Original Paper Plant Biotechnology, **18** (1), 45-54 (2001)

Comparison and Characterization of *cis*-Regulatory Regions in Some Embryo-Specific and ABA-Responsive Carrot Genes, *DcECPs*

Sukmin KO[1], Siripong THITAMADEE[2], Heping YANG[3], Chang-Ho EUN[1], Kimiyo SAGE-ONO[1], Katsumi HIGASHI[1], Shinobu SATOH[1] and Hiroshi KAMADA[1]

[1] *Gene Experiment Center, Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki, 305-8572 Japan*
[2] *Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama-cho, Ikoma, Nara, 630-0101 Japan*
[3] *Laboratory Bioactive Natural Product Chemistry, Graduate School of Bio-Agricultural Sciences, Nagoya University, Chikusa, Nagoya, Aichi, 464-8601 Japan*
Corresponding author E-mail address: sukmin@sakura.cc.tsukuba.ac.jp

Received 18 September 2000; accepted 30 October 2000

Abstract

Expression of carrot *ECP (DcECP)* genes encoding late-embryogenesis abundant proteins is embryo-specific and ABA-inducible. The expression is regulated by *C-ABI3*, a carrot homologue of *Arabidopsis ABI3*. To understand the molecular mechanisms controlling ABA-inducible and embryo-specific gene expression, we compared and analyzed the promoter regions of some *ECP* genes. The accumulation of DcECPs mRNA in response to ABA was not inhibited by an inhibitor of protein synthesis, cycloheximide. These results indicate that the ABA-inducible expression of the *DcECPs* did not require *de novo* protein synthesis. Sequence comparison among some *ECP* gene promoters revealed that the promoters contained several conserved motifs including ABRE (ABA responsive element)-like ACGT core motifs, and an Sph box (CATGCATG), which has been identified as a motif mediating gene activation of maize antocyanin regulatory gene *C1*. To investigate the promoter activity of *DcECP31* promoter region, we carried out a deletion analysis with a transient assay system using protoplasts of embryogenic cells or with a transformation system using embryogenic cells of carrot. We found that the -250 bp upstream region of the *DcECP31* promoter is sufficient for embryo-specific and ABA-inducible promoter activity. Following deletion analysis of *DcECP40* promoter, we clarified that the distal (-670~-390) and proximal regions (-140~-50), are essential for the ABA-inducible expression.

Introduction

Embryogenesis is the process that constructs the basic body system of plants. During embryogenesis, the zygote undergoes a series of morphological and cellular changes resulting in the formation of a mature embryo composed of an embryonic axis with shoot and root poles and cotyledon (West and Harada, 1993). Studies on plant embryogenesis, especially characterization of genes which are specifically expressed during embryogenesis, are necessary in order to understand the molecular mechanisms controlling plant development. However, molecular analysis on early embryogenesis is not available, because it is impossible to prepare enough embryos at the same developmental stage (Zimmerman, 1993). On the other hand, it is thought that somatic embryogenesis can be utilized as a model of zygotic embryogenesis because the spatial and temporal changes in some aspects of both somatic and zygotic embryogenesis are quite similar (Zimmerman, 1993). Carrot has been used as a model plant for somatic embryogenesis, because in carrot large amounts of somatic embryos at the same developmental stage can be readily induced by a relatively simple manipulation.

A number of genes have been isolated from somatic embryos, and several genes that are preferentially expressed in somatic embryos have characteristics of a class of protein called late embryogenesis abundant (LEA) proteins (Dure III *et al.*, 1981, 1989, Galau *et al.*, 1986). The timing of their expression in embryogenesis and their abscisic acid (ABA) inducibility have led to the postulation that in zygotic embryos, they play a role in protecting

the embryo during desiccation (Baker *et al.*, 1988). Because the expression of many LEA genes are regulated by ABA, studies on LEA genes have been directed toward understanding the molecular mechanism of hormonal regulation (Busk and Pagès, 1998). Among the LEA genes, wheat *Em*, rice *rab16A* and rice *Osem* from rice are particularly well characterized in regard to regulation by ABA (Marcotte *et al.*, 1989, Mundy *et al.*, 1990, Hattori *et al.*, 1995). From sequence comparison and functional analysis of the promoters of these genes, ABA-responsive elements (ABRE) have been identified (Mundy *et al.*, 1990). At present, more than 20 functional ABREs have been identified in ABA-inducible promoters. The element is defined as a sequence of 8-10 base pairs with the core sequence ACGT (Busk and Pagès, 1998). The ACGT core motifs have been found to have related responses to diverse environmental signals in plants, including light (Donald and Cashmore, 1990), anaerobiosis (Mckendree *et al.*, 1990), coumaric acid (Loake *et al.*, 1992), methyl jasmonate (Mason *et al.*, 1993) and UV-light (Weisshaar *et al.*, 1991). Therefore, it has been considered that combinatorial interactions between the ACGT core motifs and other regulatory sequences in the promoter may determine the signal specificity. Ho and coworkers identified *cis*-elements called coupling elements which are active in combination with an ABRE but not alone (Shen *et al.*, 1995, 1996). In the promoters of barley genes HVA22 and HVA1, the coupling elements CE1 (TGCCACCGG) and CE3 (ACGCGTGTCCTG) are required for activation by ABA. Recently, Hobo *et al.* (1999) have also demonstrated that ACGT-containing ABREs and CE3 are functionally equivalent to *cis*-acting elements in the promoter of *Osem* gene.

We previously isolated and characterized some cDNAs for embryogenic cell proteins (ECPs) which are specifically found in embryogenic cells from carrot, *DcECP31* (Kiyosue *et al.*, 1992 a), *DcECP40* (Kiyosue *et al.*, 1993), *DcECP45* (unpublished), *DcECP63* (Zhu *et al.*, 1997), and those of *Arabidopsis* homologues, *AtECP31* (Yang *et al.*, 1996) and *AtECP63* (Yang *et al.*, 1997). These genes encode LEA proteins. The transcripts of *DcECPs* are accumulated at high levels in embryogenic cells and disappear during the development of somatic embryos. The level of *DcECPs* transcripts increased after treatment with ABA in torpedo-shaped somatic embryos but not in seedlings (Kiyosue *et al.*, 1992 a, Kiyosue *et al.*, 1993, Zhu *et al.*, 1997). These results suggest that expression of *DcECP* genes is regulated by ABA and unknown embryonic factors. On the other hand, we previously isolated and characterized a carrot gene *C-ABI3*, homologous to the ABI3 gene of *Arabidopsis* from a carrot somatic embryo cDNA library (Shiota *et al.*, 1998). The expression of *C-ABI3* was detected specifically in embryogenic cells, somatic embryos and developing seeds. In transgenic mature leaves of carrot in which *C-ABI3* was ectopically expressed, expression of *DcECPs* was induced by treatment with ABA, which indicates that the expression of *DcECP* genes was controlled by the pathway(s) that involved C-ABI3 and ABA (Shiota *et al.*, 1998). These results indicate that embryo specific expression of *DcECP* genes is specified by C-ABI3, and their expression is restricted to seed tissues.

In this paper, in order to understand the molecular mechanisms of hormonal regulation of the expression of embryo-specific genes, we compared and analyzed the promoter regions of *ECP* genes.

Materials and Methods

Plant materials

Carrot (*Daucus carota* L. cv. US-Harumakigosun) seedlings were grown for 10 days at 25 ℃ with 16 h of light daily. Carrot embryogenic cells and somatic embryos were obtained as described by Satoh *et al.* (1986). Mature leaves were collected from plants that had been grown for two months at 25 ℃ with illumination as mentioned above.

Isolation of RNA and Northern blot analysis

Total RNA was isolated from somatic embryos by the phenol / SDS method (Ausubel *et al.*, 1987). The total RNA (20 μ g per lane) was loaded on an agarose (1.2 %) gel prepared with 2.2 M formaldehyde, subjected to electrophoresis and transferred to a Gene Screen Plus nylon filter (NEW Research products, Boston, MA, U.S.A.). Hybridization was performed at 60 ℃ according to the manufacturer's instructions.

Construction and screening of carrot genomic library

Carrot genomic DNA was isolated from leaves of two-month-old plants using a modified method reported by Wagner *et al.* (1987) and Bousquet *et al.* (1990). The carrot genomic library was constructed by cloning *Sau* 3AI partially digested genomic DNA into the phage vector λ DASH (Stratagene, La Jolla, CA, U.S.A., Maniatis *et al.*, 1982). Approximately 2 x 10^6 independent plaques of the primary library were screened by plaque hybridization using ^{32}P-labeled DcECP31 or 40 cDNA prepared with the random primed DNA

labeling kit (Pharmacia Biotech, Piscataway, NJ, U.S.A.). Hybridized plaques were picked up and purified by several rounds of plaque purification. Cloned lambda DNAs were prepared and the restriction sites of several enzymes were mapped. Chosen DNA restriction fragments were subcloned into pBluescript II SK^+ vector (Stratagene).

Sequencing of genomic clones

For sequencing of the genomic DNAs, deletion clones were constructed with a Kilo Sequence Deletion Kit (TaKaRa, Kyoto, Japan). Double - stranded plasmid DNAs were isolated and sequenced by the dye primer cycle sequencing method according to the protocol supplied with the Dye Primer Cycle Sequencing Kit (Applied Biosystems, Forster city, CA, U.S.A.).

Protoplast isolation

Carrot protoplasts were isolated from log phase culture of embryogenic cells using a modification of the procedure of Goodall *et al.* (1990) and Liu *et al.* (1994). After 4 days of subculture, the cell suspensions were filtered through three stainless steel meshes with 1000 μm, 63 μm and 37 μm pore sizes and the small cell clusters that remained on the 37 μm and 63 μm meshes were collected. They were sedimented at 300 x *g* for 5 min and resuspended in a solution containing 5 mM 2-(N-morpholino) ethane-sulfonic acid (MES) (pH 5.7), 0.4 M sorbitol, and 2 % ($w \cdot v^{-1}$) Driselase (Kyowa Hakko, Tokyo). The cells were transferred to a Petri dish and gently shaken at 20 ℃ for 4 to 5 h. Light microscopy was used to monitor the time course of protoplast release. The protoplasts were filtered through 60 μm nylon mesh and pelleted at 300 x *g* for 5 min. The protoplasts were gently suspended in 20 m*l* of W5 solution (Goodall *et al.*, 1990) and counted using a hemocytometer and light microscope. The protoplasts were repelleted and suspended in W5 solution at 2 x 10^6 cells $\cdot$ ml^{-1}.

Transient expression in protoplasts

Freshly prepared carrot protoplasts in W5 solution were pelleted and resuspended in MC buffer [5 mM MES (pH 5.7), 20 mM $CaCl_2$ and 0.5 M mannitol] at 6 x 10^6 cells $\cdot$ ml^{-1}. For transient assay, 2 x 10^5 cells in 0.3 ml MC buffer were mixed at room temperature with 50 μg of plasmid DNA constructs. The protoplasts and plasmid DNAs were mixed with 0.3 ml of 40 % ($w \cdot v^{-1}$) polyethylene glycol 4000 containing 0.1 mM $Ca(NO_3)_2$ and 0.4 M mannitol (pH 10.0), at room temperature for 5 min. Six ml of Murashige and Skoog's (MS) medium (pH 5.6) (Murashige and Skoog, 1962) with or without ABA was added, and protoplasts were incubated in the dark at 25 ℃ for 24 hrs. Following incubation, the protoplasts were pelleted by centrifugation at 300 x *g* for 5 min and lysed by the addition of 0.2m*l* GUS extraction buffer [50 mM NaH_2PO_4 (pH 7.0), 10 mM EDTA, 0.1% sodium lauryl sarcosine, 10 mM 2-mercaptoethanol]. After removal of insoluble materials by centrifugation, a portion of the supernatant was used to determine the protein content according to the Bradford method (Bradford *et al.*, 1976). The remainder of the supernatant was assayed for GUS activity using a fluorometric assay (Jefferson *et al.*, 1987).

Carrot transformation and GUS assay

Hypocotyl segments (10 mm) from one-week-old carrot seedlings were transformed with *Agrobacterium tumefaciens* harboring plasmids containing different chimeric genes. Hypocotyl segments were co-cultivated with *Agrobacterium* for 3 days at 28 ℃ on MS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D ; 1 mg $\cdot$ l^{-1}) (Fujii and Uchimiya, 1991). After 3 days of co-cultivation, the segments were placed on MS selection medium containing 2,4-D (1 mg $\cdot$ l^{-1}), claforan (500 mg $\cdot$ l^{-1}) and kanamycin (100 mg $\cdot$ l^{-1}). Selection for putatively transformed callus was made over 5 weeks in a 25 ℃ constant temperature room. To initiate transformed cell lines, each originating from a single cell, transgenic somatic embryos were induced from transformed callus in 2,4-D-free MS medium that contained kanamycin (100 mg $\cdot$ l^{-1}) and the induced torpedo-shaped embryos were dissected vertically. A portion of dissected embryos were assayed histochemically for GUS activity. The remaining cells of the GUS-positive embryos were placed on MS medium containing 2,4-D (1 mg $\cdot$ l^{-1}) and kanamycin (100 mg $\cdot$ l^{-1}) to establish cell lines. After 5 weeks of culture, somatic embryos were induced from the transformed cells originated from a single cell, and assayed histochemically for GUS activity. Histochemical GUS assay was carried out according to the method reported earlier (Jefferson *et al.*, 1987). Transgenic somatic embryos were dipped in a staining solution containing 1 mM X-gluc (5-bromo-4-chlolo-3-indoryl-β-D-glucuronide) and 50 mM NaH_2PO_4. They were incubated overnight at 37 ℃ , then observed under a light microscope.

Construction of promoter deletion series

The *DcECP31* or *DcECP40* promoter deletions were produced by synthesizing a set of oligo nucleotide primers and using polymerase chain reaction (PCR) to amplify the corresponding fragments.

The template for PCR reaction was the genomic clone of *DcECP31* or *DcECP40* promoter (either a 1.2 kb or 1.4 kb fragment subcloned into pBluescript II SK$^+$ vector, respectively). *Hin*dIII and *Bam*HI restriction sites were incorporated into the 5' and 3' primers, respectively. The amplified fragments were subcloned into the PCR II vectors using a TA Cloning Kit (Invitrogen, San Diego, CA, U.S.A.), and confirmed by sequencing. They were then subcloned into the *Hin*dIII and *Bam*HI sites of pBI 101 binary T - DNA vector or pBI 221 vector from which the CaMV 35S promoter had been removed (Clontech, Polo Alto, CA, U.S.A.). The recombinant pBI 101 vectors were transferred into *Agrobacterium tumefaciens* strain LBA4404 (pAL 4404) by triparental mating (Bevan, 1984).

Results

Induction of ECPs mRNA does not require de novo protein synthesis

As a first experiment, we investigated whether *de novo* protein synthesis is necessary for the ABA-induced expression of *ECP* genes by using an inhibitor of protein synthesis, cycloheximide. As shown in **Fig. 1**, the ABA-induction of *ECPs* gene expression was not inhibited by cycloheximide. On the other hand, the expression of *Dcem*, a carrot gene homologous to maize *Em* gene, was inhibited by cycloheximide and thus requires *de novo* protein synthesis. These results suggest that the induction of *ECPs* mRNA by ABA does not require *de novo* protein synthesis.

The promoter region of ECP genes contains some ACGT core motifs

As shown in **Fig. 2**, each promoter region of *ECP* genes contains some ACGT core motifs that were identified as conserved motifs in the ABA - responsive regulatory element. Especially, the *AtECP31* promoter region contains some ACGT core motifs located in the proximal (-300 to -61) and distal (-782 to -738) regions. A Sph box sequence (CATGCATG), which had been identified as a motif for mediating gene activation of maize antocyanin regulatory gene *C1* or of a number of seed storage protein genes (Hattori *et al.*, 1992, Baümlein *et al.*, 1992) was found in the *AtECP63* promoter region, from -201 to -193.

GUS activity after -250 bp upstream deletion of DcECP31 is similar to that with no deletion

Various *DcECP31* or *DcECP40* promoter deletion :: *GUS* constructs (**Fig. 3** and **4**) were introduced into carrot cells. Because the transformed callus may be a mixture of cells originating from separately transformed cells, we initiated cell lines originating from a single transformed cell by the following procedure. Somatic embryos were induced from putative transformed cells. Embryos were dissected vertically and a portion of dissected embryos were assayed histochemically for GUS activity. Embryogenic cells were induced again from the GUS-positive remainders, and the cells and somatic embryos induced from the cells were assayed again histochemically for GUS activity (**Fig. 5 A-D**). Embryogenic cells and somatic embryos at all developmental stages (globular, heart-shaped and torpedo-shaped stages) were GUS positive when they were initiated from the single somatic embryo containing constructs up to -250 bp upstream deletion of *DcECP31* promoter. GUS activity was decreased in the late-torpedo stage (**Fig. 5 E**), and could not be detected in mature leaf (data not shown). By contrast, the clones containing constructs which had the three putative

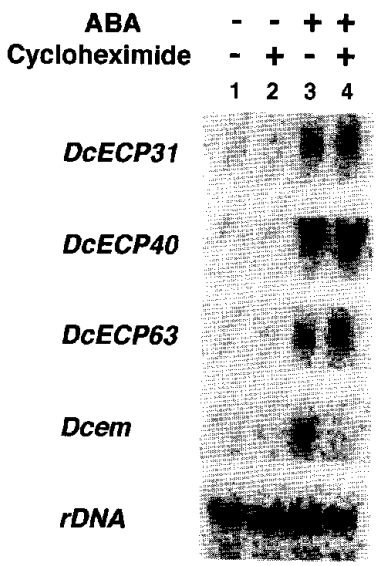

Fig. 1 Effects of cycloheximide, an inhibitor of protein synthesis, on ABA-induced accumulation of transcripts of *ECP* genes.
RNAs used were prepared from late torpedo stage somatic embryos that had been pre-incubated without (lanes 1 and 3) or with (lanes 2 and 4) 10 μM cycloheximide for 1 h and then treated without (lanes 1 and 2) or with (lanes 3 and 4) 50 μM ABA for 1 h. Twenty micrograms of total RNA were fractionated by gel electrophoresis, and transcripts were allowed to hybridize with the ^{32}P-labeled cDNA of *ECPs* as probes. ABA-inducible gene *Dcem*, a carrot gene homologous to maize *Em* gene, was used as a negative marker, of which the expression is inhibited by cycloheximide. The blot was reprobed with *18 S rDNA* to provide an internal standard.

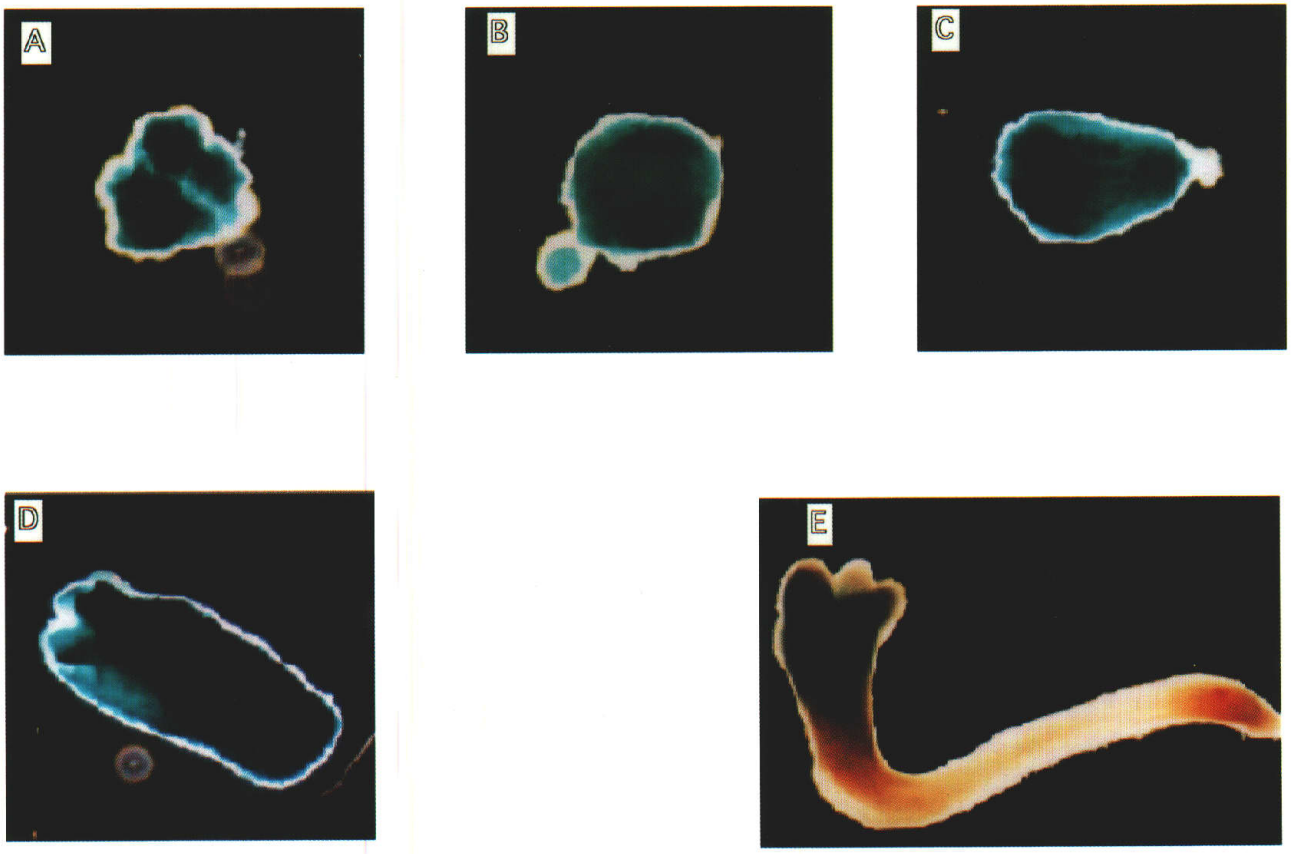

Fig. 5 Histochemical GUS assay in embryogenic cells and somatic embryos transformed with *DcECP31* promoter (−810 bp) : *GUS* chimeric gene.
Transformed somatic embryos which were prepared from single cells (see Materials and Methods) were assayed histochemically for GUS activity. Tissues were stained in X-gluc. Solution overnight as described in Materials and Methods. (A) embryogenic cell, (B) globular somatic embryo, (C) heart-shaped somatic embryo, (D) torpedo-shaped somatic embryo and (E) 20-day-old somatic embryo.

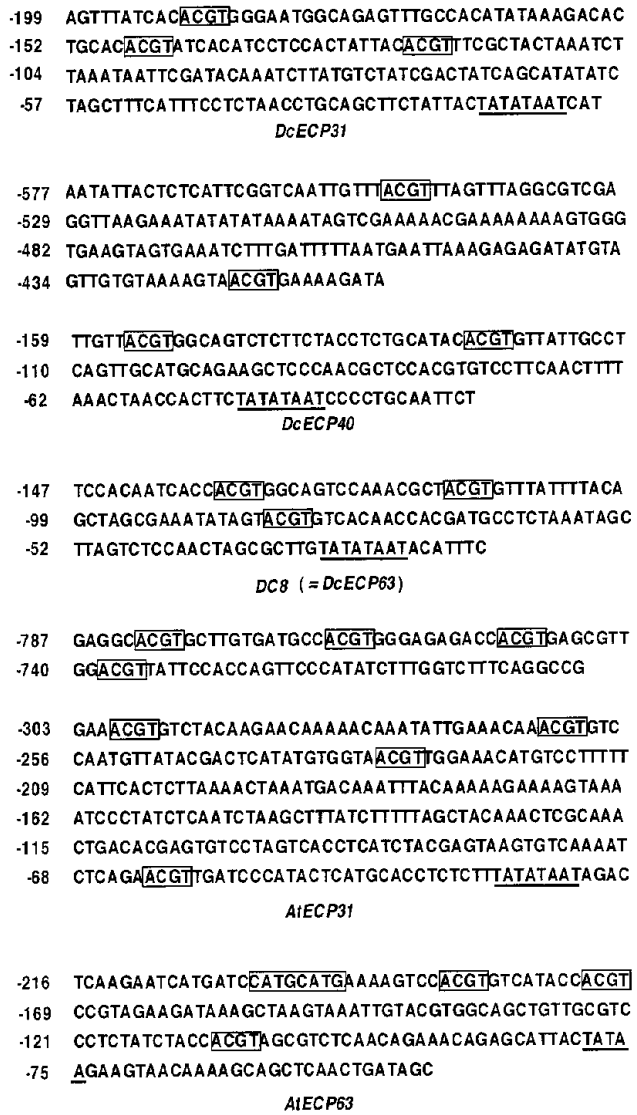

Fig. 2 Nucleotide sequences of some *ECP* and *DC8* gene promoters.
DC8 sequence was quoted from Franz *et al.* (1989) and it is same as *DcECP63* (Zhu *et al.*, 1997). Numbers are relative to the putative site of initiation of transcription determined from cDNA sequences. The positions of TATA box are underlined and ACGT core motifs are boxed. Nucleotide sequences of *ECP* gene promoters are deposited in the DDBJ under accession numbers AB050961 (DcECP31), AB050962 (DcECP40), AB050963(AtECP31) and AB050964 (AtECP-63).

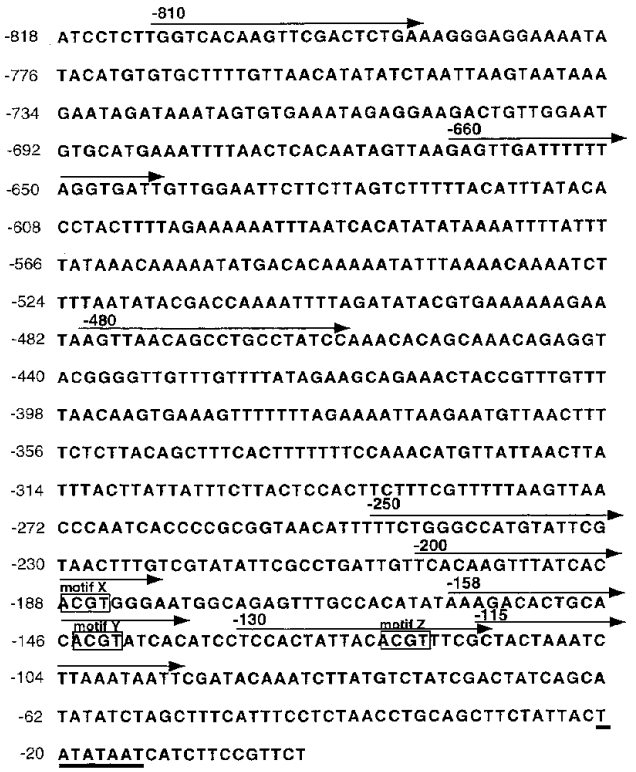

Fig. 3 Nucleotide sequence of the promoter region of *DcECP31*.
Numbers are relative to the site of initiation of transcription. The positions of TATA box are underlined and ACGT core motifs are boxed. The positions of oligonucleotide primers used for PCR amplication are indicated by arrows.

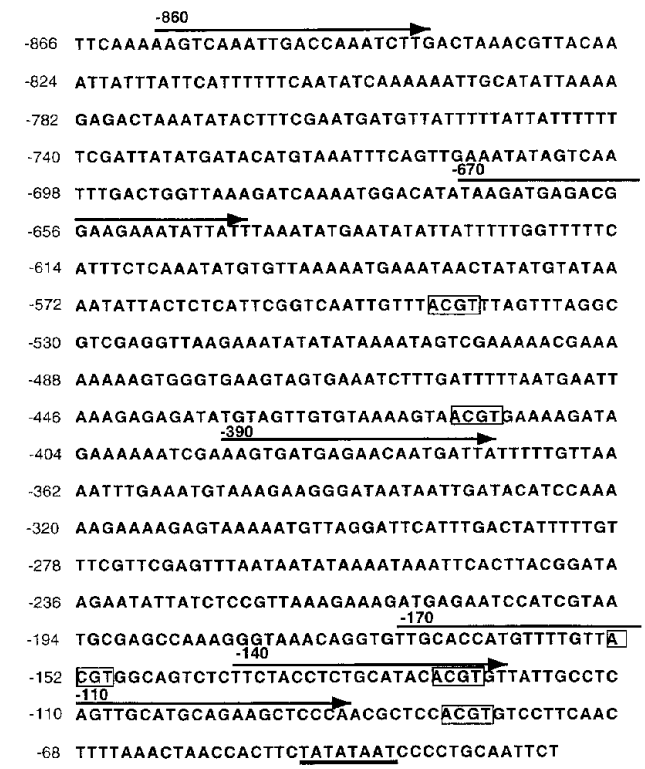

Fig. 4 Nucleotide sequence of the promoter region of *DcECP40*.
Numbers are relative to the site of initiation of transcription. The positions of TATA box are underlined and ACGT core motifs are boxed. The positions of oligonucleotide primers used for PCR amplication are indicated by arrows.

ABREs (motif X, Y and Z) deleted did not show any GUS activity in any developmental stage (data not shown). These results indicate that -250 bp upstream of the *DcECP31* promoter region is sufficient to confer *GUS* expression in embryogenic cells and somatic embryos at all developmental stages.

On the other hand, the pattern of GUS expression pattern with the region -860 bp upstream of *DcECP40* promoter was quite similar to that observed with the region -810 bp upstream of *DcECP31* promoter (data not shown).

The ability of the various promoter fragments to confer GUS expression in transformed cells was similar to that of transient expression

To determine the promoter regions required for *DcECP31* or *DcECP40* transcription, we constructed a number of 5' deletions, as shown in **Fig. 6** and **7**, and analyzed the constructs in transgenic cells that were treated for 24 h with 50 μM ABA and compared to those which were not treated. Our results showed that *DcECP31* promoter activity was retained in deletions up to -250, but was drastically decreased at -115 in which putative ABREs were deleted, as shown in **Fig. 6 A**. These results indicate that -250 bp upstream of *DcECP31* promoter region is sufficient to confer *GUS* expression. In addition to the analysis of transgenic cells, we performed transient assays using carrot protoplasts with the various deletion fragments of *DcECP31* promoter. Results of the transient assay were generally similar to those observed with transgenic cells (**Fig. 6 B**).

In the deletion analysis of *DcECP40* promoter with transient assay, the ABA -regulated expression of *GUS* was drastically reduced in the construct deleted between -670 ~ -390. Although GUS activity was reduced to 20% of the -860 construct, ABA inducible GUS activity was retained in region -140

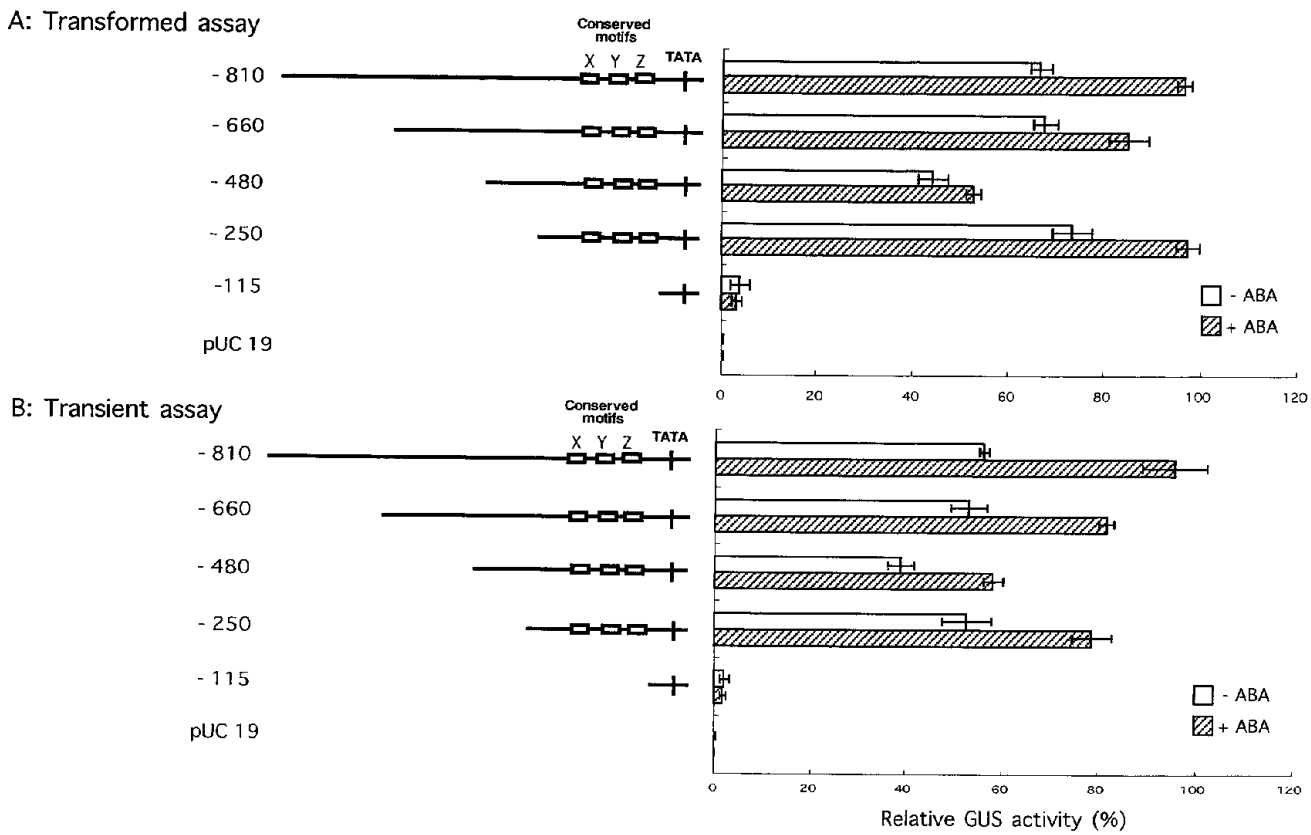

Fig. 6 Quantitative fluorometric GUS assay in transformed embryogenic cells (A) and transfected carrot protoplasts (B) on *DcECP31* promoter.
The diagram in the left shows the 5' unidirectional deletions of the *DcECP31* promoter. Deletion end points are indicated in base pairs from the relative transcription start site. pUC19 DNA was used as a negative control. GUS activity is given in relative units. (A) GUS activity was determined in tissue homogenates of each independent transgenic line cultured with (striped) or without (open) ABA (50 μM). Bar graphs represent the average GUS activity and standard deviation in three independent lines for each deletion construct. (B) GUS assays in a carrot protoplast transient expression system. Eight transfections with (striped) or without (open) ABA (50 μM) were conducted for each deletion construct. The average GUS activity and standard deviation are given for each construct.

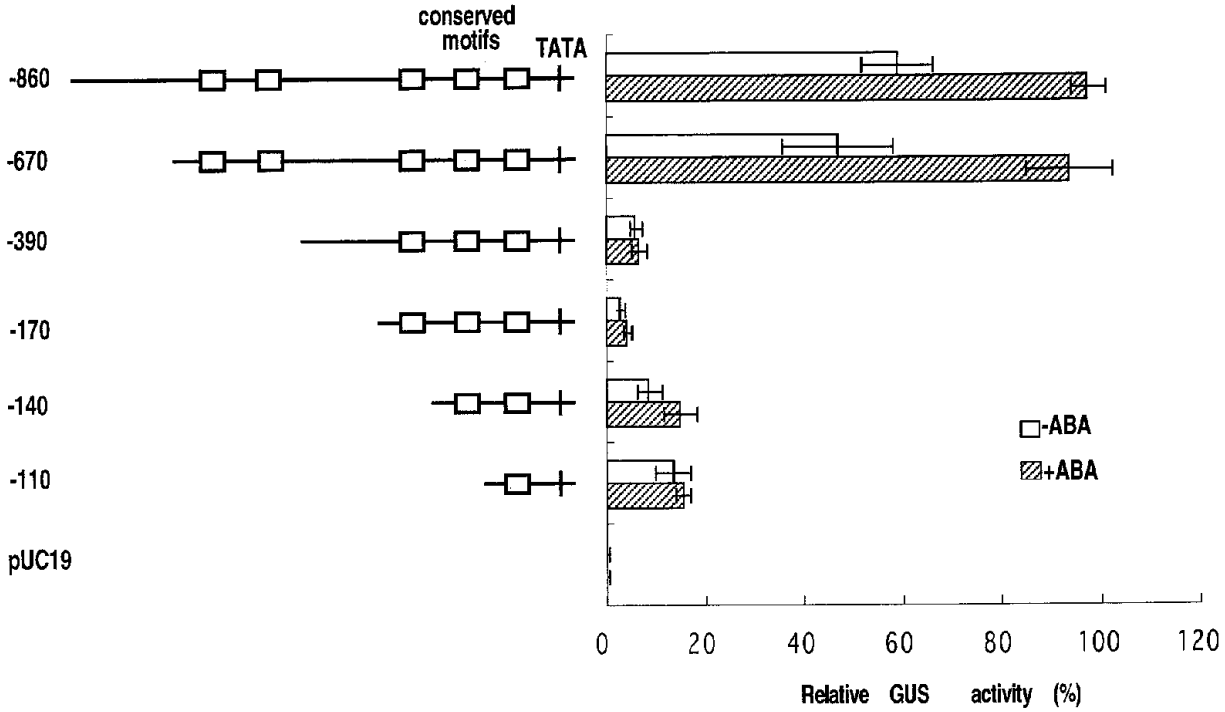

Fig. 7 Quantitative fluorometric GUS assay in transfected carrot protoplasts on *DcECP40* promoter.
The diagram in the left shows the 5' unidirectional deletions of the *DcECP40* promoter. Deletion end points are indicated in base pairs from the relative transcription start site. pUC19 DNA was used as a negative control. GUS activity is given in relative units. Three transfections with (striped) or without (open) ABA (50 μM) were conducted for each deletion construct. The average GUS activity and standard deviation are given for each construct.

upstream containing two putative ABREs (**Fig. 7**). These results suggest that *DcECP40* promoter contains some functional elements in the distal (-670 ~ -390) and proximal (-140 ~ -50) regions, which are essential for ABA-inducible expression. But, we also can not exclude the possibility that one of these regions might be an embryo-specific enhancer.

Discussion

Our previous studies have shown that the expression of *ECP* genes is embryo-specific and ABA-inducible (Kiyosue *et al.*, 1992a, 1993, Yang *et al.*, 1996, 1997, Zhu *et al.*, 1997). These characteristics led us to study the molecular mechanism of embryogenesis. As shown in **Fig. 1**, the induction of *DcECPs* mRNA by ABA does not require *de novo* protein synthesis. Furthermore, an embryo-specific transcription factor C-ABI3, a carrot homologue of *Arabidopsis* ABI3, involves in the control of the expression of *DcECP* genes (Shiota *et al.*, 1998). Thus, it is considered that C-ABI3 acts directly in the regulation of the expression of *ECP* genes. On the other hand, the induction of *Rab16* mRNA by ABA require *de novo* protein synthesis, while the induction of *Osem* mRNA depends only partially on such synthesis but wheat *Em* does not (Hattori *et al.*, 1995, Williamson and Quatrano, 1988). However, in the data presented here, the ABA - induced expression of *Dcem*, a carrot homologue of wheat *Em*, was inhibited by cycloheximide. This indicates that the ABA induction of the expression of these genes may be controlled by some different mechanisms as reported by Shinozaki *et al.* (2000).

In sequence comparison of the *ECP* promoters, all of the *ECP* promoters contain multiple, at least three ACGT-core motifs within the -300 positions. In the promoters, we found the ACGT-core motif or Sph box sequence (CATGCATG) which was identified as a motif for mediating gene activation of maize antocyanin regulatory gene *C1* or of a number of seed storage protein genes (Hattori *et al.*, 1992, Baümlein *et al.*, 1992). *DcECP63* which was found in our laboratory as an ECP (Zhu *et al.*, 1997) showed a 94 % similarity to *DC8* which is expressed in carrot seeds (Franz *et al.*, 1989). It seems that *DcECP63* is the same gene as *DC8*. In the *DC8* promoter, three ACGT-core motifs are located within -170 upstream (Franz *et al.*, 1989). ACGT-core motif of the *DcECP31* promoter (motif X, CACACGTGGG) and that of *AtECP63* (TGTA-

CGTGGC) resemble to Em1a (GACACGTGGC) of wheat *Em* and motif I (AGTACGTGGC) of rice *Rab16B*, respectively. These motifs function as essential parts of the ABRE complex (ABRC) in each promoter (Vasil *et al.*, 1995 and Ono *et al.*, 1996). From the sequence comparison of ABRC, the synergistic interaction between ACGT-core motifs is considered, and in fact, the two ACGT-core motifs in *DcECP31* promoters act as *cis*-elements (Ko *et al.*, in preparation).

Histochemical GUS assay with the *DcECP31* promoter deletions showed that GUS activity in -250 bp upstream deletion was similar to that with no deletion. On the other hand, in quantitative fluorometric assays for GUS activity with the *DcECP31* promoter deletions, the pattern of *GUS* expression in the transformed embryogenic cells was qualitatively similar to those observed with transient assay using embryogenic cell protoplasts (**Fig. 6**). However, responsiveness to exogenous ABA of the *DcECP31* promoter in the transformed embryogenic cells was not conspicuous. Because endogenous levels of ABA in carrot embryogenic cells are very high (Kiyosue *et al.*, 1992a and 1992b), it is thought that the amount of DcECP31 transcripts does not increase even by exogenous ABA treatment. In transient assay, ABA responsiveness was more conspicuous than that of transformed assay (**Fig. 6**). The report indicating that protoplasts do not synthesize ABA (Zeevaart *et al.*, 1988) may support this idea.

In some cases, stable and transient transformation analyses have led to different conclusions about the activity of the promoter region (Bustos *et al.*, 1991). Accordingly, to remove these experimental limitations, we compared both experimental systems and showed that there was no qualitative discrepancy between these systems in carrot. Thus we used the transient assay system for identification of the promoter region of *DcECP31*. In the promoter, -250 upstream region containing three ACGT motifs is sufficient for the ABA-regulated promoter activity (**Fig. 6 B**). However, on the deletion analysis of the *DcECP40* promoter with transient assay, we identified the distal (-670 ~ -390) and proximal (-140 ~ -50) regions, which are essential for ABA-induced expression (**Fig. 7**). The conserved motif is only ACGT-core sequence of -550 and -420 position (TTTACGTTTA and GTAACGTGAA) in the distal (-670 ~ -390) region. Although *DcECP31* promoter contains the ACGT core motifs at -496 position (TATACGTGAA), this region could not affect the ABA inducible promoter activity (**Fig. 6**). This suggests that there is a different regulation mechanism of gene expression between *DcECP31* and *DcECP40*, although they are controlled by the same transcription factor C-ABI3. It is known that different transcription factors control positively and / or negatively the expression of a *LEA* gene (Busk *et al.*, 1997, Chern *et al.*, 1996). Therefore we are carrying out more precise analysis of *DcECP* promoters.

Acknowledgements

This work was supported in part by a Grant-in-Aid from Ministry of Education, Science, Culture and Sports, Japan, by a Grant-in-Aid for " Research for the future " Program from the Japan Society for the Promotion of Science (JSPS-RFTF97L00601), by the Special Coordination Funds of the Science and Technology Agency of the Japanese Government, and by the Program for Promotion of Basic Research Activities for Innovative Biosciences. We wish to thank Dr. T. Hattori (Mie University), Dr. N. Fujii (Tohoku University) and Dr. H. Abe (JIRCAS) for their invaluable discussions and technical advice. Finally we would like to thank Dr. D. Macer of the University of Tsukuba for his comments on the manuscript.

References

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

Baker, J., Steele, C. and Dure III, L., 1988. Sequence and characterization of 5 LEA proteins and their genes from cotton. Plant Mol. Biol., **11** : 277 - 291.

Baümlein, H., Nagy, I., Villarroel, D., Inze, D. and Wobus, U., 1992. *Cis*-analysis of a seed protein gene promoter: the conservative RY repeat CATGCATG within the legumin box is essential for tissue specific expression of a legumin gene. Plant J., **2** : 233 - 239.

Bevan, M., 1984. Binary *Agrobacterium* vectors for plant transformation. Nucl. Acids Res., **12** : 8711 - 8721.

Bousquet, J., Simon, L. and Lalonde, M., 1990. DNA amplification from vegetative and sexual tissues of trees using polymerase chain reaction. Can. J. For. Res., **20** : 245 - 257.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., **72** : 248 - 254.

Busk, P. K., Jensen, A. B. and Pages, M., 1997. Regulatory elements *in vivo* in the promoter of the abscisic acid responsive gene *rab 17* from maize. Plant J., **11** :

1285 - 1295.

Busk, P.K. and Pagès, M., 1998. Regulation of abscisic acid-induced transcription. Plant Mol. Biol., **37** : 425 - 435.

Bustos, M. M., Begum. D., Kalkan, F. A., Battraw, M. J. and Hall, T. C., 1991. Positive and negative *cis*-acting DNA domains are required for spatial and temporal regulation of gene expression by a seed storage protein promoter. EMBO. J., **10** : 1469 - 1479.

Chern, M.-S., Bobb, A. J. and Bustos, M. M., 1996. The regulator of MAT2 (ROM2) protein binds to early maturation promoters and represses PvALF-activated transcription. Plant Cell, **8** : 305 - 321.

Donald, R.G.K. and Cashmore, A. R., 1990. Mutation of either G box or I box sequences profoundly affect expression of the *Arabidopsis rbcS-1A* promoter. EMBO J., **9** : 1717 - 1726.

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

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

Franz, G., Hatzopoulos, P., Jones, T. J., Karauss, M. and Sung, Z. R., 1989. Molecular and genetic analysis of an embryonic gene, *DC8* from *Daucus carota* L. Mol. Gen. Genet., **218** : 143 - 151.

Fujii, N. and Uchimiya, H., 1991. Conditions favorable for the somatic embryogenesis in carrot cell culture enhance expression of the *rolC* promoter-gus fusion gene. Plant Physiol., **95** : 238 - 241.

Galau, G. A., Hughes, D. W. and Dure III, L., 1986. Abscisic acid induction of cloned cotton late embryogenesis abundant (LEA) mRNAs. Plant Mol. Biol., **7** : 155 - 170.

Goodall, G., Wiebauer, K. and Filipowicz, W., 1990. Analysis of pre-mRNA processing in transfected plant protoplasts. Methods Enzymol., **181** : 148 - 155.

Hattori, T., Vasil, V., Rosenkrans, L., Hannah, L.C., McCarty, D. R. and Vasil, I.K., 1992. The *Viviparous-1* gene and abscisic acid activate the *C1* regulatory gene for anthocyanin biosynthesis during seed maturation in maize. Genes & Dev., **6** : 609 - 618.

Hattori, T., Terada, T. and Hamasuna, S., 1995. Regulation of the *Osem* gene by abscisic acid and the transcriptional activator VP1: analysis of *cis*-acting promoter elements required for regulation by abscisic acid and VP1. Plant J., **7** : 913 - 925.

Hobo, T., Asada, M., Kowyama, Y. and Hattori, T., 1999. ACGT - containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. Plant J., **19** : 679 - 689.

Jefferson, R.A., 1987. Assay for chimeric genes in plants: The GUS fusion system. Plant Mol. Biol. Rep., **5** : 387 - 405.

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

Kiyosue, T., Nakajima, M., Yamaguchi, I., Iida, Y., Satoh, S., Kamada, H. and Harada, H., 1992 (b) Endogenous level of abscisic acid in embryogenic cells and non-embryogenic cells and somatic embryos of carrot (*Daucus carota* L.). Biochem. Physiol. Pflanzen, **188** : 293 - 297.

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

Loake, G. J., Faktor, O., Lamb, C. J. and Dixon, R. A., 1992. Combination of H-box (CCTACCN7-CT) and G-box (CACGTG) cis-elements is necessary for seed-forward stimulation of a chalcone synthase promoter by the phenylpropanoid-pathway intermediate *p*-coumaric acid. Proc. Natl. Acad. Sci. USA., **89** : 9230 - 9234.

Liu, Z. B., Ulmasov, T., Shi, X., Hagen, G. and Guilfoyle, T. J., 1994. Soybean *GH3* promoter contains multiple auxin-inducible elements. Plant Cell, **6** : 645 - 657.

Maniatis, T., Fritsch, E. F. and Sambrook, J., 1982. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY.

McKendree, W. L., Jr, Paul, A.-L., DeLisle, A. J. and Ferl, R. J., 1990. *In vivo* and *in vitro* characterization of protein interactions with the dyad G-box of the *Arabidopsis Adh* gene. Plant Cell, **2** : 207 - 214.

Marcotte, W. R., Jr, Russell, S. H. and Quatrano, R. S., 1989. Abscisic acid-responsive sequences from the *Em* gene of Wheat. Plant Cell, **1** : 969 - 976.

Mason, H. S., DeWald, D. B. and Mullet, J. E., 1993. Identification of a methyl jasmonate-responsive domain in the soybean *vspB* promoter. Plant Cell, **5** : 241 - 251.

Mundy, J., Yamaguchi-Shinozaki, K. and Chua, N.-H., 1990. Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice *RAB* gene. Proc. Natl. Acad. Sci. USA , **87** : 1406 - 1410.

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

Ono, A., Izawa, T., Chua, N.-H. and Shimamoto, K., 1996. The *rab16B* promoter of rice contains two distinct abscisic acid-responsive elements. Plant Physiol., **112** : 483 - 491.

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

Shen, Q. and Ho, T.-H.D., 1995. Functional dissection of an abscisic acid (ABA)- inducible gene reveals two independent ABA-responsive complexes each containing a G-box and a novel *cis*-acting element. Plant Cell, **7** :

295 - 307.

Shen, Q., Zhang, P. and Ho, T.-H.D., 1996. Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. Plant Cell, **8** : 1107 - 1119.

Shinozaki, K. and Yamaguchi-Shinozaki, K., 2000. Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Current Opin. Plant Biol., **3** : 217-223.

Shiota, H., Satoh, R., Watabe, K., Harada, H. and 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.

Vasil, V., Marcotte, W. R. Jr., Rosenkrans, L., Cocciolone, S. M., Vasil, I.K., Quatrano, R. S. and McCarty, D. R., 1995. Overlap of Viviparous (VP1) and abscisic acid response elements in the *Em* promoter: G-box elements are sufficient but not necessary for VP1 transactivation. Plant Cell, **7** : 1511 - 1518.

Wagner, D. B., Furnier, G. R., Saghai - Maroof, M. A., Williams, S. M., Dancik, B. P. and Allard , R. W., 1987. Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc. Natl. Acad. Sci. USA., **84** : 2097 - 2100.

Weisshaar, B., Armstrong, G. A., Block, A., da Costa e Silva, O. and Hahlbrock, K., 1991. Light - inducible and constitutively expressed DNA - binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. EMBO J., **10** : 1777 - 1786.

West, M.A.L. and Harada, J. J., 1993. Embryogenesis in higher plants: An overview. Plant Cell, **5** : 1361 - 1259.

Williamson, J. D. and Quartrano, R. S., 1988. ABA - regulation of two classes of embryo - specific sequences in mature wheat embryos. Plant physiol., **86** : 208 - 215.

Yang, H., Saitou, T., Komeda, Y., Harada, H. and Kamada, H., 1996. Late embryogenesis abundant protein in *Arabidopsis thaliana* homologous to carrot ECP31. Physiol. Plant., **98** : 661 - 666.

Yang, H., Saitou, T., Komeda, Y., Harada, H. and Kamada, H., 1997. *Arabidopsis thaliana ECP63* encoding a LEA protein is located in chromosome 4. Gene, **184** : 83 - 88.

Zeevaart, JAD. and Creelman, RA., 1988. Metabolism and physiology of abscisic acid. Annu. Rev. Plant Physiol., **39** : 439-473.

Zhu, C., Kamada, H., Harada, H., He, M. and Hao, S., 1997. Isolation and characterization of a cDNA encoded an embryogenic cell protein 63 related to embryogenesis from carrot. Acta Botanica Sinica, **39** : 1091 - 1098.

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