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MOLECULAR FARMING

Continued expression of plant-made vaccines following long-term cryopreservation of antigen-expressing tobacco cell cultures

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Abstract Production of vaccines in plant cells provides an alternative system that has several advantages when compared to current vaccine production methods. Establishment of stable seed stocks for a continuous supply of a vaccine is a critical part of production systems. Therefore, a vitrification method for cryopreservation was applied to non-transgenic and three different antigen-expressing transgenic *Nicotiana tabacum* (NT-1) lines. Preculture of the suspension cultures 1 d prior to vitrification was sufficient for cell survival through the cryopreservation process. Inclusion of 0.3 M mannitol in the preculture medium was necessary for maintenance of cell viability. Cultures were also treated with and without heat shock prior to vitrification, and it was found that heat shock was unnecessary for growth recovery post cryopreservation. All cultures survived storage in liquid nitrogen at intervals ranging from 1 h to 1 yr. Antigen expression was measured by enzyme-linked immunosorbent assay for cultures that grew post cryopreservation and those that had never been cryopreserved. Expression levels in cultures derived from cryopreserved material were comparable to cultures that had not been cryopreserved. Transmission electron microscopy showed that the integrity of the cell structure was maintained post cryopreservation.

Keywords LT-B · Newcastle disease virus · NT-I · Suspension cultures · Vitrification

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Introduction

Transgenic plant cell culture is an attractive alternative for production of homologous proteins, such as pharmaceutical proteins, because it circumvents the presence of contaminants associated with mammalian or other culture systems. To conform to existing regulatory requirements, plant cell cultures expressing pharmaceutical proteins would be required to be derived from a consistent and stable master stock. The application of cryopreservation protocols offers a convenient method for stabilizing primary cell lines for timely recovery and seeding of new cultures.

Cryopreservation is the storage of biological material at ultra-low temperatures generally at −196°C by reduction or controlled cessation of all biochemical and physical processes. This method is often employed to reduce the costs and labor associated with maintenance of valuable *in vitro* cultures of plant, insect cell, or mammalian origin. Cryopreservation provides a method for retaining the original form and characteristics of master stock materials for revival and production of consistent and repeatable batches at a later time.

The process of cryopreservation can include pretreatments such as heat shock (Reinhoud et al. 1995), desiccation (Zhang et al. 2001), cold acclimation (Leunufna and Keller 2003), and vitrification of live cells (Reinhoud et al. 1995; Sarkar and Naik 1998). Vitrification occurs when the intracellular water of the plant tissue is converted to a frozen, glasslike state, thus preventing the formation of lethal ice crystals during the freeze/thaw process. When the sample is thawed, biological activity resumes. Our experiments were based on a report by Reinhoud et al. (1995), which demonstrated that both two-step freezing and vitrification methods work well with robust tobacco cell lines. The advantages of vitrification as opposed to the two-

step method are that the required preculture time is less, there is no need for expensive step-cooling apparatus, higher survival rates, and faster recovery.

Our studies with antigen-expressing transgenic cell lines focused on vitrification followed by storage under liquid nitrogen for periods ranging from 1 h to 1 yr. Antigen expression was tested following regrowth of cells from the various freeze intervals, and we found that the cells from all intervals continued to express the antigen at levels comparable to those that did not undergo cryopreservation.

Materials and Methods

Cell cultures. We generated antigen-expressing transgenic lines of *Nicotiana tabacum* L., line NT-1 (Paszty and Lurquin 1987) by *Agrobacterium tumefaciens*-mediated transformation (Smith 2002; Smith et al. 2002). The gene constructs used for transformation were designated CLT105, CLT101, and CHN. CLT105 contained cassettes for expression of the *Escherichia coli* heat labile enterotoxin (LT) protein, which is a multi-subunit protein that contains one A subunit (LT-A), which is toxic, and five B subunits (LT-B) (Sixma et al. 1991). The LT-A subunit coding sequence was modified by mutation of G192, which greatly reduces the toxicity of LT-A. CLT101 also contained cassettes for expression of the *E. coli* LT-A and LT-B subunits, however, in this construct there were no mutations made in the LT-A coding sequence, which results in the native, fully active protein. The CHN construct contained a cassette for expression of the Newcastle disease virus hemagglutinin-neuraminidase (HN) protein (Huang et al. 2004). The constitutive cassava vein mosaic virus promoter was used in all constructs to drive expression of the proteins (Verdaguer et al. 1996). The gene for phosphinothricin acetyltransferase (*pat*) from *Streptomyces viridiochromogenes* was used as the selectable marker in all constructs (Wohlleben et al. 1988). It confers resistance to the herbicide component phosphinothricin and its derivatives such as bialaphos, which is a nonselective herbicide known as Herbiace (Meiji Seika, Japan) (Ziemienowicz 2001). We used 3 mg/l of bialaphos (Gold Biotechnolgy, St. Louis, MO) as the selection agent in all culture media used for the transformations, maintenance of the transgenic lines that were generated, and cultures that recovered following cryopreservation.

Three antigen-expressing cell lines (CLT105-48, CLT101-14, and CHN-18) generated after transformation of the NT1 cell culture line were used for the cryopreservation studies. We investigated the effect of three different culture media on the growth of non-transgenic NT-1 cell lines prior to the initiation of the cryopreservation studies. The three different liquid culture media were designated LSg, KCMS, and NT-1. LSg was a modified Linsmaier and Skoog (LS) (1965) medium that included LS salts (PhytoTechnology Laboratories, Shawnee Mission, KS) and vitamins, 30 g l^{-1} glucose (Sigma-Aldrich, St. Louis, MO), and 0.05 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich). The pH of the medium was adjusted to 5.8. KCMS contained Murashige and Skoog (MS) (1962) salts (Caisson Laboratories, Sugar City, ID), 1.3 mg l^{-1} thiamine (Sigma-Aldrich), 200 mg l^{-1} KH_2PO_4 (Fisher Scientific, Pittsburgh, PA), 30 g l^{-1} sucrose (grade II; PhytoTechnology Laboratories), 0.2 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin (PhytoTechnology Laboratories). NT-1 contained MS salts, 180 mg l^{-1} KH_2PO_4, 0.5 mg l^{-1} 2-N-morpholinoethanesulfonic acid (MES; Sigma-Aldrich), 1 mg l^{-1} thiamine, 100 mg l^{-1} myoinositol (Sigma-Aldrich), 30 g l^{-1} sucrose, and 2.21 mg l^{-1} 2,4-D. The pH of KCMS and NT-1 was adjusted to 5.7.

All suspension cultures were maintained in the dark at 25°C on an orbital shaker at 100 rpm.

Preculture, heat shock, and mannitol concentration. Three days after subculture (late exponential growth period), the cells were precultured for 1, 2, or 3 d in KCMS or NT-1 liquid medium. To determine the effect of mannitol concentration, the media were supplemented with either 0.1 or 0.3 M mannitol (Fisher Scientific) for 24 or 72 h.

To test the effects of a heat shock, precultured material was placed on an orbital shaker at 100 rpm at 37°C for 2 h. They were transferred back to the shaker at 25°C for 4 h before vitrification.

Vitrification. We used a modified version of the method reported by Reinhoud et al. (1995). The vitrification solution designated PVS2/100% contained 30% glycerol (Sigma-Aldrich), 15% ethylene glycol (Sigma-Aldrich), and 15% DMSO (Sigma-Aldrich) in a 0.4 M sucrose solution. A PVS2/20% solution was made by diluting PVS2/100%. Both solutions were adjusted to a pH of 5.8, autoclaved, then stored at 4°C.

To start the vitrification process, 4 ml of ice-cold PVS2/20% was added to a 1 ml settled cell volume. The preparation was incubated on ice for 5 min, then 1 ml of cold PVS2/100% was added at five 1-min intervals for a total of 9 ml. The preparations were centrifuged for 1 min at 7,500–8,000 rpm. The supernatant was discarded, and 0.5 ml of PVS2/100% was added at two 1-min intervals while the cells remained on ice. Then 1 ml of PVS2/100% was added three times at 1-min intervals. Three milliliters of this mixture was then transferred into six 0.5 ml cryogenic straws (Continental Plastic Corporation, Delavan, WI). The straws were heat sealed at each end with hot forceps, and immersed immediately into liquid nitrogen.

Recovery. Vitrified material was stored in liquid nitrogen for 1 h, 1 d, 1 wk, 1 mo, 3 mo, 6 mo, 9 mo, and 1 yr. For thawing, straws were immersed in a 40°C water bath for 3–5 s, then immediately diluted with 7 ml of cold 1.2 M sucrose. The cells were kept on ice for 20 min, then centrifuged for 3 min at 7,500–8,000 rpm. Cells were transferred to two layers of filter paper (42.5 mm Whatman) that were overlain on NT-1 solidified medium containing 0.75% agarose (Invitrogen Life Technologies, Carlsbad, CA) in 60×15 mm tissue culture plates. The cultures were maintained at 25°C in the dark. Two days after plating, the filter papers containing the cells were transferred to fresh NT-1 solidified medium. Transgenic lines were initially plated on medium without a selection agent until cell growth was well established and covered a small plate. They were then transferred to a selective medium containing 3 mg/l bialaphos.

Thereafter, the cells were transferred at approximately 2-wk intervals to fresh culture medium. When cell growth nearly covered the surface of the medium in the plate, the cells were removed from the filter paper and transferred to larger plates (100×15 mm). When the callus again covered the plate, the cells were transferred to NT-1 selective medium solidified with 8 g l^{-1} Agar (Sigma-Aldrich) for maintenance.

Protein extraction and ELISA. Each cell culture sample was weighed and added to a microcentrifuge tube (item # 02-681-344, Fisher Scientific) prior to disrupting the cells in ice-cold extraction buffer at pH 7 [10 mM sodium phosphate (Fisher Scientific), 100 mM NaCl (Fisher Scientific), 1 mM EDTA (Fisher Scientific), 0.1% Triton X-100 (Fisher Scientific), 10 µg/ml Leupeptin (Sigma-Aldrich), and 50 mM sodium ascorbate (Sigma-Aldrich). To disrupt the cells, 0.5 ml of 710–1,180 µm acid washed beads (#G-1152, Sigma-Aldrich) and 1 ml of ice-cold extraction buffer were added to each sample. The prepared tubes were placed in a Fast-Prep machine (Bio 101 Savant; Savant Instruments, Inc., Holbrook, NY) for 30 s at speed 5. The samples were placed on ice for 5 min.

This was followed by centrifugation at 14,000 rpm at 4°C for 2 min. The supernatant was diluted 1:25 for antigen analysis. Total soluble protein (TSP) was measured by a Bradford protein assay using the Bio-Rad Protein Reagent (Bio-Rad Laboratories, Hercules, CA; Bradford 1976).

LT-B and HN transgene expression and antigen accumulation were determined using antigen-specific enzyme-linked immunosorbent assay (ELISA). For LT-B, 96-well high-binding polystyrene plates (Fisher Scientific) were coated with 100 µl of gangliosides, (EMD Biosciences, San Diego, CA) diluted to 50 µg/ml (stock was 25 mg/ml) in borate (Sigma-Aldrich) buffer pH 9, and incubated overnight at room temperature. They were washed three times with phosphate-buffered saline (PBS), pH 7.4 (all components purchased from Fisher Scientific, 140 mM sodium chloride, 1.5 mM monobasic potassium phosphate, 4 mM dibasic sodium phosphate, 3 mM potassium chloride, and 3 mM sodium azide).

The plates were blocked with 200 µl of 5% skim milk (Difco product; Voigt Global Distribution Inc. Lawrence, KS) in PBS-T [PBS + 0.05% Tween 20]. They were then incubated for 2 h at 37°C, and washed three times with PBS-T. Samples were diluted in 5% milk and LT-B standard (gift from Benchmark Biolabs, Lincoln, NE) diluted to 50 ng/ml (1:20,000, stock was 1 mg/ml), and 100 µl of each sample were added to each well. Plates were washed three times with PBS-T. The primary antibody, rabbit anti-LT-B (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was diluted 1:300,000 in 5% milk, added to the plates, and they were incubated for 1 h at 37°C, and followed by three washes with PBS-T. The goat anti-rabbit IgG (H&L)-horseradish peroxidase (HRP; Kirkegaard and Perry Laboratories) conjugate was diluted 1:2,000 conjugate with 5% milk, and added to the plates. The plates were incubated for 45 min at 37°C, and washed three times with PBS-T. Fifty microliters of one component TMB substrate (Kirkegaard and Perry Laboratories) was added and incubated at room temperature for 20 min. Fifty microliters of TMB stop solution (Kirkegaard and Perry Laboratories) was added and the OD values were read at 450 nm.

For HN quantitation, plates were coated with 100 µl/well SPAFAS chicken anti-Newcastle disease virus (NDV) polyclonal antibody (1:1,000) (gift from Benchmark Biolabs) in 0.01 M borate buffer, and incubated overnight at 2–7°C. Plates were equilibrated to room temperature for 30 min, washed three times with PBS-T at 300 µl/well, and blocked with 200 µl/well of 5% skim milk in PBS-T. The plates were incubated for 2 h at 37°C and washed one time with PBS-T at 300 µl/well. Inactivated NDV virus (gift from Benchmark Biolabs) was used as the reference antigen (diluted 1:25 in blocking buffer) at 100 µl/well was added to designated wells, and 100 µl of 5% skim milk in PBS-T was added to each of the remaining wells. Twofold serially diluted reference antigen samples were loaded down one side of each plate (7.313–234 ng/ml). The plates were incubated for 1 h at 37°C, and washed three times with PBS-T at 300 µl/well. The primary antibody, HN Mab 4A (gift from Benchmark Biolabs; diluted 1:100 in blocking buffer), was added at 100 µl/well. Plates were incubated for 1 h at 37°C, and washed three times with PBS-T at 300 µl/well. The conjugate, goat anti-mouse IgG (H&L) HRP (Kirkegaard and Perry Laboratories) diluted (1:3,000) in blocking buffer, was added at 100 µl/well. Plates were incubated for 1 h at 37°C and washed three times with PBS-T at 300 µl/well. The ABTS substrate (Kirkegaard and

Perry Laboratories) was added at 100 µl/well, plates were incubated for 30 min at room temperature for color development, and OD values were read at 405 nm.

Gelling agent comparison. Cells were thawed and plated onto NT-1 solidified medium containing either 0.75% agarose or 0.8% Agar. They were plated onto filter paper overlain on the medium in 100×20 mm Petri plates and cultured in the dark at 25°C.

Transmission electron microscopy. CHN-18 suspension cultures derived from callus that had been frozen for 1 yr and a CHN-18 control (no cryopreservation) were initiated for material for transmission electron microscopy (TEM). Two weeks after initiation, cultures were sent to the Cornell Integrated Microscopy Center (Cornell University, Ithaca, NY 14853; http://www.cimc.cornell.edu/) for TEM.

The source of materials used for TEM was Electron Microscopy Sciences, Hatfield, PA (www.emsdiasum.com). For primary fixation, one-part cells including media were mixed with one part 4% glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 6.8 and incubated for 0.5 h at room temperature followed by 1.5 h at 4°C. They were rinsed three times in buffer (0.1 M sodium cacodylate, pH 6.8) at 4°C with each rinse lasting 10 min. A secondary fixation consisted of 2% osmium tetroxide for 1 h at room temperature followed by a rinse as previously described. The samples were dehydrated in a graded ethanol series: 10%, 30%, 50%, and 70% ethanol for 10 min each at 4°C. This was followed by a second series of 90%, 100%, and 100% ethanol for 10 min each at room temperature.

The following steps were followed at room temperature for gradual infiltration with Spurr's resin:

> 4 parts ethanol:1 part resin, 2 h; 2 parts ethanol:1 part resin, 3 h; 1 part ethanol:1 part resin, overnight; 1 part ethanol:2 parts resin, 3 h; 1 part ethanol:4 parts resin, 4 h; pure resin for overnight; pure resin + accelerator for 3 h. Samples were placed in molds and cured in a 70°C oven, overnight. Thin (~70 nm) sections were taken using a Reichert Ultracut E ultramicrotome and were contrasted using uranyl acetate and lead citrate. Sections were viewed on a Tecnai 12 Biotwin transmission electron microscope, and digital images were taken using a Gatan Multiscan camera, model 791.

Results and Discussion

Protein vaccine antigens and antigen expressing-NT1 cell lines. E. coli heat-labile LT enterotoxin elicits severe diarrhea in humans and some animals. LT is a protein-based toxin that is composed of a penteric ring of 11.6 kDa identical B subunits (LT-B) and one 27 kDa A subunit (LT-A), which contains a toxic ADP ribosyl transferase activity (Spangler 1992). The five B subunits are non-toxic and are only responsible for binding of the holotoxin to ganglioside receptors such as GM1, which is found on cell membranes of eukaryotic cells. The antigen-expressing cell culture line, CLT105-48, was recovered following transformation of the NT1 cell culture line with a gene construct that contained cassettes for expression of the *E. coli* heat labile enterotoxin (LT) protein; however, the LT-A subunit coding sequence was modified by mutation of G192, to reduce the toxicity. The antigen-expressing line designated CLT101-14 also contained cassettes for expression of the *E. coli* LT-A and LT-B subunits, however, no mutations were made in the LT-A coding sequence as was the case with the CLT105 construct. This results in expression of the native, fully active protein.

Newcastle disease virus belongs to the family Paramyxoviridae and is an important pathogen of many bird species. It can have devastating effects on the commercial poultry industry throughout the world. The surfaces of NDV particles contain two functional glycoproteins, which are the fusion (F) and hemagglutinin-neuraminidase (HN) proteins (Huang et al. 2004). HN is an approximately 74 kDa protein that contains both the receptor recognition and neuraminidase (NA) activities of the virus, and plays a critical role in infection. The transgenic cell line, CHN-18, expresses the HN protein.

Effect of culture media. Initially, we tested growth of non-transgenic cell lines in three different liquid culture media designated LSg, KCMS, and NT-1 prior to cryopreservation studies of antigen-expressing cell lines. The LSg medium had a negative effect on the growth of non-transgenic cell cultures, whereas, there was vigorous growth observed on KCMS and NT-1 media. The most likely reason the cell cultures did not grow well on LSg medium as compared to KCMS and NT-1 had to do with the concentration of plant growth regulators. LSg medium contained only 0.05 mg l^{-1} 2,4-D, whereas, KCMS medium contained 0.2 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin, and the NT-1 medium contained 2.21 mg l^{-1} 2,4-D. The higher concentrations of 2,4-D in the NT-1 and KCMS media, which also contained kinetin, contributed to the vigorous growth of the cell cultures as compared to the non-vigorous growth on LSg. The selection of growth regulator and use of an optimized concentration or combination of growth regulators has been shown to influence the growth of cell cultures (Dodds and Roberts 1988).

Cells precultured in KCMS and NT-1 survived the freeze/thaw process, however, those precultured in LSg did not. Therefore, in all subsequent experiments the cell

Figure 1. Comparison of the effects of NT-1 medium containing agar and agarose on the regrowth of CHN-18 callus cultures following cryopreservation. *Left* Callus growth on agar-containing medium. *Right* Callus growth on agarose-containing medium.

lines were precultured in either KCMS or NT-1 liquid medium depending upon the medium used for previous maintenance of the specific lines.

Effect of vitrification, preculture time, and heat shock treatments. All initial experiments had been conducted with a 3-d preculture to allow comparison of vitrification with the two-step method, which requires a 3-d preculture. All cell lines tested were vitrified for 1 h and survived the freeze/thaw process as determined by the recovery of cell growth. For these initial experiments, we also included control samples treated and not treated with cryoprotectants. We did not observe any negative effects of the cryoprotectants on cell regrowth, therefore, a cryoprotectant control was not included in future experiments.

The second series of experiments focused on whether it was possible to reduce the preculture time from 3 d, eliminate the heat shock treatment, and then simultaneously eliminate both treatments. We looked at 1, 2, and 3 d of preculture, and found that reducing the preculture to 1 d, eliminating the heat shock treatment, and simultaneously eliminating both steps did allow recovery of all cell lines. Reinhoud et al. (1995) also reported that when using the vitrification method, a 1-d preculture would be sufficient, and that the heat shock treatment was unnecessary for cell lines precultured during the optimal growth phase (late exponential growth phase).

Effect of mannitol concentrations. We compared the effects of 0.1 M and 0.3 M mannitol in preculture medium on the survival of cells post cryopreservation. Cell growth routinely began 1–4 wk after thawing, and the cultures from the mannitol concentration comparison were maintained for 2 mo. However, cells precultured in medium that contained 0.1 M mannitol did not survive after cryopreservation, therefore, we continued to preculture in medium containing 0.3 M mannitol.

Effect of gelling agent. Based on a report by Reinhoud et al. (1995), we used 0.75% agarose as the gelling agent in the NT-1 medium used for cell recovery following cryopreservation. However, agarose is costly at approximately three times the price of agar. Therefore, to develop a method that would be more cost-effective, we did a comparison of cell regrowth of control NT-1 cultures that were vitrified, thawed, and plated onto NT-1 medium solidified with either agarose or agar. Cells plated on

Table 1. LT-B antigen expression determined by ELISA at various time points post cryopreservation

Cell line	Treatment	ng LT-B/g cells[x]	µg TSP/g cells
NT-1 control	None	0	128.57
CLT101-14	No cryopreservation	4,968 (0.04)[y]	307.84
	1 h	2,230 (0.01)	314.84
	1 d	NA	–
	1 wk	4,030 (0.04)	156.27
	1 mo	NA	–
	3 mo	2,869 (0.01)	67.73
	6 mo	4,927 (0.03)	110.19
	9 mo	4,163 (0.01)	192.77
	1 yr	4,786 (0.04)	96.16
CLT105-48	No cryopreservation	8,015 (0.04)	58.02
	1 h	NA	–
	1 d	5,153 (0.05)	125.21
	1 wk	3,218 (0.03)	171.33
	1 mo	NA	–
	3 mo	3,567 (0.02)	180.63
	6 mo	4,379 (0.08)	281.54
	9 mo	4,451 (0.05)	222.70
	1 yr	NA	–

TSP total soluble protein

[x] Average of three technical replicates

[y] Standard deviation

Table 2. HN antigen expression in CHN-18 determined by ELISA at various time points post cryopreservation

Treatment	ng HN/g cells[x]	µg TSP/g cells[z]
Control[x]	15,180 (0.44)[y]	100
1 d	24,016 (0.20)	131
1 wk	21,899 (0.17)	104
1 mo	18,002 (0.33)	129
3 mo	16,124 (0.36)	123
6 mo	22,555 (0.25)	97
9 mo	8,958 (0.01)	141
1 yr	12,956 (0.03)	170

TSP total soluble protein

[x] The control is CHN-18 that has not undergone cryopreservation

[y] Average of three technical replicates

[z] Standard deviation

agarose-containing medium, recovered quicker and had approximately four times the cell mass of those on agar, which made it clear that agarose was the gelling agent that should be used in culture medium used for recovery post cryopreservation (Fig. 1).

Antigen analysis. To determine if antigen expression was maintained post cryopreservation, we used ELISA to quantify the amount of antigen in non-cryopreserved cells and cells recovered after cryopreservation for different lengths of time. Recovery time or the first sign of re-growth following thawing of the cells varied from 1–4 wk. The post-cryopreservation cultures were not assayed for antigen production until they were well established on the culture medium, which ranged from 1.5 mo for rapidly recovering cells to up to 3 mo for the slowest recovering cell cultures. There was no correlation between the length of time the cells were stored in liquid nitrogen and the recovery time. The amount of antigen was determined for lines CLT101-14, CLT105-48, and CHN-18 following recovery.

LT-B alone was assayed in cultures of line CLT105-48 even though the construct was designed for expression of the holotoxin because anti-LT-B antibody was the only antibody available to us for the ELISA. LT-B continued to be expressed in CLT101-14 and CLT105-48 cultures recovered after each cryopreservation interval (Table 1). The amount of LT-B produced by line CTL105-48 varied from 3,218–8,015 ng/g cells, with the highest amount being observed in the sample that was not cryopreserved. The LT-B amounts produced by CTL105-48 following cryopreservation were not correlated with time stored in liquid nitrogen, that is, we did not observe a decrease in LT-B production as the storage time in liquid nitrogen increased. The variation could be a result of the length of time needed for LT-B to accumulate in the cells. Therefore, it is possible that more established cultures, like the original CLT105-48 recovered following transformation, having been maintained in culture longer than cultures recovered post cryopreservation, would have a higher amount of LT-B per nanograms of cells than newly established cultures following cryopreservation.

For studies on the effects of cryopreservation on HN expression, we chose the cell line designated CHN-18 because of its vigorous growth. We also observed a shorter recovery time post cryopreservation for CHN-18. Callus regrowth for CHN-18 occurred within 7–10 d post cryopreservation, whereas, regrowth for CLT101-14 and CLT105-48 took 2 to 4 wks. We attributed the faster regrowth of CHN-18 to its vigorous growth prior to cryopreservation (Table 2).

We were most interested in how long-term storage under cryopreservation conditions would affect the subsequent expression of antigen in the cell culture lines. These antigen-expressing cell lines served as experimental prototypes to determine if cell cultures could be a viable approach for the production of plant-made vaccines, which would require the routine initiation of seed stocks that were cryopreserved. There have been previous reports on the

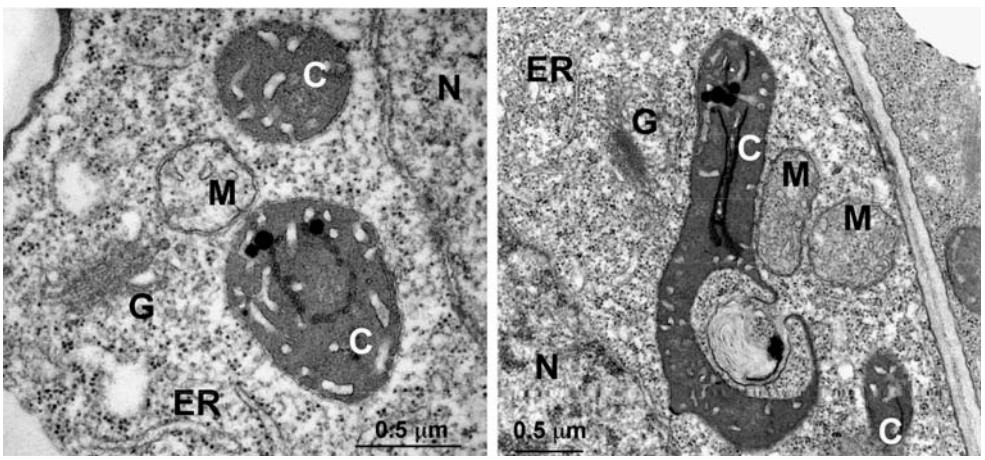

Figure 2. Transmission electron micrographs of a single cell from CHN-18 suspension cultures initiated following regrowth of cultures that underwent cryopreservation for 1 yr. *Left* control (no cryopreservation). *Right* Underwent cryopreservation for 1 yr. *C* chloroplast, *ER* endoplasmic reticulum, *G* Golgi bodies, *M* mitochondrion, *N* nucleus.

expression of proteins from transgenic cell culture lines that were cryopreserved, however, the times maintained under these conditions could be considered short-term, in that the longest time cell lines were maintained in liquid nitrogen was 1 mo. We investigated antigen expression from cultures that were maintained for longer periods of time. Cho et al. (2007) investigated the effects of cryopreservation on expression of a recombinant human cytoxic T-lymphocyte antigen 4-immunoglobulin (hCTLA4Ig) from transgenic rice suspension cultures. They found that expression of hCTLA4Ig remained stable; however, the cultures were cryopreserved for only 1 mo. In other reports on expression of proteins from cryopreserved transgenic cell culture lines of wheat (Fretz and Lörz 1995), *Papaver somniferum* (Elleuch et al. 1998), *Arabidopsis* (Menges and Murray 2004), and tobacco (Menges and Murray 2004), the times maintained in liquid nitrogen ranged from 1 h to 1 mo.

Transmission electron microscopy. CHN-18 suspension cultures were initiated from cells recovered after 1 yr of cryopreservation and control cells that had not undergone cryopreservation. They were sent to the Cornell Integrated Microscopy Center for transmission electron microscopy to determine if cryopreservation affected the integrity of the cells. There were no obvious differences between the two samples at the ultrastructural level, which demonstrated that the integrity of the cell structure was maintained following cryopreservation (Fig. 2). All organelles (nuclei, mitochondria, and chloroplasts) that normally have double membranes had intact membranes in both the control cells and the cells cryopreserved for 1 yr.

Contaminants. Periodically, we observed fungal, bacterial, and yeast contaminants in a small percentage of cultures recovered after cryopreservation. We tested for contaminants at the various steps in the process, and we determined that low levels of the contaminants were present in the source cell cultures. As a result, we recommend screening cell cultures for contaminants prior to introduction into cryopreservation. We investigated several culture media that are commonly used for growth of microorganisms. These media included Leifert and Waites Sterility Test Medium (LW; item #L476; Phytotechnology Laboratories), Sabouraud Dextrose Agar (SDS; item #210940; Becton, Dickinson, and Company, Sparks, MD), and Luria Broth (LB) [1 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l Difco Bacto Agar (Becton, Dickinson, and Company). We found all of these media to be suitable for screening cultures for contaminants.

In summary, our results demonstrate that long-term cryopreservation is a viable method for maintenance of seed stocks for plant-made vaccine cell lines based on the ability of cells to recover after long intervals in liquid nitrogen and the continued expression of the various proteins of interest to us. An additional benefit is one of labor savings over time because cryopreservation eliminates the need for repeated subcultures along with corresponding media preparation necessary to maintain cell cultures. The decrease in the labor needed for maintenance could also decrease the chances of introducing a contaminant into cultures or loss of cultures due to improperly made culture media. The reliance on conventional methods for culture maintenance can be a risky approach for maintaining plant cells that produce pharmaceuticals; therefore, cryopreservation should be investigated as a possible option. All the antigen-expressing cell lines generated and characterized for this study were developed as experimental prototypes. Results from this work will be used to develop standardized protocols for the generation and long-term cryopreservation of cell cultures for large-scale commercial production of plant-made vaccines.

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