Available online at www.sciencedirect.com
SCIENCE DIRECT®
Plant Science 166 (2004) 799–806
PLANT SCIENCE
www.elsevier.com/locate/plantsci

A cryopreservation method maintaining the genetic fidelity of a model forest tree, *Populus tremula* L. × *Populus tremuloides* Michx.

Soile Jokipii [a,*], Leena Ryynänen [b], Pauli T. Kallio [c], Tuija Aronen [b], Hely Häggman [a,b]

[a] *Department of Biology, University of Oulu, P.O. Box 3000, FIN-90014 Oulu, Finland*
[b] *The Finnish Forest Research Institute, Punkaharju Research Station, Finlandiantie 18, FIN-58450 Punkaharju, Finland*
[c] *Institute of Biotechnology, ETH-Zürich, CH-8093 Zürich, Switzerland*

Received 15 August 2003; received in revised form 19 November 2003; accepted 19 November 2003

Abstract

The aim of the present study was to develop an effective cryopreservation method for hybrid aspen (*Populus tremula* L. × *Populus tremuloides* Michx.), an economically important woody plant and widely used model system of forest scientists. Specific attention was paid to genetic fidelity of cryopreserved materials and transgene stability after retrieval. Three different kinds of cryopreservation protocols were applied, i.e. the slow cooling of PGD-treated in vitro apical segments, the vitrification of PVS2-treated in vitro buds and the slow cooling of dormant in vivo buds. The slow cooling of in vivo buds proved to be the most appropriate way to cryopreserve the samples, resulting in a generally excellent regrowth rate of 72–96%. According to random amplified polymorphic DNA (RAPD) assays the genetic fidelity of the cryostored materials was maintained during both slow cooling and vitrification. In addition, neomycinphosphotransferase II (*nptII*) gene was found to be stable in transgenic lines after cryopreservation. The results clearly indicate that it is possible to apply cryopreservation techniques to preservation of valuable clones and transgenic lines of hybrid aspen.

Keywords: Cryopreservation; Genetic fidelity; Hybrid aspen; *nptII*; *Populus tremula* L. × *P. tremuloides* Michx.

1. Introduction

Hybrid aspen is F_1 hybrid of European aspen (*Populus tremula*) and American aspen (*Populus tremuloides*) produced by artificial hybridization. In recent years, the paper industry has renewed its interest in hybrid aspen because its fibres have been found suitable for the manufacture of mechanical pulp and fine paper. In addition, hybrid aspen is favoured due to its white coloured wood, which consumes less chemicals in the paper making process, thus saving on the production costs and causing less damage to the environment [1].

Populus species and their hybrids are recognized as the model systems of forestry for a number of reasons: due to their fast growth, ease of propagation, considerable genetic variation, small genome size (550 Mb), and transformability [2,3]. *Populus* species have proved to be competent for especially *Agrobacterium*-mediated gene transfer [4,5], and to date, they are the most common tree group used in transgenic field experiments in Europe (http://biotech.jrc.it/deliberate/doc/snifs.doc). The recent advances in the development of molecular genome maps [2], large collections of expressed sequence tags (ESTs) [6], microarray analysis and the initiative to sequence the first tree genome, that of *Populus trichocarpa*, are also promoting the exploitation of *Populus* by a wide range of scientists [3,7].

The intensive research performed with poplars and aspens creates demand for the development of feasible non-genotype specific methods for a long-term maintenance. The advantages of cryopreservation, the storage of material at super-low temperature, are minimum requirements for space and labour. In the genus *Populus*, calli of *Populus euramericana* cv. *gelrica* [8], *Populus glandulosa* [9] as well

Abbreviations: BA, 6-benzyladenine; DMSO, dimethylsulfoxide; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog; PGD, mixture of polyethylene glycol, glucose and DMSO in water; PVS2, Plant vitrification solution 2, mixture of glycerol, ethylene glycol, DMSO and sucrose in MS medium; RAPD, random amplified polymorphic DNA; WPM, woody plant medium

* Corresponding author. Tel.: +358-8-553-1547; fax: +358-8-553-1061.
E-mail address: soile.jokipii@oulu.fi (S. Jokipii).

0168-9452/$ – see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2003.11.017

as in vitro shoot tips of *P. tremula* × *Populus alba* [10] and *P. alba* L. [11] have successfully been cryopreserved. In these experiments, applying both slow cooling and vitrification methods, best regrowth rates have been 62–67% [10,11], but the numbers of genetic backgrounds have been limited.

One essential aspect of cryopreservation is the genetic stability of plants recovered from cryogenic storage. So far, the use of genetic markers have generated controversial results for genetic fidelity of cryopreserved material [12–15] and the information concerning the transgenic plants is scarce [16–19]. The aim of this work was to develop an effective cryopreservation method for multiple genotypes of hybrid aspen with specific focus on genetic fidelity. Genetic fidelity was analysed using RAPD markers and in transgenic hybrid aspen lines also stable integration of transgenes was confirmed by Southern hybridizations both before and after cryostorage.

2. Materials and methods

2.1. Plant material

All the plant materials were originally derived from suckers of the three selected hybrid aspen plus trees (*P. tremula* L. × *P. tremuloides* Michx.) V613, V617 and V619, which are growing in southern Finland (61°48′N, 28°22′E). Micropropagated stock shoots were produced according to Ryynänen [20].

In vitro apical segments, i.e. the upper parts of the shoots were excised from the transgenic lines V613/14 and V613/36. The lines had been generated by *Agrobacterium tumefaciens*-mediated gene transfer using strain LBA 4404 carrying pBVHb-plasmid [21]. pBVHb includes the *Vitreoscilla* hemoglobin gene (*vhb*) driven by the 35S CaMV promoter and the neomycin phosphotransferase (*nptII*), a selectable marker gene driven by the *nos* promoter.

In vitro buds were derived from non-transgenic clones V613, V617 and V619 and transgenic lines V613/73, V617/85 and V619/92. The transgenic lines were transformed with *A. tumefaciens* strain LBA 4404 carrying pBI121-plasmid (Clontech, California, USA) with *uidA* reporter gene encoding β-glucuronidase and the *nptII* gene. The stock shoots for both the in vitro shoot and in vitro bud experiments were cultured on semisolid Murashige and Skoog (MS) [22] medium, containing 2.22 μM 6-benzyladenine (BA), 2.85 μM indole-3-acetic acid (IAA), and also 85.8 μM kanamycin for transgenic lines; the shoots were maintained at +25 °C under a 16 h photoperiod with a light intensity of 100–130 μmol photons $m^{-2}\,s^{-1}$ and subcultured every 4 weeks.

In vivo buds were collected from the 2-year-old greenhouse-grown non-transgenic clones V613, V617 and V619, and transgenic lines V613/14 and V613/36. The plants were first grown under a natural photoperiod at ambient temperature limited to a minimum of 5 °C [19]. After the growing season, the seedlings were cold stored at +2 °C in the darkness for 2 months before sampling.

2.2. Cryopreservation of in vitro apical segments and in vitro buds

The in vitro apical segments were cryopreserved using the modified slow freezing method of Ryynänen [23]. At the beginning of the experiment the in vitro grown stock shoots were transferred on kanamycin-free MS medium (containing 2.22 μM BA and 2.85 μM IAA) to minimize any potential stress caused by selection on kanamycin. The effects of both the duration from the previous subculturing and the duration of cold acclimation were studied in the following combinations: the shoots were cultured 0, 1, 2, or 3 weeks in normal growth conditions and then cold acclimated 0, 1, or 3 weeks at +5 °C under an 8 h photoperiod with light intensity of 23 μmol photons $m^{-2}\,s^{-1}$. Excised apical segments, about 0.5–1.0 cm in length, included a apical bud and 5–8 leaves with axillary buds. Fifteen segments per treatment were precultured for 3 days in the above mentioned conditions on the MS medium supplemented with 5% dimethylsulfoxide (DMSO). The samples were then transferred into 0.25 ml liquid hormone-free MS medium in 2 ml plastic cryotubes (Sarstedt, Nümbrecht, Germany) on ice. One ml PGD (10% polyethylene glycol 6000 (w/v), 10% glucose (w/v) and 10% DMSO (v/v)) was added as cryoprotectant dropwise over a period of 30 min [24]. The cryotubes were then left to stand on ice for 30 min. Thereafter, the samples were frozen slowly to −38 °C in a programmable cooling chamber (Lauda Ultra-Kryomat RUK 60, Königshofen, Germany) at a rate of $10\,°C\,h^{-1}$. After reaching the terminal temperature, the cryotubes were stored in liquid nitrogen for 3 days. Finally, the samples were thawed at 37 °C in a water bath for circa 2 min, then washed with liquid MS medium for 30 min and cultured on semisolid MS medium containing 2.22 μM BA and 2.85 μM IAA, for plant regeneration. The regrowth of the samples was observed 3 and 6 weeks after thawing. The apical segments showing organogenesis by producing new shoots and leaves were classified as regrowing.

The vitrification of in vitro buds was performed with the slightly modified method of Lambardi et al. [11]. In vitro grown stock shoots were acclimated for 3 weeks at +5 °C under an 8 h photoperiod with light intensity of 23 μmol photons $m^{-2}\,s^{-1}$. Excised 2–4 mm long unpeeled axillary and apical buds were precultured on hormone-free MS medium (0.09 M sucrose) for 3 days in the above mentioned conditions. The samples were then transferred into 0.25 ml liquid hormone-free MS medium in 2 ml plastic cryotubes (Sarstedt, Nümbrecht, Germany) on ice and pretreated for 20 min at 0 °C with a mixture of 2 M glycerol and 0.4 M sucrose [25], followed by the incubation for 30 min at 0 °C with PVS2 solution [26], which contains 30% glycerol (v/v), 15% ethylene glycol (w/v), 15% DMSO (v/v) and 0.4 M sucrose in hormone-free MS medium. The cryotubes were stored in liquid nitrogen for 3 days. After storage the samples were

thawed at 37 °C in water bath for circa 2 min, washed with liquid MS medium (1.2 M sucrose) for 20 min and cultured on the same regeneration medium as for in vitro shoots.

Twenty vitrified in vitro buds from the clone V617 and the line V617/85 and forty from the clones V613 and V619, as well as from the lines V613/73 and V619/92, were included in the experiment. For testing the effects of the cryoprotectant solutions, twenty buds per genotype, i.e. treatment controls, were handled in the same way except that they were not immersed in liquid nitrogen. The non-treated controls, 18–20 per each genotype, were kept in tissue culture during the experiment. The regrowth of the buds was recorded 3 and 6 weeks after thawing.

2.3. Cryopreservation of in vivo buds

In vivo axillary and apical buds with scales and about 2 cm piece of attached twigs were collected in 2 ml cryotubes (Sarstedt, Nümbrecht, Germany) on ice, 2–5 buds per cryotube. The bud type (axillary or apical bud) and the position of the bud on the seedling (base, middle or top), were identified. The cryopreservation of buds was carried out as described for silver birch (*Betula pendula* Roth) by Ryynänen [27]. The cryotubes were first placed at 0 °C for 36 h after which they were frozen slowly in the programmable cooling chamber from 0 to −38 °C at a rate of 10 °C h^{-1}, and then kept at the terminal prefreezing temperature for 24 h. Subsequently, they were immersed in liquid nitrogen for about 4 months. After cryostorage, buds were thawed at 37 °C in a water bath for circa 2 min and sterilized with 70% ethanol for 1.5 min. The scales, young leaves and twigs were removed from the buds and the remaining meristems were cultured on semisolid woody plant medium (WPM) [28] containing 2.22 μM BA and 0.005 μM NAA for regeneration.

The number of cryopreserved in vivo buds from transgenic lines varied between 25 and 41, the ones from non-transgenic lines varied from 35 to 57. This variability was due to different number of buds in original stock plants. The non-cryopreserved control buds, 20 for each genotype, were sterilized and cultured for regeneration as the cryopreserved samples. The regrowth of the buds was examined after 2 and 4 weeks of culturing.

2.4. Establishment of plants recovered from cryopreservation under greenhouse conditions

All shoots, about 10–25 mm in length with 5–10 leaves, both controls and those regenerated after cryopreservation were dipped in 0.5 mM indole-3-butyric acid (IBA) solution (pH 5.5) for a few seconds and potted in peat–perlite (1:1) at a high relative air humidity for 2 weeks, followed by cultivation and fertilization (once with 0.2% Superex Fertilizer, Kekkilä, Tuusula, Finland) in decreasing humidity for two more weeks. After acclimation, the plants were transferred into 0.5 l pots, containing slow-release fertilized peat (Kemira, Helsinki, Finland).

2.5. DNA analyses

The presence of transgene *nptII* in the shoots regenerated from the control and from cryopreserved materials was tested by Southern hybridization. Fourteen transgenic cryopreserved plants, five non-cryopreserved transgenic plants, two cryoprotectant-treated but non-cryopreserved plants, and three wild-type plants were used. To study genetic fidelity of cryopreserved materials, DNA samples of the shoots regenerated from in vitro buds of lines V613/73 and V619/92 and in vivo buds of clones V613, V617 and V619 were compared using random amplified polymorphic DNAs (RAPDs). Within each genotype and treatment, samples were collected from one randomly selected plant. Genomic DNA for molecular analysis was extracted from the leaves of greenhouse-grown plants, that were minimum of 5 months of age, using the method of Lodhi et al. [29] with modifications according to Valjakka et al. [30].

For Southern hybridization analysis, genomic DNA (20 μg) was digested with *Hind*III restriction enzyme having an unique cutting site within the transferred VHb construct at the 5′ end of the 35S CaMV promoter. The DNA fragments were separated on a 0.8% (w/v) agarose gel and transferred to a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) by capillary transfer. The prehybridizations and hybridizations were performed in a DIG Easy Hyb solution (Roche Diagnostics GmbH) at +42 °C with *nptII* probe. Double stranded probe of *nptII* (619 bp) was labelled with digoxigenin-11-dUTP (Roche Diagnostics GmbH) in the polymerase chain reaction. The primers used in the reaction were 5′-TGGGCACAACAGACAATCGG-3′ and 5′-CAGCAATATCACGGGTAGCC-3′ [30]. The 50 μl reaction mixture contained about 10 ng of plasmid DNA, 50 ng of both primers, 100 μM dATP, dCTP, dGTP, 65 μM dTTP, 35 μM DIG-11-dUTP and 1.0 U of Dynazyme DNA polymerase (Finnzymes, Espoo, Finland) in manufacturer's buffer. The reaction mixture was first heated at 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min with a final extension step of 72 °C for 7 min in a DNA Thermal Cycler 480 (Perkin-Elmer, Wellesley, MA, USA). After hybridization, the membranes were washed twice for 5 min in 2 × SSC, 0.1% SDS at room temperature, and twice for 15 min in 0.1 × SSC, 0.1% SDS at 68 °C. Detection was performed according to the manufacturer's (Roche Diagnostics GmbH) instructions with a chemiluminescent substrate.

For RAPD assays, 24 arbitrary 10-mers were preliminary tested, of which 10 primers generating the sharpest RAPD profiles were chosen to the final analyses (OPC-01, OPD-14, OPD-15, OPE-05, OPE-06, OPE-09, OPE-10, OPE-17, OPF-03 and OPF-06 purchased from Operon Technologies, Alameda, USA). The 25 μl reaction mixture contained about 100 ng of template DNA, 50 ng of primer, 100 μM of each of the dNTPs and 0.5 U of Dynazyme DNA polymerase (Finnzymes, Espoo, Finland) in manufacturer's buffer. The amplification of DNA was performed according

to Aronen et al. [12]. The mixture was first heated at 94 °C for 10 min, followed by 45 cycles of 94 °C for 1 min, 36 °C for 2 min and 72 °C for 2 min with a final extension step of 72 °C for 5 min in a DNA Thermal Cycler 480. In the control reactions the genomic DNA was omitted to check for their possible contaminations.

2.6. Statistical evaluation

The effect of genotype and cryopreservation treatments on the regrowth of in vitro and in vivo buds as well as the effect of the in vivo—bud type and location was examined using the logit model (SPSS 10.0).

3. Results

3.1. Cryopreservation of in vitro apical segments and in vitro buds

Cryopreservation of in vitro apical segments based on the slow-cooling method succeeded with only four samples out of 360, every recovered segment representing different preculture-cold treatment combination (data not shown). Generally, the frozen segments turned pale within 1–2 days of culture and the structures of their stems and leaves were soft. After 2–4 weeks of tissue culture, the viable samples started to form chlorophyll-containing tissues and later three of them produced new shoots.

Vitrification of in vitro buds was more successful, because all genotypes tested resumed growth after cryostorage. However, the regeneration of the buds varied significantly ($P < 0.001$) among genotypes (Table 1). The growth rate of cryopreserved buds ranged from 0 to 65% and 3 to 75% after 3 and 6 weeks of culture, respectively, which were significantly ($P < 0.001$) lower than those of the controls. Some signs of cryoinjuries, such as blanching of leaf-edges of the existing outermost leaves surrounding meristems, were found in vitrified materials 2–3 days after thawing.

3.2. Cryopreservation of in vivo buds

In vivo buds were successfully cryopreserved using the slow cooling method. Regrowth rate of the cryopreserved buds ranged from 59 to 95% and 72 to 96% after 2 and 4 weeks of culture, respectively (Table 2). Correspondingly, 89–100% and 90–100% of the control buds showed regrowth after 2 and 4 weeks of culture, respectively. The difference between the regeneration of genotypes ($P < 0.05$), and cryopreserved and control buds ($P < 0.01$) was statistically significant. The bud type or its position on the seedling did not affect the regrowth (data not shown).

Table 2
Regrowth (%) of in vivo buds of hybrid aspen cryopreserved by slow cooling method

Clone/line	After 2 weeks of culture		After 4 weeks of culture	
	Control	Cryopreserved	Control	Cryopreserved
V613	88.9	59.4	100.0	71.9
V617	100.0	68.0	90.0	80.0
V619	95.0	94.7	95.0	94.7
V613/14	100.0	78.3	100.0	95.7
V613/36	100.0	75.6	100.0	82.9

In the case of the in vitro apical segments as well as in vitro and in vivo buds, the regrowth of the cryopreserved material was obtained without any callus formation. The plantlets regenerated from the cryopreserved explants appeared to have normal phenotypes compared with the control plantlets during both in vitro cultivation and greenhouse growth.

3.3. DNA analyses

The genetic fidelity of the PVS2-treated and vitrified in vitro buds, as well as cryostored in vivo buds, was tested by comparing RAPD profiles (Fig. 1) generated arbitrary 10-mers. The 10 RAPD-primers used in the experiment yielded a total of 80 strong fragments, the size of the fragments varying from 300 to 3000 bp. The number of amplified products ranged from 5 to 11 per primer. A total of 31.3% of the fragments were polymorphic among the genotypes. No reproducible variation of the RAPD profiles within the clones and lines were observed. Occasionally, a few ghost bands were detected in the control reactions.

Integration of *nptII* gene on genomes of shoots regenerated from cryopreserved and control materials was tested by Southern hybridization. The slow cooling and vitrification methods used did not affect the copy number of the

Table 1
Regrowth (%) of in vitro buds of hybrid aspen cryopreserved by vitrification

Clone/line	After 3 weeks of culture			After 6 weeks of culture		
	Control	Treatment control	Cryopreserved	Control	Treatment control	Cryopreserved
V613	100.0	85.0	22.5	100.0	95.0	25.0
V617	100.0	90.0	65.0	100.0	90.0	75.0
V619	100.0	100.0	2.5	100.0	95.0	2.5
V613/73	100.0	100.0	0.0	100.0	100.0	10.0
V617/85	100.0	55.0	0.0	100.0	65.0	15.0
V619/92	100.0	80.0	32.5	100.0	95.0	40.0

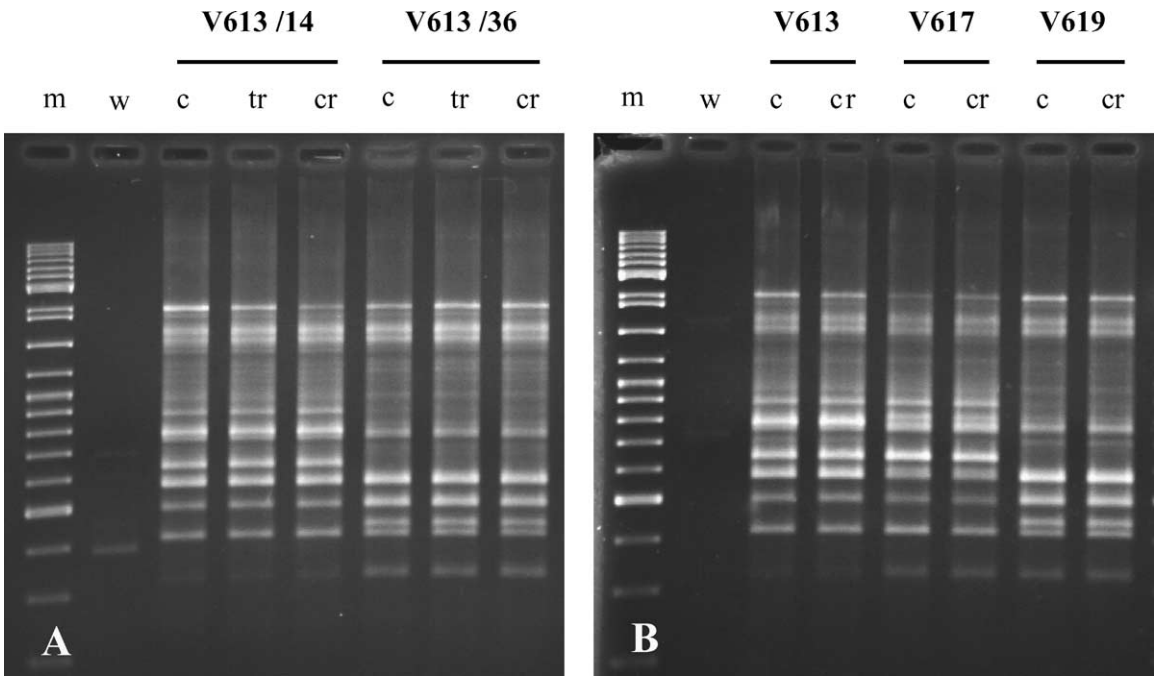

Fig. 1. Examples of the RAPD profiles generated for studying the genetic fidelity of cryopreserved hybrid aspen clones and lines. DNAs amplified with primer OPD-15. (A) Plants regenerated from vitrified in vitro buds compared with treatment controls and controls. (B) Plants regenerated from in vivo buds cryopreserved with slow cooling method compared with controls. Abbreviations: c, non-cryopreserved control; cr, cryopreserved; m, molecular marker; tr, treatment control; w, water.

transgene. There appeared to be two copies of *nptII* gene in the line V613/73 while lines V619/92, V613/14 and V613/36 had one copy of the transgene both before and after cryopreservation (Fig. 2). There was some variance in the speed that copies were carried along the gels probably because the quality of the isolated DNA varied. Line V617/85 could not be tested with Southern hybridization, because the regenerated shoots died before transfer to the greenhouse. However, the line appeared to be transgenic according to preliminary PCR-test (data not shown).

4. Discussion

4.1. Cryopreservability

In hybrid aspen, the cryopreservation of in vivo buds by the slow-cooling method was the most successful way to cryostore samples, resulting in a generally excellent regrowth rate of 72–96% after 4 weeks of tissue culture. The different in vivo bud types were appropriate for cryopreservation of hybrid aspen, because they all, i.e. apical and axillary buds and buds located in upper, middle and lower parts of the stem had similar regrowth rates. Although the differences in the regrowth between hybrid aspen genotypes were statistically significant, the method can be considered feasible for the multiple genetic backgrounds tested in the study.

All the genotypes from the in vitro bud experiment could also be preserved with the modified vitrification method of Lambardi et al. [11]. Three weeks after thawing, clone V617 had the best regrowth rate (65%), which was close to that of *P. alba* L. (62%) after 4 weeks in tissue culture [11]. As with the in vivo buds, there were significant differences between the regrowth of the hybrid aspen clones and lines.

4.2. Genetic fidelity of cryopreserved materials

One important aspect of cryopreservation is the genetic fidelity of plants recovered from cryogenic storage. This is particularly true in the case of long-living forest trees, because the effects of occasional mutations or genetic rearrangements may not be readily observed in young plants but expressed later on in mature trees [12].

So far, using genetic markers has generated controversial results for genetic fidelity of cryopreserved material. Häggman et al. [13] observed no genetic changes in RAPD profiles of cryopreserved embryogenic cultures of Scots pine (*Pinus sylvestris* L.). Similarly, Turner et al. [14] found that the genetic fidelity of *Anigozanthos viridis* was maintained during cryostorage, because no qualitative differences were seen in amplified fragment length polymorphism (ALFP) DNA fingerprints. Harding et al. [15] did not detect any variation in restriction fragment length polymorphisms (RFLP) profiles of rRNA genes of mahogany (*Swietenia macrofylla*) after cryopreservation, but they did observe chromatin and DNA methylation changes that might affect the patterns of gene expression. When assayed by RAPD

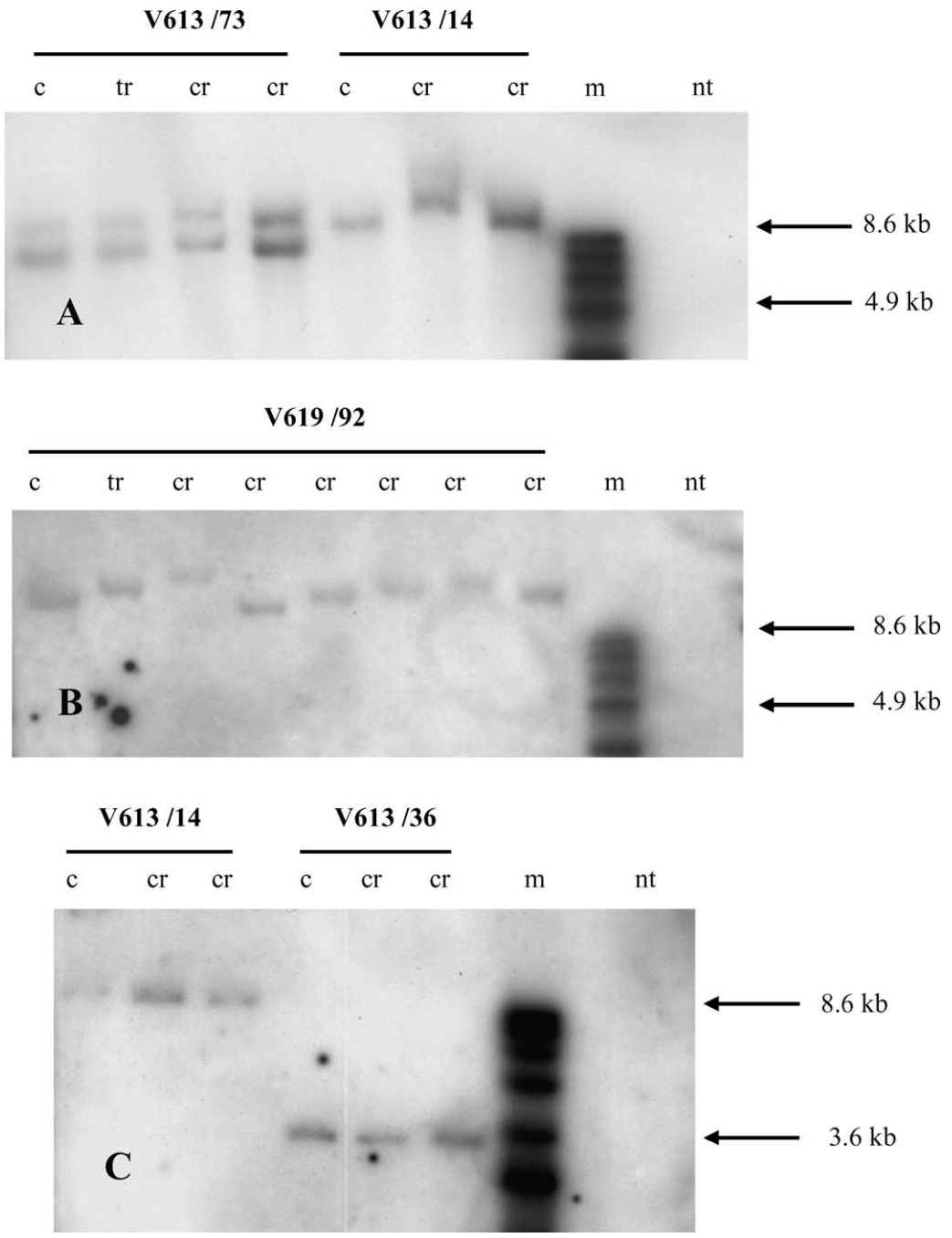

Fig. 2. Southern hybridisation analysis of *nptII* genes of the transgenic cryopreserved hybrid aspens and controls. (A) V613/14 plants regenerated from in vitro apical segments cryopreserved with slow cooling method and V613/73 plants regenerated from vitrified in vitro buds. (B)V619/92 plants regenerated from vitrified in vitro buds. (C) V613/14 and V613/36 plants regenerated from in vivo buds cryopreserved with slow cooling method. Abbreviations: c, non-cryopreserved control; cr, cryopreserved; m, molecular marker; nt, non-transformed control; tr, treatment control. The sizes (kb) of the DNA fragments are marked with arrows.

profiles, a widely used cryoprotectant, DMSO, was shown to cause considerable genetic variations in the DMSO-treated but non-frozen embryogenic cultures of *Abies cephalonica* [12]. In the same study, however, no such changes were observed in the samples stored in LN or when DMSO was used in the PGD cryoprotectant mixtures, indicating that cryostorage probably eliminates a high proportion of mutated cells and the other compounds of the cryoprotectant mixture may diminish the mutagenic potential of DMSO [12].

The RAPD assays performed in the present study suggest no genetic aberrations originated in hybrid aspen clones and lines during vitrification or slow cooling treatments. A few ghost bands detected in some of the control reactions

were possibly generated by the pairing of primers. The difference between present results and those of Aronen et al. [12] may be because of different species and cryopreservation methods but also DNA extraction protocols used. In the current study the best regrowth rates were achieved by slow cooling method of in vivo buds in which no potentially mutagenic cryoprotectants were used, thus, lowering the risk of genetic rearrangements. However, there may be certain genetic changes, e.g. extra copies of chromosomes, which molecular markers may not detect. Further, the use of genomic DNA in RAPD assays may also detect mutations in non-coding regions of the genome that do not influence the phenotype. Hence, molecular markers can be used for assaying the genetic fidelity, but preferably together with other approaches such as morphological and cytological observations [31].

As more experience is gained with transgenic plants, more attention is paid to the stable integration and expression of transgenes. Gene silencing and changes in the level of transgene expression have been observed in *Populus* species as a consequence of the integration patterns of transgene, the environmental conditions, the physiological and developmental state of material and growing season [32–34]. Trees adapt themselves to the changing environments during their long life spans, and thus, genes that cause low fitness may be silenced or eliminated [35]. Therefore, it is important to study transgene stability and expression of genetically modified trees in the short- as well as long-term experiments.

In the present work, the effort was also focused on studies of integration of the transgene i.e. selectable marker gene *nptII*. The *nptII* gene was found to be stably integrated in transgenic lines of hybrid aspen after cryopreservation regardless of the cryopreservation methods used. The result is consistent with earlier studies on herbaceous and woody plants. Benson and Hamill [16] found T-DNA-fragments in the genomes of *Beta vulgaris* and *Nicotiana rustica* to be unchanged after cryostorage. Elleuch et al. [17] reported the integration and expression structure of the *sam1* transgene in *Papaver somniferum* were not affected by cryopreservation. Kobayashi et al. [18] observed that the length and copy number of *nptII* gene were equal in vitrified and control cells of *Citrus sinensis* Osb. Ryynänen et al. [19] also found that multiple copies of *nptII* gene were stably maintained and the function of *nptII* at mRNA level was constant in silver birch after cryopreservation.

As conclusions, the results reported here show that it is possible to preserve the valuable clones and transgenic lines of hybrid aspen by using cryopreservation technology. Among the different cryopreservation protocols applied in the study, the slow cooling method of in vivo buds was applicable for all genotypes tested and resulted in the highest regeneration percentages after retrieval. The genetic fidelity and stable integration of transgene was found to be maintained during cryogenic storage.

Acknowledgements

The authors would like to thank Dr. Kelvin H. Lee for critical reading of the manuscript and Ms. Airi Huttunen, Ms. Taina Naukkarinen and Ms. Aila Viinanen for their excellent technical assistance.

References

[1] E. Beuker, Breeding of Aspen and Poplars in Finland, Reports from the Foundation for Forest Tree Breeding, vol. 1, 1991, pp. 23–27.
[2] H.D. Bradshaw Jr., R. Ceulemans, J. Davis, R. Stettler, Emerging model systems in plant biology: poplar (*Populus*) as a model forest tree, J. Plant Growth Regulat. 19 (2000) 306–313.
[3] G. Taylor, *Populus*: Arabidopsis for forestry. Do we need a model tree? Ann. Bot. 90 (2002) 681–689.
[4] J.J. Fillatti, J. Sellmer, B. McCown, B. Haissig, L. Comai, *Agrobacterium* mediated transformation and regeneration of *Populus*, Mol. Gen. Genet. 206 (1987) 192–199.
[5] M.-S. Kim, N.B. Klopfenstein, Y.W. Chun, *Agrobacterium*-mediated transformation of *Populus* species, in: N.B. Klopfenstein, Y.W. Chun, M.-S. Kim, M.R. Ahuja (Eds.), Micropropagation, Genetic Engineering, and Molecular Biology of Populus, Rocky Mountain Forest and Range Experiment Station, Fort Collins, Colorado, 1997, pp. 51–59.
[6] F. Sterky, S. Regan, J. Karlsson, M. Hertzberg, A. Rohde, A. Holmberg, B. Amini, R. Bhalerao, M. Larsson, R. Villarroel, M. Van Montagu, G. Sandberg, O. Olsson, T.T. Teeri, W. Boerjan, P. Gustafsson, M. Uhlén, B. Sundberg, J. Lundeberg, Gene discovery in the wood-forming tissues of poplar: analysis of 5,692 expressed sequence tags, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 13330–13335.
[7] S.D. Wullschleger, S. Jansson, G. Taylor, *Populus* emerges as the perenial favorite, Plant Cell 14 (2002) 2651–2655.
[8] A. Sakai, Y. Sugawara, Survival of poplar callus at super-low temperatures after cold acclimation, Plant Cell Physiol. 14 (1973) 1201–1204.
[9] S.H. Son, Y.G. Park, Y.W. Chun, R.B. Hall, Germplasm preservation of *Populus* through in vitro culture systems, in: N.B. Klopfenstein, Y.W. Chun, M.-S. Kim, M.R. Ahuja (Eds.), Micropropagation, Genetic Engineering, and Molecular Biology of Populus, Rocky Mountain Forest and Range Experiment Station, Fort Collins, Colorado, 1997, pp. 44–49.
[10] F. Accart, V. Monod, M. Poissonnier, J. Dereuddre, M. Paques, Cryoconservation d'apex de *Populus tremula* × *alba* cultives *in vitro* transformes genetiquement ou non, Ann. Res. Sylvicoles 1993–1994 (1995) 23–39.
[11] M. Lambardi, A. Fabbri, A. Caccavale, Cryopreservation of white poplar (*Populus alba* L.) by vitrification of in vitro-grown shoot tips, Plant Cell Rep. 19 (2000) 213–218.
[12] T.S. Aronen, J. Krajnakova, H.M. Häggman, L.A. Ryynänen, Genetic fidelity of cryopreserved embryogenic cultures of open-pollinated *Abies cephalonica*, Plant Sci. 142 (1999) 163–172.
[13] H.M. Häggman, L.A. Ryynänen, T.S. Aronen, J. Krajnakova, Cryopreservation of embryogenic cultures of Scots pine, Plant Cell Tiss. Org. Cult. 54 (1998) 45–53.
[14] S. Turner, S.L. Krauss, E. Bunn, T. Senaratna, K. Dixon, B. Tan, D. Touchell, Genetic fidelity and viability of *Anigozanthos viridis* following tissue culture, cold storage and cryopreservation, Plant Sci. 161 (2001) 1099–1106.
[15] K. Harding, M. Marzalina, B. Krishnapillay, N.A. Nashatul Zaimah, M.N. Normah, E.E. Benson, Molecular stability assessments of trees regenerated from cryopreserved mahogany (*Swietenia macrophylla*) seed germplasm using non-radioactive techniques to examine the chromatin structure and DNA methylation status of the ribosomal RNA genes, J. Trop. Forest Sci. 12 (2000) 149–163.

[16] E.E. Benson, J.D. Hamill, Cryopreservation and post freeze molecular and biosynthetic stability in transformed roots of *Beta vulgaris* and *Nicotiana rustica*, Plant Cell Tiss. Org. Cult. 24 (1991) 163–172.
[17] H. Elleuch, C. Gazeau, H. David, A. David, Cryopreservation does not affect the expression of a foreign *sam* gene in transgenic *Papaver somniferum* cells, Plant Cell Rep. 18 (1998) 94–98.
[18] S. Kobayashi, A. Sakai, T. Ohgawara, Y. Nakamura, Stable maintenance of an integrated gene in nucellar cells of navel orange (*Citrus sinensis* Osb.) under storage in LN_2, J. Jpn. Soc. Hortic. Sci. 63 (1994) 553–558.
[19] L. Ryynänen, M. Sillanpää, S. Kontunen-Soppela, H. Tiimonen, J. Kangasjärvi, E. Vapaavuori, H. Häggman, Preservation of transgenic silver birch (*Betula pendula* Roth) lines by means of cryopreservation, Mol. Breeding 10 (2002) 143–152.
[20] L. Ryynänen, Micropropagation of Hybrid Aspen, Reports from the Foundation for Forest Tree Breeding, vol. 1, 1991, pp. 61–68.
[21] H. Häggman, A.D. Frey, L. Ryynänen, T. Aronen, R. Julkunen-Tiitto, H. Tiimonen, K. Pihakaski-Maunsbach, S. Jokipii, X. Chen, P.T. Kallio, Expression of *Vitreoscilla* haemoglobin in hybrid aspen (*Populus tremula* × *tremuloides*), Plant Biotech. J. 1 (2003) 287–300.
[22] T. Murashige, F. Skoog, A revised medium for rapid growth and bio assays with tobacco tissue cultures, Physiol. Plant. 15 (1962) 473–497.
[23] L. Ryynänen, Cold hardening and slow cooling: tools for successful cryopreservation and recovery of in vitro shoot tips of silver birch, Can. J. Forest Res. 26 (1996) 2015–2022.
[24] K.K. Kartha, L.C. Fowke, N.L. Leung, K.L. Caswell, I. Hakman, Induction of somatic embryos and plantlets from cryopreserved cell cultures of white spruce (*Picea glauca*), J. Plant Physiol. 132 (1988) 529–539.
[25] T. Matsumoto, A. Sakai, K. Yamada, Cryopreservation of *in vitro*-grown apical meristems of wasabi (*Wasabia jabonica*) by vitrification and subsequent high plant regeneration, Plant Cell Rep. 13 (1994) 442–446.
[26] A. Sakai, S. Kobayashi, I. Oiyama, Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification, Plant Cell Rep. 9 (1990) 30–33.
[27] L. Ryynänen, Survival and regeneration of dormant silver birch buds stored at super-low temperatures, Can. J. Forest Res. 26 (1996) 617–623.
[28] G. Lloyd, B. McCown, Commercially-feasible micropropagation of Mountain Laurel, *Kalmia latifolia*, by use of shoot-tip culture, Proc. Int. Plant Propagators' Soc. 30 (1980) 421–427.
[29] M.A. Lodhi, G.-N. Ye, N.F. Weeden, B.I. Reisch, A simply and efficient method for DNA extraction from grapevine cultivars and *Vitis* species, Plant Mol. Biol. Rep. 12 (1994) 6–13.
[30] M. Valjakka, T. Aronen, J. Kangasjärvi, E. Vapaavuori, H. Häggman, Genetic transformation of silver birch (*Betula pendula*) by particle bombardment, Tree Physiol. 20 (2000) 607–613.
[31] J.-L. Fourré, P. Berger, L. Niquet, P. André, Somatic embryogenesis and somaclonal variation in Norway spruce: morphogenetic, cytogenetic and molecular approaches, Theor. Appl. Genet. 94 (1997) 159–169.
[32] G. Pilate, D.D. Ellis, S. Hawkins, Transgene expression in the field-grown poplar, in: N.B. Klopfenstein, Y.W. Chun, M.-S. Kim, M.R. Ahuja (Eds.), Micropropagation, Genetic Engineering, and Molecular Biology of Populus, Rocky Mountain Forest and Range Experiment Station, Fort Collins, Colorado, 1997, pp. 84–89.
[33] M. Fladung, Gene stability in transgenic aspen (*Populus*). I. Flanking DNA sequences and T-DNA structure, Mol. Gen. Genet. 260 (1999) 574–581.
[34] S. Kumar, M. Fladung, Gene stability in transgenic aspen (*Populus*). II. Molecular characterization of variable expression of transgene in wild and hybrid aspen, Planta 213 (2001) 731–740.
[35] M.R. Ahuja, Transgenes and genetic instability, in: N.B. Klopfenstein, Y.W. Chun, M.-S. Kim, M.R. Ahuja (Eds.), Micropropagation, Genetic Engineering, and Molecular Biology of Populus, Rocky Mountain Forest and Range Experiment Station, Fort Collins, Colorado, 1997, pp. 90–100.