THE EXAMINATION OF *HEVEA BRASILIENSIS* PLANTS PRODUCED BY *IN VITRO* CULTURE AND MUTAGENESIS BY DNA FINGERPRINTING TECHNIQUES

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Abstract

Rubber (*Hevea brasiliensis)* plants derived from anther and ovule culture as well as gamma-irradiated plants were examined by several DNA marker techniques. These include restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), sequence tagged microsatellite sites (STMS), DNA amplification fingerprinting (DAF) and amplified fragment length polymorphisms (AFLPs). Compared to control plants produced by vegetative propagation (cutting and budding), plants produced by *in vitro* culture appeared to have a reduction in the number of rDNA loci. Two RAPD protocols were compared and found to be similar in amplification of the major DNA bands. After confirmation that the RAPD method adopted was reproducible, the technique was applied to the present studies. Eight out of the 60 primers screened were able to elicit polymorphisms between pooled DNA from *in vitro* culture plants. Variations in DNA patterns were observed between pooled DNA samples of anther-derived plants as well as between anther-derived and ovule-derived plants. Comparisons of RAPD patterns obtained between anther-derived plants exposed to increasing dosages of gamma-irradiation with non irradiated anther-derived plants revealed distinct DNA polymorphisms. The changes in DNA profiles did not appear to be correlated to the dosage of irradiation. Since somaclonal variation was detected, it was difficult to identify changes which were specifically caused by irradiation. Application of the STMS technique to tag microsatellite sequences $(GA)_n$, $(TA)_n$ and $(TTA)_n$ in the hydroxymethylglutaryl coenzyme A reductase-1 (hmgr-1) gene failed to detect differences between plants derived from anther and ovule culture. Although restriction endonuclease digestions with methylation sensitive enzymes suggested that four *in vitro* culture plants examined exhibited similar digestion patterns as the controls, a change in cytosine methylation in one anther-derived plant was detected. Examination of individual *in vitro* culture plants by the DAF technique revealed genetic heterogeneity among these plants. Differences in DNA profiles between anther-derived and ovule-derived plants were also detected. In general, more somaclonal variations were detected in anther-derived than ovule-derived plants. When the DAF technique was applied to DNA obtained from leaves of irradiated budded stumps, DNA profiles were shown to be different from non irradiated controls. Whilst DNA patterns of samples irradiated at the same dosage displayed similar DNA patterns, these varied with increasing dosage. Examination of *in vitro* culture plants by AFLPs confirmed earlier results that somaclonal variations were present in *Hevea*.

1. INTRODUCTION

Hevea brasiliensis Muell Arg., a member of the *Euphorbiaceae* family, is an important economic crop of Southeast Asia. *H. brasiliensis* is traditionally cultivated for the production of natural rubber, but more recently, it has emerged as a valuable renewable source of timber. Because planting of unselected seedlings yields uneconomic plantations, all commercial cultivation is carried out through vegetative propagation, that is by budding a desirable trunk clone to a seedling root stock. For further enhancement of productivity, a further budding of a desirable crown (canopy) clone to the trunk is often practised, resulting in a three-part tree [1]. Budding however, is a skilled and labour intensive exercise. *In vitro* culture was envisaged as an alternative method for mass propagation to circumvent the requirement for skilled labour as well as an avenue for *Hevea* crop improvement. Tissue culture of *Hevea* was initiated nearly four decades ago [2,4]. Although early success was limited [5], plants produced from *in vitro*

culture have been established in field trials in China and Malaysia. As far as we are aware, these *in vitro* culture plants have not been examined at the DNA level.

The present study was undertaken to determine whether *Hevea* plants produced by *in vitro* culture were true to type (i.e., genetically identical) *via* DNA fingerprinting techniques. At the same time, DNA fingerprinting would also be employed as an early selection criterion for improved characteristics after mutagenesis through gamma-irradiation. Several DNA fingerprinting techniques were utilized. These included a hybridization-based technique namely restriction fragment length polymorphisms (RFLPs) [6] and polymerase chain reaction (PCR)-based techniques such as random amplified polymorphic DNA (RAPD) [7-8], sequence tagged microsatellite sites (STMS) [9-10], DNA amplification fingerprinting (DAF) [11] and amplification fragment length polymorphism (AFLP) [12].

2. MATERIALS AND METHODS

2.1. Plant materials

A random selection of 59 *in vitro* culture plants which were derived from both anther and ovule culture of *H. brasiliensis* clone GL1 were examined. These plants were 36 months old at the initiation of this investigation and were already field planted at the Rubber Research Institute of Malaysia Experiment Station at Sungei Buloh. Plants of the same clone, but propagated vegetatively by cutting and bud-grafting, acted as controls.

2.2. Production of mutagenic plants by gamma-irradiation

Anthers of *H. brasiliensis* clone RRIM 600 were gamma-irradiated at 1-4 gray at the therapy unit of the Malaysian Institute for Nuclear Technology (MINT). The irradiated anthers were then cultured *in vitro* for plantlet production [13]. Leaves from two irradiated (anther-derived) plants at each dosage were sampled and their DNA fingerprints were compared with non-irradiated *in vitro* culture plants as controls.

Similarly, budded stumps of *H. brasiliensis* clone RRIM 905 were gamma-irradiated at 5-25 Gy at MINT as described [14].The irradiated budded stumps were then planted out in the field. Leaves which developed subsequently were harvested and used for DNA fingerprinting studies. DNA profiles from bifoliate leaves obtained from six RRIM 905 gamma-irradiated budded stumps were compared with DNA from bifoliate leaves from two non-irradiated budded stumps of the same clone as well as regular trifoliate leaves from untreated controls.

2.3. DNA and RFLP methods

DNA samples were isolated by the procedure of Low *et al* [15], with adaptations from Institut fur Genbiologische Forschung (IGF), Berlin. Quality of the isolated DNA was ascertained by analysis in agarose gel electrophoresis as well as its susceptibility to digestion by *Eco*RI. DNA for RFLP analysis was digested overnight at 37°C with various restriction enzymes according to the manufacturer's instructions. Restriction enzyme digestion was carried out at a concentration of 10 units of restriction enzyme per μg of DNA, in the presence of 5mM spermidine and 100 μg/ml casein. Other RFLP methods were similar to those described earlier [16,17].

Simple sequence oligonucleotides were generated by concatemerisation of the basic repeating unit to sequences of several kilobases by PCR and used as probes for RFLP analysis [18]. The clone pTa71 which contains a 9 kb *Eco*RI fragment of ribosomal DNA (rDNA) from wheat, *Triticum aestivum* [19] was recloned into pUC19 and was a generous gift from Flavell and O'Dell (John Innes Centre, Norwich, UK). This was used as a hybridization probe on digested DNA of *in vitro* culture plants.

2.4. PCR-based fingerprinting techniques

All PCR-based marker techniques, except AFLP, were carried out as described [17]. Examination of genomic DNA from *in vitro* culture and control plants by the RAPD technique was carried out with two different protocols [7,20]. All amplifications were preceded by "hot start" and executed with random hexamer primers (Operon Technologies, USA). Amplification products were analyzed after electrophoresis in 2% agarose gels in 1xTBE buffer as well as in 10% denaturing polyacrylamide gels containing 1.6M urea in 1xTBE followed by silver nitrate staining.

Amplification of microsatellite regions by STS was carried out according to Low *et al* [21] with primers flanking the regions of interest [17].

The DAF technique was carried out according to the procedure of Caetano-Anolles *et al* [11] and random hexamer primers, in various combinations of primer-pairs were tested for their ability to elicit DNA polymorphism as described [17].

2.5. AFLP analysis

Hevea DNA samples were digested with *Eco*RI and *Mse*I for AFLP analysis which was carried out by the radioactive γ -^{33}P-ATP procedure [18] using an AFLP Analysis System 1 kit (Gibco BRL, USA).

3. RESULTS

3.1. Production of mutagenic plants from gamma-irradiated anthers

A total of 2281 anthers were irradiated with gamma-rays at between 1-4 Gy. *In vitro* culture of the irradiated anthers yielded 573 embryoids, representing 47% embryogenesis. Twenty-seven plantlets were produced, with eight plants being planted in the field. DNA was isolated from leaves of these plants for DNA fingerprinting analysis.

3.2. Polymorphisms between gamma-irradiated and non irradiated budded stumps

Normal *Hevea* plants have trifoliate leaves. Occasionally, transient emergence of bifoliate leaves could be observed without treatment of the plant. These bifoliate leaves would revert to their regular trifoliate form after a time, without treatment. The reason for this transient expression is unclear and remains a topic of interest.

DAF profiles of normal, non-irradiated plants which had trifoliate leaves were shown to differ from those rarer, non-irradiated plants which had bifoliate leaves (Fig. 1). Similarly, bifoliate leaves derived from gamma-irradiated budded stumps displayed a different DNA fingerprint from those obtained from bifoliate leaves of control, non-irradiated plants. Though

DNA profiles varied with increasing dosage of irradiation, they were essentially similar, at the same dosage (5 Gy).

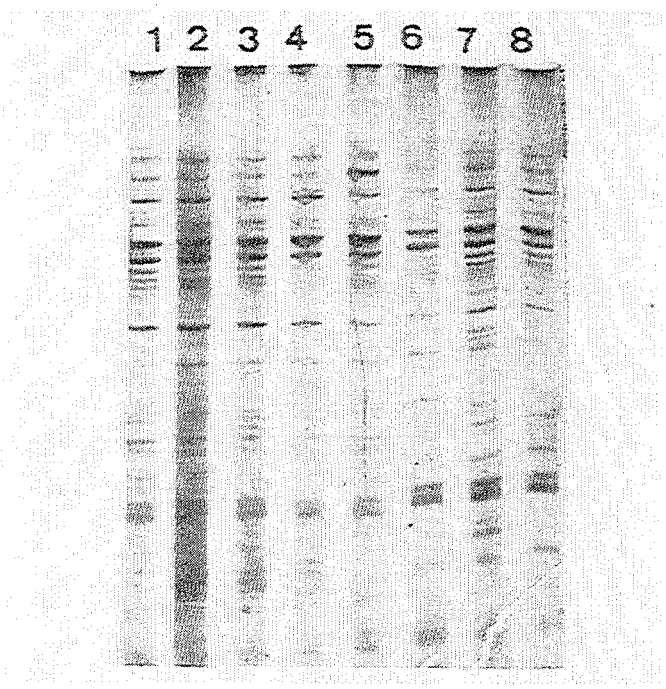

FIG.1. DNA amplification fingerprinting (DAF) profile of budded stumps after exposure to increasing dosages of gamma-irradiation.1:In vitro anther-derived plant EAC 1/7 (non-irradiated in vitro control), 2:Bifoliate leaves from gamma-irradiated budded stump (5 Gy), 3:Bifoliate leaves from gamma-irradiated budded stump (5 Gy), 4:Bifoliate leaves from gamma-irradiated budded stump (10 Gy), 5:Bifoliate leaves from gamma-irradiated budded stump (15 Gy), 6:Bifoliate leaves from gamma-irradiated budded stump (25 Gy), 7:Bifoliate leaves (non irradiated control), and 8:Trifoliate leaves (non irradiated control).

3.3. Methylation and reduction in rDNA copy number

DNA obtained from *in vitro* culture plants was digested separately with various restriction enzymes such as *Alu*I, *Hae*III, *Taq*I, *Hpa*II, *Mbo*I and *Msp*I as well as in combinations such as *Alu*I+*Hae*III. Hybridization with a rDNA probe, pTa71, revealed that DNA from *in vitro* culture plants was digested more extensively with *Alu*I than DNA from control plants (Fig. 2). Similar results were obtained when pTa71 was hybridized with DNA digested with *Hae*III as well as *Alu*I+*Hae*III in combination. No differences in hybridization results were observed when pTa71 was probed with *Hpa*II and *Msp*I digested DNA from regenerated plants (data not shown).

Sixteen oligonucleotide probes were synthesized by concatemerisation of the basic di-, tri- and tetramers to several hundred basepairs (bp) in length. In order to evaluate their methylation status, some of these SSR oligonucleotide sequences were used as hybridization probes on digested DNA from a small sample of regenerated plants. Hybridisation with $(GACA)_n$ on DNA digested with *Taq*I produced similar discreet bands with DNA from control and *in vitro* culture plants. However, one of the *in vitro* culture plants (EAC 4/91) appeared to have a more distinct fragment of approximately 2 kb than the others (*Fig. 3).* Hybridization with the same probe on DNA digested with *Hpa*II and *Mbo*I, on the other hand, elicited a polymorphism between *in vitro* culture plant EAC 4/91 and the other samples examined (*Fig. 3*). Whilst hybridization signals were similar between DNA from the control and the four regenerated plants, these were different in the case of the *in vitro* culture plant EAC 4/91 in both enzymic digests.

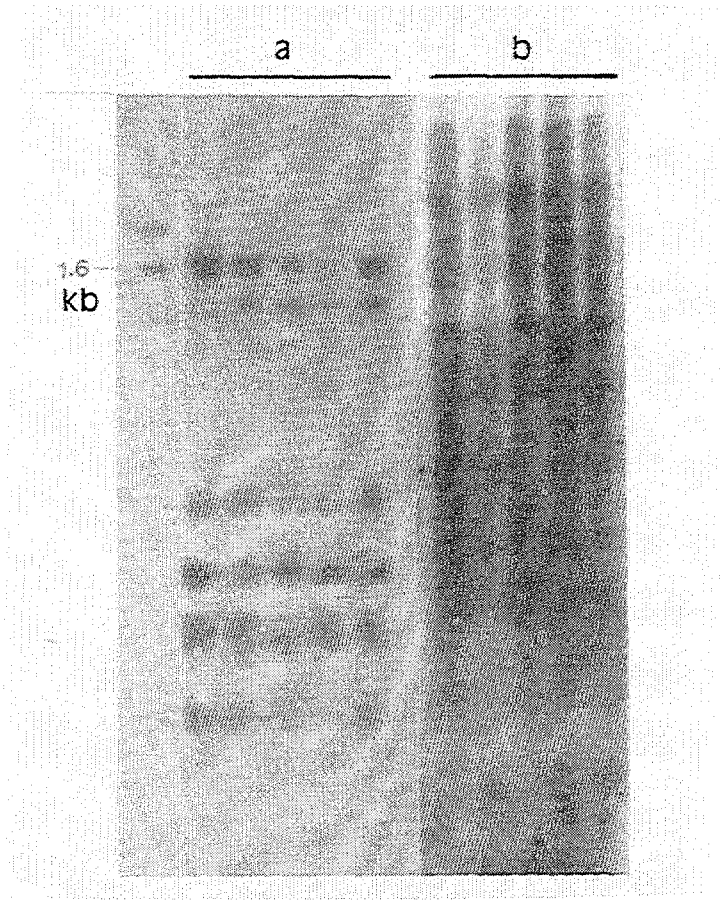

FIG. 2. A reduction in rDNA loci number as revealed by pTa71, after hybridisation with AluI-digested DNA from (a)five in vitro culture and (b) five control plants.

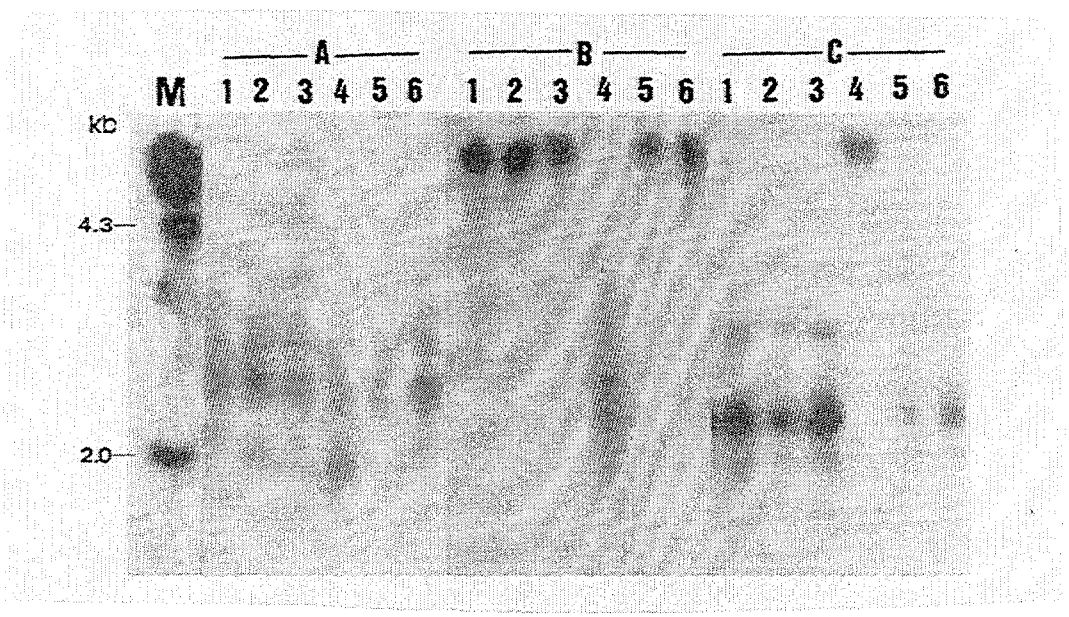

FIG. 3. Polymorphism as revealed by SSR oligonucleotide probe $(GACA)_n$ after hybridisation with DNA digested with (A) TaqI (B) HpaII and © MboI. 1: Control (cutting); 2: In vitro culture plant IVP 1; 3: In vitro culture plant IVP14; 4: In vitro culture plant EAC 4/91; 5: In vitro culture plant EAC 18/90; 6: In vitro culture plant EAC 34/90.

3.4. RAPD polymorphism in tissue culture plants

With the incorporation of "hot start", RAPD results were more reproducible and the number of amplified bands was also reduced. Reproducibility of the RAPD technique after the above modifications was demonstrated when five sets of duplicate samples of genomic DNAs, obtained from independent extractions were amplified by the RAPD technique and subjected to polyacrylamide gel electrophoresis (PAGE, Fig. 4). Comparison of the two RAPD protocols [7,20] demonstrated that all major bands were similarly amplified. However, because of shorter cycling times, the protocol of Koller [20] was adopted in all subsequent RAPD experiments.

Eight out of the 60 random primers screened were able to elicit polymorphism between *in vitro* culture plants. As a quick screen to detect somaclonal variations, the population of *in vitro* culture plants was divided into groups of nine plants. Anther-derived plants were grouped separately from ovule-derived plants. DNA isolated from these plants was pooled according to these groups and pooled DNA profiles examined. DNA polymorphisms between pooled DNA from anther-derived plants were detected as well as between anther-derived and ovule-derived plants (data not shown).

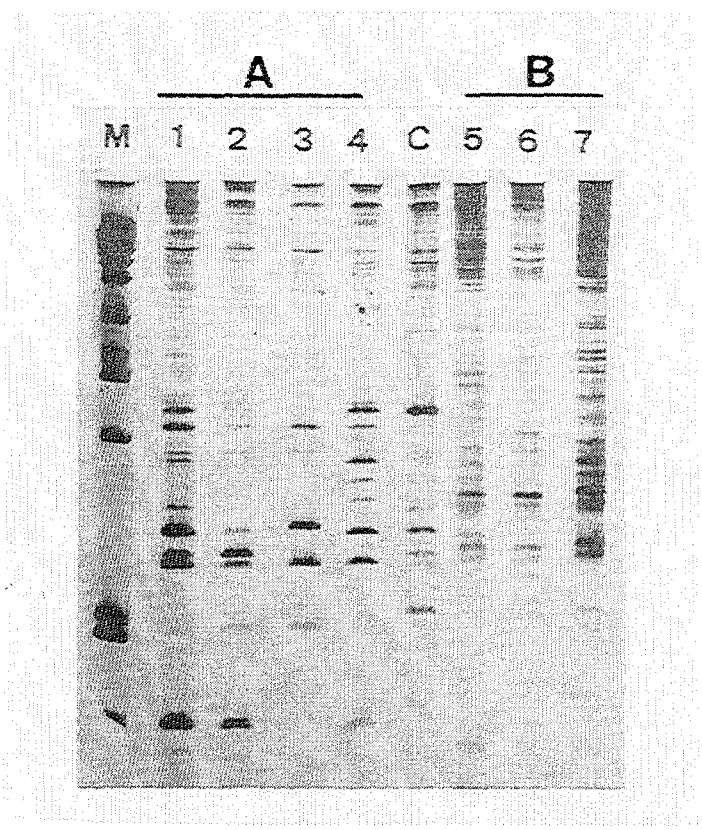

FIG. 4. RAPD polymorphisms ((A) Primer Op-F07 and (B) Primer Op-F08) between control and anther-derived plants after exposure to increasing dosages of gamma-irradiation (1-4 gray). 1: γ -irradiated anther-derived plant (1 gray); 2: γ -irradiated anther-derived plant (2 gray); 3: γ -irradiated anther-derived plant (3 gray); 4: γ-irradiated anther-derived plant (4 gray); 5: γ -irradiated anther-derived plant (2 gray); 6: γ -irradiated anther-derived plant (3 gray); 7: γ -irradiated anther-derived plant (4 gray); C: Control (non-irradiated anther-derived plant); M: Mol. wt. marker, 1 kb ladder.

Comparison of RAPD patterns obtained after amplification with primer Op-F07 between anther-derived plants exposed to increasing dosages (1-4 gray) of gamma-irradiation with non-irradiated plants from the same source (anthers) revealed distinct DNA polymorphisms (Fig 4). DNA fingerprints between the irradiated materials appeared to vary in number and size of DNA fragments. With increasing dosage of irradiation, large DNA fragments appeared to be replaced by several shorter ones.

3.5. DAF polymorphism in tissue culture plants

Twenty hexamer primers in 190 combinations of primer-pairs were tested for their ability to elicit DNA polymorphisms. Of these, only two were found to be informative. As described above (Section 3.4.), samples of pooled DNA were used. With primer-pair (OpA4/OpA19), the DNA profiles between these groups were nearly indistinguishable. On the other hand, DNA profiles of individual plants were more diverse. Anther-derived plants tended to be more polymorphic than ovule-derived plants.

3.6. STMS within the HMGR-1 gene

Twenty seven combinations of forward and reverse primers were used to tag the microsatellite region $(GA)_8$ within the hydroxymethylglutaryl coenzyme A reductase (hmgr-1) gene in tissue culture plants. Even though many fragments were amplified within this locus, no polymorphism was detected. Separate digestion of these amplified products by *Alu*I and *Hae*III failed to yield any polymorphic fragment. Similarly, no polymorphism was detected at the $(TA)_n$ and $(TTA)_n$ loci.

3.7. AFLP between *in vitro* culture plants

Four *Eco*RI primers, namely, *Eco*RI+AAC, *Eco*RI+ACG, *Eco*RI+ACT, *Eco*RI+AGG and eight *Mse* I primers were used in combination, resulting in 32 primer-pair combinations to detect somaclonal variations among a random sample of ten *in vitro* culture plants. Depending on the primer pair combination used, polymorphic bands were obtained in all the regenerated plants, in comparison to the control (data not shown). Polymorphisms were scored as presence or absence of bands, without consideration for band intensity.

4. DISCUSSION

4.1. Production of mutagenic plants by gamma-irradiation of budded stumps

The differences in DNA profiles between bifoliate and trifoliate normal leaves are not surprising, since these were phenotypically different. However, the differences in DNA profiles between non irradiated and irradiated bifoliate leaves (Fig. 1) are interesting, as these would reflect changes as a consequence of gamma-irradiation. The observation that DNA profiles were similar after exposure to the same dosage of gamma-irradiation (5 Gray), suggested that the observed changes in DNA pattern were consistent and that the DAF technique was reproducible. Although variations in DNA profiles with increasing dosage of gamma-irradiation was demonstrated, there was an absence of a distinct trend in these differences, making it difficult to assign specific DNA fragments to dosage.

4.2. Methylation and reduction in rDNA copy number

The status of DNA methylation in regenerated plants compared to control plants was studied using the high copy number probe pTa71, as well as oligonucleotide probes such as $(GACA)_n$ on DNA which was cut by methylation sensitive enzymes. Comparison of pTa71 hybridization results (Fig. 2), suggested that there was a reduction in the number of rDNA loci in plants produced from cell culture. The reduction in rDNA loci in *Hevea* could be a result of the minimization of gene redundancy, whilst maintaining the gene product [21] or culture-induced [22]. Though reduction in rDNA loci number had been reported in other plants [21-24], the phenomenon is unknown in *Hevea* and may not have adverse effects [25].

Since DNA from *in vitro* culture plant EAC 4/91 was cleaved equally by both *Msp*I and *Hpa*II, in contrast to DNA from the control and other *in vitro* culture plants, this suggested the absence of methylation in cytosine in the recognition sequence, as revealed by probe pTa71. A reduction in DNA methylation in regenerated plants is not unusual and had been reported [27-28].

Polymorphism between *in vitro* culture plant EAC 4/91 and the other samples examined, as revealed by hybridization of $(GACA)_n$ on DNA digested with *Hpa*II and *Mbo*I (Fig. 3) was unrelated to DNA quality, since all samples could be digested equally by *Taq*I. Results after *Mbo*I and *Taq*I digestion suggested the presence of hydroxymethylated cytosine in plant EAC 4/91. Spectroscopic and chemical analysis would however, be needed to confirm the above, but are beyond the scope of the present study. Tissue culture-induced changes in DNA methylation have been reported and were attributed to the wide range of changes that occur after propagation of tissue culture plants [21]. Changes in DNA methylation were reported to be frequent in maize callus, regenerated plants and the progeny of such plants [27]. The extent of DNA methylation could vary with the plant and could also fluctuate with time in the same plant, as seen in carrot root explants [28]. It remains to be determined whether DNA methylation changes in *Hevea* are reversible and would have adverse effects with time.

4.3. RAPD polymorphism in mutagenic and *in vitro* culture plants

The demonstration that all major bands were amplified similarly by the two RAPD protocols tested provided confidence in the reproducibiltiy of the RAPD technique (data not shown). This was further confirmed when 5 sets of duplicate DNA samples produced similar amplification results (data not shown).

Since polymorphism in RAPD fingerprints were detected between irradiated and non irradiated control *in vitro* plants (Fig. 4), the observed changes were probably caused by gamma-irradiation, rather than the culture method. However, because somaclonal variations had been demonstrated in the present study, and these could not be differentiated from mutagenesis, it was difficult to ascertain the origin of these changes. With increasing dosage, large DNA fragments appeared to be replaced by shorter ones, indicating possible cleavage of long DNA by gamma-irradiation.

4.4. DAF polymorphism between *in vitro* culture plants

The lack of polymorphism when pooled samples of DNA from tissue culture plants were compared with control, suggested that either somaclonal variation was absent, or was present at a very low level. However, the diverse DNA profile obtained when DNA profiles of 48 *in vitro* culture plants were compared individually, as well as with control vegetative plants

suggested the presence of somaclonal variations. Since DAF profiles from individual anther-derived plants were more polymorphic than individual ovule-derived plants, it appeared that anthers have a greater propensity for somaclonal variation than ovules. This finding, though new in rubber had been reported in other plants [22-28]. The polymorphic bands detected in *Hevea* appeared to be unusually large in contrast to those reported elsewhere [29-30]. Preliminary examination of DAF polymorphic bands in *Hevea* suggested that the short fragments (less than 1.0 kb) were less polymorphic than the longer ones.

4.5. STMS within the HMGR-1 gene

The lack of STMS polymorphism within the hmgr-1 gene between control and regenerated plants is not unexpected since only a very minute region of a specific gene was examined. Failure to elicit polymorphism after separate digestion by *Alu*I and *Hae*III of the amplified fragments indicated that neither insertion/deletion nor point mutation [31] were present among the tissue culture plants within that gene. Being a latex-enriched [32] key enzyme of rubber biosynthesis, differences in hmgr-1 gene are expected to be less in leaves than in latex.

4.6. AFLP between *in vitro* culture plants

All 10 regenerated plants displayed polymorphism when compared to control, suggesting that somaclonal variations exist in *Hevea* (data not shown). This is in confirmation to results from other fingerprinting techniques discussed above. The frequency of somaclonal variation appeared to be high, as estimated by AFLP (data not shown).

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