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TOWARDS THE DEVELOPMENT OF MICROSATELLITE MARKERS IN RUBBER (*HEVEA BRASILIENSIS*)

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ABSTRACT

Identification of microsatellites from *Hevea brasiliensis* was initiated by the construction of a genomic DNA insert library in lambda ZAP Express vector. 10000 plaques were screened in total and 113 $(CT)_n$ and 91 $(AC)_n$ positive clones/plaques were selected from the primary plates. Out of the 204 positive clones, 50 clones were selected at random and subjected to a second round of screening. After secondary screening, 30 clones were recovered individually of which 16 were $(CT/GA)_n$ and 14 $(AC/TG)_n$. *In vivo* excision of the phagemid containing the genomic DNA insert was done from these 30 positive clones. The isolated phagemids were restricted with *Xba* I and *Bam* H I to release the inserts as well as to identify the duplicate clones through restriction profiles. Screening was also performed with the dinucleotide repeat probe $(AT/TA)_{20}$ and no positive signal was obtained possibly due to the minimum occurrence of this repeat sequence within the genomic DNA. 12 positive clones were sequenced to validate the presence of microsatellites from genomic libraries of *Hevea*. In total, nine of the clones were identified with microsatellite repeats. Eight of the positive clones contained long arrays of the dinucleotide motifs CG, TG, AG, CA, CT, whereas one positive clone possessed trinucleotide repeats GAT, GTT and one had a tetranucleotide repeat AAAT. Both simple and compound repeats were observed. Identification and characterization of these SSR containing genomic clones is the initial step towards the development of microsatellite markers in *Hevea*.

INTRODUCTION

DNA sequences with short repeated motifs (less than 6 bp) are termed microsatellites, simple sequence repeats (SSRs), or short tandem repeats (STRs). Such repeats display high levels of polymorphism because of variation in repeat length. Microsatellites are useful as molecular markers because they are (i) highly abundant and uniformly distributed, (ii) highly polymorphic, (iii) have codominant Mendalian inheritance, (iv) rapidly analyzed via PCR and gel electrophoresis, (v) allow relatively simple interpretation and genetic analysis of a single-locus, and (vi) are easily accessible to other laboratories via published primer sequences (Weber, 1990; Saghai-Maroof *et al.*, 1994). In plants, SSR markers have been successfully applied to a variety of questions including the construction of genetic maps (Taramino and Tingey, 1996), the assessment of genetic diversity (Doldi *et al.*, 1997), cultivar identification and pedigree studies (Sefc *et al.*, 1998). Recently, microsatellites have been isolated and characterized in several crops like wheat (Roder *et al.*, 1998), grapevine (Sefc *et al.*, 1999), peach (Testolin *et al.*, 2000) and many other plants. The data available have shown that microsatellites are abundant and polymorphic and can be used as genetic markers in many crop plants.

The rubber tree *Hevea brasiliensis* (Wild. Ex. Adr. de. Juss. Muell. Arg), produces about 98% of the world's natural rubber. The perennial nature, long breeding and selection cycles, difficulties in raising F_2 progenies etc. make conventional genetic analysis in *Hevea* very difficult. Application of molecular markers, complementing the conventional methodologies, for genomic characterization of *Hevea* has gained momentum very recently with the development of various molecular marker techniques. Available reports described the successful application of RAPD or RFLP markers, which include assessment of genetic variability in Wickham and wild materials using RFLPs (Besse *et al.*, 1994), clonal identification and evaluation of genetic diversity in popular clones using RAPDs (Varghese *et al.*, 1997), the estimation of phylogenetic relationships from mitochondrial DNA RFLPs (Luo *et al.*, 1995) and identification of mildew resistance genes by the RAPD techniques (Shoucai *et al.*, 1994). Low *et al.* (1996) for the first time detected microsatellites in the *Hevea* genome through the database search of some *Hevea* gene sequences. DNA fingerprints in *Hevea brasiliensis* using heterologous minisatellite probes from human was reported by Besse *et al.* (1993) and the construction of a microsatellite enriched library in *Hevea brasiliensis* was reported by Atan *et al.* (1996). Lespinasse *et*

al., (2000) also described the construction of a genetic linkage map of rubber tree using different molecular markers. However, in the absence of any published information about the development of microsatellites in *Hevea*, it is felt necessary to develop this potential marker system in *Hevea* for its exploitation in the characterization of *Hevea* genome. Therefore, with the aim of producing microsatellite markers in *Hevea* genome, we have initiated a systematic isolation of microsatellites in *Hevea brasiliensis*. The present work reports the successful identification and characterization of some microsatellite/SSR bearing genomic clones that lead to the development of microsatellite markers in *Hevea*.

MATERIALS AND METHODS

Plant material and DNA extraction

Total genomic DNA was extracted from about 1.0 g of young leaves of *Hevea brasiliensis* according to the CTAB (cetyltrimethylammonium bromide) method of Doyle and Doyle, 1990.

Library construction and identification of positive clones

A small insert library was constructed in lambda ZAP Express vector (Stratagene) as follows: Genomic DNA was completely digested with the restriction enzymes *Eco* R I and *Xho* I and cloned into lambda ZAP Express vector and packaged in Gigapack III gold packaging extract according to the protocol described by Stratagene. This phage was plated after incubation with *E.coli* XL-1 Plue cells. Plaques were transferred onto positively charged Nylon membranes according to standard procedures (Sambrook *et al.*, 1989) and screened by plaque hybridization with the dinucleotide repeat probes $(CT/GA)_{20}$, $(AC/TG)_{20}$ and $(AT/TA)_{20}$ which are prevalent in other plant species. The blots were washed to a stringency of 0.5X SSC and 0.1% SDS at 65°C. The positive plaques were recovered individually and were subjected to another round of screening. After the second round of screening, the positive plaques were purified and converted into plasmids by *in vivo* excision of the kanamycin resistant pBK-CMV phagemid vector that allows insert characterization in the plasmid system. The excised phagemids were double digested with *Xba* I and *Bam* H I to identify the size of the insert. 12 positive clones [6 of $(AC)_n$ and 6 of $(CT)_n$] were sequenced using the primers T3 and T7 to validate the presence of microsatellites from genomic libraries of *Hevea*.

RESULTS AND DISCUSSION

A genomic library of *Hevea* was screened by plaque hybridization using $(AC)_{20}$, $(CT)_{20}$ and $(AT)_{20}$ oligonucleotides as radiolabelled probes. Among the 10000 plaques screened, 91 clones were found positive for $(AC)_n$ and 113 clones for $(CT)_n$. Some of these positive clones/plaques were recovered individually for second round of screening. After the second round of screening, 30 clones were recovered individually of which 16 were found positive for $(CT)_n$ and 14 positive for $(AC)_n$ (Table 1). *In vivo* excision of the phagemid containing the genomic DNA insert was done from these 30 positive plaques. The excised phagemids were double digested with *Xba* I and *Bam* H I to identify the size of the insert. Based on the screening result obtained, 12 clones [6 of $(AC)_n$ and 6 of $(CT)_n$] were sent for sequencing to Microsynth GmbH, Switzerland, using the primers T3 and T7. In total, 9 clones were identified with microsatellite repeats after DNA sequencing of the purified phagemid clones. During the sequence analysis it was noted that the majority of the isolated microsatellites were containing more than one microsatellite motifs. The repeats found were dinucleotide repeats like $(CG)_n$, $(TG)_n$, $(AG)_n$, $(CA)_n$, $(CT)_n$; trinucleotide repeats such as $(GAT)_4$, $(GTT)_3$ and a tetranucleotide repeat $(AAAT)_5$ was also noticed. The microsatellites frequently contained more than one simple sequence repeat motifs (compound microsatellites) whereas some were simple, perfect and long (Table 2). AG, AC and CT dinucleotide repeats occur at comparable frequencies in the *Hevea* genome, while the trinucleotide and tetranucleotide repeats occur at lower frequencies. Screening was also performed with the dinucleotide repeat probe $(AT)_{20}$ and no positive signal was obtained. This may be possibly due to the minimum occurrence of this repeat sequence within the genomic DNA.

From the sequence data obtained, it can be concluded that microsatellites occur in the genome of *Hevea* and it can be further developed to be a

TABLE: 1 Details of the screening of phage library for SSR-containing clones

1.	Total No. of plaques screened :	10,000
2.	First round of screening :	204 positive clones/plaques [91 $(AC)_n$ and 113 $(CT)_n$]
3.	Second round of screening of 50 $(CT/GA)_n$ and $(AC/TG)_n$ positive clones from the first round of selection :	30 positive clones [16 with $(CT/GA)_n$ and 14 with $(AC/TG)_n$] selected out of 50 clones. 12 clones [6 $(AC)_n$ and 6 $(CT)_n$] were sequenced

TABLE: 2 Microsatellites / SSRs in *Hevea*[2]

Clone	Nucleotide repeats	Type of repeats
hmac3	$(TG)_{14}$	Dinucleotide simple repeat
hmac4-1	$(CT)_{17}(CA)_3(CT)_2(GT)_{16}$	Dinucleotide compound perfect repeats
hmac4-2	$(AC)_{16}(AG)_2(TG)_3(AG)_{17}$	Dinucleotide compound perfect repeats
hmac5	$(CA)_{18}$	Dinucleotide simple repeat
hmct1	$(CT)_{12}AG(GT)_8$	Dinucleotide compound imperfect repeats
hmct3	$(CT_9(CA)_3$	Dinucleotide compound perfect repeats
hmct4	$(CG)_3(TG)_5(AG)_{11}$	Dinucleotide compound perfect repeats
hmct5	$(GAT)_4(GTT)_3(GA)_5A(AG)_{10}$	Di/trinucleotides compound imperfect repeats
hmct6	$(AAAT)_5$	Tetranucleotide simple repeat

reliable marker system in *Hevea*. It should be possible to establish a set of highly polymorphic microsatellite markers for the evaluation of the genetic diversity among the *Hevea* clones, to develop clone specific DNA fingerprints for their identification and also to develop microsatellites associated with desirable traits. Microsatellite markers for these purposes should be carefully selected for amplifying only the expected fragments, in order to avoid misinterpretations and to enable detection on polyacrylamide or agarose gels (Plaschke *et al.*, 1995). Further work is in progress on designing oligonucleotide primers based on the flanking sequences on both upstream and downstream of the microsatellite repeats.

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