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GENETIC ANALYSIS OF SOMATIC EMBRYO-DERIVED PLANTS OF *HEVEA BRASILIENSIS* (CLONE RRII 105) USING RAPD MARKERS

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Genetic stability of somatic plants developed through somatic embryogenesis from immature anther-derived callus cultures of *Hevea brasiliensis* (clone RRII 105) was examined using random amplified polymorphic DNA (RAPD) analysis. Ten somatic plants along with their mother tree and 10 monoclonal seedlings were subjected for RAPD analysis and compared. Fifteen arbitrary decamers displayed monomorphic banding profile within all somatic plants and their mother plant. However, polymorphism was detected among monoclonal seedlings with all the five primers tested. UPGMA-based dendrogram showed that all the somatic plants along with the source plant clustered together and no variation was observed among these plants. Absence of polymorphism displayed in the RAPD profile indicated a stable genome in plants developed through somatic embryogenesis.

Keywords: Genetic stability, *Hevea brasiliensis*, Polymorphism, RAPD markers, Somatic embryogenesis

INTRODUCTION

In tissue culture, genetic stability of the regenerants is an important requirement. All regenerated individuals are expected to be genetically similar with the mother plant. Despite the advantages of the regeneration system, genetic variability also has been observed among *in vitro*-derived plants (Isabel *et al.*, 1993; Salvi *et al.*, 2001; Soniya *et al.*, 2001). Genetic variability may sometimes be beneficial for crop improvement programmes. However, when the system is utilized for mass multiplication and genetic transformation of a particular variety or clone with potential characters, random genetic changes are not desirable. It is, therefore, essential to assess the genetic stability/ variability of plantlets before utilizing the system for crop improvement programmes.

Among the different methods for assessing genetic variability, molecular markers are powerful tools since they are stable and detectable in all tissues. Of all available techniques, random amplified polymorphic DNA (RAPD) analysis is a useful and rapid technique. RAPD analysis has been successfully used to determine the genetic integrity of regenerants in many plant species like turmeric (Salvi *et al.*, 2001), tomato

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(Soniya *et al.*, 2001), banana (Ray *et al.*, 2006) and arecanut (Karun *et al.*, 2008).

For a heterozygous, woody perennial tree crop like rubber (*Hevea brasiliensis*), the integration of specific desired characters through conventional breeding is both time consuming and labour intensive. Further, the propagation of this crop by bud-grafting can lead to intraclonal variation in field performance due to stock-scion interaction. Hence, an *in vitro* plant regeneration system *via* somatic embryogenesis is an essential pre-requisite for the effective employment of the protocol in rubber tree genetic improvement. At the Rubber Research Institute of India, efficient plant regeneration pathways have already been developed from different explants like immature anther, immature inflorescence and leaf explants (Jayasree *et al.*, 1999; Sushamakumari *et al.*, 2000; Kala *et al.*, 2005). However, hardly any reports are available on genetic analysis to understand the genomic stability of plantlets derived from these explants. The present work was carried out with an objective to assess the genetic integrity of plants generated *via* somatic embryogenesis.

MATERIALS AND METHODS

Development of somatic plantlets

Somatic embryogenesis and plant regeneration from immature anthers were successfully carried out employing the protocol developed by Jayasree *et al.* (2001). After 50 days of culture, friable callus was induced from immature anthers on modified MS medium supplemented with 2.0 mg/L 2, 4- dichlorophenoxyacetic acid (2,4-D) combined with 0.5 mg/L kinetin. Induced calli were sub-cultured for embryogenesis on medium containing glutamine (200 mg/L), casein hydrolysate (400 mg/L), kinetin (0.7 mg/L), naphthaleneacetic acid (0.2 mg/L) and gibberellic acid (GA_3) (2.0 mg/L). Somatic embryos developed up to the cotyledon stage were transferred to plant regeneration medium supplemented with 1.0 mg/L benzyladenine coupled with 2.0 mg/L GA_3. Plantlets were regenerated one month after transferring the mature embryos to plant regeneration medium. Well-developed plantlets were selected and transplanted to small polybags filled with sterile soilrite for hardening. Fully-hardened plants were transplanted to large polybags and maintained under shade house.

Raising monoclonal seedlings

Seeds were collected from monoclonal garden of clone RRII 105 at Hevea Breeding Sub-station, Kanyakumari District, Tamil Nadu and kept for germination in well-drained sand beds with periodic watering. Germinated seeds were planted in polybags and kept in glass house.

DNA isolation and RAPD analysis

One-year-old somatic plants (n=10) were randomly selected for genetic analysis. The mother plant of the somatic plants was used as the control. The relative genetic stability or variability of somatic plants was compared with one-year-old plants of monoclonal seedlings (n=10). DNA was isolated from leaves of all these selected plants following modified CTAB procedure (Doyle and Doyle, 1990). Samples of immature leaf tissues (2.0 g) were ground to fine powder with liquid nitrogen using sterile mortar and pestle and transferred into a 50 ml centrifuge tube. 2X CTAB extraction buffer (20 ml) was added to each powdered leaf sample and incubated in a water bath for 30 min at 55 ^{0}C with occasional shaking. The homogenate

was centrifuged at 8000 rpm for 10 min. An equal volume of Tris-saturated phenol: chloroform:isoamyl alcohol (25:24:1) was added to the supernatant and spun at 10,000 rpm for 10 min. Aqueous phase was removed and then incubated for 2 h at 37 °C with 5.0 µl of DNAase-free RNAase (10 mg/ml). Proteins were removed by adding equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged for 10 min at 10,000 rpm at room temperature. Aqueous phase was re-extracted with equal volume of chloroform and centrifuged at 10,000 rpm for 10 min. Aqueous phase was removed and DNA was precipitated with 0.6 volume of ice-cold isopropyl alcohol and tubes were kept in ice for 20 min. DNA was pelleted by centrifugation at 8,000 rpm for 10 min at 4 °C. Pelleted DNA was washed with 70 per cent ice-cold ethanol and re-suspended in TE buffer. DNA from each sample was loaded on agarose gel and electrophoresis was carried out to determine the quantity and quality needed for PCR.

A preliminary screening has been carried out with 20 decamer primers (Operon Technologies, USA) to assess the ability to produce satisfactory banding pattern and 15 primers were selected for the detailed study. PCR analysis was followed according to the standard protocol (Williams *et al.*, 1990). Amplification was performed in a DNA thermal cycler (Perkin Elmer, USA) in 20 µl volume consisting of 1X reaction buffer, 100 µM dNTPS, 0.5 units of *Taq* DNA polymerase enzyme, 20 ng of template DNA and 250 nM of RAPD primers. The reaction mixture was overlaid with one drop of sterile mineral oil. Following initial denaturation at 94 °C for 3 min, the amplification programme was set to 36 cycles of denaturation at 94 °C for 1 min, annealing at 38 °C for 1 min and extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. The reaction products were separated in 1.5% agarose gel. To each PCR tube, 6 µl of 4X loading dye was added, mixed well and was loaded on to gel and electrophoresis was performed at 50 volt. Gels bands were stained in ethidium bromide solution for 20 min and photographed under UV light. PCR amplification was repeated thrice for confirming reproducibility.

Data analysis

Amplification products were scored on the basis of their presence or absence in the gel. Pairwise comparisons based on both unique and shared amplification products were employed to calculate genetic distance (Link *et al.*, 1995). The data were subsequently used for cluster analysis using UPGMA to construct a dendrogram. All calculations were made using the Treecon programme (Van De Peer and De Wachter, 1994).

RESULTS AND DISCUSSION

RAPD profile of somatic plants was compared with the mother plant as well as with monoclonal seedlings. Out of the 20 primers screened initially, 15 primers showed clear and reproducible amplifications. For each plant, amplified DNA products were scored for the presence or absence of bands. DNA bands with low intensities which could not be distinguished clearly were not considered. Each primer produced a unique amplification pattern and was different in their size. With all the 15 primers used for the detailed investigation, no detectable variation was observed in somatic plants. All the amplicons generated was monomorphic across all the somatic plants and the mother plant. However, the number of bands varied with respect to primers (Table 1). Banding

Table 1. **Details of primers and fragment size generated with 15 RAPD primers in somatic plants**

Primer	Sequences (5'-3')	No of bands	Fragment size (kb)
OPA-04	AATCGGGCTG	8	0.4-1.5
OPA-07	GAAACGGGTG	10	0.3-5.0
OPA-08	GTGACGTAGG	5	0.4-1.5
OPA-12	TCGGCGATAG	10	0.3-5.0
OPA-20	GTTGCGATCC	8	0.4-4.0
OPB-06	TGCTCTGCCC	6	0.4-2.0
OPB-07	GGTGACGCAG	12	0.3-5.0
OPB-18	CCACAGCAGT	10	0.4-1.9
OPB-20	GGACCCTTAC	10	0.3-4.0
OPC-01	TTCGAGCCAG	9	0.3-5.0
OPC-04	CCGCATCTAC	10	0.4-1.5
OPC-07	GTCCCGACGA	7	0.5-2.0
OPC-09	CTCACCGTCC	11	0.4-5.0
OPD-08	GTGTGCCCCA	11	0.3-5.0
OPD-18	GAGAGCCAAC	11	0.3-4.0

pattern showed the minimum number of five bands with OPA-08 primer. With primers OPB-06 and OPC-07 also, the number of bands produced was found to be low with six and seven bands, respectively. A maximum number of 12 bands was produced by the primer OPB-07.

Figure 1A-C show representative amplification profiles produced by three primers (OPA-20, OPB-07 and OPB-20). The size of the monomorphic amplicons produced by the 15 primers ranged approximately from 0.3 to 5.0 kb (Table 1). No polymorphism or variation was detected within somatic plants and in the control plant. The RAPD profile of the 10 monoclonal seedlings of the clone RRII 105 showed variation with all the five tested primers (Fig. 1A-C). Majority of the bands observed with the seedlings were present in the somatic plants and their mother plant. In the monoclonal seedlings, additional bands were also present with all five primers. Number of bands for each primer varied from 9 to 14. Primer OPA-12 generated 13 bands that showed polymorphism between control and monoclonal seedlings. Three additional bands of approximately 1.4, 1.0 and 0.3 kb present in some of the seedlings were absent in other seedlings and control. Similarly with primer OPA-20, a non-parental polymorphic band of about 2.0 kb was present in some of the seedlings (Fig.1A). Primer OPB-07 produced a total of 14 bands of which bands with approximately 1.3 and 0.9 kb were absent in control and somatic plants. However, in majority of seedlings, these particular bands were present (Fig. 1B). With primer OPB-20 also, polymorphism was detected (Fig.1C). Similarly, with primer OPD-18, an additional band of approximately 1.4 kb was present in all the seedlings which was absent in control.

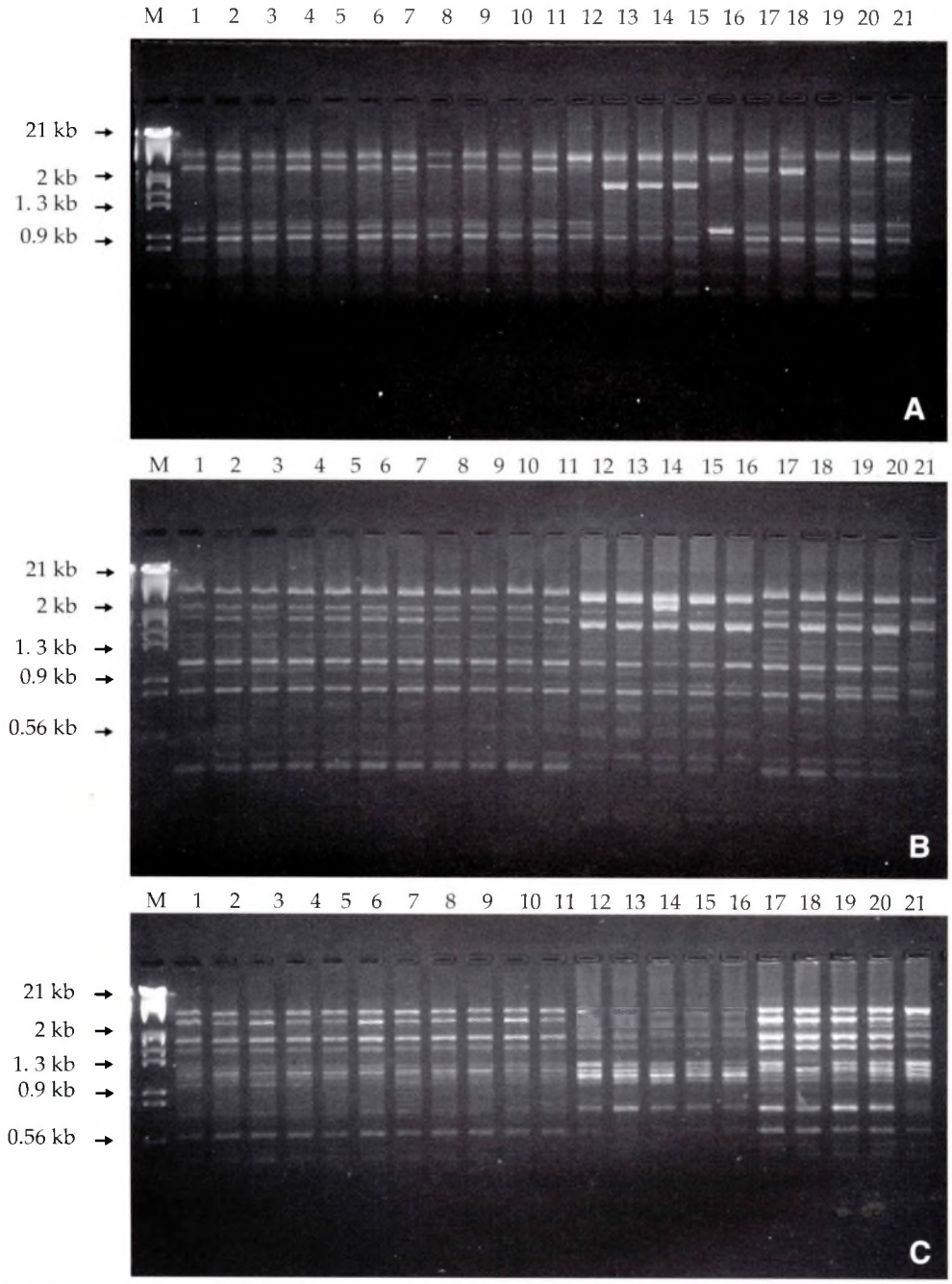

Fig. 1. A-C. RAPD profiles of somatic plants and monoclonal seedlings DNA amplification obtained with primers (A) OPA-20; (B) OPB- 07; (C) OPB-20; M: Marker DNA (λ-DNA double digested with *EcoRI* and *Hind III* restriction enzyme); Lane 1: Mother plant; Lanes 2-11: Somatic plants; Lanes 12- 21: Monoclonal seedlings.

Pairwise genetic distance was calculated by Treecon programme using the RAPD data of control, somatic plants and monoclonal seedlings and constructed UPGMA dendrogram. The dendrogram showed that all the somatic plants along with the mother plant were clustered together and there was no variation among these plants. Other seedling populations were grouped into two, consisting of five seedlings in each group at a distance coefficient of 0.1. Among the seedling population, maximum genetic distance was found to be around 25% and the minimum distance was approximately 2% between somatic plants and seedlings (Fig. 2).

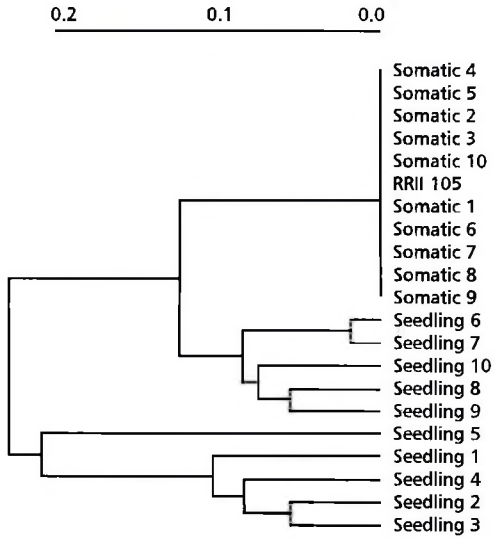

Fig. 2. UPGMA-based dendrogram showing the genetic relationship between mother plant, somatic plants and monoclonal seedlings

RAPD markers are widely used to detect somaclonal variation at the DNA level (Cloutier and Landry, 1994). In many plant species, genetic stability/variability was analyzed by RAPD technique (Shoyama *et al.*, 1997; Akhar *et al.*, 2000; Salvi *et al.*, 2001; Soniya *et al.*, 2001). The *in vitro* stress during culturing may cause the genome to respond by DNA methylation and this may modify the RAPD profile by altering the primer binding sites through the insertion or excision of transposons. Changes in the RAPD pattern may thus result from the loss/gain of a primer annealing site caused by point mutation or by the insertion or deletion of sequences or transposition elements (Peschke *et al.*, 1991). In the present study, RAPD markers were used to assess the genetic stability/variability if any, in plants developed through somatic embryogenesis.

In the present study, a preliminary attempt was made to assess the genomic stability of somatic plants with 15 primers. Considering the size of rubber genome (2n = 4 Gb), the number of primers used in this study was not sufficient to confirm whether the genome of somatic plants is uniform. However, a relative comparison was made with monoclonal seedlings of the same clone. Since *H. brasiliensis* is a predominantly cross-pollinated tree species, the monoclonal seedlings are expected to be heterozygous. No detectable variation was observed (*i.e.* polymorphism was absent) among somatic plants with all the tested primers indicating that the genome of somatic plants was relatively stable. However, monoclonal seedlings showed polymorphism with all the five primers. The results agree with the observation of Thakur *et al.* (1999) and Shoyama *et al.* (1997) where no somaclonal variation was observed in *Quercus serrata* somatic seedlings as well as in *Panax* somatic embryo-derived plants. Similar results are also reported in willow *(Salix* sp.*)* and date palm *(Phoenix dactylifera)* plants regenerated through somatic embryogenesis (Akhar *et al.*, 2000). Contradictory to this, Heinze and

Schmidt (1995) observed a low frequency of genetic instability in the population of somatic plantlets of spruce. In arecanut, plantlets derived *via* direct somatic embryogenesis showed high similarity (99%) with that of mother plant (Karun *et al.*, 2008). Polymorphism in RAPD has also been reported in many plants including turmeric (Salvi *et al.*, 2001) and tomato somatic plants (Soniya *et al.*, 2001). Plants derived from leaf base callus of turmeric showed 16.5% polymorphism with certain primers (Salvi *et al.*, 2001). In tomato, out of 10 primers, six produced polymorphism for all 11 plants (Soniya *et al.*, 2001). However, no such variation could be noticed within somatic plants of *H. brasiliensis*. In the case of monoclonal seedlings, RAPD profile displayed variation with the same primers.

CONCLUSION

The present study shows that RAPD analysis can efficiently be applied to assess the genetic stability of somatic plants. RAPD markers obtained using 15 arbitrary decamers showed no detectable variation across the somatic embryogenesis - derived plants though variation was observed in monoclonal seedlings. Cluster analysis revealed that all the somatic plants could be grouped together along with the source plant and there was no variation at all among these plants. In the case of seedling - derived plants, they formed two subgroups at a distant coefficient of 0.1 revealing variation among them. In summary, the amplification products were monomorphic for all the plants of *H. brasiliensis* regenerated by somatic embryogenesis suggesting that this system can be effectively used for mass multiplication and genetic transformation of this tree crop.

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