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Genetic stability of cryopreserved ornamental *Lilium* germplasm

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Abstract

The genus *Lilium* contains a number of ornamental crop species, which are commercially important in many countries. As they are vegetatively propagated, maintaining genetic stability is essential for their efficient conservation. In this study, we investigated the genetic stability of regenerated plants of three cultivars (*L. bolanderi* 'Lenora', *L. bolanderi* 'Mount Duckling' and *L. bolanderi* 'Mount Dragon') and one variety (*L. callosum* var. *flavum*) after cryopreservation, compared with fresh (donor) and non-cryopreserved plants using morphological traits and ISSR markers. No differences in morphological parameters including flower, stigma and pollen colour, floral spots, floral direction or polymorphic bands were observed between control (fresh and non-cryopreserved) and cryopreserved plantlets. In addition, based on the resulting UPGMA dendrogram, the four taxa were divided into different clusters. All cryopreserved, non-cryopreserved and fresh plants in each group could be grouped together in a single cluster with more than 97 or 100% similarity. The results suggest a very low level or the absence of genetic variation in terms of morphological and genetic stability among the plants regenerated after cryopreservation.

Introduction

Field conservation, which is used for vegetatively propagated plants, has risk of loss caused by disease or unexpected climate. Also, *in vitro* conservation is susceptible to contamination and somaclonal variation (Towill, 1988; Chen *et al.*, 2011). One of the most effective methods for long-term storage is cryopreservation of biological material in liquid nitrogen (LN) at a low temperature of −196 °C using *in vitro* collections (Ahuja, 2011). Previous studies reported the role of cryopreservation as a viable means of long-term preservation in various vegetatively propagated plants, such as strawberry, potato and lily and demonstrated to improve the survival and regrowth of various plants after cryopreservation (Hirai *et al.*, 1998; Bouman *et al.*, 2003; Dhital *et al.*, 2009; Yi *et al.*, 2013). However, the challenges of genetic stability in cryopreserved plants are not completely resolved (Engelmann, 2014; Vidyagina *et al.*, 2021). It is desirable to assess the genetic alterations of plants after cryogenic storage to ensure that the method is appropriate for long-term, stable preservation of genetic diversity. The most commonly used PCR-based markers in studies of genetic stability are Random Amplified Polymorphic DNA (RAPD) (Yadav *et al.*, 2013) and inter-simple sequence repeat (ISSR) (Raji *et al.*, 2018). For *Lilium* species, the assessment of genetic stability using an ISSR marker has been reported (Yin *et al.*, 2013).

Previous studies have been undertaken to examine the genetic stability of *Lilium* germplasm after cryopreservation using various markers (Liu and Yang, 2012; Khandagale *et al.*, 2014), but few studies have evaluated the differences in morphological characteristics between control and regenerated plants at the mature stage (Yin *et al.*, 2014). The aim of this study is to assess the genetic stability of four *Lilium* taxa regenerated after cryopreservation.

Experimental

One accession each of four *Lilium* taxa (*L. bolanderi* 'Lenora', *L. callosum* var. *flavum*, *L. bolanderi* 'Mount Duckling' and *L. bolanderi* 'Mount Dragon') were used in this study for cryopreservation and determination of genetic stability in *Lilium* genetic resources. The germplasm accessions used in this study are listed in online Supplementary Table S1.

Cryopreservation

The experimental procedure of cryopreservation is described in online Supplementary Table S2, which was based on the protocol of Kim *et al.* (2009) with minor modifications.

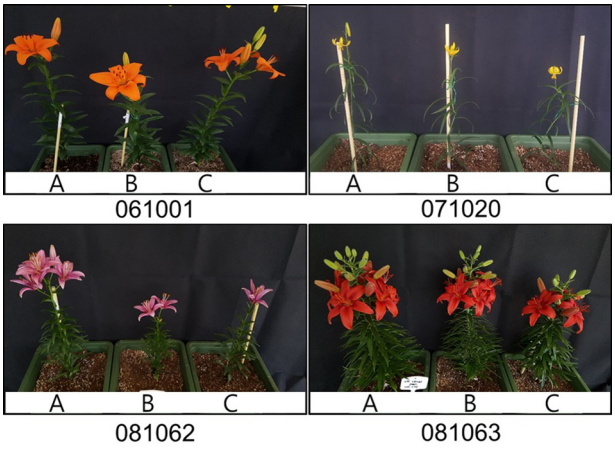

Fig. 1. Plant growth regenerated from four accessions of *Lilium* taxa, including fresh plants (A), non-cryopreserved (B), and cryopreserved (C) regenerants. Accession numbers 061001, 071020, 081062 and 081063 refer to *L. bolanderi* 'Lenora', *L. callosum* var. *flavum*, *L. bolanderi* 'Mount Duckling' and *L. bolandri* 'Mount Dragon', respectively.

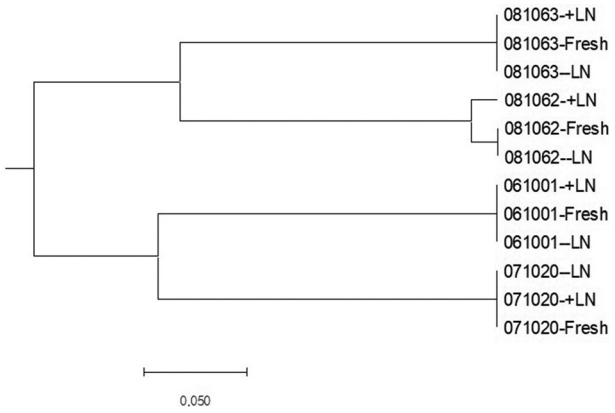

Fig. 2. UPGMA dendrogram of four accessions of *Lilium* taxa including fresh, non-cryopreserved (−LN) control and cryopreserved (+LN) plants generated using ISSR markers. Accession numbers 061001, 071020, 081062 and 081063 refer to *L. bolanderi* 'Lenora', *L. callosum* var. *flavum*, *L. bolanderi* 'Mount Duckling' and *L. bolandri* 'Mount Dragon', respectively.

Morphological characteristics

All samples (*in vitro* grown without treatment; fresh, before and after cryopreservation; −LN and +LN) were planted in a greenhouse and the morphological traits such as flower, stigma and pollen colour, floral spots and flower direction in 3 years after acclimatization were monitored (online Supplementary Table S1). We monitored the plants for a complete morphological comparison at maturity, not in the early growth stages.

Molecular analysis

To confirm the genetic fidelity using 6 ISSR markers, previously selected for their reproducible band patterns (Wang *et al.*, 2008; Bush *et al.*, 2010; Liu and Yang, 2012; Khandagale *et al.*, 2014) (online Supplementary Table S3), genomic DNA was extracted from the young leaves of the fresh plant and the plantlets (−LN and +LN) regenerated *in vitro* using a QIAGEN DNA extraction kit (QIAGEN Co. Germany). ISSR amplifications were carried out in a 25 µl total volume containing 20 ng of template DNA, 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 and 0.2 µM primer. DNA was amplified under the following thermal conditions: initial denaturation step of 5 min at 94 °C, followed by 30 to 40 cycles of each at 94 °C for 1 min, 50 °C for 45 s and 72 °C for 1 min with a final extension step at 72 °C for 7 min. Fragments were separated in 1.5% agarose gel and visualized in Gel Image Analysis System (CoreBio-MAXTM, Davinch-K, Seoul, Korea). Amplified bands were sized and scored as present (1) or absent (0) with three replications per treatment. The unweighted pair group method with an arithmetic mean (UPGMA) dendrogram of accessions was constructed with PowerMarker version 3.25 (Liu and Muse, 2005) and using the software MEGA (Tamura *et al.*, 2007).

Discussion

To confirm the genetic stability of *Lilium* germplasm after cryopreservation, the genetic integrity of four *Lilium* taxa was assessed via comparison of morphological traits and ISSR analysis under treatment conditions (fresh control, non-cryopreserved (−LN) and cryopreserved (+LN) plants). The results showed no differences in visual morphology such as flower, stigma and pollen colour, floral spots and flower direction between treatments of each accession (Fig. 1 and online Supplementary Table S1). These results are similar to those reported previously in different plants (e.g. Ahuja *et al.*, 2002; Chen *et al.*, 2011; Fki *et al.*, 2011). To validate morphological stability, we also assessed the genetic similarity between fresh and pre- and post-cryopreserved plants using ISSR analyses. Six markers were used to generate reproducible and distinguishable patterns of bands in the size range of 0.3 to 2.5 kb for the four accessions. Overall, a total of 79 bands were produced and the number of bands for each primer ranged from 10 to 16, with an average of 13 bands. The number of polymorphic bands ranged from 4 for UBC 880 to 13 for UBC 814, with a mean of 9 bands, and the rates of polymorphism ranged between 40 and 92.9% with a mean of 69.7% (online Supplementary Table S3). No differences in polymorphic bands were observed between +LN treatment plants and fresh and −LN plants. The genetic classification of fresh, −LN and +LN plants of the four *Lilium* taxa was based on ISSR markers as shown in Fig. 2. The resulting UPGMA dendrogram was used to divide the four taxa into different clusters. All plants exhibited more than 97% genetic similarity between treatments (Fresh, −LN and +LN) in 'Mount Duckling' (081062) and 100% in other accessions.

In conclusion, these results suggest a very low level or the absence of genetic variation among plants regenerated after cryopreservation. Although the cryopreservation has been successfully used for *Lilium* species, information of genetic stability in the mature plants regenerated from cryopreserved bulblets of *Lilium* has been lacking. The analysis of morphological and genetic fidelity in the current study supports the continued cryobanking of *Lilium* germplasm.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S147926212200003X

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