Characterization of aminoalcoholphosphotransferases from Arabidopsis thaliana and soybean

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(Received September 23, 1998; accepted March 30, 1999)

Abstract — Aminoalcoholphosphotransferases (AAPTs, EC 2.7.8.1 and EC 2.7.8.2) catalyze the condensation of 1,2-diacylglycerols with CDP-aminoalcohols to form phosphatidylaminoalcohols. Using a soybean (Glycine max) AAPT cDNA (GmAAPT1) as a heterologous hybridization probe, two additional plant AAPT-encoding cDNAs, designated AtAAPT1 and AtAAPT2, were isolated from an Arabidopsis thaliana cDNA library. Southern blot assays suggest that these two cDNAs may represent the only AAPT genes in this species. Heterologous expression of AtAAPT1 and AtAAPT2 in a yeast strain deficient in AAPT activities permitted the determination of substrate specificities of the two Arabidopsis enzymes (designated AtAAPT1p and AtAAPT2p). Although each AAPT isoform was capable of incorporating both CDP-ethanolamine and CDP-choline into phosphatidylethanolamine (PE) and phosphatidylcholine (PC), respectively, AtAAPT2p displayed a somewhat greater preference for CDP-choline over CDP-ethanolamine in comparison to AtAAPT1p. The previously characterized soybean AAPT, GmAAPT1p, and AtAAPT1p showed similar degrees of Ca2+ and CMP inhibition; AtAAPT2p, however, was inhibited to a lesser degree in the presence of these compounds. All three plant AAPTs are capable of catalyzing the reverse reaction, generating CDP-choline and diacylglycerol from PC in the presence of CMP. Finally, overexpression of the soybean AAPT cDNA in transgenic tobacco using a strong constitutive promoter resulted in only modest increases in enzymatic activity, suggesting the possibility of post-transcriptional regulation. © Elsevier, Paris

Cholinephosphotransferase / ethanolaminephosphotransferase / Ca2+ inhibition / CMP inhibition / phospholipid biosynthesis

AAPT, aminoalcoholphosphotransferase / CMP, cytidine monophosphate / PC, phosphatidylcholine / PE, phosphatidyl-ethanolamine

1. INTRODUCTION

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the two most abundant phospholipid constituents of most eukaryotic cellular membranes [1]. PE and PC are structurally similar and the biosynthetic pathways for their synthesis are highly coordinated. In addition to its role as a structural component of biological membranes, PC has many other important physiological and biochemical roles. These include acting as a reservoir for fatty acids and second messengers [12], 1,2-diacylglycerols [32] and as a substrate for desaturation of oleic (18:1) and linoleic (18:2) acids in plants [3]. PE also has several functions in addition to its role as a structural component of membranes. For example, PE can be N-acylated to form N-acylphosphatidylethanolamine which is believed to play a role in membrane stabilization during seed germination [5] and as a source of the signaling molecule N-acyl ethanolamine [7].

In Saccharomyces cerevisiae and mammals, two major pathways are responsible for producing PC: the methylation and the nucleotide pathways [1, 15]. For the methylation pathway, PE is methylated three times by one (mammals) or two (yeast) methyltransferase enzymes to form PC. Three reactions are involved in

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the nucleotide pathway: (a) the phosphorylation of choline by choline kinase; (b) the formation of CDP-choline by the enzyme cholinephosphate cytidylyltransferase; and (c) the condensation of diacylglycerol with CDP-choline by cholinephosphotransferase (CPT, EC 2.7.8.2) to form PC. Under standard growth conditions, the methylation pathway is primarily responsible for de novo biosynthesis of PC in yeast; in contrast, the nucleotide pathway is responsible for the great majority of PC biosynthesis in all mammalian tissues except the liver where the methylation pathway also participates. In plants, in vivo labeling studies suggest that most PC is generated by a single pathway that combines elements of both the traditional nucleotide and methylation pathways [8, 9, 24].

Biosynthesis of PE in eukaryotes can also occur through more than one mechanism. In yeast, PE is produced via the decarboxylation of phosphatidylserine or through a nucleotide pathway similar to that described above for PC [15]. In mammals, minor amounts of PE are also generated through a Ca\(^{2+}\)-dependent base exchange reaction [1]. Although PE formation through all three of these pathways has been demonstrated in higher plants, the nucleotide pathway is believed to represent the major route of synthesis [24].

The terminal steps in the nucleotide pathways of PC and PE synthesis are catalyzed by similar reactions, CPT and ethanolaminephosphotransferase (EPT, EC 2.7.8.1). These enzymes may collectively be referred to as aminoalcoholphosphotransferases (AAPTs). Even though it has been shown that animals and yeast possess distinct enzymes associated with CPT and EPT activities, the situation in plants has been the subject of some debate [22, 24, 34]. The recent characterization of a soybean cDNA (GmAAPT1) encoding for an AAPT that appears to possess comparable CPT and EPT activities suggests that a single plant enzyme can in fact be responsible for both reactions [10]. Because it is not known whether all isoforms of plant AAPTs are similarly bifunctional, an examination of additional plant AAPTs was considered worthwhile. In this study, we describe the isolation and characterization of two AAPT genes from *Arabidopsis thaliana*. By expressing these cDNAs in a yeast strain deficient in AAPT activities, we examined the substrate specificities, Ca\(^{2+}\) and CMP inhibition profiles, and the abilities of these enzymes to catalyze the reverse reaction. In addition, the consequences of overexpressing AAPT-encoding genes in transgenic plants was examined by transforming *Nicotiana tabacum* with the soybean AAPT cDNA.

### 2. RESULTS

#### 2.1. Isolation of cDNAs encoding for two AAPT isoforms from *A. thaliana*

To obtain homologues of the soybean AAPT1 gene from *Arabidopsis*, 250 000 plaques of a cDNA library prepared from aerial tissues of *Arabidopsis* were screened using the soybean cDNA as a heterologous hybridization probe. Five positive plaques were identified, purified to homogeneity, and their respective plasmids recovered. Restriction analysis revealed that four of the cDNAs shared common restriction patterns and differed from each other only in total size. The largest of this group was selected for further analysis and designated *AtAAPT1*. The remaining unique cDNA was designated *AtAAPT2*.

DNA sequence analysis revealed *AtAAPT1* and *AtAAPT2* to be 1 485 and 1 314 bp in length, respectively. Examination of the open reading frames showed that both cDNAs are predicted to encode for polypeptides of 390 amino acids in length. An alignment of the predicted protein sequences of the two *Arabidopsis* enzymes (*AtAAPT1p* and *AtAAPT2p*), soybean GmAAPT1p, and the yeast CPT1p and EPT1p polypeptides is shown in figure 1. The primary amino acid sequences for *AtAAPT1p* and *AtAAPT2p* share 80.5 and 76.9 % sequence identity, respectively, with the soybean GmAAPT1p sequence, and 87.7 % sequence identity with each other (*table I*). *AtAAPT1p* and *AtAAPT2p* also share 97.4 and 87.7 % protein sequence identity, respectively, to the recently characterized AAPT cDNA from *Brassica campestris* (*BcAAPT1* [28]). In yeast, the CDP-aminoalcohol binding domain is believed to lie within residues 79–186 of CPT1p [19]. As shown in figure 1, this is the region of the protein where the greatest sequence conservation is observed among all five AAPTs, suggesting a potential conservation of function.

The yeast CPT1 and EPT1, and soybean and *B. campestris AAPT1* gene products have previously been shown to share predicted structural and topological features. Each polypeptide is predicted to contain seven membrane spanning regions, based on their hydropathy profiles [10, 28]. When *AtAAPT1p* and *AtAAPT2p* were subjected to a similar analysis, conservation of the same predicted membrane spanning regions was also evident (data not shown).

#### 2.2. DNA and RNA blot analysis

Southern blot analysis of *Arabidopsis* genomic DNA using *AtAAPT1* as a hybridization probe sug-
gests that AAPT genes are represented by a very small multigene family in this species. As shown in figure 2A, EcoRI and BamHI digests resulted in hybridization to two and three bands, respectively. Although hybridization to AtAAP1 was not directly confirmed by Southern blotting, the two Arabidopsis cDNAs share high DNA sequence identity (76% overall identity, with up to 90% identity observed in certain conserved regions).

**Figure 1.** Amino acid sequence alignment of five AAPTs. Comparisons were made among the predicted amino acid sequences of AAPTs from Arabidopsis (AtAAP1 and AtAAP2), soybean (GmAAP1 and GmAAP2), and yeast (CPT1 and EPT1). Shaded regions correspond to residues that are conserved among three or more proteins. The region denoted with asterisks represents the putative CDP-aminoalcohol binding domain in CPT1 [19]. GenBank accession numbers for AtAAP1 and AtAAP2 are AF091843 and AF091844, respectively.
regions); therefore, it is expected that cross-hybridization to genomic AtAAP2 sequences would occur under the hybridization conditions used in this experiment. Because of the simplicity of the DNA banding patterns, it is likely that AtAAP1 and AtAAP2 represent the only AAPT-encoding genes in Arabidopsis.

A northern blot analysis of AtAAP1 and AtAAP2 was conducted using gene-specific probes corresponding to the 3′-untranslated regions of the respective cDNAs (figure 2 B). Unlike the highly conserved coding regions, the 3′-flanking sequences of AtAAP1 and AtAAP2 share almost no similarity. As we observed with our previous characterization of the GmAAPT1 cDNA of soybean [10], we were unable to detect a hybridization signal when the northern blots were conducted using total Arabidopsis RNA preparations (data not shown). Hybridization to blots containing purified poly(A)+ RNA, however, demonstrated that each AAPT isoform is expressed in young vegetative tissue of Arabidopsis (figure 2 B).

2.3. Substrate specificities of AtAAP1p and AtAAP2p

We have previously presented evidence that the GmAAPT1p enzyme of soybean appears to possess both CPT and EPT activities, showing little or no preference for either CDP-choline or CDP-ethanolamine [10]. To determine substrate preferences for the Arabidopsis AAPTs, the AtAAP1 and AtAAP2 cDNAs were cloned into a yeast vector that placed their expression under the transcriptional control of the constitutive ADH1 promoter [2]. These constructs were subsequently expressed in the AAPT-deficient yeast strain RK-ec and microsomal membrane preparations derived from these cells were used to assay for enzyme activity. CPT reactions were established by measuring the incorporation of radiolabeled cholinephosphate from [14C]-CDP-choline into PC using endogenous diacylglycerols as the lipid substrate. As a control, microsomal membranes derived from RK-ec containing the empty vector control (pDB20) were similarly assayed. Although a minor amount of CPT activity could be detected in the control preparations, it typically represented less that 5% of the activity observed in the strains expressing the plant AAPT constructs.

The ability of nonradiolabeled CDP-ethanolamine and CDP-choline to competitively inhibit the AtAAP1p and AtAAP2p-catalyzed incorporation of [14C]-CDP-choline into PC is shown in figure 3. Although the generation of [14C]-PC was inhibited for both Arabidopsis enzymes, AtAAP1p appeared to be somewhat more sensitive to CDP-ethanolamine than AtAAP2p. At nearly equimolar concentrations of

<table>
<thead>
<tr>
<th></th>
<th>EPT1p</th>
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<th>AtAAP1p</th>
<th>AtAAP2p</th>
<th>BaAAP1p</th>
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<tr>
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<tr>
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<td>76.9</td>
<td>81.0</td>
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</tr>
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<td>AtAAP1p</td>
<td>87.7</td>
<td></td>
<td>87.7</td>
<td>97.4</td>
<td></td>
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<tr>
<td>AtAAP2p</td>
<td></td>
<td></td>
<td></td>
<td>87.7</td>
<td></td>
</tr>
</tbody>
</table>

Table I. Predicted amino acid sequence identities among known AAPTs. Values shown represent percent identity.

Figure 2. Nucleic acid analysis of Arabidopsis AAPT genes. A, DNA blot using the AtAAP1 cDNA as a probe. Three micrograms of total DNA isolated from vegetative tissues of Arabidopsis were digested with EcoRI (1) or BamHI (2) and loaded on a 0.8% agarose gel. Molecular mass markers are given at the left in kilobases. B, RNA blots using mRNA isolated from young vegetative tissues of Arabidopsis. Each lane represents 5 μg poly(A)+ RNA. Probes were gene-specific and prepared from the 3′-untranslated regions of AtAAP1p (1) and AtAAP2p (2) as described in Methods. Location of the 18S ribosomal RNA is indicated.
radiolabeled CDP-choline (48 μM) and unlabeled CDP-ethanolamine inhibitor (50 μM). AtAAP1p showed a 37% reduction in activity (compared to a 42% reduction using an equivalent amount of unlabeled CDP-choline), whereas AtAAP2p activity was only reduced 29% (in comparison to a 47% reduction using cold CDP-choline).

To enable the direct comparison of the substrate affinities of AtAAP1p and AtAAP2p, CPT and EPT activities were measured simultaneously by incubating the microsomal preparations with equimolar amounts of [14C]-CDP-choline and [14C]-CDP-ethanolamine (at equivalent specific activities) in the same reaction mix. Under these conditions, AtAAP1p was 20% more efficient at catalyzing the incorporation of radiolabel from [14C]-CDP-choline into PC than [14C]-CDP-ethanolamine into PE (table II). The differential was even greater in the reactions catalyzed by AtAAP2p; approximately 44% more radiolabel was transferred from [14C]-CDP-choline into its phospholipid product than was observed with [14C]-CDP-ethanolamine. These results are very consistent with the above described competitive inhibition assays, both of which indicate that AtAAP2p has a modestly higher preference for CDP-choline over CDP-ethanolamine than is observed with the AtAAP1p enzyme.

2.4. Ca2+ inhibition of AAPT enzyme activity in vitro

Previous reports have indicated that Ca2+ can inhibit CPT activity in castor bean [25] and cottonseed [6]. To examine the Ca2+ inhibition profiles for each of the soybean and Arabidopsis AAPTs, microsomal membranes were isolated from yeast cultures expressing each of the plant cDNAs, and incubated with [14C]-CDP-choline in the presence of increasing concentrations of Ca2+ ions. As shown in figure 4, the GmAAPT1p enzyme was inhibited by 30% in the presence of 0.1 mM Ca2+, and its activity reduced by over 95% at 10 mM Ca2+. AtAAP1p showed a similar pattern, with a 30% reduction in activity apparent at 0.1 mM Ca2+ and nearly complete inhibition observed at 10 mM Ca2+. AtAAP2p showed slightly less inhibition than GmAAPT1p and AtAAP1p at 0.1 mM Ca2+ (18%), but like the other plant AAPTs, was severely inhibited at 10 mM Ca2+.

2.5. CMP inhibition of AAPT enzyme activity

AAPTs are generally inhibited by cytidine 5’-monophosphate (CMP), an end-product of AAPT re-
actions. Although both of the yeast AAPTs show CMP inhibition, EPT1p is strongly inhibited by the compound whereas CPT1p shows weak inhibition [17, 19]. Inhibition of CPT activity by CMP has also been demonstrated in animals [14] and plants [33]. As shown in figure 5, the CPT activities of GmAAPT1p, AtAAP TC1p and AtAAP TC2p were all reduced when reactions were conducted in the presence of increasing amounts of CMP. Of the three enzymes, AtAAP TC2p was the least sensitive, showing only a 14 % reduction in activity at 2 mM CMP compared with 60 % and 43 % inhibition for GmAAPT1p and AtAAP TC1p, respectively.

2.6. Reverse reaction of plant AAPT enzymes

CPTs from a variety of sources have been reported to catalyze the reverse reaction (i.e. the formation of CDP-choline utilizing PC and CMP as substrates) [14, 32, 33]. To test whether soybean and Arabidopsis AAPTs are capable of catalyzing the reverse reaction, RK-ec cells expressing the GmAAPT1 and AtAAP TC2 cDNAs (in addition to a control line expressing only the control vector pDB20) were initially incubated with [14C]-methionine to facilitate the synthesis of membranes containing [14C]-PC (see Methods). Microsomal membrane fractions were subsequently isolated, incubated in the presence of CMP, and the reaction products resolved using thin layer chromatography. As shown in table III, microsomes from cells harboring only the pDB20 control vector produced a very small amount of [14C]-CDP-choline. Because the cpt1 and ept1 defects in strain RK-ec are not knockout mutations, this minor amount of product is likely due to some residual yeast CPT activity. Using mi-
crosomes from cells expressing GmAAPT1 andAtAAPT2, much higher levels of [14C]-CDP-choline synthesis were observed than that of the control. During the 60-min duration of the experiment, approximately 13% of the [14C]-labeled PC was converted to CDP-choline in microsomes containing GmAAPT1p, and approximately 23% conversion was observed in the AtAAPT2p microsomes. Although not included in the original experiment, RK-ec cells expressing AtAAPT1 also demonstrated a significant reverse enzymatic activity (data not shown).

Additional assays were conducted using the AtAAPT2p enzyme to determine the CMP dependency of the reaction and the effects of Ca2+ and Mn2+. As shown in table IV, omission of CMP from the reaction mix resulted in nearly an 80% reduction in activity. The fact that any activity was observed is likely due to the presence of endogenous CMP in the microsomal preparations as has been proposed by Slack et al. [33]. Only 13% of the original activity was retained when assays were conducted in the presence of 1 mM Ca2+. This is similar to the level of inhibition observed when the forward reaction was assayed using this same Ca2+ concentration (figure 4). Mn2+ was previously shown to be a more effective cofactor than Mg2+ in stimulating the reverse reaction in rat liver CPT reactions [30]. In contrast to the mammalian enzyme, Mg2+ was the superior cofactor for the AtAAPT2p-catalyzed reaction.

2.7. Overexpression of GmAAPT1 in transgenic tobacco

Agrobacterium-mediated transformation methodologies were used to generate transgenic N. tabacum cv. SR1 plants in which expression of the GmAAPT1 cDNA was driven by the constitutive 35S promoter of the cauliflower mosaic virus. A total of 44 kanamycin-resistant transformants were selected and grown in soil to maturity. Since no visible phenotype was observed (or expected), RNA blots were prepared to detect lines that effectively expressed GmAAPT1. Of several plants that demonstrated significant expression of the GmAAPT1 message, four lines were chosen for further analysis and designated JG2, JG4, JG9 and JG12. These plants were allowed to flower and produce seed for subsequent analysis of the T1 generation.

The results of a northern blot analysis using total RNA isolated from the T1 generation of the four selected transgenic lines and a nontransgenic N. tabacum SR1 control plant are shown in figure 6A. A signal corresponding to endogenous tobacco AAPT mRNAs was not expected in the nontransgenic control since we have only been able to detect endogenous AAPT transcripts using poly(A)+ RNA as discussed above. For the lines overexpressing GmAAPT1, JG2 and JG12 displayed the highest steady-state mRNA levels, JG9 was intermediate, and JG4 showed the least amount of transcript accumulation. Southern blot analysis of the same materials using enzyme digestions that could be considered diagnostic for copy number suggested that each transgenic line possessed either one or two copies of the transgene (data not shown).

CPT assays were conducted on microsomal preparations from the GmAAPT1 transgenic and control

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**Table III.** Reverse reactions using [14C]-labeled microsomes. Cells harboring the control vector pDB20, GmAAPT1 and AtAAPT2 were labeled in vivo with 0.37 MBq [14C]-methyl-methionine (2.5 GBq mmol−1). Microsomal membrane fractions were isolated and 50 µg microsomal protein was reacted in the presence of 0.4 mM CMP. [14C]-PC was measured before (first column) and after a 60-min incubation at 30 °C (next two columns). [14C]-CDP-choline formed after the 60-min incubation is presented in the last two columns. Rep1 and Rep2 represent two independent replications.

<table>
<thead>
<tr>
<th>Microsome source</th>
<th>[14C]-PC (dpm 10−2)</th>
<th>[14C]-CDP-choline (dpm 10−2)</th>
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<tbody>
<tr>
<td></td>
<td>0 min Rep1 Rep2 60 min</td>
<td>0 min Rep1 Rep2 60 min</td>
</tr>
<tr>
<td>pDB20</td>
<td>92.6 85.8</td>
<td>60 min 92.2 1.87 Rep1 11.70 Rep2 2.10</td>
</tr>
<tr>
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<td>67.6 19.20 Rep1 19.20 Rep2 7.70</td>
</tr>
<tr>
<td>AtAAPT2</td>
<td>76.7 58.5</td>
<td>56.6 17.60 Rep1 17.60 Rep2 17.60</td>
</tr>
</tbody>
</table>

**Table IV.** Properties of the reverse reaction using microsomes containing AtAAPT2p. Complete reactions have all the components described in Methods. –CMP reactions have all the components except CMP. +Ca2+ reactions contain all the components of the reverse reaction plus 1 mM Ca2+. +Mn2+ reactions have 20 mM Mn2+ instead of 20 mM Mg2+. Activities are presented as DPM of [14C]-CDP-choline recovered.

<table>
<thead>
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<th>Reaction</th>
<th>[14C]-CDP-choline (dpm)</th>
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<tr>
<td>Complete</td>
<td>6 020</td>
</tr>
<tr>
<td>–CMP</td>
<td>1 310</td>
</tr>
<tr>
<td>+Ca2+</td>
<td>779</td>
</tr>
<tr>
<td>+Mn2+</td>
<td>2 316</td>
</tr>
</tbody>
</table>
plants. An initial time course study indicated that using 75 µg microsomal protein, [14C]-CDP-choline incorporation into PC was linear up to 25 min without the addition of exogenous diacylglycerols (data not shown). To compare enzyme activities among the GmAAPT1 transgenic and control plants, CPT assays were performed for 15 min at 30 °C in the presence of near saturating levels of [14C]-CDP-choline (96 µM).

As shown in figure 6 B, the CPT activity of microsomal preparations derived from JG12 was approximately 2.2-fold higher than that observed using N. tabacum SR1 control microsomes; JG9 and JG2 were about 1.7-fold higher, and JG4 showed approximately 1.4-fold greater activity than the control. The addition of exogenous diacylglycerols to the reactions did not significantly alter the results, reconfirming that the endogenous levels of this substrate were not limiting under these assay conditions (data not shown). Finally, the phospholipid compositions of these same plants were determined by a quantitative gas chromatographic analysis. No major quantitative differences were observed in the relative abundance of PC and PE in the transgenic versus control materials (data not shown), nor in the fatty acid composition of the PC extracted from these plants (table V).

### 3. DISCUSSION

The terminal step in the biosynthesis of PC and PE via the nucleotide pathway is catalyzed by CPT and EPT, respectively. Although the two enzymes catalyze similar reactions, there is considerable evidence that two separate enzymes exist in mammals and yeast. Early work by Kennedy and Weiss [23] showed that in rat liver microsomes, the EPT enzyme was more susceptible to inactivation by lyophilization than the CPT enzyme. The most convincing evidence from mammals, however, comes from the report of the partial purification of the EPT enzyme from rat brain microsomes in which the EPT activity was retained while the CPT activity was inhibited during purification [30].

Isolation of the CPT1 and EPT1 genes from yeast verified the previously obtained biochemical evidence that two separate enzymes catalyze CPT and EPT reactions in this organism. When CPT1p and EPT1p activities were assayed in vitro, it was concluded that CPT1p strictly utilizes CDP-choline as a substrate, whereas EPT1p was capable of catalyzing both CPT and EPT reactions (although CDP-ethanolamine was the preferred substrate) [18]. Initial comparisons of the kinetic parameters and relative activities of the two enzymes lead to the prediction that the EPT1 gene product was responsible for 50% of the total cellular CPT activity in yeast. Studies designed to measure the

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Figure 6. Analysis of transgenic tobacco plants expressing the soybean GmAAPT1 cDNA. A, RNA blot analysis of control (SR1) and transgenic (JG2, JG4, JG9 and JG12) tobacco plants using GmAAPT1 as a hybridization probe. Each lane represents 10 µg total cellular RNA. Location of the 18S ribosomal RNA is indicated. B, CPT assays using microsomes isolated from transgenic and control tobacco plants. Each assay was incubated at 30 °C for 15 min in a reaction mix containing 75 µg microsomal protein and 96 µM [14C]-CDP-choline.

Table V. Fatty acid composition of PC isolated from leaves of SR1, JG2, JG4 and JG12 tobacco plants. Values are expressed as mole percentages.

<table>
<thead>
<tr>
<th>Plant</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
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<td>6.09</td>
<td>9.84</td>
<td>34.3</td>
<td>21.4</td>
</tr>
</tbody>
</table>

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in vivo contribution of each enzyme, however, revealed that CPT1p was responsible for 95% of the cellular CPT activity and EPT1p accounted for only 5% [27]. From these results, it became clear that cellular factors exist that can influence the catalytic activity of EPT1p in vivo that are not apparent when the assays are conducted in vitro. Competitive binding of EPT1p to cellular CDP-ethanolamine and an increased sensitivity to CMP inhibition (compared to CPT1p) are two factors that could reduce its contribution to the overall in vivo CPT activity of the cell [18, 27].

In higher plants, several lines of evidence support the hypothesis that the CPT and EPT reactions are catalyzed by a single enzyme. These include equal inhibition of both enzyme activities after lyophilization [34] and similar CDP-aminoalcohol inhibition profiles [25, 26]. The recent isolation of the soybean AAPT1 cDNA and subsequent substrate inhibition assays resulted in the conclusion that the soybean enzyme is equally capable of utilizing both CDP-choline and CDP-ethanolamine [10]. The complexity of Southern blot profiles, however, suggested the likelihood that other AAPT genes are present in soybean, leaving open the possibility that other AAPT isoforms exist in this species that display more restrictive substrate specificities as has been observed in yeast. The isolation of two Arabidopsis AAPT-encoding cDNAs has enabled us to gain a more comprehensive understanding of the nature of AAPT enzymes in higher plants. Because of the simplicity of the Southern blotting patterns (figure 3) and the previous absence of the Arabidopsis AAPT enzyme, it is likely that AtAAPT1 and AtAAPT2 represent the only AAPT-encoding genes in Arabidopsis. We cannot exclude the possibility, however, that other isoforms may exist, encoded by genes whose nucleotide sequences are not similar enough to AtAAPT1 to have been detected in the Southern blot assay.

Our characterization of the Arabidopsis AtAAPT1p and AtAAPT2p enzymes further supports the hypothesis that higher plants lack AAPTs with very narrow substrate specificities. Similar to the soybean GmAAPT1p, each Arabidopsis AAPT enzyme utilizes both CDP-choline and CDP-ethanolamine as substrates (figure 3 and table II). Nevertheless, modest differences were observed between the two enzymes. AtAAPT2p showed a greater preference for CDP-choline over CDP-ethanolamine (table II) and was less susceptible to CMP inhibition than AtAAPT1p (figure 5). In this respect, it may be argued that AtAAPT2p more closely resembles the yeast CPT1p than does AtAAPT1p, since enhanced affinity for CDP-choline and reduced CMP inhibition are two of the hallmarks that differentiate yeast CPT1p from EPT1p. Because the differential between each of these properties for AtAAPT1p and AtAAPT2p is subtle compared to the differences in these parameters reported for CPT1p and EPT1p, however, it would be incorrect to conclude that the complement of AAPT enzymes in Arabidopsis is comparable to that observed in yeast. Instead, all three plant AAPTs examined in this report differ significantly in at least one major respect from either of their yeast counterparts. Unlike yeast EPT1p that displays a five-fold greater affinity for CDP-ethanolamine over CDP-choline [18], none of the plant AAPTs shows a higher affinity for CDP-ethanolamine; and none excludes CDP-ethanolamine as a substrate, a characteristic of the CPT1p enzyme. Cumulatively, the data suggest that higher plant AAPT reactions are conducted by dual function enzymes. It can therefore be postulated that substrate availability is likely to be the major factor that regulates the flow of metabolites through the final step of PE and PC biosynthesis through their respective nucleotide pathways. Caution should be exercised, however, in drawing conclusions based solely on enzymatic assays conducted in vitro. Until the contributions of the various plant AAPT isoforms can be independently investigated in vivo, the possibility that cellular factors may alter the specificity profile within the cell (as was observed for the EPT1p enzyme; [27]) must be acknowledged.

Net biosynthesis of PC and PE via their nucleotide pathways is believed to be regulated primarily through the enzymatic reactions preceding the AAPT reactions, cholinephosphate cytidylyltransferase and ethanolaminephosphate cytidylyltransferase, respectively [24, 35, 36]. Because AAPT reactions are readily reversible, however, the possibility of regulation via alteration of the directionality of this activity must also be considered as a factor that could influence the PE and PC composition in eukaryotic membranes. This phenomenon may be particularly relevant during seed development where there is considerable evidence that PC acts as an intermediate in the biosynthesis of storage triacylglycerols [3, 4, 32]. Slack et al. [33] proposed that diacylglycerol, a substrate for both PC and triacylglycerol biosynthesis, is in equilibrium with PC, and that this equilibrium is maintained by the reversible reaction of the CPT enzyme.

What factors then control the directionality of AAPT reactions? Calcium availability does not appear to be a good candidate due to the fact that both the forward and reverse reactions are equally inhibited by
this cation (figure 4 and table IV). Of all previously characterized AAPT activators or inhibitors, CMP appears to be the most likely to play such a role, since the availability of this compound favors the reverse reaction. Further investigation of the availability and regulation of endogenous CMP pools, particularly during seed development, is warranted.

Finally, expression of the soybean AAPT cDNA (GmAAPT1) in transgenic tobacco plants did not lead to obvious differences in the PC and PE contents or fatty acid compositions of membrane phospholipids in young tobacco leaves. This result was not surprising considering that there is no evidence in plants that AAPT enzyme levels per se are limiting and that flux through these enzymes is most likely regulated by substrate availability. What was surprising, however, was our inability to increase enzyme activity (as measured in vitro) more than two-fold, despite the fact that a high level of GmAAPT1 transcript accumulation was observed in these plants (figure 6). Because the same GmAAPT1 cDNA insert that effectively lead to high levels of AAPT activity in yeast [10] was used to generate the transgenic tobacco plants, we believe that it is unlikely that the limited increase in enzymatic activity was the result of problems with the fidelity of the GmAAPT1 cDNA. Possible explanations of this phenomenon include an increase in enzyme turnover, or the production of enzyme that is in an inactive form. These alternatives can only be tested by establishing the relative AAPT protein levels in the transgenic plant and control plants. Unfortunately, AAPT s are integral membrane proteins with very few potential antigenic regions and our attempts at raising antibodies against GmAAPT1p have thus far proven unsuccessful. Nevertheless, the transgenic plant results reveal that substantial enhancement of AAPT activities in plants is not likely to be achieved merely through the production of more transcript, suggesting that protein accumulation levels are also regulated by post-transcriptional events.

4. METHODS

4.1. Materials

The Arabidopsis thaliana cDNA library was a kind gift from Dr Ronald Davis [11]. Yeast strain RR-ec (Mata, cpl1, epl1, his3-D1, leu2-3, leu2-112, ura3-52) has been described previously [10]. Cytidine 5'-diphospho[methyl-14C]-ethanolamine (1.9 GBq mmol-1) has been described previously [10]. Cytidine 5'-diphospho[methyl-14C]-choline (2.2 GBq mmol-1) was purchased from ICN Biochemicals. [14C]-methyl-methionine (2.5 GBq mmol-1) was obtained from NEN Biochemicals.

4.2. cDNA library screening

Screening of the Arabidopsis cDNA library was conducted as described by Sambrook et al. [31]. A PstI-EcoRI fragment of pGmaapt119 [10] was used to prepare a [32P]-labeled DNA probe using the Random Prime Labeling Kit as described by the manufacturer (Boehringer Mannheim). Hybridization was conducted at 50 °C overnight in a hybridization solution containing 6× SSPE (1× SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 10× Denhardt’s solution (1× Denhardt’s solution is 0.02 % Ficoll, 0.02 % PVP, 0.02 % BSA), 1 % SDS, and 100 µg·mL-1 salmon sperm DNA. Membranes were washed twice at room temperature in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1 % SDS, and 100 µg·mL-1 salmon sperm DNA. Membranes were washed twice at room temperature in 20× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1 % SDS for 15 min each, followed by a 10-min wash in 1× SSC, 0.1 % SDS at 50 °C. Plaques corresponding to positive signals were subjected to two additional rounds of screening, and purification. Plasmids were recovered as described previously [11].

4.3. DNA sequencing

To facilitate single-stranded DNA sequencing, each candidate cDNA was initially subcloned into pUC119 or pUC120 [37] in both orientations. To obtain complete sequence data for each cDNA, subclones were constructed using internal restriction sites common to both the cDNA insert and multicloning sites of pUC119 or pUC120. For regions where there were still missing gaps in the sequence data, sequence-specific primers were prepared (Genosys). Sequencing reactions were conducted using the Sequenase Kit Version 2.0 (U.S. Biochemical Corp.) Computer analysis of the sequence data and pairwise comparisons with other AAPT s were conducted using programs of the GCG software package (University of Wisconsin Genetics Computing Group). Multiple sequence alignments were conducted using the LINEUP and PILEUP programs; pairwise sequence identities were calculated using the GAP program.

4.4. DNA and RNA gel blot analysis

Total genomic DNA from vegetative tissues of Arabidopsis thaliana cv. C24 (Lehle Seeds, AZ) was isolated as previously described [10]. Three micrograms of Arabidopsis DNA was digested with EcoRI.
and BamHI and separated electrophoretically on a 0.8% agarose gel in a Tris-phosphate-EDTA buffer. Total RNA was isolated from similar tissues and mRNA was prepared using the mRNA Separator Kit (Clontech). Five micrograms of mRNA were loaded on 1.2% formaldehyde agarose gels as described previously [31]. The DNAs were transferred to nylon membranes (Micron Separations, Inc.) and the RNAs were transferred to nitrocellulose membranes (Gibco-BRL) according to standard procedures [31]. For Southern blot hybridizations, an EcoRI fragment of AtAAPT1 representing the entire AtAAPT cDNA was labeled as described above. To generate gene-specific probes for the northern blots, AtAAPT1 and AtAAPT2 were digested with HindIII. Because each plasmid possesses a conserved HindIII site immediately adjacent to the stop codon (and an additional downstream HindIII site is found in the multiple cloning site), digestion with this enzyme enables the precise excision of the 3'-untranslated regions of the cDNAs. Hybridizations were performed at 65°C in 6× SSPE, 1× Denhardt’s, 0.1% SDS, and 100 μg/mL denatured herring sperm DNA. Blots were washed twice for 20 min in 2× SSPE, 0.1% SDS at room temperature, and once for 20 min in 1× SSPE, 0.1% SDS at 65°C.

4.5. Expression of plant AAPT cDNAs in yeast

The AtAAPT1 and AtAAPT2 cDNAs were subcloned into the XhoI site of plasmid pIC-20H. Subsequently, partial digests using HindIII enabled cloning of each complete cDNA into the HindIII site of the yeast expression vector, pDB20 [2]. Expression of foreign genes in the pDB20 vector is mediated by the strong constitutive ADH1 promoter. Restriction analysis enabled the identification of clones for which the AAPT reading frames were in the correct orientation relative to the ADH1 promoter. These constructs were subsequently transformed into the AAPT-deficient yeast strain, RK-ec, by the method of Gietz et al. [13]. Positive transformants were selected on minimal media lacking uracil and expression confirmed by colony autoradiography as described previously [10]. For the enzymatic assays described below, RK-ec transformed with the pDB20 vector alone served as a negative control. Unless otherwise stated, all liquid yeast cultures were grown in YPD media according to standard protocols [16].

4.6. Microsomal membrane isolation from yeast cells

Microsomes were isolated essentially as described [10] with minor modifications. Cultures were initiated in 200 mL YPD and grown to an OD600 of 0.5 to 1. Cells were harvested by centrifugation (3,000 × g for 10 min), washed with sterile deionized water and repelleted. The cells were then brought up in 1 mL GME buffer (20% glycerol, 50 mM MOPS, pH 7.5, 1 mM EDTA) and disrupted using a mini-beadbeater (Biospec Products, Inc., Bartlesville, OK). The homogenate was centrifuged at 14,000 × g for 10 min to remove cellular debris. After adjusting the volume of the supernatant with GME buffer to 12 mL, a microsomal pellet was prepared by centrifugation for 1.5 h at 100,000 × g. The resulting microsomal pellet was brought up in an appropriate volume of 1× GME buffer, and protein content determined using the DC Protein Assay System (BioRad).

4.7. Enzyme assays

Unless otherwise stated, CPT reactions were conducted in 200 μL reaction volumes containing 50 mM Tris-HCl (pH 7.5), 20 mM MgCl2, 2 mM DTT, 2.5 mM EGTA, 48 μM [14C]-CDP-choline (0.19 GBq·mmol⁻¹) and 50 to 100 μg of microsomal protein. Reactions were incubated at 30°C for 30 min (unless otherwise stated), and stopped by the addition of 0.7 mL 0.1 M HCl and 2 mL chloroform/methanol (2/1, v/v). After a thorough mixing and brief centrifugation (1,000 × g for 5 min), the organic layer was removed, dried under nitrogen gas, and counts obtained using scintillation spectroscopy. Thin layer chromatography analysis confirmed that over 96% of the radioactivity co-migrated with authentic PC. For the CDP-aminoacohol inhibition assays, non radiolabeled CDP-choline or CDP-ethanolamine were added as described in the text. For the substrate preference assays, [14C]-CDP-choline (0.19 GBq·mmol⁻¹) and [14C]-CDP-ethanolamine (0.19 GBq·mmol⁻¹) were each added to a final concentration of 24 μM together in a single reaction. Reaction products were loaded on Silica gel 60 plates (Whatman Laboratory Division, Clinton, NJ) and developed in chloroform/methanol/acetic acid (65/35/5, v/v/v). Labeled compounds corresponding to PC and PE were identified by running authentic standards adjacent to the samples, and quantitated using scintillation spectroscopy.

4.8. Reverse reaction

Yeast cells expressing the plant AAPTs or the pDB20 control vector alone were grown in YPD to an
OD600 of 0.2–0.3. Cells were harvested by centrifugation at 3 000 × g for 5 min and resuspended in 25 mL of a synthetic complete media lacking uracil [16]. One hundred microliters of [14C]-methyl-methionine (2.5 GBq·mmol⁻¹) were added to the media and the cells were allowed to incubate with shaking for an additional 2 h at 30 °C. Cells were pelleted and 0.5 mL of the supernatant was removed to determine the efficiency of radiotrace uptake. Typically, 70–80% of the labeled methionine was incorporated. Microsomal membrane preparations were made as described above except the resulting microsomal pellet was resuspended in a buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 0.5 mM EGTA. Reactions were stopped by the addition of an equal volume of chloroform/methanol (2/1, v/v). The aqueous layers were separated by centrifugation at 3 000 × g for 5 min and resuspended in 25 mL ddH2O, samples were loaded on Silica gel 60 plates and developed in a solvent system [29]. Regions that co-migrated with authentic CDP-choline were scraped and quantitated using scintillation spectroscopy.

### 4.9. Generation of transgenic plants

To facilitate cloning of the soybean AAPT1 cDNA (GmAAPT) into the binary vector pBI121 (Clontech), it was first subcloned into the BamHI and NotI sites of pBluescript SK+ (Stratagene). Because GmAAPT1 contains an internal SacI site, partial digests were used to produce a 1.4-kb BamHI-SacI fragment that was subsequently ligated into BamHI-SacI digested pBI121 to generate the binary vector, p35S-GmAAPT. This cloning effectively resulted in the replacement of the GUS reporter gene in pBI121 with the GmAAPT1 reading frame. Agrobacterium tumefaciens strain EH105 (a gift from Dr Stanton B. Gelvin) was subsequently transformed with this construct by the freeze-thaw method [20] and maintained on LB agar media supplemented with 50 μg·mL⁻¹ kanamycin and 10 μg·mL⁻¹ rifampicin. Leaf discs of Nicotiana tabacum cv. SR1 were transformed according to the method of Horsch et al. [21].

## Acknowledgments

We thank Bill Novitzky and Cinthia M. Williford for technical assistance and Dave Monks for critically reviewing the manuscript. This research was supported by a grant from the United Soybean Board.

## REFERENCES


