Molecular Markers for Genetic Stability of Intergeneric Hybrids Fragaria × Potentilla Derived From Tissue Culture

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Abstract. The effect of growth regulators, explant source and culture age on genetic stability of plants obtained from tissue culture propagation of ornamental strawberry “Serenata” were examined. Genomic DNAs of in vitro-derived shoots and control plant were extracted and compared by RAPD-PCR analyses. Ten primers (from 48 previously tested) were selected and used in RAPD analysis to prove the clonal fidelity (i.e. genetic stability) of the tissue culture-derived ornamental strawberry plants. The lack of polymorphisms in micropropagated plants screened through molecular markers was used to suggest genetic fidelity. Identically banding patterns of the RAPD profiles obtaining from vitroplants, regenerated via organogenesis or meristems culture, suggested that in the ornamental strawberry, variety “Serenata”, neither explant source, nor callus age or limited number of subcultures, in basal media supplemented with low concentration of growth regulators, were associated with occurrence of somaclonal variation.

Keywords: tissue culture, intergeneric hybrids, RAPDs, clonal fidelity

INTRODUCTION

In recent years, with the advent of recombinant DNA technology and PCR, molecular markers are being used for a variety of studies. The molecular markers, including RAPDs, have also been used for testing the genetic fidelity of micropropagated and organogenesis derived plants or elite genotypes. Nevertheless, abnormalities were observed in some cases of tissue-culture strawberry plants, when compared to conventionally propagated ones [11, 12, 14, 22, 30].

Although in vitro culture techniques have monopolized mass propagation and are of beneficial use in various fields, in plant breeding [31], the morphogenesis seems to be highly dependent on plant growth regulators [1], media used for culture [21, 27], or explant source [9, 22, 27], which are closely related with the genotype [14, 28]. In this context, the risk of genetic variations, induced by tissue culture, and the usefulness and suitability of RAPD markers, for assessing the genetic stability of micropropagated strawberry [6, 24], must be emphasized.

The economic consequences of somaclonal variation, can be enormous, so it is extremely important to ascertain the suitability of a particular micropropagation protocol developed for a particular species, in terms of the production of genetically identical plants [29]. Analysis of genomic DNA is commonly used in tissue culture-derived plants, allowing the characterization of genotypes, regardless of plant physiological stage or environmental conditions [18]. Furthermore, the time required for the provision of highly informative results are short. Several cytological, isozyme and molecular markers have been used to detect the variation and/or confirm the genetic stability in micropropagated strawberry plants [4, 5, 30, 33]. In different plant species, the use of PCR, especially in the form of RAPDs [3], has proved to be useful for identification of genetic variation, depending only by the number of the primers involved.

In the present study, the genetic stability and uniformity of shoots obtained in six different tissue culture conditions were assessed by using RAPD markers.

MATERIALS AND METHODS

Plant material: An intergeneric hybrid of Fragaria × Potentilla, named “Serenata”, from the Fragaria Germplasm Collection of the Research Institute for Fruit Growing, Pitesti, Mârăcineni, Romania, it was investigated. In order to determine the genetic fidelity of shoots formed by different tissue-culture methods, this variety of ornamental strawberry was established in vitro culture starting from meristems, leaf and petiole pieces (Table 1).

In this study, RAPD technique was employed, as it has the advantage of simplicity, is quickly performed and is of relatively low cost. Also, it requires very little plant material and quick DNA extraction protocols are suitable.

DNA extraction: Total genomic DNA was extracted from both tissue culture-derived plants and field grown mother plant using DNeasy Plant Mini Kit (Qiagen), following the manufacturers protocol.

To check the degradation of DNA, samples were loaded into 0.8% agarose gel in 0.5 × TBE submitted to electrophoresis, stained with ethidium bromide and observed under ultraviolet (UV). The DNA used for the amplification was of high molecular weight, with little degradation and free of RNA, as revealed by UV and gel electrophoresis.

PCR conditions: RAPD amplification was performed in a reaction volume of 25 μl containing 4 mM MgCl2, 0.4 mM of each dNTP, 0.6 units Taq DNA polymerase, 0.8 μM primer and 100-200 ng genomic DNA. The amplification reactions were carried out in an TC-512 Gradient Thermocycler (Bibby Scientific Ltd) programmed as following: preliminary denaturation of DNA at 95°C for 2 min, 45 cycles of 92°C for 30 s, 36°C for 25 s and 72°C for 74 s, and a final extension step at 72°C for 7 min. The PCR pro-
Table 1. The origin of tissue culture regenerated shoots in the intergeneric hybrid *Fragaria x Potentilla* tested for genetic fidelity.

<table>
<thead>
<tr>
<th>No.</th>
<th>Intergeneric hybrid of <em>Fragaria x Potentilla</em></th>
<th>Basic culture medium and combinations of growth regulators</th>
<th>Tissue-culture methods</th>
<th>Plant material provenance</th>
</tr>
</thead>
</table>
| 1.  | “Serenata”                                    | - Lee & Fossard [19]  
- 1.0 mg/l BAP,  
1.0 mg/l IAA,  
0.1 mg/l GA₃ | Micropropagation (starting from meristems with 2-3 leaf primordia, 0.1-0.3 mm in size) | Fourth subculture (120 days of *in vitro* culture) |
| 2.  | “Serenata”                                    | - Murashige & Skoog [23]  
- 1.0 mg/l BAP,  
1.0 mg/l IAA,  
0.1 mg/l GA₃ | Micropropagation (starting from meristems with 2-3 leaf primordia, 0.1-0.3 mm in size) | Fourth subculture (120 days of *in vitro* culture) |
| 3.  | “Serenata”                                    | - Knop [16]  
- 1.0 mg/l BAP,  
1.0 mg/l IAA,  
0.1 mg/l GA₃ | Micropropagation (starting from meristems with 2-3 leaf primordia, 0.1-0.3 mm in size) | Fourth subculture (120 days of *in vitro* culture) |
| 4.  | “Serenata”                                    | - Lee & Fossard [19]  
- 0.5 mg/l IBA,  
3.0 mg/l BAP | Organogenesis (starting from leaf explants) | 70 days of *in vitro* culture (3-4 weeks of callus phase) |
| 5.  | “Serenata”                                    | - Lee & Fossard [19]  
- 0.5 mg/l IBA,  
3.0 mg/l BAP | Organogenesis (starting from petiole explants) | 70 days of *in vitro* culture (3-4 weeks of callus phase) |
| 6.  | “Serenata”                                    | - Murashige & Skoog [23]  
- 1.0 mg/l IBA,  
3.0 mg/l BAP | Organogenesis (starting from leaf explants) | 70 days of *in vitro* culture (3-4 weeks of callus phase) |
| 7.  | “Serenata” - Control plant                    | N/A                                                                       | N/A                     | Germplasm collection |

Table 2. List of primers, their sequence and size of the amplified fragments generated by 10 RAPD primers.

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequence</th>
<th>No of scorable bands</th>
<th>No of monomorphic bands</th>
<th>Size in bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OPA02</td>
<td>TGCCGAGCTG</td>
<td>5</td>
<td>5</td>
<td>2248-810</td>
</tr>
<tr>
<td>2</td>
<td>OPA11</td>
<td>CAATCGCCGT</td>
<td>10</td>
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<tr>
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<td>5</td>
<td>OPB09</td>
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<td>CTGCTGGGAC</td>
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<td>7</td>
<td>OPB15</td>
<td>GGAGGGTGTTG</td>
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<td>8</td>
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<td>GATGACCGCC</td>
<td>4</td>
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</tr>
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<td>9</td>
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<td>GAACGGACTC</td>
<td>5</td>
<td>5</td>
<td>2140-598</td>
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<td>CTCACCGTCC</td>
<td>7</td>
<td>7</td>
<td>2774-647</td>
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The primers selected for this study gave rise to a total of 60 scorable bands ranging from 312 bp to 2774 bp in size. From the selected markers, two (OPA11 and OPB10) yielded 20 bands across the shoots tested, and another one (OPC09) yielded 7 bands. The other primers were not as efficient in generating successive PCR products. A total of 420 bands (number of plantlets analyzed X number of bands with all primers) were generated by the RAPD technique, giving rise to monomorphic patterns across all plantlets studied. All RAPD profiles from the tissue culture-derived ornamental strawberry plants were found to be analogous to those of the control plant, indicating either identity or similarity. Examples of RAPD patterns amplified with primers OPC09 and OPB09 are shown in Fig. 1 A & B.

Photographs of the gels were obtained with a Gene Flash Syngene Bio Imaging. The 100 bp DNA Ladder (Fermentas) was used as a molecular size standard and the size of the amplification products was estimated using LabImage software from the photographs of the gels.

**RESULTS**

On the basis of the number, intensity and reproducibility of RAPD bands, ten random primers (Table 2) were selected out of the 48 previously tested and used in RAPD analysis to prove the clonal fidelity (i.e. genetic stability) of the tissue culture-derived strawberry plants.

Table 2. List of primers, their sequence and size of the amplified fragments generated by 10 RAPD primers.

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Identically banding patterns of the RAPD profiles obtained from vitroplants, regenerated via direct organogenesis, and from control plant, suggested that in the ornamental strawberry, variety “Serenata”, neither explant source, nor callus age were associated with occurrence of somaclonal variation. Also, the correspondence between RAPD profiles of control plant and microshoots of intergeneric hybrids “Serenata”, after four subcultures, in basal media supplemented with low concentration of growth regulators, denote that no genetic variation had occurred.

DISCUSSIONS

In this study, using 10 RAPD primers, no polymorphism was detected among the shoots regenerated starting from meristems or leaf discs. This results were similar to those of Nehra et al. [26] who found no variants for plants derived from meristem culture or direct leaf regeneration for strawberry cultivars “Vecstar” and “Redcoat”. Among the various methods developed to micropropagate strawberry, enhanced axillary branching culture has become the most important, especially because it is generally considered to be an in vitro culture sistem with low risc of genetic instability [17, 20]. However, the occurrence of somaclonal variation should not be overruled.

Most somaclonal variations occur in plants regenerated from in vitro cultures that have undergone a dedifferentiation phase [17]. Also, high frequency of somaclonal variation was obtained among plants regenerated from 16 and 24-week-old-calli [25] or multiplicated on basal media supplemented with high concentration of growth regulators, such as BAP and 2,4-D [25, 10].

The DNA amplification products, could result from changes in either the sequence of the primer binding sites or changes which alter the size and present the successful amplification of target DNA. In the present study, the amplifies products exhibited monomorphism among micropropagated intergeneric hybrids Fragaria x Potentilla, irrespective of culture media, and were similar to those of the control plant. Sporadic occurrences of somaclonal variations were observed in some cultivars of strawberry, always after a high number of subcultures [4, 13]; therefore it is suggested to limit the number of transfers to 5-10 times.

Because there were no changes in the banding patterns observed in the tissue culture plants as compared with that of the control plant, we conclude that our micropropagation protocol can be followed without much risk of genetic instability. Furthermore, the lack of polymorphic bands among the RAPD profiles of microshoots regenerated through organogenesis, can be associated with a higher genetic stability of this genotype. Other studies have also shown that the genotype of plant can affect the amount of variability that occurs as a consequence of culturing tissues in vitro [8].

The results obtained in this study confirmed the usefulness and applicability of the RAPD method in determining genetic stability and uniformity of plants clonally propagated in vitro. The clonal fidelity of in vitro regenerated shoots has also been determined with the RAPD technique in other species, such as Begonia sp. [2], Lilium sp. [32], Drosera sp., [15], Dendrobium hybrid [7]. The informative primers identified and tested in our studies will be useful in genetic analysis of ornamental strawberry varieties in germplasm holdings.

REFERENCES


