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TECHNICAL ADVANCE

Cryopreservation of transformed and wild-type *Arabidopsis* and tobacco cell suspension cultures

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Summary

We have recently described *Arabidopsis* cell suspension cultures that can be effectively synchronised. Here, we describe procedures that allow clonal-transformed cell suspension lines to be produced using *Agrobacterium*-mediated transformation, and an optimised and straightforward procedure for the cryopreservation and recovery of both parental and transformed lines. Frozen cultures show 90% viability and rapid regrowth after recovery. We show that the cryopreservation procedure is equally applicable to the frequently used tobacco bright yellow (BY)2 cell suspension culture, and that cell cycle synchronisation capacity of parental lines is maintained after both transformation and recovery from cryopreservation. The techniques require no specialised equipment, and are suitable for routine laboratory use, greatly facilitating the handling and maintenance of cell cultures and providing security against both contamination and cumulative somaclonal variation. Finally, the ability to store easily large numbers of transformed lines opens the possibility of using *Arabidopsis* cell suspension cultures for high-throughput analysis.

Keywords: *Arabidopsis* cell suspension, tobacco BY2 cell suspension, cryopreservation, plant cell cycle, transformation.

Introduction

Cell suspension cultures are invaluable tools in plant cell biology, offering a population of near-identical cells for studies of the cell cycle in which cellular processes are decoupled from development. The recent establishment of *Arabidopsis* cell suspension cultures MM1 and MM2d and associated techniques for their effective synchronisation (de Jager *et al.*, 2001; Menges and Murray, 2002) has allowed us to extend this utility to genome-wide expression studies of cell cycle regulation of transcription and other processes using Affymetrix arrays (Menges *et al.*, 2002, 2003).

To extend further the utility of the MM1 and MM2d cell suspension lines for analysis of the effects of overexpression or downregulation of target genes using transgenic techniques, we established a transformation protocol, which was used to derive transgenic *Arabidopsis* cell lines overexpressing cell cycle-related genes. As our long-term goal is to use this cell suspension for the high-throughput analysis of gene function, and the routine maintenance of cell cultures by repeated weekly subculture is not only labour intensive but also creates the risk of transgene loss or other acquired variation in growth rate, chromosome cytology or other undesirable genetic change, we also sought a reliable method for long-term storage and regeneration of transgenic and parental cell cultures.

Despite the widespread use of storage of animal cells in liquid nitrogen (LN), few laboratories have used such methods for the routine storage of plant cells. Controlled freezing and low temperature storage of cultured plant cells in LN might enable the characteristics of newly initiated cultures to be preserved for indefinite periods. However, the limited reports of methods used with plant cells are typically characterised by low reported viabilities, long lag periods before a rapidly growing cell suspension is recovered and requirement for specialised apparatus to provide a controlled slow cooling rate (Jain *et al.*, 1996; Jekkel *et al.*, 1989; Kim *et al.*, 2001; Maddox *et al.*, 1983).

Post-thaw survival following freezing is strongly influenced by a number of factors. First, the importance of cell

size and degree of vacuolation and the stage in the growth cycle of the cell suspension cultures subjected to freezing and thawing has been explored, and actively dividing exponential stage cells show best results (Withers and Street, 1977). In addition, the importance of pre-treatment with cryoprotectant and the conditions of freezing and thawing for cell recovery have been reported (Nag and Street, 1973, 1975a,b; Withers and Street, 1977). There are two main critical factors that are known to affect the viability of frozen cells; one is the combination of chemical cryoprotectants, and the other is a freezing programme that permits extracellular freezing and the occurrence of protective dehydration. Dimethyl sulphoxide (DMSO) and sugars such as sorbitol have been proven to be effective cryoprotective agents to enable plant cells to survive deep frozen temperatures (Chen *et al*., 1984a). It has been shown that during the freezing process, ice crystal formation together with cell dehydration is the primary cause of cell death (Kim *et al*., 2001). A combination is more efficient than the use of a single cryoprotectant, as the individual toxic effect of each agent is reduced (Chen *et al*., 1984a). As a result, most reported cryopreservation protocols for plant cell cultures follow a two-step procedure consisting of pre-culture with sugar and polyols, treatment with chemical cryoprotectants, combined with slow cooling (1–2°C min^{-1}) of cells to subzero temperatures (−30 to −100°C) before quenching in LN for storage.

Early reports have shown that recovery of cultures after thawing requires not only high post-thaw viability but also further survival through a recovery period preceding renewal of normal metabolic functions and cell division (Withers and King, 1979). Cryoprotectant toxicity and rehydration/deplasmolysis injury upon thawing and cryoprotectant removal often contributes to viability losses, which may delay or sometime preclude recovery (Chen *et al*., 1984b; Maddox *et al*., 1983; Withers and King, 1979). Low post-thaw viability may result in undesirable selection for a specific subset of cells, particularly in heterogeneous cultures (Withers and King, 1979). A high degree of post-thaw viability is therefore necessary to ensure the recovery of a homogeneous cell population representative of the original culture before freezing, particularly if the aim is to preserve cell lines that have been proven to be of high quality for further use or value-added transformed cultures.

Here, we describe the development of simple and effective techniques for both transformation and cryopreservation in LN of the *Arabidopsis* cell lines MM1 and MM2d that offer a high viability rate and rapid post-thaw growth and re-establishment. We demonstrate that desirable characteristics such as synchronisation potential and transgene expression are unaffected by frozen storage and recovery. These techniques require no specialised equipment, and we show them to be equally effective with the tobacco bright yellow (BY)2 cell line, suggesting their likely general utility for the frozen storage of plant cells. These techniques create the potential for high-throughput studies using plant cell suspension cultures with potential applications in functional genomic analysis.

Results

Viability and post-thaw growth of cryopreserved Arabidopsis *MM1 cells*

Establishment of an effective cryopreservation procedure for *Arabidopsis* suspension culture cells required achieving a sufficiently high viability and post-thaw growth rate to ensure re-establishment of cell cultures equivalent to the original cultures. We therefore first optimised the concentration of cryoprotectants in the freezing protocol using cell line MM1 prior to storage in LN. Cultures were treated with different concentrations of D-sorbitol (0.1–1 M) and DMSO (0–10%) followed by a slow freezing in a cryocontainer as described in Experimental procedures. Cell viability was determined by Trypan blue staining to identify dead or dying cells immediately after rapid thawing at 40°C in a waterbath. Results shown in Figure 1(a) reveal that preincubation of cells in medium containing 0.5 M sorbitol prior to cryoprotection with 5–10% DMSO resulted in optimum immediate post-thaw viability (up to 90% with 7.5% DMSO).

To assess the effect of different freezing methods, MM1 cells were grown in 0.5 M sorbitol-enriched MSS-medium for 2 days, treated with various concentrations of DMSO (0–10%) at 4°C for 1 h and transferred into cryovials. Cryoprotected cells were either plunged directly in LN, stored at −80°C (freezer) for 5.5 h prior to transfer into LN or transferred into a cryocontainer (placed in an additional styropore box, see Experimental procedures) to ensure a controlled slow freezing rate of approximately −0.5°C min^{-1} during storage at −80°C for 5.5 h prior to transfer into LN. A controlled slow freezing rate resulted in optimum viability of up to 90% (Figure 1a,b). A maximum of only 18% viable cells were obtained post-thaw in samples that were plunged directly into LN (fast freezing rate). In cells frozen at a moderate rate (stored at −80°C prior to transfer into LN), a maximum of 48% viable cells were counted in the sample cryopreserved with 0.5 M sorbitol together with 10% DMSO (Figure 1b).

The ability of cells to re-grow rapidly following the immediate post-thaw period was tested. Although various degrees of viability were found using the Trypan blue test directly post-thaw, cells frozen at a moderate or fast rate showed no ability for re-growth on solid medium (Figure 1c). Viable MM1 cells retain their characteristic green colour, whereas cells of non-viable cultures with a high degree of cell death bleach to a creamy colour with no further growth

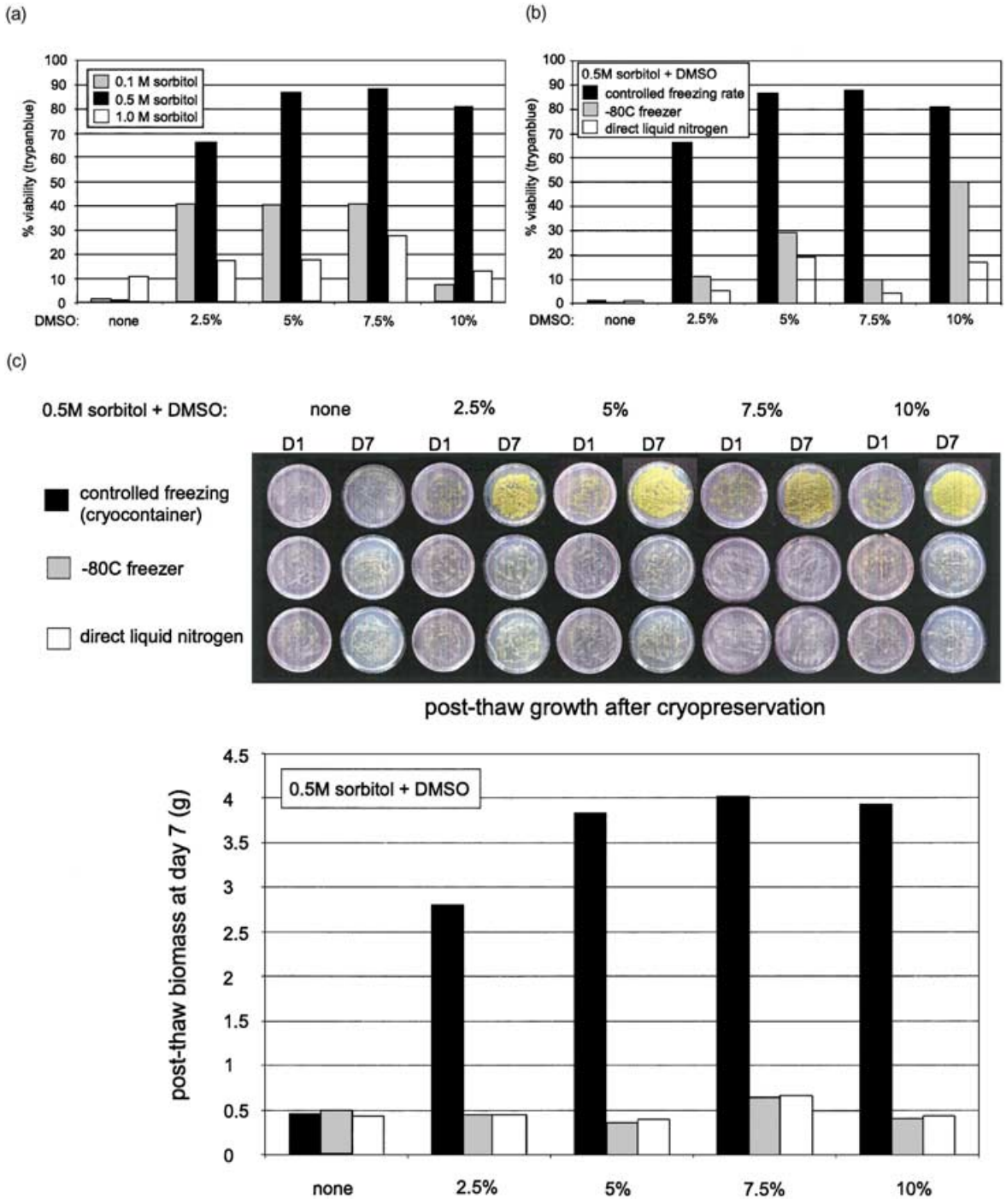

Figure 1. Post-thaw viability and growth of MM1 cell suspension after cryopreservation.
(a) Trypan blue viability test to determine the percentage of viable cells by microscopic examination in samples cryopreserved using concentration of sorbitol and DMSO as indicated, followed by a controlled slow freezing rate (0.5°C min^{-1}).
(b) Percentage of viable cells (Trypan blue test) in samples cryoprotected using 0.5 M sorbitol and DMSO concentrations as indicated using various freezing procedures (black bars, controlled slow freezing rate at 0.5°C min^{-1}; grey bars, storage at −80°C for 5.5 h prior to transfer at LN; and white bars, direct immersion in LN).
(c) Post-thaw growth ability of the same samples (as described in b) after transfer on solid medium. Post-thaw growth at days 1 and 7 (upper panel). Biomass (g) achieved 7 days post-thaw (lower).

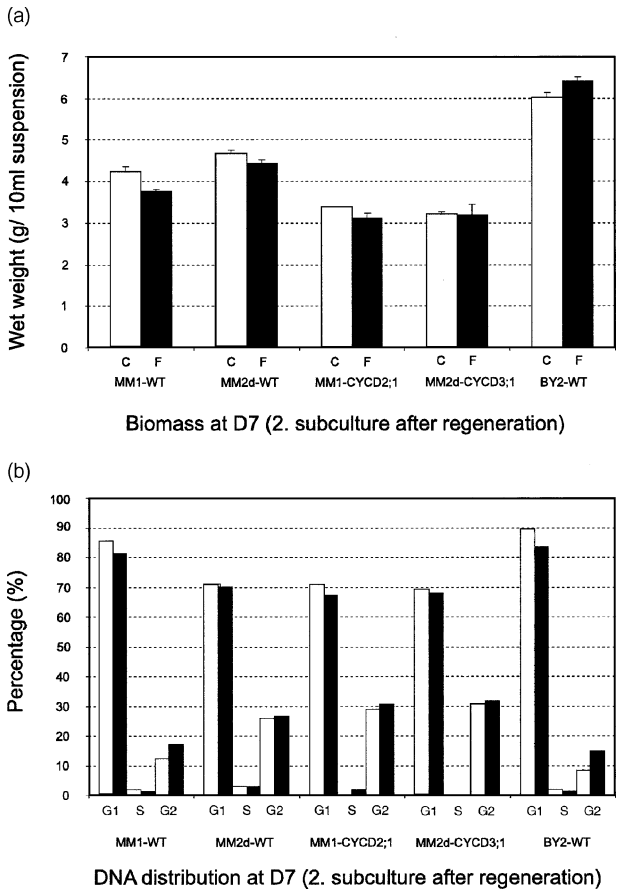

Figure 2. Growth characteristics of newly regenerated cell suspension after the second subculture into fresh medium post-thaw.
(a) Cell growth was followed of frozen/thawed cell lines (F, black bars) and directly compared to the untreated continuously subcultured control line (C, white bars) by inoculating 5 ml of an early stationary phase culture into 100 ml fresh MSS medium and incubation for further 7 days prior to determination of the reached biomass (wet weight).
(b) Determination of the DNA distribution by flow cytometry in the same samples as described in 'a' (black bars, frozen/thawed line; and white bars, control line).

visible until day 7 (Figure 1c). The bleached appearance of these cells was symptomatic of possible signs of cryoinjury, deplasmolysis and oxidative stress, and is already distinguishable from the controlled-rate frozen cells after a single day of culture (Figure 1c). In contrast, cells frozen with a controlled slow freezing rate resulted in significant rapid growth on solid medium (Figure 1c). Therefore, whilst the cell viability test can be useful as an indicator of potential survival of cells post-thaw, we conclude that the re-growth assay is essential for a reliable evaluation of full cell recovery. A summary of the biomass reached after re-growth for 7 days is given in Figure 1(c). As there is no significant difference in viability or post-thaw growth in MM1 cells protected with 0.5 M sorbitol using DMSO concentrations ranging between 5 and 10%, 5% DMSO was used for further experiments as the secondary cryoprotectant.

Cryopreservation of Arabidopsis *cell lines MM1, MM2d and transgenic derivatives*

Cells were transformed as described in Experimental procedures using *Agrobacterium*-mediated transformation and kanamycin selection to produce clonal derivatives. Derivatives of MM1 ectopically expressing the D-type cyclin *CYCD2;1* under the control of the CaMV 35S promoter and of MM2d similarly expressing *CYCD3;1* were produced. Cultures of MM1, MM2d, transgenic derivative lines (MM1-CYCD2;1 and MM2d-CYCD3;1) and a wild-type (WT) tobacco BY2 cell line were cryopreserved and stored for 1 month in LN and then re-grown as described in Experimental procedures, with the exception that BY2 cells were plated on BY2-agar supplemented with 0.2 mg l^{-1} 2.4-D post-thaw. All cell lines showed rapid growth post-thaw on solid medium (data not shown), and were transferred after 7 days to liquid culture. Microscopic examination revealed that these cells showed no obvious morphological differences compared to control lines that had been continuously subcultured for the same period of time. In each case, vigorously growing suspension cultured cells were observed 7 days after regeneration of liquid culture (data not shown).

Growth characteristics of regenerated cryopreserved cell suspensions

To assess possible detrimental effects of cryopreservation, the growth characteristics of recovered cryopreserved cells were compared to cells of untreated control lines. The cell lines MM1 and MM2d are normally subcultured after 7 days, by which time they have reached stationary phase. Cultures of cryopreserved cells reached approximately the same biomass (wet weight) as the control line, when measured 7 days after the second culture post-thaw into fresh liquid medium (Figure 2a). This further confirms the rapid post-thaw growth, and restoration of the same growth characteristics compared to lines not subjected to the freezing procedure within 2 weeks of liquid culture.

The different cell lines used have characteristic distribution of nuclear DNA content in stationary phase, reflecting cessation of division in different cell cycle phases. The nuclear DNA distribution of cryopreserved and control cultures were compared. No significant differences in the distribution of the DNA content was observed, either in total DNA content or in the distribution between different cell cycle phases (Figure 2b).

To identify whether cryopreserved transgenic cultures cells retain transgenes, the MM2d-CYCD3;1 cell line was checked for presence of the *CYCD3;1* construct by PCR (Figure 3a) and for growth ability in selective medium containing 100 μM kanamycin (Figure 3b). Again no significant differences in growth were observed compared to the

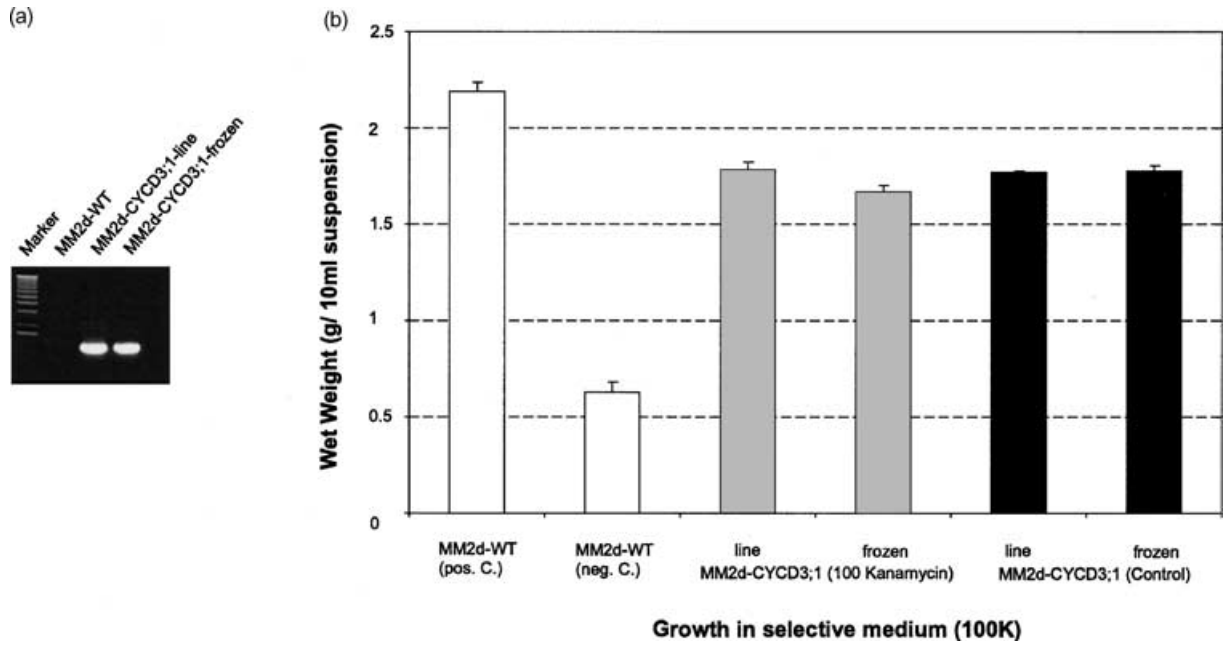

Figure 3. Characterisation of cryopreserved transgenic cell line MM2d-CYCD3;1.
(a) To confirm the existence of the introduced *At-CYCD3;1* gene construct, DNA was prepared and used in the PCR as described in Experimental procedures.
(b) Growth ability of MM2d-WT and transgenic MM2d-CYCD3;1 cell lines in selective medium (+kanamycin) compared to growth in medium lacking kanamycin, showing no difference between cryopreserved and non-frozen cultures.

control line, further confirming the maintenance of characteristics after freezing (Figure 3b). Similar experiments were performed using the MM1-CYCD2;1 overexpressing line to characterise transgenic properties after cryopreservation (data not shown).

We conclude that using the optimised cryopreservation procedure for *Arabidopsis* cell suspension cultures, cryoprotection and storage of cells in LN has no negative effect on growth characteristics or maintenance of transgenes.

Synchronisation of regenerated Arabidopsis *cell cultures after cryopreservation*

Arabidopsis cell suspension cultures have generally proved difficult to synchronise (Breyne and Zabeau, 2001; Callard and Mazzolini, 1997; Richmond and Somerville, 2000; Stals *et al.*, 2000). An important characteristic of the MM1 and MM2d cultures is their synchronisation ability (Menges and Murray, 2002). To assess whether this capacity is maintained in transgenic lines, and in control and transgenic lines after cryopreservation 4 weeks post-thaw, cell cultures were used for synchronisation experiments using aphidicolin (Menges and Murray, 2002). Removal of the toxin by washing allows synchronous resumption of the cell cycle from late G1/early S phase. The distribution of nuclear DNA content was analysed by flow cytometry over a period of 14 h by which time cells had completed S phase, G2, M and were re-entering G1 (Figure 4). As previously shown, MM1 and MM2d cells proceed synchronous through the cell cycle after block release (Menges and Murray, 2002). It is strikingly that MM1-CYCD2;1 cells proceed less synchronously through S phase compared to the WT line with a significant laggard population, whereas transgenic MM2d-CYCD3;1 cells rapidly and synchronously undertake S phase after removal of aphidicolin, and show up to 90% S phase synchrony. MM2d-CYCD3;1 cells then have an extended G2 phase, a phenomenon which is also seen in transgenic cell line MM1-CYCD3;1 (Samland *et al.*, manuscript in preparation) and in *CYCD3;1* overexpressing plants (Dewitte *et al.*, 2003).

We therefore observe characteristic differences in cell cycle distribution and progression between WT *Arabidopsis* cell lines and lines overexpressing different D-type cyclins. However, these behaviours are found to be identical between the same cell lines whether or not subject to cryopreservation, and all frozen cultures show identical synchronisation ability to their control culture. Moreover, tobacco BY2 cells that often show reduced growth rates after manipulations (see, for example, Nakagami *et al.*, 2002) show equal growth and synchronisation after cryopreservation. We conclude that cryopreservation and storage in LN has no negative effect on the synchronisation ability of *Arabidopsis* or tobacco BY2 cells after regeneration of non-transgenic or transgenic cultures.

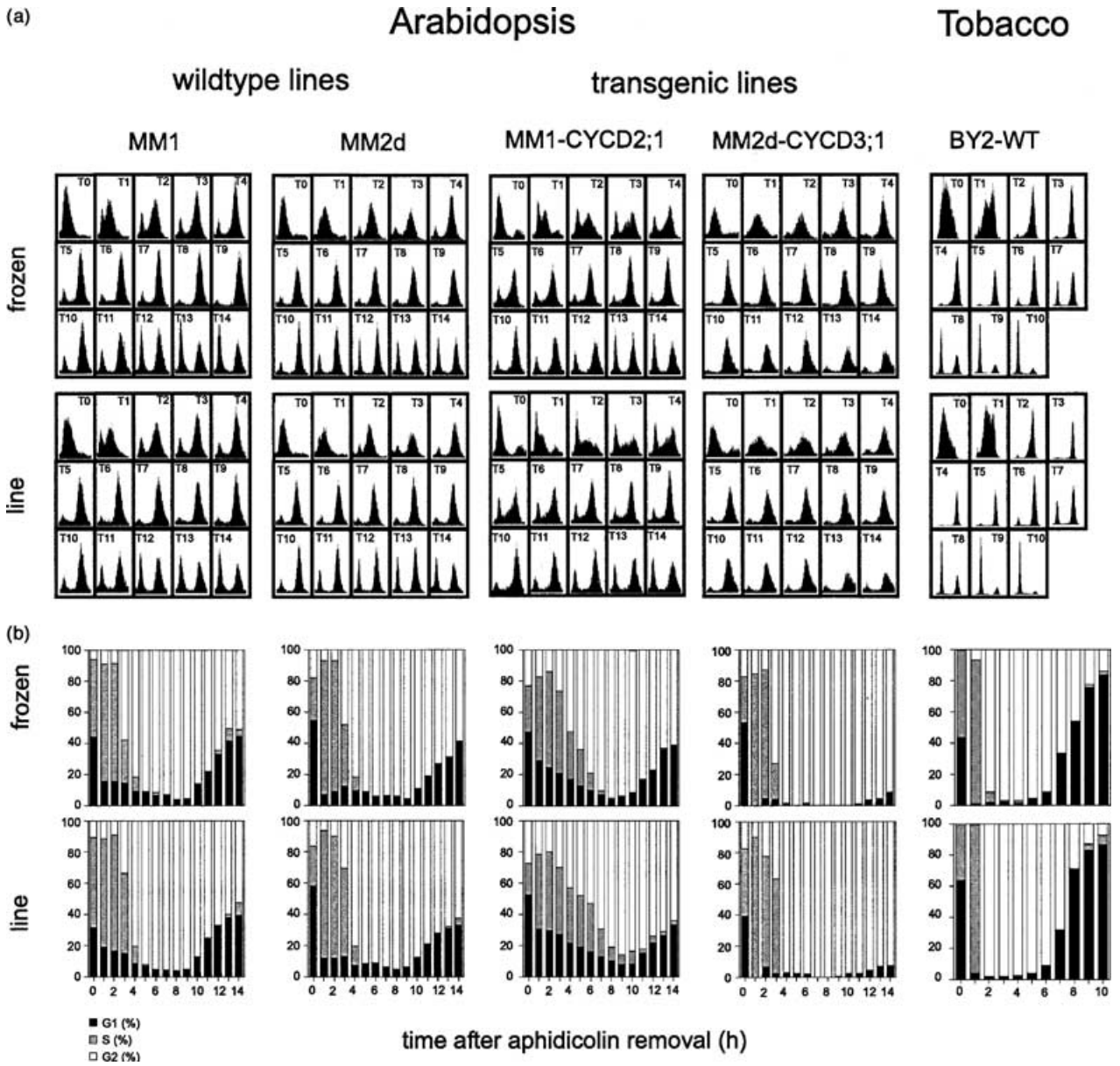

Figure 4. Aphidicolin block/release of regenerated *Arabidopsis* cell cultures after cryopreservation and storage in LN.
(a) Flow cytometry analysis after release of aphidicolin block in late G1/early S phase of regenerated cell lines MM1, MM2d, transgenic derivatives cultures (MM1-CYCD2;1, MM2d-CYCD3;1) and tobacco BY2 after cryopreservation compared to the appropriate control line (unfrozen) showing the coherent population of cells progressing synchronously through S and G2 phases. Each block represents a sample taken at hourly intervals from T_0 to T_{14}.
(b) DNA-histograms of flow analysis results in (a).

Discussion

Although cryopreservation procedures have been applied to cells and tissues from several plant species, including carrot, *Zea mays*, rice, periwinkle and tobacco (Chen *et al.*, 1984a; Finkle and Ulrich, 1982; Maddox *et al.*, 1983; Nag and Street, 1973; Withers and King, 1979), recommended cryopreservation conditions differ from species to species. In most cases, specialised equipment for controlled cooling is required, reported cell viabilities can be low and there is hence considerable risk that cultures recovered after cryopreservation may differ from the original. As a result, cryopreservation has not been adopted as routine by laboratories using plant suspension cultures. Here, we described a simple and effective cryopreservation method in LN for *Arabidopsis* cell lines MM1, MM2d and transgenic derivatives giving a high viability rate and rapid growth post-thaw after regeneration, resulting in recovered lines having identical characteristics to the parental line before freezing. We demonstrate the utility of this method for

synchronisable *Arabidopsis* cell lines MM1 and MM2d, and describe transgenic derivatives of these cells. We also show that the cryopreservation procedure is equally applicable to tobacco BY2 cells.

According to early reports of freezing protocols used in various plant species, exponential stage cultures are preferred as starting material (Kartha *et al.*, 1982; Kim *et al.*, 2001; Lynch *et al.*, 1994; Nag and Street, 1973, 1975a). Exponential growing cells are reported to survive the freezing–thawing procedure better than larger, more highly vacuolated cells because of their denser cytoplasm, smaller vacuole and hence relatively lower water content.

Previous work cites the use of a range of cryoprotectants for preservation, with sorbitol reported as the best cryoprotectant among the sugars tested (Chen *et al.*, 1984b; Gazeau *et al.*, 1998; Zhang *et al.*, 2001). Osmotica such as sorbitol acts to protect cells from freezing injury by providing conditions where cells develop maximum cytoplasmic content and minimum cell size. This can be achieved by pre-incubation of cells in media of enhanced osmotic potential (MSS + sorbitol) for 1–2 days (Chen *et al.*, 1984a,b; Maddox *et al.*, 1983; Zhang *et al.*, 2001). In periwinkle, sorbitol was reported to have toxic effects above a concentration of 0.5 M (Chen *et al.*, 1984b). Our analysis using a range of osmotic molarities (MSS + 0.1 to 1.0 M sorbitol) on *Arabidopsis* cells resulted in highest viability (up to 90%) using a concentration of 0.5 M sorbitol. Sorbitol is a non-penetrating cryoprotectant that lowers the cellular water content and reduces the rate of initial ice crystallisation during freezing (Chen *et al.*, 1984a). As a second treatment, DMSO was added after cooling cells to around 4°C. DMSO is a penetrating cryoprotectant that is believed to preserve the stability of vital cellular systems, reducing metabolic activity and toxic effects of the cryoprotectant and cellular dehydration on freezing (Finkle and Ulrich, 1982). Testing a range of DMSO concentrations between 0 and 10%, our analysis clearly demonstrated that 5% DMSO resulted in high viability and rapid growth.

It is believed that during the initial stages of freezing, a slower freezing rate of the extracellular solution reduces the stress on the plasma membrane. Additionally, dehydration by slow extracellular freezing minimises intracellular ice formation and hence protects against cryoinjury. Such partially dehydrated cells are reported to be able to survive the LN temperature (Chen *et al.*, 1984a,b; Maddox *et al.*, 1983; Withers and King, 1979). Freezing of cells with a controlled slow cooling rate has been reported using an apparatus that can reduce the temperature at the desired rate. To date, cooling rates between 0.5 and 2°C min^{-1} were used to freeze various plant species. We show that a slow freezing rate (approximately 0.5°C min^{-1}) can be achieved without the need to purchase expensive equipment by the simple expedient of placing a standard cryofreezing container inside a styrofoam (expanded polystyrene) box in a standard −80°C freezer. A further key step in the freezing phase is the temperature at which cells are transferred into the liquid phase of LN after the initial controlled slow freezing step. To date, there are various reports of so-called two-step freezing protocols that use a wide range of transfer temperatures, normally between −20 and −100°C (Chen *et al.*, 1984a; Jekkel *et al.*, 1989; Nag and Street, 1973, 1975a; Withers and Street, 1977). For convenience, we have chosen a transfer temperature of −80°C, which also avoids the risk of new ice crystal formation during the transfer into LN.

In contrast to the requirement for freezing at a slow controlled cooling rate, cell survival on thawing is best at the highest thaw rate (120°C min^{-1}). Rapid thawing was ensured by rapid transfer of vials from LN into a 40°C waterbath. This beneficial effect of rapid thawing has been reported by various groups (Jekkel *et al.*, 1989; Lynch *et al.*, 1994; Nag and Street, 1975b; Withers and Street, 1977).

During the post-thaw procedure, a minimum cell density is required for survival and re-growth as freshly frozen cells do not survive at low density (Maddox *et al.*, 1983). There are various reports about the concentration of cell suspension prior to cryoprotection, in a range of 10–60% packed cell volume (PCV; Benson *et al.*, 1992; Chen *et al.*, 1984b; Jain *et al.*, 1996; Lynch *et al.*, 1994; Maddox *et al.*, 1983). We concentrated cells to a PCV of 40% prior to cryopreservation. To avoid loss of viability because of rehydration/deplasmolysis injury, it is important to take special care in the treatment of cells directly post-thaw. It is reported that post-thaw washing of cells decreases their viability because of a rapid change of osmotic pressure (Watanabe *et al.*, 1999; Withers and King, 1979). The same effect is observed if cells are diluted too rapidly in an attempt to directly re-establish a liquid culture (Maddox *et al.*, 1983). Therefore, a compromise is necessary that minimises the time cells are exposed to toxic level of cryoprotectant. Other workers therefore used filter paper placed on solidified medium to separate toxic cryoprotectant in solution from cryopreserved cells (Lynch *et al.*, 1994). We used a nylon membrane to allow similar transfer onto a fresh agar plate after 3-h incubation period. The nylon membrane ensures good contact of cells with the solidified medium and additionally aids the loss of cryoprotectant from the recovering cell culture without the need to remove any excess of supernatant. Many groups reported low viability and long lag periods before rapidly growing cell suspension are recovered post-thaw (Jain *et al.*, 1996; Jekkel *et al.*, 1989; Kim *et al.*, 2001; Maddox *et al.*, 1983). However, following our approach, we clearly demonstrate that a significant rapid growth of freshly frozen cells was achieved and a liquid culture could

easily be regenerated after only 7 days post-thaw, which was ready to use for further characterisation experiments.

In conclusion, the work presented here provides a transformation procedure for the *Arabidopsis* cell lines MM1 and MM2d, and describes the development and use of an optimised and convenient cryopreservation procedure. This method offers particular utility for the generation and analysis of large numbers of transgenic cell lines for analysis of cell cycle phenotypes, as synchronisation potential is unaffected by the storage procedure. Cryopreservation of plant cell cultures using these methods is now a routinely used procedure in our laboratory for all cell lines generated.

Experimental procedures

Culture conditions

Suspension cultures of the fast-growing *Arabidopsis thaliana* cell line MM1 and MM2d (Menges *et al.*, 2002), selected from a cell suspension produced from Landsberg *erecta* stem explants by May and Leaver (1993) were used for transformation and cryopreservation experiments. For routine subculture, cell lines MM1, MM2d and transgenic derivatives were maintained in MSS medium containing 3% sucrose, 0.5 mg l^{-1} NAA and 0.05 mg l^{-1} kinetin as described previously (Menges and Murray, 2002). All medium used contains these additions unless otherwise stated. A suspension culture of tobacco BY2 was maintained as described by Nagata *et al.* (1992).

Transformation of Arabidopsis *cell lines MM1 and MM2d*

To stably introduce an overexpression construct (under the control of a 35S-promoter) into *Arabidopsis* cells, a modification of the *Agrobacterium*-mediated transformation method by Gallego *et al.* (1999) was used. The expression construct for *CYCD2;1* (pCEC35) carries the CYCD2;1 cDNA under a 35S promoter and leader sequence from cab22 in vector pTYG15 (den Boer, personal communication). The expression construct for *CYCD3;1* (pCRK9a) consists of the CYCD3;1 cDNA under the 35S promoter in vector pSLJ94 (Jones *et al.*, 1992; Samland *et al.*, manuscript in preparation). Both binary vectors carry for kanamycin resistance in *Arabidopsis*; full construction details are available from the authors.

For transformation, 2.5 ml of an early stationary phase cell suspension (7 days after previous subculture) was subcultured into 25 ml MSS medium (dilution 1 : 10) and incubated at 27°C, 130 r.p.m. in the conditions as described above for MM2d, except that the suspension was cultured in 100 ml narrow neck Erlenmeyer flasks for a period of 2 days. Ten millilitres of this exponential growing MM2d cell suspension was subcultured into 50 ml fresh MSS medium (dilution 1 : 5). This culture was used in part for *Agrobacterium* infection by co-cultivation and in part for production of conditioned medium. For each transformation, 10 ml of the diluted (1 : 5) MM2d cell culture was transferred into a 250-ml Erlenmeyer flask, acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone, Acros Organics, Loughborough, UK) was added to a concentration of 500 μM, and the cell suspension was infected with 100 μl of diluted (1 : 10) *Agrobacterium* culture. To prepare the *Agrobacterium* culture, 100 μl of an exponential growing culture of Luria-Bertani medium (LB)4404 containing the 35S:CYCD3;1 construct (OD_{600nm} between 1 and 1.5) was washed three times with 1 ml MSS-medium by centrifugation (14 926 ***g*** per 2 min) and re-suspended in 900 μl MSS-medium (dilution 1 : 10 relative to the harvested *Agrobacterium* culture). The *Agrobacterium*-infected MM2d culture was incubated in darkness at 27°C without agitation for 2 days. To obtain conditioned medium, the remaining diluted cell suspension was incubated at 130 r.p.m., 27°C in the dark for a further 2 days.

To prepare conditioned medium, cells were cleared from the medium by centrifugation (387 relative chemical force (rcf) per 5 min, without brake). The infected cell culture was transferred into a 50-ml Falcon tube and centrifuged for 5 min at 387 rcf, without applying brake force. The cells were then gently washed three times with 50 ml fresh MSS medium (387 rcf per 5 min, without brake) and finally re-suspended in 20 ml of the conditioned medium. Timentin (ticarcillin/clavulanic acid, 1500/100 mg; Duchefa Biochemie BV) was added to a concentration of 500 μg ml^{-1} to select against remaining agrobacteria, and the putative transformed MM2d cells were incubated for further 3 days rotating at 130 r.p.m., 27°C in the dark in a 250-ml Erlenmeyer flask.

The cell suspension was then transferred into a 50-ml Falcon tube, centrifuged for 5 min at 387 rcf, without applying brake force and re-suspended in 10 ml fresh MSS medium (supplemented with 50 μg per 10 ml NAA and 5 μg per 10 ml kinetin). Cell suspension (2.5 ml) was spread on each plate containing 0.8% MSS-agar (MSS-medium supplemented with 0.8% Bacto™ Agar, containing no additional NAA or kinetin), 250 μg ml^{-1} timentin and 100 μg ml^{-1} kanamycin. Plates were sealed with porous tape (3M, Micropore™, Bradenell, UK) and incubated in darkness, at 27°C. Initially an apparent lawn of growing cells is seen, which resolves into distinct calli. After 2–3 weeks putatively transformed callus material was transferred with a Gilson pipette tip onto new 0.8% MSS-agar plates containing hormones (250 μg ml^{-1} timentin, 100 μg ml^{-1} kanamycin, 0.5 mg l^{-1} NAA and 0.05 mg l^{-1} kinetin) and allowed to grow for a further 1–2 weeks. Note that only one clone should be chosen from each transformation to ensure independent origins of the transformation event. The same procedure can be followed with MM1 except cultures are incubated under light conditions normally uses for cell line MM1 (see Menges and Murray, 2002). In our hands, both cell lines, MM1 and MM2d are equally transformable using the procedure described above, but it is important to adhere to the details given regarding flask volumes, cell treatments and conditions.

To monitor transformation efficiencies, a construct containing the yellow fluorescent protein (YFP) reporter gene was used, which resulted in reproducibly up to 50% of transient gene expression after 48 h of co-cultivation with agrobacteria harbouring this construct (data not shown).

Establishment of transgenic liquid cultures

To re-establish liquid cultures, callus tissue was transferred to a 55-mm Petri dish, carefully squeezed with a 200-μl (yellow) micropipette tip and re-suspended in 10 ml fresh MSS medium (with 100 μg ml^{-1} kanamycin). Callus-derived cell suspensions were incubated under normal cultivation conditions in 100 ml Erlenmeyer flasks. After 10 days of cultivation, a further 10 ml MSS medium was added to the suspension cultures and left for further 7 days. Five millilitres of each putative transgenic cell suspension was then subcultured into 50 ml fresh MSS medium (100 μg ml^{-1} kanamycin). This subculturing procedure was repeated two times at 7-day intervals in the same medium. Subsequent subculturing was carried out weekly into fresh MSS medium (without

kanamycin), adjusting the transferred volume of cells to produce an inoculum of approximately the same cell number as used when diluting WT-MM2d culture (in which case 3.5 ml is weekly subcultured into 100 ml fresh medium). For example, putative transgenic MM2d-CYCD3;1 cell suspensions were maintained by subculturing weekly 3.5 ml into 100 ml fresh MSS medium (0.5 mg l^{-1} NAA and 0.05 mg l^{-1} kinetin) in 300 ml Erlenmeyer flasks.

To stably introduce a CYCD2;1 construct into MM1 cells and to establish liquid cultures of putative transgenic calli, the same procedure was followed with the exception that the culture conditions were as described for MM1 cells (23°C, 120 r.p.m., continuous light; Menges and Murray, 2002).

Cryopreservation of Arabidopsis *suspension cell cultures*

Five millilitres of early stationary phase cell suspensions (7 days after previous subculture) were pre-cultured in 100 ml fresh MSS medium under conditions as described above. After 3 days incubation, cell suspensions were transferred into 50 ml Falcon tubes and cells were harvested by centrifugation (387 rcf, 1 min, without brake). The weight of the cell pellet was determined and an appropriate volume of MSS medium (containing 0.5 M D-sorbitol; Sigma, Poole, UK) was added to reach a PCV of approximately 40%. Re-suspended cells were transferred into a 100-ml Erlenmeyer flask and incubated for a further 2 days. Cell suspensions were then pre-cooled for 25 min at 4°C (coldroom) with rotation at 140 r.p.m. in ambient light (for cultivation of dark-grown cell lines, Erlenmeyer flasks were covered with aluminium foil). An appropriate volume of pre-cooled DMSO (Sigma, Poole, UK) was added to reach a concentration of 5% DMSO. Cell lines were incubated for a further 1 h at 4°C (coldroom) with rotation at 140 r.p.m. Cell suspension (1.8 ml) was transferred each into 2 ml cryovials (Cat. No. E31100012, Starlab, Helsinki, Finnland, UK). The vials were placed into a cryofreezing-container (Nalgene™ Cryo 1C Freezing container, Cat. No. 5100-0001, Fisher Scientific, Loughborough, UK) and the container was stored in a styropore box chosen to reduce the cooling rate from −1 to approximately −0.5°C min^{-1} during the incubation period at −80°C. A standard box of about 2 l volume (width, 14 cm; length, 14 cm; height, 10 cm) and 2.5 cm wall thickness as used for the delivery of biological supplies on dry ice was found to be suitable. After cooling of cells for at least 4 h at −80°C, vials were immediately plunged in the liquid phase of LN and stored under LN (−196°C).

Recovery of viable cells and regeneration of cell suspension

Cryovials were placed immediately from LN into a waterbath at 40°C for 3 min. Thawed culture was then spread on a nylon membrane (CellMicroSieves™, 70 μm pore size, BioDesign Inc., NY, USA) which was placed on a plate containing 0.8% MSS-agar (MSS medium, containing 3% sucrose, 0.5 mg l^{-1} NAA and 0.05 mg l^{-1} kinetin, supplemented with 0.8% Bacto™ agar). Plates were sealed with tape (3M, Micropore™) and incubated for 3 h either under continuous light conditions (1300 lux) at 23°C (MM1 and derivative) or at 27°C in the dark (MM2d and derivative). The filter carrying the spread cell suspension was then transferred onto a fresh MSS-agar plate and incubated as above. After 7 days of growth on solid medium, recovered cell callus material was re-suspended in 50 ml fresh MSS medium and transferred into a 100-ml Erlenmeyer flask. Cells were rotated at 120 r.p.m. (MM1 and derivative) or 130 r.p.m. (MM2d and derivative) and incubated further as described above.

PCR analysis of regenerated transgenic cell line

Genomic DNA was prepared from *Arabidopsis* cell line MM2d-WT and transgenic derivatives using the Phytopure plant DNA extraction kit (Nucleon Biosciences, Lanarkshire, UK) according to the manufacturer's instructions. PCR analysis was performed using 35S (GTGGATTGATGTGATATCTCC) and *CYCD3;1* (CATTAACTCTATCAAGCCATGGCAC) primers to amplify the transgenic CYCD3;1 insert in cell lines MM2d (94°C for 5 min, followed by 35 cycles of 94°C (30 sec), 55°C (1 min), 72°C (1 min) and then 72°C for 5 min).

Aphidicolin-induced synchronisation in cryopreserved cell cultures

Arabidopsis MM1, MM2d and transgenic cells were reversibly blocked in late G1/early S phase with aphidicolin as described by Menges and Murray (2002). Tobacco BY2 cells were synchronised according to Nagata *et al.* (1992). To determine nuclear DNA content, a sample of frozen cell pellet was treated to release nuclei and analysed by flow cytometry as described by Menges and Murray (2002).

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