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Original Paper

Freeze-substitution transmission electron microscopy of gentian shoot tips cryopreserved at ultra low temperatures

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Abstract Transmission electron microscopy (TEM) combined with freeze substitution was employed to examine the ultrastructure of cells of gentian shoot tips cooled to the ultra-low temperature of slush nitrogen and liquid nitrogen. When shoot tips were cooled in ultra-low temperature without plant vitrification solution 2 (PVS2) treatment, massive ice formation was observed throughout the cells, indicating that severe injury occurred during cooling. In contrast, when shoot tips were treated with PVS2 and subsequently cooled to ultra- low temperatures, no ice crystals were observed in the cells. In addition, the cells of PVS2-treated shoot tips exhibited considerable plasmolysis and formation of small vesicles in cytoplasm. These results clearly demonstrate that the PVS2 treatment is essential for preventing damage caused by ice formation and for successful cryopreservation of plant shoot tips.

Key words: cryopreservation, freeze substitution, shoot tips, transmission electron microscopy, ultrastructure.

Introduction

Cryopreservation of shoot tips at the temperature of liquid nitrogen (LN) is employed for long-term preservation in a wide range of plant materials including temperate, subtropical and tropical species (Sakai et al 2008). The potential of cell survival at ultra-low temperatures was demonstrated using poplar and salix twigs, collected in winter that could survive after direct immersion into LN or liquid helium (Sakai 1960). Fahy et al. (1984) proposed that the occurrence of vitrification is requisite for survival of biological cells when cryopreserved at the temperature of LN. To achieve effective vitrification with plant shoot tips, samples are generally treated with a vitrification solution consisting of several cryoprotectants such as dimethyl sulfoxide (DMSO), glycerol, ethylene glycol and sugars, which reduces the water content of tissues and increases the cytoplasm internal viscosity. For cryopreservation, specimens to be cryopreserved must be vitrified during cooling in LN to prevent lethal ice crystal formation in cells. When vitrification occurs successfully, the specimens can be preserved in LN for a long period. In addition, cryopreserved shoot tips show high viability, without phenotypic changes on regrowth and genetic changes in succeeding generations (Hirai and Sakai 1999; Maki et al. 2015; Matsumoto et al. 2013). Thus, it is considered that there is no or little damage to cells after long-term storage using optimized vitrification methods.

Although vitrification methods have been developed for many plant species, there have been few observations of cell ultrastructure and water behavior in cells of shoot tips when stored in LN. When cryopreserved shoot tips exhibited high survival rates, no ice crystals were observed in their cells, because the concentrated water in cells of shoot tip were vitrified (in glass condition) in LN (Teixeira et al. 2013). To understand the cryopreservation mechanism, it is necessary to clarify the behavior of cells and water in plant shoot tips stored in ultra-low temperature. The objectives of the present study were: (1) To obtain clear observations of cells of cryopreserved gentian shoot tips using transmission electron microscopy (TEM) combined with freeze substitution fixation; (2) To record each step during the vitrification procedure of the cell ultrastructure of cryopreserved gentian shoot tips stored at ultra-low temperature; (3) To clarify the relationship of cell ultrastructure and shoot tip survival after cryopreservation.

Abbreviations: LN, liquid nitrogen; PVS2, plant vitrification solution 2; SN, slush nitrogen; TEM, transmission electron microscopy.

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Materials and methods

Plant material

In vitro grown gentian shoots (*Gentiana triflora*×*Gentiana scabra*, cv. Marchen) were used for this study. Stock cultures were subcultured every three weeks on solid Murashige and Skoog medium (MS; Murashige and Skoog 1962) containing a half strength of inorganic salts (thereafter referred to as 1/2 MS medium) supplemented with 0.2 mg l^{-1} benzyladenine, 2.5% (w/v) sucrose, and 0.8% (w/v) agar. Stock cultures were maintained with a 16-h photoperiod under white florescent light (50 μmol m^{-2} s^{-1}) at 25°C (standard condition, Tanaka et al. 2004).

Transmission electron microscopy combined with freeze-substitution fixation of the sample

In studies using freeze substitution, samples were cryo-fixed to preserve cell ultrastructure during cooling in slush nitrogen (SN) or LN. For cryo-fixation, rapid cooling rate of approximately 10,000°C s^{-1}, by directly plunging into SN was employed as a coolant. Shoot tips were freeze-substituted according to the method of Ramsay and Koster (2002). After immersion in SN or LN, shoot tips were transferred into a vial containing freeze-substitution medium that was prepared with 2% (w/v) OsO_4 in 100% methanol and precooled to −91°C on dry ice. Vials with specimens were kept in the freeze-substitution medium at −84°C for 7 days, during which sublimation of dry ice was observed within 2 days after incubation began. The vials were successively incubated at −18°C for 24 h, at 4°C for 24 h, and at room temperature for 3 h. The specimens were then washed three times with 100% methanol and placed into 100% propylene oxide for 30 min on a rotary shaker at 80 rpm at room temperature. Propylene oxide was changed three times during incubation.

The specimens were embedded in Spurr's resin (TAAB, Berkshire, England) (Spurr 1969). Ultra-thin sections (approximately 70 nm in thickness) were then cut with an ultramicrotome (Ultracut-S, Leica Inc., Wien, Austria) using a glass knife, collected on copper grids with 100 mesh size and stained with 1% (w/v) uranyl acetate (modified Watson's protocol, 1958) at 60°C for 15 min followed by aqueous saturated lead citrate (Reynolds 1963) for 30 min at room temperature. The sections were viewed with an H-800 electron microscope (Hitachi Co., Ltd., Tokyo, Japan) at 100 kV.

Cryopreservation procedure

Vitrification of the gentian shoot tips was performed following the procedure developed by Tanaka et al. (2004; Figure 1). Three-week-old in vitro grown shoots were cold-hardened at 5°C with an 8-h photoperiod (26 μmol m^{-2} s^{-1}) for 20 days. Subsequently, shoot tips (1.5 mm long and 1 mm diameter) were excised from cold-hardened shoots, placed on 1/2 MS medium containing 0.3 M sucrose, and precultured at 5°C for 1 day with an 8-h photoperiod (26 μmol m^{-2} s^{-1}). These shoot tips were placed into 2 ml plastic cryotubes (Wheaton Science Products, NJ, USA) and treated with 1 ml of loading solution (LS) containing 2.0 M glycerol and 0.4 M sucrose in liquid MS basal medium for 20 min at 25°C. After the LS treatment, the shoot tips were placed in 1 ml of plant vitrification solution 2 (PVS2, Sakai et al. 1990) at 25°C for 30 min. PVS2 contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose in liquid MS basal medium, pH 5.8. During PVS2 treatment, PVS2 was replaced with fresh PVS2 once. After PVS2 treatment, cryotubes containing shoot tips and 0.5 ml PVS2 were plunged in SN or LN and kept for more than 1 h. For regeneration, cryotubes with shoot tips were rapidly rewarmed in a 37°C water bath for 1 min, and PVS2 solution was then replaced with 1.0 M sucrose solution in MS medium and incubated for 20 min at room temperature. Shoot tips were then transferred onto solidified 1/2 MS medium and cultured for 4 weeks at 25°C under standard conditions. Survival of shoot tips was determined by counting the number of shoot tips developing normal shoots after 4 weeks.

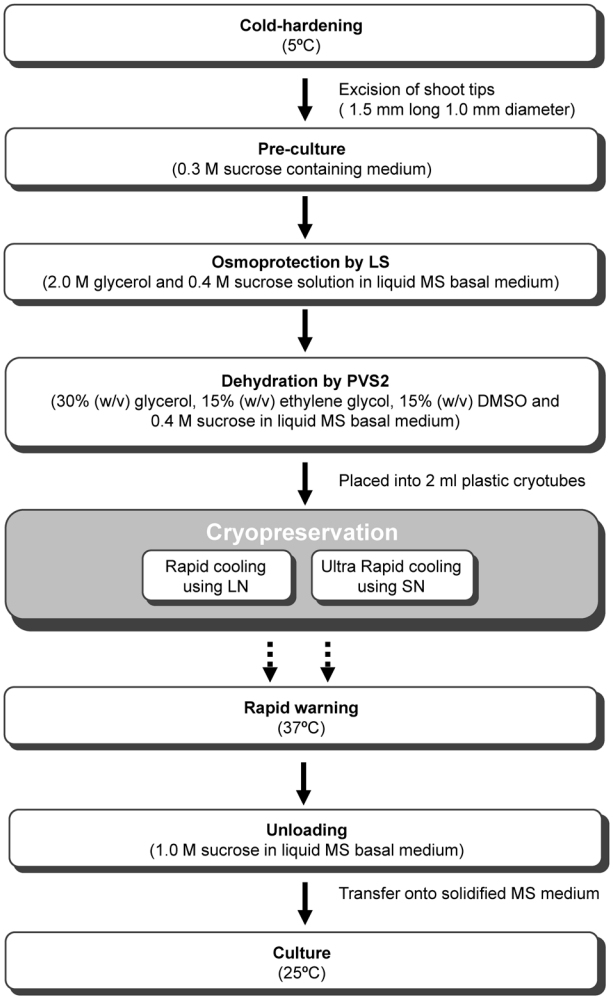

Figure 1. A flowchart of vitrification protocol.

Results

Successful cryofixation by SN or LN followed by freeze-substitution showed the fine structure of cell preservation of cells of cryopreserved gentian shoot tips by TEM (Figure 1). Freeze substitution is used to prepare dry, partially dehydrated and frozen tissues for TEM. In freeze substitution, successful preservation of tissue morphology and ultrastructure depends on rapid freezing to immobilized cell contents, followed by replacement of solidified water with chemical fixatives at low temperature (Ramsay and Koster 2002). In the present study, we applied TEM with freeze substitution to the cryopreserved shoot tips of gentian. The shoot tips treated in freeze-substitution medium with 2% (w/v) OsO_4 in 100% methanol were kept at −84°C for 7 days, at −18°C for 24 h, at 4°C for 24 h, and at room temperature for 3 h. These successive incubation treatments resulted in successful observations of the fine structure of preserved cells. However, there were no clear differences observed in cell ultrastructure between LS only and LS + PVS2 treatments (Figure 2).

The structure of gentian meristematic shoot tip cells by freeze-substitution TEM was observed after cryopreservation steps such as no treatment of vitrification procedure, after preculture, after LS treatment and after PVS2 treatment, which were cryofixed using SN (Figure 3). Many ice crystals were observed in cells of the non-treated control shoot tips cooled with SN. The ice crystals (white place in cells) were dispersed evenly in cytoplasm, vacuoles and nucleus. In addition, there were no plasmolyzed cells (Figure 3a). Similar results were obtained with precultured specimens. There were many ice crystals in cells and no plasmolyzed cells (Figure 3b). Thus, there seems to be no morphological changes with cold-hardening and preculture on cellular and water behavior during cooling in SN. These treatments resembled the non-treated control. In contrast, when samples were treated with LS or with LS+PVS2 successively, ice crystals did not form in cells. The cells in both these treatments were severely plasmolyzed, especially in treatment with LS+PVS2, and many small vesicles were observed in the cytoplasm (Figure 3c, d).

The ultrastructure of samples cooled with LN instead of SN was observed (Figure 4). With LN, the cooling rate is thought to be slower than with SN, which may result in an increased probability and frequency of ice crystal formation during cooling. Thus, it is possible that cellular ultrastructure after cooling in LN may not be the same with samples cooled in SN. When precultured samples were cooled with LN, many ice crystals were observed in cells and there were no plasmolyzed cells (Figure 4a). The same result was obtained with SN. When samples were treated with the LS and cooled with LN, ice crystals were formed in cells with moderate plasmolysis (Figure 4b). This is quite different from the result with specimens treated with SN. When samples were treated with

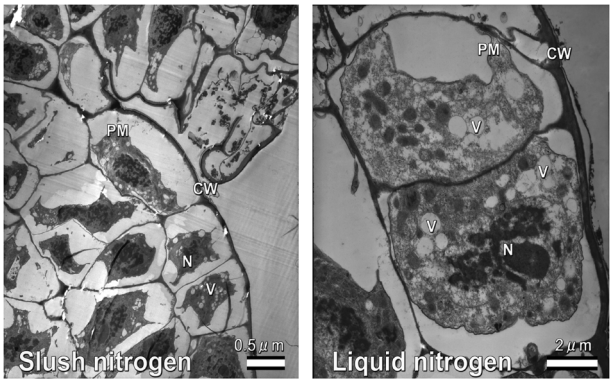

Figure 2. The structure of gentian meristematic cells cryofixed using slush nitrogen or liquid nitrogen by freeze-substitution TEM. The meristematic cells of gentian shoot tips after PVS2 treatment were cryofixed using slush nitrogen (left) or liquid nitrogen (right). Cold-hardening, 5°C for 20 days; Preculture, 0.3 M sucrose at 5°C for 1 day; LS treatment, 25°C for 20 min. Bar indicates 0.5 μm (left) and 2 μm (right). CW, N, PM and V indicate cell wall, nucleus, plasma membrane and vesicles in cytoplasm, respectively.

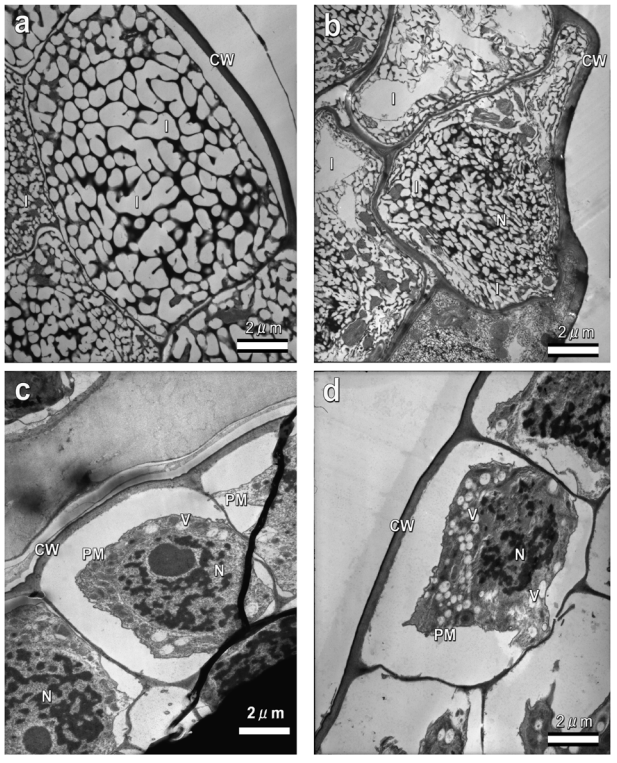

Figure 3. The structure of gentian meristematic cells by freeze-substitution TEM after each vitrification step (cryofixation using slush nitrogen). The meristematic cells of gentian shoot tips after each step were cryofixed using slush nitrogen. (a) No treatment of vitrification procedure, (b) After preculture, (c) after LS treatment and (d) after PVS2 treatment. Cold-hardening, 5°C for 20 days; preculture, 0.3 M sucrose at 5°C for 1 day; LS treatment, 25°C for 20 min. All bars indicate 2 μm. CW, N, PM, V and I indicate cell wall, nucleus, plasma membrane, vesicles in cytoplasm and ice crystals, respectively.

Table 1. Survival rates of cryopreserved shoot tips and summarized results of ice crystal and plasmolysis at each step of cryopreservation using two cryogens.

Cryopreservation steps	LN				SN			
	Ice crystal	Plasmolysis	Survival rate (%)	Remark	Ice crystal	Plasmolysis	Survival rate (%)	Remark
None	Y	N	0		Y	N	0	
Cold-hardning+Preculture	Y	N	0		Y	N	0	
LS	Y	Y	0		N	Y	0	Small vesicles
PVS2	N	Y	80	Small vesicles	N	Y	80	Small vesicles

Y means occurrence. N means no occurrence. Survival rate (%) means survival shoots/30 shoots ×100.

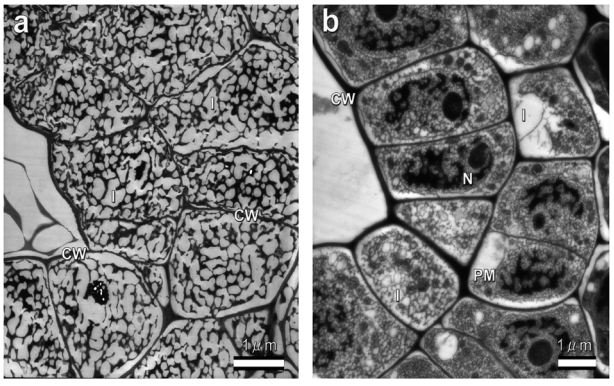

Figure 4. The structure of gentian meristematic cells by freeze-substitution TEM after preculture and the LS treatment steps (cryofixation using liquid nitrogen). The meristematic cells of gentian shoot tips after each step were cryofixed using liquid. (a) After preculture and (b) after LS treatment. Cold-hardening, 5°C for 20 days; Preculture, 0.3 M sucrose at 5°C for 1 day; LS treatment, 25°C for 20 min. Bars indicate 1 μm. CW, N, PM, V and I indicate cell wall, nucleus, plasma membrane, vesicles in cytoplasm and ice crystals, respectively.

LS+PVS2, and cooled with LN, no ice crystals observed in cells and cells were severely plasmolyzed with many small vesicles (Figure 2 right). The same result was obtained with SN.

For successful cryopreservation of the gentian shoot tips by the vitrification procedure, the treatment with PVS2 is indispensable, resulting high survival rates regardless of cooling with SN or LN (Table 1). When cooled with SN after the LS treatment only, there were no apparent ice crystals formed in cells of shoot tips. However, the cryopreserved shoot tips with SN after the LS treatment resulted in no survival at all (Table 1). The shoot tips cooled with LN after LS treatments also did not survive.

Discussion

Using TEM with freeze-substitution, it has been demonstrated in vivo that there is no apparent ice crystal formation in cells of shoot tips when properly treated with PVS2 and subsequently cooled with cryogens (Figure 2). In addition, after PVS2 treatment, many shoot tip cells exhibited considerable plasmolysis and formation of small vesicles within cells. These results strongly suggest that rapid cooling after PVS2 treatment mitigates ice formation in cells at least as far as can be observed under freeze-substitution TEM. Almost all shoot tips survived under these conditions (Table 1), these results strongly indicating that no ice formation occurred in cells when shoot tips were properly treated with PVS2 followed by immersion in a cryogen (Sakai et al. 2008; Tanaka et al. 2004). When treated with PVS2 followed by immersion into a cryogen, many plasmolyzed cells were observed in meristems. Plasmolysis is expected from PVS2 treatment because the osmotic concentration of PVS2 is estimated to be greater than 8 Osmol (Sakai 2003) and sucrose would not permeate quickly into cells, resulting in cells that are subjected to hypertonic conditions. Even after treatment with LS following SN cooling, there were many cells showing plasmolysis. However, after treatment with LS following LN cooling, there were cells showing the moderate plasmolysis with ice crystal formation. The plasmolysis after LS treatment was also observed in shoot tips of *Garcinia cowa* (Yap et al. 2011). However, LS treatment alone seemed to be insufficient for successful cryopreservation even with the rapid cooling rate obtained with SN, although TEM observations indicated no apparent ice formation in cells cooled with SN. Hypertonic treatment with PVS2, in turn, would result in osmotic dehydration of cells and an increase in viscosity of cytoplasm, which is a prerequisite for vitrification to occur during cooling (Fahy et al. 1984).

Another notable observation was that many small vesicles were observed in the cytoplasm of surviving shoot tips. Furthermore, the size of vacuoles was reduced corresponding with an increase in the number of small vesicles. It has been reported that vesiculation of the central vacuole occurs during cold treatment or preculture with a high concentration of sucrose (Bachiri et al. 2000; Sauter et al. 1996). The small vesicles in cells observed might be formed due to either of these two treatments but not due to the PVS2 treatment or cooling per se. On the other hand, there are several reports of endocytotic vesiculation of the plasma membrane occurring during a hypertonic treatment, which is irreversible and lethal (Singh and Johnson-Flanagun

1987). We believe that this is not the case in the present study because nearly all shoot tips with cells containing such small vesicles survived after cooling and showed vital regrowth after rewarming from LN. Nevertheless, further studies to determine the origin of the small vesicles in cells of survived shoot tips are needed.

The shoot tips without PVS2 treatment showed many large ice crystals throughout cells after cooling with LN and did not survive after warming (Table 1). This may be due to the rewarming process. With the D cryo-plate method of ulluco shoot tips, the encapsulated shoot tips should be rapidly warmed in the process of rewarming for obtaining high regrowth, resulting avoiding lethal tissue crystallization in the cells of shoot tips (Valle Arizaga et al. 2017). However, with the encapsulation dehydration method, rewarming is usually carried out slowly at room temperature, since samples are sufficiently dehydrated before freezing, there is no risk of ice recrystallization upon rewarming (Gonzalez-Arnao and Engelmann 2006). These results clearly indicate a direct connection between intracellular ice formation and cell death under cryopreservation conditions, showing that the PVS2 treatment is essential for cell survival after vitrification in LN. This is supported by the observations in the present study that ice formation was not observed in cells after the PVS2 treatment. In contrast, massive ice formation was frequently detected in cells when cooled without the PVS2 treatment. There are, however, a few exceptions to this pattern of ice distribution. Samples treated with LS but not with PVS2 showed no ice formation when cells were cooled with SN. In addition, most cells under these conditions were plasmolyzed and contained small vesicles in the cytoplasm. Although the cell ultrastructure seemed to be similar in cells treated with LS only and LS + PVS2, no shoot tips could survive when treated with LS alone (Table 1). There are two explanations for the differences in survival of shoot tips cryopreserved after or before the PVS2 treatment. Since the cooling rate with SN is faster than that with LN, water in cells treated with only LS could be vitrified during cooling in SN. It is known that the propensity of vitrification of water depends on both solute concentrations and cooling rate of the system. Therefore, both parameters would be satisfied for LS treated specimens to vitrify during cooling with SN, but vitrified water with less suitable concentrations of solutes might be devitrified and formed ice crystal during warming of the specimens. Another possibility is that even with faster cooling, water in cytoplasm of the LS treated cells forms small ice crystals that could not be observed under conditions employed with TEM in the present study. If this is the case, during subsequent warming, small ice crystals would grow into a size that is sufficient to result in severe injury in cells. Shoot tips treated with only LS followed by cooling with SN did not survive after rewarming. From these results, it is concluded that treatment with LS was insufficient for shoot tips to survive after cooling even though no ice crystals were observed in cells that were plasmolyzed and that contained many small vesicles.

The permeation of components of PVS2 such as DMSO and ethylene glycol into cells of plant shoot tips could occur during a short period of the PVS2 treatment. It is possible that the viscosity of cytoplasm increases and water in cells is trapped separately in small compartments, which subsequently leads to vitrification of the cytoplasm. Plasmolysis of many cells after PVS2 treatment followed by cooling indicates that the rate of permeation of PVS2 solution is not so fast that cells are exposed to hyperosomotic conditions. This is necessary to induce a reduction of water content in cells and, hence, to increase the probability of vitrification of cytoplasm. The evidence of vitrification derived from the cryo-SEM observations and the glass transition detected in differential scanning calorimetry (DSC) of mint shoot tips suggest a higher cytoplasm viscosity in specimens after PVS2 treatment than in specimens after LS treatment, that were evidently not vitrified (Teixeira et al. 2013). This viscosity increase could originate from both further water content reduction and by the entrance in cells of the viscosity enhancing PVS2 components (Teixeira et al. 2013). Tissue desiccation and chemical permeation induced by vitrification procedures affect not only cellular freezing properties but also differences of cellular responses by cell location, size or other physiological characteristics (Teixeira et al. 2013). In the osmotic responses of sweet potato suspension cell cultures, the plasma membranes were more permeable to DMSO and ethylene glycol compared to sucrose nor glycerol solutions which showed low to no membrane permeability in the timeframes studied (Volk and Caspersen 2017). Generally, smaller cells with only small vacuoles and densely staining cytoplasm (meristem) showed the least plasmolysis during osmotic dehydration treatment, resulting in high survival rates after LN exposure (Volk and Caspersen 2007).

To our knowledge, this is the first report of systematic observations of the ultrastructure of cells of plant shoot tips under conditions employed for cryopreservation with TEM combined with the freeze substitution technique. Recently, new cryopreservation methods, droplet vitrification (Panis et al. 2005) and cryopreservation method using cryo-plates, V and D cryo-plate methods (Niino et al. 2013; Yamamoto et al. 2011), have been developed. Both methods use the direct immersion of specimens in LN and in rewarming solution, resulting rapid cooling and warming and high regenerations. Viability of the recovered material showed a close relation between the dehydration time, cooling and warming rates, ice formation avoidance and tissue

vitrification (Teixeira et al. 2014). Further observations using TEM combined with freeze substitution are needed to clarify the mechanism of these new cryopreservation methods, especially the D cryo-plate method, in which air dehydration following LS treatment without osmotic dehydration by PVS2 is employed.

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