Note Plant Biotechnology, 21 (4), 309-314 (2004)

Isolation and Characterization of Six Abscisic Acid-Inducible Genes from Carrot Somatic Embryos

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Received 6 August 2004; accepted 13 October 2004 (Edited by M. Umeda)

Abstract

In carrot (*Daucus carota* L.) somatic embryos, desiccation tolerance is induced by treatment with abscisic acid (ABA). Six cDNA clones that showed ABA-enhanced expression were isolated from carrot by differential screening with ABA-treated and ABA-untreated somatic embryos, and they were named the *CAISE* (*carrot ABA-induced in somatic embryos*) genes. Five of the clones encode late embryogenesis abundant (LEA) proteins, and the other clone encodes a glucose and ribitol dehydrogenase. The expression of the *CAISE* genes was detected in maturing seeds, embryogenic cells, and ABA-treated somatic embryos in which exhibit desiccation tolerance induced by endogenous or exogenous ABA. These results indicate that ABA-induced desiccation tolerance in carrot somatic embryos may be induced by the LEA proteins and the glucose and ribitol dehydrogenase encoded by these ABA-inducible genes.

Accession numbers AB105039, AB105040, AB105041, AB105042, AB105043, AB105044.

Key words: abscisic acid (ABA), carrot (*Daucus carota* L), somatic embryogenesis, seed desiccation tolerance.

Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; ABA, abscisic acid; DAF, days after flowering; ECP, embryogenic cell protein; LEA, late embryogenesis abundant; MS, Murashige and Skoog's medium.

Somatic embryogenesis has been extensively investigated as a model system for the development of zygotic embryogenesis. Carrot (Reinert, 1958; Steward *et al.*, 1958), in particular, constitutes an important system for somatic embryogenesis studies, because numerous somatic embryos that are synchronized at various developmental stages can be readily induced by a simple procedure.

Despite their morphological similarities, zygotic embryos show desiccation tolerance and dormancy but somatic embryos do not (Iida *et al.*, 1992). In the developing seeds of several higher plants, the endogenous level of abscisic acid (ABA) increases transiently just before the initiation of desiccation (Rock and Quatrano, 1995). Furthermore, ABA-deficient and ABA-insensitive mutants of *Arabidopsis* and maize produce seeds that do not exhibit desiccation tolerance or enter dormancy (Finkelstein *et al.*, 2002). These observations indicate that ABA has important roles in the induction of desiccation tolerance and dormancy in seeds. However, the ABA content of carrot somatic embryos is low throughout the development of the embryo (Kiyosue *et al.*, 1992a), and desiccation tolerance can be induced in alfalfa and carrot somatic embryos by ABA treatment (Senaratna *et al.*, 1990; Iida *et al.*, 1992). Therefore, somatic embryos may be able to acquire desiccation tolerance through exogenous application of ABA.

In many plant species, various late embryogenesis abundant (LEA) proteins accumulate in mature seeds, and the expression of most *LEA* genes is positively regulated by ABA (Delseny *et al.*, 2001). It seems likely that the LEA proteins function to protect cells against desiccation (Hoekstra *et al.*, 2001). The VP1/ABI3 factor also plays important roles in seed-specific ABA signal transduction,

controlling expression of some ABA-inducible genes, including the LEA genes (McCarty *et al.*, 1991; Parcy *et al.*, 1994). Using the carrot somatic embryogenesis system, we found that C-ABI3, a carrot homolog of VP1 / ABI3, may be involved in ABA signal transduction, affecting the expression of certain embryo-specific ABA-inducible genes, including embryogenic cell protein (ECP) genes (Shiota *et al.*, 1998; Shiota and Kamada, 2000).

In the present project, we isolated genes whose expression is enhanced in ABA-treated somatic embryos in order to investigate the molecular mechanisms related to the ABA-induced desiccation tolerance of somatic embryos.

Carrot (*Daucus carota* L. cv. US-Harumakigosun) seedlings were grown for 10 days at 25℃ with 16 h of daily light at approximately 40 photons m^{-2} s^{-1}. Carrot embryogenic cells and non-embryogenic cells were obtained, as described by Satoh *et al.* (1986). The small cell clusters of embryogenic cells (37-63 μm in size) were rinsed three times with liquid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), resuspended in liquid MS medium at a final density of 0.2 ml packed cell volumes per liter, and cultured at 25℃ in darkness on a gyratory shaker (75 rpm). After 15 days of culture, torpedo-shaped somatic embryos were observed to have formed. Mature leaves were collected from plants that had been grown from somatic embryos for two months at 25℃ with illumination, as described above. Seeds were collected from carrot plants (*Daucus carota* L. cv. Yohmeigosun) grown in the experimental field of the Takii Seed Co., Ltd. (Ushiku, Ibaraki, Japan). Seeds and fruits were harvested on various days after flowering (DAF).

Somatic embryos in 15-day-old-cultures were transferred to 100 ml of fresh liquid MS medium containing ABA (3.7 μM or 10 μM) and cultured at 25℃ for 1, 3, 5, or 7 days in darkness on a gyratory shaker (75 rpm). Similarly, non-embryogenic cells were cultured in 100 ml of fresh liquid MS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D; 1 mg l^{-1}) and ABA (10 μM or 100 μM) at 25℃ for 24 h in darkness on a gyratory shaker (100 rpm). Mature leaves of carrot plants were sprayed with 100 μM ABA and incubated for 8 h in darkness.

For preparation of the cDNA library and Northern blot analysis, total RNA was isolated from somatic embryos, non-embryogenic cells, and mature leaves (all of which had been treated with ABA or mock-treated), embryogenic cells, and developing seeds, according to the method described by Ausubel *et al.* (1987) with modifications. Poly(A)$^+$ RNA was isolated, using the mRNA Purification Kit (Amersham Biosciences), from total RNA of somatic embryos that had been treated with ABA or mock-treated. cDNA was synthesized from the poly(A)$^+$ RNA using the cDNA Synthesis System Plus (Amersham Biosciences). A cDNA library of ABA-treated somatic embryos was constructed in lambda gt10 using a mixture of cDNAs from ABA-treated (10 μM; 1-7 days) somatic embryos and a cDNA Cloning System (Amersham Biosciences). All procedures were carried out according to the protocols provided by the suppliers.

Differential screening was performed by plaque hybridization. A total of approximately 40,000 plaques of recombinant phages were plated and transferred onto nylon filters (Hybond-N$^+$; Amersham Biosciences). [^{32}P]-labeled cDNA fragments from ABA-treated (10 μM; 1-7 days) and mock-treated somatic embryos were used as positive and negative control probes, respectively. Hybridization was performed at 65℃ , as directed by the manufacturer (Amersham Biosciences). Radioactivity on the filters was detected using the BAS 2000 system (Fuji Photo Film). The isolated lambda gt10 phage DNAs were digested with *Eco*RI and the insert fragments were subcloned into the pBluescript II SK$^+$ vector (Stratagene). To sequence the cDNA clones, double-stranded plasmid DNAs were isolated and sequenced using the dye terminator cycle sequencing method with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems).

Total RNA (20 μg) was loaded on an agarose (1.2%) gel containing 0.66 M formaldehyde, separated by electrophoresis, and transferred to GeneScreen Plus nylon filters (New Research Products). Hybridization was performed at 60℃, according to the manufacturer's instructions. Autoradiography was performed at -80℃ using Biomax MS film (Eastman Kodak).

In this project, we attempted to isolate ABA-inducible genes from ABA-treated carrot somatic embryos using differential screening. Over 200 positive clones were isolated from approximately 40,000 plaques of a cDNA library constructed from ABA-treated somatic embryos, and the clones were classified into six groups that did not cross-hybridize in dot-blot analyses (data not shown). The six positive clones were isolated and named the *CAISE* (*carrot ABA-induced in somatic embryos*) genes (*CAISE1, CAISE2, CAISE3, CAISE4, CAISE5*, and *CAISE6*) (**Table 1**). In Northern blot analysis, the *CAISE1, CAISE2, CAISE3, CAISE4, CAISE5* and *CAISE6* probes hybridized to RNAs of 0.8, 1.1, 0.8, 0.6, 1.3, and 1.2 kb, respectively (**Fig. 1**). Expression of each of the clones was detected in ABA-untreated somatic embryos and was enhanced by

ABA treatment (10 μM, 7 days) (**Fig. 1**). The *CAISE* gene expression in the ABA-untreated embryos may have been induced by endogenous ABA, since carrot somatic embryos contain low levels of endogenous ABA (Kiyosue *et al.*, 1992a).

CAISE2 and *CAISE6* were found to be identical to the carrot genes *ECP31* and *ECP40*, respectively (**Table 1**) (Kiyosue *et al.*, 1992b; Kiyosue *et al.*, 1993). *ECP31* and *ECP40* belong to the *LEA* gene families and are expressed in embryogenic cells, developing seeds, and ABA-treated somatic embryos (Kiyosue *et al.*, 1992b; Kiyosue *et al.*, 1993). *CAISE4* encodes a protein identical to that encoded by the carrot gene *EMB1* (**Table 1, Fig. 2**) (Wurtele *et al.*, 1993), although the nucleotide sequences in the 3'-untranslated regions of these genes have only 73% homology (data not shown). It is possible that these two genes are identical, with the differences in the nucleotide sequences derived from different carrot cultivars. *EMB1* has homology to the wheat *Em* gene and is expressed in developing somatic embryos, with expression gradually increasing during development (Wurtele *et al.*, 1993). The deduced amino acid sequence of *CAISE3* has significant homology to those of *Em*-like genes of several plant species (**Table 1**) (Manickam *et al.*, 1996; Delseny *et al.*, 2001). CAISE3 is also highly similar to CAISE4, although CAISE3 contains a 20-amino-acid sequence not present in CAISE4 (**Fig. 2**). In the genomes of some plant species, the presence of multiple classes of *Em*-like genes has been reported (Manickam *et al.*, 1996; Delseny *et al.*, 2001). These facts indicate that both *CAISE3* and *CAISE4* are carrot *Em*-like genes. *CAISE1* encodes a type of dehydrin protein (**Table 1**), based on the presence of three characteristic dehydrin segments: the K segment, the S segment, and the Y segment (data not shown) (Baudo *et al.*, 1996). Thus, five of the *CAISE* genes encode LEA proteins. In general, the LEA proteins help maintain the stability of cellular components and proteins through the solubilization of substances during desiccation (Hoekstra *et al.*, 2001). It seems likely that the products of these five *CAISE* genes protect cells against dehydration in ABA-treated carrot somatic embryos.

The remaining *CAISE* gene, *CAISE5*, has significant homology to glucose and ribitol dehydrogenase genes of barley and lupin (**Table 1**) (Alexander *et al.*, 1994; Francki *et al.*, 2002). These genes are expressed specifically during embryogenesis, with increased expression at the seed maturation stage (Alexander *et al.*, 1994; Francki *et al.*, 2002). During the acquisition of desiccation tolerance in seeds, sugars accumulate, maintaining the stability of membranes and functional proteins by replacing the water molecules at the charged

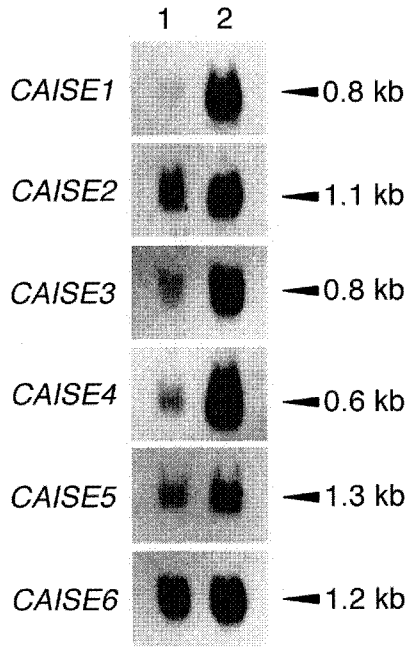

Fig. 1 Northern blot analysis of the expression of the *CAISE* genes in carrot somatic embryos.

Twenty micrograms of total RNA from torpedo-shaped embryos that were mock-treated (lane 1) or treated with ABA (lane 2) were fractionated by gel electrophoresis, and transcripts were allowed to hybridize with [^{32}P]-labeled cDNA fragments of the *CAISE* genes. For ABA treatment, 10 μM ABA was added and the samples were incubated for seven days.

Table 1. Summary of cDNA clones isolated from ABA-treated carrot somatic embryos by differential screening.

Gene name	Length of clones (bp)	Predicted size of gene products (a. a.)	Homologous protein products (species)	Accession No.
CAISE1	771	149	DHN1 (potato)	Y15813
CAISE2	982	256	ECP31 (carrot)	X60593
CAISE3	778	113	Em-like (mung bean)	U31210
CAISE4	523	92	EMB1 (carrot)	X17608
CAISE5	1179	291	pG31 (barley)	S72926
CAISE6	1120	306	ECP40 (carrot)	X61914

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CAISE3   1: MASGQEKRSELDARAKQGETVVPGGTGGKSLEAQEHLAEGRSKGGHTRKEQLGTEGY : 57
            ||| |||. |||||.||||||||||||||||.||||||||.|||||||.|||
CAISE4   1: MASQQEKK-ELDARARQGETVVPGGTGGKSLEAQQHLAEGRSKGGQTRKEQLGGEGY : 56
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||
EMB1     1: MASQQEKK-ELDARARQGETVVPGGTGGKSLEAQQHLAEGRSKGGQTRKEQLGGEGY : 56

CAISE3  58: QEIGTKGGETRREQMGKEGYEQMGRMGGLATKDKSGAERAEEEGIDIDQSKFRTKS  : 113
                                ..|||.|||...|.||.||||.||||||.||||||
CAISE4  57: --------------------HEMGRKGGLSNNDMSGGERAEQEGIDIDESKFRTKK  : 92
                                ||||||||||||||||||||||||||||||||||||
EMB1    57: --------------------HEMGRKGGLSNNDMSGGERAEQEGIDIDESKFRTKK  : 92
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Fig. 2 Alignment of the deduced amino acid sequences of the carrot *Em*-like genes *CAISE3*, *CAISE4*, and *EMB1*.

An alignment of the deduced amino acid sequences of *CAISE3*, *CAISE4*, and *EMB1* (accession no. X17608) is presented. Identical amino acid residues are shown connected with a line. Single dots indicate similar amino acids. Gaps introduced to optimize the alignments are indicated by hyphens.

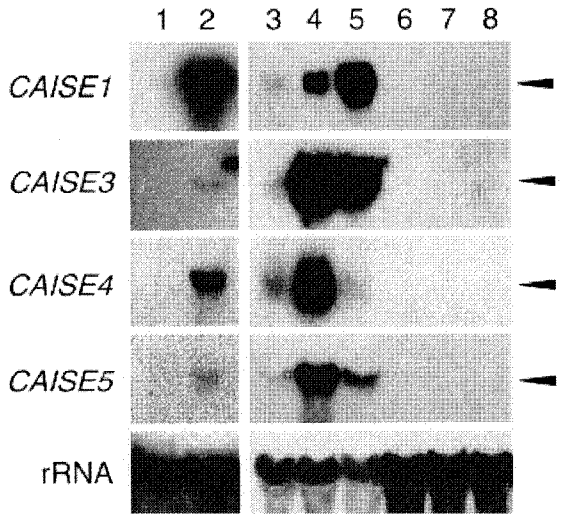

Fig. 3 Northern blot analysis of expression of the *CAISE* genes in mature leaves, somatic embryos, embryogenic cells, and non-embryogenic cells.

Total RNA was isolated from mature leaves (lanes 1 and 2), torpedo-shaped somatic embryos (lanes 3 and 4), embryogenic cells (lane 5), and non-embryogenic cells (lanes 6, 7, and 8). The leaves were either mock-treated (lane 1) or treated with ABA (100 μM for 8 h) (lane 2). Somatic embryos and non-embryogenic cells were treated with 0 M (lanes 3 and 6), 3.7 μM (lane 4), 10 μM (lane 7), or 100 μM (lane 8) ABA for 24 h. Twenty micrograms of total RNA per sample were fractionated by gel electrophoresis and allowed to hybridize with [^{32}P]-labeled cDNA fragments of *CAISE1*, *CAISE3*, *CAISE4* or *CAISE5*. The blot was reprobed with labeled 18S rRNA to provide an internal standard.

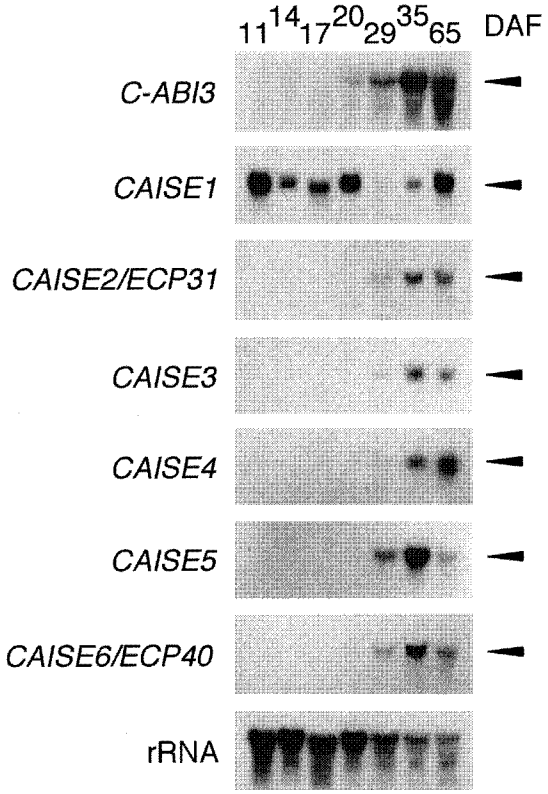

Fig. 4 Northern blot analysis of the expression of *C-ABI3* and the *CAISE* genes in developing carrot seeds.

Twenty micrograms of total RNA from seeds at various DAF, as indicated, were fractionated by gel electrophoresis and allowed to hybridize with [^{32}P]-labeled cDNA fragments of *C-ABI3*, *CAISE1*, *CAISE2/ECP31*, *CAISE3*, *CAISE4*, *CAISE5* or *CAISE6/ECP40*. The blot was reprobed with labeled 18S rRNA to provide an internal standard.

surfaces (Hoekstra *et al.*, 2001). Therefore, CAISE5 might function as a short alcohol-polyol-sugar dehydrogenase, possibly related to carbohydrate metabolism and the acquisition of desiccation tolerance in ABA-treated somatic embryos (Alexander *et al.*, 1994). On the other hand, it has been recently

proposed that sugars are involved in phytohormone signaling during plant growth and development (León and Sheen, 2003). Thus, CAISE5 might also have roles in signal transduction mechanisms.

Transcripts of each of the *CAISE* genes, were detected in embryogenic cells, somatic embryos, and seeds in the later stages of development, but not in non-embryogenic cells with or without ABA-treatment (**Figs. 1, 3** and **4**) (Shiota and Kamada, 2000). In somatic embryos, the expression of all of the *CAISE* genes was enhanced after an ABA treatment that was shorter and of a lower concentration than usual (3.7 μM, 24 h) (**Fig. 3**) (Shiota and Kamada, 2000). Maturing seeds, embryogenic cells, and ABA-treated somatic embryos exhibit desiccation tolerance induced by endogenous or exogenous ABA (Iida *et al.,* 1992; Kiyosue *et al.,* 1992a; Shiota *et al.,* 1998), and the CAISEs may be involved in ABA-induced desiccation tolerance in zygotic and somatic embryos.

Zygotic and somatic embryos that show ABA-induced desiccation tolerance may have the tolerance against water loss from cells, because the relative water content of desiccated embryos fall to about 5% (Shiota *et al.,* 1998). In carrot somatic embryos, the extent of desiccation tolerance increases gradually during the first 8 days of ABA-treatment, although the weak tolerance is induced by just 30 h of ABA-treatment (Iida *et al.,* 1992). On the other hand, the *CAISE* genes were strongly expressed in somatic embryos treated with a shorter-term (24 h) ABA, and levels of the expression were maintained during at least 7 days of ABA-treatment (**Figs. 1** and **3**). Therefore, it is suggested that the accumulation of several components whose synthesis is induced by ABA, such as the products of the *CAISE* genes, might be a prerequisite for the protection of cells against damage by water loss, and the accumulation might require at least 8 days of ABA-treatment in somatic embryos.

In developing seeds, the expression of all of the *CAISE* genes increased during the seed maturation phase (after 29 DAF) (**Fig. 4**). The *CAISE2/ECP31, CAISE3, CAISE4, CAISE5* and *CAISE6/ECP40* transcripts were confined to seeds after 29 DAF (**Fig. 4**). In carrot seeds, the water content begins to decrease after 38 DAF, and endogenous levels of ABA increase after 23 DAF, with a transient peak at 29 DAF (Shiota *et al.,* 1998). These results suggest that the *CAISE* genes are induced by a programmed increase in endogenous ABA during seed maturation. In contrast, the expression of *CAISE1* was observed throughout seed development, and the level of the expression was higher in the early stage of seed development (11-20 DAF) (**Fig. 4**). Many dehydrin genes are induced not only by ABA but also by several environmental factors (Nylander *et al.,* 2001). In fact, the expression of *CAISE1* was induced in carrot leaves by drought, salinity, and cold and heat stresses (data not shown). The transcription of *CAISE1* at 11-20 DAF may be induced by environmental factors (*e.g.* low temperature or drought), because the endogenous ABA level was low in seeds at 11-20 DAF (Shiota *et al.,* 1998). Therefore, the expression of *CAISE1* may be controlled independently by endogenous ABA and environmental factors.

The expression of all of *CAISE* genes was not detected in mature leaves without ABA-treatment (**Fig. 3**) (Shiota *et al.,* 1998). In ABA-treated leaves, strong ABA-inducible expression of *CAISE1* and *CAISE4* was detected, and weak expression of *CAISE3* and *CAISE5* was also observed (**Fig. 3**). *CAISE2/ECP31* and *CAISE6/ECP40* as embryo-specific ABA-inducible genes show no ABA-inducible expression in mature leaves (Kiyosue *et al.,* 1992b; Kiyosue *et al.,* 1993; Shiota *et al.,* 1998). These results suggest that the embryo specificity of the expression of *CAISE3* and *CAISE5* may be higher than that of *CAISE1* and *CAISE4.* In seeds of higher plants, the VP1 / ABI3 factor functions as a transcriptional factor in ABA-induced gene expression (McCarty *et al.,* 1991; Parcy *et al.,* 1994). During carrot seed development, *C-ABI3* is expressed prior to the increase in endogenous ABA levels and the ABA-induced expression of the *CAISE* genes, since *C-ABI3* transcripts were detected after 20 DAF (**Fig. 4**) (Shiota *et al.,* 1998). Therefore, C-ABI3 might control the expression of *CAISE* genes in carrot embryos. In order to clarify whether the *CAISE* genes are actually controlled by C-ABI3, expression analysis of the *CAISE* genes is now been performed using transgenic plants in which *C-ABI3* is expressed ectopically.

Based on these results, we propose that many ABA-inducible genes such as *CAISEs* and *ECPs* are involved in ABA-induced desiccation tolerance in zygotic and somatic embryos. The desiccation tolerance in these embryos may be induced by accumulation of sufficient levels of components that are involved in the protection of cells against damage by desiccation, including LEA proteins, heat shock proteins, and sugars.

Acknowledgements

This research was supported in part by a Grant-in-Aid for Research on Priority Areas and for Scientific Research from the Ministry of Education, Science, Culture, and Sports, Japan; by a Grant-in-Aid from the "Research for the Future" Program

from the Japan Society for the Promotion of Science (JSPS-RFTF00L01601); and by the Special Coordination Funds of the Science and Technology Agency of the Japanese Government.

The authors are grateful to the staff of the Ibaraki Experimental Field of the Takii Seed Co., Ltd. for collecting carrot seeds.

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