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ORIGINAL ARTICLE

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Abscisic acid and stress treatment are essential for the acquisition of embryogenic competence by carrot somatic cells

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Abstract Studies of carrot embryogenesis have suggested that abscisic acid (ABA) is involved in somatic embryogenesis. A relationship between endogenous ABA and the induction of somatic embryogenesis was demonstrated using stress-induced system of somatic embryos. The embryonic-specific genes *C-ABI3* and embryogenic cell proteins (ECPs) were expressed during stress treatment prior to the formation of somatic embryos. The stress-induction system for embryogenesis was clearly distinguished by two phases: the acquisition of embryogenic competence and the formation of a somatic embryo. Somatic embryo formation was inhibited by the application of fluridone (especially at 10^{-4} M), a potent inhibitor of ABA biosynthesis, during stress treatment. The inhibitory effect of fluridone was nullified by the simultaneous application of fluridone and ABA. The level of endogenous ABA increased transiently during stress. However, somatic embryogenesis was not significantly induced by the application of only ABA to the endogenous level, in the absence of stress. These results suggest that the induction of somatic embryogenesis, in particular the acquisition of embryogenic competence, is caused not only by the presence of ABA but also by physiological responses that are directly controlled by stresses.

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Abbreviations ABA: Abscisic acid · 2,4-D: 2,4-dichlorophenoxyacetic acid · EC: Embryogenic cells · ECP: Embryogenic cell protein · GC-SIM-MS: Gas chromatography-selected ion monitoring-mass spectrometry · IAA: Indole acetic acid · NC: Non-embryogenic cells

Introduction

The traditional approach to inducing somatic embryogenesis in plant cell cultures is to collect embryogenic cells (EC) growing on an auxin-containing medium and then transferring them to an auxin-free medium (Reinert 1959; Steward et al. 1958; Sung et al. 1984; Kiyosue et al. 1993a). Thus, it has been postulated that the induction of somatic embryogenesis is a physiological function of auxin. The artificial plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) is the most effective auxin for the induction of EC (Kamada and Harada 1979). However, 2,4-D is also a strong herbicide and the concentration of indole acetic acid (IAA) required for the induction of somatic embryogenesis is over 10^3 times the endogenous free IAA level (Kamada and Harada 1979; Ribnicky et al. 1996). Therefore, 2,4-D is thought to function as a stress substance rather than as a phytohormone, triggering the acquisition of embryogenic competence by carrot cells. It has been reported that the application of heavy metal ions (Cd^{2+}, Ni^{2+}, Cu^{2+} and Co^{2+}), high osmotic pressure (sucrose, NaCl), and high temperature (37°C) induces somatic embryogenesis in carrot, in the absence of exogenous phytohormones (Harada et al. 1990; Kiyosue et al. 1989a, b, 1990; Kamada et al. 1989, 1993, 1994).

The induction of carrot somatic embryogenesis, by treatment with various stresses, has been exploited to

isolate those proteins and genes that are thought related to the acquisition of embryogenic competence. These proteins, i.e. embryogenic cell proteins (ECPs), belong to the LEA protein groups (Kiyosue et al. 1992a, b, 1993b; Tachikawa et al. 1998). The expression of the *ECP* genes is positively regulated by abscisic acid (ABA), a phytohormone that is involved in abscission (Bentley et al. 1975), dormancy (Walton 1980), and drought tolerance (Galli and Levi 1982). Recently, the gene *ABI3* was isolated, based on studies of ABA-insensitive *Arabidopsis* mutants (Koornneef et al. 1984). This gene is believed to be related to the seed-specific signal transduction of ABA (Parcy et al. 1994). A homolog of this gene in carrot was isolated and named *C-ABI3* (Shiota et al. 1998). This gene is mainly expressed in embryonic tissue and positively regulates the expression of the *ECP* genes (Shiota et al. 1998; Shiota and Kamada 2000).

The endogenous levels of ABA also increase in response to stress treatments in various plants (Skriver and Mundy 1990), including maize (Saab et al. 1992), pea (Fedina et al. 1994), and oilseed rape (Sauser et al. 1992). It has been reported that carrot EC contain about 2.5 times more endogenous ABA than somatic embryos at the torpedo stage, and about 67.5 times more than non-embryogenic cells (NC) that have lost the ability to form somatic embryos (Kiyosue et al. 1992c). Furthermore, treatment with 10^{-4} M ABA induces embryo formation in carrot apical tip explants (Nishiwaki et al. 2000); and ABA also plays an important role in the induction of secondary somatic embryogenesis in carrot (Ogata et al. 2005). These results suggest that the stress-induced accumulation of endogenous ABA is involved in the induction of carrot somatic embryogenesis.

To examine the significance of ABA in the induction of somatic embryogenesis in carrot, the times at which somatic cells acquire embryogenic competence during the stress induction of somatic embryos were evaluated, based on the expression of *C-ABI3* and the *ECP* genes, as well as on changes in the endogenous ABA levels. Furthermore, fluridone, an inhibitor of ABA biosynthesis, was used to clarify the involvement of ABA in the acquisition of embryogenic competence.

Materials and methods

Plant materials and cell culture

Daucus carota L. cv. US-Harumakigosun was used as the plant material. The EC were induced from 9-day-old-hypocotyls, as described (Tachikawa et al. 1998). The medium used in this experiment was the Murashige and Skoog (MS) medium (Murashige and Skoog 1962), containing or lacking 2,4-D (1 mg/l). To establish NC, small cell clusters of less than 1 mm in diameter were collected from an EC suspension and subcultured at 2-week intervals, as described by Satoh et al. (1986). The NC, which had lost the ability to form somatic embryos, were then used as a negative control for the acquisition of embryogenic competence. The cultured cells were harvested, frozen immediately in liquid nitrogen, and stored at −80°C until use.

Induction of somatic embryogenesis by stress treatments

Apical tip segments (ca. 7 mm in length) were excised from surface-sterilized seedlings, as described by Tachikawa et al. (1998). The explants were cultured at 25°C in 9-cm-diameter plastic Petri dishes on 30 ml of a phytohormone-free MS solid medium (0.8% agar, w/v), containing 3% (w/v) sucrose and stress substances at final concentrations of 0.7 M sucrose, 0.3 M NaCl or 1 mg/l 2,4-D. Cultures grown in the stress-substance-free MS medium at 37°C were subjected to heat stress. Sucrose-stress treatments were carried out for 4 days or for 1, 2, 3, 4, or 6 weeks. The NaCl and heat-stress treatments were carried out for 2 and 3 weeks, respectively. The 2,4-D treatment was performed for 5 days. As a negative control, explants were cultured on a phytohormone-free MS solid medium (0.8% agar, w/v) containing 3% (w/v) sucrose, for 3 days, a length of time that is sufficiently distant from the shock induced by excision as well as the subsequent elongation of true leaves. Explants were harvested for Northern analysis at the indicated times, and immediately frozen in liquid nitrogen and stored at −80°C until use. After each treatment, 50–120 of the explants were transferred to a phytohormone-free MS medium containing 3% (w/v) sucrose and incubated at 25°C. The frequency of somatic embryo formation (%) was examined, at 4 weeks after transfer, for sucrose and 2,4-D treatments, and at 6 weeks after transfer for heat and NaCl treatments. The frequency was calculated as follows: (number of explants that formed somatic embryos/number of surviving explants) × 100.

Treatment with fluridone and ABA

Solutions of fluridone or ABA in dimethyl sulphoxide (DMSO) were added to the media after autoclaving. The final concentration of fluridone was 10^{-4} M, and the final concentrations of ABA were 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M. Cultures growing on medium containing a stress substance were treated with fluridone and ABA. The final concentration of DMSO was 0.1% (v/v) in all of the treatments. The effects of fluridone and/or ABA were estimated along with the frequency of somatic embryo formation at 4 or 8 weeks after transfer.

RNA extraction and Northern blot analysis

The phenol/SDS method (Ausubel et al. 1987) was used to isolate total RNA from EC, NC, and apical tip explants, some of which had been subjected to stress treatments. An amount of 20 μg of total RNA per lane was fractionated

by electrophoresis in formaldehyde-agarose gels (1.2%; v/v) and transferred to nylon membranes (Biodyne B, Pall BioSupport, NY, USA). cDNA fragments from each gene were labeled by random priming with [α-^{32}P]dCTP using a Multiprime labeling kit (Amersham, Tokyo, Japan). The blots were prehybridized at 42°C for 2 h in a hybridization buffer containing 50% formamide (v/v), 5x SSPE, 5x Denhardt's solution, 0.1% SDS (w/v), and 100 mg/ml a denatured herring sperm DNA; and then hybridized with 10^{-7} cpm of labeled DNA in the same buffer at 42°C for 16 h. The membrane was washed twice with 2x SSC for 10 min at room temperature and then twice with 2x SSC containing 0.1% SDS (*w*/*v*) for 10 min at 60°C. Signals were visualized and quantified using a BAS5000 Bio-Imaging plate (Fuji Photo Film, Tokyo, Japan). As an internal control, the filters were rehybridized with a [α-^{32}P]-labeled cDNA fragment encoding the 18S rRNA.

Determination of ABA

Abscisic acid was extracted from the explants and determined as described (Kuwabara et al. 2003). The explants (ca. 200 mg FW) were homogenized in 80% (v/v) acetone containing 0.1 mg/ml 2,4-di-tert-butyl-4-methylphenol. After adding $^{13}C_2$-ABA as an internal standard, the homogenate was shaken for 1 h on ice in darkness and then centrifuged at 1,200×*g* for 5 min at 4°C. The precipitate was then re-extracted, and the combined supernatant was evaporated to remove residual acetone. The ABA was partially purified from the residual aqueous solution by partitioning, using hexane and ether, followed by high performance liquid chromatography. The ABA, methylated with diazomethane, was analyzed by gas chromatography (GC) selected ion monitoring (SIM) mass spectrometry (MS). The GC-MS was performed with a mass spectrometer (QP5050A, Simadzu, Tokyo, Japan) coupled to a gas chromatograph (GC-17A, Shimadzu). The ABA was determined by monitoring the fragments with values of 192 and 190, which correspond to ^{13}C-ABA and endogenous ABA, respectively.

Results

The frequency of somatic embryo formation in stress-treated apical tip segments

Embryo formation was induced in carrot apical tip explants using the stress treatments (Fig. 1) of high osmotic pressure, high temperature, high salinity, and high concentration of 2,4-D. To estimate the relationship between the length of the stress treatment and the frequency of embryo formation (the ratio of the number of explants formed from embryos to the total number of explants), carrot apical tip explants were treated with 0.7 M sucrose, one of the most effective stresses for inducing somatic embryogenesis, for varying lengths of time (4 days or 1, 2, 3, 4, or 6 weeks). The frequency of embryo formation was investigated 4 weeks after removal of the explants from the stress condition. Embryos formed at a low rate (< 1%) on explants that had been subjected to 4 days of stress treatment, and longer stress-treatment periods resulted in increased frequencies of embryo formation. The frequency of somatic embryo formation was 81% in explants exposed to stress for 6 weeks. The frequency of embryo formation increased linearly with increases in the stress-treatment periods, from 1 to 4 weeks (Fig. 2). Treatment with NaCl or high temperatures led to a low-rate of embryo formation by apical tip explants (< 1%) after 4 weeks of recovery,

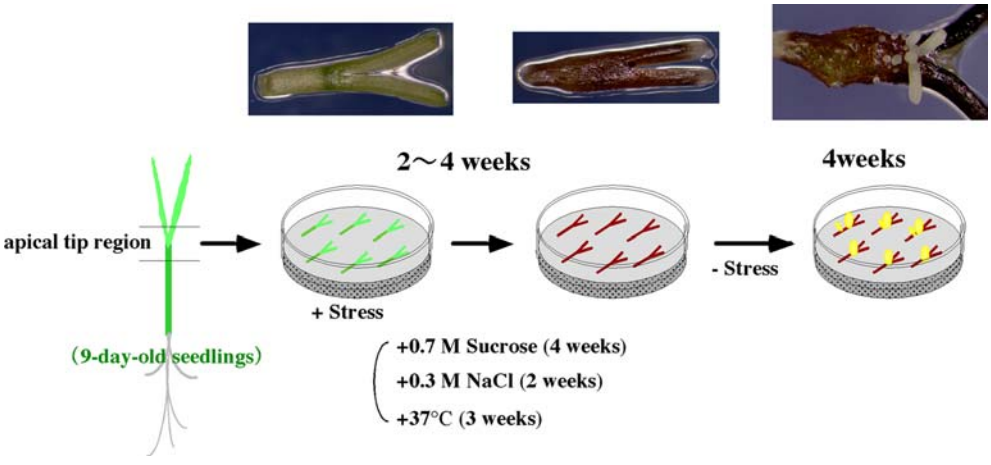

Fig. 1 Stress induction of carrot somatic embryos. Apical tip segments of 9-day-old seedlings were cultured on a phytohormone-free MS medium, under stress conditions (0.7 M sucrose, 0.3 M NaCl or a high temperature of 37°C) for 1 to 6 weeks, and then transferred to a phytohormone-free MS medium under stress-free conditions at 25°C. Somatic embryos formed on the surface of leaves and/or apical tip segments without a visible intervening callus stage (*photograph*). The *bar* indicates 1 mm. Heart- or torpedo-shaped embryos appeared on the explants as early as about 1 week after removal of the stressor. The resultant somatic embryos developed into plantlets with normal morphology

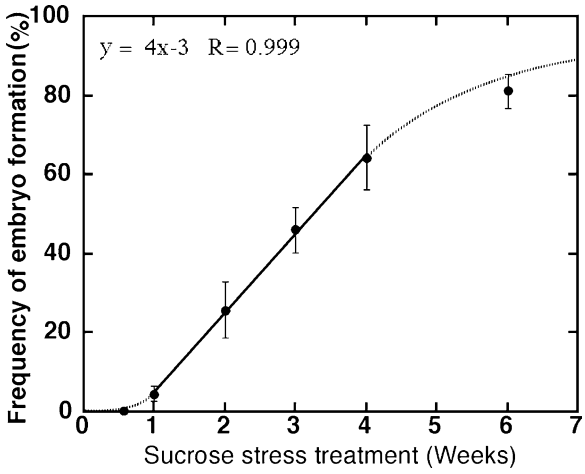

Fig. 2 Frequency of somatic embryogenesis during sucrose-stress treatment. The explants were subjected to periods of stress, of over 4 days or 1–6 weeks, in the presence of 0.7 M sucrose. As a negative control, the explants were cultured on a phytohormone-free MS solid medium (0.8% agar, w/v) containing 3% (w/v) sucrose without any stress treatment for 3 days, a time that is sufficiently distant from the shock of excision and before the elongation of true leaves. After the treatments, 100–120 of the explants were transferred to phytohormone-free MS medium containing 3% (w/v) sucrose, without any stress substances, and grown at 25°C. The frequency of somatic embryo formation was examined at 4 weeks after transfer and calculated as follows: (number of explants forming somatic embryos/number of surviving explants) × 100

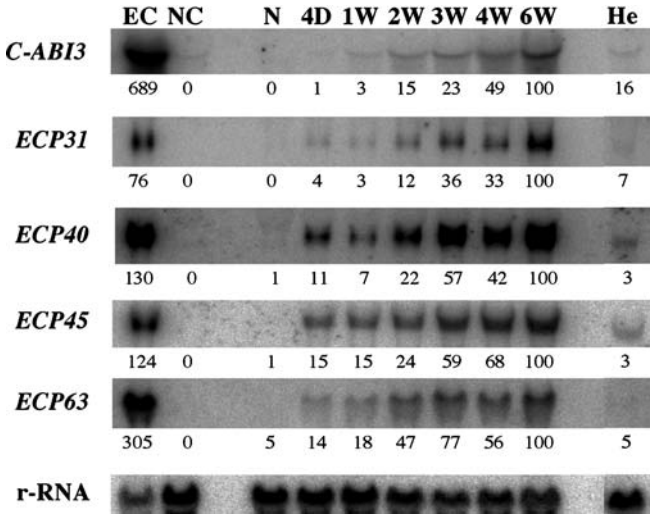

Fig. 3 Changes in the expression of *C-ABI3* and the *ECP* genes in explants during stress treatment. The expression patterns of *C-ABI3* and the *ECPs*, in stress-treated apical tip segments, were investigated using Northern hybridization. *Lanes EC* and *NC* indicate EC and NC induced with auxin, respectively. *Lane N* indicates a negative control that has been cultured for 3 days on a stress-substance-free MS medium. *Lanes 4D, 1 W, 2 W, 3 W, 4 W*, and *6 W* indicate explants subjected to sucrose-stress treatment for 4 days or 1–6 weeks, respectively. *He* indicates the stress treatments of culturing at 37°C. The *graphs* show the amounts of each mRNA that accumulated during the sucrose-stress treatment. The data were normalized to the 18S rRNA signal and the expression level in each lane is shown relative to the level of the signal in the 6 W sample, which was defined as 100

following removal of the stress. No somatic embryos formed in explants not exposed to stress treatments, even at 8 weeks after the beginning of culturing, at which point the explants were developing true leaves and roots.

Expression of *C-ABI3* and the *ECP* genes during stress treatment

The expression of the *ECP* genes and *C-ABI3* were examined during stress treatments of apical tip explants. These genes are known to be embryonic-tissue specific genes that are not expressed in seedling and mature leaves (Tachikawa et al. 1998; Shiota et al. 1998). The *C-ABI3* is related to ABA signal transduction. The *C-ABI3* was already expressed in explants after 4 days of stress treatment, and the expression increased with increasing lengths of the treatments. The *C-ABI3* expression level appeared to be associated with the frequency of embryo formation (Figs. 2, 3). All of the *ECP* genes were expressed during the stress treatments, and the expression levels increased in concert with the frequency of embryo formation, as for *C-ABI3* (Fig. 3). These results indicate that the somatic cells in the explants had already acquired embryogenic competence during the stress treatments, even before the formation of somatic embryos was visible.

Effects of ABA and/or fluridone on the induction of somatic embryogenesis

Since some stress treatments cause somatic embryogenesis in carrot cells, and the involvement of ABA in somatic embryogenesis has been reported (Kiyosue et al. 1992c; Nishiwaki et al. 2000; Ogata et al. 2005); it was expected that ABA would influence the induction of somatic embryogenesis. ABA was applied at various concentrations (10^{-7} to 10^{-5} M), during stress treatments, to examine the influence of ABA on somatic embryo formation. The frequency of somatic embryo formation increased with the ABA concentration. The application of 10^{-5} M ABA resulted in an increase in the frequency of somatic embryo formation from 18 (without ABA) to 30% (Table 1).

Various concentrations of fluridone (10^{-7} to 10^{-4} M) were applied during the stress treatments to further examine the influence of ABA on somatic embryo formation. With an effective fluridone concentration of 10^{-4} M (data not shown), the frequency of somatic embryo formation dropped from 17 (without fluridone) to 5% (Table 2). The inhibition was also observed during the induction of somatic embryos by other stresses (Table 3).

To assess the possibility that fluridone could inhibit somatic embryogenesis without inhibiting ABA bio-

Table 1 Effect of ABA application on the induction of somatic embryogenesis

ABA concentration (M)	Frequency of embryo formation[a]
Control	18.4 ± 2.2
10^{-7}	20.0 ± 2.4
10^{-6}	21.6 ± 3.6
10^{-5}	30.4 ± 1.8

Various concentrations of ABA (10^{-7}, 10^{-6}, and 10^{-5} M) were added during sucrose stress treatments using stock solutions in DMSO. The final DMSO concentration was 0.1% (*v*/*v*) in all experiments. The mean ± SE is given for five experiments, each with 100–120 explants. [a]The frequency of somatic embryo formation (%) was examined 4 weeks after transfer and calculated using the following formula: (number of explants forming somatic embryos/number of surviving explants) × 100

synthesis, the effects of simultaneous application of fluridone and ABA were examined. Both fluridone (10^{-4} M) and ABA (10^{-7} to 10^{-4} M) were applied simultaneously during stress treatments. The inhibition of somatic embryogenesis, which was caused by fluridone, was negated by the simultaneous application of ABA at all of the investigated concentrations (data not shown). The simultaneous application of fluridone (10^{-4} M) and ABA (10^{-5} M) resulted in frequencies very similar to those of the control (without fluridone or ABA; Table 2).

Changes in the endogenous ABA level during stress treatment

The initial level of endogenous ABA in the explants was 1.2 ng/g fresh weight (FW; Fig. 4). During the stress treatment, the level of endogenous ABA peaked at 3 days, at which time the ABA content was 9.2 ng/g FW, followed by a gradual decrease. The ABA content at 42 days of stress treatment was 1.5 ng/g FW, which was similar to the level before the stress treatment (Fig. 4). In contrast, in cultures incubated for 3 days in the absence of stress substances, the content was 1.8 ng/g FW (Fig. 4). After a stress treatment of 42 days, followed by a 3 days of culture in stress-free conditions, the ABA content was 1.1 ng/g FW, similar to the content after a stress treatment of 42 days (Fig. 4).

Table 2 Effect of fluridone and/or ABA application on the induction of somatic embryogenesis

Treatment	Frequency of embryo formation[a]
Control	17.0 ± 0.9
Fluridone	5.3 ± 1.1
Fluridone + ABA	19.2 ± 1.7

Fluridone (10^{-4} M) and ABA (10^{-5} M) were added simultaneously during sucrose stress treatments. The simultaneous application of fluridone and ABA resulted in a frequency of embryo formation of about the same level as in the control not treated with fluridone or ABA. The ABA and fluridone stocks were dissolved in DMSO and the final DMSO concentration was 0.1% in all experiments. The mean ± SE is given for five experiments, each with 100–120 explants. [a]The frequency of somatic embryo formation (%) was examined 4 weeks after transfer and calculated using the following formula: (number of explants forming somatic embryos/number of surviving explants) × 100

Table 3 Effect of fluridone application on the induction of somatic embryogenesis

Stress type	Ratio of somatic embryo formation[a]
Sucrose	0.4 ± 0.1
NaCl	0.0 ± 0.1
Heat (37°C)	0.1 ± 0.1
2,4-D	0.7 ± 0.1

Fluridone dissolved in DMSO was added to 10^{-4} M during each stress treatment. The final concentration of DMSO was 0.1% (*v*/*v*) in all experiments. The mean ± SE is given for three experiments, each with > 50 explants. Application of fluridone decreased the frequency of somatic embryogenesis. [a]The ratio of somatic embryo formation was calculated as the frequency of somatic embryo formation in the presence of fluridone divided by that in untreated cultures, after transfer to stress-free conditions for 4 or 6 weeks for sucrose and 2,4-D or NaCl and heat stress, respectively

Effect of fluridone on ABA biosynthesis and the expression of embryonic genes

Since fluridone treatment affected somatic embryogenesis (Table 2), the effects of fluridone at the molecular level were evaluated. The ABA levels were determined, after 3 days of culture in the presence of fluridone, when the endogenous ABA content was maximal (Fig. 4). The content of endogenous ABA after 3 days of fluridone treatment was 3.1 ng/g FW, one-third that of the level in explants cultured in the absence of fluridone (Table 4). Therefore, the fluridone treatment reduced ABA synthesis, but did not eliminate it. In addition, the expression of *C-ABI3*, *ECP31*, and *ECP63* was analyzed in the presence of fluridone and/or ABA. The expression of each of these genes was associated with the frequency of somatic embryo formation, which decreased in the presence of fluridone and was at the control level in the presence of both fluridone (10^{-4} M) and ABA (10^{-5} M; Table 5).

Table 4 Contents of endogenous ABA after 3 days of stress treatment

Application	Endogenous ABA (ng/g fresh weight)
Mock	7.99 ± 0.77
Fluridone	3.12 ± 0.05

To evaluate the effects of 10^{-4} M fluridone, ABA was quantitated at 3 days into the sucrose stress treatment, at which time the endogenous ABA level had peaked (Fig. 4). The mock sample contained 0.1% DMSO (*v*/*v*) to match that introduced into the fluridone-treated sample

Table 5 Effect of fluridone and ABA on somatic embryogenesis and the expression of embryogenesis-related genes ABA was applied in the stress-induction system for somatic embryos but no stresses were applied.

Application	Fluridone	Fluridone + ABA
Relative embryo formation[a]	0.4	1.3
Relative C-ABI3	0.4	0.9
Expression ECP31	0.6	1.3
Level[b] ECP63	0.6	1.3

The expression of *C-ABI3*, *ECP31*, and *ECP63* was analyzed in the presence of fluridone (10^{-4} M) and/or ABA (10^{-5} M) during sucrose stress treatment. The expression data were normalized to the 18S rRNA signal. The expression of all genes was associated with the frequency of somatic embryo formation, which decreased in the presence of fluridone and remained at the control level in the presence of both fluridone and ABA. The data resulted from the same batch of samples. After stress treatment with/without fluridone and/or ABA each, about 200 explants were harvested for RNA extraction and > 100 explants were used to estimate somatic-embryo formation. The same tendency was observed in two separate experiments. [a]The embryo formation in each treatment is shown as a relative value without both fluridone and ABA, which was defined as 1.0. [b]The expression level in each treatment is shown relative to the level of the signal without both fluridone and ABA, which was defined as 1.0

Induction of somatic embryogenesis by ABA treatment alone

The effect of ABA on somatic embryogenesis was verified by treatment with 0.7 M sucrose (Tables 1, 2), with ABA appearing to promote somatic embryogenesis. To determine whether ABA is the sole factor required for the induction of somatic embryogenesis, 10^{-5} to 10^{-3} M ABA was used in the stress-induction system for somatic embryos in place of the stress substances. 4 weeks after release from the ABA treatment, small somatic embryos had formed. After four more weeks of culturing, the frequencies of somatic embryo formation were 1, 5, and 29% in the presence of 10^{-5}, 10^{-4}, and 10^{-3} M ABA, respectively (Table 6).

Table 6 Effect of ABA on somatic embryogenesis.

ABA Concentration (M)	Frequency of embryo formation[a]
10^{-3}	29.0
10^{-4}	5.0
10^{-5}	1.0

At 8 weeks after release from the ABA treatment, the frequency of embryo formation was evaluated. The same tendency was observed in two separate experiments, each with 100–120 explants.[a]The frequency of somatic embryo formation (%) was examined at 8 weeks after transfer and calculated as follows: (number of explants forming somatic embryos/number of surviving explants) × 100

Discussion

Acquisition of embryogenic competence in the stress-induction system for carrot somatic embryos

In the stress-induction system for carrot somatic embryos, stress treatment followed by transfer to stress-free conditions, leads to embryo formation from cells of the explants, bypassing the callus stage (Fig. 1). The two steps in this system are the period during the stress treatment and the period after release from the stress condition. To study the acquisition of embryogenic competence, it was necessary to clarify the time at which the explant somatic cells acquired embryogenic competence. The embryo-specific genes *C-ABI3* and the *ECPs* were already expressed during the stress treatment, before the formation of somatic embryos, and their expression levels increased with the duration of the stress treatment (Fig. 3). Furthermore, the frequency of embryo formation increased linearly during stress-treatment periods from 1 to 4 weeks (Fig. 2). These results indicate that the explant somatic cells acquired embryogenic competence during the stress treatment and before the formation of somatic embryos. Therefore, this stress-induction system is clearly separated into two phases: the acquisition of embryogenic competence and the formation of somatic embryos. The former phase is suitable for studying the acquisition of embryogenic competence by somatic cells.

Involvement of ABA in somatic embryogenesis

It has been reported that stresses such as high osmotic pressure, heavy metal ions, and heat can readily induce somatic embryogenesis in carrot (Kiyosue et al. 1989a, b, 1990; Kamada et al. 1989, 1993, 1994). It was postulated that ABA was involved in these processes. In addition, ABA has been reported to play an important role in regulating the expression of carrot *ECP* genes, which are related to the acquisition and/or maintenance of embryogenic competence (Kiyosue et al. 1992b, 1993b). The endogenous ABA level in EC is higher than that in NC (Kiyosue et al. 1992c). Furthermore, somatic embryogenesis is induced by the treatment of seedlings with ABA in culture (Nishiwaki et al. 2000), and ABA also plays an important role in the induction of secondary somatic embryogenesis in carrot (Ogata et al. 2005). These observations suggest that ABA contributes to the induction of somatic embryogenesis in carrot. The application of ABA also promoted somatic embryogenesis in our system (Table 1).

In the stress-induction system, the level of endogenous ABA reached a peak within 1 week, and then gradually decreased through the rest of the stress treatment (Fig. 4). The expression of *C-ABI3* in explants exposed to stress for 4 days indicates that ABA signal transduction might be active early on in the stress treatment (Fig. 3). Furthermore, the expression of some embryonic genes was associated with the frequency of somatic embryo formation when the fluridone and/or ABA were added (Table 5). These results strongly suggest that endogenous ABA is involved in the induction

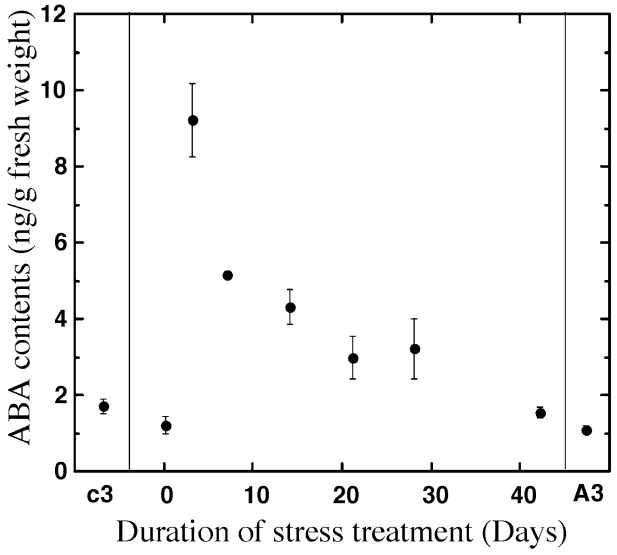

Fig. 4 Endogenous ABA levels during stress treatments. Endogenous ABA contents were determined in stress-induced somatic embryos by GC, SIM, and MS. Samples exposed to stress treatment for 0, 3, 7, 14, 21, 28, or 42 days were analyzed. *c3* indicates tissue that was cultured for 3 days in the absence of stress conditions, *A3* indicates tissue exposed to a stress treatment for 42 days and then cultured in stress-free conditions for 3 days. The *bars* represent the means ± SE for three experiments, each with over 250 explants

of carrot somatic embryogenesis, in particular the acquisition of embryogenic competence.

Effects of fluridone on somatic embryogenesis

To examine the contribution of de novo synthesis of ABA to the formation of somatic embryos, fluridone, a potent inhibitor of ABA biosynthesis, was applied during the stress treatments. The frequency of somatic embryo formation decreased proportionally due to the concentration of fluridone added under sucrose-stress conditions, especially at 10^{-4} M (Table 2). The decrease in somatic embryo formation was also observed when somatic embryos were induced by other stresses (Table 3). However, simultaneous application of fluridone and ABA revealed that the inhibitory effect of fluridone was negated by ABA (Table 2). The inhibition of ABA biosynthesis by fluridone was investigated by quantitating the endogenous ABA, which revealed that fluridone treatment decreased the endogenous ABA content to one-third of that in plants grown in the absence of fluridone (Table 4). It thus appears that fluridone treatment partially blocked ABA biosynthesis, but did not completely eliminate it. This study demonstrated that fluridone application did not completely inhibit embryo formation (Table 2).

It is known that plants treated with fluridone turn white and sometimes exhibit morphological abnormalities such as shortened petioles (Bartels and Watson 1978). Thus, it is possible that the inhibition of carrot somatic embryogenesis by fluridone might be due to a toxic effect of fluridone. However, carrot explants treated with fluridone showed the same viability as control plants not exposed to fluridone (data not shown), thus decreasing the possibility of a toxic effect. Furthermore, the expression levels of embryonic genes were associated with the frequency of embryo formation triggered by the application of fluridone and/or ABA (Table 5). Therefore, it is thought that the fluridone treatment caused a reduction in the endogenous ABA level in carrot cultures.

Significance of stress as an inducer of somatic embryogenesis

The endogenous level of ABA in the explants was about 10^{-5} to 10^{-4} M during the stress treatments (Fig. 3). In addition, the application of ABA at a concentration equivalent to the endogenous level, during the stress treatments, promoted somatic embryo formation (Table 1). However, somatic embryogenesis was not significantly induced by the application of ABA alone, at the same concentration, but without stress treatment (Table 6). It appears that ABA treatment is insufficient for the induction of somatic embryogenesis.

The formation of non-zygotic embryos can be induced by the heat treatment of *Brassica napus* microspores (Keller and Armstrong 1978) and by the starvation of immature *Nicotiana tabacum* pollen (Imamura et al. 1982; Kyo and Harada 1985). It has been thought that one of the responses of plant cells to stress is the acquisition of embryogenic competence (Kiyosue et al. 1992). In carrot, auxin treatment also induces somatic embryogenesis. The concentrations of exogenously applied auxin, required to induce somatic embryogenesis, are much higher than the endogenous auxin levels (10^{-4} to 10^{-6} M vs. 10^{-8} M) in plant tissues (Kamada and Harada 1979; Ribnicky et al. 1996). The synthetic auxin 2,4-D is more effective at inducing somatic embryogenesis than the endogenous auxin IAA, because the artificial auxin cannot be metabolized in plant cells. The 2,4-D has been used as a herbicide and induces stress responses in plant cells (Czarncka et al. 1984). These facts suggest that when exogenous auxin is applied, it acts as a stressor rather than as a plant hormone. However, it has been reported that the application of ABA alone triggers somatic embryogenesis in carrot (Nishiwaki et al. 2000). Given that, in this study, embryogenesis was induced by a high concentration of ABA (10^{-4} to 10^{-3} M), ABA might also act as a stressor and induce somatic embryogenesis, in a manner similar to the application of 2,4-D.

These results suggest that the induction of somatic embryogenesis is caused not only by the presence of ABA, but also by the physiological responses that are directly controlled by stresses. Both ABA-responsive and ABA-independent reactions to environmental stresses have been documented (Shinozaki-Yamaguchi and Shinozaki 1994). Various stress substances or

conditions might stimulate the biosynthesis of ABA and other responses in the absence of exogenous ABA, and the induction of somatic embryogenesis might require both pathways. However, it is unclear whether the induction of somatic embryogenesis requires auxin or other phytohormones. The tissue in the vicinity of the shoot meristem in seedlings is the region in which embryos form most frequently, following stress treatment (Kamada et al. 1993); it is also an auxin-rich region (Li et al. 1999).

This study has demonstrated that somatic cells acquire embryogenic competence during stress treatment in the stress-induction system for carrot somatic embryogenesis, and that both the stresses and the appearance of ABA are essential for this acquisition. Further studies on ABA-independent and ABA-dependent physiological responses are needed to clarify the molecular mechanisms that trigger the acquisition of embryogenic competence. The involvement of other phytohormones is also important for somatic embryogenesis. Since no phytohormone application is required in the stress-induction system for somatic embryogenesis, this system should be useful for studying the relationships between embryogenesis and phytohormones.

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References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1987) Phenol/SDS Method for plant RNA preparation. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York, pp 4.3.1–4.3.4

Bartels PG, Watson CW (1978) Inhibition of carotenoid synthesis by fluridone and norflurazon. Weed Sci 26:198–203

Bentley B, Morgan CB, Morgan DG, Sand FA (1975) Plant growth substances and effects of phytoperiod on flower and flower bud development in *Phaseolus vulgaris*. Nature 256:121–122

Czarncka E, Edelman L, Schoffl F, Key JL (1984) Comparative analysis of physical stress responses in soybean seedlings using cloned heat shock cDNAs. Plant Mol Biol 3:45–58

Fedina IS, Tsonev TD, Guleva EI (1994) ABA as modulator of the response of *Pisum sativum* to salt stress. J Plant Physiol 143:245–249

Galli MG, Levi M (1982) Increased drought resistance induced by pretreatment with abscisic acid in germinating embryos of *Haplopappus gracilis*. Physiol Plant 54:425–430

Harada H, Kiyosue T, Kamada H, Kobayashi K (1990) Stress-induced carrot somatic embryogenesis and their application to synthetic seeds. In: Sangwan RS, Sangwan-Norreel BS (eds) The impact of biotechnology in agriculture. Kluwer Academic Publishers, Dordrecht, pp 129–157

Imamura J, Okabe E, Kyo M, Harada H (1982) Embryogenesis and plantlet formation through direct pollen culture of isolated pollen of *Nicotiana tabacum* cv. Samsun and *Nicotiana rustica* cv. Rustica. Plant Cell Physiol 23:713–716

Kamada H, Harada H (1979) Studies on organogenesis in carrot tissue culture. I. Effects of growth regulation on somatic embryogenesis and root formation. Z Pflanzenphysiol 91:225–266

Kamada H, Kobayashi K, Kiyosue T, Harada H (1989) Stress-induced somatic embryogenesis in carrot and its application to synthetic seed production. In Vitro Cell Dev Biol 25:1163–1166

Kamada H, Ishikawa K, Saga H, Harada H (1993) Induction of somatic embryogenesis in carrot by osmotic stress. Plant Tissue Cult Lett 10:38–44

Kamada H, Tachikawa Y, Saitou T, Harada H (1994) Heat stress induction of carrot somatic embryogenesis. Plant Tissue Cult Lett 11:229–232

Keller WA, Armstrong KC (1978) High-frequency production of microspore-derived plants from *Brassica napus* anther cultures. Z Pflanzenphysiol 80:100–108

Kiyosue T, Kamada H, Harada H (1989a) Induction of somatic embryogenesis from carrot seeds by hypochlorite treatment. Plant Tissue Cult Lett 6:138–143

Kiyosue T, Kamada H, Harada H (1989b) Induction of somatic embryogenesis by salt stress in carrot. Plant Tissue Cult Lett 6:162–164

Kiyosue T, Takano K, Kamada H, Harada H (1990) Induction of somatic embryogenesis in carrot by heavy metal ions. Can J Bot 68:2021–2033

Kiyosue T, Satoh S, Kamada H, Harada H (1992a) Purification and immunohistochemical detection of an embryogenic cell protein in carrot. Plant Physiol 95:1077–1083

Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K, Higashi K, Satoh S, Kamada H, Harada H (1992b) Partial amino-acid sequence of ECP31, a carrot embryogenic-cell protein, and enhancement of its accumulation by abscisic acid in somatic embryos. Planta 186:337–342

Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K, Higashi K, Satoh S, Kamada H, Harada H (1992c) Isolation and characterization of a cDNA that encodes *ECP31*, an embryogenic-cell protein from carrot. Plant Mol Biol 19:239–249

Kiyosue T, Nakajima M, Yamaguchi I, Satoh S, Kamada H, Harada H (1992) Endogeneous levels of abscisic acid in embryogenic cells, non-embryogenic cells and somatic embryos of carrot (*Daucus carota* L.). Biochem Physiol Pflanzen 188:343–347

Kiyosue T, Satoh S, Kamada H, Harada H (1993a) Somatic embryogenesis in higher plants. J Plant Res Special Issue 3:75–82

Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K, Kamada H, Harada H (1993b) cDNA cloning of *ECP40*, an embryogenic-cell protein in carrot, and its expression during somatic and zygotic embryogenesis. Plant Mol Biol 21:1053–1068

Koornneef M, Reuling G, Karessen CM (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. Plant Physiol 61:377–383

Kuwabara A, Ikegami K, Koshiba T, Nagata T (2003) Effects of ethylene and abscisic acid upon heterophylly in *Ludwigia arcuata* (*Onagraceae*). Planta 217:880–887

Kyo M, Harada H (1985) Studies on conditions for cell division and embryogenesis in isolated pollen culture of *Nicotiana rustica*. Plant Physiol 79:90–94

Li Y, Wu YH, Hagen G, Guilfoyle T (1999) Expression of the auxin-inducible *GH3* promoter/GUS fusion gene as a useful molecular marker for auxin physiology. Plant Cell Physiol 40:675–682

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nishiwaki M, Fujino K, Koda Y, Masuda K, Kikuta Y (2000) Somatic embryogenesis induced by the simple application of abscisic acid to carrot (*Daucus carota* L.) seedlings in culture. Planta 211:756–759

Ogata Y, Iizuka M, Nakayama D, Ikeda M, Kamada H, Koshiba T (2005) Possible involvement of abscisic acid in the induction of secondary somatic embryogenesis on seed coat-derived carrot somatic embryos. Planta 221:417–423

Parcy F, Valon C, Raynal M, Gaubier-Comella P, Delseny M, Giraudat J (1994) Regulation of gene expression programs during Arabidopsis seed development: roles of the ABI3 locus and of endogenous abscisic acid. Plant Cell 6:1567–1582

Reinert J (1959) Uber die Kontrolle der morphogenese und die induction von adventivembryonen an Gewebekulturen aus Carotten. Planta 53:318–333

Ribnicky DM, Ilic N, Cohen JD, Cooke TJ (1996) The effects of exogenous auxins on endogenous indole-3-acetic acid metabolism. The implications for carrot somatic embryogenesis. Plant Physiol 112:549–558

Saab IN, Sharp RE, Pritchard J (1992) Effect of inhibition of abscisic acid accumulation on spatial distribution of elongation in the primary root and mesocotyl of maize at low water potentials. Plant Physiol 99:26–33

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

Sauser C, Kwiatkowski J, Jung J, Grossamann K (1992) Accumulation of abscisic acid in cell suspension cultures of oilseed rape treated with the growth retardant BAS111.W: effects on osmotic potential and potassium, water and sugar contents. J Plant Physiol 140:747–753

Shinozaki-Yamaguchi K, Shinozaki K (1994) A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 6:251–264

Shiota H, Satoh R, Watabe K, Harada H, Kamada H (1998) *C-ABI3*, the carrot homologue of *Arabidopsis ABI3*, is expressed during both zygotic and somatic embryogenesis and functions in the regulation of embryo-specific ABA-inducible genes. Plant Cell Physiol 39:1184–1193

Shiota H, Kamada H (2000) Acquisition of desiccation tolerance by cultured carrot cells upon ectopic expression of *C-ABI3*, a carrot homologue of *ABI3*. J Plant Physiol 156:510–515

Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell 2:503–512

Steward FC, Mapes MO, Mears K (1958) Growth and organized development of cultured cells. I. Growth and division of freely suspended cells. Am J Bot 45:693–703

Sung ZR, Fienberg A, Chorneau R, Borkird C, Furner I, Smith J (1984) Developmental biology of embryogenesis from carrot culture. Plant Mol Biol Rep 2:3–14

Tachikawa Y, Saitou T, Kamada H, Harada H (1998) Changes in protein pattern during stress-induction of carrot (*Daucus carota* L.) somatic embryogenesis. Plant Biotechnol 15:17–22

Walton DC (1980) Biochemistry and physiology of abscisic acid. Annu Rev Plant Physiol 31:453–489