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Cloning and Expression of a cDNA Encoding Aminoalcoholphosphotransferase from *Pimpinella brachycarpa*

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Aminoalcoholphosphotransferase catalyzes the synthesis of phosphatidylcholine and phosphatidylethanolamine from diacylglycerol plus CDP-choline or CDP-ethanolamine as the phosphobase donor. We screened a cDNA library to isolate a clone for use in studying the structure and expression pattern of this enzyme from *Pimpinella brachycarpa*. The *P. brachycarpa* aminoalcoholphosphotransferase cDNA contains an open reading frame of 1,170 bp coding for a protein of 389 amino acids. The deduced amino acid sequence shares over 90% similarity with other aminoalcoholphosphotransferase sequences. Hydropathy profile analysis suggests that the secondary structure of *P. brachycarpa* aminoalcoholphosphotransferase is very similar to that of soybean and Chinese cabbage enzymes, having an overall hydrophobicity and the same number of predicted transmembrane helices. The catalytic domain contains the CDP-alcohol phosphotransferase motif with two aspartate residues. Reverse transcriptase-PCR analysis indicates that the expression of *P. brachycarpa AAPT* is regulated by temperature.

Keywords: aminoalcoholphosphotransferase, phosphatidylcholine, phosphatidylethanolamine, phospholipid, *Pimpinella*, temperature

Phosphatidylcholine and phosphatidylethanolamine are abundant phospholipids, comprising more than 80% of the total in most eukaryotic membranes. They are mainly synthesized de novo by homologous nucleotide pathways consisting of three consecutive reactions (Kennedy and Weiss, 1956; Moore, 1982; Vance, 1989; Kinney, 1993). The terminal step of each pathway involves the conversion of diacylglycerol to phospholipid using cytidine diphosphate (CDP)-choline or -ethanolamine as the source of the head group (Weiss et al., 1958). In yeast, the enzymes responsible for this reaction, collectively called aminoalcoholphosphotransferases, are separate: cholinephosphotransferase for phosphatidylcholine biosynthesis and ethanolaminephosphotransferase for phosphatidylethanolamine biosynthesis (Hjelmstad and Bell, 1991). In plants, however, it has been speculated that both enzyme activities are catalyzed by a single aminoalcoholphosphotransferase with dual substrate specificity (Macher and Mudd, 1974; Lord, 1975; Sparace et al., 1981; Justin et al., 1985; Dewey et al., 1994). The product of a single gene has been shown to be responsible for both cholinephosphotransferase and ethanolaminephosphotransferase activities in soybean (*Glycine max*; Dewey et al., 1994) and *Arabidopsis thaliana* (Goode and Dewey, 1999). The deduced amino acid sequence of *Brassica rapa* L. ssp. *pekinensis* (Chinese cabbage) *AAPT1* has also suggested that it encodes an aminoalcoholphosphotransferase having 81% identity and 94% similarity with that of soybean (Min et al., 1997).

In the context of mammalian aminoalcoholphosphotransferases, it had been assumed that separate cholinephosphotransferase and ethanolaminephosphotransferase activities exist, but the situation has become complicated. Recent studies have revealed that, while an ethanolaminephosphotransferase specific enzyme purified near homogeneity was reported from bovine liver (Mancini et al., 1999), two human isoforms have been detected: one, the product of the *hCEPT1* gene, having the ability to synthesize both phosphatidylcholine and phosphatidylethanolamine in vitro and in vivo (Henneberry and McMaster, 1999); and the other, the product of *hCPT1*, exhibiting only cholinephosphotransferase activity (Henneberry et al., 2000).

We have been studying the molecular aspects of phospholipid metabolism in plants, especially in relation to the effects of temperature on the biosynthesis of phosphatidylcholine. The effect of temperature on the metabolism of phosphatidylcholine is well docu-

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mented. For example, in rye (*Secale cereale*) roots the incorporation of choline into phosphatidylcholine was higher in 5°C-grown than 20°C-grown roots (Kinney et al., 1987). All three enzymes of the nucleotide pathway, including cholinephosphotransferase, showed higher activities in roots grown at low temperatures. In soybean the activity of cholinephosphotransferase was higher in 20°C-grown than in 35°C-grown cotyledons (Cho and Cheesbrough, 1990). The higher enzyme activity at lower temperatures may have resulted from increased synthesis of the enzyme rather than from the involvement of isozymes or metabolic effectors. Because data are lacking on the molecular aspects of a temperature-control mechanism, we have undertaken a series of experiments to study the effects of cold treatment on phosphatidylcholine biosynthesis in various plants. Herein we report the results from our analysis of *Pimpinella brachycarpa*, an important oilseed plant, whose cDNA reveals close identity with soybean *AAPT1* and Chinese cabbage *AAPT1*.

MATERIALS AND METHODS

Plant Material and Construction of cDNA Library

P. brachycarpa (Kom.) Nakai, induced from somatic embryogenic callus, was cultured on MS medium (Murashige and Skoog, 1962) as described by Kim et al. (1996). For cold treatment, the plants were transferred to a 5°C growth chamber and further grown for 1, 2, 4, or 6 d. A cDNA library from shoot tips induced from somatic embryogenic callus was constructed by using the λ ZAPII cDNA Synthesis Kit and ZAP-cDNA Gigapack II Gold Cloning Kit (Stratagene).

Screening the cDNA Library

The cDNA library was transformed into *Escherichia coli* strain XL1-Blue MRF' (Stratagene). A total of 3.0×10^5 plaques were blotted onto Hybond-N+ filters (Amersham). Our probe was synthesized by PCR amplification using a digoxigenin (DIG) labeling system (Boehringer Mannheim) with the Chinese cabbage *AAPT1* cDNA as a DNA template; forward primer (5'-CTTTGCCTGCTA-CTAACCACG-3'); and reverse primer (5'-CAAAACA-CCTCCAAGAAGAACAG-3'). The filters were prehybridized for about 1 h at 42°C in 5× SSC, 50% formamide, 0.1% sodium-lauroylsarcosine, 0.02% SDS, and 2% blocking reagent. Hybridization was carried out in prehybridization solution plus a DIG-labeled probe for 18 h at 42°C. The filters were washed with 2× SSC, 0.1% SDS at room temperature twice for 5 min each, and then with 0.5× SSC, 0.1% SDS at 68°C twice for 15 min each.

Sequencing and Sequence Analysis

The phage DNA isolated from cDNA-library screening was excised in vivo with ExAssist helper phage to recover pBluescript SK(–), according to the manufacturer's instructions (Stratagene). DNA sequencing was performed by using Sequenase Version 2.0 (United States Biochemical) with the T3 and T7 primers, and gene-specific primers. Analysis of the nucleotide and deduced amino acid sequences was performed according to Kyte and Doolittle (1982), DNASIS (Hitachi), and CLUSTAL V (Higgins et al., 1992).

Reverse Transcriptase-PCR (RT-PCR) with an Endogenous Standard

Total RNA was extracted from the leaves of normal (25°C) or cold-treated (5°C) plants with the RNeasy Plant Mini Kit (Qiagen). With one μg total RNA as template, reverse transcription was performed using the Reverse Transcription System (Boehringer-Mannheim) for 1 h at 42°C. Using 2 μL of the total 20-μL reaction mixture as template, PCR was performed with forward and reverse primers (forward primer, 5'-GGTTCATGTTGCTCACGG-3'; reverse primer, 5'-TA-CTATTATGGGTTTTCC-3') to synthesize a 1192-bp fragment that corresponded to nucleotides 251 to 1442. As an internal standard, a 315-bp fragment from 18S rRNA was amplified in the same reaction mixture, as specified by the manufacturer (Quantum-RNA 18S Internal Standards, Ambion).

RESULTS AND DISCUSSION

To isolate the *AAPT* cDNA from *P. brachycarpa*, we screened approximately 3.0×10^5 plaques using the DIG-labeled 858-bp fragment as a probe synthesized by PCR from the sequence of Chinese cabbage *AAPT1* (Min et al., 1997). After secondary screening, 15 positive phage clones were selected and excised in vivo to recover pBluescript SK(–). The longest positive clone was then sequenced. The sequence of *P. brachycarpa* aminoalcoholphosphotransferase cDNA is 1,600 bp long and contains an open reading frame encoding 389 amino acids (Fig. 1) with molecular mass of 44.2 kD. The length of the coding sequence is exactly the same as that of soybean *AAPT1* (Dewey

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AAAAAGAGTAAGAGATGGGTTATATAGGGGCTCATGGTGTTGCTGCCTTGCATAAGCACA   60
              M  G  Y  I  G  A  H  G  V  A  A  L  H  K  H  K
AGTACAGTGGGGTTGATCACTCTTATCTTGCTAAATATGTCTTGCAACCCTTTTGGACTA  120
 Y  S  G  V  D  H  S  Y  L  A  K  Y  V  L  Q  P  F  W  T  K
AATGTGTTACCTTCTTCCCTCTTTGGATGCCACCCAATATGATTACTCTCGTGGGATCTA  180
 C  V  T  F  F  P  L  W  M  P  P  N  M  I  T  L  V  G  S  M
TGTTCTTGGTCACTTCTGCTGCTCTTGGCTTTATATATTCACCTCACTTAGATTCGCCTC  240
 Y  L  V  T  S  A  A  L  G  F  I  Y  S  P  H  L  D  S  P  P
CACCCAGATGGGTTCATGTTGCTCACGGTCTACTTCTATTCCTGTACCAGACTTTTGATG  300
 P  R  W  V  H  V  A  H  G  L  L  L  F  L  Y  Q  T  F  D  A
CTGTGGATGGCAAGCAAGCAAGAAGGACCAATTCTTCTAGTCCATTGGGAGAGCTGTTTG  360
 V  D  G  K  Q  A  R  R  T  N  S  S  P  L  G  E  L  F  D
ATCATGGATGTGATGCACTTGCATGTACCTTTGAGGCGTTAGCCTTTGGGAGCACTGCCA  420
 H  G  C  D  A  L  A  C  T  F  E  A  L  A  F  G  S  T  A  M
TGTGTGGGAAAGATACTTTCTGGTTTTGGGTGATATCAGCTGTTCCATTTTATGGTGCCA  480
 C  G  K  D  T  F  W  F  W  V  I  S  A  V  P  F  Y  G  A  T
CATGGGAACACTTTTTCACCAATACACTTATCCTTCCCGTTGTTAATGGACCTACAGAGG  540
 W  E  H  F  F  T  N  T  L  I  L  P  V  V  N  G  P  T  E  G
GTCTCATGCTGATATACGTGGGCCATATCTTTACAGCTATAGTTGGGGCCGAGTGGTGGG  600
 L  M  L  I  Y  V  G  H  I  F  T  G  P  T  E  G  L  M  L  I
TCCATCAGTTTGGAAAATCTGTACCGTTCTTGAGTTGGGTCCCAATTTTAAGTGAAGTTC  660
 Y  V  G  H  I  F  T  P  F  L  S  W  V  P  I  L  S  E  V  P
CGACATATCGAGCAGTATTGTATTTGATGATTGCTTTTGCTGTTATTCCGACGTTGACAT  720
 T  Y  R  A  V  L  Y  L  M  I  A  F  A  V  I  P  T  L  T  F
TCAATGTGCAAAATGTTTACAAAGTTGTCCAGGCAAGAAAAGGAAGCATGCTTCTAGCTT  780
 N  V  Q  N  V  Y  K  V  V  Q  A  R  K  G  S  M  L  L  A  L
TAGCAATGCTTTACCCATTTGTGGTGCTAATGGCAGGGATCTTGATCTGGGATTATTTGT  840
 A  M  L  Y  P  F  V  V  L  M  A  G  I  L  I  W  D  Y  L  S
CTCCATATGATATAATGGTGAATTATCCATATATGGTTGTGTTGGGAACTGGACTTGCTT  900
 P  Y  D  I  M  V  N  Y  P  Y  M  V  V  L  G  T  G  L  A  F
TTGGGTTTCTGGTGGGGAGAATGGTTCTGGCTCACTTGTGTGATGAACCAAAGGGATTGA  960
 G  F  L  V  G  R  M  V  L  A  H  L  C  D  E  P  K  G  L  K
AAACTAATATGTGCCTGTCACTGTTGTATCTCCCATTTGCCATTGCAAATACACTCACTG 1020
 T  N  M  C  L  S  L  L  Y  L  P  F  A  I  A  N  T  L  T  A
CCAGACTAAATGATGGGGTTCCATTGGTTGAAGAGAAATGGGTTCTCCTTGGTTATTGTG 1080
 R  L  N  D  G  V  P  L  V  E  E  K  W  V  L  L  G  Y  C  V
TATACACAGGTGCACTTTATCTGCATTTCGCAACATCAGTTATCCACGAAATAACGACTG 1140
 Y  T  G  A  L  Y  L  H  F  A  T  S  V  I  H  E  I  T  T  A
CTCTCGGAATCTATTGCTTCAGGATAACAAGAAAGGAGGCTTGAGAATGTGCACTTGTTC 1200
 L  G  I  Y  C  F  R  I  T  R  K  E  A  *
TTTACTTCTTTACTATAGGAACCTCAGATTCAGGTGGCAGATTGGTCTGAAAGGTTTATC 1260
TGTGAATTCAACTGGAGAAGTATTGTCAATCAACGAAAATTTTGGGTTCTTGCAGAATGC 1320
AGTAGAAAATCTATCACAATTACTTGATCTAGTTGATTCCGTGATTCATTATATATGTAC 1380
TTGGCTATATGCCATTCATGACATCTTATTCCTCATTAATTGATGGAAAACCCATAATAG 1440
TAATACTTCCTTGTATTTTGCTTCAGGGATAGTAGAAGTGGTGATGGAAAATCGTCTTTT 1500
GTAATCTCTTTGGAAAGTTTGCTTTGGCTGCAACCTAATACTTTCGTCACCAATATTATG 1560
TGTGCTTCTTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1. Nucleotide and predicted amino acid sequences of *P. brachycarpa* aminoalcoholphosphotransferase cDNA. The GenBank accession number of the cDNA sequence is U96439. Underlined sequences and those marked with ^ are putative FUE and NUE sites, respectively, of the polyadenylation signal.

et al., 1994) and Chinese cabbage *AAPT1* (Min et al., 1997).

The first ATG codon, at position 15, is preceded by a short A-rich segment (Fig. 1). The nucleotides surrounding the start codon (TAAGAGATGGCT) are closely related to the consensus sequence (TAAACAATGGCT) for plant translation initiation (Joshi, 1987). The short open reading frame consisting of 11 amino acids, suggested to be responsible for the negative regulation of soybean *AAPT1* gene activity (Dewey et al., 1994), could not be evaluated in the *P. brachycarpa* cDNA due to the short length of the 5'-nontranslation region. The 3' end of the cDNA has a TGA stop codon at position 1,182, followed by an untranslated sequence of 416 bp that includes a poly(A) terminus of 29 bp. A putative polyadenylation signal sequence, AATATT, is located 20 bp upstream from the poly(A) sequence. The 3'-untranslated region also contains a far upstream element (FUE) motif (Rothine, 1996), TTTGTA, at position 1,498, and another similar sequence, TTTGGA, at position 1,509. Several repeats of the second FUE motif (Rothine, 1996), GATT, are present at positions 1,227, 1,240, 1,355, and 1,363.

The hydropathy profile revealed in Kyte and Doolittle's analysis (1982) shows a pattern very similar to those of the soybean and Chinese cabbage enzymes (data not shown). The overall hydrophobicity for *P. brachycarpa* is +57%, comparable to +57% and +61% for the soybean and Chinese cabbage proteins, respectively. All three sequences contain seven membrane-spanning helices, demonstrating that aminoalcoholphosphotransferase is an integral membrane protein.

Analysis of the *P. brachycarpa* aminoalcoholphosphotransferase primary sequence also indicated very high similarity to the soybean and Chinese cabbage enzymes. As shown in Figure 2, aminoalcoholphosphotransferase from *P. brachycarpa* exhibited 79% identity and 91% similarity with soybean aminoalcoholphosphotransferase, and 83% identity and 92% similarity with Chinese cabbage aminoalcoholphosphotransferase at the deduced amino acid level. It is notable that the CDP-aminoalcohol binding domain

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P   MGYIGAHGVAALHKHKYSGVDHSYLAKYVLQPFWTKCVTFFPLWMPPNMI   50
S   MGYIGTHGVAALHRYKYSGVDHSYVAKYVLQPFWSRFVNFFPLWMPPNMI
C   MGYIGAHGVAALHRYKYSGVDHSYLAKYVLQPFWTRFVKVFPLWMPPNMI
    ***** ******* ********* *********   *  **********

P   TLVGSMFLVTSAALGFIYSPHLDSPPPRWVHVAHGLLLFLYQTFDAV■■K  100
S   TLMGFMFLLLSALLGYIYSPQLDTAPPRWVHFAHGLLLFLYQTFDAV■■K
C   TLMGFMFLVTSSLLGYIYSPQLDSPPPRWVHFAHGLLLFLYQTFDAV■■K
    ** * ***  *  ** **** **  ****** ******************

P   Q■RTNSSSPL■ELF■HGC■ALACTFEALAFGSTAMCGKDTFWFWVISAV  150
S   Q■RTNSSSPL■ELF■HGC■ALACTFEALAFGSTAMCGRTTFWWWLISAI
C   Q■RTNSSSPL■ELF■HGC■ALACAFEAMAFGSTAMCGRDTFWFWVISAI
    ************************ *** ********** *** * ***

P   PFYGATWEHFFTNTLILPVVNGPTEGLMLIYVGHIFTAIVGAEWWVHQFG  200
S   TFYGATWEHYFTNTLILPVINGPTEGLMIIYICHFFTAIVGAEWWVQQFG
C   PFYGATWEHYFTNTLILPVINGPTEGLALIFVSHFFTAIVGAEWWVQQLG
     ******** ********* *******  *   * ********** * *

P   KSVPFLSWVPILSEVPTYRAVLYLMIAFAVIPTLTFNVQNVYKVVQARKG  250
S   KSLPFLNWLPYLGGIPTFKAILCLMIAFGVTPTVTCNVSNVYKVVKGKNG
C   QSIPLFSWVPFVNAIQTSRAVLYMMIAFAVIPTVAFNVSNVYKVVQSRKG
     * *   * *       * *  **** * **   ** ******    *

P   SMLLALAMLYPFVVLMAGILIWDYLSPYDIMVNYPYMVVLGTGLAFGFLV  300
S   SMPLALAMLYPFVVLVGGVLVWDYLSPSDIMGKYPHLVVIGTGLTFGYLV
C   SMLLALAMLYPFVVLLGGVLIWDYLSPINLIETYPHLVVLGTGLAFGFLV
    ** ************  * * ******      **  ** **** ** **

P   GRMVLAHLCDEPKGLKTNMCLSLLYLPFAIANTLTARLNDGVPLVEEKWV  350
S   GRMILAHLCDEPKGLKTGMCMSLMFLPLAIANVLASRLNDGVPLVDERLV
C   GRMILAHLCDEPKGLKTNMCLSLVYLPFALANALTARLNDGVPLVDELWV
    *** ************** ** **  ** * ** *  ********* *  *

P   LLGYCVYTGALYLHFATSVIHEITTALGIYCFRITRKEA             389
S   LLGYCAFSVTLYLHFATSVIHEITNALGIYCFRITRKEA
C   LLGYCIFTVSLYLHFATSVIHEITTALGIYCFRITRKEA
    *****     *************** **************
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Figure 2. Alignment of the amino acid sequences derived from the *P. brachycarpa AAPT* (P), soybean *AAPT1* (S), and Chinese cabbage *AAPT1* (C) cDNAs. Identical amino acid residues are shown by asterisks (*). Underlined residues indicate the positioning of the predicted amphipathic helix. Identical residues of a conserved CDP-alcohol phosphotransferase motif of $DGX_2ARX_8GX_3DX_3D$ are boxed.

spanning amino acids 69-139 [as revealed by studies with chimeric enzymes of cholinephosphotransferase and ethanolaminephosphotransferase from yeast (McMaster and Bell, 1997)], shares 92% identity and 99% similarity among the three species. That site is believed to confer substrate specificity to the enzymes for CDP-choline or CDP-ethanolamine. This implies that all three plant aminoalcoholphosphotransferases presented in Figure 2 have the same specificity for CDP-aminoalcohol, that is, the ability to synthesize both phosphatidylcholine and phosphatidylethanolamine from diacylglycerol moieties (Dewey et al., 1994). However, the precise definition of the substrate specificity of *P. brachycarpa* aminoalcoholphosphotransferase awaits detailed biochemical and genetic analyses.

An amphipathic helix with highly asymmetric distribution of hydrophilic and hydrophobic residues was identified within the CDP-aminoalcohol binding domain (Figs. 2 and 3). The polar face of the helix was heavily charged with two Asp (–) and Glu (–) residues each and one His (+) residue. It was hypothesized that the amphilicity of this helix is required to allow interfacing of the hydrophilic CDP-aminoalcohol substrate with that of the hydrophobic diacylglycerol (Henneberry and McMaster, 1999). In this catalytic domain we also identified a conserved motif of phospholipid-synthesizing enzymes that catalyze CDP-alcohol phosphotransferase reactions, Asp Gly X_2 Ala Arg X_8 Gly X_3 Asp X_3 Asp (Fig. 2). Williams and McMaster (1998) have suggested that the final two Asp residues

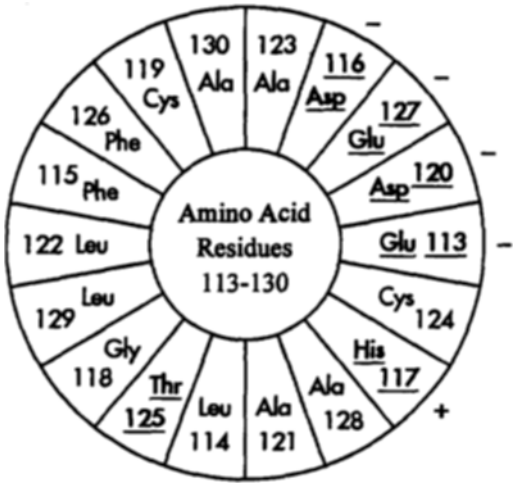

Figure 3. A predicted amphipathic helix within the CDP-aminoalcohol binding domain of *P. brachycarpa* aminoalcoholphosphotransferase. Hydrophilic residues are in bold characters and underlined.

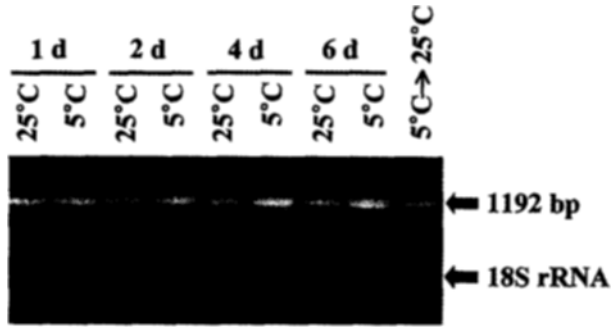

Figure 4. Detection of *P. brachycarpa* aminoalcoholphosphotransferase expression in response to low temperature by RT-PCR. Plants were maintained at 25°C, and then either remained or were transferred to a growth chamber at 5°C for 1, 2, 4, or 6 d. Four-day cold-treated plants were returned to 25°C and further grown for 2 d for comparison with 6-d cold-treated plants. Total RNA was extracted from the leaf tissue and subjected to RT-PCR for the amplification of a 1,192-bp fragment with an endogenous standard 18S rRNA.

of this motif are involved in a general base reaction that does not proceed through an enzyme-bound intermediate.

Using an internal standard with RT-PCR, we examined the expression pattern of *P. brachycarpa* aminoalcoholphosphotransferase in response to low temperature. The anticipated size of the PCR product is 1,192 bp, which includes the 3'-untranslated region. Our data indicated that cold-treated leaves accumulated aminoalcoholphosphotransferase mRNA after a 1-d 5°C treatment, and that these levels continuously increased thereafter up to those found with the 6-d treatment (Fig. 4). This increase in expression that resulted in the elevated amount of phospholipids in cellular membranes at low temperatures will confer cold resistance to plants by increasing their membrane fluidity (Horvath et al., 1981; Williams et al., 1987; Harwood et al., 1994; Harwood, 1998). In addition, we detected an attenuation of mRNA when the cold-treated plants were returned to a normal ambient temperature and grown for 2 d (Fig. 4). This decrease in phospholipid-synthesizing enzyme activities at higher temperatures may be necessary to maintain appropriate membrane fluidity. It is also noteworthy that animal cells compensate for elevated membrane phospholipid production by degrading phosphatidylcholine to glycerophosphocholine in order to sustain membrane phospholipid homeostasis (Baburina and Jackowski, 1999).

We have recently described that the level of Chinese cabbage aminoalcoholphosphotransferase mRNA is

markedly increased within 1 d of cold treatment (Choi et al., 2000). The expression level of *CCT* encoding CTP:phosphocholine cytidylyltransferase, which catalyzes the preceding and cardinal step of the nucleotide pathway of phosphatidylcholine biosynthesis, also increases as early as the first 30 min of treatment at 5°C (Choi et al., 2001). In the work presented here, the level of expression of *P. brachycarpa* aminoalcoholphosphotransferase appeared to be not only up-regulated by low temperatures, but also down-regulated by higher temperatures (Fig. 4). Therefore, we might conclude that expression of phosphatidylcholine-synthesizing enzymes is regulated in response to various temperatures in plants. These results are also consistent with previous reports that the enzyme activities of the nucleotide pathway are greater in plant tissues grown at low temperatures (Kinney et al., 1987; Cho and Cheesbrough, 1990).

The increased level of aminoalcoholphosphotransferase expression will not only confer cold resistance to plants, but production of seed oil might also be improved, because phosphatidylcholine provides acyl components for synthesis of triacylglycerol in developing seeds (Stymne and Stobart, 1987). This will be important for oil crops like *P. brachycarpa*, whose seeds are used as a raw material for producing anise oil that lends a unique fragrance to many commercial products such as liquor, toothpaste, and medicine.

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