Available online at www.sciencedirect.com
ScienceDirect
Plant Physiology and Biochemistry 46 (2008) 550–558
Plant Physiology and Biochemistry
www.elsevier.com/locate/plaphy

Research article

A carrot G-box binding factor-type basic region/leucine zipper factor DcBZ1 is involved in abscisic acid signal transduction in somatic embryogenesis

Hajime Shiota [a,*], Sukmin Ko [b], Shinko Wada [c], Claudia Tomiko Otsu [c], Ichiro Tanaka [a], Hiroshi Kamada [c]

[a] *International Graduate School of Arts and Sciences, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan*
[b] *Eugentech Research Center, Korea Research Institute of Bioscience and Biotechnology, 52 Oun-dong, Yusong, Daejon 305-333, South Korea*
[c] *Gene Research Center, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan*

Received 14 August 2007
Available online 2 March 2008

Abstract

Carrot (*Daucus carota*) somatic embryogenesis has been extensively used as an experimental system for studying embryogenesis. In maturing zygotic embryos, abscisic acid (ABA) is involved in acquisition of desiccation tolerance and dormancy. On the other hand, somatic embryos contain low levels of endogenous ABA and show desiccation intolerance and lack dormancy, but tolerance and dormancy can be induced by exogenous application of ABA. In ABA-treated carrot embryos, some ABA-inducible genes are expressed. We isolated the *Daucus carota bZIP1* (*DcBZ1*) gene encoding a G-box binding factor-type basic region/leucine zipper (GBF-type bZIP) factor from carrot somatic embryos. The expression of *DcBZ1* was detected in embryogenic cells, non-embryogenic cells, somatic embryos, developing seeds, seedlings, and true leaves. Notably, higher expression was detected in embryogenic cells, true leaves, and seedlings. The expression of *DcBZ1* increased in seedlings and true leaves after ABA treatment, whereas expression was not affected by differences in light conditions. During the development of zygotic and somatic embryos, increased expression of *DcBZ1* was commonly detected in the later phase of development. The recombinant DcBZ1 protein showed specific binding activity to the two ABA-responsive element-like motifs (motif X and motif Y) in the promoter region of the carrot ABA-inducible gene according to results from an electrophoretic mobility shift assay. Our findings suggest that the carrot GBF-type bZIP factor, DcBZ1, is involved in ABA signal transduction in embryogenesis and other vegetative tissues.
© 2008 Elsevier Masson SAS. All rights reserved.

Keywords: Abscisic acid; Basic region/leucine zipper factor; *Daucus carota*; Seed maturation; Somatic embryogenesis

1. Introduction

Somatic embryogenesis has been extensively investigated as an experimental system for examinations of the development of zygotic embryogenesis. In the carrot system, numerous physiological, biochemical, and molecular biological studies have been done, although somatic embryogenesis can be achieved in several other plant species [1–4]. Somatic embryos develop into seedlings after the expression of morphological changes similar to those that occur in zygotic embryos (passage through a globular stage, heart-shaped stage, and torpedo-shaped stage). However, some differences in physiological and biochemical features exist between somatic and zygotic embryos [1,2,5].

In developing seeds of several higher plants, desiccation tolerance and dormancy are induced by the effect of the phytohormone abscisic acid (ABA), which is synthesized during the seed maturation phase [6]. In contrast, the endogenous ABA

Abbreviations: ABA, abscisic acid; ABRE, ABA-responsive element; DAF, days after flowering; bZIP, basic region/leucine zipper; CAISE, carrot ABA-induced in somatic embryos; ECP, embryogenic cell protein.

* Corresponding author. Tel.: +81 45 787 2318; fax: +81 45 787 2413.
E-mail address: hshiota@yokohama-cu.ac.jp (H. Shiota).

0981-9428/$ - see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2008.02.010

content of carrot somatic embryos is low throughout development, and desiccation tolerance and dormancy are not observed [7]. Desiccation tolerance can be induced in somatic embryos of alfalfa and carrot by exogenous ABA treatment [8,9]. In ABA-treated carrot somatic embryos, as well as in maturing seeds, several ABA-induced genes [*embryogenic cell protein* (*ECP*) and *carrot ABA-induced in somatic embryos* (*CAISE*) genes] are expressed, and they are suggested to be involved in induction of desiccation tolerance and dormancy [10].

Generally, several transcriptional factors strictly regulate gene expression in numerous physiological phenomena. Basic region/leucine zipper (bZIP) factors are known as a group of transcriptional factors, and several kinds of bZIP factors exist in the plant genome [11]. The basic region in the N-terminal region binds to DNA sequences with an ACGT core, and the leucine zipper is involved in protein-protein interactions [11]. In the plant genome, 75 kinds of bZIP factors have been identified and are classified into ten groups (groups A–I and S) according to similarity of the basic region and additional conserved motifs [11]. Interestingly, among these groups, group G bZIP factors [G-box binding factor (GBF)-type bZIP factors] are involved in different physiological phenomena, such as light and phytohormone signal transduction [11].

It has been reported that group G bZIP factors are involved in light signal transduction in *Arabidopsis* (GBF1–GBF3) and parsley (CPRF1–CPRF7) [12–14]. They regulate light-dependent gene expression by binding to the so-called G-box (CC**ACGT**GG) with an ACGT core in light-responsive promoters. It has also been indicated that some kinds of GBF-type bZIP factors are regulated at the transcriptional level by light level. The parsley *CPRF1* is up-regulated under high-light conditions, whereas the *Arabidopsis GBF1* is down-regulated in darkness [12,15]. In general, however, the light-dependent functional activity of GBF-type bZIP factors is modulated in a phosphorylation-dependent manner, without transcriptional regulation of genes encoding the bZIP factors [14,16].

It has also been reported that group G bZIP factors are involved in ABA signal transduction in wheat (EmBP1), bean (ROM1 and ROM2), rice (OSBZ8), and maize (ZmBZ-1 and EmBP-2) [17–21]. The ABA-responsive element (ABRE; Py**ACGT**GGC) containing the ACGT core sequence in the promoter region of numerous ABA-inducible genes is involved in regulation of ABA-induced expression as a *cis*-acting element [6,22]. GBF-type bZIP factors mentioned above regulate ABA-induced transcription by binding to ABRE [17–21].

Additionally, group A bZIP factors are known to regulate transcription of ABA-inducible genes by binding to ABRE [11]. According to research using mutants or transgenic plants, it has been reported that ABI5, ABFs, ABRFs, and DPBFs of *Arabidopsis* and TRAB1 of rice are the main bZIP factors involved in ABA signal transduction during seed maturation or environmental stress responses [23–28]. Thus, complicated systems in which the different groups of bZIP factors (groups A and G) are involved might function in plant cells.

In the carrot system, many ABA-inducible genes (*ECP*s and *CAISE*s) are expressed in ABA-treated somatic embryos [10]. In the case of *DcECP31*, it has been reported that ABRE-like sequences, motif X (CAC**ACGT**GGG) and motif Y (CAC**ACGT**ATC) in the promoter region, are involved in regulation of ABA-induced gene expression [29,30]. According to a transit assay and gain-of-function analysis, both motif X and motif Y are essential for regulation of ABA-induced gene expression, and motif X functions as an enhancer-like element while motif Y participates in ABA responsiveness [29,30]. Certain bZIP factors are predicted to interact with these motifs as *trans*-acting factors.

In this report, we describe the isolation of a carrot gene encoding a GBF-type bZIP factor, *DcBZ1*, from carrot somatic embryos. *DcBZ1* showed very high homology to parsley *CPRF5*. We also indicate the possible involvement of DcBZ1 in ABA signal transduction during ABA-induced gene expression in the carrot system by analyzing gene expression profiles and biochemical properties.

2. Materials and methods

2.1. Plant materials

Carrot (*Daucus carota* L. cv. US-Harumakigosun) seedlings were grown for 10 days at 25 °C with 16 h of light (approximately 40 μmol photons $m^{-2} s^{-1}$) daily. Carrot embryogenic cells, non-embryogenic cells, and somatic embryos were obtained as described by Satoh et al. [31]. True leaves were collected from plants that had been grown for 2 months at 25 °C with the illumination regime mentioned above. True leaves grown under other light conditions (24 h of light, 24 h of dark, or 6 h of dark after 16 h of light) were also prepared. ABA treatment of somatic embryos (3.7 μM ABA for 24 h), non-embryogenic cells (10 μM ABA for 24 h), seedlings (100 μM ABA for 24 h), and true leaves (100 μM ABA for 8 h) was performed as described by Shiota et al. [10]. Carrot seeds and fruits were harvested from carrot (*Daucus carota* L. cv. Yohmeigosun) plants grown in the experimental field of Takii Seed Co., Ltd. (Ushiku, Ibaraki, Japan) on various days after flowering (DAF). These were stored at −80 °C prior to use.

2.2. Isolation of RNA from carrot cells and reverse transcription PCR (RT-PCR)

Total RNA was isolated from embryogenic cells, non-embryogenic cells, somatic embryos, seedlings, true leaves, and developing seeds by the phenol/SDS method [32]. The total RNA was treated with RNase-free DNase I. First-strand cDNA mixtures were prepared from 1 μg of total RNA with oligo (dT_{15}) primer and AMV reverse transcriptase XL (Takara Bio, Ohtsu, Japan). The cDNA synthesized from 30 ng of total RNA and 12 pmol of the PCR primers was used for the PCR analysis.

To isolate partial cDNA fragments of carrot genes for GBF-type bZIP factors, RT-PCR was performed using mRNA from somatic embryos. We constructed PCR primers GBFS1 (5′-GATGAACGAGAGCTCAAGC-3′) and GBFA4 (5′-TTCG CATTCGGCCTGCTTC-3′) corresponding to the nucleotide sequence of the basic region of GBF-type bZIP factors from

several plant species. DNA was amplified under the following conditions: 1 cycle at 94 °C for 2 min; and 40 cycles at 94 °C for 40 s, 58 °C for 1 min, and 72 °C for 3 min. The PCR amplified products were subcloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA).

To analyze expression of the carrot genes, specific PCR primers were designed (BZ43-3S: 5′-GTTAACACATAAA ATTTACTTG-3′, BZ43-3A: 5′-GGTTATAAAATGAGACG TC-3′). PCR amplification was performed using these primers and cDNA from embryogenic cells, non-embryogenic cells, somatic embryos, seedlings, true leaves, and developing seeds as templates under the following conditions: 1 cycle at 94 °C for 2 min; and 25 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. The primer pairs yielded a strong single band for the gene (246 bp).

2.3. Screening of the cDNA library and sequencing of cDNA clones

cDNA screening was performed by plaque hybridization using a cDNA library prepared from carrot somatic embryos [33]. Approximately 15,000 phages were screened by hybridization with a [^{32}P]-labeled amplified cDNA fragment, CG5-1, as the probe. The lambda gt10 phage DNAs were isolated and digested with NotI or EcoRI. The inserted fragments in phage DNAs were subcloned into the pBluescript II SK^{+} vector (Stratagene, La Jolla, CA, USA) and sequenced according to the protocol of the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

2.4. Quantitative PCR analysis

The cDNA synthesized from 25 ng of total RNA was used for Real time RT-PCR analysis. Real time RT-PCR was performed on a Smart Cycler II (Cepheid, Sunnyvale, CA, USA) using the SYBR Premix Ex Taq kit (Takara Bio) according to the manufacturer's instructions. Values were normalized with the amplification rate of *Ubiquitin* as a constitutively expressed internal control. Three determinations were performed for each sample.

2.5. Expression and purification of the recombinant protein by the Escherichia coli expression system

To produce the recombinant protein from *Escherichia coli*, a DNA construct was made in the pGEX-4T-2 vector (GE Healthcare, Piscataway, NJ, USA). The open-reading frame of the carrot gene with additional EcoRI and XhoI sites at each end was obtained by PCR amplification using BZ43ECO5 (5′-GGAATTCCCATGGGGGCTGGGGAA-3′) and BZ43XHO3 (5′-CCGCTCGAGTACTGTCACCTCCGTC-3′) as forward and reverse primers, respectively. After restriction enzyme digestion, the fragment of cDNA was subcloned into the pGEX-4T-2 plasmid as a translational fusion to glutathione *S*-transferase (GST).

Expression and purification of recombinant fusion proteins were performed according to the instructions from GE Healthcare. The recombinant fusion protein was induced by isopropyl-beta-D-thiogalactopyranoside (IPTG; a final concentration of 0.1 mM) at 37 °C for 3 h after a 30-min pre-incubation. Extraction and purification of the fusion protein were performed using Glutathione-Sepharose (GE Healthcare) according to the methods described by Torres-Schumann et al. [34]. Thrombin (GE Healthcare, 0.02 units μl^{-1}) cleavage was achieved by incubation at 22 °C for 40 min, and then the thrombin was removed by Benzamidine-Sepharose (GE Healthcare).

2.6. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed according to the protocol of the DIG gel shift kit (Roche, Mannheim, Germany). The partial DNA fragment of the promoter region of *DcECP31* (accession no. AB050961) containing two ABRE-like motifs, motif X and motif Y, was used [29]. The double-stranded DNA probe was end-labeled with digoxigenin (DIG) using terminal transferase. Fifty nanograms of purified recombinant protein from an *E. coli* expression system were incubated with end-labeled oligonucleotide (12 ng) at 20 °C for 15 min in the presence or absence of the unlabeled oligonucleotides as a specific competitor. Binding reactions were carried out in a reaction solution (Roche) containing 1 µg of poly (dIdC) as a non-specific competitor. Reaction products were separated electrophoretically in 6% non-denaturing polyacrylamide gel [acrylamide/bisacrylamide (9/1, v/v) containing 0.5 × TBE]. After electrophoresis (8 V cm^{-1} at 4 °C), DIG-labeled nucleotides were electrotransferred to nylon filters (Biodyne Plus; Pall BioSupport, East Hills, NY, USA) and then detected with a DIG detection system (Roche).

3. Results

3.1. Isolation of the gene encoding a GBF-type bZIP factor from carrot somatic embryos

To isolate partial cDNA fragments of carrot genes for the GBF-type bZIP factors, PCR primers corresponding to the conserved basic region of GBF-type bZIP factors in several plant species were constructed. An amplified DNA fragment, whose size was approximately 90 bp, was obtained after PCR using the PCR primers mentioned above and cDNA from carrot somatic embryos (data not shown). The amplified cDNA fragment (CG5-1, 89 bp) showed high homology to genes for the GBF-type bZIP factors of several plant species (data not shown). Six positive clones were isolated from the cDNA library (approximately 15,000 phages) prepared from carrot somatic embryos by hybridization screening using CG5-1 as a probe. These clones encoded the same cDNA (data not shown). One of the clones, clone 4-3 (1354 bp long), contained an open-reading frame encoding 353 amino acids, and was named *Daucus carota bZIP1* (*DcBZ1*). The sequence was deposited in the EMBL/GenBank/DDBJ database (accession number AB334113).

The predicted amino acid sequences revealed that *DcBZ1* showed structural features typical of GBF-type bZIP factors

(Group G bZIP factors) (Fig. 1) [11]. The deduced amino acid sequence showed high homology to the GBF-type bZIP factors of several plant species: parsley CPRF1, CPRF3, CPRF4a, and CPRF5 [12–14]; kidney bean ROM1 and ROM2 [18–19]; wheat HBP1a [35]; rice OSBZ8 [20]; and *Arabidopsis* GBF1, GBF2, and GBF3 [15] in the basic region, leucine zipper, and additional conserved domains (Fig. 2). Notably, carrot *DcBZ1* had very high homology (93%) to parsley *CPRF5* at an open-reading frame. *DcBZ1* was likely a carrot homolog to *CPRF5*, because the nucleotide sequence of *DcBZ1* showed 88% homology to *CPRF5* in the 3′ untranslated region (data not shown).

3.2. Expression of DcBZ1 in the carrot system

To specifically detect *DcBZ1* transcription, RT-PCR and quantitative RT-PCR were performed using the specific PCR primers corresponding to the 3′ untranslated region. The single DNA fragment (246 bp) was amplified after PCR (Figs. 3 and 4). The expression of *DcBZ1* was detected in embryogenic cells, non-embryogenic cells, somatic embryos, mature seeds (65 DAF), seedlings, and true leaves (Figs.3A,B and 4A,B).

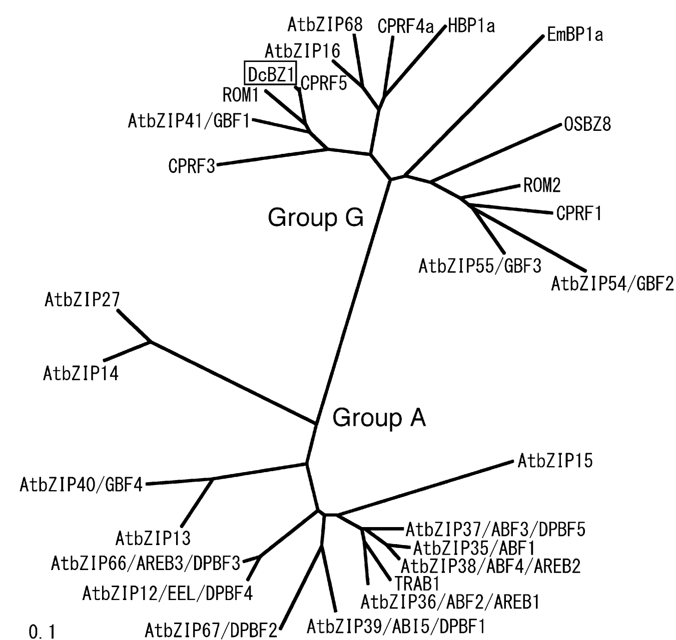

Fig. 1. A phylogenetic tree for the predicted amino acid sequences of group A and group G of bZIP factors in several plant species. An unrooted neighbor-joining (N-J) tree was constructed with ClustalW using predicted sequences from the database. The accession numbers are as follows: AB334113 for DcBZ1, X58575 for CPRF1, X58576 for CPRF3, Y10809 for CPRF4a, AJ292743 for CPRF5, U57389 for ROM1, U41817 for ROM2, AB023288 for TRAB1, U42208 for OSBZ8, U07933 for EmBP1a, X56781 for HBP1a, AF334209 for AtbZIP12/EEL/DPBF4, BN000023 for AtbZIP13, BN000021 for AtbZIP14, AJ419599 for AtbZIP15, BT022055 for AtbZIP16, BN000022 for AtbZIP27, AF093544 for AtbZIP35/ABF1, AF093545 for AtbZIP36/ABF2/AREB1, AF093546 for AtbZIP37/ABF3/DPBF5, AF093547 for AtbZIP38/ABF4/AREB2, AF334206 for AtbZIP39/ABI5/DPBF1, U01823 for AtbZIP40/GBF4, X63894 for AtbZIP41/GBF1, AF053228 for AtbZIP54/GBF2, U51850 for AtbZIP55/GBF3, AB017162 for AtbZIP66/AREB3/DPBF3, AJ419600 for AtbZIP67/DPBF2, and BT004147 for AtbZIP68.

Notably, higher expression in embryogenic cells and true leaves and slightly higher expression in seedlings were detected. The expression levels of *DcBZ1* in somatic embryos and non-embryogenic cells is likely to be sufficient, when compared to that of *Ubiquitin* (Fig. 3B), whereas the expression in somatic embryos and non-embryogenic cells was lower than that in embryogenic cells and true leaves after RT-PCR (Fig. 3A). Northern blot analysis also detected *DcBZ1* expression in somatic embryos and non-embryogenic cells (data not shown).

During seed development, expression of *DcBZ1* was detected throughout the globular (14 DAF), heart-shaped (17 DAF), and torpedo-shaped (26–65 DAF) developmental stages (Fig. 4A,B) [33]. The level of expression increased in the later phase of seed development (late torpedo-shaped stage, 47–65 DAF) (Fig. 4A,B). In the case of somatic embryos, the expression of *DcBZ1* was detected in globular embryos (5-day-old embryos, G5), heart-shaped embryos (7-day-old embryos, H7), and torpedo-shaped embryos (10-day-old embryos, T10; 14-day-old embryos, T14), indicating that *DcBZ1* was expressed throughout the developmental stages of embryos (Fig. 4C,D) [33]. As well as during seed development, expression increased in the later phase of somatic embryo development (late torpedo-shaped stage, T14) (Fig. 4C,D). It was also found that *DcBZ1* may be expressed not only in zygotic embryos but also in tissues that surround zygotic embryos (endosperms and seed coats), because the level of expression in developing seeds was higher than that in developing somatic embryos (Fig. 4).

GBF-type bZIP factors have been reported to be involved in light and phytohormone (ABA) signal transduction [11]. Therefore, we analyzed the effects of illumination or ABA on expression of the *DcBZ1* gene. No change in expression levels was observed in true leaves grown in 24 h light (L.24 h), 24 h darkness (D.24 h), or 6 h darkness after 18 h light (D.6 h) (Fig. 3C,D). On the other hand, expression increased markedly in seedlings and true leaves after ABA treatment (Fig. 3A,B). In somatic embryos and non-embryogenic cells, slightly increased expression was detected after ABA treatment (Fig. 3A,B).

3.3. Binding activity of DcBZ1 to the promoter region of DcECP31

The recombinant GST-DcBZ1 fusion protein (approximately 75 kDa) was induced by 0.1 mM of IPTG at 37 °C for 3 h (Fig. 5, lane 2). After the purification procedure, the recombinant full-length DcBZ1 proteins (approximately 45 kDa) were successfully obtained (Fig. 5, lane 3).

The 65-bp partial DNA fragment (E31XY) containing motif X (CAC**ACGT**GGG) and motif Y (CAC**ACGT**ATC) in the promoter region of *DcECP31* was end-labeled with DIG (Fig. 6A). EMSAs were performed using the recombinant DcBZ1 proteins and the DIG-labeled E31XY as a probe. The three shifted bands (two stronger and larger bands, and a weaker and smaller band) indicating specific DNA-protein complexes were detected after incubation (Fig. 6B, lane 2), indicating that the recombinant DcBZ1 proteins produce multiple

```
A
DcBZ1          25: PPSYPDWSSSMQAYYGAGAAP-AFYASTVAPPTPHPYMWGGQHPLMPPYGTPIPYPVYPPGG--MYAH-PSIATNPSMV : 99
CPRF5          25: PPSYPDWSSSMQAYYGAGAAP-AFYASTVAPPTPHPYMWGGQHPLMPPYGTPIPYPVYPPGG--MYAH-PSIATNPSIV : 99
ROM1           24: VPAYPDWSSSMQAYYAPGAAPPPFFASTVASPTPHPYLWGSQHPLMPPYGTPVPYPALYPPG-SIYAHHPSMAVTPSVV :101
AtbZIP41/GBF1  24: PTPYPDWQNSMQAYYGGGGSPNPFFPSPVGSPSPHPYMWGAQHHMMPPYGTPVPYPAMYPPG-AVYAH-PSMPMPPNSG :100
CPRF3          25: TTPFPDLLSSMQAYYGG-AAPAAFYASTVGSPSPHPYMWRNQHRFILPYGIPMQYPALFLPG-GIFTH-PIVPTDPNLA :101
AtbZIP16       39: MAT-PDWSGFQAYS---PMPPPHGYVASSPQ--PHPYMWGVQH-MMPPYGTPPHPYVAMYPPGGMYAH-PSMPPGSYPY :109
AtbZIP68       43: VAVTQDWSGFQAYS---PMPP-HGYVASSPQ--PHPYMWGVQH-MMPPYGTPPHPYVTMYPPGGMYAH-PSLPPGSYPY :113
CPRF4a         34: GPVTPDWSGFQAYS---PMPPHGYMASSPQA--PHPYMWGVQH-MMPPYGTPPHPYV-MYPHGGIYAH-PSMPPGSYPF :104
HBP1a          30: APVYPEWPGFQGYP---AMPPHGFFPPPVAAGQAHPYMWGPQH-MVPPYGTPPPPYM-MYPPGTVYAH-PTAP-GVHPF :101
AtbZIP54/GBF2  28: HVYHHDWAAMQAYYGPRVGIPQYYNSNLAPGHAPPPYMWASPSPMMAPYGAPYPPFCPPGG---VYAH-PGVQMGSQPQ :102
AtbZIP55/GBF3  28: HVYP-DWAAMQAYYGPRVAMPPYYNSAMAASGHPPPYMWWNPQHMMSPSGAPYAAVYPHGGG--VYAH-PGIPMGSLPQ :102
CPRF1          30: HVYP-DWAAMQAYYGPRVALPPYFNPAVASGQSPHPYMWGPPQPVMPPYGVPYAALYAHGG---VYAH-PGVPLAASPM :103
ROM2           31: HVYP-DWAAMQYYG-PRVNIPPYFNSAVASGHAPHPYMWGPPQPMMPPYGPPYAAFYSPGG---VYTH-PAVAIGPHSH :103
OSBZ8          25: LSPYLDWSNMQAYYGPGILPPTFFSPGIAAGHTPPPFILGPQPLVPSAFGKPYAAIYPPGG---AFSH-PFMPLMVSPL : 99
EmBP1a         24: AQAHAEWAASMHAYYAAAASAAGHPYAAWPLPPQAQQHGLVAAGPGAAYGAPVPFPMYHHPAAAYYAHAHASMAAGVP- :101
                        :              .                          . * *             ::*

B
                                                    #      #      #      #      #      #      #
DcBZ1         257: NQDERELKRQKRKQSNRESARRSRLRKQAECEELQGRVETLNNENRSLRDELKRLSEECEKVTSENNTIKEELIRVY :333
CPRF5         257: NQDERELKRQKRKQSNRESARRSRLRKQAECEELQGRVETLNNENRSLRDELKRLSEECEKVTSENNTIKEELIRVY :333
ROM1          254: IQDERELKRQKRKQSNRESARRSRLRKQAECEDLQKRVETLGSENRTLREELQRLSEECEKLTSENSSIKEELERMC :330
AtbZIP41/GBF1 217: VKDERELKRQKRKQSNRESARRSRLRKQAECEQLQQRVESLSNENQSLRDELQRLSSECDKLKSENNSIQDELQRVL :293
CPRF3         191: VNDERELKRQRRKQSNRESARRSRLRKQAKSDELQERLDNLSKENRILRKNLQRISEACAEVTSENHSIKEELLRNY :267
AtbZIP16      300: LQDDRELKRQRRKQSNRESARRSRLRKQAECDELAQRAEVLNEENTNLRAEINKLKSQCEELTTENTSLKDQLSLFP :376
AtbZIP68      290: VSDEREIKRQRRKQSNRESARRSRLRKQAECDELAQRAEVLNGENSSLRAEINKLKSQYEELLAENSSLKNKFSSAP :366
CPRF4a        304: LQDERELKRQKRKQSNRESARRSRLRKQAECDELAQRAEALKEENASLRAELSRFRTEYEKIVAQNEVLKEKIREVP :380
HBP1a         247: QWDERELKKQKRKLSNRESARRSRLRKQAECEELGQRAEALKSENSSLRIELDRIKKEYEELLSKNTSLKAKLGESG :323
AtbZIP54/GBF2 244: PWNEKEVKREKRKQSNRESARRSRLRKQAETEQLSVKVDALVAENMSLRSKLGQLNNESEKLRLENEAILDQLKAQA :320
AtbZIP55/GBF3 254: LQNERELKRERRKQSNRESARRSRLRKQAETEELARKVEALTAENMALRSELNQLNEKSDKLRGANATLLDKLKCSE :330
CPRF1         264: LHNDRDLKRERRKQSNRESARRSRLRKQAEAEELAIKVDSLTAENMALKAEINRLTLTAEKLTNDNSRLLEVMKNAQ :340
ROM2          276: LQNERELKRERRKQSNRESARRSRLRKQAETEELARKVEMLTAENVSLKSEITQLTEGSEQMRMENSALREKLRNTQ :352
OSBZ8         220: TKDDKESKRERRKQSNRESARRSRLRKQAETEELARKVELLTAENTSLRREISRLTESSKKLRLENSALMEKLTETG :296
EmBP1a        248: QMDERELKRERRKQSNRESARRSRLRKQQECEELAQKVSELTAANGTLRSELDQLKKDCKTMETENKQLMGKILSHD :324
                     :::: *:::** ************** : ::*  : . *   *  *: :: ::      :   *  :   :
```

Fig. 2. Comparison of the amino acid sequences of group G bZIP factors in several plant species. Multiple alignment of the amino acid sequences of group G bZIP factors in several plant species. The alignment was constructed with ClustalW. (A) The regions containing the proline-rich activation domain and the additional conserved motifs (boxed) are indicated. (B) The basic regions (boxed) and the leucine zippers (#) are indicated. Common amino acid residues are indicated with asterisks. Similar amino acid residues are indicated with colons or periods.

complexes. The proline-rich domain of soybean SGBFs and *Arabidopsis* GBFs have also been reported to have high-binding activities that form multiple complexes [36].

The weaker shifted bands were detected after incubation with unlabeled E31XY as a competitor, depending on the concentration (Fig. 6B, lane 3–4). Therefore, we performed a competition assay using E31mm containing the mutated motif X (CAC**GGGG**GGG) and mutated motif Y (CAC**GGGG**ATC), E31mY containing mutated motif X (CAC**GGGG**GGG) and normal motif Y (CAC**ACGT**ATC), or E31Xm containing normal motif X (CAC**ACGT**GGG) and mutated motif Y (CAC**GGGG**ATC) as competitors (Fig. 6A). The weaker shifted bands were detected after incubation with unlabeled E31mY or E31Xm as competitors (Fig. 6B, lane 6–7). On the other hand, the shifted bands were detected strongly after incubation with unlabeled E31mm as a competitor (Fig. 6B, lane 5). These results indicate that the binding of DcBZ1 protein and E31XY is in competition with E31XY, E31mY, or E31Xm, but not with E31mm. Thus, DcBZ1 binds specifically to both motif X and motif Y.

4. Discussion

In this study, we isolated a gene for a GBF-type bZIP factor (Group G bZIP factor) from carrot somatic embryos, and proposed that DcBZ1 is involved in transcriptional regulation of ABA-inducible genes. This is the first report of the function of bZIP factors, not only in somatic embryogenesis but also in carrot cells.

Generally, it has been reported that GBF-type bZIP factors are involved in light signal transduction [11]. Carrot DcBZ1 was predicted to be involved in light signal transduction because the expression of *DcBZ1* was higher in seedling and true leaves, which were grown under light regimes. The expression of *DcBZ1* in true leaves was not affected by differences in light conditions. It has been suggested that the expression of the genes for GBF-type bZIP factors is not regulated by light, and that the functional activities of the factors are regulated by light-dependent phosphorylation [14,16]; however, parsley CPRF1 and *Arabidopsis* GBF1 are reportedly regulated transcriptionally by light [12,15]. In parsley, *CPRF5*, a putative homolog to carrot *DcBZ1*, is not regulated transcriptionally by light, although CPRF5 may be involved in ultraviolet or blue light signal transduction [14]. As in CPRF5, DcBZ1 may be involved in light signal transduction without regulation of its transcription. On the other hand, it is likely that DcBZ1 may have some roles apart from light signal transduction, because *DcBZ1* was expressed in embryogenic cells, somatic embryos, and non-embryogenic cells cultured in darkness.

It has also been reported that GBF-type bZIP factors are involved in ABA signal transduction [11]. The expression of *DcBZ1* was increased markedly by ABA treatment in

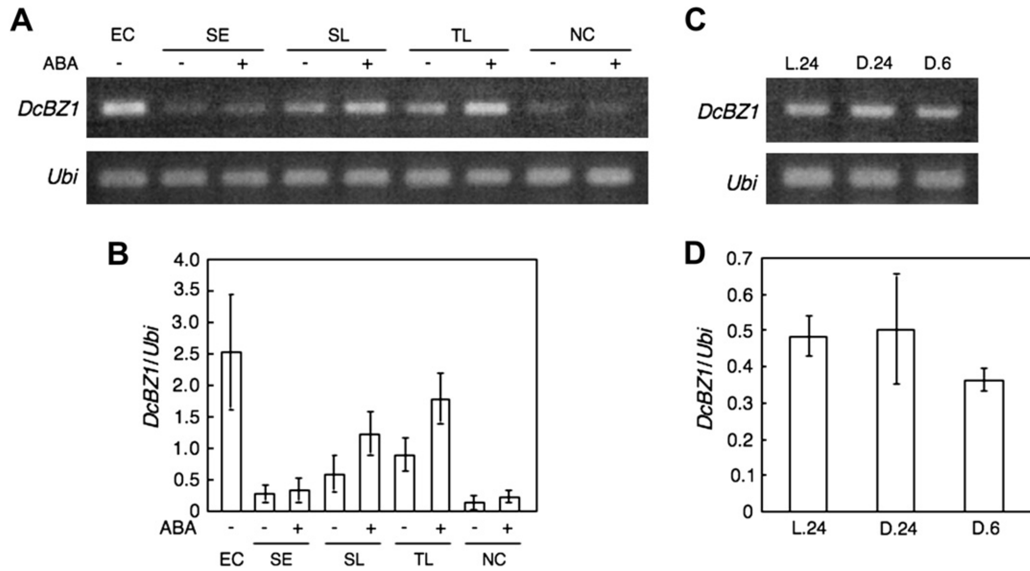

Fig. 3. Expression of *DcBZ1* in several carrot tissues and cells. The expression of *DcBZ1* in carrot tissues and cells was analyzed by RT-PCR (A, C) or quantitative RT-PCR (B, D). (A, B) mRNAs from embryogenic cells (EC), torpedo-shaped somatic embryos (SE), seedlings (SL), true leaves (TL), and non-embryogenic cells (NC) were analyzed. ABA treatment was performed under the following conditions: 3.7 μM (SE), 10 μM (NC), or 100 μM (SL) of ABA for 24 h, and 100 μM ABA for 8 h (TL). (C, D) mRNAs from true leaves grown with 24 h light (L.24), 24 h dark (D.24), or 6 h dark after 18 h light (D.6) were analyzed. RT-PCR was performed using an equal volume of cDNA as template DNA and specific primers for *DcBZ1*. The products resulting from 25 PCR cycles were fractionated on a 1.5% agarose gel and visualized using ethidium bromide staining. *Ubiquitin* (*Ubi*) was used as a transcriptional control. The data shown are representative of multiple experiments. On quantitative RT-PCR, values were normalized with the amplification rate of *Ubi* as a constitutively expressed internal control. Data are presented as mean values ± SE of three independent measurements.

seedlings and true leaves. This indicates that DcBZ1 may be involved in ABA signal transduction. This is supported by the higher expression of *DcBZ1* in embryogenic cells, which contain high levels of endogenous ABA and express several ABA-inducible genes [7,10]. In *Arabidopsis* and rice, it has been reported that group A bZIP factors are the main factors for ABA signal transduction [11,37]. Some, *ABI5/DPBF1*, *TRAB1*, *ABF2/AREB1*, *ABF3/DPBF5*, *ABF4/AREB2*, and *EEL/DPBF4*, are up-regulated transcriptionally by ABA [24,28,38,39]. In the case of group G bZIP factors, the

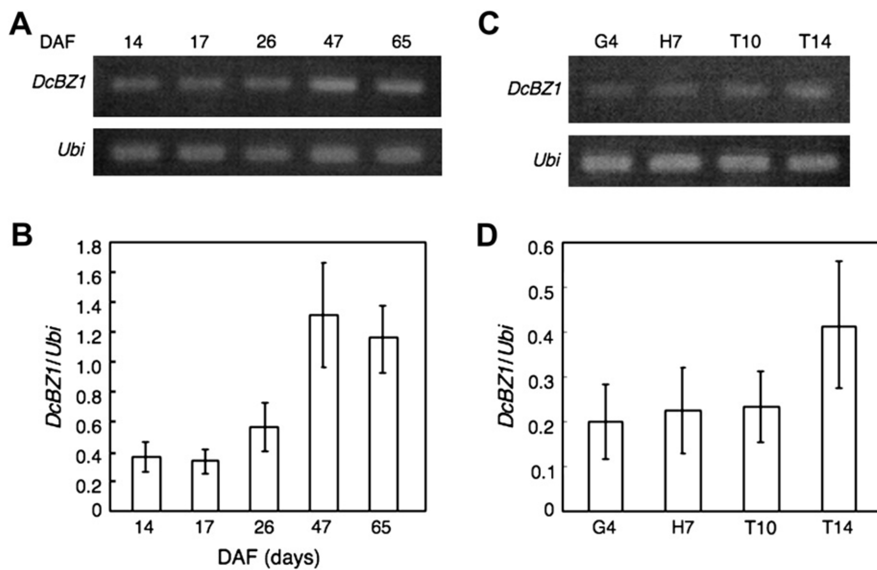

Fig. 4. Expression of *DcBZ1* during seed and somatic embryo development. The expression of *DcBZ1* during seed and somatic embryo development was analyzed by RT-PCR (A, C) or quantitative RT-PCR (B, D). (A, B) mRNAs from developing seeds (14, 17, 26, 47, and 65 days after flowering) were analyzed. (C, D) mRNAs from globular embryos (G5), heart-shaped embryos (H7), and torpedo-shaped embryos (T10 and T14) were analyzed. RT-PCR was performed using an equal volume of cDNA as template DNA and specific primers for *DcBZ1*. The products resulting from 25 PCR cycles were fractionated on a 1.5% agarose gel and visualized using ethidium bromide staining. *Ubiquitin* (*Ubi*) was used as a transcriptional control. The data shown are representative of multiple experiments. On quantitative RT-PCR, values were normalized with the amplification rate of *Ubi* as a constitutively expressed internal control. Data are presented as mean values ± SE of three independent measurements.

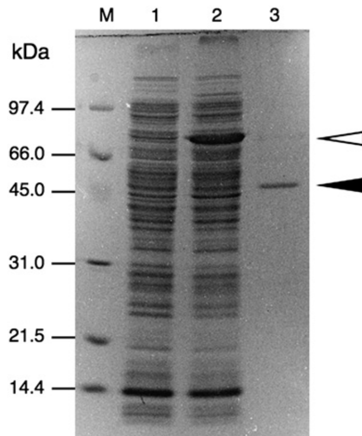

Fig. 5. Expression and purification of the recombinant DcBZ1 proteins. The recombinant GST-DcBZ1 fusion protein was obtained using an *Escherichia coli* expression system. Soluble proteins were extracted from bacteria after incubation with 0 M (lane 1) or 0.1 mM (lane 2) isopropyl-beta-D-thiogalactopyranoside. The recombinant fusion proteins (open arrowhead) were affinity purified with Glutathione-Sepharose, and then the recombinant DcBZ1 proteins (solid arrowhead) were eluted from Sepharose after thrombin cleavage (lane 3). Proteins were separated by SDS-PAGE (10%) and stained with Coomassie Brilliant Blue. M: molecular weight markers.

expression of the genes for *Arabidopsis* GBF3, rice OsBZ8, and maize ZmBZ-1 is also up-regulated by ABA in embryos, shoots, and roots [20,21,39,40].

The recombinant DcBZ1 protein bound specifically to the DNA fragment containing ABRE-like *cis* elements, motifs X and Y, in the promoter regions of a carrot ABA-inducible gene, *DcECP31*. The results of the competition assay confirmed that DcBZ1 binds to both motifs X and Y independently. Ko et al. [29,30] reported that both motifs are essential for regulating *DcECP31* gene expression, and the motif X functions as an enhancer-like element and the motif Y participates in ABA responsiveness. It is generally thought that a single copy of ABRE is insufficient for ABA-responsive transcription, and that ABRE requires another ABRE or the so-called coupling element (CE) for ABA-responsive transcription [17,41]. Therefore, it is strongly suggested that DcBZ1 may regulate ABA-dependent gene expression by binding to the ABRE-like *cis* elements in carrot cells.

In somatic embryos and developing seeds, it was common to find increased expression of *DcBZ1* in the later phase of development. The increased expression likely occurs through the developmental process, but not by hormonal regulation, because *DcBZ1* gene expression was slightly enhanced by ABA in somatic embryos. During carrot seed development, endogenous levels of ABA increase after 23 DAF, with a transient peak at 29 DAF, and some ABA-inducible genes (*ECP*s and *CAISE*s) are expressed after 29 DAF [10,33]. Furthermore, the increased expression of *ECP*s and *CAISE*s is detected in ABA-treated torpedo-shaped somatic embryos [10]. These results indicate that the expression profile of *DcBZ1* is similar to that of carrot ABA-inducible genes. GBF-type bZIP factors, bean ROM1 and ROM2, rice OsBZ8, and maize ZmBZ-1 and EmBP-2, are reportedly involved in transcriptional

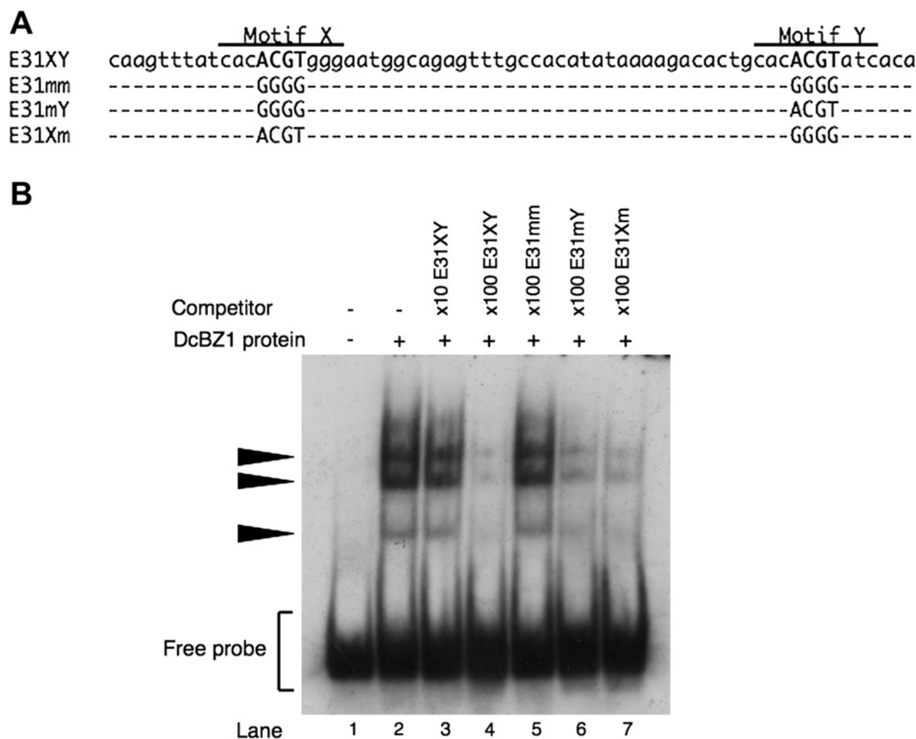

Fig. 6. Interaction of DcBZ1 with the promoter region of *DcECP31*. (A) Physical map of a part of the *DcECP31* promoter. The partial DNA fragment of the promoter region containing both the X motif (CAC**ACGT**GGG) and the Y motif (CAC**ACGT**ATC) in *DcECP31* was used as a probe (E31XY). The DNA fragments, including base-substituted motifs, were used as non-specific competitors (E31mm, E31mY, and E31Xm). (B) Electrophoretic mobility shift assay (EMSA). The double-stranded oligonucleotides (E31XY) were end-labeled with digoxigenin. Fifty nanograms of the recombinant DcBZ1 protein were incubated with 12 ng of digoxigenin-labeled probes. In the competition assay, 10- or 100-fold molar excess unlabeled oligonucleotides were added. Arrowheads indicate positions of shifted bands.

regulation of ABA-inducible genes and seed maturation [18–21]. Therefore, it is suggested that DcBZ1 is one of the bZIP factors that regulate ABA-dependent gene expression relating to dormancy and maturation in late embryogenesis.

Thus, our findings suggest that the carrot GBF-type bZIP factor, DcBZ1, is involved in ABA signal transduction in embryogenesis and other vegetative tissues, rather than in light signal transduction.

Acknowledgments

This research was supported in part by Grants-in-Aid for Research on Priority Areas and for Scientific Research from the 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-RFTF00L01601); and by the Special Coordination Funds of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government. The authors are grateful to all members of the Ibaraki Experimental Field of Takii Seed Co., Ltd. for collecting carrot seeds.

References

[1] R.H. Racusen, F.M. Schiavone, Positional cues and differential gene expression in somatic embryos of higher plants, Cell Differ. Dev. 30 (1990) 159–169.

[2] M. Ikeda, M. Umehara, H. Kamada, Embryogenesis-related genes; its expression and roles during somatic and zygotic embryogenesis in carrot and *Arabidopsis*, Plant Biotechnol. 23 (2006) 153–161.

[3] K. Yazawa, H. Kamada, Identification and characterization of carrot HAP factors that form a complex with the embryo-specific transcription factor C-LEC1, J. Exp. Bot. 58 (2007) 3819–3828.

[4] T. Takeda, M. Mizukami, H. Matsuoka, Characterization of two-step direct somatic embryogenesis in carrot, Biochem. Eng. J. 38 (2008) 206–211.

[5] P. Christou, N.S. Yang, Developmental aspects of soybean (*Glycine max*) somatic embryogenesis, Ann. Bot. 64 (1989) 225–234.

[6] R.R. Finkelstein, S.S.L. Gampala, C.D. Rock, Abscisic acid signaling in seeds and seedlings, Plant Cell 14 (2002) S15–S45.

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

[8] T. Senaratna, B.D. McKersie, S.R. Bowley, Artificial seeds of alfalfa (*Medicago sativa* L.). Induction of desiccation tolerance in somatic embryos, In Vitro Cell Dev. Biol. 26 (1990) 85–90.

[9] Y. Iida, K. Watabe, H. Kamada, H. Harada, Effects of abscisic acid on the induction of desiccation tolerance in carrot somatic embryos, J. Plant Physiol. 140 (1992) 356–360.

[10] H. Shiota, G. Yang, S. Shen, C.H. Eun, K. Watabe, I. Tanaka, H. Kamada, Isolation and characterization of six abscisic acid-inducible genes from carrot somatic embryos, Plant Biotechnol. 21 (2004) 309–314.

[11] M. Jakoby, W. Dröge-Laser, J. Vicente-Carbajosa, J. Tiedemann, T.K.F. Parcy, bZIP transcription factors in *Arabidopsis*, Trends Plant Sci. 7 (2002) 106–111.

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

[13] S. Kircher, S. Ledger, H. Hayashi, B. Weisshaar, E. Schäfer, H. Frohnmeyer, CPRF4a, a novel plant bZIP protein of the CPRF family: comparative analyses of light-dependent expression, post-transcriptional regulation, nuclear import and heterodimerisation, Mol. Gen. Genet. 257 (1998) 595–605.

[14] A. Rügner, H. Frohnmeyer, C. Näke, F. Wellmer, S. Kircher, E. Schäfer, K. Harter, Isolation and characterization of four novel parsley proteins that interact with the transcriptional regulators CPRF1 and CPRF2, Mol. Genet. Genomics 265 (2001) 964–976.

[15] U. Schindler, A.E. Menkens, H. Beckmann, J.R. Ecker, A.R. Cashmore, Heterodimerization between light-regulated and ubiquitously expressed *Arabidopsis* GBF bZIP proteins, EMBO J. 11 (1992) 1261–1273.

[16] F. Wellmer, E. Schäfer, K. Harter, The DNA binding properties of the parsley bZIP transcription factor CPRF4a are regulated by light, J. Biol. Chem. 276 (2001) 6274–6279.

[17] M.J. Guiltinan, W.R. Marcotte, R.S. Quatrano, A plant leucine zipper protein that recognizes an abscisic acid response element, Science 250 (1990) 267–271.

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

[19] M.S. Chern, H.G. Eiben, M.M. Bustos, The developmentally regulated bZIP factor ROM1 modulates from lectin and storage protein genes in bean embryos, Plant J. 10 (1996) 135–148.

[20] H. Nakagawa, K. Ohmiya, T. Hattori, A rice bZIP protein, designated OSBZ8, is rapidly induced by abscisic acid, Plant J. 9 (1996) 217–227.

[21] C. Nieva, P.K. Busk, E. Domínguez-Puigjaner, V. Lumbreras, P.S. Testillano, M.C. Risueño, M. Pagès, Isolation and functional characterisation of two new bZIP maize regulators of the ABA responsive gene *rab28*, Plant Mol. Biol. 58 (2005) 899–914.

[22] M. Riera, C. Valon, F. Fenzi, J. Giraudat, J. Leung, The genetics of adaptive responses to drought stress: abscisic acid-dependent and abscisic acid-independent signalling components, Physiol. Plant. 123 (2005) 111–119.

[23] S.Y. Kim, H.J. Chung, T.L. Thomas, 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 (1997) 1237–1251.

[24] T. Hobo, Y. Kowyama, T. Hattori, A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 15348–15353.

[25] H. Choi, J. Hong, J. Ha, J. Kang, S.Y. Kim, ABFs, a family of ABA-responsive element binding factors, J. Biol. Chem. 275 (2000) 1723–1730.

[26] R.R. Finkelstein, T.J. Lynch, The *Arabidopsis* abscisic acid response gene *ABI5* encodes a basic leucine zipper transcription factor, Plant Cell 12 (2000) 599–609.

[27] L. Lopez-Molina, N.H. Chua, A null mutation in a bZIP factor confers ABA-insensitivity in *Arabidopsis thaliana*, Plant Cell Physiol. 41 (2000) 541–547.

[28] Y. Uno, T. Furihata, H. Abe, R. Yoshida, K. Shinozaki, K. Yamaguchi-Shinozaki, *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 11632–11637.

[29] S. Ko, C.H. Eun, S. Satoh, H. Kamada, Analysis of *cis*-regulatory elements in carrot embryo-specific and ABA-responsive gene, *DcECP31*, Plant Biotechnol. 18 (2001) 55–60.

[30] S. Ko, H. Kamada, Motif X (CACACGTGGG) and motif Y (CACACG TATC) of the *DcECP31* promoter have different functions in the ABA-response pathway, J. Plant Physiol. 159 (2002) 1159–1161.

[31] S. Satoh, H. Kamada, H. Harada, T. Fujii, Auxin-controlled glycoprotein release into the medium of embryogenic carrot cells, Plant Physiol. 81 (1986) 931–933.

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

[33] H. Shiota, R. Satoh, K. Watabe, H. Harada, H. Kamada, *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 (1998) 1184−1193.

[34] S. Torres-Schumann, C. Ringli, D. Heierli, N. Amrhein, B. Keller, *In vitro* binding of the tomato bZIP transcriptional activator VSF-1 to a regulatory element that controls xylem-specific gene expression, Plant J. 9 (1996) 283−296.

[35] T. Tabata, T. Nakayama, K. Mikami, M. Iwabuchi, HBP-1a and HBP-1b: leucine zipper-type transcription factors of wheat, EMBO J. 10 (1991) 1459−1467.

[36] J.C. Hong, Y.H. Cheong, R.T. Nagao, J.D. Bahk, J.L. Key, M.J. Cho, Isolation of two soybean G-box binding factors which interact with a G-box sequence of an auxin-responsive gene, Plant J. 8 (1995) 199−211.

[37] S. Bensmihen, S. Rippa, G. Lambert, D. Jublot, V. Pautot, F. Granier, J. Giraudat, F. Parcy, The homologous ABI5 and EEL transcription factors function antagonistically to fine-tune gene expression during late embryogenesis, Plant Cell 14 (2002) 1391−1403.

[38] S.Y. Kim, J. Ma, P. Perret, Z. Li, T.L. Thomas, *Arabidopsis* ABI5 subfamily members have distinct DNA-binding and transcriptional activities, Plant Physiol. 130 (2002) 688−697.

[39] M. Suzuki, M.G. Ketterling, Q.B. Li, D.R. McCarty, Viviparous1 alters global gene expression patterns through regulation of abscisic acid signaling, Plant Physiol. 132 (2003) 1664−1677.

[40] G. Lu, A.L. Paul, D.R. McCarty, R.J. Ferl, Transcription factor veracity: is GBF3 responsible for ABA-regulated expression of *Arabidopsis Adh*? Plant Cell 8 (1996) 847−857.

[41] Q. Shen, T.H.D. Ho, 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 (1995) 295−307.