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RESEARCH NOTE

Evaluation of the genetic fidelity of in vitro-propagated gerbera (*Gerbera jamesonii* Bolus) using DNA-based markers

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Abstract The genetic fidelity of in vitro-raised gerbera clones was assessed by using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers. Out of 35 RAPD and 32 ISSR primers screened, only 12 RAPD and 10 ISSR primers produced clear, reproducible and scorable bands. The 12 RAPD primers produced 54 distinct and scorable bands, with an average of 4.5 bands per primer. The number of scorable bands for ISSR primers varied from 3 (ISSR-14) to 9 (ISSR-07), with an average of 5.5 bands per primer. The number of bands generated per primer was greater in ISSR than RAPD. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant. A similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between the mother and the in vitro-raised plantlets was 1, indicating 100% similarity. This confirmed the true-to-type nature of the in vitro-raised clones.

Keywords Gerbera · Micropropagation · Genetic fidelity · DNA markers

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Introduction

Gerbera (*Gerbera jamesonii* Bolus), commonly known as Transvaal Daisy, is an important cut flower both in the domestic and the international markets. It ranks fifth in the international cut flower trade. Micropropagation is one of the viable alternatives for large-scale multiplication of gerbera (Bhatia et al. 2008). Over the years, gerbera has been propagated by direct or indirect organogenesis using various explants, including stem tips, floral buds, leaf, capitulum etc. (Kanwar and Kumar 2008). True-to-type clonal fidelity is one of the most important pre-requisites in the micropropagation of crop species. The occurrence of cryptic genetic defects arising via somaclonal variation in the regenerates can seriously limit the broader utility of the micropropagation system (Salvi et al. 2001). It is, therefore, imperative to establish genetic uniformity of micropropagated plants to confirm the quality of the plantlets for its commercial utility.

Polymerase chain reaction (PCR)-based techniques such as random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) are immensely useful in establishing the genetic stability of in vitro-regenerated plantlets in many crop species (Lakshmanan et al. 2007; Joshi and Dhawan 2007). RAPD and ISSR markers are very simple, fast, cost-effective, highly discriminative and reliable. They require only a small quantity of DNA sample and they do not need any prior sequence information to design the primer. They do not use radioactive probes as in restriction fragment length polymorphism (RFLP) (Lakshmanan et al. 2007), thus, they are suitable for the assessment of the genetic fidelity of in vitro-raised clones.

In this study, we assessed the clonal fidelity of in vitro-raised gerbera plants using RAPD and ISSR markers. This is very useful for establishing a particular

micropropagation system for the production of genetically identical and stable plants before it is released for commercial purposes.

Materials and methods

Plant material and culture conditions

The capitulum explants of gerbera cv. Cabana were obtained from the Centre for Protected Cultivation Technology, Indian Agricultural Research Institute, New Delhi. Murashige and Skoog (MS) medium supplemented with 10 mg l^{-1} 3-benzyl adenine (BA) and 2 mg l^{-1} indole-3-acetic acid (IAA) was used for culture establishment. The in vitro-derived shoots were cultured on medium [MS + BA (1 mg l^{-1}) + IAA (0.1 mg l^{-1})] for proliferation and multiplication. The rooting was successfully obtained on 0.5 MS medium containing 1 mg l^{-1} indole-3-butyric acid and sucrose (45 mg l^{-1}). The rooted plantlets were hardened on peat and soilrite mixture. The cultures were maintained at 25 ± 1°C under fluorescent white light (47 µmol $m^{-2}s^{-1}$) during a photoperiod of 16:8 h light and dark cycles.

DNA extraction and PCR amplification conditions

Clonal fidelity of in vitro-raised clones was tested using RAPD and ISSR markers. For this purpose, 15 in vitro-raised, hardened plants were chosen randomly from the population and compared with the mother plant from which the explants were taken. Total genomic DNA of the mother plant and in vitro-raised clones was extracted from young leaf tissue by using the modified cetyl trimethyl ammonium bromide (CTAB) method as described by Murray and Thompson (1980). Thirty-five RAPD (Operon Technologies Inc., Germany) and 32 ISSR primers (XXIDT Integrated DNA Technologies Inc., Coralville, IA) were used for initial screening. PCR amplifications were carried out in a total volume of 25 µl containing 2 µl (25–30 ng) of genomic DNA. The reaction buffer consisted of 2.5 µl of 10× PCR buffer, 1.5 µl $MgCl_2$ (2 mM), 0.5 µl dNTPs (10 mM each of dATP, dGTP, dTTP and dCTP), 1 µl primer, 0.3 µl DNA Taq polymerase and 17.2 µl water. The primers showing polymorphic bands were then used for analysing the clonal fidelity of micropropagated plants. PCR amplification was performed in a DNA thermal cycler (Biometra), which was programmed for initial DNA denaturation at 95°C for 4 min, followed by 40 cycles of 1 min denaturation at 95°C, 1 min annealing (temperature specific to the primer) and 2 min extension at 72°C, with a final extension at 72°C for 10 min. Amplified products were resolved by electrophoresis on 1.4% agarose gel in tris-borate EDTA (TBE) buffer stained with 0.5 µg/ml ethidium bromide and photographs were taken by using the gel documentation system (Alpha Innotech Corporation, San Leandro, CA, USA).

Data scoring and analysis

Consistent, well-resolved fragments in the size range of 100 bp to 2.5 kb were manually scored. Each band was treated as a marker. The scoring of bands was done on the basis of their presence (‘1’) or absence (‘0’) in the gel and missing data was denoted by ‘9’. The genetic associations were evaluated by calculating the Jaccard’s similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to the cluster analysis of unweighted pair group method with arithmetic averages (UPGMA) and a dendrogram was generated by using NTSYS-pc version 2.1 software (Rohlf 2000).

Results and discussion

A total of 35 RAPD primers were used for initial screening with the mother plant of gerbera but only 12 RAPD primers gave clear and reproducible bands. The number of scorable bands for each RAPD primer varied from 2 (OPE-1) to 7 (OPF-12) (Table 1). The 12 RAPD primers produced 54 distinct and scorable bands, with an average of 4.5 bands per primer. Each primer generated a unique set of amplification products ranging in size from 350 bp (OPG-18) to 2,200 bp (OPA-18 and OPC-12). No polymorphism was detected during the RAPD analysis of in vitro-raised clones (Fig. 1a). The absence of genetic variation using RAPD has been reported in micropropagated shoots of *Pinus thunbergii* (Goto et al. 1998), in vitro-regenerated turmeric (Salvi et al. 2001) and in vitro-raised bulblets of Lilium (Varshney et al. (2001).

Out of 32 ISSR primers used in the initial screening, only ten primers produced clear and reproducible bands. The optimum annealing temperature for ISSR markers varied from 48.7 to 55.0°C (Table 2). The ten ISSR primers produced 55 distinct and scorable bands in the size range of 100 bp (ISSR-31) to 2,400 bp (ISSR-07). The number of scorable bands for each primer varied from 3 (ISSR-14) to 9 (ISSR-07), with an average of 5.5 bands per primer. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant (Fig. 1b). Similar results have been reported by Martin et al. (2004) in almond, Lakshmanan et al. (2007) in banana and Joshi and Dhawan (2007) in *Swertia chirayita*.

Two PCR-based techniques, RAPD and ISSR, were used to test clonal fidelity because of their simplicity and

Table 1 List of primers, their sequences, number and size of the amplified fragments generated by random amplified polymorphic DNA (RAPD) primers

Serial no. (Sl. No./ S. No.)	Primer code	Primer sequence (5′–3′)	Number of scorable bands per primer	Total number of bands amplified	Range of amplification (bp)
1	OPA-07	GAAACGGGTG	5	80.00	500–1,600
2	OPA-08	GTGACGTAGG	5	80.00	450–1,500
3	OPA-11	CAATCGCCGT	3	48.00	600–1,000
4	OPA-15	TTCCGAACCC	4	64.00	500–2,000
5	OPA-19	CAAACGTCGG	5	80.00	550–2,200
6	OPC-1	TTCGAGCCAG	5	80.00	600–1,800
7	OPC-5	GATGACCGCC	6	96.00	450–1,400
8	OPC-12	TGTCATCCCC	4	64.00	700–2,200
9	OPE-01	CCAAGGTCCC	2	32.00	700–1,000
10	OPF-12	ACGGTACCAG	7	112.00	400–2,000
11	OPG-09	CGATCCGCGC	4	64.00	600–1,300
12	OPG-19	CCCGACTGCC	4	64.00	350–1,300
Total			54.00	864.00	

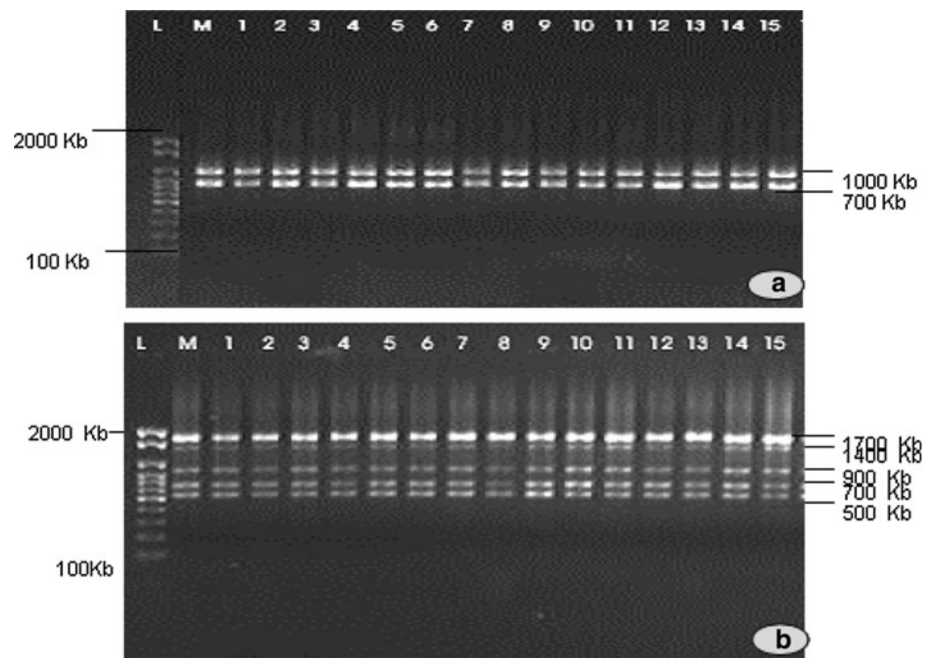

Fig. 1 Polymerase chain reaction (PCR) amplification products obtained with **a** random amplified polymorphic DNA (RAPD) primer (OPE-01) and **b** inter-simple sequence repeat (ISSR) primer (ISSR-06). *Lane L* represents 100-bp ladder, *lane M* represents the mother plant and *lanes 1–15* represent in vitro-raised clones derived from capitulum explant

cost-effectiveness. The use of two markers, which amplify different regions of the genome, allows better chances for the identification of genetic variations in the clones (Martin et al. 2004). The number of bands generated per primer was greater in ISSR (5.5) than RAPD (4.5). These differences could possibly be due to the high melting temperature for the ISSR primers, which permits much more stringent annealing conditions and, consequently, more specific and reproducible amplification. Devarumath et al. (2002) also revealed that ISSR fingerprints detected more polymorphic loci than RAPD fingerprinting.

A total of 1,744 bands (number of plants analysed × number of scorable bands with all RAPD and ISSR primers) were generated from the mother plant and 15 in vitro-raised clones. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant (Fig. 1). A similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between the mother plant and the plantlets derived from different explants was 1, indicating 100% similarity. An UPGMA dendrogram was generated from the Jaccard's similarity values using NTSYS-pc software. A phenetic

Table 2 List of primers, their sequences, number and size of the amplified fragments generated by inter-simple sequence repeat (ISSR) primers

Serial no. (Sl. No./ S. No.)	Primers	5′–3′ motif	Annealing temperature (°C)	Number of scorable bands/primer	Total number of bands amplified	Range of amplification (bp)
1	ISSR-04	$(GA)_8Y$	49.0	6	96.00	400–2,100
2	ISSR-06	$(AG)_8Y$	49.0	6	96.00	500–1,700
3	ISSR-07	$(AG)_8Y$	50.0	9	144.00	500–2,400
4	ISSR-14	$(AG)_8Y$	49.0	3	48.00	500–1,000
5	ISSR-15	$(AG)_8$	54.0	5	80.00	600–1,900
6	ISSR-17	$(GA)_8$	53.0	4	64.00	300–800
7	ISSR-18	$(GA)_8$	54.0	6	96.00	350–1,000
8	ISSR-21	$(TC)_8$	48.7	5	80.00	300–1,600
9	ISSR-25	$(TG)_6R$	48.7	4	64.00	300–900
10	ISSR-31	$(AG)_8V$	55.0	7	112.00	100–1,200
Total				55	880.00	

dendrogram based on UPGMA analysis further confirmed the true-to-type nature of clones. Similar results were obtained by Sreedhar et al. (2007) during the clonal fidelity analysis of long-term micropropagated shoot cultures of vanilla (*Vanilla planifolia* Andrews) by RAPD and ISSR markers. Earlier, Reynoird et al. (1993) also did not observe any phenotypic variations during vegetative and reproductive phases among the regenerates of gerbera. They also confirmed this by chromosome counts and DNA flow cytometry that all of the regenerates, as well as the mother plants, were typically diploid.

The presence or absence of variations during in vitro propagation depends upon the source of explants and the method of regeneration (Goto et al. 1998). The sub- and supra-optimal levels of plant growth substances, especially synthetic ones, have also been associated with somaclonal variation (Martin et al. 2004). Even at optimal levels, long-term multiplication may often lead to somaclonal or epigenetic variations in micropropagated plants, thus, questioning the very fidelity of their clonal nature. In our study, plantlets were obtained from the sprouting of dormant buds situated in the axils of the bracts. These findings support the fact that a meristem-based micropropagation system is much more stable genetically than those in which regeneration occurs via the callus phase. Plants regenerated from adventitious buds around axillary buds or from other well-developed meristematic tissue showed the lowest tendency for genetic variation (Joshi and Dhawan 2007). Even plants derived from organised meristems are not always genetically true to the type in many crops (Devarumath et al. 2002). Hence, it becomes imperative to regularly check the genetic purity of the micropropagated plants in order to produce clonally uniform progeny while using different techniques of micropropagation.

In this study, the true to the type nature of the in vitro-raised clones was confirmed using DNA-based markers. No variability was detected among the tissue culture-raised plantlets, hence, capitulum explants can be successfully employed for the commercial multiplication of gerbera without much risk of genetic instability.

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