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Cryopreservation of Cultured Plant Cells

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ABSTRACT

Undifferentiated cell cultures of higher plants are important genetic resources used extensively in cytological, physiological, and molecular biological studies. The development of a reliable and cost-effective method for the maintenance of cultured cells is required to minimize the risk of losing cell lines to disease, contamination, and technical errors, as well as the risk of changes in morphological, biochemical, and physiological properties of cultured cells by somaclonal variations. Cryopreservation in liquid nitrogen at –197°C is appropriate for this purpose. Recently, several research groups have established simple cryopreservation protocols that are suitable for routine laboratory use. The use of these protocols not only helps to secure against loss of cell lines, but also enables long-term maintenance of a large number of transgenic cell lines. However, the optimization of cryostorage conditions for each cell line is necessary for efficient cryopreservation. In this review, we describe recent advances in cryogenic techniques for the preservation of undifferentiated cell cultures and discuss the optimal conditions for the cryopreservation of different cell lines.

Keywords: air dehydration, encapsulation, slow prefreezing, undifferentiated cell cultures, vitrification

CONTENTS

INTRODUCTION

Undifferentiated cell-suspension cultures are important genetic resources used extensively in cytological, physiological, biochemical, and molecular biological studies. Cell-suspension cultures are homogeneous populations of nearly identical cells and provide advantages for investigating cellular processes (Menges and Murray 2006). Their *in vitro* culture allows strict control of culture conditions and growth environments. Many cell lines have been established from a wide variety of plant species, including *Arabidopsis thaliana*, *Oryza sativa*, and *Nicotiana tabacum* (Baba *et al*. 1986; Nagata *et al*. 1992; Menges and Murray 2002). Cell-suspension cultures and associated techniques for synchronization, transformation, and live cell imaging are used as valuable tools for analysis of gene function, signal transduction, cell division cycle, cytokinesis, membrane trafficking, and the production of secondary metabolites.

The establishment of a convenient method for stable and cost-effective preservation of cell cultures is required to extend the utility of cultured cells to postgenomic research. The routine maintenance of cultured cells by weekly or biweekly subculture is cumbersome and labor intensive (Menges and Murray 2004). The number of cell lines that can be maintained depends on the capacity of facilities. Furthermore, the long-term maintenance of cell cultures by repeated subculture entails the risk of losing cell lines to disease, contamination, and technical errors, as well as the risk of changes in morphological, biochemical, and physiological properties of cultured cells by somaclonal variations. Cryopreservation of cell cultures in liquid nitrogen at –197°C is appropriate for this purpose and provides an easy and reliable method for long-term preservation of cell cultures (Engelmann 2000; Sakai 2000).

Considerable efforts have been made to develop convenient and practical methods for cryopreservation of plant genetic resources. Cryopreservation of plant cells is considered to be more difficult than that of animal cells, because plant cells, which have large amounts of cellular water, are prone to freezing injury when placed in liquid nitrogen (Engelmann 2000; Sakai 2000). Further, the cell lines re-established from cryopreserved stocks have to retain characteristics identical to those before cryopreservation (Harding 2004). The recent development of efficient cryopreservation protocols enables the storage of cultured cells in liquid nitrogen for practical routine use. The application for ornamental germplasm conservation has been recently reviewed by Wang and Perl (2006). In this review, we describe advances in cryogenic techniques for the preservation of undifferentiated cell cultures and discuss the optimal conditions for the cryopreservation of different cell lines.

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Invited Mini-Review

Table 1 Cryopreservation of undifferentiated cell cultures.

Cell culture	Method	Reference
alfalfa	air dehydration + encapsulation	Shibli *et al*. (2001)
Arabidopsis thaliana MM1	simplified slow prefreezing	Menges and Murray (2004)
A. thaliana MM2d	simplified slow prefreezing	Menges and Murray (2004)
A. thaliana T87	air dehydration + encapsulation	Bachiri *et al*. (2000)
bromegrass	slow prefreezing	Ishikawa *et al*. (1996)
Catharanthus roseus	slow prefreezing	Kartha *et al*. (1982)
C. roseus	air dehydration + encapsulation	Bachiri *et al*. (1995)
Chrysanthemum cinerariaefolium	slow prefreezing	Hitmi *et al*. (1997)
Digitalis lanata	slow prefreezing	Diettrich *et al*. (1982)
D. thapsi	simplified slow prefreezing	Moran *et al*. (1999)
Dioscorea deltoidea	slow prefreezing	Butenko *et al*. (1984)
Linum usitatissimum	slow prefreezing	Quatrano (1968)
maize	slow prefreezing	Withers and King (1979)
Panax ginseng	slow prefreezing	Butenko *et al*. (1984)
Papaver somniferum	slow prefreezing	Friesen *et al*. (1991)
P. somniferum	slow prefreezing + encapsulation	Gazeau *et al*. (1998)
Soybean SB-M	slow prefreezing	Luo and Widholm (1997)
sycamore	slow prefreezing	Sugawara and Sakai (1974)
Taxus chinensis	slow prefreezing	Kim *et al*. (2001)
tobacco LT	vitrification	Reinhoud *et al*. (1995)
tobacco BY-2	simplified slow prefreezing	Menges and Murray (2004)
tobacco BY-2	simplified slow prefreezing + encapsulation	Kobayashi *et al*. (2005)
tobacco BY-2	vitrification + encapsulation	Kobayashi *et al*. (2006a)
Vaccinium pahalae	air dehydration + encapsulation	Shibli *et al*. (1998)

RECENT ADVANCES IN CRYOPRESERVATION TECHNIQUES FOR CELL CULTURES

Table 1 lists reports of the cryopreservation of undifferentiated cell cultures. Cultured cells have traditionally been cryopreserved by a slow prefreezing method (Kartha and Engelmann 1994; Schrijnemakers and van Iren 1995; Withers and Engelmann 1997). Cells are cooled at a slow, controlled rate (0.5 to 2.0°C min^{-1}) to a defined temperature (–30 to –80°C) in a programmable freezer in the presence of cryoprotectant solutions, which consist of various combinations of cryoprotective chemicals such as dimethyl sulfoxide, glycerol, ethylene glycol, sugars, and sugar alcohols. The cryoprotective chemicals might protect cells by stabilizing cellular membranes and proteins during slow cooling and cryopreservation, and also mitigate freezing injury by reducing growth of ice crystal, changing shape of ice crystal, and allowing stable supercooling (Reinhoud *et al*. 2000). However, the function of cryoprotective chemicals have not been fully elucidated. Most of studies empirically determined the best combination of cryoprotective chemicals and their optimal concentrations. High concentrations of cryoprotective chemicals can damage cells because of their chemical toxicity or osmotic shock. The prefrozen cells are then rapidly cooled by immersion in liquid nitrogen and preserved safely at the temperature of liquid nitrogen for an indefinite time. Because this method requires the use of an expensive programmable freezer, the use of cryopreservation of cell cultures is still limited to a few laboratories (Menges and Murray 2004).

Recent reports, however, demonstrated that cell cultures can be successfully cryopreserved by simplified slow prefreezing methods that do not require the use of a programmable freezer. The slow prefreezing is performed instead with simple cooling in a laboratory freezer (Sakai *et al*. 1991). Menges and Murray (2004) demonstrated that a slow, controlled-rate cooling at approximately 0.5°C min^{-1} can be achieved in a laboratory freezer at –80°C by using a Nalgene Cryo 1°C freezing container placed in a polystyrene box. We established a simple method in which cryogenic vials containing cells are simply put into a freezer at –30°C (Kobayashi *et al*. 2005). Tobacco BY-2 cells encapsulated in alginate gel are treated with medium containing 2 M glycerol and 0.4 M sucrose, and then placed in a freezer at –30°C. The cells are cooled at approximately 2°C min^{-1}, and then held at –30°C for 1 to 1.5 h (Sakai *et al*. 1991).

Undifferentiated cell cultures have also been successfully cryopreserved by either vitrification or air dehydration. The recent development of these alternative techniques has extended the applicability of cryopreservation to a wide array of plant materials, including apical tips (Niino *et al*. 1992; Takagi *et al*. 1997; Hirai and Sakai 1999; Thinh *et al*. 1999; Moges *et al*. 2004; Gupta and Reed 2006; Reed *et al*. 2006), hairy roots (Yoshimatsu *et al*. 1996), and embryogenic cultures (Sakai *et al*. 1990; Wang *et al*. 2000, 2004; Danso and Ford-Lloyd 2004), from various species. Reinhoud *et al*. (1995) reported the cryopreservation of tobacco LT cells by vitrification. Cells are incubated in a vitrification solution, Plant Vitrification Solution 2 (PVS2; Sakai *et al*. 1990), at nonfreezing temperature and directly stored in liquid nitrogen. In contrast, *Catharanthus roseus* and *A. thaliana* T87 cells were cryopreserved by air dehydration with an encapsulation technique (Bachiri *et al*. 1995, 2000). Cells are precultured in sugar-enriched medium for several days, dried in silica gel to optimal water content, and finally preserved in liquid nitrogen.

ENCAPSULATION OF SUSPENSION CELLS IN ALGINATE GEL

The encapsulation technique, which involves immobilizing cultured cells in hydrophilic alginate gel, is used in combination with slow prefreezing, vitrification, or air dehydration (**Table 1**). An advantage of the use of alginate gel is that heat-independent gelation of sodium alginate solution is easily induced by the addition of calcium ions (Bachiri *et al*. 2000). The alginate gel has little effect on the viability and proliferation of cells and protects the embedded cells from mechanical damage due to manipulation, direct exposure to cryoprotectant solution, and severe changes in osmotic pressure (Draget *et al*. 1988; Bachiri *et al*. 1995; Hirai and Sakai 2003).

The encapsulation allows easy handling of a large number of suspension cell lines in a short time and ensures rapid regrowth of cell cultures. Cells encapsulated in alginate beads regrow faster than those in non-encapsulated cells (Gazeau *et al*. 1998). Rapid regrowth of thawed cells requires a minimum cell density (Menges and Murray 2004). Because a low cell density markedly suppresses mitogenic activity, immediate dispersion of cells into a liquid culture results in arrest of cell division and loss of cell viability. The encapsulation in alginate gel allows maintenance of cultured

suspension cells without loss of cells and decrease in cell density during the process of cryoprotectant dilution and regrowth. In addition, cells encapsulated in alginate gel can be protected from rehydration injury caused by rapid reduction in osmotic pressure. To dilute cryoprotectant solution after thawing, gel beads containing cultured cells are simply transferred to fresh medium without centrifugation (Kobayashi *et al*. 2006b). Cell suspensions are initiated by directly incubating the beads in liquid culture. Previous methods required complicated procedures for removing the cryoprotectant solution, because cultured cells suspended in highly concentrated cryoprotectant solution are difficult to sediment even by centrifugation. Thawed cell cultures are spread on filter paper or on a nylon membrane on semisolid agar medium, and the filter paper or nylon membrane carrying cells is transferred to fresh agar medium (Lynch *et al*. 1994; Menges and Murray 2004). The cells are incubated on the agar medium for several weeks to allow them to recover their growth capacity and then transferred into liquid culture.

COMPARISON BETWEEN CRYOPRESERVATION METHODS

Comparative studies of different cryogenic techniques revealed that slow prefreezing is generally appropriate for cryopreservation of undifferentiated cell cultures. Gazeau *et al*. (1998) demonstrated that cultured *Papaver somniferum* cells were efficiently cryopreserved by slow prefreezing. Slow prefreezing was also more effective for cryopreservation of bromegrass and tobacco BY-2 cells than vitrification (Ishikawa *et al*. 1996; Kobayashi *et al*. 2006a). In contrast, Reinhoud *et al*. (1995) showed that cryopreservation of tobacco LT cells by vitrification retained higher viability and gave faster regrowth of cell cultures than slow prefreezing. However, their protocol required prolonged preculture in mannitol medium and heat-shock treatment to enhance the capacity of LT cells to tolerate a highly concentrated vitrification solution.

The physiological characteristics of cultured suspension cells are relevant to cryopreservation. Undifferentiated cell cultures consist of vacuolated large cells that are sensitive to environmental stresses. The cells are injured more severely by the osmotic dehydration in vitrification solution than by the moderate freezing-induced dehydration during slow cooling (Kobayashi *et al*. 2006b). Direct exposure of dispersed cells in suspension to highly concentrated vitrifycation solution causes lethal damage due to a drastic change in osmotic pressure or to the chemical toxicity of the cryoprotectants.

The method recommended for cryopreservation differs between plant materials. Many reports demonstrated that apical tips are cryopreserved more effectively with vitrification than with the traditional slow prefreezing and air dehydration (Hirai and Sakai 1999; Halmagyi *et al*. 2003; Hirai and Sakai 2003; Moges *et al*. 2004). Vitrification yielded a high regeneration rate and rapid plant regrowth (Engelmann 2000; Sakai 2000; Hirai and Sakai 2003). Cryopreservation of apical tips must allow the survival of meristematic tissues for plant regeneration. Severe osmotic dehydration with the highly concentrated vitrification solution is preferable for the cryopreservation of apical meristems, which are surrounded by many layers of differentiated cells (Sakai 2000). The vitrification solution rapidly penetrates the apical organs and thus sufficiently dehydrates the meristematic cells. Moreover, the apical tips are constituted of various types of cells that have different sensitivities to freezing. In the slow prefreezing method, such complex organs are prone to freezing injury during slow cooling (Sakai 2000).

Embryogenic cultures also are more efficiently cryopreserved by vitrification than by slow prefreezing or air dehydration (Wang *et al*. 2004; Mikula 2006; Wang and Perl 2006). Morphological and physiological properties of embryogenic cells are remarkably different from those of undifferentiated cells: the embryogenic cultures contain small, cytoplasmic-rich meristematic cells that are more tolerant to environmental stresses than large, vacuolated cells (Aguilar *et al*. 1993; Häggman *et al*. 1998; Wang *et al*. 2000; Wang and Perl 2006). Embryogenic cells can survive exposure to vitrification solution and cooling in liquid nitrogen. Vitrification permits rapid regrowth of embryogenic cultures and their subsequent regeneration.

APPLICATION OF CRYOPRESERVATION TO A VARIETY OF CELL LINES

The applicability of a cryogenic technique to a wide variety of cell suspension cultures is important for practical use of cryopreservation. Menges and Murray (2004) established a simple protocol and successfully cryopreserved tobacco cell line BY-2 and *A. thaliana* cell lines MM1 and MM2d. Transgenic cell lines derived from these cultured cells were cryopreserved under the same conditions as the parental cell lines. In contrast, we developed an encapsula-tion–simple prefreezing method for the cryopreservation of tobacco BY-2 cell cultures (Kobayashi *et al*. 2005) that also worked with *Arabidopsis* T87 and rice Oc cell lines (our un-published results). Additional studies are necessary to assess the applicability of these cryopreservation protocols to other cell lines.

OPTIMIZATION OF CONDITIONS FOR CRYOPRESERVATION

The determination of optimal cryostorage conditions is indispensable for the successful preservation of undifferentiated cell cultures in liquid nitrogen. Careful optimization of cryopreservation conditions in each suspension cell line is necessary for achieving high viability of cells after thawing, because lines differ markedly in cell morphology, proliferation potential, and physiological properties, including tolerance to environmental stresses. The highest level of cell viability is required for rapid and secure re-establishment of cultured cell lines. The regrowth of suspension cell cultures depends primarily on the number of cells that survive cryopreservation (Kobayashi *et al*. 2006a). A high density ensures the rapid proliferation of cells, while a low density markedly suppresses mitogenic activity (Stuart and Street 1969). Moreover, the maintenance of high cell viability can minimize the risk of changes in cell characteristics caused by genetic variation or by inadvertent selection of particular types of cells.

The encapsulation–simple prefreezing method is composed of three critical steps: treatment with cryoprotectant solution, prefreezing in a freezer at –30°C, and rapid cooling in liquid nitrogen (Kobayashi *et al*. 2005). The optimal times of cryoprotectant treatment and prefreezing differed among suspension cell lines (**Table 2**). The slow cooling to –30°C causes the freezing-induced dehydration of cells. Sufficiently dehydrated cells are vitrified by rapid cooling in liquid nitrogen without cryoinjury due to intracellular ice crystal formation. The appropriate cooling time might depend on the amount of cellular water, size of individual cells, and aggregation of cells. In addition, the prefreezing procedure is influenced by the tolerance of cultured cells to freez-

Table 2 Optimal conditions for cryopreservation of cell cultures by the encapsulation–simple prefreezing method.

Cell culture	Cryoprotectant treatment (min)	Prefreezing (h)
Tobacco BY-2[1]	45	2
Arabidopsis T87[2]	40	3
Rice Oc[2]	60	4

[1] Kobayashi *et al*. (2005)
[2] Kobayashi *et al*. (unpublished results)
The optimal times of cryoprotectant treatment and prefreezing were determined based on viability of cells after thawing. All suspension-cell lines were subcultured from stock cultures preserved by the National Bioresource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

ing and low temperature. The cryoprotectant treatment enhances the capacity of cells to tolerate freezing, dehydration, and low temperatures (Hitmi *et al*. 1999; Menges and Murray 2004; Kobayashi *et al*. 2005). The cryoprotected cells can survive freezing to –30°C and preservation in liquid nitrogen (Kobayashi *et al*. 2005). However, long exposure to cryoprotectant solution damages cells, especially those that are susceptible to changes in osmotic pressure or to high osmotic stress.

The physiological condition and growth phase of cell-suspension cultures are crucial to cryopreservation. Cells at the exponential growth phase are generally used as starting materials (Bachiri *et al*. 2000; Menges and Murray 2004; Kobayashi *et al*. 2006b), because they have many characteristics that help to reduce freezing injury, such as small size, small vacuoles, dense cytoplasm, and low water content compared with cells at the lag or stationary phases. Ishikawa *et al*. (2006) recently showed that bromegrass suspension cells at late exponential or early stationary phase are more suitable for cryopreservation than cells at the late lag or early exponential phase. This result suggests that the growth phase at which cultured cells are harvested should be precisely optimized for efficient cryopreservation.

FUTURE PERSPECTIVES

The establishment of simple cryopreservation techniques promises to facilitate the use of undifferentiated cell cultures for postgenomic research. Our encapsulation–simple prefreezing protocol may be a good technique for routine cryopreservation of cell-suspension cultures in laboratories. The cryopreservation procedure is simple and does not require specialized equipment or reagents. The use of cryopreservation not only helps to secure against loss of cell lines, but also enables maintenance of a large number of transgenic cell lines. Although transgenic derivatives appear to be cryopreserved under the same conditions as the parental lines, the cryopreservation conditions should be well optimized for each original cell line.

The main concern with the cryopreservation of undifferentiated cell cultures is whether the cell lines regrown after storage in liquid nitrogen retain the same characteristics as the original cell lines (Harding 2004). Many reports demonstrate that particular physiological and biochemical characteristics of cultured cells were maintained after cryopreservation. Suspension cell lines re-established from cryopreserved stocks retained their capacity to produce and accumulate some secondary metabolites (Chen *et al*. 1984; Shibli *et al*. 1998; Moran *et al*. 1999; Kim *et al*. 2001; Suhartono *et al*. 2005). Elleuch *et al*. (1998) reported that transgenic *Papaver somniferum* cells maintained expression of the foreign gene and enzymatic activity of its product after cryopreservation. Menges and Murray (2004) also demonstrated that cryopreservation had no negative effect on expression of transgene, distribution of nuclear DNA contents, growth characteristics, and cell-cycle synchronization capacity in transformed *Arabidopsis thaliana* and tobacco cell lines. However, each study examined the stability of only limited properties. The genetic stability of individual plants regenerated from cryopreserved apical tips or embryogenic cultures are assessed by DNA polymorphism analysis and chromosome number analysis (Urbanová *et al*. 2002; Liu *et al*. 2004). In contrast, our knowledge of the genetic stability of undifferentiated cell-suspension cultures is still limited. Moreover, a general method for analyzing the stability of cultured cells have not been established. Although it is known that cultured suspension cells frequently suffer from changes in their characteristics caused by somaclonal variation during long-term maintenance by sequential subculture, most cell-suspension lines have not been routinely evaluated by genetic, physiological, or biochemical analysis. It is difficult to confirm the genetic identity of undifferentiated cell-suspension cultures that are maintained as populations of cells proliferating actively. In addition, epigenetic changes can not be detected by DNA polymorphism analysis. The development of techniques for monitoring cell characteristics is necessary to assess the effect of long-term maintenance by either cryopreservation or repeated subculture on cell suspension cultures.

Cultured suspension cells are useful for analyzing cellular and molecular biological events during the process of cryopreservation and recovery from storage in liquid nitrogen. But the mechanisms by which plant cells survive exposure to the temperature of liquid nitrogen remain to be fully elucidated. Basic research may help to improve cryogenic techniques for reliable and efficient conservation of plant genetic resources recalcitrant to cryopreservation.

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JAPANESE ABSTRACT

植物培養細胞は細胞レベルでの解析に利用される重要なリソースである。培養細胞は継代を繰り返すことによって維持されているが、その定期的な継代操作は煩雑である。また、コンタミネーション・人為的ミスによる細胞株の喪失や体細胞突然変異による形質の変化の危険性を常に伴っている。液体窒素中での超低温保存は培養細胞の安全な維持を可能にするだけでなく、長期保存の低コスト化を図ることもできる。最近、複数のグループが簡便で信頼性の高い培養細胞の超低温保存法を確立した。これらの方法はプログラムフリーザーなどの特殊な機器を必要とせず、日常的な活用が期待できる。超低温保存の実用化には多様な培養細胞株への適用が要求される。細胞株によって形態や生理的性質が著しく異なるため、それぞれの細胞株において保存条件の最適化が必須である。本総説では、培養細胞の超低温保存法を解説するとともに、効果的な保存のために考慮すべき諸条件について議論する。