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THE APPLICATION OF DNA MOLECULAR MARKER TECHNIQUES IN HEVEA BRASILIENSIS

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THE APPLICATION OF DNA MOLECULAR MARKER TECHNIQUES IN *HEVEA BRASILIENSIS*

By

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LIST OF ABBREVIATIONS

APS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
bp	Base-pair
BPB	Bromo-phenol blue
BSA	Bovine serum albumin
cm	Centimeter
cpm	Counts per minute
DAF	DNA Amplified Fragments
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Dinucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EDTA Na	Sodium salt of EDTA
EMBL	European Molecular Biology Library
EtBr	Ethidium bromide
EtOH	Ethanol
g	Gram
GL 1	Clone Glensheild 1 of *Hevea brasiliensis*
hr	Hour

HCl	Hydrochloric acid
HMGR-1	Hydroxymethylglutaryl coenzyme A reductase-1
IPTG	Isopropyl-β-D-Thiogalacpyranoside
kb	Kilobase
L	Litre
M	Molar
m	meter
min.	Minutes
M13	A filamentous lysogenic bacteriophage containing a single-stranded DNA
mM	Millimolar
mm	Millimeter
M.W.	Molecular weight
Na citrate	Sodium citrate
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
ng	Nanogram
OD_{260}	Optical density measured at 260 nm wavelength
OD_{280}	Optical density measured at 280 nm wavelength
PAGE	Polyacrylamide gel electrophoresis

[α-^{32}P]dCTP	Phosphorous-32 alpha labelled deoxyCTP
[γ-^{32}P]ATP	Phosphorous-32 gamma labelled ATP
PP	Polypropylene
RAPD	Random amplified polymorphic DNA
RE	Restriction enzyme
RF	Replicating form
RNase	Ribonuclease
rpm	Revolutions per minute
RRIM	Rubber Research Institute of Malaysia
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulphate
sec	Second
ss	Single stranded
STS	Sequence-tagged-sites
T4 PNK	T4 polynucleotide kinase
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TEMED	N,N,N',N',-Tetramethylethylenediamine
TE	Tris EDTA buffer
Tris	Tris(hydroxymethyl)aminoethane
UV	Ultra violet light

µg	Microgram
µl	Microlitre
V	Volt
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyronoside, a lactose analogue

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DNA was extracted from several *Hevea* sources; namely, various *Hevea* species, several cultivars from within the *Hevea brasiliensis* species such as clones and *in vitro* cultured *H. brasiliensis.* Four DNA molecular marker techniques were used to analyze the DNA. These techniques included a hybridization-based marker technique called restriction fragment length polymorphism (RFLP) and three polymerase chain reaction (PCR)-based techniques *viz.* random amplified polymorphic DNA (RAPD), DNA amplified fingerprinting (DAF) and sequence-tagged microsatellite sites (STMS). In the RFLP study, a wheat ribosomal DNA, pTa71 (rDNA) probe was able to detect a reduction in rDNA loci number in DNA from *in vitro* cultured plants compared to DNA from control plants. Hybridization with M13 DNA fragments revealed inter- and intraspecific variations among the DNA samples. Neither of these

hybridization probes could detect somaclonal variation within a sample of *in vitro* cultured plants. On the other hand, RAPD and DAF were able to detect somaclonal variation within the *in vitro* cultured plants. The polymorphic patterns produced by RAPD could be neither correlated with any particular morphological trait nor the source of calli i.e. anther or ovule. Meanwhile, DAF proved to be more sensitive as it was able to detect a high degree of variation in the DNA extracted from anther derived calli. STMS could not detect any variation nor insertion/deletion mutation at the HMGR-1 gene within the *in vitro* culture DNA. RAPD and DAF molecular markers were found to be dominant while RFLP and STMS markers were co-dominant in all of the *H. brasiliensis* crosses tested in this study. No change in the methylation sites for both *in vitro* culture and control plants were detected when the DNAs were digested with both isoschizomeric restriction enzymes *Hpa*II and *Msp*I. A microsatellite enriched library was constructed and was found to be enriched with $(GA)_n$ repeats (39%). Hybridization with one of these clones revealed inter- and intraspecific variations with *Dpn*II-restricted DNAs. This clone was subsequently sequenced and found to be an imperfect repeat.

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APLIKASI TEKNIK PENANDA-PENANDA MOLEKUL DNA KE ATAS *HEVEA BRASILIENSIS*

Oleh

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DNA telah diekstrak daripada beberapa spesis *Hevea*, kultivar dan kultur *in vitro Hevea brasiliensis*. Empat teknik penanda molekul DNA telah digunakan untuk menganalisis DNA tersebut. Teknik tersebut termasuk teknik yang berasaskan penghibridan iaitu 'restriction fragment length polymorpism' (RFLP) dan tiga teknik berasaskan 'tindakbalas polimeras berangkai' (PCR) iaitu 'random amplified polymorphic DNA' (RAPD), 'DNA amplified fingerprinting' (DAF) dan 'sequence-tagged microsatellite sites' (STMS). Dalam kajian RFLP, DNA ribosom gamdum, pTa71, (rDNA) telah digunakan sebagai prob dan telah dapat mengesan pengurangan jumlah loci rDNA pada DNA yang didapati dari pokok yang telah dikultur secara *in vitro* berbanding kepada DNA dari pokok kawalan. Fragmen DNA M13 pula telah dapat menunjukkan variasi inter- dan intraspesifik dan diantara sampel-sampel DNA yang digunakkan. Teknik RFLP gagal

mengesan variasi somaklonal dalam suatu populasi pokok yang telah dikultur secara *in vitro* dengan menggunakan kedua-dua prob tersebut diatas. Sementara itu, teknik-teknik RAPD dan DAF telah dapat mengesan variasi somaklonal dalam populasi pokok-pokok yang dikultur secara *in vitro*. Corak polimorfik yang dihasilkan oleh RAPD tidak boleh dikaitkan dengan mana-mana ciri morfologikal atau sumber kalli (anter atau ovul). DAF terbukti lebih peka, oleh kerana ia dapat mengesan variasi pada kadar yang tinggi dalam DNA yang diekstrak daripada kalli anter. Walaubagaimanapun, STMS tidak berjaya mengesan apa-apa variasi ataupun mutasi sisipan/pemadaman pada gen HMGR-1 dalam DNA daripada pokok yang dikultur secara *in vitro*. Penanda molekul RAPD dan DAF didapati adalah penanda dominan sementara penanda-penanda RFLP dan STMS adalah kodominan pada semua kacukan *H. brasiliensis* yand dikaji. Tidak ada perubahan pada tapak-tapak metilasi untuk DNA dari pokok yang dikultur secara *in vitro* mahupun pada pokok kawalan dapat dikesan apabila sampel DNA tersebut dicerna dengan enzim-enzim isoskitzomer *Hpa*II dan *Msp*I. Satu koleksi klon-klon yang diperkaya dengan mikrosatelit telah dibina dan didapati mempunyai banyak jujukan DNA ulangan-ulangan $(GA)_n$ (39%). Satu klon terpilih telah digunakan sebagai prob dan telah dapat menunjukkan variasi inter- dan intraspesis apabila dihibridisasikan kepada DNA yang telah dicernakan dengan enzim *Dpn*II. Klon ini telah ditentukan jujukan DNAnya dan didapati ianya adalah ulangan jujukan DNA yang tidak sempurna.

CHAPTER I

INTRODUCTION

Marker assisted selection (MAS) is a strategy that enables one to follow a selected trait utilizing a linked genetic marker in a breeding programme. It also facilitates the selection of heritable traits that may not be expressed among individuals at any particular time. Such a programme has yet to be implemented in the breeding programme of *Hevea brasiliensis.* Unlike crop plants such as tomato and lettuce where genetic linkage maps were not only constructed (Helentjaris *et al.*, 1986a; Landry *et al.*, 1987), but also had genetic markers linked to quantitative trait loci in high density genetic maps, the genetic studies of *H. brasiliensis* are still at its infancy.

Cultivated *H. brasiliensis* is a perennial plant that has a long generation time as well as a narrow genetic base. It is believed that with a narrow genetic base, there is a low variability among the cultivated clones due to high levels of inbreeding. However, Chevallier (1988) and Besse *et al.* (1993;1994) found these to be otherwise based on isozyme and restriction fragment length polymorphism (RFLP) techniques, respectively. Variability of the Wickham material as demonstrated by

the isozyme technique was thought to be dependent upon the number and choice of loci sampled e.g. the loci for esterases and phosphatases (Chevallier, 1988). Thus, the variability that was encountered was overestimated whereas that of the germplasm was under estimated. The same argument could be said of the RFLP study.

Several new DNA molecular marker techniques that were successfully developed and used in other plants like maize and tomato were also applied to *Hevea* studies. These new techniques are polymerase chain reaction (PCR)-based which include (a) random amplified polymorphic DNA (RAPD) (Low, 1991), (b) sequence-tagged sites (STS) or sequence-tagged microsatellite sites (STMS) (Low *et al.*, 1994 a, b), (c) DNA amplified fingerprinting (DAF) (Low and Safiah, 1995; Low *et al.*, 1995 a, b) and (d) simple sequence repeats (SSRs) (Safiah *et al.*, 1996).

PCR-based marker techniques promise to be more versatile and robust than RFLP markers as only minute amounts or degraded DNA samples can be used for analysis (Li *et al.*, 1988). These techniques are also much faster than RFLP. Therefore screening of a large number of samples e.g. for mapping a large population, is more feasible with these techniques than with RFLP analysis. PCR-based techniques are less labourious and in the long run much more economical especially when this programme is extended (Ragot and Hoisington, 1993).

Cultivated rubber plants were initially seedling progeny from clonal parents that were randomly crossed. Grafted clones of selected seedlings were later established (Whycherley, 1976). *Hevea* is still propagated today by grafting and breeding programs that are based on hand pollination. But clonal assessment of