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Plasmolysis and recovery of different cell types in cryoprotected shoot tips of *Mentha* × *piperita*

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Summary. Successful cryopreservation of plant shoot tips is dependent upon effective desiccation through osmotic or physical processes. Microscopy techniques were used to determine the extent of cellular damage and plasmolysis that occurs in peppermint (*Mentha* × *piperita*) shoot tips during the process of cryopreservation, using the cryoprotectant plant vitrification solution 2 (PVS2) (30% glycerol, 15% dimethyl sulfoxide, 15% ethylene glycol, 0.4 M sucrose) prior to liquid-nitrogen exposure. The meristem cells were the smallest and least plasmolyzed cell type of the shoot tips, while the large, older leaf and lower cortex cells were the most damaged. When treated with cryoprotectant solutions, meristem cells exhibited concave plasmolysis, suggesting that this cell type has a highly viscous protoplasm, and protoplasts have many cell wall attachment sites. Shoot tip cells were most severely plasmolyzed after PVS2 treatment, liquid-nitrogen exposure, and warming in 1.2 M sucrose. Successful recovery may be dependent upon surviving the plasmolytic conditions induced by warming and diluting treated shoot tips in 1.2 M sucrose solutions. In peppermint shoot tips, clumps of young meristem or young leaf cells survive the cryopreservation process and regenerate plants containing many shoots. Cryoprotective treatments that favor survival of small, meristematic cells and young leaf cells are most likely to produce high survival rates after liquid-nitrogen exposure.

Keywords: Vitrification; Cryopreservation; Plasmolysis; Cryoprotectant; Liquid nitrogen; *Mentha* × *piperita*.

Abbreviations: LN liquid nitrogen; PIPES piperazine-N,N′-bis(2-ethanesulfonic acid); PVS2 plant vitrification solution 2.

Introduction

Cryopreservation methods have been successfully used to back up collections of field, greenhouse, and tissue culture collections of plant genetic resources. Explants of vegetatively propagated plant materials can be cryoprotected and then stored for extended lengths of time in liquid nitrogen (LN). Different methods for cryopreserving in vitro shoot tips, suspension cell cultures and dormant buds have all been successfully implemented for germplasm collections (Engelmann 2004, Sakai 2000, Withers 1985). Successful cryopreservation methods dehydrate explants to limit ice formation during cooling to and warming from −196 °C (Fahy et al. 1987, Steponkus et al. 1992).

Although desiccation is essential for surviving LN exposure, excessive drying can damage plant cells. Cells that are filled with dry matter reserves are often more able to tolerate desiccation stresses since they do not contract as much as highly vacuolated cells (Walters et al. 2002).

Shoot tips can be desiccated by either osmotic or physical processes. Exposure to concentrated solutions containing relatively impermeant solutes, such as glycerol, sucrose, glucose, and polyethylene glycol, effectively removes water from the cytoplasm. A subsequent slow cooling (0.1 to 1 °C per min to −35 °C) prior to LN exposure freeze dehydrates explants even further. Alternatively, osmotically treated shoot tips can be rapidly cooled to −196 °C, where ice nucleation is prevented during a vitrification, or glass-forming, process.

Excessive desiccation in unprotected cells can damage cellular integrity by affecting the ability of cells to reassemble membranes and perform electron transport, DNA repair, and lipid, protein, and RNA synthesis (Oliver 1996). Furthermore, structural integrity of membranes and plasmodesmata connectivity may be irreversibly damaged. Some of these cellular damages may be prevented by the use of permeant chemicals in cryoprotective solutions. For mint shoot tips, ethylene glycol and dimethyl sulfoxide

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have protective benefits and have low toxicity at cryoprotection temperatures (0 to 25 °C) and time intervals (0 to 60 min) (Volk et al. 2006). Plant vitrification solution 2 (PVS2), containing 30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide, and 0.4 M sucrose in MS salts (Murashige and Skoog 1962, Sakai et al. 1990), is frequently used as a cryoprotectant solution. While effective in cryoprotectant solutions, extended exposures to glycerol can be toxic to mint plant cells (Volk et al. 2006). The ultrastructural responses of shoot tips after treatment with PVS2 have not been described.

Mentha × *piperita* L. is a model plant system for cryopreservation research (Towill 1990, 2002). Axenic 1 mm long axillary buds from nodal sections can be easily excised and cryopreserved. Cryoprotectant toxicity and biophysical vitrification events resulting from PVS2 exposure have been previously described for peppermint (Volk et al. 2006, Volk and Walters 2006). A 30 min PVS2 treatment dehydrates shoot tips from 1.5 to 0.6 g of water per g of dry mass (Volk and Walters 2006). This is considerably wetter than orthodox seed water contents (0.02 to 0.08 g of water per g of dry mass) and comparable to water contents that recalcitrant seeds can tolerate (Walters et al. 2002).

The purpose of this study was to determine the structural changes of cells that occur during the process of osmotic dehydration, LN exposure, and recovery when PVS2 is used as a cryoprotectant in a shoot tip vitrification system. The cellular specificity of these structural changes enables us to determine the sensitivity of cell types within the shoot tip during cryoprotection.

Material and methods

Plant material

Mentha × *piperita* cv. Todd Mitcham peppermint (PI 557973) plants were maintained in vitro and propagated on MS basal mineral and vitamin medium (Murashige and Skoog 1962) with 3% sucrose and 1% agar. Shoot tips (1 mm^3) were excised from lateral buds that emerged from nodal sections cultured on growth medium for 3 days.

Sample preparation for sucrose treatments

Shoot tips were treated with 0.3 to 2.1 M sucrose for 2 h at 22 °C to ensure adequate time for plasmolysis and then fixed overnight in a mixture of 1.25% glutaraldehyde and 2% paraformaldehyde containing 0.05 M PIPES buffer (piperazine-N,N′-bis(2-ethanesulfonic acid); 1.2 M sucrose treatment only), 0.3 M sucrose (all treatments), or the plasmolyzing concentration of sucrose (all treatments) to determine the effects of fixative molarity on deplasmolysis during shoot tip fixation. In another experiment, shoot tips were treated with either 0.6 or 1.2 M sucrose for 2.5 h to induce plasmolysis and were then deplasmolyzed by treatment with 0.3 M sucrose for 2.5 h prior to fixing in fixative with 0.05 M PIPES buffer.

Sample preparation for cryopreservation

Mentha × *piperita* shoot tips were treated using a vitrification method modified from Towill (2002). Shoot tips were first cultured overnight in 0.3 M sucrose and then immersed in 2 M glycerol plus 0.6 M sucrose for 20 min at 22 °C, which was followed by treatment with PVS2 for 30 min at 0 °C. Three shoot tips were placed within three drops of PVS2 on an aluminum foil strip. Foil strips were then plunged directly into LN and held for at least an hour. Foils were warmed by immersion in 1.2 M sucrose in half-strength MS salts for 20 min prior to plating onto full-strength MS salt and vitamin medium containing 0.5 mg of benzyladenine and 0.1 mg of indole-3-butyric acid per liter, 3% sucrose, and 0.7% agar, pH 5.7, for regeneration. Recovering shoot tips were embedded 24 h or 7 days or 14 days after warming from LN.

A minimum of ten shoot tips each from two replicate trials were placed into fixative (1.25% glutaraldehyde, 2% paraformaldehyde, and 0.05 M PIPES buffer) containing the cryoprotectant immediately after each step in the cryopreservation protocol. Shoot tips were also fixed with fixative containing 0.3 M sucrose in place of the buffer and this method of formulation was continued for each of the different cryoprotectant solutions.

Microscopy

Fixed samples were dehydrated with a graded series of acetone and infiltrated with Spurr resin over several days. Samples were polymerized at 70 °C overnight and sectioned (1 μm thickness) with glass knives on an RMC MT-X microtome (Ventana Medical Systems Inc., Tucson, Ariz., U.S.A.). Sections were counterstained with Stevenel's Blue (del Cerro et al. 1980) and visualized with an Olympus BH-2 microscope (Olympus Optical Co., Tokyo, Japan). Images were digitally captured with a Magnafire model 560800 camera (Optronics, Goleta, Calif., U.S.A.) using Magnafire 2.1C application software (Optronics) with 1300 by 1030 active pixels and saved as 24 bit TIFF files. Cell and protoplast cross-sectional areas were measured by Scion Image for Windows (www.scioncorp.com; Scion Corporation, Frederick, Md., U.S.A.). Multiple shoot tips were sectioned and eight different cell types were identified. Ten cells of each cell type were measured in three shoot tips for each treatment. On the basis of our observations, we reduced the eight cell types to five cell types. Cells within the three layers of the meristem had the same size and the same extent of plasmolysis; therefore, data from these cell types were pooled for analysis. Cells from the first leaf primordium and from the upper portion of the second and third leaf primordia were identical in size and extent of plasmolysis and were pooled for analysis. These cell types were termed the young leaf cells. The lower region of the second and third leaf primordia was termed the older-leaf region (see Fig. 1). The cellular region immediately below the meristem cells was classified as the upper cortex and the lower region, in which vascular cells were undergoing differentiation, was classified as the lower cortex (see Fig. 1). Provascular cells in the cortex region were not selected for quantification.

The extent of plasmolysis within a cell was determined by calculating the ratio of the protoplast cross-sectional area to the cell wall cross-sectional area (plasmolysis ratio) using digital tracer measurements. Shoot tips with a plasmolysis ratio of 1 do not exhibit plasmolysis and lower plasmolysis ratio values indicate greater extents of plasmolysis. Analyses of variance and means separation tests (Tukey–Kramer honestly significant difference) were performed by the JMP software package (SAS Institute Inc., Cary, N.Y., U.S.A.).

For transmission electron microscopy, thin sections (70 to 90 nm) were cut with a diamond knife, mounted on formvar-coated copper grids (150 mesh) and stained with 3.2% uranyl acetate in 50% methanol and 50% ethanol for 15 min and Reynolds lead citrate (Bozzola and Russell 1991) for 15 min. Thin sections were observed with a JEOL 2000 EXII transmission electron microscope (Jeol. Ltd., Tokyo, Japan) at 100 kV accelerating voltage.

Results

Five distinct cell types within peppermint (*Mentha* × *piperita*) shoot tips were examined (Fig. 1): meristem, young

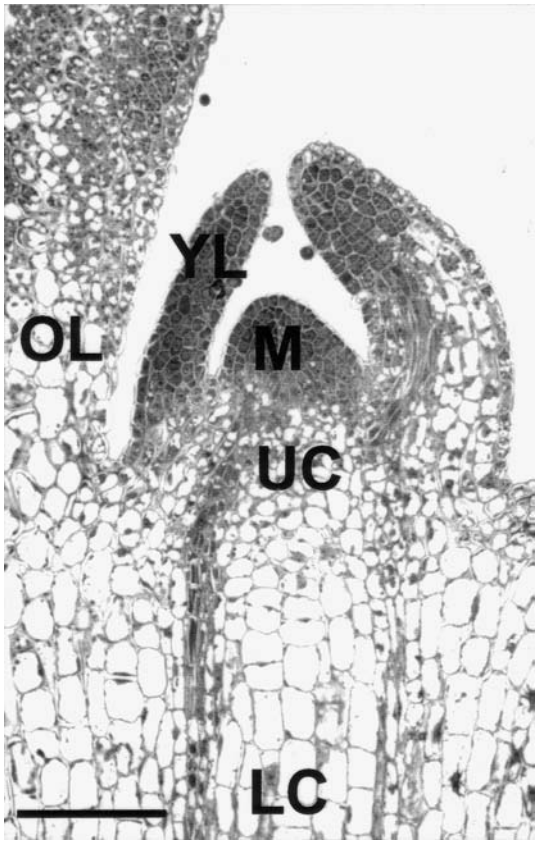

Fig. 1. Meristem (*M*), young leaf (*YL*), older leaf (*OL*), upper cortex (*UC*), and lower cortex (*LC*) cell types in a freshly harvested and embedded shoot tip of *Mentha* × *piperita*. Bar: 100 μm

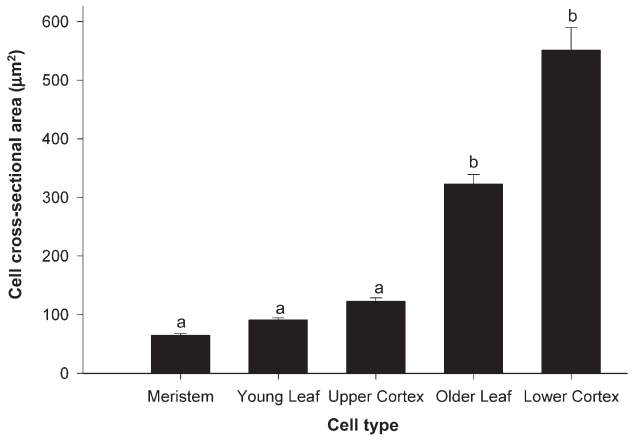

Fig. 2. Cell cross-sectional areas of five cell types within peppermint shoot tips (means with standard error). Ten cells of each cell type were measured in three shoot tips for each treatment. Letters denote significant differences in a means separation test ($\alpha < 0.05$)

Table 1. Ratio of protoplast cross-sectional area to cell wall cross-sectional area for five cell types within peppermint shoot tips[a]

Cell type and concn of plasmolysis sucrose solution (M)	Plasmolysis ratio for cells with the following compound in fixative:[b]		
	0.05 M PIPES	0.3 M sucrose	Sucrose at concn of plasmolysis solution
Meristem cells			
0.3		1.00 ± 0 a	1.00 ± 0 a
0.6		0.66 ± 0.04 b	0.70 ± 0.01 b
0.9		0.70 ± 0.05 bc	0.59 ± 0.05 bc
1.2	0.71 ± 0.04	0.58 ± 0.02 c	0.50 ± 0.02 c
1.5		0.65 ± 0.03 bc	0.62 ± 0.01 bc
1.8		0.64 ± 0.04 bc	0.61 ± 0.01 bc
2.1		0.52 ± 0.03 b	0.69 ± 0.06 b
Young leaf cells			
0.3		1.00 ± 0 a	1.00 ± 0 a
0.6		0.77 ± 0.02 b	0.70 ± 0.04 b
0.9		0.74 ± 0.05 bc	0.62 ± 0.04 b
1.2	0.76 ± 0.06	0.67 ± 0.04 bc	0.61 ± 0.04 b
1.5		0.65 ± 0.01 bc	0.61 ± 0.01 b
1.8		0.62 ± 0.01 bc	0.62 ± 0.03 b
2.1		0.58 ± 0.08 c	0.67 ± 0.09 b
Upper cortex cells			
0.3		0.55 ± 0.04 a	0.55 ± 0.04 ab
0.6		0.63 ± 0.01 a	0.62 ± 0.01 a
0.9		0.63 ± 0.08 a	0.57 ± 0.03 a
1.2	0.62 ± 0.01	0.56 ± 0.03 a	0.41 ± 0.02 b
1.5		0.56 ± 0.03 a	0.49 ± 0.05 ab
1.8		0.46 ± 0.01 a	0.51 ± 0.03 ab
2.1		0.48 ± 0.05 a	0.53 ± 0.04 ab
Older leaf cells			
0.3		0.69 ± 0.03 a	0.69 ± 0.03 a
0.6		0.57 ± 0.06 abc	0.58 ± 0.04 ab
0.9		0.64 ± 0.03 ab	0.54 ± 0.03 b
1.2	0.74 ± 0.06	0.47 ± 0.04 bc	0.38 ± 0.02 c
1.5		0.52 ± 0.05 abc	0.39 ± 0.04 c
1.8		0.38 ± 0.01 bc	0.38 ± 0.03 c
2.1		0.46 ± 0.05 c	0.42 ± 0.01 c
Lower cortex cells			
0.3		0.68 ± 0.07 a	0.68 ± 0.07 a
0.6		0.49 ± 0.02 a	0.45 ± 0.03 bc
0.9		0.52 ± 0.06 a	0.54 ± 0.09 ab
1.2	0.66 ± 0.05	0.46 ± 0.02 a	0.34 ± 0.01 c
1.5		0.45 ± 0.06 a	0.40 ± 0.02 bc
1.8		0.38 ± 0.07 b	0.41 ± 0.05 bc
2.1		0.53 ± 0.05 a	0.42 ± 0.01 bc

[a] Shoot tips were treated wit 0.3 to 2.1 M sucrose for 2 h prior to fixing in fixative containing either 0.05 M PIPES buffer, 0.3 M sucrose, or the sucrose concentration of the plasmolysis solution

[b] Values are means with standard error. Letters denote significant differences of the results for each cell type at $\alpha = 0.05$

leaf, lower region of the older leaf, upper cortex, and lower cortex. The meristem cells were within the most compact region, with no visible intercellular spaces and cells that were less than 100 μm^2 in cross section (Fig. 2). The cells of the adjacent upper cortex region were not significantly larger than the meristem cells but exhibited larger vacuoles. The lower cortex cells were large and highly vacuolated. The young leaf cells were compact and not significantly larger than the meristem cells, while the older leaf cells had statistically the same cross-sectional areas as the lower cortex cells (Figs. 1 and 2).

Sucrose treatments

Peppermint shoot tips can survive exposure to sucrose solutions that cause plasmolysis. All shoot tips continued to grow when they were returned to growth medium after exposure to 0 to 2.1 M sucrose for 2 h (data not shown). Within a few days, shoot tips from all the sucrose treatments achieved the same size and growth rate. After exposure to sucrose treatments, shoot tips were fixed with fixative containing 0.05 M sucrose, 0.3 M sucrose, or the plasmolyzing concentration of sucrose to determine the extent of deplasmolysis that occurs during fixation. Fixative containing 0.05 M PIPES buffer resulted in deplasmolysis; however, for most cell types, no significant differences were observed between the extent of plasmolysis observed in shoot tips fixed with 0.3 M sucrose or with the plasmolysing concentration of sucrose in the fixative, indicating that deplasmolysis during fixation using 0.3 M sucrose was minimal (Table 1).

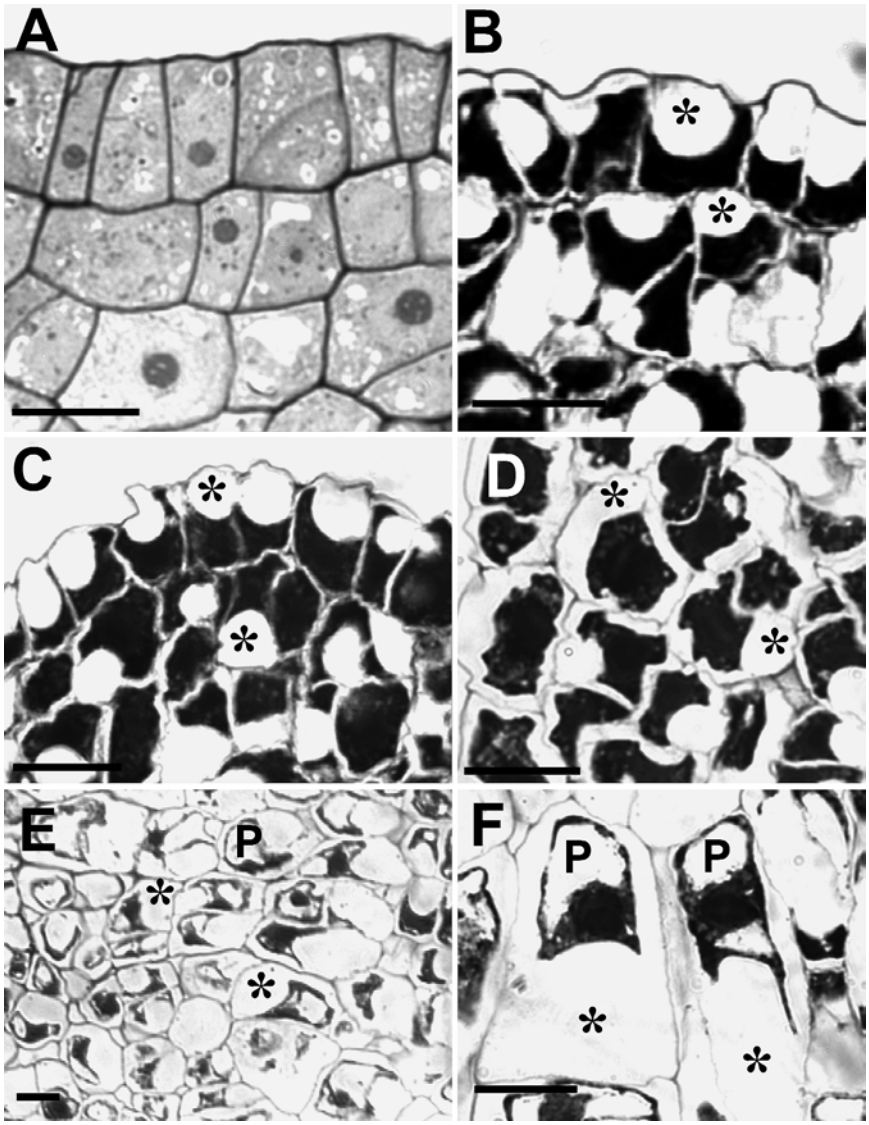

Fig. 3 A–F. Peppermint shoot tips treated with sucrose solutions and then fixed with the plasmolyzing concentration of sucrose included in the fixative. **A** Meristem cells, 0.3 M sucrose; **B** meristem cells, 1.2 M sucrose; **C** meristem cells, 2.1 M sucrose; **D** young leaf cells, 2.1 M sucrose; **E** older leaf cells, 2.1 M sucrose; **F** lower cortex cells, 2.1 M sucrose. *P* Protoplast; asterisks, regions where plasmolysis has occurred. Bars: 10 μm

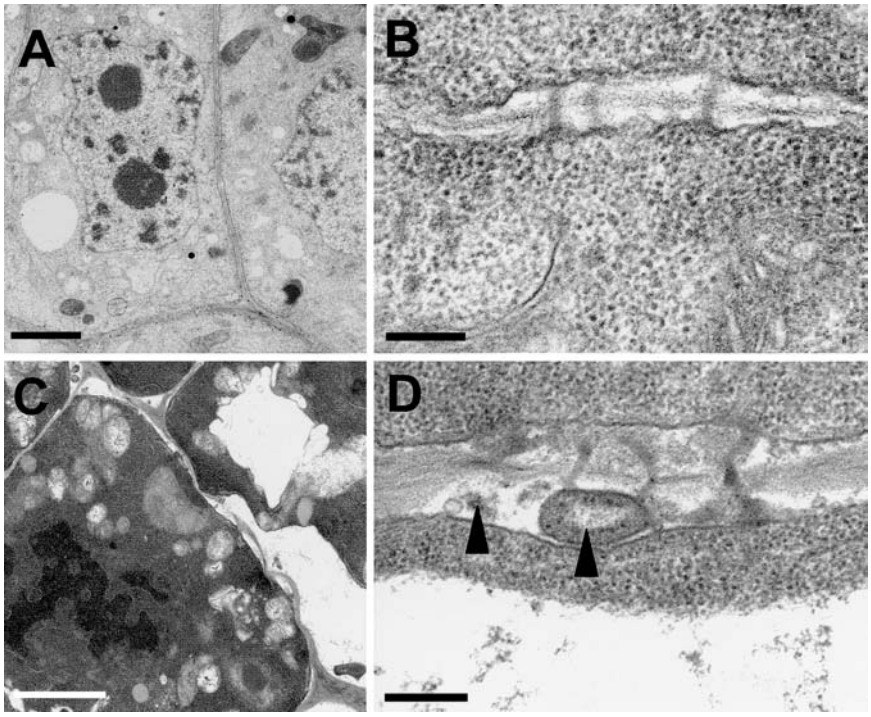

Fig. 4. Transmission electron micrographs of meristem cells from peppermint shoot tips treated with 0.3 M sucrose (**A** and **B**) or 1.2 M sucrose (**C** and **D**). Arrowheads indicate cytoplasmic vesicles adjacent to cell wall. Bar: A, 1 μm; C, 2 μm; B and D, 200 nm

Meristems of peppermint shoot tips treated with 0.3 M sucrose did not appear plasmolyzed (Figs. 3A and 4A). Meristem, young leaf, and upper cortex cells treated with 0.6 to 1.2 M sucrose frequently experienced concave plasmolysis, where the cytoplasm was pushed away from one side or corner of the cells (Fig. 3B). Plasmolyzed cells in the outermost layer of the meristem had protoplasts that pulled away from only the exterior surface of the cells when treated with 1.2 or 2.1 M sucrose (Fig. 3B, C). In interior layers of the meristem or young-leaf tissues, protoplasm contracted from various sides of the cell during plasmolysis (Figs. 3C, D and 4C). Protoplasts in severely plasmolyzed older leaf and lower cortex cells pulled away from most of the cell walls (Fig. 3E, F). In concentrated solutions, older leaf and lower cortex cells had protoplasts that had few, if any, visible cell wall connections and a rough, compressed appearance (Fig. 3E, F).

The plasmodesmata between meristem cells in shoot tips treated with 0.3 M sucrose appeared to remain intact (Fig. 4B). Plasmodesmata between plasmolyzed cells in shoot tips treated with 1.2 M sucrose persisted, although vesicles of cytoplasm formed adjacent to the cell walls in regions where plasmolysis occurred (Fig. 4D).

The extent of plasmolysis was dependent upon cell type (Table 1). In plasmolyzing solutions with sucrose concentrations greater than 0.3 M, the plasmolysis ratio in meristem cells was 0.5 to 0.7, with a general trend of higher levels of plasmolysis in more concentrated solutions. A similar trend was observed in the compact cells within the young leaves of the shoot tips (Table 1). The slightly larger

Table 2. Ratio of protoplast cross-sectional area to cell wall cross-sectional area for five cell types within peppermint shoot tips

Plasmolysis sucrose solution[a]	Plasmolysis ratio for cells[b]				
	Meristem	Young leaf	Upper cortex	Older leaf	Lower cortex
0.6 M	0.84 ± 0.02 a	0.84 ± 0.03 a	0.74 ± 0.03 b	0.69 ± 0.03 b	0.72 ± 0.02 a
0.6 M to 0.3 M	0.85 ± 0.05 a	0.83 ± 0.07 a	0.77 ± 0.01 b	0.77 ± 0.02 b	0.75 ± 0.04 a
1.2 M	0.71 ± 0.04 a	0.76 ± 0.06 a	0.62 ± 0.01 a	0.66 ± 0.05 a	0.74 ± 0.06 a
1.2 M to 0.3 M	0.89 ± 0.05 a	0.86 ± 0.04 a	0.76 ± 0.01 b	0.84 ± 0.03 b	0.78 ± 0.05 a

[a] Shoot tips were treated with 0.6 or 1.2 M sucrose for 2.5 h and then either fixed or placed into 0.3 M sucrose for 2.5 h prior to fixation
[b] Values are means with standard error. Letters denote significant differences within a column at $\alpha = 0.05$

cells of the upper-cortex region plasmolyzed to a greater extent than the meristem and young-leaf cells. The upper-cortex cells were most plasmolyzed when they were treated with solutions of ≥1.2 M sucrose and fixed with the fixative containing the plasmolyzing solution of sucrose. The vacuolated older-leaf and lower-cortex regions had plasmolysis ratios of 0.34 to 0.38 when treated with solutions of ≥1.2 M sucrose (Table 1). Increasing the plasmolysis solution to concentrations greater than 1.2 M sucrose did not statistically increase the extent of plasmolysis when shoot tips were fixed with fixative containing the plasmolyzing concentration of sucrose (Table 1).

In another experiment, peppermint shoot tips were immersed in 0.3 M sucrose solutions for 2.5 h to deplas-

Table 3. Ratio of protoplast cross-sectional area to cell wall cross-sectional area for five cell types within peppermint shoot tips[a]

Cell type and treatment solution[b]	Plasmolysis ratio for cells with the following compound in fixative:[c]		Cell type and treatment solution[b]	Plasmolysis ratio for cells with the following compound in fixative:[c]	
	0.05 M PIPES	PVS2		0.05 M PIPES	PVS2
Meristem cells			Lower cortex cells[e]		
0.3 M suc (24 h)	0.94 ± 0.07 a	1.00 ± 0 a	0.3 M suc (24 h)	0.31 ± 0.05 c	0.80 ± 0.02 a
2 M glyc + 0.6 M suc	0.89 ± 0.11 ab	0.86 ± 0.06 abc	2 M glyc + 0.6 M suc	0.72 ± 0.01 a	0.55 ± 0.12 ab
PVS2 (5 min)		0.95 ± 0.04 ab	PSV2 (5 min)		0.68 ± 0.03 ab
PVS2 (15 min)		0.88 ± 0.04 abc	PVS2 (15 min)		0.71 ± 0.04 ab
PVS2 (30 min)	0.95 ± 0.05 a	0.81 ± 0.04 abc	PVS2 (30 min)	0.59 ± 0.04 ab	0.65 ± 0.07 ab
PVS2 (30 min) + LN	0.94 ± 0.06 a	0.76 ± 0.01 bc	PVS2 (30 min) + LN	0.50 ± 0.05 bc	0.61 ± 0.03 ab
PVS2 (30 min) + LN + 1.2 M suc	0.73 ± 0.03 ab	0.65 ± 0.10 c	PVS2 (30 min) + LN + 1.2 M suc	0.53 ± 0.04 b	0.58 ± 0.06 ab
PVS2 (30 min) + 1.2 M suc (no LN)	0.70 ± 0.02 ab	0.65 ± 0.03 c	PVS2 (30 min) + 1.2 M suc (no LN)	0.50 ± 0.01 bc	0.45 ± 0.02 b
Recovery (24 h)[d]	0.77 ± 0.03 ab				
Recovery (7 days)	0.64 ± 0.05 b		Older leaf cells[e]		
			0.3 M suc (24 h)	0.37 ± 0.01 b	0.80 ± 0.02 a
Young leaf cells			2 M glyc + 0.6 M suc	0.63 ± 0.06 a	0.62 ± 0.07 ab
0.3 M suc (24 h)	1.00 ± 0 a	1.00 ± 0 a	PVS2 (5 min)		0.74 ± 0.02 ab
2 M glyc + 0.6 M suc	0.73 ± 0.04 abc	0.88 ± 0.04 ab	PVS2 (15 min)		0.77 ± 0.03 a
PVS2 (5 min)		0.85 ± 0.06 ab	PVS2 (30 min)	0.42 ± 0.02 b	0.68 ± 0.03 ab
PVS2 (15 min)		0.79 ± 0.09 abc	PVS2 (30 min) + LN	0.69 ± 0.02 a	0.63 ± 0.03 ab
PVS2 (30 min)	0.88 ± 0.12 abc	0.80 ± 0.05 ab	PVS2 (30 min) + LN + 1.2 M suc	0.43 ± 0.02 b	0.57 ± 0.11 ab
PVS2 (30 min) + LN	1.00 ± 0 a	0.63 ± 0.04 bc	PVS2 (30 min) + 1.2 M suc (no LN)	0.50 ± 0.05 ab	0.50 ± 0.03 a
PVS2 (30 min) + LN + 1.2 M suc	0.47 ± 0.11 c	0.61 ± 0.05 c			
PVS2 (30 min) + 1.2 M suc (no LN)	0.77 ± 0.05 abc	0.67 ± 0.02 bc			
Recovery (24 h)	0.75 ± 0.01 abc				
Recovery (7 days)	0.69 ± 0.09 abc				
Upper cortex cells					
0.3 M suc (24 h)	0.52 ± 0.05 a	0.86 ± 0.07 a			
2 M glyc + 0.6 M suc	0.68 ± 0.05 a	0.58 ± 0.09 bc			
PVS2 (5 min)		0.75 ± 0.05 ab			
PVS2 (15 min)		0.60 ± 0.06 abc			
PVS2 (30 min)	0.63 ± 0.04 a	0.61 ± 0.04 abc			
PVS2 (30 min) + LN	0.68 ± 0.10 a	0.58 ± 0.03 bc			
PVS2 (30 min) + LN + 1.2 M suc	0.47 ± 0.06 a	0.64 ± 0.03 abc			
PVS2 (30 min) + 1.2 M suc (no LN)	0.60 ± 0.10 a	0.49 ± 0.01 c			
Recovery (24 h)	0.67 ± 0.01 a				
Recovery (7 days)	0.60 ± 0.02 a				

[a] Shoot tips were fixed after each step of the cryopreservation protocol in fixative containing either 0.05 M PIPES buffer or the cryoprotectant solution
[b] Suc, sucrose; glyc, glycerol
[c] Values are means with standard error. Letters denote significant differences of the results for each cell type within each column at $\alpha = 0.05$
[d] Recovery medium did not contain cryoprotectant solutions; no data for cryoprotectant in fixative could be collected for these treatments
[e] Lower cortex and older leaf cells were not intact after LN exposure and no data could be collected for recovery treatments

molyze after 2.5 h treatments in either 0.6 or 1.2 M sucrose. These treatments did not result in significant levels of deplasmolysis in the meristem, young-leaf, or lower-cortex cell types (Table 2). The upper cortex and older leaf cells treated with 1.2 M sucrose were more plasmolyzed than those immersed in 0.6 M sucrose or in the 0.3 M sucrose deplasmolysis solution (Table 2).

Plasmolysis with cryoprotectants

Nearly all peppermint shoot tips form healthy shoots when returned to medium after cryoprotection treatment but before LN exposure. Approximately 75% survival is obtained when peppermint shoot tips are treated with cryoprotectant, exposed to LN, thawed in 1.2 M sucrose, and plated onto medium (Volk et al. 2006).

The process of cryopreservation differentially affects the five cell types within the shoot tips. Within a cell type, the same trends were identified with respect to the extent of plasmolysis during the cryopreservation process when shoot tips were fixed in fixative containing 0.05 M PIPES buffer or the cryoprotectant solution (Table 3). The protoplasts in meristem and young-leaf cells had a plasmolysis ratio of 0.79 or greater through the PVS2 treatments

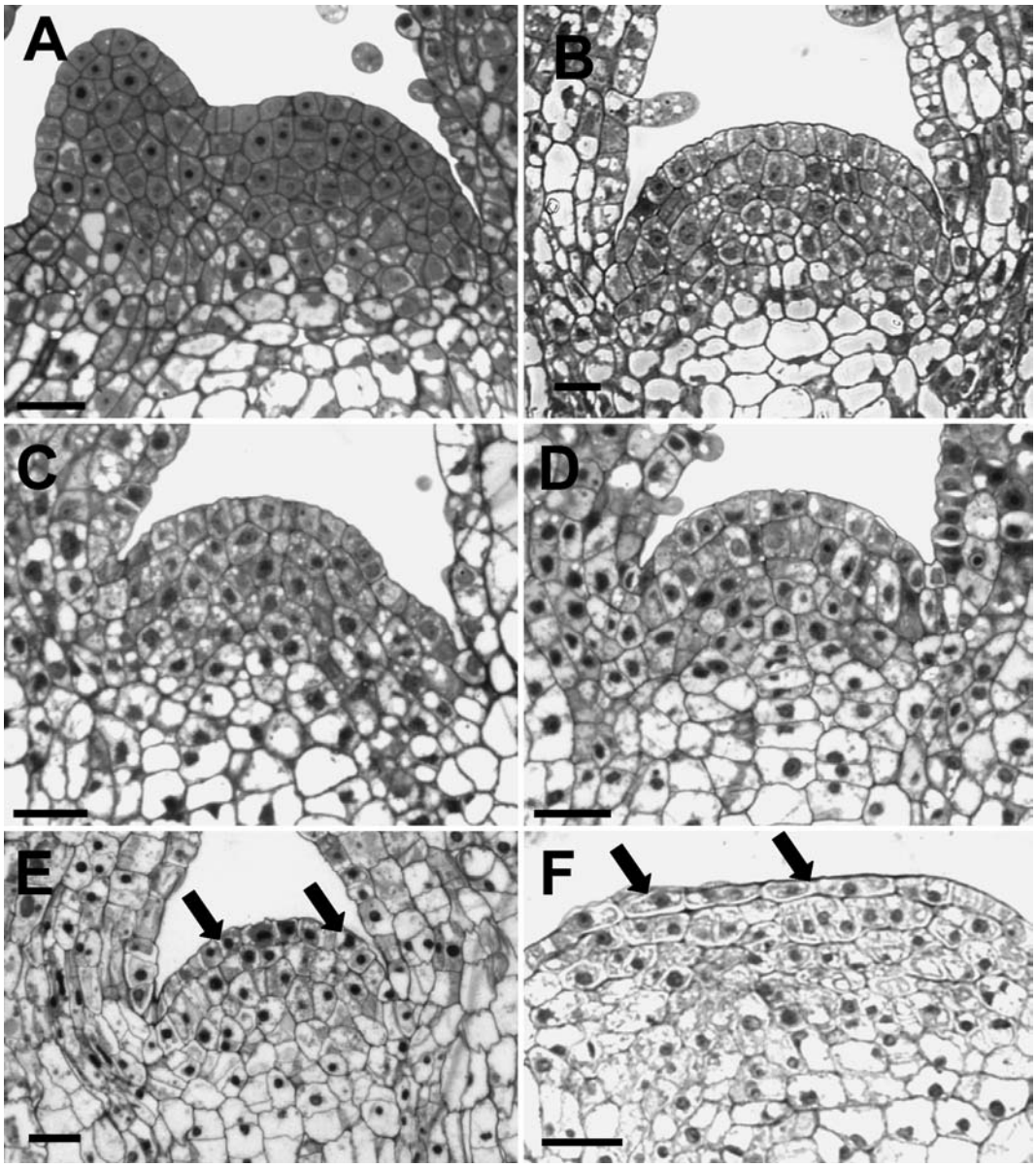

Fig. 5 A–F. Meristems of peppermint shoot tips after successive steps in the cryopreservation procedure. **A** 0.3 M sucrose, **B** 2 M glycerol plus 0.6 M sucrose, **C** PVS2, **D** PVS2 plus LN, **E** PVS2 plus LN plus 1.2 M sucrose, **F** 7-day recovery after LN treatment. Arrows indicate regions where plasmolysis has occurred. Bars: 100 μm

(Table 3). These cell types became more plasmolyzed as the cryopreservation procedure progressed, with the plasmolysis ratio dropping to levels as low as 0.47 after LN exposure and warming in 1.2 M sucrose (Table 3). The original meristem cells did not regain full protoplast sizes even after 7 days in culture (Fig. 5F). After PVS2

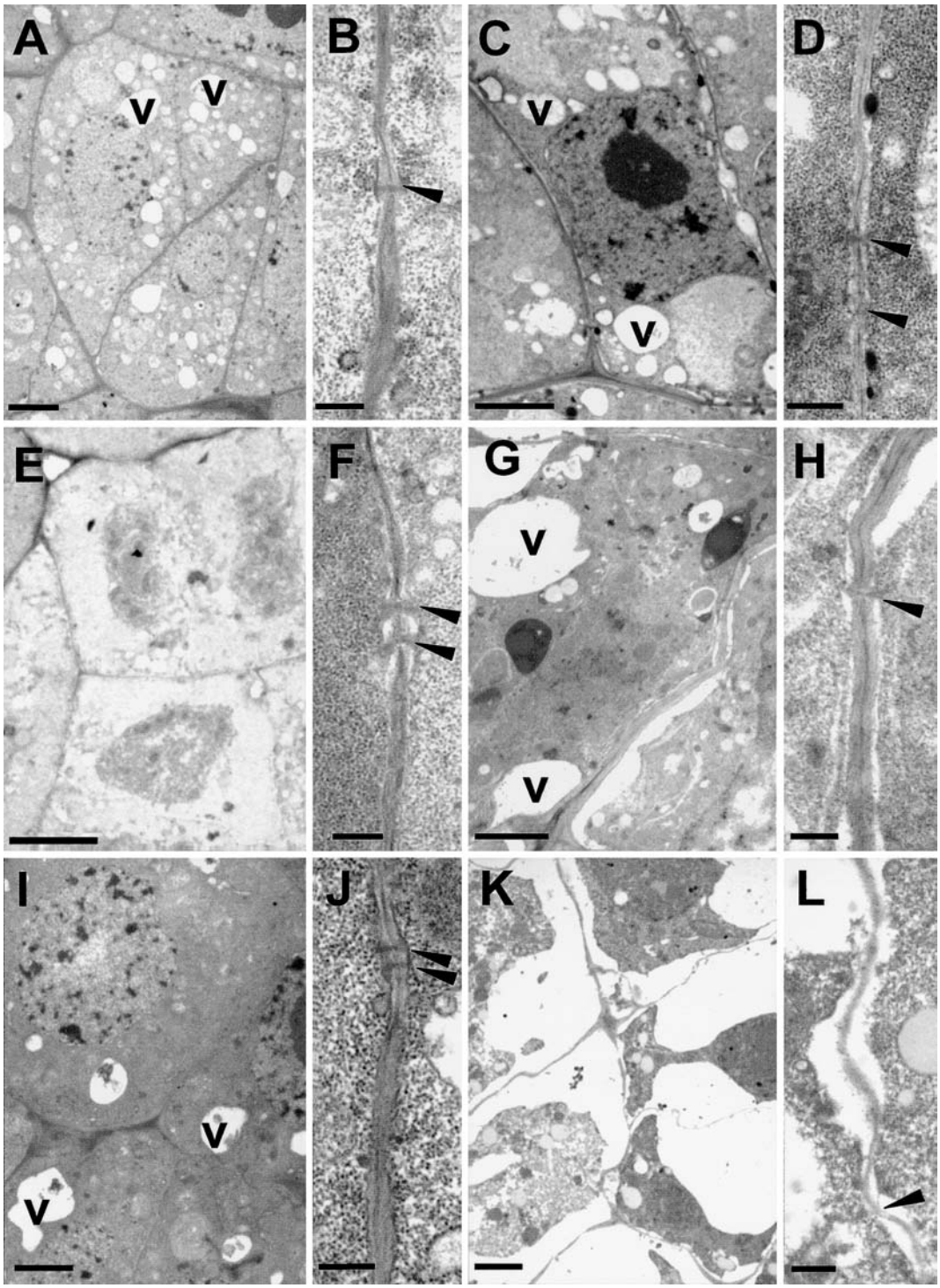

Fig. 6. Transmission electron micrographs of peppermint meristems from shoot tips treated with 2 M glycerol plus 0.6 M sucrose (**A** and **B**), PVS2 for 30 min (**C** and **D**), PVS2 plus LN (**E** and **F**), PVS2 plus LN plus 1.2 M sucrose (**G** and **H**), PVS2 plus 1.2 M sucrose (no LN) (**I** and **J**), or 7-day recovery after LN treatment (**K** and **L**). *v* Vacuoles. Arrowheads indicate plasmodesmata. Bar: A and K, 5 μm; C, E, G, and I, 2 μm; B, D, H, J, and L, 200 nm; F, 300 nm

treatments either with or without LN, young-leaf and upper-cortex cells often contained darkly staining nuclei with protoplasts that separated from the cell wall at multiple sites.

We used transmission electron microscopy to observe the meristem cells of peppermint shoot tips treated with cryoprotectants. As expected, very little plasmolysis was observed within the meristem cells of shoot tips treated with 2 M glycerol plus 0.6 M sucrose (Fig. 6A). Simple plasmodesmata appeared to connect the protoplasts of adjacent cells in all the cryoprotective treatments except those observed after a 7-day recovery interval (Fig. 6B, D, F, H, J, L). Small vacuoles were observed in meristem cells up until LN exposure (Fig. 6A, C). After immersion in LN (and thawing into fixative), the vacuoles appeared to coalesce into larger vacuoles within the meristem cells (Fig. 6G).

Larger and more vacuolated cell types, such as the older-leaf and lower-cortex cells, experienced greater extents of plasmolysis during cryoprotective treatments. In the glycerol-sucrose and PVS2 treatments, the lower-cortex and older-leaf cells showed convex plasmolysis (data not shown). After 24 h or 7 days of culture, recovering shoot tips infiltrated poorly during embedding. These cell types appeared to be severely damaged by the cryopreservation process and plasmolysis data could not be collected. They did not appear to recover after LN exposure.

Our data reveal that recovering peppermint shoots originate from multiple regions within a single cryopreserved shoot tip. New meristems form either from outer cell layers of the original meristem or from the axil of the shoot and young leaf. Most shoot tips that remained green after 7 days of recovery formed organized meristems that resulted in small green shoots apparent after 14 to 28 days (Fig. 7A–C, G, H). The original meristem cells from green shoot tips observed after 7 days frequently appeared severely damaged in embedded shoot tips (Figs. 5F and 6K, L).

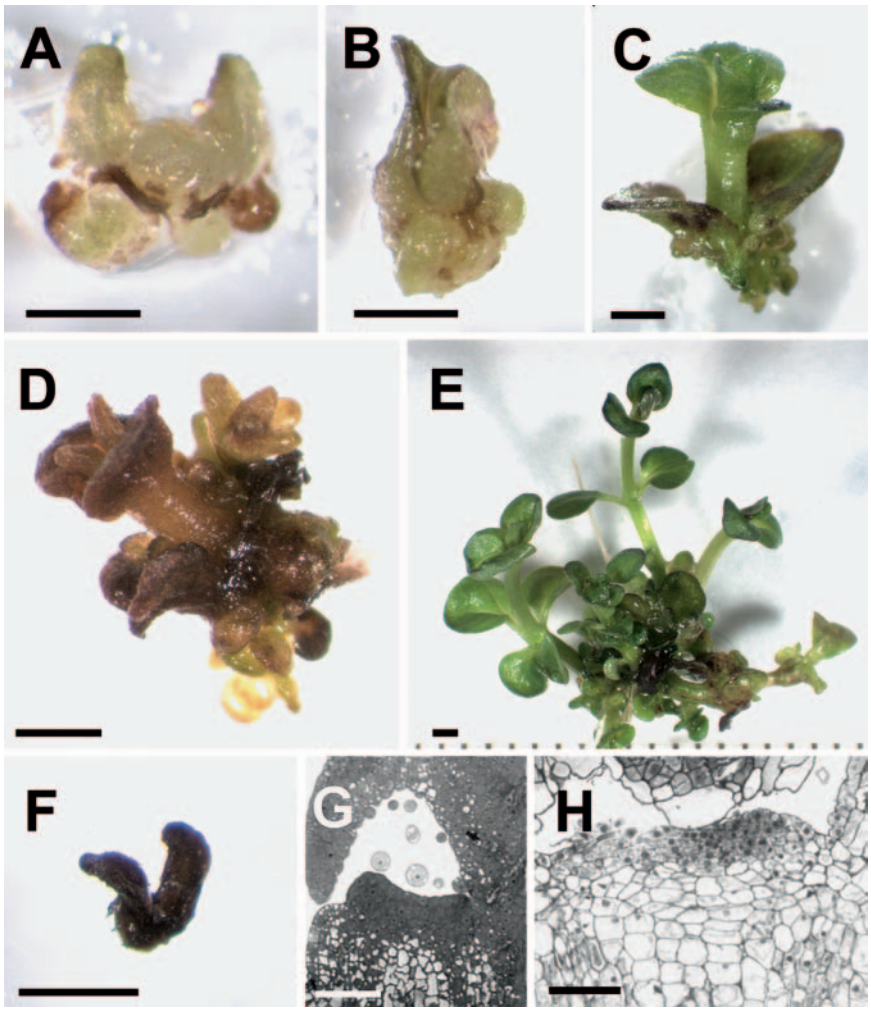

Fig. 7 A–H. Peppermint shoot tip regrowth after cryoexposure. **A** and **B** 7 days after warming. **C** 14 days after warming. **D** and **E** 28 days after warming. **F** 14-day peppermint shoot tip that did not survive cryoexposure. **G** Healthy axillary bud meristem formed on cryoexposed 14-day peppermint shoot tip. **H** Primary peppermint meristem exhibiting cell division and recovery 14 days after cryoexposure. Bar: A–F, 1 mm; G and H, 50 μm

Discussion

Tissue desiccation and chemical permeation induced by PVS2 affect cellular freezing properties (Volk and Walters 2006). PVS2 has components that are permeant (dimethyl sulfoxide and ethylene glycol) and impermeant (glycerol and sucrose). This complex solution serves to dehydrate shoot tips and to change the behavior of water remaining within shoot tips (Volk and Walters 2006). The complex nature of PVS2 may provide the mechanism needed for meristem survival during cryopreservation.

The extent of plasmolysis within cells is highly dependent upon cell type. These differences in cellular response to plasmolyzing solution and LN exposure may be attributed to cell location, size, or other physiological characteristics. Smaller cells with only small vacuoles and densely staining cytoplasm, such as the meristem, upper cortex, and younger leaf cells, showed the least plasmolysis during osmotic dehydration treatments. There appears to be a limit to how far these cells contract, which is likely to be associated with the compressibility of the remaining cellular constituents and a lower water content. Large, vacuolated cells, such as the older leaf and lower cortex cells, exhibited high levels of plasmolysis with either sucrose or cryoprotectant treatment. The quantity of water lost from the protoplasm of these larger cells appeared to be lethal since these cell types did not generally survive the imposed osmotic stresses. These observations are in agreement with those made by Withers (1980), who stated that cell suspensions of highly cytoplasmic cells are preserved better than larger, vacuolated cells.

All cell types exhibited concave plasmolysis at low sucrose solution concentrations. Even at high sucrose concentrations, the meristem cells had concave plasmolysis patterns consistent with highly viscous protoplasm and many cell wall attachment sites, as described by Attree and Sheffield (1985). These results suggest that relatively little water moves during the plasmolysis of meristematic cell types. Older leaf and lower cortex cells with large vacuoles exhibited plasmolysis patterns under highly osmotic conditions that left the protoplast in only a small portion of the cell. These convex-plasmolysis patterns, also described by Oparka (1994), suggest that lower protoplasmic viscosity and weaker levels of plasma membrane–wall adhesion occur within these more mature cell types.

In most cases, inclusion of cryoprotectant solution components in the fixative solutions did not significantly increase the extent of plasmolysis measured. Our aqueous fixation method is comparable to those reported in the literature and suitable for transmission electron microscopy observations (Haskins and Kartha 1980, Mari et al. 1995). The plasmodesmata between meristem cells appear to remain intact during the early stages of plasmolysis. Primary plasmodesmata that remain intact between the M1, M2, and M3 cell layers of the meristem may remain functional for solute, RNA, and protein intercellular transport and signaling (Rinne and van der Schoot 1998, Roberts and Oparka 2003). The presence of plasmodesmata may prevent plasmolysis from occurring in specific cell wall regions, and extreme conditions may result in the formation of vesicles containing cytoplasm at the sites of plasmodesmata. These pieces of protoplasm attached to plasmodesmata are similar to those observed by Bayer et al. (2004) in plasmolyzed *Arabidopsis thaliana* cell suspensions. Similarly, membrane vesicles were observed during exposure of rice embryogenic suspension cells and coffee shoot tips to cryoprotectants (Mari et al. 1995, Wang et al. 1998). The functionality of the plasmodesmata during plasmolysis and cryopreservation of meristems has not been evaluated; however, other researchers have determined that plasmodesmata can become plugged with callose and dysfunctional during extreme plasmolysis (Bayer et al. 2004, Drake et al. 1978, Ruan et al. 2004).

The fact that plasmolysis was most evident in cryopreserved shoot tips after thawing and 1.2 M sucrose treatment suggests that early recovery is a critical timepoint which helps dictate shoot tip survival. Similarly, during the first three days after LN exposure, control and frozen sugarcane embryogenic calluses exhibited differences with respect to electrolyte leakage, lipid peroxidation products, and membrane protein content (Martinez-Montero et al. 2002). Membrane recovery is critical to survival after cryopreservation (Finkle et al. 1985).

The observations made after cryoprotection with PVS2 differed from those reported for encapsulation and two-step cooling cryopreservation methods. Peppermint shoot tips did not appear to accumulate large quantities of starch during the imposed treatments. In contrast, starch and osmophilic particle accumulation were observed during coffee and sugarcane shoot tip encapsulation cryopreservation methods after pretreatments with 0.5 to 1 M sucrose (Mari et al. 1995, Gonzalez-Arnao et al. 1993). The osmophilic granules and clear cytoplasmic vesicles that were observed between the plasmalemma and cell wall after dehydration in peppermint cells were also observed in coffee cells (Mari et al. 1995). Similar to our observations in peppermint shoot tips, the meristems of date palm shoot tips treated with a two-step slow-cooling protocol exhibited some plasmolysis and damage after LN exposure (Bagniol et al. 1992).

We observed numerous shoots growing from recovering peppermint shoot tips, suggesting that multiple groups of cells serve as independent meristematic regions, each regenerating a new meristem. The observation that undifferentiated meristematic cells survive cryopreservation is consistent with observations made by Wesley-Smith et al. (1995) using pea embryonic axes. Similarly, Haskins and Kartha (1980) observed minimal damage in the outermost meristem cell layers when pea meristem cells were treated with 5% dimethyl sulfoxide and then slowly cooled to −40 °C prior to LN exposure. Bagniol et al. (1992) reported that meristem cells of date palm shoot tips survived cryopreservation, while vacuolated cells near the base of the explant did not survive LN exposure when two-step cryopreservation methods were used. Others have identified systems in which entire meristems grow directly into new shoot tips after cryopreservation using two-step cooling or encapsulation methods (Gonzalez-Arnao et al. 1993, Chang and Reed 1999, Mari et al. 1995).

The differences between our results with PVS2 and those obtained with other cryoprotection methods suggest that the physiological response of shoot tips is dependent upon the cryopreservation method. Microscopic tools provide valuable insights into the degree of plasmolysis within specific shoot tip cell types. These studies demonstrate that the cellular nature of undifferentiated cells makes them less vulnerable to the damages incurred during the osmotic stresses of cryoprotectant treatments. Cryoprotective treatments that favor survival of the small, meristematic and young leaf cells of the shoot tip are most likely to produce high survival rates after LN exposure.

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References

Attree SM, Sheffield E (1985) Plasmolysis of *Pteridium* protoplasts: a study using light and scanning-electron microscopy. Planta 165: 151–157

Bagniol S, Engelmann F, Michaux-Ferriere N (1992) Histo-cytological study of apices from in vitro plantlets of date palm (*Phoenix dactylifera* L.) during a cryopreservation process. CryoLetters 13: 405–412

Bayer E, Thomas CL, Maule AJ (2004) Plasmodesmata in *Arabidopsis thaliana* suspension cells. Protoplasma 223: 93–102

Bozzola JJ, Russell LD (1991) Electron microscopy. Jones and Bartlett, Boston

Chang Y, Reed BM (1999) Extended cold acclimation and recovery medium alteration improve regrowth of *Rubus* shoot tips following cryopreservation. CryoLetters 20: 371–376

del Cerro M, Cogen J, del Cerro C (1980) Stevenel's blue, an excellent stain for optical microscopical study of plastic embedded tissues. Microsc Acta 83: 117–121

Drake GA, Carr DJ, Anderson WP (1978) Plasmolysis, plasmodesmata, and the electrical coupling of oat coleoptile cells. J Exp Bot 29: 1205–1214

Engelmann F (2004) Plant cryopreservation: progress and prospects. In Vitro Cell Dev Biol Plant 40: 427–433

Fahy GM, Levy DI, Ali SE (1987) Some emerging principles underlying the physical properties, biological actions, and utility of vitrification solutions. Cryobiology 24: 196–213

Finkle BJ, Zavala ME, Ulrich JM (1985) Cryoprotective compounds in the viable freezing of plant tissues. In: Kartha KK (ed) Cryopreservation of plant cells and organs. CRC Press, Boca Raton, Fla, pp 75–114

Gonzalez-Arnao MT, Engelmann F, Huet C, Urra C (1993) Cryopreservation of encapsulated apices of sugarcane: effect of freezing procedure and histology. CryoLetters 14: 303–308

Haskins RH, Kartha KK (1980) Freeze preservation of pea meristems: cell survival. Can J Bot 58: 833–840

Mari S, Engelmann F, Chabrillange N, Huet C, Michaux-Ferriere N (1995) Histo-cytological study of apices of coffee (*Coffea racemosa* and *C. sessiliflora*) in vitro plantlets during their cryopreservation using the encapsulation-dehydration technique. CryoLetters 16: 289–298

Martinez-Montero ME, Mora N, Quinones J, Gonzalez-Arnao MT, Engelmann F, Lorenzo JC (2002) Effect of cryopreservation on the structural and functional integrity of cell membranes of sugarcane (*Saccharum* sp.) embryogenic calluses. CryoLetters 23: 237–244

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

Oliver MJ (1996) Desiccation tolerance in vegetative plant cells. Physiol Plant 97: 779–787

Oparka KJ (1994) Plasmolysis – new insights into an old process. New Phytol 126: 571–591

Rinne PL, van der Schoot C (1998) Symplasmic fields in the tunica of the shoot apical meristem coordinate morphogenic events. Development 125: 1477–1485

Roberts AG, Oparka KJ (2003) Plasmodesmata and the control of symplastic transport. Plant Cell Environ 26: 103–124

Ruan YL, Xu SM, White R, Furbank RT (2004) Genotypic and developmental evidence for the role of plasmodesmatal regulation in cotton fiber elongation mediated by callose turnover. Plant Physiol 136: 4104–4113

Sakai A (2000) Development of cryopreservation techniques. In: Engelmann F, Takagi H (eds) Cryopreservation of tropical plant germplasm: current research progress and application. Japan International Research Center for Agricultural Sciences, Tsukuba; International Plant Genetic Resources Institute, Rome, pp 1–7

Sakai A, Kobayashi S, Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Plant Cell Rep 9: 30–33

Steponkus PL, Langis R, Fujikawa S (1992) Cryopreservation of plant tissues by vitrification. In: Steponkus PL (ed) Advances in low-temperature biology. JAI Press Ltd., Greenwich, Conn, pp 1–61

Towill LE (1990) Cryopreservation of isolated mint shoot tips by vitrification. Plant Cell Rep 9: 178–180

Towill LE (2002) Cryopreservation of *Mentha* (mint). In: Towill LE, Bajaj YPS (eds) Cryopreservation of plant germplasm II. Biotechnology in agriculture and forestry, vol 50. Springer, Berlin Heidelberg New York, pp 151–163

Volk GM, Walters C (2006) Plant vitrification solution 2 lowers water content and alters freezing behavior in shoot tips during cryoprotection. Cryobiology 52: 48–61

Volk GM, Harris JL, Rotindo KE (2006) Survival of mint shoot tips after exposure to cryoprotectant solution components. Cryobiology 52: 305–308

Walters C, Farrant JM, Pammenter NW, Berjak P (2002) Desiccation stress and damage. In: Black M, Pritchard HW (eds) Desiccation and survival in plants: drying without dying. Oxford University Press, Oxford, pp 263–291

Wang J-H, Ge J-G, Liu F, Huang C-N (1998) Ultrastructural changes during cryopreservation of rice (*Oryza sativa* L.) embryogenic suspension cells by vitrification. CryoLetters 19: 49–54

Wesley-Smith J, Berjak P, Pammenter NW, Vertucci CW (1995) Ultrastructural evidence for the effects of freezing in embryonic axes of *Pisum sativum* L. at various water contents. Ann Bot 76: 59–64

Withers LA (1980) The cryopreservation of higher plant tissue and cell cultures – An overview with some current observations and future thoughts. CryoLetters 1: 239–250

Withers LA (1985) Cryopreservation of cultured plant cells and protoplasts. In: Kartha K (ed) Cryopreservation of plant cells and organs. CRC Press, Boca Raton, Fla, pp 243–267