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Technical Note

Cryopreservation of tobacco BY-2 suspension cell cultures by vitrification with encapsulation

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Abstract Tobacco BY-2 suspension cells were successfully cryopreserved by a vitrification method combined with an encapsulation technique. Cell cultures cryopreserved using the optimal conditions established in this study could be thawed and grown enough to subculture in fresh medium within 14 days. However, the vitrification method was less effective for cryopreservation of BY-2 than a simplified slow prefreezing method.

Key words: Cryopreservation, encapsulation, slow prefreezing method, tobacco BY-2, vitrification method.

The tobacco BY-2 suspension cell line is an important genetic resource used extensively in cytological and molecular studies (Nagata et al. 1992). However, the maintenance of BY-2 cells by weekly subculture is laborious and entails the risk of losing the line because of contamination, somaclonal variation, or technical error. Cryopreservation in liquid nitrogen (LN) provides a reliable method for long-term storage of BY-2 cells and helps to secure against loss of the cell line (Engelmann 2000; Sakai 2002). We recently reported successful cryopreservation of BY-2 cells by a simplified slow prefreezing method combined with an encapsulation technique using alginate gel (Kobayashi et al. 2005).

Vitrification method is another cryopreservation procedure and has extended the applicability of cryopreservation to a broad range of plant materials from various species, including embryogenic cultures (Sakai et al. 1990; Nishizawa et al. 1993; Huang et al. 1995), somatic embryos (Fang et al. 2004), apical tips (Niino et al. 1992; Takagi et al. 1997; Takagi 2000; Hirai and Sakai 2003; Charoensub et al. 2004; Vidal et al. 2005; Wang et al. 2005), and hairy roots (Yoshimatsu et al. 1996). The vitrification method uses highly concentrated plant vitrification solution 2 (PVS2), by which cells are osmotically dehydrated at a nonfreezing temperature (Sakai et al. 1990). The dehydrated cells are completely vitrified after immersion in LN and preserved safely at the temperature of LN (−196°C). In the present study, we cryopreserved BY-2 cells by vitrification with encapsulation and compared this method with the simplified slow prefreezing method.

Suspension cultures of BY-2 cells derived from *Nicotiana tabacum* L. cv. 'Bright Yellow-2' were subcultured from stock cultures preserved by the National BioResource Project (NBRP) of Ministry of Education, Culture, Sports, Science and Technology, Japan, and were maintained in modified Linsmaier and Skoog (mLS) medium (Nagata et al. 1992). Cells were collected on day 3 after subculture and encapsulated in alginate gel beads (approximately 4 mm in diameter) at a cell density of 0.2 ml packed cells ml^{-1} as described previously (Kobayashi et al. 2005). Cryopreservation using the vitrification procedure with encapsulation was performed as described by Hirai and Sakai (1999). We omitted a preculture step, because preculture of BY-2 cells in medium containing 0.3 M sucrose, mannitol, or sorbitol for 1 day resulted in loss of cell viability (Kobayashi et al. 2005). In many reports, osmoprotection has been carried out at a room temperature (Nishizawa et al. 1993; Huang et al. 1995; Takagi et al. 1997; Takagi 2000; Hirai and Sakai 1999, 2003; Charoensub et al. 2004; Vidal et al. 2005; Wang et al. 2005), and PVS2 treatment has been at 0°C to reduce osmotic shock and toxic effect of PVS2 (Nishizawa et al. 1993; Takagi 2000; Hirai and Sakai 1999, 2003; Vidal et al. 2005; Wang et al. 2005). Beads containing BY-2 cells were soaked in osmoprotectant solution (mLS medium containing 2 M glycerol and 0.4 M sucrose) at 25°C. The

Abbreviations: LN, liquid nitrogen; mLS medium, modified Linsmaier and Skoog medium; PVS2, plant vitrification solution 2; PVS2(50), half-strength PVS2.

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osmoprotected beads were preincubated in a half-strength PVS2 [PVS2(50); mLS medium containing 15% (w/v) glycerol, 7.5% (w/v) ethylene glycol, and 7.5% (w/v) dimethylsulfoxide with 0.4 M sucrose] at 0°C and then incubated in PVS2 [mLS medium containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethylsulfoxide with 0.4 M sucrose] at 0°C. Three beads were transferred to a 2.0-ml cryovial containing 0.3 ml PVS2. Finally, the vials were plunged into LN. After more than 30 min storage, test vials were warmed in a water bath at 40°C. To dilute PVS2, the beads were incubated in 3 ml of mLS medium containing 1.2 M sucrose for 15 min at 25°C. The medium was replaced with 3 ml of mLS medium containing 0.5 M sucrose and then with 3 ml of normal mLS medium at 15 min intervals. Three beads were suspended in 3 ml of mLS medium in one well of a 12-well microplate and cultured with rotary shaking (130 rpm) at 27°C in the dark.

To determine the optimal time of exposure to PVS2, the beads containing BY-2 cells were treated with osmoprotectant solution for 60 min at 25°C, and then incubated in PVS2 for 0–80 min at 0°C before they were plunged into LN. We also examined the effect of preincubation in PVS2(50) on cryopreservation. The osmoprotected beads were incubated in PVS2(50) and PVS2 for 0–50 min at 0°C, and then immersed in LN. To determine the optimal time of osmoprotection, the encapsulated cells were treated with osmoprotectant solution for 0–60 min at 25°C, incubated in PVS2(50) for 40 min at 0°C and PVS2 for 40 min at 0°C, and then immersed in LN. In the control experiments, the beads were cultured in mLS medium without any treatment (encapsulated control), and the PVS2-treated beads were transferred to dilution medium without immersion in LN (treated control). The viability of the cells was determined by an Evans blue exclusion method 1 day after thawing (Kobayashi et al. 2005). This method is effective in measuring the viability rate of dispersed suspension cells such as BY-2.

The osmotic dehydration process with PVS2 is crucial for successful cryopreservation of plant cells (Sakai et al. 2002). Direct exposure of cells to PVS2 markedly reduced cell viability (Figure 1A). Cell viability after storage in LN was less than 20%. This severe damage to the cells may have been due to an excessively rapid change in osmotic pressure. The stepwise increase in PVS2 concentration mitigated this toxic effect (Figure 1B). Most of cells preincubated in PVS2(50) survived after dehydration with PVS2. Preincubation in PVS2(50) also yielded high cell viability after storage in LN, compared with that after direct exposure to PVS2. The highest viability of cells stored in LN was 41%, which was achieved after preincubation in PVS2(50) for 40 min and dehydration with PVS2 for 40 min.

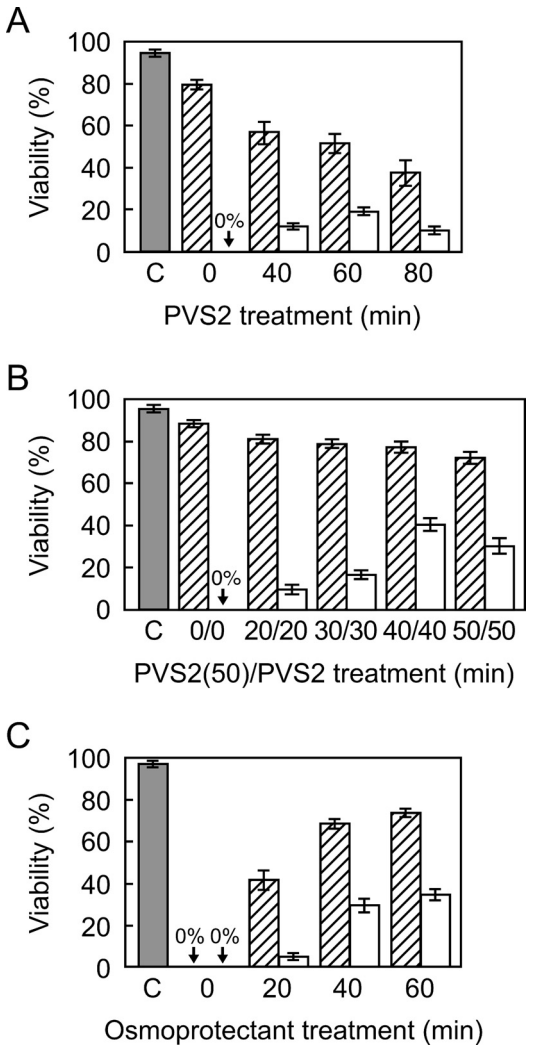

Figure 1. Determination of optimum conditions for cryopreservation of BY-2 cells. (A) Effects of duration of PVS2 treatment on the cell viability after cryopreservation. The encapsulated cells were treated with osmoprotectant solution for 60 min at 25°C, incubated in PVS2 for the indicated times at 0°C, and then immersed in LN. (B) Effects of duration of PVS2(50) and PVS2 treatments on cell viability after cryopreservation. The encapsulated cells were treated with osmoprotectant solution for 60 min at 25°C, incubated in PVS2(50) and PVS2 at 0°C for the indicated times, and then immersed in LN. (C) Effects of duration of treatment with osmoprotectant solution on the cell viability after cryopreservation. The encapsulated cells were treated with osmoprotectant solution at 25°C for the indicated times, incubated in PVS2(50) and PVS2 at 0°C for 40 min each, and then immersed in LN. Gray column, cells without treatment (encapsulated control); hatched columns, cells treated with PVS2 (treated control; not immersed in LN); open columns, cells stored in LN. Results represent the means and standard errors of 3 independent experiments.

Many reports have shown that osmoprotection with 2 M glycerol and 0.4 M sucrose is effective in enhancing the capacity of cells to tolerate severe dehydration with PVS2 (Hirai and Sakai 1999, 2003; Thinh et al. 1999; Matsumoto and Sakai 2003). BY-2 cells treated with osmoprotectant solution survived after dehydration with PVS2 and even after storage in LN (Figure 1C). The viability of cells stored in LN increased with increasing

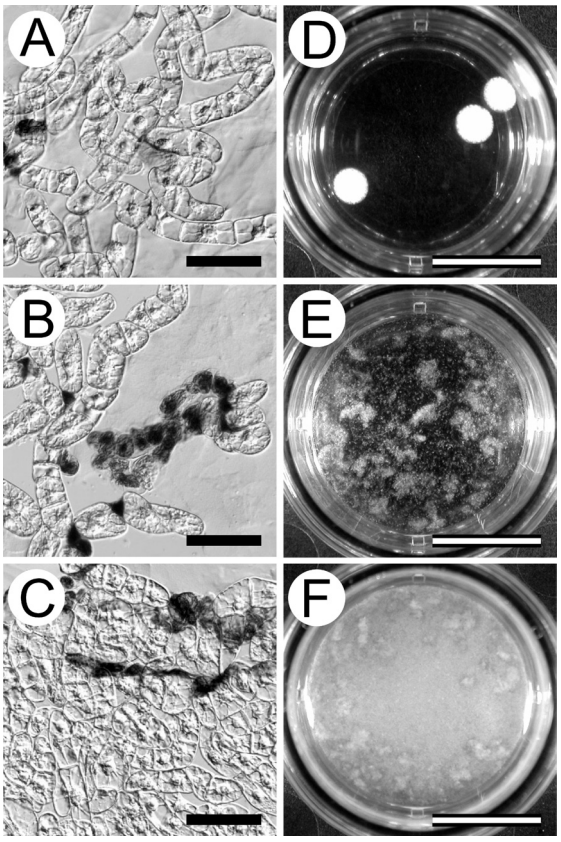

Figure 2. Regrowth of BY-2 cells after cryopreservation and re-establishment of cell suspension cultures. (A) Encapsulated cells before cryopreservation. (B) Cryopreserved cells on day 1 of culture. (C) The cryopreserved cells on day 7 of culture. The dead cells were stained black by Evans blue. Bars in A–C, 100 μm. (D) Alginate beads on day 7 of culture. (E) Beads crushed to release the embedded cells. (F) Suspension cultures after a further 7 days. Bars in D-F, 1 cm.

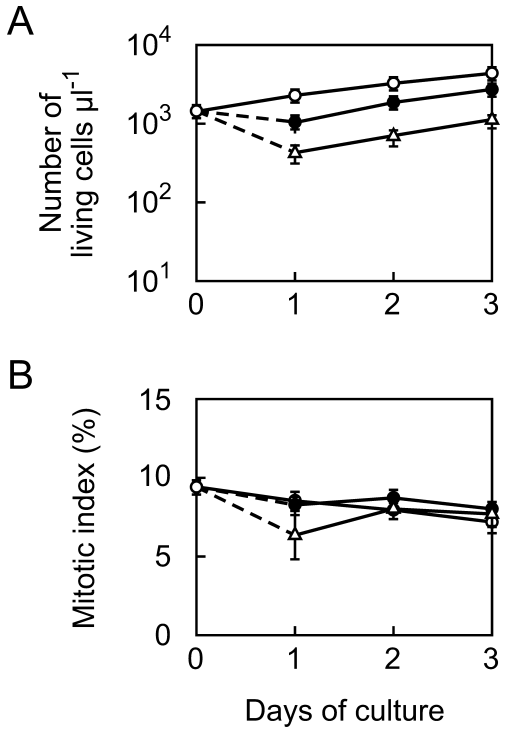

Figure 3. Recovery of proliferation potential and mitotic activity by BY-2 cells after cryopreservation with two cryostorage protocols. (A) Number of living cells. (B) Mitotic index. In the vitrification method, the encapsulated cells were treated with osmoprotectant solution for 60 min at 25°C, incubated with PVS2(50) and PVS2 for 40 min each at 0°C, and then immersed in LN. In the slow prefreezing method, the encapsulated cells were treated with osmoprotectant solution for 60 min at 25°C, frozen in a freezer at −30°C for 2 h, and then immersed in LN (Kobayashi et al. 2005). Cell number and mitotic index were determined as described previously (Kobayashi et al. 2005). Open circles, cells without treatment (encapsulated control); filled circles, cells cryopreserved by the slow prefreezing method; open triangles, cells cryopreserved by the vitrification method. Results represent the means and standard deviations of 3 independent experiments.

duration of the treatment with osmoprotectant and reached approximately 35% after treatment for 60 min. In contrast, all of the cells not treated with osmoprotectant treatment were unviable after either dehydration with PVS2 or storage in LN.

BY-2 cells were effectively cryopreserved in LN by using the optimal conditions established in this study: treatment with osmoprotectant solution for 60 min at 25°C; preincubation in PVS2(50) for 40 min at 0°C; and exposure to PVS2 for 40 min at 0°C. When we compared the results of cryopreservation experiments shown in Figures 1B, 1C, and 3, the mean value and standard error of cell viability under the optimal conditions was 36.1±2.0%. There were no statistically significant differences between experiments with one-way analysis of variance (P=0.329). After warming of the vial and dilution of PVS2, cells in alginate beads resumed proliferation (Figure 2A, B, C). The cells were then released into culture medium by gently crushing the beads on day 7 of culture (Figure 2D, E), and cell suspension cultures were grown enough to subculture in fresh medium within 14 days (Figure 2F).

We compared the proliferation potential and mitotic activity of BY-2 cells cryopreserved by the described vitrification method with those of cells cryopreserved by the slow prefreezing method. Viabilities of cells cryopreserved by the vitrification and the slow prefreezing method were 29% and 63%, respectively. The marked decrease in the number of live cells after cryopreservation retarded the regrowth of cell cultures (Figure 3A). The regrowth of cell cultures cryopreserved by the vitrification method was delayed for 2 days compared with that of cell cultures cryopreserved by the slow prefreezing method. However, the growth rate of the cells did not differ between these methods. Mitotic index was calculated as the percentage of cells with condensed chromatin after staining the cells with an acetocarmine solution (Kobayashi et al 2005). The mitotic index of cells cryopreserved by either vitrification or the slow prefreezing method was equal to that of encapsulated control cells, indicating that the cells recovered their growth capacity during day 1 of

Table 1. Cryopreservation efficiency of BY-2 cells by two methods with encapsulation.

Methods	Cell viability (%)[a]	Regrowth rate (%)[b]	Growth of cell suspensions (days)[c]
Vitrification	29.0±4.7	93.5	<14
Slow prefreezing	63.1±6.5	100.0	<7

[a] The viability of cells was determined by an Evans blue exclusion method 1 day after thawing. Results represent the means and standard errors of 3 independent experiments.

[b] Each cell suspension was grown from 3 beads (1 cryovial) in 3 ml mLS medium. The regrowth rates were calculated from 30 vials.

[c] Time required for growth of cell suspensions enough to subculture in fresh medium.

culture (Figure 3B). This result reveals that the regrowth of cell cultures primarily depends on the number of cells that survive after cryopreservation.

Table 1 summarizes a comparison between two cryopreservation methods with encapsulation. Both vitrification and simplified simple prefreezing methods required no specialized equipment and completed their procedures within a few hours. However, the slow prefreezing method was suitable for cryopreservation of BY-2 cells encapsulated in alginate beads, because the slow prefreezing method promised retention of high viability during cryopreservation, high regrowth rate after thawing, and rapid regrowth of cell suspension cultures compared with the vitrification method (Table 1). Menges and Murray (2004) demonstrated that the effective cryopreservation of suspension cell cultures including BY-2 was achieved with a slow controlled-rate cooling at approximately $-0.5°C\,min^{-1}$ using a cryofreezing container inside a styrofoam box in a laboratory freezer at −80°C. Previous studies assessing different protocols for cryopreservation of suspension cells from bromegrass (Ishikawa et al. 1996) and *Papaver somniferum* (Gazeau et al. 1998) also concluded that the slow prefreezing method was more effective than the vitrification method. Undifferentiated cell cultures that are sensitive to environmental stresses may be injured more severely by osmotic dehydration with vitrification solutions than the moderate freezing-induced dehydration during cooling to −30°C. To examine the effect of long-term cryopreservation in LN on the viability and genetic stability of cultured cells, we are now conducting cryopreservation of BY-2 cells by a simplified slow prefreezing method with encapsulation.

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