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Partial Characterization of Soybean cDNA Encoding CTP : Phosphocholine Cytidylyltransferase

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As the first step to elucidate the relationship between the structure and function of CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) in plants, the partial nucleotide sequence of soybean cytidylyltransferase cDNA was determined using a polymerase chain reaction (PCR). Degenerate oligonucleotide primers were synthesized from the conserved region revealed from the rat and yeast cytidylyltransferase DNA sequences. The catalytic domain region showed 78 and 76% homology with the rat and yeast amino acid sequences, respectively. The hydropathy profile indicated that the C-terminal non-catalytic portion of the protein was very hydrophilic, and in the region between the catalytic domain and the C-terminal region, there was a large amphipathic α-helical domain that was believed to bind the membrane surface in the active formation. There are 7 potential sites for phosphorylation by protein kinase C and 4 potential sites for phosphorylation by Ca^{2+}/calmodulin kinase within the determined sequence.

Keywords : soybean, CTP:phosphocholine cytidylyltransferase, phosphatidylcholine, amphipathic helix

CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) catalyzes the key regulatory reaction of the nucleotide pathway of phosphatidylcholine synthesis in higher eukaryotes (Vance, 1989). In mammals, cytidylyltransferase exists both as an active membrane bound form and as an inactive cytosolic form. The interconversion between the two forms is believed to regulate the overall formation of phosphatidylcholine, the major membrane phospholipid (Vance and Pelech, 1984; Vance, 1989). The mammalian cytidylyltransferase structure predicted from the cDNA sequence indicated an amphipathic α-helical domain at the C-terminal end, within which an 11-mer motif was repeated three times in tandem (Kalmar *et al.*, 1990, 1994; Sweitzer and Kent, 1994). It was suggested that this domain binds the membrane surface in the active formation of cytidylyltransferase (Craig *et al.*, 1994).

In most plant tissues studied so far, cytidylyltransferase has been shown to be ambiquitous like its animal equivalent (Morré, 1970; Lord *et al.*, 1972; Price-Jones and Harwood, 1986; Kinney and Moore, 1987; Kinney *et al.*, 1987; Cheesbrough, 1989). The regulation of cytidylyltransferase activity in plants, however, bears somewhat discrepant results. The enzyme from castor bean endosperm is regulated in a manner similar to that from animal tissues. The activity of the solubilized enzyme was greatly stimulated by phospholipids and phosphatidylcholine-oleate vesicles, suggesting that the membrane-bound form is active (Wang and Moore, 1990). The cytidylyltransferase activity appeared to be also regulated by phosphorylation (Wang and Moore, 1989). The enzyme from pea stems, in contrast, was found to be very hydrophilic and active in its cytosolic form, and not regulated by the translocation mechanism (Price-Jones and Harwood, 1986). In its structure the pea cytidylyltransferase may be more or less like the yeast enzyme, which, although located mainly in the membrane fraction, was predicted from its gene analysis to be rather hydrophilic protein (Tsukagoshi

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et al, 1987). The cytidylyltransferase from yeast lacks the amphipathic membrane-binding domain found in mammals.

Soybean has shown to be an interesting system to study the metabolism of phosphatidylcholine in plant tissues. In developing seeds phosphatidylcholine is actively involved in the synthesis of triacylglycerol by providing polyunsaturated fatty acids (Ohlrogge *et al*., 1991), and in soybean the nucleotide pathway for phosphatidylcholine synthesis seems to be affected by seed growth temperatures (Cheesbrough, 1989; Cho and Cheesbrough, 1990). The activity of cytidylyltransferase along with that of cholinephosphotransferase was two to five times higher in seeds grown at 20℃ than in seeds grown at 35℃. Moreover, the preliminary results showed that the distribution of cytidylyltransferase activities between the cytosol and the postchloroplast membrane fraction changed with alteration of temperatures (Cheesbrough, 1989). As the first step to elucidate the relationship between the structure and function of cytidylyltransferase in plants, we have tried to clone the cDNA for cytidylyltransferase from soybean and report its partial sequence and predicted polypeptide structure in this paper.

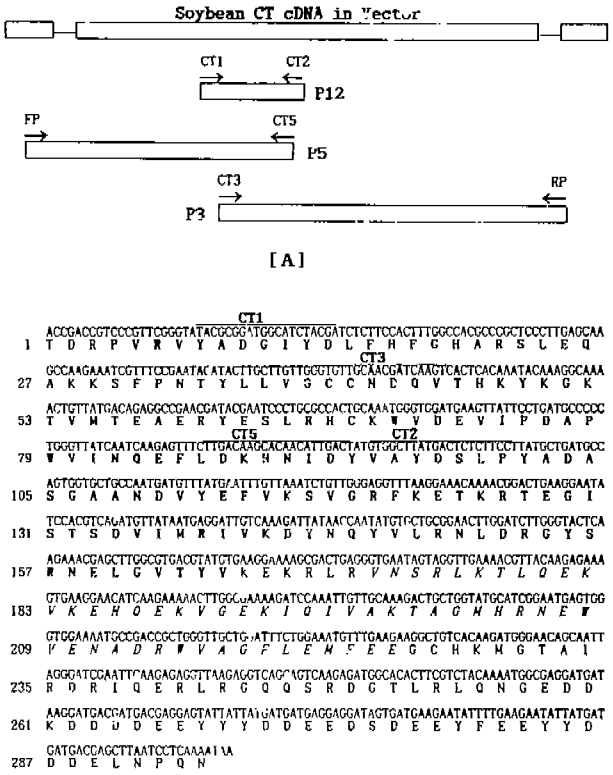

Fig. 1. (A) Polymerase chain reaction strategy to clone soybean cytidylyltransferase cDNA. (B) Nucleotide sequence and deduced amino acid sequence of the cytidylyltransferase cDNA. The sequences used for PCR amplification are indicated by arrows. The amino acid sequences of the proposed catalytic domain are in boldface, and those of the amphipathic helix are in italics. See text for detail.

MATERIALS AND METHODS

Plant material, RNA extraction and cDNA library construction

The total RNA was extracted from somatic embryos obtained from tissue culture of young soybean cotyledons (*Glycine max* cv. Whangkeum) (Choi, 1995). The poly(A$^+$)RNA was isolated using oligo (dT) cellulose column (New England Biolabs) or poly(U) Sepharose affinity paper (Amersham). cDNAs were synthesized and ligated in *Eco*RI-digested λgt11, and cDNA library was tansformed into *Escherichia coli* Y1090.

Polymerase chain reaction (PCR), sequencing and cloning

A partial cDNA was cloned by PCR using degenerate oligonucleotide primers. First strand cDNA was synthesized from mRNA with oligo(dT) primer using cDNA Cycle Kit (Invitrogen) and used directly for PCR as a template according to the instructions by the manufacturer. Sense primer CT1 [5′-TA(CT)GCNGA(CT)GGN(AG)TNTT(CT)GA-3′] and antisense primer CT2 [5′-GG(AGT)AT(AG)TC(AG)TC(AG)TGNGCNAC-3′] were designed based on the common sequences of rat cDNA (Kalmar *et al*., 1990) and yeast genomic DNA (Tsukagoshi *et al*., 1987) (Figs. 1A and 2). The PCR reaction mixture (25 μL) contained 500 ng template, 2.5 μL 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 50 pmol degenerate primers, 200 μM dNTP's, 3 mM $MgCl_2$ and 2.5 units of Taq polymerase. Reactions were annealed for 2 min at 53℃, extended for 2 min at 72℃ and denatured for 1 min at 95℃. The amplified product (P12) was cloned into pT7 Blue T-vector (Novagen) and sequenced by using DNA Sequencing Kit from United States Biochemical with T7

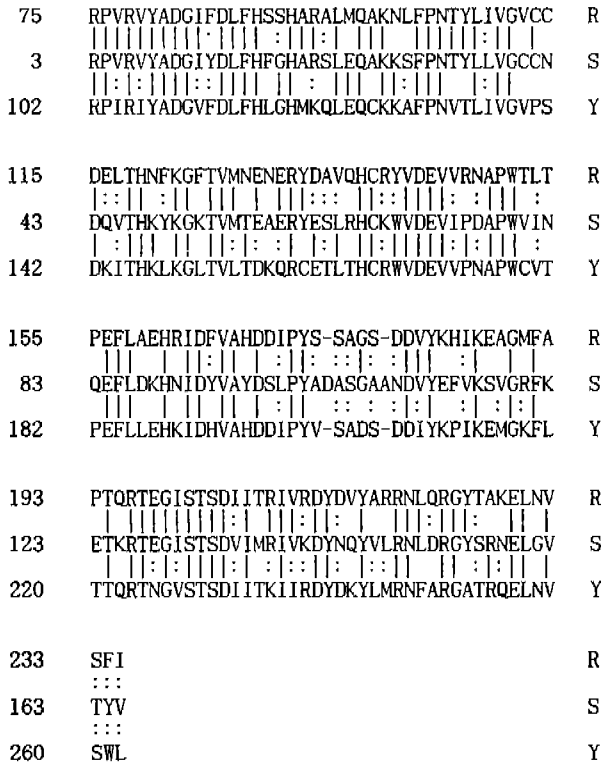

Fig. 2. Comparison of the catalytic domain of soybean cytidylyltransferase protein sequence (S) with corresponding rat liver (R) and yeast sequences (Y). Identical amino acid residues are indicated by vertical lines, and conservative changes by colons.

and U19 or SP6 as sequencing primers.

The 5′ and 3′ ends of cytidylyltransferase cDNA were obtained by PCR using the gene-specific nested primers CT5 (5′-AGTCAATGTTGTGCTTGTCAA-3′) and CT3 (5′-GTGTTGCAACGATCAAGT-3′), respectively, with the combination of λgt11 forward and reverse primers (Figs. 1A and 1B). The fragments amplified (P5 and P3) were also cloned into pT7 Blue T-vector and sequenced as above.

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).

RESULTS AND DISCUSSION

cDNA cloning by PCR

The product (P12) of PCR with degenerate primers had the same length of 278 bp as was expected from the rat and yeast sequences (Tsukagoshi *et al.*, 1987; Kalmar *et al.*, 1990) (Fig. 1(B) and 2). The predicted polypeptide shared 58 and 56% identity and 73 and 69% similarity (excluding the primer sequences) with the rat and yeast protein sequence, respectively, and turned out to be located within the region corresponding to the catalytic domain of rat cytidylyltransferase (Kalmar *et al.*, 1994).

Based on P12 sequence were synthesized two specific primers CT5 and CT3. Each of these primers was used with the combination of λgt11 forward and reverse primers to generate the 5′ and 3′ fragments (P5 and P3), respectively, by PCR (Figs. 1A and 1B).

Characterization of soybean cytidylyltransferase sequence

The determined sequence of soybean cytidylyltransferase cDNA is 884 bp long and contains an open reading frame (ORF) of 294 amino acids (Fig. 1(B)). Based on the ORF's of 367 amino acids of the mammalian cytidylyltransferases (Kalmar *et al.*, 1990) and 424 amino acids of the yeast enzyme (Tsukagoshi *et al.*, 1987), we assume that the determined soybean sequence corresponds to 70-80% of the total sequence.

The basic structure of soybean cytidylyltransferase was revealed to be same as that of mammalian cytidylyltransferase. It consists of three main regions, namely the catalytic domain in the central region, the very hydrophilic C-terminal region, and the amphipathic membrane-binding domain between them.

The conserved region between residues 3 and 165 represents for the proposed catalytic domain of cytidylyltransferase (Figs. 1B and 2), housing the active site of the enzyme, as proposed by Cornell and her colleagues (Craig *et al.*, 1994). This region shows 78% similarity with the rat cytidylyltransferase and 76% with the yeast sequence (Fig. 2). The homology of the region between the mammalian and yeast proteins was 75% (Kalmar *et al.*, 1990). By contrast, the N- and C-terminal domains shared only 23 and 37% homology, respectively, between the rat and yeast cytidylyltransferases. According to the secondary structure prediction by Chou-Fasman algorithm (Chou and Fasman, 1978), the catalytic domain

contains many short α-helices and β-sheets connected by turns, suggesting that it is a tightly folded, globular structure.

The analysis of hydropathy profile of soybean cytidylyltransferase reveals that the sequence of soybean cytidylyltransferase, like the mammalian and yeast enzymes, does not contain a hydrophobic region long enough to be a transmembrane domain (data not shown). This suggests that cytidylyltransferase is not a membrane-spanning enzyme, although it is bound to the membrane fraction in the active formation. The profile of soybean cytidylyltransferase also shows that the 3′ end of the enzyme encompasses a large domain of very strong hydrophilicity. Sequence analysis shows that the hydrophilic nature of this region is mainly due to the enrichment of positively charged residues, like aspartic acid and glutamic acid (Fig. 1(B)).

The most intriguing feature of the soybean cytidylyltransferase sequence is that between the catalytic domain and the C-terminal hydrophilic region is an α-helical region between amino acid residues 172 and 225 (Fig. 1(B)), as predicted by the Chou-Fasman algorithm (Chou and Fasman, 1978). The helical projection, as shown in Fig. 3, clearly demonstrates the amphipathic nature of this unbroken α-helical region with the highly asymmetric distribution of hydrophilic and hydrophobic residues. The polar face is highly enriched in positively charged glutamic acid and negatively charged lysine. It was a longtime unsolved question how the plant cytidylyltransferases were activated as compared to the mammalian counterparts. The presence of the α-helical amphipathic structure strongly suggests that the activity of soybean cytidylyltransferase may be also controlled by reversible translocation of the enzyme between the cytosol and the microsomal membranes like mammalian enzyme. Cornell and her colleagues suggested from the studies of the rat liver cytidylyltransferase that this amphipathic helix was involved in binding the membranes, with its axis parallel to the plane of the membrane and with the hydrophobic side chains intercalating into the phospholipid bilayer and the polar face contacting the cytosol (Kalmar *et al.*, 1990; Craig *et al.*, 1994; Johnson and Cornell, 1994). The high enrichment of the 3′-end region of the soybean cytidylyltransferase with negatively charged glutamic acid and aspartic acid residues, suggestive of strong polar interaction of this region of the enzyme with the negatively charged phospholipids of the membrane, also could help the right positioning of the enzyme in the active conformation.

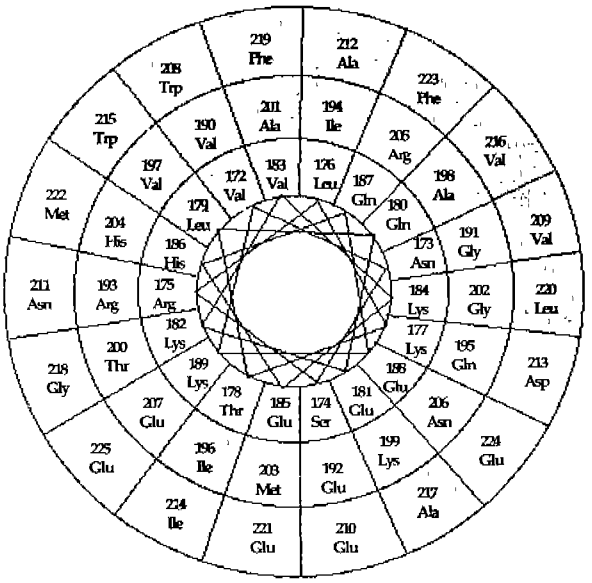

Fig. 3. Helical wheel projection of soybean cytidylyltransferase in the amphipathic helix region. Hydrophobic residues are shaded gray.

Potential sites for phosphorylation

Since it was shown that cytidylyltransferase was phosphorylated at multiple sites *in vivo* and that phosphorylation played an important role in the regulation of cytidylyltransferase activity in mammalian tissues (Watkins and Kent, 1991; Wang *et al.*, 1993; MacDonald and Kent, 1994), the consensus sequence of phosphorylation was searched in soybean cytidylyltransferase. Throughout the determined sequence, there are 7 potential sites for phosphorylation by protein kinase C [Xaa-(Arg/Lys_{1-3}, Xaa_{0-2})-(Ser/Thr)-($Xaa_{0\ 2}$, Arg/$Lys_{1\ 3}$)-Xaa] with Ser/Thr at positions 63, 117, 124, 156, 174, 232 and 251, and 4 potential phosphorylation sites by Ca^{2+}/calmodulin protein kinase II [Xaa-Arg-Xaa-Xaa-(Ser/Thr)-Xaa] with Ser/Thr at positions 63, 156, 174 and 251 (Kemp and Pearson, 1990; Kennelly and Krebs, 1991). Two of these sites (positions 232 and 251) are found in the hydrophilic C-terminal region. It is expected that when considered the multiple

phosphorylation sites in the C-terminal region of mammalian cytidylyltransferases there are more potential phosphorylation sites in the C-terminal region of soybean cytidylyltransferase, the sequence of which is to be determined. The sites for phosphorylation in the C-terminal region have been implicated to be important in the regulation of the enzyme translocation, since removal of the C-terminal tail resulted in loss of phosphorylation of overexpressed rat liver cytidylyltransferase by kinases endogenous to chinese hamster ovary cells (Kalmar *et al.*, 1994). In the latest study with rat mutants, it was suggested that the phosphorylation of the C-terminal domain interferes with the activation of cytidylyltransferase by lipid regulators (Yang and Jackowski, 1995). Ser-Pro motif (Ser-Pro-Ser-Ser-Ser-Pro and Ser-Pro-Ser-Pro-Ser), which is the site for phosphorylation by p34cdc2 kinase and was repeated three times in the C-terminal region of mammalian cytidylyltransferases (Kalmar *et al.*, 1994; MacDonald and Kent, 1994), is not found within the determined soybean cytidylyltransferase sequence. It lacked in the yeast cytidylyltransferase (Tsukagoshi *et al.*, 1987).

The presence of multiple potential sites for phosphorylation in the soybean cytidylyltransferase sequence suggests that the phosphorylation-dephosphorylation also plays an important role in the regulation of the enzyme activity in soybean. There is no evidence yet that the soybean cytidylyltransferase is phosphorylated either *in vivo* or *in vitro*, but the activity of cytidylyltransferase from castor bean has been reported to be regulated by phosphorylation (Wang and Moore, 1989).

It will be very interesting to investigate in detail the biochemical nature of purified soybean cytidylyltransferase in terms of activity regulation. It has not been demonstrated whether the membrane-bound soybean enzyme is the active form and the phosphorylation and dephosphorylation are actually involved in the translocation. Because the distribution of cytidylyltransferase activities between the cytosol and the postchloroplast membrane fraction in developing soybean seeds changed with alteration of temperatures (Cheesbrough, 1989), it may also provide a model system to study the regulation mechanism of plant cytidylyltransferases in response to environmental factors.

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LITERATURE CITED

Cheesbrough, T.M. 1989. Changes in the enzymes for fatty acid synthesis and desaturation during acclimation of developing soybean seeds to altered growth temperature. *Plant Physiol.* **90**: 760-764.

Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from source enriched in ribonuclease. *Biochemistry* **18**: 5294-5299.

Cho, S.H. and T.M. Cheesbrough. 1990. Warm growth temperatures decrease soybean cholinephosphotransferase activity. *Plant Physiol.* **93**: 72-76.

Choi, S.-B. 1995. Molecular cloning and characterization of somatic embryogenesis-related genes, *SGE5* and *SGE8*, in soybean. Ph.D. Dissertation, Seoul National University, 150 pp.

Chou, P.Y. and G.D. Fasman. 1978. Emperical predictions of protein conformation. *Ann. Rev. Biochem.* **47**: 251-276.

Craig, L., J.E. Johnson and R.B. Cornell. 1994. Identification of the membrane-binding domain of rat liver CTP:phosphocholine cytidylyltransferase using chymotrypsin proteolysis. *J. Biol. Chem.* **269**: 3311-3317.

Johnson, E.J. and R.B. Cornell. 1994. Membrane-binding amphipathic α-helical peptide derived from CTP: phosphocholine cytidylyltransferase. *Biochemistry* **33**: 4327-4335.

Kalmar, G.B., R.J. Kay, A. LaChance, R. Aebersold and R.B. Cornell. 1990. Cloning and expression of rat liver CTP:phosphocholine cytidylyltransferase: an amphipathic protein that controls phosphatidylcholine synthesis. *Proc. Natl. Acad. Sci. USA.* **87**: 6029-6033.

Kalmar, G.B., R.J. Kay, A.C. LaChance and R.B. Cornell. 1994. Primary structure and expression of a human CTP:phosphocholine cytidylyltransferase. *Biochim. Biophys. Acta* **1219**: 328-334.

Kemp, B.E. and R.B. Pearson. 1990. Protein kinase recognition sequence motifs. *Trends Biochem. Sci.* **15**: 342-346.

Kennelly, P.J. and E.G. Krebs. 1991. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266**: 15 555-15558.

Kinney, A.J. and T.S. Moore. 1987. Phosphatidylcholine biosynthesis in castor bean endosperm: the localization and control of CTP:cholinephosphate cytidylyltransferase activity. *Arch. Biochem. Biophys.* **259**: 15-21.

Kinney, A.J., D.T. Clarkson and B.C. Loughman. 1987. The

regulation of phosphatidylcholine biosynthesis in rye roots. Stimulation of the nucleotide pathway by low temperature. *Biochem. J.* **242**: 755-759.

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

Lord, J.M., T. Kagawa and H. Beevers. 1972. Intracellular distribution of enzymes of the cytidine diphosphate choline pathway in castor bean endosperm. *Proc. Natl. Acad. Sci. USA.* **69**: 2429-2432.

MacDonald, J.I.S. and C. Kent. 1994. Identification of phosphorylation sites in rat liver CTP:phosphocholine cytidylyltransferase. *J. Biol. Chem.* **269**: 10529-10537.

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

Price-Jones, M.J. and J.L. Harwood. 1986. The control of CTP:cholinephosphate cytidylyltransferase activity in pea. *Biochem. J.* **240**: 837-842.

Tsukagoshi, Y., J. Nikawa and S. Yamashita. 1987. Molecular cloning and characterization of the gene encoding cholinephosphate cytidylyltransferase in Saccharomyces cerevisiae. *Eur. J. Biochem.* **169**: 477- 486.

Vance, D.E. 1989. CTP:cholinephosphate cytidylyltransferase. *In* Phosphatidylcholine Metabolism. D.E. Vance (ed.). CRC, Boca Raton, FL, USA, pp. 34-45.

Vance, D.E. and S.L. Pelech. 1984. Enzyme translocation in the regulation of phosphatidylcholine biosynthesis. *Trends Biochem. Sci.* **9**: 17-20.

Wang, X. and T.S. Moore. 1989. Partial purification and characterization of cytidine 5′-triphosphate: choline-phosphate cytidylyltransferase. *Arch. Biochem. Biophys.* **274**: 338-347.

Wang, X. and T.S. Moore. 1990. Phosphatidylcholine biosynthesis in castor bean endosperm. Purification and properties of cytidine 5′-triphosphate: choline-phosphate cytidylyltransferase. *Plant Physiol.* **93**: 250-255.

Wang, Y., J.I.S. MacDonald and C. Kent. 1993. Regulation of CTP:phosphocholine cytidylyltransferase in Hela Cells. *J. Biol. Chem.* **268**: 5512-5518.

Watkins, J.D. and C. Kent. 1991. Regulation of CTP:phosphocholine cytidylyltransferase activity and subcellular location by phosphorylation in chinese hamster ovary. *J. Biol. Chem.* **266**: 21113-21117.

Yang, W. and S. Jackowski. 1995. Lipid activation of CTP: phosphocholine cytidylyltransferase is regulated by the phosphorylated carboxyl-terminal domain. *J. Biol. Chem.* **270**: 16503-16506.

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大豆 CTP : Phosphocholine Cytidylyltransferase cDNA의 部分的 特徵

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적 요

식물의 CTP:phosphocholine cytidylyltransferase(EC 2.7.7.15)의 구조와 기능과의 관계를 밝히기 위한 첫단계로서 대두 cytidylyltransferase cDNA의 부분 염기서열을 PCR 방법을 이용하여 결정하였다. 쥐와 효모의 유전자 염기서열 중 보존된 부분으로부터 degenerate primer를 합성하여 PCR에 이용하였다. 촉매 도메인은 쥐 및 효모의 아미노산 서열과 각각 78%와 76%의 상동성을 나타내었다. Hydropathy 분석에 의하면, C-말단의 비촉매 부분은 매우 강한 친수성을 보였으며, 활성화된 상태에서 막표면과 결합하는 것으로 믿어지는 큰 양매성의 α-helix 도메인이 촉매 도메인과 C-말단 사이에 발견되었다. 결정된 아미노산 서열내에서 protein kinase C에 의한 인산화 가능 부위가 7곳, 그리고 Ca^{2+}/calmodulin kinase에 의한 인산화 가능 부위가 4곳이 있었다.

주요어 : 대두, CTP:포스포콜린 시티딜기전이효소, 포스파티딜콜린, 양매성 나선구조

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