CLONING AND BIOCHEMICAL CHARACTERIZATION OF A PHOSPHOBASE *N*-METHYLTRANSFERASE FROM *ARABIDOPSIS* INVOLVED IN CHOLINE AND PHOSPHOLIPID METABOLISM

By

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ABSTRACT

In plants, phosphocholine (PCho) is a precursor to the membrane component phosphatidylcholine (PtdCho) and free choline (Cho). A mutant *Saccharomyces cerevisiae* yeast strain unable to produce PtdCho without exogenous choline was used for transformation with an *Arabidopsis* cDNA library cloned in the yeast expression vector pFL61. A plant cDNA associated with locus At1g48600 functionally complemented the mutant by restoring growth on minimal synthetic medium lacking choline but containing the phosphobase phosphomethylethanolamine (PMEA). Crude extracts prepared from the yeast showed a novel capacity to convert PMEA to phosphodimethylethanolamine (PDEA) and PCho and hence this enzyme has been named *Arabidopsis S*-adenosyl-L-methionine (AdoMet): phosphomethylethanolamine *N*-methyltransferase (AtPMEAMT).

AtPMEAMT is a bipartite enzyme containing tandem N- and C- terminal AdoMet-binding domains. The predicted amino acid sequence shows an 87% identity to the previously characterized AdoMet: phosphoethanolamine N-methyltransferase (AtPEAMT) from *Arabidopsis*. An important distinction between AtPMEAMT and AtPEAMT is that the former enzyme is unable to methylate phosphoethanolamine (PEA). However, both AtPEAMT and AtPMEAMT can methylate PMEA and PDEA, two phosphobase intermediates of PCho synthesis. The apparent K_m values were determined for AtPEAMT and AtPMEAMT toward PMEA and PDEA and found to be 0.32 and 0.14 mM, respectively, for PEAMT and 0.16 and 0.03 mM, respectively, for PMEAMT. The N- and C-terminal AdoMet-domains of PEAMT and PMEAMT were cloned separately into a pET30a(+) vector for protein expression and extracts containing recombinant

proteins were assayed for phosphobase methyltransferase activity. Only the gene product encoding the domain associated with the C-terminal half of PMEAMT methylated both PMEA and PDEA, an activity found with the native protein. A chimera was produced by combining the N-terminal half of PEAMT and the C-terminal half of PMEAMT. The chimeric protein is able to methylate PEA, PMEA and PDEA indicating that a feature associated with the N-terminal half of PEAMT is required for PEA methylation. This result suggests that differences associated with the N-terminal domain are likely responsible for the inability of PMEAMT to use PEA as a substrate.

An *Arabidopsis* mutant line with a T-DNA insertion in the promoter region of *PMEAMT* (SALK 006037) was obtained and RT-PCR analysis of plants homozygous for the insert showed that the mutant lacks transcripts associated with this gene. Relative to wild-type plants grown under identical conditions the mutant plants showed no visible difference in morphological or developmental phenotype. However, shotgun lipidomics using electrospray ionization tandem mass spectrometry showed a 2.1-fold greater abundance of a 34:3 phosphatidylmethylethanolamine (PtdMEA) molecular species in mutant plants compared to wild-type. One biological role of PMEAMT may be to reduce the likelihood for PtdMEA incorporation into phospholipids of membranes. PtdMEA incorporation in membranes is associated with reduced viability of yeast but its effect on the physiology of plants is, as yet, unknown.

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ABBREVIATIONS

AdoHcy	S-adenosyl-L-homocysteine
AdoMet	S-adenosyl-L-methionine (see SAM)*
At	*Arabidopsis thaliana*
BADH	Betaine aldehyde dehdrogenase
bp	Base pairs
BSA	Bovine serum albumin
CDH	Choline dehydrogenase
CDP	Cytidine diphosphate
Cho	Choline
CMO	Choline monooxygenase
d	Day(s)
DEA	Dimethylethanolamine
DTT	Dithiothreitol
EA	Ethanolamine
EDTA	Ethylenediaminetetraacetic acid
Fd	Ferridoxin
gsp	Gene-specific primer
h	Hour(s)
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hmz	Homozygous

Htz	Heterozygous
K_m	Michaelis-Menten constant
MEA	Methylethanolamine
min	Minute(s)
mL	Millilitre
mM	Millimolar
mmol	Millimoles
NAD/NADP	Nicotinamide adenine dinucleotide/phosphate
NMT	(Phosphobase) *N*-methyltransferase
OD	Optical density
P-Base	Phosphobase
PAGE	Polyacrylamide gel electrophoresis
PCho	Phosphocholine
PCR	Polymerase chain reaction
PDB	Protein data bank
PDEA	Phosphodimethylethanolamine
PEA	Phosphoethanolamine
PEAMT	Phosphoethanolamine *N*-methyltransferase
PLD	Phospholipase D
PMEA	Phosphomethylethanolamine
PMEAMT	Phosphomethylethanolamine *N*-methyltransferase
Ptd	Phosphatidyl

rpm	Rotations per minute
SAM	S-adenosyl-L-methionine (see AdoMet)*
s	Second(s)
SD	Synthetic dextrose minimal media
SDS	Sodium dodecyl sulfate
T-DNA	Transfer DNA
TE	Tris EDTA buffer
TLC	Thin-layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
Trp	Tryptophan
WT	Wild-type

* SAM is used in place of AdoMet in Chapter 4 in keeping with the journal used for formatting the content of this section.

CHAPTER ONE

Literature Review

Plant environmental stress

Plants commonly grow and reproduce in environments where they are prevented from expressing their genetic potential to attain maximum productivity (Boyer 1982; Bray *et al.*, 2000). The environmental stresses imposed on plants include biotic factors that originate through interactions between organisms (e.g. parasites and herbivores) and abiotic factors that depend on the interaction between organisms and the physical environment (e.g. metal toxicity, drought, salinity and temperature extremes) (Bray *et al.*, 2000). Since plants are sessile organisms they must be able to tolerate local changes by developing physical or chemical changes to withstand pressures. Alternatively, some plants avoid stresses by completing their entire life-cycle prior to being exposed to detrimental conditions (Price *et al.*, 1998).

Biotic Stress

Biotic stresses result from living organisms that harm plants and include herbivores such as insects or mammals and pathogens (Maffei and Bossis, 2006). Types of pathogens that can cause biotic stress in plants are viruses, bacteria, fungi and nematodes that may be transferred to plant tissues by herbivores (Kluth *et al.*, 2002). To combat these stresses plants can develop physical barriers such as a thick cuticle or thorns or chemical defenses such as phytoalexins that are toxic or distasteful to the invasive organisms (Lui *et al.*, 1992). These are general responses referred to as basal and non-host resistance that can defend against a wide range of biotic stresses. Some pathogens are able to circumvent these general defenses causing disease (Nomura *et al.*, 2006).

Plants have countered adverse interactions by evolving the ability to recognize specific pathogen elicitors via proteins encoded by plant resistance (R) genes that initiate a cascade of defense responses (Jones *et al*., 2004) leading to the production of anti-microbial compounds specific to the infecting pathogen (Pitzschke *et al*., 2009). Similarly, pathogenic elicitors can induce a hypersensitive response to increase concentrations of the reactive oxygen species H_2O_2 at the area of infection (oxidative burst) activating plant defenses including the induction of programmed cell death (Apostol *et al*., 1989; Lui *et al*., 1992; Bolwell *et al*., 2002).

Abiotic Stress

Abiotic stress can result from unfavourable environmental conditions such as drought, salinity and temperature extremes preventing plants from attaining their maximum productivity (Chen and Murata, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006).

Temperature Stress

Each plant species has an optimal growth temperature that largely determines where it can survive and when the temperature falls outside this optimal range the plant becomes stressed (Iba, 2002). At elevated temperatures photosynthetic reactions are hindered posing limitations to plant growth and reproduction (Seemann *et al*., 1984). Specifically, damage can occur to chloroplast membranes and stomatal closure reduces gas exchange required for photosynthesis (Seemann *et al*., 1984; Garcia-Mata and

Lamattina, 2001). Other damage initiated by high temperatures is the alteration of cell membrane composition causing membrane fragility and deterioration (Smolenska and Kuiper, 1977; Pike *et al.*, 1979). To tolerate increased temperatures plants can form heat shock proteins (HSP) that act as chaperones to preserve the function of heat labile proteins (Boston *et al.*, 1996; Iba, 2002). For example, Queitsch *et al.* (2000) demonstrated that HSP101 is involved in *Arabidopisis* thermotolerance. *Arabidopsis* plants with reduced levels of HSP101 were unable to recover from exposure to 45°C with a conditioning pre-treatment at 38°C compared to plants constitutively expressing HSP101.

Low temperatures can result in morphological, physiological, and biochemical changes that relate to plant freezing tolerance and survival (Charron *et al.*, 2002). Reduced temperatures affect membrane fluidity and phase transitions that can cause ion leakage and deactivate membrane proteins (Iba, 2002). To counter these effects plants have evolved mechanisms to increase the degree of fatty acid unsaturation in phospholipids to reduce the chance of freezing-induced phase transformation (Uemura *et al.*, 1995; Wang, 2006). Plants may also increase tolerance against freezing by increasing the solute concentration of the cytoplasm with compounds including soluble carbohydrates and proline (Wanner and Junttila, 1999). These cytosolic compounds may increase freezing tolerance by sequestering toxic ions and/or reducing the rate and extent of cellular dehydration preventing protein denaturation (Steponkus, 1984).

Osmotic stress

Osmotic stress is a change in cellular water potential that adversely affects plant growth or survival (Xiong and Zhu, 2002). The osmotic stress imposed on plants by drought and salinity typically reduces crop yields by at least half (Boyer, 1982; Bray *et al.*, 2000) and thus considerable research is focused on the understanding of plant tolerance towards these conditions (reviewed by Sakamoto and Murata, 2000; Cushman, 2001; Wang *et al.*, 2003; Bartels and Sunkar, 2005).

Osmotic stress can initiate a cascade of interacting events including an increase in ABA concentration and decrease of xylem pH and conductivity, all of which may act as signalling mechanisms mediating stress responses (Wilkinson and Davies, 1997; Bahrum *et al.*, 2002). Furthermore, under water-limiting conditions, reactive oxygen species that are normally produced as byproducts of aerobic metabolism can be present in increased amounts (Apel and Hirt, 2004). These oxygen species include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^•$) (Apel and Hirt, 2004). Hydrogen peroxide and superoxide radicals are relatively unreactive but they can form hydroxyl radicals that can oxidize proteins, lipids, and DNA, thereby disrupting cellular functions (Dat *et al.*, 2000). Peroxidation of lipids also disrupts the membrane of the plant cell causing essential solutes to leak from organelles and from the cell resulting in metabolic imbalances (Fridovich, 1986; Blokhina *et al.*, 2003).

Mechanisms involved in alleviating osmotic stress

An initial physiological response to drought or salinity is the closure of stomata to prevent water loss (Garcia-Mata and Lamattina, 2001) but because this limits gas exchange and thus photosynthesis, this response is only a temporary solution. Older and mature leaves may accumulate large amounts of salts in response to increased salinity compared to younger and actively photosynthesizing leaves (Munns, 1993; Hasegawa *et al.*, 2000). Plants can also lower salt accumulation in transpiring organs by the mechanism known as salt exclusion (Munns, 2005). Roots must exclude most of the Na^+ and Cl^- dissolved in the soil solution or the salt in the shoot will accumulate to toxic levels. Changes of selectivity for K^+/Na^+ exchange at the plasma membrane (Reimann, 1992) and the accumulation of Na^+ in the vacuoles to prevent toxicity in the cytoplasm (Munns, 2002) are examples of mechanisms leading to salt localization.

Other molecular mechanisms have also evolved in plants to promote growth and survival under water-limiting conditions (Xiong *et al.*, 2002). Phosphatidic acid (PA) is released by the hydrolysis of phosphatidylcholine (PtdCho) by the enzyme phospholipase D (PLD). PA can act as a secondary messenger in plants and its synthesis can be induced in times of environmental stresses (Munnik, 2001). When *Arabidopsis* plants were subjected to wounding and freezing stresses, wild-type plants produced approximately twice the amount of PA than when PLD activity was suppressed by RNA interference (Fan *et al.*, 1997). This finding indicates that PLD activity increases under stress leading to higher levels of PA (Zien *et al.*, 2001; Welti *et al.*, 2002). One way in which PA can alleviate the impact of osmotic stress in *Arabidopsis* is by interacting with a protein

phosphatase 2C to close stomata in response to ABA thereby minimizing water loss (Zhang *et al*., 2004; Mishra *et al*., 2006).

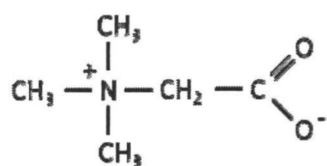
Many plants accumulate low molecular weight organic compounds termed osmolytes or compatible solutes in response to drought or increased salinity (Rhodes and Hanson, 1993; Rontein *et al*., 2002). These compounds are also present in non-stressed plants with concentrations typically ranging between 5 and 50 μmol g^{-1} fresh weight (FW) but under stress conditions can reach levels greater than 200 μmol g^{-1} FW without imparting cellular toxicity (Cushman, 2001; Rontein *et al*., 2002).

Compatible solutes

Compatible solutes can lower the water potential of a cell promoting the uptake and retention of water to maintain cell turgor when the quality or quantity of water is low (Wyn Jones and Storey, 1981; Weretilnyk *et al*., 1989). Compatible solutes are chemically diverse and include polyols and sugars (e.g. mannitol and pinitol), amino acids (e.g. proline) and methylamines (e.g. glycine betaine, proline betaine) (Fig. 1.1) (Nuccio *et al*., 1999; Yancey, 2005). At average concentrations (5-50 μmol g^{-1} FW) these organic solutes may not significantly contribute to osmotic adjustment but may act to reduce the effect(s) of abiotic stresses in other ways (Cushman, 2001). For example, Shen *et al*. (1997) demonstrated that mannitol is involved in alleviating oxidative stress. In their study the hydroxy radicals generated by illumated thylakoids led to a reduction in the *in vitro* activity of an enzyme involved in photosynthetic carbon fixation, phosphoribulokinase (PRK), by 65% relative to assays that included 125 mM mannitol.

Figure 1.1 Compatible organic solutes show chemical diversity. Examples of organic solutes found among plants include quaternary ammonium compounds typified by (a) glycine betaine and (b) proline betaine, sugar alcohols (c) mannitol and (d) pinitol, and the amino acid (e) proline.

(a)



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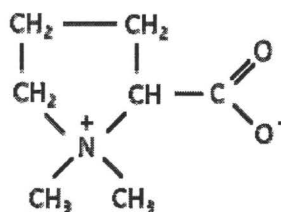

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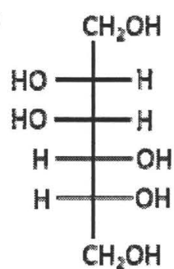

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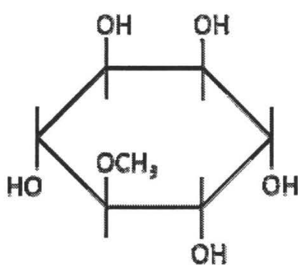

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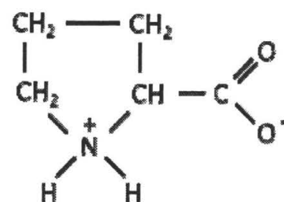
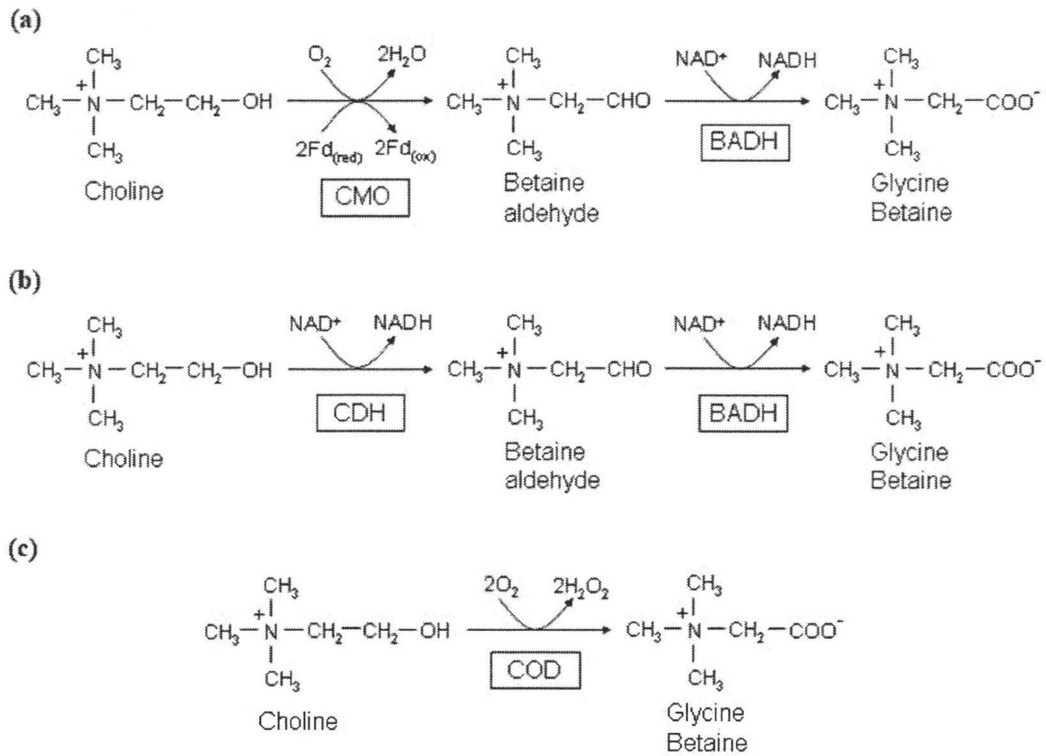


Mannitol was proposed to play a role in scavanging hydroxy radicals and, in so doing, protect PRK *in planta*. Certain osmolytes are also capable of stabilizing protein conformation under combined heat and salt stress and maintaining cell membrane integrity by acting as chemical chaperones (Gorham, 1995; Nakayama *et al*., 2000; Diamant *et al*., 2001). Given the reported protective effects of compatible organic osmolytes, genetic engineering has been used to increase the capacity of crop species such as rice, soybeans and potatoes to accumulate these osmoprotectants in order to increase their stress tolerance and productivity (McNeil *et al*., 1999).

Glycine betaine synthesis

Glycine betaine is found in organisms representing every Kingdom of life (Yancey, 1995). In plants, glycine betaine can accumulate to osmotically significant levels (40 to 400 mM final concentration) in at least ten different families (Weretilnyk *et al*., 1989; Rhodes and Hanson, 1993). In spinach plants, glycine betaine is synthesized in the chloroplast by a two-step oxidation of choline (Weigel *et al*., 1986; Brouquisse *et al*., 1989). The first step in glycine betaine synthesis involves the ferredoxin-dependent enzyme choline monooxygenase (CMO) that converts choline to the intermediate betaine aldehyde (Fig. 1.2) (Brouquisse *et al*., 1989; Rathinasabapathi *et al*., 1997). In *Escherichia coli* and mammalian cells this step is catalyzed by an NAD^+-dependent choline dehydrogenase (CDH) (Sakamoto and Murata 2001). In plants and *E. coli* the enzyme betaine aldehyde dehydrogenase (BADH) oxidizes betaine aldehyde to glycine

Figure 1.2 Routes of glycine betaine synthesis in different organisms. Pathways shown for (a) plants, (b) *E. coli* and mammalian cells and (c) *Arthrobacter globiformis*. Abbreviations: CMO, choline monoxygenase; CDH, choline dehydrogenase; COD, choline oxidase; BADH, betaine aldehyde dehydrogenase; $Fd_{(red)}$, reduced ferredoxin; $Fd_{(ox)}$, oxidized ferredoxin


(a)
CH3
CH3–N–CH2–CH2–OH
CH3
Choline
O2
2H2O
2Fd(red)
2Fd(ox)
CMO
CH3–N–CH2–CHO
Betaine aldehyde
NAD+
NADH
BADH
CH3–N–CH2–COO-
Glycine Betaine
(b)
Choline
NAD+
NADH
CDH
Betaine aldehyde
NAD+
NADH
BADH
Glycine Betaine
(c)
Choline
2O2
2H2O2
COD
Glycine Betaine

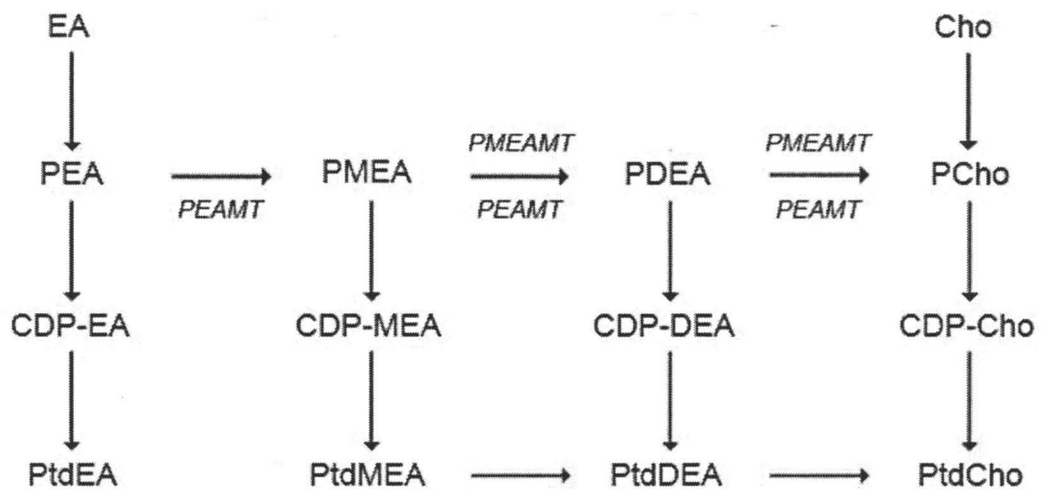
betaine using NAD^+ or $NADP^+$ as a cofactor (Weigel *et al.*, 1986; Weretilnyk and Hanson, 1989). In the soil bacterium *Arthrobacter globiformis* the enzyme choline oxidase (COD) is able to convert choline to glycine betaine in a single step (Fig. 1.2) (Ikuta *et al.*, 1977). The understanding of the biochemical pathways involved in choline synthesis has helped develop genetic engineering approaches for improved crop resistance to environmental stress (Bohnert *et al.*, 1995).

Pathways of choline and phosphocholine synthesis

Few plant species have been studied with respect to choline metabolism. However, biochemical studies using spinach leaves are consistent with choline being derived from the precursor phosphocholine (PCho) produced in the cytosol of the plant cell (Weretilnyk *et al.*, 1995). Choline produced from PCho is likely transported to the chloroplast for oxidation to glycine betaine (Weigel *et al.*, 1986; Nuccio *et al.*, 1998). PCho is also present in all plants as a component of PtdCho, an integral part of membrane phospholipids (Summers and Weretilnyk, 1993). Once synthesized, PCho may be present in the cytoplasm as free PCho or be directed toward the synthesis of choline for storage in vacuoles and/or PtdCho (Fig. 1.3) (Bligny *et al.*, 1989; Summers and Weretilnyk, 1993).

Radiotracer evidence show different routes of PtdCho synthesis depending on the plant species. PtdCho synthesis can occur at the phosphobase level (P-base), phosphatidyl base (Ptd-base) level or a combination of the two (Fig. 1.3) (Datko and Mudd, 1988a,b). For plants studied to date, regardless of the pathway used the committing step appears to be the *N*-methylation of phosphoethanolamine (PEA) to

Figure 1.3 Pathways of phosphatidylcholine (PtdCho) synthesis in plants. Ethanolamine (EA) is phosphorylated to produce phosphoethanolamine (PEA). Phosphocholine (PCho) synthesis proceeds at the phosphobase (P-base) level by three sequential methylations of PEA producing the intermediates phosphomethylethanolamine (PMEA) and phosphodimethylethanolamine (PDEA). Phosphoethanolamine *N*-methyltransferase (*PEAMT*) is able to catalyze all three methylations while the methylation of PMEA and PDEA is postulated to be catalyzed by a second enzyme, phosphomethylethanolamine *N*-methyltransferase (*PMEAMT*). PCho can then be directed toward the synthesis of choline (Cho) or PtdCho via a cytidine 5'-diphosphate (CDP) intermediate. PtdCho synthesis may then also proceed at the phosphatidyl-base (Ptd-base) level involving the methylation of the substrates phosphatidylmethylethanolamine (PtdMEA) and phosphatidyldimethyl-ethanolamine (PdtDEA).


EA
Cho
PMEAMT
PMEAMT
PEA
PMEA
PDEA
PCho
PEAMT
PEAMT
PEAMT
CDP-EA
CDP-MEA
CDP-DEA
CDP-Cho
PtdEA
PtdMEA
PtdDEA
PtdCho

produce phosphomethylethanolamine (PMEA). Subsequent methylations can occur at the P-base level using PMEA and phosphodimethylethanolamine (PDEA) as substrates to produce PCho or at the Ptd-base level involving the sequential methylation of the substrates phosphatidylmethylethanolamine (PtdMEA) and phosphatidyldimethylethanolamine (PtdDEA) to produce PtdCho (Fig. 1.3). For example, in *Lemna paucicostata* methylations take place primarily at the P-base level whereas for soybean they occur at the Ptd-base level. In carrot, the methylations of PMEA and PtdMEA producing PCho and PtdCho, respectively, occur along both P- and Ptd-base routes (Datko and Mudd, 1988a). Choline can also be produced by the hydrolysis of PtdCho as mediated by PLD (Munnik, 2001).

Enzymes involved in phosphocholine synthesis

Methyltransferases

Methyltransferases are enzymes that catalyze the transfer of a methyl group from one compound to another and hence are involved in the methylation of many substances including nucleic acids, proteins and carbohydrates (reviewed by Moffat and Weretilnyk, 2001). *S*-adenosyl-L-methionine (AdoMet) is by far the most common methyl donor molecule used by all types of methyltransferases (Cheng and Roberts, 2001). Methyltransferases are found in all organisms from bacteria to humans and are involved in many processes central to cellular biochemistry including transcriptional regulation and protein modification (Zubieta *et al.*, 2001). The characterization and organization of these enzymes have received considerable attention in view of their diverse biological

roles including metabolism and signal transduction (Joshi and Chiang, 1998; Ibrahim *et al.*, 1998; Martin and McMillan, 2002).

Phosphoethanolamine N-*methyltransferase and phosphomethylethanolamine* N-*methyltransferase*

Among the products synthesized by methyltransferases is PCho, a precursor to the metabolite glycine betaine. In the chenopods spinach and sugar beet, PCho synthesis proceeds via the P-base route. A single enzyme, phosphoethanolamine *N*-methyltransferase (PEAMT), is capable of catalyzing all three methyl-transfer reactions (Weretilnyk *et al.*, 1995; Nuccio *et al.*, 2000). In plants, PEAMT has been cloned and biochemically characterized from *Arabidopsis*, spinach and wheat plants (Bolognese and McGraw, 2000; Nuccio *et al.*, 2000; Charron *et al.*, 2002). It has also been cloned from *Zea mays* (Wu *et al*, 2007) and the translated product has recently been shown to be able to methylate PEA (Peel *et al.*, 2010). Ye *et al.* (2005) reported the cloning of a PEAMT from *Brassica napus* that shares a 93% identity at the amino acid level to the characterized *Arabidopsis* PEAMT, however its biochemical activity was not determined.

A second methyltransferase, designated phosphomethylethanolamine *N*-methyltransferase (PMEAMT), is believed to be involved in choline metabolism and its activity has been found in diverse dicot plants (Lorenzin *et al.*, 2001). The PMEA→ PDEA activity associated with PMEAMT has been detected and partially purified from spinach leaves (Weretilnyk *et al.*, 1995; Smith *et al.*, 2000; Burian, 2000) but a gene encoding this enzyme has not been identified from any plant species. Evidence for the

existence of PMEAMT as a distinct enzyme from PEAMT includes the presence of PMEA and PDEA methylating activities in extracts prepared from dark-exposed spinach leaves that lack an enzyme with PEA methylating activity (Weretilnyk *et al.*, 1995). Smith *et al.*, (2001) also observed that during the purification of spinach PEAMT the ratios of specific activities associated with PEA:PMEA:PDEA methylation changed as PEAMT was purified from approximately 1:1.5:1.2 in crude extracts to 1:0.9:0.7 in more highly purified preparations. The authors stated that this change is consistent with more than one enzyme contributing towards the three activities in crude preparations and at least one other enzyme capable of methylating PMEA and PDEA being depleted as PEAMT is purified and enriched.

Regulation of PEAMT

PEAMT and PMEAMT use AdoMet as the methyl donor and PEA or PMEA as the initial substrate. They have been designated in this thesis as PEAMT and PMEAMT, respectively, in accordance with the substrates used. As described earlier, PEAMT can catalyze all three P-base methylation steps shown in Figure 1.3 (Smith *et al.*, 2000) and its activity and protein level decrease in the dark while transcripts associated with *PEAMT* show a circadian pattern with highest levels during the dark period (Drebenstedt, 2001). The activity of this enzyme is salt- and light-responsive and increased activity is associated with increased protein levels (Weretilnyk *et al.*, 1995). How PEAMT activity is regulated by light remains unknown. In contrast to PEAMT, PMEAMT activity does

not appear to be light-responsive (Summers and Weretilnyk, 1993). This observation was taken as additional evidence that PMEAMT is likely a distinct enzyme from PEAMT.

The committing step in choline synthesis is the methylation of PEA to PMEA and so PEAMT is considered to be the regulatory enzyme of the pathways involved in choline and PtdCho synthesis (Datko and Mudd 1988a,b; Mou *et al.*, 2002). The activity of this enzyme has been shown to be feedback inhibited by the reaction products *S*-adenosyl-L-homocysteine (AdoHcy) and PCho with this inhibition leading to reduced levels of choline produced *in planta* (Mudd and Datko, 1989a,b; Smith *et al.*, 2000; Nuccio *et al.*, 2000; Jost *et al.*, 2009). For example, the *in vitro* activity of 400-fold purified spinach PEAMT was reduced by 47% and 71% in the presence of 0.01 mM AdoHcy and 1 mM of PCho, respectively, compared to controls (Smith *et al.*, 2000). PEAMT activity is also inhibited by choline addition to cell cultures or tissue culture plants as evidenced by a reduced accumulation of radiolabeled P-base and Ptd-base products by 98 and 77% for carrot and soybean compared to controls, respectively, in the presence of 50 μM PCho (Mudd and Datko, 1989b).

Importance of PEAMT *in planta*

The physiological role of PEAMT activity in *Arabidopsis* has been examined *in vivo* using transgenic plants with suppressed expression of *PEAMT* by antisense technology and a T-DNA insertion line silenced with respect to *PEAMT* expression (Mou *et al.* 2002; Cruz-Ramirez *et al.* 2004). The silencing of *PEAMT* expression by RNA interference produced a plant with a substantially altered phenotype compared to wild-

type including a 64% reduction in choline levels compared to wild-type plants, pale-green leaves, early senescence, and shorter siliques producing fewer seeds (Mou *et al.*, 2002). This plant line also showed increased sensitivity to stress compared to the wild-type as noted by reduced growth on media containing 200 mM NaCl and temperature-sensitive male sterility when grown at 26°C. In contrast, the T-DNA insertion line deficient in *PEAMT* gene expression showed fewer abnormalities but the ones reported included altered root development, root epidermal tissue cell death, and a significant 23% decrease in PtdCho content relative to wild-type (Cruz-Ramirez *et al.*, 2004).

Engineering glycine betaine synthesis in non-accumulators

The trait of glycine betaine synthesis has been introduced into *Arabidopsis*, a plant that does not naturally accumulate this compound (Hayashi *et al.*, 1997). *Arabidopsis* was transformed with the bacterial *codA* gene encoding choline oxidase from *Arthobacter globiformis*. Levels of glycine betaine increased from 0 μmol g^{-1} FW in wild-type plants to 1 μmol g^{-1} FW in transgenic lines. While this change in glycine betaine content seems modest, the authors report that this change was associated with improved stress tolerance. The seeds of transformed lines showed 100% germination on media containing 200 mM NaCl whereas only 20% of wild-type seeds were able to germinate on the same media. These researchers also exposed plants to continuous light at 5°C for one week followed by 2 days at 22°C and found that leaves from wild-type plants became chlorotic while leaves of the transgenic line were unaffected.

The use of *CMO* and *BADH* genes from plants that naturally accumulate glycine betaine has been suggested to be better suited for transgenic approaches to plant stress tolerance improvement than the *COD* gene (Rathinasabapathi *et al*., 1997). The reason given is that CMO activity is coupled to reduced ferredoxin that is formed in photosynthetic light reactions when there is presumably a greater demand for glycine betaine accumulation. As such, glycine betaine production under the activity of CMO is linked to the availability of energy and the need of the product for growth. Also, unlike CMO, COD releases the reactive oxygen species H_2O_2 as a byproduct that could hinder enzymatic functions in plants transformed with this gene (Rathinasabapathi *et al*., 1997).

An initial step in the engineering of glycine betaine by introducing the two-step oxidation of choline in a non-accumulating plant was performed by Rathinasabapathi *et al*. (1994) who over-expressed BADH in the chloroplasts of transgenic tobacco. The transformed plants were able to produce glycine betaine in amounts comparable to those plants that naturally accumulate this osmolyte but only when supplied with exogenous betaine aldehyde. To complete the enzymatic pathway oxidizing choline to glycine betaine, the spinach CMO was also introduced into chloroplasts of transgenic tobacco already expressing BADH (Rathinasabapathi *et al*., 1997; Nuccio *et al*., 1998). The glycine betaine content was determined in leaves of the transgenic plants and was found at levels up to 0.05 μmol g^{-1} FW, a content that is very low compared to that of a natural glycine betaine accumulator (5 to 50 μmol g^{-1} FW). Nuccio *et al*. (1998) concluded that the endogenous choline supply is a major limitation for glycine betaine synthesis in

transgenic tobacco and therefore interest shifted to the enzymatic pathway(s) involved in choline synthesis.

In order to increase the supply of choline, McNeil *et al.* (2001) over-expressed spinach PEAMT in tobacco already expressing the *CMO* and *BADH* gene products. This approach led to a 50-fold increase in free choline. However, elevated choline content only yielded a small percentage increase (1 to 5%) in the total accumulation of glycine betaine when compared to plants that naturally accumulate this osmoprotectant under similar conditions. The authors suggested that constraints including a low capacity to import choline into the chloroplast, inhibition of PEAMT by PCho and/or an inadequate supply of ethanolamine as possible reasons for the inability of these plants to produce higher levels of glycine betaine.

S-adensoyl-L-methionine binding domains and motifs

To bind a molecule of AdoMet, a series of three unique, semi-conserved, motifs (called AdoMet-binding domains) are required (Kagan and Clarke, 1994; Joshi and Chiang, 1998) (Table 1.1). All three semi-conserved motifs are found in each of the N- and C-terminal bipartite domains found in PEAMT enzymes from spinach, wheat and *Arabidopsis* (Bolognese and McGraw, 2000; Nuccio *et al*., 2000; Charron *et al*., 2002). Nuccio *et al*. (2000) determined that the portion of spinach PEAMT carrying the N-terminal domain is only responsible for the methylation of PEA and suggested that the half carrying the C-terminal domain must be involved in the methylation of PMEA and PDEA. When the enzymatic properties of the AdoMet-binding domains of wheat

Table 1.1 Three semi-conserved AdoMet-binding motifs are found among confirmed and predicted non-DNA methyltransferases. Motifs are compiled from Kagan and Clarke 1994, Joshi and Chiang 1998, and Ibrahim *et al.*, 1998.

Motif	Amino acid sequence
I	(V/I/L)(V/L)(D/K)(V/I)GGXX(G/A)
II	(P/G)(Q/T)(F/Y/A/V)DA(I/V/Y)(F/I)(C/V/L/S)
III	(A/P/G/S)(L/I/V)(A/P/G/S)XX(A/P/G/S)(K/R)(V/I)(E/I)(L/I/V)

PEAMT were compared by *in vitro* activity determinations it was shown that the portion of the enzyme containing the N-terminal domain is responsible for the methylation of PEA, PMEA and PDEA while the C-terminal domain methylates only PMEA and PDEA (Charron *et al.* 2002). In *Arabidopsis* the enzymatic function of each domain has not been reported.

P-base methyltransferases from other organisms

PEAMT has been cloned from *Plasmodium falciparum*, the causative agent of mammalian malaria, and the nematode *Caenorhabditis elegans* (Pessi *et al.*, 2004; Palavalli *et al.*, 2006; Brendza *et al.*, 2007). PEAMT from *P. falciparum* (PfNMT) is approximately half the size of spinach PEAMT and contains a single catalytic domain that is homologous to both the N- and C- terminal AdoMet-binding domains found in plant PEAMTs studied to date. PfNMT is able to methylate the three P-base intermediates involved in PCho synthesis (Pessi *et al.*, 2004). *P. falciparum* produces PtdCho via the P-base route as well as the CDP-Cho pathway using choline as an initial substrate (Witola *et al.*, 2008). When the P-base pathway was disrupted, *P. falciparum* showed alterations in development and survival. Parasite infection by P-base methylation defective strains was reduced by a minimum of 50% relative to wild-type and when choline was included in the culture medium at physiological concentrations (~20 μM) there was no increase in infection indicating that the mutant could not compensate for the loss of PfNMT (Witola *et al.*, 2008).

C. elegans contains at least two PEAMT enzymes, CeNMT-1 and CeNMT-2, that are similar in molecular mass to characterized plant PEAMTs but each contains only one methyltransferase domain (Brendza *et al.*, 2007). CeNMT-1 methylates PEA and has only one AdoMet-binding domain with consensus sequence motifs that are found in the N-terminal region of the protein. CeNMT-2 also contains a single AdoMet-binding domain however it is located in the C-terminal end of the protein. CeNMT2 methylates PMEA and PDEA, similar to the proposed PMEAMT from plants (Weretilnyk *et al.*, 1995; Smith *et al.*, 2001; Palavalli *et al.*, 2006; Brendza *et al.*, 2007). CeNMT-1 and CeNMT-2 share less than 15% amino acid identity to each other and when RNA interference experiments were performed there were severe developmental defects and reduced fertility in the resulting mutants. The phenotypes were identical for CeNMT-1- and CeNMT-2-deficient mutants and the addition of choline reversed the RNAi-generated phenotype (Pallavalli *et al.*, 2006; Brendza *et al.*, 2007).

In mammals, yeast, and some bacteria, PtdCho is produced via the CDP-choline pathway (Kennedy pathway) that converts choline to PtdCho (Kent, 2005). Some strains of bacteria can also produce PtdCho by the reaction of choline and CDP-diacylglycerol (Sohlenkamp *et al.* 2003). Yeast and mammalian liver cells can also use the Ptd-base pathway that involves three sequential methylations of PtdEA to produce PtdCho (Kanipes *et al.*, 1998; Li *et al.*, 2005). There are no known homologues of the *P. falciparum* and *C. elegans* PEAMT enzymes in mammals and other eukaryotes (Pessi *et al.*, 2004) and thus these gene products are considered to be potential targets for the development of compounds able to treat malaria and nematicidal compounds of medicinal

and agricultural value (Chaudhary and Roos, 2005; Palavalli *et al.*, 2006; Witola *et al.*, 2008).

Objectives of this thesis research

A hypothesis regarding the physiological role of PMEAMT in plants is that this enzyme is necessary to complete the conversion of P-base intermediates (PMEA and PDEA) to PCho. This cellular role would be best accommodated if PMEAMT co-localizes with PEAMT. A second hypothesis regarding PMEAMT is that it may be involved in plant acclimation to environmental stress as has been documented for spinach and wheat PEAMTs (Nuccio *et al.* 2000; Charron *et al.* 2002).

An obstacle to addressing these hypotheses is that an enzyme consistent with PMEAMT activity has not been unequivocally demonstrated to operate in plants. As such, the objectives of this thesis research are to address the following:

- To determine if *Arabidopsis* has a PMEAMT enzyme and, if so, clone *PMEAMT* from *Arabidopsis*, compare its sequence to *PEAMT* and over-express the gene product for biochemical characterization
- To provide basic kinetic properties of *Arabidopsis* PMEAMT and PEAMT
- To determine the substrate(s) used by the N- and C-terminal AdoMet- domains of PEAMT and PMEAMT. This analysis may help identify factors that regulate substrate specificity of P-base methyltransferases in general.

- To determine the *in planta* physiological role of PMEAMT through identification and analysis of a T-DNA insertion mutant associated with the gene encoding PMEAMT

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CHAPTER TWO

Materials and Methods

Materials

Unless stated otherwise, chemicals were purchased from Sigma-Aldrich (Oakville, ON). All solutions were prepared with de-ionized water purified by a Barnstead NANOpure II water purification system (SYBRON / Barnstead, Dubuque, IA).

Media components bacto-agar, bacto-yeast extract and bacto-peptone were purchased from BD Biosciences (Mississauga, ON). Yeast Nitrogen Base with amino acids with ammonium sulfate and without vitamins was from US Biological (Burlington, ON). D-glucose, NaCl, and agarose were from BioShop Canada Inc (Burlington, ON). Evergreen columns used in enzyme assays were purchased from Diamed Lab Supplies Inc (Mississauga, ON). ReadySafe fluor used for scintillation counting was purchased from Beckman-Coulter Instruments Inc. (Mississauga, ON). *S*-Adenosyl-L-[*methyl*-^{3}H and ^{14}C] methionine (3 TBq $mmol^{-1}$ and 2 GBq $mmol^{-1}$, respectively) were purchased from Perkin Elmer, New England Nuclear (Waltham, MA). Thin-layer chromatography (TLC) plates were made by Polygram® SIL G (Macherey-Nagel, Germany) and the x-ray film XAR-5 was from Kodak (Rochester, NY). PMEA and PDEA were prepared by phospholipase C treatment of PtdMEA (Cat. No. 850851P) or PtdDEA (Cat. No. 850854P) purchased from Avanti Polar Lipids (Alabaster, AL) following the method of Datko and Mudd (1988).

DNA restriction digest enzymes and Pfu DNA polymerase were from Fermentas Canada Ltd. (Burlington, ON). Qiagen Qiaprep® Spin Miniprep kits were used for plasmid extractions (Cat. No. 27104). RNAeasy Qiagen RNeasy Mini Kit (50) (Cat. No.74104) and Qiagen QIAshredder™ (Cat. No. 79654) were used for RNA isolation

following the protocol described by the manufacturer (Qiagen Canada, Mississauga, ON). All primers were synthesized by The Institute for Molecular Biology and Biotechnology (MOBIX) at McMaster University.

The *Arabidopsis* cDNA library in vector pFL61 was acquired from the American Type Culture Collection (Manassas, VA, Cat. No. 77500). The *Saccharomyces cerevisiae* yeast mutant CPBY19 (*cho2 opi3*) with the genotype *ura3-52 leu2Δ1 his3 Δ 200 trp Δ 63 opi3::HIS3 cho2::LEU2* was generously provided to me by Dr. Cindy Bolognese (Bolognese and McGraw, 2000).

Plant growth and tissue preparation

Spinach

Spinacia oleracea L. (spinach cv. Savoy Hybrid 612) seeds (Harris Moran Seeds, Rochester, NY, USA) were planted in trays containing moist vermiculite and allowed to germinate for 3 to 5 d under 8 h/24°C light, 16h/19°C dark photoperiod with 350 µmol $m^{-2}s^{-1}$ photon flux density. Seedlings were transplanted to 350 mL plastic pots filled with vermiculite and watered daily with half-strength Hoagland's solution (Hoagland and Arnon, 1950). Salt-treated plants were irrigated with half-strength Hoagland's solution containing 200 mM NaCl at the beginning of the dark period and leaves were collected just prior to the light photoperiod for further analysis.

For protein extraction, 2 g of de-veined leaves from five-week-old spinach plants were harvested at the end of the dark cycle. All extraction procedures were carried out at 4°C. Two mL of 100 mM Hepes, 1 mM EDTA, 5 mM DTT pH 7.8 (HED) was added to

a mortar containing coarsely chopped spinach leaves. The leaves were ground using a pestle to produce a fine slurry and the solution was transferred to a clean 15 mL Corex® test tube. The mortar and pestle were rinsed with 2 mL HED and this solution was added to the same tube. The tube was centrifuged for 10 min at 10000 *g* and the supernatant transferred to a labeled microcentrifuge tube. The concentration of total protein was determined by Bradford's assay (Bradford, 1976). Extracts were frozen in liquid nitrogen and stored at -80°C.

Arabidopsis

Wild-type *Arabidopsis* seeds (CS 60000) and seeds reported to have a T-DNA insertion associated with the gene at locus At1g48600 (SALK 006039, SALK 144248, CS 856087) were purchased from the *Arabidopsis* Biological Resource Center at the University of Ohio. Seeds were sterilized in 1.5 mL microcentrifuge tubes to which 1 mL of 70% v/v ethanol was added and tubes were incubated for 2 min with gentle inversion. The ethanol was removed and 1 mL of 30% v/v bleach and 0.1 % v/v Tween 20 was added and incubated with the seeds for 10 min with periodic inversions. The seeds were rinsed five times with 1 mL of sterile H_2O and then 1 mL of 0.1 % w/v sterile Phytoblend (Cat. No. PTC 001, Caisson Laboratories, Logan, UT) was added to suspend the seeds and the tubes were stored overnight at 4°C. The following day, seeds were transferred to Murashige and Skoog (MS) plates (Murashige and Skoog, 1962) containing 1.0 % w/v sucrose, 1.0 mL MS vitamins (Sigma, M3900), and 0.215 g Murashige and Skoog Salt Mixture (Sigma, M5524-10L). The plates were incubated for 10 d at 22°C with a 24 h

light photoperiod (light intensity of 100 μmol m^{-2} s^{-1}). Seedlings were transplanted to Promix BX soil (Premier Horticulture Inc., Quakertown, PA) and grown at 22°C with a 12 h photoperiod and a light intensity of 120 μmol m^{-2} s^{-1}. Leaf tissue (50 – 100 mg) was harvested approximately four weeks after transplant and immediately frozen in liquid nitrogen and stored at -80°C.

Immunopurification of PMEAMT

Polyclonal antibodies specific for PEAMT (Drebenstedt, 2000) were used to isolate PMEAMT by immunoprecipitation.

Antibody coupling to protein-A agarose

Polyclonal antibodies prepared using spinach PEAMT as an antigen were covalently linked to protein A agarose beads (Roche Diagnostics, Cat. No. 1719408) using a modified procedure from Harlow and Lane (1988). In a 1.5 mL microcentrifuge tube, 200 μL of beads were washed three times with 400 μL phosphate buffered saline (PBS, 10 mM sodium phosphate pH 7.2 and 0.9% (w/v) NaCl). Twenty μL of PEAMT antiserum (containing 2 mg protein) was added to the beads and the volume brought to 400 μL with PBS. The suspension was mixed on an end-over-end rotator overnight at 4°C. Four 1-mL aliquots of 200 mM sodium borate (pH 9) were used to wash and transfer the beads to a 15 mL Falcon tube that was then centrifuged at 2500 *g* for 4 min at 22°C. The supernatant was discarded and the beads washed again with 4 mL sodium borate solution. To covalently bind the antibody to the beads, 20.8 mg (final

concentration 20 mM) of dimethylpimelimidate was added to the 4 mL solution and incubated at room temperature for 30 min on an end-over-end rotator. The beads were centrifuged as above and the supernatant removed. Four mL of 200 mM ethanolamine were added and mixed on an end-over-end rotator for 2 h. The beads were washed twice with 4 mL of PBS and stored at 4°C as a 1:1 suspension with 0.1% (w/v) of the antibacterial compound sodium azide.

Immunoprecipitation and immunoblotting with PEAMT antibodies

The protein A agarose beads coupled to anti-PEAMT were washed three times with HED containing either 154, 75, 25, or 0 mM NaCl. Lower salt concentrations decrease the stringency of antigen binding (Harlow and Lane, 1988) that would favour the binding of proteins similar to PEAMT. A volume of cell-free leaf extract that contained 240 µg of protein was added to the beads and the total volume was brought up to 300 µL with HED containing the concentration of NaCl used for the wash (either 154, 75, 25, or 0 mM NaCl) The tubes were incubated at 4°C for 3 h on an end-over-end rotator. Following centrifugation for 5 s at 14000 *g* the supernatant was removed for enzyme analysis. The beads were washed three times each with 200 µL of 150 mM NaCl wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate) followed by two, 200 µL washes with 500 mM NaCl wash buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% (v/v) Nonidet P-40, 0.05% (w/v) sodium deoxycholate). Finally the beads were washed once with 200 µL of no-salt wash buffer (50 mM Tris-HCl pH 7.5, 0.1% (v/v) Nonidet P-40, 0.05% (w/v) sodium deoxycholate)

(Harlow and Lane, 1988). The supernatant was removed and 50 μL of SDS-solubilizing buffer (60 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 1% (w/v) SDS, 1% (w/v) DTT, 0.002% (w/v) bromophenol blue) was added and the mixture was heated at 90°C for three min before loading onto an SDS-PAGE gel.

SDS-PAGE was performed according to Smith *et al.* (2000) and Western blotting based on the Trans-Blot® SD DNA/RNA Blotting Kit Instruction Manual (Bio-Rad, Mississauga, ON, Cat. No. 170-3957). Briefly, samples were separated by electrophoresis on a 7.5 - 15% gradient SDS-PAGE gel and the proteins transferred to Hybond™-P PVDF membranes (Amersham-Pharmacia, Montreal, PQ, Cat. No RPN303F). Membranes were blocked with 100 mL 1% (w/v) BSA and hybridized for 2 h with 2000-fold diluted PEAMT antiserum. A second hybridization was then done using 3000-fold diluted goat anti-rabbit IgG (Bio-Rad, Mississauga, ON, Cat. No. 170-6518) as described for the primary antibody. Proteins hybridized to anti-PEAMT were visualized with a colour development solution containing alkaline phosphate/BCIP/NBT (Sambrook *et al.*, 1989).

To determine the efficiency of antigen binding to the activated Protein A agarose beads, P-base enzyme assays were performed using desalted aliquots collected at various stages of the procedure. Specifically, assays were performed with the supernatant obtained following the 3 h incubation of the beads with the crude protein extract as well as from each of the wash buffers and elution buffers collected. P-base methylating activities have been shown to increase in salinized spinach plants (Summers and Weretilnyk, 1993; Weretilnyk *et al.*, 1995) and therefore extracts from plants salt-shocked

to 200 mM NaCl were used for immunoprecipitation. To reduce the possible competition between PEAMT and PMEAMT for binding to activated anti-PEAMT Protein A agarose beads, a refinement to this protocol was devised. In this procedure, immunoprecipitation using stringent coupling and elution conditions was used to first deplete PEAMT protein from a sample. Next the supernatant containing PMEAMT activity was recovered and the entire immunopreciptation procedure repeated using the activated Protein A agarose beads was repeated as described above. Finally, immunoprecipitation was also carried out using leaf extracts from spinach plants subjected to 40 h continuous dark. PEAMT activity is greatly reduced by exposure of plants to prolonged dark periods but PMEAMT activity is not reduced (Weretilnyk *et al.*, 1995).

Cloning by heterologous functional complementation

Cloning plant genes by functional complementation of heterologous biosynthetic pathways in yeast or *Escherichia coli* has proven to be a successful and valuable investigative approach (e.g. Minet *et al.*, 1992). This technique was used to clone both spinach and *Arabidopsis PEAMT* (Nuccio *et al.*, 2000; Bolognese and McGraw, 2000). The yeast mutant strain CPBY19 (*ura3-52 leu2Δ1 his3 Δ 200 trp Δ 63 opi3::HIS3 cho2::LEU2*) was used to clone the gene encoding PMEAMT by functional complementation. The *cho2* gene product is a phospholipid methyltransferase (PLMT) that catalyzes the first methylation (PtdEA → PtdMEA) involved in PtdCho synthesis and also has a limited ability to catalyze the two terminal methylations of PtdMEA and PtdDEA (McGraw and Henry, 1989; Bolognese and McGraw, 2000) (Fig. 1.3). The gene

opi3 encodes the methyltransferase that catalyses the two terminal methylations and therefore only mutants carrying both *opi3* and *cho2* mutations show a clear requirement for choline to survive. A lithium acetate (LiAc)-mediated method was used to transform the yeast with an *Arabidopsis* (Landsberg erecta ecotype) whole seedling cDNA library constructed in the yeast expression vector pFL61 (Minet *et al.*, 1992).

The pFL61 vector confers uracil prototrophy and therefore yeast expression of a cDNA encoding a plant-derived gene involved in choline biosynthesis should functionally complement the mutant phenotype on tryptophan-containing media. Transformed yeast cells were plated first on synthetic dextrose minimal media (SD) supplemented with 0.1 mM tryptophan (Trp) and 1 mM choline and incubated at 30ºC. The sugar alcohol inositol was omitted from the media as it has been shown to reduce the activity of phospholipid *N*-methyltransferases (Yamashita *et al.*, 1982). Transformed colonies were replica-plated onto SD medium supplemented with 0.1 mM Trp and 1 mM MEA. Growth of CPBY19 is temperature sensitive on media containing MEA and so plates were incubated at 37ºC to screen for any colonies growing under non-permissive conditions. Work reported by McGraw and Henry (1989) showed that choline production rescues this mutant and permits growth at 37ºC and so any colonies growing at 37ºC could contain a plasmid responsible for choline production. Colonies showing growth under selection were streaked out on fresh SD plates supplemented with 1 mM EA and grown at 37°C. Prior to enzyme or plasmid DNA extraction, a single colony was used to inoculate 50 mL of liquid SD media containing 1mM Trp and 1 mM choline and grown

overnight at 30°C with shaking at 200 rpm. Cells were harvested by centrifugation at 3000 *g* for 5 min at 4°C.

DNA was extracted from mutant yeast strains able to grow at 37°C and used for PCR amplification with pFL61 vector-specific primers JST46 (5'-CGTAGTTTTTCA AGTTCTTAGATGC-3') and JST47 (5'-TTTAGCGTAAAGGATGGGG-3') (Stolz, 2003). The PCR mixture underwent 35 cycles of denaturing for 1 min at 94°C followed by annealing for 1 min at 46.5°C and a 2 min extension at 72°C. Strains showing production of an amplified insert were subject to further experimentation. A 40 mL volume of SD media containing 0.1 mM Trp and 1 mM choline was inoculated by a single colony isolate and grown overnight at 30°C with shaking at 200 rpm. The cells were pelleted by centrifugation in Oakridge centrifuge tubes at 10000 *g* and suspended in 300 μL HED buffer. The suspended cells were transferred to a 1.5 mL microfuge tube and broken by vortexing for 2 min with 0.5 mm glass beads (Biospec Products, Inc. Bartlesville, OK, Cat. No. 11079105). The supernatant was desalted through Sephadex G25 microcentrifuge columns (Weigel *et al*., 1986) and then a 25 μL aliquot was assayed for PEAMT or PMEAMT activity.

Transformations

Saccharomyces cerevisiae

The transformation of *S. cerevisiae* was completed following a protocol in the Clonetech Yeast Protocols Handbook (PT3034-1). One mL of YPD (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose) was inoculated with a colony (2-3 mm

in diameter) of CPBY19 and vortexed for 5 min to remove any clumps. This suspended cell preparation was added to a 250 mL flask containing 50 mL of YPD and allowed to incubate overnight at 30°C at 250 rpm (OD_{600} >1.5). The next day a volume of the overnight culture (approximately 8 mL) was added to a flask containing 300 mL of fresh YPD until an OD_{600} of 0.2 to 0.3 was obtained. This culture was incubated at 30°C and 200 rpm for 3 h until reaching an OD_{600} of 0.4 to 0.6 and then the media with cells was transferred to 50 mL Falcon tubes. The Falcon tubes were centrifuged at 1000 *g* for 5 min at room temperature and each pellet was suspended in approximately 5 mL H_2O and combined in a single Falcon tube. After centrifugation for 5 min at 1000 *g* the supernatant was discarded and the pellet suspended in 1.2 mL 10 mM Tris-HCl, 1 mM EDTA/100 mM lithium acetate pH 7.5 (TE/LiAc). Plasmid DNA (1 µg) and 0.1 mg of herring testes carrier DNA (Sigma D-7290) was added to a sterile 15 mL Falcon tube and mixed. One hundred µL of yeast competent cells were added to each tube containing 1 µg of plasmid DNA and the contents vortexed briefly to mix. Next 0.6 mL LiAc/40% polyethylene glycol 3350 was added and the tubes incubated at 30°C for 30 min at 200 rpm. Seventy µL of dimethyl sulfoxide was added to the tubes, the contents were gently mixed and then the cells were heat-shocked at 42°C for 15 min then chilled on ice for 2 min. This suspension was transferred to a sterile microcentrifuge tube and centrifuged at 14000 *g* for 5 s at room temperature. The supernatant was removed and the cell pellet suspended in 0.5 mL of sterile TE buffer. A volume of 0.1 mL TE with yeast cells was plated on appropriate media for screening. Plates were incubated at either 30°C or 37°C for 2 to 4 d until colonies appeared.

Escherichia coli

The *E. coli* strain DH5α was used for routine cloning while the BL-21 strain was used for protein expression. In each case, 50 mL of Luria-Bertani (LB) (Bertani, 1951) broth containing 30 μg mL^{-1} kanamycin (kan_{30}) was inoculated with 100 μL of an overnight culture and incubated at 37°C for 2 h with shaking at 200 rpm. Media containing cells were transferred to sterile tubes and centrifuged at 3000 *g* for 5 min at 4°C. The bacterial pellets were washed once with 10 mL of chilled 0.1 M $MgCl_2$, centrifuged as described above and then cells were suspended in 10 mL of chilled 0.1 M $CaCl_2$ (Sambrook *et al*. 1989). The bacteria were incubated on ice for 20 min, centrifuged, and the pellet suspended in 1 mL 0.1 M $CaCl_2$. Using a round bottom Falcon tube (Becton Dickinson Labware, Franklin Lakes, NJ, Cat. No. 352059), 200 μL of suspended bacteria were added to approximately 0.4 μg plasmid DNA and then the mixture was placed on ice for 40 min. The tubes containing bacteria and plasmid DNA were incubated at 42°C for 1 min to heat-shock the cells and then the tubes were moved to room temperature. Following transformation, 1.8 mL of super optimal broth (2% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM-KCl, 10 mM-$MgCl_2$, 10 mM-$MgS0_4$ pH 7.0 supplemented with 20 mM glucose) (Hanahan, 1983) was added to the cells and tubes with contents were incubated at 37°C for 2 h with shaking at 200 rpm. One hundred μL of cells were then plated on LB_{kan30} agar and then plates were incubated overnight at 37°C.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the clone encoding PMEAMT. Nucleotide sites selected at the N-terminus of *PMEAMT* corresponding to AdoMet-binding motifs II and III were mutagenized so that the motifs of the translated product would be identical to those of PEAMT. The primers used for mutagenesis of motif II were 5'-TCCAGACTTGAAATTCAAAGATGGATCTCTCGACTTGATTTTCTC-3' (sense) and 5'-GAGAAAATCAAGTCGAGAGATCCATCTTTGATTTTCAAGTCTGGA-3' (antisense). The primers used to modify motif III were 5'- GCAGAGAGAATGATTG-GATGGATCAAGGTAGGGGGATACATTTTCTTCAG-3' (sense) and 5'-CTGAAGAAAATGTATCCCCCTACCTTGATCCATCCAATCATTCTCTCTCTGC-3' (antisense) (mutated residues are underlined). Resulting sequences were confirmed as having been changed correctly by DNA sequencing.

Cloning

The *PMEAMT* gene was amplified by PCR using primers incorporating nucleotide changes to create the restriction sites *NcoI* at the putative translational initiation site and *BamH I* at the end of the longest open reading frame. The modified *PMEAMT* insert was PCR-amplified with Pfu polymerase and the PCR mixture was heated for 3 min at 94°C followed by 3 cycles of 60 s denaturation at 94°C, 60 s annealing at 47°C and 90 s extension at 72°C. Thirty additional cycles were performed with an annealing temperature of 50°C and a final extension time of 10 min at 72°C. The PCR products were subjected to restriction enzyme digestion with *NcoI* and *BamH1* and the fragment

was ligated into the *Nco1* and *BamH1* restriction sites of pET30a (+) (Novagen) following the protocol supplied with the T4 DNA ligase (Fermentas, Cat. No. EL0011). pET30a (+) confers kanamycin resistance, has a T7 *lac* promoter and adds an N-terminal polyhistidine tag on the translated, over-expressed protein (Novagen, pET System Manual 11th ed.).

Following ligation, the plasmid containing the modified *PMEAMT* cDNA was transformed into *E. coli* (DH5-α), a strain that is more easily transformed than BL-21 (Moffatt, personal communication) but lacks the gene for T7 RNA polymerase. A sterile toothpick was used to pick colonies and transfer cells first to fresh plates and then to 10 μL of H_2O. Cells representing10 colonies were pooled in the microcentrifuge tube containing the 10 μL of H_2O. The cells were lysed by heating at 95°C for 5 min and the insoluble material was pelleted at room temperature by centrifugation for 1 min at 10000 *g*. Two μL of the supernatant was used to screen for transformed strains containing the insert by PCR amplification using the T7 terminator (5'-GCTAGTTATTGCTCAGCGG-3') and T7 promoter (5'-TAATACGACTCACTATAGGG-3') primers (Wallace *et al.*, 1981). PCR was conducted with an initial denaturing step for 3 min at 94°C followed by 30 cycles of denaturing for 30 s at 94°C, annealing for 50 s at 58°C, and elongation for 1 min at 72°C and a final extension time of 10 min at 72°C.

Three strains were found to show an amplified product corresponding to the predicted length of the *PMEAMT* gene. Plasmids from these strains were used to transform an *E. coli* strain BL21 in order to over-express the recombinant protein for purification. A restriction digest using *Nco1* and *BamH1* was performed with the plasmid

DNA used for transformation of BL21 and sequencing of the cloned DNA confirmed the identity of the gene as encoding the entire predicted open reading frame of *Arabidopsis* PMEAMT.

Arabidopsis PEAMT genes cloned by functional complementation of CPBY 19 following transformation using the pFL61 cDNA library were PCR-amplified using primers 5'- TTCCGCCATGGCTGCATCG-3' and 5'-GTAGATTTGGATCCGCTT-AATTCTTG-3' to incorporate N-terminus *Nco1* and C-terminus *BamH1* restriction sites, respectively. Primers were also designed with the same restriction sites to sub-clone each AdoMet-binding domain. For the N-terminus domain the right primer 5'-GAGCT-CTCCATGGTCGGATG-3' was designed to introduce a stop codon with the *BamH1* restriction site while the C-terminus left primer 5'-GAGCTCTCCATGGTCGGATG-3' introduced an *Nco1* restriction site with a translation start codon.

Following the same strategy, the same restriction sites were incorporated into the cloned *Arabidopsis PMEAMT* cDNA using the gene-specific olignucleotide primers 5'-GCTACTCCATGGAGCATTCTAG-3' (left primer) (*Nco1*) and 5'-ACAGGATCCTT-ACTTCTTGTCGG-3' (right primer) (*BamH1*). The N-terminus of *PMEAMT* was amplified using the gene-specific left primer with the right primer 5'-CTGGGGATCCT-TGTCATTCTAC-3' (*BamH1* and stop codon) and the C-terminus was amplified using the gene-specific right primer and the left primer 5'-GCTCTCCATGGTTGGCTGC-3' (*Nco1* and start codon).

To create a mutant chimeric gene containing the N-terminus of *Arabidopsis PEAMT* and C-terminus of *Arabidopsis PMEAMT*, left 5'-GAACAAGAAGAATCCG-

AATCAGATTTG-3' and right 5'-CAAATCTGATTCGGATTCTTCTTGTTC-3' primers were designed with each incorporating an *EcoR1* restriction site (underlined). Pfu polymerase was used for amplification with the same PCR conditions as described earlier for *PMEAMT*.

PCR-amplified products (50 µL) were precipitated overnight at -20°C in 125 µL ethanol and 5 µL 3M sodium acetate (NaAc) pH 6. The DNA was pelleted by centrifugation at 12000 *g* for 10 min, the pellet was air-dried and then dissolved in 20 µL H_2O. A 20 µL volume of the plasmid DNA preparation was used for restriction digestion with the enzymes *Nco*1, *Eco*R1, or *Bam*H1 depending on the flanking restriction sites for the plasmid under study. The DNA fragments were recovered by overnight precipitation in 75 µL ethanol and 3 µL of 3M NaAc pH 6 at -20°C. Following centrifugation the pellet was air-dried and suspended in 10 µL H_20 and this preparation was used for ligation into an appropriately digested pET30a(+) expression vector. Procedures used for ligation, transformation and screening were as described above using *E. coli* DH5-α competent cells first and then plasmids bearing the correct products (as verified by DNA sequence analysis) were isolated and used to transform competent *E. coli* BL21 (DE3) cells. In the case of the fused chimeric gene the regions encoding the two domains were ligated first and then the fused product was subsequently ligated to the pET30a(+) vector that had been subjected to restriction digestion with *Nco1* and *BamH1*. Recombinant genes were subjected to DNA sequencing to ensure that PCR amplification did not introduce unintended modifications.

In all cases, cell-free extracts were prepared from transformed bacteria that were then used in assays for P-base enzyme activity.

Protein expression

For protein expression, a single colony of *E. coli* (BL21) transformed with a control (unmodified) or modified pET30a(+) plasmid was used to inoculate 1 mL of LB_{kan30} and grown overnight at 37°C with shaking at 200 rpm. One hundred µL of the overnight culture was used to inoculate 50 mL of LB_{kan30} and the mixture was shaken at 200 rpm at 37°C until reaching an OD_{600} of 0.6. At this point, 1 mM of isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM and the cells were grown for another 3 h. The cells were then harvested by centrifugation at 3000 *g* for 5 min and the pellets were suspended using 1 mL of HED. The suspended cells were sonicated three times at 10 s each time at setting 5 using a Branson Sonifer Cell Disruptor 350. Unbroken cells and cell debris were removed by centrifugation at 12000 *g* for 10 min and the supernatant was used for protein purification or desalted before being used for enzyme assays.

Protein purification

PMEAMT was purified using HIS-Select™ nickel charged spin columns (Sigma H7787). Crude cell extract was first desalted with Sephadex G-25 (Pharmacia) equilibrated with buffer 50 mM Hepes-KOH pH 8.0, 300 mM NaCl and 1 mM DTT (HND). Six hundred µL of desalted extract containing approximately 80 mg total protein

was added to the spin column and centrifuged at 1000 *g* for 1 min at room temperature. To maximize binding of HIS-tagged proteins to the column, the flow-through was run through the column a second time. To remove non-specifically bound proteins the column was rinsed twice with 600 µL of HND. The HIS-tagged protein was eluted from the column with 500 µL of HND buffer containing 250 mM imidazole and 1 mg mL^{-1} BSA. To help prevent any protein inactivation due to exposure to metal ions (Smith *et al.*, 2000) EDTA was immediately added to the eluate at a final concentration of 1 mM.

Enzyme assays

Enzyme assays were performed using desalted, cell-free extracts prepared from either *E. coli*, yeast, or spinach leaves to determine whether the presence and expression of cDNA gene products could methylate substrates involved in PCho synthesis (Datko and Mudd 1988; Summers and Weretilnyk 1993). Crude cell extracts were desalted by gel filtration chromatography using Sephadex G-25 medium beads equilibrated with HED buffer. Eluates containing purified HIS-tagged proteins were also desalted prior to use in assays. The final assay volume was 150 µL and contained 100 mM Hepes-KOH (pH 7.8), 1 mM Na_2EDTA, 250 µM P-base, 200 µM [^{3}H] AdoMet, and 25 µL extract. Upon addition of extract the contents were incubated for 30 min at 30°C. The reaction was then stopped by dilution with 1 mL of cold water and, after mixing, 1 mL of the stopped mixture was added to an Evergreen column containing DOWEX 50W (H^+) X8-200 resin. The column matrix was then washed with three 0.5 mL aliquots of cold water and P-bases were eluted with 10 mL of 0.1 N HCl. Two mL of the eluate were added to 10 mL of

ReadySafe fluor and vortexted to mix. A scintillation counter was then used to determine the radioactivity for a count time of 10 min (Summers and Weretilnyk 1993). Michaelis–Menten constants (Km) were calculated for *Arabidopsis* PEAMT, PMEAMT and the chimeric protein containing the N-terminus from PEAMT and the C-terminus from PMEAMT. Concentrations of 15, 7.5, 3.75, 2.5, 1.5, and 0.75 mM PEA, PMEA, and PDEA were used when determining apparent Km values for enzymes (Henderson, 1992). All assays were performed in duplicate and repeated at least twice.

Product identification by thin layer chromatography

To 8 mL of assay eluate, 300 nmol each of PEA, PMEA and PDEA were added as carrier compounds. Assay products were dried down with nitrogen gas and dissolved in 10 µL of 0.1 N HCl. TLC plates pre-equilibrated in methanol: n-butanol: H_2O (5:5:1 v/v/v) were spotted with 4 µL of products dissolved in 0.1 N HCl and then they were developed in methanol: n-butanol: conc. HCl: H_2O; (10:10:1:1 v/v/v/v). P-bases radiolabelled with ^{14}C-AdoMet were detected by exposure to X-ray film (Smith *et al.*, 2000).

Nucleic acid preparations

E. coli

Bacterial plasmid DNA was isolated using Qiagen QIAprep® Spin Miniprep Kit (Cat. No. 27104). Overnight cultures were grown in 2 mL of LB_{kan30} and the cells pelleted by centrifugation for 1 min. The pellet was resuspended in 250 µL of 50 mM

Tris HCl (pH 8.0), 10 mM EDTA. All steps to extract nucleic acids were performed at room temperature. To the suspension 250 µL of a solution containing 200mM NaOH and 1% (w/v) SDS were added and the tube mixed 4 to 6 times by inversion. Immediately after mixing, 350 µL of 3.0 M KOAc (pH 5.5) were added and the mixture inverted as above to neutralize the solution. The tube was centrifuged at 13000 *g* for 10 min at room temperature and the supernatant transferred to a QIAprep spin column that was then centrifuged for 1 min at 13000 *g*. The column was washed with 75% EtOH, 25 mM NaCl, 5 mM Tris-HCl (pH 7.5) and then plasmid DNA was eluted with 50 µL of H_2O.

Yeast

Yeast nucleic acid isolation was performed using lyticase (Sigma L2524) (5 units μL^{-1}) based on the methods outlined in the Clonetech yeast protocol handbook (PT3024-1). Cells from overnight yeast cultures (20 mL) were pelleted in Oakridge tubes by centrifugation at 10000 *g* for 10 min at 4°C. The supernatant was discarded and the cells were suspended in 1 mL of sterile SD media and transferred to a 1.5 mL microcentrifuge tube. The suspension was centrifuged for 2 min at 14000*g* at 4°C and the cells suspended in 50 µL of the same media. Twenty µL of lyticase solution were added and the tubes incubated at 37°C for 1 h with shaking at 200 rpm. Ten µL of 20% (w/v) SDS were added and the tubes vortexed vigorously for 1 min and then the contents of the tube was frozen at -20°C. The solution was then thawed and vortexed to ensure complete lysis of cells. The sample volume was brought to 200 µL in 10 mM Tris, 0.1 mM EDTA pH 8.0 (TE) buffer and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v)

was added and the contents vortexed for 5 min. The tube was centrifuged at 14000 *g* for 10 min and the aqueous (upper) phase was transferred to a fresh tube. Nucleic acids were precipitated overnight at -20°C after the addition of 10 µL 7.5 M ammonium acetate and 500 µL ethanol. The tube was then centrifuged as above, the supernatant was discarded and the nucleic acid pellet was dissolved in 30 µL of H_2O.

Arabidopsis

Approximately 50 mg of *Arabidopsis* fully expanded rosette leaf tissue was harvested from 4 week-old plants. The tissue was placed into a sterile microcentrifuge tube and frozen in liquid nitrogen prior to nucleic acid extraction. The frozen tissue was ground in 400 µL of extraction buffer at room temperature until completely macerated (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS). The contents of the tube was then briefly mixed by votexing and left at room temperature for 1 h. The mixture was centrifuged for 1 min at 15000 *g* and then a 300 µL aliquot of the supernatant was transferred into a new microcentrifuge tube. Nucleic acids were precipitated from the supernatant with 300 µL of isopropanol for 5 min and recovered by centrifugation as above. After removing the supernatant the pellet was air-dried and then dissolved in 100 µL of sterile H_20 then incubated at 65°C for 1 min. Insoluble material was removed by centrifugation at 15000 *g* for 2 min and the supernatant transferred to another new microfuge tube. Nucleic acids were re-precipitated with 10 µL of 3M NaAc pH 6.0 and 250 µL of ethanol for 5 min. After centrifuging for 5 min at 15000 *g* the

supernatant was removed, the pellet was air-dried and then dissolved in 25 µL of H_2O and stored at -20°C.

Screening for *Arabidopsis* T-DNA insertions

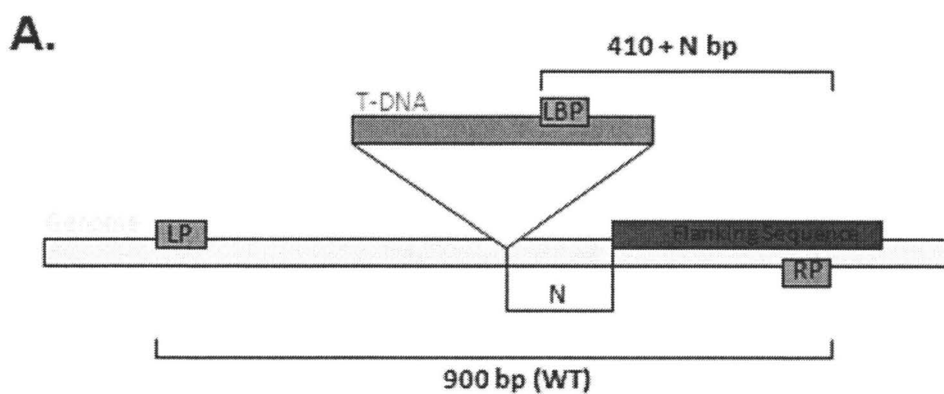
To characterize the physiological role of PMEAMT *in vivo*, three T-DNA insertion lines and their wild-type line were obtained from the *Arabidopsis* Biological Resource Center (ABRC) based at the University of Ohio (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). These lines (CS856087, SALK 006037, SALK 144248) are reported to have a T-DNA insert associated with At1g48600, the locus identified in this research as encoding *PMEAMT* (Alonso *et al*., 2003). To determine if these lines are homozygous for the insertion, a series of PCR reactions were performed involving three oligonucleotide primers. A gene-specific right and left primer (RP and LP) are designed to amplify an approximately 900 bp region of the gene of interest while a third primer (MP) is specific to the left border of the T-DNA insertion and will amplify an insert of approximately 700 bp in length. Therefore, a PCR reaction performed with all three primers in the presence of wild-type DNA (no insertion) should give a single product of about 900 bp. With DNA from plants homozygous for the presence of T-DNA insertions (insertions in both chromosomes) a single band should be produced having a size of about 700 bp and DNA from plants heterozygous for the T-DNA will yield bands of both sizes (Fig. 2.1).

Primers designed for screening for the presence of T-DNA insertions were specific for individual lines and are listed in Table 2.1. These primers were used for PCR

amplification using plant genomic DNA with an initial denaturation for 3.5 min at 94°C followed by 30 cycles of 30 s denaturation at 94°C, 50 s annealing at 58°C, 1 min extension at 72°C, and a final extension for 10 min at 72°C.

Plants identified as homozygous for the T-DNA insertion were allowed to flower and yield seed. Progeny were grown and the RNA extracted from leaves to determine if *PMEAMT* transcripts could be detected in plants of the various wild-type and mutant lines. Leaf tissue weighing approximately 50 mg was harvested and transferred to sterile microfuge tubes and frozen immediately in liquid nitrogen. RNA extraction was performed using a Qiagen RNeasy MiniKit (Cat. No. 74104). Gene specific primers (gsp) (Table 2.2) were used to reverse transcribe the RNA by PCR. At least one gsp was designed to span an intron preventing the amplification of contaminating genomic DNA. A control PCR reaction using ubiquitin specific primers (Table 2.3) was used to assess the quantity and quality of the RNA. The PCR conditions were the same as for the T-DNA insertion screening of genomic DNA by PCR amplification except the annealing temperature was 67°C for the ubiquitin primers.

Figure 2.1 Screening of *Arabidopsis* lines to detect the presence of T-DNA insertions. (A) For wild-type (WT) lines, left (LP) and right (RP) gene specific primers were used to amplify a DNA fragment by PCR using DNA extracted from *Arabidopsis* leaves. N is the difference between the actual insertion site and the flanked sequence position (~300 bp). (B) Lines heterozygous (HtZ) for the T-DNA insert produce one 900 bp gene specific PCR product and one ~700 bp PCR product amplified with the RP and the left border primer of the T-DNA insertion (LB). Finally, lines homozygous (HmZ) for the T-DNA insertion show a single PCR product of ~700 bp amplified with the RP and LB primers. Figures are adapted from those on the Salk Institute Genomic Analysis Laboratory website (http://signalsalk.edu/tdnaprimers.2.html).


A.
410 + N bp
T-DNA
LBP
LP
Flanking Sequence
RP
N
900 bp (WT)

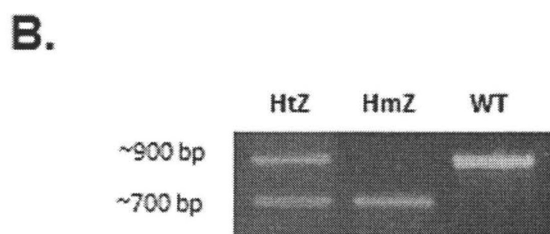

B.
HtZ
HmZ
WT
~900 bp
~700 bp

Table 2.1 Left (LP) and right (RP) primers and the left border primer (LB) for the T-DNA insertion screening of At1g48600 from three *Arabidopsis* T-DNA insertion lines. The LB primer is the same for each line.

Plant line	Primer (5' – 3')
SALK144248	LP – TGGAGGAGAAAAGACAAAGAAC
	RP – GCTCACTTCTATAATTCTAAGTTTATG
SALK006037	LP – AGAAAACAGTTTGGACTTTTCG
	RP – TGTGATGGGAGATTTCAATGG
CS856087	LP – CCTCAAAAGGTTAGAGAGATTACCA
	RP – TGCTCCATCCAGTAGCTTTTC
	LB – TGGTTCACGTAGTGGGCCATCG

Table 2.2 *Arabidopsis* At1g48600gene specific primers (gsp) and ubiquitin 10 gene primers used for RT-PCR.

Gene	Primer (5' – 3')
At1g48600	LP – GATTGGATGGGTCAAGCCAG
	RP – GAATAGAGCTGGCTTGTCTTGG
Ubiquitin10	LP – GATCTTTGCCGGAAACAATGGAGGATGGT
	RP –CGACTTGTCATTAGAAAGAAAGAGATAACAGG

Lipid profiling

Lipids were extracted from leaf tissue following a modified Bligh and Dyer (1959) protocol. Approximately 100 mg of fully expanded rosette leaf tissue was harvested from each of four, four-week-old *Arabidopsis* wild-type and mutant plants and immediately frozen in liquid nitrogen. The tissue was then transferred to a mortar containing liquid nitrogen and ground to a frozen powder with a pestle. To this frozen powder, 700 µL of chilled methanol and 58 µL of 1 M NaCl were added and the slurry was transferred to a 15-mL glass Corex® tube. The mortar was rinsed with another 700 µL of methanol and combined with the slurry. The mixture was heated for 15 min at 70°C with shaking at 100 rpm followed by centrifugation at 4°C for 3 min at 14000 *g*. The supernatant was removed and transferred to a 16 x 100 mm glass test tube. Lipid components were extracted from the pellet using 750 µL of chloroform by heating for 5 min at 37°C while shaking and the insoluble material pelleted by centrifugation as for the methanol solution. The chloroform supernatant was pooled with the methanol supernatant and 1.4 mL H_2O added. The tube was centrifuged at room temperature for 15 min at 5000 *g* to separate polar and lipid phases and each phase was transferred separately to 2-mL glass screw cap vials and dried under a stream of nitrogen gas.

Lipidomics analysis was conducted using ESI/MS/MS by the McMaster Regional Centre for Mass Spectrometry according to the methods reported by Basconcillo *et al.* (2009). The lipid phase was dissolved in 200 µL methanol:chloroform (1:1 v/v) and prior to analysis aliquots were diluted 5-fold with methanol. Lithiated lipid adducts were analyzed using neutral loss scans of 189, 175, 161, and 147 that correspond to PtdEA,

PtdMEA, PtdDEA and PtdCho polar head groups, respectively. A Waters Quattro Ultima triple quadrapole mass spectrometer and microelectrospray ionization source and MassLynx software were used to analyze the extracts and the data.

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CHAPTER 3

Preface

This chapter describes the cloning of *PMEAMT*, a gene encoding an enzyme capable of methylating two phospho-base substrates involved in phosphocholine synthesis. Under my supervision, enzyme assays using yeast extracts were performed by Katie Tchourliaeva as a part of her undergraduate thesis project and her technical contribution is acknowledged in the manuscript. Mitchell Macleod performed assays on His-tagged purified AtPMEAMT in the presence of various inhibitors and this data is given in Table 3.1. The lipidomics samples were prepared by me and they were analysed by the McMaster Regional Centre for Mass Spectrometry. With the guidance of Elizabeth Weretilnyk, I have developed the entire project and performed all the experiments. I wrote the first draft of the manuscript and subsequent drafts were prepared with editing input by Drs. Peter Summers, Brian McCarry and Elizabeth Weretilnyk. Data shown in Figure 3.7 of the manuscript and the figure itself was prepared by Dr. Peter Summers. This research was originally published in The Journal of Biological Chemistry:

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Identification of phosphomethylethanolamine *N*-methyltransferase from *Arabidopsis* and its role in choline and phospholipid metabolism[*]

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Running Title

Arabidopsis Phosphomethylethanolamine *N*-Methyltransferase

Three sequential methylations of phosphoethanolamine (PEA) are required for the synthesis of phosphocholine (PCho) in plants. A cDNA encoding an *N*-methyltransferase that catalyses 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 (PMEA) 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 PEAMT 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 phosphatidyl-methylethanolamine (PtdMEA) to be altered relative to wild-type with the content of a 34:3 lipid molecular species two-fold higher in mutant plants. In *Saccharomyces 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, 2, 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 anti-malarial and nematicidal properties for use in medicine and agriculture (6, 7, 8).

Radiotracer studies provide evidence that choline and hence PtdCho synthesis can proceed along various pathways in plants. Figure 3.1A shows that synthesis of choline can involve intermediates at the level of phosphobases (P-base), phosphatidyl bases (Ptd-base) 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, 11, 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. 3.1A) (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) offer 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 likely 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 over-expression 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), likely 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 towards 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 towards 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 head group of membrane phospholipids.

EXPERIMENTAL PROCEDURES

Media- Minimal synthetic defined (SD) media 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, 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 his3 Δ 200 trp Δ 63 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 Cat. No. 77500; 22). Following transformation, cells were plated on media 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 media supplemented with 1 mM MEA at 37°C. Single colonies that were recovered on MEA at 37°C were tested for growth on media 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 sub-cloned into a pET30a expression vector by incorporating N-terminus Nco1 and C-terminus BamH1 restriction sites by PCR. The primers for *PMEAMT* were 5'-GCTACTCCATGGAGCATTCTAG-3' (Nco1) and 5'-ACAGGATCC-TTACTTCTTGTCGG-3' (BamH1) and those for *PEAMT* were 5'-TTCCGCCATGGCTGCATCG-3' '(Nco1) and 5'-GTAGATTTGGATCCGCT-TAATTCTTG-3' (BamH1). PCR-amplified products were ligated into the expression

vector and the resulting plasmids transformed into *E. coli* BL21 (DE3) cells for expression and DNA sequence analysis.

Protein expression and purification- Fifty ml cultures of SD 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 10000 g for 10 min at 4°C and the pellet suspended in 0.3 ml 100 mM Hepes-KOH (pH 7.8), 1 mM Na_2EDTA, 5 mM DTT (HED). The yeast cells were disrupted by vortex action with glass beads (18). The supernatant was recovered following centrifugation at 12000 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 (Sigma H7787). 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^{-1} 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-labelled 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 four-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'-TGGTTC-ACGTAGTGGGCCATCG-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 denaturation at 94°C, 50 s annealing at 58°C, 1 min 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 (Cat. 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 ubiquitin10 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 to 120 mg) was harvested from four-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 1M 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 14000 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 pooled with the methanolic supernatant. To the pooled supernatants was added 1.4 ml H_2O; the mixture was vortexed 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 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^{-1} into a Waters Quattro Ultima triple quadrupole mass spectrometer equipped with a micro-electrospray 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 head groups 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).

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 media with EA suggesting that complementation was associated with more than one plant cDNA (Fig. 3.2). Using vector-specific primers, 89 isolates gave a PCR-amplified product of around 2100 bp in length and three each showed insert lengths ~2050 bp, and 1900 bp. The cDNAs associated with three random independent clones carrying 2100 bp length 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. 3.3). 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. 3.1B). The translated sequence of the ca 1900 bp cDNA insert is shown in Figure 3.3 and it corresponds to the *Arabidopsis* gene at locus At1g48600 that is

annotated as a putative *PEA N*-methyltransferase 2, (National Center for Biotechnology Information, NCBI). Strains containing this insert were able to grow on SD supplemented with MEA but not on media containing EA (Fig. 3.2). 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 bp, 2050 bp 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 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 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) and 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 to PCho (Fig. 3.4). 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. 3.2) and cell-free extracts prepared from this strain showed no methyltransferase enzyme activity towards 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, 32, 33) (Fig. 3.3). 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. 3.3). Specifically, single amino acid substitutions occur between PEAMT and PMEAMT in SAM-binding motifs post-I 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 towards 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 Ni ion

matrix. In view of this difficulty, apparent K_ms were also calculated for crude, desalted cell-free yeast extracts to determine if exposure to the Ni 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 over-expression 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 towards 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 towards PMEA and a 5-fold higher affinity for PDEA compared to 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 of 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 to 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. 3.5). 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.

In view of the possible role of AtPMEAMT in membrane synthesis (Fig. 3.1A), a lipidomics approach was used to compare the phospholipid composition between the wild-type and *atpmeamt* lines. Figure 3.6 shows representative phospholipid profiles in positive scan mode with neutral-losses corresponding to the mass of the polar head group (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 head groups 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 towards the total leaf phospholipids were equal (data not shown). Also, PtdEA and PtdCho carrying a variety of acyl chains are detected with the 34:3 and 34:2 lipid molecular species comprising the most abundant classes for these Ptd-bases (Fig. 3.6). PtdMEA components are frequently near or below the level of detection and PtdDEA lipid molecular species were not statistically different from the detection limits. While absolute quantitative estimates for components in profiles can be difficult to make (30, 36), the peak heights associated with PtdMEA is 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 (Fig. 3.6B; Fig. 3.7). Other than the 34:3-PtdMEA species we found no significant 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. 3.4).

In many, if not all plants, the enzyme PEAMT catalyses 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 substrates. 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 likely reflects its low abundance or absence in the library. Since 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. 3.3). 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 54018 Da with a theoretical pI of 5.04 while the properties of AtPEAMT are 56102 Da with a theoretical pI of 5.39 (18, 40). Neither gene product is predicted to contain a chloroplast or mitochondrion-targeting signal (41, 42) suggesting that both are likely 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 (12, 18, 31, 47).

Our estimates of K_m values determined using PMEA and PDEA show that AtPMEAMT has a higher affinity towards PMEA and PDEA than does AtPEAMT. If these enzymes co-localize 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 to wild-type plants (Fig. 3.6, 3.7). 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 *opi*3 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 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 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 modelling almost invariably support the proposal that the flux rates associated with the choline biosynthetic pathways (Fig. 3.1) 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 radiolabelled PMEA (25). McNeil *et al.* (2) show that over-expression 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 towards PCho. Whereas 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 towards PCho than TaPEAMT1 (16). A truncated form of spinach PEAMT (ΔPEAMT) showed half the sensitivity towards PCho than the recombinant

wild-type gene product (12). By way of comparison, AtPMEAMT inhibition at 47% by 1 mM PCho is comparable to that reported for ΔPEAMT from spinach (IC_{50} of 1 mM) and is less sensitive to PCho than the TaPEAMT1 isoform (12, 16). Thus AtPMEAMT is less sensitive towards inhibition by PCho than plant PEAMTs characterized to date. This feature and the high affinity towards PMEA should allow AtPMEAMT to more efficiently remove PMEA 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 while PEAMT activity does (11, 14). Thus while 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 house-keeping 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 head groups 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. While 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 (Fig. 3.6B, Fig. 3.7). 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 head group 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. 3.1). 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 remodelling could incorporate PMEA post 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 by 53, 54). 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 towards 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 towards 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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FOOTNOTES

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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

Acknowledgements: Thank you to Katie Tchourliaeva for her work determining K_m values from yeast extracts. The CPBY yeast strain was generously donated by Dr. Cindy Bolognese.

FIGURE LEGENDS

Figure 3.1. 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 while 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

Figure 3.2. Heterologous complementation of PtdCho synthesis in *Saccharomyces cerevisiae* CPBY19 by *Arabidopsis* cDNAs encoding P-base methyltransferases. CPBY19 (*cho2, opi3*) mutant strain grown on SD media supplemented with 1 mM EA or 1 mM MEA. Yeast was untransformed (1) or transformed with pFL61carrying cDNA encoding either (2) AtPMEAMT or (3) AtPEAMT.

Figure 3.3. 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 3.4. 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.

Figure 3.5. 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 ubiquitin10 gene were used as a control. *B*, Four 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. Seven-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 was no overt phenotypic differences between the SALK 006037 and wild-type *Arabidopsis* lines.

Figure 3.6. Phospholipid profiles show PtdMEA in leaves of *atpmeamt Arabidopsis* plants. Positive mode electrospray mass spectra of crude lipid extracts from leaves of (A) wild-type and (B) *atpmeamt* (SALK 006037) *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).

Figure 3.7. 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 per cent 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 a Student's t test ($p < 0.05$). n = 4 for each genotype. *Error bars*, S.E.

TABLES

Table 1. Inhibition of recombinant AtPMEAMT by PCho and AdoHcy. Methyltransferase activity was assayed using PMEA as P-base substrate. His-tagged PMEAMT was purified by a Ni affinity matrix with BSA and DTT present. Assays were linear with respect to time (n = 4; ± SE) and the control activity was 1.8 nmol ml $^{-1}$ min $^{-1}$.

Addition	mM	PMEAMT Activity (% of Control)
PCho	1 .0	47 ± 2
	5 .0	17 ± 2
Choline	5.0	101 ± 9
AdoHcy	0.01	73 ± 1
	0.20	18 ± 1

FIGURES

Figure 3.1.

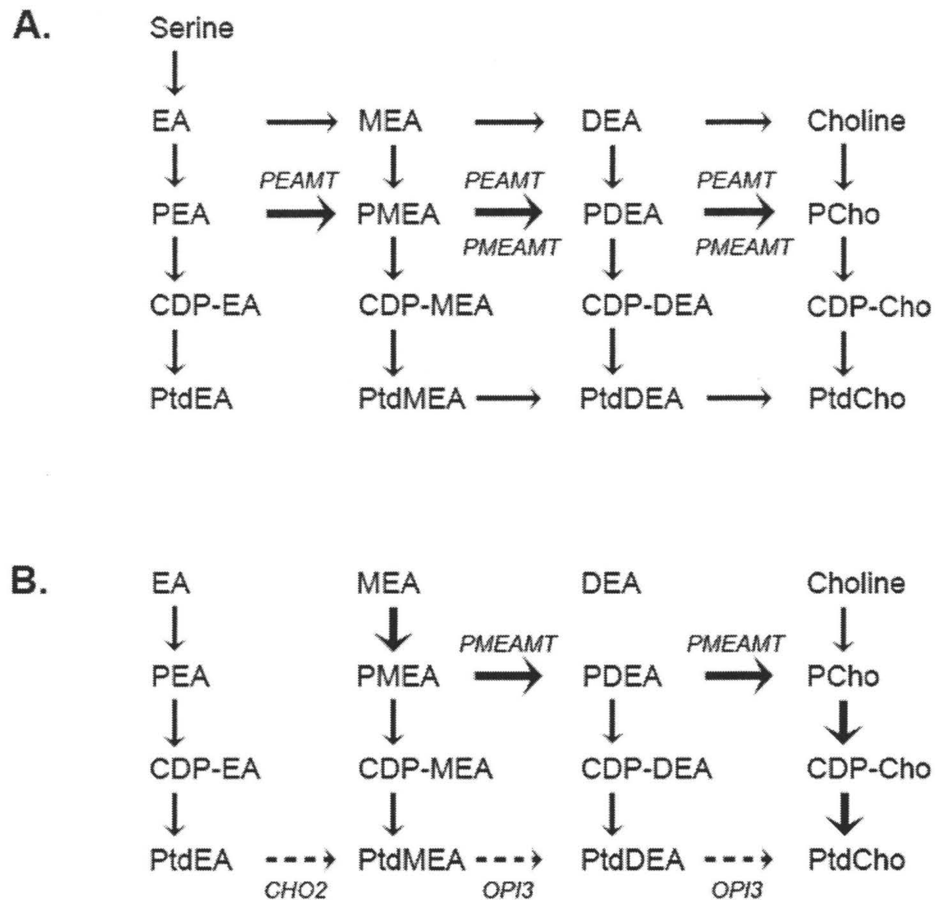


Figure 3.2.

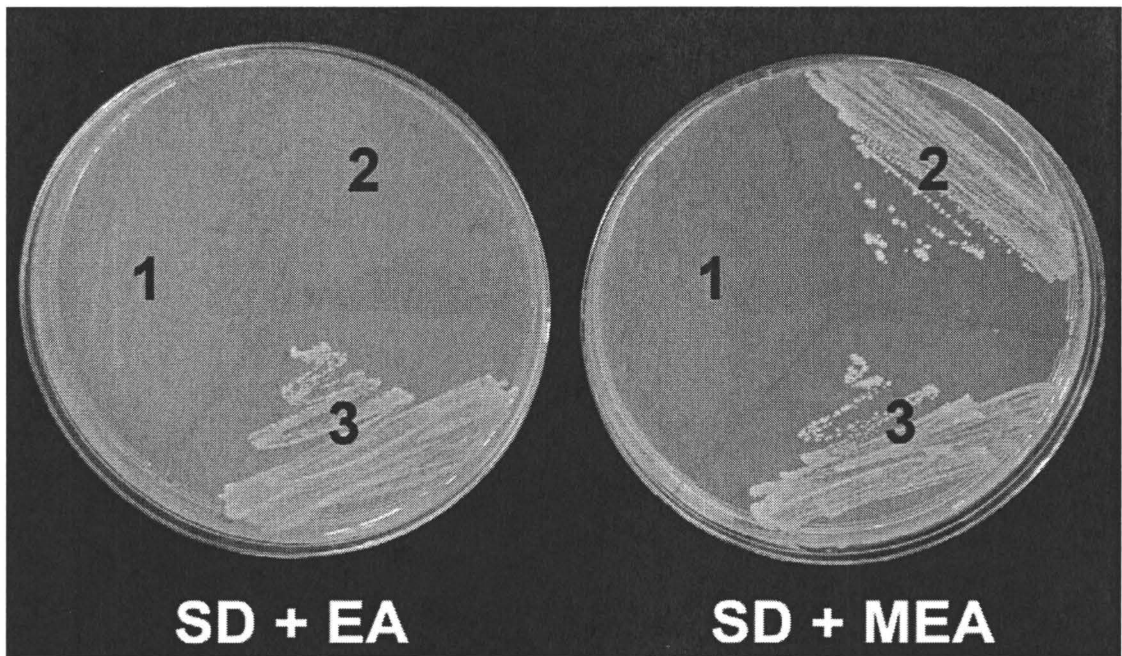


Figure 3.3.

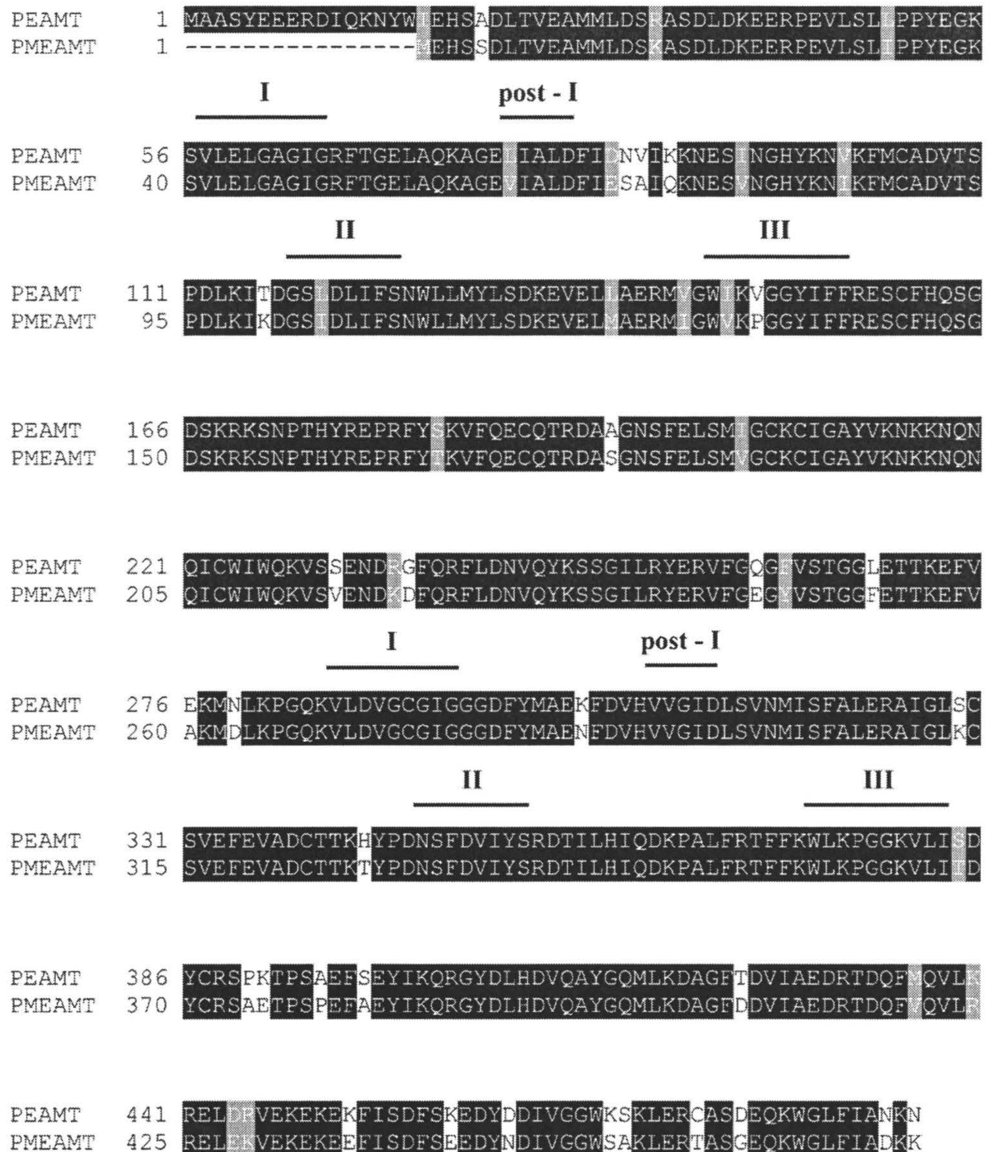


Figure 3.4.

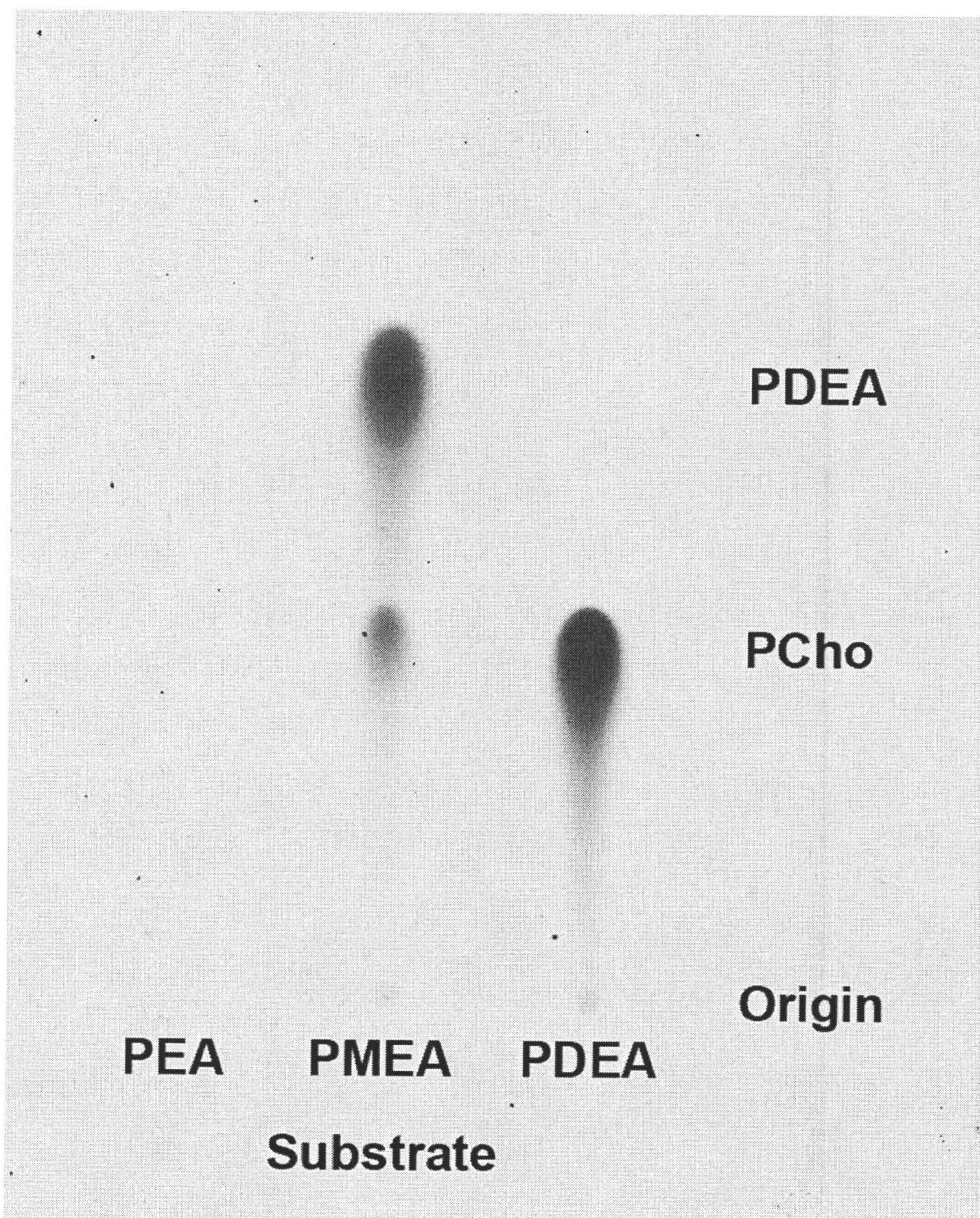
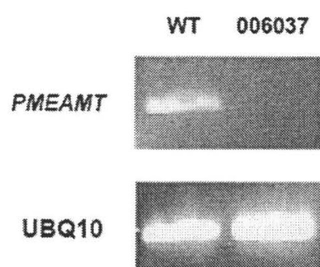


Figure 3.5.

A.



B.

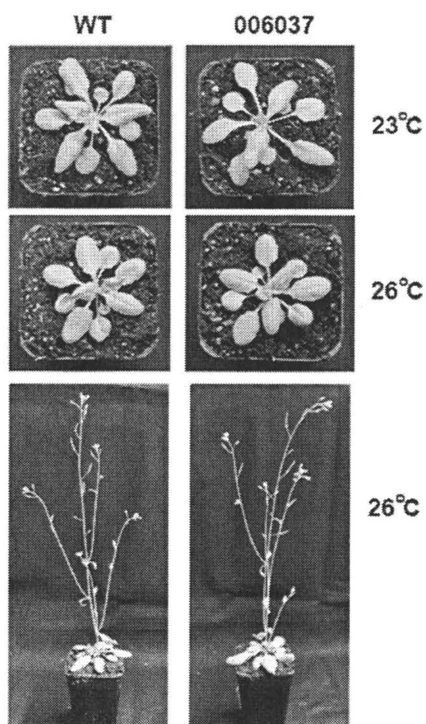


Figure 3.6.

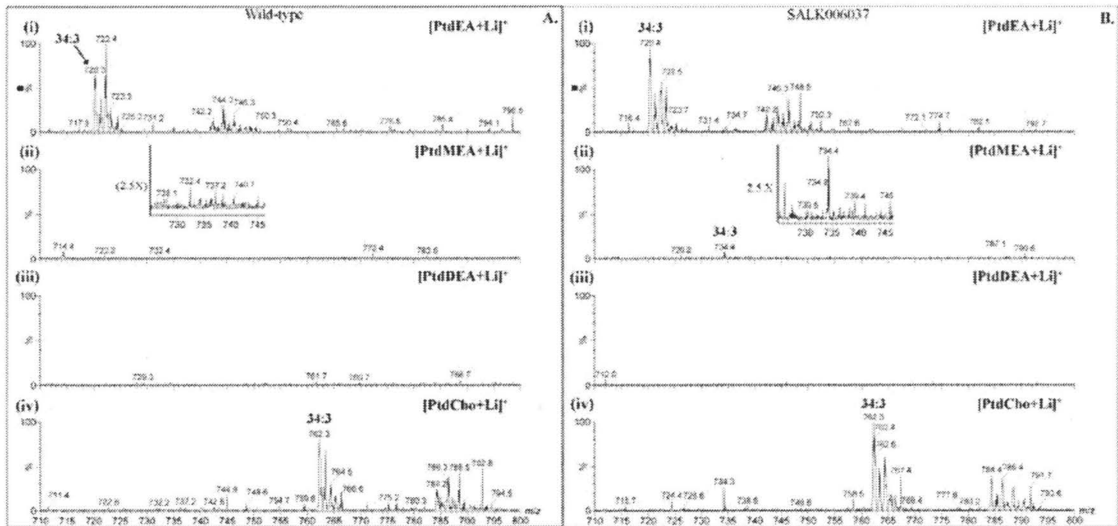
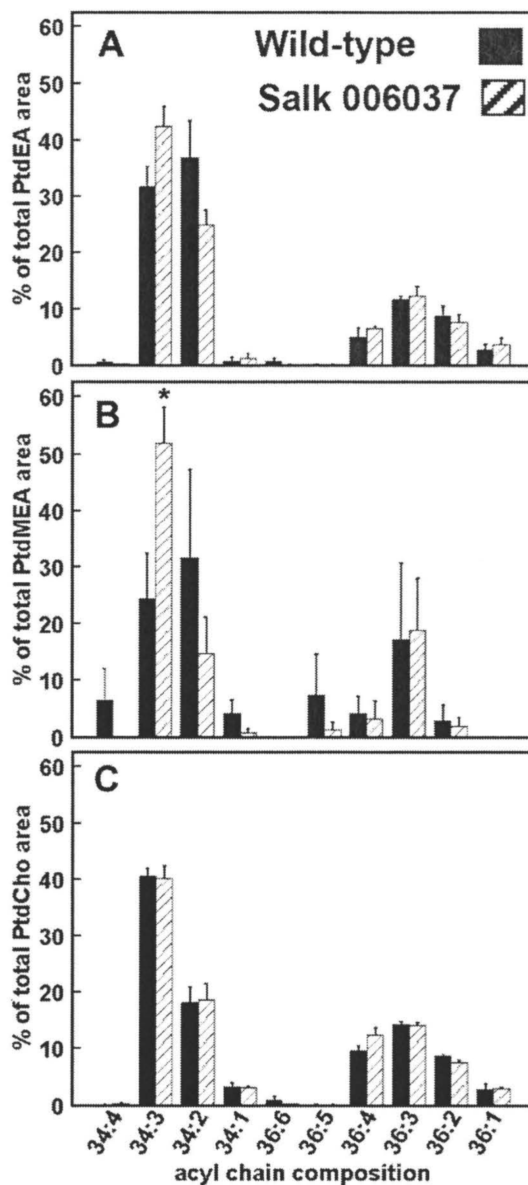


Figure 3.7.



CHAPTER 4

Preface

This chapter was co-authored by myself and Mitchell MacLeod with editing by Drs Elizabeth Weretilnyk and Peter Summers. My work consisted of the cloning of each S-adenosyl-L-methionine (SAM)-binding domain for both *AtNMT1* and *AtNMT2* and a chimeric gene consisting of the N-terminal half of *AtNMT1* and C-terminal half of *AtNMT2*. I assayed these gene products in the presence of PEA, PMEA and PDEA to determine which (if any) of these phosphobase substrates were methylated by each recombinant protein. Furthermore, I determined the Michaelis-Menten constants (K_m) for the translated chimeric protein and AtNMT3 for each of the three substrates and verified the products produced by the chimeric enzyme by thin-layer chromatography. Mitchell was responsible for the cloning of AtNMT3 as well as the protein modelling. k_{cat} values would be preferred to accompany K_m values however the inclusion of BSA in protein purification precluded this possibility. Vmax values for AtNMT2 were calculated to be 720 and 544 $nmol \cdot \mu g^{-1} \cdot min^{-1}$ for PMEA and PDEA, respectively. The template used for formatting follows that of the journal FEBS Letters.

Title: Functional characterization of domains for related bipartite phosphobase *N*-methyltransferases from Arabidopsis

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Abstract:

Phosphocholine synthesis in plants requires three sequential methylations of phosphoethanolamine catalyzed by phosphoethanolamine *N*-methyltransferases (NMTs). In *Arabidopsis,* AtNMT1 catalyzes all three reactions and AtNMT2 the two final transfers. AtNMTs have two *S*-adenosyl-L-methionine binding domains and recombinant proteins derived from AtNMT1 and AtNMT2 show that the N-terminal domain of AtNMT1 is required for phosphoethanolamine methylation. *AtNMT3* was cloned and the product, like AtNMT1, catalyzes all three reactions. Homology modelling shows that an alpha helix present at the N-termini of AtNMT1 and AtNMT3 is absent from AtNMT2 implicating this region in the phosphobase-*N*-methyltransferase activity of the N-terminal domain.

Keywords:

Methyltransferase

Choline metabolism

Homology modeling

Arabidopsis

Abbreviations: AtNMT, Arabidopsis *N*-methyltransferase; NMT, *N*-methyltransferase; PCho, phosphocholine; PDEA, phosphodimethylethanolamine; PEA, phosphoethanolamine; PMEA; phosphomethylethanolamine; PtdCho, phosphatidylcholine; SAM, *S*-adenosyl-L-methionine

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1. Introduction

Choline is found in plants as free choline, phosphocholine (PCho) and the predominant phospholipid phosphatidylcholine (PtdCho). In many organisms studied to date including plants, choline biosynthesis occurs via water-soluble phosphobase intermediates [1,2]. In this pathway, three sequential *N*-methylations of phosphoethanolamine (PEA) through phosphomethylethanolamine (PMEA) and phosphodimethylethanolamine (PDEA) yields PCho, the immediate precursor of free choline and an intermediate in PtdCho biosynthesis. The genes encoding phosphobase *N*-methyltransferase (NMT; E.C. 2.1.1.103) enzymes responsible for catalyzing these reactions have been cloned and their products characterized from several plants [3,4,5,6], from *Caenorhabditis elegans* [7,8] and the protozoan parasite *Plasmodium falciparum* [2].

Biochemically characterized NMT enzymes from phylogenetically diverse plant species including spinach, wheat, Arabidopsis, and corn show a minimum of 75% identity at the amino acid level to Arabidopsis AtNMT1 [3,4,5,6,9]. Plant *NMT*s encode products deduced to be approximately 500 amino acids in length with two catalytic domains, each one associated with a series of three, semi-conserved *S*-adenosyl-L-methionine (SAM)-binding motifs [10,11]. Each domain of a single plant NMT can be different with respect to substrate specificity. In the case of wheat NMT (corresponding to TaPEAMT1), the N-terminal half of the enzyme methylates PEA, PMEA and PDEA while the C-terminal end only methylates PMEA and PDEA [5]. In contrast, the N-terminal half of spinach NMT appears to only use PEA as a substrate [3]. Unlike the bipartite domain organization

found for plant phosphobase NMTs, the enzymes catalyzing these reactions in *C. elegans* (PMT-1 and PMT-2) [7,8] and *P. falciparum* (PfPMT) have only a single methyltransferase domain [2]. This structural diversity exemplifies the difficulty of identifying methyltransferase enzymes as orthologs in the absence of biochemical validation [12].

In *Arabidopsis*, AtNMT1 is encoded by the gene at locus At3g18000 and it catalyzes all three methylations required to convert PEA to PCho [4]. *Arabidopsis* has two other NMT-like enzymes, one encoded by a gene at locus At1g48600 annotated as encoding a putative NMT 2 (AtNMT2) and the third at locus At1g73600 encoding AtNMT3. AtNMT2 is biochemically distinct from AtNMT1 in its inability to use PEA as a substrate [6]. Properties with respect to substrate use by AtNMT3 have not been determined so its role in PCho synthesis remains to be confirmed.

We report on the use of site directed mutagenesis and recombinant enzymes to determine the functional significance of the bipartite structure of AtNMTs. Site directed mutagenesis to render SAM-binding motifs of the N-terminal catalytic domain of AtNMT2 more similar to AtNMT1 did not produce an AtNMT2 variant able to use PEA as a substrate. Assays of isolated N- and C-terminal halves of AtNMT1 and AtNMT2 and a chimeric protein produced by fusion of the N terminal half of AtNMT1 with the C-terminal half of AtNMT2 shows that the capacity of AtNMT1 to methylate PEA is a property of the N-terminal domain. Homology modeling shows that the N-terminal halves of NMTs that use PEA as a substrate have an alpha helix predicted to be in close association with the SAM binding cleft. The sequence encoding this alpha helix is absent

from AtNMT2 and may explain why the N-terminal portion of AtNMT2 lacks phosphobase methylation activity.

2. Materials and methods

2.1 Genes encoding AtNMT1, AtNMT2 and AtNMT3

The cloning and characterization of genes encoding AtNMT1 and AtNMT2 have been described previously [4,6]. For cloning *AtNMT3*, total RNA was prepared with the RNeasy (QIAGEN) kit and 1 μg was used for the cDNA synthesis reaction [3,4]. cDNA copies were produced using KlenTaq® LA DNA polymerase (Sigma) with the primers 5'-TCCAGACTCGCTATCGAAGG-3' and 5'- CAGGGTACTGGGTTTTGATG-3' in the presence of 5 μmol D-trehalose for 60 min at 55°C [13, 14]. The primers used to amplify *AtNMT3* were 5'- GTTCTCAAGGACCATGGCTTCGTATGGCGA-3' and 5'-AGGGTACTGGGTTTTGAGGATCCACATACATAC -3' incorporating an N-terminal *Nco1* with a start codon and C-terminal *BamH1* restriction site with a stop codon, respectively. PCR conditions included heating 1 min at 94°C followed by five cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C, and 2 min extension at 68°C. This series was followed by twenty-five cycles with annealing and extension at 68°C for 3 min and concluded with a final extension time of 10 min at 68°C. PCR-amplicons were used for restriction with a combination of *Nco*1 and *Bam*H1 and the DNA released was ligated to restriction sites of a pET30a+ expression vector (Novagen).

2.2 Domain cloning and gene recombination

AtNMT1 cloned in the pFL61 yeast expression vector [15] was amplified by PCR using primers specific to the N- and C-terminal halves. The primers used for cloning the N-terminal half were, 5'- TTCCGCCATGGCTGCATCG-3' and 5'-GAGCTCTCCATGGTCGGATG-3' incorporating an N-terminal *Nco*1 with a start codon and C-terminal *BamH*1 restriction site with a stop codon, respectively. The primers 5'-GAGCTCTCCATGGTCGGATG-3' and 5'-GTAGATTTGGATCCGCTTAATTCTTG-3' were used to clone the C-terminal half incorporating the same restriction sites as for the N-terminal half.

Each half of *AtNMT2* was cloned using the same strategy as for *AtNMT1*. The N-terminal region was amplified using the primers 5'-GCTACTCCATGGAGCATTCTAG-3' (*Nco*1) and 5'-CTGGGGATCCTTGTCATTCTAC-3' (*BamH*1) and the C-terminal amplified with 5'-GCTCTCCATGGTTGGCTGC-3' (*Nco1*) and 5'-ACAGGATCCTTACTTCTTGTCGG-3' (*BamH*1). PCR conditions included heating for 3 min at 94°C followed by three cycles each composed of 1 min denaturation at 94°C, 1 min annealing at 47°C and 1.5 min extension at 72°C. This series was followed by thirty cycles with a change in the annealing temperature to 50°C and concluded with a final extension time of 10 min at 72°C.

To construct a recombinant gene comprised of the N-terminal half of *AtNMT1* and C-terminal half of *AtNMT2*, left 5'-GAACAAGAAGAATCCGAATCAGATTTG-3' and right 5'-CAAATCTGATTCGGATTCTTCTTGTTC-3' primers were used to incorporate an overlapping *EcoR*1 restriction site for ligation of the two halves without altering the original amino acid sequence in this region. PCR conditions were as described above.

Following the *EcoR*1 restriction of each product, the hybrid construct was ligated at their shared *EcoR*1sites. The full-length product was then cloned into the expression vector at the *Nco*1 and *BamH*1 restriction sites as described above.

DNA sequencing was used to confirm the nucleotide sequence for all plasmid constructs both before and after transformation into *E. coli* BL21 (DE3) for protein over-expression.

2.3 Site-directed mutagenesis

The *AtNMT2* SAM-binding motifs II and III were altered by site-directed mutagenesis to match the corresponding motifs of *AtNMT1*. The primers (with mutated residues underlined) used for motif II were 5'-TCCAGACTTGAAATTCAAAGATGGATCTCTCGACTTGATTTTCTC-3' (left) and 5'- GAGAAAATCAAGTCGAGAGATCCATCTTTGATTTCAAGTCTGGA-3' (right) and for motif III were 5'- GCAGAGAGAATGATTGGATGGATCAA-GGTAGGGGGATACATTTTCTTCAG-3' (left) and 5'-CTGAAGAAAATGTATCCCCCTACCTTGATCCATCCAATCATTCTCTCTCTGC-3' (right). Modifications were confirmed by DNA sequencing.

2.4 Protein preparation

Protein over-expression was performed as described by BeGora *et al*. [6]. Fifty ml of LB medium containing 30 μg ml^{-1} kanamycin was inoculated with cells from a single colony and expression was induced by the addition of isopropyl-β-D-

thiogalactopyranoside to a final concentration of 0.4 mM prior to incubation for 3 h. Cells were harvested by centrifugation at 3000 *g* for 5 min and the pellet was suspended in 1 ml of 100 mM HEPES-KOH (pH 7.8), 1 mM Na_2EDTA, 5 mM DTT buffer. Cells were broken by sonicating 3 x 10 s at setting 5 using a Branson Sonifier Cell Distruptor 350, the cell debris was removed by centrifugation at 4°C for 10 min at 10000 *g* and the desalted supernatant was assayed for enzyme activity [6].

2.5 Enzyme assays

Phosphobase methyltransferase enzyme assays were performed using PEA, PMEA or PDEA following the assay conditions outlined previously [16]. Assay products were confirmed by TLC and autoradiography of the plates following the method reported by Smith *et al*., [17]. The assay contained 100 mM HEPES-KOH (pH 7.8), 1 mM Na_2EDTA, 250 µM phosphobase, 200 µM [^{3}H] SAM, and enzyme extract and the contents incubated for 30 min at 30°C. All assays were performed in triplicate and repeated at least twice.

2.6 Homology modelling of Arabidopsis NMTs

Homology models of the N- and C-terminal halves of the AtNMT proteins were based upon templates from *Leishmania major* and *Pyrococcus horikoshii* (Protein Data Bank (PDB): 1XTP and 1VE3) that share 32 and 36% amino acid sequence identity, respectively, to the N- and C-terminal domains of AtNMT1 The primary amino acid sequence of the N- and C- terminal portions for each AtNMT was aligned to the structural

templates and uploaded to the Swiss model server to generate a PDB file of all three AtNMT proteins [18].. The PDB files were visualized with Deepview/Swiss-PDB viewer® (www.expasy.org/spdbv).

3. Results and Discussion

3.1 SAM binding regions

The alignments of predicted amino acid sequences for AtNMT1, AtNMT2, and AtNMT3 show a high degree of identity at the amino acid level (Fig. 4.1). AtNMT1 and AtNMT2 share the greatest sequence identity at 87% while the proteins with the lowest, albeit still high identity at 78%, are AtNMT1 and AtNMT3. This alignment also shows that a striking property of AtNMT2 is that it lacks 16 and 15 amino acids found at the N-termini of AtNMT1 and AtNMT3, respectively.

AtNMT1 is able to methylate the three phosphobases (PEA, PMEA, PDEA) in the PCho biosynthesis pathway [4] while AtNMT2 is unable to methylate PEA but can use both PMEA and PDEA as substrates [6]. Given the high degree of homology between AtNMT1 and AtNMT2 we sought to identify structural features associated with the difference in substrate use of these gene products. In our first approach we compared the amino acid sequences associated with the SAM-binding motifs and looked for differences between AtNMT1 and AtNMT2 with particular attention to any divergence between these sequences and those of comparable motifs from biochemically characterized NMTs in other species.

Figure 4.1 shows that the three gene products each have two repeated domains containing three, semi-conserved SAM-binding motifs (InterPro accession: IPR013216). These domains, found in N- and C-terminal halves of the enzymes, are comprised of 33 amino acids and belong to the methyltransf_11 protein superfamily (PDB - PF08241). With the exception of the conservative substitution of T346 in AtNMT3 in place of serine at a comparable position in AtNMT1 and AtNMT2, the amino acid sequences of the SAM-binding motifs for the C-terminal halves are identical for the remaining 32 amino acids. In contrast, there are seven differences among the predicted amino acid sequences of the SAM-binding motifs found at the N-terminal end and in five cases only one AtNMT has a different amino acid from the other two. Of the seven differences found among the N-terminal motifs of AtNMT1, AtNMT2, and AtNMT3, four occur between AtNMT1 and AtNMT2. The post-I SAM-binding motif of AtNMT1 has a leucine (L79) in place of valine found in AtNMT2. This post-I valine is found in spinach [3] and wheat [5] NMTs, two enzymes shown to methylate PEA making this difference unlikely to account for the variation in enzyme activity between AtNMT1 and AtNMT2. Amino acid differences in motifs II and III between AtNMT1 and AtNMT2 were targeted for site-directed mutagenesis because these differences could not be easily reconciled by comparison with activities of known NMTs. In motif III, P132 of AtNMT2 was changed to valine as found in the corresponding sequence position of AtNMT1. This substitution is non-conservative and predicted to have a greater effect on protein function compared to conservative substitutions [19,20]. Two other alterations introduced to motifs II and III from AtNMT2 to render them more like AtNMT1 with respect to sequence are shown in

Figure 4.1 (I →L in II and V→ I in III). The change in motif II alone or combined with the changes in motif III did not lead to the production of an AtNMT2 capable of PEA methylation. This outcome suggests that the inability of AtNMT2 to methylate PEA does not likely involve differences associated with SAM-binding motifs but could involve any of several amino acid differences found in either or both halves of the enzyme.

3.2 Cloning and recombinant enzyme properties

Testing the longer N- and C-terminal domains individually for methyltransferase activity with phosphobase substrates is an approach that has been used by others in order to identify domain-specific substrate use by NMTs from spinach (SoPEAMT) and wheat (TaPEAMT1) [3,5]. Using this strategy, we cloned genes encoding recombinant proteins comprised of partial AtNMT proteins as well as a chimeric product consisting of the N-terminal half of AtNMT1 and the C-terminal half of AtNMT2 and these recombinant enzymes were used in assays of NMT activity using PEA, PMEA or PDEA as substrates (Fig. 4.2). When the C-terminal half of AtNMT1 and the N-terminal halves of AtNMT1 and AtNMT2 were assayed *in vitro* for methyltransferase activity, none was detected. This approach did not provide an answer regarding the lack of PEA methylation by AtNMT2. However, we detected low rates of methylation activity for the C-terminal half of AtNMT2 using PMEA and PDEA but not PEA as substrates. This suggested that the capacity to methylate PMEA and PDEA resides with the C-terminal domain. It is possible that sensitivity of the assay conditions compromised our ability to detect enzyme activities associated with the other half-NMT products tested but it is also likely that the

domains of the truncated proteins are non-functional. To overcome this difficulty in testing the individual domains we recombined the N-terminal half of AtNMT1 with the C-terminal half of AtNMT2 and tested the chimeric recombinant enzyme with the three phosphobase substrates. This strategy yielded an active product resembling AtNMT1 in the ability to methylate all three substrates. This outcome is consistent with the AtNMT1 N-terminal domain catalyzing the methylation of PEA and the absence of this activity in AtNMT2 being a consequence of an inactive N-terminal half.

We previously reported the apparent K_m values of AtNMT1 and AtNMT2 towards PMEA and PDEA [6]. The apparent K_m of AtNMT1 towards PEA is 0.21 mM and a similar substrate affinity is shown by the chimeric product at 0.19 mM. The affinity of this fusion protein towards PMEA and PDEA was 0.16 mM and 0.35 mM, respectively. Thus the apparent K_m towards PMEA was identical to that given by the full-length AtNMT2 used as a source of the C-terminus but the substrate affinity towards PDEA was almost 10-fold lower (0.35 mM) compared to native AtNMT2 (0.03 mM). TLC analysis of the assay products shows conversion of PEA to PMEA, PMEA to PDEA, but comparatively poor methylation of PDEA to PCho in the same time period (Supplemental Fig. 4.1). This autoradiograph of the TLC also suggests that the chimera has a lower affinity towards PDEA than either PEA or PMEA because the same substrate concentrations were used for each assay.

The observation the chimeric protein product has different substrate affinities towards PMEA and PDEA than either AtNMT1 or AtNMT2 suggests that recombination of domains from different AtNMT isoforms could be used to generate recombinant gene

products with novel kinetic properties. In fact, duplication involving two related methyltransferase genes has been cited as a probable mechanism giving rise to the naturally occurring bipartite NMT isoforms found in plants [3]. This natural process of recombination can explain the existence of NMT isoforms with different catalytic properties. For example, wheat has two NMT isoforms with different affinities toward PEA and their distinct patterns of expression are consistent with non-overlapping biochemical and physiological roles [21]. With this consideration, the biochemical activities associated with the remaining Arabidopsis NMT isoform, AtNMT3, were tested for comparison to AtNMT1 and AtNMT2 (Fig. 4.2). Given that the predicted amino acid sequence for AtNMT3 shows less identity to AtNMT1 than AtNMT2 and both AtNMT1 and AtNMT2 have different biochemical properties, direct testing of AtNMT3 is required to identify suitable substrates with confidence. Thus the gene encoding AtNMT3 was cloned and in vitro assays show the full length AtNMT3 gene product to resemble AtNMT1 with respect to the use of PEA, PMEA and PDEA as substrates although this would appear to be a distinct isoform in that the affinity of AtNMT3 for PMEA and PDEA is 8- and 5-fold lower, respectively, than those for AtNMT1 (Fig. 4.2). Whether this difference with respect to substrate affinities is important for the physiological role of AtNMT3 as has been proposed for the wheat NMT isoforms remains to be determined.

Two variants of AtNMT3 were cloned and showed premature stop codons yielding products of 266 and 330 amino acids. Methyltransferase activity was detected for the 330 amino acid long product but only with PEA as a substrate (Fig. 4.2). This indicates that PMEA and PDEA methylation properties are associated with the C-terminal

half of the enzyme and that a length exceeding 266 amino acids in the N-terminal half of AtNMT3 is necessary for PEA methylation activity. Moreover, this observation is consistent with the results shown in Figure 4.2 in that we did not detect PEA methyltransferase activity for the truncated N-terminus of AtNMT1 when it was not fused with its own C-terminal half or one provided by AtNMT2.

3.3 Homology modelling

The AtNMTs provide six templates for homology modeling that can be compared in light of their different domain specific activities. Modelling the C-terminal halves of the AtNMTs provides near identical structures for the three isoforms (Supplemental Fig. 4.2). This outcome is not surprising given the high degree of amino acid identity for the C-terminal domains shown in the alignment of Figure 4.1 and their functional equivalence in that this domain is responsible for the methylation of PMEA and PDEA but not PEA (Fig. 4.2). A notable difference in the predicted structures for the AtNMT proteins is that the N-terminal half of AtNMT2 is missing an alpha helix found in the models of AtNMT1 and AtNMT3 (Fig. 4.3). Rotation of the models shows that the alpha helix is positioned over the SAM-binding cleft indicating possible interaction with either SAM alone or between SAM and the phosphobase substrate. If the position of the alpha helix relative to the SAM binding cleft is important for enzyme function, the absence of the helix could account for the lack of PEA methylation by the N-terminal half of AtNMT2. However, these differences do not help explain why PEA is a suitable

substrate for the N-terminal domains of AtNMT1 and AtNMT3 and not the C-terminal halves of the same enzymes.

Further biochemical and structural comparisons using homologous and recombinant NMTs will be useful in building predictive models that can help design NMTs with specific properties. For example, plant NMTs are inhibited by the products PCho and S-adenosyl-L-homocysteine and the activity of some NMTs are light and/or salt-regulated [22]. Design of NMTs with altered substrate affinities that are less susceptible to inactivation by product inhibition or protein turnover offers opportunities for making plants with a heightened capacity for choline biosynthesis [23]. This trait would be considered valuable in two important applications. Choline is critical in human nutrition [24] so an increase in choline content would improve the nutritive value of plants. Also, to date the genetic engineering of crops with improved stress tolerance through an enhanced capacity to accumulate choline-derived osmoprotective solutes like glycine betaine has met with limited success [23,25]. An insufficient supply of choline to sustain osmolyte accumulation is regarded as one challenge that needs to be overcome and given the important role of phosphobase NMTs in choline synthesis they are obvious targets for metabolic engineering.

4. References

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Figure legends:

Figure 4.1. Amino acid sequence alignment of AtNMT1, AtNMT2, and AtNMT3 showing SAM-binding motifs. Identical amino acids are shaded in black and conservative substitutions are shaded in gray. *Asterisks* mark the positions where site-directed mutagenesis of *AtNMT2* was used to match the sequence of AtNMT1.

Figure 4.2. Methyltransferase substrates for AtNMT isoforms and their recombinant derivatives. AtNMT1 (open), AtNMT2 (black), and AtNMT3 (grey) N and C terminal halves are shown. All recombinant enzymes were tested for activity using the three phosphobase substrates but apparent K_m values were only determined for bipartite proteins. The symbol "-" denotes no activity detected. For recombinant proteins carrying a single domain the symbol "✓" indicates that enzyme activity was confirmed by radioassay. The inclusion of BSA as a stabilizing agent during purification precludes the determination of k_{cat}. [a] K_m values previously reported by BeGora *et al*., [6].

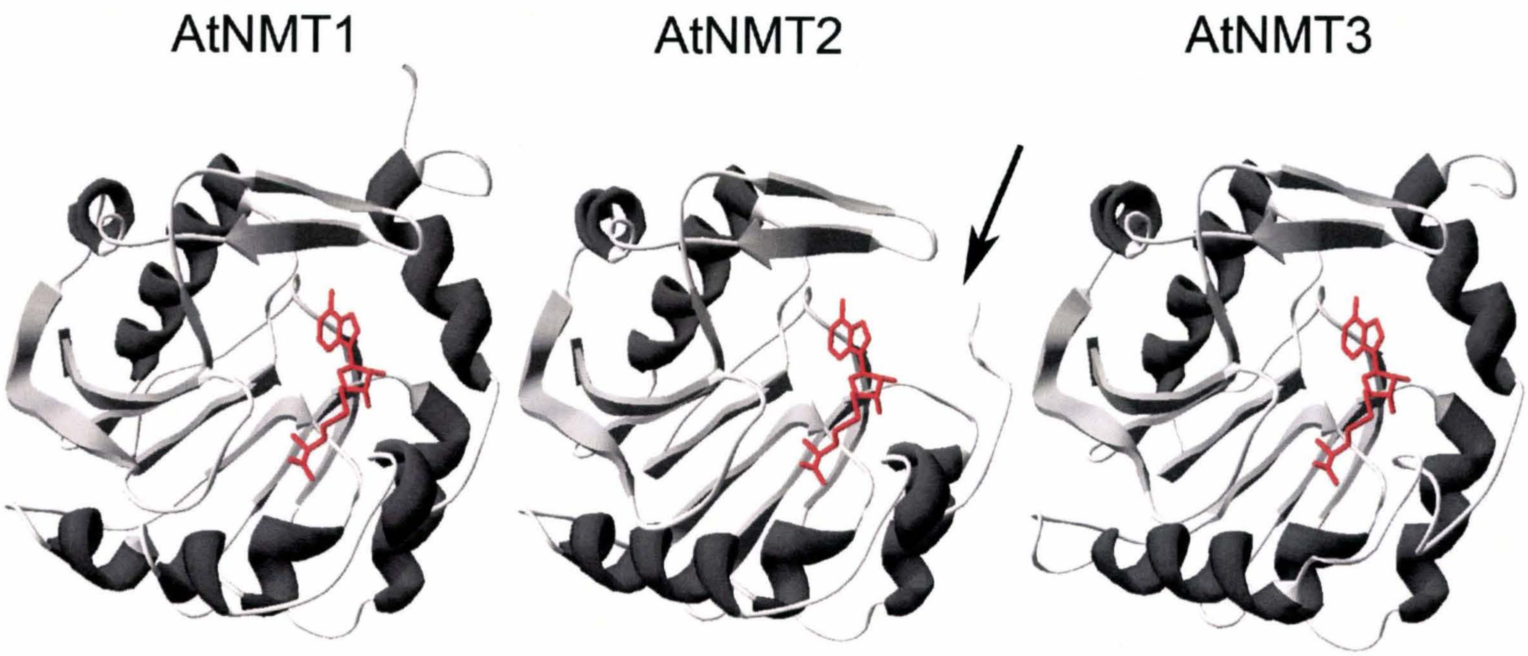
Figure 4.3. Model structures of N-terminal halves of AtNMT proteins illustrating binding cleft conformation incorporating S-adenosyl-L-homocysteine (red). The arrow indicates the region of the missing alpha helix in AtNMT2 that is present in AtNMT1 and AtNMT3.

Figure 4.1.

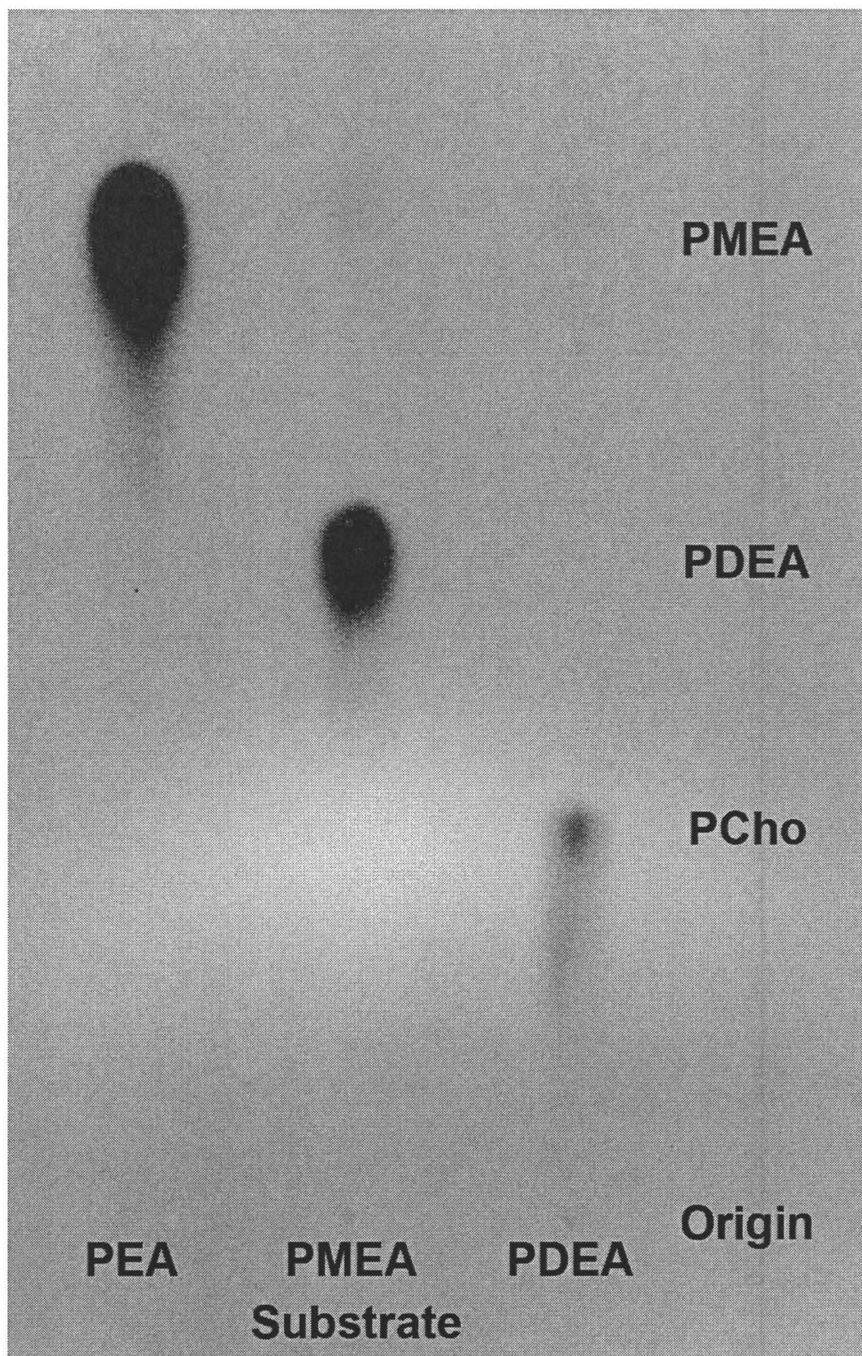
```
                                                                         I
AtNMT1    1 MAASYEEERDIQKNYWIEHSADLTVEAMMLDSRASDLDKEERPEVLSLLPPYEGKSVLEL
AtNMT2    1 ----------------MEHSSDLTVEAMMLDSKASDLDKEERPEVLSLIPPYEGKSVLEL
AtNMT3    1 -MASYGEEREIQKNYWKEHSVGLSVEAMMLDSKASDLDKEERPEILAFLPPIEGTTVLEF

               I          post-I                                         II
                                                                            *
AtNMT1   60 GAGIGRFTGELAQKAGELIALDFIDNVIKKNESINGHYKNVKFMCADVTSPDLKITDGSL
AtNMT2   44 GAGIGRFTGELAQKAGEVIALDFIESAIQKNESVNGHYKNIKFMCADVTSPDLKIKDGSI
AtNMT3   59 GAGIGRFTTELAQKAGQVIAVDFIESVIKKNENINGHYKNVKFLCADVTSPNMNFPNESM

               II                        III
                                        *  *
AtNMT1  120 DLIFSNWLLMYLSDKEVELLAERMVGWIKVGGYIFFRESCFHQSGDSKRKSNPTHYREPR
AtNMT2  104 DLIFSNWLLMYLSDKEVELMAERMIGWVKPGGYIFFRESCFHQSGDSKRKSNPTHYREPR
AtNMT3  119 DLIFSNWLLMYLSDQEVEDLAKKMLQWTKVGGYIFFRESCFHQSGDNKRKYNPTHYREPK

AtNMT1  180 FYSKVFQECQTRDAAGNSFELSMIGCKCIGAYVKNKKNQNQICWIWQKVSSENDRGFQRF
AtNMT2  164 FYTKVFQECQTRDASGNSFELSMVGCKCIGAYVKNKKNQNQICWIWQKVSVENDKDFQRF
AtNMT3  179 FYTKLFKECHMNDEDGNSYELSLVSCKCIGAYVRNKKNQNQICWLWQKVSSDNDRGFQRF

                                                             I
AtNMT1  240 LDNVQYKSSGILRYERVFGQGFVSTGGLETTKEFVEKMNLKPGQKVLDVGCGIGGGDFYM
AtNMT2  224 LDNVQYKSSGILRYERVFGEGYVSTGGFETTKEFVAKMDLKPGQKVLDVGCGIGGGDFYM
AtNMT3  239 LDNVQYKSSGILRYERVFGEGFVSTGGLETTKEFVDMLDLKPGQKVLDVGCGIGGGDFYM

                post-I                                       II
AtNMT1  300 AEKFDVHVVGIDLSVNMISFALERAIGLSCSVEFEVADCTTKHYPDNSFDVIYSRDTILH
AtNMT2  284 AENFDVHVVGIDLSVNMISFALERAIGLKCSVEFEVADCTTKTYPDNSFDVIYSRDTILH
AtNMT3  299 AENFDVDVVGIDLSVNMISFALEHAIGLKCSVEFEVADCTKKEYPDNTFDVIYSRDTILH

                          III
AtNMT1  360 IQDKPALFRTFFKWLKPGGKVLISDYCRSPKTPSAEFSEYIKQRGYDLHDVQAYGQMLKD
AtNMT2  344 IQDKPALFRTFFKWLKPGGKVLITDYCRSAETPSPEFAEYIKQRGYDLHDVQAYGQMLKD
AtNMT3  359 IQDKPALFRRFYKWLKPGGKVLITDYCRSPKTPSPDFATYIKKRGYDLHDVQAYGQMLRD

AtNMT1  420 AGFTDVIAEDRTDQFMQVLKRELDRVEKEKEKFISDFSKEDYDDIVGGWKSKLERCASDE
AtNMT2  404 AGFDDVIAEDRTDQFVQVLRRELEKVEKEKEEFISDFSEEDYNDIVGGWSAKLERTASGE
AtNMT3  419 AGFEEVIAEDRTDQFMKVLKRELDAVEKEKEEFISDFSKEDYEDITGGWKSKLLRSSSGE

AtNMT1  480 QKWGLFIANKN
AtNMT2  464 QKWGLFIADKK
AtNMT3  479 QKWGLFIAKRN
```

Figure 4.2.

Protein	K_m PEA (mM)	K_m PMEA (mM)	K_m PDEA (mM)
AtNMT1-N / AtNMT1-C	0.21	0.32[a]	0.14[a]
AtNMT1-N	—	—	—
AtNMT1-C	—	—	—
AtNMT2-N / AtNMT2-C	—	0.16[a]	0.03[a]
AtNMT2-N	—	—	—
AtNMT2-C	—	✓	✓
AtNMT1-N / AtNMT2-C	0.19	0.16	0.35
AtNMT1-N	—	—	—
AtNMT3-N	✓	—	—
AtNMT3-N / AtNMT3-C	0.58	2.56	0.75

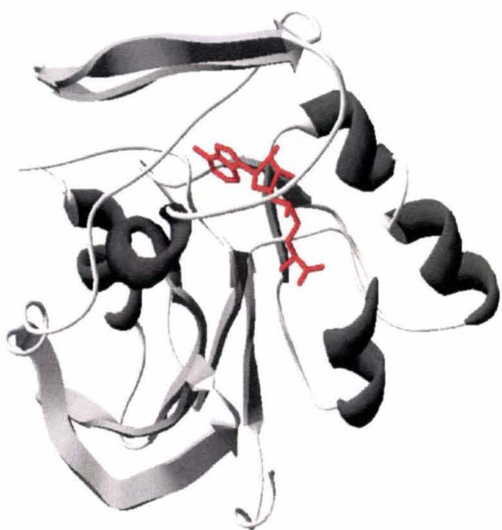

AtNMT1
AtNMT2
AtNMT3

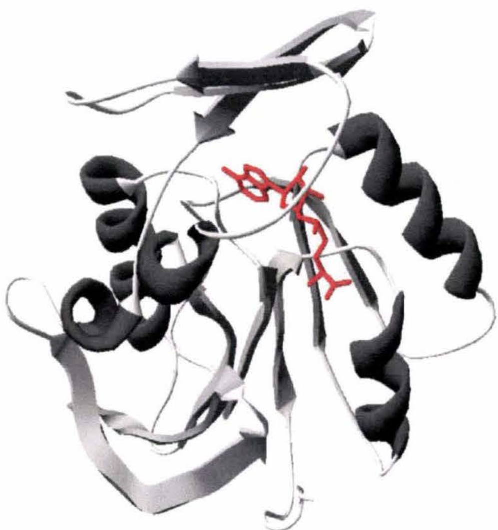
Supplemental Figure 4.1. Autoradiograph showing TLC separation of phosphobase *N*-methyltransferase assay products of the chimeric protein consisting of the N-terminal half of AtNMT1 and C-terminal half of AtNMT2.


PMEA
PDEA
PCho
Origin
PEA
PMEA
PDEA
Substrate

Supplemental Figure 4.2. Model structures of C-terminal halves of AtNMT proteins showing binding cleft conformation incorporating SAM (red).

AtNMT1


AtNMT2


AtNMT3

CHAPTER 5

General Results and Discussion

Computational analysis

This study involved cloning a previously uncharacterized plant gene (*PMEAMT*) and characterizing the properties of its translated product, a methyltransferase involved in PCho synthesis. Using the biochemically characterized *Arabidopsis* PEAMT as the basis of inquiry, a protein basic local alignment search tool (BLAST) (National Center for Biotechnology Information) was used to look for similar proteins in a non-redundant database that includes all organisms. The top matches are shown in Table 5.1 and each had an expected value (E-value) of 0. The E-value reflects the chance that protein matches would be found in a database due to chance alone (Newman *et al.* 1994). Interestingly, all the proteins found with high similarity were from plants.

The most similar protein to *Arabidopsis* PEAMT with respect to amino acid sequence is found in *Brassica napus* with a 93.4% identity (Table 5.1). The next two proteins most similar to *Arabidopsis* PEAMT are gi 15221909 and gi 24212080; both were found in the *Arabidopsis* database and are similar in length consisting of 475 and 490 amino acids, respectively. These proteins are encoded by genes at loci At1g48600 and At1g73600, respectively, and are described as putative PEAMT proteins. Both of these gene products have a high amino acid sequence identity to the biochemically characterized *Arabidopsis* PEAMT (At3g18000) at 86.6% and 81.5% for gi 15221909 and gi 24212080, respectively (Table 5.1). An unnamed protein from *Vitis vinifera* shows 80.2% amino acid sequence identity while the other proteins annotated as PEAMT, putative PEAMT or Adomet-methyltransferase (AdoMet-MT) and found on this list range

Table 5.1. NCBI BLAST results using *Arabidopsis* PEAMT (GI 11890406) as a query against a non-redundant database of proteins from all organisms. The top 24 matches are shown including the GI number, description, number of amino acids (AA) and the percent AA identity compared to the query sequence.

Species	GI	Description	Length (AA)	% identity
Arabidopsis thaliana[a]	11890406	PEAMT	491	100
Brassica napus	32478660	PEAMT	491	93.4
Arabidopsis thaliana[b]	15221909	putative PEAMT	475	86.6
Ricinus communis	255582633	putative PEAMT	492	83.5
Arabidopsis thaliana[c]	24212080	putative PEAMT	490	81.5
Vitis vinifera	225456147	AdoMet-MT	491	80.9
Vitis vinifera	147765575	unnamed protein	490	80.2
Aster tripolium	28804509	PEAMT	493	79.1
Spinacea oleracea	24212082	PEAMT	494	78.7
Gossypium hirsutum	159895667	PEAMT	475	78.7
Beta vulgaris	71000457	PEAMT	494	78.5
Solanum lycopersicum	12584943	PEAMT	491	78.2
Populus trichocarpa	224135553	PEAMT	485	77.6
Suaeda japonica	28436074	PEAMT	494	77.4
Oryza sativa	115439355	PEAMT	499	77.4
Atriplex nummularia	56692311	PEAMT	503	77.3
Oryza sativa	45272584	PEAMT	499	77.2
Salicornia europaea	110277465	PEAMT	494	76.9
Suaeda japonica	28436074	PEAMT	494	76.7
Zea mays	226510341	PEAMT	502	76.5
Triticum aestivum	259018725	PEAMT	505	76.5
Suaeda liaotungensis	117607053	PEAMT	494	76.5
Sorgum bicolor	242054097	PEAMT	501	76.2
Triticum aestivum	17887465	PEAMT	498	75.2
Zea mays	112866285	PEAMT	501	75.1

a = At3g18000, b = At1g48600, c = At1g73600

in amino acid identity from 75.1% to 83.5% including the biochemically characterized PEAMTs from spinach and wheat (Table 5.1).

Antibodies and immunoprecipitation

With the high conservation of amino acid sequence identity among plant PEAMTs (Table 5.1) we expected that polyclonal antibodies prepared against spinach PEAMT as the antigen (Drebenstedt, 2001) would likely immunoprecipitate a spinach PMEAMT. We also expected that anti-spinach PEAMT polyclonal antibodies might cross-react with *Arabidopsis* PEAMT and PMEAMT given the high degree of identity among their predicted amino acid sequences with spinach PEAMT. As such, use of these antibodies in immunoprecipitation experiments was an early approach to PMEAMT identification and purification.

In our immunoprecipitation procedure anti-PEAMT antibodies were coupled to Protein A Sepharose and then the coupled beads were used to purify PEAMT from crude extracts (Harlow and Lane, 1988; Drebenstedt, 2001). However, while PEAMT activity was bound by the activated beads and depleted from the supernatant we discovered that PMEAMT activity remained in the supernatant. We tried a few approaches to improve the likelihood of PMEAMT coupling to the beads. For example, after the initial coupling of spinach PEAMT to anti-PEAMT polyclonal antibodies coupled to Protein A Sepharose beads, the beads were pelleted and the supernatant was used for a second immunoprecipitation step. The rationale for this step was that the supernatant should be depleted of PEAMT during the first passage over the Protein A Sepharose beads thereby

reducing the competition for antibody coupling between fresh beads and any residual PEAMT-like enzymes including PMEAMT. However, this approach was not successful in immunoprecipitating PMEAMT from spinach leaves. Since PEAMT activity is high and potentially difficult to deplete from leaf cell-free extracts, growth conditions and harvesting time for plants used to prepare extracts were also adjusted to minimize PEAMT activity and maximize PMEAMT activity relative to residual PEAMT activity. Specifically, spinach PEAMT activity is reduced in leaves subjected to 40 h dark exposure whereas the activity of PMEAMT is not affected by this treatment (Weretilnyk *et al.*, 1995). P-base methylating activities increase in salt-treated spinach plants (Summers and Weretilnyk, 1993; Weretilnyk *et al.*, 1995) so extracts prepared from leaves of salt-treated plants were also tested in immunoprecipitation trials in order to elevate the initial content of PMEAMT activity. Antibody-antigen binding conditions were also modified by using different salt concentrations during antibody-antigen coupling in order to alter the stringency of antigen binding to the antibody-bead complex. Higher salt concentrations increase the stringency of antibody-antigen binding by favouring hydrophobic bonds involved in the binding complex (Harlow and Lane, 1988). Therefore, decreasing salt concentrations were used to reduce the stringency of binding in order to couple related proteins including PMEAMT to anti-PEAMT. Surprisingly, none of these strategies or conditions was successful in immunprecipitating spinach PMEAMT (data not shown).

Immunoprecipitation protocols typically involved enzyme activity measurements to track the contributions of P-base methyltransferases and protein detection by Western

blot hybridizations. For example, the methylating activity using PEA and PMEA as substrates was tested using desalted leaf extracts of spinach plants subjected to 40 h continuous dark and was found to be 0.01 nmol min^{-1} and 0.16 nmol min^{-1}, respectively. Following the first immunoprecipitation of this extract with anti-PEAMT beads residual PEA-methylating activity in the supernatant was not detected while the PMEA-methylating activity decreased to only 0.15 nmol min^{-1}. Otherwise stated, ~91% of the original PMEA-methylating activity remained in the supernatant. Following the second immunopreciptation step on beads using the PEAMT-depleted supernatant, PMEA-methylating activity decreased from 0.15 nmol min^{-1} to 0.09 nmol min^{-1} indicating that 60% of the activity was unbound and 40% of the activity was lost and presumably coupled to the antibodies on the beads. However, no bands were detected following polyacrylamide gel electrophoresis and Western blot hybridization of protein immunopurified by the beads (data not shown). This loss of enzyme activity and protein may be due to an apparent instability of PMEAMT when diluted by buffer. We later determined that the activity of His-tagged purified *Arabidopsis* PEAMT and PMEAMT is reduced (or lost completely) unless the purified recombinant proteins are recovered in buffer supplemented with BSA (see Chapter 3).

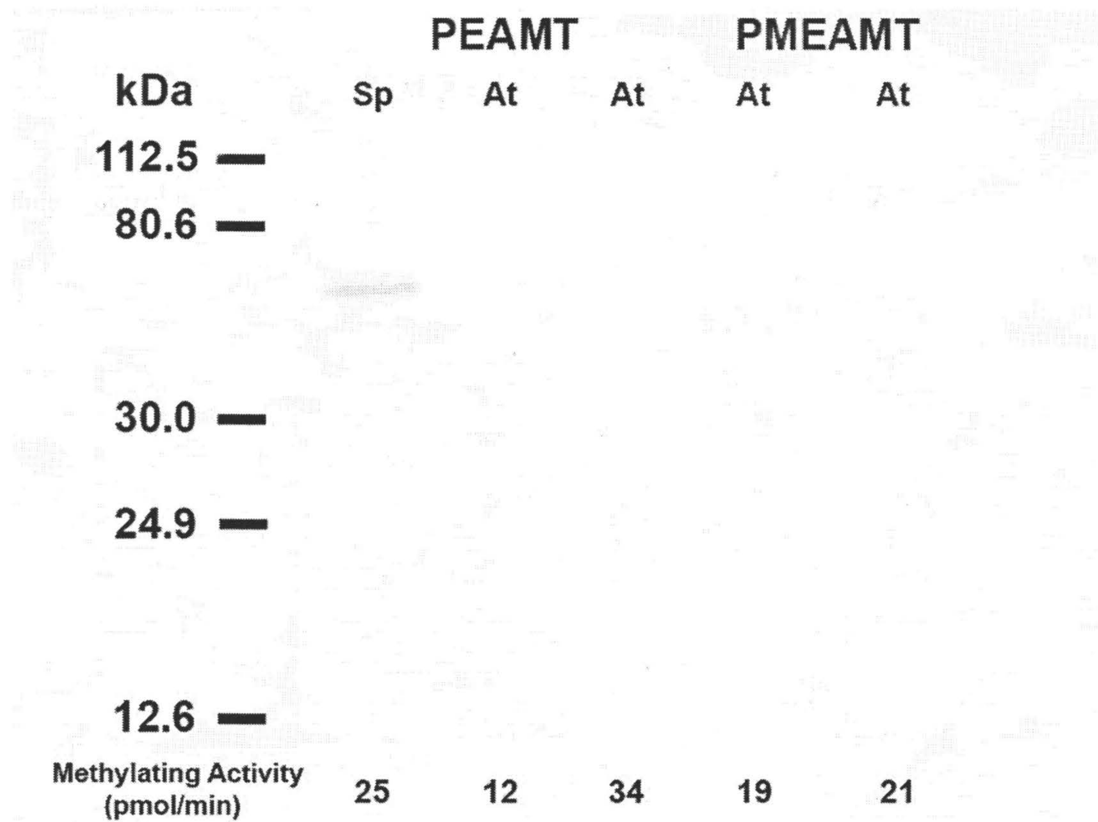
The spinach anti-PEAMT also failed to cross-react with *Arabidopsis* PEAMT or PMEAMT over-expressed in yeast (Fig. 5.1) despite the fact that these enzymes share a 79% and 78% amino acid identity to spinach PEAMT, respectively. Although these enzymes are expected to be similar based upon their use of comparable substrates, the spinach anti-PEAMT antibodies did not cross-react with *Arabidopsis* PEAMT or

PMEAMT enzymes preventing their use in cross-immunoprecipitation. Due to the difficulty in isolating PMEAMT by immunoprecipitation using spinach anti-PEAMT antibodies, an alternative approach involving functional complementation was adopted to clone the gene encoding *Arabidopsis* PMEAMT.

Cloning of *Arabidopsis* PMEAMT

Heterologous functional complementation of the *opi3*, *cho2* mutant *S. cerevisieae* strain was performed using an *Arabidopsis* cDNA library prepared in the yeast expression vector pFL61. Transformants were first screened for uracil prototrophy at 30°C on SD media containing choline. Any growth under these conditions showed that yeast was successfully transformed with a plasmid. Transformants were then transferred and screened at 37°C on media containing MEA to determine whether the gene product encoded a protein capable of supporting growth in the absence of choline. We expected that yeast transformed with plant cDNAs encoding either PEAMT or PMEAMT could grow on media containing MEA as both products might allow the mutants to methylate PMEA to PCho. As such, all clones that were able to grow on MEA were also screened on SD containing EA at 37°C. This screening process allowed us to differentiate strains expressing enzymes able to methylate PEA and PMEA or only PMEA (i.e. PEAMT in the first instance and PMEAMT in the second scenario). That is, a PMEAMT-expressing strain should only grow on SD media supplemented with MEA whereas one with PEAMT would also grow with EA (Fig. 3.2).

Figure 5.1 Immunodetection of *Arabidopsis* PEAMT and PMEAMT using spinach anti-PEAMT polyclonal antibodies. Protein blot hybridization using spinach anti-PEAMT against spinach (Sp) PEAMT over-expressed in *E. coli* as a control and *Arabidopsis* (At) PEAMT and PMEAMT from yeast extracts. The position of molecular mass standards is shown on the left in kDa and the band present corresponds to spinach PEAMT with a molecular mass of approximately 54 kDa. The total methylating activity (pmol min^{-1}) loaded into each lane is shown with PEA as a substrate for PEAMT and PMEA as a substrate for PMEAMT.


PEAMT
PMEAMT
kDa
Sp
At
At
At
At
112.5
80.6
30.0
24.9
12.6
Methylating Activity (pmol/min)
25
12
34
19
21

Following screening, only three of 103 clones were found to encode a product consistent with AtPMEAMT activity. This outcome suggests that full-length *AtPMEAMT* cDNA is less abundant than those of *AtPEAMT* in the *Arabidopsis* cDNA library and, by inference, presumably a reflection of transcript abundance in the seedlings used for the library. When sequenced, the plant cDNA conferring growth on only MEA was found to match the gene at locus At1g48600. Clones that were complemented by EA and MEA-supplemented media were found to carry a cDNA that matched locus At3g18000, the gene identified biochemically by Bolognese and McGraw (2000) as encoding a product with PEAMT activity. No clones were found that corresponded to the gene at locus At1g73600, a gene also annotated as encoding a putative PEAMT. An absence of transcripts associated with this gene at the seedling stage could explain why we found no clones complemented by this product. It is possible that the protein encoded by the gene at locus At1g73600 is toxic to yeast or the high, 81.5% amino acid sequence identity, places it in the methyltransferase class of enzymes but it does not methylate P-bases. Regardless of the exact reason, yeast colonies with cDNAs corresponding to At1g73600 were not rescued using this selection strategy.

Site-directed mutagenesis of PMEAMT

The AdoMet-binding motifs in PEAMT and PMEAMT were identified based on the conservation of these motifs to those reported necessary for binding AdoMet in non-DNA methyltransferases (Kagan and Clarke, 1994; Joshi and Chiang, 1998) (Table 1.1). Charron *et al*. (2002) showed that a single substitution of glycine by glutamic acid in

motif I in the N-terminal half of a wheat P-base NMT (TaPEAMT1) eliminated the PEA-methylation activity of the mutant enzyme. They found, however, that enzyme activity was unaffected when this same amino acid substitution was performed on the C-terminal AdoMet-binding domain. This was explained by the authors as being a consequence of the N-terminal AdoMet-binding domain methylating three P-base substrates (PEA, PMEA, PDEA) while the C-terminal can only methylate two (PMEA and PDEA). In the study of TaPEAMT1, differences in amino acid composition within motifs, including a single amino acid change, was shown to abolish PEAMT activity (Charron et al., 2002).

In the case of *Arabidopsis* methyltransferases, the C-terminal AdoMet-binding motifs are identical between AtPEAMT and AtPMEAMT as is motif I of the N-terminal half of these enzymes. However, amino acid sequence differences are found between N-terminal motifs II and III of AtPEAMT and AtPMEAMT (Fig. 3.3). To determine if PEA-methylating activity could be acquired by AtPMEAMT, site-directed mutagenesis was performed on amino acids from motif II and III of AtPMEAMT to make these motifs more similar to those of AtPEAMT. These differences first included a conserved substitution of valine to isoleucine in motif II. A conserved and non-conserved substitution of valine to isoleucine and proline to valine, respectively, were then performed in motif III of this enzyme (Fig. 3.3). In each case, no PEA-methylating activity could be detected for the modified AtPMEAMT showing that these amino acid changes are insufficient to lead to a gain-of-function outcome. There are many documented examples in the literature where a single amino acid substitution affects enzyme activity (e.g. Furman-Matarasso *et al*., 1999; Cahoon and Shanklin, 2000; Marcus

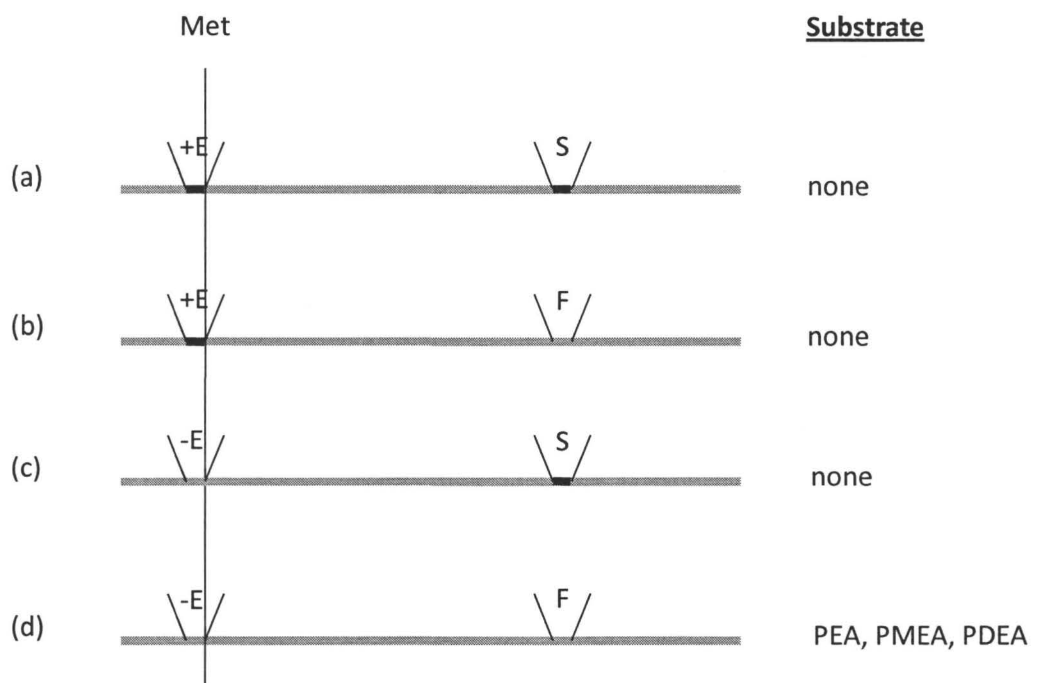
et al., 2003). For example, in tobacco, Furman-Matarasso *et al.* (1999) showed that the activity of β-1,4-endoxylanase, an enzyme involved in defense responses, was inhibited when glutamic acid-86 or -177 was substituted with glutamine (Gln), aspartic acid (Asp), or glycine (Gly). Conversely, a single amino acid change in a murine phospholipase C resulted in a gain-of-function of this enzyme in the presence of epidermal growth factor causing spontaneous inflammation and autoimmunity (Everett *et al.*, 2009).

When an *AtPEAMT* cDNA was first sub-cloned into a pET30a(+) vector, sequencing the cloned plant gene showed the predicted translated product to have two amino acid changes compared to the predicted sequence of the native protein. These changes included an extra glutamic acid immediately ahead of the start codon and a serine in place of the phenylalanine at position 438 in the C-terminal end (Fig. 5.2). Other than these two differences, PEAMT was in-frame and identical in predicted amino acid sequence to the AtPEAMT cDNA previously characterized (Bolognese and McGraw, 2000). Although the phenylalanine to serine substitution was not a part of an AdoMet-binding motif, the recombinant mutant enzyme was unable to methylate any of the P-base substrates involved in PCho synthesis (data not shown). Site-directed mutagenesis was performed on this cloned DNA to correct both mutations and the products were expressed in order to determine which change led to the loss of AtPEAMT activity. However, individual substitutions had no effect and PEAMT activity was only restored when both mutations were corrected (Fig. 5.3). It is possible that the conformational changes as a result of both substitutions leads to different charges or bonding affinities within the altered relative to unaltered proteins (Marcus *et al.*, 2003; Yu *et al.*, 2005).

Figure 5.2 Alignment of predicted amino acid sequence for AtPEAMTs showing conserved regions (black) and the positions of amino acid changes (white) for recombinant clones isolated in this project. Changes include an extra glutamic acid between residues 45 and 46 of the unaltered version and a phenylalanine substituted by serine at position 438.

```
AtPEAMT     HHHHHHSSGLVPRGSGMETKETAAAKFERQHMETDSPDLGTDDDDK-AMETAASYEEERD
AtPEAMT*    HHHHHHSSGLVPRGSGMETKETAAAKFERQHMETDSPDLGTDDDDKAEMETAASYEEERD

AtPEAMT     IQKNYWIEHSADLTVEAMETMETLDSRASDLDKEERPEVLSLLPPYEGKSVLELGAGIGR
AtPEAMT*    IQKNYWIEHSADLTVEAMETMETLDSRASDLDKEERPEVLSLLPPYEGKSVLELGAGIGR

AtPEAMT     FTGELAQKAGELIALDFIDNVIKKNESINGHYKNVKFMETCADVTSPDLKITDGSLDLIF
AtPEAMT*    FTGELAQKAGELIALDFIDNVIKKNESINGHYKNVKFMETCADVTSPDLKITDGSLDLIF

AtPEAMT     SNWLLMETYLSDKEVELLAERMETVGWIKVGGYIFFRESCFHQSGDSKRKSNPTHYREPR
AtPEAMT*    SNWLLMETYLSDKEVELLAERMETVGWIKVGGYIFFRESCFHQSGDSKRKSNPTHYREPR

AtPEAMT     FYSKVFQECQTRDAAGNSFELSMETIGCKCIGAYVKNKKNQNQICWIWQKVSSENDRGFQ
AtPEAMT*    FYSKVFQECQTRDAAGNSFELSMETIGCKCIGAYVKNKKNQNQICWIWQKVSSENDRGFQ

AtPEAMT     RFLDNVQYKSSGILRYERVFGQGFVSTGGLETTKEFVEKMETNLKPGQKVLDVGCGIGGG
AtPEAMT*    RFLDNVQYKSSGILRYERVFGQGFVSTGGLETTKEFVEKMETNLKPGQKVLDVGCGIGGG

AtPEAMT     DFYMETAEKFDVHVVGIDLSVNMETISFALERAIGLSCSVEFEVADCTTKHYPDNSFDVI
AtPEAMT*    DFYMETAEKFDVHVVGIDLSVNMETISFALERAIGLSCSVEFEVADCTTKHYPDNSFDVI

AtPEAMT     YSRDTILHIQDKPALFRTFFKWLKPGGKVLISDYCRSPKTPSAEFSEYIKQRGYDLHDVQ
AtPEAMT*    YSRDTILHIQDKPALFRTSFKWLKPGGKVLISDYCRSPKTPSAEFSEYIKQRGYDLHDVQ

AtPEAMT     AYGQMETLKDAGFTDVIAEDRTDQFMETQVLKRELDRVEKEKEKFISDFSKEDYDDIVGG
AtPEAMT*    AYGQMETLKDAGFTDVIAEDRTDQFMETQVLKRELDRVEKEKEKFISDFSKEDYDDIVGG

AtPEAMT     WKSKLERCASDEQKWGLFIANKN
AtPEAMT*    WKSKLERCASDEQKWGLFIANKN
```

Figure 5.3 The effect of amino acid differences in AtPEAMT (a-d) on the methylation of P-bases involved in PCho synthesis. All gene products were tested for activity using PEA, PMEA, and PDEA. Met signifies the position of the translation starting codon and + denotes position of an additional amino acid, S and F refer to serine and phenylalanine, respectively.


Met
Substrate
(a)
+E
S
none
(b)
+E
F
none
(c)
-E
S
none
(d)
-E
F
PEA, PMEA, PDEA

Enzymes with partially overlapping substrates

Unlike plant PEAMTs that catalyze the methylation of three P-base substrates (PEA, PMEA, and PDEA), PMEAMT only methylates the substrates PMEA and PDEA. The presence of two enzymes that can methylate overlapping substrates may occur for several reasons. Firstly, redundancy due to gene duplication is believed to have occurred in *Arabidopsis* at least four times since flowering plants evolved (~200 million years ago) (Vision *et al.*, 2000). Thus multiple copies on the same and different chromosomes are expected as an outcome. AtPEAMT and AtPMEAMT do not have the same activities and this property may have diverged during evolution. Perpetuation of both enzymes may be because they are specific to or more predominant in certain tissues or cellular compartments. Thus a comparison of the distribution and expression pattern of *PEAMT* and *PMEAMT* transcripts could be used to predict a biological role for their gene products although a potential caveat is that transcript abundance is not necessarily positively correlated to protein activity. This is true for spinach PEAMT where it was shown that the lowest protein and activity of PEAMT is found during the dark period, a time when transcript abundance is highest (Drebenstedt, 2001). However, if transcripts are found to increase (or decrease) in abundance in a given environment, it could lead to predictions that certain metabolite(s) associated with the activity of these gene products would be immediately or imminently required under certain conditions. For example, enzymes may be differentially regulated by external cues including temperature or light as is true for the light and salt-responsive changes in spinach PEAMT activity (Weretilnyk *et al.*, 1995; Smith *et al.*, 2001).

Enzymes may show differences with respect to kinetic properties and these differences may make them more or less suited to specific roles. Kinetic analysis from this research shows that PMEAMT has a 2- and 5-fold greater affinity for PMEA and PDEA, respectively, compared to PEAMT (Fig. 4.2). Similarly, two isoforms of PEAMT from wheat were shown to have different affinities toward PEA but their affinities towards PMEA and PDEA were not tested (Jost *et al.*, 2009). These differences in substrate affinity could contribute towards changes in PCho production under varying environmental conditions.

For plants that oxidize choline to glycine betaine under stress, increasing PtdCho or choline content could be important in environmental stress tolerance (Nuccio *et al.*, 1998; McNeil *et al.*, 2001; Tasseva *et al.*, 2004). During stress, PtdCho can be degraded by phospholipase D (PLD) to yield phosphatidic acid (PA) that can act as a secondary messenger activating stress responses (reviewed by Munnik, 2001). The importance of PA in *Arabidopsis* plants exposed to salt or osmotic stress was tested by Bargmann *et al.* (2009) using *Arabidopsis* PLD T-DNA knock-out lines that contained only 30% of PA compared to wild-type plants. The mutant lines showed reduced root growth in the presence of either 150 mM NaCl or 300 mM mannitol compared to wild-type (40 and 50%, respectively). PA has also been implicated in alleviating other plant stresses including oxidative (Zhang *et al.*, 2003), freezing (Li *et al.*, 2004), and phosphate starvation conditions (Li *et al.*, 2005). The relative contributions that AtPEAMT and AtPMEAMT make towards choline and PtdCho production under optimum growth

conditions or under stress are unknown but are not likely equal given that they are not functionally redundant gene products.

Comparison of P-base NMTs from different organisms

P-base *NMT*s have been cloned and their products characterized from *Arabidopsis*, spinach, and wheat plants (Bolognese and McGraw, 2000; Nuccio *et al.* 2000; Charron *et al.* 2002; Jost *et al.* 2009), from the nematode *C. elegans* (Palavalli *et al.* 2006; Brendza *et al.* (2007), and from the protozoan *P. falciparum* (Pessi *et al.* 2004). *P. falciparum* NMT (PfNMT) is approximately half the molecular mass (31 kDa) of the other characterized NMT proteins that range from 50 to 57 kDa. PfNMT methylates all three P-base substrates involved in PCho synthesis (PEA, PMEA, and PDEA) and its activity is reduced by 50% relative to control activity (IC_{50}) at 50 μM concentrations of the reaction products PCho or AdoHcy. In the case of the two *C. elegans* P-base NMTs, only one catalyzes the methylation of PEA (CePMT1) while the second NMT (CePMT2), uses PMEA and PDEA. CePMT 1 and 2 each have only a single AdoMet-binding domain and are virtually insensitive to inhibition by PCho (IC_{50} CePMT1 ~ 7000 μM and IC_{50} CePMT2 ~1500 μM) (Palavalli *et al.*, 2006; Brendza *et al.*, 2007). Plant NMTs each contain two AdoMet-binding domains and PCho sensitivity lies between that of the highly sensitive PfNMT and comparably insensitive CeNMTs (e.g. spinach IC_{50} 250–490 μM) (Smith *et al.*, 2000; Jost *et al.*, 2009). Jost *et al.* (2009) suggests that the duplicated AdoMet-binding domain structure of plant proteins may contribute to the intermediate sensitivity toward PCho. In this work, AtPMEAMT was found to be inhibited by PCho

with an estimated IC_{50} ~1000 µM compared to other plant NMTs (BeGora *et al.*, 2010). This lower sensitivity towards PCho might allow a more complete conversion of PMEA and PDEA to PCho.

The sensitivity of P-base NMTs towards AdoHcy is more consistent between different species and ranges from an IC_{50} of ~10 µM for spinach and *C. elegans* NMTs to 70 and 130 µM in TaPEAMT1 and TaPEAMT2, respectively (Smith *et al.*, 2000, Palavalli *et al.*, 2006; Jost *et al.*, 2009). Compared to spinach PEAMT, AtPMEAMT is less inhibited by AdoHcy as 10 µM AdoHcy only reduced activity by 27% instead of 50% for spinach PEAMT.

Differences among the sensitivities of various NMTs towards feed-back inhibition by AdoHcy and PCho indicate that enzymes such as AtPEAMT and AtPMEAMT may have distinct roles in addition to the need for AtPEAMT in PMEA production. Both enzymes may convert PMEA to PCho but this overlap in capability may be necessary to respond to an increased demand for PCho and/or prevent the accumulation of intermediates in PCho synthesis. Enzymes with different regulatory properties could assist in engineering NMTs with increased affinities for substrates and reduced inhibition by reaction products. For example, combining the PfNMT with an NMT AdoMet-binding domain from another species could produce a fusion protein less inhibited to both PCho and AdoHcy. Similarly, the manipulation of individual amino acids within each domain could engineer an NMT with specific enzymatic properties.

***Arabidopsis* T-DNA screening**

A total of 80 plants representing three different lines were screened for the presence of a T-DNA insertion associated with the gene encoding AtPMEAMT at locus At1g48600 (Table 5.2). Of the thirty-five plants from the SALK 144248 line, five were wild-type, 30 were heterozyous for the T-DNA insertion and none were found to be homozygous (Table 5.2). All 27 plants screened from the CS856087 line were wild-type (Table 5.2). Only the SALK 006037 line was found to contain wild-type and mutant plants homozygous for a T-DNA insertion and so this line was selected for further studies including RNA extraction and lipid profiling. Gene-specific primers were used and RT-PCR showed that no transcripts associated with AtPMEAMT could be detected in RNA extracted from these plants (Supplemental Fig. 3.1). The lack of an overt phenotype associated with suppressed expression of *PMEAMT* was, at first, unanticipated due to the features ranging from aberrant root development to male sterility already reported for plants deficient in PEAMT activity (Mou *et al.*, 2002; Cruz-Ramirez *et al.*, 2004). The use of shot-gun lipidomics (Basconcillo *et al.*, 2009) that we applied to the study of *Arabidopsis* was particularly valuable in revealing an important distinction between wild-type and mutant *atpmeamt* plants. A change in the phospholipid composition shown by a significantly higher content of 34:3-PtdMEA in membranes of the mutant would not have been identified without application of this highly sensitive and novel profiling strategy.

Dissecting and functionally testing parts of an enzyme is one way of identifying determinants that may biochemically distinguish closely related enzymes (e.g. reviews Kormander *et al.*, 2009; Kolychev, 2010). As such, this strategy can reveal any unique

Table 5.2 Screening of three putative *AtPMEAMT* knock-out seed lines reported to have a T-DNA insert associated with the gene at locus At1g48600.

	T-DNA Mutant Line		
Genotype	006037	144248	CS856087
		No. of plants	
Wild-type	10	5	27
Heterozygous for T-DNA insertion	3	30	0
Homozygous for T-DNA insertion	5	0	0
Total Screened	18	35	27

contributions seemingly identical enzymes may make to the metabolism of an organism. This information can be used to predict kinetic properties including suitable substrates for unidentified enzymes or creating enzymes for novel applications. In this study, I tested the different AdoMet binding domains, separately and in combination, in order to determine how these gene products are organized and how this organization translates into biochemical activities (Chapter 4). Most importantly, this approach showed that PEA methylation is most likely a property of the N-terminal half of AtNMTs.

Future Research

There are several genetic tools already in place upon which to base future studies. To determine possible roles of these *Arabidopsis* NMTs *in planta*, knockout plants corresponding to each gene could be crossed (in all combinations) to generate lines with plants showing suppressed P-base methylation capacities. The phenotypes associated with these genotypes could be compared with particular attention to effects known to be associated with their activities such as root development, fertility, and membrane phospholipid composition.

While suitable plant genetic models may be difficult to find, ideally PEAMT and PMEAMT enzymes should be compared in glycine betaine accumulating species. Unfortunately, plants such as spinach that have been used in biochemical studies of this pathway are not likely to be amenable to genetic analyses due to a lack of transformability and genome sequence information. However, comparison of P-base methyltransferase activities in these species may help determine why this trait has proven

difficult to introduce in non-accumulating plant species (Nuccio *et al*., 1998; McNeil *et al*., 2001). Transgenic tobacco and *Arabidopsis* fall short of providing the necessary choline to oxidize to glycine betaine (Nuccio *et al*., 1998; Huang *et al*., 2008) and it is possible that this deficiency can be resolved when factors regulating PEA methylation are fully understood. As discussed earlier, understanding how primary structure affects enzyme-substrate specificity as well as product inhibition could help redesign enzymes that are more suited to the production of choline for glycine betaine synthesis in non-accumulating crop species. In this regard, the determination of protein tertiary structure by X-ray crystallography could also help predict the effect of structure versus function and this information would be useful in targeted engineering of NMTs with specific biochemical properties.

Conclusions

An important outcome of this research was the identification of the biochemical function of the gene product associated with locus At1g48600 of *Arabidopsis thaliana.* This study identifies this enzyme as a P-base NMT that methylates PMEA→ PDEA→PCho and corrects its current annotation as a putative PEA-NMT2, a gene product that would also use the P-base substrate PEA and catalyse the reaction sequence PEA→PMEA→PDEA→PCho. In recognition of the first substrate used by this gene product it has been designated PMEAMT (hence AtPMEAMT). With respect to affinities towards P-base substrates, AtPMEAMT has a higher affinity toward PMEA and PDEA than AtPEAMT.

In common with other plant P-base NMTs, AtPMEAMT shows bipartite organization but no methylating capacity was detected for the N-terminal AdoMet-binding domain. The C-terminal AdoMet-binding domain was shown to be responsible for methylating both substrates, namely PMEA and PDEA. Production of a recombinant hybrid enzyme using the N-terminal half of AtPEAMT and the C-terminal half of AtPMEAMT allowed identification of the N-terminal portion of the protein as the half conferring the capacity of AtPEAMT to methylate PEA, an organizational feature in common with that reported for wheat and spinach PEAMT where the N-terminal AdoMet-binding domain methylates PEA and the C-terminal half uses PMEA and PDEA as substrates.

No *AtPMEAMT* transcripts were detected in leaf extracts from an *atpmeamt* mutant and PtdMEA was clearly detected among the mutant phospholipids. Thus suppressed expression of *AtPMEAMT* was associated with a statistically significant increase in 34:3-PtdMEA relative to wild-type. Based on these observations, a proposed role for AtPMEAMT is to promote the complete conversion of PMEA and PDEA to PCho in order to reduce the likelihood of PMEA or PDEA incorporation into membrane phospholipids. Whether an increased content of PtdMEA or PtdDEA has a detrimental effect on plants remains a question for future research.

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