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Analysis of *cis*-Regulatory Elements in Carrot Embryo - Specific and ABA-Responsive Gene, *DcECP31*

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Abstract

Expression of carrot embryogenic cell specific protein genes (*DcECPs*) that are late-embryogenesis abundant proteins is ABA-inducible and embryo-specific. The expression of some *DcECPs* is controlled by an embryo-specific transcription factor *C-ABI3*, a carrot homologue of *Arabidopsis ABI3*. In order to understand the molecular mechanisms regulating ABA-inducible and embryo-specific expression of *DcECP* genes, we carried out a detailed analysis of *DcECP31* promoter with deletion analysis, base-substitution mutagenesis and electrophoretic mobility shift assay (EMSA). We identified two important elements in the promoter of *DcECP31*, motif X (CACACGTGGG) and motif Y (CACACGTATC), which are sufficient for the embryo-specific and ABA-inducible promoter activity. By precise EMSA analysis, it was demonstrated that a nuclear protein which has sequence specific-binding ability binds to the flanking ACGT core motifs.

Introduction

We previously isolated and characterized some cDNAs for embryogenic cell proteins (ECPs) belonging to late embryogenesis abundant (LEA) protein group, from carrot, *DcECP31* (Kiyosue *et al.*, 1992), *DcECP40* (Kiyosue *et al.*, 1993), *DcECP45* (unpublished) and *DcECP63* (Zhu *et al.*, 1997), and *Arabidopsis, AtECP31* (Yang *et al.*, 1996) and *AtECP63* (Yang *et al.*, 1997). The expression pattern of *ECP* genes is ABA-inducible and embryo-specific. Furthermore, the ABA-inducible expression of carrot *ECPs* (*DcECPs*) is controlled by C-ABI3, a homologue of *Arabidopsis* ABI3, without *de novo* protein synthesis (Shiota *et al.*, 1998, Ko *et al.*, in preparation). These characteristics led us to study the molecular mechanisms of embryogenesis.

In our previous study (Ko *et al.*, in preparation), sequence comparison among some *ECP* gene promoters revealed that the promoters contained several conserved motifs, including ABA responsive element (ABRE)-like ACGT core motifs or Sph box, the motifs reported to mediate gene activation of maize antocyanin regulatory gene *C1* (Hattori *et al.*, 1992, Baümlein *et al.*, 1992). Generally, it has been considered that combinatorial interactions between the different ACGT core motifs or between the ACGT core motifs and other regulatory sequences in the promoter create the diversity in the regulatory mechanisms (Shen *et al.*, 1995, 1996, Vasil *et al.*, 1995, Hattori *et al.*, 1995).

In a previous study, we demonstrated that -250 bp upstream of *DcECP31* promoter contains three putative ABREs which are sufficient for the embryo-specific and ABA-inducible promoter activity (Ko *et al.*, in preparation). In this paper, we carried out a precise analysis of *DcECP31* promoter with deletion analysis, base-substitution mutagenesis and electrophoretic mobility shift assay (EMSA).

Materials and Methods

Plant materials and transient expression

Carrot (*Daucus carota* L. cv. US-Harumakigosun) seedlings were grown for 10 days at 25 ℃ with 16 h of light daily. Carrot embryogenic cells were obtained as described by Satoh *et al.* (1986).

Protoplast isolation and transient expression in protoplasts were performed as described elsewhere (Ko *et al.*, in preparation).

Construction of base-substituted promoter

The H1 wild-type fragment (-207 to -107) of the *DcECP31* promoter and its mutant fragments (M2, M3, M4 and M6) were prepared by PCR using a genomic clone of *DcECP31* as a template (Ko *et

al., in preparation) and were fused to *GUS* gene (-115 *DcECP31-GUS* or -130 *DcECP31-GUS* constructs). The primers used for the amplification of the H1 fragment were 5'-GTTTATCA-CACGTGGGAATGGC and GTAGCGAAACGTG-TAATAGTGG-3'. The primers used for the amplification of the mutant fragments were 5'-GGATCCTTATCACCCCCGGGAATG and GTA-GCGAAACGTGTAATAGTGG - 3' (M2), 5' - AAGCTTATCACACGTGGGAATGGCAGAGTT-TG and AAGCTTAGGATGTGATGGGGGTGCA-GTGTCTT-3' (M3), 5'-GTTTATCACACGTGG-GAATGGC and AAGCTTAGCGAAGGGGGTA-ATAGTGGAGGATG-3' (M4), 5'-GTTTATCA-CACGTGGGAATGGC and AAGCTTAGCGAA-GGGGGTAATAGTGGAGGATGTGATGGGGG-TGCAGT - 3' (M6). The amplified fragments were subcloned into the PCR II vectors using a TA Cloning Kit (Invitrogen), and confirmed by sequencing. The *Hin*dIII-*Xba* I fragments were then subcloned into the *Hin*dIII and *Xba* I sites of pBI 221 vector to substitute the CaMV 35S promoter with *DcECP31* promoter (Clontech). The structure of the fusion construct was confirmed by sequencing.

Preparation of nuclei and nuclear extracts

Nuclei were prepared by the method described by Masuda *et al.* (1991) with slight modifications. Suspension-cultured carrot embryogenic cells (20 m*l* packed cell volume at 100 x *g*) were obtained at day 6 of subculture. The cells were washed twice with buffer A [0.5 M sorbitol, 25 mM MES (pH 5.6), 5 mM $CaCl_2$] and suspended in 3 volumes of enzyme solution containing 2% Driselase (Kyowa Hakko, Tokyo) in buffer A. The suspension was incubated at 25 ℃ for 30 min with gentle agitation. After the partial digestion of cell walls, the cells were washed three times with buffer A by resuspension and centrifugation at 200 x*g* for 5 min. The pellet was carefully resuspended on ice in 60 m*l* of buffer B [25 mM MES (pH 5.6), 5 mM $MgCl_2$, 10 mM KCl, 0.4% ($w \cdot v^{-1}$) triton X-100, 0.35 M sucrose, 30% ($v \cdot v^{-1}$) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT)]. The suspension was then homogenized in a teflon homogenizer. The homogenates were strained through two layers of Miracloth (Calbiochem, La Jolla, CA) and finally filtered through a series of nylon meshes with decreasing pore size (44 μm, 32 μm and 22 μm).

Nuclear extracts were prepared by the method described by Green *et al.* (1987) with minor modifications. The above filtrates were pelleted by centrifugation at 3,500 x *g* for 10 min and the pellet was gently resuspended on ice in 40 m*l* of nuclear lysis buffer [110 mM KCl, 15 mM HEPES / KOH (pH 7.6), 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF]. One tenth volume of 4 M $(NH_4)_2SO_4$ was added and incubated at 4 ℃ for 30 min with gentle agitation. The mixture was centrifuged at 36,000 x*g* for 60 min in a Beckman Ti 70 rotor. Proteins were precipitated by addition of 0.3 g $\cdot ml^{-1}$ freshly ground $(NH_4)_2SO_4$ with stirring for 30 min at 4 ℃. Following centrifugation at 10,000 x*g* for 15 min, the pellet was resuspended in 1 m*l* of nuclear extract buffer [NEB ; 25 mM Hepes / KOH (pH 7.6), 40 mM KCl, 0.1 mM EDTA, 10 % ($v \cdot v^{-1}$) glycerol, 1 mM DTT, 1 mM PMSF] and dialyzed against NEB without PMSF. Insoluble material was removed by centrifugation at 25,000 x*g* for 30 min. Extracts were aliquoted, frozen in liquid nitrogen and stored at -80 ℃. Protein concentration was determined by the method of Bradford *et al.* (1976) using BSA as a standard.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed essentially as described by Yanai *et al.* (1996). Different DNA fragments (45 bp) that corresponded to each motif's flanking regions were used. Single stranded oligonucleotides were annealed, and the double stranded DNA probe (100 ng) was end-labeled using[γ -^{32}P]ATP and T_4 polynucleotide kinase. Eight micrograms of nuclear extracts were incubated with 1 μg poly (dIdC) (Boehringer Mannheim) as nonspecific competitor and end - labeled oligo nucleotide (0.3 ng, approximately 10,000 cpm) at 20 ℃ for 20 min in the presence or absence of the unlabeled oligonucleotides as a specific competitor. Binding reactions (10 μ l) were carried out in a solution containing 40 mM KCl, 25 mM HEPES / KOH (pH 7.6), 1 mM EDTA, 5% ($v \cdot v^{-1}$) glycerol, 0.5 mM DTT. Reaction products were then separated electrophoretically in 4.5 % nondenaturing polyacrylamide gel (9 : 1 acrylamide / bisacrylamide) containing 1 x TBE [90 mM Tris-HCl (pH 8.0), 89 mM boric acid, 2 mM EDTA] that had been pre-electrophoresed for 20 min. Following electrophoresis (~ 10 V cm^{-1} with constant recirculation at 4 ℃), gels were dried and autoradiographed with an intensifying screen.

Results

Motif X is essential for the ABA-regulated activity of the DcECP31 promoter

To determine the promoter regions required for *DcECP31* transcription, we constructed a deletion series for the -250bp upstream and performed transient assays (**Fig. 1**). The ABA-regulated ex-

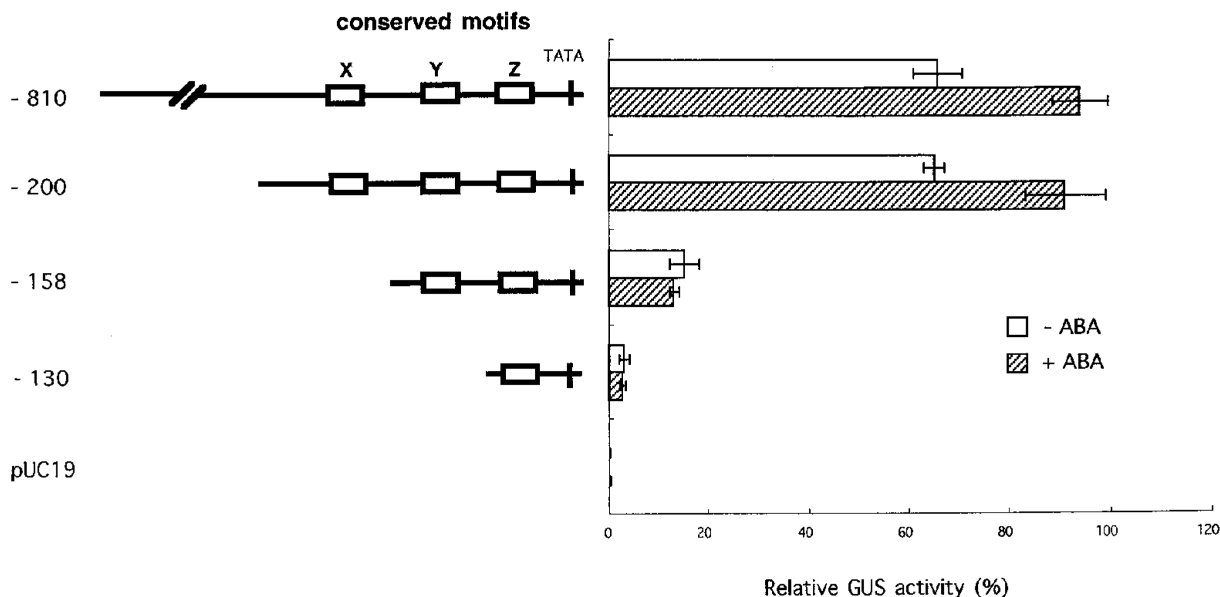

Fig. 1 Quantitative fluorometric GUS assay in transfected carrot protoplasts.
The diagram on the left shows the 5' unidirectional deletions of the *DcECP31* promoter. Deletion end points are indicated in base pairs from the relative transcription start site. pUC19 DNA was used as a negative control. GUS activity is given in relative units. 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.

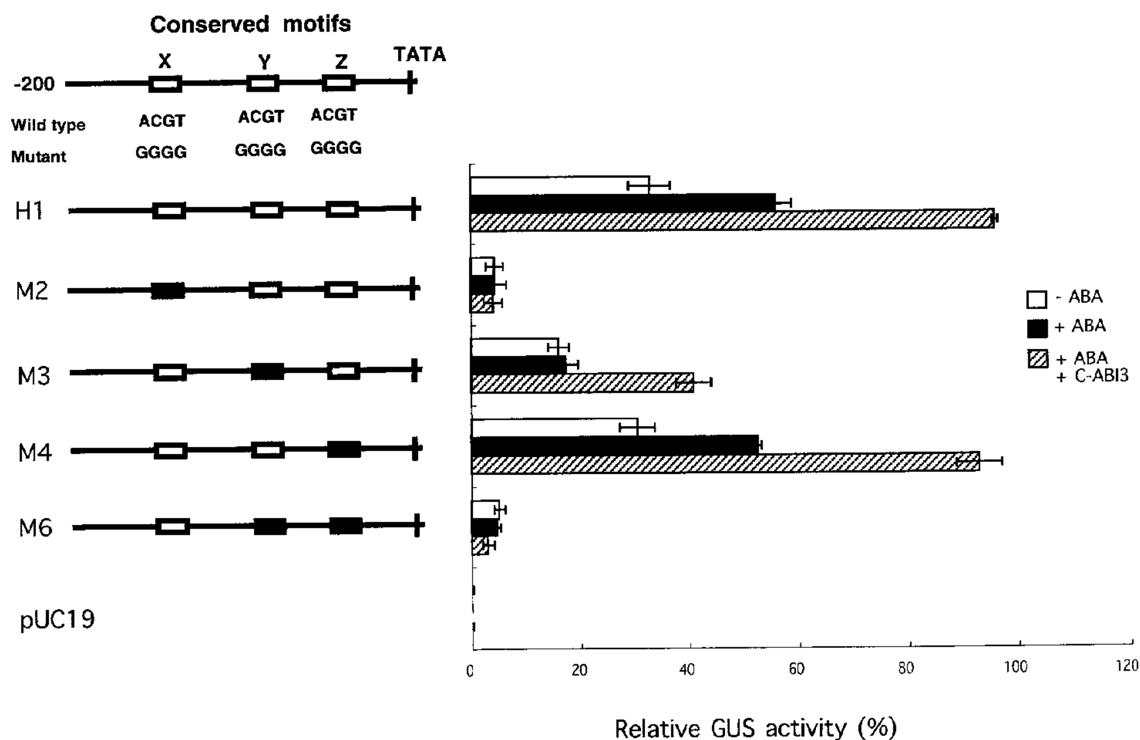

Fig. 2 Effects of base - substitution mutagenesis in the -200 promoter region.
Three motifs and mutated sequence are represented by open boxes and closed boxes, respectively, on the left. Transactivation of a GUS reporter gene fused to various base-substitution derivatives in the -200 promoter region by over expression of C-ABI3 was determined by cotransfection with *35S :: C-ABI3* and reporter plasmids into carrot protoplasts. Five transfections with ABA (50 μM, black), with both ABA (50 μM) and C-ABI3 (transactivation ; striped) or without both treatments (open) were conducted for each construct. Bars represent the average GUS activity and standard deviation for each construct.

pression of GUS was retained in deletions up to -200 which contains the three putative ABREs (Motif X, Y and Z). But the ABA-regulated expression was drastically reduced at -158 which lacks the motif X (CACACCTGGG). These results indicate that the -200 bp sequence of the *DcECP31* promoter is sufficient for the ABA-regulated expression of *GUS* and that motif X is also essential for the ABA-regulated promoter activity. However, we could not exclude the possibility that motif X cooperates with other motifs, so we further analyzed the -200 bp sequence with base-substitution mutagenesis.

Combination of motif X (CACACGTGGG) with motif Y (CACACGTATC) is most important for the ABA-regulated activity of the DcECP31 promoter

To detect the motifs functioning for ABRE, we constructed the mutant series which were base substituted in ACGT core region and performed transient assays (**Fig. 2**). The most notable reduction of the GUS activity occurred when motif X was mutated (M2). This result indicates that motif X is critical for ABA-regulated activity of the *DcECP31* promoter. On the other hand, the mutation of both the motif Y and Z (M6) greatly reduced the GUS activity even in a combination treatment with ABA and C-ABI3. In contrast, the mutation at the motif Z (M4) alone had no significant effect. These results indicate that the motif X is certainly essential for the promoter activity, but motif X alone is not functionally sufficient. Thus, it is concluded that the motif X functions in combination with motif Y.

Three conserved motifs of DcECP31 promoter bind to nuclear factors in a sequence-specific manner

To determine whether the *DcECP31* -200 promoter region, which is sufficient for the ABA-regulated promoter activity, binds specifically to nuclear proteins in embryogenic cell extracts, we performed EMSA. When the -200 fragment was used as a probe, a specific shifted band could not be detected. Thus, dissected DNA fragments of -200 were used (**Fig. 3A**). Different double-stranded oligonucleotides were synthesized corresponding to each motif, or to the base-substituted motifs' flanking region. Incubation of the nuclear extracts with probes produced one major binding complex with a similar pattern in each lane (**Fig. 3B, lanes 2, 7** and **12**). The specificity of the binding was shown by a competition experiment. The specific competitors, corresponding to probe DNA, competed with the labeled probe for binding in extracts. Non-specific competitors including base-substituted motifs in each ACGT core motifs did not compete for binding to the probe (**Fig. 3B, lanes 5, 10** and **15**). These results indicate that a DNA binding nuclear protein bound to the flanking ACGT core motifs in a specific way.

Discussion

Our previous studies have shown that -250 bp upstream of the *DcECP31* promoter containing three putative ABREs is sufficient for the embryo-specific and ABA-inducible promoter activity (Ko *et al.*, in preparation). In this study, we conducted

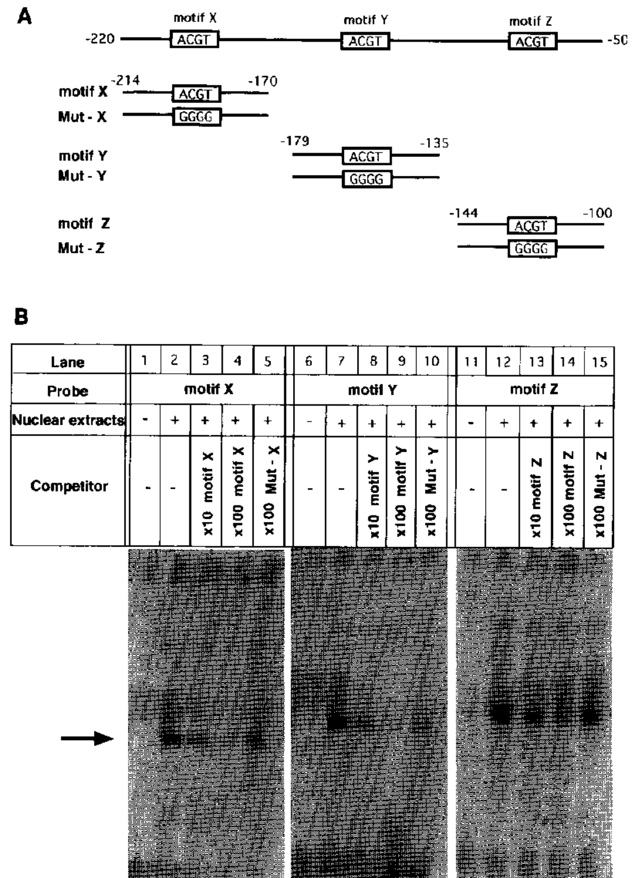

Fig. 3 Interaction of nuclear factors with the *DcECP31* promoter region
(A) Physical map of a part of the *DcECP31* promoter. The top represents the region containing three motifs. The maps below are the probe and base-substituted motifs showing nonspecific competitors used for electrophoretic mobility shift assay [corresponding to each column in (B)]. (B)Electrophoretic mobility shift assay. The double stranded oligonucleotides were end-labeled with T_4 polynucleotide kinase using [γ -^{32}P] ATP. Eight micrograms of nuclear extracts were incubated with 0.3 ng of ^{32}P-labeled probes. In competition assay, 10 - or 100 - fold molar excess unlabeled oligonucleotides were added. Positions of shifted bands are indicated by arrows.

further deletion analysis in the -250 bp region and clarified that motif X (CACACGTGGG) is essential for the promoter activity (**Fig. 1**). Motif X resembles Em1a (GACACGTGGC) of Wheat *Em*, that has been identified as an essential part of the ABRE complex and typical strong ABRE (YACGTGGC) as well (Vasil *et al.*, 1995, Busk and Pagès, 1998). Generally, these motifs are active in combination with other sequences but not alone (Shen *et al.*, 1995, 1996, Hattori *et al.*, 1995, Hobo *et al.*, 1999 a). Therefore, since we could not exclude the possibility that motif X cooperates with other motifs such as Em1a (GACACGTGGC) and Em1b (GACACGTGCC) ABRE complex of Wheat *Em* (Guiltinan *et al.*, 1990), we used base-substitution mutagenesis to clarify this. As shown in **Fig. 2**, the most notable reduction of promoter activity was found when motif X was mutated (M2). Motif X is most active in combination with motif Y (CAC ACGTATC) but not alone. In contrast, half of wild type promoter activity remained (H1) when the motif Y had been mutated and the motif Z was native (M3). This suggests that motif Z (TACACGTTTC) may be involved in the promoter activity but is redundant. These results indicate that motif X and Y function together. Combinatorial effects between the functional motifs are well known. Thus, it is likely that some kinds of patterns in the combinatorial interaction between functional motifs are involved in the ABA-based regulation mechanisms. One interaction is between two ACGT-containing motifs and the other is between ACGT- containing motifs and ABRE like motifs. Interaction between Em1a (GACACGTGGC) and Em1b (GACACGTGCC) of wheat *Em* is a good example of the former case (Guiltinan *et al.*, 1990). Combination between motif X and Y described in this paper may also belong to this case. The latter case, interaction between ACGT-containing motifs and ABRE like motifs, is well illustrated in the promoter of rice *Rab16B* which contains two separate functional ABRE, ACGT-containing motif I (AGTACGTGGC) and ABRE-like motif III (GCCGCGTGGC) (Ono *et al.*, 1996). In many cases, ABRE-like motifs that are functional coupling elements show similar structural characteristics, with a single base change at the *A* position of the ACGT core (Hobo *et al.*, 1999 b).

ACGT-core motif is a typical and important sequence for the binding of plant basic leucine-zipper (bZIP) transcription factor, except in some cases as TGACTCA binding opaque-2 (de Pater *et al.*, 1994) or ACACNNG binding factor in *Dc3* promoter (DPBF) (Kim *et al.*, 1997). However, plant bZIP exhibits a relaxed DNA-binding specificity for DNA sequence motifs containing an ACGT core (Izawa *et al.*, 1993). By the EMSA data in this work, we demonstrate that a nuclear protein binds specifically to ACGT core motif corresponding to the base-substitution in each ACGT core motif (**Fig. 3**). The nuclear protein which binds to each motif may be the same kind of bZIP and if it is true, bZIP has wide DNA-binding specificity.

In this paper, we identified the functional ABREs in the promoters of the ABA-inducible and embryo-specific gene, *DcECP31*. It was reported that transgenic carrot plants showing ectopic expression of *C-ABI3* have the ability to accumulate mRNA of *ECP* genes in the mature leaves when they were treated with ABA (Shiota *et al.*, 1998). This observation shows that embryo-specificity of *DcECP* gene expression is restricted by C-ABI3.

C-ABI3 has a conserved B3 domain (Shiota *et al.*, 1998) and the B3 domain is known to bind to Sph box (Suzuki *et al.*, 1997). The B3 domain of C-ABI3 did not bind directly to *DcECP31* promoter (unpublished data). These results support the concept that VP1/ABI3 factor, including C-ABI3, may function as a coactivator that interacts with the G-box via protein-protein contacts with G box-specific DNA binding proteins (Vasil *et al.*, 1995). Recently, Hobo *et al.* (1999 b) identified rice bZIP, TRAB1, that interacts with OsVP1, and showed that TRAB1 belongs to the class of ABA-inducible and embryo-specific transcription activators. Now we are conducting experiments to identify the carrot bZIP which binds to motif X and motif Y of *DcECP31* promoter and C-ABI3.

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References

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.

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

de Pater, S., Katagiri, F., Kijne, J. and Chua, N.-H., 1994. bZIP proteins bind to a palindromic sequence without an ACGT core located in a seed-specific element of the pea lectin promoter. Plant J., **6** : 133 - 140.

Green, P. J., Kay, S. A. and Chua, N.-H., 1987. Sequence-specific interactions of a pea nuclear factor with light responsive elements upstream of the rbcS-3A gene. EMBO J., **6** : 2543 - 2549.

Guiltinan, M. J., Marcotte, W. R. Jr. and Quatrano, R. S., 1990. A plant leucine zipper protein that recognizes an abscisic acid response element. Science, **250** : 267 - 271.

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 (a). ACGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. Plant J., **19** : 679 - 689.

Hobo, T., Kowyama, Y. and Hattori, T., 1999 (b). A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription. Proc. Natl. Acad. Sci. USA, **96** : 15348 - 15353.

Izawa, T., Foster, R. and Chua, N.-H., 1993. Plant bZIP protein DNA binding specificity. J. Mol. Biol., **230** : 1131 - 1144.

Kim, S. Y., Chung, H. J. and Thomas, T. L., 1997. Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the *Dc3* promoter using a modified yeast one-hybrid system. Plant J., **11** : 1237 - 1251.

Kiyosue, T., Yamaguchi-Shinozaki, K., Shinozaki, K., Higashi, K., Satoh, S., Kamada, H. and 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. 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.

Masuda, K., Takahashi, S., Nomura, K. and Inoue, M., 1991. A simple procedure for the isolation of pure nuclei from carrot embryos in synchronized cultures. Plant Cell Rep., **10** : 329 - 333.

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.

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.

Suzuki, M., Kao, C.Y. and McCarty, D. R., 1997. The conserved B3 domain of viviparous1 has a cooperative DNA binding activity. Plant Cell, **9** : 799 - 807.

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.

Yanai, K., Nibu, Y., Murakami, K. and Fukamizu, A., 1996. A *cis*-acting element located between TATA box and transcription initiation site is critical in response to regulatory sequences in human angiotensinogen gene. J. Biol. Chem., **271** : 15981 - 15986.

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.

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.