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Genetically modified carrot cells acquiring desiccation tolerance

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Abstract

To obtain direct evidence for the involvement of C-ABI3, a carrot (*Daucus carota* L.) homolog of VP1/ABI3, seed-specific transcription factor, in the acquisition of desiccation tolerance, transgenic carrot non-embryogenic cells (NC) in which the *C-ABI3* gene was expressed ectopically was prepared. Non-transgenic NC, in which expression of *C-ABI3* was not detected, did not exhibit desiccation tolerance even after treatment with abscisic acid (ABA). In transgenic NC that expressed *C-ABI3*, embryo-specific ABA-inducible genes (*ECP* genes) were expressed upon ABA-treatment. Furthermore, the transgenic NC became desiccation-tolerant upon ABA-treatment, but did not tolerate desiccation without ABA-treatment. These results provide direct evidence for the involvement of C-ABI3 in the ABA-induced acquisition of desiccation tolerance.

Abbreviations: ABA, abscisic acid; ECP, embryogenic cell protein; FDA, fluorescein diacetate; LEA, late embryogenesis abundant; NC, non-embryogenic cells

In seeds of higher plants, abscisic acid (ABA) is a key factor in acquisition of desiccation tolerance and in dormancy, and it acts via induction of the expression of genes for various late embryogenesis abundant (LEA) proteins [4]. It has been suggested that expression of some of these genes is mediated by the seed-specific transcription factor VP1/ABI3 in some plant species [3, 17]. It has been reported that the *C-ABI3* gene, a carrot homolog of the gene for VP1/ABI3, is expressed in somatic embryos, embryogenic cells and developing seeds, but not in seedlings, mature leaves and non-embryogenic cells (NC) which do not tolerate desiccation [18]. Cells and tissues that express the *C-ABI3* gene acquire desiccation tolerance upon exposure to endogenous or exogenous ABA [7, 6, 9, 18]. In addition, expression of embryo-specific ABA-inducible genes, such as *ECP31*, *ECP40* and *ECP63*, can be induced by ABA in mature leaves of transgenic carrot that ectopically express the *C-ABI3* gene [18]. However, no evidence has yet been reported for the ABA-induced acquisition of desiccation tolerance by transgenic plants or tissues that express VP1/ABI3 ectopically. Then, we induced ectopic expression of the *C-ABI3* gene in carrot NC and demonstrated the involvement of C-ABI3 in the ABA-induced acquisition of desiccation tolerance.

Carrot (*Daucus carota* L. cv. US-Harumakigosun) embryogenic cells and NC were obtained and maintained as described by Satoh *et al.* (1986).

The DNA construct was made in the pBE2113-GUS binary T-DNA vector, which includes a kanamycin-resistance gene as a selective marker [13]. A full length carrot *C-ABI3* cDNA was cloned in pBE2113-GUS as described by Shiota *et al.* (1998). The cDNA was placed under the control of the El2_ promoter [13]. The binary vector was transferred to *Agrobacterium tumefaciens* C58C1Rif^r (pGV2260) by triparental mating [2].

Cells in a seven-day-old culture of NC in liquid MSD medium [Murashige and Skoog (1962) (MS) medium that contained 2,4-dichlorophenoxyacetic acid (2,4-D; 1 mg/L)] were co-cultivated with *Agrobacterium* at 25 °C on a gyratory shaker (100 rpm) for 3 days. Then, Claforan™ (500 mg/L; Hoechst) was added to the culture and the culture was maintained for 4 days under the same conditions. Thereafter transgenic NC were selected by successive subculturing 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

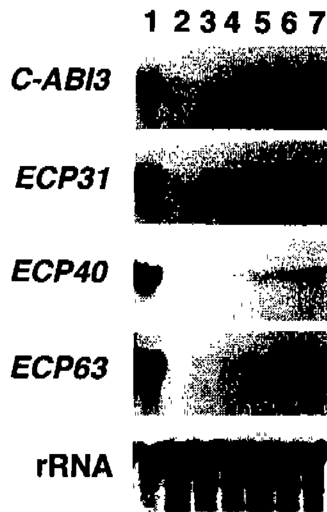

Figure 1. Northern blotting analysis of the expression of the *C-ABI3* gene and three *ECP* genes in transgenic and non-transgenic NC with and without ABA-treatment. Total RNA was isolated from embryogenic cells as a positive control (lane 1), from non-transgenic NC (lanes 2, 3 and 4), and from transgenic NC (491S1) (lanes 5, 6 and 7). NC were cultured for 24 h in medium that contained 0 M (lanes 2 and 5), 1×10^{-5} M (lanes 3 and 6) or 1×10^{-4} M (lanes 4 and 7) 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 [^{32}P]-labeled cDNAs that corresponded to the *C-ABI3*, *ECP31*, *ECP40* and *ECP63* genes as probes. The blot was reprobed with 18S rRNA to provide an internal standard.

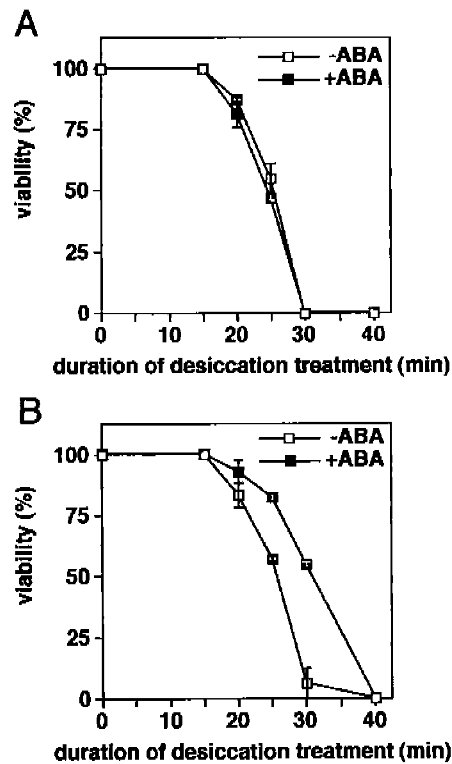

Figure 2. The viability of transgenic and non-transgenic NC after treatment with ABA and desiccation. The viability of non-transgenic NC (A) and transgenic NC, 491S1 (B), with and without ABA-treatment is shown. The viability of NC with and without ABA-treatment is shown by closed and open squares, respectively. ABA-treatment consisted of culture in the presence of 1×10^{-4} M for 14 days. Desiccation treatment was performed for 0, 15, 20, 25, 30 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.

selected NC were maintained by subculturing at two-week intervals in MSD that contained 100 mg/L kanamycin under the same conditions.

NC were transferred to 100-mL aliquots of liquid MSD medium that contained ABA at 1×10^{-5} M or 1×10^{-4} M and cultured on a gyratory shaker (100 rpm) at 25 °C for 24 h, 7, 10, 12 or 14 days in darkness, as indicated.

NC with or without ABA-treatment were washed three times with distilled water by centrifugation (100 xg). The NC were suspended in distilled water at 250 ml packed cell volume (at 100 xg) /L. 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.

The viability of desiccated NC was determined by staining with fluorescein diacetate (FDA) [20]. After desiccation treatment, the NC on the paper were rehydrated and cultured on semi-solidified (0.2% Gelrite™; Monsant) MSD medium at 25 °C for 7 days in darkness. After 7 days in culture, the NC were transferred to MS liquid medium containing FDA (0.2 mg/L) and incubated at 25 °C for 10 min. The stained NC were washed with MS liquid medium three times by centrifugation (100 xg). They were observed with a fluorescence microscope (model BHS-RFK with filter system B; OLYMPUS). 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.

Total RNA was isolated from NC with or without ABA-treatment and from embryogenic cells by the phenol/SDS method [1]. Total RNA (20 μg/lane) was separated by agarose (1.2%) gel electrophoresis and transferred to a Biodyne B nylon filter (Pall BioSupport). Hybridization was performed at 65 °C accord-

ing to the instructions from the manufacturer of the filter using [^{32}P]-labeled cDNAs as probes.

For the selection of transgenic cells, the concentration of kanamycin was increased gradually. Finally, we obtained successfully two batch cultures of transgenic NC (491S1 and 491S2) that were resistant to kanamycin (100 mg/L). In non-transgenic NC, no expression of the *C-ABI3* gene was detected with or without ABA-treatment (Figure 1). By contrast, in transgenic NC (491S1), expression of the *C-ABI3* gene was detected with and without ABA-treatment and the level of expression of the transcript was not increased by ABA-treatment (Figure 1). Therefore, the ectopic expression of the *C-ABI3* gene was driven by the El2Ωpromoter, a modified version of the 35S promoter of cauliflower mosaic virus, in NC strongly.

Using cDNAs that corresponded to three *ECP* genes as probes and mRNA from non-transgenic NC, we failed to detect any expression of the corresponding transcripts with or without ABA-treatment (Figure 1). In transgenic NC (491S1), the expression of these transcripts was detected with and without ABA-treatment and the levels of expression depended on the concentration of ABA applied (Figure 1). These results support the observations in mature leaves of transgenic plants that ectopically expressed the *C-ABI3* gene [18]. Thus, it appears that expression of *ECP* genes might be controlled by ABA and C-ABI3. By contrast, in transgenic NC without ABA-treatment, lower levels of expression of *ECP* genes were observed (Figure 1). This expression might be induced by the small amount of endogenous ABA, because the endogenous level of ABA in NC is very low, but some ABA is still detectable [9].

The *ECP* genes encoded the ECPs which are members of the family of LEA proteins that appear to protect cells against injury during desiccation [10, 11, 4, 21, 19]. The cells and tissues in which the various *ECP* genes express develop desiccation tolerance upon exposure to endogenous or exogenous ABA [7, 6, 9].

After treatment of cells with ABA at 1×10^{-4} M for 14 days, the cells were subjected to staining with FDA. NC with and without ABA-treatment did not survive 30-min of desiccation treatment (Figure 2A). The viability of transgenic NC (491S1) with ABA-treatment 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 (Figures 2A and 2B). After desiccation treatment for 40 min, non-transgenic NC, 491S1 with or without ABA-treatment did not survive (Figures 2A and 2B).

Thus, the transgenic NC (491S1) acquired desiccation tolerance upon ABA-treatment (Figure 2). Furthermore, another batch culture of transgenic NC (491S2) also showed same level of ABA-induced desiccation tolerance as 491S1 (data not shown). These observations provide direct evidence for the involvement of C-ABI3 in the ABA-induced acquisition of desiccation tolerance. It also strongly suggests that C-ABI3 might be involved in desiccation tolerance not only in carrot zygotic embryos but also in somatic embryos via the expression of *ECP* genes, as judged from the pattern of expression of the *C-ABI3* gene [18].

The ABA-induced desiccation tolerance in transgenic NC was weaker than that in somatic embryos [6], because transgenic NC required long-term exposure to a high concentration of ABA and could not tolerate to 40 min of desiccation treatment (Figure 2B). In *Arabidopsis*, ABI4, ABI5, FUS3 or LEC1 are considered to be the factors which involve in seed-specific ABA-signal transduction and desiccation tolerance [5, 8, 12, 15]. Thus, it is possible that ABA-induced desiccation tolerance in transgenic NC was weaker because these factors might not exist or might not function in NC and only the components controlled by C-ABI3 might function in 491S1. Then, ectopic expression of ABI4, ABI5, FUS3 or LEC1 in NC might be able to induce stronger dedication tolerance in NC.

The transgenic NC might be useful as a system for studies of the ectopic expression of the *C-ABI3* gene because the level of ABA is very low and the ectopic expression of the *C-ABI3* gene is strong. It is easy to obtain large amounts of such cells in tissue culture and to monitor the physiological effects of ABA by exogenous application of ABA. This system might also be useful for analysis of molecular mechanisms, such as protein-protein interactions, during ABA-signal transduction that involves the VP1/ABI3 factor.

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