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Development of Molecular Markers for Hevea

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The development of restriction fragment length polymorphisms (RFLP) techniques as molecular markers for Hevea *is described. Two* Hevea *libraries containing genomic DNA sequences (genomic library) and complementary DNA (cDNA) sequences (cDNA library) were generated. Using an anonymous DNA probe from the* Hevea *genomic library, polymorphisms between* Hevea *species and between* Hevea *clones were detected. The potential usefulness of RFLP techniques in* Hevea *breeding programmes is discussed.*

Restriction fragment length polymorphisms (RFLP) have been proposed as potentially useful molecular markers for genetic analysis in higher plants[1-5]. RFLP are differences in fragment lengths when genomic deoxyribonucleic acids (DNA) of genetically distinct individuals are digested with a particular restriction endonuclease (RE). These differences are the result of heritable changes in the DNA. Point mutations create or destroy restriction endonuclease sites, while DNA rearrangements, insertions or deletions alter their relative positions.

As genetic markers, RFLP promise to be numerous[6]. RFLP have several characteristics which are of particular ultility in genetic improvement programmes. These include co-dorminant expression, multiple allelic forms, developmental stability and the absence of pleiotropic effect on other characters. RFLP analysis is a potentially powerful tool for assaying genetic variability. It is also useful in the study of inheritance of quantitative characters. If RFLP analysis can be applied as successfully to *Hevea* as to tomato[4,7,8], barley[9], pea[10] and lettuce[11], it will then provide the *Hevea* plant breeder with an efficient and powerful early selection tool. The present screening of *Hevea* by conventional methods can only be performed at least two years after planting. Since RFLP analysis can be performed much earlier, the technique is of value in culling and in shortening the selection time for *Hevea*. Ultimately, RFLP will be developed as genetic markers for the construction of a total genetic map of the *Hevea* genome.

MATERIALS AND METHODS

Plant Material

Four *Hevea* genotypes which were cultivated in the RRIM Experiment Station at Sungei Buloh were used. These consisted of two *Hevea brasiliensis* clones, RRIM 600 *(Field 19C)* and RRIM 628 *(Field 14)* and two *Hevea* species, *H. pauciflora* (HHB 12/3, *Field 67C*) and *H. camargoana* (1/4, 1/5, *Field 45N*).

DNA Analysis

Genomic DNA from the above genotypes were isolated by the method described by Sharp *et al.*[12] Young *Hevea* leaves were pulverised after lyophilisation and DNA was extracted from them by digestion at 37°C for 1–2 h in extraction buffer [100 mM Tris.Cl pH 8.5, 100 mM NaCl, 50 mM EDTA, 2% sodium dodecylsulphate (SDS), 0.05 mg/ml proteinase K]. This was followed by extraction with phenol/chloroform and precipitation with isopropanol. The precipitate was dissolved in TE (10 mM Tris.Cl pH 8.0, 1 mM EDTA). After the removal of ribonucleic acid (RNA) contamination by digestion at 37°C for 30 min with boiled ribonuclease A (RNase A) at 1 μg/ml, the DNA was again precipitated with isopropanol and dissolved in TE.

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Nineteen restriction endonucleases (RE) were studied *(Table 1)*. Aliquots (5 μg) of DNA were digested for 16–20 h with the selected RE under the conditions recommended by the supplier (Bethesda Research Laboratories). Genomic digests with these enzymes were electrophoresed through large agarose (0.8% for six-base cutters and 1.2% for four-base cutters) slabs with TAE (0.04 *M* Tris-acetate, 0.001 *M* EDTA) as the electrophoresis buffer. These were then transferred to nylon membranes (Genescreen Plus, Dupont) by the alkaline Southern blotting technique of Reed and Mann[13].

TABLE 1. RESTRICTION ENDONUCLEASES USED FOR RFLP DETECTION

6-base cutters	4-base cutters
*Apa*I	*Alu*I
*Bam*HI	*Dde*I
*Bgl*II	*Hae*III
*Dra*I	*Hha*I
*Eco*RI	*Hinf*I
*Eco*RV	*Mbo*I
*Hind*III	*Msp*I
*Sal*I	*Rsa*I
*Sst*I	*Taq*I
*Pvu*II	

Hybridisation and Washing Conditions

The conditions for hybridisation and washing were similar to those described[12]. Following Southern blotting, nylon membranes were prehybridised at 65°C in hybridisation solution (0.6 *M* NaCl, 20 mM Pipes pH 6.8, 4 mM EDTA, 0.2% Ficoll 400, 0.2% polyvinyl-pyrollidone (PVP), 1% SDS, 0.5% sodium pyrophosphate containing 500 μg/ml auto-claved salmon sperm DNA). After 4–5 h of prehybridisation, the prehybridisation solution was replaced by a similar solution containing the ^{32}P-labelled probe. Hybridisation was then allowed to proceed for 16 h at 65°C. After hybridisation, the membranes were washed sequentially with 2XSSC (0.3 *M* NaCl/0.03 *M* sodium citrate, pH 6.8), 0.1% SDS, and 0.5XSSC, 0.1% SDS for 30 min each at 65°C. Autoradiography was carried out at −70°C by exposure of the membrane to KODAK XAR-5 film between two intensifying screens for four days.

Construction of Libraries

DNA fragments of 500–2000 basepairs (bp) obtained from PstI digestion of *H. brasiliensis* clone RRIM 600 genomic DNA were isolated. These were successfully cloned into pUC18 plasmids and transformed into *E. coli* strain MC1022 to generate a genomic library. Clones were screened by ampicillin resistance. Clones carrying recombinant plasmids were purified and the recombinant plasmids were isolated by the proteinase K mini-preparation plasmid isolation method of Pruitt[14].

Total messenger RNA (mRNA) was isolated from young *Hevea* leaves by the method of Baulcombe and Buffard[15]. Double-stranded complementary DNA (cDNA) was synthesised by the cDNA synthesis system from Amersham. The cDNA was subsequently cloned into the *Pst*I site of plasmid vector *p*UC18 with the help of dephosphorylated 8-mer linkers (New England Biolabs). Following transformation into *E. coli* strain MC1022, a small cDNA library was created. Recombinant plasmids from the cDNA library were isolated from each clone by the proteinase K mini-preparation plasmid isolation procedure of Pruitt[14]. After digestion with *Pst*I, the sizes of these cDNA were determined by electrophoresis on agarose with lambda DNA digested with *Hind*III, as molecular weight markers.

Hybridisation Probes

Several named heterologous probes like wheat chlorophyll a/b binding (cab) protein (from N. H. Chua, Rockefeller University, New York), wheat ribulose carboxylase small sub-unit (from Plant Breeding Institute, Cambridge) and rice nitrate reductase (from A. Kleinhofs, Washington State University, Pullman) were used as probes.

Besides the above-named heterologous probes, a genomic DNA (gDNA) and complementary DNA (cDNA) clone, isolated from the newly generated *Hevea* genomic and cDNA libraries respectively were also used. These were labelled with P^{32} by the method of Feinberg and Vogelstein[16] and used as an anonymous probe against genomic digests of *Hevea* with the same conditions of hybridisation and washings as that used for the named heterologous probes.

RESULTS AND DISCUSSION

Size of DNA Inserts

After electrophoresis on agarose and with the assistance of molecular size markers generated from a *Hind*III digest of lambda DNA, the size of cDNA inserts was estimated to be 300–2000 basepairs (bp) in length *(Figure 1)*. The size of the gDNA probe used in the above hybridisation was deduced similarly to be approximately 800 bp in length.

Named Probes

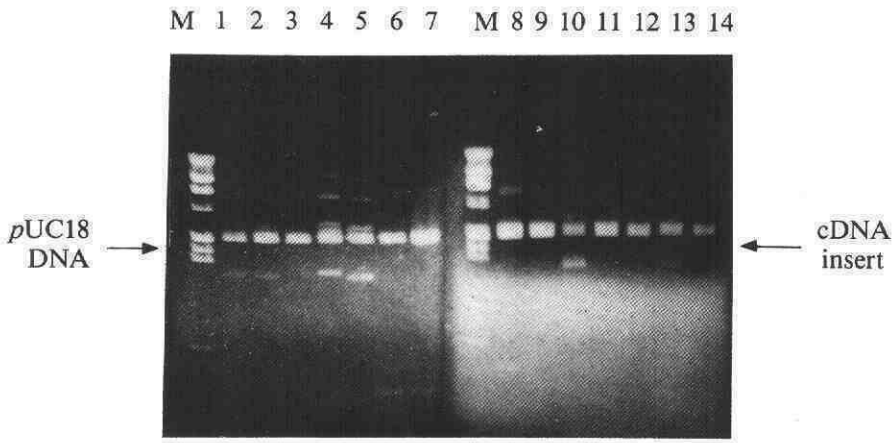
Hybridisations with the above-named cDNA probes *viz.* wheat cab protein, small sub-unit of wheat ribulose carboxylase and rice nitrate reductase, with genomic digests of *Hevea* DNA did not show any homology (results not shown). The lack of homology with these heterologous probes might be because all these probes were from monocotyledonous plants. A higher homology to *Hevea* genomic sequences might be obtained with cDNA probes from dicotyledonous plants.

Demonstration of Polymorphisms

When genomic digests of *Hevea* DNA were hybridised with an anonymous gDNA probe, polymorphisms within *H. brasiliensis* clones (RRIM 600 and RRIM 628) as well as between *Hevea* species were seen (*Figures 2* and *3*). DNA from barley (a monocotyledonous plant) which was included into one of the blots *(Figure 2)* as a control, was shown to have no homology with the gDNA clone of *Hevea* (this is not unexpected as the probes were derived from monocotyledonous plants).

Similar hybridisations with one of the cDNA clones (*p*CP 7) showed less distinct results *(Figure 4)*.

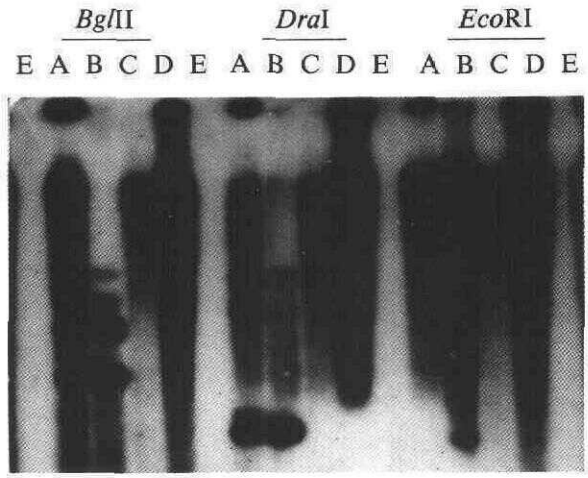
Screening of the rest of the genomic and cDNA libraries has just been completed and will be discussed elsewhere.



M = Molecular weight marker (λDNA/*Hind*III digest containing *p*UC18 DNA)

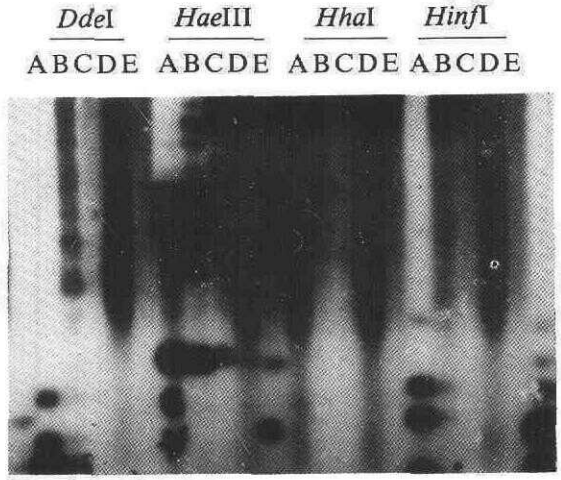
1–14 = *p*UC18 recombinants harbouring cDNA inserts

Figure 1. Size determination of cDNA clones.



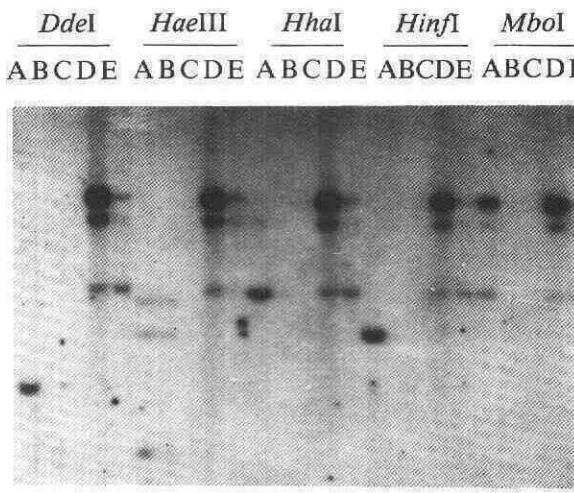
A = RRIM 600 B = RRIM 628
C = *H. camargoana* D = *H. pauciflora*
E = Barley (control)

Figure 2. Hybridisation of a Hevea *gDNA clone with* Hevea *genomic DNA digested with RE containing six-base recognition sites.*



A = RRIM 600 B = RRIM 628
C = *H. camargoana* (T4) D = *H. pauciflora*
E = *H. camargoana* (T5)

Figure 3. Hybridisation of a Hevea *gDNA clone with* Hevea *genomic DNA digested with RE containing four-base recognition sites.*



A = RRIM 600 B = RRIM 628
C = *H. camargoana* (T4) D = *H. pauciflora*
E = *H. camargoana* (T5)

Figure 4. Hybridisation of a Hevea *gDNA clone with* Hevea *genomic DNA digested with RE containing four-base recognition sites.*

Preliminary results suggested that polymorphisms could be detected between *Hevea* clones and possibly between *Hevea* species. These results are encouraging but need confirmation. If RFLP in *Hevea* are confirmed, then their usefulness as a selection tool for the *Hevea* breeder could be exploited.

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