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Short communication

Assessment of clonal fidelity of micropropagated gerbera plants by ISSR markers

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ABSTRACT

True-to-type clonal fidelity is one of the most important pre-requisites in micropropagation of crop species. Genetic fidelity of *in vitro* raised 45 plants of gerbera (*Gerbera jamesonii* Bolus) derived from three different explants, *viz.*, capitulum, leaf and shoot tips, was assessed by 32 ISSR markers, for their genetic stability. Out of 32 ISSR markers, 15 markers produced clear, distinct and scorable bands with an average of 5.47 bands per marker. The markers designed from AG motif amplified more number of bands. The markers anchored at 3′ ends produced high number of consistent bands than unanchored markers. Fifteen ISSR markers generated a total of 3773 bands, out of which 3770 were monomorphic among all the clones. The Jaccard's similarity coefficient revealed that out of 45 clones derived from different explants, 44 were grouped into a single large cluster alongwith the mother plant with a similarity coefficient value of 1.00, whereas one clone (C38) remained ungrouped. The clones derived from capitulum and shoot tip explants did not show any genetic variation, whereas, one of the leaf-derived clones exhibited some degree of variation.

1. Introduction

Gerbera (*Gerbera jamesonii* Bolus) commonly known as Transvaal Daisy, ranks fifth in the international cut flower trade. Its commercial propagation through division of clumps and other conventional methods of propagation is slow and inadequate for the production of large number of uniform propagules. Micropropagation from different explants such as shoot tips, capitulum and leaf segments is a viable approach for large-scale multiplication of gerbera (Aswath and Choudhary, 2002; Jerzy and Lubomski, 1991; Reynoird et al., 1993). A major problem associated with micropropagation is occurrence of somaclonal variations among the sub-clones of parental line, arising as a direct consequence of *in vitro* culture of plant cells, tissue and organs. The frequency of these variations varies with the source of explant and their pattern of regeneration (somatic embryogenesis/organogenesis/axillary bud multiplication), media composition and cultural conditions (Damasco et al., 1996; Salvi et al., 2001). The cryptic genetic defects arising via somaclonal variation in the regenerants seriously limits the utility of micropropagation system. ISSR markers were chosen because the technique is very simple, fast, cost effective, highly discriminative, reliable, require small quantity of sample DNA, do not need any prior primer sequence information and non-radioactive (Lakshmanan et al., 2007; Reddy et al., 2002). The use of ISSR primers for assessment of genetic fidelity is well documented (Leroy et al., 2001; Joshi and Dhawan, 2007; Lakshmanan et al., 2007). Here clonal fidelity of *in vitro* raised gerbera plants have been assessed using ISSR markers for establishing a particular micropropagation system for the production of genetically identical and stable plants before it is released for commercial purposes.

Objective of present investigation was to study the effect of source of explants on genetic integrity of *in vitro* raised plants using ISSR markers.

2. Materials and methods

2.1. Plant material and in vitro culture

Gerbera cultivar 'Cabana' used in present study was grown under polyhouse conditions at Centre for Protected Cultivation Technology, Indian Agricultural Research Institute, New Delhi. Three types of explants, *viz.*, shoot tip, capitulum and leaf were used for culture initiation *in vitro*. MS medium containing BAP (10 mg/l) and IAA (2 mg/l) was used for culture establishment. For shoot bud regeneration from the leaf-derived calli, MS + BAP

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(3 mg/l) + NAA (0.5 mg/l) was used. The *in vitro*-derived shoots were cultured on medium [MS + BAP (1 mg/l) + IAA (0.1 mg/l)] for proliferation and multiplication. For rooting the individual shoots were cultured on 1/2 MS medium containing IBA (1 mg/l) and sucrose (45 mg/l) after 8 weeks of culture at 25 ± 1 °C under fluorescent white light (47 μ mol/(m^2 s)) at 16:8 h light and dark cycles. The rooted plantlets were hardened on peat and soilrite mixture. A set of 15 *in vitro* raised clones (total 45), each derived from three different explants, *viz*., capitulum, leaf and shoot tips were randomly selected for assessment of clonal fidelity by ISSR markers.

2.2. DNA extraction and PCR amplification conditions

Total genomic DNA was extracted from young leaf tissue of each *in vitro*-derived clones by using modified CTAB method as described by Murray and Thompson (1980). Quality and quantity of genomic DNA was assessed by co-electrophoresis of the genomic DNA along with λ uncut DNA.

A factorial experiment with varying concentrations of genomic DNA (12.5, 25 and 50 ng/μl), $MgCl_2$ (1.5, 2.0 and 2.5 mM) and *Taq* DNA polymerase (0.5, 0.75 and 1 U) was performed to optimize PCR conditions. Thirty-two ISSR primers (XXIDT Integrated DNA Technologies Int., Coralville, IA) were used for standardization of optimum annealing temperature. PCR amplifications were used as described in Alizadeh et al. (2008). All the PCR components used in this study were purchased from MBI, Fermentas. PCR amplification was performed in a DNA thermal cycler (*T-Gradient Biometra, GmBh*, Göttingen, Germany). Amplified products were resolved by electrophoresis on 1.4% agarose gel in TBE (1×) buffer stained with ethidium bromide and photographs were taken by using Gel documentation system (Alpha Innotech Corporation, USA).

2.3. Data 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. 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 Ver. 2.1 software (Rohlf, 2000).

3. Results and discussion

The regenerant plants analyzed in this study were obtained from three different sources of explants, i.e. capitulum, shoot tip and leaf. The capitulum- and shoot tip-derived plants were obtained via direct regeneration, however, leaf-derived plants were regenerated via callus through indirect regeneration.

The conditions of PCR amplification such as the concentration of template DNA, *Taq* DNA polymerase, $MgCl_2$ and annealing temperature are very crucial for molecular analysis. The genomic DNA at 25 ng/μl was found to be optimum for PCR amplification. More spurious products were amplified at lower concentration with low intensity and were difficult to score. Some products that were amplified at 25 ng were not amplified at higher concentration (50 ng). Among the three concentration of *Taq* DNA polymerase, IU yielded sharp and consistent bands, whereas, other concentrations produced either faint bands or smears. Sharpness of bands was improved by increasing $MgCl_2$ concentration to 2.5 mM. The optimum concentration of PCR components was standardized on the basis of reproducibility of the bands after repeating the experiments three times.

The ISSR technique is a PCR-based method, which involves amplification of DNA segment present at amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction (Reddy et al., 2002). The technique uses microsatellite, usually 16–25 bp long as primer in single PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes.

In our study, out of 32 ISSR markers used for initial screening, only 15 markers produced reproducible and scorable bands. The optimum annealing temperature for these ISSR markers varied from 48.7 to 55 °C (Table 1). In present study, dinucleotide SSRs motifs AC, TC, GA, AG and TG were used. Primers based on AG motif amplified clear and more number of bands, thus revealed more coverage of the genome. Similar results have been reported by Fang et al. (1997) in trifoliate orange, Ratnaparkhe et al. (1998) in chickpea, Blair et al. (1999) in rice, Lakshmanan et al. (2007) in banana and Joshi and Dhawan (2007) in *Swertia chirayita*.

Both unanchored and 3′-anchored primers were used in this study. More number (26%) of bands were obtained with 3′-anchored primers than the unanchored primers. The extent of polymorphism varies with the nature of primer (unanchored, 3′-anchored, or 5′-anchored) used for amplification. When unanchored, i.e. only the SSRs were used as primers, the primer tends to slip within the repeat units during amplification leading to smears

Table 1
List of primers, their sequence motifs, nature, annealing temperatures, number and size of the amplified fragments generated by ISSR primers in gerbera cv. Cabana

S. no.	Primers	5′–3′ motif	Anchoring	Annealing temperature (°C)	Number of scorable bands per primer	Total number of bands amplified	Size range (bp)
1	ISSR-03	$(GA)_6Y$	3′ anchor	49.0	5	230	600–2200
2	ISSR-04	$(GA)_8Y$	3′ anchor	49.0	6	276	400–2100
3	ISSR-06	$(AG)_8Y$	3′ anchor	49.0	6	276	500–1500
4	ISSR-07	$(AG)_8Y$	3′ anchor	50.0	9	414	500–2400
5	ISSR-08	$(AC)_8Y$	3′ anchor	50.0	5	230	600–1750
6	ISSR-09	$(AC)_8Y$	3′ anchor	55.0	7	321	300–1800
7	ISSR-14	$(AG)_8Y$	3′ anchor	49.0	3	139	500–1000
8	ISSR-15	$(AG)_8$	Unanchored	54.0	5	231	600–1900
9	ISSR-17	$(GA)_8$	Unanchored	53.0	4	184	300–800
10	ISSR-18	$(GA)_8$	Unanchored	54.0	6	276	350–1000
11	ISSR-19	$(GA)_8$	Unanchored	53.0	4	184	300–1000
12	ISSR-21	$(TC)_8$	Unanchored	48.7	5	230	400–1750
13	ISSR-22	$(TC)_8$	Unanchored	48.7	6	276	400–2300
14	ISSR-25	$(TG)_6R$	3′ anchor	48.7	4	184	300–900
15	ISSR-31	$(AG)_8V$	3′ anchor	55.0	7	322	100–1200
Total					82	3773	