Planta (2003) 217: 547–558
DOI 10.1007/s00425-003-1031-6

ORIGINAL ARTICLE

Qungang Qi · Yong-fen Huang · Adrian J. Cutler
Suzanne R. Abrams · David C. Taylor

Molecular and biochemical characterization of an aminoalcoholphosphotransferase (AAPT1) from *Brassica napus*: effects of low temperature and abscisic acid treatments on *AAPT* expression in *Arabidopsis* plants and effects of over-expression of *BnAAPT1* in transgenic *Arabidopsis*

Received: 10 December 2002 / Accepted: 15 March 2003 / Published online: 9 May 2003

Abstract Aminoalcoholphosphotransferases (AAPT, EC 2.7.8.1 and EC 2.7.8.2) catalyze the transfer of CDP-aminoalcohols to *sn*-1, 2 diacylglycerol (DAG) to form phosphatidylaminoalcohols with the release of CMP. The *Brassica napus* L. *AAPT1* gene (designated *BnAAPT1*) was identified from cDNA libraries of seedlings and developing seeds. Functional characterization was accomplished by heterologous expression of *BnAAPT1* in a yeast strain deficient in AAPT activities. BnAAPT1 exhibited a greater preference for utilizing CDP-choline as a substrate with V_{max} of 35 [^{14}C]phosphatidylcholine nmol h^{-1} mg^{-1} protein and apparent K_m of 32 μM while CDP-ethanolamine had a V_{max} of 13 [^{14}C]phosphatidylethanolamine nmol h^{-1} mg^{-1}protein and an apparent K_m of 127 μM. The enzyme was activated by Mg^{2+}, Mn^{2+} and phospholipid mixtures, and inhibited by Ca^{2+}. A CDP-alcohol phosphotransferase motif, Asp^{99}-Gly^{100}-(X_2)-Ala^{103}-Arg^{104}-(X_8)-Gly^{113}-(X_3)-Asp^{117}-(X_3)-Asp^{121}, was completely conserved in BnAAPT1 and its catalytic role was confirmed by scanning alanine mutagenesis. Over-expression of BnAAPT1 under the control of the double 35S promoter in transgenic *Arabidopsis thaliana* (L.) Heynh. plants led to elevated levels of the corresponding transcript and enzyme activity. In four of the high over-expression transgenic lines, phospholipid and fatty acid composition analyses revealed that chloroplastidic and extrachloroplastidic membranes isolated from transgenic leaves had about a 25% increase in phosphatidylcholine and in the proportions of polyunsaturated fatty acids [18:2 + 18:3], relative to the control. There were also consistent, but small differences observed in the proportions of 18:3 in transgenic green siliques and in 20:1 in mature transgenic seeds of these lines. Induction of *Arabidopsis AAPT* transcription in response to (+)-abscisic acid and low-temperature treatments, and the cold tolerance in *BnAAPT1* transgenic seedlings implies that AAPT may play a role in resistance to damage at low growth temperatures.

Keywords Abscisic acid sensitivity · Aminoalcohol phosphotransferase · *Brassica* · Low-temperature tolerance · Membrane fatty acid composition · Transgenic *Arabidopsis*

Abbreviations AAPTs: aminoalcoholphosphotransferases (EC 2.7.8.1 and EC 2.7.8.2) · ABA: (+)-abscisic acid · CPCT: choline phosphate cytidylyltransferase · CPT: CDP-choline phosphotransferase (EC 2.7.8.1) · EPT: CDP-ethanolamine phosphotransferase (EC 2.7.8.2) · PC: phosphatidylcholine · PE: phosphatidylethanolamine · WT: wild type

Introduction

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major structural phospholipids found in most eukaryotic cellular membranes (Donaldson et al. 1972; Ansell and Spanner 1982). These zwitterionic phospholipids are the products of aminoalcoholphos-

The GenBank accession number for the aminoalcoholphosphotransferase (AAPT1) from *Brassica napus* is AY179560

Q. Qi · A.J. Cutler · S.R. Abrams · D.C. Taylor (✉)
Plant Biotechnology Institute,
National Research Council of Canada,
110 Gymnasium Place,
Saskatoon, SK S7N 0W9, Canada
E-mail: david.taylor@nrc-cnrc.gc.ca
Fax: +1-306-9754839

Y. Huang
Biology Department, Harbin Normal University,
50 Hexing Road, 150080 Harbin, China

Present address: Q. Qi
Monsanto Company, 800 North Lindbergh Blvd.,
St. Louis, MO 63141, USA

photransferase (AAPT) reactions, which convert *sn*-1, 2-diacylglycerol (DAG) to PC and PE using CDP-choline and CDP-ethanolamine, respectively, as phosphoaminoalcohol donors. As the final step of the Kennedy pathway (Kennedy and Weiss 1956), AAPTs are integral membrane proteins whose activity occurs in microsomal fractions of animal, yeast and plant cells (Wilgram and Kennedy 1963; Bowden and Lord 1975; Bell et al. 1981; Dewey et al. 1994). In addition to its role in the maintenance of membrane structure and function, PC acts as a reservoir for fatty acids and second messengers (Billah and Anthes 1990; Exton 1990), a source of acylated glycerol backbone to alter DAG pools, and as a substrate for unsaturation of oleic and linoleic acids in plants (Browse and Somerville 1991).

Studies of yeast and animal AAPTs have supported the presence of distinct diacylglycerol cholinephosphotransferase (CPT, EC 2.7.8.2) and ethanolaminephosphotransferase (EPT, EC 2.7.8.1) enzymes (Bell and Coleman 1980; Percy et al. 1984; McMaster and Bell 1994). In plants, however, it remains to be determined whether separate AAPTs corresponding to PC and PE synthesis exist or whether CPT and EPT activities reside in a single protein. Several studies using microsomal membrane fractions of plant cells suggested that the same enzyme is responsible for the synthesis of both PC and PE (Lord 1975; Sparace et al. 1981; Justin et al. 1985). The recent characterization of *Arabidopsis* AtAAPT1p and AtAAPT2p and soybean AAPT1 suggests that higher-plant AAPTs appear to be dual-function enzymes (Dewey et al. 1994; Goode and Dewey 1999), although AtAAPT2p showed a modestly higher preference for CDP-choline over CDP-ethanolamine in comparison with AtAAPT1p. However, examination of additional plant AAPT isoforms and a comparison of their structures and functions are necessary before reaching this conclusion.

Alignment of the predicted protein sequences derived from the known DNA sequences of yeast *CPT1* and *EPT1*, soybean *AAPT1*, *Arabidosis AtAAPT1p* and *AtAAPT2p*, and *Brassica campestris AAPT1* revealed high conservation at all levels, especially in the first soluble loop involving the CDP-aminoalcohol-binding domain (McMaster and Bell 1997; Goode and Dewey 1999). Furthermore, database searches for known proteins with homology to the CDP-choline-binding region of CPT1 also identified sequences within prokaryotic phosphatidylglycerolphosphate synthase and phosphatidylserine synthase, and yeast and mammalian phosphatidylinositol synthase (McMaster and Bell 1997). Each of these enzymes catalyzes the synthesis of a phospholipid by the displacement of CMP from a CDP-alcohol by a second alcohol with formation of a phosphoester bond. Comparison of the sequences within each of the above enzymes identified a completely conserved motif Asp-Gly-$(x)_2$-Ala-Arg-$(x)_8$-Gly-$(x)_3$-Asp-$(x)_3$-Asp, referred to as the CDP-alcohol phosphotransferase motif (McMaster and Bell 1997; Williams and McMaster 1998). The motif of yeast CPT1 has been demonstrated to play a role in catalysis of the enzyme by scanning alanine mutagenesis (Williams and McMaster 1998). It is unknown whether this motif of animal and plant AAPTs has a similar function.

Although the precise molecular mechanisms regulating AAPT activity remain to be defined, some biochemical properties of the enzyme have been studied using microsomal preparations of yeast, animal and plant cells. Activation of CTP by cations Mg^{2+}, Mn^{2+}, and Co^{2+} and phospholipid have been observed in yeast (McMaster et al. 1996), animals (Cornell 1989) and plants (Jolliot et al. 1982). Inhibition of AAPT by Ca^{2+} and CMP has been demonstrated in yeast (McMaster et al. 1996), animal (Cornell 1989) and plant (Chapman and Trelease 1990; Goode and Dewey 1999) systems. CPT activity showed a dramatic change during castor bean endosperm development (Moore and Kinney 1989), implying a role in plant developmental regulation. CPT-specific activities of rye (*Secale cereale*), soybean (*Glycine max*) and canola (*Brassica napus*) were significantly stimulated by low growth temperature (Kinney et al. 1987; Cho and Cheesbrough 1990; Itzhaki et al. 1991).

The present work describes the isolation of a *Brassica napus AAPT1* cDNA, and the molecular and biochemical characterization of its encoded product, BnAAPT1. We examined its functionality, substrate specificity, and regulation by cations and phospholipids by in vitro enzyme assays using recombinant BnAAPT1 expressed in a yeast strain deficient in AAPT activities. The role of the conserved CDP-alcohol phosphotransferase motif of BnAAPT1 in catalysis was demonstrated by mutational analysis. The response of the plant AAPT to low growth temperatures and the phytohormone (+)-abscisic acid (ABA) was also studied at a molecular level. Effects of its overexpression on lipid and fatty acid compositions of cell membranes and seeds were also examined in transgenic *Arabidopsis* plants.

Materials and methods

Chemical reagents

Cytidine 5′-diphospho [methyl-^{14}C]choline (1.9 GBq/mmol) and [α-^{32}P]dATP (111 MBq/mmol) were purchased from American Radiolabeled Chemicals. Cytidine 5′-diphospho [methyl-^{14}C]ethanolamine (2.2 GBq/mmol) was obtained from ICN Biochemicals. Phospholipid standards were purchased from Avanti Polar Lipids. The phytohormone ABA was obtained by resolution of the racemic methyl esters of ABA, as described by Dunstan et al. (1992). All other reagents were of the highest quality commercially available.

Construction of canola cDNA library, isolation of *BnAAPT1* cDNA and DNA sequence analyses

cDNAs were synthesized from mRNA isolated from 14-day-old canola (*Brassica napus* L. cv. WestStar) leaves using a ZAP-cDNA Synthesis Kit (Stratagene). Screening of the cDNA library was performed using standard protocols as described by Sambrook et al. (1989). The canola cDNA library was screened with a ^{32}P-labeled cDNA fragment of the *Arabidopsis* expressed

sequence tag (EST; GenBank accession number T42269; obtained from ABRC, Ohio) under highly stringent hybridization conditions. The cDNA inserts from plaque-purified phage clones were cloned into the pBluescript SK(+) vector. One clone (pSKBnAAPT1), which contained the largest cDNA, was the focus for further analyses. DNA was sequenced using an Applied Biosystems model 373 DNA Sequencer System with a *Taq* DyeDeoxyTM Terminator Cycle Sequencing Kit (Applied Biosystems). The nucleotide and deduced amino acid sequences of *BnAAPT1* were compared with sequences available in data banks using the FASTA and BLAST research programs. The sequence of *BnAAPT1* was submitted to GenBank and assigned Accession No. AY179560.

Expression of recombinant *BnAAPT1* in yeast and isolation of microsomal membranes

The *BnAAPT1* cDNA encoding an open reading frame (ORF) of 389 amino acids (aa) was inserted into a yeast expression vector (pYES2, Invitrogen) at *Bam*HI and *Xba*I sites to create the derivative recombinant plasmid pYES2BnAAPT1. Gene expression in the pYES2 vector is under the control of the *Gal1* promoter. The recombinant plasmid was subsequently transformed into the AAPT-deficient yeast strain HJ091 (a *ura3-52, his3-1, leu2-3, 112trp1-289 cpt1::LEU2 ept1*$^{-}$) by the method of Gietz et al. (1992). Strain HJ091 was kindly provided by Dr. C.R. McMaster, Dalhousie University, Halifax, Canada. The transformed yeast cells with pYES2BnAAPT1 or pYES2 alone (plasmid only) were grown at 30°°C in YPD (1% yeast extract, 2% peptone, 2% dextrose) or synthetic dextrose minimal media according to standard protocols (Guthrie and Fink 1991). The overnight cultures were used to inoculate 400 ml of SD-urea supplemented with 2% galactose to give an initial OD_{600} of 0.4. Then cultures were grown at 30 °C to an OD_{600} of 1.0–2.5.

Microsomal membrane fractions were prepared from cell cultures containing the appropriate supplements (400 ml) grown to mid-log phase and harvested by centrifugation (3,000 *g* for 10 min), washed two times with 1 ml of GME buffer [20% glycerol, 50 mM MOPS/NaOH (pH 7.5, 1 mM EDTA] and re-pelleted. Following glass-bead disruption, the homogenate was removed and beads were rinsed three times with GME buffer. The homogenate and washes were centrifuged for 10 min at 16,000 *g*. The resulting supernatant was centrifuged at 100,000 *g* for 70 min in a Ti 65 rotor in a Beckman ultracentrifuge (L8-70 M). The membrane pellet was suspended in 1 ml of GME buffer for enzyme assays. The membrane protein concentration was determined by the method of Bradford (Bradford 1976) using a Bio-Rad kit and BSA as a standard.

Enzyme assays and kinetics

AAPT activities in microsomal membranes isolated from yeast or plants were determined by incorporation of label from [^{14}C]CDP-choline or [^{14}C]CDP-ethanolamine into PC and PE, respectively, using a Triton X-100 mixed micelle assay as described by Hjelmstad and Bell (1991). For the standard enzyme assay, the reaction mixture contained 50 mM MOPS/NaOH (pH 7.5), 20 mM $MgCl_2$, 0.5% Triton X-100, 10 mol% dioleoyl-PC, 10 mol% dipalmitoleoylglycerol (di-16:1 DAG) and 40–80 μg of membrane protein in a total volume of 250 μl. The reaction was initiated by the addition of 100 μM [^{14}C]CDP-choline (0.22 GBq/mmol) and incubated at 30 °C for 30 min, and then stopped by adding 1 ml of 0.15 N HCl and 2 ml chloroform/isopropanol (2:1, v/v). Labeled products, PC or PE, were extracted and quantified by thin-layer chromatography analysis as described by Taylor et al. (1992). Kinetic studies were carried out by varying the reaction time, or protein and substrate concentrations. The utilization of labeled CDP-ethanolamine by the recombinant BnAAPT1 was also examined by replacing labeled CDP-choline with labeled CDP-ethanolamine in the standard assay. Where indicated, effectors on enzyme activity were added to the assays at the concentrations indicated in Table 2.

Site-directed mutagenesis

Scanning alanine mutagenesis of the conserved residues within BnAAPT1 motif was performed. DNAs encoding BnAAPT1 chimeras with a desired residue mutation were produced using the Quickchange site-directed mutagenesis (SDM) kit (Stratagene). The PCR reaction mixture contained SDM buffer, 1 mM deoxynucleotide mix (dATP, dCTP, dTTP, dGTP), 5 ng of *BnAAPT1* cDNA template, 1.5 μM of sense and antisense primers with the desired mutation (Table 1), and 2.5 U of *pfu* DNA polymerase in a total volume of 50 μl. The extension parameters used for SDM were as follows: after denaturation at 96 °C for 2 min, 16 cycles at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 1.5 min using PTC100 Programmable Thermal Controller (MJ Research, Watertown, MA, USA). Following the PCR reaction, the rest of the steps were performed according to the manufacturer's recommendation for the SDM kit. All BnAAPT1/pYES constructs were sequenced to verify that only the intended point mutations were present. The desired chimeras were transformed into the yeast strain HJ091 for production of the recombinant mutant BnAAPT1 enzymes.

Table 1 Mutagenic oligonucelotides used in this study. The mutant triplet codons on each primer are *underlined in lower case font*

Mutation	Mutant oligonucleotide sequence
Wild type	Forward primer: 5′-CAGACATTTGATGCGGTTGATGGAAAGCAAGCACG-3′
	Reverse primer: 5′- CGTGCTTGCTTTCCATCAACCGCATCAAATGTCTG-3′
$Asp^{99} \rightarrow Ala$	Forward primer: 5′-CAGACATTTGATGCGGTTgctGGAAAGCAAGCACG-3′
	Reverse primer: 5′-CGTGCTTGCTTTCCagcAACCGCATCAAATGTCTG-3′
$Gly^{100} \rightarrow Ala$	Forward primer: 5′-GCGGTTGATgctAAGCAAGCACGAAGAACAAACTCC-3′
	Reverse primer: 5′-GGAGTTTGTTCTTCGTGCTTGCTTagcATCAACCGC-3′
$Ala^{103} \rightarrow Gly$	Forward primer: 5′-GCGGTTGATGGAAAGCAAggtCGAAGAACAAACTCC-3′
	Reverse primer: 5′-GGAGTTTGTTCTTCGaccTTGCTTTCCATCAACCGC-3′
$Arg^{104} \rightarrow Ala$	Forward primer: 5′-GGAAAGCAAGCAgctAGAACAAACTCCAG-3′
	Reverse primer: 5′-CTGGAGTTTGTTCTagcTGCTTGCTTTCC-3′
$Gly^{113} \rightarrow Ala$	Forward primer: 5′-CCAGTAGCCCACTCgctGAGCTCTTTGATCATGG-3′
	Reverse primer: 5′-CCATGATCAAAGAGCTCagcGAGTGGGCTACTGG-3′
$Asp^{117} \rightarrow Ala$	Forward primer: 5′-CCACTCGGAGAGCTCTTTgctCATGGTTGTGATGC-3′
	Reverse primer: 5′-GCATCACAACCATGagcAAAGAGCTCTCCGAGTGG-3′
$Asp^{121} \rightarrow Ala$	Forward primer: 5′-GCTCTTTGATCATGGTTGTgctGCACTTGCTTGTGCG-3′
	Reverse primer: 5′-CGCACAAGCAAGTGCagcACAACCATGATCAAAGAGC-3′

Table 2 Biochemical properties of the recombinant BnAAPT1 expressed in yeast (*Saccharomyces cerevisiae*) strain HJ091. The cell membrane preparation and assay conditions were as described in Materials and methods

Apparent K_m for CDP-choline	32 μM
Apparent $V_{max}$for CDP-choline	33.6 [^{14}C]PC nmol·h^{-1}·mg^{-1} membrane protein
Optimal pH	7.6
Activity as % of control value	
Control	100
+ 5 mM Mg^{2+}	134
+ 5 mM Mn^{2+}	125
+ 2 mM Ca^{2+}	66
+ phospholipid mixture	131

Construction of *BnAAPT1* binary vector and transformation of *Arabidopsis*

The *Hin*dIII and *Eco*RI restriction fragment including the double 35S promoter, AMV enhancer and NOS terminator in the corresponding T-DNA cassette was excised from a shuttle vector pBI524 (Datla et al. 1993) and cloned into the respective restriction sites of plant transformation vector pRD400 (Datla et al. 1992) to generate a new binary vector pRD401. The full-length of *BnAAPT1* cDNA was amplified by a pair of primers (5′-CATGCCATGGGATACATAGGAGCACATG-3′ with a *Nco*I site at the 5′ end of the cDNA, and 5′-CGGGATCCTTGAGCTTCCTTCAAGCTTCCTTACG-3′ with a *Bam*HI site at its 3′ end) using *Pfu* polymerase (Stratagene). The resulting PCR product was sequenced to ensure fidelity during the PCR reaction, and digested with *Nco*I and *Bam*HI and cloned into the respective sites of pRD401 in a sense orientation, under the control of a tandem 35S promoter. After digestion and sequence confirmation, the desired plasmid was transformed into *Arabidopsis thaliana* (ecotype Columbia) plants by a vacuum-infiltration method (Bechtold et al. 1992). Transgenic T_1 plants were selected on MS medium containing 50 mg/l kanamycin and further verified by PCR and Southern blot analyses using leaves of T_2 plants. The T_3 transgenic plants were used for molecular and biochemical characterization.

Plant growth conditions and ABA and low temperature treatments

Arabidopsis thaliana (L.) Heynh. plants (ecotype Columbia), both wild type (WT) and transgenic, were grown at the same time in controlled growth chambers under a photosynthetic flux of 100–120 μmol m^{-2} s^{-1}, at 20 °C with a photoperiod of 16 h light/8 h dark.

To analyze the effects of ABA on expression of *Arabidopsis AAPTs*, 12 day-old seedlings grown in pots were cleared of soil with water, and then floated in 0.1×-strength Murashige–Skoog–Gamborg medium without sucrose and hormones. ABA treatment was carried out in a solution containing 30 μM ABA for 24 h. For cold treatment, the seedlings were transferred from 20 °C to a growth chamber set at 2 °C for a further 5 days. Seedling samples were harvested after various treatment intervals for further analysis.

Preparation of chloroplastidic and extrachloroplastidic membranes from *Arabidopsis*

All operations were carried out at 4 °C unless otherwise stated. About 40 g of freshly harvested 2-week-old *Arabidopsis* leaves were homogenized in 4 vol. of ice-cold extraction buffer consisting of 50 mM HEPES (pH 7.5) containing 0.4 M sucrose, 2.5% Ficoll, 2.5% dextran, 4 mM EDTA, 2 mM EGTA, 2 mM DTT, 10 mM KCl, and protease inhibitor cocktail tablets (Roche, Germany). The homogenate was filtered through four layers of cheesecloth and one layer of Miracloth. Subcellular fractions of the tissue homogenate were obtained according to the method of Weiss et al. (1997), which uses a sucrose step gradient and sequential differential centrifugation using an SS 34 rotor in a Sorvall RC5C centrifuge (NEN Life Science Products) and a Ti 65 rotor in a Beckman ultracentrifuge (L8-70 M). All isolated fractions of chloroplast, plasma and microsomal membranes were washed twice with the extraction buffer and resuspended in 50 mM HEPES (pH 7.5) containing 0.2% Triton X-100, 4 mM EDTA, 2 mM DTT, and 40 mM KCl. Membrane fractions were frozen in liquid nitrogen or stored in −80 °C prior to analysis.

RNA isolation and northern blot analysis

Total RNA was isolated from both WT and transgenic leaves using TRIzol reagent (Invitrogen). mRNA was isolated from plant tissues using an mRNA Isolation Kit (Promega) according to the instructions provided by the manufacturer. For northern analyses, total RNA (15 μg) or mRNA (7 μg) was fractionated in denaturing formaldehyde (6.7%), agarose (1.2%) gels and transferred onto Hybond-N+ nylon membrane (Amersham). The blots were hybridized with a α-[^{32}P]dCTP-labeled *BnAAPT1* cDNA as a probe using QuickHyb Solution as described in Stratagene's protocol. Quantification of hybridized signal was performed using Molecular Dynamics Phosphorimager and accompanying software.

Analysis of phospholipid and fatty acid composition from *Arabidopsis* plants

Total acyl lipids were extracted from WT and transgenic leaves, green siliques at mid-development and mature seeds according to the method described by Mudd and Dezacks (1981). A portion of total lipid extracts (TLE) was transmethylated directly for assay of total acyl composition. An internal standard of 17:0 free fatty acid was added. Gas chromatography (GC) was performed on a Hewlett-Packard model 5880 instrument fitted with a DB-23 column (30 m × 0.25 mm; film thickness 0.25 μm; J&W Scientific, Folsom, CA, USA). The GC conditions were as described by Holbrook et al. (1992). The remaining portion of the TLE was further separated into its polar and neutral lipid components by TLC on Silica Gel H as described previously (Taylor et al. 1992), and individual lipid classes were transmethylated and analyzed for acyl composition by GC.

Results

Isolation of a *Brassica napus* cDNA encoding AAPT (*BnAAPT1*) and its predicted structural features

To identify AAPT homologues in *B. napus*, approximately 500,000 plaques of a cDNA library prepared from seedlings of *B. napus* were screened, using an *Arabidopsis* partial EST cDNA (GenBank accession number T42269) as a heterologous hybridization probe. Seven clones from the screen were obtained, sequenced, and shown to represent two genes encoding AAPT homologues. Alignment of the two partial cDNA sequences with the *BnAAPT1* revealed that they have several different amino acid residues, indicating that at least two AAPT genes exist in *B. napus*. Furthermore, the restriction enzymes *Bam*HI, *Xba*I, and *Eco*RI, which

do not have recognition sites within *BnAAPT1* cDNA, were used to digest the genomic canola DNA. These digests produced three hybridizing bands using BnAAPT1 cDNA as a hybridization probe suggesting that the *B.napus* genome likely contains three copies of APPT (data not shown). Five of the clones shared coding symmetry but differed from each other in the size of their 5′ or 3′-untranslated (UTR) regions. The largest of this group was selected for further analysis and designated *BnAAPT1* (GenBank Accession No. AY179560).

The remaining two clones appeared to be *N*-terminus-truncated forms of AAPT and contained some different amino acid residues within the coding region as compared to the other five *BnAAPT* sequences (data not shown). The sequencing of the largest cDNA revealed a putative 1,170-bp ORF flanked by 112 and 246 bp of 5′- and 3′-UTRs, respectively. This ORF encodes a predicted polypeptide of 389 aa with a calculated molecular mass of 43 kDa (data not shown). Sequence comparison revealed that BnAAPT1 showed extensive homology with AAPTs from other species at the primary structure level. The predicted BnAAPT1 exhibited a 33.3% and 30.1% identity with yeast CPT1 and EPT1, respectively; 62.8% with soybean AAPT1; 60.9% and 62% with *Arabidopsis* AtAAPT1p and AtAAPT2p, respectively; 66% and 65.3% with *B. rapa* AAPT1 and 3, respectively; and 61.5% with *Pimpinella brachycarpa* AAPT. A high degree of sequence conservation was also found throughout these primary sequences, including the CDP-aminoalcohol-binding domain, seven membrane-spanning regions, and the active site of the enzyme (Dewey et al. 1994; McMaster and Bell 1997; Williams and McMaster 1998; Goode and Dewey 1999; Choi et al. 2000). This cDNA was further characterized and used in transgenic studies.

Biochemical properties of recombinant BnAAPT1

To examine the functionality of the cloned *BnAAPT1*, the gene was inserted into a yeast vector pYES2 under the control of the galactose-inducible *GAL1* promoter, and expressed in the AAPT-deficient yeast strain HJ091. The microsomal membrane fractions from the induced cells were used to measure the enzyme activities. The specific activity of the recombinant BnAAPT1 was 33.6 [^{14}C]PC nmol h^{-1} mg^{-1}protein with apparent $K_m \approx 32$ μM in the presence of [^{14}C]CDP-choline. The non-transformed or empty vector HJ901 controls did not show any detectable enzyme activity. The enzyme was active at a pH range of 6–8.5, with a maximum activity at pH 7.6 (Table 2). The metal ions Mg^{2+} and Mn^{2+} stimulated BnAAPT1 activity by 34% and 25%, respectively. However, addition of Ca^{2+} in the reaction mixture resulted in inhibition of the enzyme activity by 34%. Phospholipid showed a stimulatory effect (31% increase) on the activity. To investigate the substrate preference of BnAAPT1, competitive inhibition experiments and utilization of the labeled CDP-choline or CDP-ethanolamine as substrates were performed by using the recombinant yeast membrane fractions. As shown in Table 3, BnAAPT1 can utilize both [^{14}C]CDP-choline and [^{14}C]CDP-ethanolamine as substrates. However, the enzyme activity using [^{14}C]CDP-ethanolamine was only 36% of the activity using [^{14}C]CDP-choline as a substrate, showing its preference for CDP-choline. Addition of 60 μM unlabeled CDP-choline or CDP-ethanolamine into the enzyme assay containing an equimolar amount (60 μM) of labeled CDP-choline led to 65% and 32% reduction in the respective activities. Furthermore, using labeled CDP-ethanolamine (60 μM) as a substrate, adding unlabeled CDP-choline or CDP-ethanolamine (60 μM) caused 74% and 55% reduction in the production of the labeled PE, respectively. The results from these substrate competitive inhibition experiments further indicate that BnAAPT1 enzyme has a dual function as a CPT and an EPT, but clearly prefers CDP-choline to CDP-ethanolamine as a substrate.

Mutational analysis of the BnAAPT1 conserved motif

The amino acid sequence of BnAAPT1 was compared with that of other known AAPTs. This comparison revealed the presence of a conserved CDP-alcohol phosphotransferase motif, Asp^{99}-Gly^{100}-$(x)_2$-Ala^{103}-Arg^{104}-$(x)_8$-Gly^{113}-$(x)_3$-Asp^{117}-$(x)_3$-Asp^{121} (Fig. 1). In order to examine whether this motif has a catalytic function in higher-plant AAPTs, we used scanning alanine site-directed mutagenesis to study its effect on BnAAPT1 activity. The substitutions of $Asp^{99} \rightarrow Ala$,

Table 3 Substrate preference of the recombinant BnAAPT1. Microsomal membrane fractions isolated from the BnAAPT1 recombinant HJ091 cells were used for assays in the presence or absence of 60 μM non-radiolabeled CDP-choline or CDP-ethanolamine. In one assay, 60 μM [^{14}C]CDP-choline was substituted by 60 μM [^{14}C]CDP-ethanolamine as a substrate

Substrate	**[^{14}C]PC nmol·h^{-1}·mg^{-1} protein**	**% Of the control**
+ [^{14}C]CDP-choline	35.2 ± 1.2	100
+ [^{14}C]CDP-choline, + cold CDP-choline	12.5 ± 0.3	35
+ [^{14}C]CDP-choline, + cold CDP-ethanolamine	23.9 ± 0.8	68
Substrate	**[^{14}C]PE nmol·h^{-1}·mg^{-1} protein**	**% Of the control**
+ [^{14}C]CDP-ethanolamine, – [^{14}C]CDP-choline	12.7 ± 0.7	100
+ [^{14}C]CDP-ethanolamine, + cold CDP-ethanolamine	5.7 ± 0.6	45
+ [^{14}C]CDP-ethanolamine, + cold CDP-choline	3.3 ± 0.4	26

AY179560 BnAAPT1	D G K Q A R R T N S S S P L G E L F D H G C D
AF446089 BrAAPT3	D G K Q A R R T N S S S P L G E L F D H G C D
AF091844 AtAAPT2	D G K Q A R R T N S S S P L G E L F D H G C D
AF091843 AtAAPT1	D G K Q A R R T N S S S P L G E L F D H G C D
P17898 yeastCPT1	D G M H A R R T G Q Q G P L G E L F D H C I D
U96713 BrAAPT1	D G K Q A R R T N S S S P L G E L F D H G C D
U12735 GmAAPT1	D G K Q A R R T N S S S P L G E L F D H G C D
U96439 PrAAPTase	D G K Q A R R T N S S S P L G E L F D H G C D
S48967 yeast EPT1	D G V H A R R I N Q S G P L G E L F D H S I D

Fig. 1 Known AAPTs possessing a highly conserved CDP-alcohol phosphotransferase motif. BnAAPT1c is the *Brassica napus* protein (encoded by *BnAAPT1*, GenBank accession number AY179560) characterized in the current study. AtAAPT1 and AtAAPT2 represent the *Arabidopsis thaliana* AAPTs (GenBank accession numbers: AF091844 and AF091843). BrAAPT1 and BrAAPT3 are the *Brassica rapa* AAPTs (GenBank accession numbers: U96713 and AF446089, respectively). GmAAPT1 is the soybean (*Glycine max*) AAPT1 (GenBank accession number: U12735). PrAAPTase is the *Pimpinella brachycarpa* AAPT (GenBank accession number: U96439). CPT1 and EPT1 are *Saccharomyces cerevisae* CPT1 and EPT1 (GenBank accession numbers: P17898 and S48967, respectively). The conserved key amino acid residues in the CDP-alcohol phosphotransferase motif are *underlined* and in *bold* in the aligned protein sequences

Table 4 BnAAPT1 activities in microsomal preparations from transgenic yeast cell lysates. Microsomes were prepared from HJ091 yeast cells expressing WT or BnAAPT1 mutant proteins. Enzyme activity was measured using the standard assay conditions described in Materials and methods

BnAAPT1 mutants	[^{14}C-]PC nmol·h^{-1}·mg^{-1} protein	% Of control activity
Wild type	29.3 ± 1.5	100
Asp^{99} → Ala	25.2 ± 1.1	86
Gly^{100} → Ala	6.6 ± 0.5	22
Ala^{103} → Gly	16.6 ± 0.9	56
Arg^{104} → Ala	10.3 ± 0.7	35
Gly^{113} → Ala	3.6 ± 1.5	12
Asp^{117} → Ala	Undetectable	–
Asp^{121} → Ala	Undetectable	–

Gly^{100} → Ala, Ala^{103} → Gly, Arg^{104} → Ala, and Gly^{113} → Ala resulted in 14%, 78%, 44%, 65%, and 88% reduction in the BnAAPT1 enzyme activity, respectively (Table 4). Mutation of Asp^{117} → Ala and Asp^{121} → Ala effectively abolished BnAAPT1 activity.

Induction of *Arabidopsis AAPT* transcription by low temperature and ABA

Since the synthesis of cell membrane phospholipids are regulated by low temperature (Gombos et al. 1994; Welti et al. 2002) and ABA (Farkas et al. 1982), and since the stimulation of AAPT activity by cold temperature has been observed in several species (Kinney et al. 1987; Cho and Cheesbrough 1990; Itzhaki et al. 1991), it was of interest to determine whether *AAPT* transcription of genes endogenous to *Arabidopsis* were responsive to low temperature and to the plant phytohormone ABA. Since *BnAAPT1* shares high homology (83.8% and 88% identity) with *Arabidopsis AAPT1p* and *AAPT1p2p*, the *BnAAPT1* cDNA was used as a hybridization probe for northern blot analysis. As shown in Fig. 2, an mRNA blot analysis clearly reveals that exposure of 14-day-old *Arabidopsis* seedlings to low temperature (2 °C) for 5 days resulted in an increase in transcription of *Arabidopsis AAPT* in comparison with non-acclimated plants (at 20 °C). A similar induction in transcription of *Arabidopsis AAPT* was observed by exogenous application of 30 μM ABA to *Arabidopsis* seedlings for 24 h. It should be noted that the level of *Arabidopsis AAPT* transcription detected in the experiment may be contributed by both *Arabidopsis AAPT1p* and *AAPT2p*, because the two *Arabidopsis AAPT* genes show 83.3% identity at the nucleotide sequence level.

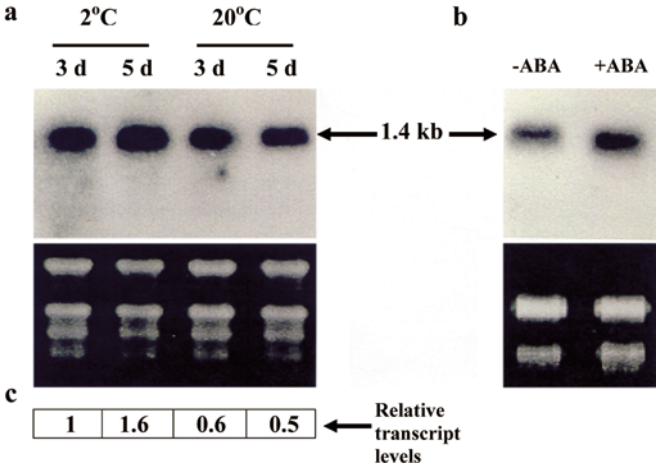

Fig. 2a–c Effect of low growth temperature and ABA treatments on *Arabidopsis* endogenous *AAPT* transcripts in leaf tissue. *Arabidopsis* (ecotype Columbia) seedlings (12-day-old) were treated with cold temperature (2 °C) and 30 μM ABA as described in Materials and methods. mRNA (7 μg) was used for RNA gel electrophoresis. The *top panels* of **a** and **b** show that the blot was hybridized with ^{32}P-labeled *BnAAPT1* cDNA under high-stringency conditions. The *bottom panels* show the corresponding ethidium bromide-stained ribosomal RNA bands as a control for loading. **a** Cold treatment. **b** ABA treatment: *+ABA* and its corresponding control −ABA. **c** Quantification of *Arabidopsis* AAPT transcripts in leaves grown at 2 °C compared to those grown at 20 °C. The intensity of hybridization band of leaves 3 days after the cold treatment was set at 1

Overexpression of *BnAAPT1* in transgenic *Arabidopsis* plants

To determine if the *BnAAPT1* over-expression has any biological significance, we generated 40 transgenic lines of *Arabidopsis* with *BnAAPT1* in a sense orientation under the control of a tandem CaMV 35S promoter. Results of PCR and DNA gel blot analyses confirmed that all of the transgenic lines had inserts from the

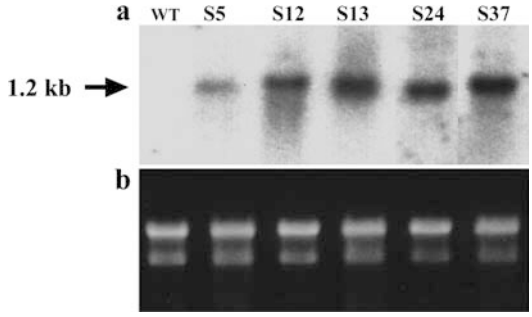

Fig. 3a, b RNA gel blot analysis of *BnAAPT1* transcript in the *Arabidopsis* transgenic leaves. Total RNA was extracted from *Arabidopsis* T_3 transgenic lines S5, S12, S13, S24, S37, and non-transformed wild-type (*WT*) control plants. Fifteen micrograms of total RNA was loaded onto each lane. **a** The blot was hybridized with ^{32}P-labeled *BnAAPT1* cDNA under high-stringency conditions. **b** The corresponding ethidium bromide-stained ribosomal RNA bands were used as a control for loading

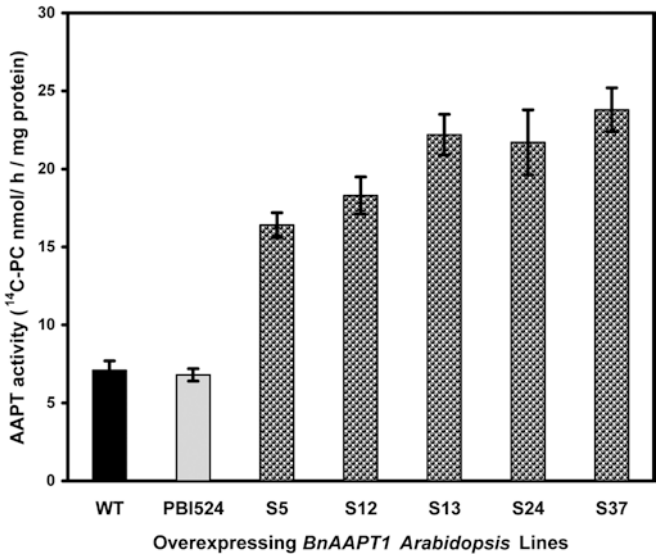

Fig. 4 AAPT activity in the *BnAAPT1* transgenic *Arabidopsis* leaves. CPT (AAPT) assays were performed using microsomal fractions (100 μg protein) from transgenic or wild-type (*WT*) plants and plasmid-only (*pBI524*) control plants in the presence of 100 μM [^{14}C]CDP choline

BnAAPT1 (data not shown). Under normal growth conditions the growth phenotypes of the transgenic and WT plants were indistinguishable. Northern blot analyses using total RNA isolated from young seedlings were performed to determine the relative level of *BnAPPT1* transcript in the transgenic plants. RNA blots of 25 transgenic lines identified a single hybridizing band of ca. 1.2 kb corresponding to the transcript of *BnAAPT*.

Several transgenic lines showed a relatively substantial increase in the steady-state level of the *BnAAPT1* transcript (five are shown in Fig. 3). It is important to note that WT control plants did not show a signal corresponding to the endogenous *Arabidopsis AAPT*, which is not unexpected given that total RNA was utilized in the northern blot in this case (i.e. the relative abundance of the *BnAAPT1* in the total RNA pool would be diluted significantly).

To evaluate whether the increase in transcript levels corresponds to trends in the enzyme activity, CPTase assays were carried out using microsomal preparations from 20-day-old leaves of the *BnAAPT1* transgenic and WT plants. As shown in Fig. 4, the microsomal preparations derived from the five selected transgenic plants displayed approximately 2- to 3.5-fold higher CPTase activity relative to the control. The results from the RNA blot analyses and CPTase enzyme assays clearly indicate an overexpression of *BnAAPT1* in leaves of the four selected transgenic *Arabidopsis* lines.

Polar lipid and fatty acid compositions in the transgenic *Arabidopsis*

Fatty acid compositions were analyzed from crude extracts of leaves, green siliques and mature seeds of the 22 transgenic lines and the WT (complete data not shown). About 20% of the transgenics i.e. four of the transgenic lines, S12, S13, S24 and S37, exhibited consistent proportional changes in polyunsaturated and long-chain fatty acids (Table 5). For instance, the content of 18:2 in the S37 leaves was increased from 9.4 to 13.9 mol% total fatty acids (47% increase). The green siliques of the same transgenic line showed an increase in content of 18:3 from 21.8 to 26.9 mol% total fatty acids (23% increase) and in content of 22:1c15 from 3.7 to 15.8 mol% of total fatty acids (4-fold increase). Similarly, the mature seeds of the S37 transgenic line showed an increase in 20:1c11 from 7.5 to 12 mol% of total fatty acids. Lines S12, S13 and S24 exhibited similar trends: i.e. increases in the proportions of 18:2 in the leaves, 18:3 in green siliques and 20:1 in mature seeds (Table 5).

Since the Kennedy pathway plays an important role in phospholipid synthesis and these polar lipids are the major components of plant cellular membranes, more detailed studies of lipid and fatty acid profiles were conducted in lines S13 and S37, specifically addressing changes in the extrachloroplastidic and chloroplastidic membranes of the *BnAAPT1* transgenic plants. The extrachloroplastidic and chloroplastidic membrane fractions were separately isolated from 14-day-old leaves of transgenic lines, S13 and S37, and from WT for analyses of polar lipid and fatty acid proportions. The chloroplastidic membranes in the transgenic plants showed substantial (18–20%) increases in the PC content while phosphatidylglycerol content exhibited a 15–25% decrease, relative to that of WT (Table 6). For extrachloroplastidic membrane fractions, the PC content in the S37 transgenic line exhibited an increase (20%) from 37.4 to 45.1 mol% of total phospholipids, while phosphatidic acid content in the S37 line decreased (38%) from 9.5 to 5.9 mol% of total phospholipids. The contents of the other phospholipids in the transgenic extrachloroplastidic membranes did not show significant differences compared to WT. Furthermore, analyses of

Table 5 Fatty acid composition in total lipid extracts from leaves, green siliques and mature seeds of the *BnAAPT1* transgenic *Arabidopsis* plants. Values are expressed as mole percentages. Consistent trends in proportions as cited in the Results are marked in *bold font*. *n.d.* Not detected

Tissue	Line	16:0	$16:1^{9}$	16:3	18:0	$18:1^{9}$	18:2	18:3	20:0	$20:1^{11}$	$20:1^{13}$	22:0	$22:1^{13}$	$22:1^{15}$
Leaf	WT	16.1	3.5	11.3	1.5	1.2	9.4	55.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	S13	15.3	4.3	10.8	1.5	1.4	**12.5**	54.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	S37	16.9	3.8	10.1	1.7	2.0	**13.9**	52.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	S12	14.7	3.4	11.8	1.5	1.6	**12.9**	54.2	–	–	–	–	–	–
	S24	16.6	4.1	10.1	1.4	1.1	**10.3**	56.8	–	–	–	–	–	–
Green siliques	WT	10.2	0.6	0.9	3.1	11	27.8	21.8	2.2	13.7	1.6	1.8	1.5	3.7
	S13	12.7	1.0	2.3	2.9	7.8	28.2	**23.9**	1.8	7.5	1.2	1.3	0.9	8.5
	S37	16.6	1.7	3.8	2.6	4.1	25.0	**26.9**	1.2	1.6	0.3	0.4	0	15.8
	S12	11.4	0.8	1.3	3.1	9.1	21.7	**29.4**	2.2	11.1	1.9	1.9	1.4	5.9
	S24	9.5	0.6	1.2	3.0	11.4	21.0	**28.3**	1.7	14.8	1.6	1.0	1.0	5.2
Mature seeds	WT	14.4	1.4	2.0	2.9	8.6	28.3	24.8	1.5	7.5	1.8	2	1.7	2.6
	S13	12.3	1.0	1.1	2.9	8.8	27.6	22.6	1.9	**11.4**	1.7	2.0	1.9	3.8
	S37	11.6	1.1	0	3.0	10.4	28.3	22.4	1.7	**12.0**	1.9	1.7	2.4	4.2
	S12	12.3	1.2	1.3	2.9	9.8	28.8	22.2	1.6	**10.8**	1.4	2.2	1.9	2.8
	S24	12.7	1.2	0.8	2.8	9.6	27.2	25.1	1.6	**11.1**	1.5	2.3	1.4	2.9

Table 6 Lipid composition of extrachloroplastidic and chloroplastidic membrane fractions isolated from *BnAAPT1* transgenic leaves. S13 and S37 are two BnAAPT1 *Arabidopsis* transgenic lines. Membranes used for lipid analyses were isolated from 20-day-old leaves. Lipids: *DGDG* digalactosyl diacylglycerol, *MGDG* monogalactosyl diacylglycerol, *PA* phosphatidic acid, *PG* phosphatidylglycerol, *PI* phosphatidylinositol, *PS* phosphatidylserine, *SQDG* sulfoquinovosyl diacylglycerol. *n.d.* Not detected

Lipid	Extrachloroplastidic (mol% of total phospholipids)			Chloroplastidic (mol% of total lipids)		
	WT	S13	S37	WT	S13	S37
MGDG	n.d.	n.d.	n.d.	34.5 ± 0.6	36.5 ± 0.8	37.2 ± 1.5
DGDG	n.d.	n.d.	n.d.	31.6 ± 0.5	32.7 ± 0.3	34.1 ± 1.0
SQDG	n.d.	n.d.	n.d.	5.8 ± 0.2	4.2 ± 0.2	3.9 ± 0.5
PC	37.4 ± 1.1	44.3 ± 2.1	45.1 ± 2.3	16.7 ± 0.4	20.1 ± 0.6	19.6 ± 1.3
PE	31.4 ± 0.8	32.5 ± 1.4	30.6 ± 1.0	0.8 ± 0.2	0.4 ± 0.1	0.5 ± 0.2
PG	10.4 ± 0.7	8.7 ± 1.2	7.8 ± 0.4	8.1 ± 0.5	6.9 ± 0.3	5.2 ± 0.8
PI + PS	11.3 ± 0.6	9.8 ± 0.4	10.6 ± 1.6	2.1 ± 0.7	1.0 ± 0.4	1.4 ± 0.3
PA	9.5 ± 0.7	4.7 ± 0.3	5.9 ± 0.3	0.5 ± 0.2	0.3 ± 0.1	0.5 ± 0.1

Table 7 Distribution of 18:1, 18:2 and 18:3 in the extrachloroplastidic and chloroplastidic membranes from the *BnAAPT1* transgenic leaves. The same total lipid extracts used in Table 5 were used in this experiment

Fatty acid	Extrachloroplastidic (mol% of total fatty acids)			Chloroplastidic (mol% of total fatty acids)		
	WT	S13	S37	WT	S13	S37
18:1	7.6 ± 0.4	4.3 ± 0.5	5.5 ± 0.7	6.4 ± 0.4	4.5 ± 0.8	4.1 ± 0.3
[18:2 + 18:3]	22.6 ± 1.7	28.7 ± 1.2	27.4 ± 2.7	30.3 ± 1.5	37.7 ± 0.3	35.1 ± 1.6

the unsaturated fatty acids (oleic (18:1), linoleic (18:2), linolenic (18:3) acids) revealed that both extrachloroplastidic and chloroplastidic membranes in the S13 and S37 transgenic plants had an approximately 25% increase in total proportions of [18:2 + 18:3] in comparison to the WT (Table 7).

Response of *BnAAPT1* transgenic *Arabidopsis* plants to low temperature

As described above, the *Arabidopsis* endogenous *AAPT* gene was induced by low temperature (Fig. 2). It was interesting to test the response of *BnAAPT1* transgenic *Arabidopsis* seedlings to cold treatment. The S13 and S37 transgenic T_3 seeds along with WT seeds were initially germinated and grown for 10 days at 20 °C as described in Materials and methods. Forty seedlings each for the transgenic lines and WT were then transferred into a growth chamber at 2 °C for 20 days. The same numbers of the transgenic and WT seedlings continued to grow at 20 °C. The data are summarized in Table 8. When exposed to 2 °C for 20 days, both the transgenic and WT plant exhibited decreases in plant biomass and chlorophyll content. The dry weight and total chlorophyll content of the WT were reduced by 13- and 2.3-fold, respectively, in comparison with those of non-cold-treated WT seedlings, whereas the transgenic

Table 8 Vegetative growth characteristics in *BnAAPT1* transgenic and WT*Arabidopsis* plants. Plants were germinated, grown for 10 days at 20 °C, and then transferred to 2 °C for 20 days. Chlorophyll content of *Arabidopsis* leaves was measured according to the method described by Wu et al. (1997)

	Dry weight (mg per plant)			Total chlorophyll ($mg \cdot g^{-1}$ fresh weight)		
	WT	S13	S37	WT	S13	S37
20 °C	124.5 ± 3.2	129.6 ± 4.0	132.5 ± 1.2	1.6 ± 0.2	1.6 ± 0.1	1.8 ± 0.3
2 °C	9.6 ± 1.4	15.7 ± 2.3	17.1 ± 1.6	0.7 ± 0.1	1.1 ± 0.2	1.3 ± 0.1

lines, S13 and S37, showed 8- and 1.5-fold reduction in the dry weight and total chlorophyll content, respectively, at the low growth temperature, relative to 20 °C growth temperature. When all plants were exposed to the 2°C treatment, the biomass and total chlorophyll content of the transgenic plants were higher than those of WT by 2- and 1.6-fold, respectively.

Discussion

CPTase and EPTase catalyze the final reactions of the Kennedy (CDP-choline or CDP-ethanolamine) pathway for de novo synthesis of PC and PE, respectively. Kennedy and co-workers first described these enzymes during their seminal observation of the universal use of cytidine nucleotides for the synthesis of phospholipids (Kennedy and Weiss 1956; Weiss et al. 1958). The two enzymes are integral membrane-bound entities utilizing both an insoluble substrate (diacylglycerol) and a soluble substrate (CDP-alcohol). These enzymes are collectively referred to as aminoalcoholphosphotransferases (AAPTs; Bell and Coleman 1980; Dewey et al. 1994; Goode and Dewey 1999).

Although CPTase and EPTase catalyze similar reactions, the detailed genetic, molecular and biochemical studies of yeast and mammalian AAPTs have established that two distinct enzymes exist in these tissues (Percy et al. 1984; Hjelmstad and Bell 1991; McMaster and Bell 1994). However, in higher plants, the situation remains unclear and additional studies in vitro and in vivo are necessary to settle this debate, even though several studies have claimed that plants possess a single enzyme (AAPT), which catalyzes both CPT and EPT reactions (Macher and Mudd 1974; Lord 1975; Sparace et al. 1981; Justin et al. 1985).

More recently, molecular and biochemical characterization of soybean *AAPT1* and *Arabidopsis AtAAPT1p* and *AtAAPT2p* revealed that these plant AAPTs can utilize both CDP-choline and CDP-ethanolamine as substrates but with different preferences and CMP inhibition properties (Dewey et al. 1994; Goode and Dewey 1999). These results further suggest that higher-plant AAPT is a dual-function enzyme. However, as pointed out by the authors of those studies, this conclusion still needs to be supported by contributions from independent in vitro and in vivo studies of various plant AAPT isoforms.

A full-length cDNA isolated from *Brassica napus* was confirmed to encode an AAPT by its enzymatic activity in a heterologous yeast expression system. The primary structure of BnAAPT1 generally resembles that of AAPTs from yeast and other plant species in that it shows a high similarity to these AAPTs and possesses amino acid residues essential for the CDP-alcohol binding domain and the conserved predicted membrane-spanning regions. Furthermore, our characterization of BnAAPT1 indicates that it has similar biochemical properties to other enkaryotic AAPTs in terms of cation (Mg^{2+} or Mn^{2+}) and phospholipid requirements for the activation of the enzyme activity (Table 2). The evidence presented in this study clearly demonstrates that BnAAPT1 possesses a significant CPTase activity (Table 3). However, BnAAPT1 shows a significant difference from yeast CPT1 with respect to CDP-alcohol substrate specificity. The BnAAPT1 can utilize both CDP-choline and ethanolamine as substrates (Table 3), while yeast CPT1 was shown to be capable of utilizing only CDP-choline (Percy et al. 1984).

Two lines of evidence support our finding that BnAAPT1 shows a greater preference for CDP-choline over CDP-ethanolamine as a substrate. First, utilization of the radiolabeled CDP-ethanolamine as a substrate gave only 36% of the activity observed using [^{14}C]CDP-choline. Secondly, the substrate dilution assays demonstrated that non-radiolabeled CDP-choline was more effective than non-radiolabeled CDP-ethanolamine in diluting the enzyme activity (measured as incorporation of labeled CDP-choline or CDP-ethanolamine into PC or PE, respectively; Table 3).

The existence of more than one *BnAAPT* gene in *B. napus* adds to the complexity of BnAAPT function in plants. A more precise investigation of the functional differences, if any, between these AAPT isoforms in canola can be pursued when the full-length additional AAPT cDNAs are isolated. Interestingly, multiple copies of AAPTs in higher plants were also found in soybean (Dewey et al. 1994), *Arabidopsis* (Goode and Dewey 1999), and *B. rapa* (Choi et al. 2000).

Multiple alignment of various orthologous AAPT amino acid sequences reveals a conserved CDP-alcohol phosphotransferase motif Asp-Gly-$(X)_2$-Ala-Arg-$(X)_8$-Gly-$(X)_3$ (Fig. 1). This motif was also completely conserved in enzymes that catalyze the same reaction type (Williams and McMaster 1998). These enzymes include prokaryotic phosphatidylglycerolphosphate synthase

and phosphatidylserine (PtdSer) synthase, and yeast and mammalian phosphatidylinositol synthase and PtdSer synthase (McMaster and Bell 1997). The structure/function study of yeast CPT1 indicated that the CDP-alcohol phosphotransferase motif plays an intimate role in catalysis (Williams and McMaster 1998). However, it has been observed that this motif is not essential for *Escherichia coli* PtdSer synthase. The *E. coli* enzyme possesses a separate motif, $H(x)K(U)_4D(X)_4UUGO$, that also appears capable of the formation of the identical phosphoester bond (Koonin 1996; Ponting and Kerr 1996). To test whether the higher eukaryotic (e.g. higher plant) AAPTs have a similar function for this motif as does yeast CPT1, scanning alanine site-directed mutagenesis of the conserved amino acid residues within the motif Asp^{99}-Gly^{100}-$(x)_2$-Ala^{103}-Arg^{104}-$(x)_8$-Gly^{113}-$(x)_3$-Asp^{117}-$(x)_3$-Asp^{121} was conducted as shown in Table 1. The ability of each mutant to catalyze the BnAAPT1 reaction was assessed in vitro using a mixed-micelle enzyme assay by determining the capacity of each mutant to incorporate radiolabeled choline into PC in *CPT1*- and *EPT1*- deficient strains. Overall results of the current study generally agree with data from the yeast CPT1 study (McMaster and Bell 1997), but also show certain differences (Table 4). For instance, mutation at Asp^{99} to Ala displayed a minor effect (14% reduction) on the current enzyme activity, while the same mutation in CPT1 did not result in any effect. The substitution of Gly^{100} with Ala caused a 78% decrease in the BnAAPT1 enzyme activity, whereas this mutation of the CPT1 completely ablated detectable activity both in vitro and in vivo. The Gly^{100} has been suggested to be involved in protein stability or folding. BnAAPT1 mutations at $Ala^{103} \rightarrow Gly$, $Arg^{104} \rightarrow Ala$, and $Gly^{113} \rightarrow Ala$ showed a significant reduction in enzyme activity. In vitro studies on yeast CPT1 had demonstrated that mutations at Ala^{117} and Arg^{118} of the CPT1 motif led to 5- to 10-fold decreased apparent V_{max} and 2- to 4-fold increased apparent K_m, suggesting that the two residues play a role in substrate binding. However, when expressed in CPT1 null yeast cells in vivo, the Ala^{117} to Gly and Arg^{118} to Ala mutants showed incorporation rates of the exogenously supplied radiolabeled choline into PC that were similar to those of their parent enzyme. This indicated that substrate availability plays a regulatory role in the flow of metabolites through the nucleotide pathway to PC synthesis in this organism. The mutations of $Asp^{117} \rightarrow Ala$ and $Asp^{121} \rightarrow Ala$ in he current study eliminated BnAAPT1 activity. These results indicated that the Asp residues at positions 117 and 121 are crucial for BnAAPT1 activity. They are consistent with results obtained in studies of the yeast CPT1 protein wherein it was suggested that AAPT enzymes most likely utilize general base catalysis via nucleophilic attack by the hydroxyl group of the free alcohol on one of the final two Asp residues within the motif (McMaster and Bell 1997).

The levels of endogenous *Arabidopsis* AAPT transcripts increased in plants grown at a low temperature and in seedlings treated with ABA (Fig. 2). It should be noted that the transcripts measured in the control and the treated *Arabidopsis* seedlings may come from both *AtAAPT1p* and *AtAAPT2p* (Goode and Dewey 1999), because they are highly homologous. Therefore, the current study cannot distinguish which isoform of *AtAAPTs* was induced by the treatments.

The up-regulation of plant CPTase activity by low temperature has been observed in rye roots (Kinney et al. 1987) and soybean cotyledons (Cho and Cheesbrough 1990). More recently, it has been reported that the level of *AAPT2* transcript increased at low temperature in different organs of Chinese cabbage (Choi et al. 2000). The same group also reported an induction of choline phosphate cytidylyltransferase (CPCT) transcription by low temperature (Choi et al. 2001). The higher expression of plant AAPTs, coordinated with CPCT, could lead to an increase in PC and PE content or in the proportions of polyunsaturated fatty acids in cellular membranes, thus enhancing membrane fluidity and the ability of cold-sensitive young seedlings to resist low temperature. Several lines of evidence support this hypothesis. The exposure of wheat seedlings to aminoalcohols such as choline and ethanolamine can enhance their ability to resist low-temperature stress (Horvath et al. 1981; Williams et al. 1987). In the present study, our *BnAAPT1* transgenic *Arabidopsis* seedlings exhibited higher chlorophyll content and biomass than those of WT seedlings when grown at 2 °C (Table 8), indicating an increase in cold tolerance. *BnAAPT1* transgenic tomato plants also showed a similar resistance to cold temperature (our unpublished observation). Further- more, analyses of extrachloroplastidic and chloroplastidic membrane lipids revealed a 25% increase in PC and in polyunsaturated fatty acids [18:2 + 18:3].

The plant hormone ABA, has been shown to regulate expression of many fatty acid-related genes, including oleosin, Δ15 desaturase, and 3-ketoacyl-Coenzyme A synthase, phospholipid transfer protein (Holbrook et al. 1992; Zou et al. 1995; Qi et al. 1998; Hughes et al. 1992, respectively). In addition, it was observed that pea plant CPTase activity was stimulated by the auxin indol-3-ylacetic acid. However, the molecular mechanism for the induction of *Arabidopsis* AAPT by ABA as reported here, is currently unknown.

Expression of *BnAAPT1* in *Arabidopsis* plants led to elevated levels of the corresponding transcript and enzyme activity (cf. Figs. 3 and 4, respectively). While most of the transgenic lines did not exhibit substantial changes in PC content or fatty acid composition in the total lipid extracts of transgenic leaves, siliques and seeds compared to the WT control, four transgenic lines, namely S12, S13, S24 and S37, showed consistent changes in the proportions of certain fatty acids in these three organs (Table 5). Interestingly, the increase in 20:1 in the mature seeds of the *BnAAPT1* transgenics may, for the first time, directly support the hypothesis that PC may play a role in the elongation of oleoyl moieties to very long-chain fatty acids (VLCFAs), and, via the

reverse activity of CPTase, produce a diacylglycerol pool enriched in VLCFAs (e.g. 20:1 moieties) to be redirected to the storage lipid (triacylglycerol) pool. Further detailed analyses of leaf phospholipids and fatty acids using extrachloroplastidic and chloroplastidic membranes isolated from S13 and S37 and WT leaves revealed a 25% increase in PC and in polyunsaturated fatty acids [18:2 + 18:3] in the *BnAAPT*1 transgenics (Tables 6 and 7, respectively). In contrast, it has been reported that overexpression of soybean AAPT1 in transgenic tobacco plants did not result in significant changes in lipid and fatty acid composition in the transgenic tobacco leaves relative to the control tissue (Goode and Dewey 1999).

Studies in animals suggest that PC synthesis via the Kennedy pathway is normally regulated at the level of CDP-choline supply via CPCT (McMaster and Bell 1997). However, analyses of the correlation between PC synthesis and enzyme activities (CPCT, AAPT and choline kinase) of the Kennedy pathway during the postgermination period of castor bean concluded that the coordination of membrane CPCT and AAPT plays a role in regulation of PC synthesis (Kinney and Moore 1989). In addition, regulation of AAPT activity by low temperature, plant hormones, and developmental stage of rose petals (Itzhaki et al. 1998) and bean cotyledons (Brown et al. 1994) implies that AAPT may play a role in controlling the nucleotide pathway. However, a more precise molecular investigation of plant AAPTs is necessary to uncover the regulatory mechanism of PC biosynthesis.

In conclusion, a *BnAAPT1* gene isolated from *Brassica napus* shows high homology with AAPTs from other plant species and has similar protein structures including the conserved CDP-aminoalcohol-binding and membrane-binding domains. It shares similar biochemical properties with other AAPTs but with different substrate preferences, clearly preferring CDP-choline, but also able to use CDP-ethanolamine. The first molecular characterization of plant CDP-alcohol phosphotransferase motifs of BnAAPT1 revealed that the motif is involved in catalysis of the enzyme, similar to the conclusion drawn from studies of the yeast CPT1. Cold induction of *Arabidopsis AAPT* expression and an increase in the cold tolerance of *Arabidopsis* transgenic seedlings transformed with *BnAAPT1*, suggest that plant AAPT plays a role in resistance to low growth temperature. Finally, overexpression of *BnAAPT1* in *Arabidopsis* plants resulted in four transgenic lines showing relatively high over-expression (transcript intensity) and about a 2- to 3.5-fold elevation in CDP-choline AAPT activity. These changes were correlated with increases in PC content and in the proportions of [18:2 + 18:3] in leaf membrane fractions. Data from a number of studies suggest that a coordinated expression of CPCT and AAPT is probably required to maximally enhance the production of PC.

Acknowledgments The authors acknowledge the excellent support of Dr. Lawrence Pelcher, Barry Panchuk and Don Schwab in PBI's DNA Technologies Unit and Drs. Elżbieta Miętkiewska and Fawzy Georges for their helpful comments when reviewing this manuscript prior to submission. This is NRCC Publication No. 45249.

References

Ansell GB, Spanner S (1982) Phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine. In: Neuberger A, van Deenen LLM (eds) New comprehensive biochemistry, vol 4. Elsevier, New York, pp 1–50

Bechtold N, Ellis J, Pelletier G (1993) In planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C R Acad Sci Ser III Sci Vie 316:1194–1199

Bell RM, Coleman RA (1980) Enzymes of glycerolipid synthesis in eukaryotes. Annu Rev Biochem 49:459–487

Bell RM, Ballas LM, Coleman RA (1981) Lipid topogenesis. J Lipid Res 22:391–403

Billah MM, Anthes JC (1990) The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem J 269:281–291

Bowden L, Lord JM (1975) Development of phospholipid synthesizing enzymes in castor bean endosperm. FEBS Lett 9:369–371

Bradford MM (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

Brown JH, Chambers JA, Ghosh S, Froese CD, Huff AM, Thompson JE (1994) Phospholipid metabolism in membranes of senescing bean cotyledons. J Exp Bot 45:1513–1522

Browse J, Somerville C (1991) Glycerolipid synthesis: biochemistry and regulation. Annu Rev Plant Physiol Plant Mol Biol 42:467–506

Chapman KD, Trelease RN (1990) Inhibition of cottonseed choline- and ethanolaminephosphotransferases by calcium during postgerminative growth. Plant Physiol 93:1525–1529

Cho SH, Cheesbrough TM (1990) Warm growth temperatures decrease soybean cholinephosphotransferase activity. Plant Physiol 93:72–76

Choi YH, Lee JK, Lee CH, Cho SH (2000) cDNA Cloning and expression of an aminoalcoholphosphotransferase isoform in Chinese cabbage. Plant Cell Physiol 41:1080–1084

Choi YH, Lee JK, Cho SH (2001) Structure and expression of a CPT: phosphocholine cytidyltransferase gene from *Arabidopsis thaliana*. Mol Cells 11:95–99

Cornell R (1989) Cholinephosphotransferase. In: Vance DE (ed) Phosphatidylcholine metabolism. CRC Press, Boca Raton, FL, pp 47–64

Datla RSS, Hammerlidl JK, Panchuk B, Pelcher LE, Keller, WA (1992) Modified binary plant transformation vectors with the wild-type gene encoding NPTII. Gene 211:383–384

Datla RSS, Bekkaoui F, Hammerlidl JK, Pilate G, Dunstan DI, Crosby WL (1993) Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. Plant Sci 94:139–149

Dewey RE, Wilson RF, Novitzky WP, Goode JH (1994) The AAPT1 gene of soybean complements a cholinephosphotransferase-deficient mutant of yeast. Plant Cell 6:1495–1507

Donaldson RP, Tolbert NE, Schnarrenberger C (1972) A comparison of microbody membranes with microsomes and mitochondria from plant and animal tissue. Arch Biochem Biophys 152:199–215

Dunstan DI, Bock CA, Abrams GD, Abrams SR (1992) Metabolism of (+)- and (−)abscisic acid by somatic embryo cultures of white spruce. Phytochemistry 31:1451–1454

Exton JH (1990) Signaling through phosphatidylcholine breakdown. J Biol Chem 265:1–4

Farkas T, Nemecz G, Singh B (1982) Abscisic acid-induced changes in physical state and lipid composition of plant membranes. Dev Plant Biol 8:471–474

Gietz D, St Jean A, Woods RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 20:1425

Gombos Z, Wada H, Murata N (1994) The recovery of photosynthesis from low temperature photoinhibition is accelerated by the unsaturation of membrane lipids: a mechanism of chilling tolerance. Proc Natl Acad Sci USA 91:8787–8791

Goode JH, Dewey RE (1999) Characterization of aminoalcoholphosphotransferases from *Arabidopsis thaliana* and soybean. Plant Physiol Biochem 37:445–457

Guthrie C, Fink GR (1991) Guide to yeast genetics and molecular biology: methods in enzymology, vol 42. Academic Press, New York

Hjelmstad RH, Bell RM (1991) sn-1,2-Diacylglycerol choline- and ethanolaminephosphotransferases in *Saccharomyces cerevisiae*: mixed micellar analysis of the *CPT1* and *EPT1* gene products. J Biol Chem 266:4357–4365

Holbrook LA, Magus JR, Taylor DC (1992) Abscisic acid induction of elongase activity, biosynthesis and accumulation of very long chain monounsaturated fatty acids and oil body proteins in microspore-derived embryos of *Brassica napus* L. cv Reston. Plant Sci 84:99–115

Horvath I, Vigh I, Farkas T (1981) The manipulation of polar head group composition of phospholipids in the wheat Miranovskaja 808 affects frost tolerance. Planta 151:103–108

Hughes MA, Dunn MA, Pearce RS, White AJ, Zhang L (1992) An abscisic-acid-responsive, low temperature barley gene has homology with a maize phospholipids transfer protein. Plant Cell Environ 15:861–865

Itzhaki H, Pauls KP, Borochov A (1991) Effects of cold hardening on microsomal membrane properties and phosphatidylcholine biosynthesis in canola (*Brassica napus*) leaves. J Plant Physiol 138:75–79

Itzhaki H, Mayak S, Borochov A (1998) Phosphatidylcholine turnover during senescence of rose petals. Plant Physiol Biochem 36:457–462

Jolliot A, Justin AM, Bimont EB, Mazliak P (1982) Regulation by lipids of plant microsomal enzymes. III. Phospholipid dependence of the cytidine-diphospho-choline phosphotransferase of potato microsomes. Plant Physiol 70:206–210

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

Kennedy EF, Weiss SB (1956) The function of cytidine coenzymes in the biosynthesis of phospholipids. J Biol Chem 222:193–214

Kinney AJ, Moore TS (1989) Phosphatidylcholine biosynthesis in castor bean endosperm: mechanisms of regulation during the immediate postgermination period. Phytochemistry 28:2635–2639

Kinney AJ, Clarkson DT, Loughman CB (1987) The regulation of phosphatidylcholine biosynthesis in rye (*Secale cereale*) roots. Stimulation of the nucleotide pathway by low temperature. Biochem J 242:755–759

Koonin EV (1996) A duplicated catalytic motif in a new superfamily of phosphohydrolases and phospholipid synthases that included poxvirus envelope proteins. Trends Biol Sci 21:242–243

Lord JM (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 BA, Mudd JB (1974) Biosynthesis of phosphatidylethanolamine by enzyme preparations from plant tissue. Plant Physiol 53:171–175

McMaster CR, Bell RM (1994) Phosphatidylcholine biosynthesis in *Saccharomyces cerevisiae*: regulatory insights from studies employing null and chimeric sn-1,2-diacylglycerol choline- and ethanolaminephosphotransferase. J Biol Chem 269:28010–28016

McMaster CR, Bell RM (1997) CDP-choline: sn-1,2-diacylglycerol cholinephosphotransferase. Biochim Biophys Acta 11348:100–110

McMaster CR, Morash SC, Bell RM (1996) Phospolipid and cation activation of chimaeric choline/ethanolamine phosphotransferase. Biochem J 313:729–735

Moore JR, Kinney AJ (1989) Phosphatidylcholine biosynthesis in castor bean endosperm. Mechanisms of regulation during the immediate postgermination period. Phytochemistry 28:2635–2639

Mudd JB, Dezacks R (1981) Synthesis of phosphatidylglycerol by chloroplasts from leaves of *Spinacia oleracea* L. (spinach). Arch Biochem Biophys 209:584–591

Percy AK, Carson MA, Moore, JF, Waechter CJ (1984) Control of phosphatidylethanolamine metabolism in yeast: diacylglycerol ethanolaminephosphotransferase and diacylglycerol cholinephosphotransferase are separate enzymes. Arch Biochem Biophys 230:69–81

Ponting CP, Kerr ID (1996) A novel family of phospholipase D homologs that include phospholipid synthases and putative endonucleases: identification of duplicated repeats and potential active site residues Protein Sci 5:914–922

Qi Q, Rose PA, Abrams GD, Taylor DC, Abrams SR, Cutler AJ (1998). (+)-Abscisic acid metabolism, 3-ketoacyl-Coenzyme A synthase gene expression, and very-long-chain monounsaturated fatty acid biosynthesis in *Brassica napus* embryos. Plant Physiol 117:979–987

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sparace SA, Wagner LK, Moore TS (1981) Phosphatidylethanolamine synthesis in castor bean endosperm. Plant Physiol 67:922–925

Taylor DC, Barton DL, Rioux KP, Mackenzie SL, Reed DW, Underhill EW, Pomeroy MK, Weber N (1992) Biosynthesis of acyl lipids containing very-long chain fatty acids in microspore-derived and zygotic embryos of *Brassica napus* L. cv Reston. Plant Physiol 99:1609–1618

Weiss CA, White E, Huang H, Ma H (1997) The G protein α subunit (GPα1) is associated with the ER and the plasma membrane in meristematic cells of *Arabidopsis* and cauliflower. FEBS Lett 407:361–367

Weiss SB, Smith SW, Kennedy EP (1958) The enzymatic formation of lecithin from cytidine diphosphate choline and D-1,2-diglyceride. J Biol Chem 231:53–64

Welti R, Li W, Li M, Sang Y, Biesiada H, Zhou HE, Rajashekar CB, Williams TD, Wang X (2002) Profiling membrane lipids in plant stress responses. Role of phospholipase Dα in freezing-induced lipid changes in *Arabidopsis*. J Biol Chem 277:31994–32002

Wilgram GF, Kennedy EP (1963) Intracellular distribution of some enzymes catalyzing reactions in the biosynthesis of complex lipids. J Biol Chem 236:2615–2619

Williams JG, McMaster CR (1998) Scanning alanine mutagenesis of the CDP-alcohol phosphotransferase motif of *Saccharomyces cerevisiae* cholinephosphotransferase. J Biol Chem 273:13482–13487

Williams WP, Horvath I, Quinn PJ, Thomas PG, Vigh L (1987) Freezing resistance and lipid changes in choline-treated wheat seedlings. In: Stumpf PK, Mudd JB, Nes WD (eds) The metabolism, structure, and function of plant lipids. Plenum Press, New York pp 201–204

Wu J, Lightner J, Warwick N, Browse J (1997) Low-temperature damage and subsequent recovery of *fab1* mutant *Arabidopsis* exposed to 2 °C. Plant Physiol 113:347–356

Zou J, Abrams GD, Barton DL, Taylor DC, Pomeroy MK, Abrams SR (1995) Induction of lipid and oleosin biosynthesis by (+)-abscisic acid and its metabolites in microspore-derived embryos of *Brassica napus* L. cv Reston. Plant Physiol 108:563–571