Physiological and Molecular Biological Studies on Involvement of C-ABI3 on Acquisition of Abscisic Acid-Induced Desiccation Tolerance

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Abbreviations

aa: amino acid
ABA: abscisic acid
ABI: ABSCISIC ACID-INSENSITIVE
b: nucleotide base(s)
bp: base pair(s)
C-ABI3: carrot-ABI3
CAIŒE: carrot ABA-inducible genes in somatic embryos
CaMV: cauliflower mosaic virus
cDNA: complementary DNA
CTAB: cetyltrimethylammonium bromide
cv.: cultivar
2,4-D: 2,4-dichlorophenoxyacetic acid
DAF: days after flowering
DAP: days after pollination
DNA: deoxyribonucleic acid
DTT: dithiothreitol
DW: distilled water
ECP: embryogenic cell protein
EDTA: ethylenediaminetetraacetic acid
FDA: fluorescein diacetate
FUS: FUSCA
GA: gibberellin or gibberellic acid
GUS: β-glucuronidase
HPRI: human placenta ribonuclease inhibitor
IB: isolation buffer
kb: kilo base(s)
kbp: kilo base pair(s)
L.: Lynne
LB medium: Luria-Bertani medium
LEA: late embryogenesis abundant
LEC: LEAFY COTYLEDON
mRNA: messenger RNA
MOPS: 3-((N-morpholino)propanesulfonic acid
MS medium: Murashige and Skoog's medium
MSD medium: Murashige and Skoog's medium containing 1 mg/l 2,4-D
NC: non-embryogenic cells
ORF: open reading frame
PCR: polymerase chain reaction
Rif: rifampicin
RNA: ribonucleic acid
rpm: revolutions per minute
rRNA: ribosomal RNA
SDS: sodium dodecyl sulfate
SM: 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 8 mM MgSO₄ with 0.01% gelatin
SSC: 150 mM NaCl with 15 mM sodium citrate
T-DNA: transferred-DNA
TE: 10 mM Tris-HCl (pH 8.0) with 1 mM EDTA
Tris: tris(hydroxymethyl)aminomethane
VP: VIVIPAROUS
wt: weight
xg: centrifugal force (x unit gravitational field)
Abstract
Generally, in higher plants, mature seeds show dormancy and desiccation tolerance. At the later phase of seed development, level of abscisic acid (ABA; a kind of phytohormones) increases transiently and then desiccation starts. In some plant species, it is known that the VP1/ABI3 factor functions as a transcriptional factor on seed-specific ABA-signal transduction. Recently, it is considered that the mechanisms of ABA-signal transduction involving the VP1/ABI3 factor and other factors might be very complicated. Therefore, it is thought that the analyses at tissue, cellular and protein levels are necessary. However, zygotic embryos in seeds are not useful materials, because it is difficult to observe zygotic embryos owing to the surrounding maternal tissues (nucellus tissues) of seeds and to isolate embryos as a large quantity synchronizing the developmental stage.

On the other hand, it is known that carrot somatic embryogenesis which appears from somatic cells, but not from fertilized eggs, is as a morphological model of zygotic embryogenesis, because carrot somatic embryos develop through the same morphological change as zygotic embryos. Furthermore, a large quantity of somatic embryo at the same developmental stage can be collected using tissue culture method. However, somatic embryos differ to zygotic embryos in the functional aspect, especially in desiccation tolerance and dormancy, because carrot somatic embryos contain low level of endogenous ABA. Desiccation tolerance of carrot somatic embryos can be acquired by ABA-treatment. Thus, it is considered that carrot somatic embryos may have the same function on desiccation tolerance and dormancy as zygotic embryos,
but the mechanisms of expression of the function remain to be clarified.

To know the mechanisms of expression of the function, especially induction of desiccation tolerance, I isolated a carrot homolog of the VPI/ABI3 gene from a cDNA library of somatic embryos and designated C-ABI3 (carrot-ABI3). The sequence of C-ABI3 was very similar to those of Arabidopsis ABI3, maize VPI, rice OsvPI and kidney bean PvaAlf in the four conserved regions. Expression of the C-ABI3 gene was detected by Northern blot analysis in embryogenic cells, somatic embryos and developing seeds, but not in non-embryogenic cells nor seedlings. Thus, expression of the C-ABI3 gene was limited to tissues and cells that acquired desiccation tolerance in response to endogenous or exogenous ABA, suggesting that C-ABI3 might be involved in acquisition of ABA-induced desiccation tolerance in somatic and zygotic embryos. Furthermore, the level of endogenous ABA in seeds increased transiently from 23 days after flowering (DAF) to 32 DAF and then desiccation of seeds started from 38 DAF. Thus, expression of the C-ABI3 gene in developing seeds was observed prior to the increase in the level of endogenous ABA (from 14 DAF; globular stage). This result also suggests that C-ABI3 might be involved in acquisition of ABA-induced desiccation tolerance in zygotic embryos.

In transgenic carrot mature leaves in which C-ABI3 was ectopically expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter, the expression of ECP31, ECP63, ECP40 and DcEm was induced by ABA-treatment, indicating that expression
of the *DcEm* and *ECP* genes might be controlled by the pathway(s) that involves *C-ABI3* and ABA. However, the expression of *DcDHNL*, *DcEMB1* and *CAISE5* was induced by ABA-treatment in mature leaves of both transgenic and non-transgenic carrot, and expression of them in non-transgenic carrot was higher than that in transgenic carrot. This indicates that expression of *DcDHNL*, *DcEMB1* and *CAISE5* might be controlled by the pathway(s) that involves other unknown factor(s) and ABA, and *C-ABI3* might repress the ABA-induced expression of *DcDHNL*, *DcEMB1* and *CAISE5*. In transgenic carrot non-embryogenic cells (NC) as another ectopic expression system of the *C-ABI3* gene, the expression of *ECP31*, *ECP63*, *ECP40* and *DcEm* was induced by ABA-treatment, but the expression of *DcDHNL*, *DcEMB1* and *CAISE5* was not. This supports the experimental results in transgenic mature leaves. Furthermore, in the transgenic NC, desiccation tolerance was induced by ABA-treatment. These results suggest strongly the function of *C-ABI3* as an ortholog of the *VP1/ABI3* factor on embryo-specific ABA-induced desiccation tolerance through induction of some ABA-inducible genes not only in zygotic embryos but also in somatic embryos. It is also suggested that somatic embryogenesis might be a morphological and functional model of zygotic embryogenesis.
General Introduction
Generally, in higher plants, zygotic embryos develop from fertilized eggs through the globular, heart-shaped and torpedo-shaped stages in seeds. In most plant species, zygotic embryos show desiccation tolerance and dormancy in the later phase of seed development (Zimmerman, 1993 for a review). After dormancy, seeds rehydrate, germinate and grow to seedlings under appropriate conditions. It is thought that seed dormancy is a very important system for its survival by avoiding the bad environmental condition.

In higher plants, some phytohormones, such as auxin, gibberellin (GA), cytokinin, ethylene and abscisic acid (ABA) are involved in most of phenomena in several developmental phases (Kende and Zeevaart, 1997 for a review). Especially, it is known that ABA is an important phytohormone that involves in acquisition of desiccation tolerance and dormancy, because endogenous ABA increases rapidly in mature plants in response to desiccation or drought stresses, then guard cells close to keep water content in leaves, and because level of endogenous ABA is high in dormant buds and dry seeds, generally (Kende and Zeevaart, 1997 for a review). Furthermore, just before the initiation of seed desiccation, the level of endogenous ABA has been reported to increase transiently in developing seeds of Arabidopsis, cotton, alfalfa, rape, soybean, wheat, tomato and tobacco (Karssen et al., 1983; Ackerson, 1984; Finkelstein et al., 1985; Galau et al., 1987; Napier et al., 1989; Xu et al., 1990; Berry and Bewley, 1992; Imai et al., 1995).

At the later phase of seed development in many plant species,
various late embryogenesis abundant (LEA) proteins accumulate in mature seeds and the expression of almost all of their genes is positively regulated by ABA. It is suggested that the LEA genes are induced by endogenous ABA in mature seeds. Thus, it seems likely that the LEA proteins function to protect cells against desiccation, because LEA proteins are very hydrophilic ones (Chandler and Robertson 1994 for a review).

Recently, genetic analysis using mutants has produced various valuable results in the field of plant physiology. Several mutants (loci) which show deficiencies in seed dormancy (resulting to precocious seed germination) and desiccation tolerance, have been reported in Arabidopsis, maize and others. (Robertson, 1955; Karssen et al., 1983; Koornneef et al., 1984), because these mutants were easy to select by their typical phenotypes of precocious germination (vivipary). They are mutants (loci) concerning to ABA-biosynthesis and ABA-signal transduction (Karssen et al., 1983; Koornneef et al., 1984; Robichaud and Sussex, 1986).

In maize, nine viviparous mutants exhibiting vivipary phenotype were isolated (Robertson, 1955). Eight of them are ABA-deficient mutants, because they showed no or lower level of endogenous ABA and the vivipary phenotype was recovered to wild phenotype when they were treated with exogenously applied ABA (Robichaud and Sussex, 1986). One of the viviparous maize mutants, viviparous1 (vp1), is a seed-specific ABA-insensitive mutant. The ABA content of the seeds is same as that of wild type and the phenotype cannot be recovered by exogenous ABA (Robertson, 1955;
Robichaud and Sussex, 1986 and 1987). Seeds of vpl undergo viviparous germination; they exhibit desiccation intolerance, as well as the absence of dormancy and of accumulation of anthocyanin pigments and storage proteins (Neill et al., 1987). The VPl gene was cloned by transposon tagging (McCarty et al., 1991), and its expression was shown to be specific to the embryo and the endosperm. Moreover, expression of the Em (one of LEA proteins) gene, C1 (a regulator of anthocyanin biosynthesis) gene, Globulin (seed storage proteins) genes and KDI6 (a major protein specific in embryo) gene (McCarty et al., 1989; Kriz et al., 1990; Butler and Cuming, 1993; Paiva and Kriz, 1994), which is controlled by ABA, was not observed in the vpl mutant. Furthermore, a fusion gene composed of a part of the gene for the VPl protein and the gene for the GAL-4 DNA-binding domain had high transcriptional activity in carrot protoplasts (McCarty et al., 1991). Thus, the VPl protein might function as an activator of transcription that is involved in transduction of the ABA-signal during seed maturation (McCarty et al., 1991; Vasil et al., 1995). VPl might also repress the expression of the gene for alpha-amylase that is expressed after break of seed dormancy (Hoecker et al., 1995).

In Arabidopsis thaliana, five ABA-insensitive mutants, abil, abi2, abi3, abi4 and abi5, have been isolated (Koornneef et al., 1984; Finkelstein 1994). In these mutants, the level of endogenous ABA is comparable to or higher than that in the wild type (Koornneef et al., 1984). The vegetative tissues and seeds of the abil and abi2 mutants show low sensitivity to ABA (Koornneef et al., 1984). By contrast, in abi3, abi4 and abi5 mutants, only
seeds exhibit low sensitivity to ABA and the seeds fail to exhibit seed dormancy, desiccation tolerance, accumulation of seed storage proteins and lipids (Finkelstein and Somerville, 1990; Nambara et al., 1992; Finkelstein, 1993 and 1994), and breakdown of chlorophyll (Ooms et al., 1993). The ABI3 gene was cloned by genomic walking and showed high similarity to the VPI gene of maize in the A1 domain in the acidic amino-terminal domain and in three basic domains (B1, B2 and B3 domains; Giraudat et al., 1992; Suzuki et al., 1997). Moreover, expression of the genes for some LEA proteins and some seed storage proteins, which is controlled by ABA, was not observed in the abi3 mutant (Parcy et al., 1994). Thus, ABI3 might be an important mediator involving in the seed-specific transduction of the ABA-signal in Arabidopsis.

Thus, it is considered generally that VPI/ABI3 is an important factor which is involved in seed-specific ABA-signal transduction (Giraudat, 1995 for a review). In addition, several factors, such as putative transcriptional factors, DNA binding proteins, protein phosphatases, cyclic ADP-riboses and protein farnesyl transferases, appear to be involved in ABA-signal transduction by genetic and molecular biological analysis (Guilluiian et al., 1990; Leung et al., 1994; Meyer et al., 1994; Cutler et al., 1996; Abe et al., 1997; Leung et al., 1997; Wu et al., 1997; Finkelstein et al., 1998; Lotan et al., 1998; Luerßen et al., 1998). Recently, it is also suggested that these factors might function in ABA-signal transduction by interacting with each other in a complex manner (Hattori, personal communication). Thus, it is thought that genetic analysis using mutants could not
clarify the complex mechanisms completely, and biochemical and physiological analyses are necessary to clarify the mechanisms. However, investigations using seeds or isolated zygotic embryos from seeds are not realistic, because seeds contain other tissues in addition to zygotic embryos. Because in the early developmental stage, zygotic embryos occupy only a limited part of the seeds and are surrounded by endosperms and seed coats, it is difficult to isolate zygotic embryos at the same developmental stage in large quantities. Especially, seeds of Arabidopsis (a model plant of higher plant species) are very small. Therefore, somatic embryos which are formed from somatic cells seem to be useful to analyze the mechanisms of embryogenesis by biochemical, physiological and molecular biological methods. However, somatic embryos of Arabidopsis are not easy to induce successfully (Sangwan et al., 1992; Wu et al., 1992; Pillon et al., 1996).

Since the first reports in 1958 (Reinert, 1958; Steward et al., 1958), somatic embryogenesis has been used extensively as an experimental system for examinations of the physiological, biochemical and molecular biological events that occur during zygotic embryogenesis. Among the numerous plant species in which somatic embryogenesis can be achieved, carrot has frequently been used as a model plant in studies of somatic embryogenesis because carrot somatic embryos can be readily generated in large quantities by transferring embryogenic cells from auxin-containing medium to auxin-free medium. Moreover, growth of carrot somatic embryos can be synchronized by selecting small clusters of embryogenic cells and transferring them to auxin-free medium at a low cell density (Zimmerman, 1993 for a review).
Somatic embryos develop into seedlings after morphological changes that are similar to those occurring in zygotic embryos (passage through globular, heart-shaped and torpedo-shaped stages). However, there are some differences in the physiological features between somatic and zygotic embryos. One of the most important differences is the lack of desiccation tolerance in somatic embryos (Senaratna et al., 1990; Iida et al., 1992). Somatic embryos cannot survive after drying because they lack desiccation tolerance, whereas zygotic embryos exhibit desiccation tolerance and dormancy occurs after seed desiccation at the later phase of seed development. In seeds, ABA is synthesized in tissues of the mother plant that surround the embryos, is transported to the embryos, and induces changes that protect the embryos from the harmful effects of desiccation (Dure III, 1975 for a review). Thus, ABA appears to be involved in acquisition of desiccation tolerance and dormancy in seeds. The level of endogenous ABA in carrot somatic embryos is low (Kamada and Harada 1981; Kiyosue et al., 1992a) and desiccation tolerance in somatic embryos can be induced by exogenous ABA in carrot and alfalfa (Kitto and Janick, 1985; Senaratna et al., 1990; Iida et al., 1992). These observations suggest that ABA is a key factor in the acquisition of desiccation tolerance, even in somatic embryos.

Although some ABA-inducible genes have been isolated from ABA-treated carrot somatic embryos by differential screening (Watabe, 1994), it has not been reported that there is a VP1/ABI3-like factor in somatic embryogenesis in any other plant species. By considering the facts mentioned above, it is likely that
VP1/ABI3-like genes might exist in the carrot genome and might be expressed and function in the transduction of the ABA-signal not only during seed maturation but also during somatic embryogenesis in carrot. In this study, I describe the isolation of a carrot homolog(s) of ABI3 and its possible involvement in the transduction of the ABA-signal during the acquisition of ABA-induced desiccation tolerance in zygotic and somatic embryos of carrot. Furthermore, in this study, I suggest that somatic embryogenesis might be not only a morphological model but also a functional model of zygotic embryogenesis.
Part 1.

Isolation and sequence analysis of a homolog to VP1/ABI3 in carrot
Introduction

It has been reported that the level of endogenous ABA in carrot somatic embryos is low (Kamada and Harada 1981; Kiyosue et al., 1992a) and that desiccation tolerance in somatic embryos can be induced by exogenously applied ABA in carrot (Iida et al., 1992). Generally the VP1/ABI3 factor is thought to play an important role in seed-specific ABA-signal transduction in zygotic embryos of higher plants. Therefore, it seems likely that VP1/ABI3-like genes exist in the carrot genome and they express and function in the transduction of the ABA-signal during seed maturation and during somatic embryogenesis in carrot. First, I tried to identify the existence of carrot homolog(s) of the VP1/ABI3 gene and to isolate it (them) by using two partial cDNA fragments for Arabidopsis ABI3 and a full cDNA fragment for rice OSVPl as probes. In this part, I studied the following; detection of signal of the homolog(s) in carrot genome, determination of cells or tissues in which the homolog(s) was expressed, construction of a cDNA library from mRNA of the cells or tissues, isolation of cDNA clones for the homolog(s), sequencing of the clones, characterization of the protein deduced from the cDNA sequence, determination of copy number(s) of the homolog(s) in carrot genome, and analysis of the genomic clones of the homolog(s).
Materials and Methods

Plant materials

Seedlings of carrot (Daucus carota L. cv. US-Harumakigosun) were grown from dry seeds for 15 days on vermiculite at 25°C with 16 hours of light daily (approximately 40 photons· m⁻²· s⁻¹). Mature plants of carrot were grown from dry seeds for two months on the same condition mentioned above.

Induction of embryogenic cells, somatic embryos and non-embryogenic cells

Carrot embryogenic cells, non-embryogenic cells and somatic embryos were obtained as described by Satoh et al. (1986). Ten-day-old seedlings of carrot were soaked in 70% (v/v) ethanol for 30 seconds and then sterilized with sodium hypochlorite solution (1% of available chlorite concentration) containing approximately 1% Tween™ 20 for 15 min. Then they were rinsed five times with sterilized distilled water (DW). Segments of hypocotyls of 10 mm in length were cut from seedlings and cultured on semi-solidified (0.8% agarose) Murashige and Skoog’s medium (MS medium; Murashige and Skoog, 1962) that contained 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) at 25°C in darkness. Embryogenic cells were formed after 1-2 months of culture and suspended into 100 ml of liquid MS medium that contained 2,4-D (1 mg/l) in 300 ml flasks. The embryogenic cells were cultured on a gyratory shaker (75 rpm) at 25°C in darkness and subcultured at 14-day intervals.

Clusters of embryogenic cells of 37-63 μm in diameter were collected from a 2-week-old suspension by passing the suspension
through three stainless-steel sieves (pore diameter, 37 µm, 63 µm and 1,000 µm, respectively) and they were washed three times with 2,4-D-free liquid MS medium by centrifugation at 100 xg. The rinsed clusters of cells were suspended in 100 ml of liquid MS medium without 2,4-D at a final density of 0.2 ml packed cell volume (at 100 xg) per liter of medium and cultured at 25°C in darkness on a gyratory shaker (75 rpm) for 15 days, by which time torpedo-shaped somatic embryos had formed.

When cell clusters of embryogenic cells smaller than 1 mm were subcultured every two weeks in MS liquid medium containing 2,4-D (1 mg/l) for over 6 months, non-embryogenic cells which could not form somatic embryos even after transferring to auxin-free media were obtained.

These cells and tissues were stored at -80°C prior to use.

**Isolation of genomic DNA from carrot somatic embryos**

Carrot genomic DNA was isolated from 14-day-old somatic embryos (approximately 5 g). A frozen sample was ground in liquid nitrogen and homogenized with extraction buffer [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM EDTA and 15% sucrose]. The mixture was centrifuged at 42 xg at 4°C for 3 min. The precipitate was dissolved in 2T-1E solution composed of 500 mM Tris-HCl (pH 8.0) with 250 mM EDTA and 13% SDS. After incubation at 70°C for 15 min and mixing with 1/2 volume of 7.5 M ammonium acetate, the mixture was incubated at 4°C for 30 min and centrifuged at 7,600 xg for 25 min at 4°C. After adding 2/5 volume of isopropanol, the aqueous layer was stirred gently with a grass stick and sticked DNA was picked up with a grass stick. DNA was washed with 75% ethanol and
dissolved in TE buffer. After RNase (10 µg/ml) treatment at 37°C for 10 min and Proteinase K (50 µg/ml) treatment at 37°C for 60 min, the DNA solution was mixed with TE saturated phenol/chloroform (1:1, v/v) solution and centrifuged at 13,800 xg for 1 min at 25°C. This procedure was repeated more than four times and then rinsed with chloroform/isoamyl alcohol (24:1, v/v) solution. Finally, DNA was precipitated by ethanol and the precipitate was dissolved in TE buffer.

**Isolation of genomic DNA from carrot mature leaves**

Carrot genomic DNA was isolated from two-month-old leaves (approximately 1 g) by a modified version of the methods reported by Wagner et al. and Bousquet et al. (Wagner et al., 1987; Bousquet et al., 1990). Frozen sample was ground in liquid nitrogen and homogenized with 10 times volume of the sample of ice cold isolation buffer [IB; 10% polyethylene glycol 6,000, 0.35 M sorbitol, 0.1 M Tris-HCl (pH 8.0), 0.5% spermidine, 0.5% spermine and 0.5% β-mercaptoethanol]. The mixture was centrifuged at 11,850 xg at 4°C for 20 min. The precipitate was dissolved in 10 times volume of the sample of ice cold IB and centrifuged at 11,850 xg at 4°C for 20 min again. The precipitate was dissolved in 5 times volume of the sample of ice cold lysis buffer [0.35 M sorbitol, 0.1 M Tris-HCl (pH 8.0), 0.5% spermidine, 0.5% spermine and 0.5% β-mercaptoethanol] and 1/10 volume of 10% sarcosine was added. The mixture was incubated at room temperature for 10 min and 1/2 volume of 2 x CTAB solution [2% cetyltrimethylammonium bromide (CTAB), 0.1 M Tris-HCl (pH 9.5), 20 mM EDTA, 1.4 M NaCl and 0.5% β-mercaptoethanol] was added. The mixture was incubated at 65°C
for 10 min and equal volume of chloroform/isoamyl alcohol (24:1, v/v) solution was added. The mixture was centrifuged at 11,850 xg at room temperature for 20 min and the aqueous layer was transferred into a new centrifuge tube. After adding equal volume of isopropanol, the aqueous layer was stirred gently with a grass stick and stucked DNA was picked up with a grass stick. DNA was washed with 75% ethanol and dissolved in TE buffer. The DNA solution was mixed with equal volume of TE saturated phenol and centrifuged at 13,800 xg for 1 min at 25°C. The aqueous layer was mixed with equal volume of TE saturated phenol/chloroform (1:1, v/v) solution and centrifuged at 13,800 xg for 1 min at 25°C. After adding equal volume of isopropanol and mixing gently, stucked DNA was picked up with a grass stick, washed with 75% ethanol, transferred into a new centrifuge tube and dissolved in TE buffer.

**Genomic Southern blot analysis**

Samples (10 μg) of carrot genomic DNA from mature leaves were separately digested with EcoR I, Hind III and Xba I. The digested DNA was fractionated by agarose (0.7%) gel electrophoresis and transferred to Hybond-N nylon filters (Amersham, Buckinghamshire, UK) after alkałization and neutralization. Hybridization buffer contained 5 x SSC, 5 x Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA according to the instructions from Amersham.

Four cDNA fragments were used as probes. pNH401 (739 bp) and pNH402 (330 bp) were partial cDNA fragments of *Arabidopsis ABI3* which contains several positively charged stretches with helix-
forming potential that might interact with DNA (McCarty et al., 1991) and the B3 conserved domain, respectively. cOSVPl was a full cDNA fragment of rice OSVPl (Hattori et al., 1994). Another was an insert of cDNA clone 409, isolated from carrot in this study. In a high-stringency condition, the nylon membranes were pre-hybridized at 65°C for 2 hours and hybridized at 65°C for 12 hours or more by adding each of the [32p]-labeled probe. In a low-stringency condition, the same procedure was performed at 45°C. After the hybridization, the membranes were washed with 2 x SSC for 15 min at room temperature twice and in 2 x SSC with 0.1% SDS at 65°C (high-stringency) or 45°C (low-stringency) for 30 min. The signals were detected with the BAS 2000 system (Fuji Photo Film, Tokyo, Japan) and X-ray films (Biomax MS; Eastman KODAK, Rochester, NY, USA).

Genomic PCR

In order to confer whether carrot homolog(s) of ABI3 exists or not, genomic PCR was performed. Two forward and two reverse primers were designed after comparing the sequences of ABI3 and VP1 (Fig. 1). AB31S (TGAAGCAAAGCGACGTGG) and AB32S (TACTCCGTTTGTGTTCCTCAGC) were constructed as forward primers (Fig. 1). AB31A (AATCACCTCTTTGGAGCCC) and AB32A (GCCAAAACTGTAGCGCATGTT) were constructed as reverse primers (Fig. 1). Amplification was carried out using 70 pmol primers and 1 μg carrot genomic DNA from somatic embryos as a template in the following conditions: 1 cycle of 97°C for 5 min, 60°C for 5 min and 72°C for 3 min; 2 cycles of 97°C for 1 min, 60°C for 2 min and 72°C for 3 min; 40 cycles of 95°C for 1 min, 60°C for 2 min and 72°C for 3 min. The PCR amplified products were checked by agarose (1.0%) gel or poly-acrylamide gel (5.0%)
electrophoresis and visualized by ethidium bromide staining. In addition, the products were checked by Southern blot analysis using the $^{32}$P-labeled pNH402 as a probe.

For analysis of intron(s) in carrot homolog(s) of ABI3, genomic PCR was performed again. CABI31S (CAAAAGGTGTGAAGCAGA), CABI32S (CGAATAACAAAAAGCAGGAT), CABI33S (GACTCCAAGAGGTGATT) and CABI3in1S (AAAGCAGAGCAATCGT) were constructed as forward primers (Fig. 8). CABI31A (AATCACCTCTTTGAGTCC), CABI32A (TCTGCTTC AACACCTTTTG), CABI33A (ATCCTGCTTTTGTATTCC), CABI3in1A (AGTCCCTATA TCTTCCATGA) and CABI3in5A (TATGGTGTCTTCTCGTTAC) were constructed as reverse primers (Fig. 8). Amplification was carried out using 70 pmol primers and 1 μg carrot genomic DNA from mature leaves as a template in the following conditions: 1 cycle of 97°C for 5 min, 57°C for 5 min and 72°C for 3 min; 2 cycles of 97°C for 1 min, 57°C for 2 min and 72°C for 3 min; 40 cycles of 95°C for 1 min, 57°C for 2 min and 72°C for 3 min. In the case using CABI33S and CABI3in5A as primers, amplification was carried out in the following conditions: 1 cycle of 97°C for 5 min, 52°C for 5 min and 72°C for 3 min; 2 cycles of 97°C for 1 min, 52°C for 2 min and 72°C for 3 min; 40 cycles of 95°C for 1 min, 52°C for 2 min and 72°C for 3 min. The PCR amplified products were checked by poly-acrylamide gel (5.0%) electrophoresis and visualized by ethidium bromide staining. They were subcloned into pCR II vector (Invitrogen, Carlsbad, CA, USA) or into the EcoRV site of pBluescript II SK+ vector (Stratagene, La Jolla, CA, USA) after blunting the ends of the amplified fragments.
Isolation of RNA from carrot cells

Total RNA was isolated from embryogenic cells, non-embryogenic cells, somatic embryos and seedlings by the phenol/SDS method (Ausubel et al., 1987). Frozen samples (approximately 1-10 g) were ground in liquid nitrogen and homogenized with a mixture (1:1, v/v) of TE saturated phenol and extraction buffer composed of 200 mM Tris-HCl (pH 9.0), 100 mM NaCl, 10 mM EDTA, 0.5% SDS and 14 mM β-mercaptoethanol. After adding equal volume of chloroform/isoamyl alcohol (24:1, v/v), the solution was mixed well and centrifuged at 1,500 xg for 30 min at 25°C. The aqueous layer was subjected three times to the same procedure after adding TE saturated phenol and the final aqueous layer was rinsed with chloroform/isoamyl alcohol. After incubation on ice for 20 min with sodium acetate (final 300 mM) to precipitate polysaccharides, the aqueous layer was precipitated by ethanol and centrifuged at 17,000 xg for 20 min at 4°C. The precipitate was dissolved in TE-HPRI buffer composed of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT and 5 units/ml HPRI. After the aqueous layer was mixed with 1/4 volume of 10 M LiCl and incubated on ice for 1 hour, it was centrifuged at 17,000 xg for 20 min at 4°C. The precipitate was dissolved in TE-HPRI buffer and the solution was put on TE-HPRI buffer containing 5.7 M CsCl and ultra-centrifuged at 211,000 xg for 16 hours at 16°C. The precipitate was dissolved in TE-HPRI buffer. Poly (A)⁺ RNA was purified by chromatography on oligo (dT) cellulose (Pharmacia, Piscataway, NJ, USA).21
Northern blot analysis

Total RNA (20 μg/lane) or poly (A)$^+$ RNA was loaded on an agarose (1.2%) gel prepared with 2.2 M formaldehyde. It was subjected to electrophoresis and transferred to a Gene Screen Plus nylon filter (NEW Research Products, Boston, MA, USA). Hybridization buffer contained 1% SDS, 1 M sodium chloride, 10% dextran sulfate and 100 μg/ml denatured salmon sperm DNA. After baking at 80°C for 2 hours, the nylon membranes were pre-hybridized at 60°C for 30 min and hybridized with the $^{32}$P-labeled pNH402 as a probe at 60°C for more than 12 hours. They were washed with 2 x SSC for 5 min at room temperature twice and 2 x SSC with 1.0% SDS for 20 min at 60°C. The signals were detected with the BAS 2000 system and X-ray films (Biomax MS).

Construction of cDNA library

Poly (A)$^+$ RNA was purified from a population of 16-day-old somatic embryos including globular, heart-shaped and torpedo-shaped embryos. Double-stranded cDNAs were synthesized from the poly (A)$^+$ RNA by cDNA Synthesis System Plus (Amersham). After addition of EcoR I/Not I adaptors (Pharmacia), cDNAs were size-fractionated on a Size-sep 400 spun column (Pharmacia). cDNAs were inserted into the EcoR I site of the lambda gt10 vector and packaged in lambda gt10 with a cDNA cloning system (Amersham).

Screening of cDNA library

Approximately 150,000 phages were plated on LB plates and incubated at 37°C for 10 hours. Amplified phages were transferred to Hybond-N nylon filters with alkalization and neutralization. A PCR-amplified cDNA fragment from pNH402 was used as a $^{32}$P-
labeled probe. AB33S (ATTTGCGGTTTCTCTTGC) as a forward primer and AB33A (AAATCACCTTCTTGGAGC) as a reverse primer were constructed to amplify 330-bp fragment of ABI3 cDNA which contained the B3 region conserved between ABI3 and VP1 (Fig. 1). Only one fragment was amplified using pNH402 as a template (25 cycles of 97°C for 30 seconds, 50°C for 1 min and 72°C for 1 min). Hybridization buffer was prepared as mentioned above according to the instructions from Amersham. The nylon membranes were pre-hybridized at 65°C for 2 hours and hybridized with the probe DNA at 65°C for more than 12 hours. They were washed with 2 x SSC for 5 min at 65°C twice and 2 x SSC with 1.0% SDS for 20 min at 65°C. The signals were detected with the BAS 2000 system.

In the first screening, 41 phages showing positive signals were picked up with pipet tips and resuspended in SM buffer composed of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 8 mM MgSO4 and 0.01% gelatin. In the second screening, 22 phages were isolated. The sizes of inserts in the recombinant phages were checked with PCR amplification using the lambda forward (GCTGGGTAGTCCCCACCTTT) and lambda reverse (CTTATGAGTATTTCTTCCAGGGTA) primers (25 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min). The Lambda gt10 phage DNA of clone 491 (insert size: 2,500 bp), clone 4121 (insert size: 2,700 bp) and clone 411 (insert size: 2,400 bp) were isolated and digested with Not I or EcoRI I. The inserted fragments were subcloned into pBluescript II SK⁺ vector.
Sequencing of cDNA clones

For sequencing of the cDNA clones, deletion clones were constructed from clone 491 and clone 4121 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 (PE Applied Biosystems, Foster City, CA, USA) using a ABI 373A auto sequencer (PE Applied Biosystems).

Sequencing of the fragments amplified by genomic PCR

Double-stranded plasmid DNAs were isolated and sequenced by the dye terminator cycle sequencing method according to the protocol supplied with the Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems) using a ABI 373A auto sequencer. Over three clones were sequenced on each fragment.
Results

Genomic Southern blot analysis using cDNAs for ABI3 and OSVP1 as probes

To examine whether there is an ABI3 homolog(s) in carrot, genomic Southern blot analysis was carried out. When pNH401 (a partial cDNA fragment of ABI3) was used as a probe, some faint bands were detected only under low-stringency conditions (data not shown). When pNH402 (a partial cDNA fragment of ABI3) and cOSVP1 (a full cDNA fragment of OSVP1) were used as probes, no signal was detected under either low- or high-stringency conditions (data not shown). The weak signals detected using pNH401 as a probe are unlikely to represent the presence of an ABI3 homolog(s) in carrot, because the sequence of pNH401 covers only a region that is not conserved between Arabidopsis ABI3 and maize VPI (Fig. 1). On the other hand, pNH402 might be unable to hybridize with the carrot genomic DNA, because the genomic sequence corresponding to pNH402 consists of five exons separated by four introns (Fig. 9; Giraudat et al., 1992). cOSVP1 was thought to fail to hybridize with the carrot genomic DNA for the same reasons as pNH401 and pNH402. Therefore, I then attempted to isolate the genomic DNA for ABI3/VPI-like factor(s) by the genomic PCR method.

Genomic PCR

Although PCR was performed under some conditions, fragments of approximately 190 bp, 770 bp and 870 bp were amplified only when AB31S and AB32A were used as primers (Fig. 2a). On the other hand, strong signals were detected at approximately 190 bp, 200 bp
and 290 bp by Southern blot analysis with pNH402 as a probe (Fig. 2b).

**Northern blot analysis using ABI3 as a probe**

To determine the appropriate tissues for cDNA screening, Northern blot analysis was carried out using pNH402 as a probe. A band of approximately 2.9-kb was detected in somatic embryos and embryogenic cells, but not in non-embryogenic cells nor seedlings (Fig. 3). The band is likely to represent a transcript of a carrot ABI3 homolog, because the size of Arabidopsis ABI3 cDNA is 2,960 bp, and because ABI3 is expressed in siliques containing seeds but not in leaves nor stems (Giraudat et al., 1992). Because a strong signal was detected for poly (A)^+ RNA purified from 16-day-old carrot somatic embryos (data not shown), a cDNA library was constructed from poly (A)^+ RNA of 16-day-old carrot somatic embryos and screened with a partial cDNA fragment of Arabidopsis ABI3 (pNH402) as a probe.

**Isolation and nucleotide sequencing of cDNA clones**

From approximately 150,000 phages of the somatic embryos cDNA library, 22-positive clones were obtained. Among them, two clones with relatively long inserts (clone 491, approximately 2,500 bp; and clone 4121, approximately 2,700 bp) were subjected to sequence analysis. Clone 491 contained an open reading frame (1,992 b) encoding 663-amino acid protein (Fig. 4). One (AGAGATGAA) of the possible translation initiation sites of clone 491 was similar to that of the ABI3 gene (AACGATGAA), and it fits the consensus sequence for translation initiation in plants (Fig. 4; Lütcke et al., 1987; Giraudat et al., 1992). Thus, it was the most
probable for the initiation codon of translation. The sequence of clone 4121 was the same as that of clone 491 except for the length of the 5'-untranslated region (data not shown). I named these clones as C-ABI3 (carrot-ABI3). Comparison of the overall amino acid sequence of the open reading frame revealed that C-ABI3 shows a low but significant similarity to Arabidopsis ABI3 (Giraudat et al., 1992), maize VP1 (McCarty et al., 1991), rice OSVP1 (Hattori et al., 1994) and kidney bean PvAlf (Phaseolus vulgaris; Bobb et al., 1995) (Table 1a, Fig. 5). However, in the B3 region (360 bp) that is conserved between Arabidopsis ABI3 and maize VP1, the sequence of the C-ABI3 gene was very similar to those of the ABI3, VP1, OSVP1 and PvAlf genes (Table 1b, Fig. 5).

**Genomic Southern blot analysis using C-ABI3 as a probe**

To determine the copy number of the gene that corresponded to C-ABI3, I carried out genomic Southern blot analysis under high-stringency conditions. Digestion with EcoR I and Xba I gave only one fragment of approximately 17.0 kbp and 8.0 kbp, respectively (Fig. 6). Fragments of approximately 8.0 kbp and 5.4 kbp were detected after digestion with Hind III (Fig. 6) because there is a Hind III site in the C-ABI3 cDNA (data not shown). These results indicate that only one copy of the C-ABI3 gene was present in the carrot genomic DNA.
**Sequence analysis of the fragments amplified by genomic PCR**

Genomic DNA fragments for C-ABI3 was obtained by PCR using carrot genomic DNA as a template. By using CABI31S and CABI31A as primers, a major fragment of approximately 600 bp was amplified and named as G600 (Fig. 7a). By using CABI3in1S and CABI32A as primers, a major fragment of approximately 190 bp was amplified and named as A194 (Fig. 7b). By using CABI3in1S and CABI3in1A as primers, a major fragment of approximately 450 bp was amplified and named as B451 (Fig. 7b). By using CABI33S and CABI3in5A as primers, a major fragment of approximately 240 bp was amplified and named as D240 (Fig. 7b). In contrast by using any other combination of primers on any condition of amplification, no fragment was amplified (data not shown). G600 was subcloned into the EcoRV site of pBluescript II SK⁺ vector after blunting of the ends, and A194, B451 and D240 were subcloned into pCR II vector.

Sequence analysis revealed that A194 included 2 exons and one intron (Fig. 8) by comparing with the sequence of cDNA clone 409. The size of the intron was 100 bp and named it as intron 1 (in1; Fig. 8). B451 included 3 exons and 2 introns (Fig. 8). The sizes of the first and second introns were 100 bp (in1) and 95 bp (intron 2: in2) (Fig. 8). G600 included 4 exons and 3 introns (Fig. 8). The sizes of the first, second and third introns were 95 bp (in2), 123 bp (intron 3: in3) and 83 bp (intron 4: in4), respectively (Fig. 8). D240 included 2 exons and one intron (Fig. 8). The size of the intron was 112 bp and named it as intron 5 (in5; Fig. 8).
The sequences of the introns were not similar to those of Arabidopsis ABI3 and rice OSVP1 (data not shown; Hattori et al., 1994). The 5′- and 3′-sequences of in1, in2, in3, in4 and in5 fit the consensus sequence for splicing: GT at 5′-end and AG at 3′-end; GTAAGT at 5′-end and CAG at 3′-end; GTA at 5′-end and AG at 3′-end; GTA at 5′-end and CAG at 3′-end; GTA at 5′-end and CAG at 3′-end, respectively (Fig. 8). In comparison with VP1, ABI3 and OSVP1, the insertion position of the introns of C-ABI3 was conserved in the B3 region (Fig. 9; McCarty et al., 1991; Giraudat et al., 1992; Hattori et al., 1994). The other intron was not observed in the genomic clones of the C-ABI3 gene (data not shown).
Discussion

In this part, cDNA clones of a carrot homolog of Arabidopsis ABI3 were isolated and designated C-ABI3. C-ABI3 had an open reading frame that encoded 663 amino acids (Fig. 4). The amino acid sequence of C-ABI3 shows a high similarity to those of ABI3, VP1, OSVP1 and PvAlf in four conserved domains (A1, B1, B2 and B3; Table 1a and 1b, Fig. 5). The four conserved domains are important for these factors as the transcriptional factors (Giraudat et al., 1992; Suzuki et al., 1997). The A1 domain (amino acid 33-49 in C-ABI3) corresponds to the region responsible for activation of transcription of the Em gene in maize (McCarty et al., 1991). The B1 domain (amino acid 167-222) contains putative positively charged stretches with helix-forming potential that might interact with DNA (McCarty et al., 1991). The B2 domain (amino acid 376-409) that contains the putative nuclear targeting signal might alternatively have other functions (McCarty et al., 1991; Giraudat et al., 1992). The B2 domain can enhance the DNA-binding activity of the basic leucine zipper proteins (bZIP proteins) EmBP-1 and also has relatively weak nonspecific DNA-binding activity (Hill et al., 1996). The B3 domain (amino acid 499-619) is most conserved and is a putative DNA-binding domain resembling the DNA-binding and dimerization domain of the human CTF/NF1 transcriptional activator (Giraudat et al., 1992). The B3 domain of VP1 also has strong DNA-binding activity that is specific for the maize CI promoter (Suzuki et al., 1997). Recently, the B3 domain of VP1 has been reported to activate expression of the Em gene without
binding to the *Em* promoter directly (Carson et al., 1997).

The B2 domain has been shown to contain the putative nuclear targeting signal (RKKR; Giraudat et al., 1992). However, the corresponding amino acid sequence in C-ABI3 is RQKR, though the sequence in other homologs (RKKR) is conserved (Fig. 4 and 5). Mechanisms of nuclear transport are thought to be common in animal and plant cells and the nuclear localization signals are conserved as lysine- and arginine-rich sequences (Raikhel, 1992). The lysine- and arginine-rich sequences as putative nuclear localization signals were observed in the B1 and B2 domains of C-ABI3 (FKKSTIECAYKRMGSSKEGGKLILEWYEQLQKLR in B1 domain and RVVRLASATKEARQKRKMKR in B2 domain) (Fig. 4 and 5; Raikhel, 1992). Thus, it is likely that putative nuclear localization signals might be in both the B1 and B2 domains of C-ABI3. In fact, nuclear localization of rice OSVP1 is observed using fusion protein of OSVP1 and β-glucuronidase (GUS) (Hattori, personal communication).

The length of C-ABI3 (663 amino acids; Fig. 4 and 5) is a little shorter than those of homologs in other plant species (ABI3, 721 amino acids; VP1, 691 amino acids; OSVP1, 728 amino acids; PvAlf, 751 amino acids) (Fig. 5). The "missing" amino acid residues may not affect the functions of C-ABI3, because the "missing" amino acid residues are outside the conserved domains (Fig. 5).

As shown in Fig. 2a and 2b, three fragments were amplified by genomic PCR and three bands were detected by Southern blot analysis. The amplified fragments of approximately 190 bp was detected as positive signals by Southern blot analysis, but others
(approximately 770 bp and 870 bp) were not detected (Fig. 2a and 2b). It is likely that the smaller fragment is derived from the C-ABI3 gene itself and the two bigger fragments are non-specific ones, because this genomic PCR was performed at low stringency conditions using primers designed from sequence of Arabidopsis ABI3. On the other hand, all the three bands detected by Southern blot analysis may be C-ABI3, because amplified fragments whose sizes were 200 bp and 290 bp might not be able to be detected successfully with ethidium bromide staining (Fig. 2a and 2b). However, it is suggested that only one copy of the C-ABI3 gene exists in the carrot genome according to the results of genomic Southern blot analysis and genomic PCR using C-ABI3 specific primers (Fig. 6, 7a and 7b). Copy number of the C-ABI3 gene was conserved between carrot and Arabidopsis (Giraudat et al., 1992).

It is noteworthy that insertion positions of five introns in the B3 region were conserved among VP1, OSVP1, ABI3 and C-ABI3 (Fig. 9), because the B3 region, which is most conserved, consists of four exons being conserved between monocotyledon and dicotyledon. These results indicate that the situational style of the C-ABI3 gene in carrot genome might be conserved among those of the other homologs in each genome.

In summary of Part 1, the C-ABI3 gene in carrot is a homolog of the VP1/ABI3 gene and might have the same function in zygotic and somatic embryos, because the four important regions were conserved and only one copy of C-ABI3 existed in carrot genome.
Part 2.

Expression analysis of the C-ABI3 gene
Introduction

In part 1, I isolated the C-ABI3 gene as a carrot homolog of the VPI/ABI3 gene from carrot somatic embryos, and it showed high homology to the homologs of other plant species at four conserved domains (Table 1; Fig. 5).

It is reported that expression of the VPI gene is specific in zygotic embryos and endosperm, that of the ABI3 gene is specifically observed in zygotic embryos and disappears during germination, and that of the PvAlf gene is specific in developing seeds (McCarty et al., 1989 and 1991; Giraudat et al., 1992; Bobb et al., 1995). These reports support the idea that the VPI/ABI3 factor is a transcriptional factor on seed-specific ABA-signal transduction.

In order to know the expression pattern of the C-ABI3 gene, I tried to analyze its expression by Northern blot analysis in carrot cultured cells such as embryogenic cells, non-embryogenic cells and somatic embryos, in several tissues such as seedlings and several stages of developing seeds, and during stress-induced somatic embryogenesis (Kamada et al., 1989, 1993 and 1994; Kiyosue et al., 1989 and 1990). It has not been reported the expression analysis of the VPI/ABI3 gene in cultured cells such as somatic embryos and embryogenic cells in other plant species. In the carrot system, several types of cultured cells are available as materials. In this part, I tried to analyse the expression pattern of C-ABI3 using various materials in carrot.
Materials and Methods

Plant materials

Seedlings of carrot (*Daucus carota* L. cv. US-Harumakigosun) were grown from dry seeds for 15 days on vermiculite at 25°C with 16 hours of light daily (approximately 40 photons·m⁻²·s⁻¹), harvested, weighed and stored at -80°C prior to use.

*Induction of embryogenic cells, somatic embryos and non-embryogenic cells*

Carrot embryogenic cells, non-embryogenic cells and somatic embryos were obtained as described by Satoh et al. (1986). Globular somatic embryos (4 days), heart-shaped somatic embryos (7 days) and torpedo-shaped somatic embryos (10 days and 14 days) were collected from the cultures which were cultured in phytohormone-free liquid MS medium on a gyratory shaker (75 rpm) from clusters of embryogenic cells of 37-63 μm in diameter for some duration times mentioned above. These cells and tissues were stored at -80°C prior to use.

*Plant materials for seed sampling*

Carrot (*Daucus carota* L. cv. Yohmeigosun) plants grown in the experimental field of Takii Seed Co., Ltd. (Ushiku, Ibaraki, Japan) were used for sampling of seeds. Seeds and fruits were harvested on various days after flowering (DAF), weighed and stored at -80°C prior to use.

*Treatment with high concentration of sucrose as stress*

Ten-day-old seedlings of carrot were sterilized as mentioned in Part 1. Segments of apical tips of 5 mm in length were cut from
seedlings and cultured on semi-solidified (0.8% agarose) MS medium that contained 0.7 M of sucrose at 25°C with 16 hours of light daily (approximately 40 photons· m⁻²· s⁻¹) for 4 days and 14 days. After 14-day-culture with 0.7 M sucrose, apical tips were transferred onto semi-solidified stress-free MS medium and cultured under the same condition as mentioned above for 14 days. On the other hand, apical tips were cultured on semi-solidified stress-free MS medium and cultured under the same condition as mentioned above for 14 days as a negative control. Cultured apical tips were collected, weighed and stored at -80°C prior to use.

**Isolation of RNA from carrot cells**

Total RNA was isolated from embryogenic cells, non-embryogenic cells, somatic embryos, developing seeds, seedlings and apical tips by the phenol/SDS method (Ausubel et al., 1987).

**Northern blot analysis**

Total RNA (20 μg/lane) was loaded on an agarose (1.2%) gel prepared with 2.2 M formaldehyde. It was subjected to electrophoresis and transferred to a Gene Screen Plus nylon filter (NEW Research Products). Hybridization buffer contained 1% SDS, 1 M sodium chloride, 10% dextran sulfate and 100 μg/ml denatured salmon sperm DNA. After baking at 80°C for 2 hours, the nylon membranes were pre-hybridized at 60°C for 30 min and hybridized with the [³²P]-labeled cDNA of clone 491 as a probe at 60°C for more than 12 hours. They were washed with 2 x SSC for 5 min at room temperature twice and 2 x SSC with 1.0% SDS for 20 min at 60°C. The signals were detected with the BAS 2000 system (Fuji Photo Film) and X-ray films (Biomax MS; Eastman KODAK).
Results

Northern blot analysis of expression of the C-ABI3 gene in cultured tissues

Using clone 491 as a probe, I detected the expression of the C-ABI3 gene in embryogenic cells and somatic embryos (Fig. 10). However, no expression was detected in seedlings and non-embryogenic cells (Fig. 10). In developing somatic embryos, expression was detected at all stages of development (Fig. 10). Relatively strong expression of the C-ABI3 gene was observed in 10-day-old torpedo-shaped somatic embryos (Fig. 10).

Northern blot analysis of expression of the C-ABI3 gene in developing seeds

In developing seeds, expression of the C-ABI3 gene was clearly detected after 20 DAF and maintained until dry seeds (62 DAF; Fig. 11c). Weak expression was also observed at 14 DAF and 17 DAF (Fig. 11c).

Northern blot analysis of expression of the C-ABI3 gene during stress-induced somatic embryogenesis

Expression of the C-ABI3 gene was detected in apical tips which were treated with sucrose (0.7 M) for 14 days and also in apical tips which were cultured on stress-free MS medium for 14 days after 14-day-treatment with sucrose (0.7 M) (Fig. 12). However, no signal of the expression was detected in apical tips which were treated with sucrose (0.7 M) for 4 days and also in apical tips which were cultured on stress-free MS medium for 14 days (Fig. 12). The signals in apical tips were weaker than those in embryogenic cells and torpedo-shaped somatic embryos (Fig. 12).
Discussion

The expression of the C-ABI3 gene was observed in embryogenic cells and somatic embryos, but not in non-embryogenic cells nor seedlings (Fig. 10). In embryogenic cells, the level of ABA is high and desiccation tolerance is observed (Kaimori and Ishihara, 1991; Kiyosue et al., 1992a). In somatic embryos, the level of endogenous ABA is low (approximately 32-50% to embryogenic cells; Kamada and Harada, 1981; Kiyosue et al., 1992a) and no desiccation tolerance is acquired without application of exogenous ABA (Iida et al., 1992). However, desiccation tolerance in carrot somatic embryos can be induced by exogenous application of ABA (Iida et al., 1992). In non-embryogenic cells and seedlings, the level of ABA is very low (approximately 1.3-1.7% to embryogenic cells; Kiyosue et al., 1992a) and no desiccation tolerance is acquired even upon treatment with exogenous ABA (Kaimori and Ishihara, 1991; my unpublished data). These results indicate that cells and tissues expressing the C-ABI3 gene acquire desiccation tolerance in response to endogenous or exogenous ABA.

Expression of the ABI3 gene of Arabidopsis is observed in siliques containing seeds but not in leaves nor stems (Giraudat et al., 1992). Expression of the VPl gene of maize is observed specifically in embryos and endosperms (McCarty et al., 1989). Expression of the OSVPl gene of rice and the PvAlf gene of kidney bean is specifically observed in embryos (Hattori et al., 1994; Bobb et al., 1995). These reports support the results in carrot described here (Fig. 10 and 11c). The ABI3/VPl factor functions as
a key factor in the transduction of the ABA-signal in seeds, and vpl mutant and abi3 mutant show no desiccation tolerance of seeds (McCarty et al., 1991; Giraudat et al., 1992). Therefore, C-ABI3 might be involved in acquisition of ABA-induced desiccation tolerance in somatic embryos, and the function of C-ABI3 in carrot somatic embryos might be same as that of VP1, ABI3, OSVP1 and PvAlf in zygotic embryos (McCarty et al., 1991; Hattori et al., 1994; Parcy et al., 1994; Bobb et al., 1995; Hill et al., 1996; Carson et al., 1997; Suzuki et al., 1997).

In developing seeds, expression of the C-ABI3 gene was observed through the globular stage (14 DAF), the heart-shaped stage (17 DAF) and the torpedo-shaped stage (after 20 DAF) (Fig. 11b and 11c; Shiota et al., 1998). The level of endogenous ABA increased after 23 DAF, with a transient peak at 29 DAF (Fig. 11a; Shiota et al., 1998). Desiccation of seeds began at 38 DAF (Fig. 11a; Shiota et al., 1998). Thus, the C-ABI3 gene was expressed prior to the increase in level of endogenous ABA, which was followed by desiccation of seeds. This observation supports the hypothesis that C-ABI3 might function in signal transduction on the ABA-induced acquisition of desiccation tolerance in seeds. However, the level of expression was low at the globular (14 DAF) and heart-shaped (17 DAF) stages, when whole seeds were used as materials (Fig. 11b and 11c; Shiota et al., 1998). It is likely that the relative level of C-ABI3 mRNA is very low in young seeds, and it might be difficult to detect the expression of the C-ABI3 gene in young seeds by Northern blot analysis because the young embryo occupies only a very small part of each seed (Fig. 11b;
Expression of the ABI3 gene is observed at all stages of the development of Arabidopsis embryos, from early to late embryogenesis (Parcy et al., 1994) and the expression of C-ABI3 was observed throughout the development of carrot somatic embryos (Fig. 10). A detailed examination of expression of the C-ABI3 gene in carrot seeds is now being performed by in situ hybridization.

During stress-induced somatic embryogenesis, ability of formation of somatic embryos is obtained after 14 days of sucrose (0.7 M)-treatment as a stress, but not after 4 days of sucrose (0.7 M)-treatment (Kamada et al., 1989 and 1993). During sucrose (0.7 M)-treatment, no development nor growth of apical tips was observed (data not shown; Kamada et al., 1989 and 1993). During culture on stress-free MS medium, leaves develop and grow from apical tips, and somatic embryos are formed on developed leaves after culture on stress-free medium for at least 4 weeks (Kamada et al., 1989 and 1993). Expression of the C-ABI3 gene was observed in apical tips which were treated with sucrose (0.7 M) for 14 days and also in those which were cultured on stress-free MS medium for 14 days after 14-day-treatment with sucrose (0.7 M) (Fig. 12). In this condition, apical tips might obtain competence of somatic embryogenesis but no somatic embryo has formed (Kamada et al., 1989 and 1993). Expression of the C-ABI3 gene was not detected in apical tips which were treated with sucrose (0.7 M) only for 4 days and also in those which were cultured on stress-free MS medium for 14 days as a negative control (Fig. 12). In this condition, apical tips might not obtain competence of somatic
embryogenesis (Kamada et al., 1989 and 1993). These results indicate that C-ABI3 expresses very early in the cells which obtained competence of somatic embryogenesis. Expression of the C-ABI3 gene was detected as a weaker signal in apical tips than that in embryogenic cells and somatic embryos (Fig. 12). It is suggested that the number of cells which express the C-ABI3 gene might be very small among cells of apical tips, because somatic embryos which were induced by stress-treatment are formed only on the limited surface of the elongated leaves after transferring to stress-free medium (Kamada et al., 1989 and 1993).

Summarizing the results and discussions in Part 2, it is suggested that C-ABI3 might have the same function as that of the VP1/ABI3 factor in zygotic and somatic embryos of carrot because expression of the C-ABI3 gene was observed specifically in cells and tissues which acquire ABA-induced desiccation tolerance, and C-ABI3 might be involved in ABA-signal transduction in somatic and zygotic embryos because expression of the C-ABI3 gene was shown prior to increase of endogenous ABA during seed development.
Part 3.

Functional analysis of C-ABI3 using the transgenic plants
Introduction

In part 2, I showed that the pattern of expression of the C-ABI3 gene was related to the pattern of acquisition of ABA-induced desiccation tolerance (Fig. 10 and 11c), and suggested a possibility that C-ABI3 might be involved in ABA-induced acquisition of desiccation tolerance not only in zygotic embryos but also in somatic embryos and embryogenic cells. In seeds of Arabidopsis, ABI3 functions on induction of expression of ABA-inducible genes such as At2s3, CRC, AtEm6 and some genes for seed storage protein (Nambara et al., 1992; Finkelstein, 1993; Parcy et al., 1994), on breakdown of chlorophyll (Ooms et al., 1993), on repression of germination with maintaining dormancy (Nambara et al., 1992), and on accumulation of anthocyanin pigments (Neill et al., 1987; Nambara et al., 1994). In seeds of maize, VP1 functions on induction of expression of ABA-inducible genes such as Em, Cl and Glbl (McCarty et al., 1989; Kriz et al., 1990; Butler and Cuming, 1993; Paiva and Kriz, 1994), on accumulation of anthocyanin pigments, and on repression of germination with maintaining dormancy (Hoecker et al., 1995). In seeds of kidney bean and rice, PvAlf and OsVP1 function on induction of expression of ABA-inducible genes such as PHSβ, DLEC2 and OsEm (Hattori et al., 1994 and 1995; Bobb et al., 1995).

In order to know directly the suggested functions of C-ABI3, I tried to analyze transgenic plants in which the C-ABI3 gene was over-expressed. The putative functions of C-ABI3 are thought to be
comparable to those of the VP1/ABI3 factor as mentioned above. The analysis of transgenic plants was performed as follows: analysis of expression of carrot ABA-inducible genes as makers in carrot developing seeds, analysis of mature leaves of transgenic carrots which showed ectopic expression of the C-ABI3 gene, and analysis of transgenic non-embryogenic cells (NC) which showed ectopic expression of the C-ABI3 gene.

In carrot, some ABA-inducible genes were isolated by differential screening from ABA-treated somatic embryos and named as Carrot ABA-Inducible genes in Somatic Embryos (CAISE; Watabe, 1994; my unpublished data). Moreover, some genes for Embryogenic Cell Protein (ECP) were also isolated from embryogenic cells or somatic embryos as makers of somatic embryogenesis (Kiyosue et al., 1992b and 1993; Yang et al., 1997; Tachikawa et al., 1998). In this part, I used six CAISE genes and three ECP genes showing enhanced expression in ABA-treated somatic embryos (Kiyosue et al., 1992b and 1993; Yang et al., 1997; Tachikawa et al., 1998; my unpublished data) as probes and tried to clarify the involvement of C-ABI3 in ABA-induced expression of these genes in relation to acquisition of desiccation tolerance.
Materials and Methods

Plant materials

Carrot (Daucus carota L. cv. Yohmeigosun) plants grown in the experimental field of Takii Seed Co., Ltd. were used for sampling of seeds. Seeds and fruits were harvested on various DAF, weighed and stored at -80°C prior to use.

Mature leaves of carrot (Daucus carota L. cv. US-Harumakigosun) were grown for two months from somatic embryos on semi-solidified (0.2% Gelrite™; Monsanto, St. Louis, MO, USA) MS medium at 25°C with 16 hours of light daily (approximately 40 photons·m⁻²·s⁻¹).

Sequencing of the CAISE genes

Double-stranded plasmid DNAs were isolated and sequenced by the dye terminator cycle sequencing method according to the protocol supplied with the Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems) using an ABI 373A auto sequencer (PE Applied Biosystems).

Isolation of RNA from carrot cells

Total RNA was isolated from embryogenic cells, non-embryogenic cells, somatic embryos, developing seeds and mature leaves of carrot by the phenol/SDS method (Ausubel et al., 1987).

Northern blot analysis

Total RNA (20 µg/lane) was loaded on an agarose (1.2%) gel prepared with 2.2 M formaldehyde. It was subjected to electrophoresis and transferred to a Gene Screen Plus nylon filter (NEW Research Products). Hybridization buffer contained 1% SDS, 1
M sodium chloride, 10% dextran sulfate and 100 μg/ml denatured salmon sperm DNA. After baking at 80°C for 2 hours, the nylon membranes were pre-hybridized at 60°C for 30 min and hybridized with the [32P]-labeled probe DNAs at 60°C for more than 12 hours. They were washed with 2 x SSC for 5 min at room temperature twice and 2 x SSC with 1.0% SDS for 20 min at 60°C. The signals were detected with the BAS 2000 system (Fuji Photo Film) and X-ray films (Biomax MS; Eastman KODAK).

**Construction of the vector for transformation of carrot hypocotyls**

The DNA construction was made in the pBI121 binary T-DNA vector (Clontech, Polo Alto, CA, USA; Fig. 13a), which has a kanamycin-resistance marker gene for the selection of transformed plant cells. A carrot cDNA fragment (189-2,509 bp of the C-ABI3 gene; Fig. 4) that included the putative open reading frame was cloned in pBI121 by replacing the Xba I-Sac I fragment of the coding region of the gene for β-glucuronidase (GUS). The cDNA fragment was put under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The binary vector, pBI121-ABI3, was transferred into E. coli strain HB101 (Fig. 13b).

The binary vectors in E. coli were transferred into Agrobacterium tumefaciens strain LBA4404 (pAL4404) by triparental mating (Bevan, 1984). Agrobacterium tumefaciens strain LBA4404 was cultivated in LB medium at 28°C for 24 hours. E. coli strain HB101 (pRK2013) and E. coli strain HB101 (pBI121-ABI3 or pBI121) were cultivated in LB medium containing kanamycin (100 mg/l) at 28°C for 24 hours. Each bacterial culture was collected by
centrifugation (at 7,000 xg), resuspended with sterilized cold 10 mM MgSO₄ and collected by centrifugation (at 7,000 xg) again. Each bacterial pellet was resuspended with a small quantity of cold 10 mM MgSO₄, equal aliquots of each bacterial suspension were mixed gently on LB plate and incubated at 28°C for 16 hours. After incubation, the bacterial colony was resuspended with a small quantity of 10 mM MgSO₄, diluted with 10 mM MgSO₄, spread onto Min A [12.1 mM K₂HPO₄, 6.6 mM KH₂PO₄, 1.5 mM (NH₄)₂SO₄, 0.8 mM MgSO₄ and 0.2% glucose] plates containing kanamycin (400 mg/l) and incubated at 28°C for 3 days. The colonies were picked up, transferred onto a new Min A plate containing kanamycin (400 mg/l) and incubated at 28°C for 3 days again. The colonies were picked up, transferred onto a LB plate containing kanamycin (100 mg/l) and incubated at 28°C for 3 days. The clones of Agrobacterium which contained both pAL4404 and pBI121-CABI3 or pBI121 were used for infection.

Construction of the vector for transformation of carrot non-embryogenic cells

The DNA construction was done in the pBE2113-GUS binary T-DNA vector, which includes a kanamycin-resistance gene as a selective marker (Fig. 13c; Mitsuhrara et al., 1996). A fragment of carrot cDNA of the C-ABI3 gene mentioned above (Fig. 4) that included the putative open reading frame was cloned in pBE2113-GUS by replacement of the Xba I-Sac I fragment of the coding region of the gene for GUS. The cDNA fragment was placed under the control of the El2Ω promoter. The binary vector, pEl2Ω-CABI3, was transferred into E. coli strain DH5 alpha (Fig. 13d).
By triparental mating using *E. coli* strain HB101 (pRK2013) as described above (Bevan, 1984), the binary vectors were transferred to *Agrobacterium tumefaciens* strain LBA4404 (pAL4404) and *Agrobacterium tumefaciens* strain C58ClRif*E* (pGV2260) which shows tolerance to ampicillin (100 mg/l) and rifampicin (100 mg/l) (Deblaere et al., 1985). The clones of *Agrobacterium* which contained both pGV2260 and pBI121-CABI3 or pBE2113-GUS were selected by tolerance to kanamycin (200 mg/l), ampicillin (100 mg/l) and rifampicin (100 mg/l). The clones of *Agrobacterium* which contained both pAL4404 or pGV2260 and pBI121-CABI3 or pBE2113-GUS were used for infection.

**Transformation of carrot hypocotyls**

Hypocotyl segments (1 cm) from carrot seedlings were infected with *Agrobacterium* and then kanamycin-resistant embryogenic cells were induced from the infected hypocotyl segments on MS medium that contained kanamycin (100 mg/l) and 2,4-D (1 mg/l) (Fujii and Uchimiya, 1991). Transgenic somatic embryos were induced from the embryogenic cells in phytohormone-free MS medium that contained kanamycin (100 mg/l). Mature leaves of transgenic carrot plants were grown for two months from the transgenic somatic embryos on semi-solidified (0.2% Gelrite™) MS medium at 25°C with 16 hours of light daily (approximately 40 photons·m⁻²·s⁻¹).

**Transfer of the gene to carrot non-embryogenic cells**

I had established newly the method for transformation of carrot non-embryogenic cells (NC) using *Agrobacterium*. Cells in a seven-day-old culture of NC in liquid MS medium containing 2,4-D (1 mg/l) (MSD), were co-cultivated at 25°C on a gyratory shaker
(100 rpm) for 3 days with Agrobacterium that had been cultured at 28°C for 24 hours. Then, Claforan™ (500 mg/l) (Hoechst, Tokyo, Japan) was added to the culture and the culture was continued for 4 days under the same condition as mentioned above. Then, kanamycin-resistant NC were selected by successive subculture at two-week intervals in the following media: MSD containing 500 mg/l Claforan™ and 20 mg/l kanamycin twice; MSD containing 500 mg/l Claforan™ and 50 mg/l kanamycin twice; MSD containing 50 mg/l kanamycin twice; and MSD containing 100 mg/l kanamycin four times. The selected NC were maintained by subculture at two-week intervals in MSD medium that contained 100 mg/l kanamycin under the same condition as mentioned above.

Treatment with ABA

A stock solution of ABA (1 x 10⁻² M) in distilled water (DW) was sterilized by a passage through filter with 0.20-μm pores.

Mature leaves of transgenic and non-transgenic plants were sprayed with 1 x 10⁻⁴ M ABA and incubated for 8 hours in darkness (Kiyosue et al., 1992b). Mature leaves without ABA-treatment were sprayed with DW and incubated for 8 hours in darkness.

Transgenic and non-transgenic NC were transferred to 100 ml aliquots of liquid MSD medium that contained ABA at 1 x 10⁻⁵ M or 1 x 10⁻⁴ M and cultured on a gyratory shaker (100 rpm) at 25°C for 24 hours, 7 days, 10 days, 12 days or 14 days in darkness, as indicated.

Somatic embryos were transferred to 100 ml aliquots of liquid MS medium that contained ABA at 3.7 x 10⁻⁶ M and cultured on a gyratory shaker (75 rpm) at 25°C for 24 hours in darkness (Kiyosue et al., 1992b).
Desiccation treatment and determination of viability

The viability of desiccated NC was determined by staining with fluorescein diacetate (FDA) (Widholm, 1972).

Transgenic and non-transgenic NC with or without ABA-treatment were transferred to centrifuge tubes, collected by centrifugation (at 100 xg) and washed three times with sterilized DW. The NC were suspended in DW at 250 ml of packed cell volume (at 100 xg) per liter. One ml of the suspension of NC was spread on a sterilized filter paper (diameter, 7 cm). The NC on the filter paper were placed on silica gel (approximately 10 g) in a Petri dish (diameter, 9 cm) and incubated for several minutes. After desiccation treatment, the NC on the paper were rehydrated and cultured on semi-solidified (0.2% Gelrite™) MSD medium at 25°C for 7 days with 16 hours of light daily (approximately 40 photons·m⁻²·s⁻¹). After 7 days in culture, the NC were transferred to a centrifuge tube with phytohormone-free MS liquid medium, a stock solution of FDA in acetone was added (final concentration, 0.2 mg/l) and the tube was incubated at 25°C for 10 min. The stained NC were washed with MS liquid medium three times by centrifugation (at 100 xg) and then suspended in MS medium. They were observed with a fluorescence microscope (model BHS-RFK with filter system B; OLYMPUS, Tokyo, Japan). Only cells emitting the brightest green fluorescence were considered to be surviving cells. The viability of cells was determined by averaging the percentage survival of randomly selected populations of more than 200 cells.
Results

Nucleotide sequence of cDNA clones of the CAISE genes

CAISE1, CAISE2, CAISE4 and CAISE6 were highly homologous to carrot genes, GEA6 (putative Dehydrin), ECP31, EMB1 and ECP40, respectively (Table 2; Ulrich et al., 1990; Kiyosue et al., 1992b and 1993; Wurtele et al., 1993; Lin et al., 1996). Then, I also renamed or recognized CAISE1, CAISE2, CAISE4 and CAISE6 as DcDhhydrin1 (DcDHN1), ECP31, DcEMB1 and ECP40, respectively (Table 2). Putative amino acid sequence of CAISE3 showed significant homology to those of the Em genes of mung bean, Arabidopsis and wheat, and then I renamed it as DcEm (Table 2; Futers et al., 1993; Gaubier et al., 1993, Manickam et al., 1996). CAISE5 showed significant homology to glucose and ribitol dehydrogenase homologs, pG31 of barley and TLPCB6 of Trollius ledebourii (Table 2; Alexander et al., 1994; Bailey et al., 1996).

Northern blot analysis of expression of the ECP and CAISE genes

In developing seeds, strong expression of the C-ABI3 gene was observed after 20 DAF, and saturated on 35 DAF (Fig. 14). ECP31, DcEm, DcEMB1, CAISE5, ECP40 and ECP63 expressed on 29 DAF, 35 DAF and 65 DAF in developing seeds, but not on 11 DAF, 14 DAF, 17 DAF and 20 DAF in seeds (Fig. 14). Expression of the ECP31 and DcEMB1 gene increased gradually during seed development, but expression of the DcEm, CAISE5, ECP40 and ECP63 gene showed a peak on 35 DAF (Fig. 14). DcDHN1 expressed throughout seed
development and the expression was very strong on 11 DAF, 17 DAF and 20 DAF (Fig. 14). The signals on 11 DAF and 17 DAF were smaller than those on other DAF (Fig. 14). After 29 DAF, expression of the DcDHNI gene increased gradually (Fig. 14).

**Northern blot analysis in transgenic carrot**

I obtained eight transgenic cell lines and analyzed three of them (line 1, 2 and 3). The morphological features of the transgenic plants were comparable to those of non-transgenic plants (data not shown).

In non-transgenic plants, no expression of the C-ABI3 gene was detected in mature leaves irrespective of ABA-treatment (Fig. 15). By contrast, in transgenic plants, its expression was detected in mature leaves irrespective of ABA-treatment (Fig. 15), and the level of expression was not increased by exogenously applied ABA (Fig. 15).

Using ECP31 and ECP63 as probes and mRNA from non-transgenic plants, I detected no or slight expression of the corresponding transcripts in mature leaves with or without ABA-treatment (Fig. 15). In transgenic plants, strong expression of these transcripts was detected in ABA-treated mature leaves but not in ABA-untreated mature leaves (Fig. 15).

Using ECP40 as a probe and mRNA from non-transgenic plants, I detected one band in the ABA-treated mature leaves, but not in ABA-untreated ones (Fig. 15). By contrast, using mRNA from transgenic plants, I detected two bands in the ABA-treated mature leaves, but not in the ABA-untreated ones (Fig. 15). The smaller mRNA (approximately 0.9 kb) was the same size as the mRNA detected
in ABA-treated non-transgenic mature leaves (Fig. 15), and the
bigger mRNA (approximately 1.1 kb) was the same size as the mRNA
detected in embryogenic cells and ABA-treated somatic embryos
(Fig. 15; data not shown).

Using DcEm as a probe, in both non-transgenic and transgenic
plants, the expression was detected in mature leaves with ABA-
treatment, but not in those without ABA-treatment (Fig. 15). In
transgenic plants, the level of expression of the DcEm gene in
mature leaves with ABA-treatment was same or stronger as compared
to that in non-transgenic plants (Fig. 15).

Using DcDHNI, DcEMBl and CAISE5 as probes, the expression
of them was detected in mature leaves with ABA-treatment, but not
in those without ABA-treatment in both transgenic and non-
transgenic plants (Fig. 15). In transgenic plants, expression of
them was weaker than that in non-transgenic plants (Fig. 15).

Transgenic non-embryogenic cells

Non-transgenic non-embryogenic cells (NC) were resistant to
Claro® (500 mg/l), but not to kanamycin (20–200 mg/l; data not
shown). Non-transgenic NC did not grow in MSD medium containing
kanamycin (100 mg/l) at 25°C on a gyratory shaker (100 rpm) in
darkness and did not accumulate yellow pigments (Fig. 16a). For
selection of transgenic cells, I increased the concentration of
kanamycin gradually, because in my preliminary experiments, when
Agrobacterium-treated NC were directly cultured in medium with a
high concentration of kanamycin (100 mg/l), no proliferation of
cells occurred (data not shown). Finally, by gradual increase of
the concentration of kanamycin and using Agrobacterium strain
C58ClRif\(^{}\) (pGV2260), I obtained a batch culture of transgenic NC that was resistant to kanamycin (100 mg/l) (line 491S1; Fig. 16a). Under certain culture conditions, the appearance and growth characteristics of the transgenic NC were same as those of non-transgenic NC (Fig. 16b and 16c).

After ABA-treatment, any difference of the morphological aspects was not observed between non-transgenic and transgenic NC (Fig. 16b, 16c, 16d and 16e).

**Northern blot analysis in transgenic or non-transgenic non-embryogenic cells**

In non-transgenic NC, no expression of the C-ABI3 gene was detected irrespective of ABA-treatment (Fig. 17). By contrast, in transgenic NC, expression of the C-ABI3 gene was detected irrespective of ABA-treatment and the level of expression of the transcripts did not increase by ABA-treatment (Fig. 17).

Using ECP31, ECP40 and ECP63 as probes and mRNA from non-transgenic NC, any expression of the corresponding transcripts was not observed irrespective of ABA-treatment (Fig. 17). In transgenic NC, expression of these transcripts was detected irrespective of ABA-treatment and the level of the expression increased with higher concentration of ABA applied (Fig. 17).

Using DcEm as a probe, the expression was detected in transgenic NC with ABA-treatment, but not in transgenic NC without ABA-treatment nor in non-transgenic NC irrespective of ABA-treatment (Fig. 17). The expression in transgenic NC treated with 1 x 10\(^{-4}\) M ABA was stronger than that in transgenic NC treated with 1 x 10\(^{-5}\) M ABA (Fig. 17).
Using *DcDHN1*, *DcEMBl* and *CAISE5* as probes, no expression of them was detected in non-transgenic and transgenic NC irrespective of ABA-treatment (Fig. 17).

Using total mRNA from embryogenic cells as a positive control, I detected expression of the *C-ABI3*, *ECP31*, *ECP40*, *DcEm*, *DcDHN1*, *DcEMBl*, *CAISE5* and *ECP63* genes (Fig. 17).

Using total mRNA from somatic embryos with or without ABA-treatment, expression of the *C-ABI3* gene was detected irrespective of ABA treatment, and the level of the expression did not increase by ABA-treatment (Fig. 17). The expression of the *ECP* and *CAISE* genes was detected in somatic embryos irrespective of ABA-treatment and the level of the expression increased by ABA-treatment (3.7 x 10^{-6} M, 24 hours) (Fig. 17).

**Desiccation tolerance of non-embryogenic cells**

Both transgenic and non-transgenic NC treated with ABA at 1 x 10^{-5} M for 7-14 days and at 1 x 10^{-4} M for 7-10 days did not proliferate after 25-min desiccation treatment (data not shown). Transgenic NC treated with ABA at 1 x 10^{-4} M for 12 days or 14 days showed cell proliferation even after 30-min desiccation treatment (Fig. 19i; data not shown). By contrast, transgenic NC without ABA-treatment and non-transgenic NC with or without ABA-treatment did not proliferate after 30-min desiccation treatment (Fig. 19d and 19i; data not shown). Transgenic NC treated with ABA at 1 x 10^{-4} M for 14 days grew normally after 20-min desiccation treatment, while transgenic NC without ABA-treatment and non-transgenic NC with or without ABA-treatment proliferated in a limited extent after 20-min desiccation treatment (Fig. 19b and 19g).
Non-transgenic and transgenic NC treated with ABA at $1 \times 10^{-4}$ M for 14 days were subjected to staining with fluorescein diacetate (FDA) after desiccation treatment. Non-transgenic NC with or without ABA-treatment did not survive after 30-min of desiccation treatment (Fig. 20a). The viability of transgenic NC treated with ABA was higher than that of transgenic NC without ABA-treatment and non-transgenic NC with or without ABA-treatment after desiccation treatment for 20 min, 25 min or 30 min (Fig. 20a and 20b). Specifically, the viability of transgenic NC treated with ABA was very high after desiccation treatment for 25 min or 30 min as compared to that of other samples (80% and 50%; Fig. 20a and 20b). After desiccation treatment for 40 min, both transgenic NC and non-transgenic NC with or without ABA-treatment did not survive (Fig. 20a and 20b).
Discussion

Sequence and expression analysis of the ABA-inducible genes

Six CAISE genes showing enhanced expression in ABA-treated somatic embryos has been isolated by differential screening (Table 2; Fig. 17). Four of them showed high homology to corresponding carrot genes (Table 2). It was reported that ECP31 and ECP40 were isolated from carrot embryogenic cells, and expression of them was observed in embryogenic cells, later phase of developing seeds and ABA-treated somatic embryos (Fig. 14 and 17; Kiyosue et al., 1992b and 1993). ECP31 showed high homology to cotton LEA D34 (Kiyosue et al., 1992b), and ECP40 showed high homology to rice RAB16, maize Dehydrin, barley Dehydrin and cotton LEA D11 (Kiyosue et al., 1993). DcDHN1 might be one of Dehydrin/Rab gene family, because it showed very high homology to GEA6 which shows homology to some Dehydrin genes (Table 2; Lin et al., 1996). GEA6 expressed in embryogenic callus and somatic embryos, and the expression is stronger in callus and heart-shaped embryos (Lin et al., 1996). DcEMBl might be a same gene as carrot EMB1, because they showed very high homology each other and were isolated from the different cultivars of carrot (Table 2; Ulrich et al., 1990). It was reported that the expression of the EMB1 gene begins in globular somatic embryos and increases in torpedo-shaped somatic embryos, and the sequence of the EMB1 gene shows homology to cotton LEA D19 and wheat Em (Wurtele et al., 1993). In general, expression and accumulation of LEA proteins appear in later phase
of seed development, and LEA protein might function on maintaining the stability of cellular components during desiccation (Delseny et al., 1993 for a review). Generally, it is thought that LEA proteins and stress proteins such as heat shock proteins might maintain the stability of cellular membrane and proteins by correctly refolding proteins which are denatured on account of water loss (Delseny et al., 1993 for a review). Thus, the four genes isolated in this work belong to LEA or Dehydrin/Rab gene family, and their products might protect cells against dehydration.

CAISE5 showed homology to glucose and ribitol dehydrogenase homologs, barley pG31 and Trollius ledebourii TLPCB6 (Table 2; Alexander et al., 1994; Bailey et al., 1996). Expression of the pG31 gene was observed specifically during embryogenesis, but did not increase by endogenous or applied ABA during maturation phase (Alexander et al., 1994). Expression of the TLPCB6 gene was observed in dry seed and decreased during germination, and the decrease of the expression was enhanced by the treatment with gibberellic acid (GA) (Bailey et al., 1996). In barley, products of the pG31 gene had an activity as a glucose specific dehydrogenase which produces D-glucoic acid from D-glucose with NAD(P)⁺, but the physiological function is not clear (Bailey et al., 1996). Moreover, in barley, it was reported that an ABA-related aldose reductase which produces glycerol from glyceraldehyde with NADP⁺, and suggested that aldose reductase is considered as a key enzyme in the polyol pathway leading to the accumulation of sorbitol as a common cell osmolyte which helps to
balance the osmotic strength of the cytoplasm (Bartels et al., 1991). One of some interesting observations which is associated with acquisition of desiccation tolerance in seeds is accumulation of several sugars which maintain the stability of membranes by replacing the water molecules at charged membrane surfaces (Crowe et al., 1984). Furthermore, it is also suggested that carbohydrates and sugars might conserve structures of tissues and cells influencing the occurrence of glasses, liquid of high viscosity, which stop all chemical reactions relating to molecular diffusion during seed dehydration (Williams and Leopold, 1989; Bruni and Leopold, 1991). Thus, it is considered that CAISE5 might function as a glucose (or ribitol) dehydrogenase with other enzymes which might be related to carbohydrate metabolism, and involve in acquisition of desiccation tolerance in ABA-treated somatic embryos (Alexander et al., 1994).

The ECP63 and five of CAISE genes without the DcDHMI gene expressed after 29 DAF specifically in developing seeds, and the expression of the ECP63 and all CAISE genes increased during seed maturation after 29 DAF (Fig. 14). In carrot seeds, desiccation started from 38 DAF (Fig. 11a; Shiota et al., 1998). Level of endogenous ABA increased from 23 DAF and showed a transient peak on 29 DAF in carrot seeds, and zygotic embryos developed to the torpedo-shaped stage on 26 DAF (Fig. 11a and 11b; Shiota et al., 1998). These facts indicate that the ECP63 and all CAISE genes might be induced by endogenous ABA which increased in developing seeds. Thus, it is suggested that these genes might be involved in ABA-induced desiccation tolerance in somatic and zygotic embryos.
On the other hand, the expression patterns of these genes in developing seeds was classified into two groups. One of them showed a peak of expression on 35 DAF (ECP40, ECP63, DcEm and CAISE5) and another showed a peak of expression on 65 DAF (ECP31, DcEMB1 and DcDHNI) (Fig. 14). This fact suggests the possibility that these groups might share the functions during seed maturation.

In the case using DcDHNI as a probe in developing seeds, detected signals might show the presence of at least two different transcripts which could be cross-hybridized each other, because detected signals showed slightly different sizes (Fig. 14). In higher plants, Dehydrin/Rab gene family was generally consisted of several Dehydrin/Rab genes. In fact, ECP40 is able to be recognized to one kind of Dehydrin/Rab gene family in carrot (Kiyosue et al., 1993). Therefore, the expression pattern of DcDHNI in developing seeds might be occurred by cross-hybridization to transcripts of other Dehydrin/Rab genes. On the other hand, the detected signals after 29 DAF might be transcripts which might be induced by programmed increase of endogenous ABA during seed development, because expression pattern of DcDHNI after 29 DAF was very similar to that of ECP31 and DcEMB1 (Fig. 14). The expression on 11-20 DAF might be induced by environmental factor(s) directly, because the seeds (fruits) of carrot were consisted of pericarp, nucelli, endosperms and embryos, and the exterior pericarp might be affected by several environmental factors (e.g. low temperature or slightly drought). It is reported that the expression of maize RAB17 and Arabidopsis RAB18, two of
Dehydrin/Rab gene family, in vegetative tissues was induced by not only ABA but also osmotic stress directly, and suggested alternative pathways involving different molecular mechanisms in vegetative tissues and embryos (Vilardell et al., 1994). Therefore, it is also considered that the expression of the DcdHNI gene on 11-20 DAF might relate to environmental conditions, but that on 29-65 DAF might relate to seed maturation program.

The CAISE and ECP genes which I analyzed in this work expressed in ABA-treated somatic embryos, embryogenic cells and later phase of seed development (Fig. 14 and 17). These cells and tissues show desiccation tolerance which is induced by endogenous or exogenous ABA (Kaimori and Ishihara, 1991; Kiyosue et al., 1992a; Iida et al., 1992). These results suggest that CAISEs and ECPs might be involved in ABA-induced desiccation tolerance in zygotic and somatic embryos.

In maize, expression of the VPI gene was observed at least after 10 days after pollination (DAP) in zygotic embryos, and showed a peak at 16 DAP and then decreased gradually until seeds grew to maturity (McCarty et al., 1991). Expression of the Em gene was observed at 16 DAP and kept at relatively high levels throughout the late stage of embryo development (McCarty et al., 1991). These results suggest that VPI controls expression of the Em gene at the transcriptional level. In carrot developing seeds, the strong expression of the C-ABI3 gene was observed after 20 DAF and kept high level until dry seeds (62 DAF or 65 DAF; Fig 11c and 14). Expression of the CAISE and ECP genes in developing seeds was shown after 29 DAF with gradual increase (Fig. 14). During seed development, C-ABI3 was expressed prior to increase of
endogenous ABA and expression of the CAISE and ECP genes (Fig. 11a, 11c and 14; Shiota et al., 1998). These results indicate the possibility that the all CAISE and ECP genes might be controlled by C-ABI3 and ABA in developing seeds at the transcriptional level.

**Transgenic carrot plants**

In mature leaves of transgenic plants named line 1, 2 and 3, ectopic expression of the C-ABI3 gene was induced successfully by CaMV 35S promoter (Fig. 15). The function of CaMV 35S promoter was not affected by ABA (Fig. 15).

Expression of the ECP31 and ECP63 genes was induced in transgenic mature leaves by ABA-treatment but not in non-transgenic leaves even after ABA-treatment (Fig. 15). The expression of the ECP31 and ECP63 genes was specifically detected in embryogenic cells, maturing seeds and ABA-treated somatic embryos in which C-ABI3 expressed (Kiyosue et al., 1992b; Shiota et al., 1998; Tachikawa et al., 1998; unpublished data). These results indicate that the expression of the ECP31 and ECP63 genes might be controlled by same pathway(s) that depends on C-ABI3 and ABA (Fig. 18b). Expression of the ECP40 gene was detected as two signals in transgenic mature leaves after ABA-treatment (Fig. 15). One of them, the smaller transcript, was not controlled by C-ABI3 but controlled by other unknown factor(s), because it was also detected in non-transgenic mature leaves after ABA-treatment (Fig. 15, 18a and 18b). On the other hand, the presence of the larger transcript depended on C-ABI3 and ABA, because it was detected only in transgenic leaves with ABA-treatment, but not in non-
transgenic leaves even after ABA-treatment (Fig. 15, 18a and 18b). These results were supported by the fact that the transcript detected in embryogenic cells and ABA-treated somatic embryos was the same size as the larger transcript (data not shown; Kiyosue et al., 1993). These results strongly indicate that C-ABI3 is involved in the control of the expression of some embryo-specific ABA-inducible genes, such as the ECP genes, in carrot somatic and zygotic embryos (Fig. 18e), and support the findings obtained in zygotic embryos of maize, Arabidopsis, rice and kidney bean (McCarty et al., 1991; Hattori et al., 1994; Parcy et al., 1994; Bobb et al., 1995; Hill et al., 1996; Carson et al., 1997).

Expression of the DcDHN1, DcEMBL and CAISE5 genes was induced by ABA-treatment in mature leaves of both non-transgenic and transgenic plants (Fig. 15). This indicates that C-ABI3 might not be involved in ABA-induced expression of the DcDHN1, DcEMB1 and CAISE5 genes, and other unknown factor(s) might exist and function on ABA-induced expression of them (Fig. 18a and 18b). On the other hand, it is suggested the possibility that C-ABI3 might function as a repressor on ABA-induced expression of the DcDHN1, DcEMB1 and CAISE5 genes (Fig. 18b), because expression of the DcDHN1, DcEMB1 and CAISE5 genes in transgenic plants was weaker than that in non-transgenic plants (Fig. 15). In maize, it was reported that VP1 function as a repressor on expression of alpha-amylase (Hoecker et al., 1995).

Expression of the DcEm gene was induced by ABA in mature leaves of both non-transgenic and transgenic plants, and the expression level in transgenic plants was the same as or stronger
than that in non-transgenic plants (Fig. 15). These results indicate that the expression of the \textit{DcEm} gene might be controlled by certain factor(s) and ABA, and C-ABI3 might be enhanced the expression (Fig. 18b). In maize, rice and Arabidopsis, the \textit{Em} or \textit{Em}-like genes are under the control of the VPI/ABI3 factor (McCarty et al., 1991; Hattori et al., 1994; Parcy et al., 1994). On the other hand, it was reported that two types of \textit{Em}-like genes exist and ABI3 involves in the expression of only one of them (Parcy et al., 1994). Thus, \textit{DcEm} and \textit{DcEMBl} might be \textit{Em}-like genes of carrot, and \textit{DcEm} might be a type of which the expression depends on C-ABI3 and \textit{DcEMBl} might be another type of which the expression does not depend on C-ABI3, judging from the results shown here and the facts reported in Arabidopsis (Fig. 15, 18a and 18b; Parcy et al., 1994).

Furthermore, all results which I reported in this work strongly suggest that the progression of maturation program and acquisition of desiccation tolerance required exogenous application of ABA in somatic embryos as in zygotic embryos.

\textbf{Transgenic non-embryogenic cells}

In transgenic NC, ectopic expression of the C-ABI3 gene was induced strongly and successfully by E12\cell{\omega} promoter (Fig. 17). The function of E12\cell{\omega} promoter was not affected by ABA (Fig. 17).

In transgenic NC, expression of the \textit{ECP31}, \textit{ECP40}, \textit{ECP63} and \textit{DcEm} gene was induced by ABA-treatment and level of the expression depended on the concentration of ABA applied (Fig. 17). This result supports the suggestion mentioned above that C-ABI3 involves in the control of the expression of some embryo-specific
ABA-inducible genes, such as ECP genes, in carrot somatic and zygotic embryos (Fig. 18d and 18e). Then, it is also supported that DcEm might be a type of Em-like gene of which the expression depends on C-ABI3 and DcEMBl might be another type of which the expression does not depend on C-ABI3. ABA-inducible expression of the DcDHN1, DcEMBl and CAISE5 genes was not exhibited in both non-transgenic and transgenic NC even after ABA-treatment (Fig. 17). This result indicates that C-ABI3 might not be involved in ABA-induced expression of the DcDHN1, DcEMBl and CAISE5 genes (Fig. 18c and 18d). Additionally, other unknown factor(s) which might control ABA-induced expression of the genes in mature leaves might not exist in NC, and then available function of C-ABI3 as a repressor in ABA-induced expression of the DcDHN1, DcEMBl and CAISE5 genes might be blind in transgenic NC (Fig. 18b, 18c and 18d). These facts also suggest that this other unknown factor(s) might be mainly involved in ABA-signal transduction in developed vegetative tissues (Fig. 18a).

By contrast, in transgenic NC that had not been treated with ABA, lower level of the expression of the ECP genes was observed (Fig. 17). The level of endogenous ABA in NC is very low (1.3-1.7% to embryogenic cells), but the level is still detectable (Kiyosue et al., 1992a). Therefore, expression of the ECP genes in transgenic NC in the absence of ABA-treatment might be induced by the small amount of endogenous ABA. It seems very likely that C-ABI3 in the transgenic NC might play an important role in expression of the ECP genes.

The transgenic NC acquired desiccation tolerance upon ABA-
treatment (Fig. 19 and 20b). This observation provides direct evidence for the involvement of C-ABI3 in the ABA-induced acquisition of desiccation tolerance. It also strongly suggests that C-ABI3 is involved in desiccation tolerance not only in carrot zygotic embryos but also in somatic embryos via expression of the ECP genes, as judged from the pattern of expression of the C-ABI3 gene (Fig. 10, 11c, 14 and 17).

The transgenic NC acquired desiccation tolerance by ABA-treatment at high concentration (1 x 10^{-4} M) for long duration (14 days) (Fig. 19 and 20b). This desiccation tolerance is weaker than that in somatic embryos induced by ABA-treatment at lower concentration (1 x 10^{-5} M) for shorter duration (7 days), because ABA-treated somatic embryos survived after at least 3 hours of desiccation, but ABA-treated transgenic NC did not survive after desiccation more than 30 min (Fig. 19 and 20b; Iida et al., 1992). In ABA-treated somatic embryos, ECP31, ECP40, ECP63 and DcEm of which the expression was controlled by C-ABI3, and DcDHNI1, DcEMB1 and CAISE5 of which the expression was not controlled by C-ABI3 were expressed and accumulated by ABA-treatment (Fig. 17 and 18e). However, in ABA-treated non-transgenic NC, ECP31, ECP40, ECP63 and DcEm of which the expression was controlled by C-ABI3 were expressed and accumulated by ABA-treatment, but DcDHNI1, DcEMB1 and CAISE5 of which the expression was not controlled by C-ABI3 were not (Fig. 17 and 18d). These results suggest that the difference on strength of ABA-induced desiccation tolerance between somatic embryos and transgenic NC might be the difference on expression pattern of these ABA-inducible genes and on accumulation of their products (Fig. 18d and 18e). Therefore, weak
desiccation tolerance in transgenic NC might be acquired by ABA-treatment at high concentration for long duration through insufficient accumulation of some components which involve in protection of cells against damages by desiccation. Furthermore, it is also suggested that the main reason for the absence of desiccation tolerance in NC might be loss of C-ABI3 and endogenous ABA (Fig. 10, 17 and 18c; Kaimori and Ishihara, 1991; Kiyosue et al., 1992a), and ABA-induced desiccation tolerance in zygotic and somatic embryos might be induced by enough accumulation of several kinds of components which are involved in protection against cell damage by desiccation such as LEA proteins, heat shock proteins and sugars. In order to recognize other factor(s) which controls expression of the DcDHNL, DcEMBL and CAISE5 genes, expression analysis of them in some Arabidopsis mutant (e.g. abi4, abi5, fus3 or lecl; Finkelstein, 1994; Keith et al., 1994; Meinke et al., 1994) should be examined.

Summarizing the results and discussions in Part 3, it is suggested that C-ABI3 involves in ABA-induced desiccation tolerance through controlling of expression of some ABA-inducible genes in not only zygotic embryos but also somatic embryos and embryogenic cells, and C-ABI3 is an ortholog of the VP1/ABI3 factor in carrot.
General Discussion
In this study, I suggested that C-ABI3 involves in ABA-induced desiccation tolerance as an ortholog of the VP1/ABI3 factor in not only zygotic embryos but also somatic embryos and embryogenic cells. It is a novel suggestion that the VP1/ABI3 factor exists and functions in somatic embryos and cultured cells. Especially, I showed that the transgenic NC in which C-ABI3, as one of the VP1/ABI3 factor, expressed ectopically acquired desiccation tolerance by ABA-treatment (Fig. 19 and 20b), and it is the first direct evidence on acquisition of desiccation tolerance involving the VP1/ABI3 factor and ABA in cellular level.

In the field of investigation of ABA-signal transduction, it has been reported that there are several trans-acting factors binding to cis-acting regulatory elements (ABA responsive element; ABRE) on promoter regions of ABA-inducible genes, such as wheat EmBP-1 binding to Em promoter (Guiltinan et al., 1990), tobacco TAF-1 binding to rice RAB16 promoter (Oeda et al., 1991), tobacco TGAla binding to pea PSL promoter (Pater et al., 1993), rice OSBZ8 binding to OsEm promoter (Nakagawa et al., 1996), kidney bean ROM2 binding to MAT2, PHSB and DLEC2 promoter (Chern, et al., 1996), sunflower DPBF-1, 2 and 3 binding to carrot Dc3 promoter (Kim, et al., 1997; Kim and Thomas, 1998) and others (Vilardell et al., 1991; Goupil et al., 1992; Pla et al., 1993). These all trans-acting factors (proteins) are some kinds of basic leucine zipper proteins (bZIP proteins). In addition, EmBP-1, TAF-1, OSBZ8, ROM2, DPBF-1, -2 and -3 belong to G-box binding factor type bZIP proteins (GBF-type bZIP proteins). Thus GBF-type bZIP
proteins might be main trans-acting factors on ABA-signal transduction (Busk and Pagès, 1998; Schwechheimer and Bevan, 1998 for reviews). Furthermore, it is considered that the conserved sequence of ABRE might be 8-10 bp with central ACGT (Busk and Pagès, 1998 for a review). The binding style of GBF-type bZIP proteins to ABRE is not clear and it is thought the possibility that homo-dimer or hetero-dimer of GBF-type bZIP proteins or hetero-dimer of GBF-type bZIP proteins and other types of bZIP proteins might be formed to bind to ABRE (Hattori, personal communication; Busk and Pagès, 1998 for a review).

Recently, it was reported that other cis-acting regulatory elements, such as coupling element (CE1 on barley HVA22 promoter and CE3 on barley HVA1 promoter), motifIII on rice RAB16b promoter, motifA on rice OsEm promoter, RY on winged bean WCl-3b promoter etc., are shown on promoter regions of ABA-inducible genes with ABRE (Shen and Ho, 1995; Ono et al., 1996; Shen et al., 1996; Sakata et al., 1997; Busk and Pagès, 1998 for a review). It is suggested that both ABRE and these other cis-acting elements might be necessary for seed-specific ABA-signal transduction (Hattori, personal communication).

DNA binding activity of the VP1/ABI3 factor has been reported only in one case that the B3 domain of VP1 binds to Sph-box on C1 promoter (Suzuki et al., 1997). Generally, it is considered that the VP1/ABI3 factor might not bind directly to the promoter regions of LEA genes, and might function as an activator on the expression in combination with other trans-acting factor(s) such as GBF-type bZIP proteins which bind to ABRE and other cis-acting
elements (Busk and Pagès, 1998; Schwechheimer and Bevan, 1998 for reviews). In fact, there are some reports indicating that binding activity and transcriptional activity of EmBP-1 or ROM2, GBF-type bZIP proteins, are affected by VP1 or PvALF (Chern et al., 1996; Hill et al., 1996; Busk and Pagès, 1997; Carson et al., 1997; Hollung et al., 1997; Razik and Quatrano, 1997).

On the other hand, the acquisition of desiccation tolerance in carrot somatic embryos is stage-specific during embryo development (Iida et al., 1992). Acquisition of the tolerance occurred when torpedo-shaped somatic embryos are treated with ABA but not when globular or heart-shaped somatic embryos are treated with ABA (Iida et al., 1992). In developing somatic embryos, expression of the C-ABI3 gene was observed throughout the development of somatic embryos (Fig. 10). These results suggest that the acquisition of ABA-induced desiccation tolerance in somatic and zygotic embryos might be controlled by the simultaneous action of C-ABI3 and some other unknown factor(s) which determines the stage specificity of the acquisition of desiccation tolerance. It is reported the successful isolation of some mutants (loci) showing seed-specific ABA-insensitivity such as abi4 and abi5, and seed-specific desiccation intolerance and no seed dormancy such as fus3 and lec1 in Arabidopsis (Finkelstein, 1994; Keith et al., 1994; Meinke et al., 1994). It is also suggested that FUS3 and LEC1 act with ABI3 in concert to control aspects of seed dormancy regulating gene expression (Parcy et al., 1997). More recently, ABI4, LEC1 and FUS3 are reported to be a putative transcriptional factor which contains DNA binding
domain APETALA2, a putative transcriptional factors which contain CCAAT box-binding factor HAP3 subunit and a putative transcriptional factor which contains B3-like domain of the VP1/ABI3 factor, respectively (Finkelstein et al., 1998; Lotan et al., 1998; Luerßen et al., 1998). Thus, these factors such as ABI4, ABI5, FUS3, LECl or other unknown factor(s) might determine stage-specificity of desiccation tolerance, or be involved in ABA-signal transduction not relating to the VP1/ABI3 factor. It is also considered that this might be one of the reasons why ABA-induced desiccation tolerance of transgenic NC expressing C-ABI3 were weaker than that of somatic embryos (Fig. 19 and 20b).

To investigate seed-specific desiccation tolerance and ABA-signal transduction, I have to consider involvement of other (unknown) factors not only the VP1/ABI3 factor. Furthermore, the VP1/ABI3 factor and other (unknown) factors might function by interacting each other complicatedly. For physiological analysis and analysis of protein-protein interactions, carrot somatic embryos are provided as a useful system because a large quantity of embryos which are synchronized to the same stage can be collected easily, and somatic embryos respond to exogenous ABA (Iida et al., 1992). Therefore, I propose this study as the first step on investigation of embryo-specific ABA-signal transduction at cellular and protein level, because somatic embryos are suggested to have the same function as zygotic embryos on ABA-signal transduction. Thus, somatic embryogenesis can be used as not only a morphological model but also a functional model of zygotic embryogenesis.
There are some reports indicating that the VP1/ABI3 factor functions as an inducer of ABA-inducible genes in maize, Arabidopsis, rice and kidney bean (McCarty et al., 1989; Kriz et al., 1990; Nambara et al., 1992; Butler and Cuming, 1993; Finkelstein, 1993; Hattori et al., 1994 and 1995; Paiva and Kriz, 1994; Parcy et al., 1994; Bobb et al., 1995). In carrot, C-ABI3 functioned as an inducer of at least four ABA-inducible genes (Fig. 15, 17 and 18). On the other hand, there is a report indicating that the VP1/ABI3 factor functions as an repressor of gene expression for alpha-amylase in maize (Hoecker et al., 1995). The function is reasonable, because the expression of alpha-amylase is induced by GA-treatment and repressed by ABA-treatment (Hoecker et al., 1995). In carrot, C-ABI3 functioned as a repressor on expression of at least three ABA-inducible genes which were non-embryo-specific (Fig. 15, 18b and 18e). These facts suggest a novel hypothesis that C-ABI3 functions not only as an inducer on expression of embryo-specific ABA-inducible genes but also as a repressor on expression of non-embryo-specific ABA-inducible genes (Fig. 18b and 18e). The function as a repressor might mean to avoid over-expression of the non-embryo-specific ABA-inducible genes whose expression is induced strongly in mature plantlets, and to make a balanced level of expression of embryo-specific ABA-inducible genes and that of non-embryo-specific ABA-inducible genes.

Desiccation tolerance in somatic and zygotic embryos is specific and different to that in mature plantlets, because that
in zygotic and somatic embryos is very strong (Iida et al., 1992), but seedlings and mature leaves of carrot could not tolerate to hard desiccation (data not shown). Generally, dry seeds of higher plants can be stored under the desiccated conditions for a very long during by maintaining themselves in dormancy situation, but mature plantlets can not. In the case of carrot somatic embryos, desiccation tolerance was induced by ABA-treatment and level of water content in desiccated somatic embryos was very low (5-10%; Iida et al., 1992; Shiota et al., 1999). Furthermore, the desiccated somatic embryos could be stored at -25°C under the desiccated condition for long duration (at least 169 weeks; Shiota et al., 1999). Therefore, the desiccation tolerance in somatic and zygotic embryos is suggested to be caused by tolerance to water loss of cells. On the other hand, mature plantlets could not tolerate to hard water loss. In leaves of higher plants, stomatal closure is occurred by desiccation stress or ABA which was induced by desiccation, in order to decrease water loss. Thus, desiccation tolerance in mature plantlets might function mainly for avoiding water loss and keeping water content.

ABA in mature plantlets is biosynthesized by environmental stresses such as drought, low temperature, and others (Kende and Zeevaart, 1997 for a review). On the other hand, ABA in seeds was biosynthesized in maternal tissues according to the developmental program or the seed maturation program, and transferred into embryos (Dure III, 1975 for a review). Thus, in higher plants, ABA might be involved in two different situation. In this study, it is suggested that C-ABI3 is involved in embryo-specific ABA-signal
transduction, and C-ABI3 can function also in mature leaves when it was expressed ectopically (Fig. 15 and 18b). These results indicate that C-ABI3 might be able to be involved in two different ABA-signaling and suggest that C-ABI3 might give embryo-specific characters on ABA-signal transduction to cells and tissues.
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Tables and Figures
Table 1. The exhibited identity at the nucleotide (nuc.) and amino acid (aa) levels among C-ABI3, ABI3, VP1, OSVP1 and PvAlf.

(a) The entire open reading frame

<table>
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<th>Arabidopsis</th>
<th>Maize</th>
<th>Rice</th>
<th>Kidney bean</th>
<th>Carrot</th>
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<tr>
<td></td>
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<td>OSVP1</td>
<td>PvAlf</td>
<td>C-ABI3</td>
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<td>aa nuc.</td>
<td>aa nuc.</td>
<td>aa nuc.</td>
<td>aa nuc.</td>
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<td>35.4 48.3</td>
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<tr>
<td>Maize</td>
<td>VP1</td>
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<td>—</td>
<td>64.0 71.6</td>
<td>40.6 50.1</td>
</tr>
<tr>
<td>Rice</td>
<td>OSVP1</td>
<td>—</td>
<td>—</td>
<td>—     35.8 50.5</td>
<td>34.6 50.6</td>
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<tr>
<td>Kidney bean</td>
<td>PvAlf</td>
<td>—</td>
<td>—</td>
<td>—     —</td>
<td>—     41.7 55.9</td>
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(b) The B3 conserved region

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<td>OSVP1</td>
<td>PvAlf</td>
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</tr>
<tr>
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<td>aa nuc.</td>
<td>aa nuc.</td>
<td>aa nuc.</td>
<td>aa nuc.</td>
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<td>VP1</td>
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<td>93.6 92.1</td>
<td>84.5 73.0</td>
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<tr>
<td>Rice</td>
<td>OSVP1</td>
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<td>—</td>
<td>—     80.9 72.3</td>
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<tr>
<td>Kidney bean</td>
<td>PvAlf</td>
<td>—</td>
<td>—</td>
<td>—     —</td>
<td>—     78.4 74.2</td>
</tr>
<tr>
<td>Carrot</td>
<td>C-ABI3</td>
<td>—</td>
<td>—</td>
<td>—     —</td>
<td>—     —</td>
</tr>
</tbody>
</table>

(a): Identity (%) over the entire open reading frames.
(b): Identity (%) in the B3 conserved regions.
Table 2. Characterization of the CAISE genes.

<table>
<thead>
<tr>
<th>clone name</th>
<th>size of clone</th>
<th>size of mRNA</th>
<th>gene name</th>
<th>size of ORF</th>
<th>homologous protein product (species)</th>
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<td>CAISE1</td>
<td>771 bp</td>
<td>0.8 kb</td>
<td>DcDHN1</td>
<td>149 aa</td>
<td>GEA6 (carrot)</td>
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<tr>
<td>CAISE2</td>
<td>976 bp</td>
<td>1.1 kb</td>
<td>ECP31</td>
<td>256 aa</td>
<td>ECP31 (carrot)</td>
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<tr>
<td>CAISE3</td>
<td>778 bp</td>
<td>0.8 kb</td>
<td>DcEm</td>
<td>113 aa</td>
<td>EM (mung bean, Arabidopsis, wheat)</td>
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<td>0.6 kb</td>
<td>DcEMB1</td>
<td>91 aa</td>
<td>EMB1 (carrot)</td>
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<tr>
<td>CAISE5</td>
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<td>1.3 kb</td>
<td>CAISE5</td>
<td>291 aa</td>
<td>PG31 (barley), TLPCB6 (Trollius ledebouri)</td>
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<tr>
<td>CAISE6</td>
<td>1,120 bp</td>
<td>1.1 kb</td>
<td>ECP40</td>
<td>306 aa</td>
<td>ECP40 (carrot)</td>
</tr>
</tbody>
</table>

ORF: open reading frame
Fig. 1. Comparison of the nucleotide sequences between ABI3 and VP1 and design of the primers.

Common nucleotide sequences are indicated by asterisks. The regions as pNH401 (upper) and pNH402 (lower) are underlined. Each designed primer is indicated by each arrow.
Fig. 2. PCR amplification using carrot genomic DNA as a template and Southern blot analysis of the PCR product.

(a): Five-μl of the PCR product amplified from 1 mg carrot genome DNA as a template using AB31S and AB32A as primers was fractionated by poly-acrylamide (5.0%) gel electrophoresis and visualized by ethidium bromide staining (Lane 2). Hae III digested ØX174 DNA was used as a molecular weight marker (Lane 1).

(b): The fractionated PCR product was allowed to hybridize with the [32P]-labeled pNH402 (a partial cDNA fragment of ABI3) as a probe.
Fig. 3. Northern blot analysis of the expression of ABI3 homolog in carrot cells.

Twenty-μg samples of total RNA from somatic embryos (Lane 1), seedlings (Lane 2), non-embryogenic cells (Lane 3) and embryogenic cells (Lane 4) were fractionated by agarose (1.2%) gel electrophoresis and transcripts were allowed to hybridize with the $[^{32}P]$-labeled pNH402 (the B3 region of the ABI3 gene) as a probe. The blot was reprobed with 18S rRNA to provide an internal standard.
Fig. 4. Nucleotide sequence of the C-ABI3 cDNA and the deduced amino acid sequence.

The region cloned in pBI121 for plant transformation is single-underlined. One of the possible sites of initiator of translation is double-underlined.
Fig. 5. Comparison of the deduced amino acid sequences encoded in C-ABI3, ABI3, PvPAlf, VP1 and OSVP1.

Conserved and consensus amino acid sequences are shown as red and blue letters, respectively. Four conserved domains (A1, 33-49 amino acid; B1, 167-222 amino acid; B2, 376-409 amino acid; and B3, 499-619 amino acid in C-ABI3) are boxed.
Fig. 6. Southern blot analysis of carrot genomic DNA.

Ten-µg aliquots of carrot genomic DNA were digested with EcoRI, Hind III and Xba I and fractionated by agarose (0.7%) gel electrophoresis. Fragments were allowed to hybridize with the $^{32}$P-labeled cDNA of clone 491 as a probe.
Fig. 7. PCR amplification using carrot genomic DNA as a template.

(a): Five-μl of the PCR product amplified from 1 mg carrot genome DNA as a template using CABI31S and CABI31A as primers was fractionated by poly-acrylamide (5.0%) gel electrophoresis and visualized by ethidium bromide staining (Lane 2). Hae III-digested øX174 DNA was used as a molecular weight marker (Lane 1).

(b): Each 5-μl of the PCR product amplified from 1 mg carrot genome DNA as a template using CABI3in1S and CABI32A (lane 2), CABI3in1S and CABI3in1A (lane 3), and CABI3in3S and CABI3in5A (lane 4) as primers was fractionated by poly-acrylamide (5.0%) gel electrophoresis and visualized by ethidium bromide staining. Hae III digested øX174 DNA was used as a molecular weight marker (Lane 1).
Fig. 8. Nucleotide sequences of introns of the C-ABI3 gene in carrot genomic DNA.

Summary of carrot genomic clones which were amplified by PCR in Fig. 7 is shown. Five introns are exhibited as in1, in2, in3, in4 and in5. The consensus sequences for splicing at each end of the introns are underlined. The arrows show the primers for PCR amplification.
1836  CABI3in1S
AAAAAGCAGACAGATCGAGACGGGACACACAAACGACAGAAAGAAAAGA
K Q S N R R P A Q Q Q Q K K Q

1884  CABI31S  CABI32A
GGTTAAAAGGTGAGAAAGCAGGGAATTCGTTCAAAAGGTTGAGCAGATGTTGGG
G F K G E K N L K F L L Q K V L K Q S D V G

1885  CABI32A
TGTCTTGGAAGAATTGTAATGCGCTAAA  1977
C L G R I V W L P K

in1  GTTTGGCATTGTATGATTTTCTCCAGGCCTGACCAATGCGAGGAA
TGAATGTGTTCTGGTGTTTATTTAG

in2  GTAAAGTGTCCTGATCTTTCAAAAAATTTTGTTCATAGTTTAAGTTTCTTTTAGAATTATCTCAGTCTCAACCCGCTTTGATTAG
CATGCTAAAACCGCTTTGATTAG

in3  GTTACCAATGCTGAAAATTTTGAGATTTATATTATATATATATGTAATGCGAAATTACATTACGTTTGAC
ACTTACCTGTCCTCTTAAATACATTACATTACATTTTGGCTTTTAGAATCTGAG

in4  GTACAAATGCTTTCAAAAATTTGCGATATTATATTATATTATATATATTATGTAATGCGAATTACATTACGTTTGAC
TGTTTTAACTTTTACAG

in5  GTACGGGATATTTGAGATCTGCAATACATCTATATATAGTTGCTATTGTGGTCTGGTCTCTTATCCAAAGATGCGATTATGC
CTTATACGAGGGAGTTGGTTGGGAGGAAACTGAGCGGCAAGTACAGGAAA
L I R G V K V R Q P V K G K L E A K V T R K
CACCATA  2281  H H
Fig. 9. Schematic model of intron insertion positions among the VP1/ABI3 homologs.

Triangles show five intron insertion positions. Four conserved regions are boxed.
Fig. 10. Northern blot analysis of expression of the C-ABI3 gene in several tissues and developing somatic embryos.

Twenty-μg samples of total RNA from non-embryogenic cells (lane 1), embryogenic cells (lane 2), globular embryos (5-day-old somatic embryos, lane 3), heart-shaped embryos (7-day-old somatic embryos, lane 4), torpedo-shaped embryos (10-day-old somatic embryos, lane 5; 14-day-old somatic embryos, lane 6) and seedlings (lane 7) were fractionated by gel electrophoresis and transcripts were allowed to hybridize with the \(^{32}\mathrm{P}\)-labeled cDNA of clone 491 as a probe. The blot was reprobed with 18S rRNA to provide an internal standard.
C-ABI3

rRNA

2.9 kb
Fig. 11. Changes in levels of water and endogenous ABA during seed development and Northern blot analysis of expression of the C-ABI3 gene in developing seeds.

(a) Fresh weight (mg fresh weight per seed; closed circles), water content (percentage; open squares) and ABA content (pmol per seed fresh weight; open circles). The water content was calculated as the ratio of dry weight to fresh weight. ABA was extracted from seeds at various DAF and quantitated by the ELISA (Shiota et al., 1998).

(b) Morphological changes in zygotic embryos. Bars show 100 µm (Shiota et al., 1998).

(c) Northern blot analysis. Twenty-µg samples of total RNA from seeds at various DAF were fractionated by gel electrophoresis and allowed to hybridize with the $^{32}$P-labeled cDNA of clone 491 as a probe. The blot was reprobed with 18S rRNA to provide an internal standard.
Fig. 12. Northern blot analysis of expression of the C-ABI3 gene during stress-induced somatic embryogenesis.

Timing of sampling is shown as a schematic model. Twenty-μg samples of total RNA from embryogenic cells (lane 1), torpedo-shaped embryos (14-day-old somatic embryos, lane 2), apical tips after 4-day-treatment with 0.7 M sucrose (lane 3), apical tips after 14-day-treatment with 0.7 M sucrose (lane 4), apical tips of 14-day-culture on MS medium after 14-day-treatment with 0.7 M sucrose (lane 5) and apical tips of 14-day-culture on MS medium (lane 6) were fractionated by gel electrophoresis and allowed to hybridize with the [32P]-labeled cDNA of clone 491 as a probe. The blot was reprobed with 18S rRNA to provide an internal standard.
Fig. 13. Schematic drawings of the plasmids being constructed for transformation of plant cells.

(a): pBI121, (b): pBI121-CABI3 in which the GUS gene of pBI121 was replaced to the C-ABI3 gene, (c): pBE2113-GUS in which CaMV 35S promoter of pBI121 was replaced to El2Ω promoter, (d): pEl2Ω-CABI3 in which the GUS gene of pBE2113-GUS was replaced to the C-ABI3 gene.
Fig. 14. Northern blot analysis of expression of the C-ABI3, ECP and CAISE genes during seed development of carrot.

Twenty-µg samples of total RNA from seeds at various DAF were fractionated by gel electrophoresis and allowed to hybridize with the $^{32}$P-labeled cDNA of clone 491, ECPs and CAISES as probes. The blot was reprobed with 18S rRNA to provide an internal standard.
Fig. 15. Northern blot analysis of expression of the C-ABI3, ECP and CAISE genes in transgenic mature leaves with or without ABA-treatment.

Transgenic plants (S1, S2 and S3 lines) were made by transformation with pBI121-CABI3 vector.

Total RNA was isolated from mature leaves of non-transgenic plants (lanes 1 and 2), mature leaves of the transgenic S1 line (lanes 3 and 4), mature leaves of the transgenic S2 line (lanes 5 and 6), and mature leaves of the transgenic S3 line (lanes 7 and 8). The leaves were untreated (lanes 1, 3, 5 and 7) or treated with ABA (lanes 2, 4, 6 and 8). Twenty-μg aliquots of total RNA in each lane were fractionated by gel electrophoresis and allowed to hybridize with the [32P]-labeled cDNAs of clone 491, ECPs and CAISEs as probes. The blot was reprobed with 18S rRNA to provide an internal standard.
Fig. 16. Morphological aspects of transgenic and non-transgenic non-embryogenic cells (NC).

(a): Transgenic NC which was transformed with pEl2Ω-CABI3 vector (line 491S1; right) and non-transgenic NC (left) were cultured in MS medium containing 2,4-D (1 mg/l) and kanamycin (100 mg/l) for 14 days on a gyratory shaker (100 rpm) in darkness.

(b-e): Non-transgenic NC which were cultured with (d) or without ABA (b) and transgenic NC (line 491S1) which were cultured with (e) or without ABA (c) were observed under a light microscope. ABA-treatment was performed at $1 \times 10^{-4}$ M for 14 days.
Fig. 17. Northern blot analysis of expression of the C-ABI3, ECP and CAISE genes in transgenic and non-transgenic NC with or without ABA-treatment.

Total RNA was isolated from somatic embryos (lane 1), somatic embryos treated with ABA (3.7 x 10^{-6} M, 24 hours; lane 2), embryogenic cells (lane 3), non-transgenic NC (lanes 4, 5 and 6), and transgenic NC (line 491S1; lanes 7, 8 and 9). NC were cultured for 24 hours in medium that contained 0 M (lanes 4 and 7), 1 x 10^{-5} M (lanes 5 and 8) or 1 x 10^{-4} M (lanes 6 and 9) ABA, respectively. Twenty-μg aliquots of total RNA were loaded in each lane and fractionated by electrophoresis. The RNA were then allowed to hybridize with the [^{32}P]-labeled cDNAs of clone 491, ECPs and CAISEs as probes. The blot was reprobed with 18S rRNA to provide an internal standard.
Fig. 18. Schematic drawings showing involvement of C-ABI3 and other factor(s) on expression of ABA-inducible genes.

Each drawing showed the schematic model in mature leaves of non-transgenic plants (a), mature leaves of transgenic plants (b), non-transgenic NC (c), transgenic NC (d), and embryogenic cells including somatic embryos, zygotic embryos and embryogenic cells (e).
Fig. 19. The growth of transgenic and non-transgenic NC after treatment with ABA and desiccation.

Non-transgenic NC (a-e) and transgenic NC (line 491S1) (f-j) were rehydrated and cultured for 14 days after treatment with ABA and desiccation. ABA-treatment consisted of culture in the presence of 0 M (left cultures) or $1 \times 10^{-4}$ M (right cultures) for 14 days. Desiccation treatment was performed for 0 min (a and f), 20 min (b and g), 25 min (c and h), 30 min (d and i) and 40 min (e and j).
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Fig. 20. The viability of transgenic and non-transgenic NC after treatment with ABA and desiccation.

The viability of non-transgenic NC (a) and transgenic NC (line 491S1) (b) with or without ABA-treatment is shown. The viability of NC with or without ABA-treatment is shown by closed or open squares, respectively. ABA-treatment consisted of culture in the presence of $1 \times 10^{-4}$ M for 14 days. Desiccation treatment was performed for 0 min, 15 min, 20 min, 25 min, 30 min and 40 min. After desiccation treatment, NC were rehydrated and cultured for 7 days. Then NC were stained with FDA and the viability of the cells was determined by averaging the percentage survival of randomly selected populations of more than 200 cells. The standard errors (bars) are based on results from three replicate samples for each treatment.
(a) 

(b)
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