Journal of Plant Biology, March 2005, 48(1) : 16-24 **RESEARCH ARTICLE**

cDNA Cloning and Expression Analysis of a CTP:Phosphoethanolamine Cytidylyltransferase from Barley

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To elucidate the relationship between the structure and function of CTP:phosphoethanolamine cytidylyltransferases (ECT, EC 2.7.7.14) in plants, we cloned and characterized the cDNA of an *ECT* from *Hordeum vulgare*. This *HvECT1* cDNA is 1783 bp long, and includes an open reading frame (ORF) of 1263 b that encodes a protein of 421 amino acids. The predicted protein sequence of HvECT1 is 73% identical to and 84% similar with *Arabidopsis thaliana* AtECT1; it also shares 57, 55, and 37% similarities with human, rat, and yeast ECTs, respectively. Its 252-b 5′-noncoding leader contains a putative upstream ORF of 36 nucleotides, possibly encoding a putative peptide enriched in Ala and Pro residues. Alignment of the N-terminal and C-terminal halves of HvECT1 revealed a large internal repetitive sequence. Both halves contain the HXGH motif known to be involved in the catalytic activity of cytidylyltransferases. The RTXGVSTT sequence and Asp residues are also conserved. Our hydropathy profile showed that HvECT1 contains a signal sequence that is absent in yeast or animal ECTs. Results from reverse transcriptase-PCR indicated that *HvECT1* is expressed highly in the leaves, stems, and roots of one-week-old plants; its expression is not regulated by low temperatures. After transforming a yeast mutant *ect1* and labeling those transformants with radioactive ethanolamine, we identified *HvECT1* cDNA through *in vivo* analyses of the enzymatic reaction products.

Keywords: Hordeum vulgare, low temperature, membrane, phosphatidylethanolamine, phospholipid

Phosphatidylethanolamine (PE) is a major phospholipid component of biological membranes. Its nucleotide pathway for *de novo* synthesis includes three consecutive reactions (Kennedy and Weiss, 1956). CTP:phosphoethanolamine cytidylyltransferase (ECT, EC 2.7.7.14) catalyzes the second reaction -- conversion of phosphoethanolamine to CDP-ethanolamine -- and is generally regarded as the key regulatory step (Wang and Moore, 1991; Bladergroen and van Golde, 1997).

This biosynthetic pathway is analogous to that for the production of phosphatidylcholine (PC), another important phospholipid constituent of eukaryotic membranes (Kennedy and Weiss, 1956). The terminal step in each pathway involves conversion of diacylglycerol to phospholipid using CDP-ethanolamine or CDP-choline as the source of the head group of PE or PC, respectively. This action is believed to be catalyzed by a single aminoalcoholphosphotransferase with dual substrate specificity in plants (Macher and Mudd, 1974; Lord, 1975; Sparace et al., 1981; Justin et al., 1985; Dewey et al., 1994; Kim et al., 2004). However, Wang and Moore (1991) have reported that synthesis of CDP-ethanolamine and CDP-choline is catalyzed by separate enzymes, and have demonstrated the presence of ECT in castor bean endosperm. That enzymatic activity is associated with membrane fractions of the endoplasmic reticulum and mitochondria, although 80% of the activity is found in the latter. Studies with yeast also have suggested that ECT is associated with membranes of the endoplasmic reticulum, the site of phospholipid formation (Rho et al., 1996). In rat liver cells, ECT has been purified from a post-microsomal supernatant (Sundler, 1975), and immunogold electron microscopy studies have shown that ECT is localized in areas of the cytoplasm that are rich in rough endoplasmic reticulum (van Hellemond et al., 1994). The molecular structures of ECT from yeast and animals, however, have revealed neither hydrophobic regions long enough to span a membrane nor any α-helical amphipathic structures that could play a role in reversible translocation of the enzyme between the cytosol and a membrane fraction, as is the case with the analogous enzyme CCT (Rho et al., 1996; Nakashima et al., 1997; Bladergroen et al., 1999).

CCT (CTP:phosphocholine cytidylyltransferase) has been relatively well studied in plants with regard to its molecular structure and expression pattern in response

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to low temperature (Nishida et al., 1996; Choi et al., 1997; Inatsugi et al., 2002). It contains an amphipathic α-helical domain, as found in its animal counterparts. Moreover, in *Arabidopsis*, the expression of one isoform is up-regulated during chilling (Inatsugi et al., 2002). Cold treatment also induces the expression of *AAPT* (encoding aminoalcoholphosphotransferase) in Chinese cabbage (Choi et al., 2000), *Pimpinella brachycarpa* (Lee et al., 2001), and *Brassica napus* (Qi et al., 2003). It is well documented that the amount of phospholipid increases at low temperatures in various species (Kinney et al., 1987; Lynch and Steponkus, 1987; Harwood et al., 1994; Harwood, 1998). Unlike CCT, however, there is a paucity of data on the molecular structure and expression of ECT in plants. Recently, cDNAs encoding ECT from *A. thaliana* (Mizoi et al., 2003) and *Chlamydomonas* (Yang et al., 2004) have been cloned. Here, we report the cloning and characterization of the cDNA of *HvECT1* encoding an ECT from barley, and describe its expression pattern in different organs and in relation to low temperatures. We also present a possible down-regulatory role for the upstream open reading frame (uORF) of *HvECT1* in the expression of ECT in a heterologous system.

MATERIALS AND METHODS

Plant Material and Isolation of RNA

Barley seeds (*Hordeum vulgare* L. cv. Paldo) were germinated on an MS Duchefa agar medium in sterile Erlenmeyer flasks. After the plants were grown for one to three weeks at either 25°C (normal condition) or 4°C (low-temperature treatment), their leaves, stems, and roots were collected and stored in liquid nitrogen at −70°C.

Polymerase Chain Reaction with Degenerate Primers

cDNA was synthesized using total RNA that had been extracted from both control and treated plant tissues. It served as template for the PCR, which was performed with two degenerate oligonucleotide primers: ETF1 (sense primer, 5′-CC[A/T/C]GATGG[A/T]AC TGATGC[A/T/G]TATGC-3′) and ETR1 (antisense primer, 5′-AGC[C/T]TCATG[A/G]TTGGCTAC[A/T]ATCC-3′). These primers were synthesized based on homologous sequences from *Arabidopsis* (GenBank accession AC005499), tomato (GenBank accession AW 031599), and soybean (GenBank accession AI794768) to produce a 729-bp fragment. The PCR reaction mixture (25 µL) contained 500 ng template, 2.5 µL 10x PCR buffer [50 mM KCl, 1 mM EDTA, 0.1% Triton X-100, and 10 mM Tris-HCl (pH 8.0)], plus 20 pmol each of the degenerate primers, 200 µM dNTPs, and 5 units of *Taq* polymerase (TaKaRa, Japan). Reactions were annealed for 90 s at 46°C, extended for 1 min at 72°C, and denatured for 1 min at 95°C, for 35 cycles. The amplified product was cloned into the pGEM T-Easy vector (Promega, USA).

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

The 5′ and 3′-ends of *HvECT1* cDNA were obtained by RACE (Frohman et al., 1988; Belyavsky et al., 1989), using the MarathonTM cDNA Amplification Kit (Clontech, USA) and the 3′-AmpliFinderTM Race Kit (Clontech) according to the manufacturer's instructions. The 3′-RACE gene-specific primer was ETF2 (5′-CACTCTTCACTACAAAGACAG-3′); the 5′-RACE gene-specific primers were ETR2 (5′-GCCTATCATG-CATCAAGATTGC-3′) and ETR3 (nested primer, 5′-GTCTTCTGAGAAGATCTATCC-3′). A cDNA containing the full coding sequence was constructed by PCR using cDNA as template, ETFL (5′-CGTCCAGATCTC-CCGGATCCTCCCAC-3′) for the 5′-noncoding region, and ETR3 for the 3′-noncoding region. The reaction products were also cloned into the pGEM T-Easy vector and then sequenced.

Sequence Analysis

Hydropathy analysis of the predicted polypeptide was performed according to Kyte and Doolittle (1982). We based our prediction of secondary structures from the deduced amino acid sequence on the Chou-Fasman algorithm (Chou and Fasman, 1978). SignalP 3.0 was used for predicting the signal peptide and signal anchor (Bendtsen et al., 2004).

Reverse Transcriptase-PCR (RT-PCR)

Total RNA was extracted from the leaves and roots of normal and cold-treated plants. Using 3 µg total RNA as template, reverse transcription was conducted with MMLV reverse transcriptase (Promega) for 1 h at 42°C and 10 min at 65°C. Then, with 2 µL of a total 20 µL reaction mixture as template, PCR was performed using ETF2 and ETR3 to synthesize a 693-bp fragment that corresponded to nucleotides 805

through 1497. The reactions were annealed for 30 s at 59°C, extended for 1 min at 72°C, and denatured for 30 s at 95°C, for 30 cycles. As a standard, an 829-bp fragment from 18S rRNA was amplified for 25 cycles under the same PCR conditions.

Transformation of Yeast Mutant and Detection of ECT Activity

The mutant yeast strain NA9, devoid of ECT activity, was cultured and maintained in a YPD medium (Hosaka and Yamashita, 1980; Nakashima et al., 1997). A cDNA fragment harboring the open reading frame, with or without uORF, was amplified by PCR. The product was then digested with an appropriate restriction enzyme, and the resulting fragment was cloned into the expression vector pAC1 (Innis et al., 1985). Plasmid pAC1 contains ECT cDNA with or without a uORF, and is designated as pHvECT1L or pHvECT1S, respectively. This plasmid was transformed into the mutant yeast strain by the lithium acetate method (Hill et al., 1991).

Thin-layer chromatography (TLC) was carried out for our yeast cell extracts after they were incubated with [^{14}C]ethanoloamine. Cells were grown to the mid-exponential phase in a synthetic dextrose medium containing appropriate nutritional supplements to ensure plasmid maintenance. The cells were then harvested, washed, and disrupted with a beadbeator according to Hosaka and Yamashita (1980). The lyophilized extract was re-suspended in 10% trichloroacetic acid and subjected to TLC on a Silica Gel 60 plate (Merck, Germany), using ethanol and 2% ammonia (1:1, v/v) as the developing solvent. Radioactive materials were located by autoradiography.

RESULTS

Cloning Strategy of *HvECT1* cDNA

The product from PCR with degenerate primers had the expected length of 729 bp, based on the sequence of the *Arabidopsis* gene (data not shown). For 3′-RACE, a gene-specific primer ETF2 was used in combination with the manufacturer's PCR-anchor primer to generate a 980-bp 3′-fragment. For 5′-RACE, gene-specific primers ETR2 and ETR3 were synthesized based on the sequence of the noncoding region of the 3′-RACE product. These 5′-RACE primers were used in combination with the d(G)-*Eco*RI primer to synthesize a 997-bp PCR product that contained the 5′-noncoding region. A cDNA fragment including the entire open reading frame of *HvECT1* was amplified by PCR, using primers ETFL and ETR3 as sense and antisense primers, respectively. ETFL, which corresponds to the nucleotide sequence 8 to 30 of *HvECT1* cDNA, was designed to contain *Bam*HI and *Bgl*II sites for heterologous expression.

The PCR product was sequenced to verify the entire coding sequence of *HvECT1*, which is 1783 b long and contains an ORF of 1263 b. It encodes a protein of 421 amino acids (Fig. 1), and has a deduced molecular mass of 46.7 kD and a pI of 7.3. The length of this coding sequence is approximately the same as that of *A. thaliana*, but longer than that of yeast ECT (323 residues; Rho et al., 1996), human ECT (389 residues; Nakashima et al., 1997), and rat ECT (404 residues). This difference in length can be detected in the N-terminal region (Fig. 2).

Structure of the 5′- and 3′-Noncoding Sequences

The start codon is in a context consistent with the ideal for translation initiation (Joshi, 1987a). Although only one A residue (see Fig. 1) exists -- at position −4 (instead of the ideal case of A residues at −1, −3, −4, and −5) -- the downstream triplet GAT (at +4, +5, +6) closely matches the consensus sequence (GCT). The 5′-noncoding region is relatively long (252 b) and contains a short uORF comprising 36 b capable of coding for a putative peptide of 12 amino acids. The upstream open reading frame (uORF) is characterized by four Ala and four Pro residues. The 3′ end of the cDNA has a TGA stop codon (starting at nucleotide 1456) followed by an untranslated sequence of 225 b, including a poly (A) stretch of 17 b. A putative polyadenylation signal sequence (Joshi, 1987b), ATATA, is located 17 b upstream (starting at nucleotide 1750) from the polyadenylation start site. Although a TG-rich far-upstream element appears at nucleotides 1630 through 1643, a probable consensus motif TTGTA was not found (Hunt, 1994). The sequence has 86% of its nucleotides as G and T, implying that this distinctive TG-rich region is a *cis* element of plant polyadenylation signals.

Structure of the Predicted Protein

ECT proteins from barley and *A. thaliana* exhibit 73% identity and 84% similarity over the entire protein sequence (Fig. 2). HvECT1 also shares 57 and 55% similarity with human and rat ECTs, respectively, but only 37% similarity with yeast ECT. Similarities

between plant ECTs and CCTs are approximately 30% (data not shown).

Alignment of its N-terminal and C-terminal halves revealed a large internal repetitive sequence, as is the case with yeast and animal ECTs (Fig. 2B). Sequence identity between these two halves of barley ECT is

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acagctccgtccgtctccgagacctcccacccatctcccatggcgaaggcccctccagcc   60
                                        m  a  k  a  p  p  a
gcccgcccgcatccctaaccctaggctagatcccgccgcgcacctcgcgcgctcgcgagacc  120
a  r  p  h  p  *
aaacacacgcctctgaccactccccccccagccccgctgactcctctgggaccgtgagtcc  180
attgctttacccatggattcggctagtagcagcgcgcggacggtggcggcgtgcgtgatc  240
                M  D  S  A  S  S  S  A  R  T  V  A  A  C  V  I   16
ggcgggatcgtgctgggcgcctcggttgtcgcgctgcacctcggcgctggccccgcggcc  300
 G  G  I  V  L  G  A  S  V  V  A  L  H  L  G  A  G  P  A  A   36
ccgagtctgccaccggtcgaggccctccggcggcgcttccgccgccgctgccgccccgtg  360
 P  S  L  P  P  V  E  A  L  R  R  R  F  R  R  R  C  R  P  V   56
cgggtctacatggacggctgcttcgacatgatgcactacggtcactgcaacgcgctgcgc  420
 R  V  Y  M  D  G  C  F  D  M  M  H  Y  G  H  C  N  A  L  R   76
caggcacgggcgctcggggacgagctcgtcgtcggcgtcgtcagcgacgacgagatcacc  480
 Q  A  R  A  L  G  D  E  L  V  V  G  V  V  S  D  D  E  I  T   96
gccaacaagggaccccccgtcacgccgctccacgagagaatgaaaatggtccgtgctgtc  540
 A  N  K  G  P  P  V  T  P  L  H  E  R  M  K  M  V  R  A  V  116
aaatgggttgacgatgtcattccagatgcaccatatgccataaccgaagatttcatgaac  600
 K  W  V  D  D  V  I  P  D  A  P  Y  A  I  T  E  D  F  M  N  136
aagctattcaatgagtacaatattgattacattattcatggtgatgatccttgcctgctc  660
 K  L  F  N  E  Y  N  I  D  Y  I  I  H  G  D  D  P  C  L  L  156
ccagatggtactgatgcatatgcccttgccaaaaaggctggccgatataagcagattaaa  720
 P  D  G  T  D  A  Y  A  L  A  K  K  A  G  R  Y  K  Q  I  K  176
aggactgaaggagtgtcaacaacagatattgttggaagaatgctcctttgtgttagagag  780
 R  T  E  G  V  S  T  T  D  I  V  G  R  M  L  L  C  V  R  E  196
agaccagtttctgataataacagccactcttcactacaaagacagttcagtcatgggcat  840
 R  P  V  S  D  N  N  S  H  S  S  L  Q  R  Q  F  S  H  G  H  216
ggtcagaatattgatgatagtggatttggaagtggaacaaaaatatctcatttttcttccc  900
 G  Q  N  I  D  D  S  G  F  G  S  G  T  K  I  S  H  F  L  P  236
acatctcggcggatagtacagttctcaaatggcaggggtccaggaccaaattcccgggta  960
 T  S  R  R  I  V  Q  F  S  N  G  R  G  P  G  P  N  S  R  V  256
gtgtacatagatggtgcatttgatctgttccatgctggtcatgtcgagatactgcgactt 1020
 V  Y  I  D  G  A  F  D  L  F  H  A  G  H  V  E  I  L  R  L  276
gctcgagggcttggagatttcttgcttgtgggcattcacacagatcagaccataagttca 1080
 A  R  G  L  G  D  F  L  L  V  G  I  H  T  D  Q  T  I  S  S  296
actcgaggaccacatcgcccaatcatgaatctccatgagcgcagtttgagtgttttggct 1140
 T  R  G  P  H  R  P  I  M  N  L  H  E  R  S  L  S  V  L  A  316
tgccgttatgttgacgaagtgatcattggtgctccctggcacatttcgaaagacatggtt 1200
 C  R  Y  V  D  E  V  I  I  G  A  P  W  H  I  S  K  D  M  V  336
accactttaatatttcattggttgttcatgggactatagctgagaacatggactataca 1260
 T  T  F  N  I  S  L  V  V  H  G  T  I  A  E  N  M  D  Y  T  356
gaggatgattcaaatccatatgctgttccagttgctatgggcatttatcataagctggag 1320
 E  D  D  S  N  P  Y  A  V  P  V  A  M  G  I  Y  H  K  L  E  376
agccctttggacatcaccactagtactattataaggaggatagtttctaaccatgaagcc 1380
 S  P  L  D  I  T  T  S  T  I  I  R  R  I  V  S  N  H  E  A  396
tatcagaagcggaacgagaagaagaaggaagccagtgagaagaagtactacgacagtaaaagc 1440
 Y  Q  K  R  N  E  K  K  E  A  S  E  K  K  Y  Y  D  S  K  S  416
tttgtcaatggagagtagttacctatggatagatcttctcagaagactggtcctaaatct 1500
 F  V  N  G  E  *                                             421
tcgaggctctaacacaggtcacaaatgaagccattaggtgatctacagttttacccgctc 1560
cattggcatttttgctatatagcttagtagccttcaaatgcaatcttgatgcatgatagg 1620
caatggcgctgagttgggtgcggaacctggatgaatttccaatgcccacttccagtgcta 1680
gtacctaccctattcccaccctgtataccggcttccagagctgccgttaccggctgagtt 1740
ctttttgagatatactgttgttcgagaaaaaaaaaaaaaaaaa                  1783
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Figure 1. Nucleotide and deduced amino acid sequences of *HvECT1*. GenBank accession number for cDNA sequence is AY198340. Amino acid sequence of uORF motif in 5′-leader of transcript is underlined. Sequences underlined and marked within 3-noncoding region are putative far-upstream and near-upstream elements, respectively, of polyadenylation signal. Motifs involved in catalytic activity of ECT are in bold typeface and underlined.

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B  MDSASSSARTVAACVIGG--IVLGASVVALHLG----AGPAAPSLPPVEALRRRFRRRCR
A  --MVWEKEKIVGSCIVGGAAFAVGASFLHLFLKGELPLGLGLGLSCPWRILRKRK-----
Y  ------------------------------------------------------MTVNLD
R  ----------MIRNGHGAGGAAGLK----------GPGG----------------QR
H  ----------MIRNGRGAAGGAEQP----------GPGG----------------RR

B  PVRVYMDGCFDMMHYGHCNALRQARALG----DQLVVGVVSDDEITANKGPPVTPLHERM
A  PVRVYMDGCFDMMHYGHCNALRQARALG----DQLVVGVVSDEEIIANKGPPVTPLHERM
Y  PDKVWIDGCFDFTHHGHAGAILQARRTVSKENGKLFCGVHTDEDIQHNKGTPVMNSSERY
R  TVRVWCDGCYDMVHYGHSNQLRQARAMG----DYLIVGVHTDEEIAKHKGPPVFTQEERY
H  AVRVWCDGCYDMVHYGHSNQLRQARAMG----DYLIVGVHTDEEIAKHKGPPVFTQEERY
     :*: ***:*: *:**   : ***         *  ** :*::*  :** **    **

B  KMVRAVKWVDDVIPDAPYAITEDFMNKLFNEYHIDYIIHGDDPCLLPDGTDAYALAKKAG
A  TMVKAVKWVDEVISDAPYAITEDFMKKLFDEYQIDYIIHGDDPCVLPDGTDAYALAKKAG
Y  EHTRSNRWCSEVVEAAPYVTDPNWMDKYQ----CQYVVHGDDITIDANGEDCYKLVKEMG
R  KMVQAIKWVDEVVPAAPYVTTLETLDKHN----CDFCVHGNDITLTVDGRDTYEEVKQAG
H  KMVQAIKWVDEVVPAAPYVTTLETLDKYN----CDFCVHGNDITLTVDGRDTYEEVKQAG
     :: :*  :*:  ***    : : *       :: :**:*  :' :* * *  *: *

B  RYKQIKRTEGVSTTDIVGRMLLCVRERPVS-------DNNSHSSLQRQFSHGHGQNID--
A  RYKQIKRTEGVSSTDIVGRMLLCVRERSIS-------DTHSRSSLQRQFSHGHSSPKF--
Y  RFKVVKRTYGVSTTEIIHRILTKKSLPPTHPDYYPTTQELSFYS----------------
R  RYRECKRTQGVSTTDLVGRMLLVTKAHHSS-------QEMSSEYREYADSFGKPPHPTPA
H  RYRECKRTQGVSTTDLVGRMLLVTKAHHSS-------QEMSSEYREYADSFGK-------
   *::  *** ***:*::: *:*                 :  *

B  -----------DSGFGS-GTKISHFLPTSRRIVQ--FSNGRGPGPNSRVVYIDGAFDLFH
A  -----------EDGASSAGTRVSHFLPTSRRIVQ--FSNGKGPGPDARIIYIDGAFDLFH
Y  ----------------VAQDAVSKHCYVFQRDLDNVLVNGGYKFDAEDCVYVDGDFDLFH
R  GDTLSSEVSSQCPGGQSPWTGVSQFLQTSQKIIQ--FASGKEPQPGETVIYVAGAFDLFH
H  -----------CPGGRNPWTGVSQFLQTSQKIIQ--FASGKEPQPGETVIYVAGAFDLFH
                        :*:    :: ::  :  *          :*: * *****

B  AGHVEILR-LARGLGD--FLLVGIHTDQTISSTRGPHRPIMNLHERSLSVLACRYVDEVI
A  AGHVEILR-RARELGD--FLLVGIHNDQTVSAKRGAHRPIMNLHERSLSVLACRYVDEVI
Y  MGDIDQLRKLKMDLHPDKKLIVGITTSDYSS-------TIMTMKERVLIVLSCKYVDAVI
R  IGHVDFLQ-EVHKLAKRPYVIAGLHFDQEVNRYKGKNYPIMNLHERTLSVLACRYVSEVV
H  IGHVDFLE-KVHRLAERPYIIAGLHFDQEVNHYKGKNYPIMNLHERTLSVLACRYVSEVV
     * :: *     *    :: *:  :           ** ::** * **:*:** *:

B  IGAPWHISKDMVTTFNISLVVHGTIAENMDYTEDDSNPYAVPVAMGIYHKLESPLDITTS
A  IGAPWEVSRDTITTFDISLVVHGTVAESDDFRKEEDNPYSVPISMGIFQVLDSPLDITTS
Y  IDA----DATSMSQYNCEKYHIGT----------------AVLTAAGKFSEY-----LTKE
R  IGAPYSVTAELLNHFKVDLVCHGKTEIVPDR--DGSDPYEEPKRRGIFCQIDSGSDLTTD
H  IGAPYAVTAELLSHFKVDLVCHGKTEIIPDR--DGSDPYQEPKRRGIFRQIDSGSNLTTD
   * *       :  :      *                        * :       :*

B  TIIRRIVSNHEAYQKRNEKKEASEKKYYDSKS----FVNGE-----
A  TIIRRIVANHEAYQKRNAKKEASEKKYYEQKS----FVSGD-----
Y  LIVKRVESQREVYIARNQKKGMSI----------------------
R  LIVQRIIKNRLEYEARNQKKEAKELAFLEALRQQEAQPRGETD---
H  LIVQRIITNRLEYEARNQKKEAKELAFLEAARQQAAQPLGERDGDF
   *::*:  ::  *  ** **

(A)

N  50 RRCRPVRVYMDGCFDMMHYGHCNALRQARALGDELVVGVVSDDEITANKGP--PVTPLHERM
C 250 PGPNSRVVYIDGAFDLFHAGHVEILRLARGLGDFLLVGIHTDQTISSTRGPHRPIMNLHERS
           **:** **::* ** : ** ** *** *:**: :*: *:: :**  *:  ****
N 111 KMVRAVKWVDDVIPDAPYAITEDFMNKLFNEYNIDYIIHG---DDPCLLPDGTDAYALAKKA
C 312 LSVLACRYVDEVIIGAPWHISKDMVT----TFNISLVVHGTIAENMDYTEDDSNPYAVPVAM
        * * ::**:** **: *::*::       :**  ::**   ::     * :: **:

(B)
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Figure 2. (A) Alignment of amino acid sequences derived from barley *HvECT1*: *A. thaliana AtECT1* (A), yeast *ECT* (Y), rat *ECT* (R), and human *ECT* (H) cDNAs. Identical amino acid residues are denoted by asterisks (*) and conserved amino acid residues by colons (:) in bottom line. Motifs involved in catalytic activity of ECT are in bold typeface. **(B)** Alignment of N-terminal (N) and C-terminal (C) halves of *HvECT1*.

37%, with both containing an HXGH motif that is involved in the binding of CTP and transition-state stabilization of cytidylyltransferases (Veitch et al., 1998). This suggests that barley ECT also has two catalytic domains like animal and yeast ECTs (Nakashima et al., 1997). We also detected the conserved sequence of RTXGVSTT (residues 177 to 184) and amino acid residues Asp-65 and Asp-264 that are believed to play an important role in catalytic activity of the cytidylyltransferase family (Park et al., 1997; Veitch et al., 1998).

Hydropathy profile analysis (Fig. 3) showed that the sequence of barley ECT contains one hydrophobic region (residues 11 to 28), long enough to be a transmembrane domain in an N-terminal region that is lacking in animal or yeast ECT (Rho et al., 1996; Nakashima et al., 1997; Bladergroen et al., 1999). This region may form an α-helix, as predicted by Chou and Fasman (1978). According to our SignalP analysis (Bendtsen et al., 2004), the sequence has 54% probability of a signal peptide or 43% probability of a signal anchor or a stop-transfer signal, i.e., an uncleaved signal peptide. No α-helical amphipathic domain was found in ECT, which had been suggested to reversibly bind the membrane (Kalmar et al., 1990; Craig et al., 1994).

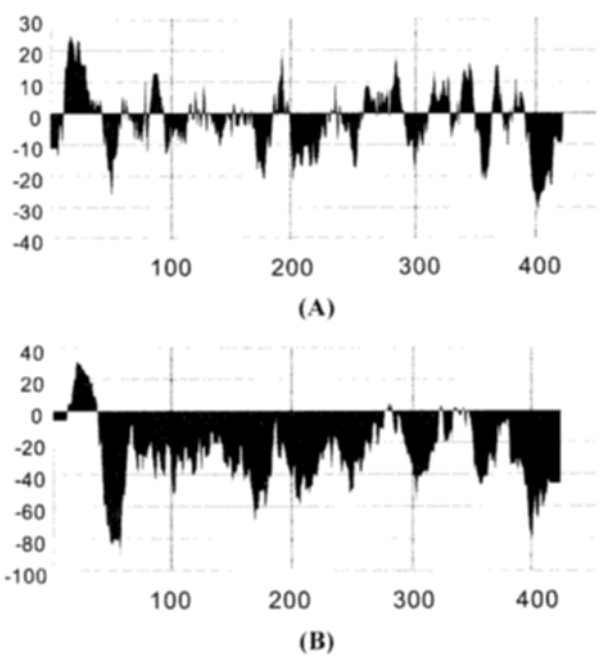

Figure 3. Hydropathy plot of deduced amino acid sequence of *HvECT1*. **(A)** Hydropathy profile analyzed via method of Kyte and Doolittle (1982). Horizontal scale shows amino acid residues; vertical scale indicates free energy (kcal/mole amino acid) for transfer from hydrophobic to hydrophilic environment. **(B)** Transmembrane helix analysis by the method of Engelman et al. (1986).

Expression Pattern of *HvECT1*

Expression of *HvECT1* in different organs and its response to low temperature were assessed via PCR by using equal amounts of total RNA (3 μg) from control and cold-treated plants (Fig. 4). *ECT* was expressed highly in the leaves, stems, and roots of one-week-old plants grown at 25°C. This pattern was expected for such rapidly expanding tissues that are actively involved in the synthesis of cellular membranes and, hence, the production of phospholipids. To investigate the response of *HvECT1* to low temperatures, we exposed 5-d-old plants to 4°C for 1 or 2 d and then measured the accumulation of transcript in their leaves and roots. Expression levels remained the same for tissues at either temperature (Fig. 4). Therefore, our data demonstrate that the expression of *HvECT1* is not changed significantly by chilling.

Transformation of a Yeast Mutant Deficient of ECT

To determine the ECT activity of our cDNA clone,

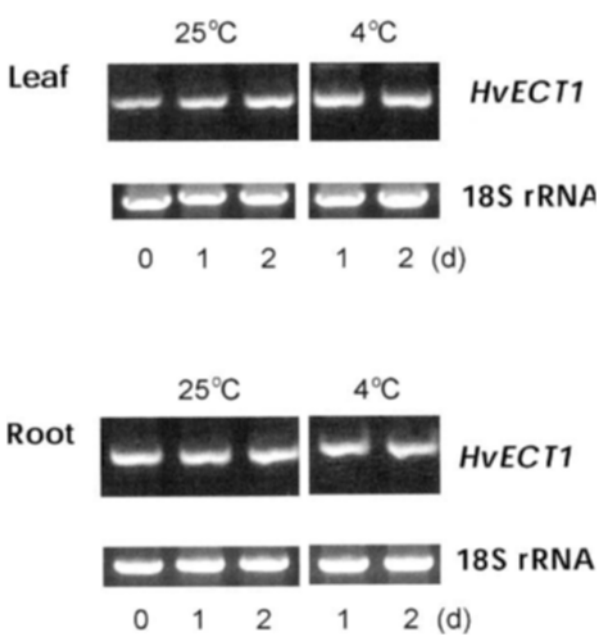

Figure 4. Detection by RT-PCR of *HvECT1* expression in response to low temperature. Barley seedlings were grown at 25°C for 5 d, then either maintained normally or transferred to growth chamber at 4°C for 1 and 2 d. Total RNA was extracted from leaves and roots, and subjected to RT-PCR for amplification of a 693-bp fragment.

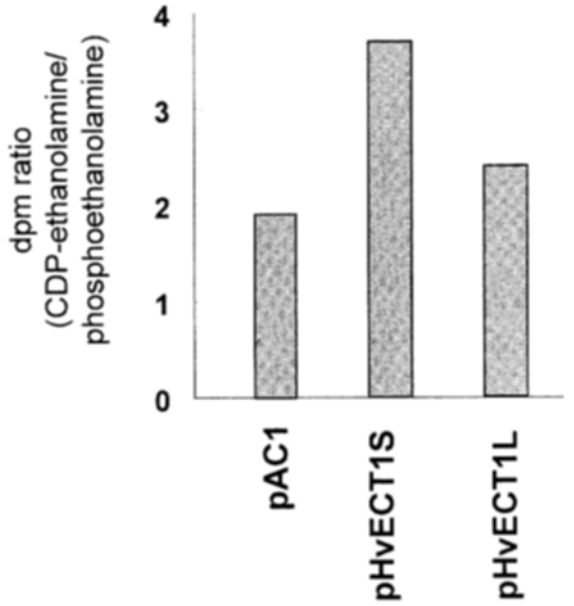

Figure 5. Trichloroacetic acid-soluble metabolites were extracted from yeast mutants harboring pAC1, pHvECT1S, and pHvECT1L after labeling cells with [^{14}C]ethanolamine for 60 min at 30°C. Individual spots were scraped from TLC plates and counted by scintillation spectroscopy. Activity reported is average of two independent assays.

we transformed yeast *ect1* mutants using plasmids pHvECT1L and pHvECT1S, which contain *HvECT1* cDNA with and without a uORF, respectively. The mutant harboring either a vector-only plasmid pAC1, pHvECT1L, or pHvECT1S was grown in the presence of 37 kBq [^{14}C]ethanolamine for 60 min at 30°C. Trichloroacetic acid-soluble materials were then analyzed by thin-layer chromatography. Mutant cells transformed with pHvECT1S (Fig. 5) accumulated higher amounts of CDP-ethanolamine, i.e., the product of ECT activity, than those harboring pAC1 that exhibited basal ECT activity, as had been suggested by Nakashima et al. (1997). However, the level of CDP-ethanolamine was very low in mutants with pHvECT1L that contained the *HvECT1* cDNA with a uORF. These results suggest a possible regulatory function for the uORF in the translation of *HvECT1*.

DISCUSSION

Phosphatidylethanolamine is not only the major structural component of cellular membranes, but also plays several important physiological roles. For example, it is 1) a substrate of phospholipase D in the production of secondary messengers during signal transduction in animal cells (Mukherjee et al., 1996); 2) a donor of the ethanolamine residue to the phosphoethanolamine bridge between the glycosylphosphatidylinositol anchor and the surface glycoproteins of yeast (Menon and Stevens, 1992); and 3) a substrate for the synthesis of *N*-acylphosphatidylethanolamine, which is involved in signal transduction and membrane protection in plants (Chapman and Moore, 1993; Chapman, 2000). Despite these important metabolic roles, our understanding of the molecular aspects of PE biosynthesis in plants remains rudimentary. Genes have been cloned from yeast, humans, and rats, but only recently from plants. Mizoi et al. (2003) have reported the cloning of *ECT* cDNA from *A. thaliana*. Our present study is only the second to describe *ECT* cDNA cloned from plants, and goes further in determining its expression patterns in different organs and in response to a low temperature.

The predicted structure of plant ECTs differs from that of yeast and animal enzymes, especially in the N-terminal region of the protein (Fig. 2). Longer polypeptides in the plant ECT result from the presence of additional residues in that region, in which a hydrophobic domain can adequately span the membrane. Two possible roles for this structure might be proposed based on analytical results from the SignalP program showing that this structure probably is either a signal peptide or a signal anchor (Bendtsen et al., 2004). If the latter, the structure should be retained in the membrane. Our prosite database analyses revealed four potential myristoylation sites; one in the N-terminal region is GAGPAA (residues 31 to 36). This site may keep the catalytic domain of ECT close to the membrane (Chow et al., 1992). Such anchoring might then facilitate the transfer of the enzymatic product CDP-ethanolamine to aminoalcoholphosphotransferase, which requires a lipid, diacylglycerol, as the other substrate. In castor seed endosperms, CDP-ethanolamine is synthesized on the outer leaflet of the endoplasmic reticulum (Shin and Moore, 1990). Therefore, it is possible that plant ECTs may be membrane-associated proteins, with their N-termini protruding into the lumen of the endoplasmic reticulum, while the other parts of the enzymes, including their catalytic domains, are located on the cytoplasmic side. Phosphoethanolamine, the substrate of the ECT reaction, is provided by the action of a soluble ethanolamine kinase (Kinney, 1993).

However, it is also possible that plant ECTs are soluble enzymes associated with the membrane, as is true in their yeast and animal counterparts (Rho et al., 1996; Nakashima et al., 1997; Bladergroen et al.,

1999). The predicted structures of ECT from yeast and animals contain neither transmembrane domains nor α-helical amphipathic structures. Bladergroen et al. (1999) have speculated that animal ECTs associate with the endoplasmic reticulum membrane through protein-protein interaction with ethanolaminephosphotransferase. In castor bean endosperm (Wang and Moore, 1991) and *Chlamydomonas* (Yang et al., 2004), ECT activity is primarily associated with the membrane fractions of mitochondria. In the case of the yeast, its ECT activity is correlated with that of fumarase, a marker enzyme for mitochondria, suggesting that the N-terminal hydrophobic sequence is a subcellular targeting signal to mitochondria in plant ECTs.

Another important feature of barley ECT is the occurrence of an upstream ORF within the cDNA (Fig. 1). The putative peptide has 12 amino acid residues and is enriched in Ala and Pro residues. A uORF is present in less than 10% of all eukaryotic mRNAs, but the frequency is much higher for regulatory genes, including those that control growth and development (Kozak, 1987a). Studies have demonstrated that uORFs usually impede the translation initiation of the downstream major ORF (Kozak, 1987b; Morris and Geballe, 2000). Likewise, our yeast mutant cells transformed with a plasmid containing barley *ECT* cDNA (and with a uORF) exhibited only a basal level of ECT activity (Fig. 5). Interestingly, Dewey et al. (1994) have reported that a uORF located in soybean aminoalcoholphosphotransferase mRNA down-regulates the expression of *AAPT* in a heterologous system. Because this enzyme is responsible for the last step in the nucleotide pathway of PC and PE synthesis, we speculate that post-transcriptional regulation by the translation of the uORF might be important in controlling phospholipid formation in plants.

The particular expression pattern of *HvECT1* in different organs of our barley seedlings was expected, and was based on the assumption that rapidly expanding tissues are actively involved in the synthesis of cellular membranes, a process that requires an appropriate amount of phospholipids. The molecular aspects of phospholipid metabolism in response to low temperature have recently become a target of extensive research, especially concerning PC biosynthesis. For example, the expression of *CCT* and *AAPT* has been shown to increase during cold treatments in plants, suggesting that phospholipids function in response to environmental stress (Choi et al., 2000; Lee et al., 2001; Inatsugi et al., 2002; Qi et al., 2003). However, our results demonstrated that *HvECT1* expression remained almost unchanged after tissues were exposed to low temperature (Fig. 4). These findings are supported by recent reports for other plant species, in which no increase in *ECT* expression was detected after up to 12 d of cold treatment (H. Okuyama, Hokkaido University, personal communication). We suppose that the increase of PE in membranes might not be as important for improving membrane fluidity at low temperatures, because PE has a much higher transition temperature than PC for the same molecular composition (Phillips et al., 1972; Houslay and Stanley, 1982; Harwood, 1998). An increase in the PC to PE ratio in response to low temperature has been seen in a number of organisms, including plants (Harwood, 1998). Moreover, such overproduction of PE might even be harmful to cells because polyunsaturated molecular species of PE, not PC (with its small head group and wide acyl chains), could form a nonbilayer hexagonal II phase structure in the membranes (Hauser et al., 1981; Gurr et al., 2002). Those hexagonal II structures formed by PE overproduction would then disturb the normal functioning of cell membranes in a bilayer structure.

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