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SUCROSE INCUBATION INCREASES FREEZING TOLERANCE OF ASPARAGUS (*Asparagus officinalis* L.) EMBRYOGENIC CELL SUSPENSIONS

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Abstract

The freezing tolerance of asparagus (*Asparagus officinalis* L.) embryogenic cells, as determined by electrolyte leakage, was increased by the incubation of samples in medium containing 0.8 M sucrose. To elucidate the mechanism involved, we investigated the changes in soluble carbohydrates, cell ultrastructure and proteins accompanying the increase in freezing tolerance following incubation in sugar-rich medium. During sugar incubation, the intracellular sucrose content increased from 67 $\mu mol \cdot g^{-1}$FW to 429 $\mu mol \cdot g^{-1}$FW; it was also metabolized into fructose and glucose, as determined by high-performance liquid chromatography. Microscopy revealed that sugar incubation induced plasmolysis of embryogenic cells and drastic changes in cell ultrastructure with the appearance of rough endoplasmic reticulum (rER). Furthermore, immunoblotting analysis with anti-dehydrin antiserum revealed that a dehydrin-like protein appeared only when maximal freezing tolerance was induced by sugar incubation. These results suggest that freezing tolerance of asparagus embryogenic cells is increased by a complex mechanism involving notably changes in cell ultrastructure and accumulation of certain sugars and proteins during sugar incubation.

Keywords : *Asparagus officinalis* L.; cryopreservation; sugar incorporation; ultrastructural changes; plasmolysis; protein accumulation; late-embryogenesis abundant proteins; dehydrins.

INTRODUCTION

The repeated subculture of plant cell suspensions is one of the methods used for the conservation of plant germplasm. However, the maintenance of cell lines is time-consuming and involves risks of contamination, somaclonal variation, and loss of regenerative-potential as the culture duration is extended. Cryopreservation is a very efficient method for long-term conservation of plant genetic resources (19, 32). However, regeneration from cryopreserved cultured cells requires the minimization of freezing injury that causes a reduction in cell survival after a freeze-thaw cycle.

Plant cell suspensions have been successfully cryopreserved using incubation with various osmoticums prior to freezing. The methods include the conventional two-step freezing procedure, vitrification or encapsulation-dehydration (38). In most cases, the incubation of cells in medium with high concentration of mannitol or a sugar such as sucrose is required. Thus, the intracellular

accumulation of sugar during sugar incubation seems to be one of the most important phenomena in preventing freezing injury. Actually, it has been shown that successful cryopreservation is related to intracellular accumulation of sugar during sugar incubation in oil palm embryogenic culture (5), and cell suspensions of *Eucalyptus gunnii* (22, 33) and *Chrysanthemum cinerariaefolium* (13).

It has also been shown that structural changes occur in plant cells during pretreatment (7). Plasmolysis, one of these structural changes, is closely related to an increase in freezing tolerance of plant cells (17, 29). In addition, osmotic stress and/or dehydration stress induce protein accumulation accompanying specific gene expression (39). These genes are strong candidates for a freezing-tolerance gene (31). Proteins of the dehydrin family, which are induced by environmental stresses such as dehydration or cold acclimation of plant cells, may have a certain protective function against freezing stress (37).

In nature, most temperate perennial plants have developed systems to tolerate freezing stress induced by exposure to low temperatures in late autumn. This increase in freezing tolerance due to cold acclimation is correlated with a number of modifications, including changes such as sugar (28), specific protein (1, 25) and lipid (34) contents, and with morphological changes in cell structures (26, 28). Interestingly, these changes accompanying cold acclimation have some similarities with those occurring in plant cells during incubation in a sugar-rich medium. However, in both sugar-incubated and cold-acclimated samples, it is still not clear which change is the main factor for increasing the freezing tolerance of plant cells.

In this study, we examined changes in intracellular sugar content, ultrastructure of cells and intracellular protein content in relation to an increase in the freezing tolerance of asparagus embryogenic cells following incubation in a sugar-rich medium. The results obtained in this study may enhance one understanding of the mechanism of freezing injury and contribute to improving cryopreservation procedures.

MATERIALS AND METHODS

Plant material: Embryogenic cell suspension cultures of asparagus (*Asparagus officinalis* L. cv. Mary Washington 500W) were used in this study. Asparagus green spears were sprouted from 12-year-old seedlings and grown to about 150 mm in height in a glasshouse at temperatures of 24°C (day) and 16°C (night) (14 h light / 10 h dark photoperiod). Non-developed, small lateral shoot apices (approximately 1.5 mm in length) were excised from the green spears and incubated in a standard liquid medium at 24°C under continuous light. Light-yellow granular calli (#0.2 -1 mm diameter) that appeared on the cut ends of the shoot apices and were released into the liquid medium after incubation for 1 month were used as the experimental material. The standard liquid medium consisted of a modified Murashige and Skoog (MS) medium (24) with half the concentration of NH_4NO_3 and KNO_3, 0.09 M sucrose, 2 $mg \cdot L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 $mg \cdot L^{-1}$ folic acid, 0.05 $mg \cdot L^{-1}$ biotin and 5 mM 2-(n-morpholino) ethanesulfonic acid (MES) (pH 5.8) as a buffering reagent. After 1 month of incubation at 24°C under continuous light on agar-solidified hormone-free MS medium plates containing 0.09 M sucrose, the suspension cells (granular calli) easily formed somatic embryos. The cell suspensions that acquired the ability of embryogenesis were called embryogenic cells.

Sugar incubation and cold treatment: The embryogenic cells were incubated in standard liquid medium supplemented with sucrose to a final concentration of 0.8 M for various periods at 24°C (continuous light). Some embryogenic cells were incubated in standard medium for various periods with a thermoperiod of 4°C for 12 h / 2°C for 12 h under continuous light. Embryogenic cells subcultured in the standard medium for 1 week at 24°C (continuous light) were used as control samples.

Measurement of freezing tolerance by electrolyte leakage: Embryogenic cells (approximately 300 mg FW) were drained on filter paper, placed in a 5-mL test tube and equilibrated at -2.5°C for 30 min. The freezing was initiated at -2.5°C by inoculating small ice crystals. After 1-h holding at -2.5°C, the samples were cooled at a rate of 0.5°C·min^{-1} to the desired temperatures (from -5°C to -30°C) using a programmable freezer [Ultra Low MDF-192AT deep freezer (Sanyo Electric Co., Osaka, Japan) equipped with a programming controller (Tajiri Machine Manufacturing Co., Sapporo, Japan)]. Frozen samples were thawed in a water bath maintained at 34°C, and then 3 mL 0.09 M sucrose solution buffered with 5 mM MES (pH 5.8) (for control samples and cold-treated samples) or 0.8 M sucrose solution (pH 5.8) (for sugar-incubated samples) was added. After 12-h shaking of the tubes to promote the release of electrolytes, the electrolyte conductivity of each solution was measured using a B-173 conductivity meter (Horiba Co., Kyoto, Japan). The percentage of electrolyte leakage was calculated according to the method previously described (8). T_{EL50} was defined as the freezing temperature at which 50 % electrolyte leakage occurred.

Sugar analysis: Sugar analysis was performed as described previously (30). The embryogenic cells (approximately 200 mg FW) incubated with the sugar solutions were quickly rinsed 3 times in a solution containing 0.8 M polyethylene glycol (PEG) 400 (pH 5.8) and drained on filter paper. Control samples and cold-treated samples were rinsed in 0.09 M PEG 400 solutions (pH 5.8). These samples were homogenized in the presence of 2 mL 0.2 N $HClO_4$, 200 mg sea sand, and 1 mL 20 mM lactose (as an internal standard) at 0°C. After centrifugation of the homogenate at 14,000 g for 5 min at 4°C, the supernatant was adjusted to pH 4.0 with $KHCO_3$ and freeze-dried with an FD-5 freeze dryer (Tokyo Rikakikai Co., Tokyo, Japan). The residues were solubilized in 0.5 mL distilled water and centrifuged at 14,000 g for 5 min. Measurement of the sugar contents was performed with 10 µl of the supernatant using a high-performance liquid chromatograph (HPLC) equipped with a NH_2P-50 4E column (Shodex, Tokyo, Japan), L-3300 RI-monitor (Hitachi Co., Tokyo, Japan) and a L-5020 column oven (Hitachi Co.) regulated at 30°C. HPLC was conducted using 75% (v/v) HPLC grade acetonitrile (Kanto Chemical Co., Tokyo, Japan) as the mobile phase at a flow rate of 0.7 mL·min^{-1}.

Confocal laser scanning microscopy: For observation of living cells under a confocal laser scanning microscope (CLSM), the embryogenic cells were stained in 10 µM fluorescein diacetate (FDA) (Sigma Chemical Co., St. Louis, MO, USA) solution buffered with 5 mM MES (pH 5.8) for 30 min at 24°C. When the embryogenic cells were incubated in standard liquid medium containing 0.8 M sucrose, FDA staining was performed in the presence of 0.8 M sucrose. Sections were observed under the CLSM after rinsing 3 times with standard liquid medium (for control samples) or standard liquid medium containing 0.8 M sucrose (for sugar-incubated samples) to remove extracellular FDA.

A MRC-1024 CLSM (Japan Bio-Rad Laboratories Inc., Tokyo, Japan) utilizing a 15 mW argon/krypton laser (488 nm) equipped with an Axioplan light-fluorescence microscope (Carl Zeiss Co., Oberkochen, FRG) was used to observe fluorescent staining of the cells. A series of images obtained with the CLSM was reconstructed as a 3-dimensional image using a Laser Sharp 3.2 software (Japan Bio-Rad Laboratories Inc.) installed in a MRC-1024. The FDA was excited by a 488-nm line, and a 540/30-nm emission filter was used.

Chemical fixation, freeze-substitution and ultra-thin sectioning: For preparing ultra-thin sections, embryogenic cells (approximately 40 mg FW) were drained on filter paper and fixed chemically with 2.5% (w/v) glutaraldehyde and 3.7% (w/v) paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.2) for 24 h at 4°C. Post-fixation with 2% (w/v) osmium tetroxide was carried out for 2 h at 4°C. In order to observe the real cell structure in the sugar-incubated samples, some sugar-incubated samples were prepared for observation with the transmission electron microscope (TEM) by a freeze-substitution method (15). Samples smashed against a pure copper block at liquid nitrogen temperature using a JFD-RFA rapid freezing apparatus (JEOL Co., Tokyo, Japan) were transferred into liquid nitrogen and put in vials containing a freeze-substitution fluid [2% (w/v) osmium tetroxide in 100% acetone] that had previously been cooled to -80°C. The substitution was carried out

at -80°C for 2 days. Substituted samples were kept at -20°C for 2 h, then at 4°C for 1 h.

Both the chemically-fixed samples and freeze-substituted samples were dehydrated in a graded series of ethanol [50 -100% (v/v)] for 1 h and propylene oxide for 30 min, and then infiltrated with Spurr embedding resin (TAAB Laboratories Equipment Ltd., Aldermaston, Berks, UK) at 4°C. Polymerization of resin was carried out at 60°C for 3 days. Thin sectioning (70-80 nm thickness) of the samples was performed using an Ultracut omU4 ultramicrotome (Reichert, Wien, Austria) with a diamond knife (Diatomo Ltd., Switzerland). Sections were stained with 4% (w/v) aqueous uranyl acetate for 20 min and with 0.2% (w/v) aqueous lead citrate for 5 min. They were observed under a JEM 1200 EX transmission electron microscope (TEM) (JEOL Co.) at 100 kV.

Protein extraction and immunoblotting analysis: The embryogenic cells (approximately 100 mg FW) were drained on filter paper and homogenized with 75 mM 2-(n-monopholino) sulfonic acid (MOPS)-KOH (pH 7.6) containing 0.2 M sucrose, 5 mM ethylenediamide tetraacetic acid (EDTA), 10 mM ethyleneglycol bis (2-aminoethylether)-tetraacetic acid (EGTA), 5% (w/v) polyvinyl polypyrolidone (PVPP), 4 mM $Na_2S_2O_5$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 $mg \cdot L^{-1}$ leupeptin, 100 $mg \cdot L^{-1}$ pepstatin, 1 mM benzamidine and 5 mM e -aminocaproic acid at 4°C. Homogenized samples were centrifuged at 156,000 g for 30 min at 4°C. The resultant supernatant was used as soluble protein fraction. The soluble protein fraction was assayed for protein content by the Bradford assay (3). A part of the soluble protein fraction was boiled at 100°C for 10 min and then centrifuged at 14,000 g for 10 min. The resultant supernatant was used as a boiling-stable protein fraction. The boiling-stable protein fraction was analyzed by immunoblotting after one-dimensional sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE) (21).

Boiling-stable protein fractions equivalent to the content of proteins corresponding to 0.7 mg FW of the cells were solubilized in an SDS-solubilization buffer containing 4% (w/v) SDS, 20 mM Tris-HCl (pH 6.8), 10% (w/v) ß-mercaptoethanol, 20% (w/v) glycerol and 0.01% (w/v) bromophenol blue (BPB). Solubilized samples were heated at 70°C for 15 min prior to electrophoresis. Electrophoresis was performed using 4% stacking gel and 13% running gel at a constant current of 15 mA. After electrophoresis, the gels were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Co., Bedford, MA, USA) for 2 h at 200 mA in a transfer solution containing 48 mM Tris base, 39 mM glycine, 0.037% (w/v) SDS and 20% (w/v) methanol. The proteins were probed with a rabbit polyclonal antibody, anti-dehydrin (1:1000 dilution) (StressGen Biotechnologies Co., Victoria, BC, Canada), and developed using an alkaline phosphatase-conjugated secondary antibody (1:3000 dilution) (Sigma Chemical Co.) and a 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt (BCIP)/*p*-nitroblue tetrazolium chloride (NBT) reagent system.

RESULTS

Changes in freezing tolerance following sugar incubation or cold treatment

As determined by the measurement of electrolyte conductivity, the asparagus embryogenic cells incubated in standard liquid medium for 1 week (control samples) exhibited a freezing tolerance of –7°C (T_{EL50}) (Fig. 1). By contrast, the incubation of the samples in medium containing 0.8 M sucrose resulted in a remarkable increase in freezing tolerance. The maximum freezing tolerance of –24°C (T_{EL50}) was achieved after 2 days of sugar incubation.

However, further extension of the incubation resulted in a gradual reduction in freezing tolerance. Cold treatment for up to 12 days did not result in an increase in the freezing tolerance of the asparagus embryogenic cells.

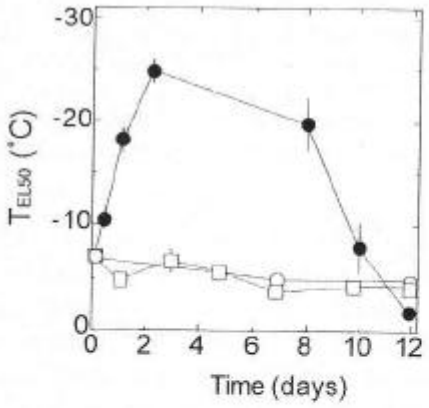

Fig. 1. Changes in freezing tolerance of asparagus embryogenic cells following incubation for various durations at 24°C in standard medium (○), in medium containing 0.8 M sucrose (●), or cold treatment at 4 / 2°C (□) as revealed by T_{EL50}. Time 0 corresponds to controls without sugar incubation or cold treatment. The data represent the mean from 3 replicates, and the bars represent SE.

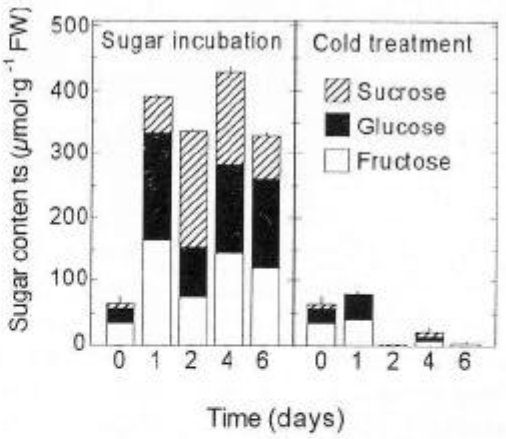

Fig. 2. Changes in intracellular sugar contents and the composition of sugars in asparagus embryogenic cells following incubation for various durations (1-6 days) at 24°C in medium containing 0.8 M sucrose or cold treatment at 4 / 2°C. Time 0 corresponds to controls without sugar incubation or cold treatment. The data represent the mean from 3 replicates, and the bars represent SE.

Changes in intracellular sugar contents following sugar incubation or cold treatment

The intracellular sugar contents in the asparagus embryogenic cells after incubation for various periods in medium containing 0.8 M sucrose or after cold treatment were measured (Fig. 2). The total content of sugars in the control samples was 67 μmol·g^{-1}FW. In the case of the sugar-incubated samples, the total intracellular content of sugars, including sucrose, glucose and fructose, remained high (322 - 429 μmol·g^{-1}FW) during the sugar incubation. In the case of cold-treated samples, sugar contents gradually decreased during the cold treatment.

Ultrastructural changes in cells following sugar incubation

The ultrastructure of the living asparagus embryogenic cells stained with FDA was observed under a CLSM (Fig. 3). The FDA fluorescence was emitted from the cytoplasm and nuclei of the embryogenic cells. The embryogenic cells incubated in standard liquid medium for 1 week appeared regular in shape (Fig. 3A), but the living embryogenic cells incubated in medium containing 0.8 M sucrose for 2 days showed plasmolysis (Fig. 3B). Plasmolysed cells were observed throughout the sugar incubation.

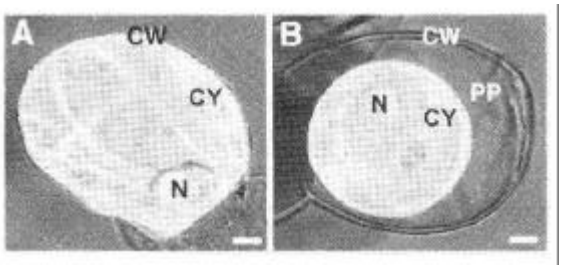

Fig. 3. CLSM images showing FDA vital staining in asparagus embryogenic cells. After treatment, samples were stained with 10 μM FDA for 30 min. Spherical-white areas correspond to cytoplasm in the living cells. (A) Control samples were incubated in standard medium at 24ºC for 1 week. (B) Samples were incubated in medium containing 0.8 M sucrose at 24ºC for 2 days. Sugar-incubated cells were drastically plasmolysed, and spaces between the cell wall (CW) and cytoplasm (CY), the periprotoplasmic spaces (PP) were observed. N, nuclei. Scale bars = 1 μm.

The ultrastructure of the cytoplasm of the embryogenic cells was observed under TEM using an ultra-thin sectioning method (Fig. 4). In the control samples, normal structural organization was seen in the cytoplasm (Fig. 4A). In the case of the plasmolysed cells that had been incubated in a medium containing 0.8 M sucrose for 2 days, however, a large quantity of rough endoplasmic reticulum (rER) was observed in the contracted cytoplasm by the freeze-substitution method (Fig. 4B). These freeze-substituted cells seemed to be free of ice crystals, and the membranes of some organelles such as the rER and plastids appeared negatively stained. The rER in the sugar-incubated samples was prominent in the cytoplasm, occasionally forming layers immediately below the plasma membrane (Fig. 4C).

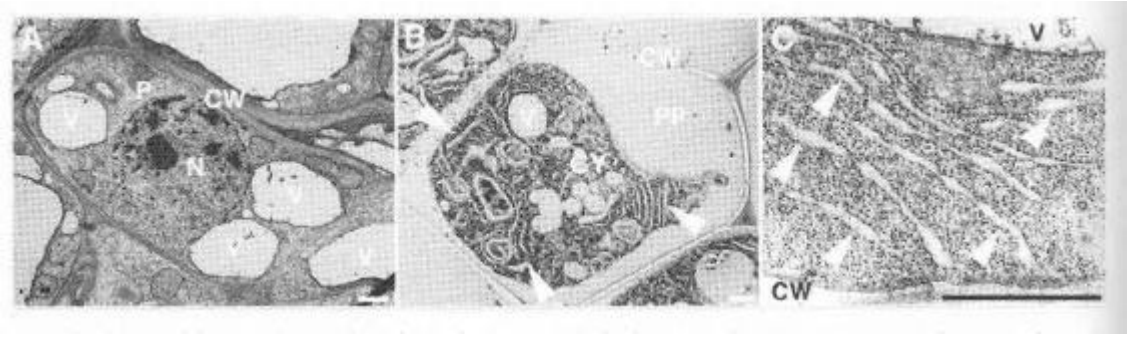

Fig. 4. Ultra-thin sections showing ultrastructural changes in asparagus embryogenic cells following incubation in medium containing 0.8 M sucrose. (A) Control samples were incubated in standard medium at 24°C for 1 week and fixed chemically. (B) Samples were incubated in medium containing 0.8 M sucrose at 24°C for 2 days, then freeze-fixed and freeze-substituted. Sugar-incubated cells were plasmolysed, and large spaces between the cell walls (CW) and cytoplasm (CY), the periprotoplasmic spaces (PP) were noted. (C) Highly magnified image of samples incubated in medium containing 0.8 M sucrose for 2 days. Arrowheads show developed rER. CW, cell walls; N, nuclei; P, plastid; V, vacuoles. Scale bars = 1 μm.

Changes in soluble protein contents and electrophoretic patterns of dehydrin-like proteins following sugar incubation

The intracellular soluble protein contents in the asparagus embryogenic cells were analyzed during incubation in liquid medium containing 0.8 M sucrose or during the cold treatment (Fig. 5). During sugar incubation, the most notable change in protein content was an increase in proteins from the soluble protein fraction rather than from the microsome protein fraction (data not shown). The content of soluble proteins in the asparagus embryogenic cells increased drastically immediately after the beginning of sugar incubation (0.5 day), then decreased gradually (Fig. 5). When boiling-stable soluble proteins were analyzed by SDS-PAGE, the most apparent change in the protein profile of the asparagus embryogenic cells during sugar incubation was the accumulation of a 42-kD polypeptide

(data not shown).

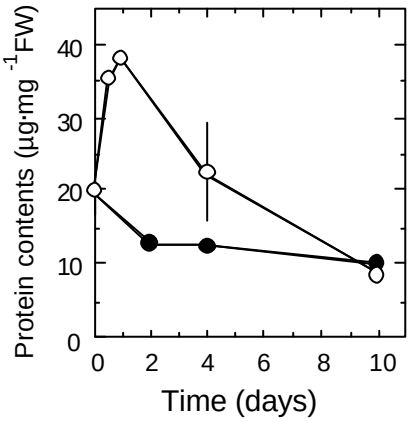

Fig. 5. Changes in soluble protein contents in asparagus embryogenic cells following incubation for various durations at 24°C in medium containing 0.8 M sucrose (?) or cold treatment at 4 / 2°C (?). Two hundred mg FW of the cells were used for each replicate. Time 0 corresponds to controls without sugar incubation or cold treatment. The data represent the mean from 3 replicates, and the bars represent SE.

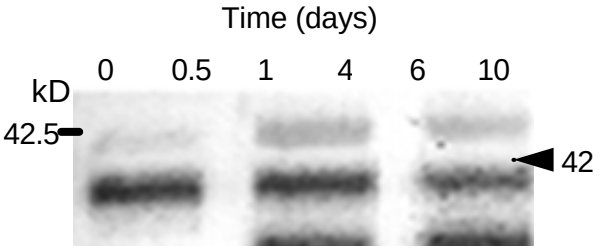

Fig. 6. Immunoblot analysis of boiling-stable proteins in samples following incubation for various durations (0-10 days) in medium containing 0.8 M sucrose at 24°C. Antiserum against anti-LEA group 2 proteins (dehydrin) was used. Arrowheads show proteins that increased predominantly during sugar incubation. Each lane contained proteins corresponding to 0.7 mg FW of cells. Time 0 corresponds to controls without sugar incubation.

Immunoblot analysis revealed that the 42-kD boiling-stable proteins which accumulated in the asparagus embryogenic cells during sugar incubation, are immunologically related to the late-embryogenesis abundant (LEA) group 2 proteins, the dehydrins (Fig. 6). Interestingly, although a 42-kD band was detected in the samples incubated in liquid standard medium, the intensity of this band reached its maximum soon after the beginning of sugar incubation (0.5 day), maintained a similar level for 4 days, and then decreased gradually with prolonged sugar incubation. No such band was detected in the cold-treated samples (data not shown). The immunoblot analysis of the soluble proteins was repeated 3 times with similar results.

DISCUSSION

Because of their regeneration capacities, embryogenic cell suspension cultures are potentially important in biotechnological breeding, propagation of elite material and the conservation of genetic resources. Therefore, cryopreservation techniques are necessary to conserve such materials. To successfully achieve cryopreservation of plant cells using conventional or vitrification procedures, an increase in the freezing tolerance of cells is necessary to prevent the occurrence of freezing injury. It is well known that the survival rate of embryogenic cells of herbaceous and arboreous plants after cryopreservation is improved by incubation in medium containing high concentration of osmoticums such as sugars (2, 6, 22, 27). Incubation in medium containing high sugar concentration for a long period induces sugar accumulation (13, 22) and changes in cell structures (7). Furthermore, it is widely accepted that increases in the levels of proteins (31), proline (36) and endogenous abscisic acid (16) represent some of the early physiological responses in osmotically-stressed plant cells. All these responses of plant cells before cryopreservation may be related to increasing freezing tolerance.

Cryopreservation of *Asparagus officinalis* L. shoot apices has already been reported (20). The authors suggested that the freezing tolerance of meristematic tissues could be increased by incubation in medium containing high sugar concentration. In this study, we succeeded in increasing the freezing tolerance of asparagus embryogenic cell suspensions by incubation in sugar-rich medium. However,

cold treatment had no such effect. In addition, cold treatment did not cause changes in cell structure (data not shown) or accumulation of sugars and proteins. In many plants exposed to severe winter frost, various changes occur at both the biochemical and structural levels, accompanying the acquisition of freezing tolerance (26, 28). Although the asparagus embryogenic cells used in this study did not display similar changes in soluble carbohydrates, cell ultrastructure and proteins when exposed to cold, there remains the possibility that the period of low-temperature treatment used in this study (12 days) was too short to induce cold acclimation. Thus, additional studies using embryogenic cells cold-treated for a longer period are needed.

By contrast, preincubation of embryogenic cells in sugar-rich medium caused various changes, including the accumulation of sugars and proteins and changes in the structure. The intracellular content of sugars increased dramatically during sugar incubation. The sucrose incorporated into the cells was converted into monosaccharides from the beginning of sugar incubation; however, the total intracellular content of sugars was only roughly correlated to the level of freezing tolerance. It is widely accepted that an increase in endogenous sugar levels is closely related to an increase in freezing tolerance in many plant tissues (13, 22). However, the finding that the maximum of sugar incorporation did not coincide with the maximum freezing tolerance indicates that sugar incorporation is not the only mechanism involved in increasing the freezing tolerance of embryogenic cells. In asparagus shoot apices and cabbage petioles, in which the freezing tolerance was increased by sugar incubation, a small portion of sugars accumulated through sugar transporters over a long period was sufficient to increase the freezing tolerance of tissue cells, rather than a large quantity of sugars incorporated by fluid-phase endocytosis over a short period (18). Sugars incorporated directly into the cytoplasm have also been shown to reduce the occurrence of a close apposition of membranes caused by freezing injury in extracellularly-frozen cells (10).

Incubation of embryogenic cells in a sugar-rich medium also caused changes in the cell structure. Plasmolysis was observed in asparagus embryogenic cells during incubation in sugar-rich medium. Studies using wheat suspension cells have shown that plasmolysis of cells incubated in a highly concentrated sucrose solution increased freezing tolerance, reducing protoplast deformation during freezing and preventing structural changes in the plasma membrane (29). In the case of cabbage mesophyll tissue cells, plasmolysis may reduce the deformation of protoplasts caused by freezing, thus suppressing the close apposition of membranes, which is a major cause of freezing injury (17).

The appearance of developed rER was also observed in asparagus embryogenic cells during incubation in the sugar-rich medium. In cold-acclimated plant cells, particularly of woody plants, which exhibit a high freezing tolerance, remarkable changes in cell ultrastructure have been found to occur during cold acclimation (9, 26, 28). Interestingly, an ultrastructural change in the rER is one of such changes. In twigs of *Robinia pseudoacacia*, the form of ER cisternae changed to numerous shorter units in winter in comparison with summer (26). The ER cisternae formed multiplex lamellae (MPL) on the underside of the plasma membranes. MPL are supposed to protect plasma membranes from the close apposition of membranes that causes freezing injury by extracellular freezing in cortical parenchyma cells of mulberry (9). During sugar incubation, the rER of embryogenic cells also formed layers similar to the MPL observed in mulberry cortical parenchymatic cells. It is reasonable to assume that the developed rER may play a role in increasing freezing tolerance in embryogenic cells after sugar incubation.

Sugar incubation of asparagus embryogenic cells also induced the accumulation of a 42-kD boiling-stable polypeptide that cross-reacted with the antiserum against anti-LEA group 2 proteins, the dehydrins. Although the precise mechanism responsible for the acquisition of freezing tolerance is still not clear, it has been suggested that some specific genes might be expressed during cold acclimation, and that many of these genes code for proteins that could potentially contribute to the enhancement of freezing tolerance. Both quantitative and qualitative changes have been reported in relation to change in freezing tolerance in many cold-acclimated cells of many plant species that have a high freezing

tolerance (11, 31). Particularly, LEA proteins and/or cold-induced hydrophilic proteins such as cold-responsive (COR) proteins are supposed to play roles in increasing freezing tolerance in plant cells (31). In recent studies, it has been assumed that a cold-responsive gene, the COR 15am protein from cold-acclimated *Arabidopsis thaliana*, has the function of suppressing freezing injury in the liposomes of rye leaves (35). These proteins are rich in Ala, Gly, Lys, Glu and Asp residues, and are therefore extremely hydrophilic and remain soluble upon boiling (boiling-stable). In this study, the concentration of a boiling-stable protein increased during sugar incubation.

LEA proteins are very hydrophilic and are thought to bind or replace water during osmotic stress (14). In particular, dehydrins are presumed to play a role in suppressing injury caused by cellular dehydration (12). Furthermore, a fundamental biochemical role of dehydrins is considered to be related to the stabilization of cytoplasmic proteins against denaturation or of the cytoplasmic surface of membranes against deformation in dehydrated plant tissues (4). Thomashow suggested that the accumulation of dehydrin-like proteins causes the stabilization of biological macromolecules under freeze-induced dehydration (31). A recent study has suggested that high molecular-weight dehydrins (PCA60) from bark tissues of cold-acclimated peach twigs have antifreeze activity (37). As mentioned above, dehydrins have been associated with increasing the freezing tolerance of plant cells. Therefore, in the case of asparagus embryogenic cells, the accumulation of a dehydrin-like protein during incubation in a sugar-rich medium may have the main or subsidiary effect of increasing freezing tolerance. In this study, the role of the 42-kD dehydrin-like boiling-stable protein during freezing was not elucidated. Further experiments focusing on the properties and localization of this protein are needed to elucidate its function.

In conclusion, the freezing tolerance of asparagus embryogenic cells incubated with sugar-rich medium may be increased by a complex mechanism consisting of many effects, which include notably: 1) the effect of sugar accumulation in the cytoplasm; 2) the effect of plasmolysis in a hypertonic solution; and 3) the effect of the accumulation of specific hydrophilic proteins, such as dehydrins. However, it has been reported that osmolytes other than sugars, such as proline (36) and glycine betaine (23), also accumulate in plant cells during treatment with a hypertonic solution or during dehydration. Therefore, in order to understand the precise mechanisms underlying the increase in freezing tolerance during sugar incubation, changes in osmolytes other than sugars during sugar incubation should also be studied.

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