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Somaclonal variations were not induced by the cryopreservation: Levels of somaclonal variations of in vitro and thawed protocorms of *Dendrobium* Bobby Messina analysed by SCoT and TRAP DNA markers

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ABSTRACT

The present study was conducted to develop targeted region amplification polymorphism (TRAP) and start codon targeted polymorphism (SCoT) DNA markers for the identification of somaclonal variation in cryopreserved *Dendrobium* Bobby Messina. With reference to previous orchid cryopreservation via PVS2 (plant vitrification solution) vitrification methods, regenerated explants were assessed in order to determine the genetic similarity in comparison to the mother plant. 3 different samples were selected involving stock culture PLBs (protocorm-like bodies), non-cryopreserved PLBs and thawed cryopreserved PLBs. During the study, eight pairs of (8) TRAP primers (TRAP CMS TRAP 8-4A, FLS TRAP 2-2A, TRAP 14-5A, TRAP 20-6B, TRAP 16-3B, ChIC TRAP 5-5B, TRAP3-1D and TRAP 9-5A) produced unambiguous and reproducible bands ranging from 100 to 2000 bp. In cryopreserved PLBs, all TRAP primers displayed polymorphic bands. In the non-cryopreserved PLBs, primer TRAP 20-6B produced monomorphism and the remaining 7 showed both complete and partial polymorphism, respectively. SCoT markers indicated that four primers were able to generate reproducible and clear bands at the sizes of 500 to 3000 bp. SCoT primers (S26, S32, S33 and S36) showed polymorphism for both cryopreserved and non-cryopreserved PLBs of *Dendrobium* Bobby Messina. The TRAP and SCoT DNA markers designed were found to be an efficient tool to evaluate potential rate of somaclonal variations of regenerated PLBs following cryopreservation.

1. Introduction

Orchidaceae is the largest family of the phylum of Angiosperm, a category of flowering plants (Weston et al., 2005; Bustam et al., 2013). It is estimated to have a range from 17,000 up to 35,000 species from this phylum worldwide (Dressler, 1993). Orchids are categorized as cut flowers where out of all orchid types worldwide, only a few genera are commercially grown as cut flowers. This includes *Cattleya*, *Dendrobium*, *Phalaenopsis*, *Cymbidium* and *Vanda* (Salunkhe et al., 1990; Ching et al., 2012; Poobathy et al., 2012).

Despite high market demands, breeding of *Dendrobium* orchids through sexual hybridization is not profitable in terms of commercialization due to the long generation period which generally takes 3 years for seeds to produce to flowers and also due to the lack of useable genetic variability (Kuehnle and Sugii, 1992; Yin et al., 2011; Poobathy et al., 2012). Tissue culture propagation in greenhouses and agricultural cultivation techniques has been developed for mass production to overcome this matter. It is therefore important to establish a competent method in preservation of *Dendrobium* orchids (Kuehnle and Sugii, 1992; Bustam et al., 2013). There are two major approaches in preserving endangered plant species: in situ conservation, where conservation the plant under their natural habitat, and ex situ conservation, where plants are maintained under artificial environment (Hirano et al., 2005; Bustam et al., 2013).

One of the protocols adopted for orchid ex situ conservation is cryopreservation. Cryopreservation is useful for the safe long term storage of plant tissues with particular characteristics and significance in conserving genetic resources (Panis and Lambardi, 2006; Antony et al., 2014). Successful cryopreservation involves treating selected explants to a series of stress inducing treatments, eg; such as pre-culture, cryoprotection, dehydration, quick immersion in liquid nitrogen, defrosting, recovery and regeneration of plant (Antony et al., 2011; Gantait et al., 2015). Nevertheless, explants that were exposed to excessive physical environment such as very low temperatures, high osmotic force, dehydration and highly toxic additives (e.g., cryoprotectant solution) during cryopreservation resulted in physiological stress (Panis and Lambardi, 2006; Antony et al., 2011, 2012; Poobathy et al., 2012; Gantait et al., 2015). This in return gave rise to genetic instability of orchids and could also induce changes that lead to development of desirable traits. Somaclonal mutation with

Abbreviations: PLB, protocorm-like bodies; TRAP, targeted region amplification polymorphism; SCoT, start codon targeted polymorphism; MS, Murashige and Skoog; PCR, polymerase chain reaction; LN, liquid nitrogen; EST, expressed sequence tag; RAPD, random amplified polymorphic DNA; SSR, simple sequence repeats; AFLP, amplified fragment length polymorphism.

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beneficial traits could be introduced in the regenerated plants especially after a successful cryopreservation procedure that may lead to desirable traits being coupled (Antony et al., 2012).

Apart from that, the manifestation of somaclonal variation occurs also during in vitro propagation, industrial production of phytochemicals by cell culture or regeneration of plants from genetically engineered cells which have serious drawbacks in the practical applications of plant tissue culture technique (Rathore et al., 2014). Therefore, understanding the reasons and the mechanisms involved in somaclonal variation in addition to assessment of genetic stability of regenerated plants at earlier stage has aided reducing or eliminating the problem of genetic variations during micropropagation. In reality, the variability detected in tissue culture derived plants is a joint effect of genetic and epigenetic variations arising during the course of culture and the genetic heterogeneity of the cells of explants (Rathore et al., 2014). Genetic stability of tissue culture plants is greatly dependent upon the mode of regeneration method, tissue culture environment and culture conditions (Rani and Raina, 2000; Bairu et al., 2011). Previously, evaluation of tissue culture induced variation was based on cytological and phenotypic analyses. In the last two decades, a number of DNA-based molecular markers such as RAPD, ISSR, AFLP and SSR have been successfully applied to assess genetic fidelity or variability in tissue culture raised plants (Rathore et al., 2011; Rai et al., 2012; Nayak et al., 2013; Singh et al., 2013; Rathore et al., 2014).

Additionally, molecular markers are beneficial for DNA fingerprinting as well as germplasm evaluation. (Pujar et al., 1999; Antony et al., 2012; Yao et al., 2012; Poobathy et al., 2013a,b; Yuan et al., 2013; Liu et al., 2015). The newly developed TRAP (target region amplified polymorphism) technique utilises the existence of the public express sequence tag EST databases to design PCR primers against annotated EST sequences for the detection of polymorphic markers in order to link the EST sequences with its respective phenotypes (Hu and Vick, 2003; Liu et al., 2015). TRAP possibly will yield more precise estimates of genetic diversity when compared with other markers (Liu et al., 2015). On the other hand, another genetic marker being efficiently utilised for the detection of somaclonal mutation is the start codon targeted (SCoT) marker (Collard and Mackill, 2009). SCoT markers are gaining popularity over other dominant DNA marker systems like RAPD and ISSR for higher polymorphism and better marker resolvability (Gorji et al., 2011; Satya et al., 2015). This marker was established based on the conserved short-range region flanking the ATG start codon in the plant genes. It shares the principle of using a single primer like RAPD and ISSR. The marker system has been successfully employed in genetic diversity analysis and fingerprinting of a number of agricultural and horticultural crop species (Luo et al., 2010; Xiong et al., 2011; Mulpuri et al., 2013) and in population structure analysis of mushrooms (Zhao et al., 2013). At current, the development of new methods in generating DNA markers has led to the preference of TRAP and SCoT markers being used in plant molecular genetics studied for various gene targeting applications.

2. Materials and methods

2.1. Planting materials

Protocorm-like bodies (PLBs) and plantlets of *Dendrobium* Bobby Messina were obtained from the PVS2 (plant vitrification solution) cryopreservation protocol established previously by our group (Antony et al., 2013). These plant materials were used as the explant to initiate the propagation of PLBs for the subsequent experiments. The plantlets were subcultured in half strength of Murashige and Skoog (MS) media and the cultures were incubated at 25 ± 2 °C in a 16 h photoperiod under white fluorescent lamps (Philips TLD, 36 W) at 150 μmol m^{-2} s^{-1}. Explants were subcultured once in every three weeks.

2.2. DNA extraction

The plant genomic DNA was extracted from the PLBs of stock culture (control), thawed cryopreserved PLBs and non-cryopreserved PLBs of *Dendrobium* Bobby Messina. Extraction was carried out using plant extraction kit following the protocol of Wizard® Genomic DNA Purification Kit (Promega, USA). The extracted DNA was stored at 4 °C prior to its usage.

2.3. PCR amplification

TRAP primers were based on (Saleh et al., 2013) and SCoT primers based on Bhattacharyya et al.(2013) as depicted in (Table 1–2). These primers were used to assess the genetic fidelity of regenerated cryopreserved and non-cryopreserved PLBs of *Dendrobium* Bobby Messina. The PCR reaction was performed in a total volume of 20 μL. Each mixture consists of 2 μL of 10× PCR buffer (10× Dream *Taq*™ Buffer) with potassium hydrochloride (KCL), 4.8 μL magnesium chloride ($MgCL_2$), 4 μL primer accordingly, 1.6 μL dNTP mix, 1 μL *Taq* DNA polymerase (Thermoscientific), 3 μL DNA and deionised distilled water to a final volume of 20 μL. The PCR was conducted in a MyCycler™ thermocycler (Bio-Rad Laboratories, Inc., USA), programmed for 3 min at 95 °C for initial denaturation, 35 cycles of denaturation at 95 °C for 30 s, 35 cycles of annealing for 30 s at 5 °C below melting temperature (Tm) of each primer, followed by 35 cycles of extension at 72 °C for 1 min and lastly with the final extension at 72 °C for 10 min. All PCR amplification products were separated on 1.2% agarose gels in TBE buffer stained with ethidium bromide and visualized under the UV light.

2.4. Evaluation of DNA fragments

PCR products from every sample of cryopreserved and non-cryopreserved PLBs were then evaluated by counting the value of similarity index (SI) of the PLBs treated in comparison with PLBS from the stock culture. Reproducible bands were evaluated manually and scored '1' for the presence of band and '0' for the absence of band. Coefficient similarity between stock cultures and treated cultures (cryopreserved or non-cryopreserved PLBs) was calculated using the formula by Nei and Li (1979) as shown below:

$$SI = 2Nij/Ni + Nj.$$

Table 1
TRAP primers used in the amplification of DNA segments of PLB samples of *Dendrobium* Bobby Messina.

No.	Primers	Sequence (5′–3′)	GC content (%)	T_M (°C)
1.	ChIC TRAP 5-5B_F	TGAGTCCAAACCGGAAT	47.0	50.7
	ChIC TRAP 5-5B_R	CAGGCAAGACGCAAGGTG	61.1	56.9
2.	TRAP 14-5A_F	GAGTCCAAACCGGAGC	62.5	53.0
	TRAP 14-5A_R	CCCTCCACCAATCACAAT	50.0	52.1
3.	TRAP 20-6B_F	TGAGTCCAAACCGGTAA	47.0	50.3
	TRAP 20-6B_R	GAGGAAGACGACGAGGAG	57.8	55.8
4.	FLS TRAP2-2A_F	TGAGTCCAAACCGGAAT	47.0	50.7
	FLS TRAP2-2A_R	CGGACAGTGGCGGAGTTA	61.1	57.1
5.	TRAP 3-1D_F	TGAGTCCAAACCGGAAT	47.0	50.7
	TRAP 3-1D_R	GGCGAACTCCGACATCTT	55.5	54.9
6.	TRAP 15-2D_F	TGAGTCCAAACCGGAGC	58.8	54.8
	TRAP 15-2D_R	TCCTACAAACATTGCCTACT	40.0	51.0
7.	TRAP 16-3B_F	TGAGTCCAAACCGGAGC	58.8	54.8
	TRAP 16-3B_R	TTCTTCCTCCCGCTCATCCT	55.0	58.0
8.	CMS TRAP8-4A_F	TGAGGTCCAAACCGGAAT	47.0	50.7
	CMS TRAP8-4A_R	TTCTTCCTCCCGCTCATCCT	55.0	58.0
9.	TRAP 9-5A_F	TGAGTCCAAACCGGAGC	58.8	54.8
	TRAP 9-5A_R	TCACCCGCACCTTCTTCC	61.1	57.5
10.	TRAP 37-3B_F	AGTAACCCACCGCCTCCTTC	60.0	59.2
	TRAP 37-3B_R	CAGGCAAGACGCAAGGTG	61.1	56.9

TRAP primers developed by Hu and Vick, 2003.