

High-Throughput Cryopreservation of Plant Cell Cultures for Functional Genomics

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(Received November 18, 2011; Accepted March 14, 2012)

Suspension-cultured cell lines from plant species are useful for genetic engineering. However, maintenance of these lines is laborious, involves routine subculturing and hampers wider use of transgenic lines, especially when many lines are required for a high-throughput functional genomics application. Cryopreservation of these lines may reduce the need for subculturing. Here, we established a simple protocol for cryopreservation of cell lines from five commonly used plant species, *Arabidopsis thaliana*, *Daucus carota*, *Lotus japonicus*, *Nicotiana tabacum* and *Oryza sativa*. The LSP solution (2 M glycerol, 0.4 M sucrose and 86.9 mM proline) protected cells from damage during freezing and was only mildly toxic to cells kept at room temperature for at least 2 h. More than 100 samples were processed for freezing simultaneously. Initially, we determined the conditions for cryopreservation using a programmable freezer; we then developed a modified simple protocol that did not require a programmable freezer. In the simple protocol, a thick expanded polystyrene (EPS) container containing the vials with the cell–LSP solution mixtures was kept at –30°C for 6 h to cool the cells slowly (pre-freezing); samples from the EPS containers were then plunged into liquid nitrogen before long-term storage. Transgenic *Arabidopsis* cells were subjected to cryopreservation, thawed and then re-grown in culture; transcriptome and metabolome analyses indicated that there was no significant difference in gene expression or metabolism between cryopreserved cells and control cells. The simplicity of the protocol will accelerate the pace of research in functional plant genomics.

Keywords: *Arabidopsis thaliana* • Cryopreservation • Functional genomics • High-throughput • Suspension-cultured cells • Tobacco BY-2.

Abbreviations: EPS, expanded polystyrene; FDR, false discovery rate; FT-ICR/MS, Fourier transform ion cyclotron resonance mass spectrometry; GC/TOF-MS, gas chromatography time-of-flight mass spectrometry; GUS, β-glucuronidase; Hm, hygromycin; MEPM, meropenem

Introduction

With the recent, rapid advance of next-generation DNA sequencing technologies, a vast amount of information on genomic sequences and transcripts from many plant species has become available, and generation of these types of data is faster and easier than ever before. Therefore, there are efforts to utilize the information in efficient ways, preferably using high-throughput strategies, to accelerate our understanding of plant gene function. For example, high-throughput vector construction utilizing a large set of full-length *Arabidopsis* cDNA clones was reported (Ogawa et al. 2008a). Over the past few decades, research in functional genomics has accelerated with the use of transgenic plants. However, generating robust transgenic plants is time consuming (often requiring a year or more) and is limiting (often prohibitive) for high-throughput functional genomics applications. Cultured plant cells are a useful alternative to whole plants for high-throughput genetic engineering, because transgenic cell lines that are ready to use in experiments can be generated within a few weeks. Suspension-cultured cell lines have been established from several plant species, and several such lines, e.g. *Nicotiana tabacum* BY-2 (Nagata et al. 1992) and *Arabidopsis thaliana* T87 (Axelos et al. 1992), have been maintained for many years. The virtually homogeneous nature of cells in these cultures gives rise to reproducible and reliable results; moreover, the

Plant Cell Physiol. 53(5): 943–952 (2012) doi:10.1093/pcp/pcs038, available online at www.pcp.oxfordjournals.org

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cells are maintained and grown under strictly controlled conditions. Cultured plant cells have been successfully used for genetic analyses (e.g. Callard et al. 1996, Mitsukawa et al. 1997, Uno et al. 2000, Takahashi et al. 2001, Stolc et al. 2005). However, continuous culturing with periodic refreshment of medium is laborious; continuous culturing also increases the risk of microbial contamination and loss of the culture. The labor-intensive maintenance associated with continuous cell culture of many transgenic cell lines can cause a bottleneck in functional genomic studies.

Cryopreservation of transgenic cell lines removes the need for frequent culturing and, therefore, reduces the chance of microbial contamination. Several protocols for cryopreservation of cultured plant cells and tissues have been developed since the initial cryopreservation of flax (*Linum usitatissimum*) suspension-cultured cells (Quatrano 1968). Techniques, such as pre-freezing (Sakai 1960, Withers and King 1980), desiccation (Nitzsche 1980) and vitrification (Langis et al. 1989, Uragami et al. 1989), have been used to avoid cell damage during freezing. Encapsulation of plant tissues or cells in alginate beads has been combined with these techniques to improve cryopreservation (Fabre and Dereuddre 1990, Matsumoto et al. 1995, Kobayashi et al. 2005). However, these protocols include time-consuming procedures (e.g. drop-wise addition of a toxic cryoprotectant to the cell suspension) that limit their use for high-throughput handling of many transgenic cell lines. Although some protocols for cryopreservation of cultured cells were designed to meet the demands of functional genomics research (Menges and Murray 2004, Ogawa et al. 2008b), further simplicity would be advantageous.

Here, we developed a simple protocol for cryopreservation of suspension-cultured cells from five commonly used plant species—*A. thaliana*, *Daucus carota*, *Lotus japonicus*, *N. tabacum* and *Oryza sativa*. Transcriptome and metabolome analyses indicated that the transgenic *Arabidopsis* cells that had been cryopreserved using this simple protocol, thawed and then re-grown over a few cycles of subculture were not significantly different from control cells. Thus, cryopreservation was a suitable alternative to continuous culture for maintaining cell lines in a stable way. This simple protocol allowed us to cryopreserve ≥ 100 cell lines simultaneously in a single day; therefore, it will contribute to high-throughput functional genomics research.

Results

In preliminary experiments, we found that LS solution (2 M glycerol, 0.4 M sucrose), which was used as a protectant during cryopreservation of *Citrus sinensis* cells (Sakai et al. 1991), resulted in higher cell viability when cell solution mixtures were incubated at room temperature for up to 2 h, while without replacement of the culture medium mJPL3 with LS, no viable cells were recovered after cryopreservation. Thus, many cell samples could be handled in a high-throughput manner

prior to freezing. Here, we optimized conditions for cryopreservation using a modified LS solution (see below) and a programmable freezer; we then modified these conditions to simplify the protocol. Using the simple protocol, only standard laboratory equipment, such as tube containers and a -30°C freezer, were required to process ≥ 100 cell samples for cryopreservation (Fig. 1).

Cryopreservation through slow pre-freezing

We examined the effects of LS solution on cell viability of *A. thaliana* T87 cells, which were subjected to cryopreservation under strictly controlled cooling conditions using a programmable freezer. Cells in exponential phase were suspended in LS solution and then kept for 0, 30, 60, 90 or 120 min at room temperature with or without shaking; cells were cooled at a rate of $-0.5^{\circ}\text{C min}^{-1}$ down to -35°C (i.e. pre-freezing). Cooled samples were then plunged into liquid nitrogen. Cell viability was defined as the ratio of the percentage of viable cells after freezing to that of unfrozen cells as shown in the Materials and Methods. Incubation in LS solution for up to 120 min with or without shaking showed high cell viability of 45–55% without a significant difference at $P < 0.05$ (Fig. 2). As 0 min of incubation with LS gave almost the same viability as 30–120 min, it is likely that the cooling rate of the cells with LS ($-0.5^{\circ}\text{C min}^{-1}$) is slow enough to protect the cells for cold damage without incubation at room temperature. As incubation in LS for up to 120 min is enough to prepare > 100 sample vials, LS solution is suitable for high-throughput cryopreservation of *Arabidopsis* cells.

To optimize pre-freezing conditions, the cells suspended in LS were cooled to -35°C at a rate of -0.5 , -1 or $-2^{\circ}\text{C min}^{-1}$ using a programmable freezer. After reaching -35°C , cells were kept at -35°C for 0, 30 or 60 min, and then plunged into liquid nitrogen (Fig. 3). Cell viability was significantly high when cells were cooled at $-0.5^{\circ}\text{C min}^{-1}$, and the holding times at -35°C had no significant effect on cell viability when cells were cooled at $-0.5^{\circ}\text{C min}^{-1}$. In contrast, samples cooled at $-2^{\circ}\text{C min}^{-1}$ and immediately plunged into liquid nitrogen (duration at $-35^{\circ}\text{C} = 0$ min) had much lower cell viability (Fig. 3). These results indicated that slower cooling resulted in higher cell viability and that the cooling at $-0.5^{\circ}\text{C min}^{-1}$ eliminated the need to hold cells at -35°C for 30 or 60 min, which was required when cells were cooled at -2 or $-1^{\circ}\text{C min}^{-1}$.

To increase the viability of frozen cells further, we examined the effect of adding proline to LS solution because proline is reportedly a cryoprotectant (Withers and King 1979). We found that supplementation with proline $> 8.7\text{ mM}$ significantly increase cell viability, and LS solution with 86.9 mM proline, designated LSP solution, supported 70% cell viability—the highest viability associated with any proline-supplemented LS solution (Fig. 4). All subsequent cryopreservation experiments were carried out using LSP solution (2 M glycerol, 0.4 M sucrose and 86.9 mM proline).

To simplify the protocol, we assessed whether enclosing the sample vials containing cell–LSP mixtures in a commercial tube

Cryopreservation

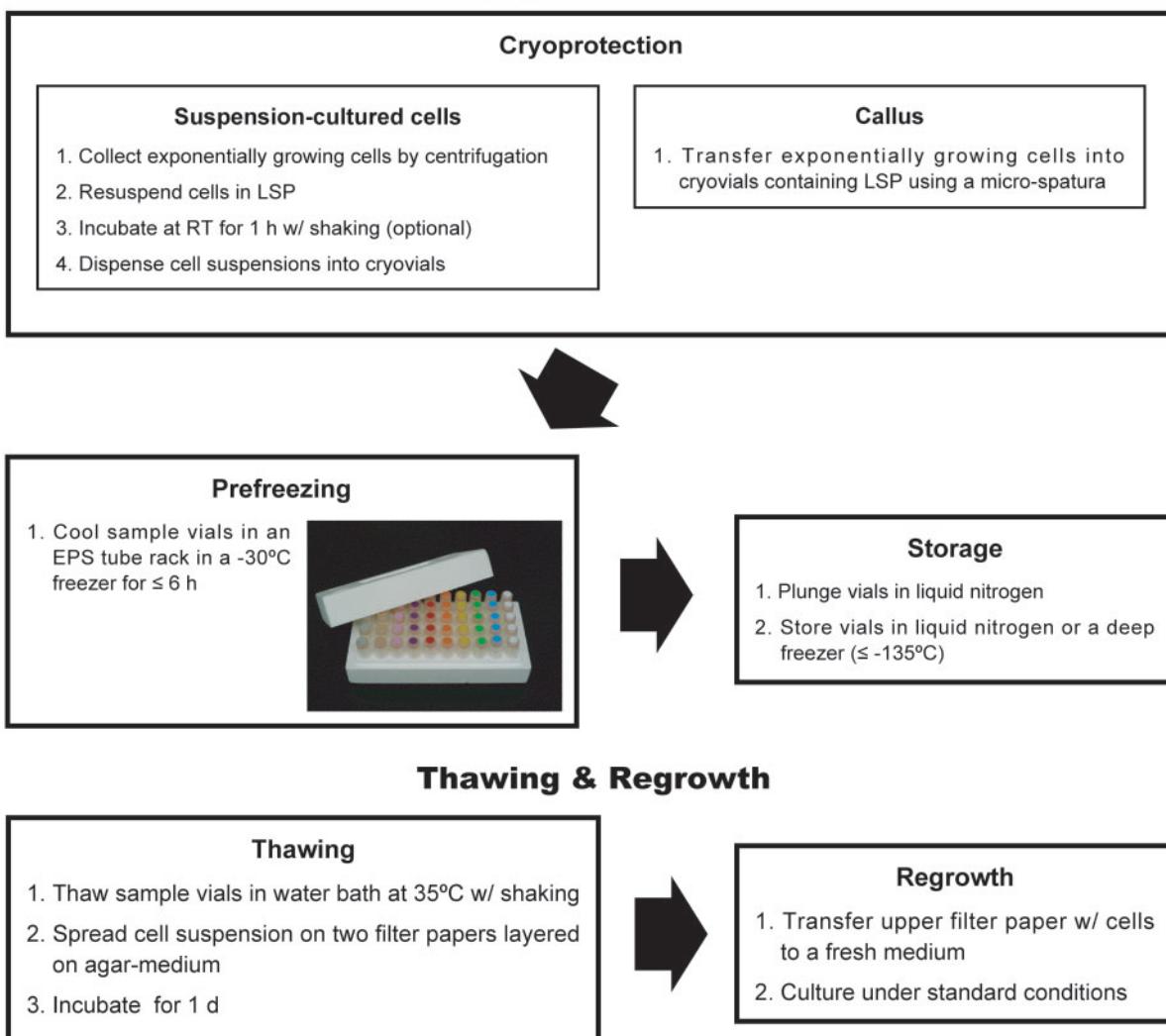


Fig. 1 A schematic diagram of the simple protocol used for cryopreservation of suspension-cultured plant cells.

container would eliminate the need for programmed pre-freezing. First we tested a commercial freezing container (Mr. Frosty, Nalge Nunc), which is designed for cryopreservation of animal cells and holds a maximum of 18 vials. The vials with *A. thaliana* T87 cells suspended in LSP solution were placed in the container, which was then kept in a -30°C freezer for 6 h; the cell viability upon thawing was approximately 60% (**Fig. 5a**). However, when sample vials in the container were kept in a -80°C freezer for 8 h, as recommended for animal cells by the supplier, the cell viability was significantly low (<10%; **Fig. 5a**). Next, we tested two types of commercially available tube containers made of thick expanded polystyrene (EPS), HS4283 (W 210 mm × D 108 mm × H 67 mm for 50 vials, Heathrow Scientific) and SD-14 (W 175 mm × D 175 mm × H 81 mm for 64 vials, Maruemu Co. Ltd.). Samples in these containers were kept in a -30°C freezer for >4 h and had a significantly high viability, approximately 70%, upon thawing; this viability was comparable with that achieved with programmed

pre-freezing (**Fig. 5b**). The cooling rate inside these containers was estimated to be $-0.25^{\circ}\text{C min}^{-1}$ under these conditions; therefore, the vials were likely to reach -30°C within 4 h.

Cryopreservation of calli maintained on solidified medium was further simplified by scratching a spoonful of calli (approximately 50 µl) with a micro-spatula, mixing it into 450 µl of LSP solution in a cryovial and then subjecting the vial to slow pre-freezing (**Fig. 1**).

From here on, we will refer to the procedure that involved programmed pre-freezing as the programmable freezer method and the protocol that involved EPS containers as the EPS tube container method.

Cryopreservation of commonly used plant cell lines

Using the simple cryopreservation protocol established for *A. thaliana* cell line T87, we optimized the conditions for

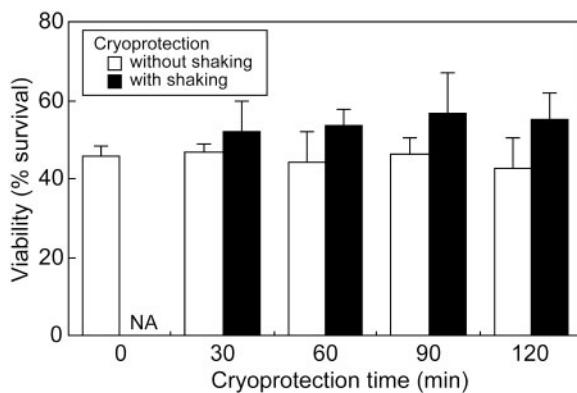


Fig. 2 Effect of cryoprotection on the viability of cryopreserved *A. thaliana* T87 cells. T87 cells from 7-day-old cultures were incubated in LS solution at room temperature for 0–120 min with or without shaking. Cells were then cooled to -35°C at $-0.5^{\circ}\text{C min}^{-1}$. Data are presented as the mean \pm SD of three independent experiments, each of which was examined using three vials. NA, not available. No significant difference was observed among values by Kruskal–Wallis test ($P = 0.194$).

commonly used plant suspension-cultured cell lines from four other plant species, *D. carota*, *L. japonicus*, *N. tabacum* and *O. sativa* (Fig. 6a–e). We also optimized conditions for another *A. thaliana* Co-0 suspension-cultured cell line, called the Alex line here. The cells were mixed with LSP solution at room temperature and then subjected to slow pre-freezing either immediately or after incubation at room temperature for 60 min with shaking. Samples were slowly pre-frozen in an EPS tube container (HS4283) in a -30°C freezer for 2, 4 or 6 h; for each cell line, the highest cell viability was observed when the samples were pre-frozen for 6 h. The *A. thaliana* and *N. tabacum* lines had nearly maximum viability when pre-frozen for just 4 h (Fig. 6a, d). The maximum cell viability for all cell lines varied from 50% (*L. japonicus*) to 95% (*A. thaliana* Alex and *N. tabacum* BY2). Pre-freezing in EPS containers for 2 h followed by immediate freezing in liquid nitrogen consistently resulted in low cell viability for all lines; this observation indicated that vial temperature did not reach -30°C within 2 h (see above). For the *L. japonicus* (Fig. 6c) and *N. tabacum* BY2 (Fig. 6d) cell lines, incubation in LSP for 60 min prior to slow pre-freezing was crucial to high viability, indicating that these cell lines required time for the cryoprotectant to impregnate the cells. Apparently, there is no relationship between this requirement and cell proliferation rates or the sizes of cell clumps because the *L. japonicus* cells proliferate slowly and form large coarse cell clumps, but the BY2 cells proliferate rapidly and form small fine clumps.

Assessment of gene expression and metabolism of cryopreserved cells

To assess the effects of cryopreservation on gene expression and metabolism, we analyzed three independent transgenic

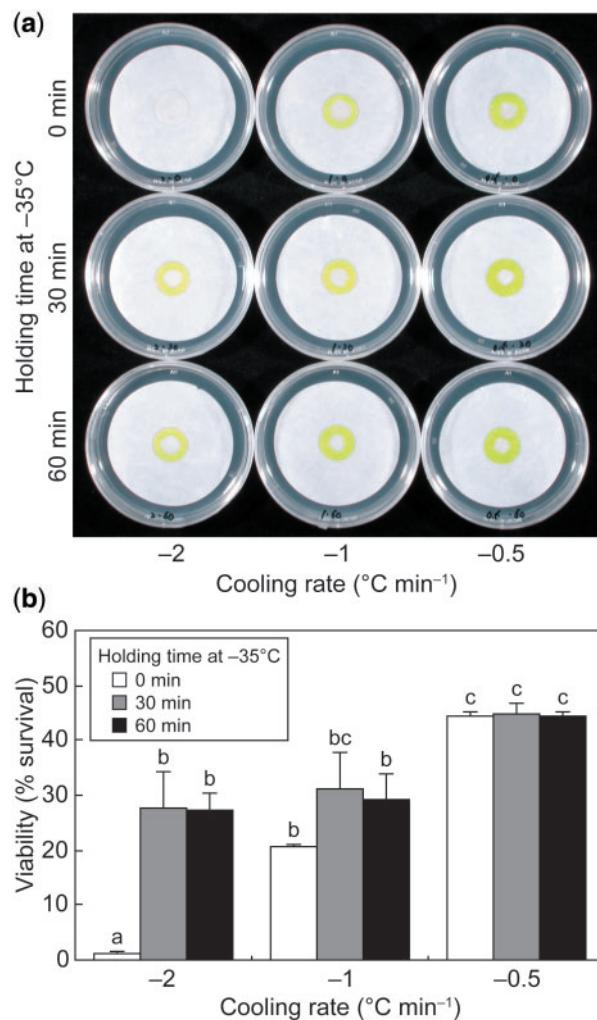


Fig. 3 Effect of the cooling rate on cell viability of cryopreserved *A. thaliana* T87 cells. Exponentially growing cells from 7-day-old cultures of T87 were mixed with LSP solution and incubated with shaking at room temperature for 30 min. The cells were cooled to -35°C at a rate of -2 , -1 or $-0.5^{\circ}\text{C min}^{-1}$ using a programmable freezer. After reaching -35°C , the cells were kept at -35°C for 0, 30 or 60 min before freezing in liquid nitrogen. The cryopreserved cells were thawed and plated on filter paper on agar plate for re-growth (a). The viability of frozen cells was measured in three independent samples (b). Values with the same letter are not significantly different according to Scheffé's F-test ($P < 0.05$).

lines of *Arabidopsis* T87 cells carrying the β -glucuronidase (GUS) gene. Expression of the GUS transgene differed substantially between lines, ranging >6 -fold. Cryopreservation was carried out by either the programmable freezer method or the EPS tube container method. Thawed cells that had been cryopreserved were subjected to three cycles of subculturing (2 weeks each), and cells from the exponentially growing 7-day-old cultures from the third subculture were analyzed.

The GUS activity per fresh weight was not significantly different ($P < 0.01$) after cryopreservation for all cell lines when compared with the control cells (Fig. 7).

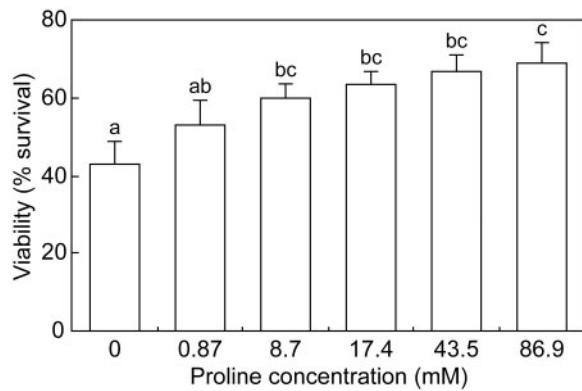


Fig. 4 Effect of proline in the cryoprotectant on viability of cryopreserved *A. thaliana* T87 cells. T87 cells from 7-day-old cultures were suspended in LS solution supplemented with 0–86.9 mM proline; these samples were cooled to -35°C at $-0.5^{\circ}\text{C min}^{-1}$. Data are presented as the mean \pm SD of three independent experiments, each of which was examined using three vials. Values with the same letter are not significantly different according to Scheffé's F-test ($P < 0.05$).

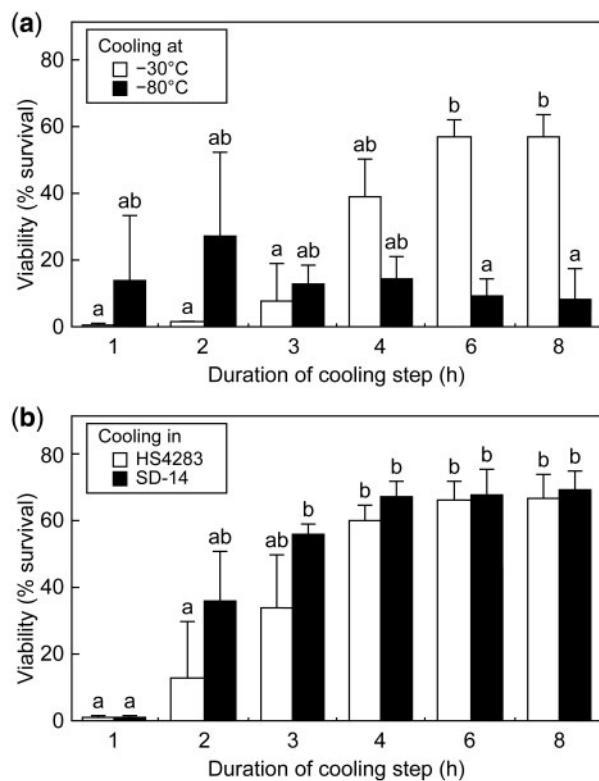


Fig. 5 Cryopreservation of *A. thaliana* T87 cells using commercial tube containers. The vials containing T87 cells in LSP solution were placed in a Mr. Frosty container and cooled at -30 or -80°C for 1–8 h (a). The vials containing T87 cells in LSP solution were placed in an EPS tube container (HS4283 or SD-14) and cooled at -30°C for 1–8 h (b). Data are presented as the mean \pm SD of three independent experiments, each of which was examined using three vials. Values with the same letter are not significantly different according to Scheffé's F-test ($P < 0.05$).

Non-targeted analysis of metabolites in these three lines was carried out using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR/MS) and gas chromatography time-of-flight mass spectrometry (GC/TOF-MS). The FT-ICR/MS analysis of the cells reproducibly detected 377 and 512 independent ions in the positive mode and the negative mode, respectively (Supplementary Table S1); the GC/TOF-MS analysis detected 481 reliable and reproducible peaks (Supplementary Table S2). The significance of the differences in metabolic ions between cryopreserved cells and control cells was estimated using the false discovery rate (FDR) for the P-values of the Student's t-test. Of 889 and 481 ions identified by FT-ICR/MS and GC/TOF-MS, respectively, 885 ions (99.55%) and 476 ions (98.97%), respectively, showed no significant changes between cryopreserved and control cells. The ions that were determined to have changed significantly (FDR < 0.05) showed no reproducible change among the three transgenic lines.

To evaluate the effects of cryopreservation on gene expression in *Arabidopsis* cells in a non-targeted manner, microarray analyses of three independent transgenic cell lines carrying the GUS gene were performed using the oligo DNA microarray (Agilent Technologies), which comprises 21,500 oligo 60-mer DNA probes (Supplementary Table S3). Microarray hybridization was carried out once for each cell line; therefore, the statistical reliability of the array experiments was lower than that of the metabolite analyses, which were performed in triplicate. Differences in gene expressions between cells cryopreserved using the programmable freezer method and control cells (exp1) and between cells cryopreserved using the EPS tube container method and control cells (exp2) were estimated. For all three lines, 16,222 and 16,660 probes (exp1 and exp2, respectively) showed significant signal values, and 16,217 (99.77%) and 16,643 (99.90%) of these probes (exp1 and exp2, respectively) showed no significant changes between cryopreserved and control cells. The small percentage of genes that were determined to have changed significantly (FDR < 0.05) did not have obviously related biological functions.

Taken together, these results indicated that the cryopreservation procedures we describe here did not affect gene expression or metabolism in cryopreserved cells.

Discussion

Here we established a simple high-throughput protocol for cryopreservation of cultured plant cells derived from five commonly used plant species, *A. thaliana*, *D. carota*, *L. japonicus*, *N. tabacum* and *O. sativa* (Fig. 1). A key component of the high-throughput procedure was the use of LSP solution, a cryoprotectant; >100 cell samples in LSP solution could be prepared at room temperature before freezing without significant loss of cell viability. The addition of proline to LS solution up to 86.9 mM was effective on cell viability after cryopreservation (Fig. 4). Although Withers and King (1979)

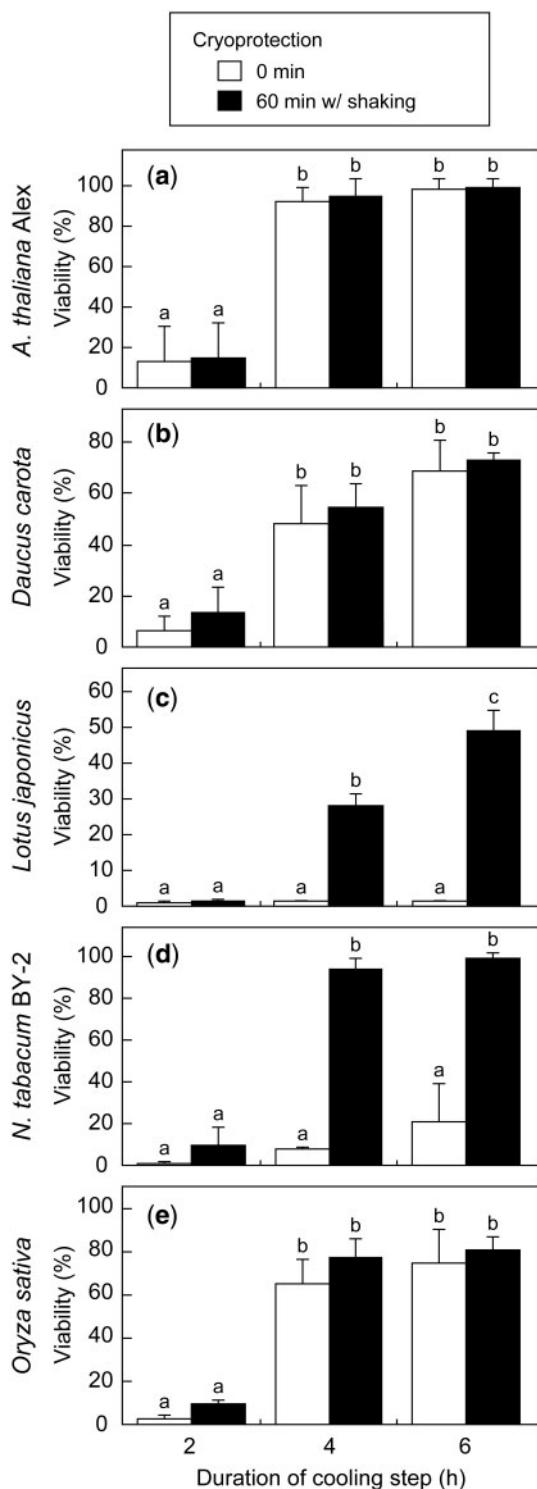


Fig. 6 Cryopreservation of five suspension-cultured cell lines using the EPS tube container method. Suspension-cultured cells of *A. thaliana* Alex (a), *Daucus carota* (b), *Lotus japonicus* (c), *Nicotiana tabacum* BY-2 (d) or *Oryza sativa* (e) were suspended in LSP solution for 0 or 60 min with shaking. The cells were then cooled to -30°C over 2–6 h in an EPS tube container HS4283. Data are presented as the mean \pm SD of three independent experiments, each of which was examined using three vials. Values with the same letter are not significantly different according to Scheffé's F-test ($P < 0.05$).

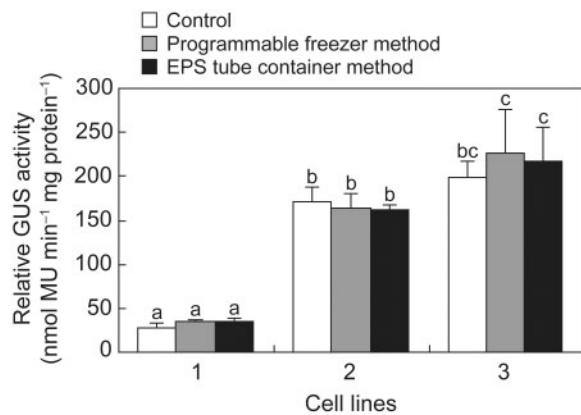


Fig. 7 Relative GUS activities of cryopreserved *A. thaliana* T87 cells. Cryopreservation of three independent T87 transgenic cell lines, which carry the β -glucuronidase gene, was performed using the programmable freezer method or the EPS tube container method with the HS4283 container. As a control, the GUS activity of the cells before cryopreservation is also shown. Data are presented as the mean \pm SD of three independent experiments. Values with the same letter are not significantly different according to Fisher's PLSD ($P < 0.01$).

reported that proline was an effective cryoprotectant of cultured cells of *Zea mays* L., pre-growth of the cells for 3–4 d in medium containing proline was necessary for freezing tolerance. In contrast, no pre-growth is required when LSP solution is used for cryopreservation. LSP solution differs from other cryoprotectants (e.g. dimethylsulfoxide, ethylene glycol and propylene glycol) that have been used for cryopreservation of various plant cell lines (Reinhoud et al. 2000). The other cryoprotectants must be added to samples drop-wise within a short time period and at low temperature to minimize direct contact between the concentrated solution and cells. The protocol described in this study is simpler than previously reported protocols, including the protocols that were designed for functional genomics applications (Menges and Murray 2004, Ogawa et al. 2008b). The simplicity of this protocol makes it suitable for preservation of a large number of transgenic cell lines for high-throughput functional genomics experiments. We are currently working on preparing a large set of *Arabidopsis* T87 transgenic cell lines, each of which harbors an *Arabidopsis* full-length cDNA encoding a transcription factor; the cDNAs were introduced using a high-throughput vector construction procedure (Ogawa et al. 2008a). These transgenic lines were cryopreserved using the protocol described in this study.

With our protocols, cells did not require conditioning before cryopreservation or after thawing, which contributed to the simplicity of the protocols. Many cryopreservation protocols require that cells are pre-conditioned before freezing; for some protocols, cells must be cultured for days or weeks at low temperature or in medium containing concentrated sugars, sugar alcohols, amino acids and/or ABA (Reinhoud et al. 2000). The thawing and re-growth processes in our protocol were also much simpler than those in previously reported

protocols. The cryopreserved cells were thawed at 35°C for 2 min and then transferred to a paper filter and kept on agar with growth medium for 1 d; cells were then subcultured routinely from the next day on. In contrast, thawing following many cryopreservation protocols (Reinhoud et al. 2000) requires that cells should be washed immediately and thoroughly with medium before cultivation to eliminate the toxic cryoprotectant used. Several reports also recommend additional manipulation after cell thawing to improve cell growth; for example, activated charcoal (Kuriyama et al. 1990) or surfactant Pluronic F-68 (Lowe et al. 2001) is added to medium, or cells are incubated in the dark or under dim lighting (Benson and Noronha-Dutra 1988). However, these manipulations did not significantly improve growth or viability of thawed *A. thaliana* T87 cells that were cryopreserved using our simple protocol (data not shown).

Our findings indicated that pre-freezing, prior to freezing in liquid nitrogen, to -30°C was preferable to pre-freezing to -80°C. Menges and Murray (2004) included pre-freezing to -80°C in the protocol for cryopreservation of *A. thaliana* and *N. tabacum* cultured cell lines, in which they placed the Mr. Frosty container in a thick styrofoam box to reduce the freezing rate to lower than $-1^{\circ}\text{C min}^{-1}$ in a -80°C freezer. In contrast, slow pre-freezing to -30°C using the Mr. Frosty container or the EPS tube container was attained without further manipulation of the cooling rate, which also contributes to the simplicity of our protocol. Kobayashi et al. (2005) also reported that tobacco BY-2 cells encapsulated in alginate beads were successfully cryopreserved by slow pre-freezing to -30°C.

No significant changes in transcription or metabolism of transgenic *Arabidopsis* T87 cell lines before and after cryopreservation were observed. To assess the transcription and metabolism under normal growth conditions, frozen cells were thawed and re-grown over two cycles of subculturing and a final 7 d culture before the analyses. Most transcripts and metabolites detected in the assays were not significantly different in cells before and after cryopreservation. Thus, wounding or injury that might have occurred during cryopreservation and that could affect transcription and/or metabolism was resolved or reversed during the post-thaw culturing. In a review, Harding (2004) states that plant cells and tissues are stable after cryopreservation at the genetic, phenotypic, cytological, biochemical and molecular levels, even though these cells had suffered physical and/or physiological stresses, including wounding or injury. Our results were consistent with this view.

The protocol established in this study was used successfully to preserve six commonly used cultured plant cell lines from five plant families Brassicaceae (*A. thaliana*), Leguminosae (*L. japonicus*), Solanaceae (*N. tabacum*), Apaceae (*D. carota*) and Poaceae (*O. sativa*). This set of plants was phylogenetically diverse, with representatives from the subclasses Dicotyledonae and Monocotyledonae, and individual cell lines differed with regard to phenotypic characteristics (e.g. growth rates and sizes of cell clumps). The phylogenetic and phenotypic diversity of

these cell lines indicated that the protocol may be used with a wider array of plant species, although experimental trials must be carried out for each species.

Materials and Methods

Suspension-cultured cell lines

We obtained suspension-cultured cells of *A. thaliana* (L.) Heynh. ecotype Columbia cell line T87 (Axelos et al. 1992) and *N. tabacum* L. cell line BY-2 (Nagata et al. 1992) from the Experimental Plant Division of RIKEN Bioresource Center. The Alex line derived from *A. thaliana* Col-0 (Mathur et al. 1998) was a gift from Masaaki Umeda (The University of Tokyo), *D. carota* L. cells were a gift from Hiroyuki Koyama (Gifu University), *L. japonicus* (Regal) Larsen ecotype Gifu was a gift from Toshio Aoki (Nihon University) and *O. sativa* L. cv. Sasanishiki (Hayakawa et al. 1990) was a gift from Toshihiko Hayakawa (Tohoku University). Detailed information on each cell line is listed in **Supplementary Table S4**.

Cryopreservation of suspension-cultured cells

Exponentially growing cells (4-day-old cultures for *A. thaliana* Alex, *D. carota* and *N. tabacum* BY-2; 7-day-old cultures for *A. thaliana* T87 and *O. sativa*; and 12-day-old cultures for *L. japonicus*) were transferred into 15 ml tubes and centrifuged at 100 × g for 1 min. Cell suspensions were handled using micropipets with large orifice tips (QSP 119, Porex Corporation). The supernatant was removed, and cells were then suspended in cryoprotectant solution (LS: 2 M glycerol, 0.4 M sucrose; Sakai et al. 1991) supplemented with 0–86.9 mM L-proline at the cell density of 10% (v/v), and incubated at room temperature for 0–120 min with and without shaking at 60 r.p.m. Aliquots (0.5 ml) of cell suspensions were dispensed into 1.2 ml cryovials (Fisher Scientific). Cryovials containing cell suspension were cooled to -35°C at a rate of -0.5, -1 or $-2^{\circ}\text{C min}^{-1}$, and cooled cells were held at -35°C for 0–60 min in a programmable freezer (Kryo 560-16, Planer PLC). This method is referred to as the programmable freezer method herein. Pre-freezing was also examined in the freezing container (Nalgene 'Mr. Frosty' Cryo 1°C Freezing Container, Nalge Nunc International) or an EPS tube container (50-well EPS Tube Rack HS4283, Heathrow Scientific or the Tube Holder SD-14, Maruemu Co. Ltd.). The freezing container and the EPS tube containers were placed into a -30°C freezer or a -80°C freezer and left for 1–8 h. The pre-freezing method is referred as the EPS tube container method herein. After pre-freezing, vials were immediately plunged into liquid nitrogen and held for at least 1 h before subsequent experiments.

Thawing and re-growth of cryopreserved cells

The cryovials with frozen cells were placed in a water bath at 35°C for 2 min with vigorous shaking (approximately 180 r.p.m.). The thawed cells were dropped onto double-layered

filter papers (85 mm diameter) that were on culture medium solidified with 7 g l⁻¹ agar. To obtain nearly uniform cell density on the filter paper, we took care to spread the cells within a 20 mm circle (marked with a pencil) when dropping thawed cells onto the filter paper. The cells were incubated for 1 d at the temperature for each cell type shown in **Supplementary Table S4**. The upper filter paper with the cells was transferred to fresh medium for further growth.

Measurement of cell viability after thawing

Viability of cryopreserved cells was assessed using an assay developed by Ishikawa et al. (1995). The assay is based on the fact that, for exponentially growing cells, there is a linear relationship between the viability of the initial cells and the cell mass after re-growth when the relationship is assessed by the least square method ($R^2 > 0.9$, $P < 0.05$). Frozen control cells (0% viability), which were prepared by rapid freezing and thawing in cryoprotectant-free medium, and unfrozen control cells (100% viability) were mixed to obtain a series of cells having different viability (0, 25, 50, 75 or 100%). These cells were cultured in the medium for each type shown in **Supplementary Table S4**, and cell mass (g wet weight) was measured after various culture periods in the exponentially growing phase. From these experiments, we determined that the culture periods required to measure cell viability varied accurately among cell lines; *N. tabacum* BY-2 cultures required 5 d of growth; *A. thaliana* Alex and T87, *D. carota* and *O. sativa* cultures required 7 d; and *L. japonicus* required 12 d. Cell viability was calculated by comparing the cell mass of cryopreserved cells with the cell mass of frozen and unfrozen control cells. In each experiment, three vials per treatment were examined, and each experiment was repeated three times. The data were statistically analyzed using the non-parametric Kruskal–Wallis test followed by the post-hoc Scheffé's F-test using Excel 2003 software (Microsoft) with add-in software Statcel (Yanai, 1998).

Preparation and cryopreservation of transgenic *Arabidopsis* T87 cells

Arabidopsis T87 cells were transformed with *Agrobacterium tumefaciens* EHA101 (Hood et al. 1986) carrying a binary vector, pLG121-Hm, using the method described by Ogawa et al. (2008a). pLG121-Hm contains a selectable marker, the hygromycin phosphotransferase gene *hpt*, and a reporter intron-containing *uidA* (Ohta et al. 1990). Transgenic T87 cells were maintained on mJPL3 medium supplemented with 25 mg l⁻¹ meropenem (MEPM; Meropen, Dainippon Sumitomo Pharma Co. Ltd.) and 20 mg l⁻¹ hygromycin (Hm) and solidified with 3 g l⁻¹ gellan gum. Suspension cultures were established by transferring 0.5 g wet weight of cells into 100 ml of mJPL3 medium supplemented with 12.5 mg l⁻¹ MEPM and 5 mg l⁻¹ Hm; these cell suspensions were cultured under the standard conditions (**Supplementary Table S4**). Samples from 7-day-old cultures were cryopreserved using the programmable freezer method or the EPS tube container method; the

remaining cells were collected by centrifugation, rinsed once with distilled water, frozen in liquid nitrogen and then stored at –80°C until use as control cells. Cryopreservation of the transgenic T87 cell lines was carried out using the EPS tube container method and, after thawing, the cells were cultivated in the medium with 25 mg l⁻¹ MEPM and 20 mg l⁻¹ Hm. Suspension cultures of cryopreserved transgenic T87 cells were grown by transferring 0.5 g wet weight of cells into a 300 ml flask containing 100 ml of mJPL3 medium supplemented with 12.5 mg l⁻¹ MEPM and 5 mg l⁻¹ Hm, and cultured under the conditions shown in **Supplementary Table S4**. For further analyses, 7-day-old cultures from the third subculture period were collected.

Fluorometric GUS analysis

Histochemical GUS assay performed with transgenic *Arabidopsis* T87 cells maintained on Hm^r agar medium as described by Jefferson (1987). Analysis of variance (ANOVA) of the data was carried out using the one-factor ANOVA method followed by Fisher's protected least significant difference (PLSD) test using Microsoft Excel 2003 software.

FT-ICR/MS analysis

Metabolite analysis by FT-ICR/MS and subsequent data analysis were performed as described by Oikawa et al. (2006). Briefly, transgenic T87 cells and leaves of 21-day-old *Arabidopsis* plants were extracted with methanol, and the extracts were filtered through DISMIC-13JP (Advantec Co. Ltd.), evaporated in an N₂ atmosphere and stored at –80°C. For FT-ICR/MS analysis, the extracts were dissolved in 50% (v/v) acetonitrile/water. As internal mass standards, a set comprising lidocaine, prochloraz, reserpine and bombesin was used for the positive ion mode analysis, and a set comprising 2,4-D, ampicillin, CHAPS and tetra-N-acetylchitotetraose was used for the negative ion mode analysis. Mass analysis was performed using an IonSpec Explorer FT-ICR/MS and the Omega8 software (IonSpec Co.). Independent electrospray ionization (ESI) spectra ($n = 10$) were acquired from each sample in broad and detection mode over an *m/z* range of 55–1,000. Data processing on raw MS data sets from FT-ICR/MS was performed using the Dr.DMASS software (Oikawa et al. 2006) with the default settings. Ion intensities were transferred to log base 10 and normalized by median. The Student's *t*-test was performed using Microsoft Excel software, and the calculation of the FDR of the *P*-values for the *t*-test was performed using the software R v2.13.0 with the q value package, which is available free from the web site (<http://www.r-project.org/>).

GC/TOF-MS analysis

Analysis of metabolites by GC/TOF-MS was performed as described by Ogawa et al. (2008b). Briefly, metabolites were extracted from transgenic T87 cells that had been passed through cryopreservation and control transgenic T87 cells using methanol that contained ribitol as an internal standard;

additional metabolites were extracted from the cells using chloroform. For each sample, the methanol and chloroform extracts were mixed and water was added. The samples were then mixed with a vortex and centrifuged; the methanol/water layer (polar fraction) of each sample was collected. Extracts were derivatized with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide and then fractionated using a 6890 GC (Agilent Technologies Co. Ltd.). Mass spectra data were acquired on a Pegasus III TOF-MS with ChromaTOF software (LECO Co.). Raw MS data sets were processed with the MATLAB 6.5.2 software (MathWorks Inc.) with custom script (Jonsson et al. 2004). Peak areas associated with metabolites were transformed to log base 10 and normalized by median. The Student's *t*-test and calculation of FDR were performed as described for the FT-ICR/MS analysis.

Microarray analysis

The microarray analysis was performed as described by Nakamura et al. (2007). Total RNA was extracted using the FastRNA Pro Green Kit (Qbiogene), and triplicate samples from each cell line were combined for analysis. Hybridizations were performed with the Arabidopsis2 Oligo DNA Microarray (Agilent Technologies Co. Ltd.) using Cy3-labeled (cells passed through cryopreservation) and Cy5-labeled (control cells) cRNAs. Signal detection and quantification were conducted with a DNA Microarray Scanner and the Feature Extraction 6.1.1 software (Agilent Technologies Co. Ltd.), respectively. Probes that were flagged with both 'IsPosAndSignif' and 'IsWellAboveBG' for significant array signals were excluded from further analysis. Student's *t*-test and calculation of the FDR were performed as described for the FT-ICR/MS analysis.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the New Energy and Industrial Technology Development Organization (NEDO) [performed as a technology development project in the 'Green Biotechnology Program'].

Acknowledgments

The authors thank Drs. Csaba Koncz (Max-Planck-Institut für Züchtungsforschung) and Masaaki Umeda (The University of Tokyo) for providing the *Arabidopsis* Col-0 cell suspension line, Alex. We also thank Dr. Hiroyuki Koyama (Gifu University) for the *Daucus carota* cell line, Dr. Toshihiko Hayakawa (Tohoku University) for the *Oryza sativa* cell line, and Dr. Toshio Aoki (Nihon University) for the *Lotus japonicus* cell line. We are grateful to Dr. Hiroyuki Ohta (Tokyo Institute of Technology)

for technical advice on the GUS analysis, Drs. Par Jonsson and Thomas Moritz (Swedish University of Agricultural Sciences) for providing scripts and source code for the MATLAB software, and Dr. Miyako Kusano (RIKEN Plant Science Center) for technical advice on data processing using the MATLAB software.

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