The Isolation and Characterization in Yeast of a Gene for Arabidopsis *S*-Adenosylmethionine:Phospho-Ethanolamine *N*-Methyltransferase[1]

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Saccharomyces cerevisiae opi3 mutant strains do not have the phospholipid *N*-methyltransferase that catalyzes the two terminal methylations in the phosphatidylcholine (PC) biosynthetic pathway. This results in a build up of the intermediate phosphatidylmonomethylethanolamine, causing a temperature-sensitive growth phenotype. An Arabidopsis cDNA library was used to isolate three overlapping plasmids that complemented the temperature-sensitive phenotype. Phospholipid analysis showed that the presence of the cloned cDNA caused a 65-fold reduction in the level of phosphatidylmonomethylethanolamine and a significant, though not equivalent, increase in the production of PC. Sequence analysis established that the cDNA was not homologous to *OPI3* or to *CHO2*, the only other yeast phospholipid *N*-methyltransferase, but was similar to several other classes of methyltransferases. *S*-adenosyl-Met:phospho-base *N*-methyltransferase assays revealed that the cDNA catalyzed the three sequential methylations of phospho-ethanolamine to form phospho-choline. Phospho-choline is converted to PC by the CDP-choline pathway, explaining the phenotype conferred upon the yeast mutant strain by the cDNA. In accordance with this the gene has been named *AtNMT1.* The identification of this enzyme and the failure to isolate a plant phospholipid *N*-methyltransferase suggests that there are fundamental differences between the pathways utilized by yeast and by some plants for synthesis of PC.

Phosphatidylcholine (PC) is a major membrane lipid in plants, accounting for 40% to 60% of lipids in non-plastid plant membranes (Moore, 1990). Studies of this major lipid have revealed several important findings: the synthesis of PC is affected by the plant growth regulator indole-3-acetic acid (Price-Jones and Harwood, 1983), PC is a substrate for the 18:2 fatty acid desaturase (Somerville and Browse, 1991), and freezing tolerance has been correlated with changes in the amount of PC (Kinney et al., 1987) and degree of polyunsaturation in PC (Sikorska and Kacperska-Palacz, 1980). It is clear that PC is necessary for a wide array of structural and biochemical functions. The enzymes involved in the biosynthesis of PC in plants have been investigated at the biochemical level and investigations have shown that the biosynthetic pathway utilized may vary between species (Marshall and Kates, 1973; Price-Jones and Harwood, 1983; Kinney and Moore, 1987, 1988; Kinney et al., 1987; Datko and Mudd, 1988a, 1988b, 1989a, 1989b, 1989c; Wang and Moore, 1991; Prud'homme et al., 1992a, 1992b; Kinney, 1993; Moore, 1993; Rhodes and Hanson, 1993; Summers and Weretilnyk, 1993; Williams and Harwood, 1994; Weretilnyk et al., 1995; McNeil et al., 2000; Smith et al., 2000). The committed step to PC synthesis in plants is considered to be the methylation of phospho-ethanolamine (P-EA; Datko and Mudd, 1988a, 1988b).

To gain a better understanding of PC biosynthesis in plants, investigation at a molecular genetic level is necessary. Cloning plant genes by complementation of heterologous biosynthetic pathways is a valuable investigative approach. An overview of the *Saccharomyces cerevisiae* PC biosynthetic route is given in Figure 1. The de novo pathway is the major pathway for production of PC in *S. cerevisiae* (Carman and Henry, 1989). The decarboxylation of phosphatidylserine to form phosphatidylethanolamine (PE) is the first step in de novo PC synthesis in *S. cerevisiae* (Fig. 1). The sequential methylation of PE to form PC in *S. cerevisiae* is a well-characterized pathway (Yamashita et al., 1982; Summers et al., 1988; Carman and Henry, 1989; McGraw and Henry, 1989; Gaynor and Carman, 1990; Gaynor et al., 1991; Paltauf et al., 1992; Preitschopf et al., 1993) and is catalyzed by the *S*-adenosyl-Met-dependent (SAM) phospholipid *N*-methyltransferases (PLMTs) encoded by the *CHO2* and *OPI3* genes (Summers et al., 1988; McGraw and Henry, 1989). Unlike yeast, direct methylation of PE to form phosphatidylmonomethylethanolamine (PM) has not been observed in plants (Marshall and Kates, 1973; Datko and Mudd, 1988a, 1988b; Williams and Harwood, 1994).

The CDP-choline pathway for PC biosynthesis found in yeast and other eukaryotes is also found in plants (Kinney and Moore, 1987; Kinney et al., 1987; Nishida et al., 1996; Jones et al., 1998). This is considered to be the main biosynthetic pathway for PC production in plants (Kinney and Moore, 1987; Kinney et al., 1987; Nishida et al., 1996; Jones et al., 1998). In this pathway PC is made from reactions that make

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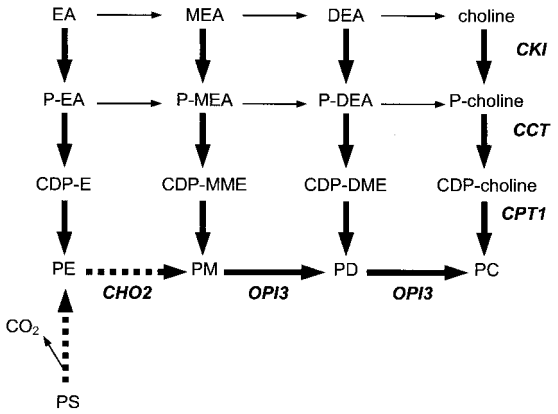

Figure 1. PC biosynthesis in yeast and plants. The methyl donor for each methylation step is SAM. Pathways occurring only in yeast are dashed lines; pathways found in yeast and plants are large arrows; plant pathways are small arrows. Adapted from Moore (1993) and McGraw and Henry (1989). PS, Phosphatidylserine.

use of the availability of free choline, the soluble head-group precursor of PC (Fig. 1). The enzymes that catalyze this three-step process have been cloned (Dewey et al., 1994; Monks et al., 1996; Nishida et al., 1996; Choi et al., 1997; Jones et al., 1998).

The experiments reported here were initiated to acquire biochemical and molecular genetic information on the methylation pathway for PC biosynthesis in Arabidopsis. The accumulation of PM in the yeast *opi3* mutant strain is correlated with an inability to tolerate high temperature and this phenotype is aggravated if the PM precursor monomethylethanolamine (MEA) is also provided in the growth medium (McGraw and Henry, 1989). Three overlapping plasmids were isolated that complemented the temperature-sensitive phenotype of the *opi3* mutants. The cloned DNA was sequenced and found to contain a previously uncharacterized gene 1,860 bp in size, which was named Arabidopsis SAM:phosphoethanolamine *N*-methyltransferase (P-EA-Met) one (*AtNMT1*). This gene encodes a protein 491 amino acids in length, which contains two SAM-binding domains and is less than 10% identical to the yeast Cho2 and Opi3 PLMTs. The *AtNMT1* cDNA was characterized and found to reduce the level of PM in the *opi3* strain over 65-fold. It is surprising that the reduction in PM did not result in an equivalent increase in PC, as would be expected if the plasmids encoded PLMTs. Instead the AtNmt1p was found to catalyze three sequential methylations of P-EA to form phospho-choline, in this manner providing a substrate for the synthesis of PC by the CDP-choline pathway.

RESULTS

Heterologous Complementation of *opi3*

The Arabidopsis cDNA library from Minet and colleagues (Minet et al., 1992) was amplified in *Escherichia coli* and the plasmid DNA isolated and transformed into the yeast phospholipid *N*-methyltransferase mutant *opi3-5* (CPBY182) as described in "Materials and Methods." The vector in this library, pFL61 (Fig. 2A, inset), has the yeast orotidine-5′-phosphate decarboxylase (*URA3*) gene, which is required for the biosynthesis of uracil. The *opi3-5* strain is also an uracil auxotroph (Ura^-) due to a mutation in the endogenous *URA3* gene. Positive transformants were obtained by utilizing media that lacked uracil, to select for Ura^+ transformants. The transformants were then screened for the ability to grow at 37°C on media containing MEA. Twenty-one Ura^+ MEA^+ transformants were isolated. Three of the 21 isolates maintained their Ura^+ MEA^+ phenotypes upon rescreening and are referred to as pCB1, pCB2, and pCB3.

To confirm that complementation was due to the cloned cDNA, plasmid loss experiments were performed. This is accomplished through the use of 5-fluoro-orotic acid (5-FOA). 5-FOA is a pyrimidine analog that reacts with orotidine-5′-phosphate decar-

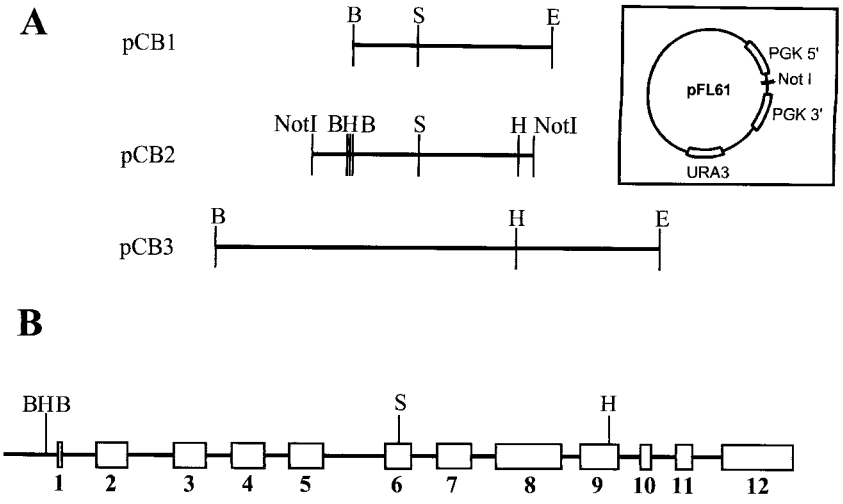

Figure 2. Sequence analysis. A, Partial restriction map of the complementing cDNAs. pCB1 is approximately 1,800 bp, pCB2 is approximately 2,000 bp, and pCB3 is approximately 4,000 bp. N, *Not*I; B, *Bgl*II; H, *Hin*dIII; S, *Sac*I; Inset, pFL61. B, Genomic organization of *AtNMT1*. The *AtNMT1* gene has 11 introns. Boxes denote exons 1 through 12. The coding sequence is 1,673 bp.

boxylase (the *URA3* gene product) to produce a toxic product, 5-fluorouracil (Boeke et al., 1984). *ura3* mutant strains containing a plasmid-borne *URA3* are unable to grow on medium containing 5-FOA because of the toxic byproduct (Boeke et al., 1984). Thus, 5-FOA provides a strong selection pressure in favor of cells that have lost the plasmid-borne *URA3*. The *opi3-5* strains containing pCB1, pCB2, or pCB3 were grown in the presence of 5-FOA as described in "Materials and Methods." Colonies were replica-plated to Ura$^-$ media and to media containing MEA. All three strains had segregants that lost the ability to grow at 37°C on media lacking uracil and containing MEA, indicating that the plasmids had been lost and that the strains' ability to grow on MEA at 37°C cosegregated with the plasmids (data not shown).

Sequence Analysis

Figure 2A is a partial restriction map of the pCB1, pCB2, and pCB3 clones that shows the clones are overlapping. Restriction analyses indicated that pCB1 and pCB3 no longer had the two vector *Not*I sites that should flank the insertions of cDNA (Fig. 2A), whereas pCB2 retained the *Not*I sites (Fig. 2A). The *Not*I sites should be retained in the pFL61 library clones, so pCB1 and pCB3 have undergone some rearrangement in the regions flanking the inserts (Minet et al., 1992). The first 575 bases of the inserts in pCB2 and pCB3 were sequenced using a primer complementary to vector sequence (described in "Materials and Methods") and were found to be identical (data not shown). Sequence for the pCB1 clone was not obtained, indicating that the plasmid flanking sequences may no longer have the site complementary to the primer. However, the restriction maps indicate that pCB1 overlaps pCB2. In addition, pCB1, pCB2, and pCB3 complement the *opi3-5* mutation in an identical manner as described later (see Fig. 4).

The entire sequence from the pCB2 clone (GenBank accession no. AF197940) was used in a DNA BLAST search (Altschul et al., 1997) of the GenBank database (at NCBI http://www.ncbi.nlm.nih.gov/BLAST/). The search revealed 12 sequences from the same region of Arabidopsis chromosome III with similarity to pCB2. Each sequence was identical to part of the pCB2 cDNA clone (data not shown). The probability of all 12 of these sequences occurring in the correct order in the same DNA fragment (pCB2) randomly is so small as to be zero. Therefore, the retrieved sequences must be exons from the genomic copy of the gene. The sequence for this region of chromosome III (GenBank accession no. AB019230) was analyzed using the Arabidopsis Sequence Table for chromosome III provided by The Arabidopsis Database (AtDB). The gene was found to be in the MEB5 region, as sequenced by the Kazusa team (http://genome-www.stanford.edu/Arabidopsis/). The Kazusa Genome Displayer at AtDB helped to identify 11 introns and 12 exons that when linked together matched the sequence of the pCB2 cDNA clone (Fig. 2B). The gene encoded by pCB1, pCB2, and pCB3 will be referred to as Arabidopsis P-EA-Met one (*AtNMT1*) and the GenBank accession number for the complete cDNA is AF197940.

The predicted sequence of 491 amino acids from the pCB2 cDNA is shown in Figure 3. The amino acid sequence contains two potential SAM-binding domains according to the criteria determined by van Gemen and van Knippenberg, one located near the N-terminal and referred to as the N-terminal SAM-binding domain, and one located closer to the C-terminal and referred to as the C-terminal domain (van Gemen and van Knippenberg, 1990). A SAM-binding domain is composed of four motifs: I, post-I, II, and III. Each motif is separated by a conserved number of amino acids (van Gemen and van Knippenberg, 1990). The consensus sequence for motif I is (LIV)-(VL)-(ED)-x-G-(APC)-G-x-G-(LI)-x-(LIM) (van Gemen and van Knippenberg, 1990), where x is any amino acid. Multiple amino acids within the parentheses means that at that position in the protein, any one of the amino acids within the parentheses satisfies the requirement for the consensus sequence. It is surprising that the SAM-binding domains or the *AtNMT1* protein were not similar to either of the PLMTs encoded by the *S. cerevisiae OPI3* and *CHO2* genes (Summers et al., 1988; McGraw and Henry, 1989) or the PLMT encoded by the *Schizosaccharomyces pombe CHO1* gene (Kanipes et al., 1998; Table I). The *AtNMT1* sequence was used in a protein BLAST search (Altschul et al., 1997) of the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). The *AtNMT1* protein was somewhat similar to more than 70 methyltransferases (data not shown). Homology was limited to the SAM-binding domain and the highest similarity to the methyltransferases was in motif I of the SAM-binding domain. Eleven of the methyltransferases that are the most similar, 40% identical or more, are shown in Table I.

Phenotypic Analysis of *opi3* and *cho2 opi3* Strains Transformed with the Complementing Plasmids

If the plasmids do indeed encode a complementing DNA, then they should be able to complement an *opi3* null mutation. Therefore, the complementing plasmids were transformed back into the *opi3-5* mutant strain (CPBY182) and into another strain containing the deletion mutation *opi3*Δ*2* (PMY231). The phenotypes of these transformants can be seen in Figure 4. The complementing plasmids allowed strains containing an *opi3* mutation to grow on media containing MEA at 37°C (Fig. 4A).

There is a second growth phenotype that *opi3* strains exhibit. Ongoing synthesis of PC or its immediate precursor phosphatidyldimethylethanolamine (PD) is necessary for repression of the gene for inositol-1-phosphate synthesis (*INO1*). If synthesis of

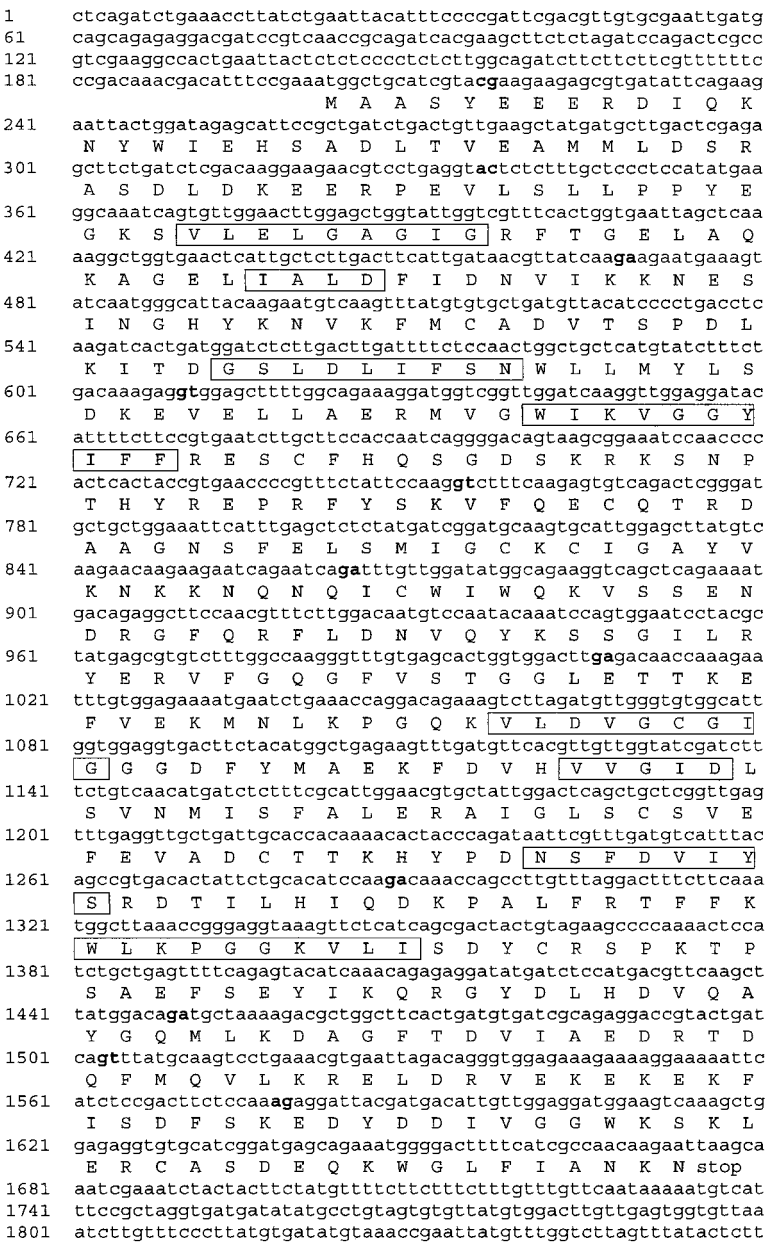

Figure 3. AtNMT1 nucleotide sequence. Shown is the nucleotide sequence of the *AtNMT1* gene (complete cDNA sequence GenBank accession no. is AF197940) and the deduced amino acid sequence using the one-letter abbreviations for the amino acids. The boxed sequences of the *AtNMT1* gene product are two putative SAM-binding domains. The first domain is in the N-terminal portion of the protein from amino acid 57 to 156. Within the SAM-binding domain, there are four motifs: Motif I (positions 57–65), post-I (positions 79–82), Motif II (positions 118–126), and Motif III (positions 147–156). The second putative SAM-binding domain is in the C-terminal portion of the protein from amino acid 286 to 383: Motif I (positions 286–294), post-I (positions 308–312), Motif II (positions 347–354), and Motif III (positions 374–383). The bold letters in the nucleotide sequence denote the intron/exon boundary.

PC or PD is not taking place at a substantial rate, as in *cho2* or *opi3* mutants, *INO1* transcription is not repressed and inositol is excreted. This creates a screenable growth phenotype referred to as Opi$^-$ (Greenberg et al., 1982; Summers et al., 1988; McGraw and Henry, 1989). Because the *opi3* strain is excreting inositol, an inositol auxotroph can grow in a ring around a patch of the *opi3* strain. If the *opi3* mutation has been complemented, then the ring is eliminated because regulation of *INO1* has been restored. When the *opi3*Δ*2* (PMY231) strain transformed with the cloned cDNAs was analyzed for the Opi$^-$ phenotype, the complementing plasmids did not eliminate the Opi$^-$ ring (Fig. 4B).

The Cho2 PLMT has a limited ability to catalyze the two terminal Opi3 PLMT methylations, so a low level of methylated lipid is produced that appears to be adequate for survival of *opi3* mutant strains (McGraw and Henry, 1989). The Opi3 PLMT can substitute at low efficiency for the Cho2 PLMT and produce reduced levels of PC. Only strains containing *opi3* and *cho2* mutations are choline auxotrophs. The double mutation causes a complete block in synthesis of methylated lipids. As a consequence, the strain does not grow unless some capacity for synthesis of methylated lipids is restored or the strain is provided with one or more of the soluble precursors of the methylated phospholipids so that the CDP-choline pathway can be utilized (Summers et al., 1988; McGraw and Henry, 1989). Therefore, the complementing plasmids were further characterized in a double mutant strain.

Table I. *Comparison of known methyltransferases*

Bold letters indicate amino acids that are conserved between proteins; lower-case letters indicate amino acids that do not match the consensus sequence; capital letters indicate amino acids that match the SAM-binding-domain Motif I consensus sequence (van Gemen and van Knippenberg, 1990). SCMT, Sterol *C*-methyl transferase; NAMT, *N*-adenosyl (rRNA) dimethyl transferase; PLMT, phospholipid *N*-methyl transferase; Sc, *S. cerevisiae*; Sp, *S. pombe*; Kvl, *Kluyveromyces lactis*; At, Arabidopsis; Nt, tobacco; Ta, wheat; Gm, soybean; Zm, maize; Os, rice; sub. japonica; Rc, *Ricinus communis*.

Species/qa(Genbank no.)	SAM-Binding Domain Motif I	Function	% Identity to *AtNMT1* *N*-terminal	% Identity to *AtNMT1* *C*-terminal
SpCHO1 (CAA21286)	LtklanGdskkacyM	PLMT	6.7%	6.7%
ScCHO2 (A28443)	LLEkcialAiISnff	PLMT	13.4%	20%
ScOPI3 (AAA34851)	LtkmcgGarkgcymL	PLMT	6.7%	13.4%
ScDIM1 (AAA57357)	VLEvGPGtGnLTvrI	NAMT	46.7%	40%
SpDIM1 (CAB58154)	VLEvGPGtGnLTvrM	NAMT	46.7%	40%
KvlDIM1 (CAA92586)	VLEiGPGtGnLTvrI	NAMT	46.7%	33.3%
AtPFC1 (AAC09322)	VLElGAGkGkLTtmL	NAMT	66.7%	33.3%
AtNMT1 N-terminal	VLElGAGiGrfTgeL		–	40%
AtNTM1 C-terminal	VLDvGCGiGgggdfy		40%	–
NtSMT (U81312)	VLDvGCGiGgplreI	SCMT	40%	66.7%
TaSMT (U60755)	VLDvGCGiGgplreI	SCMT	40%	66.7%
GmSMT1 (U43683)	VLDvGCGiGgplreI	SCMT	40%	66.7%
ZmSMT (AF045570)	VLDvGCGiGgplreI	SCMT	40%	66.7%
OsSMT (AF042332)	VLDvGCGiGgplreI	SCMT	40%	66.7%
RcSMT (U81313)	VLDvGCGiGgplreI	SCMT	40%	66.7%
Motif I Consensus	VLExGAGxGxLTxxL IVD..P..A.IS..I L..... C........M			

A strain containing the *cho2*Δ*1* deletion mutation was mated with a strain containing the *opi3*Δ*2* deletion mutation. Diploids were selected and sporulated. A haploid spore was selected that contained the *cho2*Δ*1* and *opi3*Δ*2* mutations (CPBY19). The *cho2*Δ*1 opi3*Δ*2* strain was transformed with the pCB plasmids and single transformants were isolated and characterized. The strains were analyzed on media with and without choline and the results are shown in Figure 5. The *cho2*Δ*1 opi3*Δ*2* strain alone did not grow on media lacking choline, but did grow on media supplemented with choline (Fig. 5). In contrast, the *cho2*Δ*1 opi3*Δ*2* strain transformed with the pCB plasmids did grow in the absence of choline (Fig. 5). Therefore, the plant gene encoded by the plasmids conferred the capacity to synthesize methylated phospholipid(s).

Phospholipid Analysis

Yeast cells with PLMTs incorporate the ^{14}C-methyl group from exogenously added [*methyl*-^{14}C]Met into the lipid head group to form methylated lipids, most notably PC. To more clearly define the action of the *AtNMT1* cDNA gene product, a 30-min pulse-labeling analysis of methylated lipids was performed on wild type, *cho2*, *opi3-5*, *opi3-5* (pCB2), and *opi3*Δ*2 cho2*Δ*1* (pCB2). Figure 6A shows that the cloned *AtNMT1* cDNA dramatically reduced the level of PM in the *opi3-5* strain. It is curious that the amount of PC produced was not significantly higher than in the *opi3-5* control (without the plasmid copy of *AtNMT1*).

The determination of methylated lipid composition requires only a 30-min pulse labeling. If the cloned gene encodes a plant *N*-methyltransferase that is not working at peak efficiency in a heterologous yeast system, 30 min may not be enough time to produce labeled PC. A determination of PC content after incorporation of radiolabel has reached a steady state would be more reliable in detecting a slower production of PC. Therefore, *opi3-5* cells containing *AtNMT1* were grown in selective medium in the presence of ^{32}P-orthophosphate overnight, as described in "Materials and Methods. This allows labeling of phospholipids to reach a steady state.

Figure 6, B and C show autoradiographs of phospholipids separated on a two-dimensional paper chromatogram (described in "Materials and Methods"). The *opi3-5* strain (Fig. 6B) had PM as the major contributor to total phospholipid content and contained no detectable amounts of PC, in agreement with previously published results (McGraw and Henry, 1989). In contrast, *opi3-5* containing *AtNMT1* (Fig. 6C) produced significant PC, yet also had a high level of PE. No PM was detected.

SAM:Phospho-Base Methyltransferase Activity

The intermediates in PC production, PM and PD, were not produced in the *opi3-5* strain containing *AtNMT1* at a level comparable to wild type (Fig. 6A). Therefore, it was unlikely that AtNmt1p would be catalyzing the sequential methylation of PE to form PC. Yet the presence of *AtNMT1* in the *opi3*Δ*2 cho2*Δ*1* strain relieved choline auxotrophy (Fig. 5). It is clear

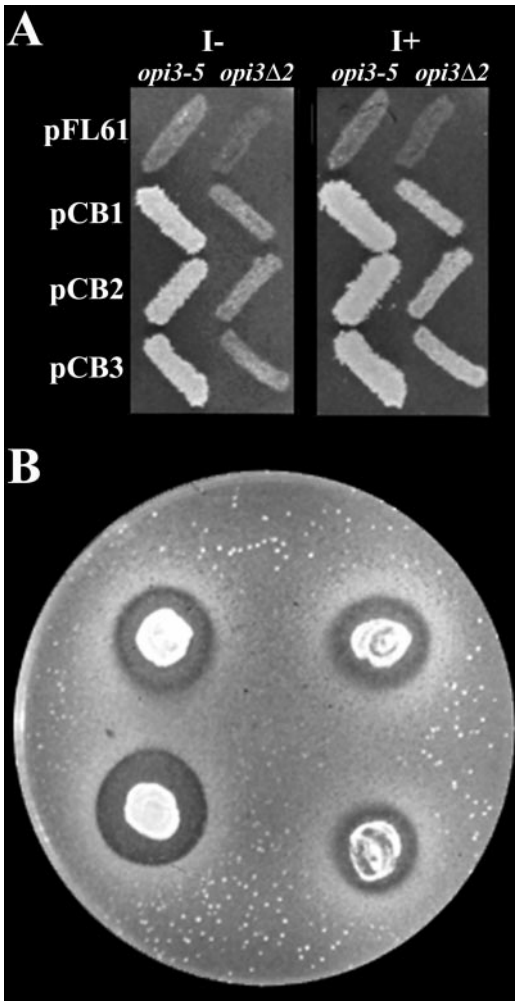

Figure 4. Complementation analysis of Opi3$^-$ phenotypes. A, The *opi3* strains transformed with the pCBs grow at 37°C in the presence of MEA. The pCBs were transformed into two separate yeast strains as described in "Materials and Methods," each of which contained a different mutation in *opi3*. The *opi3-5* strain is CPBY182 and the *opi3*Δ*2* is PMY231. Media composition is described in "Materials and Methods." B, The pCBs do not restore inositol regulation in an *opi3*Δ*2* strain. Yeast strain *opi3*Δ*2* containing the pCBs was grown on I$^-$ medium (see "Materials and Methods") at 30°C for 16 h. The plate was then sprayed with an inositol auxotroph (see "Materials and Methods") and was incubated another 16 h at 30°C as described in "Materials and Methods."

that some PC was being produced from a pathway other than the sequential methylation of PE. Methylation of P-EA has been demonstrated in each plant system studied, leading Datko and colleagues to postulate that the methylation of P-EA may be the common starting point for PC biosynthesis in higher plants (Datko and Mudd, 1988a, 1988b; Fig. 1). Therefore, the *opi3*Δ*2* *cho2*Δ*1* strain containing *AtNMT1* was analyzed for the ability to methylate P-EA, phospho-monomethylethanolamine (P-MEA), and phospho-dimethylethanolamine (P-DEA).

Figure 7 is an autoradiograph of the products from the SAM:phospho-base *N*-methyltransferase assay. The *opi3*Δ*2* *cho2*Δ*1* (pCB2) and wild-type strains with no substrate added (water was added in place of substrate) produced no phospho-bases (Fig. 7, lanes 1 and 5). The *opi3*Δ*2* *cho2*Δ*1* (pCB2) strain using the substrate P-EA produces all three phospho-bases (Fig. 7, lane 2). The *opi3*Δ*2* *cho2*Δ*1* (pCB2) strain using the substrate P-MEA produces P-DEA and phosphocholine (Fig. 7, lane 3) and using the substrate P-DEA, produces only phospho-choline (Fig. 7, lane 4). The rate of methyltransferase activity corresponding to these products is summarized in Table II. The highest rate of methyltransferase activity, 13.4 nmol min^{-2} mg^{-1} protein, occurred with P-EA as the substrate. The lowest rate of methyltransferase activity, 8.5 nmol min^{-2} mg^{-1} protein, occurred with P-DEA as the substrate.

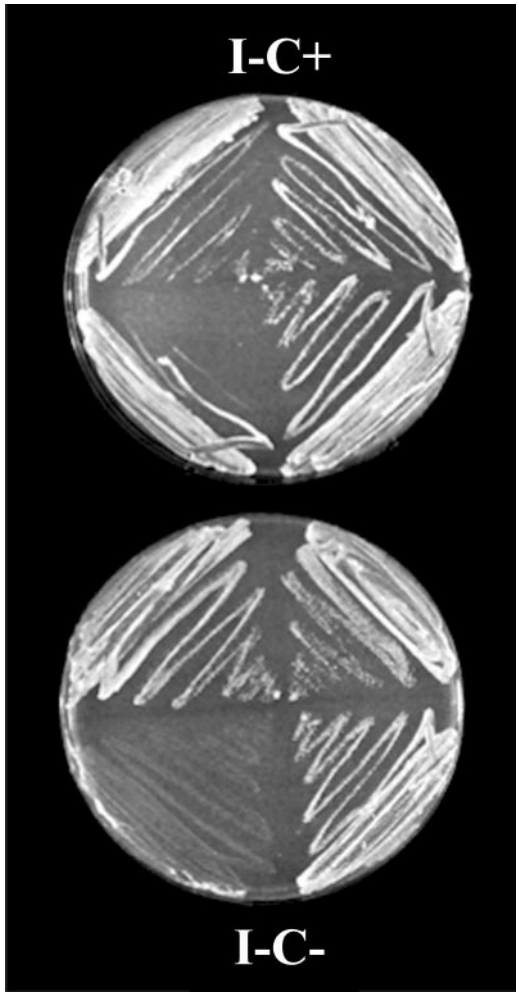

Figure 5. The complementing plasmids relieve choline auxotrophy in a *cho2*Δ*1* *opi3*Δ*2* strain. The *cho2*Δ*1* *opi3*Δ*2* strain (CPBY19) was constructed and transformed with the pCBs as described in "Materials and Methods." Each plate contains (from top left, clockwise): *cho2*Δ*1* *opi3*Δ*2* (pCB2), *cho2*Δ*1* *opi3*Δ*2* (pCB1), *cho2*Δ*1* *opi3*Δ*2* (pCB3), and *cho2*Δ*1* *opi3*Δ*2* (pFL61). Where indicated, the media is supplemented with 1 mM choline (C$^+$).

A

[methyl-^{14}C]Methionine Pulse Labelings

Strain	Media	nmole-^{14}C/μg cells PM	PD	PC
Wild type	I+	22.40	109.00	874.00
cho2	I+	9.83	4.45	172.00
opi3-5	I+	449.00	9.26	18.00
opi3-5 (pCB2)	I+	6.90	0.65	2.18
opi3Δ2 cho2Δ1 (pCB2)	I-C-	0.84	1.68	29.80
opi3Δ2 cho2Δ1 (pCB2)	I-C+	0.81	3.59	1.42

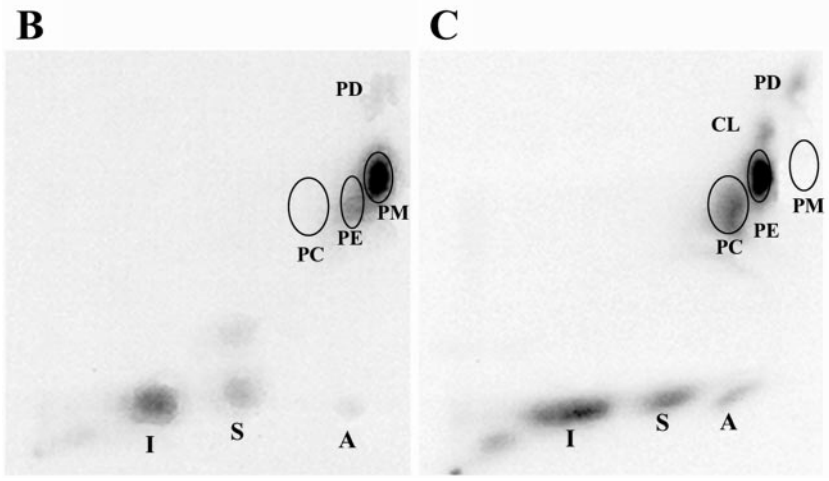

Figure 6. Phospholipid analysis of *opi3-5* strains with and without *AtNMT1*. A, Synthesis of methylated lipids. Cells from the indicated strains were subjected to a 30-min pulse of 0.5 μCi/mL [methyl-^{14}C]Met. The incorporation into methylated lipids was determined as described in "Materials and Methods." B, *opi3-5* and C, *opi3-5* (pCB1), are autoradiographs of two-dimensional paper chromatograms of lipids extracted from the indicated cells after labeling to steady state with 10 μCi of ^{32}P-orthophosphate at 30°C overnight (see "Materials and Methods" for details). I, Phosphatidylinositol; S, phosphatidylserine; CL, cardiolipin; A, phosphatidic acid.

DISCUSSION

The cDNA cloned encodes a previously uncharacterized gene, *AtNMT1*. The deduced protein has a calculated M_r of 56,102 with a predicted pI of 5.39 (Bjellqvist et al., 1993; Bjellqvist et al., 1994; Wilkins et al., 1998). The evidence presented supports the conclusion that AtNmt1p is a SAM:P-EA-Met with capacity to methylate P-MEA and P-DEA in addition to P-EA. This conclusion results from the following interpretations of the data.

Pulse labeling of methylated lipids in the *opi3* (pCB2) strain showed a large decrease in the level of [*methyl*-^{14}C]PM without a concomitant increase in the level of [*methyl*-^{14}C]PC. In addition, the intermediate PD was not produced. Therefore, a mechanism other than restoration of PLMT function was responsible for the decrease in PM (Fig. 6A). When the *AtNMT1* gene was present, the *cho2Δ1 opi3Δ2* cells no longer required supplements for growth, providing strong molecular genetic evidence that methylated lipids are being produced. In addition, the *cho2Δ1 opi3Δ2* strain containing the AtNmt1 methyltransferase produced more PC in the absence of choline (29.8 nmol-^{14}C/μg cells) than in the presence (1.42 nmol-^{14}C/μg cells; Fig. 6A). This is consistent with the findings of Mudd and Datko that the specific activity for P-EA-Met in carrot, soybean, and *Lemna paucicostata* is reduced in the presence of choline (Mudd and Datko, 1989b, 1989c). Phospholipid composition at steady state also showed that AtNmt1p promoted the production of [^{32}P]PC in the *opi3-5* (pCB1) strain. It is therefore curious that in the *opi3-5* (pCB1) strain, [^{32}P]PM is undetectable, whereas [^{32}P]PE accumulates in high amounts. The AtNmt1p cannot be methylating PE because no [^{32}P]PM is detected. Additional support comes from the inability of the AtNmt1p to eliminate the Opi$^-$ phenotype in an *opi3* strain (Fig. 4), indicating that AtNmt1p did not catalyze production of PC or PD at a rate that was fast enough to restore regulation of the *INO1* gene (McGraw and Henry, 1989). Taken together, the data suggest that the action of AtNmt1p is not to degrade the PM, but rather to utilize a precursor to PM in some other way to produce PC.

Plants can produce PC in various ways depending on the species. The main pathway for PC synthesis in the endosperm of *R. communis* (castor bean) is the sequential methylation of EA (Prud'homme et al., 1992). The main pathway in *L. paucicostata* is the sequential methylation of P-EA (Datko and Mudd, 1988a). In carrot, sequential methylation of P-EA and sequential methylation of PM occur at about the same levels (Datko and Mudd, 1988b). In yeast, PC can be made only by the CDP-choline pathway or by sequential methylation of PE. This, combined with the data above, leads to the postulation that the substrate for AtNmt1p is a PE precursor, such as EA or P-EA. Phospho-base methyltransferase assays indi-

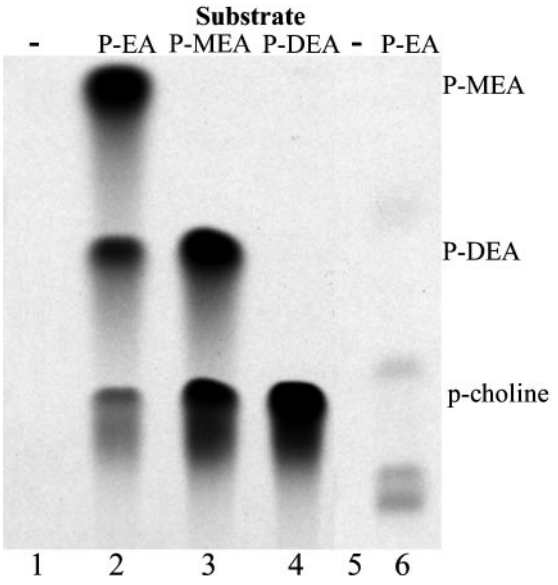

Figure 7. Autoradiograph of radiolabeled P-EA-Met assay products separated and identified by TLC. Lanes 1 through 4 are *opi3*Δ*2 cho2*Δ1 (pCB2); lanes 5 and 6 are wild type (PMY168). The substrate added and product(s) produced are as indicated. Media lacking inositol was inoculated with the *opi3*Δ*2 cho2*Δ*1* (pCB2) yeast strain or wild type (PMY168). Yeast cell extract was prepared and subjected to the assay for phospho-base activity and TLC as described in "Materials and Methods."

cate that AtNmt1p can catalyze the sequential methylation of P-EA to form phospho-choline (Fig. 7A). The rate of production of P-MEA is the highest. The remaining methylations occur at a slower rate (Table II), suggesting that P-EA is the primary substrate for the AtNmt1 (P-EA-Met). In addition, the trend seen here is consistent with the metabolic fluxes determined by McNeil and colleagues (McNeil et al., 2000).

The most direct hypothesis for the in vivo function of AtNmt1p is that it catalyzes the sequential methylation of P-EA to form phospho-choline that is subsequently used in the CDP-choline pathway for production of PC. This model is diagramed in Figure 8.

Table II. *In vitro phospho-base N-methyltransferase activities*

Yeast cell-free extract from the *opi3*Δ*2 cho2*Δ*1* (pCB2) strain was prepared as described in "Materials and Methods." The phosphobase *N*-methyltransferase assays and quantification of radiolabelled methylated phospho-base products were performed as described in "Materials and Methods." The amount of radioactivity corresponding to the phospho-bases was measured by liquid scintillation counting. Each measurement is a sum of all possible phosphobase products.

Substrate	Activity (mean ± SE, $n = 3$)
	nmol min^{-1} *mg*$^{-2}$ *protein*
P-EA	13.4 ± 0.5
P-MEA	10.2 ± 0.1
P-DEA	8.5 ± 0.4

Several features of the unusual metabolic configuration, part yeast and part plant, that are the basis for the "pseudocomplementation" are noteworthy. Based on the fact that the substrate for the AtNmt1p is P-EA, the model postulates that the normal flux of the pathway from P-EA to PE is reversed in a thermodynamic process driven by the removal by methylation of P-EA and the "road block" in the forward direction due to the absence of the PLMTs. This model accounts for the reduced level of PM in the *opi3-5* strain containing *AtNMT1*, as well as the PC produced. In addition, it explains why the level of PC in the *opi3-5* strain is higher than in the same strain carrying *AtNMT1*. In *opi3* mutant strains, the Cho2 PLMT inefficiently catalyzes the two terminal methylations; if the concentration of PM is decreased, the rate of these reactions would also be expected to decrease.

No other gene that might be a PLMT was isolated. Therefore, the in vivo pathway for PC production in Arabidopsis very likely begins with P-EA as outlined here. During final revisions to this submitted manuscript, the cloning of a gene for a P-EA *N*-methyltransferase from spinach was reported (Nuccio et al., 2000). The spinach enzyme also methylates P-EA, P-MEA, and P-DEA, as did the Arabidopsis enzyme reported here. Thus a deeper understanding of the significance and function in vivo of this important class of enzymes is surely imminent.

Hydropathy analysis indicates that AtNmt1p is not hydrophobic (Kyte and Doolittle, 1982; Fig. 9A), does not have an N-terminal chloroplast-targeting signal (von Heijne et al., 1989), and is predicted to have a 65% probability of being a cytoplasmic protein (Nakai, 1991; Nakai and Kanehisa, 1992) consistent with the cytoplasmic localization reported for the characterized P-EA-Mets (Prud'homme and Moore, 1992b; Weretilnyk et al., 1995; Smith et al., 2000). In contrast, all known PLMTs are membrane proteins (Vance and Vance, 1985). The Opi3 PLMT and the Cho2 PLMT

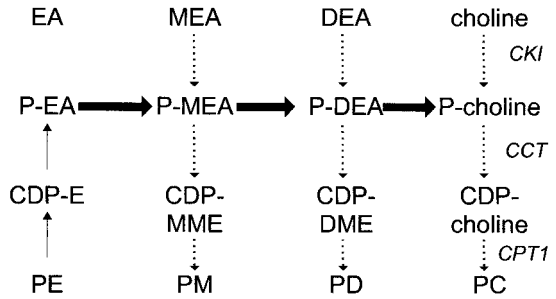

Figure 8. Model for sequential methylation catalyzed by the AtNmt1 *N*-methyltransferase in yeast. Thick arrows indicate steps in sequential methylation that are catalyzed by the AtNmt1 methyltransferase in yeast. Small, dashed arrows indicate the yeast CDP-choline pathway (also CDP-EA, CDP-MEA, and CDP-DEA pathways). Reverse arrows indicate a yeast pathway that has become thermodynamically favorable because the AtNmt1 methyltransferase is using (removing) the P-EA as a substrate for sequential methylation.

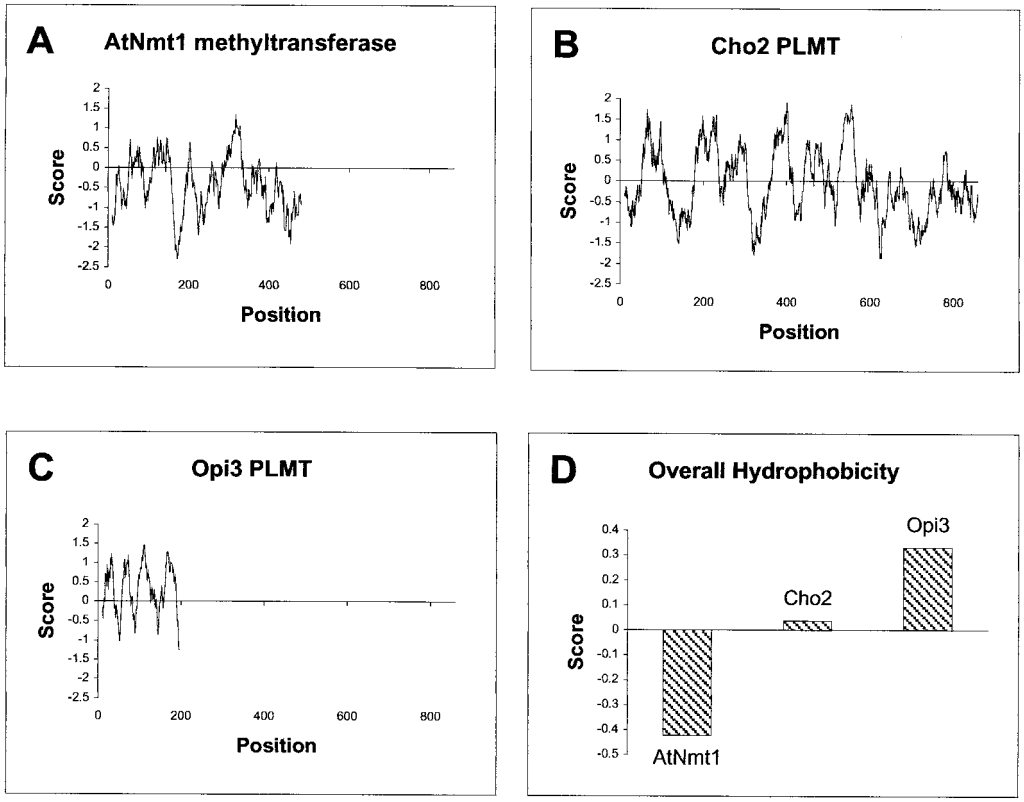

Figure 9. Hydrophobicity analysis. A through C, The hydropathy plots were generated according the criteria established by Kyte and Doolittle (1982) using ProtScale at the Expert Protein Analysis Systems proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/cgi-bin/protscale.pl). Positive hydropathy scores indicate hydrophobic regions. D, The average hydrophobicity was calculated from the values used in A through C to compare the overall hydrophobicity of the methyltransferases.

are very hydrophobic (Kodaki and Yamashita, 1987; Fig. 9, B and C) and have been localized to the endoplasmic reticulum (Kuchler et al., 1986). Cellular localization of AtNmt1p will eventually determine if it is cytoplasmic, as predicted.

A single seven-stranded β-sheet encompassing the SAM-binding domain is characteristic of all methyltransferases (Hodel et al., 1996). According to the criteria determined by McClelland and Rumelhart (McClelland and Rumelhart, 1988), AtNmt1p is predicted to have two β-sheet structures, with the sheet structures grouped as two distinct seven-stranded β-sheets, each encompassing a SAM-binding domain. The presence of SAM-binding domains in the form of a seven-stranded β-sheet indicates that AtNmt1p is capable of using SAM as a methyl donor.

Motif I of the SAM-binding domains in the Opi3 PLMT, the Cho2 PLMT, and the *S. pombe* Cho1 PLMT bear only a slight resemblance to the consensus sequence, whereas the AtNmt1p sequence strongly resembles the consensus sequence. It is therefore not unexpected that little similarity was found between either of the two AtNmt1p SAM-binding domains and the Opi3 and Cho2 PLMTs, or the *S. pombe* Cho1 PLMT, SAM-binding domain.

Two proteins that are quite similar to AtNMT1p, *Caenorhabdits elegans* U64834 and *C. elegans* U39998, which are 50% and 36% identical to AtNMT1p, respectively, have been identified. The function of these proteins is not known; however, they may constitute *C. elegans* P-EA-Mets. Similarity of other proteins to the AtNmt1p was limited to Motif I of the SAM-binding domain (Table I). Motif I of the N-terminal SAM-binding domain of AtNmt1p was most similar to the *N*-adenosyl (rRNA) dimethyl transferase encoded by *AtPFC1*, whereas the C-terminal motif I portion of the SAM-binding domain was most similar to the sterol *C*-methyltransferases (Table I). Although the similarity is strictly limited to motif I of the SAM-binding domain, this does raise the possibility that an *N*-adenosyl (rRNA) dimethyl transferase or a sterol *C*-methyltransferase could substitute for a PLMT. However, there is also evidence against this interpretation. When the *CHO2* and *OPI3* genes were cloned, only the two PLMTs were isolated in two separate cloning endeavors, both using libraries made with high copy number vectors (Summers et al., 1988; McGraw and Henry, 1989). *S. cerevisiae* contains an *N*-adenosyl (rRNA) dimethyl transferase encoded by the *DIM1* gene

(Lafontaine et al., 1994), as well as a sterol *C*-methyltransferase encoded by the *ERG6* gene (Hardwick and Pelham, 1994). If either of these enzymes could substitute for a PLMT at low efficiency and allow complementation, then the genes encoding these enzymes would also have been cloned. Because they were not, an *N*-adenosyl (rRNA) dimethyl transferase or a sterol *C*-methyltransferase is probably not capable of catalyzing the production of methylated lipids.

The present study provides a starting point for the investigation in Arabidopsis of a pathway for sequential methylation of P-EA. In addition, this study motivates the question of whether Arabidopsis uses PLMTs, because it is curious that no PLMTs were cloned. This could be because Arabidopsis does not use PLMTs or it could be due to the growth conditions of the plants that were the source of the mRNA. The presence of choline and inositol in the media are known to regulate the yeast PLMTs (Summers et al., 1988; McGraw and Henry, 1989). The growth media of the Arabidopsis plants used to create the cDNA library did include inositol (555 μM), but not choline (Minet et al., 1992). However, the *SpCHO1* and the *ScCHO2* and *ScOPI3* genes were isolated from libraries made from cells grown on media containing inositol and choline, suggesting that the failure to identify Arabidopsis PLMTs may be because the organism does not have them.

In conclusion the isolated cDNAs encode a SAM: P-EA-Met that catalyzes methylation of P-EA and to a lesser extent P-MEA and P-DEA. The results here demonstrate heterologous complementation is useful as an analytical tool that provided a way to isolate and functionally characterize genes from a higher plant. A better understanding of the metabolic capacity of both organisms has resulted from this work, of interest not only to yeast and plant scientists, but also to bioengineers specializing in quantifying and altering the flux of metabolic pathways.

MATERIALS AND METHODS

Materials

All restriction endonucleases and their corresponding buffers were purchased from New England Biolabs (Beverly, MA). Components for media were purchased from Difco (Detroit), whereas the phospholipid precursors (MEA, DEA, and choline) and *myo*-inositol were purchased from Sigma (St. Louis). Phospholipids were purchased from Avanti (Alabaster, AL) and stored at −20°C as chloroform solutions. The [*methyl*-^{14}C]Met powder (55 mCi/mmol) was purchased from Amersham-Pharmacia Biotech (APBiotech, Uppsala).

Media

Media for growth and sporulation of yeast were as described in McGraw and Henry (McGraw and Henry, 1989). Glc was the carbon source in media, including complete synthetic defined media (SD). Soluble phospholipid precursors were added to the media, where indicated as follows: 1 mM EA, MEA, DEA, or choline. Media containing inositol (I^+) was SD media containing inositol at a concentration of 100 μM. Selective media to retain plasmids was SD media lacking uracil.

Strains

The yeast strain genotypes are described in Table III. CPBY182 was constructed by mating NO155 and PMY168. The strains CPBY34, CPBY35, and CPBY36 were constructed by transforming CPBY19 with pCB1, pCB2, and pCB3.

The Arabidopsis cDNA library from Minet et al. (1992) contained cDNA produced from complete Arabidopsis (Landsberg *erecta* ecotype) seedlings (stage two leaves), including roots, and the seeds were germinated and grown as described by Minet et al. (1992). The library was amplified in *Escherichia coli* and the plasmid DNA isolated and transformed into the yeast phospholipid *N*-methyltransferase mutant *opi3-5* (CPBY182). Transformants were iso-

Table III. *Strain genotypes and plasmid descriptions*

Strain	Genotype	Reference or Source
NO155	*opi3-5 ade5 Mat alpha*	McGraw and Henry (1989)
PMY168	*ura3-52 leu2Δ1 his3Δ200 trpΔ63 Mat a*	Lai and McGraw (1994)
CPBY182	*opi3-5 ura3-52 leu2Δ1trpΔ63 ade5*	This study
PMY231	*opi3Δ2::HIS3 ura3-52 leu2Δ1 his3Δ200 Mat alpha*	K. Robinson
CPBY19	*opi3Δ2::HIS3 cho2Δ1::LEU2 ura3 his3 leu2Δ1 trpΔ63*	This study
CPBY34	CPBY19 (pCB1)	This study
CPBY35	CPBY19 (pCB2)	This study
CPBY36	CPBY19 (pCB3)	This study
PMY179	*ade1/ade1 ino1/ino1 MATa/alpha*	McGraw and Henry (1989)
pFL61	*URA3* yeast/*E. coli* vector	Minet et al. (1992)
pCB1	pFL61 containing Arabidopsis cDNA	This study
pCB2	pFL61 containing Arabidopsis cDNA	This study
pCB3	pFL61 containing Arabidopsis cDNA	This study

lated using selective SD media lacking uracil. The transformants were screened for their ability to grow at 37°C on selective SD media containing 1 mM MEA.

Assay for Methylated Lipid Synthesis

The 250 μCi of [*methyl*-^{14}C]Met powder (APBiotech) was suspended in 500 μL water and stored at 4°C. Two milliliters of exponentially growing cells were labeled for 30 min with 0.5 μCi/mL [*methyl*-^{14}C]Met. Cells were then washed and resuspended in water and were disrupted using a Mini-Bead-Beater (Biospec Products, Bartlesville, OK) and glass beads (0.45–0.52 μm, B. Braun Biotech International, Melsungen, Germany). Lipids were extracted with 2 mL of chloroform:methanol (2:1, v/v). The organic layer was evaporated to dryness by heating to 50°C under a stream of nitrogen. Lipids were resuspended in chloroform and non-radioactive phospholipid standards (Avanti) were added to each sample immediately prior to performing chromatography. One-dimensional chromatography was done as by Waechter and Lester (Waechter and Lester, 1971). The position of radioactively labeled lipids was determined by staining the chromatogram with iodine. The labeled areas corresponding to specific lipids were removed, counted by liquid scintillation, and normalized to the dry cell weight. Dry cell weight was determined by collecting the cells on glass filter paper (GF/B, Whatman, Clifton, NJ) and drying in a vacuum oven (Precision Scientific, Chicago, IL) under vacuum at 80°C for approximately 16 h.

Phospholipid Composition

A culture was prepared in 2 mL of selective SD media lacking inositol (I^-) and containing 10 μCi of ^{32}P-orthophosphate (APBiotech). The culture was incubated at 30°C overnight. The cells were harvested by centrifugation when in exponential phase of growth and resuspended in 1 mL of water. The cells were disrupted with glass beads and lipids were extracted with 2 mL of cholorform:methanol (2:1, v/v). The amount of radioactivity in the organic layer was quantified by liquid scintillation counting of a 50-μL sample. The lipids were then evaporated to dryness under a stream of nitrogen and resuspended in chloroform. The samples were separated by two-dimensional chromatography using the method of Steiner and Lester (Steiner and Lester, 1972).

Preparation of Yeast Cell Extract

Cell extract was prepared by a procedure modified from that described by Carman and colleagues (Klig et al., 1985). I^- media was inoculated with the CPBY35 or wild-type (PMY168) yeast strains and incubated overnight at 30°C. Cells were collected by centrifugation and resuspended in 1 mL methyltransferase buffer 100 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.5, 1 mM disodium EDTA, 0.3 M Suc, and 10 mM 2-mercaptoethanol) per 1 g of cells (Summers and Weretilnyk, 1993). Cells were disrupted using a Mini-Bead-Beater in 30-s bursts with glass beads followed by a 30-s chilling on ice. This was repeated for a total of 1.5 min of disruption. Glass beads and cell debris were removed by centrifugation at 1,500*g* for 5 min. The supernatant was used as the cell extract. The cell extract was immediately frozen in liquid nitrogen. Samples were stored at −80°C.

SAM:P-EA-Met Assay

Yeast cell extract was de-salted by centrifugation through sephadex G25 prior to performing the methyltransferase assay (Smith et al., 2000). SAM:P-EA-Met activities were assayed by P. Summers and E. Weretilnyk using P-EA, P-MEA, or P-DEA as substrates and following their published procedure (Summers and Weretilnyk, 1993). The extracts were suitably diluted with methyltransferase buffer to achieve linear estimates of activity. Thin-layer chromatography (TLC) was used to identify the P-EA-Met assay products using the method described by Smith and colleagues (Smith et al., 2000) with [*methyl*-^{14}C]*S*-adenosyl-Met in the assay.

Inositol Excretion Assay

The *opi3* strains excrete inositol (McGraw and Henry, 1989). To assay this, the strains are gown on I^- media. If the patches of *opi3* strains are sprayed with a yeast strain that is an inositol auxotroph (PMY179), it will be able grow around the *opi3* strain that is excreting inositol (Preitschopf et al., 1993). Therefore, the strains of interest were patched onto I^- plates and allowed to grow for 16 h at 30°C. The plates were then sprayed with the inositol auxotroph (PMY179) and allowed to grow for another approximately 16 h at 30°C, viewed, and photographed.

Plasmid Segregation

The cells were grown overnight in complete SD media to allow a percentage of the population to lose the plasmid. Dilutions were then plated on complete SD media plates containing inositol such that single colonies were easily visible. The cells were then replica plated to medium containing 5-FOA at a concentration of 1g/L to select for the cells that had lost the plasmid (Boeke et al., 1984). Following growth the cells were then replica plated to check the markers, the MEA phenotype, and the inositol excretion phenotype.

Yeast Plasmid Transformation

Exponentially growing cells were used in a polyethylene glycol/LiAc transformation protocol (Schiestl and Gietz, 1989), where 10 μL (approximately 5 μg) of plasmid DNA was used. The transformed cells were plated on selective SD media and incubated at 30°C until single colonies formed.

Plasmid Isolation from Yeast

Yeast cultures were grown overnight in selective SD. The cells were disrupted using a Mini-Bead-Beater and glass beads (0.45–0.52 μm) in 200 μL lysis buffer (2% [v/v] Triton X-100, 1% [w/v] SDS, 100 mM NaCl, 10 mM Tris [tris(hydroxymethyl)-aminomethane]-HCl, pH 8, and 1 mM EDTA). The suspension was then extracted with 1:1 (v/v) phenol:chloroform. A second extraction of chloroform only was performed. The aqueous phase then had an equal volume 5 M NH_4OAc and 2× volume ethanol added to precipitate the nucleic acids. After mixing, the suspension was placed at −20°C overnight. The following day, the suspension was centrifuged, washed with 70% (v/v) ethanol, and resuspended in sterile water.

DNA Sequencing

DNA sequence was obtained by cycle sequencing using the dye terminator method of analysis. The Center of Marine Biotechnology's BioAnalytical Services Laboratory performed all sequencing using a PE Biosystems (Foster City, CA) 310 capillary sequencer. The vector-based primer used was synthesized by Operon (5′-GCATCTAAGAAC TTGA-3′) and was complimentary to the sequence from position 1,167 to 1,182 of the pFL61 plasmid (79 bases prior to the *Not*I site). The primers for sequencing the remainder of the *AtNMT1* cDNA were *AtNMT1* sequenced-based primers.

E. coli Methods

Plasmid DNA isolated from yeast was transformed into $CaCl_2$-competent *E. coli* (RR1; Maniatis, 1982). DNA was isolated (Maniatis, 1982) and subjected to restriction endonuclease digestion for 1 h such that 1 unit of enzyme was used for every 1 μg of DNA (Maniatis, 1982). The digested DNA was separated on a 0.8% (w/v) Tris-borate/EDTA/agarose gel run at approximately 25 V (Maniatis, 1982) for approximately 16 h along with the 1-kb DNA Ladder (BRL, Gaithersburg, MD).

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