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Cloning of an Aminoalcoholphosphotransferase cDNA from Chinese Cabbage Roots

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Aminoalcoholphosphotransferase is the enzyme that catalyzes the synthesis of phosphatidylcholine and phosphatidylethanolamine from diacylglycerol using CDP-aminoalcohol such as CDP-choline and CDP-ethanolamine. To determine its cDNA structure from roots of Chinese cabbage, *Brassica campestris* L. ssp. *pekinensis*, degenerate primers were designed from the regions showing high amino acid homology between yeast *CPT1* and soybean *AAPT1* and used for PCR amplification of Chinese cabbage DNA. Chinese cabbage aminoalcoholphosphotransferase cDNA (*AAPT*) contains an open reading frame of 1,167 bp coding for a protein of 389 amino acids. It shared 81% identity and 94% similarity with soybean *AAPT1* at the predicted amino acid level. Hydropathy profile analysis suggested that the predicted protein structure of Chinese cabbage aminoalcoholphosphotransferase was very similar to the soybean enzyme, showing an overall hydrophobicity and having the same number of predicted transmembrane domains. Southern analysis indicated that there might be close isoforms of the enzyme. *AAPT* was expressed equally well in young shoots and roots.

Keywords: aminoalcoholphosphotransferase, Chinese cabbage, phospholipid, membrane

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major phospholipid components of most eukaryotic membranes. Together they comprise more than 80% of total phospholipids in most of these membranes. Besides their structural roles, they have been also implied in the signal transduction mechanism in animal tissues, especially in the case of PC (Exton, 1990).

PC and PE are mainly synthesized *de novo* by homologous nucleotide pathways consisting of three consecutive reactions (Vance, 1989; Kinney, 1993). The final step of each pathway involves the conversion of diacylglycerol to phospholipid using cytidine diphosphate (CDP)-aminoalcohol, namely CDP-choline or CDP-ethanolamine, as the source of the lipid head group. In animals and yeast, the enzymes responsible for this reaction, collectively called aminoalcoholphosphotransferases, are separate ones for PC and PE biosynthesis (Bell and Coleman, 1980; Percy *et al.*, 1984; Hjelmstad and Bell, 1991a). Thus CDP-choline: *sn*-1,2-diacylglycerol cholinephosphotransferase (E.C. 2.7.8.2) catalyzes the conversion of diacylglycerol to PC, while the biosynthesis of PE is catalyzed by CDP-ethanolamine: *sn*-1,2-diacylglycerol ethanolaminephosphotransferase (E.C. 2.7.8.1).

In plants, however, it was speculated that both enzyme activities are catalyzed by a single aminoalcoholphosphotransferase (Macher and Mudd, 1974; Lord, 1975; Sparace *et al.*, 1981; Justin *et al.*, 1985; Dewey *et al.*, 1994; Monks *et al.*, 1997). The supporting evidence was that the biosynthesis of PC and PE was competitively inhibited by CDP-ethanolamine and CDP-choline, respectively. It was revealed that the product of a single gene, *AAPT1*, was responsible for both cholinephosphotransferase and ethanolaminephosphotransferase activities in soybean (Dewey *et al.*, 1994). Moreover, the amino acid sequence of soybean aminoalcoholphosphotransferase showed almost equal homology to the yeast cholinephosphotransferase (33.0% identity and 58.6% similarity) and the yeast ethanolaminephosphotransferase (32.2% identity and 57.0% similarity) (Hjelmstad and Bell, 1990, 1991b; Dewey *et al.*, 1994). It was the first nucleotide sequence known

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for aminoalcoholphosphotransferase gene in higher eukaryotic organisms.

We were very interested in phosphatidylcholine metabolism in plants, mainly because of the effects of temperature on the biosynthesis of this particular phospholipid. For example, in rye roots the incorporation of choline into PC 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 temperature. In soybean the activity of cholinephosphotransferase was higher in 20°C-grown than in 35°C-grown cotyledons (Cho and Cheesbrough, 1990). It was suggested that the higher enzyme activity at lower temperature could be attributed to a higher level of the enzyme rather than to the involvement of isozymes or metabolic effectors. We have undertaken a series of researches to study the effects of temperature on PC biosynthesis in Chinese cabbage, one of the most important crop plants in Korea. As a first result, we report the cloning of aminoalcoholphosphotransferase cDNA from Chinese cabbage roots.

MATERIALS AND METHODS

Plant Material

Seeds of Chinese cabbage (*Brassica campestris* L. var. *pekinensis* Makino) were germinated on moistened filter paper in sterile petri dish, and plants were grown at greenhouse conditions for 3-4 weeks. Roots were collected into liquid nitrogen and stored at -70 °C prior to use.

Isolation of RNA and Genomic DNA

mRNA was isolated from roots using Messenger RNA Isolation Kit from Stratagene according to the instructions provided by the company. Genomic DNA was isolated from shoots and roots of seedlings (2-3 days after germination) according to the modified protocol of Rogers and Bendich (1988).

Polymerase Chain Reaction (PCR)

PCR was performed using cDNA (synthesized using mRNA from roots) as template and degenerate oligonucleotides, CPT1 and CPT2, synthesized based on homologous sequences from soybean and yeast, as primers. CPT1 (5'-[TC]TNGGNGA[AG][TC]TNTT-[TC]GA[TC]C-3') is a sense primer and corresponds to nucleotide sequences 492-510 of soybean cDNA (Dewey *et al.*, 1994) and 901-919 of yeast genomic DNA (Hjelmstad and Bell, 1990). CPT2 (5'-TGNG-CNA[AGT][AGT]ATCATN[GT]NCC-3') is an antisense primer, corresponding to nucleotide sequences 1063-1081 of soybean and 1484-1502 of yeast DNA. The PCR reaction mixture (25 μL) contained 500 ng template, 2.5 μL 10x PCR buffer (15 mM $MgCl_2$, 50 mM KCl, 100 mM Tris-HCl, pH 8.0), 50 μM degenerate primers, 200 μM dNTP's, and 2.5 units of Dynazyme (Finnzymes OY). Reactions were annealed for 2 min at 50-55°C, extended for 2 min at 72°C and denatured for 1 min at 95°C, and repeated for 35 cycles. The amplified product was cloned into pT7 Blue T-vector (Novagen) and sequenced by using Sequenase Version 2.0 from United States Biochemical.

Rapid Amplification of cDNA Ends (RACE) and Construction of a cDNA Containing the Full Coding Sequence

The 5' and 3' ends of aminoalcoholphosphotransferase cDNA were obtained by RACEs (Frohman *et al.*, 1988; Belyavsky *et al.*, 1989) using MarathonTM cDNA Amplification Kit (Clontech) and 3'-AmpliFinderTM Race Kit (Clontech) according to the instructions of the manufacturer. The 5'-gene specific primer was 5'-CAAAACACCTCCAAGAAGAACCACG-3' (nucleotide sequence 875-899) and 3'-specific gene primer was 5'-TTTCACCAACACACTTATTCTTCCG-3' (nucleotide sequence 569-593). A cDNA containing the full coding sequence was constructed by PCR using cDNA as template and the gene-specific primers FCPT1 (5'-CTTTGCCTGCTACTAACCACCG-3') and FCPT2 (5'-TCCACACAGAAAAACAGCACCC-3') for the 5' and 3' noncoding regions, respectively. The products of these reactions were also cloned into pT7 Blue T-vector (Novagen) and sequenced as above.

Sequence Analysis

The hydropathy analysis of the predicted polypeptide was performed according to Kyte and Doolittle (1982). The prediction of secondary structure from deduced amino acid sequence was based on the Chou-Fasman algorithm (Chou and Fasman, 1978).

Genomic Southern Blot Analysis

Genomic Southern analysis was performed according to the instructions for digoxigenin (DIG)-labeling

provided by the manufacturer (Boehringer-Mannheim). Total genomic DNA (10 μg) was digested with *Hin*dIII and fractionized by electrophoresis in a 0.8% agarose gel. DNA was transferred to positively charged Hybond nylon membrane (Amersham) by capillary blotting in a 10X SSC solution, and then cross-linked by baking for 2 h at 80°C. Prehybridization (5X SSC, 50% formamide, 7% SDS, 2% blocking reagent, 50 mM sodium phosphate, pH 7 and 0.1% N-laurylsarcosine) was performed for 2 h at 42°C, and hybridization was carried out for 18 h at 42°C in a solution with the identical composition after the addition of a DIG-labeled, PCR-synthesized probe (392 bp, nucleotide sequence 507-898). Following hybridization, filters were washed in a 2X SSC, 0.1% SDS at 25°C for 5 min, and subsequently 0.5X SSC (0.1% SDS) at 42°C for 15 min.

Reverse Transcriptase-PCR (RT-PCR) and Southern Blot Analysis

Using one μg total RNA as templates, reverse transcription was performed using the Reverse Transcription system (Boehringer-Mannheim) for 1 h at 55°C. Using 3 μL of total 20 μL reaction mixture as templates, PCR was performed using forward and reverse primers (forward primer, 5'-TTTCACCAACACACTTATTCTTCCG-3'; reverse primer, 5'-ACCATATTGATCATTCGCCG-3') to synthesize a fragment corresponding nucleotide sequence 569-1328. Gel blot analysis was performed basically same as above. The probe (426 bp) for RT-PCR and Southern blot analysis was synthesized by PCR using forward primer (5'-CCTTTCGTGGTTCTTCTTGG-3'; nucleotide sequence 870-889) and reverse primer (FCPT2; nucleotide sequence 1274-1295).

RESULTS

The determined sequence of Chinese cabbage aminoalcoholphosphotransferase cDNA is 1,530 bp long and contains an open reading frame of 1,167 bp coding for a protein of 389 amino acids (Fig. 1) with an estimated molecular mass of 44.2 kD. The length of the coding sequence is exactly same as that of soybean (Dewey *et al.*, 1994), the only plant whose aminoalcoholphosphotransferase cDNA is cloned so far besides Chinese cabbage.

The first ATG codon is relatively well consistent with the ideal context for translation initiation (Joshi, 1987a). There are A residues at positions -3 and -4 (instead of ideal A residues at -1, -3, -4 and -5) (Fig. 1). The downstream triplet GGT (at +4, +5, +6) closely matches the consensus sequence (GCT). The short open reading frame observed in the upstream untranslated region of soybean aminoalcoholphosphotransferase cDNA (Dewey *et al.*, 1994) was not detected in case of Chinese cabbage cDNA.

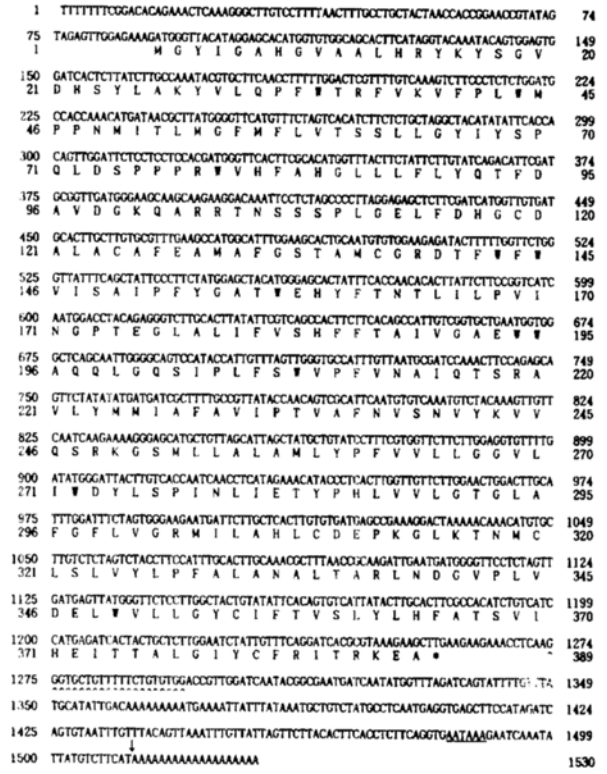

Fig. 1. Nucleotide sequence and deduced amino acid sequence of Chinese cabbage aminoalcoholphosphotransferase cDNA. Stop codon (*) and far-upstream element (^) are indicated below the respective nucleotide sequences. Polyadenylation signal is underlined. Polyadenylation site (↓) is indicated above the nucleotide sequence. The GenBank accession number for the sequence is U96713.

The 3' end of the cDNA has a TGA stop codon (starting at nucleotide 1257) followed by an untranslated sequence of 271 bp including a poly(A) terminus of 19 bp. A putative polyadenylation signal sequence (Joshi, 1987b), AATAAA, is located at 22 bp upstream (starting at nucleotide 1484) from the polyadenylation start site. There is a TG-rich far-upstream element (Hunt, 1994), although a probable consensus motif TTGTA was not found. The sequence (nucleotide sequence 1274-1294) has 90.5% of its nucleotides as G's and T's. This distinctive TG-rich region has been implied to be a *cis* element of plant polyadenylation signals.

As shown in Fig. 2, the enzymes from soybean

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CAAPT    MGYIGAHGVAALHRYKYSGVDHSYLAKYVLQPFWTRFVKVFPLWMPPNMI  50
SAAPT1   MGYIGTHGVAALHRYKYSGVDHSYVAKYVLQPFWSRFVNFFPLWMPPNMI

CAAPT    TLMGFMFLVTSSLLGYIYSPQLDSPPPRWVHFAHGLLLFLYQTFDAVDGK 100
SAAPT1   TLMGFMFLLLSALLGYIYSPQLDTAPPRWVHFAHGLLLFLYQTFDAVDGK

CAAPT    QARRTNSSSPLGELFDHGCDALACAFEAMAFGSTAMCGRDTFWFWVISAI 150
SAAPT1   QARRTNSSSPLGELFDHGCDALACTFEALAFGSTAMCGRTTFWWWLISAI

CAAPT    PFYGATWEHYFTNTLILPVINGPTEGLALIFVSHFFTAIVGAEWWAQQLG 200
SAAPT1   TFYGATWEHYFTNTLILPVINGPTEGLMIIYICHFFTAIVGAEWWVQQFG

CAAPT    QSIPLFSWVPFVNAIQTSRAVLYMMIAFAVIPTVAFNVSNVYKVVQSRKG 250
SAAPT1   KSLPFLNWLPYLGGIPTFKAILCLMIAFGVTPTVTCNVSNVYKVVKGKNG

CAAPT    SMLLALAMLYPFVVLLGGVLIWDYLSPINLIETYPHLVVLGTGLAFGFLV 300
SAAPT1   SMPLALAMLYPFVVLVGGVLVWDYLSPSDIMGKYPHLVVIGTGLTFGYLV

CAAPT    GRMILAHLCDEPKGLKTNMCLSLVYLPFALANALTARLNDGVPLVDELWV 350
SAAPT1   GRMILAHLCDEPKGLKTGMCMSLMFLPLAIANVLASRLNDGVPLVDERLV

CAAPT    LLGYCIFTVSLYLHFATSVIHEITTALGIYCFRITRKEA             389
SAAPT1   LLGYCAFSVTLYLHFATSVIHEITNALGIYCFRITRKEA
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Fig. 2. Comparison of the deduced amino acid sequences between Chinese cabbage *AAPT* (*CAAPT*) and soybean *AAPT1* (*SAAPT1*) cDNA. Identical amino acid residues are indicated by vertical lines, and conservative changes by colons. The two aminoalcoholphosphotransferases have the same 389 amino acids and share 81% identity and 94% similarity.

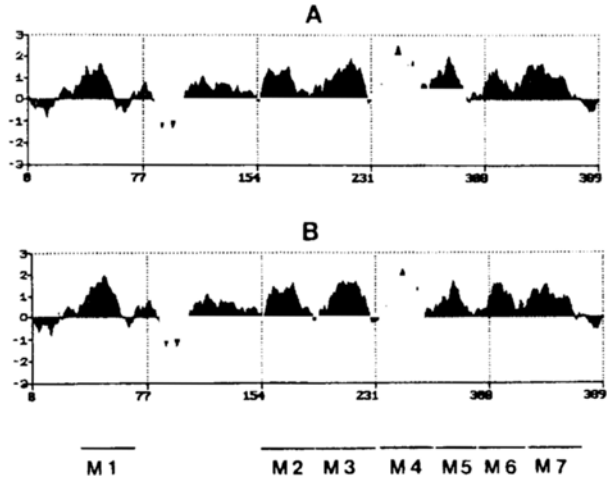

Fig. 3. Comparison of the hydropathy profiles of the predicted protein sequence of aminoalcoholphosphotransferases from Chinese cabbage (A) and soybean (B). Values in positive numbers represent hydrophobicity. The seven membrane-spanning domains are indicated as M1 to M7.

and Chinese cabbage exhibited 81% identity and 94% similarity between them over the entire protein

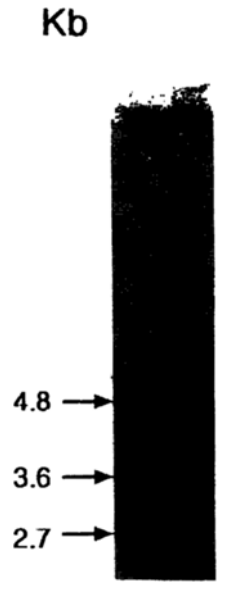

Fig. 4. Southern blot analysis of genomic DNA. DNA was isolated from Chinese cabbage tissues, and digested with *Hind*III. DNA (10 μg) was fractionated in a 0.8% agarose gel, and the transferred membrane was hybridized with a DIG-labeled, PCR-synthesized fragment (392 bp, nucleotide sequence 507-898) as a probe.

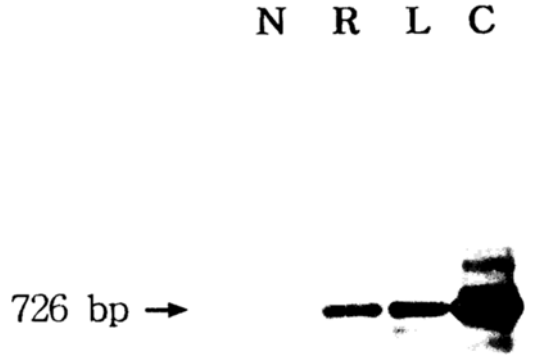

Fig. 5. RT-PCR and Southern blot analysis using the 426-bp fragment (nucleotide sequence 870-1295) as a probe. C, *AAPT* cDNA PCR product as a control; L, shoots; R, roots; N, negative control (PCR with total RNA mixture without reverse transcription).

sequence. The hydropathy profile of Chinese cabbage aminoalcoholphosphotransferase revealed from Kyte and Doolittle analysis (1982) also shows an almost identical pattern with that of soybean enzyme (Fig. 3). Both sequences contained seven helices which have sufficient hydrophobicity and length to span the membrane, demonstrating that aminoalcoholphosphotransferase is a membrane-bound protein.

To determine the number of genes encoding the

enzyme in Chinese cabbage, genomic DNA was isolated from the tissues and analyzed by Southern blot with a DIG-labeled probe (392 bp, nucleotide sequence 507-898). The probe identified two hybridizing fragments of different intensities (Fig. 4) that might indicate the existence of two close isoforms.

RT-PCR and Southern hybridization were performed to investigate the expression pattern of *AAPT*, using the 426-bp fragment including 3'-noncoding region (nucleotide sequence 870-1295) as a probe. As shown in Fig. 5, *AAPT* is expressed equally well in young shoots and roots as expected.

DISCUSSION

Based on the high sequence homology and close structural resemblance with the soybean enzyme, the current Chinese cabbage cDNA-encoded protein is believed to be an aminoalcoholphosphotransferase. Despite of its importance as an enzyme involved in the synthesis of the major phospholipid components of biological membranes, Chinese cabbage is only the second organism whose aminoalcoholphosphotransferase cDNA has been studied in higher eukaryotes after soybean (Dewey *et al.*, 1994), being only the third as a whole. The only other organism is yeast, in which two distinguished enzymes cholinephosphotransferase and ethanolaminephosphotransferase are responsible for the biosynthesis of PC and PE, respectively (Hjelmstad and Bell, 1991a, 1991b).

Although there are only two organisms to compare, aminoalcoholphosphotransferase seems to be a well-conserved enzyme in plants. It has the same length of 389 amino acids with an estimated molecular weight of 44.2 kD (Fig. 1) in both soybean and Chinese cabbage. The homology was 94% between the two plant species (Fig. 2) and the hydropathy analysis revealed almost the same profiles with seven membrane-spanning domains in the structure (Fig. 3).

Southern blot analysis of genomic DNA identified two possible isoforms of *AAPT* cDNA (Fig. 4). In soybean, it was suggested that *AAPT1* exists as a small multigene family (Dewey *et al.*, 1994). It is very interesting to note that a partial cDNA, which shows 95% identity and 99% similarity with *AAPT* at amino acid level within the determined sequence, has been cloned along with AAPT in Chinese cabbage (data not shown). It is very likely that two different isoforms of the gene are responsible for the syhthesis of storage oil in developing seeds and for the production of membrane lipids in young tissues respectively, being expressed at different stages of the plant development, as suggested for soybean.

The result of RT-PCR and Southern hybridization for expression pattern is well expected, since rapidly developing tissues are actively involved in the synthesis of membranes and hence in the synthesis of phospholipids. Basically the same results were obtained in soybean, in which *AAPT1* transcripts accumulated in all tissues, roots, leaves, stems and developing seeds (Dewey *et al.*, 1994).

In relation to the characteristics of the Chinese cabbage aminoalcoholphosphotransferase, the most important feature to be determined is its substrate specificity to CDP-choline and CDP-ethanolamine. It is of our main current interest whether the enzyme of Chinese cabbage utilizes either both CDP-aminoalcohols, as in soybean and other plants, or only one of them. We are now investigating the biochemical characteristics of the protein product of the cloned coding sequence of Chinese cabbage *AAPT* cDNA.

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