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The *AAPT1* Gene of Soybean Complements a Cholinephosphotransferase-Deficient Mutant of Yeast

Ralph E. Dewey,[a,1] Richard F. Wilson,[b] William P. Novitzky,[b] and John H. Goode[a]

[a] Department of Crop Science, North Carolina State University, Raleigh, North Carolina 27695-7620
[b] United States Department of Agriculture, Agricultural Research Service, Raleigh, North Carolina 27695-7620

Aminoalcoholphosphotransferases (AAPTases) utilize diacylglycerols and cytidine diphosphate (CDP)-aminoalcohols as substrates in the synthesis of the abundant membrane lipids phosphatidylcholine and phosphatidylethanolamine. A soybean cDNA encoding an AAPTase that demonstrates high levels of CDP-choline:*sn*-1,2-diacylglycerol cholinephosphotransferase activity was isolated by complementation of a yeast strain deficient in this function and was designated *AAPT1*. The deduced amino acid sequence of the soybean cDNA showed nearly equal similarity to each of the two characterized AAPTase sequences from yeast, cholinephosphotransferase and ethanolaminephosphotransferase (CDP-ethanolamine:*sn*-1,2-diacylglycerol ethanolaminephosphotransferase). Moreover, assays of soybean *AAPT1*-encoded enzyme activity in yeast microsomal membranes revealed that the addition of CDP-ethanolamine to the reaction inhibited incorporation of ^{14}C-CDP-choline into phosphatidylcholine in a manner very similar to that observed using unlabeled CDP-choline. Although DNA gel blot analysis suggested that *AAPT1*-like sequences are represented in soybean as a small multigene family, the same *AAPT1* isoform isolated from a young leaf cDNA library was also recovered from a developing seed cDNA library. Expression assays in yeast using soybean *AAPT1* cDNAs that differed only in length suggested that sequences in the 5′ leader of the transcript were responsible for the negative regulation of gene activity in this heterologous system. The inhibition of translation mediated by a short open reading frame located 124 bp upstream of the *AAPT1* reading frame is one model proposed for the observed down-regulation of gene activity.

INTRODUCTION

Phosphatidylcholine (PC) is the major phospholipid constituent of most eukaryotic cellular membranes (Ansell and Spanner, 1982). Investigation of the mechanisms of PC biosynthesis and its regulation in higher plants has been considered important not only because of its role as a structural component of cellular membranes but also because of its involvement in the processes that determine the desaturation content of both membrane lipids and storage triacylglycerols (Ohlrogge et al., 1991; Somerville and Browse, 1991). One of the essential steps in the predominant pathway of PC biosynthesis in higher plants, commonly referred to as the nucleotide pathway, involves the utilization of an aminoalcoholphosphotransferase (AAPTase) localized in the endoplasmic reticulum to convert *sn*-1,2-diacylglycerol to PC using a cytidine diphosphate (CDP)-aminoalcohol as the head group donor. Although traditionally this reaction was considered simply in terms of a single CDP-aminoalcohol substrate (CDP-choline) as the source of the lipid head group, recent work has revealed a more complex picture. In higher plants, it has been shown that CDP-methylethanolamine and CDP-dimethylethanolamine are also physiologically relevant substrates for the AAPTase-mediated reaction. The resultant products (phosphatidylmethylethanolamine and phosphatidyldimethylethanolamine) are then rapidly converted to PC by the action of *N*-methyltransferases (Datko and Mudd, 1988a, 1988b; Prud'homme and Moore, 1992). The specific CDP-aminoalcohol substrate(s) preferentially used in the synthesis of PC can differ significantly among plant species. It is not known whether the same AAPTase is capable of utilizing any of the three CDP-aminoalcohols to synthesize the corresponding phosphatidyl derivative or whether distinct enzymes are involved.

Adding to the complexity of establishing the precise role and defining substrates of AAPTases in plants is the current confusion regarding whether the same enzyme responsible for the production of PC can also utilize CDP-ethanolamine as a substrate in the synthesis of the closely related membrane lipid phosphatidylethanolamine (PE). Although it is clear that separate AAPTases are involved in PC and PE biosynthesis in animals and yeast (Bell and Coleman, 1980; Percy et al., 1984), several studies have suggested that the same enzyme is utilized in the synthesis of both PC and PE in higher plants (Macher and Mudd, 1974; Lord, 1975; Sparace et al., 1981; Justin et al., 1985).

In yeast, two genetic loci have been identified that encode AAPTase activities: *CPT1*, encoding CDP-choline:*sn*-1,2,-diacyl-

[1] To whom correspondence should be addressed.

glycerol cholinephosphotransferase (EC 2.7.8.2), and *EPT1*, encoding CDP-ethanolamine:*sn*-1,2-diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1). The cholinephosphotransferase product of the *CPT1* gene demonstrates minimal ethanolaminephosphotransferase activity (Hjelmstad and Bell, 1987, 1990). The ethanolaminephosphotransferase encoded by *EPT1*, however, has been shown to also possess significant cholinephosphotransferase activity (Hjelmstad and Bell, 1988, 1991). Both enzymes are localized in the membranes of the endoplasmic reticulum. The two yeast gene products share 54% amino acid identity and are predicted to be very similar in secondary structure and membrane topography.

By genetic complementation of a yeast mutant lacking cholinephosphotransferase activity, we isolated a cDNA encoding a soybean AAPTase based on its ability to utilize radiolabeled CDP-choline as a substrate in the synthesis of PC. The predicted amino acid sequence of the soybean gene shows homology similar to both of the yeast AAPTase enzymes: 32.2% identity with the yeast CPT1 protein and 33.0% with the EPT1 polypeptide. The identification of a higher plant AAPTase gene will now enable us to study the function, substrate specificities, and regulation of this important enzymatic reaction.

RESULTS

Complementation of a Yeast Strain Deficient in Cholinephosphotransferase and Ethanolaminephosphotransferase Activities

Previously, yeast strains deficient in cholinephosphotransferase and ethanolaminephosphotransferase activities were obtained from ethyl methanesulfonate–mutagenized cells, and their respective genes were isolated by genetic complementation (Hjelmstad and Bell, 1987, 1988, 1990, 1991). Because mutations at these loci do not result in an easily discernible phenotype, the use of a colony autoradiographic assay was required for both mutant identification and subsequent complementation. The assay for cholinephosphotransferase activity is based on the incorporation of radiolabeled phosphorylcholine from CDP-choline into PC using permeabilized yeast colonies bound to filter paper. Diacylglycerols endogenous to yeast membranes provide the phosphorylcholine acceptors. Radiolabeled phospholipids are fixed to the filter paper, and the unincorporated CDP-choline radiolabel is removed with dilute trichloroacetic acid washes.

The initial goal of this study was to isolate a soybean cDNA that encodes an AAPTase responsible for PC biosynthesis by genetic complementation of a yeast mutant deficient in cholinephosphotransferase activity. Because the yeast *EPT1* gene product displays a cholinephosphotransferase activity that is ~60% as active as its ethanolaminephosphotransferase activity (Hjelmstad and Bell, 1988), it was important that the yeast strain be mutant at both the *CPT1* and *EPT1* loci to eliminate all endogenous cholinephosphotransferase activities. Yeast strains HJ110 and HJ729 are mutant in *CPT1* and *EPT1* gene activities, respectively (Hjelmstad and Bell, 1987, 1988). Because the strains are of identical mating type (MATa), HJ110 was initially crossed to a yeast strain of the opposite mating type (KT1115, MATα), and selected haploids from this cross (*cpt1*, MATα) were crossed to HJ729. A 2:2 segregation (weak signal versus no signal) was observed when haploids from this final cross were screened using radiolabeled CDP-choline in the colony autoradiographic assay (data not shown). Haploids demonstrating a weak signal were presumed to have retained wild-type ethanolaminephosphotransferase activity (*cpt1*, *EPT1*), and those showing no significant signal were presumed to be mutant at both loci (*cpt1*, *ept1*). One of the double-mutant haploids was designated RK-ec and selected for use in the complementation experiments. RK-ec provided an additional advantage in that it is capable of growing on media containing galactose as the sole carbon source, a quality lacking in both HJ110 and HJ729 (R. Dewey, unpublished observation). This trait (obtained from the KT1115 parent) permits the screening of RK-ec using yeast expression vectors that possess galactose-inducible promoters.

Two cDNA libraries were constructed in the yeast expression vector pYES2, one using mRNAs isolated from young soybean leaves and the other using developing seed tissue 25 days after flowering (DAF). The latter material was chosen because of observations that cholinephosphotransferase activity in oilseeds is significantly induced during seed development, concomitant with triacylglycerol accumulation (Slack et al., 1985). The cloning of cDNA inserts adjacent to the galactose-inducible *GAL1* promoter of pYES2 facilitates regulated gene expression. The double-mutant yeast strain RK-ec was transformed with the soybean cDNA libraries and plated onto selective media at a density of ~3000 colonies per 90-mm Petri dish. To repress expression from the *GAL1* promoter, the plating media contained glucose as the sole carbon source. Transformed colonies were replica plated onto galactose-containing media to induce expression of the transgene. Yeast colonies were subsequently transferred to filter paper and subjected to the colony autoradiographic assay using ^{14}C-CDP-choline as the radiolabeled substrate.

Transformed RK-ec yeast colonies were screened for cholinephosphotransferase activity by colony autoradiography. Approximately half of the screenings were conducted using the young leaf cDNA library and half using the 25-DAF developing seed cDNA library. Because the colonies were plated at a high density, the area of the plate corresponding to a putative positive signal was restreaked onto fresh selective media, and the colony autoradiographic procedure was repeated. This second screen eliminated the few false positive signals that occasionally appeared after the initial assay. After screening more than one million yeast transformants, a colony from an experiment using the young leaf library was observed that maintained a positive signal throughout a second and even

a third colony autoradiographic assay. To ensure that the positive signal was attributable to expression of the soybean cDNA and not the result of a genomic reversion of the RK-ec host, the transforming plasmid (designated pGmaapt2) was isolated and retransformed into fresh RK-ec cells. As shown in Figure 1A, the cholinephosphotransferase-deficient phenotype of yeast strain RK-ec was successfully complemented in cells transformed with pGmaapt2. Furthermore, the complemented phenotype showed strict inducibility by galactose, confirming again that the factor responsible for cholinephosphotransferase activity is borne on the pGmaapt2 plasmid.

To verify that the positive autoradiographic signal observed in the pGmaapt2-transformed RK-ec cells was a result of the synthesis of radiolabeled PC, the filter-bound cells were chloroform–methanol extracted, and the in situ reaction products were characterized by thin-layer chromatography. Greater than 98% of the radiolabeled product comigrated with authentic PC (data not shown).

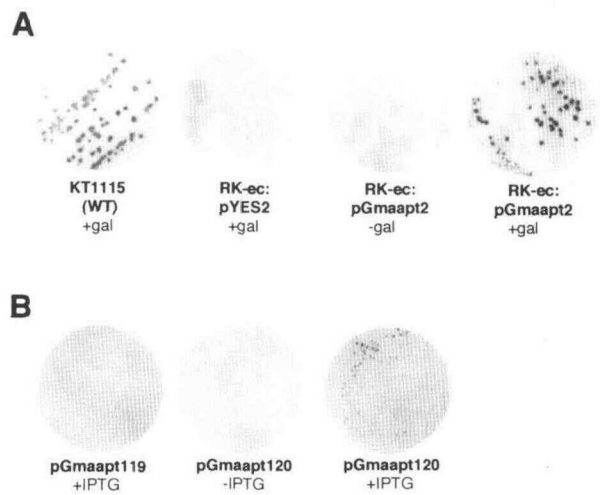

Figure 1. Colony Autoradiographic Assays for Cholinephosphotransferase Activity in Yeast and *E. coli*.

(A) Complementation of yeast strain RK-ec using the soybean *AAPT1* cDNA cloned into yeast expression vector pYES2 (pGmaapt2). Yeast strains KT1115 (wild type for both *CPT1* and *EPT1*) and RK-ec (transformed with pYES2) represent positive and negative controls, respectively. To demonstrate the inducibility of the complementing phenotype, colonies were grown on media containing either glucose (−gal) or galactose (+gal) as the sole carbon source. Filters were exposed to x-ray film for 24 hr.
(B) Synthesis of radiolabeled PC in *E. coli* transformed with the soybean *AAPT1* cDNA cloned into plasmid vector pUC120 (pGmaapt120). *AAPT1* cDNA in the antisense orientation in relation to the *lacZ* promoter was used as a negative control (pGmaapt119). Inducibility of the positive phenotype was demonstrated by the addition of isopropyl β-D-thiogalactopyranoside (+IPTG) to the plating media. Filters were exposed to x-ray film for 5 days. −IPTG, no IPTG added.

Nucleotide Sequence Analysis of pGmaapt2

The nucleotide sequence of the cDNA insert of pGmaapt2, designated *AAPT1*, is shown in Figure 2. A single large open reading frame (ORF) capable of encoding a 389–amino acid long polypeptide was identified within the cDNA. Data base searches comparing the predicted amino acid sequence against the major protein data banks revealed extensive homology to only two different sequences, the *CPT1* and *EPT1* gene products of yeast. Because these two yeast sequences share 54% identity at the predicted amino acid level, it is not surprising that the *AAPT1* gene product would show similarity to both. Interestingly, the soybean polypeptide showed almost equal homology to each of the yeast proteins: 33.0% identity (58.6% similarity) to the yeast *EPT1* gene product and 32.2% identity (57.0% similarity) to the yeast *CPT1* polypeptide. An alignment of the three amino acid sequences is shown in Figure 3A.

Computer analyses of the yeast CPT1 and EPT1 predicted amino acid sequences have previously been used to construct models for the membrane topography and secondary structure of these proteins (Hjelmstad and Bell, 1990, 1991). Hydropathy profile analysis of the yeast proteins suggested that each possesses seven transmembrane domains. As shown in Figure 3B, the hydropathy profile of the soybean AAPT1 polypeptide is very similar to the yeast proteins; the polypeptide contains seven segments of sufficient hydrophobicity and length to span the membrane. In addition to having the same number of predicted transmembrane domains, the three polypeptides share similarity of the relative location of each domain, suggesting a high degree of conservation of membrane topography.

DNA gel blot analysis of genomic soybean DNA using *AAPT1* cDNA as a hybridization probe suggested that the gene may represent a member of a small multigene family. A typical hybridization pattern is shown in Figure 4A. When the stringency of the hybridization and wash conditions was lowered, an additional band was consistently observed.

RNA gel blot analysis of poly(A)$^+$ RNA isolated from young leaves, young stems, young roots, and developing seeds of soybean 25 DAF revealed the presence of a 1.6-kb transcript corresponding to *AAPT1* in each tissue (Figure 4B). Considering the necessity of AAPTases in producing membrane phospholipids in virtually all cell types, it was not surprising that *AAPT1* transcripts were found in each tissue tested. Interestingly, steady state accumulation of *AAPT1* mRNAs was repeatedly observed to be greatest in the roots and stems of young plants.

Expression of Soybean *AAPT1* in *Escherichia coli*

Hjelmstad and Bell (1987, 1988) generated considerable genetic and enzymological data providing evidence that the yeast *CPT1* and *EPT1* loci encode the structural enzymes responsible for cholinephosphotransferase and ethanolaminephospho-

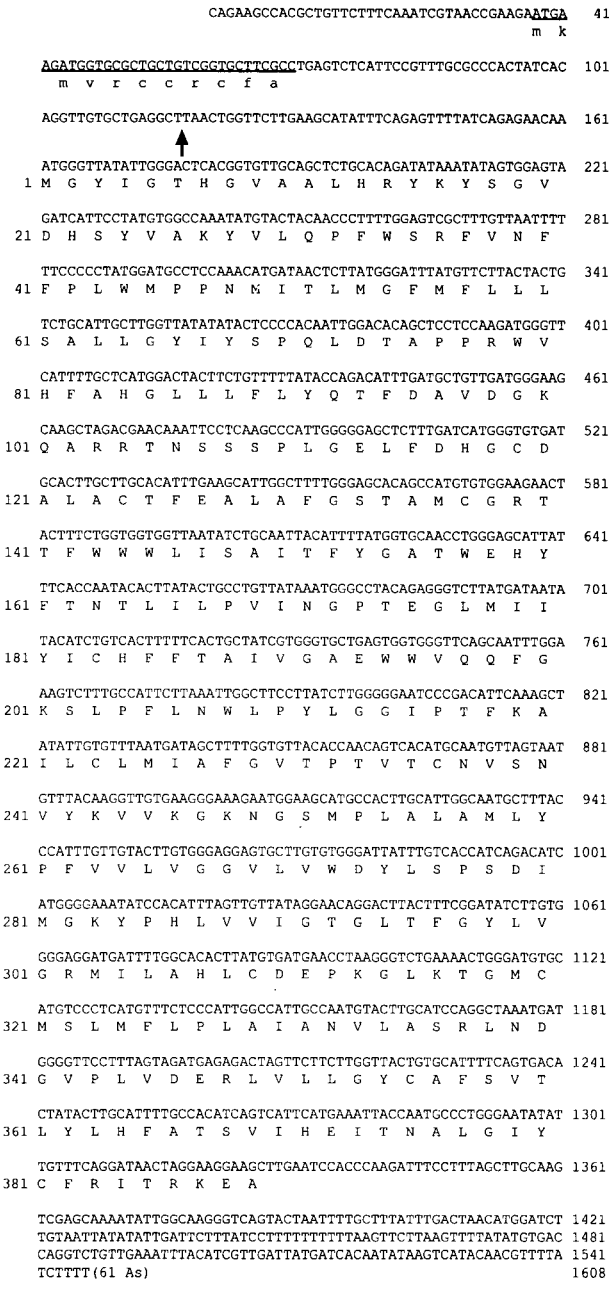

Figure 2. Nucleotide and Predicted Amino Acid Sequences of a Soybean *AAPT1* cDNA.

The sequence presented corresponds to the longest *AAPT1* cDNA characterized in this study (from pBSaapt1.6). An arrow indicates the beginning of the pGmaapt2 cDNA that was identified by complementation of yeast strain RK-ec. The nucleotide sequence of the uORF motif located in the 5′ flanking region of the transcript is underlined (predicted amino acid sequence is in lowercase letters). The GenBank accession number of the *AAPT1* cDNA sequence is U12735.

transferase activities rather than secondary loci that either regulate the activities of the structural genes or act by altering substrate availability. An additional approach for demonstrating gene function is to express the product in a heterologous organism that does not possess the activity of interest. *E. coli* membranes do not contain PC, and the organism completely lacks AAPTase activities.

To facilitate the nucleotide sequencing of the soybean pGmaapt2 clone, the cDNA insert was initially subcloned into *E. coli* vectors pUC119 and pUC120 (see Methods). From the sequence, it was determined that the *AAPT1* reading frame in pUC120 (designated pGmaapt120) was in the sense orientation in relation to the plasmid's *lacZ* promoter and in the antisense orientation in pUC119 (designated pGmaapt119). To test for the ability of the *AAPT1* reading frame to confer cholinephosphotransferase activity in *E. coli*, cells were transformed with pGmaapt120 (and pGmaapt119 as a negative control), transferred to filter paper, and assayed using colony autoradiography. As shown in Figure 1B, pGmaapt120-transformed *E. coli* colonies demonstrated a weak, yet positive signal in the assay; no signal was detected using the pGmaapt119 vector. Furthermore, the cholinephosphotransferase activity was inducible by isopropyl β-D-thiogalactopyranoside, as would be expected from a gene expressed from the *lacZ* promoter. Thin-layer chromatography analysis of the radiolabeled products confirmed that the radiolabel was incorporated into PC (data not shown). The ability of the soybean cDNA to direct synthesis of PC in *E. coli* using ^{14}C-CDP-choline provided compelling evidence that the *AAPT1* gene encodes a structural AAPTase enzyme.

Inhibition of Soybean *AAPT1*-Mediated Cholinephosphotransferase Activity by CDP-Ethanolamine

Several studies have presented evidence suggesting that cholinephosphotransferase and ethanolaminephosphotransferase activities in higher plants may be catalyzed by a single enzyme (Macher and Mudd, 1974; Lord, 1975; Sparace et al., 1981; Justin et al., 1985). Among the data supporting this hypothesis are observations that CDP-choline and CDP-ethanolamine are competitive inhibitors of ethanolaminephosphotransferase and cholinephosphotransferase activities, respectively. By the expression of pGmaapt2 in the heterologous yeast system, we have demonstrated that the *AAPT1*-encoded polypeptide possesses significant cholinephosphotransferase activity (Figure 1). The most direct approach to establishing the degree to which the *AAPT1* gene product may also possess ethanolaminephosphotransferase activity would be to assay its ability to use radiolabeled CDP-ethanolamine in synthesizing labeled PE. Because radiolabeled CDP-ethanolamine is currently unavailable commercially, we tested the ability of unlabeled CDP-ethanolamine to inhibit the cholinephosphotransferase activity of the AAPT1 enzyme as a preliminary step

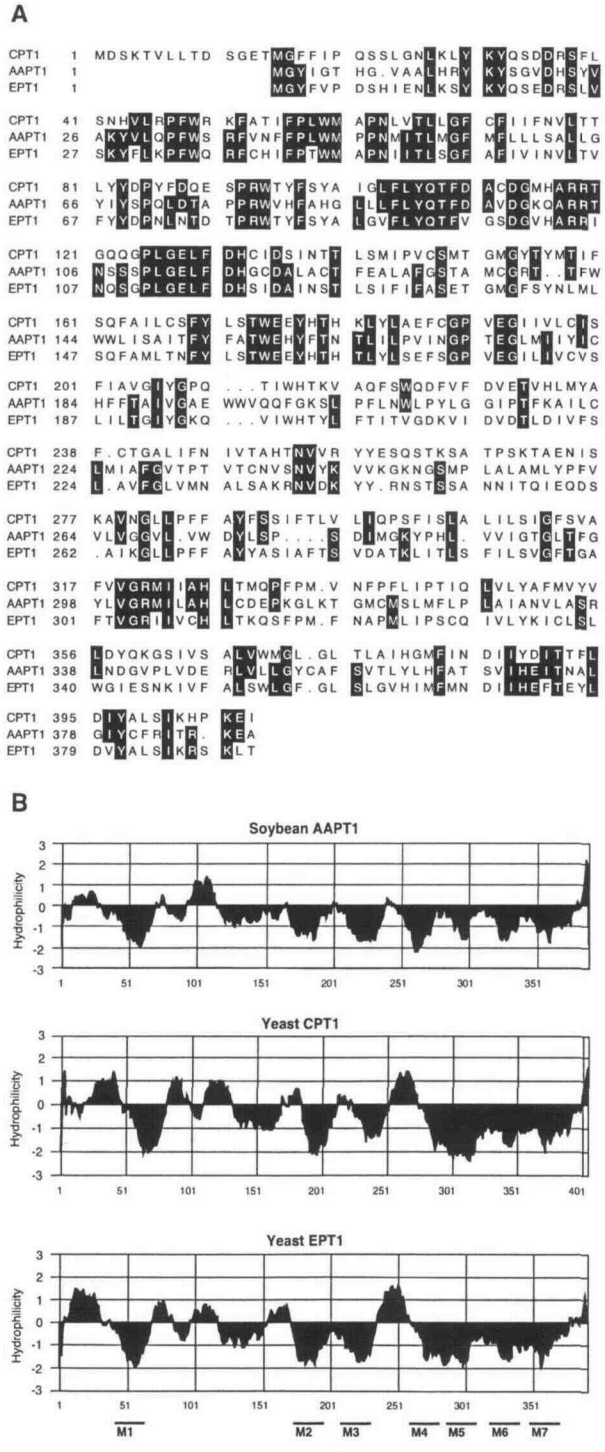

Figure 3. Comparison of AAPTases from Soybean and Yeast.

(A) Alignment of the amino acid sequences derived from the yeast *CPT1*, *EPT1*, and soybean *AAPT1* cDNAs. Identical residues for the soybean AAPT1 protein and the yeast CPT1 protein or the soybean AAPT1 protein and the yeast EPT1 polypeptide are shown on a black background.

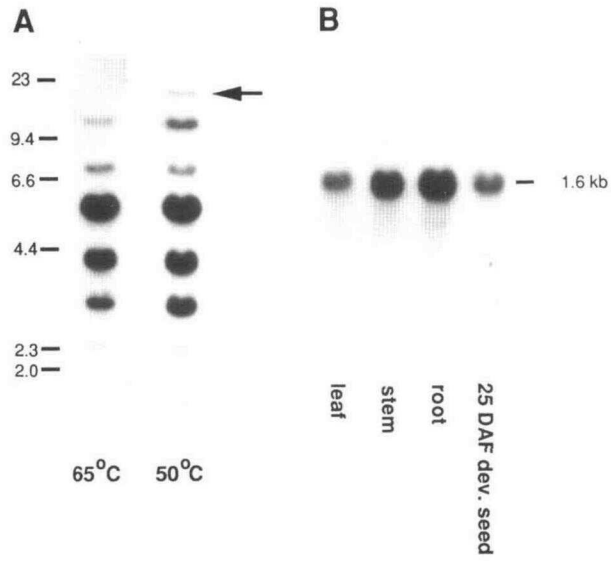

Figure 4. Gel Blot Analyses of Soybean Using an *AAPT1* Probe.

(A) Genomic DNA gel blot of *AAPT1* in soybean. Each lane represents 10 μg of EcoRI-digested genomic soybean DNA hybridized to the *AAPT1* probe under conditions of high (65°C) or low (50°C) hybridization stringencies. The arrow indicates a hybridizing band that is visualized only under low-stringency conditions. Molecular length markers are given at the left in kilobases.

(B) RNA gel blot of *AAPT1* transcripts in different tissues of soybean. Ten micrograms of poly(A)$^+$ RNA was used for each lane. The molecular length of the hybridizing transcript was estimated using RNA size standards (data not shown). 25 DAF dev. seed, developing seeds 25 DAF. The mature 1.6-kb *AAPT1* transcript is indicated.

toward addressing the question of whether the soybean enzyme can catalyze both of the AAPTase reactions.

Microsomal membrane preparations of yeast strain RK-ec transformed with pGmaapt2 were assayed for their ability to synthesize radiolabeled PC using ^{14}C-CDP-choline and endogenous diacylglycerols as substrates. Increasing amounts of nonradiolabeled CDP-choline or CDP-ethanolamine were added to compare the relative effects of each compound in reducing the incorporation of radiolabeled CDP-choline into PC. These results are shown in Figure 5. As expected, reducing the specific activity of the reaction (i.e., increasing quantities of unlabeled CDP-choline) resulted in a continual reduction in the synthesis of radiolabeled PC. Interestingly, a very similar pattern was observed when nonradiolabeled CDP-ethanolamine was used in the assay.

(B) Hydropathy profiles of the predicted amino acid sequences of the soybean and yeast AAPTases. Hydropathic values were calculated and plotted according to the method of Kyte and Doolittle (1982) using a window size of 20 residues. The seven candidate membrane-spanning domains for the yeast EPT1 polypeptide proposed by Hjelmstad and Bell (1991) are indicated as M1 to M7.

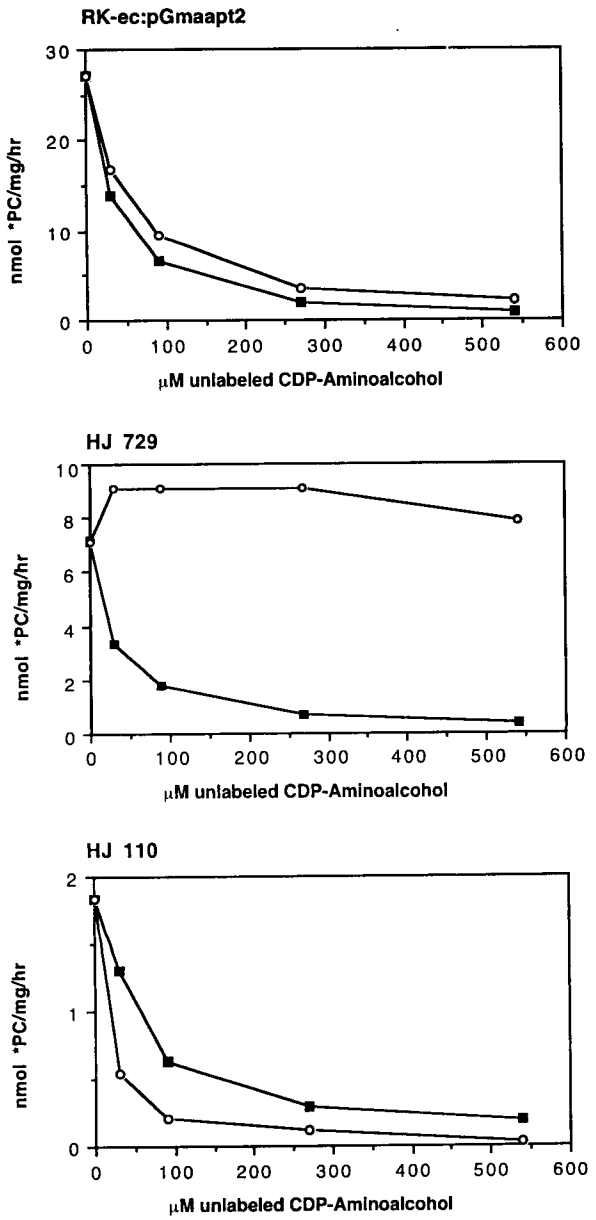

Figure 5. Effect of CDP-Choline and CDP-Ethanolamine on the Incorporation of ^{14}C-CDP-Choline into Phospholipid by Soybean and Yeast AAPTases.

Microsomal membrane fractions were isolated from the following yeast strains: RK-ec expressing pGmaapt2 (*AAPT1*, *cpt1*, *ept1*), HJ729 (*CPT1*, *ept1*), and HJ110 (*cpt1*, *EPT1*). Cholinephosphotransferase assays were conducted for each membrane preparation in the presence of increasing concentrations of unlabeled CDP-choline (■) or unlabeled CDP-ethanolamine (○). Enzyme activity was measured as nanomoles of radiolabeled PC (*PC) produced per milligram of membrane protein per hour.

When the same assay was used to measure inhibition of the cholinephosphotransferase activity mediated by the yeast *CPT1* gene, a much different result was obtained. Yeast strain HJ729 is mutant at the *EPT1* locus but contains a wild-type *CPT1* gene (Hjelmstad and Bell, 1988). Total cholinephosphotransferase activity (nanomoles of ^{14}C-PC per milligram of protein per hour) was approximately four times greater in the RK-ec cells transformed with the soybean gene than was observed using HJ729 (Figure 5). This is most likely a result of the fact that the soybean sequence was expressed from a multiple copy plasmid vector (and a different promoter) in contrast to the single chromosomal copy of *CPT1* in HJ729. Similar to the RK-ec:pGmaapt2 results, increasing concentrations of nonradiolabeled CDP-choline decreased the production of radiolabeled PC. The addition of CDP-ethanolamine to the reaction, however, failed to inhibit synthesis of radiolabeled PC in yeast strain HJ729 (Figure 5). This result demonstrated that the cholinephosphotransferase activity encoded by the yeast *CPT1* gene differs radically from the cholinephosphotransferase activity of soybean *AAPT1* with regard to the ability of CDP-ethanolamine to inhibit the respective reactions.

Yeast strain HJ110 is mutant at the *CPT1* locus, but contains a wild-type *EPT1* gene (Hjelmstad and Bell, 1987). Although *EPT1* encodes an enzyme that is believed to function primarily as an ethanolaminephosphotransferase, significant cholinephosphotransferase activity has also been attributed to this gene product. The rate of ^{14}C-PC synthesis measured using HJ110 microsomal membranes was approximately fourfold less than that observed from HJ729 and 15 times less than the RK-ec:pGmaapt2 membrane preparations. Similar to the results obtained from the CDP-aminoalcohol inhibition profiles of the soybean *AAPT1* gene product, both CDP-choline and CDP-ethanolamine were effective in reducing incorporation of ^{14}C-CDP-choline into PC in assays conducted using HJ110 membranes. In contrast to the soybean *AAPT1*-encoded enzyme, however, the assays for cholinephosphotransferase activity mediated by *EPT1* appeared to be inhibited to a greater extent by CDP-ethanolamine than CDP-choline; at any given inhibitor concentration, the rate of ^{14}C-PC synthesis was two- to threefold less in assays using CDP-ethanolamine as the competitive inhibitor than was observed using CDP-choline (Figure 5).

Cloning of *AAPT1* cDNAs from a Developing Seed cDNA Library

Current models of triacylglycerol biosynthesis in oilseeds predict a specialized role for the enzyme cholinephosphotransferase during seed development by facilitating the temporary mobilization of fatty acids to PC, thereby allowing the polyunsaturation of the acyl chains by the 18:1 and 18:2 desaturases (Ohlrogge et al., 1991; Somerville and Browse, 1991). Because the soybean *AAPT1* cDNA was capable of expressing high levels of cholinephosphotransferase activity in yeast, we considered it

likely that either the *AAPT1* gene or a gene closely related to it is responsible for the cholinephosphotransferase activity observed during this important stage of development. DNA gel blot assays suggested that *AAPT1*-like sequences may exist as a small multigene family (Figure 4A); therefore, it is possible that a different isoform of the gene is expressed during storage oil biosynthesis than that used for the production of membrane lipids in young leaves (the tissue from which the pGmaapt2 cDNA was derived).

To determine whether a different isoform of *AAPT1* is expressed during seed development, a λZAPII cDNA library generated from 25-DAF developing soybean seed mRNAs was screened using the pGmaapt2 cDNA insert as a hybridization probe. Hybridizations were conducted at low stringency in case significant divergence had occurred between the leaf- and seed-specific members of the gene family (and because the DNA gel blot data indicated that an additional band could be visualized at low-stringency conditions). *AAPT1* hybridizing plaques were fairly abundant in the library, occurring at a frequency of approximately one in every 7000 plaques screened (data not shown). Eight positively hybridizing plaques were purified to homogeneity, and their corresponding plasmids were excised. Although the cDNA inserts varied somewhat in total length (ranging from 1.2 to 1.6 kb), a detailed restriction enzyme analysis revealed no differences in the restriction profiles of the various clones for any enzyme tested, and neither were differences in hybridization intensities observed using the *AAPT1* cDNA probe in DNA gel blot assays (data not shown). To further characterize the seed-specific *AAPT1* cDNAs, each clone was subjected to limited nucleotide sequence analysis. More than 350 bases of nucleotide sequence information were obtained from both the 3′ and the 5′ ends for each clone (data not shown). Three of the eight individuals were assumed to be duplicate copies of other of the cDNAs (resulting from the amplification of the cDNA library) as judged by perfect identity in both size and sequence. For each of the five unique cDNAs, the length of the poly(A)$^+$ tails varied, and some minor variation in the fidelity of where the polyadenylation initiated was observed. At the 5′ end of the cDNAs, the only variations detected were in total length. Otherwise, the nucleotide sequences of each clone were identical not only to each other but also to the original *AAPT1* cDNA isolated from the young leaf–specific library.

The complete nucleotide sequence of the longest *AAPT1* clone isolated from the developing seed library, designated pBSaapt1.6, was determined and is shown in Figure 2. The *AAPT1* cDNA sequence from pBSaapt1.6 differed from the pGmaapt2 insert only in the lengths of the 5′ leader sequence and poly(A)$^+$ tail. The inability to detect nucleotide sequence differences between pGmaapt2 and pBSaapt1.6 and in more than 700 bases of nucleotide sequence (5′ and 3′ end information combined) among each of four additional independent cDNAs suggests that the same *AAPT1* isoform recovered from a young leaf–specific cDNA library is likely to be the predominant, if not sole, *AAPT1* gene that is expressed during seed development.

Evidence of Post-Transcriptional Regulation of *AAPT1*

In addition to providing evidence that the AAPT1 polypeptides produced in the leaf and developing seeds are likely to be the same, analysis of the seed-specific cDNAs also provided interesting information regarding the 5′ noncoding sequence of the *AAPT1* mRNA. Four of the five seed-specific clones examined had cDNA inserts that were somewhat longer than that originally characterized from pGmaapt2 (1.44 kb for the leaf clone versus ~1.6 kb for the longest cDNA from the developing seed cDNA library). Moreover, the RNA gel blot assays shown in Figure 4B revealed a mature *AAPT1* transcript of ~1.6 kb in all tissues examined.

The cDNA insert of pGmaapt2 contains 44 bp of 5′ noncoding sequence upstream of the Met initiation codon (Figure 2). The authenticity of this proposed initiator start site is supported by the following observations: (1) there is an in-frame stop codon (TGA) 33 bp upstream of the putative translational start site and (2) the proposed *AAPT1* Met initiation codon aligns perfectly with the yeast *EPT1* start codon (Figure 3). Sequence analysis of pBSaapt1.6 revealed that the full-length mRNA transcript contains an additional short ORF (11 codons in length) 124 bp upstream of the *AAPT1*-encoding reading frame (Figure 2). Three of the five characterized *AAPT1* cDNAs from the seed-specific library contain 5′ leader sequences that possess this small reading frame, one terminated within it, and the final cDNA terminated within the *AAPT1* reading frame (data not shown).

In eukaryotes, translational initiation typically proceeds from the first AUG codon encountered by the ribosome. For the great majority of eukaryotic genes, the first AUG codon of the mature mRNA represents the beginning of the reading frame of the encoded protein product. Several examples have been documented, however, where one or more short upstream ORFs (uORFs) will precede the larger, primary reading frame of the transcript (for review, see Kozak, 1989). In systems where their effect on gene expression has been examined, these uORFs typically result in a decrease in translational efficiency of the principal ORF.

To determine whether sequences present in the 5′ flanking region play a role in the post-transcriptional regulation of *AAPT1*, the insert from pBSaapt1.6 was cloned (in the sense orientation with respect to the *GAL1* promoter) into the yeast expression vector pYES2. Mutant yeast strain RK-ec was transformed with this plasmid, designated pGmaapt1.6, and assayed for cholinephosphotransferase activity using the colony autoradiographic procedure. As shown in Figure 6, a great decrease in cholinephosphotransferase activity was seen in RK-ec cells transformed with pGmaapt1.6 when compared with the original pGmaapt2 vector that lacks the extended 5′ flanking region. To provide an estimate of the decrease in enzyme activity attributable to the 5′ flanking region of clone pGmaapt1.6, the filter-bound yeast colonies from Figure 6 were chloroform-methanol extracted, and the ^{14}C-PC was analyzed by thin-layer chromatography after each sample was standardized according to total lipid content (data not shown). Quantitation of the

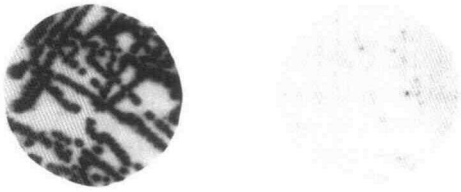

Figure 6. Colony Autoradiographic Assay Using *AAPT1* cDNAs Containing 5′ Leader Sequences That Differ in Length.

AAPT1 cDNAs cloned into the pYES2 expression vector were transformed into strain RK-ec and plated onto inducing media (containing galactose). pGmaapt2 is the original *AAPT1* construct identified by its ability to complement the mutant yeast phenotype. pGmaapt1.6 possesses the longest *AAPT1* cDNA characterized in this study. Length differences in the 5′ flanking regions of the two cDNAs are shown in Figure 2.

radiolabeled products was conducted using an imaging scanner and demonstrated that RK-ec cells transformed with pGmaapt1.6 were ~20-fold less effective in synthesizing radiolabeled PC than cells transformed with pGmaapt2.

DISCUSSION

Current models describing PC and PE biosynthesis suggest that the role of AAPTases in this process may differ somewhat between plant and animal or fungal systems. In animals and yeast, CDP-choline and CDP-ethanolamine are believed to be the primary substrates for two separate AAPTases, cholinephosphotransferase and ethanolaminephosphotransferase, respectively (Bell and Coleman, 1980; Percy et al., 1984; Hjelmstad and Bell, 1991). In higher plants, however, the question of whether a single or separate enzyme(s) catalyzes the respective reactions remains unanswered. Several studies in plants have presented compelling evidence suggesting that the same enzyme catalyzes both reactions (Macher and Mudd, 1974; Lord, 1975; Sparace et al., 1981; Justin et al., 1985). The strongest data supporting this hypothesis are observations that CDP-choline and CDP-ethanolamine are competitive inhibitors of ethanolaminephosphotransferase and cholinephosphotransferase activities, respectively, and the failure to find inhibitors that can differentiate the two activities. Reports suggesting that separate enzymes catalyze the respective reactions are based largely on molecular species labeling studies (Dykes et al., 1976; Harwood, 1976).

The issue is further complicated by observations that CDP-choline and CDP-ethanolamine are not the exclusive substrates for the AAPTase reactions in higher plants. Current models predict that CDP-methylethanolamine and CDP-dimethylethanolamine can also be utilized by AAPTases to produce phosphatidylmethylethanolamine and phosphatidyldimethylethanolamine, respectively (Datko and Mudd, 1988a, 1988b; Prud'homme and Moore, 1992). These products are subsequently converted to PC by the action of *N*-methyltransferases. The preferred route of PC biosynthesis also appears to be very different among the various plant species examined. In soybean leaves and cell cultures, it appears that CDP-methylethanolamine is the primary substrate used by the AAPTase reaction that leads to the production of PC. In contrast, the principal physiological substrate is likely to be CDP-choline in *Lemna* and castor bean endosperm, and all three CDP-aminoalcohols (methylethanolamine, dimethylethanolamine, and choline) appear to be involved in the biosynthesis of PC in carrot (Datko and Mudd, 1988b; Prud'homme and Moore, 1992).

The evidence presented in this study clearly demonstrates that the *AAPT1* gene product of soybean possesses significant cholinephosphotransferase activity (Figures 1 and 5). What remains to be determined is the degree to which the enzyme may also utilize CDP-ethanolamine in catalyzing the synthesis of PE, and CDP-methylethanolamine and CDP-dimethylethanolamine in producing PC via phosphatidylmethylethanolamine and phosphatidyldimethylethanolamine intermediates. As a preliminary investigation to address these questions, the synthesis of ^{14}C-PC using ^{14}C-CDP-choline was measured in microsomal membranes of yeast cells expressing the soybean gene in the presence of increasing amounts of nonradioactive CDP-choline or CDP-ethanolamine. The rate of reduction in synthesis of radiolabeled PC observed by lowering the specific activity of the radiolabeled substrate (i.e., increasing nonradiolabeled CDP-choline concentration) was very similar to that observed when nonradiolabeled CDP-ethanolamine was added to the reaction mix (Figure 5). In this respect, the soybean enzyme differed dramatically from the yeast cholinephosphotransferase encoded by the *CPT1* gene that failed to be inhibited at even very high concentrations of CDP-ethanolamine. The inhibition profile of the soybean *AAPT1*-encoded enzyme more closely reflected that of the yeast *EPT1* gene product, an enzyme that has been shown to possess both cholinephosphotransferase and ethanolaminephosphotransferase activities. Consistent with its presumed primary function of catalyzing the ethanolaminephosphotransferase reaction, however, CDP-ethanolamine appeared to be more effective than CDP-choline in the competitive inhibition of the cholinephosphotransferase activity of the *EPT1* gene product (Figure 5).

One model that is compatible with the above observations is that the soybean *AAPT1* gene product displays similar substrate binding affinities to both CDP-choline and CDP-ethanolamine and is capable of synthesizing either PC or PE, depending upon substrate availability. A more direct enzymological characterization, however, will be necessary to determine the relative degree to which the *AAPT1*-encoded enzyme may catalyze not only the ethanolaminephosphotransferase reaction using CDP-ethanolamine, but also its ability to utilize CDP-methylethanolamine and CDP-dimethylethanolamine as substrates to help establish its role in catalyzing each of the four physiologically relevant AAPTase-mediated reactions involved in the synthesis of PE and PC in higher plants. With the isolation of the soybean *AAPT1* cDNA and the ability to express

its product in yeast, we are now in a position to conduct investigations to definitively establish substrate specificities and more precisely determine its role in phospholipid biosynthesis. In addition, to help determine whether other *AAPT1*-like genes exist and encode AAPTases with different substrate specificities, the *AAPT1* sequence could be useful as a hybridization probe in the screening of cDNA libraries.

The scanning model is widely accepted as the mechanism by which translation of eukaryotic mRNAs is initiated (for review, see Kozak, 1991). Simply stated, eukaryotic ribosomes initially bind to the 5′ end of an mRNA and initiate translation at the first AUG codon in a "good" context. Although many of the nucleotides surrounding the initiation AUG codon are capable of affecting the translational efficiency of a given mRNA, generally positions −3 and +4 (relative to the A of the AUG codon) have the greatest influence. The presence of a purine residue at position −3 appears to be the single most important feature that is required for optimal translation of a message (Kozak, 1986). In the absence of a purine at −3, however, a G residue at position +4 becomes essential for efficient translation, and the effects of other positions are more pronounced (Kozak, 1987).

Several examples have been described in which one or more small uORFs precede the primary reading frame of an mRNA. When the translational efficiencies of these mRNAs were tested, it was found that a small peptide is produced from the uORF and that translation of the larger downstream reading frame is greatly reduced (Khalili et al., 1987; Werner et al., 1987; Damiani and Wessler, 1993; Han et al., 1993). The fact that any translational initiation is allowed to proceed from a downstream ORF is believed to be a result of a small percentage of reinitiation of the ribosomal complex, allowing those ribosomes to "scan" to the next AUG (Kozak, 1989). The proposed initiator AUG codon of the uORF in the 5′ leader sequence of the *AAPT1* transcript contains an A residue at position −3 (Figure 2) and would appear to be in a good context for translational initiation. Furthermore, the third codon of the uORF also specifies an AUG codon and therefore may act as an additional site of translational initiation.

Expression in yeast of the longest soybean *AAPT1* cDNA characterized in this study demonstrated that sequences in the 5′ flanking region of the full-length transcript have a negative effect on gene expression in this heterologous environment (Figure 6). Although it cannot be concluded from these data that the uORF was responsible for this phenomenon, similarities between the organization of the *AAPT1* transcript and genes that have been found to be negatively regulated via short 5′ proximal ORFs suggest that this motif is a likely candidate as the agent that mediates the observed repression in gene activity. Alternatively, strong secondary structure in the 5′ flanking region of some eukaryotic mRNAs has been proposed as a mechanism of negative translational regulation (Kozak, 1991). Computer analysis of the predicted optimal RNA secondary structure of the *AAPT1* mRNA, however, failed to detect any contiguous stretches of sequence complementarity longer than 6 bp in the 5′ flanking region of the transcript (data not shown).

Evidence of potential negative post-transcriptional regulation of the soybean *AAPT1* gene may have parallels with two other genes in the glycerolipid biosynthetic pathway of plants. Hannapel and Ohlrogge (1988) demonstrated that the acyl carrier protein (ACP)/mRNA ratio in developing soybean seeds was significantly less than that observed for lectin, causing them to suggest that reduced translational efficiency may constitute one aspect of ACP regulation. Long runs of CT repeats and/or the conservation of a 7-bp GC-rich motif in the 5′ noncoding region of the mRNA was considered to be responsible for reducing translational efficiency of the ACP gene (Ohlrogge et al., 1991). Recently, evidence suggesting the negative post-transcriptional regulation of the 18:1 desaturase of Arabidopsis was also presented (Okuley et al., 1994). A T-DNA insertion mutation in the 5′ flanking region of the 18:1 desaturase resulted in a nearly 90% reduction of its steady state mRNA levels. This low level of transcript, however, was sufficient to confer greater than 50% of the 18:1 desaturation attributable to the wild-type gene. One possible explanation of these results was that sequences in the wild-type 5′ flanking region of the 18:1 desaturase mRNA may confer negative translational regulation.

The negative post-transcriptional regulation observed when a "full-length" soybean *AAPT1* cDNA was expressed in yeast also provides a likely explanation regarding the great number of colonies (more than one million) that were necessary to screen before we were successful in isolating the gene using genetic complementation. When the pGmaapt2 insert was used as a hybridization probe in screening the developing seed cDNA library, it was revealed that cDNAs corresponding to *AAPT1* were represented in the library at a frequency of approximately one in 7000 transformants. By comparing the intensity of hybridization signals of *AAPT1* transcripts in developing seed versus young leaf tissue using RNA gel blot assays (Figure 4B), one would also predict the representation of *AAPT1* cDNAs to be somewhat similar in a young leaf cDNA library. Given the fact that a cDNA possessing a long 5′ flanking region produced a signal that was barely detectable using this assay (Figure 6), it is unlikely that such a cDNA would have been identified in the original screening. If the uORF in the 5′ leader sequence proves to be responsible for the observed repression of gene activity, it is possible that the only cDNAs in the expression libraries that could have been effectively isolated by this technique were ones derived from mRNAs long enough so that they contained the entire *AAPT1* reading frame, yet not so long as to also possess the uORF (in addition to being in correct orientation with respect to the vector's *GAL1* promoter). Because of the increasing number of genes involved in glycerolipid biosynthesis that are appearing to demonstrate negative post-transcriptional regulation (*AAPT1*, ACP, and the 18:1 desaturase), future attempts to isolate genes of this pathway by genetic complementation should be conducted with the understanding that such a form of gene regulation could present significant obstacles when using this strategy.

The role of AAPTases in mediating the synthesis of PC is of particular interest during seed development. The majority of polyunsaturated lipid biosynthesis in the developing seed

occurs via 18:1 and 18:2 desaturases located on the endoplasmic reticulum (Ohlrogge et al., 1991; Somerville and Browse, 1991). The substrate for these desaturase enzymes has been shown to be PC (Slack et al., 1979; Stymne and Appelqvist, 1980; Browse and Slack, 1981). The fatty acids of PC that have become polyunsaturated may subsequently be made available for incorporation into storage triacylglycerols by two mechanisms: (1) reentry into the acyl-coenzyme A pool by specific acyltransferases that exchange acyl groups at position *sn*-2 of PC with those of acyl-coenzyme A (Stymne et al., 1983; Griffiths et al., 1988) or (2) reentry into the diacylglycerol pool by the reversible reaction of the cholinephosphotransferase enzyme (Slack et al., 1983; Stymne and Stobart, 1984). Consistent with this model is the observation that cholinephosphotransferase activity increases dramatically during seed development in safflower, concomitant with increases in PC and triacylglycerol accumulation (Slack et al., 1985). Two observations suggest that the *AAPT1* product is the predominant (and possibly the only) AAPTase responsible for the cholinephosphotransferase activity in developing soybean seeds: (1) *AAPT1* cDNAs were well represented within the 25-DAF developing seed cDNA library and (2) even under low-stringency hybridization conditions, no *AAPT1*-like sequences different from our original *AAPT1* cDNA probe were recovered from the seed-specific cDNA library.

RNA gel blot analysis demonstrated that the *AAPT1* gene is expressed in each tissue examined (Figure 4B). Because of the role of AAPTases in the synthesis of membrane phospholipids in virtually all cell types, this result was expected. It is interesting, however, that the greatest accumulation of steady state transcript was observed in the root and stem tissues. One possible explanation for the greater abundance of *AAPT1* mRNAs in root than in leaf tissue may be the fact that the major membrane lipids in leaves are the galactolipids of the chloroplast, whereas in nonphotosynthetic tissues PC and PE are the predominant cellular lipids. Although there are studies that report increases in cholinephosphotransferase activity during seed development (Slack et al., 1985), it is difficult to discuss them in the context of our RNA gel blot data because they did not include comparison of the cholinephosphotransferase activity in developing seeds with the activity found in other plant tissues such as roots or leaves. Moreover, although 25 DAF represents a developmental stage during which triacylglycerol accumulation in soybeans is expected to be maximal (Wilson, 1987), RNA gel blot analyses using mRNA isolated from developing seed tissue at numerous time points would be required to determine whether this stage represents the period of maximal *AAPT1* transcript accumulation. Finally, any attempts to correlate *AAPT1* mRNA accumulation with enzyme activity must be interpreted in the context that post-transcriptional control may also play an important role in gene regulation. Should *AAPT1* in soybean show the same negative post-transcriptional control of gene expression that was observed in the heterologous yeast system, it would be possible for enzyme activity to increase in a given tissue that was capable of overcoming the mechanism of negative control without necessarily increasing steady state accumulation of the *AAPT1* mRNA.

In conclusion, the cloning and characterization of the *AAPT1* cDNA of soybean has provided valuable information concerning the structure and regulation of a gene encoding an essential step in the glycerolipid biosynthetic pathway. In addition to providing a tool that will facilitate the study of substrate specificities and the kinetics of a higher plant AAPTase, the soybean *AAPT1* clone will also provide an opportunity to study the effects of manipulating AAPTase activity in transgenic plants by either overexpression or antisense-mediated inhibition of gene activity. Such studies will help further elucidate the role of phospholipid composition and function during plant growth and development.

METHODS

Yeast Strains and Growth Conditions

Haploid yeast (*Saccharomyces cerevisiae*) strains HJ110 (*MATa, cpt1, gal*$^-$, *his3-Δ1, leu2-3, leu2-112, ura3-52, trp-289*) and HJ729 (*MATa, ept1, gal*$^-$, *his3-Δ1, leu2-3, leu2-112, ura3-52, trp-289*) were obtained from R.M. Bell (Duke University Medical Center, Durham, NC). Strain KT1115 (*MATα, leu2-3, leu2-112, ura3-52*) was obtained from K. Tatchell (North Carolina State University, Raleigh, NC). Yeast was cultured in either YPD (1% yeast extract, 2% peptone, 2% dextrose) or synthetic minimal media (0.67% yeast nitrogen base without amino acids, 2% dextrose) supplemented with amino acids at 20 μg/mL. Induction of expression from the *GAL1* promoter of pYES2 was conducted by culture of the appropriate transformed yeast strain in YP-Gal media (1% yeast extract, 2% peptone, and 2% galactose).

Haploid strain RH-6D (*MATα, cpt1, his3-Δ1, leu2-3, leu2-112, ura3-52*) was recovered from a cross between HJ110 and KT1115. Haploid strain RK-ec (*MATα, cpt1, ept1, his3-Δ1, leu2-3, leu2-112, ura3-52*) was selected from a cross between RH-6D and HJ729. All yeast manipulations and crosses were conducted according to standard protocols (Guthrie and Fink, 1991).

Construction of Soybean cDNA Libraries

Size-selected cDNA (>500 bp) was synthesized from mRNA isolated from young soybean (*Glycine max* cv Dare) leaves and developing soybean seeds 25 days after flowering (DAF) using moloney murine leukemia virus reverse transcriptase as described by the manufacturer (Gibco BRL). cDNA libraries corresponding to each of the two developmental stages were constructed in the yeast expression vector pYES2 (Invitrogen, San Diego, CA). pYES2 is designed to utilize the high-efficiency inverted BstXI cloning strategy of Aruffo and Seed (1987). BstXI adapters were ligated to the soybean cDNA and cloned into BstXI-digested pYES2 vectors as outlined by Becker et al. (1991). Ligation reactions were phenol–chloroform extracted, precipitated, and transformed into electrocompetent *Escherichia coli* Top10F′ cells according to the manufacturer's protocol (Invitrogen). Transformed cells were plated at a density of ~2 × 10^6 colonies per 150-mm Petri dish. Colonies were scraped directly from the surface of the Petri dishes, and plasmids were recovered by alkaline lysis and CsCl gradient centrifugation as described previously (Sambrook et al., 1989). For each

library, we obtained >5 × 10^7 primary transformants per μg of input cDNA. As judged by the frequency of plasmids containing inserts (obtained from 40 randomly selected colonies), we estimated that >90% of the clones from the developing seed cDNA library and >75% of the young leaf cDNA library plasmids contained soybean cDNA inserts.

For the isolation of soybean aminoalcoholphosphotransferase (AAPTase) cDNAs expressed during seed development using the leaf-specific *AAPT1* cDNA as a hybridization probe, an additional cDNA library was constructed in λZAPII. cDNAs from developing soybean seeds 25 DAF were ligated to EcoRI adapters and cloned into the EcoRI-digested, phosphatase-treated arms of λZAPII according to the manufacturer's instructions (Stratagene).

Screening of Yeast Strain RK-ec Transformed with a Soybean cDNA Library Using Colony Autoradiography

The soybean cDNA libraries constructed in the pYES2 expression vector were transformed into strain RK-ec according to the protocol of Gietz et al. (1992). Transformants were plated at a density of ~3000 colonies per 90-mm Petri dish on selective media (synthetic minimal media supplemented with amino acids, minus uracil). After growth for 3 days at 30°C, colonies were replica plated onto YP-Gal media and grown for an additional 24 hr at 30°C to induce expression of the transgene.

To screen for mutant complementation, a diacylglycerol cholinephosphotransferase colony autoradiographic assay was conducted essentially as described by Hjelmstad and Bell (1987). Briefly, the transformed, induced yeast colonies were transferred to Whatman No. 42 filter paper (90-mm circles) and frozen at −80°C for a minimum of 2 hr. Yeast colonies were permeabilized by drying in a direct stream of air from an electric fan for 30 min at room temperature. Individual filters were incubated in Petri dishes in 1.2 mL of a reaction solution containing 50 mM 3-(*N*-morpholino)propanesulfonic acid (Mops)-NaOH, pH 7.5, 20 mM $MgCl_2$, 1.5 mg/mL BSA (fatty acid free), 1 mM dithiothreitol, and 5 μM ^{14}C-cytidine diphosphate (CDP)-choline (58 mCi/mmol; Amersham Corp.). After a 1-hr incubation at 30°C, filters were transferred to a new Petri dish containing 1 mL of cold (4°C) 10% trichloroacetic acid to stop the reaction and fix the labeled phosphatidylcholine (PC) to the filter paper. After a 30-min incubation at 4°C, the filters were washed six times with 25 mL of cold (4°C) 2% trichloroacetic acid using a Buchner funnel. Air-dried filters were sprayed with three light coats of a fluorographic enhancer (EN^{3}HANCE; Du Pont–New England Nuclear) and exposed to Kodak XAR-5 film for 1 to 5 days. Colony autoradiographic assays for cholinephosphotransferase activity in *E. coli* (strain MV1190) were conducted exactly as described for yeast cells with the exception that the filter-bound *E. coli* colonies were initially permeabilized using a 10 mg/mL lysozyme, 10 mM EDTA solution as described by Raetz (1975).

Radioactive products were extracted by soaking the filter papers in 10 mL of 1:1 chloroform–methanol overnight at 4°C. After the addition of 6.25 mL of 1 N HCl, the chloroform phase was recovered, dried to completion, redissolved in 50 μL of 2:1 chloroform–methanol, and analyzed by thin-layer chromatography on Silica Gel 60 plates (Analtech, Newark, DE) using a developing solvent of 25:15:4:2 chloroform–methanol–water–acetic acid. Comigration of radiolabeled products with authentic PC run as a standard was verified using a Bioscan System 400 Imaging Scanner (Bioscan Inc., Washington, DC).

Sequence Analysis

To determine the nucleotide sequence of the soybean cDNA from plasmid pGmaapt2, the insert was initially subcloned into vectors pUC119 and pUC120 (Vieira and Messing, 1987), designated pGmaapt119 and pGmaapt120, respectively. This cloning strategy placed the inserts in the respective vectors in opposite orientation in relation to the universal primer attachment site. To facilitate sequencing of the entire cDNA, a nested set of deletions was generated using the Erase-A-Base kit according to the manufacturer's protocol (Promega). The complete cDNA sequence was determined in both orientations using Sequenase Version 2.0 (U.S. Biochemical Corp.).

Computer data base searches and hydropathy plots of the soybean *AAPT1* cDNA and predicted amino acid sequences were conducted using the MACVECTOR software package (IBI, North Haven, CT). Pairwise comparisons to establish percent identity between the soybean *AAPT1*-encoded polypeptide and the yeast *CPT1* and *EPT1* gene products were calculated using the GAP program of the University of Wisconsin (Madison) Genetics Computing Group (UWGCG) software package. The multiple sequence alignment of the three protein sequences (Figure 3A) was deduced using the UWGCG PILEUP program (gap weight = 3.0, gap length weight = 0.1).

DNA and mRNA Gel Blot Analyses

Total genomic DNA from young soybean leaves was isolated as previously described (Murray and Thompson, 1980). Ten micrograms of soybean DNA was digested with EcoRI and separated by electrophoresis on a 1% agarose gel. Total cellular RNA was isolated from various soybean tissues as outlined by Grimes et al. (1992). Poly(A)$^+$ RNA was recovered using oligo(dT) columns as outlined by the manufacturer (Gibco BRL) and separated electrophoretically on a 1.2% formaldehyde gel (Sambrook et al., 1989).

Nucleic acids were transferred to nylon membranes (Gibco BRL) according to Sambrook et al. (1989). Both RNA and DNA gel blots were hybridized overnight to ^{32}P-labeled *AAPT1* cDNA in 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% (w/v) SDS, and 100 μg/mL of denatured herring sperm DNA at 65°C. Blots were washed twice at room temperature in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% (w/v) SDS for 15 min, once at 65°C in 1 × SSC, 0.1% SDS for 15 min, and once at 65°C in 0.2 × SSC, 0.1% SDS for 15 min. All ^{32}P-labeled DNA probes were generated using random primers according to the method of Feinberg and Vogelstein (1983). To ensure that each lane of the RNA gel blots contained similar amounts of undegraded RNA, filters that had been hybridized to an *AAPT1* probe were subsequently stripped and rehybridized with a ^{32}P-labeled 25S ribosomal RNA gene from pea (data not shown).

Low-stringency hybridizations were conducted in the same hybridization solution described above using an incubation temperature of 50°C. Filters for the low-stringency hybridizations were washed twice using 2 × SSC, 0.1% (w/v) SDS at room temperature for 15 min and once with a 1 × SSC, 0.1% SDS solution at 50°C for 15 min.

Isolation of *AAPT1* cDNAs from a Developing Seed Soybean cDNA Library

The developing seed soybean cDNA library in λZAPII was plated at a density of 4 × 10^4 plaque-forming units per 150-mm Petri dish according to the procedures outlined by Stratagene. Plaques were transferred to nylon membranes (Gibco BRL) and hybridized to ^{32}P-labeled insert DNA from pGmaapt2 using the conditions of low-hybridization stringency described above. Plaques showing positive

hybridization signals were purified to homogeneity and their corresponding plasmids excised as described in the λZAPII cloning kit (Stratagene). Nucleotide sequence information for 350 to 400 bp was obtained from each end of the selected clones using the universal and reverse primers with the Sequenase Version 2.0 sequencing kit.

Cholinephosphotransferase Assays of Yeast Microsomal Membranes

Yeast colonies were inoculated into 200 mL of YPD (HJ110 and HJ729) or YP-Gal (RK-ec:pGmaapt2) and grown to an A_{600} of 1.0 to 2.0. Cells were harvested by centrifugation at 1000*g* for 10 min and washed once with 100 mL of water and once with 25 mL of 20% glycerol, 50 mM Mops-NaOH, pH 7.5, 1 mM EDTA (GME buffer). The final pellet was resuspended in 1 mL of GME buffer, and the cells were disrupted using a mini-beadbeater as described by Tillman and Bell (1986). The resulting homogenate was centrifuged for 15 min at 14,000*g* to remove unbroken cells. GME buffer was added to the supernatant to a final volume of 12 mL and centrifuged for 1.5 hr at 100,000*g*. The pellet was resuspended into 0.5 mL of GME buffer using a Teflon (Du Pont) homogenizer, aliquoted, and frozen at −80°C. All centrifugations were conducted at 4°C, and the samples were maintained on ice for all other steps of the procedure. Total membrane protein was determined as described by Bradford (1976) using BSA as the standard.

To determine the abilities of the various yeast microsomal membrane fractions to incorporate radiolabel from ^{14}C-CDP-choline into PC, 75 μg of total membrane protein was incubated in an assay mixture containing 50 mM Mops-NaOH, pH 7.5, 20 mM $MgCl_2$, 1.5 mg/mL BSA, and 25 μM ^{14}C-CDP-choline. Using these assay conditions, microsomal membranes from HJ729, HJ110, and RK-ec:pGmaapt2 showed linear incorporation of radioactivity into PC over a 45-min time course; microsomal membranes isolated from mutant strain RK-ec (nontransformed) showed no detectable synthesis of ^{14}C-PC (data not shown). For the inhibition assays, nonradioactive CDP-aminoalcohols were added to the various reaction mixtures as indicated in Figure 5 in a final reaction volume of 0.1 mL. After incubation at 30°C for 20 min, 0.7 mL of 0.1 N HCl was added to stop the reaction. ^{14}C-PC was extracted with 2 mL of 2:1 chloroform–methanol. Analysis of the organic phase by thin-layer chromatography (as described above) revealed that more than 96% of the chloroform-soluble radioactivity comigrated with authentic PC run as a standard. Radiolabeled PC produced in the microsomal membrane assays was quantitated using liquid scintillation spectrometry. Each inhibition experiment was repeated independently three times; the data shown in Figure 5 are from a single representative experiment.

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REFERENCES

Ansell, G.B., and Spanner, S. (1982). Phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine. In New Comprehensive Biochemistry, Vol. 4, A. Neuberger and L.L.M. van Deenen, eds (New York: Elsevier), pp. 1–50.

Aruffo, A., and Seed, B. (1987). Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proc. Natl. Acad. Sci. USA **84,** 8573–8577.

Becker, D.M., Fikes, J.D., and Guarente, L. (1991). A cDNA encoding a human CCAAT-binding protein cloned by functional complementation in yeast. Proc. Natl. Acad. Sci. USA **88,** 1968–1972.

Bell, R.M., and Coleman, R.A. (1980). Enzymes of glycerolipid synthesis in eukaryotes. Annu. Rev. Biochem. **49,** 459–487.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Browse, J.A., and Slack, C.R. (1981). Catalase stimulates linoleate desaturase activity in microsomes from developing linseed cotyledons. FEBS Lett. **131,** 111–114.

Damiani, R.D., and Wessler, S.R. (1993). An upstream open reading frame represses expression of *Lc*, a member of the *R/B* family of maize transcriptional activators. Proc. Natl. Acad. Sci. USA **90,** 8244–8248.

Datko, A.H., and Mudd, S.H. (1988a). Phosphatidylcholine synthesis: Differing patterns in soybean and carrot. Plant Physiol. **88,** 854–861.

Datko, A.H., and Mudd, S.H. (1988b). Enzymes of phosphatidylcholine synthesis in *Lemna*, soybean, and carrot. Plant Physiol. **88,** 1338–1348.

Dykes, C.W., Kay, J., and Harwood, J.L. (1976). Incorporation of choline and ethanolamine into phospholipids in germinating soya bean. Biochem. J. **158,** 575–581.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132,** 6–13.

Gietz, D., St. Jean, A., Woods, R.A., and Schiestl, R.H. (1992). Improved method for high efficiency transformation of intact yeast cells. Nucl. Acids Res. **20,** 1425.

Griffiths, G., Stymne, S., and Stobart, A.K. (1988). The utilization of fatty-acid substrates in triacylglycerol biosynthesis by tissue-slices of developing safflower (*Carthamus tinctorius* L.) and sunflower (*Helianthus annuus* L.) cotyledons. Planta **173,** 309–316.

Grimes, H.D., Overvoorde, P.J., Ripp, K., Franceschi, V.R., and Hitz, W.D. (1992). A 62-kD sucrose binding protein is expressed and localized in tissues actively engaged in sucrose transport. Plant Cell **4,** 1561–1574.

Guthrie, C., and Fink, G.R., eds (1991). Guide to Yeast Genetics and Molecular Biology: Methods in Enzymology, Vol. 194. (New York: Academic Press).

Han, S., Navarro, J., Greve, R.A., and Adams, T.H. (1993). Translational repression of *brlA* expression prevents premature development in *Aspergillus*. EMBO J. **12,** 2449–2457.

Hannapel, D.J., and Ohlrogge, J.B. (1988). Regulation of acyl carrier protein messenger RNA levels during seed and leaf development. Plant Physiol. **86,** 1174–1178.

Harwood, J.L. (1976). Synthesis of molecular species of phosphatidylcholine and phosphatidylethanolamine by germinating soya bean. Phytochemistry **15,** 1459–1463.

Hjelmstad, R.H., and Bell, R.M. (1987). Mutants of *Saccharomyces cerevisiae* defective in *sn*-1,2-diacylglycerol cholinephosphotransferase. J. Biol. Chem. **262,** 3909–3917.

Hjelmstad, R.H., and Bell, R.M. (1988). The *sn*-1,2-diacylglycerol ethanolaminephosphotransferase activity of *Saccharomyces cerevisiae.* J. Biol. Chem. **263,** 19748–19757.

Hjelmstad, R.H., and Bell, R.M. (1990). The *sn*-1,2-diacylglycerol cholinephosphotransferase of *Saccharomyces cerevisiae.* J. Biol. Chem. **265,** 1755–1764.

Hjelmstad, R.H., and Bell, R.M. (1991). *sn*-1,2-Diacylglycerol choline- and ethanolaminephosphotransferases in *Saccharomyces cerevisiae.* J. Biol. Chem. **266,** 5094–5103.

Justin, A.M., Demandre, C., Tremolieres, A., and Mazliak, P. (1985). No discrimination by choline- and ethanolaminephosphotransferases from potato tuber microsomes in molecular species of endogenous diacylglycerols. Biochim. Biophys. Acta **836,** 1–7.

Khalili, K., Brady, J., and Khoury, G. (1987). Translational regulation of SV40 early mRNA defines a new viral protein. Cell **48,** 639–645.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44,** 283–292.

Kozak, M. (1987). At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J. Mol. Biol. **196,** 947–950.

Kozak, M. (1989). The scanning model for translation: An update. J. Cell Biol. **108,** 229–241.

Kozak, M. (1991). An analysis of vertebrate mRNA sequences: Intimations of translational control. J. Cell Biol. **115,** 887–903.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157,** 105–132.

Lord, J.M. (1975). Evidence that phosphatidylcholine and phosphatidylethanolamine are synthesized by a single enzyme present in the endoplasmic reticulum of castor-bean endosperm. Biochem. J. **151,** 451–453.

Macher, B.A., and Mudd, J.B. (1974). Biosynthesis of phosphatidylethanolamine by enzyme preparations from plant tissues. Plant Physiol. **53,** 171–175.

Murray, M.G., and Thompson, W.F. (1980). Rapid isolation of high molecular weight plant DNA. Nucl. Acids Res. **8,** 4321–4325.

Ohlrogge, J.B., Browse, J., and Somerville, C.R. (1991). The genetics of plant lipids. Biochim. Biophys. Acta **1082,** 1–26.

Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E., and Browse, J. (1994). Arabidopsis *FAD2* gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell **6,** 147–158.

Percy, A.K., Carson, M.A., Moore, J.F., and Waechter, C.J. (1984). Control of phosphatidylethanolamine metabolism in yeast: Diacylglycerol ethanolaminephosphotransferase and diacylglycerol cholinephosphotransferase are separate enzymes. Arch. Biochem. Biophys. **230,** 69–81.

Prud'homme, M.-P., and Moore, T.S., Jr. (1992). Phosphatidylcholine synthesis in castor bean endosperm: Free bases as intermediates. Plant Physiol. **100,** 1527–1535.

Raetz, C.R.H. (1975). Isolation of *Escherichia coli* mutants defective in enzymes of membrane lipid synthesis. Proc. Natl. Acad. Sci. USA **72,** 2274–2278.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Press).

Slack, C.R., Roughan, P.G., and Browse, J.A. (1979). Evidence for an oleoyl phosphatidylcholine desaturase in microsomal preparations from cotyledons of safflower (*Carthamus tinctorius*) seed. Biochem. J. **179,** 649–656.

Slack, C.R., Campbell, L.C., Browse, J.A., and Roughan, P.G. (1983). Some evidence for the reversibility of the cholinephosphotransferase-catalyzed reaction in developing linseed cotyledons in vivo. Biochim. Biophys. Acta **754,** 10–20.

Slack, C.R., Roughan, P.G., Browse, J.A., and Gardiner, S.E. (1985). Some properties of cholinephosphotransferase from developing safflower cotyledons. Biochim. Biophys. Acta **833,** 438–448.

Somerville, C., and Browse, J. (1991). Plant lipids: Metabolism, mutants, and membranes. Science **252,** 80–87.

Sparace, S.A., Wagner, L.K., and Moore, T.S. (1981). Phosphatidylethanolamine synthesis in castor bean endosperm. Plant Physiol. **67,** 922–925.

Stymne, S., and Appelqvist, L. (1980). The biosynthesis of linoleate and α-linolenate in homogenates from developing soya bean cotyledons. Plant Sci. Lett. **17,** 287–294.

Stymne, S., and Stobart, A.K. (1984). The biosynthesis of triacylglycerols in microsomal preparations of developing cotyledons of sunflower (*Helianthus annuus* L.). Biochem. J. **220,** 481–488.

Stymne, S., Stobart, A.K., and Glad, G. (1983). The role of the acyl-CoA pool in the synthesis of polyunsaturated 18-carbon fatty acids and triacylglycerol production in the microsomes of developing safflower seeds. Biochim. Biophys. Acta **752,** 198–208.

Tillman, T.S., and Bell, R.M. (1986). Mutants of *Saccharomyces cerevisiae* defective in *sn*-glycerol-3-phosphate acyltransferase. J. Biol. Chem. **261,** 9144–9149.

Vieira, J., and Messing, J. (1987). Production of single-stranded plasmid DNA. Methods Enzymol. **153,** 3–11.

Werner, M., Feller, A., Messenguy, F., and Pierard, A. (1987). The leader peptide of yeast gene *CPA1* is essential for the translational repression of its expression. Cell **49,** 805–813.

Wilson, R.F. (1987). Seed metabolism. In Soybeans: Improvement, Production and Uses, J.R. Wilcox, ed (Madison, WI: American Society of Agronomy), pp. 643–686.