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Research Note

Polymorphism analysis of cryopreserved *Dendrobium* Bobby Messina protocorm-like bodies (PLBs) using RAPD markers

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Abstract

Dendrobium Bobby Messina, a sympodial orchid, is a new *Dendrobium* orchid hybrid from Indonesia with the parentage of (Imelda Romualdez × Jaquelyn Thomas). This orchid is cultivated for its cut flowers and potted plants, and is valued for its attractive flowers. Cryopreservation is a feasible long term storage method, whereby plants are stored at ultra low temperature using liquid nitrogen (-196ºC). Protocorm-like bodies (PLBs) are an attractive target explant for orchid cryopreservation. In this study, cryopreservation using plant vitrification solution 2 (PVS2) was carried out on PLBs of *Dendrobium* Bobby Messina using optimum conditions, and growth recovery was obtained at 40%. In the screening of suitable primers to detect polymorphism in both cryopreserved and non-cryopreserved PLBs in comparison with the stock culture, 20 primers were used but only 10 primers were able to generate bands. However, the number of bands produced in cryopreserved and non-cryopreserved PLBs from 3 primers and 1 primer indicated polymorphism and partial polymorphism respectively. The RAPD results from 6 selected primers indicated that the genetic stability of PLBs following cryopreservation was maintained. Hence, this method can be utilised as a potential cryopreservation approach for the germplasm conservation of *Dendrobium* Bobby Messina orchid.

Keywords: *Dendrobium* Bobby Messina, PLBS, PVS2, RAPD.
Abbreviations:PLBs-Protocorm like-bodies; PVS2-Plant Vitrification Solution; RAPD- Random Amplification of Polymorphic; AFLP-Amplified Fragment Length Polymorphism; DNA-Deoxyribonucleic acid; PCR-Polymerase Chain Reaction; RFLP-Restriction Fragment Length Polymorphism; SI-Similarity Index; VNTR-Variable Number Tandem Repeat.

Introduction

Successful cryopreservation procedures involves exposing selected explants to a series of stressing procedures such as preculture, cryoprotection, dehydration, rapid immersion in liquid nitrogen, thawing, recovery and plant regeneration (Reed, 2001). The freezing process may give rise to the formation of intracellular ice which could damage the cellular components such as disruption of the nucleus, chloroplast and mitochondrion (Coger and Toner, 1995). In addition, dehydration injury causes cell shrinkage and damage to the plasma membrane (Dowgert and Steponkus, 1984; Fleck et al., 2006; Ushendu et al., 2009). Exposure to excessive harsh physical environment such as very low temperatures, high osmotic pressure, dehydration and exposure to certain chemicals such as cryoprotectants during cryopreservation may cause physiological stress and in return gives rise to genetic instability (Panis and Lambardi, 2005). This stress produces the formation of free radicals, which leads to genetic alterations (Benson and Bremner, 2004). Examples of free radicals are reactive oxygen species (ROS) which include superoxides, hydrogen peroxide and hydroxyl radicals (Mittler, 2002). Potential somaclonal variation could occur in the regenerated plants especially after a cryopreservation procedure. Consequently, the long-term genetic consequences of dehydration and freezing injury for *in vitro* conservation need to be evaluated (Harding, 1996; Gagliardi et al., 2007). Plants recovered following cryogenic treatments should be genetically identical to the starting materials and genetic fidelity needs to be considered in the cryopreservation process (Ashmore, 1997). Random amplification of polymorphic DNA (RAPD) method is fast, simple, and efficient method for evaluating the genetic stability of plant materials (Nair and Reghunath, 2009). RAPD method have been effectively used to evaluate genetic relationship in numerous plants such as in apple (Bernardo Royo and Itoiz, 2004), sorghum (Singh et al., 2006) and sugar cane (Devarumath et al., 2007). RAPD analysis was successfully done in forty nine cumin ecotypes (*Cuminum cyminum* L.) in order to study the genetic diversity of the plant (Bahraminejad et al., 2012). In addition, RAPD method is useful in examining tissue culture induced variation such as seen in the identification of somaclonal variants in peach (Hashmi et al., 1997), sugarcane (Taylor et al., 1995), moth orchids (Chen et al., 1998) and bananas (Bairu et al., 2006). Genetic similarity of various orchid species of *Vanda sanderiana* and *Ascocentrum miniatum* was determined using this method (Lim et al., 1999). This technique has been used in classifications of various orchid genera such as *Phalaenopsis* (Chen et al., 1994; Goh et al., 2005; *Goodyera* (Wong and Sun, 1999) and *Zeucine* (Sun and Wong, 2001). It was also used in detecting the genetic alterations on clonally propagated *Dendrobium* hybrid which indicated the nonoccurrence of somaclonal variation (Ferreira et al., 2006). In the present work, the genetic genetic fidelity of the cryopreserved PLBs of *Dendrobium* Bobby Messina was determined using RAPD analysis with suitable primers. Therefore, the objective of this study was to evaluate the genetic fidelity of regenerated cryopreserved and non-cryopreserved PLBs of *Dendrobium* Bobby Messina

conserved using PVS2, in comparison with stock PLBs, using the RAPD method with selected primers.

Results

A total of 9 primers produced amplifications in DNA samples from the stock PLBs, and 8 primers produced amplifications products in DNA samples from both cryopreserved and non-cryopreserved PLBs. The 10 primers produced 44 bands for stock PLBs, 20 bands for cryopreserved PLBs and 19 bands for non-cryopreserved PLBs. The size of these amplification products range from between 100bp to 3000 bp (Tables 1, 2). When compared for genetic fidelity between cryopreserved PLBs and stock PLBs, a total of 20 DNA bands were produced for cryopreserved PLBs. A total of 18 bands (90%) were monomorphic and 2 bands (10%) were polymorphic, indicating that the genetic fidelity was maintained between the cryopreserved PLBs and stock PLBs. Six primers scored the similarity index (SI) value of 1.0 while 3 other primers scored as 0. Primer OPB18 produced an SI value of 0.4 indicating partial polymorphism. Primers OPA04 and OPAW17 produced 8 and 12 bands respectively from the DNA sample of stock PLBs while no products could be obtained in the DNA sample of the cryopreserved PLBs. Primer OPG15 did not resulted in any amplification product in DNA sample of PLBs stock culture while it amplified a total 2 DNA bands in cryopreserved PLBs (Table 1). To assess the genetic fidelity between the non-cryopreserved PLBs and stock PLBs, a total of 19 DNA bands were produced from non-cryopreserved PLBs. A total of 18 bands (94.7%) were monomorphic and 1 band (5.3%) was polymorphic indicating that the genetic fidelity was maintained between the non-cryopreserved PLBs and PLBs stock culture. Six primers scored the similarity index (SI) value of 1.0 and 3 other primers scored as 0. Primer (OPB18) produced an SI value of 0.4 indicating partial polymorphism. Primers, OPA04 and OPAW17 produced 8 and 12 bands respectively in the DNA sample from stock PLBs while no products could be obtained from the DNA sample of non-cryopreserved PLBs. Primer OPG15 did not result in any amplification products in the DNA sample of stock PLBs while it was observed to amplify 1 DNA band in non-cryopreserved PLBs (Table 2).

Discussion

The RAPD analysis results in the amplification of any locus by which the band may be present or absent, and the brightness or intensity of the band may be different. Band intensity differences are due to the copy number or relative sequence abundance and may serve to distinguish homozygote dominant individuals from heterozygotes. The fact that fainter bands are generally less robust in RAPD suggests that varying degrees of primer mismatch may account for many band intensity differences. Since the sources of band intensity differences are uncertain, such as due to copy number or primer mismatch, most studies disregard scoring differences in band intensity (Samagn et al., 2006). There are many factors that can influence the structural DNA and methylation variations following cryopreservation (Peschke and Phillips, 1992). However, the design of the cryopreservation protocol in the present study reduces the possibility of occurrence of somaclonal variation. The plant material used during the cryopreservation procedure was designed to utilise only PLBs as explant. This indeed eliminates the possible formation of undifferentiated callus tissue which has long been associated with somaclonal variants (Peschke and Phillips, 1992). Thus, eliminating callus formation at the micropropagation, explant excision, treatment, recovery and regenerating stages of the cryopreservation experiment reduced the potential for somoclonal variation to occur. Apart from that, age of the explants could increase the possibility of somaclonal variation (Peschke and Phillips, 1992). This problem was circumvented by utilising healthy and actively growing PLBs grown for between 3 to 4 weeks as the source of explants in the cryopreservation procedure. RAPD analysis is necessary to verify the genetic stability of cryopreserved plants. No genomic variations were detected in the regenerated material from most cryopreservation experiments such as embryogenic cultures of Scots pine (*Pinus sylvestris* L.) precultured using three different mixtures as cryoprotectants (Haggman et al., 1998), potato plants (*Solanum tuberosum* L.) derived from meristems cryopreservation by encapsulation vitrification (Hirai and Sakai, 2000), *Prunus* Ferlenain plants (Helliot et al., 2002), *Arachis* species (Gagliardi et al., 2003), *Diascorea bulbifera* (Dixit et al., 2003), *Vitis vinefera* (Zhai et al., 2003) and *Indigofera tinctoria* (Nair and Reghunath, 2009). Success of cryopreservation may be assessed not only at the level of cell viability and the capability to recover and differentiate into whole plants but also at the DNA level, indicating genetic fidelity of the recovered plants. Therefore, the importance of the genetic stability of the plant materials becomes an important aspect during the establishment of the cryopreservation protocol (Zhai et al., 2003; Liu et al., 2004; Dalamu et al., 2012). Several molecular methods such as Random Amplification of Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and Variable Number Tandem Repeat (VNTR) can be used to evaluate DNA stability (Zhai et al., 2003; Lui et al., 2004; Agarwal et al., 2008; Mallon et al., 2010). However, alterations in DNA sequences following cryopreservation are very rarely observed (Zhai et al., 2003; Liu et al., 2004). Reviews indicate that generally no DNA alterations were detected before and after cryopreservation (Zhai et al., 2003; Liu et al., 2004; Nair and Reghunath, 2009) although it is well recognized that cryopreservation may induce DNA alterations, especially at the epigenetic level accomplished by altered DNA methylation status (Harding et al., 2000; Hao et al., 2001). Ahuja et al. (2002) identified minor alteration in the RAPD amplification pattern in *Dioscorea floribunda* plant following cryopreservation. Even though the survival rate following cryopreservation significantly decreased, only minor genomic changes were detected. These alterations at molecular level did not influence the survival rate, vigour or biosynthetic ability of the cryopreserved plants (Ahuja et al., 2002). Therefore, asssessment of genetic stability available techniques is still restricted to the small part of the genome. This is due to the non-coding DNA sequences reveal variability that may not be associated with any important physiological trait or function but may specify certain degree of genome instability. Therefore, the application of sequences that encode for proteins which are associated with phenotypes of interest would be more appropriate (Ahuja et al., 2002). RAPD technology has previously successfully been used to detect occurrence of genetic alterations. However, this method possesses certain limitations with reproducibility and it is currently being replaced by technique such AFLP (Finkle et al., 1985; Harding, 1997; Aronen et al., 1999; Ahuja et al., 2002; Urbanova et al., 2005; Agarwal et al., 2008; Castillo et al., 2010; Mallon et al., 2010). These techniques should in future be used to consider more

Table 1. Distribution of monomorphic and polymorphic bands in cryopreserved PLBs and PLBs stock culture.

Primers	Total number of bands in PLBs stock culture	Total number of bands in cryopreserved PLBs	Total number of monomorphic bands	Total number of polymorphic bands	Length of amplified DNA fragments	SI Index
OPA04	8	0	0	0	100-1500	0
OPAW17	12	*0*	*0*	*0*	300-2000	*0*
OPB11	2	*2*	*2*	*0*	1000-1500	*1*
OPB12	2	*2*	*2*	*0*	900-1200	*1*
OPB18	8	*2*	*2*	*0*	200-2000	*0.4*
OPD01	1	*1*	*1*	*0*	3000	*1*
OPG13	6	*6*	*6*	*0*	500-1500	*1*
OPG14	4	*4*	*4*	*0*	600-1000	*1*
OPG15	0	*2*	*0*	*0*	900-1200	*0*
OPB 14	1	*1*	*1*	*2*	1000	*1*
Total number of bands	*44*	*20*	*18*	*2*	-	-

Note: SI index means similarity index.

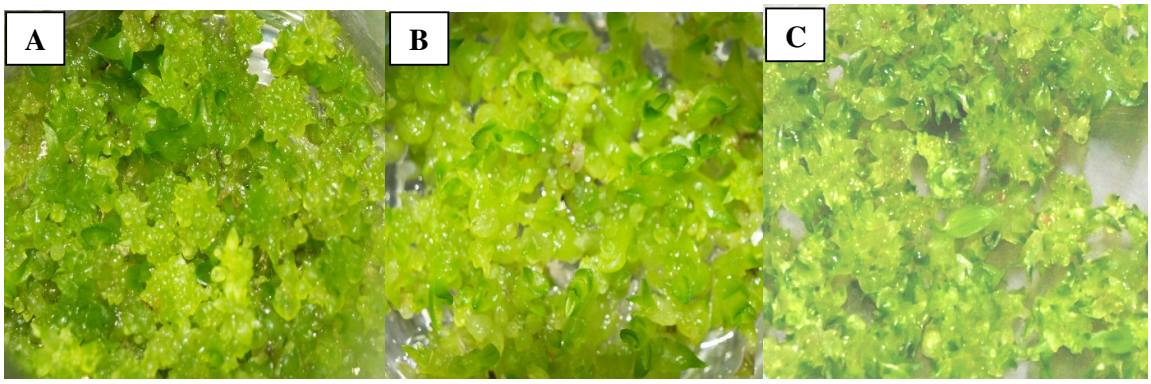

Fig 1. Sample material used in RAPD analysis. **(A)** Cryopreserved PLBs ;(**B**) Non-cryopreserved PLBs ; **(C)** PLBs stock culture.

Table 2. Distribution of monomorphic and polymorphic bands in cryopreserved PLBs and PLBs stock culture.

Primers	Total number of bands in PLBs stock culture	Total number of bands in cryopreserved PLBs	Total number of monomorphic bands	Total number of polymorphic bands	Length of amplified DNA fragments	SI Index
OPA04	8	0	0	0	100-1500	0
OPAW17	12	*0*	*0*	*0*	300-2000	*0*
OPB11	2	*2*	*2*	*0*	1000-1500	*1*
OPB12	2	*2*	*2*	*0*	900-1200	*1*
OPB18	8	*2*	*2*	*0*	200-2000	*0.4*
OPD01	1	*1*	*1*	*0*	3000	*1*
OPG13	6	*6*	*6*	*0*	500-1500	*1*
OPG14	4	*4*	*4*	*0*	600-1000	*1*
OPG15	0	*1*	*0*	*1*	900	*0*
OPB 14	1	*1*	*1*	*0*	1000	*1*
Total number of bands	*44*	*19*	*18*	*1*	-	-

carefully the issue of genetic fidelity following cryo-preservation.

Materials and methods

Plant Materials

In vitro protocorm-like bodies (PLBs) (100mg) of *Dendrobium* Bobby Messina from stock, cryopreserved and non-cryopreserved cultures, with the last two obtained from PVS2 vitrification treatment were selected (Antony et al., 2010, 2011). Figure 1 showed the cultures used in this study.

DNA extraction

The DNA extraction kit, Genomic DNA Mini Kit (Geneaid Biotech Ltd., Taipei County, Taiwan, Republic of China) was used to extract the DNA from PLBs stock culture, cryopreserved and non-cryopreserved PLBs. The extraction method was based on the protocol provided by the kit. For RAPD analysis, 20 oligonucleotide primers were chosen but however only 10 primers produced bands (Table 1). The primers were obtained from 1st BASE (Operon Technologies, Alameda, CA). The PCR consumables and buffers used in the RAPD analysis were obtained from Fermentas Life Sciences (Vilnius, Lithuania).

RAPD- PCR

The total volume of RAPD-PCR reaction was 20 µL. The mixture consists of 3 µL DNA, one unit of *Taq* DNA polymerase (DreamTaq™ DNA Polymerase, 5U.µL-1), 0.5µL of 10mM deoxyribonucleotide triphosphate (dNTP;

dNTP Mix), 2µL of 10× PCR buffer (10× DreamTaq™ Buffer) containing 100mM Tris-Hydrochloride (Tris-HCl) at pH 8.8, 500mM potassium hydrochloride (KCl), 0.8% (v/v) Nonidet P40 and 20mM magnesium chloride ($MgCl_2$), 1µL from 10µM of a single primer (Khosravi et al., 2009) and autoclaved distilled deionised water was used to top up the PCR reaction volume up to 20µL. The MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc., USA) was used to amplify the DNA samples. The PCR conditions were set with initial denaturation at 95°C for 3 minutes. The PCR was performed for 35 cycles with denaturation at 95°C for 30 seconds, annealing at 5°C below each primer's melting temperature (Tm) for 30 seconds and extension at 72°C for 1 minute followed by a final extension cycle at 72°C for 10 minutes. The PCR product was kept at 4˚C and finally stored at -40°C prior to electrophoresis.

DNA Electrophoresis

The DNA fragments produced were visualized in a 1.5% agarose gel. The gel was visualized with the Gel documentation transilluminator (Molecular Imager® Gel Doc™ XR+ System with Image Lab™ and Quantity One 1-D Analysis Softwares, Bio-Rad Laboratories, Inc., USA) (Khosravi et al. 2009). The evaluation of the DNA fragment patterns of each sample in the cryopreserved PLBs or non-cryopreseerved PLBs was performed by calculating the Similarity Indices of the cryopreserved PLBs or non-cryopreserved PLBs as compared to the DNA bands of stock PLBs. The reproducible bands were scored manually as 1 or 0 for the presence or absence of the bands. The coefficients of similarity between the treatment and stock culture were calculated using the formula below (Nei and Li, 1979; Asnita and Norzulaani, 2006; Khosravi et al., 2009).

$$SI = 2Nij \quad / \quad N_i + N_j$$

Where, SI= Similarity Index; Nij= Number of monomorphic bands between the stock culture and cryopreserved or non cryopreserved PLBs; N*i* = Total number of bands in the PLBs stock culture; N*j* = Total number of bands in the cryopreserved or non-cryopreserved PLBs

Conclusion

The RAPD results from 6 selected primers obtained from cryopreserved and non-cryopreserved PLBs by the PVS2 vitrification method indicated that the genetic stability of PLBs following cryopreservation was maintained. Hence, this method can be utilised as a potential cryopreservation protocol for the germplasm conservation of *Dendrobium* Bobby Messina orchid.

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