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Cloning and Characterization of *ECPP44*, a cDNA Encoding a 44-kilodalton Phosphoprotein Relating to Somatic Embryogenesis in Carrot

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Abstract

Somatic embryogenesis in *Daucus carota* can be induced by the treatment of shoot apices with various kinds of stress chemicals (Tachikawa *et al.*, 1998). Using this system, we previously revealed the presence of a phosphoprotein (ECPP44) of which the phosphorylation was specific to embryogenic cells (EC), stress-treated shoot apices and somatic embryos, but not in non-embryogenic cells (NC). We then obtained a 141 bp fragment of ECPP44 cDNA by RT-PCR with some primers based on the partial amino acid sequence. In this research, the fragment was used as a probe to screen the cDNA library which was compiled from somatic embryos. A full-length cDNA (925 bp) corresponding to ECPP44 was isolated and sequenced. The putative amino acid sequence revealed that ECPP44 contained poly-serine consensus and nuclear targeting signal of Group II *LEA* genes. Phylogenetic analysis of the amino acid sequence encoded by ECPP44 showed that ECPP44 is a distant homology of the dehydrin family in carrot. *ECPP44* is a single copy gene as evidenced by Southern blot analysis under high stringency. Northern blot analysis revealed that *ECPP44* mRNA accumulates in EC, stress-treated and non-treated shoot apices, and somatic embryos, but not in NC. We discuss here the accumulation and/or phosphorylation of ECPP44 as it relates to the acquisition of embryogenic competence.

Key words: *Daucus carota*, Embryogenic competence, LEA Protein, Phosphorylated protein, nucleotide sequence, Somatic embryogenesis.

Introduction

The discovery of somatic embryo formation in carrot by Steward *et al.* (1958) and Reinert (1959) has led to model studies of the regulatory and morphogenetic events in zygotic embryogenesis. By using the somatic embryogenesis system, genes expressed during the different stages of embryogenesis have been isolated for a large number of plant species [Dure III 1993; Baker *et al.*, 1988; Zimmerman 1993). Although the regulation of expression and function of most of those genes remain to be clarified, many of them have been grouped into classes known as late-embryogenesis abundant (LEA). LEA protein expressions and their proposed biochemical properties have led to the suggestion that they play a role as protectants during seed dessication (Ingram *et al.*, 1996). On the other hand, we previously reported that carrot somatic embryogenesis could be induced by culturing shoot apices on the medium containing various kinds of stress compounds (Kamada *et al.*, 1981; Tachikawa *et al.*, 1998) without auxin treatment. In this system, several proteins were identified as embryogenic cell proteins (ECPs) and the corresponding genes were isolated.

Little is known about proteins that are involved in the cellular signal transduction pathway via protein phosphorylation during induction of embryogenic competence. It has been reported that several genes involved in protein phosphorylation in relation to bacterial sporulation (Najafi *et al.*, 1997), mating of yeast cells (Ballard *et al.*, 1991) and the development of vertebrate embryos (Louis *et al.*, 1988), have been isolated and characterized at the molecular level. We previously reported a phosphoprotein that was first identified by *in vivo* labeling of poly-

peptide with ^{32}P-phosphorus that is expressed in embryogenic competent cells and tissues (Tan *et al.*, 2000). In this report, we isolated the cDNA encoding *ECPP44* and studied the expression pattern of *ECPP44* in various tissues and cells in relation to the acquisition of embryogenic competence.

Materials and Methods

Plant material and cell culture

Daucus carota L. cv. US-Harumakigosun was used as the plant material. The induction of embryogenic cells (EC) and the formation of somatic embryos were according to the method described earlier (Tachikawa *et al.*, 1998). The basal medium used in this experiment was Murashige and Skoog's medium (1962) with or without 2,4-D (1mg/*l*). For the establishment of non-embryogenic cells (NC), small cell clumps of less than 1 mm in diameter from embryogenic cell suspension were collected and subcultured at two-week intervals as described by Satoh *et al.* (1986). NC that has lost the ability to form somatic embryos and were used as a negative control of embryogenic event (Satoh *et al.*, 1986).

Somatic embryos were produced by transfer of embryogenic cells (37 to 63 μm in diameter) to 2,4-D-free MS medium as described earlier (Satoh *et al.*, 1986). Torpedo-shaped somatic embryos were harvested, immediately frozen in liquid nitrogen and stored at -80 ℃ until use.

Induction of somatic embryogenesis by stress compounds

Shoot apices (ca. 1 cm in length) were excised from surface-sterilized seedlings as described by Tachikawa *et al.* (1998) They were cultured for two or four weeks on semi solidified-agar (0.8%) MS medium with stress compounds to which no phytohormones were added, and then they were transferred onto phytohormone-free MS medium without addition of stress compounds. The effective chemicals and duration of treatment for somatic embryogenesis induction were as follows: 0.7 M sucrose for four weeks (Kamada *et al.*, 1993); 0.3 M NaCl for four weeks (Kiyosue *et al.*, 1989); 0.6 mM $CdCl_2$ for four weeks (Kiyosue *et al.*, 1990); 10^{-4} M ABA for two weeks (Kamada *et al.*, 1981); and 1 mg/*l* 2,4-D for four weeks. Cells and stress-treated shoot apices were harvested at the indicated times, and immediately frozen in liquid nitrogen and stored at -80 ℃ until use.

Isolation of RNA and construction of cDNA library from carrot somatic embryos

Total RNA was isolated from a mix population of 16-day-old somatic embryos consisting of globular, heart-shaped and torpedo-shaped embryos by the phenol/SDS method (Ausubel *et al.*, 1987). Poly $(A)^+$ RNA was purified by oligo (dT) cellulose column chromatography (Pharmacia, Piscataway, NJ, U.S.A.) was used to construct the cDNA library according to cDNA Synthesis System Plus protocols (Amersham, Buckinghamshire, U.K.). After the addition of *Eco*RI / *Not*I adaptors (Pharmacia), cDNAs were size-fractionated on a Size-sep 400 spun column (Pharmacia). cDNAs were inserted into the *Eco*RI site of the λgt10 vector and packaged in λgt10 according to the cDNA Cloning System protocols (Amersham) (Shiota *et al.*, 1998).

cDNA library screening and subcloning

pECPP-S+A cDNA fragment was obtained by PCR amplification of the cDNA library using two degenerate primers, ECPP-S (5'-GAYTGY-AARGTIGTIGARGARGARG-3') and ECPP-A (5'-TTYTTYTTYTTYTTYTCICCICC-3') which were designed based on the partial amino acid sequences of ECPP44 (Tan *et al.*, 2000). Approximately 1.2×10^5 phages were plated on solid LB medium, transferred to nylon membrane filter (Biodyne B; Nihon Pall Ltd., Tokyo, Japan) followed by the alkalization and neutralization procedures described by suppliers (Amersham). They were screened by hybridization with a [α-^{32}P]-labeled cDNA fragment from pECPP-S+A as a probe. The probe was a 141 bp fragment which contained a stretch of poly-serine cluster and lysine-rich motif conserved in dehydrin family (Tan *et al.*, 2000). Hybridization was performed at 65 ℃ according to the instructions from Amersham. The filters were washed twice with 2 × SSC for 15 min at room temperature and 2 × SSC with 0.1% SDS for 15 min at 65 ℃. Hybridization was visualized by bio-imaging analyzer with an imaging plate (BAS 2000; Fuji Photo Film Co., Tokyo, Japan). Plaques giving a signal with the cDNA probe were picked up and checked with PCR amplification using λgt10 forward and reverse primers. The PCR products were separated on 1.5% agarose gel, blotted, and hybridized again with the radio-labeled cDNA fragment of pECPP-S+A. Several clones showing positive signals were purified and subcloned into TA-cloning vector (Invitrogen, U.S.A).

Sequencing of cDNA clones

For sequencing of the cDNA clones, double-strand plasmid DNAs were isolated and sequenced by the Dye Primer Cycle Sequencing method

according to the protocol of the Dye Primer Cycle Sequencing Kit (Applied Biosystems, U.S.A), or by the ABI PRISM Dye Terminator Cycle Sequencing method according to the protocol of the Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, U.S.A).

Isolation of genomic DNA and Southern blot analysis

Genomic DNA was extracted from leaves of greenhouse-grown adult carrot plants using the Nucleon Phytopure DNA Extraction Kit (Nucleon biosciences, Amersham LIFE SCIENCE, England) according to the manufacturer's instruction. Genomic DNA (20 μg) was digested with *Hin*dIII, *Eco*RI and *Xba*I, separated on 1% agarose gel, and transferred to nylon membrane filter (Biodyne B) after the alkalization and neutralization steps. The DNA on the filter was allowed to hybridize with a 774 bp fragment of ECPP44 cDNA which was labeled by random priming with [α-^{32}P]-dCTP using a BcaBEST Labeling Kit (Takara, Japan). The filter was pre-incubated in pre-hybridization buffer (6 × SSC, 5 × Denhardt's solution, 0.1% SDS and 100 μg/m*l* denatured Herring sperm DNA) at 55 ℃ for 16 h. Following hybridization, the filter was washed with washing buffer (2 × SSPE, 0.15 M NaCl, 0.01 M sodium phosphate and 1 mM Na_2EDTA, pH 7.4) twice for 5 min at room temperature, and then twice for 15 min with 2 × SSPE and 0.1% SDS at 65 ℃ (low-stringency conditions). After exposure to an imaging plate for an appropriate time, the same filter was washed twice for 15 min with 0.1 × SSPE and 0.1% SDS at 65 ℃ (high-stringency conditions). For visualization of bands on the filter, we used a bio-imaging plate (BAS 5000, Fuji Photo Film Co., Ltd., Tokyo, Japan).

RNA extraction and Northern blot analysis

Total RNA was isolated from embryogenic cells (EC), non-embryogenic cells (NC), somatic embryos and shoot apices with or without stress chemical treatments using RNeasy Plant Mini kit (QIAGEN, Germany). A total RNA (20 μg) per lane was fractionated by electrophoresis on a formaldehyde-agarose gel (1.2%), and the bands of RNA were transferred to nylon membrane filter (Biodyne B). The cDNA fragment containing 5'-coding region (246 bp) of ECPP44 was labeled by random priming with [α-^{32}P]dCTP using a BcaBEST Labeling Kit (Takara, Japan). Pre-hybridization of RNA blots were done at 42 ℃ for 3 h in hybridization buffer containing 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS and 100 μg/m*l* denature Herring sperm DNA, and then hybridized with the same hybridization buffer at 42 ℃ for 16 h with 10^7 cpm labeled DNA probe. The membrane was washed 3 times with 2 × SSC and 0.1% SDS for 10 min at room temperature and then with 0.1 × SSC and 0.1% SDS for 12 min at 42 ℃. Signals were visualized by BAS 5000 bio-imaging plate for 2 days. To provide an internal control, the same filter was rehybridized with ^{32}P-labeled PnrRNA cDNA fragment which encodes 16S rRNA of *Pharbitis nil*.

Results

Isolation and sequence analysis of cDNA clone encoding ECPP44

A total of 1.3 × 10^5 phages were screened at the lst screening and from which 14 positive clones were obtained. Seven positive cDNA clones were selected at the 2nd screening and sequenced. Only 1 clone (clone 43) contained the full-length cDNA of ECPP44 (925 bp) **(Fig. 1)** that encoded three partial amino acid sequences (Regions I, II and III) identical to those which were previously determined in ECPP44 protein (Tan *et al.*, 2000). Protein database search using NCBI BLAST algorithm revealed that the full-length cDNA of ECPP44 **(Fig. 1)** is homologous with dehydrin genes. The ECPP44 cDNA contains an open reading frame of 774 bp flanked by 5'- and 3'- untranslated sequences of 89 bp and 62 bp, respectively. The predicted 258 amino acids has a calculated molecular mass of 28,8 kDa. Computational analysis of the primary sequence of the protein revealed a poly-serine cluster (residues 110-121) and three lysine-rich domains (residues 127-142, 167-180 and 211-224) **(Fig. 1 and 2)**. These are the structural features of a dehydrin family. The lysine-rich repeats are similar to the nuclear localization signal sequence (NLS) found in yeast mating type factor α2 that functions in nuclear transport (Hall *et al.*, 1984; Thomas *et al.*, 1997).

The amino acid sequence of ECPP44 was compared to carrot ECP40 (Kiyosue *et al.*, 1993), DcDhn1 [unpublish data] and *Arabidopsis* ERD14 (Kiyosue *et al.*, 1994) which are known as Group II LEA proteins, and we found that ECPP44 exhibits 50%, 20%, 23% homology to ERD14, ECP40 and DcDhn1, respectively **(Fig. 2)**. ECPP44 protein had characteristically two different types of repeated amino acid sequences which have already been described above: A stretch of serine-rich repeat (RSGSSSSSSSSDEE) near the middle part of the polypeptide, and the lysine-rich repeats, (GGEKKKKKEKKGLKEK, KKGFMEKIKEKLPG, KKGILEKIKEKIPG). These repeats are also present in the amino acid sequences of *Zea mays*

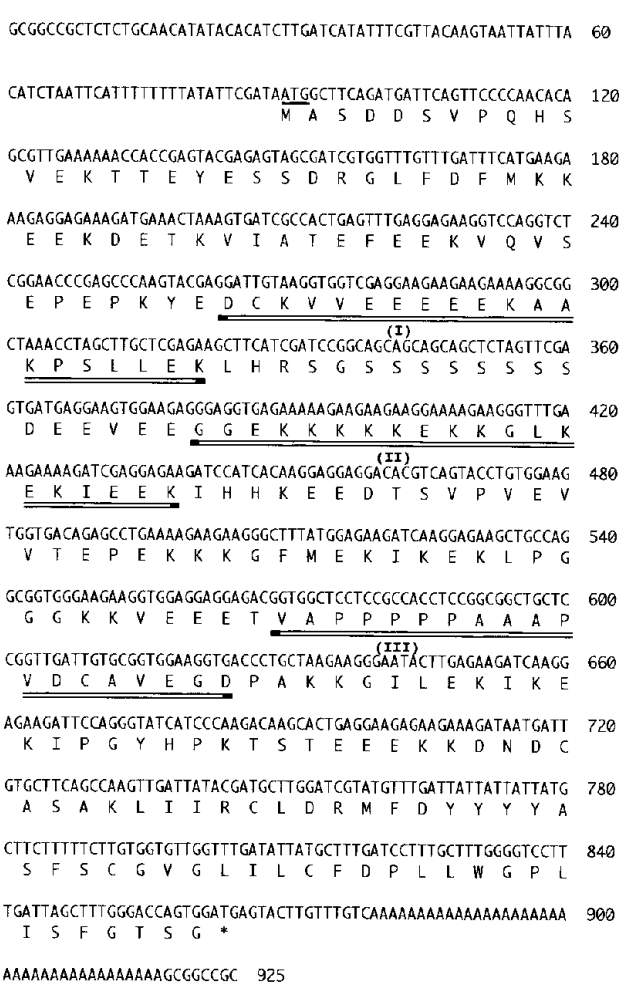

Fig. 1 Nucleic acid and deduced amino acid sequences of the carrot *ECPP44* (DDBJ accession number AB010898). The initiation codon is underlined. The asterisk indicates the stop codon. Regions I, II and III (double underlined) indicate three partial amino acid sequences which were determined by peptide mapping analysis of ECPP44 protein.

(DHN3), *Hordeum vulgare* (DHN18), *Triticum aestivum* (WCOR410), *Oryza sativa* (rab21), *Solanum tuberosum* (c17), *Arabidopsis thaliana* (ERD14) and *Pisum sativum* (dhn-cog).

Southern blot analysis of the ECPP44 gene

In order to evaluate the copy number of *ECPP44* in the carrot genome, Southern blot analysis was performed using ^{32}P-labeled ECPP44 cDNA containing one restriction site for *Hin*dIII to probe identical blots of restriction enzyme digested carrot genomic DNA. The result showed that *ECPP44* is a single copy gene (**Fig. 3**). **Fig. 3** shows the full-length ECPP44 cDNA probe also hybridized with some extra bands under a low-stringency conditions, suggesting the presence of additional genes related to *ECPP44* in the carrot genome.

Expression analysis of ECPP44 mRNA

ECPP44 gene expression was examined in response to treatment with various stress chemicals. The accumulation of the *ECPP44* mRNA were detected in shoot apices treated with stress chemicals (1 mg/*l* 2,4-D, 0.7 M sucrose, 0.3 M NaCl, 0.6 mM $CdCl_2$ and 10^{-4} M ABA), EC, and somatic embryos (**Fig. 4**). However, no *ECPP44* mRNA was detected in non-embryogenic cells. On the other hand, the levels of the *ECPP44* transcript did not show any significant difference between stress-treated and non-treated shoot apices.

Dicussion

In our previous study, we showed that ECPP44 protein is exclusively phosphorylated in EC and somatic embryos, but not in NC (cells that had lost the ability to form somatic embryos), by two-dimensional gel electrophoresis of *in vivo* ^{32}P-labeled polypeptide (Tan *et al.*, 2000). In this study, we succeeded in isolating the full-length cDNA clone encoding the ECPP44 protein. ECPP44 was specifically phosphorylated during induction and formation of somatic embryogenesis. ECPP44 protein shows similarity to the dehydrin family associated with the late-embryogenesis abundant (LEA) protein belonging to Group II (**Fig. 1**). The comparison of *ECPP44* gene with Group II *LEA* genes allowed us to highlight the serine cluster and lysine-rich sequences (**Fig. 5**), which share identical amino acids among dehydrin gene family from several mono- and di-cotyledonous plants (Kiyosue *et al.*, 1994; Kirch *et al.*, 1997; Close *et al.*, 1989; Kiyosue *et al.*, 1992).

To find out more about the possible involvement of ECPP44 in somatic embryogenesis, its expression was analyzed in response to various stress compounds in culture of carrot shoot apices. *ECPP44* transcript was detected in EC and somatic embryos, but not in NC (**Fig. 4**). Similarly, the accumulation of *ECPP44* mRNA was detected not only in all stress-treated shoot apices but also in non-treated shoot apices. We previously reported that the ECPP44 protein was detected in the control culture (non-treated shoot apices), but no phosphorylated spot was detected (Tan *et al.*, 2000). Thus, phosphorylation of ECPP44 protein is thought to be involved in the acquisition of embryogenic competence.

Southern blot analysis under low stringency condition suggested that the *ECPP44* gene belongs to a small gene family since one to three hybridized bands were also detected (**Fig. 3**). It was also shown that *ECPP44* shares considerable sequence homo-

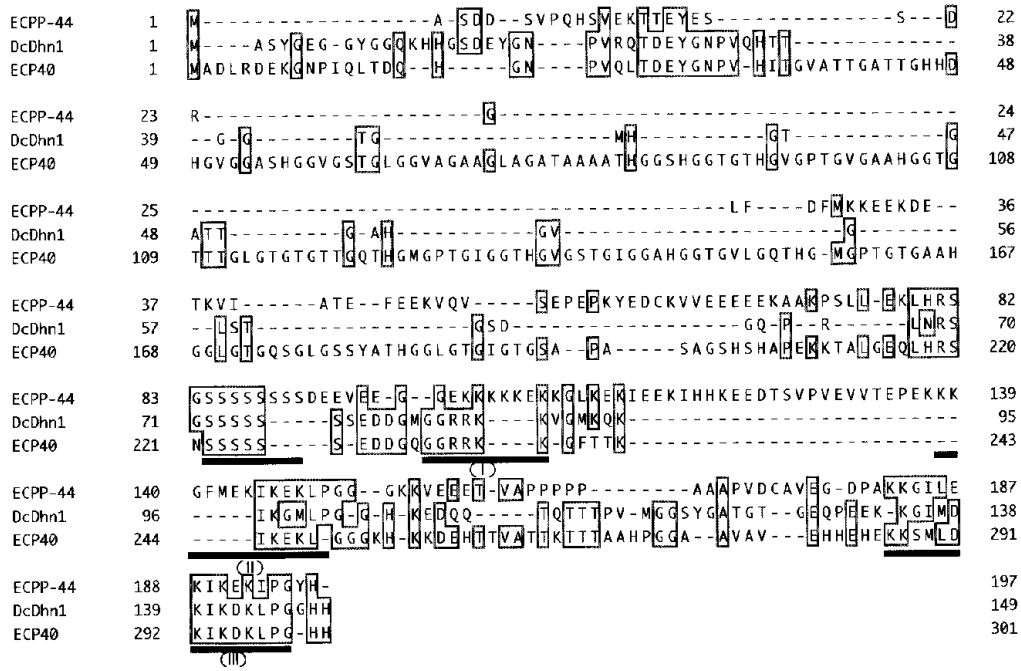

Fig. 2 Comparison of the deduced amino acid sequence of the ECPP44 with the amino acid sequences of ECP40 and DcDhn1. Open boxes represent amino acids identical to each other. The black lines represents the serine cluster followed by a putative phosphorylation site for a protein kinase ck2 recognition (amino acid residues DEE in position 92-94), The black box (I, II and III) represents three of basic lysine-rich motifs.

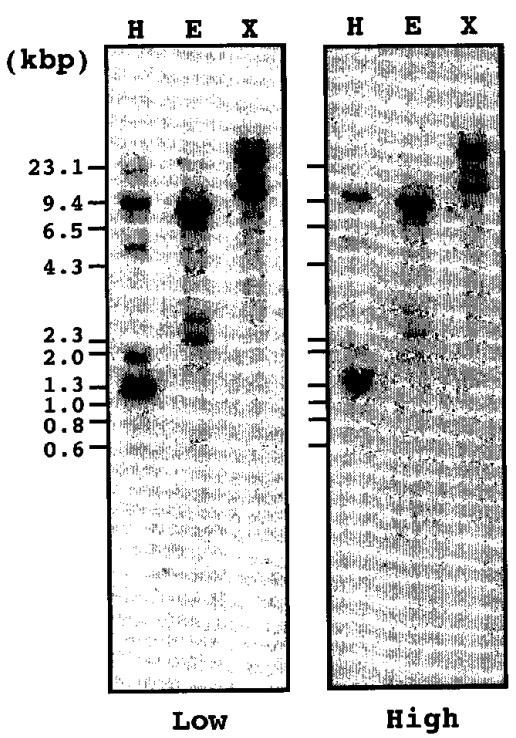

Fig. 3 Genomic Southern blot analysis of *ECPP44*. Genomic DNA was digested with *Xba*I (X), *Eco*RI (E) and *Hin*dIII (H), and probed with *ECPP44* full-length cDNA (774 bp). The hybridization was done under high (high) or low (low) stringency condition. The sizes of DNA markers are in kbp.

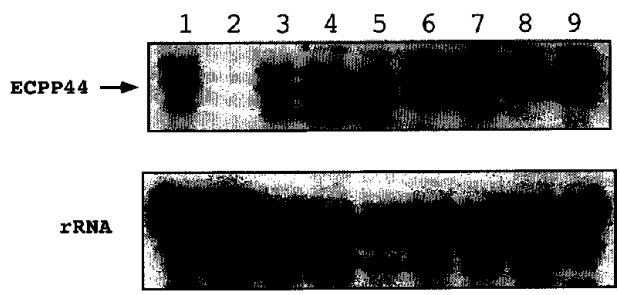

Fig. 4 Northern blot analysis of *ECPP44*. Total RNA was isolated from various tissues [lane 1: torpedo-shaped somatic embryos induced in MS medium, 2: NC cultured in MS medium with 2,4-D, 3: EC cultured in MS medium with 2,4-D, 4: shoot apices cultured in MS medium without any stress chemicals, 5: shoot apices cultured in MS medium with 2,4-D, 6: shoot apices cultured in MS medium with 0.3 M NaCl, 7: shoot apices cultured in MS medium with 0.7 M sucrose, 8: shoot apices cultured in MS medium with 0.6 mM $CdCl_2$, 9: shoot apices cultured in MS medium with 10^{-4} M ABA] and hybridized with ECPP44 cDNA 5'-coding region (246 bp) as a probe. Each lane contains 20 μg of total RNA.

logy with some of the repeats in *ECP40* (Kiyosue *et al.*, 1993) and *DcDhn1* [unpublish data] in carrot (**Fig. 2**). In **Fig. 6**, we present a phylogenetic tree based on the amino acid substitutions of various

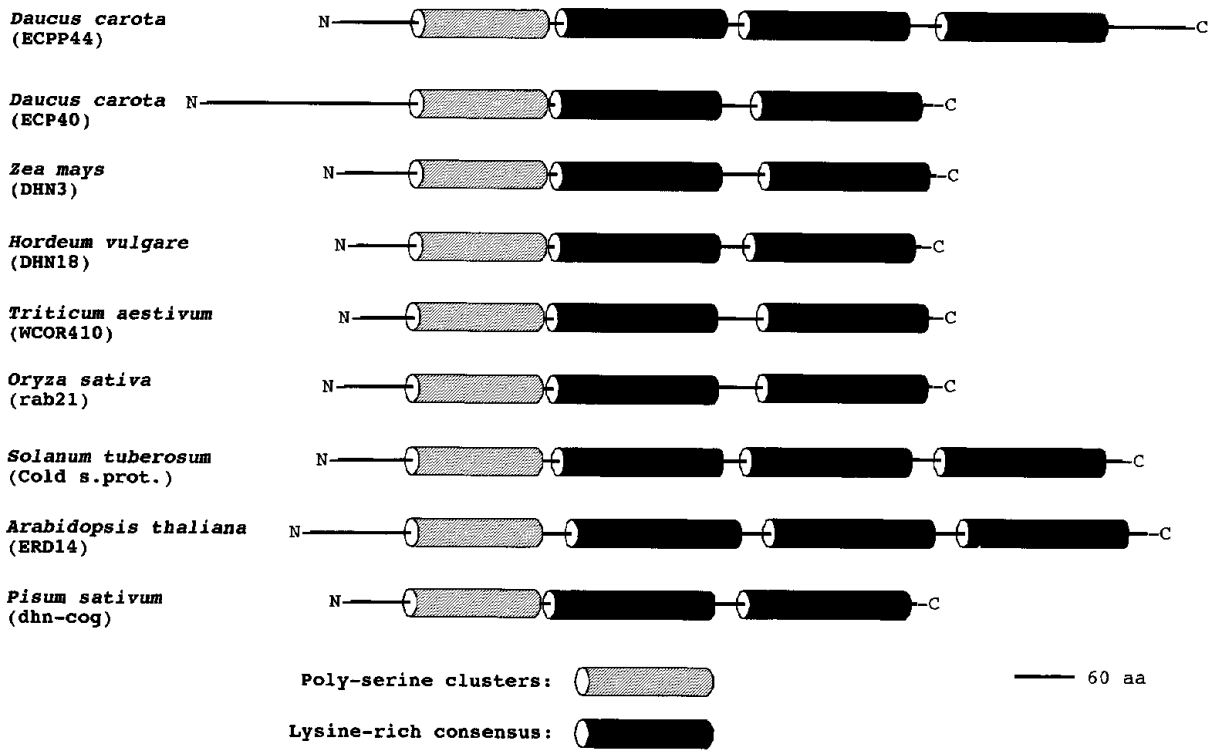

Fig. 5 Comparison of the putative structure of carrot ECPP44, carrot ECP40, maize DHN3, barley DHN18, wheat WCOR410, rice RAB21, potato C17, *Arabidopsis* ERD14 and pea DHN-COG. ECPP44 ORF contains fifteen of the lysine-rich repeats characteristic of Group II LEA proteins and a poly-serine cluster at the N-terminal region.

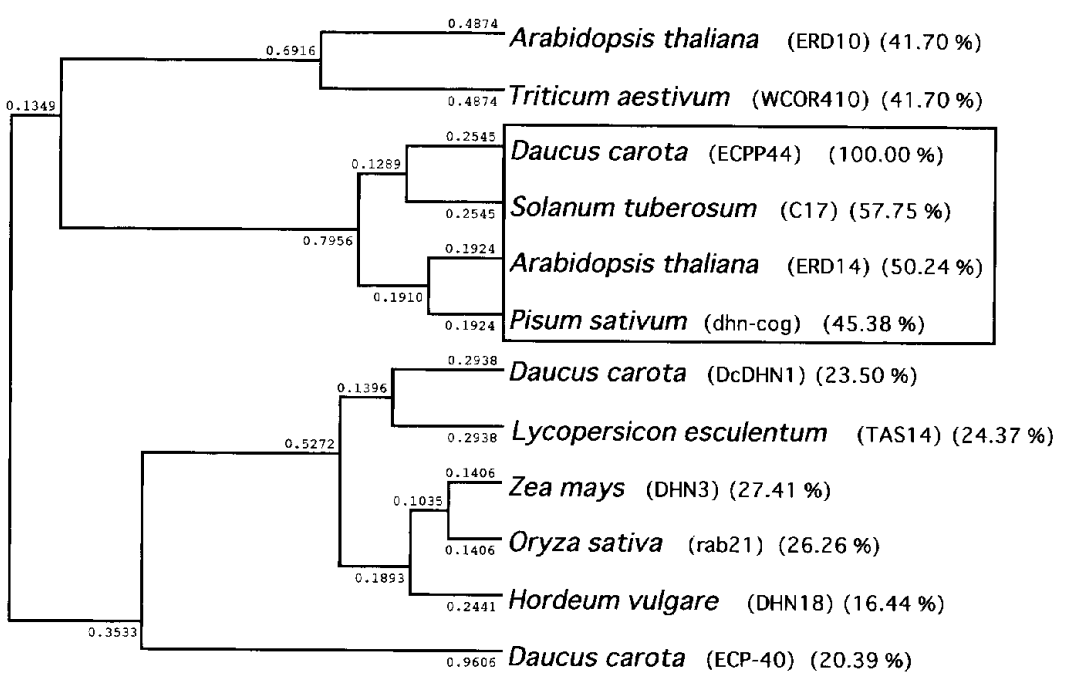

Fig. 6 A phylogenetic tree showing the evolutionary distance between ECPP44 and other related plant proteins. The tree was constructed by the UPGMA method using GENETYX-MAC software. The ECPP44 shows homology to dhn-cog (Z14145; 45.38%) from *Pisum sativum*, C17 (U69633; 57.75%) from *Solanum tuberosum*, ERD10 (D17714; 41.70%) and ERD14 (D17715; 50.24%) from *Arabidopsis thaliana*, WCOR410 (L29152; 40.00%) from *Triticum aestivum*, DHN18 (X15287; 16.44%) from *Hordeum vulgare*, DHN3 (X15290; 27.41%) from *Zea mays*, rab21 (Y00842; 26.26%) from *Oryza sativa*, Dhn1 (23.50%) and ECP40 (X61914; 20.39%) from *Daucus carota*, and TAS14 (X51904; 24.37%) from *Lycopersicon esculentum*.

dehydrin proteins. ECPP44 (carrot), C17 (potato), ERD14 (*Arabidopsis*) and Dhn (pea) (Robertson *et al.*, 1992) are in the same subfamily. Although there are sequence similarities, ECPP44 are most likely to play a significant role in embryogenesis other than functions in response to dehydration and low temperature stress. Thus, ECPP44 could be catagorized to a new type of dehydrin in carrot.

The expression of *LEA* genes (Dure III *et al.*, 1981) has been reported to be induced by cold, osmotic, drought, and salt stresses, and ABA in the vegetative tissues (Ingram *et al.*, 1996; Finch-Savage *et al.*, 1994; Close *et al.*, 1993; Dure III *et al.*, 1989; Kasuga *et al.*, 1999) of both mono- and di-cotyledonous plant species during the late embryogenesis, when the seeds start to dehydrate (Ingram *et al.*, 1996; Finch-Savage *et al.*, 1994; Lynn *et al.*, 1995; Espelund *et al.*, 1992). Most of these LEA proteins are quite hydrophilic and are believed to play a role in directly protecting plant cells from these stresses (Dure III 1993; Close *et al.*, 1993; Tachikawa *et al.*, 1998). It has been suggested that dehydrin proteins prevent cellular damage during desiccation and have also been implicated in the acquisition of desiccation tolerance in seeds (Finch-Savage *et al.*, 1994; Bradford *et al.*, 1992; Han *et al.*, 1997; Blackman *et al.*, 1992). Unlike the other LEA proteins, ECPP44 contains putative nuclear targeting signal (NLS). Thus, it is possible that ECPP44 has a different physiological role apart from the widely known dehydration tolerance.

To further expound on the role ECPP44 plays, we are making a specific antibody against ECPP44 protein and will analyze the relationship between phosphorylation of ECPP44 and acquisition of embryogenic competence. We are currently transforming carrot with ECPP44 cDNA for overexpression and/or antisense repression. These studies should provide more information about the role and function of ECPP44 in relation to somatic embryogenesis.

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References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. 1987. Phenol/SDS method for plant RNA preparation. *In Current* Protocols in Molecular Biology. Eds. by Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. pp. 4.3.1-4.3.4. Greene Publishing Associates and Wiley-Interscience, New York.

Baker, J, Steele, C., Dure III, L.S. 1988. Sequence and characterization of 6 Lea proteins and their genes from cotton. Plant Mol. Biol., **11**:277-291.

Ballard, M.J., Tyndall, W.A., Shingle, J.M., Hall, D.J., Winter, E. 1991. Tyrosine phosphorylation of a yeast 40kDa protein occurs in response to mating pheromone. EMBO J., **10**:3753-3758.

Blackman, S.A., Obendorf, R.L., Leopold, A.C. 1992. Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. Plant Physiol., **100**:225-230.

Bradford, K.J., Chandler, P.M. 1992. Expression of dehydrin-like proteins in embryos and seedlings of *Zizania palustris* and *Oryza sativa* during dehydration. Plant Physiol., **99**:488-494.

Close, T.J., Kortt, A.A., Chandler, P.M. 1989. A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. Plant Mol. Biol., **13**:95-108.

Close, T.J., Fenton, R.D., Yang, A., Asghar, R. DeMeson, D.A., Crone, D.E., Meyer, N.C., Moonan, F. 1993. Plant responses to cellular dehydration during environmental stress. Current Topics in Plant Physiology: (Eds. by Close T.J., Bray E.A.) Rockville, MD, Am. Soc. Plant Physiol., **10**:104-118.

Dure III, L.S., Greenway, S.C., Galau, G.A. 1981. Developmental biochemistry of cotton seed embryogenesis and germination: changing messenger ribonucleic acid populations as shown by *in vitro* and *in vivo* protein synthesis. Biochem., **20**:4162-4168.

Dure III, L.S., Crouch, M., Harada, J., Ho, T.H.D., Mundy, J., Quatrano, R., Thomas, T., Sung, Z.R. 1989. Common amino acid sequence domains among the LEA protein of higher plants. Plant Mol. Biol., **12**:475-486.

Dure III, L.S. 1993. A repeating 11-mer amino acid motif and plant desiccation. Plant J., **3**:363-369.

Espelund, M., Saeboe-Larssen, S., Hughes, D.W,, Galau, G.A., Larsen,F., Jakobsen, K.S. 1992. Late embryogenesis-abundant genes encoding proteins with different numbers of hydrophilic repeats are regulated differentially by abscisic acid and osmotic stress. Plant J., **2**:241-252.

Finch-Savage, W.E., Pramanik, S.K., Bewley, J.D. 1994. The expression of dehydrin proteins in desiccation-sensitive (recalcitrant) seeds of temperate trees. Planta **193**:478-485.

Hall, M.N., Hereford, L., Herskowitz, I. 1984. Targeting of *E. coli* β-galactosidase to the nucleus in yeast. Cell, **36**:1057-1065.

Han, B., Hughes, D.W., Galau, G.A., Bewley, J.D., Ker-

mode, A.R. 1997. Changes in late-embryogenesis-abundant (LEA) messenger RNAs and dehydrins during maturation and premature drying of *Ricinus communis* L. seeds. Planta, **201**:27-35.

Ingram, J., Bartels, D. 1996. The molecular basis of dehydration tolerance in plants. Plant Mol. Biol., **47**:377-403.

Kamada, H., Harada, H. 1981. Changes in endogenous level and effects of abscisic acid during somatic embryogenesis of *Daucus carota* L. Plant Cell Physiol. **22**:1423-1429.

Kamada, H., Ishikawa, K., Saga, H., Harada, H. 1993. Induction of somatic embryogenesis in carrot by osmotic stress. Plant Tissue Culture Lett., **10**:38-44.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. 1999. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotechnol., **17**:287-291.

Kiyosue, T., Kamada, H., Harada, H. 1989. Induction of somatic embryogenesis by salt stress in carrot. Plant Tissue Culture Lett., **6**:162-164.

Kiyosue, T., Takano, K., Kamada, H., Harada, H. 1990. Induction of somatic embryogenesis in carrot by heavy metal ions. Can. J. Bot., **68**:2301-2303.

Kiyosue, T., Yamaguchi-Shinozaki, K., Higashi, K., Satoh, S., Kamada, H., Harada, H. 1992. Isolation and characterization of a cDNA that encodes ECP31, an embryogenic-cell protein from carrot. Plant Mol. Biol., **19**: 239-249.

Kiyosue, T., Yamaguchi-Shinozaki, K., Shinozaki, K., Kamada, H., Harada, H. 1993. cDNA cloning of ECP40, an embryogenic-cell protein in carrot, and its expression during somatic and zygotic embryogenesis. Plant Mol. Biol., **21**:1053-1068.

Kiyosue, T., Yamaguchi-Shinozaki, K., Shinozaki, K., 1994. Characterization of two cDNAs (ERD10 and ERD14) corresponding to genes that respond rapidly to dehydration stress in *Arabidopsis thaliana*. Plant Cell Physiol., **35**:225-231.

Kirch, H.H., van Berkel, J., Glaczinski, H., Salamini, F., Gebhardt, C. 1997. Structural organization, expression and promoter activity of a cold-stress-inducible gene of potato (*Solanum tuberosum* L.). Plant Mol. Biol., **33**:897-909.

Louis, J.M., McFarland, V.W., May, P., Mora, P.T. 1988. The phosphoprotein p53 is down-regulated post-transcriptionally during embryogenesis in vertebrates. Biochim. Biophys. Acta, **950**:395-402.

Lynn, D.H., Walker-Simmons, M.K. 1995. The wheat abscisic acid-responsive protein kinase mRNA PKABA 1, is up-regulated by dehydration, cold temperature and osmotic stress. Plant Physiol., **108**:1203-1210.

Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant., **15**:473-497.

Najafi, S.M., Harris, D.A., Yudkin, M.D. 1997. Properties of the phosphorylation reaction catalyzed by SpoIIAB that help to regulate sporulation of *Bacillus subtilis*. J. Bacteriol., **179**:5628-5631.

Reinert, J., 1959. Uber die kontrolle der morphogenese und die induction von adventivembryonen an gewebeculturen aus karroten. Planta, **53**: 318-333.

Robertson, M., Chandler, P.M. 1992. Pea dehydrins: identification, characterisation and expression. Plant Mol. Biol. **19**:1031-1044.

Satoh, S., Kamada, H., Harada, H., Fujii, T. 1986. Auxin-controlled glycoprotein release into the medium of embryogenic carrot cells. Plant Physiol., **81**:931-933.

Shiota, H., Satoh, R., Watabe, K., Harada, H., Kamada, H. 1998. *C-ABI3*, the carrot homologue of the *Arabidopsis ABI3*, is expressed during both zygotic and somatic embryogenesis and functions in the regulation of embryo-specific ABA-inducible genes. Plant Cell Physiol., **39**:1184-1193.

Steward, F.C., Mapes, M.O., Smith, J. 1958. Growth and organized development of cultures cells. I. Growth and division of freely suspended cells. Amer. J. Bot., **45**:693-703.

Tachikawa, Y., Saito, T., Kamada, H., Harada, H. 1998. Changes in protein pattern during stress-induction of carrot (*Daucus carota* L.) somatic embryogenesis. Plant Biotechnol., **15**:17-22.

Tan, S.K., Kamada, H. 2000. Initial identification of phosphoproteins that appear to be involved in the induction of somatic embryogenesis in carrot. Plant Cell Rep., in press.

Thomas, M., Ferenc, N. 1997. Nuclear import of proteins: putative import factors and development of *in vitro* import systems in higher plants. Trends in Plant Science, **2**:458-463.

Zimmerman, J.L. 1993. Somatic Embryogenesis: A model for early development in higher plants. Plant Cell, **5**:1411-1423.