, as residues on this loop provide multiple contacts with pCho.

In the MT1 reaction complex, these structural transitions position the methyl group of SAM into proximity of pEA amine with Tyr-131 located between the two substrates. There is also a water molecule bound between the phosphate group of the pCho and the hydroxyl of Tyr-131. The active-site arrangement of AtPMT1 was also observed in the structure of the HcPMT1-SAHPCho complex (17), which suggests a similar substrate-assisted reaction in the MT1 domain of the plant enzyme. The position of the substrate phosphate relative to the bound water molecule could provide a proton relay to activate the hydroxyl group of Tyr-131, which then promotes deprotonation of the substrate’s primary amine for transfer of the methyl group from SAM (28). Site-directed mutagenesis of Tyr-131 confirms the importance of this residue, as substitutions resulted in a loss of pEA methylation (Fig. 7, left). Although pCho, the fully methylated product of the phosphobase pathway, can bind in the MT1 active site as an inhibitor,
pMME and pDME are not substrates for this active site in either the nematode or plant PMT (8, 11, 17). The chemical mechanism of the MT1 domain likely takes advantage of the reactivity and/or pK_a differences of the amine group of pEA versus the methylated phosphohydroxides. Ultimately, the MT1 active site of the plant and nematode PMT is tuned for the conformational and chemical steps needed for the first methylation to pCho.

Following product release from the MT1 domain, pMME (and later pDME) is methylated in the MT2 domain of the plant and nematode PMT. In contrast to the localized structural changes observed in the MT1 domain, rearrangements leading to a functional MT2 active site require large-scale movements in the di-domain structure (Fig. 4). In the AtPMT2 structure, the open MT2 active site provides access for substrate binding (Fig. 5A). Kinetic studies of C. elegans PMT2 using pDME and SAM as substrates indicate a random sequential addition (10), which given the conserved domain structures likely holds true with the plant PMT. As with the MT1 domain, we suggest that binding of both substrates favors the conformational changes that lead to functional active-site formation, in particular the breaking of the extended α9-helix seen in AtPMT2 into the two shorter α-helices (α9 and α10) that enclose the active site to bring Tyr-246 into proximity of the substrates and within interaction distance of His-360 in the AtPMT1 structure (Figs. 4, B and C, and 5). Substitutions of Tyr-246 in AtPMT2 eliminate the catalytic activity of the MT2 domain and confirm the key role of this residue for methylation chemistry (Fig. 7, right). Extensive structural and mechanistic studies of the P. falciparum PMT identified a tyrosine–histidine dyad (corresponding to Tyr-246 and His-360 of AtPMT1 and AtPMT2) as essential for catalysis (15). Chemically, the histidine acts as a general base to abstract a proton from the tyrosine to polarize the amine group and facilitate methyl group transfer. The dyad was also proposed to act as a “catalytic latch” to lock substrates into the active site. Based on the structures presented here, the observed conformational changes appear linked to substrate binding and positioning of these key catalytic residues.

Multiple studies on a variety of methyltransferases imply a connection between conformational changes and catalysis (29–33); therefore, the structures of the plant PMT for the first time reveal the details of how those transitions occur and contribute to the growing appreciation of how dynamics and chemistry are linked (34, 35). How structural rearrangements occur both within modular domains and between distinct domains in biosynthetic “assembly line” systems is still a poorly understood topic. For example, how interdomain architecture links modules of polyketide synthases, non-ribosomal peptide synthetases, and other engineered modular proteins is critical for efficient production of a variety of natural products (36–41). In summary, the structures of the plant PMT, along with previous biochemical and structural studies of these enzymes from other organisms, indicate an important role for dynamic conformational processes that are linked to the reaction sequence in each methyltransferase domain and between those domains for the efficient synthesis of pCho.

Experimental procedures

Materials

E. coli Rosetta II (DE3) cells were purchased from Novagen. Ni^{2+}-nitrilotriacetic acid-agarose beads were obtained from Qiagen. The HiLoad 26/60 Superdex-200 FPLC column was from Amersham Biosciences. Radiolabeled [methyl-^{14}C]SAM (50.4 mCi/mmol) was bought from MP Biomedicals, and the pMME and pDME substrates were synthesized by Gateway Chemical. Kanamycin and isopropyl-β-D-galactopyranoside (IPTG) were purchased from Research Products International. All other reagents were from Sigma. Seeds of A. thaliana ecotype Col-0 and T-DNA knock-out lines AtPMT1 (Salk_036291), AtPMT2 (Salk_204065), and AtPMT3 (Salk_016929C) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus).

Cloning of AtPMT isoforms, generation of bacterial expression vectors, and site-directed mutagenesis

The coding regions of AtPMT1 (At3g18000), AtPMT2 (At1g48600), and AtPMT3 (At1g73600) were amplified by PCR from A. thaliana seedling cDNA with isoform-specific oligonucleotides containing Ncol (forward primer) and NotI (reverse primer) restriction sites. The primers used were as follows (restriction sites are underlined and start/stop codons are in bold): AtPMT1-F, 5′-dTTCATGGGGCTGCATCGTACGAAG-3′, and AtPMT1-R, 5′-dTTTTGGCCGGCTTTACATTCTTGTTGGCAGT-3′; AtPMT2-F, 5′-dTTTCATGGGGCAGACACCATAAAGGAAG-3′, and AtPMT2-R, 5′-dTTTTGGCCGGCTTTACTCTTTGTTGCAGT-3′; and AtPMT3-F, 5′-dTTCATGGCATGGCTCACAGTCACACT-3′, and AtPMT3-R, 5′-dTTCATGGCATGGCTCACAGTCACACT-3′. Each PCR product (~1.5 kb) was cloned into the pCRII-TOPO vector (Invitrogen) for confirmation of sequence by automated nucleotide sequencing. Digestion of the TOPO vector with Ncol and NotI released the DNA fragment, which was then ligated into pHI8 (42), a modified version of pET-28a, to generate bacterial protein expression constructs containing an N-terminal octahistidine tag followed by a thrombin cleavage site. Site-directed mutants of the putative catalytic tyrosine residues of the MT1 (Y131A and Y131F) and MT2 (Y246A and Y264F) domains of AtPMT2 were generated using the QuikChange PCR method (Agilent).

Protein expression and purification

The pHI8-AtPMT1, -AtPMT2, and -AtPMT3 expression vectors were transformed into E. coli Rosetta II (DE3) cells. Isolated colonies were used to inoculate Terrific broth containing 50 µg ml^{-1} kanamycin and 35 µg ml^{-1} chloramphenicol and were grown (37 °C) until A_600nm = 0.8–1.2. After addition of IPTG (1 mM final), cultures were grown at 20 °C overnight. Cells were harvested by centrifugation (10,000 × g; 10 min) and resuspended in lysis buffer (50 mM Tris (pH 8.0), 500 mM NaCl, 25 mM imidazole, 10% (v/v) glycerol, 1% (v/v) Tween 20). After sonication and centrifugation (45,000 × g; 1 h), the supernatant was passed over a 3–5-ml Ni^{2+}-nitrilotriacetic acid column. Following washing with lysis buffer and wash buffer (lysis buffer without Tween 20), His-tagged PMT was isolated from the col-
Structure of Arabidopsis PMT

...other experiments were performed on a VP-ITC calorimeter (Microcal). Injections (10–12 μl) of ligand were added to a sample solution containing AtPMT2 using a computer-controlled 250-μl microsyringe at an interval of 5–6 min. Control experiments using buffer determined the heat of dilution for each injection, which was used as a correction in experimental titrations. Estimates of ΔHobs and ΔH were obtained by fitting the experimental data using the Microcal software to the following equation: $Q_{\text{obs}}^{\text{tot}} = V_0 \Delta H_{\text{obs}} / (K_{\text{obs}}L(1 + K_{\text{obs}}L))$, where $Q_{\text{obs}}^{\text{tot}}$ is total heat after the ith injection; $V_0$ is the volume of the calorimetric cell; $K_{\text{obs}}$ is the equilibrium constant; $\Delta H$ is the enthalpy change; $L$ is the ligand concentration; and $E_{\text{obs}}$ is the protein concentration. Values for the change in free energy were calculated from $\Delta G = -RT \ln(K_{\text{obs}})$, where $R$ is the gas constant (1.9872 cal K$^{-1}$ mol$^{-1}$), and $T$ is absolute temperature. Changes in entropy were calculated using $\Delta G = \Delta H - T \Delta S$.

Plant growth and quantitative real-time PCR tissue-specific expression in Arabidopsis

For each T-DNA knock-out line, seeds were sterilized with 50% (v/v) bleach for 10 min and washed three times with sterilized water. Seeds were germinated on half-strength Murashige-Skoog agar medium containing 1% (w/v) sucrose and transferred to a growth chamber (16 h light at 22 °C and 8 h dark at 18 °C with 70% relative humidity) for 10 days. Seedlings were then transferred to soil and grown under the same conditions. For genotyping of T-DNA knock-out lines, genomic DNA was extracted from 3-week-old rosette leaves in 100 mM Tris-HCl (pH 9.5), 1 M KCl, and 10 mM EDTA. After incubation (70 °C; 30 min), the extract was centrifuged (30,000 × g; 10 min), and the supernatant was transferred into a new tube and diluted 2-fold with MilliQ water (Millipore). PCRs (melting: 95 °C; 60 s; annealing: 58 °C; 60 s; extension: 72 °C; 60 s; 35 cycles) were performed in a total volume of 50 μl with 1× PCR buffer, 1.5 mM MgCl₂, 200 μM dNTPs, 50 pmol forward and reverse primers, and 1 unit of Taq DNA polymerase (Invitrogen). The T-DNA-specific primer LBa1 (5′-dTTGTTCACCT-GATGACGGCCCATCG-3′) and AtPMT1–3-specific primers (AtPMT1-F, 5′-dACCCGTAATACAAACCATC-3′, and AtPMT1-R, 5′-dCATGATCTCCTTCTCGCATGAG-3′) and AtPMT2-F, 5′-dTCCACCAATCGTAGATCAAGG-3′, and AtPMT2-R, 5′-dTGTGCCATCCATCTACCTC-3′, and AtPMT3-F, 5′-dtCAACGGTTCAACAAACCCCG-3′, and AtPMT3-R, 5′-dATCAGCCGCACAGAAATTGAC-3′) were used for genotyping analysis.

For qRT-PCR analysis, total RNA was isolated from different tissues of Arabidopsis using TRIzol reagent (Invitrogen). First-strand synthesis of cDNA from RNA (1 μg) was performed with the Superscript II reverse transcriptase kit (Invitrogen). Isoform-specific primers used for qRT-PCR were as follows: AtPMT1-F, 5′-dCTGGAATTACAGACCGGGAG-3′, and AtPMT1-R, 5′-dGTCCCATTTCTGCTATC-3′, and AtPMT2-F, 5′-dAGTGGCTAGAGTTTGTCGG-3′, and AtPMT2-R, 5′-dATGTCCAGGGGCTGATGAG-3′, and AtPMT3-F, 5′-dATGTGGCTAGGTTGTGCTG-3′, and AtPMT3-R, 5′-dTGTACGGCTTATGACCCCG-3′. The qRT-PCR analysis was performed according to the manufacturer’s instructions on a Bio-Rad iCycler iQ system with platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The Arabidopsis actin7 (ACT7) gene was used as an internal control (43). The fold change of transcripts was calculated based on an efficiency-calibrated model and compared with the transcript level under normal conditions. Statistical differences between samples were evaluated by Student’s t test. In each experiment, the mean of three biological replicates was used to generate means and statistical significance. Two experiments on independently grown plant material were performed to confirm that the results are reproducible.

Polar lipid analysis of wild-type and atpmt3 plants

Four-week-old wild-type and T-DNA knock-out plants were grown at the same time and in the same growth chamber. Dry weight of each leaf sample ranged from 13 to 35 mg. Total polar lipids were extracted using a published protocol recommended by the Kansas Lipidomics Research Center Analytical Laboratory (44). Briefly, leaves were quickly immersed in isopropyl alcohol containing 0.01% (v/v) butylated hydroxytoluene to prevent generation of phosphatidic acid by phospholipase D upon tissue damage. Polar lipid extracts were prepared using a series of chloroform/methanol and KCl extractions and then frozen for mass spectrometric analysis to provide a global profile of digalactosyldiacylglycerol, monogalactosyldiacylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and phos-
phatidic acid levels. Data analyzed were from three independent biological replicates.

**Protein crystallography**

Crystals of AtPMT1-SAHPCho complex were grown by the vapor diffusion method at 4 °C in hanging drops of a 1:1 mixture of protein (10 mg ml⁻¹) and crystallization buffer (16% (w/v) PEG-8000, 0.04 M potassium phosphate (monobasic), 20% (w/v) glycerol, 5 mM pCho, and 0.5 mM SAH). Crystals of AtPMT2-SA
complex were grown by the vapor diffusion method at 4 °C in hanging drops of a 1:1 mixture of protein (10 mg ml⁻¹) and crystallization buffer (200 mM potassium formate, 20% (w/v) PEG-3350, and 0.5 mM SAH). Prior to data collection, crystals were frozen in cryoprotectant (mother liquor supplemented with 25% glycerol). All diffraction data were collected at beamline 19ID of the Argonne National Laboratory Advanced Photon Source. HKL3000 was used to index, integrate, and scale the data sets. For solution of the AtPMT1 structure, molecular replacement using two independent search models was performed in PHASER (46). The structures of the *H. contortus* PMT1 N-terminal domain (PDB code 4KRH; residues 1–256; 17) and the *H. contortus* PMT2 C-terminal domain (PDB code 4KRH; residues 170–431; 17) were used as independent search models. COOT (47) and PHENIX (48) were used for iterative rounds of manual model building and refinement, respectively. Molecular replacement for the AtPMT2 dataset used a similar strategy. The final model of the AtPMT1-SAHPCho complex includes residues Glu-7–Asn-491, two SAH molecules, two pCho molecules, and 650 waters. The final model of the AtPMT2-SA
complex includes residues Glu-7–Glu-18 and Arg-42–Lys-490 in chain A, residues Glu-7–Ser-20 and Glu-44–Lys-490 in chain B, two SAH molecules, and 1175 waters. Table 2 summarizes the data collection and refinement statistics for both of the determined structures. Coordinates and structure factors for the AtPMT1-SAHPCho (PDB code 5WP4) and AtPMT2-SA
complexes were deposited in the PDB.

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