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Original Paper

Optical isomers of abscisic acid in carrot somatic embryos have the same effect on induction of dormancy and desiccation tolerance

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Abstract Carrot (*Daucus carota*) somatic embryos have been extensively used as an experimental system for studying plant embryogenesis. In maturing zygotic embryos, the phytohormone abscisic acid (ABA) is involved in induction of dormancy and desiccation tolerance. Somatic embryos contain only low levels of endogenous ABA and lack desiccation tolerance and dormancy, but both tolerance and dormancy can be induced by application of exogenous ABA. We analyzed the effects of the optical isomers of ABA, (*S*)-(+)-2-*cis*-ABA [(+)-ABA] and (*R*)-(−)-2-*cis*-ABA [(−)-ABA], and a racemic mixture of these two isomers (racemic ABA) on the carrot system. Somatic embryos treated with (+)-ABA, (−)-ABA, or racemic ABA showed the same levels of growth inhibition and desiccation tolerance. Similarly, each ABA isomer and racemic ABA had the same effect on accumulation of transcripts for ABA-inducible genes. The results indicate that both (+)-ABA and (−)-ABA cause the same amount of activity in these physiological phenomena. Our findings suggest that strict steric requirements of the ABA signaling pathways are absent in carrot somatic embryos, and we propose that commercially available racemic ABA is as effective as the natural isomer (+)-ABA in induction of dormancy and desiccation tolerance in carrot somatic embryos.

Key words: Abscisic acid isomers, desiccation tolerance, dormancy, somatic embryogenesis.

Somatic embryogenesis has been extensively used as an experimental system to investigate the development of zygotic embryogenesis. In the carrot (*Daucus carota*) system in particular, and also in other plant species, numerous physiological, biochemical, and molecular biological studies have examined somatic embryogenesis (Ikeda et al. 2006). As somatic embryos develop into seedlings they undergo morphological changes similar to those that occur in zygotic embryos (passage through a globular stage, heart-shaped stage, and torpedo-shaped stage). However, some differences in physiological features exist between somatic and zygotic embryos (Ikeda et al. 2006).

In developing seeds of several higher plants, dormancy and desiccation tolerance are induced by the phytohormone abscisic acid (ABA), which is synthesized during the seed maturation phase (Finkelstein et al. 2002). The endogenous ABA content of carrot somatic embryos is naturally low throughout development, and dormancy and desiccation tolerance are not observed at this stage (Kiyosue et al. 1992a). Desiccation tolerance can be induced in somatic embryos of alfalfa, spruce, and carrot by treatment with exogenous ABA (Attree et al. 1991; Iida et al. 1992; Kitto and Janick 1985; Senaratna et al. 1990). In ABA-treated carrot somatic embryos, as well as in maturing seeds, several ABA-induced genes [*embryogenic cell protein* (ECP) and *carrot ABA-induced in somatic embryos* (CAISE) genes] that encode late embryogenesis abundant (LEA) proteins or enzymes for sugar metabolism are expressed, and these are thought to be involved in induction of desiccation tolerance and dormancy (Shiota et al. 2004). This system can be used to preserve cell lines of somatic embryos under desiccation conditions at very low temperatures (−80°C) for long periods (169 weeks; Shiota et al. 1999).

In plant tissues, ABA is generally biosynthesized as (*S*)-(+)-2-*cis*-ABA [(+)-ABA], and the mirror image form, (*R*)-(−)-2-*cis*-ABA [(−)-ABA], has not been found in most plant tissues (Lin et al. 2005).

Abbreviations: ABA, abscisic acid; CAISE, carrot ABA-induced in somatic embryos; ECP, embryogenic cell protein; HPLC, high-performance liquid chromatography; LEA, late embryogenesis abundant

This article can be found at http://www.jspcmb.jp/

Commercially available ABA, which is obtained by chemical synthesis, consists of a 1 : 1 mixture of the two enantiomers, (+)-ABA and (−)-ABA. This racemic ABA has been used extensively in many experiments because it is the most economical chemical form of ABA. The natural isomer (+)-ABA is the most active physiological isomer in plants, whereas (−)-ABA may have the same or much lower physiological activity (Lin et al. 2005).

Focusing on physiological phenomena in seeds or embryos, (+)-ABA and (−)-ABA have the same effect on inhibition of embryo germination in wheat and barley, and on growth inhibition of microspore-derived embryos in oilseed rape (Abrams et al. 1993; Rose et al. 1996; Wilmer et al. 1998). However, (−)-ABA has much less effect on inhibition of germination in *Arabidopsis*, cress, tomato, and lettuce, and has no effect in yellow cedar (Gusta et al. 1992; Lin et al. 2005; Nambara et al. 2002; Schmitz et al. 2002; Toorop et al. 1999). Thus, in terms of inhibition of seed germination, the two ABA isomers have different activity in different plant species.

We examined the physiological activity of (+)-ABA and (−)-ABA on induction of dormancy and desiccation tolerance in carrot somatic embryos. We compared the effects of (+)-ABA, (−)-ABA, or racemic ABA on growth inhibition and acquisition of desiccation tolerance in these carrot somatic embryos. The embryo growth inhibition can be regarded as a part of dormancy, because the inhibition is controlled by the embryo-specific ABA signal transduction system involved in the inhibition of germination and acquisition of desiccation tolerance (Robichaud et al. 1980). We further analyzed the accumulation of transcripts for ABA-inducible genes in the carrot somatic embryos treated with the two ABA isomers or racemic ABA. Finally, we discuss the activity of racemic ABA compared to (+)-ABA in inducing dormancy and desiccation tolerance in carrot somatic embryos.

Materials and methods

Plant materials

Carrot (*Daucus carota* L. cv. US-Harumakigosun) embryogenic cells and somatic embryos were obtained as described by Satoh et al. (1986). To obtain somatic embryos, small cell cultures (37–63 μm) of embryogenic cells were cultured in phytohormone-free liquid Murashige and Skoog (MS) medium at 25°C in darkness on a gyratory shaker at 75 rpm. After a 15-day culture, torpedo-shaped somatic embryos had formed.

Chemicals

(*S*)-(+)-2-*cis*-ABA [(+)-ABA], (*R*)-(−)-2-*cis*-ABA [(−)-ABA], and racemic ABA consisting of a 1 : 1 mixture of (+)-ABA and (−)-ABA were used (Figure 1). (+)-ABA and (−)-ABA were a gift from Dr. Eiji Nambara, RIKEN Plant Science Center. Racemic ABA was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Preparation of these chemicals was performed under green safe light.

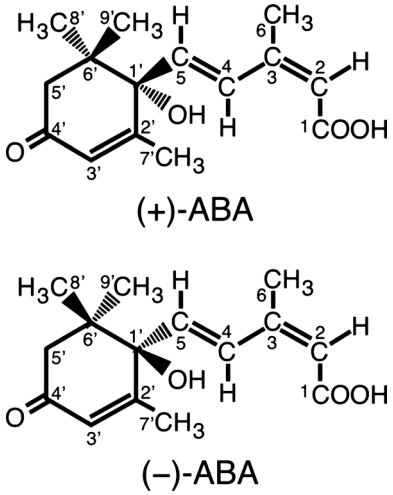

Figure 1. Structures of abscisic acid (ABA). Structures of (*S*)-(+)-2-*cis*-ABA and (*R*)-(−)-2-*cis*-ABA are indicated as (+)-ABA and (−)-ABA, respectively.

ABA treatment

Nine- or fifteen-day cultures of somatic embryos (heart-shaped or torpedo-shaped stages, respectively) were washed three times in fresh MS medium. The washed embryos were cultured in fresh MS liquid medium containing (+)-ABA, (−)-ABA, or racemic ABA (0, 0.1, 1, 10, or 100 μM) at 25°C for 3, 5, 7, 10, 14, and 18 days in darkness on a gyratory shaker at 75 rpm. ABA stock solutions were prepared in aqueous NaOH solution. Control groups were cultured in MS liquid medium containing 100 μM NaOH.

Desiccation treatment

Desiccation treatment was performed as described by Iida et al. (1992). Embryos that had been desiccated in silica gel for 3 h were rehydrated on semi-solid (0.2% Gelrite; Monsanto, St. Louis, MO, USA) MS medium and cultured at 25°C for 14 days under a 16-h day length (approximately 40 μmol photons m^{-2} s^{-1}). The percentage of embryos that had regrown was considered the viability.

Multiple comparisons between groups were made using the Tukey–Kramer test. Statistical analysis was performed with Excel 2004 (Microsoft, Redmond, WA, USA) with the add-in software Statcel 2 (OMS, Tokyo, Japan).

RNA extraction and Northern blot analysis

Total RNA was isolated from somatic embryos treated with (+)-ABA, (−)-ABA, or racemic ABA (at 0, 10, or 100 μM for 7 days) by the phenol/SDS method (Ausubel et al. 1987). Total RNA (20 μg/lane) was subjected to electrophoresis (1.2% agarose gel) and transferred to a Biodyne B nylon filter (Pall BioSupport, East Hills, NY, USA). Hybridization was performed at 65°C according to the manufacturer's instructions for the filter using [^{32}P]-labeled cDNAs that corresponded to eight carrot genes, *CAISE1* (GenBank/EMBL/DDBJ accession no.

AB105039), *CAISE2*/*DcECP31* (AB105040), *CAISE3* (AB105041), *CAISE4* (AB105042), *CAISE5* (AB105043), *CAISE6*/*DcECP40* (AB105044), *DcECP63* (X16131), and *C-ABI3* (AB005558) as probes (Kiyosue et al. 1992b, 1993; Shiota et al. 1998, 2004).

Extraction and separation of ABA isomers by high-performance liquid chromatography (HPLC)

The extraction and separation of ABA was performed under a dim green safe light. After the duration of the culture with each isomer or mixture of isomers at each concentration, MS media were filtered through filter paper (No. 2; Advantec, Tokyo, Japan). The medium was adjusted to pH 2.5 with H_2SO_4 and extracted with ethyl acetate. Ethyl acetate in the extraction was evaporated, and the evaporated sample was dissolved in organic solvent (hexane : 2-propanol, 8 : 2, v/v). The sample was applied to an HPLC column (Opti-Pak XC; 3.9×300 mm; Waters, Milford, MA, USA) and eluted with the same organic solvent at a flow rate of 0.5 ml per min at 25°C. The signal was detected at 240 nm (Okamoto et al. 1988).

We used HPLC to monitor the conformation of each ABA isomer in the medium to confirm that they were not converted to other isomers during the culture period (data not shown).

Results

The effects of ABA isomers and racemic ABA on embryo growth inhibition

During the 9 days of culture in MS medium without exogenous ABA, embryogenic cells developed into heart-shaped or torpedo-shaped somatic embryos, with a long-axis length of less than 0.1 mm (Figure 2A). After an additional 7 days of culture in MS medium, the cells developed into torpedo-shaped embryos, with a long-axis length of 0.8–1.0 mm; some of these embryos germinated and had elongating roots (Figure 2B). However, in MS medium containing 10 μM (+)-ABA, (−)-ABA, or racemic ABA, although the embryos became torpedo-shaped, they were smaller than in the control treatment (0.1–0.5 mm long-axis length; Figure 2C–E). No germination of embryos treated with the ABA isomers and mixture of isomers was observed (Figure 2C–E).

After an additional 7 days of culture in MS medium alone, the long-axis of 15-day cultures of torpedo-shaped somatic embryos increased approximately two-fold (Figure 2F). In contrast, during the same time period but with 10 μM (+)-ABA, (−)-ABA, or racemic ABA, the embryos increased approximately 1.4-fold (Figure 2F). No embryos germinated after treatment with the ABA isomers and racemic ABA, whereas most embryos germinated after the control treatment (data not shown). Each ABA isomer and mixture had the same effect on growth of somatic embryos (Figure 2F).

The effects of ABA isomers and racemic ABA on acquisition of desiccation tolerance

After 3 h of desiccation and continuous rehydration, 15-day cultures of somatic embryos treated with 10 μM racemic ABA for 7 days germinated and grew, whereas

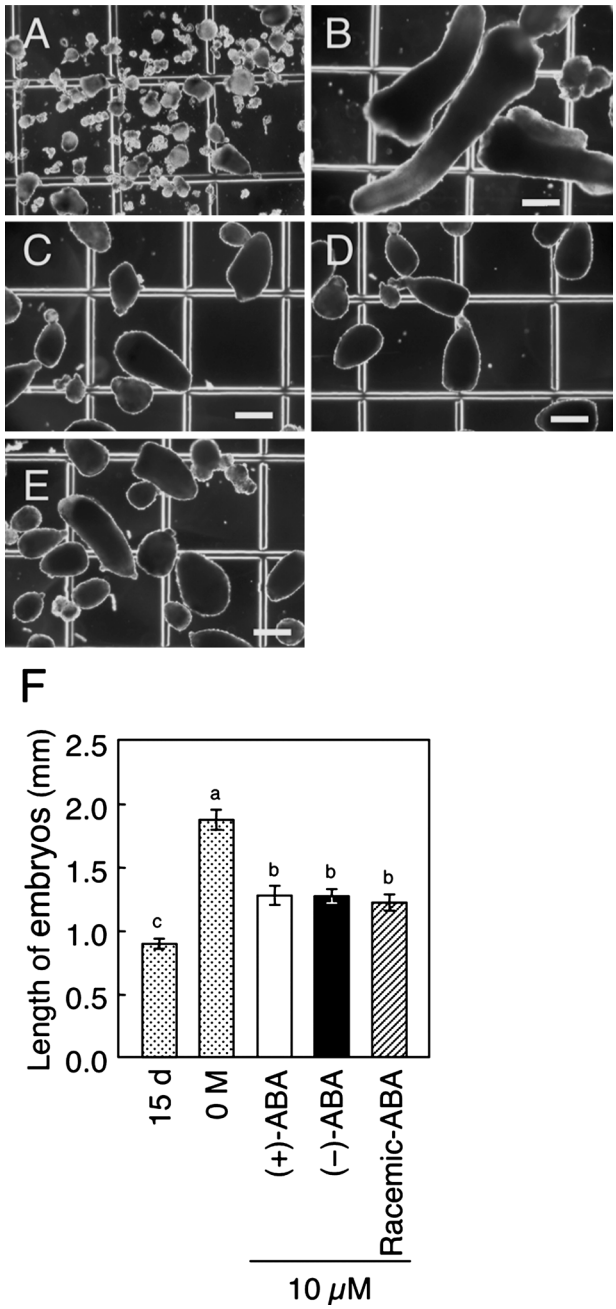

Figure 2. Effects of exogenous ABA on growth inhibition of somatic embryos. (A–E) Morphology of somatic embryos. Nine-day culture of somatic embryos (A) cultured for an additional 7 days in MS medium containing 0 M ABA (B), 10 μM (+)-ABA (C), 10 μM (−)-ABA (D), or 10 μM racemic ABA (E). Bars indicate 0.1 mm. (F) Lengths of the long-axes of somatic embryos. Fifteen-day culture of somatic embryos (15 d) cultured for an additional 7 days in medium containing 0 M ABA, 10 μM (+)-ABA, 10 μM (−)-ABA, or 10 μM racemic ABA. Values were determined by averaging the lengths of 50 non-germinating somatic embryos. Error bars indicate ±SE. Significant differences at $P<0.05$ are indicated by different letters.

no embryos in the control treatment survived and germinated (Figure 3A, B). The duration of exogenous ABA (10 μM) treatment increased the viability of somatic embryos after desiccation, and the effect of ABA on viability was saturated within 10 days of ABA treatment (Figure 3C). This suggests that it takes 10 days to saturate the accumulation of gene products and osmolytes involved in desiccation tolerance and induced by ABA (Shiota and Kamada 2000). The effects of (+)-ABA and racemic ABA on the viability of desiccated somatic embryos were the same (Figure 3C). Furthermore, the effects of ABA on desiccation tolerance increased in a concentration-dependent manner, and the effects of (+)-ABA, (−)-ABA, or racemic ABA were the same (Figure 3D). As shown in Figure 3D, the viability of embryos treated with 10 μM ABA for 7 days was higher compared to that shown in Figure 3C, indicating that the response of cells to ABA may differ among cell lines.

The effects of ABA isomers and racemic ABA on accumulation of transcripts

The carrot *ECP* and *CAISE* genes are ABA-inducible in somatic embryos (Kiyosue et al. 1992b, 1993; Shiota et al. 2004). These genes encode LEA proteins, except for *CAISE5*, which encodes glucose dehydrogenase (Kiyosue et al. 1992b, 1993; Shiota et al. 2004). Sufficient accumulation of LEA proteins may be required for ABA-inducible desiccation tolerance in somatic embryos (Shiota et al. 2004).

To confirm the effects of ABA isomers and racemic ABA on the accumulation of transcripts for ABA-inducible genes, Northern blot analysis was performed (Figure 4). Expression of *CAISE2*/*DcECP31*, *DcECP63*, *CAISE6*/*DcECP40*, *CAISE1*, and *CAISE5* was detected in the control-treated embryos (without exogenous ABA). Higher expression of all seven *ECP* and *CAISE* genes was detected in somatic embryos treated for 7 days with ABA isomers or racemic ABA. ABA concentration

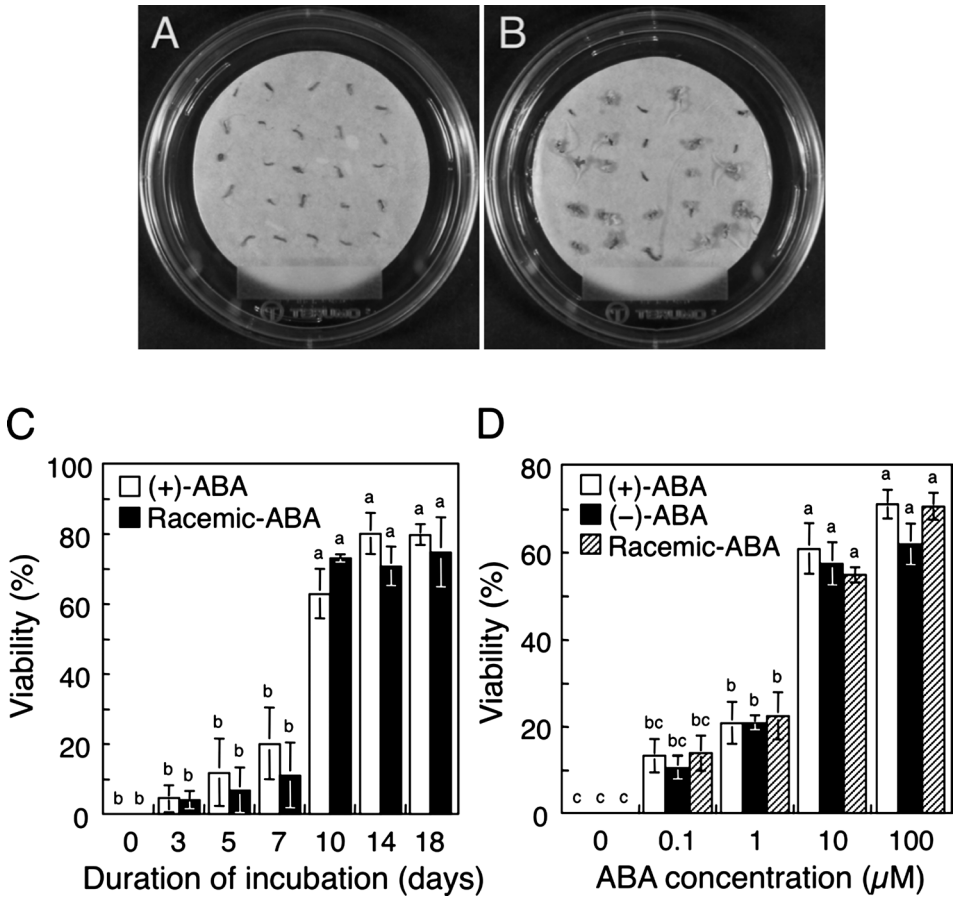

Figure 3. Effects of exogenous ABA on acquisition of desiccation tolerance in somatic embryos. (A–B) Morphology of regrown somatic embryos after rehydration. Fifteen-day culture of somatic embryos treated with 0 (A) or 10 μM (B) racemic ABA for 7 days and then desiccated for 3 h. (C) The effects of duration of ABA treatment on acquisition of desiccation tolerance. Fifteen-day culture of somatic embryos cultured for an additional 0, 3, 5, 7, 10, 14, or 18 days in medium containing 10 μM (+)-ABA or racemic ABA. (D) The effects of ABA concentration on acquisition of desiccation tolerance. Fifteen-day culture of somatic embryos cultured for an additional 7 days in medium containing 0, 0.1, 10, or 100 μM (+)-ABA, (−)-ABA, or racemic ABA. After ABA treatment, the embryos were desiccated for 3 h and then cultured on MS semi-solid medium for 14 days. The viability of embryos is indicated as the percentage of regrown embryos to the total number of embryos examined in each treatment consisting of 100 individuals. The values represent the average of three (C) or eight (D) independent examinations. The error bars indicate ±SE. Significant differences at $P<0.05$ are indicated by different letters.

dependence on the accumulation of transcripts was not observed in somatic embryos treated with (+)-ABA, (−)-ABA, or racemic ABA, indicating that accumulation of the gene transcripts may be saturated after a sufficient length (7 days) of ABA treatment.

In addition, expression of the *C-ABI3* gene encoding the C-ABI3 transcription factor, which is thought to be involved in ABA signal transduction (Shiota et al. 1998), was detected at the slightly higher level in somatic embryos with ABA isomers or racemic ABA. No significant difference in the accumulation of the transcripts was observed among embryos treated with (+)-ABA, (−)-ABA, or racemic ABA. These indicate that each ABA isomer has the same effect on the ABA signal transduction system involving the VP1/ABI3 transcription factor.

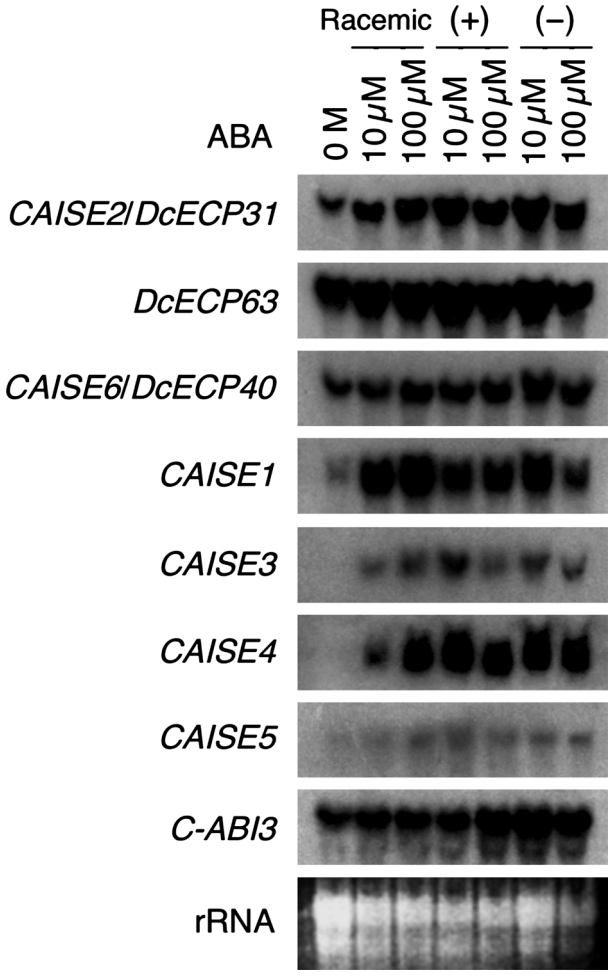

Figure 4. Effects of exogenous ABA on accumulation of transcripts for ABA-inducible genes in somatic embryos. Fifteen-day culture of somatic embryos cultured for an additional 7 days in medium containing 0, 10, or 100 μM (+)-ABA, (−)-ABA, or racemic ABA. Aliquots of 20 μg of total RNA isolated from the embryos were loaded into each lane and fractionated by electrophoresis. The RNAs were then allowed to hybridize with [^{32}P]-labeled cDNAs that corresponded to the *CAISE1*, *CAISE2*/*DcECP31*, *CAISE3*, *CAISE4*, *CAISE5*, *CAISE6*/*DcECP40*, *DcECP63*, and *C-ABI3* genes as probes. RNA loading was visualized by ethidium bromide staining.

Discussion

Generally, natural (+)-ABA shows high activity in several physiological phenomena, whereas unnatural (−)-ABA has the same or much less activity (Lin et al. 2005). We examined the effects of (+)-ABA and (−)-ABA on dormancy and desiccation tolerance in carrot somatic embryos by analyzing growth inhibition and viability.

After ABA treatment, carrot somatic embryos develop to the torpedo-shaped stage and growth is then inhibited (Iida et al. 1992). Inhibition of development and growth (long-axis length) was observed in carrot somatic embryos treated with (+)-ABA, (−)-ABA, or racemic ABA, and germination of these embryos was completely inhibited. Thus, the two ABA isomers or racemic ABA have the same effect on growth and development of carrot somatic embryos, suggesting that (+)-ABA and (−)-ABA have the same activity. These results are supported by reports describing inhibition of growth and germination of embryos treated with (+)-ABA and with (−)-ABA in oilseed rape, wheat, and barley (Abrams et al. 1993; Rose et al. 1996; Wilmer et al. 1998). On the other hand, (−)-ABA has no or much less effect on inhibition of germination in *Arabidopsis*, cress, tomato, lettuce, and yellow cedar (Gusta et al. 1992; Lin et al. 2005; Nambara et al. 2002; Schmitz et al. 2002; Toorop et al. 1999). Therefore, the effects of different ABA isomers on inhibition of seed germination are distinct in different plants.

In somatic embryos of alfalfa, spruce, and carrot, desiccation tolerance can be induced by exogenous ABA (Attree et al. 1991; Iida et al. 1992; Kitto and Janick 1985; Senaratna et al. 1990). Desiccation tolerance was induced in carrot somatic embryos treated with (+)-ABA, (−)-ABA, or racemic ABA, and each ABA isomer and the mixture had the same effect on this phenomenon. These results suggest that (+)-ABA and (−)-ABA have the same activity in terms of acquisition of desiccation tolerance in carrot somatic embryos. In calli of *Craterostigma plantagineum*, (+)-ABA and (−)-ABA also had the same effects on induction of desiccation tolerance (Chandler et al. 1997). On the other hand, (+)-ABA affects induction of maturation of white spruce somatic embryos, whereas (−)-ABA does not (Dunstan et al. 1992; Kong and von Aderkas 2007). Considering that maturation is related to desiccation tolerance in embryos, the responsiveness of somatic embryos to the ABA isomers may differ between plant species.

All seven carrot ABA-inducible genes analyzed here were upregulated by ABA in somatic embryos. No difference in the accumulation of transcripts was observed among somatic embryos treated with (+)-ABA, (−)-ABA, or racemic ABA. This suggests that each ABA isomer has the same effect on regulation of

these ABA-inducible genes in carrot embryos, confirming that each ABA isomer has the same effects on growth inhibition and acquisition of desiccation tolerance.

Not all plant species show upregulation of ABA-inducible genes in response to application of endogenous ABA isomers (Lin et al. 2005). (+)-ABA and (−)-ABA have the same effects on gene expression in the calli of *C. plantagineum* (*LEA* genes), somatic embryos of white spruce (*LMW hsp* genes), and suspension cells of brome grass (*Dehydrin* and *RAB* genes) (Chandler et al. 1997; Dong and Dunstan 1996; Wilen et al. 1996). On the other hand, *Em* gene expression in dormant embryos of wheat and *RAB18* gene expression in suspension cell cultures of *Arabidopsis* are induced by (+)-ABA but not by (−)-ABA (Jeannette et al. 1999; Walker-Simmons et al. 1992). In somatic embryos and embryogenic suspension cultures of white spruce, expression of the *Em* gene and *LEA*-like genes is highly induced by (+)-ABA compared to (−)-ABA (Bommineni et al. 1998; Dong and Dunstan 1997). Recently, Huang et al. (2007) reported that (−)-ABA regulates less than 20% of the genes that are regulated by (+)-ABA.

We found that natural [(+)-ABA] and unnatural isomers [(−)-ABA] of ABA have the same effects on the induction of dormancy and desiccation tolerance in carrot somatic embryos. Our results suggest that strict steric requirements of the ABA signaling pathways (e.g., ABA receptors), which have been previously proposed by Nakano et al. (1995) and Nambara et al. (2002), are absent in carrot somatic embryos. Therefore, commercially available racemic ABA should be as effective as endogenous (+)-ABA for the induction of dormancy and desiccation tolerance in carrot somatic embryos. We propose the carrot system as a convenient and economical technique for saving space and for preservation of artificial seeds.

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