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Original Research Article

Morphological and Genetic Stability of Dormant Apple Winter Buds After Cryopreservation

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Abstract - Twenty apple germplasm accessions from the Korean Genebank were successfully cryopreserved using two-step freezing to back up genetic resources maintained by field collections. This study examined the morphological and genetic stability of cryopreserved dormant apple buds that were stored in liquid nitrogen, and then rewarmed and regrown. Whole plants were regenerated directly from dormant buds through budding without an intermediary callus phase. The cryopreserved buds produced high levels of shoot formation (76.2-100%), similar to those of noncryopreserved buds (91.3-100%), with no observed differences between cryopreserved and noncryopreserved materials. Three of the twenty cryopreserved apple germplasm accessions were used to assess morphological and genetic stability. No differences in morphological characteristics including shoot length, leaf shape, leaf width/length ratio, and root length were observed between controls (fresh control and noncryopreserved) and cryopreserved plantlets. The genetic stability of regenerants (before and after cryopreservation) was investigated using inter simple sequence repeat (ISSR) markers. The ISSR markers produced 253 bands using four primers, ISSR 810, SSR 835, ISSR 864, and ISSR 899. These markers showed monomorphic banding patterns and revealed no polymorphism between the mother plant and regenerants before and after cryopreservation, suggesting that cryopreservation using two-step freezing does not affect the genetic stability of apple germplasm. These results show that two-step freezing cryopreservation is a practical method for long-term storage of apple germplasms.

Key words - Apple, Cryopreservation, Dormant bud, Two-step freezing, Genetic stability

Introduction

Apple is a fruit tree found in orchards throughout the world. In general, the conservation of apple genetic resources involves field planting (field gene banks) for vegetative propagation of plants, but this approach typically requires large amounts of space and extensive maintenance. Tissue culture has distinct advantages, and it is used for short-term preservation (Withers and Engelmann, 1997), but it does not provide for long-term preservation. The occurrence of genetic variation in plant material produced from tissue culture has been described for many species such as horseradish, alfalfa, and pecan trees (Rostiana *et al.*, 1999; Vendrame *et al.*, 1999; Piccioni *et al.*, 1997). Cryopreservation is an alternative approach for the long-term storage of plant germplasm and is the preferred option for the long-term preservation of clonally propagated germplasm. Under cryopreservation, plant materials are stored at ultra-low temperatures (-196℃) in liquid nitrogen (LN). At this temperature, cell division and metabolic activity are suspended, and the material remains unchanged for a long period, thereby conferring genetic stability to clonal germplasm, including materials produced from apple. Somaclonal variation in cryopreserved plant material has been assessed in potato, Scots pine, cassava, and sugarcane, and no genetic differences were found in regenerated plantlets following LN immersion (Harding and Benson, 2000; Haggman *et al*., 1998; Gonzalez-Amao *et al*., 1999). Studies of genetic alteration in apple have been limited (Wu *et al*., 1999), and no studies on simultaneous changes in phenotype and genotype have been published.

Cryopreservation of dormant vegetative apple buds was first described by Sakai and Nishiyama (1978). Protocols based on endogenous production of cryoprotectants in dormant apple buds were refined by Stushnoff (1987), Tyler and Stushnoff (1988), and Tyler *et al*. (1988) with recovery by grafting. Subsequently, an effective protocol for cryopreservation

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Table 1. Details of ISSR primers, locus, sequence, annealing temperature, and cycles used in analysis of apple germplasms

Locus	Sequence (5'-3')	Ta[z]	Cycle
810	GAGAGAGAGAGAGAGAT	50℃	30
835	AGAGAGAGAGAGAGAGYC	50℃	30
864	ATGATGATGATGATGATG	50℃	30
899	CACACACACACARY	50℃	30

[z]Ta: annealing temperature.

of dormant winter apple buds was developed at the National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, CO, USA, using material grown in the continental climate (Forsline *et al*., 1998; Forsline *et al*., 1999; Towill and Ellis, 2008; Towill *et al*., 2004). Previous studies have detailed the improvement of a standard 'two-step freezing' method to induce a higher rate of shoot formation from cryopreserved vegetative apple buds and to implement long-term conservation of apple germplasm in the Korean Genebank (Yi *et al*., 2013). In this study, we cryopreserved 20 accessions of apple genotypes using a two-step freezing method adapted for Korean genotypes. Subsequently, we observed morphological characteristics and examined genetic variation at the molecular level using inter-simple sequence repeat (ISSR) markers to assess the genetic stability of plantlets recovered from three cryopreserved apple germplasms.

Materials and Methods

Plant materials

Stems containing the current season of growth from 20 apple cultivars were collected in early January 2013 when the buds were quiescent in a field at the National Institute of Horticultural and Herbal Science (NIHH), Daegu, Republic of Korea. The stems were collected after the temperature had been below approximately 0℃ for at least 72 h. The sampled scions were wrapped and stored at -5℃ for cold acclimation for 3 weeks.

Cryopreservation procedures using two-step freezing

The stems were cut into 35 ㎜ long, single-node sections with the bud in the central position, and then spread on trays and kept unsealed in a freezer at -5℃ to dehydrate. When the stem sections reached the target moisture content of 35%, they were double wrapped in moisture-proof plastic to eliminate further desiccation and maintained at -5℃ until treatment with liquid nitrogen (LN). The sections were packaged in polyolefin tubes (Fig. 1-A) and cooled to -35℃ at a rate of 1℃/h and maintained for 24 h using a programmable refrigerator (Dasol Scientific, Hwaseong, South Korea). The tubes were then moved quickly from the cooling unit and placed in liquid nitrogen vapor for at least 24 h (Fig. 1-B). Warming was accomplished by transferring the tubes to a 4℃ room in containers with moist peat, where they were kept for 7 d.

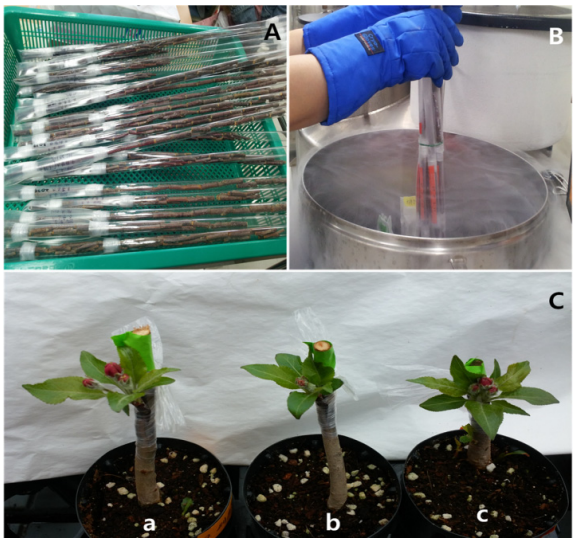

Fig. 1. Procedure of 'two-step freezing' for *Malus domestica* A: Single bud segments in polyolefin tubes desiccated to 35% water content. B: Second freezing of frozen single bud segments from -35℃ to -196℃ (liquid nitrogen). C: Shoot formation from a grafted bud obtained from a section that was cooled at 1℃/h to -35℃, held for 24 hrs, transferred to liquid nitrogen, and thawed at 4℃. a: fresh control (30 days after grafting), b: noncryopreserved (30 days after grafting), c: cryopreserved (55 days after grafting).

Viability test

Stem viability was tested by grafting buds to seedling rootstock. Grafting was performed using a chip budding technique with 1 year old apple seedling rootstock. Rootstocks for grafting used in this study were standard seedlings (Dongbu Nursery, Kyungsan). The budded rootstocks were kept in a greenhouse and examined over a 2-month period for growth of the bud. We defined viability as the formation of a shoot from the grafted bud. For evaluating viability, each accession

was repeated in three replicated experiments with 10 plants. The data are presented as mean ± standard error (Mean ± SE).

Observation of morphological characteristics

Because the shoot tips surviving after cryopreservation exhibited a lag phase of 25 d after budding, we used control shoots 30 d after grafting and surviving cryopreserved shoots 55 d after grafting to observe morphological characteristics including shoot length, leaf shape, leaf width/length ratio, and root length. Morphological analysis was performed on 10 plants with three replicates of each treatment.

DNA extraction and inter simple sequence repeat (ISSR)-PCR analysis

Total genomic DNA was isolated from young leaves using a Gentra Puregene Cell Kit for plants (Qiagen, Hilden, Germany). The isolated DNA quality and concentration were determined using a spectrometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA), and the DNA was diluted to a working concentration of 20 ng/μl. Of 99 ISSR primers from a UBC primer set (University of British Columbia, Vancouver, Canada), four primers revealed reproducible and clear amplicons, and these primers were used for further study. The sequences of these four primers were shown in Table 1. PCR amplification was carried out in a 25 μl reaction volume containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 1.0 U *Taq* DNA polymerase (Inclone Biotech, Republic of Korea), 0.3 μM primer, and 20 ng genomic DNA. The amplification was performed in a PTC-200 thermocycler (MJ Research, MA, USA) with reaction conditions programmed for an initial predenaturation at 94℃ for 5 min followed by 45 cycles of denaturation at 94℃ for 1 min, annealing at 50℃ for 45 s, and extension at 72℃ for 2 min, with a final extension at 72℃ for 5 min. Amplification products were separated by electrophoresis in 1.5% agarose gel stained with non-toxic LoadingSTAR (Dynebio, Republic of Korea) and visualized with ultraviolet (UV) light.

Results and Discussion

Survival of cryopreserved dormant buds and shoot formation

In preliminary experiments to determine optimal conditions, the two-step freezing procedure proved to be effective for the cryopreservation of apple dormant buds. We previously developed a protocol adapted for Korean winter dormant apple buds, which promotes the preservation of valuable germplasms for successful apple breeding (Yi *et al*., 2013). In this study, 20 apple germplasm accessions were preserved using this protocol. As shown in Table 2, the cryopreserved buds produced high levels of shoot formation (76.2-100%, mean 87.29%) similar those of the noncryopreserved buds (91.3-100%, mean 97.79%), with no differences observed between the cryopreserved and noncryopreserved materials. Most of the cryopreserved stems regrew and produced new leaves by 55 d after grafting, in contrast to the noncryopreserved stems, which required only 30 d. The lag phase in the cryopreserved stems may have been caused by damage from the ultra-low temperature of the LN during cryopreservation.

Morphological stability

The shoots surviving after cryopreservation grew well and developed normal plantlets and roots (Fig 1-C). The morphological markers observed in this study included shoot length, leaf shape, leaf width/length ratio, and root length. Compared with the unfrozen controls (fresh control and noncryopreserved), no significant morphological differences were observed in the cryopreserved shoots (Table 3).

The aim of conserving plant genetic resources is not only to store germplasm but also to limit the introduction of variation at the lowest level possible during the procedures being used. Thus, any conservation method should preserve the genetic stability of the plant material. In this study, both the unfrozen controls (fresh control and noncryopreserved) and the cryopreserved shoots exhibited the same regrowth patterns, indicating normal growth and development in the cryopreserved shoots.

Assessment of genetic stability using ISSR markers

The genetic fidelity of the apple material after freezing in LN appeared to be maintained based on the lack of morphological differences in the materials. However, morphological evaluation is an indirect test method that can provide only a partial assessment of genetic stability. Molecular markers provide a more direct method by examining genetic variation

Table 2. Comparison of shoot formation rates between noncryopreserved and cryopreserved stems using grafting

Accession No.	Cultivar (Species)	Regeneration rates (%)		No. of stems conserved
		Noncryopreserved	Cryopreserved	
225487	Gukgwang (*Malus domestica*)	99.8 ± 8.34[z]	76.2 ± 3.61	140
225509	Sansa (*Malus domestica*)	99.3 ± 4.19	80 ± 4.78	172
225573	Cheonchu (*Malus domestica*)	98.0 ± 11.55	77.3 ± 4.22	149
225578	Chukwang (*Malus domestica*)	91.3 ± 5.11	81.5 ± 4.41	100
225596	Hongro (*Malus domestica*)	95.3 ± 3.33	85.4 ± 6.01	100
225602	Hwahong (*Malus domestica*)	98.4 ± 5.32	79.3 ± 3.56	100
225783	Geneva Black (*Malus domestica*)	98.5 ± 7.53	90.4 ± 4.11	114
225839	Hongok (*Malus domestica*)	99.7 ± 5.77	97.7 ± 4.29	168
225987	Pink Lady (*Malus domestica*)	99.4 ± 9.62	91.3 ± 2.65	157
226079	Spur Earliblaze (*Malus domestica*)	100.0	93.3 ± 5.44	29
249906	Alps Otome (*Malus domestica*)	98.9 ± 6.36	79.3 ± 3.01	297
249917	Arkansas Black (*Malus domestica*)	98.2 ± 6.76	82.6 ± 3.33	154
249985	Gala (*Malus domestica*)	99.1 ± 7.78	93.2 ± 4.32	176
253667	Red Field (*Malus domestica*)	100.0	100.0	107
253668	Fuji (*Malus domestica*)	100.0	95.8 ± 7.22	209
253737	Tsugaru (*Malus domestica*)	91.3 ± 2.31	84.7 ± 16.84	193
254788	Sinano Gold (*Malus domestica*)	98.3 ± 3.66	89.2 ± 10.1	74
255522	Summer Dream (*Malus domestica*)	91.3 ± 2.48	85.7 ± 24.74	140
255555	Honggeum (*Malus domestica*)	99.6 ± 2.45	91.1 ± 7.78	214
255855	Hwayoung (*Malus domestica*)	99.3 ± 5.78	91.7 ± 14.43	222
	Average	97.79	87.29	150.8

[z]Standard error.

at the DNA level. In this study, we used four selected ISSR primers to analyze genetic stability during the cryopreservation procedure based on the amplification of PCR fragments ranging in length from 200 to 2000 bp (Fig. 2). A total of 253 DNA fragments were produced equally in the fresh control, noncryopreserved, and cryopreserved groups. Three cultivars exhibited distinct genotypes using the ISSR 835, 864, and 899 primers, whereas the band pattern acquired using the ISSR 810 primer did not differ by cultivar (Fig. 2). No differences in bands were detected between control (fresh control and noncryopreserved) and cryopreserved shoots using the four ISSR primers, whereas two bands were detected specifically in the noncryopreserved group (Table 4, Fig. 2). Although cryopreservation of apple dormant buds does not appear to cause genetic variation, there is the possibility of point mutations outside of priming sites investigated that may go undetected. According to Castillo *et al*. (2010), the variation detected in long cultured *in vitro* plants of *Rubus* germplasms was no longer observed after one year of growth in the field, it is believed that the variation was transient. In a similar research, De Verno *et al.* (1999) reported that somaclonal variation after cryopreservation appeared during spruce embryogenic cultures, but the variations were no longer detected in the regrown trees.

For cryogenically derived material, most studies have identified no differences at the chromosome or DNA levels following cryopreservation. Zhang *et al*. (2001) tested plantlets regenerated from *Amorphophallus* shoot tips cryopreserved by vitrification and found no DNA alteration using random amplified polymorphic DNA (RAPD) markers. In another study, adventitious buds of rice haploids were successfully cryopreserved, and subsequent analysis of surviving regenerated shoots using RAPD markers showed no genetic variation (Zhang and Hu, 2000). Moreover, studies on potato by Harding

Table 3. Comparison of morphological characteristic of control shoots 30 days after grafting and of cryopreserved shoots 55 days after grafting

Cultivar	Condition	Shoot length (㎝)	Leaf shape	W/V[z]	Root Length (㎝)
Hongro	Fresh control	6.6 ± 0.21 ab[y]	Ellipse-like	0.57 ± 0.11	10.3 ± 0.33
	Noncryopreserved	7.0 ± 0.33 a	Ellipse-like	0.57 ± 0.14	10.1 ± 0.25
	Cryopreserved	6.8 ± 0.17 a	Ellipse-like	0.56 ± 0.12	10.1 ± 0.26
Hwahong	Fresh control	8.8 ± 0.15 a	Ellipse-like	0.46 ± 0.10	10.9 ± 0.15
	Noncryopreserved	8.9 ± 0.42 a	Ellipse-like	0.44 ± 0.06	10.9 ± 0.22
	Cryopreserved	8.8 ± 0.31 a	Ellipse-like	0.46 ± 0.05	10.7 ± 0.25
Chukwang	Fresh control	9.5 ± 0.36 a	Ellipse-like	0.51 ± 0.11	11.3 ± 0.32
	Noncryopreserved	9.4 ± 0.16 a	Ellipse-like	0.49 ± 0.02	11.0 ± 0.31
	Cryopreserved	9.6 ± 0.19 a	Ellipse-like	0.50 ± 0.07	11.1 ± 0.07

[z]W/V represents width/length of leaves, ten shoot or leaves with three replicate tests.
[y]Mean separation within columns by Duncan's multiple range test at 5% level by R project (R version 3.0.1) for statistical computing.

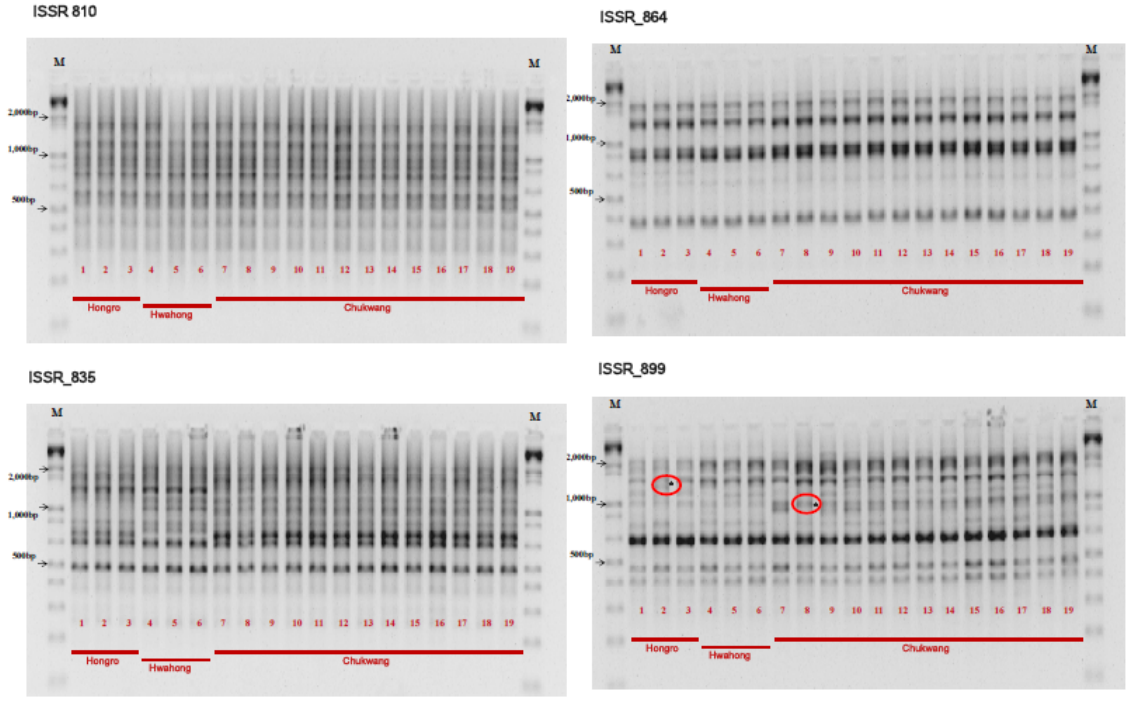

Fig. 2. ISSR profiles of fresh control, noncryopreserved, and cryopreserved plants with four primers (ISSR 810, ISSR 835, ISSR 864, and ISSR 899). Lanes 1, 4, and 7, fresh control; Lanes 2, 5, and 8, noncryopreserved; Lanes 3, 6, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, and 19, cryopreserved; M, DNA marker; *, detected specific bands.

(1991), Harding and Benson (2000), Ward *et al.* (1993), and Benson *et al*. (1996) found no differences between cryopreserved materials and controls. Maintenance of the genetic stability of cryopreserved germplasm has been also reported in *Melia* (Scocchi *et al.,* 2004), *Dioscorea* (Dixit *et al*., 2003), and in grape and kiwi (Zhai *et al*., 2003). Similarly, in our study, plants regenerated from cryopreserved dormant buds using two-step freezing were 100% genetically similar, suggesting that no DNA polymorphisms induced by cryopreservation accumulated (Harding, 2004).

The goal of the conservation of plant genetic resources is not only to store germplasm but to also minimize genetic

Table 4. The total number of amplified bands produced by four ISSR markers in three cultivars

Cultivar	Fresh control	Noncryopreserved	Cryopreserved
Hongro	32	33 (+1)	32
Hwahong	36	36	36
Chukwang	185	184 (-1)	185
Total	253	253	253

variation to the maximum extent possible during conservation. Maintenance of true-to-type clonal fidelity is an important factor to be monitored during conservation of vegetatively propagated species. In conclusion, we did not find genetic changes in the cryopreserved shoots as assessed using several morphological and molecular markers, thereby demonstrating that cryopreservation using two-step freezing of dormant buds is a practical method for the long-term storage of apple germplasm. To overcome the limitations of this study, a larger population is needed for further investigation of cryopreserved shoots.

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