In Vitro Cell.Dev.Biol.—Plant (2010) 46:246–256
DOI 10.1007/s11627-009-9265-z

SOMATIC CELL GENETICS

Genetic stability of cryopreserved shoot tips of *Rubus* germplasm

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Received: 22 March 2009 / Accepted: 2 December 2009 / Published online: 21 February 2010 / Editor: F. A. Krens

Abstract Questions often arise concerning the genetic stability of plant materials stored in liquid nitrogen for long time periods. This study examined the genetic stability of cryopreserved shoot tips of *Rubus* germplasm that were stored in liquid nitrogen for more than 12 yr, then rewarmed and regrown. We analyzed the genetic stability of *Rubus grabowskii*, two blackberry cultivars ("Hillemeyer" and 'Silvan'), and one raspberry cultivar ("Mandarin") as *in vitro* shoots and as field-grown plants. No morphological differences were observed in greenhouse-grown cryopreserved plants when compared to the control mother plants. In the field, cryopreserved plants appeared similar but were more vigorous than mother plants, with larger leaves, fruit, and seeds. Single sequence repeats (SSR) and amplified fragment length polymorphism (AFLP) analyses were performed on shoots immediately after recovery from cryopreservation and on shoots subcultured for 7 mo before analysis. Ten SSR primers developed from "Marion" and "Meeker" microsatellite-enriched libraries amplified one to 15 alleles per locus, with an average of seven alleles and a total of 70 alleles in the four genotypes tested. No SSR polymorphisms were observed between cryopreserved shoots and the corresponding mother plants regardless of subculture. Although no polymorphisms were detected in shoots analyzed immediately after recovery from cryopreservation, AFLP polymorphisms were detected in three of the four *Rubus* genotypes after they were subcultured for 7 mo. Field-grown plants from the polymorphic shoot tips of *R. grabowskii* and 'Silvan' displayed the same AFLP fingerprints as their corresponding mother plants. Only long-cultured *in vitro* shoot tips displayed polymorphisms *in vitro*, and they were no longer detected when the plants were grown *ex vitro*. The transitory nature of these polymorphisms should be carefully considered when monitoring for genetic stability.

Keywords Genetic fidelity · *In vitro* culture · Somaclonal variation · Taq DNA polymerase

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Introduction

The genus *Rubus* belongs to the family Rosaceae and contains cultivated raspberries, blackberries, hybrid berries, and a large number of species (Jennings 1988). Raspberry and blackberry fruits are produced for the fresh fruit market and for use in a number of processed food items and are important worldwide (Donnelly and Daubeny 1986). The Pacific Northwest (Oregon, Washington, and British Columbia) is a major production area for both blackberries and raspberries. Many breeding programs are actively working on releasing cultivars with excellent quality, high yields, greater adaptation to adverse environmental conditions, and increased pest and disease resistance. The wide diversity of *Rubus* species provides a potential source of novel traits. The United States Department of Agriculture-Agricultural Research Service, National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon, is responsible for collecting, maintaining, characterizing, and distributing *Rubus* accessions. *Rubus* species and cultivars are clonally propagated

and maintained in greenhouses, screenhouses, field collections, and as tissue-cultured plants and cryopreserved shoot tips (Gupta and Reed 2006). The NCGR *Rubus* collection consists of 2,094 accessions representing 193 species from 64 countries and is a unique source of genetically diverse genotypes for use by plant breeders and scientists across the world (Thompson 1995).

Cryopreservation is used for long-term storage at ultra-low temperatures (−196°C; Engelmann 2004). Advantages of cryopreservation include low maintenance costs and small storage space (Helliot et al. 2002). Most cryopreservation techniques utilize tissue culture methods in the production and regrowth of the frozen material. Cell division and metabolic activities are stopped when plants are exposed to ultra-low temperatures, allowing storage without alteration for an indefinite period of time.

Maintenance of genetic fidelity is essential for a successful cryopreservation strategy and requires tools for evaluating genetic stability of cryopreserved plants. The development of molecular techniques in recent year provides additional means for assessing genetic fidelity in plants. Single sequence repeats (SSRs) are tandemly repeated motifs of one to six bases present in coding and non-coding regions and highly polymorphic (Zane et al. 2002). SSRs were utilized to assess genetic fidelity of post-cryopreserved *Solanum* plants (Harding and Benson 2001) and to identify somaclonal variation in tissue cultured *Actinidia* (Palombi and Damiano 2002), *Theobroma* (Rodriguez et al. 2004), and *Populus* (Rahman and Rajora 2001). SSR analysis was utilized in *Solanum* to detect DNA sequence length variation in encapsulation-dehydration cryopreserved cultivars "Brodick" and "Golden Wonder." Identical SSR profiles were observed in plants regrown from cryopreserved apices, parental plants, and their progeny (Harding and Benson 2001). This indicates stable somatic inheritance of microsatellite genomic sequences. SSRs can detect somaclonal variation as was seen in *Actinidia* (Palombi and Damiano 2002). One SSR marker showed genetic variation between *in vivo* and *in vitro* plants of the cultivar "Tomuri." Somaclonal variation was also detected in *Populus* at two SSR loci (Rahman and Rajora 2001). Variation at the PTR2 locus resulted in the appearance of a new allele of increased size while variation at the PTR5 locus resulted in the appearance of a third allele.

Amplified fragment length polymorphism (AFLP) is a DNA fingerprinting technique based on the amplification of subsets of genomic restriction fragments using polymerase chain reaction (PCR; Vos et al. 1995). The major advantages of using AFLP are the ability to inspect an entire genome for polymorphism and its relative reproducibility (Blears et al. 1998). AFLP was utilized to assess genetic fidelity of *Anigozanthos* (Turner et al. 2001), *Carica papaya* L. (Kaity et al. 2008), *Cosmos atrosanguineus* Hook. (Wilkinson et al. 1998), *Fragaria* (Hao et al. 2002b), and *Prunus* (Helliot et al. 2002) after cryopreservation. While no detectable genetic variation was observed in *Anigozanthos* (Turner et al. 2001) or *Cosmos* using AFLP (Wilkinson et al. 2003), an additional fragment was detected in cryopreserved *in vitro* samples of "Joho" strawberry but not found in non-cryopreserved *in vitro* cultures (Hao et al. 2002b). This additional fragment was attributed to a change in DNA methylation status using the methylation sensitive amplified polymorphism (MSAP) assay that relies on the differential sensitivity of restriction enzymes to methylated DNA sequences (Hao et al. 2002b). AFLP analysis of cryopreserved *Prunus* plants and the non-frozen control revealed two polymorphic fragments (30 and 135 bp; Helliot et al. 2002). The frequency of the polymorphic pattern increased from 18% for the non-frozen plants to 37% for the *in vitro* plants regrown from cryopreserved apices. Recently, changes in DNA primary structure using PCR-based randomly amplified DNA fingerprinting as well as epigenetic changes caused by methylation modifications using the amplified DNA methylation polymorphism (AMP) techniques were reported in cryopreserved papaya shoot tips (Kaity et al. 2008).

Since DNA markers have different efficiencies in detecting polymorphism and different levels of DNA changes, the use of more than one marker should be more reliable during genetic stability studies. In poplar, somaclonal variation was detected in shoots regenerated from calluses using restriction fragment length polymorphisms (RFLP), Random Amplified Polymorphic DNAs (RAPDs), and SSRs (Wang et al. 1996), while Goto et al. (1998) did not find any differences using only RAPD markers in the same species. Therefore, the use of more than one molecular marker increases the reliability of genetic stability assessments.

Maintenance of genetic fidelity of meristem-derived plants following cryopreservation was supported by RAPD studies in many genera (Harding 2004). No genetic changes were detected by RAPD analysis after cryopreservation of shoot tips of *Betula pendula* Roth (Ryynanen 1998), *Vitis* and *Actinidia* (Zhai et al. 2003), *Arachis* (Gagliardi et al. 2003), *Humulus* (Peredo et al. 2008) and *Prunus* (Helliot et al. 2002), *Pinus sylvestris* L. embryogenic cultures (Haggman et al. 1998), and *Citrus* callus cultures (Hao et al. 2002a). AFLP analysis showed no differences in *Prunus* (Helliot et al. 2002), *Diospyros virginiana* L. (Ai and Zhengrong 2005), *Humulus* (Peredo et al. 2008), and *Anigozanthos viridis* Endl. (Turner et al. 2001). Harding and Benson (2001) used SSRs to examine *Solanum tuberosum* L. and found no differences between cryopreserved and control shoots. Haggman et al. (2008)

noted that currently, there is no evidence of cryopreservation-induced genetic or morphological changes in forest trees.

Since cryopreservation uses *in vitro* methods, associated risks to the genetic fidelity in stored plants are possible. Callus formation during recovery from cryopreservation may lead to genetic instability and somaclonal variation (Keller et al. 2008). Somaclonal variation may result in modification of chromosome number or methylation pattern, chromosome breakage, transposon activation, deletion, genome rearrangement, polyploidy, or nucleotide substitution (Bhatia et al. 2005). Variations may pre-exist in the natural population of plants from field collection or genebank or it may develop *de novo* as a result of tissue culture conditions. In a study by Dixit et al. (2003) on *Dioscorea bulbifera* L., RAPDs of plants regenerated from cryopreserved embryogenic tissues were identical to those of *in vitro*-grown control plants for nine of ten primer pairs tested. Only one of the 4,960 bands obtained from ten primer pairs varied in one of 60 cryopreserved plants tested. However, the extremely low frequency of variation (0.02%) detected was reported to arise during the induction and maintenance of embryogenic tissues through repeated subculturing before cryogenic treatments (Dixit et al. 2003). In *Saccharum officinarum* L. meristem culture, tissue culture was found to be responsible for the generation of phenotypic and genetic variation. A sevenfold increase in the rate of polymorphism was observed by the RAPD analysis (Zucchi et al. 2002).

Most genetic variability studies involve tissues with relatively brief exposure to liquid nitrogen and analysis immediately after recovery from liquid nitrogen. The objective of this study was to use SSR and AFLP markers and comparisons of morphology to evaluate genetic stability of *Rubus* stored in liquid nitrogen for more than 12 yr, 1 mo after rewarming, after 7 mo of *in vitro* culture, and after 1 yr of field growth.

Materials and Methods

Plant materials. *In vitro*-grown shoot tips of a wild European blackberry, *Rubus grabowskii* Weihe ex Gunther et al. (PI 379534); two blackberry cultivars, Hillemeyer (PI 553275) and Silvan (PI 553308); and one *Rubus idaeus* red raspberry cultivar Mandarin (PI 553493) were cryopreserved between 1990 and 1993 by slow cooling (Reed 1993). The plants were rewarmed and regrown in 2005 and analyzed for genetic stability using morphological and DNA markers (Table 1). Ten plants were analyzed immediately after recovery from cryopreservation (short-cultured (SC) plants) and consisted of eight plants of "Hillemeyer" and two plants of 'Silvan.' Eleven regrown plants subcultured for 7 mo (long-cultured (LC) plants) were used for molecular analysis: four in *R. grabowskii*, five in "Hillemeyer", and one each in 'Silvan' and "Mandarin." LC plants were then grown in the greenhouse for 10 mo, and four plants of each were evaluated for phenotypic differences in leaf shape and spinelessness. LC greenhouse plants and control plants propagated in the greenhouse (not *in vitro* cultured or cryopreserved) were field grown for 1 yr before evaluation.

Cryopreservation and regrowth of Rubus plants. Shoot tips from these *Rubus* plants were cryopreserved between 1990 and 1993 with the controlled-cooling cryopreservation protocol (Reed 1988, 1993). Storage was under liquid nitrogen until 2005. To recover the *Rubus* plants, cryovials were warmed for 1 min in 45°C water and 1 min in 25°C water, rinsed in liquid medium, and plated on recovery medium (Reed 1988, 1993). Plants were micropropagated on NCGR *Rubus* medium to produce enough tissue for analysis (Reed 1990).

Morphological analysis of field-grown plants. Greenhouse-grown LC plants (*R. grabowskii* cryopreserved plants A, B, and C; 'Silvan' A) that displayed differences in AFLP-based profiles *in vitro* and *R. grabowskii* D that showed no polymorphism were planted in the field alongside their control screenhouse-grown counterparts for comparing their morphological characteristics. One control (screenhouse propagated in the greenhouse) plant and two plants of each recovered shoot tip (greenhouse propagated) were planted. Data for leaf number, leaf length and width, fruit length and width, drupelet size and number, seed length and width, secondary petiole length, primocane internode length, and plant vigor were taken on six leaves/fruits/stems of each plant. Data were analyzed with SAS (2003).

DNA extraction. Genomic DNA was extracted from leaves of: (1) regrown cryopreserved *Rubus* LC *in vitro* plants prior to transplanting to the greenhouse, (2) screenhouse-grown mother plants, (3) field-transplanted polymorphic LC plants, and (4) field-transplanted control plants. Leaves were extracted with the PUREGENE kit (Gentra Systems Inc., Big Lake, MN) using the optional RNAse A treatment followed by phenol:chloroform extraction (Sambrook et al. 1989).

SSR analysis. Ten primer pairs isolated from "Meeker" *(R. idaeus*) and "Marion" (*Rubus* hybrid) were used for assessment of genetic stability of regrown cryopreserved *Rubus*: RhM001, RhM003, RhM011, RhM018, RhM021, RhM023, RhM043, RiM015, RiM017, and RiM036 (Castillo 2006). Fluorescent forward primers were ordered from Sigma-Proligo (Sigma-Aldrich Co., St. Louis, MO). PCR reactions were performed separately for each primer

Table 1. *Rubus* accessions used for genetic fidelity assessment of *in vitro* plants after cryopreservation

Genotypes	Name	Crop type	Propagation origin
48 P	*R. grabowskii*	Wild blackberry	Screenhouse mother plant
48 Pf	*R. grabowskii*	Wild blackberry	Field-grown mother plant
48 A	*R. grabowskii*	Wild blackberry	Cryopreserved, LC, field
48 B	*R. grabowskii*	Wild blackberry	Cryopreserved, LC, field
48 C	*R. grabowskii*	Wild blackberry	Cryopreserved, LC, field
48 D	*R. grabowskii*	Wild blackberry	Cryopreserved, LC, field
252 P	Hillemeyer	Blackberry cultivar	Screenhouse mother plant
252 E	Hillemeyer	Blackberry cultivar	Cryopreserved, LC
252 F	Hillemeyer	Blackberry cultivar	Cryopreserved, LC
252 H	Hillemeyer	Blackberry cultivar	Cryopreserved, LC
252 I	Hillemeyer	Blackberry cultivar	Cryopreserved, LC
252 L	Hillemeyer	Blackberry cultivar	Cryopreserved, LC
252-1	Hillemeyer	Blackberry cultivar	Cryopreserved, SC
252-2	Hillemeyer	Blackberry cultivar	Cryopreserved, SC
252-3	Hillemeyer	Blackberry cultivar	Cryopreserved, SC
252-4	Hillemeyer	Blackberry cultivar	Cryopreserved, SC
252-5	Hillemeyer	Blackberry cultivar	Cryopreserved, SC
252-6	Hillemeyer	Blackberry cultivar	Cryopreserved, SC
252-7	Hillemeyer	Blackberry cultivar	Cryopreserved, SC
252-8	Hillemeyer	Blackberry cultivar	Cryopreserved, SC
633 P	Silvan	Blackberry cultivar	Screenhouse mother plant
633 P	Silvan	Blackberry cultivar	Field-grown mother plant
633 A	Silvan	Blackberry cultivar	Cryopreserved, LC, field
633-1	Silvan	Blackberry cultivar	Cryopreserved, SC
633-2	Silvan	Blackberry cultivar	Cryopreserved, SC
743 P	Mandarin	Raspberry cultivar	Screenhouse mother plant
743 B	Mandarin	Raspberry cultivar	Cryopreserved, LC

LC long culture, analyzed after 7 mo of subculture following removal from cryopreservation; *SC* short culture, *in vitro* plant analyzed immediately following removal from cryopreservation; *Screenhouse* mother plant grown in a pot in the screenhouse; *Field* plant also propagated in the greenhouse and planted in the field

pair using a fluorescently labeled forward primer and an unlabeled reverse primer. Reactions were performed in 15 μL volumes containing 1× reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 μM of each primer, 0.025 U of Biolase Taq DNA polymerase (Bioline USA Inc., Taunton, MA), and 3 ng genomic DNA. Fragment analysis was determined after separation on a Beckman CEQ 8000 genetic analyzer (Beckman Coulter Inc., Fullerton, CA). Up to two primer pairs were multiplexed after PCR. Allele sizing and visualization were performed using the fragment analysis module of the CEQ 8000 software.

AFLP analysis. Genomic DNA (200 to 500 ng) was digested with *Eco*RI and *Mse*I. After adaptor ligation and double-digestion, pre-amplification was performed using A and C as selective nucleotides (*Eco*RI+A and *Mse*I+C, respectively). PCR was carried out in a total volume of 25 μL containing 2.5 μL of 10× biolase buffer, 0.75 μL of 50 mM $MgCl_2$, 2.5 mM of each dNTP, 0.75 μL of 10 μM each of primer *Eco*RI+A and *Mse*I+C, 0.125 μL of 5 U Biolase, and 5 μL of DNA. The MJ thermocycler (MJ Research Inc., Reno, NV) was used with the following cycling parameters: 20 cycles of 30 s at 94°C, 60 s at 56°C, and 120 s at 72°C. For selective amplification, the pre-amplified DNA was diluted 50-fold with TE buffer and used as template DNA. *Eco*RI and *Mse*I primers with three selective bases at the 3′ end were used for selective amplification. For detection, the *Eco*RI-based primers were fluorescently labeled with Well-Red D4 (Sigma-Aldrich, St. Louis, MO) fluorescent dye. Sixteen primer pair combinations or 64 primer pairs were initially tested to select for the most polymorphic set of primer pairs. The PCR amplification mixture (15 μL final volume) consisted of 1.5 μL of 10× PCR buffer, 1.2 μL of 25 mM $MgCl_2$, 2.5 mM of each dNTP, 0.938 μL of labeled 10 μM *Eco*RI+3 primer, 0.938 μL of unlabeled 10 μM *Mse*I+3 primer, 0.075 μL of either 0.75 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) in LC plants or 0.75 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) in SC plants, and 3 μL of diluted pre-amplification product.

Selective amplification was carried out in an MJ thermocycler using the following temperature profile: an initial denaturation step of 94°C for 2 min; nine cycles of 94°C for 30 s, 65°C for 30 s which decreases by 1°C/cycle for those nine cycles; 72°C for 2 min followed by 24 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, with one final cycle of 72°C for 3 min. Ten primer pairs that amplified up to 547 bands were chosen for AFLP analysis (Table 2).

The fluorescently labeled amplified fragments were analyzed by capillary gel electrophoresis using the CEQ 8000 Genetic Analyzer (Beckman Coulter, Brea, CA). The inclusion of internal size CEQ-600-size standard in each lane enabled accurate scoring (presence/absence) of DNA fragments that are 85–500 base pairs (bp) in size.

Genetic analysis of field-grown polymorphic plants. Genetic analysis of greenhouse-grown and field-transplanted LC *in vitro* cryopreserved plants and control screenhouse mother plants (non-cryopreserved or tissue cultured) was evaluated using primers that generated AFLP polymorphisms in *R. grabowskii* (E-AAC/M-CTA, E-AAG/M-CTC, and E-ACG/M-CTT) and in 'Silvan' (E-ACG/M-CAC and E-AAG/M-CTC).

Analysis of SSR and AFLP data. SSR and AFLP products were scored as present (1) or absent (0) to create a binary matrix. A Perl script converted the dominant data into a binary data format. Genetic distance matrices (Euclidean distance) were computed using NTSYS-PC (Numerical Taxonomic System, Exeter Software), version 2.1 (Rohlf 2000). The Euclidean distances were calculated as follows:

$$E_{ij} = \left[\sum_k \left(x_{ki} - x_{kj} \right)^2 \right]^{1/2}$$

where E_{ij} is the genetic distance between individuals i and j; x_{ki} and x_{kj} are the ith band scores (1 or 0) for individuals i and j. Cluster analysis was performed on standardized data based on the Euclidean distance coefficient and unweighted pair-group method. The dendrogram was generated using the TREE sub-program of the software package NTSYS-PC.

Table 2. Sequences of primers and adaptors used in amplified fragment length polymorphism analysis (primer pairs used in the analysis included: E-AAG/M-CAA, E-ACG/M-CAC, E-ACT/M-CAT, E-AGC/M-CAA, E-AAG/M-CTC, E-ACG/M-CTT, E-AGG/M-CAA, E-AAC/M-CTA, E-AAG/M-CTC, and E-AGG/M-CTT)

Primer	Nucleotide[z]
*Eco*RI preselective primer	5′-GACTGCGTACCAATTC**A**-3′
*Mse*I preselective primer	5′-GATGAGTCCTGAGTAA**C**-3′
*Eco*RI adaptor	5′-GACTGCGTACCAATTC-3′
*Eco*RI selective primers (+3)	5′-GACTGCGTACCAATTC**AAC**-3′
*Eco*RI selective primers (+3)	5′-GACTGCGTACCAATTC**AAG**-3′
*Eco*RI selective primers (+3)	5′-GACTGCGTACCAATTC**ACT**-3′
*Eco*RI selective primers (+3)	5′-GACTGCGTACCAATTC**ACG**-3′
*Eco*RI selective primers (+3)	5′-GACTGCGTACCAATTC**AGC**-3′
*Eco*RI selective primers (+3)	5′-GACTGCGTACCAATTC**AGG**-3′
*Mse*I adaptor	5′-GATGAGTCCTGAGTAA-3′
*Mse*I selective primers (+3)	5′-GATGAGTCCTGAGTAA**CAA**-3′
*Mse*I selective primers (+3)	5′-GATGAGTCCTGAGTAA**CAC**-3′
*Mse*I selective primers (+3)	5′-GATGAGTCCTGAGTAA**CAT**-3′
*Mse*I selective primers (+3)	5′-GATGAGTCCTGAGTAA**CTA**-3′
*Mse*I selective primers (+3)	5′-GATGAGTCCTGAGTAA**CTC**-3′
*Mse*I selective primers (+3)	5′-GATGAGTCCTGAGTAA**CTT**-3′

[z] The three selective nucleotides in the selective primers are in bold

Results

SSR analysis. The ten primer pairs amplified one to 15 alleles per locus, with an average of seven alleles and a total of 70 alleles in the four genotypes evaluated. Primer RhM018 was monomorphic, and primer RhM043 was the most polymorphic of the ten primers showing 15 alleles. SSR analysis of LC and SC cryopreserved *in vitro* plants did not show any variation compared to the screenhouse-grown mother plants at the ten SSR loci examined (data not shown).

AFLP analysis. Ten AFLP primer pairs were used to evaluate genetic stability in 11 LC plants and ten SC plants. These AFLP primer pairs produced 547 amplified fragments in *R. grabowskii*, 400 in "Mandarin": 530 in 'Silvan' and 521 in "Hillemeyer" LC plants with Platinum Taq polymerase. An appreciably lower number of amplified fragments were generated in SC plants using Gold Taq polymerase: 331 in "Hillemeyer" and 379 in 'Silvan.' Average number of fragments per primer pair was 55 in *R. grabowskii*, 40 in "Mandarin," 53 in 'Silvan,' and 52 in "Hillemeyer" LC plants. The average number of fragments per primer pair was lower in SC plants: 33 in "Hillemeyer" and 38 in 'Silvan.' All LC cryopreserved *in vitro* plants except "Hillemeyer" showed polymorphism when compared to the screenhouse-grown mother plants (Table 3). SC *in vitro* plants did not show any variation compared to the mother plants with the selected primer pair combinations (Table 3 or not shown).

Cluster analysis. In the SSR-based dendrogram, the accessions grouped with their respective cultivar irrespective of cryopreservation or duration in culture (not shown). In the AFLP-based dendrogram (Fig. 1), "Hillemeyer" LC and SC

Table 3. Amplified fragment length polymorphism (AFLP) polymorphisms of cryopreserved, long-cultured (LC) *in vitro Rubus* accessions when compared to screenhouse-grown mother plants using ten AFLP primer pairs

Primers/plant sample	*R. grabowskii* A	*R. grabowskii* B	*R. grabowskii* C	Silvan A	Mandarin B
E-AAC/M-CTA	−126, −166, −193, −365	−126, −132, −166, −181, −193, −365	−126, −166, −193, −365		
E-AAG/M-CTC		−246, +247, −353	−240, −246, +247, −353	−336	
E-ACG/M-CTT	−107, −202	−107, −202	−107, −202		
E-ACG/M-CAC				+88, −89, +111, +178	+88, +118, +125, +130, −163, +193, +198, −256, +322, −441
E-ACT/M-CAT					+114, −116, +117, +135, +146, −147, +195, +219, +248, +270
E-AGG/M-CTT					−90, −128, +163, +307
E-AGC/M-CTA					+108, +119, −124, +165, +167, +188, −194, +320, −438

Polymorphism was observed with seven AFLP primer pairs

in vitro plants did not differ from the screenhouse-grown mother plants. For 'Silvan,' the SC plants were identical to the mother plant, but the LC plant was different. Within *R. grabowskii*, three of four cryopreserved LC plants (48A, 48B, and 48C) were different from the screenhouse plant. The one recovered "Mandarin" LC shoot tip had a different AFLP fingerprint from the screenhouse mother plant.

Genetic analysis of field-grown plants. Greenhouse-grown plants from the polymorphic LC *in vitro*-grown shoots showed no obvious morphological variation from the greenhouse-grown controls. These plants (except for "Mandarin") were then transplanted to the field and grown for 1 yr. No differences in AFLP-generated fingerprints were detected between field-grown cryopreserved plants

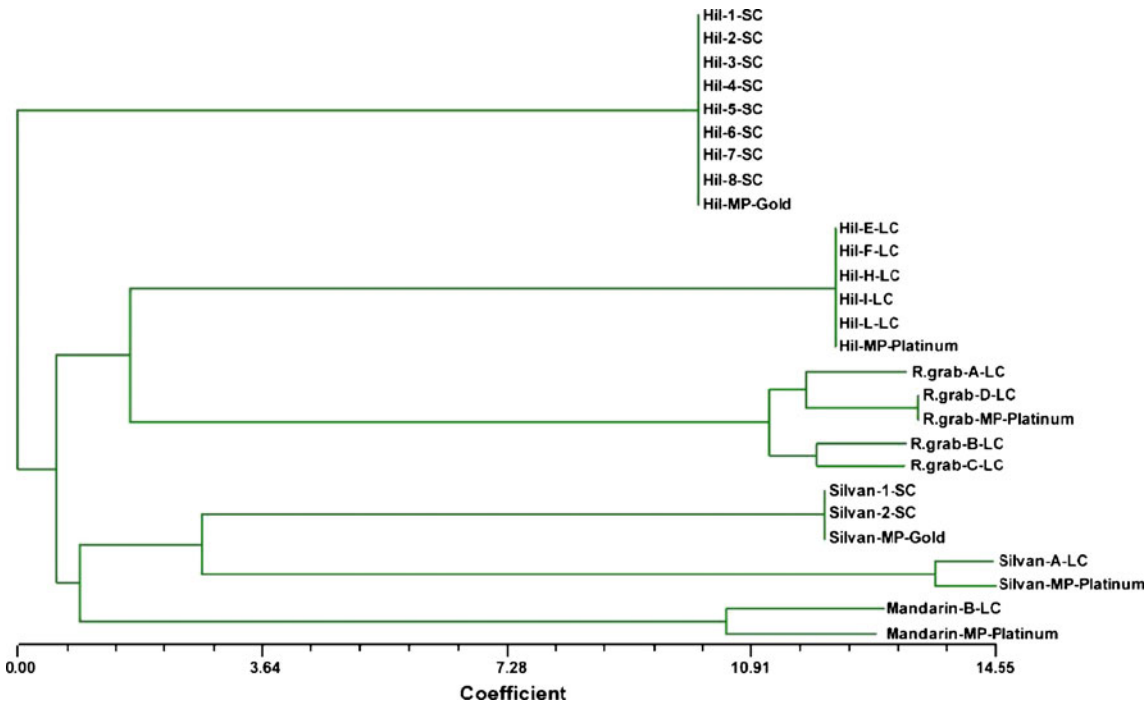

Figure 1. Neighbor joining (NJ) dendrogram of cryopreserved and screenhouse-grown mother *Rubus* plants based on amplified fragment length polymorphism data. Screenhouse plant: *MP* for mother plant. Long-cultured and short-cultured plants of "Hillemeyer" (*Hil*) and 'Silvan' and only LC of *Rubus grabowskii* (*R. grab*) and "Mandarin."

and control plants of either accession. The stability of genetic variation observed in "Mandarin" could not be confirmed due to the loss of the plants.

Morphological analysis of greenhouse and field-grown plants. In vitro plants that displayed AFLP-based polymorphism were transplanted to the greenhouse and later to the field along with plants propagated from the screenhouse mother plants. Visual assessments of approximately 10-mo-old greenhouse-grown LC cryopreserved plants and screenhouse-grown mother plants showed no gross morphological differences. All greenhouse-grown plants had the same leaf shape and presence or absence of spines (data not shown). After 1 yr in the field, all the plants generally appeared true to type; however, some specific morphological variations were observed between the LC plants and the screenhouse mother plants (Table 4). In general, the cryopreserved plants were more vigorous, as is common with tissue culture-derived plants. For the one LC 'Silvan' shoot tip studied, the number of leaflets, leaf width, and the length of the secondary petiole were all significantly larger ($P<0.05$) in the polymorphic LC cryopreserved plant than in the control screenhouse plant. Plants from three polymorphic LC shoot tips (A, B, and C) and one normal LC shoot tip (D) of *R. grabowskii* were analyzed (Table 4). There were more leaflets per leaf in all of the cryopreserved LC plants than the screenhouse mother plant control. Leaves were shorter and narrower, and the secondary petiole was shorter in cryopreserved plant A than in the control but not significantly different from the other cryopreserved plants. Cryopreserved plant C had longer primocane internodes than the control or other cryopreserved plants. Seeds of all the cryopreserved plants were longer but narrower than the control plant, while the berries (aggregate fruit) from cryopreserved plants were wider and sometimes longer. Individual fruit size (drupelet) and number of drupelets per compound fruit varied greatly and were significantly different in one or two plants and not the others.

Discussion

The various types of DNA markers detect different levels of polymorphism and different amounts of DNA change. For this reason, the use of more than one marker can increase the probability of variation detection. Genetic stability is the norm in most studies of possible plant genetic variation following cryopreservation (Harding 2004). In the same way, no differences were observed between *Rubus* screenhouse-grown mother plants and *in vitro* cryopreserved LC or SC plants using ten SSR loci (data not shown). This lack of variation suggests that there were no changes in the genetic fidelity of the plants due to cryopreservation. This was also the case in *Solanum*, in which the microsatellite sequences of plants regrown from cryopreserved apices were identical to the profiles of the parent plants and their progeny (Harding and Benson 2001). No structural changes were observed in the *in vitro* control or the *Solanum* plants grown from the cryopreserved germplasm, indicating

Table 4. Morphological analysis of field-grown *Rubus* plants derived from polymorphic cryopreserved shoot tips

	Leaflet number	Leaf length	Leaf width	Secondary petiole	Primocane internode	Fruit width	Fruit length	Drupelet number	Drupelet size	Seed width	Seed length
R. grabowskii											
Control	3.5 b	21.28 a	20.42 a	4.20 a	5.02 b	1.50 c	1.49 b	17.67 bc	0.55 c	0.45 a	0.32 b
Cryo plant A	5.0 a	17.81 b	17.42 b	3.50 b	4.50 b	2.00 ab	1.48 b	24.33 a	0.57 bc	0.31 bc	0.48 a
Cryo plant B	5.0 a	20.58 a	19.25 ab	4.00 ab	5.22 b	1.88 b	1.48 b	22.17 ab	0.70 a	0.30 c	0.45 a
Cryo plant C	5.0 a	19.42 ab	19.00 ab	4.03 ab	6.27 a	1.88 b	1.82 a	17.00 c	0.63 abc	0.34 bc	0.47 a
Cryo plant D	5.0 a	20.58 a	19.83 a	4.07 a	5.27 b	2.03 a	1.83 a	21.00 abc	0.67 ab	0.32 bc	0.48 a
LSD	0.44	1.73	2.37	0.18	0.71	0.14	0.1	4.55	0.12	0.03	0.04
Silvan											
Control	3.50 b	15.83 a	16.83 b	2.47 b	6.17 a						
Cryo plant	5.17 a	17.00 a	19.75 a	2.75 a	6.47 a						
LSD	1.02	1.9	1.78	0.28	0.53						

Means separation by Duncan's multiple range test; $n=6$. Means in a *column* followed by the same *letter* are not significantly different ($P<0.05$)

Only *R. grabowskii* had fruit at the time of evaluation. An aggregate fruit is composed of multiple drupelets (small fleshy fruits with a hard pit surrounding a single seed)

Only one 'Silvan' shoot tip was recovered from cryopreservation

LSD least significant difference

stable inheritance of SSR sequences in the somatic progeny (Harding and Benson 2001). The low coverage of the genome is one criticism of molecular techniques. Despite being highly polymorphic and co-dominant, SSRs may be clustered and distributed unevenly in certain chromosome locations. Lower coverage of the genome or a low number of primers may also fail to detect variability as reported by Wolff et al. (1995), who failed to detect RAPD- or SSR-based differences between phenotypically different members of a *Chrysanthemum* family that were either tissue cultured or vegetatively propagated. Regardless of the method used, Sharma et al. (2007) reports that much less than 1% of the genome can be assayed with these molecular markers. De Verno et al. (1999) also concluded that more markers were needed after generating identical RAPD profiles in freshly thawed embryogenic cultures of *Picea glauca* using ten primers. Detection of somaclonal variation would probably require an extremely high frequency of mutation for detection. Because of this, we also chose to look at AFLP markers in *Rubus*.

In this study, AFLP was able to detect differences in three out of the four *in vitro* LC *Rubus* accessions studied. AFLP polymorphism was observed in the *in vitro* LC plants of *R. grabowskii*, 'Silvan,' and "Mandarin" but not in "Hillemeyer." In *R. grabowskii*, three of four cryopreserved plants LC had different AFLP fingerprints when compared to the screenhouse mother plant, as did one 'Silvan' and one "Mandarin" LC plant. Such genotype-dependent variation has been observed in other cryopreserved plants like papaya (Kaity et al. 2008). SC plants of "Hillemeyer" and 'Silvan' showed no differences based on AFLP analysis (Fig. 1). RAPD markers detected somaclonal variation in some cryopreserved embryogenic cultures of white spruce 2 and 12 mo after they were reestablished *in vitro* following cryopreservation. However, variation was no longer observed after the trees were grown in the field (De Verno et al. 1999). We also observed no AFLP variation in LC *Rubus* cryopreserved plants of *R. grabowskii* or 'Silvan' after 1 yr of growth in the field. These results suggest that transient variation due to the *in vitro* culture process exists, but it may not affect the stability of regenerated plants.

Morphological markers depend on the growth stage and are easily affected by the environment, so visual evaluation may not accurately reflect variations that may occur within the plants. Morphological markers require extensive observation of the plants until maturity. Visual assessment of greenhouse plants grown from the polymorphic *Rubus* shoots and screenhouse-grown mother plants showed no phenotypic differences. These plants were not tested by AFLP until after growth in the field.

General evaluation of the polymorphic *Rubus* plants after 1 yr in the field showed no gross differences other than increased vigor in the shoot-tip-derived plants. Evaluation of specific morphological characteristics of field plants indicated variation in the vigor and size of the plants, leaves, fruit, and seeds (Table 4). Evaluation of this comparison is difficult because of the increased vigor shown by plants propagated in tissue culture compared to traditionally propagated plants. Many examples of *in vitro*-stimulated vigor of regrown plants are noted in the literature (Harding 2004). A longer (2 to 3 yr) evaluation of these plants is likely needed for a complete morphological comparison. The AFLP analysis of these field-grown *Rubus* plants showed no difference between the formerly polymorphic LC plants and those grown from screenhouse mother plants.

Cryopreservation causes many stresses that could potentially injure or affect the genetic stability of plants or result in epigenetic changes (Harding 2004). A recent study of oak embryogenic cultures found that the steps in the vitrification protocol did not affect the genetic stability of the cultures, and that storage in liquid nitrogen did not cause genetic change (Sanchez et al. 2008). However, oxidative stress during the vitrification protocol, in the form of lipid peroxidation, impacts the recovery of *Rubus* shoot tips from cryopreservation (Uchendu et al. 2010). The cryopreservation procedure may not cause genetic change; however, regrowth from calluses or the effect of plant growth regulators in culture might affect the genetic stability. Previous studies showed that blackberry and raspberry genotypes can be cryopreserved but sometimes with a lower percentage of shoot formation and with some genotypes producing calluses (Reed and Lagerstedt 1987; Reed 1993). Extended cold acclimation and optimization of the composition of the recovery medium with a reduction in IBA were found to improve the regrowth of cryopreserved *Rubus* shoot tips and reduce or eliminate callus formation (Chang and Reed 1999). Departure from organized meristematic growth makes plants more prone to genetic variation. It may also be possible that genetically altered plants do not survive to produce plantlets. "Hillemeyer" had a high regrowth rate following thawing, and we found no variation in either SC or LC plants; the other genotypes had less regrowth, and variation was seen in LC plants. The genotypic influence on variation also explains the different susceptibility of genotypes to change as in *Coffea* where genetic variation frequency was observed to vary by family (Etienne and Bertrand 2003).

The variation observed in LC *in vitro Rubus* shoots was illustrated in the dendrogram constructed using AFLP markers (Fig. 1). While SSR-based analysis indicated genetic stability of the cryopreserved plants for each of the *Rubus* genotypes, AFLP analysis revealed differences within LC *R. grabowskii*, 'Silvan' and "Mandarin" plants *in*

vitro (Fig. 1). The ability of AFLP markers to detect variability may be attributed to their high marker or diversity index (Russell et al. 1997; Vendrame et al. 1999). The index reflects the efficiency of these markers to simultaneously analyze a larger number of bands and is considered more powerful in comparison to RFLP, RAPDs, and SSRs (Russell et al. 1997). Cryopreserved yam (*Dioscorea*) shoots were genetically stable when compared to the original *in vitro* cultures (Mandal et al. 2008), and similar results were seen for apple shoot cultures (Liu et al. 2008).

The changes noted are likely due to methylation. After cryopreservation, changes were observed in the methylation status of the genome of several *in vitro* cultured plants (Hao et al. 2002a, b; Kaity et al. 2008; Johnston et al. 2009). Polymorphisms representative of demethylation sites were detected in cryopreserved strawberry when compared to the *in vitro* cultures from which they were derived (Hao et al. 2002b). Using MSAP, three demethylation sites and one *de novo* methylation were found in a single cell line of cryopreserved *Citrus* callus, compared to the original culture (Hao et al. 2002a). Using the AMP technique, methylation ranged from 0.52% to 6.62% of detected markers in cryopreserved shoot tips of *C. papaya* as compared to the original cultures (Kaity et al. 2008). Cryopreserved and cold stored hop (*Humulus*) plants showed no genetic variation with AFLP, but MSAP indicated that about 36% of the plants were polymorphic when compared to screenhouse-grown mother plants, and about half the polymorphisms were shared by the two treatments, while the remainder were not identical for the two types of cold stresses (Peredo et al. 2008). Changes in methylation status of sucrose pretreated or cryopreserved *Ribes* shoot tips were not persistent and returned to their original levels after additional subcultures (Johnston et al. 2009). None of these plants was evaluated in the field, so postcryopreservation phenotypic and genotypic evaluations of these plants are needed to assess both the stability and the significance of these changes.

Since the variation detected in LC *in vitro* plants of 'Silvan' and *R. grabowskii* was no longer observed after 1 yr of growth in the field, we believe that the variation was transient. In a similar study, spruce embryogenic cultures showed somaclonal variation after cryopreservation, but the variations were no longer detected in the regrown trees (De Verno et al. 1999).

The results of this study of cryopreserved *Rubus* shoot tips show the need for genetic analysis of cryopreserved plants after regrowth of the plants in the greenhouse or the field rather than *in vitro*. Variation observed in tissue-cultured plants after cryopreservation can be transient as was observed in 'Silvan' and *R. grabowskii* in this study. AFLP appears more sensitive and effective at detecting variations than SSR markers, especially when using such a small number of unmapped SSRs (ten in this study). In this case, the variations may have been because of methylation, so future evaluations should include methods for detection of methylation modifications such as the MSAP or AMP techniques.

After more than 12 yr of storage in liquid nitrogen shoot tips of several *Rubus* genotypes were regrown in culture and then transplanted to the greenhouse and then into the field. Although some shoot tips produced AFLP polymorphisms after several months of *in vitro* culture, AFLP analysis of the polymorphic plants that were grown in the field no longer detected variation. This analysis of the regrown plants and the change in the amount of variation detected also highlights the need for careful assessments. This includes testing later stages of growth, since epigenetic changes may be transient as seen with these *Rubus* shoot tips. This study confirms that long-term storage of shoot tips in liquid nitrogen successfully retains the genetic stability of the original plants.

Acknowledgements This study was supported by United States Department of Agriculture-Agricultural Research Service CRIS project 5358-150-21000-3300D. N.R.F. Castillo thanks the Fulbright Fellowship Program for support of her MS study. The technical assistance of Barbara Gilmore and April Nyberg was greatly appreciated.

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