Identification of a DNA marker associated with dwarf trait in *Hevea brasiliensis* (Muell.) Arg. through random amplified polymorphic DNA analysis

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Abstract :

The current study was conducted to identify DNA markers linked to genes controlling dwarf character in rubber tree (Hevea brasiliensis) using random amplified polymorphic DNA (RAPD) analysis. Hybrid progenies were developed between dwarf parent (natural variant) and cultivated clone RRII 118. Genomic DNA was subjected to PCR analysis using 85 random sequence 10-mer primers, of which 9 produced clear, scorable bands. Among the 9, one showed polymorphism which was used for dwarf character analysis. An initial comparison was made between two parents and later the dwarf parent along with their hybrid progenies. In the dwarf parent alone with the primer OPB-12, a DNA fragment with molecular size of 1.3 kb was amplified. This primer amplified the same DNA fragment (1.3 kb) with the segregated 6 F1 hybrid progenies and it was absent in the other parent. The segregation data for the marker and the dwarf trait in the F1 hybrids indicate that the DNA marker identified is linked to dwarf character. Analysis of RAPD loci by southern blot hybridization indicated that this is a specific loci of the same gene fragment. DNA markers associated with dwarf trait genes are useful to facilitate the introgression of these genes in breeding materials for the development of high yielding clones with dwarf nature to avoid wind damage of this important perennial tree crop.

Key words: Rubber tree, *Hevea brasiliensis*, RAPD analysis, DNA markers, Hybrid progenies, Dwarf variant

Introduction

Rubber tree (*Hevea brasiliensis* 2n=36), is a perennial tropical crop which belongs to the genus *Hevea* and the family Euphorbiaceae. Rubber (cis-1,4-polyisoprene) is synthesized in at least 2000 plant species. The genus *Hevea* encompasses ten species, all originating from Amazon (Schultes 1990). The genetics of rubber tree has been poorly investigated. This lack of knowledge is due to the heterozygous nature of the crop, its long growing cycle that includes 6-7 years before latex collection, and its low seed set per pollination. As a rule, rubber tree displays inbreeding depression, making it difficult to develop appropriate progenies for classical studies (Lespinasse et al. 2000). Introgression of natural *Hevea* germplasm coding for economic traits has been an important component of *Hevea* improvement programmes. During this process, genes or chromosome segments from natural variants will be introgressed into cultivated *Hevea* clones. To exploit this variability, specific markers presumably could be used to tag and follow introgression of chromosome segments. Association between markers detecting introgressed chromosome segments and traits controlled by genes on introgressed segments could then provide the basis for marker-assisted selection experiments. One of the PCR based molecular markers called RAPD has recently been shown to be very useful for DNA fingerprinting (Williams et al. 1990), detection of genetic polymorphism and varietal identification and mapping. RAPD markers have also been used to evaluate levels of gene flow between species

(Arnold et al. 1991) and detection of gene introgression in various plant species (Waugh et al. 1992; McCoy and Echt 1993; Orozco Castillo et al 1994; Garcia et al. 1995; Gomez et al. 1996) but much work has not been done in *Hevea* in this direction. However, isozymes, RFLPs, microsatellite and RAPD markers have been developed in *Hevea* for varietal identification and genetic studies (Besse et al. 1993a, b; Besse et al. 1994; Chevallier 1988; Seguin et al. 1995, 1996; Low and Gale 1996; Luo et al. 1995; Lespinasse et al. 2000; Shoucai et al. 1994, Varghese et al. 1997).

A natural variant with short stature (dwarf) which was confirmed to be a genetic variant was identified from PBIG seedling population for the first time in *Hevea* (Markose et al. 1981). Subsequently, these genetic variants were further characterized by John et al. (1995). Since *Hevea brasiliensis* is the only cultivated and high latex yielding rubber crop, there is an urgent need to develop improved clones with compact canopy. Incorporation of dwarf character into *Hevea* breeding programme will be useful for generating a tree with desirable architecture. Thus information on the genetic and molecular basis of the dwarf character in this species could provide insights on the development of high yielding dwarf clones which will eventually lead to overcome wind damage and high density planting of rubber tree.

In view of the above, in the present study, F_1 hybrid progenies (controlled cross and natural cross) derived from a natural dwarf variant and a cultivated *Hevea* clone (RRII118) were used for identification of RAPD marker associated with dwarf character.

Materials and methods

Plant material

All plant material used in this study was collected from the experimental field as well as nursery of the Rubber Research Institute of India (RRII). For detection of introgressed gene segments, three hybrid progenies each from a controlled cross [Natural Dwarf Variant (E) x RRII 118 (Γ)] and natural cross [Natural Dwarf Variant (E) x random cultivated clone of *Hevea* (Γ)] along with two parents (Natural Dwarf Variant and RRII 118) were used. RRII 118 is a cultivated clone and Dwarf is naturally occurred variant. In addition, four different morphotypes namely dwarf, semidwarf, intermediate and normal trees were also used for PCR analysis to confirm a dwarf linked DNA marker. Young leaves from the selected *Hevea* plants (disease-free) were harvested and stored immediately in polybags. The polybags containing leaf samples were packed in ice and transported to the laboratory where DNA was extracted immediately.

Genomic DNA isolation

Total cellular genomic DNA was isolated and purified from young leaf material of individual samples by the modified CTAB extraction procedure (Doyle and Doyle 1990). Young leaves (2 gms) were ground to a fine powder in liquid nitrogen in a mortar with pestle and transferred into a 50 ml centrifuge tube. 20 ml of 2x CTAB buffer [(2% hexadecyltriethylammonium bromide), 1.4M NaCl, 20 mM EDTA (pH 8.0), 0.1 M Tris-HCl (pH 8.0), 1% polyvinyl polypyrolidone (PVPP), 1% 2-mercaptoethanol] was added and incubated the extract in a water bath at 65°C for 30 min with occasional swirling. The suspension was mixed gently and spun at 8000 rpm to pellet the cell debris. The supernatant was transferred to a new centrifuge tube and mixed thoroughly with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 10,000 rpm for 10 min. The aqueous phase was transferred to a new tube and again reextracted with an equal volume of chloroform and isoamyl alcohol (24:1) and spun for 10 min at 10,000 rpm. The supernatant was carefully removed to a fresh centrifuge tube where RNA was eliminated by treatment with DNase-free RNase and the solution was incubated at 37°C for 3 h. After RNase treatment, the suspension was reextracted with an equal volume of chloroform and centrifuged (10,000 rpm, 10 min). Following centrifugation, the aqueous phase was removed to a clean tube and DNA was precipitated by the addition of 0.6 volume of isopropyl alcohol (100%). DNA was pelleted at 10,000 rpm for 10 min and

the supernatant was discarded. After adding 5 ml of 70% ethanol to the DNA pellet, it was mixed thoroughly and then spun at 8000 rpm for 5 min. The supernatant was decanted and the pellet was air dried for 20 min. The air-dried DNA pellet was resuspended in 1 ml of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). The DNA quality was checked on 0.8 % agarose gel electrophoresis. DNA samples were stored at 4°C for short term or at -20°C for long-term storage.

PCR amplification using random primers

Random decamer primers were purchased from Operon Technologies Inc., Alameda, CA, USA and were used as single primers for the amplification of genomic DNA. Initially, about 85 primers were screened for PCR amplification. The primers, which exhibited clear banding profiles after amplification, were selected for further RAPD analysis of hybrid progenies as well as parents. DNA was extracted from six hybrid lines along with two parents and RAPD was carried out using 10 base primers. In order to detect whether the amplicons are reliable, three replications were performed for each primer. The nucleotide sequence of selected primers used is shown in Table 1. Amplifications were carried out in a 20µl reaction volume, which contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5mM $MgCl_2$, 100µM dNTPs (dATP, dGTP, dCTP and dTTP), 0.5 unit of Taq DNA Polymerase enzyme, 25 ng of template DNA and 250 nM of RAPD primer. The reaction mixture was overlaid with two drops of mineral oil in order to avoid evaporation. The amplifications were carried out in a Perkin-Elmer DNA Thermal Cycler 480, USA. Tubes containing all the reaction components except for the DNA template were included as a control for each primer used. The PCR amplification profile consisted of a first cycle at 94°C for 4 min. followed by 38 cycles at 94°C for 1 min/38°C for 1.5 min/72°C for 2 min and a last cycle at 72°C for 7 min. After completion of the PCR cycles, loading buffer was added to each reaction tube. The RAPD products were separated by electrophoresis using 1.5% agarose gels containing 0.5µg/ml ethidium bromide in 0.5X TBE buffer (Sambrook et al. 1989). Electrophoresis was performed at 50V power supply for about 4 h until the bromophenol blue dye front had migrated to the bottom of the gel. The molecular standard used was the lambda DNA double digested by EcoRI/HindIII. The gels were visualized and photographed under UV-light with Canon Camera, Japan. The reproducibility of the amplification products was tested at least twice for each experiment.

Southern blot hybridization

Amplified DNA fragments were electrophoresed and transferred from 1.5% agarose gels onto nylon membrane (Hybond N^+, Amersham, UK) in 10X SSC (1X SSC is 0.15 M NaCl, 0.015 M trisodium citrate) for 16h (Sambrook et al. 1989). After DNA transfer, the membranes were UV-crosslinked and stored at 4°C until use. The selected introgressed RAPD fragment was excised from a 1% low-melting point agarose gel and purified according to standard procedure (Sambrook et al. 1989) and used for radio labelling. Radioactive probes were generated using the random primer labelling method with α-^{32}PdATP (BARC, Trombay, Mumbai, India, 4000 Ci/mmol). Hybridization was carried out in 6XSSC, 5X Denhardt's, 0.5% SDS at 65°C in a rotary hybridization oven (Amersham-Pharmacia, UK). After hybridization, filters were washed at low stringency at room temperature twice in 2X SSC + 0.1% SDS for 5 min and 1X SSC + 0.1% SDS for 15 min and high stringency at 65°C, twice in 0.5 XSSC + 0.1% SDS for 30 min and 0.1X SSC + 0.1% SDS for 30 min, followed by signal detection. The labelled blots were then exposed to X-ray film (X-Omat, Kodak) with intensifying screens at -80°C.

RAPD marker scoring and analysis

Banding patterns or DNA fingerprints were analysed by bistate type scoring. The occurrence of a specific band of amplified DNA was recorded as (+) and absences as (–) for all prominent bands within a fingerprint. Therefore, a sequence of 1's, and 0's was generated for each primer/species to form a data matrix. Individual amplified bands were indicated by the primer used and its size in bp. Fragment sizes of RAPD were estimated from the gel by comparison with molecular weight marker. Only the major fragments, which unambiguously present were considered. Thus, a single profile for

one parent clone along with hybrids was subjected to the comparison with RAPD patterns of another parent clone. A clone that had a particular band in a RAPD pattern was given a score of '+' for that trait. A clone was given a score of '–' for that trait if the band was absent from the RAPD pattern. Polymorphic index content (PIC) was calculated as follows: $PIC=1-\Sigma Pi^2$ where Pi is the band frequency of the i-th allele. In the case of RAPD, PIC was calculated following the formula [$PIC=1-p^2-q^2$], Where p= band frequency, q=no-band frequency (Ghislain et al. 1999). The data were used to generate complementation of Jaccard's similarity coefficients for RAPD bands. The Jaccard's coefficients, which considers 1–1 matches (number of 1–1 band matches divided by 1–1 matches plus total on non-matching bands) between each pair of accession were used to construct a dendrogram according to the unweighted pair group method with arithmatic averages (UPGMA) using TREECON.

Results and discussion

In the present experiment, three hybrid lines each from controlled cross and natural cross along with their parents (Natural dwarf variant and RRII 118) were selected for identification of DNA marker linked to dwarf trait based on differences found in RAPD banding patterns of the parents. A total of 85 random oligonucleotide primers from Operon (Operon Technologies Inc., USA), were screened with the DNA samples to identify best primers that generated clear banding pattern. In total 9 primers (10.58%) were found to be best for amplification, 35 primers (41.17%) generated light bands with streaking pattern and the remaining primers (41) failed to amplify bands (48.23%). The PCR analysis of individual plants resulted in the amplification of 6-18 DNA fragments and the size of bands ranged from 300 to 3500 base pairs.

To characterize the capacity of each marker to reveal polymorphic loci of clones, we used the polymorphic index content (PIC). The PIC values for each RAPD marker generated by the same primer were summed up and named it RAPD primer index (Table-1). This index reveals the information content of the RAPD primer per assay. Therefore, primers with 2.2 RAPD primer index value were preferably used in subsequent fingerprint analysis in *Hevea* species. Nine primers produced DNA polymorphisms and segregation between parents and hybrids. Results indicated that the RAPD technology effectively reveals genetic similarity or difference between hybrid lines and their respective parents. These primers produced a total of 103 bands, of which 50 (48.5%) were found to be polymorphic (Table 1). Among the 9 primers, easily detectable, well resolved polymorphic bands which were reproducible over repeated runs, with sufficient intensity were observed with 4 primers. However, primer OPB-12 revealed distinct polymorphic and segregating banding patterns that could be inferred for gene introgression between parents and their F_1 hybrid progenies. For a given cross and primer combination, the number of segregated or inherited bands that had or did not have a specific fragment transmitted from one or both parents was scored to detect the introgressed gene segment.

With this format, parental origin of the markers, as well as their allelic state, was directly inferred from the presence of the fragment in one parent, the absence in the other, and segregation (presence/absence) in the F_1 progeny samples. RAPD marker was counted only if the phenotypic classes were consistent and clearly distinguishable. The present study describes the detection of segregated or inherited markers of F_1 hybrids between natural dwarf variant and cultivated *Hevea* clones. Given that dwarf (natural) accession can now be considered as potential germplasm donors through the use of bridge crosses, molecular marker technology may prove very useful for identification of introgressed gene segments in *Hevea*.

The use of RAPD markers to detect alien gene segment introgression or to confirm the composition of interspecific hybrids has also been reported earlier in other crop species (Arnold et al. 1991; Barid et al. 1992; McCoy and Echt 1993). Garcia et al. (1995) and Gomez et al. (1996) opined that the probable mechanism of introgression is chromosome recombination and not chromosome substitution. In the present study, few introgressed gene fragments were identified using PCR technique. Thus either recombination occurred subsequently to a translocation event or transfers resulted from several independent recombination events, which account for this cumulative introgressed gene fragments.

This is consistent with earlier observations of multivalent formation that eventually could lead to homologous chromosome pairing and introgression (Frello et al. 1995).

With OPB-12 primer one specific DNA band (1.3kb) was detected and it was segregated in all F_1 hybrid progenies (Fig.1a). This DNA fragment (1.3kb) was present only in natural dwarf parent whereas it was absent in other RRII 118 parent. However, this DNA band was found in both controlled as well as natural hybrid lines. This result suggested that 1.3kb RAPD band may be linked to dwarf trait since dwarf is one of the parents in both the hybrid lines. In order to make sure that this DNA marker is really associated with dwarf trait, four different morphotypes were also used for PCR amplification. In the RAPD banding pattern, 1.3 kb band was present in dwarf, semidwarf and intermediate morphotypes but absent in normal plant. This indicates that 1.3 kb RAPD marker is linked to dwarf character in *Hevea*. For further confirmation of this dwarf DNA marker, the 1.3kb DNA fragment of dwarf parent was radio labelled and hybridized with RAPD profiles of two parents as well as six F_1 hybrid progenies. The hybridization signals illustrated in Fig.1b clearly demonstrate that the 1.3kb fragment was detected in all hybrid progenies and dwarf parent, but not in RRII 118 parent. These results inferred that this DNA marker may be part of a gene linked to dwarf trait of *Hevea*.

RFLP analysis based on Southern blot technique reveals only the crude forms of DNA sequence variation, i.e. those resulting in changes in the size of DNA fragments on the order of 10% or greater (Helentjaris 1989). Therefore, instead of concentrating on the relatively crude methods of the Southern blot, we focused our attention to more powerful methods to fine tune the detection of introgressed gene segment in *Hevea* through PCR based RAPD technology. Molecular analysis of the present study indicates that the amplified DNA fragment was linked to a specific trait (dwarf) and introgressed from dwarf parent not from the cultivated one (RRII118). These results demonstrate that RAPD markers have the potential to complement both conventional and biotechnological approaches to *Hevea* improvement programmes in the future.

The dissimilarity matrix obtained using Jaccard's coefficient is depicted in Table 2. These dissimilarity coefficients were used to generate a dendrogram (Fig. 2) by UPGMA analysis in order to determine the grouping of F_1 hybrid lines and their parents. Average linkage cluster analysis of the dissimilarity coefficients indicates that distance separating CH1 and CH2 hybrids are most dissimilar hybrids with other hybrids as well as parents. The dendrogram further indicates that there is a basic similarity among natural F_1 hybrid progenies since these three hybrids clustered together.

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