Chapter 3

Development of PVS-Based Vitrification and Encapsulation–Vitrification Protocols

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3.1 Introduction

Cryopreservation is a very important tool for the long-term storage of plant genetic resources for future generations, requiring only a minimum of space and maintenance. With increasing interest in the genetic engineering of plants, the preservation of cultured cells and somatic embryos with unique attributes is assuming greater importance. Recently, cryopreservation was reported to offer real hope for enhancing the preservation of endangered and rare plants (Touchell 1995; Touchell and Dixon 1996). This chapter describes protocols for successful vitrification using plant vitrification solution 2 (PVS2), and highlights some of the factors contributing to high levels of post-LN recovery. The development of a simple and reliable method for cryopreservation would allow the widespread storage of cultured cells, meristems, and somatic embryos. Vitrification involving vitrification solutions (Langis et al. 1990; Sakai et al. 1990; Yamada et al. 1991) and encapsulation–dehydration techniques (Fabre and Dereuddre 1990) were developed in the 1990s, and the number of cryopreserved species has increased markedly since then (Sakai 1995, 1997; Engelmann and

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*

Takagi 2000). A vitrification procedure using an ethylene glycol-based vitrification solution and French straws was presented by Steponkus and colleagues. They reported successful cryopreservation of *Dianthus* and *Chrysanthemum* (Langis et al. 1990; Schnabel-Preikstas et al. 1992), and potato (Lu and Steponkus 1994). This chapter will outline the development and uses of PVS2 developed by Sakai and associates.

3.2 The Concept of Vitrification

To maintain the viability of hydrated cells and tissues it is essential to avoid the lethal intracellular freezing that occurs during rapid cooling in LN (Sakai and Yoshida 1967). Cells and tissues that are to be cryopreserved in LN need to be sufficiently dehydrated before being immersed in LN.

There are two types of liquid–solid phase transitions in aqueous solutions. Ice formation is the phase transition from liquid to ice crystals, and vitrification is a phase transition from a liquid to amorphous glass that avoids crystallization. Water is very difficult to vitrify because the growth rate of crystals is very high, even just below the freezing point. However, highly concentrated cryoprotective solutions such as glycerol are very viscous and are easily supercooled below –70°C. This allows them to be vitrified on rapid cooling. Vitrification refers to the physical process by which a highly concentrated cryoprotective solution supercools to very low temperatures and finally solidifies into a metastable glass without undergoing crystallization at a practical cooling rate (Fahy et al. 1984). Vitrification was proposed as a method for the cryopreservation of biological materials because it would avoid the potentially detrimental effects of extracellular and intracellular freezing (Luyet 1937). Thus, vitrification is an effective freeze-avoidance mechanism. As glass fills space in a tissue it may prevent additional tissue collapse, solute concentration, and pH alteration during dehydration. Operationally, glass is expected to exhibit a lower water vapor pressure than the corresponding crystalline solid, thereby preventing further dehydration. Because glass is exceedingly viscous and stops all chemical reactions that require molecular diffusion, its formation leads to dormancy and stability over time (Burke 1986).

In controlled rate cooling methods, slow freezing to about –40°C results in sufficient concentration of the unfrozen fraction of the suspending solution and cytosol to enable vitrification upon rapid cooling in LN (partial vitrification). Vitrification can also be achieved by direct immersion in LN without the freeze-concentration step by exposing cells and tissues to extremely concentrated solutions (7–8 M) of cryoprotectants. This technique is referred to as complete vitrification, and is distinct from controlled rate cooling methods.

3.3 Vitrification Procedure

In vitrification procedures, cells and meristems must be sufficiently dehydrated with a vitrification solution to avoid lethal injury from immersion in LN. We used glycerol-based vitrification solutions designated PVS2 (Sakai et al. 1990, 1991b) and PVS3 (Nishizawa et al. 1993). Plant vitrification solution 2 contains 30% glycerol (w/v), 15% ethylene glycol (w/v), and 15% dimethylsulfoxide (DMSO; w/v) in basal culture medium (without growth regulators) containing 0.4 M sucrose (pH 5.8), and PVS3 consists of 40% glycerol (w/v) and 40% sucrose (w/v) in basal medium. PVS2 easily supercools below –100°C upon rapid cooling and solidifies into a metastable glass at about –115°C. Upon subsequent slow warming, differential scanning calorimetry records the vitrified PVS2 as displaying a glass transition (Tg) at about –115°C, with an exothermic devitrification (crystallization) (Td) at about –75°C and an endothermic melting (Tm) at about –36°C (Sakai et al. 1990). In mulberry apical meristems treated with PVS2, no freezing exotherm occurred during cooling. During the subsequent warming (10°C min^{-1}) a series of changes in the thermal behavior of the vitreous solid were observed. Such changes included glass transition, exothermic devitrification, and endothermic melting (Niino et al. 1992b). These results indicate that meristems sufficiently dehydrated with PVS2 became vitrified during rapid cooling. Crystallization during the warming process can be prevented if warming occurs rapidly. The complete vitrification method for unencapsulated tissues involves the following steps:

A. Preculture of excised meristems on solidified medium with 0.3 M sucrose for a specified duration at 25°C or 0°C (Fig. 3.1A).

B. Osmoprotection (loading treatment) (Fig. 3.1B). Precultured meristems are placed in a 2 ml cryotube and osmoprotected with a mixture of 2 M glycerol and 0.4 M sucrose for 20–30 min at 25°C. An osmotic loading treatment increases the osmolarity of the cell and minimizes osmotic damage caused by the vitrification solution.

C. Dehydration with a vitrification solution (PVS2 or PVS3; Fig. 3.1C). After removing the osmoprotection solution using a Pasteur pipette, 1–2 ml of PVS2 is added and gently mixed. Five minutes later, PVS2 is replaced with 1–2 ml of fresh PVS2 and held at 25°C or 0°C for different periods of time. Wrapping several dissected meristems together in a small piece of tissue paper (1.5 cm × 1.5 cm) may facilitate the implementation of the osmoprotection, dehydration, cooling, warming, and dilution steps (Thinh 1997).

D. Rapid cooling (Fig. 3.1D). The meristems are suspended in 0.5 ml PVS2 in cryotubes, and the cryotubes are then directly plunged into LN (cooling rate: about 300°C min^{-1}) and held at –196°C for a minimum of 1 h.

E. Rapid warming (Fig. 3.1E). Cryotubes are rapidly transferred to sterile distilled water in a water bath at 40°C (warming rate: 250°C min^{-1}). The cryotubes are vigorously shaken during rewarming for 1.5 min.

F. Dilution (unloading, dilution of the vitrification solution) (Fig. 3.1F). Immediately after warming, the PVS2 is drained from the cryotubes, replaced with 2 ml of basal culture medium with 1.2 M sucrose (Sakai et al. 1991b), and held for 20 min.

G. Plating (Fig. 3.1G). Meristems are then transferred onto a double sterile filter paper disc over a Petri dish filled with culture medium. After 1 day, the meristems are transferred to fresh filter paper in a Petri dish containing the same medium. Shoot formation is recorded as the percentage of the total number of meristems forming normal shoots 3–4 weeks after plating.

Table 3.1 presents (nonexhaustive) lists of plant species to which PVS2 or PVS3 vitrification was successfully applied. Table 3.2 lists encapsulation–vitrification successes.

Table 3.1 Examples of plant species cryopreserved using vitrification techniques. Unless specified otherwise the developed protocols are for shoot tips

Plant species	Treatments	Recovery	Reference
Allium porrum	CA, PC, LD, PVS2, RF	80%	Niino et al. 2003
Allium sativum	PC, PVS2, RF	100%	Niwata 1995
Allium sativum	PC, LD, PVS3, RF	0–100%	Makowska et al. 1999
Allium sativum	PC, PVS3, RF	72–95%	Kim et al. 2004
Allium sativum	PC, LD, PVS2, RF	0–75%	Volk et al. 2004
Allium wakegi	CA, PC, PVS2, RF	80%	Kohmura et al. 1994
Ananas comosus	PC, LD, PVS2, RF	25–65%	Gonzalez-Arnao et al. 1998
Ananas comosus	PC, LD, PVS2, RF	35.7%	Thinh et al. 2000
Arachis spp.	PC, LD, PVS2, RF	40–75%	Gagliardi et al. 2003
Armoracia rusticana	PC, PVS2, RF	69%	Phunchindawan et al. 1997
Artocarpus heterophyllus (EA)	PC, LD, PVS2, RF	50%	Thammasiri 1999
Asparagus	PVS2, RF	90%	Kohmura et al. 1992
Asparagus officinalis (EC)	LD, PVS3, RF	80–90%	Nishizawa et al. 1993
Atropa belladonna(R)	PC, PVS2, RF	50%	Kamiya et al. 1995
Beta vulgaris	PC, LD, PVS2, RF	60–100%	Vandenbussche et al. 2000.
Bletilla striata (ZE)	PC, LD, PVS2, RF	60%	Ishikawa et al. 1997
Brassica campestris (CS)	LD, EG+sor+BSA, RF	40%	Langis et al. 1989

Camellia sinensis	CA, PC, PVS2, RF	60%	Kuranuki and Sakai 1995
Castanea sativa (SE)	PC, PVS2, RF	68%	Corredoira et al. 2004
Citrus madurensis (EA)	PC, LD, PVS2, RF	85%	Cho et al. 2002
Citrus sinensis (NC)	PVS2, RF	70–75%	Sakai et al. 1990
Citrus 3 species (NC)	PVS2, RF	90–97%	Sakai et al. 1991b
Citrus spp.	PC, LD, PVS2, RF	70.5–95.6%	Wang and Deng 2004
Chrysanthemum	CA, EG+sor+BSA, RF	84%	Schnabel-Preikstas et al. 1992
Colocasia esculenta	PC, LD, PVS2, RF	80%	Takagi et al. 1997
Cymbidium spp.	PC, LD, PVS2, RF	93.3%	Thinh and Takagi 2000
Cymbopogon	PC, LD, PVS2, RF	56.6%	Thinh et al. 2000
Daucus carota (CS)	PC, LD, PVS2, RF	83.3%	Chen and Wang 2003
Daucus carota (PP)	PC, LD, PVS2, RF	47%	Chen and Wang 2003
Dendranthema	PC, PVS2, RF	31.8%	Fukai 1992
Dianthus caryophyllus	CA, LD, EG+sor+BSA, RF	100%	Langis et al. 1990
Dianthus caryophyllus	PC, PVS2, RF	50.9%	Fukai 1992
Dioscorea spp.	CA, PC, LD, PVS2, RF	0–50%	Leunufna and Keller 2005
Dioscorea rotundata	PC, LD, PVS2, RF	63%	Takagi et al. 1998
Diospyros kaki	PC, LD, PVS2, RF	89%	Matsumoto et al. 2001
Doriteanopsis (CS)	PC, LD, PVS2, RF	64%	Tsukazaki et al. 2000
Doritis pulcherrima (S)	PVS2, RF	62%	Thammasiri 2000
Fragaria x ananassa	CA, PC, LD, PVS2, RF	93%	Niino et al. 2003
Gentiana spp.	CA, PC, LD, PVS2, RF	16.7–76.7%	Tanaka et al. 2004
Grevillaria scapigera	PC, PVS2, RF	65%	Touchell and Dixon 1996
Hyosciamus niger (R)	PC, LD, PVS2, RF	93.3%	Jung et al. 2001
Ipomoea batatas	LD, PVS2, RF	0–64%	Towill and Jarret 1992
Ipomoea batatas	PC, LD, PVS2, RF	93%	Pennycooke and Towill 2001
Lilium japonicum	PC, LD, PVS2, RF	80%	Matsumoto et al. 1995b
Limonium	PC, PVS2, RF	75%	Matsumoto et al. 1998
Macropidia fuliginosa (SE)	PC, PVS2, RF	90.6%	Turner et al. 2000
Malus spp.	CA, PC, PVS3, RF	27–94%	Wu et al. 1999
Malus spp.	PVS3, RF	60%	Wu et al. 2001
Manihot esculenta	PC, LD, PVS2, RF	75%	Charoensub et al. 1999
Mentha aquatica x M. spicata	PC, LD, EG+DMSO+PEG, RF	31–75%	Towill 1990
Mentha spp.	PC, LD, PVS2, RF	8–89%	Towill and Bonnart 2003
Morus bombycis	CA, PC, PVS2, RF	65%	Niino et al. 1992b
Musa spp.	PC, LD, PVS2, RF	70%	Takagi et al. 1998
Musa spp.	PC, LD, PVS2, RF	69%	Thinh et al. 1999
Musa spp.	PC, LD, PVS2, RF	0–85%	Agrawal et al. 2004
Nicotiana tabaccum (CS)	PC, PVS2, RF	55%	Reinhoud 1996
Oryza sativa (EC)	PC, LD, PVS2, RF	45%	Huang et al. 1995
Oryza sativa (CS)	LD, DMSO+sorbitol, RF	1%	Watanabe and Steponkus 1995
Panax ginseng (R)	PC, PVS2, RF	69%	Yoshimatsu et al. 1996
Picrorhiza kurroa	CA, PC, PVS2, RF	35%	Sharma and Sharma 2003
Populus alba	CA, PC, LD, PVS2, RF	60%	Lambardi et al. 2000
Prunus domestica	PC, LD, PVS2, RF	57%	De Carlo et al. 2000
Prunus dulcis	CA, PC, LD, PVS2, RF	10%	Shatnawi et al. 1999
Prunus spp.	CA, PC, PVS2, RF	80%	Niino et al. 1997
Pyrus spp.	CA, PC, PVS2, RF	80%	Niino et al. 1992a
Quercus robur (EC)	PC, PVS2, RF	70%	Martinez et al. 2003
Quercus suber (SE)	PC, PVS2, RF	88–93%	Valladares et al. 2004
Ribes	CA, DMSO, PVS2	24–57%	Reed 1992; Luo and Reed 1997

Secale cereale (PP)	CA, LD, EG+sorb+BSA, RF	38%	Langis and Steponkus 1991
Solanum spp.	PC, EG+sor+BSA, RF	30–60%	Golmirzaie and Panta 2000
Solemostemon rotundifolius	PC, LD, PVS2, RF	85%	Niino et al. 2000a
Trifolium repens	PC, PVS2, RF	80%	Yamada et al. 1991
Vitis vinifera	PC, LD, 50/100% PVS2, RF	80%	Matsumoto and Sakai 2003
Wasabia japonica	PC, LD, PVS2, RF	85%	Matsumoto et al. 1998
Xanthosoma spp.	PC, LD, PVS2, RF	62–67%	Thinh 1997

CA: cold acclimation; *PC*: preculture; *LD*: osmoprotection (loading); *PVS2*: treatment with PVS2; *PVS3*: treatment with PVS3; *RF*: rapid freezing; *CS*: cell suspension; *PP*: protoplasts; *EA*: embryonic axes; *SE*: somatic embryos; *ZE*: zygotic embryos; *S*: seeds; *NC*: nucellar cells; *R*: roots; *EC*: embryogenic cultures; *EG*: ethylene glycol; *sor*: sorbitol; *BSA*: bovine serum albumin; *DMSO*: dimethylsulfoxide; *suc*: sucrose; *PEG*: polyethylene glycol.

Table 3.2 Examples of plant species cryopreserved using encapsulation–vitrification techniques. Unless specified otherwise, the developed protocols are for shoot tips

Plant species	Treatments	Recovery	Reference
Ananas comosus	PC, LD, PVS3, RF	54–83%	Gamez-Pastrana et al. 2004
Fragaria x ananassa Duch	CA, LS, PVS2, RF	70.3–90.0%	Hirai et al. 1998
Gentiana spp.	CA, PC, LS, PVS2, RF	43.3–93.3%	Tanaka et al. 2004
Ipomoea batatas	PC, LD, PVS2, RF	82–95%	Hirai and Sakai 2003
Malus domestica	sucr+EG, RF	64–77%	Paul et al. 2000
Manihot esculenta	PC, PVS2, RF	57–86%	Charoensub et al. 2004
Mentha spicata	LD, PVS2, RF	73–97%	Hirai and Sakai 1999
Olea europaea (SE)	LD, PVS2, RF	64%	Shibli and Al-Juboory 2000
Poncirus trifoliata x Citrus sinensis	LD, PVS2, RF	100%	Wang et al. 2002
Prunus domestica	PC, PVS2, RF	47.5%	De Carlo et al. 2000
Solanum tuberosum	PC, LD, PVS2, RF	41–71%	Hirai and Sakai 1999
Wasabia japonica	LD, PVS2, RF	95%	Matsumoto et al. 1995a

CA: cold acclimation; *PC*: preculture; *LD*: osmoprotection (loading); *PVS2*: treatment with PVS2; *PVS3*: treatment with PVS3; *RF*: rapid freezing; *CS*: cell suspension; *PP*: protoplasts; *EA*: embryonic axes; *SE*: somatic embryos; *ZE*: zygotic embryos; *S*: seeds; *NC*: nucellar cells; *R*: roots; *EC*: embryogenic cultures; *EG*: ethylene glycol; *sor*: sorbitol; *BSA*: bovine serum albumin; *DMSO*: dimethylsulfoxide; *suc*: sucrose; *PEG*: polyethylene glycol.

3.4 Preconditioning

In vitrification methods cells and meristems must be sufficiently dehydrated with PVS2 at 25°C or 0°C without causing injury to enable vitrification upon rapid cooling in LN. Many papers demonstrated that cells and meristems conditioned to withstand the dehydration of PVS2 (the treated

control without cooling to –196°C) survived subsequent rapid cooling and rewarming during the vitrification procedure with little or no additional loss (Yamada et al. 1991; Niino et al. 1992a, b; Matsumoto et al. 1994; Reinhoud 1996). It is still unclear if the constituents of PVS2 penetrate cells during the dehydration process. Steponkus et al. (1992) stated that neither sucrose nor glycerol could penetrate the cytoplasmic domain with such a short duration of loading treatment. Kim (2004) indicated that DMSO might penetrate the cell wall and plasmalemma: it was unclear if DMSO penetrated the cytoplasm during treatment with PVS2.

In any vitrification method, procedures such as preconditioning, preculture, osmoprotection (loading treatment), PVS2 exposure time, and post-LN handling are vital. The cells and tissues to be cryopreserved must be in a physiologically optimal status for the acquisition of dehydration tolerance and to produce vigorous recovery of growth (Withers 1979; Dereuddre et al. 1988). Thinh (1997) demonstrated the importance of the structure of shoot tips used as explants for the cryopreservation of tropical monocot species. Shoot tips with the apical dome partially covered (PC type) are a key for attaining high post-LN regrowth (Fig. 3.2). In addition, the growth stage of shoot tips grown *in vitro* was a key factor in obtaining more regrowth after cryopreservation.

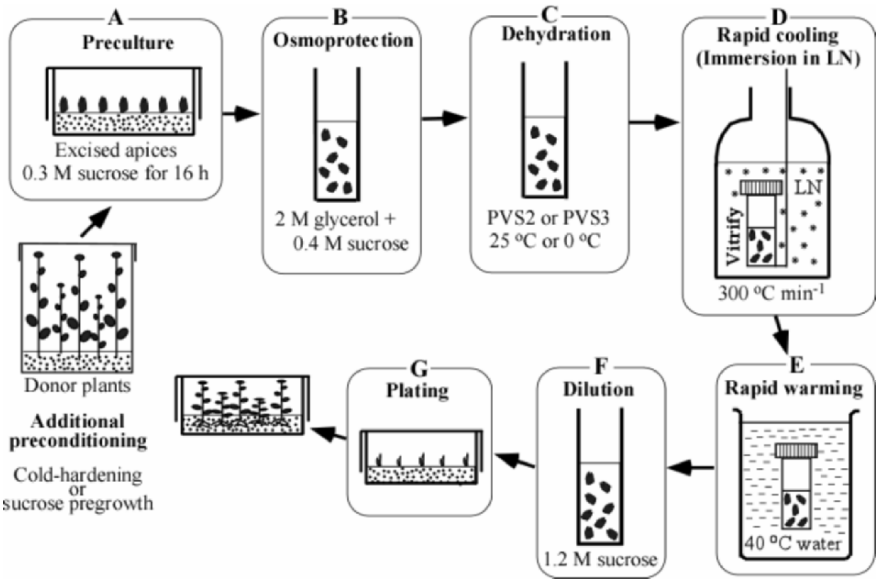

Fig. 3.1 Vitrification procedure for *in-vitro* grown meristems

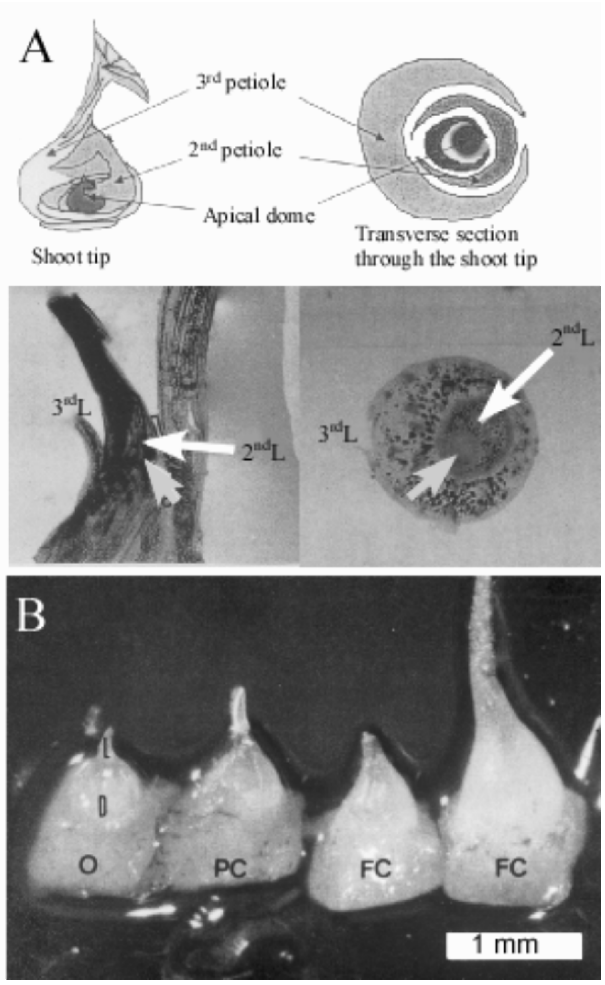

Fig. 3.2 A: Longitudinal (left) and transverse (right) hand-cut sections (lower) and diagrams (upper) across the apical dome of taro. (Thinn 1997)
B: Banana shoot tips. O: Meristems with apical domes uncovered; PC: partly covered; and FC: fully covered. PC-type shoot tips showed the highest rate of dehydration tolerance to PVS2 and recovery of growth after rapid cooling in LN. (Thinh 1997)

3.5 Preculture

3.5.1 Herbaceous Plants

Preculturing meristems excised from plantlets grown *in vitro* on solid medium with 0.3–0.7 M sucrose for 1–2 days was very effective in improving the regrowth of cryopreserved meristems (Dereuddre et al. 1988; Niino et al. 1992a). During preculture the sucrose concentrations in the plants increase significantly (Uragami et al. 1990; Dereuddre et al. 1991). The accumulation of endogenous cryoprotectants such as sugar and sugar alcohol may increase the stability of membranes under conditions of severe dehydration (Crowe et al. 1984a, b). Reinhoud (1996) cryopreserved cultured tobacco cells by vitrification, and clearly demonstrated that the development of tolerance to PVS2 in tobacco cells during preculture with 0.3 M mannitol solution for 1 day was because of the response of cells to mild osmotic stress caused by the preculture. During preculture, there is production of abscisic acid, proline, and certain proteins (late embryogenesis abundant proteins), and the uptake of mannitol. Preculture of innala buds in 0.3 M sucrose for 2 days at 25°C produced the greatest regrowth of shoots (Table 3.3) (Niino et al. 2000a).

Table 3.3 Effect of the duration of preculturing on the survival of lateral buds of innala nodal segments following PVS2 vitrification (Niino et al. 2000a, revised). Nodal segments were precultured with 0.3 M sucrose for various periods of time at 25°C. These precultured segments were osmoprotected with a mixture of 2 M glycerol and 0.4 M sucrose for 20 min at 25°C, and then dehydrated with PVS2 for 18 min at 25°C before being plunged into LN. S.E.: standard error

Preculture duration	Survival (% ± S.E.)
No preculture	0.0 ± 0.0
0.3 M sucrose 1 day	33.3 ± 5.4
0.3 M sucrose 2 days	78.3 ± 2.0
0.3 M sucrose 3 days	40.0 ± 3.5
0.3 M sucrose 4 days	8.3 ± 5.4

3.5.2 Woody Plants

Vitrification was successfully applied to cold-acclimated and precultured apical meristems of woody plants grown *in vitro* (Tables 3.1; 3.2). Cold acclimating at 5°C for 3 weeks under an 8 h photoperiod significantly

improved the recovery of shoot tips from apple, pear, and mulberry cooled to –196°C by vitrification (Niino et al. 1992a, b). Preculture of apple shoot tips on 0.7 M sucrose at 5°C for 1 day after an 8 days cold acclimation was effective at producing high levels of shoot formation (Table 3.4). Five apple species or cultivars, eight pear cultivars (Niino et al. 1992a), *Ribes* (1 week cold acclimation and subsequent 2 days preculture with 5% DMSO; Reed 1992; Luo and Reed 1997), tea plants (Kuranuki and Sakai 1995), five cultivars of cherry (Niino et al. 1997), and 11 species or cultivars of mulberry including southeast Asian cultivars were cryopreserved with PVS2 (Niino et al. 1992b). Thus, in these temperate woody plants, preculturing following cold acclimation appears to be a necessary step in producing a high level of shoot formation.

Table 3.4 Effect of cold acclimation and preculturing on the shoot formation of vitrified apple shoot tips (Niino et al. 1992a, revised). Cold acclimation: 3 weeks at 5°C (8 h day photoperiod). Preculture: on MS medium with 0.7 M sucrose at 5°C. Shoot tips (2 mm in length) were treated with PVS2 for 80 min at 25°C. Shoot formation: percentage of shoot tips producing shoots 40 d after plating. Material: *Malus domestica* cv. Fuji. S.E.: standard error

Preculture (days)	Shoot formation (% ± S.E.)	
	Non hardened	Hardened
0	10 ± 5	62.5 ± 7.5
1	25 ± 5	80.0 ± 0.0
2	10 ± 5	57.5 ± 2.5

Cryogenic protocols for shoot tips were investigated and optimized by Touchell and Dixon (1996) using rare and endangered Australian woody plants such as *Grevillea scapigera*. Meristems were precultured with 0.6 M sorbitol for 2 days at 23°C, dehydrated with PVS2 for 30 min at 0°C, and then plunged into LN. This successful vitrification protocol developed for *G. scapigera* was tested on another 18 rare and endangered Australian species. The shoot tips of 12 species were successfully cryopreserved, with 6 species showing a high capacity to regenerate shoots.

3.5.3 Tropical Plants

In some tropical plants, the preconditioning of meristem-donor shoots on solidified medium enriched with sucrose was very important in producing a high regrowth after cryopreservation (Engelmann 1991; Dumet et al. 1993a, b; Panis 1995). Thinh (1997) demonstrated that taro meristems cryopreserved by vitrification using PVS2 produced nearly 100% recovery of shoot growth when the meristem donor shoots were cultured on MS medium with 60–120 g l^{-1} sucrose for one month, and osmoprotected before dehydration with PVS2.

The same preconditioning was successfully applied to the meristems of banana (two genotypes: Cavendish AAA and Vietnamese Chuoi Huong AAB). The regrowth of vitrified banana meristems cooled to –196°C was 60–80%. Thus pregrowth is an additional promising step to be included in the vitrification procedure of some tropical plants. This vitrification procedure, with slight modification, was successfully applied by Thinh (1997) to about 20 tropical monocotyledonous plants including taro (8 cv.), banana (10 cv.), pineapple (2 cv.), and orchid (2 cv.). It is very interesting to note that the same vitrification procedure, with slight modification, is applicable to a wide range of tropical plants from several genera and families.

3.6 Osmoprotection (Loading Treatment)

For many herbaceous species, preculture with only 0.3 M sucrose for 1–5 days does not produce high recovery following vitrification. A solution of 2 M glycerol and 0.4 M sucrose in growth medium is very effective at inducing tolerance to freeze dehydration or cryoprotectant induced dehydration (Sakai et al. 1991a; Nishizawa et al. 1992). This osmoprotective solution and 50% PVS2 produced the greatest shoot formation in vitrified meristems of wasabi (Table 3.5) (Matsumoto et al. 1994). Similar results were seen with mint axillary shoot-tip meristems cooled to –196°C by encapsulation vitrification. This treatment was successfully applied to other meristems such as lily (Matsumoto et al. 1995b), statice (Matsumoto et al. 1998), hairy roots (Yoshimatsu et al. 1996), taro (Takagi et al. 1997), and innala (Niino et al. 2000a). Pretreatment was very effective in tropical monocotyledonous species (meristems) such as taro, tannia, banana, pineapple, and orchid (Thinh 1997). Several osmoprotectants were effective for *Ribes* shoot tips including sucrose and bovine serum albumen (Luo and Reed 1997).

Table 3.5 Effect of the type of osmoprotective solution used on the shoot formation of PVS2 vitrified wasabi meristems. (Matsumoto et al. 1994, revised). Meristems were precultured with 0.3 M sucrose for 16 h and treated with different solutions for 20 min at 25°C before dehydration with PVS2 for 10 min at 25°C. They were then plunged into LN. S.E.: standard error

Osmoprotection solution	Shoot formation (% ± S.E.)
2 M glycerol	46.7 ± 8.8
0.4 M sucrose	20.0 ± 0.0
2 M glycerol + 0.4 M sucrose	86.3 ± 3.2
40% PVS2	50.0 ± 11.5
50% PVS2	80.0 ± 5.8
60% PVS2	33.3 ± 14.5

3.7 Exposure Time to PVS2

The one of the keys to successful cryopreservation by vitrification is the careful control of dehydration and prevention of injury by chemical toxicity or excess osmotic stresses during treatment with PVS2. Optimizing the time of exposure, or the temperature during exposure to PVS2, is important for producing a high level of shoot formation after vitrification. To determine the optimal time of exposure to PVS2, precultured wasabi meristems (1 mm) were treated with PVS2 at 25°C or 0°C for different periods of time before being plunged into LN. Exposure to PVS2 produced a time-dependent shoot formation rate (Fig. 3.3). With exposure at 25°C, shoot formation increased greatly with increasing dehydration and reached the highest (about 95%) at 10 min. There was a slight decrease at 20 min, followed by a rapid decrease due to toxic effects. In meristems treated at 0°C, the treated controls (without cooling in LN) retained very high shoot formation up to 60 min. In vitrified meristems shoot formation was best during 30–50 min. These results demonstrate that vitrification does not cause additional loss of shoot formation beyond that produced during dehydration by PVS2 (Fig. 3.3). Thus tolerance to PVS2 is sufficient for meristems to survive vitrification under optimized conditions. In the meristems of taro and banana the injurious effects caused by PVS2 were eliminated or reduced by treatment with PVS2 at 0°C (Thinh 1997). In many vitrified cells and meristems, PVS2 treatment at 0°C was required to reduce injurious effects. Such treatment produced high recovery, even in tropical materials (Nishizawa et al. 1993; Kuranuki and Sakai 1995; Yoshimatsu et al. 1996; Thinh 1997). In many herbaceous plants the optimum exposure time to PVS2 is 10–25 min at 25°C (Matsumoto et al. 1994; Takagi et al. 1997). In excised wasabi meristems,

the optimal exposure time to PVS2 was 10 min at 25°C or 30–60 min at 0°C (Fig. 3.3). In apple shoot tips (2 mm long, 1.5 mm base diameter) consisting of the apical meristem and four or five leaf primordia, the optimum exposure time to PVS2 was 80–90 min at 25°C (Niino et al. 1992a) (Fig. 3.4). In tea (*Camellia sinensis*) shoot tips (2 mm long) excised from cold-acclimated plantlets, similar results were obtained (Kuranuki and Sakai 1995). Exposure time to PVS2 may be associated with the size of excised meristems (Niwata 1995; Niino et al. 2000a, 2003a). It also appears to be considerably species specific at the same temperature (Niino et al. 1992a, 1997; Matsumoto et al. 1994). PVS2 treatment to meristems smaller than 0.5 mm often results in toxic effects and meristems larger than 3 mm require a much longer time for dehydration.

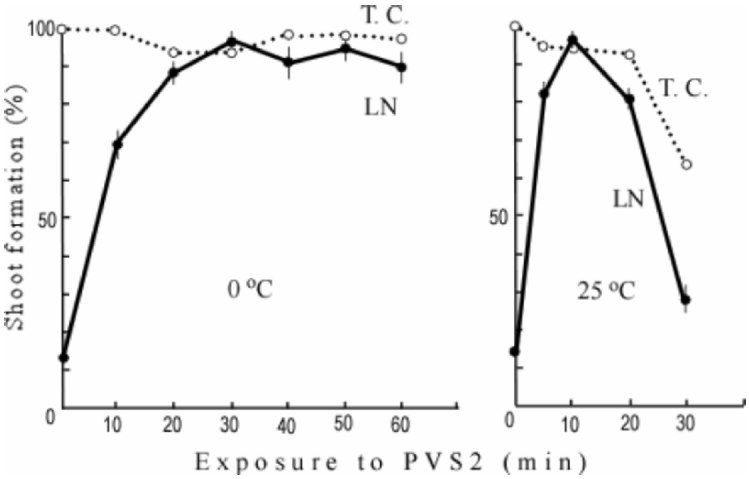

Fig. 3.3 Effect of exposure time to PVS2 at 0°C or 25°C on the shoot formation of wasabi 'Shimane No.3' meristems cryopreserved by vitrification. Meristems (1 mm size) were precultured with 0.3 M sucrose and then osmoprotected with a mixture of 2 M glycerol plus 0.4 M sucrose for 25 min at 20°C. These meristems were treated with PVS2 at 25°C or 0°C for different lengths of time before being plunged into LN. TC: treated control, same as treated with PVS2 without cooling to –196°C (Matsumoto et al. 1994, revised). Bars represent standard error

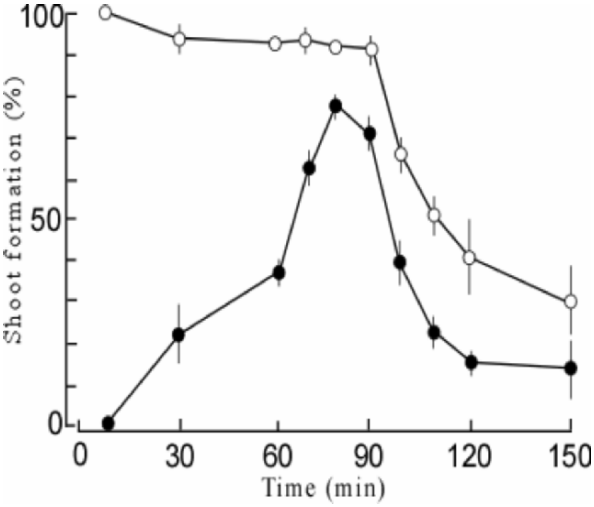

Fig. 3.4 Effect of PVS2 exposure at 25°C on the shoot formation of apple (*Malus domestica* cv. Fuji) shoot tips cryopreserved by vitrification. Cold acclimated (for 3 weeks at 5°C) shoot tips precultured with 0.7 M sucrose at 5°C for 1 day were treated with PVS2 for different lengths of time at 25°C before being plunged into LN (Niino et al. 1992a, revised). o control, ● vitrified (LN). Bars represent standard error

3.8 Regrowth

It is particularly important that cryopreserved meristems produce plants identical to their untreated phenotypes. A callus phase before shoot formation is undesirable because callusing potentially increases the frequency of genetic variants. Successfully vitrified and warmed meristems of many plants tested with the PVS-based vitrification protocol remained green after plating, resumed growth within a few days, and developed shoots directly without intermediary callus formation (Yamada et al. 1991; Niino et al. 1992a, b; Matsumoto et al. 1994, 1995b). Fluorescence microscopic examinations of longitudinal sections through the meristematic dome of vitrified meristems after 3 d of reculture revealed that the domes in most meristems appeared to be viable. This was based on fluorescein diacetate staining (Yamada et al. 1991; Matsumoto et al. 1994). In addition, little or no morphological abnormalities were observed in plants that developed from vitrified apical meristems (Yamada et al. 1991; Niino et al. 1992a, b; Matsumoto et al. 1994, 1995a, b). Touchell and Dixon (1996) reported that vitrification of the shoot tips of *G. scapigera* produced high-quality regrowth (70–80%). The same results were obtained with *Dianthus* and *Chrysanthemum* (Fukai 1992).

3.9 Encapsulation Vitrification

It order to carefully treat a large number of meristems at the same time with vitrification protocols, we developed an encapsulation-vitrification method (Matsumoto et al. 1995a). In this procedure, meristems precultured on 0.3 M sucrose were trapped within alginate beads (diameter: 3–4 mm) containing 2–3% (w/v) sodium alginate, 2 M glycerol, and 0.4 M sucrose in culture medium without calcium (Fabre and Dereuddre 1990). Encapsulated meristems were placed in a solution of 2 M glycerol and 0.4 M sucrose in liquid medium for 30 min. They were dehydrated with PVS2 in a 100 ml glass beaker on a rotary shaker at 100 rpm for 70–100 min at 0°C. About 10 encapsulated-dehydrated meristems were suspended in 0.7 ml PVS2 in 2 ml cryotubes and the cryotubes were then plunged into LN. For warming, the cryotubes were transferred to a 40°C water bath for 1 min. PVS2 was drained from the tubes and 2 ml of 1.2 M sucrose solution was added and replaced once with fresh solution. Washed encapsulated–vitrified meristems were transferred onto solidified 1/2 MS medium (Murashige and Skoog 1962) for regrowth (Table 3.6). The encapsulation–vitrification method is easy to manage and eliminates the time required for air desiccation. This method was successfully applied to the meristems of statice (Matsumoto et al. 1998), lily (Matsumoto et al. 1995b), strawberry (Hirai et al. 1998), mint (Hirai and Sakai 1999a), potato (Hirai and Sakai 1999b), gentian (Tanaka et al. 2004), and horseradish hairy roots (Phunchindawan et al. 1997). Thus, the encapsulation–vitrification procedure shows promise as a practical cryopreservation method.

Table 3.6 Shoot formation of wasabi meristems cooled to –196°C by three cryogenic protocols. (Matsumoto et al. 1995a, revised)

Cryogenic protocol	Shoot formation	Shoot length
	(% ± S.E.)	(mm size ± S.E.)
Vitrification[1]	97.5 ± 1.0	10.6 ± 4.0
Encapsulation–vitrification[2]	96.7 ± 2.9	12.2 ± 3.6
Encapsulation–dehydration[3]	67.1 ± 8.9	6.3 ± 3.6

Preculture: 0.3 M sucrose for 1 d; [1, 2]: Osmoprotected with 2 M glycerol plus 0.4 M sucrose for 20 min at 25°C; PVS2 treatment for 50 and 100 min at 0°C; [3]: treated with 0.8 M sucrose for 16 h before air-drying for about 7 h. Shoot length: 30 d after reculture. S.E.: standard error

3.10 A Personal View of the Development of PVS2 by Akira Sakai

Until about the middle of 1980, the controlled rate cooling method (Sakai 1960; Sakai and Nishiyama 1978) was used for storing cultured cells and apical meristems in liquid nitrogen. The materials were first cooled to –30 to –40°C for sufficient freeze-dehydration before cooling in liquid nitrogen. A programmable freezer is needed in this method because the material needs to be cooled at a constant rate for freeze dehydration. The method could not be used for materials that were not frost hardy. There was thus an urgent need to develop a relatively simple and efficient method that could replace the conventional method, especially for storing the genetic resources of tropical plants.

3.10.1 The Ultimate Goal—Storing Tropical Plants

The practical problem that I set myself to solve was the development of a technique in which 1 mm long apical meristems removed from a cultured plant were dehydrated osmotically for several minutes at room temperature in a concentrated vitrifying solution containing glycerol and sugar, cooled rapidly from room temperature by plunging into liquid nitrogen for vitrification, while they were still alive, and regrown into plants when required. In 1988, as a retired scientist I had no possibility of obtaining government aid for scientific research so I decided to work on this project using my own financial resources. It was the spring of my 68th year (5 years after retirement from Hokkaido Univ.). I had neither a laboratory nor co-workers. The first thing I did after returning was to purchase a PC and software so that I could prepare documents in Japanese and English. I was handling a PC for the first time. At home I read and understood the unfamiliar manual little-by-little and worked on the keyboard with two fingers. I did not want to become a "*Technobsolete*" a name given in Japan to a technologically obsolete person. This effort enabled me to prepare papers in English at home and exchange information with scientists within and outside the country through e-mail.

3.10.2 The Core of the Technology Development

To keep the about 1 mm long apical meristems excised from cultured plants alive at –196°C, they must be vitrified by cooling in liquid nitrogen after dehydration in a concentrated vitrification solution. Two problems

needed to be solved before this could be accomplished. The first was to develop an osmotic dehydrating solution (vitrification solution) that caused minimum chemical damage to the tissue. The other challenge was to provide dehydration resistance to plants that were cultured under optimum growth conditions at 25°C and therefore did not have such resistance.

Thus, firstly I had to find an effective vitrification solution. I sought the assistance of Dr. S. Kobayashi, an old acquaintance of mine working at in Akitsu a substation of the National Fruit Tree Research Station. I went to this substation and made combinations of several solutes in different proportions to prepare several vitrification solutions. I immersed nucellar embryo cells of orange in these solutions for several minutes for dehydration and plunged them into liquid nitrogen for rapid cooling. The suitability of these solutions was assessed from the survival of the cells. (Dr. Kobayashi is a specialist of nucellar cells, of *Citrus*). After cryopreservation of nucellar cells, Dr. Kobayashi determined the viability under the fluorescence microscope. I traveled from Sapporo where I lived, to Akitsu (by air and train) five times in a year, staying in a hotel each time for about 5 days and conducted these experiments. Initially, the experiments were one failure after another, but on the last day of my 5th visit to Akitsu at the end of October, 1988, I could finally get 90% survival. I named the vitrification solution that gave this result Plant Vitrification Solution 2 (PVS2). This was a glycerol-based concentrated (approximately 8 molar) solution.

Luckily the nucellar embryo cells of orange had a high level of dehydration resistance. When the cells were immersed in PVS2 for 2 min for dehydration and then cooled rapidly in liquid nitrogen, both the cells and the PVS2 vitrified. When this was rapidly thawed in warm water, about 90% of the cells were viable and regenerated by Kobayashi. However, many other cultured cells and apical meristems which did not have dehydration resistance could not survive this procedure. The next challenge was to impart dehydration resistance to excised apical meristems. First I tried culturing such meristems for 16 h in an agar medium containing 0.3M sucrose so that a large amount of sugar was taken up by the cells of the meristem. But adding sugar to the culture medium alone was not sufficient to make these cultured meristematic cells withstand the strong osmotic dehydration in the highly concentrated PVS2. I then treated the meristems cultured in the sugar-containing medium, in an osmoprotective solution (LS) with 2 M glycerol and 0.4 M sucrose per liter for 20 min. The dehydration resistance of the apical meristems could be sufficiently increased by this treatment in LS and they could be kept viable at –196°C for the first time. I first tried cryopreservation of apical meristem cultures of white clover by PVS2 protocol. Firstly, meristems (about 1 mm long) were cryopreserved by

PVS2 protocol. About 90% of the meristems were viable and grew normally (90% mean recovery).

3.10.3 Success with Vitrification of Tropical Plants

Unlike those of temperate plants, the cultured apical meristems of tropical plants were considered difficult to store at –196°C. There were only a few cases of success with tropical plants. At the end of March 1995, at the request of Dr. Takagi of the Ishigakijima branch of the Japan International Research Center for Agricultural Sciences (JIRCAS), I delivered a lecture and gave a practical demonstration on vitrification. At that time, one Mr. N.T. Thinh from Vietnam was on a research assignment at the Center as was Mr. Hirai from Hokkaido. About 1 month after that I received a communication from Mr. Thinh that meristems of *Colocasia esculenta* L. taro (an aroid) cooled to –196°C by vitrification could be regrown after warming with good success. Thereafter Mr. Thinh succeeded in liquid nitrogen storage of the meristems of about 30 tropical monocotyledonous crops one after the other, including banana (eight varieties), pineapple, and orchids, using the vitrification method developed by us. With his success, liquid nitrogen storage became possible with tropical plants using the vitrification process. Thus the apical meristems of cultured plants, whether temperate or tropical, could be vitrified by more or less the same method and stored for a long time in liquid nitrogen. In short, it became clear that the dividing tissue in the apical meristem of both tropical and temperate plants could exhibit a high level of dehydration resistance if treated suitably.

3.10.4 Fifteen years of Research

These studies that I started in 1988 without a laboratory or research grant luckily interested about 15 bright young researchers working in agricultural research stations in different regions in Japan and continued for about 15 long years. As a result of these studies more than 200 species and varieties could be successfully stored in liquid nitrogen by vitrification. I believe that these positive results could be achieved because I took the plunge into this line of research and persisted in my efforts. I had the good fortune of having many brilliant research collaborators both in Japan and abroad. I could exchange information with my collaborators in different parts of the world easily through fax and e-mail while sitting at home. These advances in information technology acted like a wind on our back and I felt the force of the changing times. Forty-six years have passed after

I first succeeded, in 1956, in cryopreserving willow shoots in liquid nitrogen, which had opened the possibility of long-term storage of plants.

My wish, as I approach the twilight of my life, is to have a few more years of active life and to contribute to the training of researchers from the tropical regions, including Southeast Asia, and also to engage in international collaborative work in order to contribute to putting international collaborative projects on the storage and development of tropical plant germplasm on proper tracks.

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