CONSTRUCTION OF RECOMBINANT PLASMIDS HARBOURING SINGLE COPY GENOMIC DNA OF OIL PALM IN *Escherichia coli*

Keywords: RFLP; Recombinant plasmids; Oil palm

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Nuclear DNA was extracted from destarched leaves of oil palm Elaeis guineensis, *varieties* tenera, dura *and* pisifera *and from leaves of* E. oleifera. *The nuclear DNA samples were digested with Pst I restriction endonuclease. It was observed that there is some C-methylation in* tenera *and* E. oleifera *but not in* pisifera *and* dura. *The presence of C-methylation in* tenera *was of interest and fragments generated from this digestion were utilized in the construction of the library. Restricted fragments of 0.4 to 1.0kb were obtained by gradient ultracentrifugation in 10%-40% sucrose. These fragments were cloned into pUC19 plasmid vector and transformed into* E. coli *DH5α as host. Colony hybridization and dot blot hybridization were carried out on all the clones with labelled nuclear DNA. Seventy six percent of the 250 clones analyzed contained single or low copy sequences and 24% contained repetitive sequences.*

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is an important economic crop of Malaysia. For years, there has been a large effort in plant breeding programmes to improve economic traits such as high yield, disease resistance, high fruit-to-bunch ratio and others. Selection for these traits takes a long time. The application of molecular biology techniques is now thought to be important in shortening the time taken during selection programmes. Over the last few years, plant geneticists, breeders and molecular biologists have begun to develop restriction fragment length polymorphism (RFLP) maps for important crop plants, and such maps are available for at least seven plant species. These include crops with long histories of genetic studies and gene mapping, such as tomato and maize, and also crops for which no genetic

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maps previously existed, such as lettuce and potato (Landry *et al.*, 1987; Bonierbale *et al.*, 1988). For tomato, the linkage map has been correlated to quantitative trait loci (QTL). RFLP maps are important for plant breeding programmes: plants can be screened for a desired trait at an early stage, *e.g.* as seedlings (Tanksley *et al.*, 1987; Tanksley *et al.*, 1989).

The use of restriction fragment length polymorphisms as genetic markers was first proposed in the context of human genetics (Botstein *et al.*, 1980). The molecular basis of RFLP is the loss or gain of a restriction site because of a point mutation within the recognition sequences of enzymes, or a molecular event leading to insertion, deletion or inversion. Both situations result in a difference in the length of genomic restriction fragments, which is detectable on Southern blots.

In view of the fact that the oil palm is a crop of major economic importance, it is timely that work should commence on a long-term project to generate RFLP linkage maps in oil palm with the hope that eventually they may be used in oil palm breeding.

The construction of a linkage map using RFLPs involves three stages. The first is the development of sources of probes: there are two types of probes that can be used, cDNA clones and genomic single or low copy clones. The second stage is the screening of probes that detect polymorphism and the third, segregation analysis of RFLP.

This report documents the construction of a single copy or low copy gene library. It is a preliminary study towards the eventual goal of using some of the inserts as probes in the mapping of RFLP loci in oil palm.

EXPERIMENTAL

Materials

Leaves from the oil palm species *E. guineensis, tenera, dura* and *pisifera* and *E. oleifera* were destarched and harvested.

Extraction of Oil Palm Nuclear DNA

Oil palm nuclear DNA was extracted using a procedure modified from Timberlake (1978) and Fisher and Goldberg (1982). Forty grams of freeze dried oil palm leaves were blended in a stainless steel Waring blender in 250ml of ice-cold H buffer (4mM spermidine, 1mM spermine, 10mM EDTA, 10mM Tris, 80mM KCl, 0.1% phenol, 500mM sucrose, 0.2% v/v 2- mercaptoethanol, pH 9.5) at maximum speed. The homogenate was filtered through four layers of cheesecloth and one layer of Miracloth (Calbiochem) into a 250ml centrifuge bottle. After centrifuging at 2000g for 20 min at 4°C, the supernatant was discarded and the pellet resuspended into 100ml of ice cold HT buffer (H buffer with 0.5% Triton X-100). The suspension was again centrifuged at 2000g, for 20 min at 4°C, and the supernatant discarded. Resuspension of the pellet in HT buffer and centrifugation were repeated (4-5 times) until the pellet of nuclei was grey to white. It was then resuspended in 25ml HT buffer; 25ml lysis buffer (100mM Tris, 40mM EDTA, 2% sarkosyl, pH 9.5) was added followed by incubation at 60°C for one hour, with occasional inversions. After incubation, the lysate was centrifuged at 2500g, for 30 min at 4°C. The supernatant was transferred to a new 250ml tube. 25ml of 7.5M ammonium acetate and 100ml cold ethanol were added, and the mixture was kept overnight at - 70°C. The precipitate which formed was centrifuged at 6000g for 30 min at 4°C. The DNA pellet was washed in 80% ethanol and resuspended in TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0).

Preparation of DNA Fragments

Fifty micrograms of oil palm nuclear DNA were digested with *PstI* restriction endonuclease for eight hours at 37°C. Restricted fragments were size-fractionated in a 10%-40% sucrose gradient by ultracentrifugation at 25000 rpm (Beckman SW40) for 25 hours at 25°C. Fractions (0.5ml each) were collected into microcentrifuge tubes, 250µl 7.5M ammonium acetate, and 0.5ml isopropanol were added for precipitation overnight at -70°C. The precipitate was centrifuged at 15000g for 15 min at 4°C, washed with 80% ethanol, vacuum-dried and dissolved in 25µl TE. DNA solutions (5 µl) were electrophoresed with lambda DNA-*Hind* III and lambda DNA-*Kpn* I as molecular size markers.

Cloning of Nuclear DNA Fragments

pUC19 plasmid vector was digested with *PstI* and dephosphorylated with alkaline phosphatase.

Digested DNA fragments (0.4-1.0 kb) and vector were ligated according to Perbal (1984) and transformed into DH5α (BRL) *E. coli* host, following the method suggested by the supplier.

Screening of Recombinant Clones

Transformants were spread on agar plates containing ampicillin (100μg/ml), isopropyl-B-D-thiogalactosidase (1mM IPTG) and X-gal (50μg/ml in dimethyl formamide). Selection for recombinant clones were based on the colony colour. The recombinant bacteria were cultured in LB media overnight at 37°C. Plasmids were isolated following the method of Birnborm and Doly (1979). Isolated plasmid DNA was electrophoresed with a control (pUC19) on 1% agarose gel.

Colony Hybridization

Bacterial colonies were prepared on nitrocellulose filters according to Grunstein and Hogness (1975). Total *tenera* nuclear DNA was labelled with alpha [^{32}P]-dCTP (Amersham) according to the supplier's method and hybridized according to Maniatis *et al.* (1982). Nitrocellulose filters were washed according to Tanskley *et al.* (1988) and autoradiographed (Fuji-RX) at -70°C.

Dot Blot Hybridization

Plasmids isolated from colonies were resuspended in 80μl of 2M NaOH and 120μl ddH2O. The membrane was boiled in ddH2O for 30 seconds and transferred to a dish containing 200ml 1M ammonium acetate. Before loading each sample on to the dot blot apparatus, 200μl 2M ammonium acetate was added to each sample. Samples were loaded into the wells of the apparatus and air bubbles were avoided in the wells. Two internal controls were included, total DNA as a positive control, and plasmid pUC19 as negative control. The membrane was washed in 2X SSC (gently rubbing each side with gloved fingers) and rinsed in 2X SSC. Membranes were blotted dry with paper towels and baked *in vacuo* at 80°C for two hours. Total DNA was labelled with [^{32}P]-dCTP and hybridized to the membrane according to the method of Maniatis *et al.* (1982). Membranes were washed according to Tanskley *et al.* (1988) and autoradiographed (Fuji-RX) at -70°C.

RESULTS AND DISCUSSION

While cDNA clones have been used to identify polymorphisms in some crop plants such as maize, other crops were found to show polymorphisms using single or unique or low copy sequences (Helentjaris *et al.*, 1986; Gebhardt *et al.*, 1989). The use of cDNA probes allow analyses for the coding regions of the genome or the sequences immediately flanking such regions. However, single copy genomic clones are expected to detect more polymorphism because of the absence of selection pressures in the maintenance of the sequence of non-transcribed regions.

However at present there is no evidence to show the advantage of cDNA clones over genomic clones as probes, or *vice versa*. A cDNA library has been constructed for use as a source of probes (Cheah, personal communication). This communication is the first to report on the construction of single copy genomic library from oil palm for use as a source of probes for the detection of RFLP in oil palm.

Preparation of Nuclear DNA

Extraction of DNA from freeze-dried oil palm leaves yielded 20μg/gram. The method employed was successful in isolating nuclear DNA with little fragmentization.

DNA digestion and size fractionation of fragments

DNA samples from three *E. guineensis* varieties *tenera, dura* and *pisifera* as well as from *E. oleifera*, were digested with *Pst*I and *Hind*III. *Hind*III was used to confirm purity of samples by observation of complete digestibility of samples. *Pst*I was chosen as it is a methylation sensitive restriction enzyme which recognizes the sequence 5'-CTGCAG-3' and sensitive to cytosine methylation in the 5' location (Nelson and Mc Clelland, 1987). It has been observed in maize (Burr *et al.*, 1988) and tomato (Tanskley *et al.*, 1987) that undermethylated areas are enriched for single copy sequences. Since methylation occurs mostly in non-coding regions, clones derived from fragments generated from *Pst*I will represent largely single or low copy sequences that are located throughout the genome.

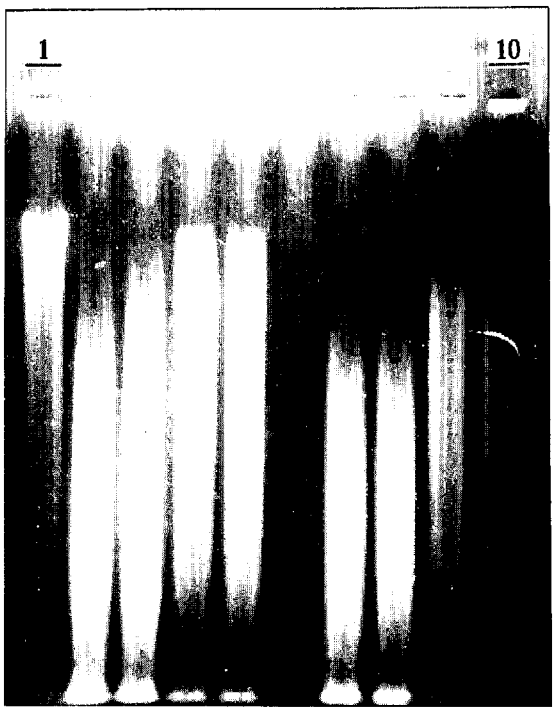

Figure 1. Nuclear DNA digested with PstI *and* Hind *III. Undigested DNA (lane 1).* dura, pisifera, oleifera *and* tenera *digested with* PstI *(lanes 2-5) and* Hind*III (lanes 7-10).*

Figure 2. 1% agarose gel electrophoresis of fractions after sucrose ultracentrifugation. Lanes 2- 16: fractions 2, 4, 8, 10, 11, 12 - 21. Lanes 1 and 17 had Lambda DNA digested with Hind *III and* Kpn *I respectively, as controls.*

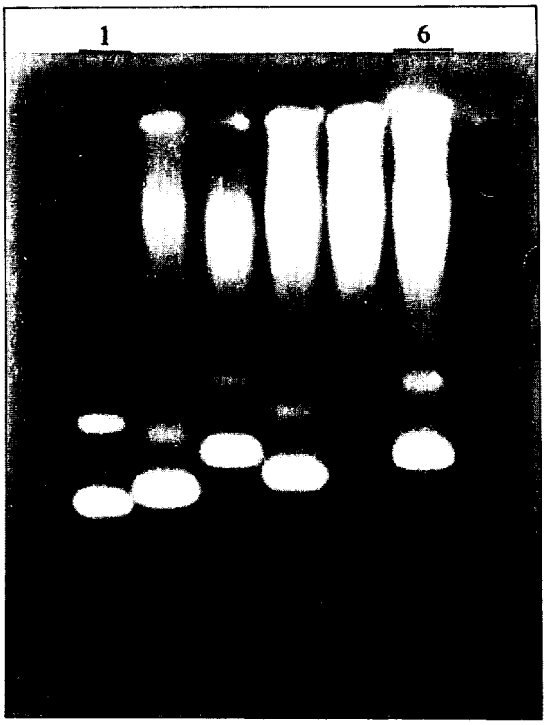

Figure 3. 1% agarose gel electrophoresis of recombinant plasmids. Lane 1: pUC 19 vector (as control). Lanes 2-6: had recombinant plasmids which migrated more slowly than the vector.

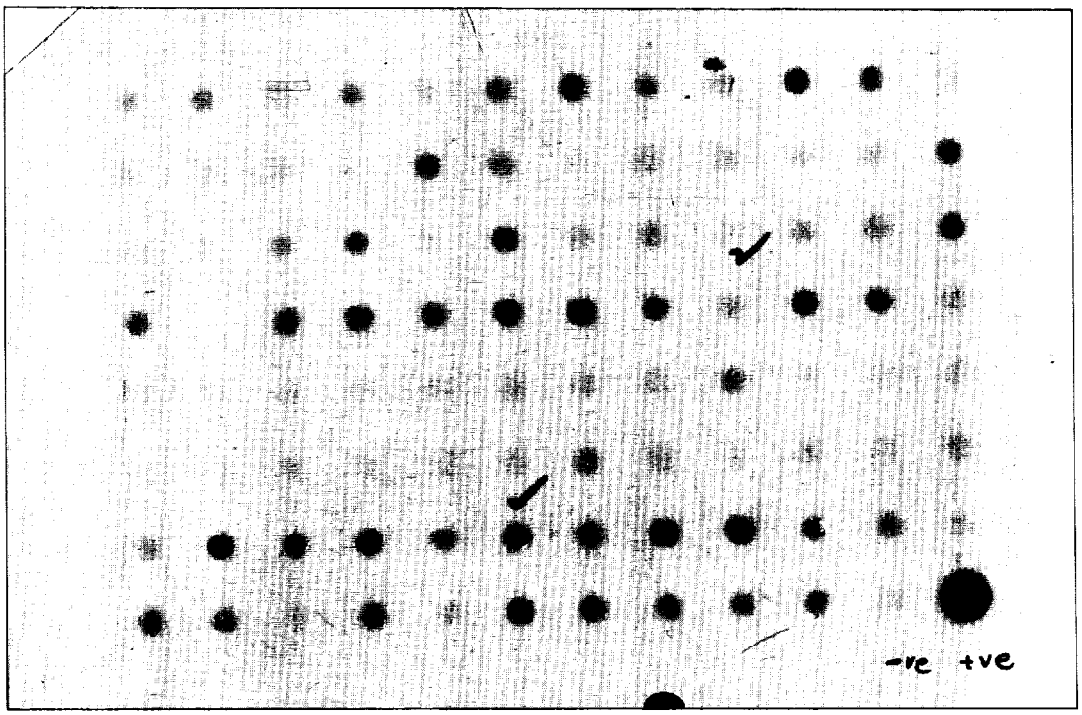

Figure 4. Autoradiography of dot blots with total nuclear DNA as probe. The dot marked '+ve' is a positive control (total nuclear DNA) and the marked '-ve' is a negative control (pUC 19).

It was observed that there is some degree of methylation in *tenera* and *E. oleifera*, but less in *pisifera* and *dura* (*Figure 1*). To ensure that there was complete digestion and that the incomplete digestion was not due to impurities, increasing amounts of *Pst*I was added to samples (data not shown). Also, to confirm these observations, the samples were also digested with *Hpa*II and *Msp*I enzymes (results to be published elsewhere) which showed differing degree of methylation. HPLC analyses of methylated cytosine of the samples showed that *tenera* has 44% methylated cytosine compared to 12% and 20% for *dura* and *pisifera* respectively (results to be published elsewhere). Therefore, *E. guineensis, tenera* displayed the highest degree of methylation and was chosen to isolate *Pst*I fragments for cloning procedures. The clones produced should have a higher percentage of single copy/low copy sequences. The *Pst*I fragments were separated by ultracentrifugation, and fractions (numbers 15-18), which represented fragment sizes of 0.4 to 1 kb, were pooled and used for cloning (*Figure 2*).

Cloning of 0.4kb-1kb nuclear DNA fragments

Nuclear DNA fragments (0.4 - 1kb fragments) were successfully cloned into pUC19 vector and transformed into DH5α host. The transformation efficiency was 4×10^3 CFU/μg. Recombinant colonies which appeared as white colonies (250 clones) were isolated. Plasmid DNA isolation of some recombinants showed presence of inserts, as shown by the slower migration in agarose gel compared to the vector alone (*Figure 3*).

Screening for single or low copy sequences

Selection of clones which contained single or low copy sequences is of importance to enable their use as probes in RFLP screening. Initially screening for single or low copy sequences was carried out with colony hybridization on 250 clones. High stringency washing (0.05 x SSC, 0.1% SDS) and differential exposure were carried out to confirm single or low copy genes. At such a stringency, there should be 80% homology to ensure stable hybridization (Beltz *et al.*, 1981). In these washing conditions, only multicopy genes remain; single or low copy sequences, which have low homology, become unstable. Therefore only clones representing repetitive sequences will give signals. Colony hybridization showed 96% single/low copy sequences. However since it has been shown that colony hybridization is not necessarily a reliable method for detection of single copy sequences, hybridization to dot blots was also carried out to confirm this result. Landry and Michelmore (1985) and Helentjaris *et al.* (1985) failed to select genomic clones which were suitable as probes using only colony hybridization.

Using dot blot hybridization on the same 250 clones it was observed that 76% were single or low copy sequences and 24% represented repetitive sequences (*Figure 4*).

This relatively high percentage of single or low copy clones is expected because of the cloning strategy. DNA from *tenera* oil palm which displayed a high degree of methylation, was digested with methylation sensitive *Pst*I. This would automatically exclude most repetitive sequences, which are usually methylated.

Studies on tomato which has high C-methylation, yielded 92% single/low copy clones when cloned with *Pst*I fragments (Tanskley *et al.*, 1987). When cloned with *Eco*RI (non-sensitive to C-methylation), only 48% of the library was found to contain single or low copy sequences. On the other hand, rice which has low methylation yielded 58% single or low copy clones with *Pst*I (Tanskley *et al.*, 1988). The *tenera-Pst*I clones in this study yielded 76% single/low copy clones. The data suggest that the *tenera* may have a level of methylation between that of tomato and rice. Work is at present under way to screen for probe/enzyme combinations that will detect RFLP in oil palm, and to detect the percentage of methylation.

The role of DNA methylation in plants is not well understood (Doefler, 1983), but there is evidence that it plays a role in gene regulation and may be an important factor to consider when analysing differences between and within species. The hypothesis is that the total genome size is positively correlated with the size of the repetitive regions of DNA (Flavell, 1989).

It is interesting to note that *tenera*, which is the hybrid of *dura* and *pisifera* showed a higher degree of methylation, although the significance of this is not apparent at present. It raises a question as to why *tenera* should have a higher degree of methy-

lation when compared with both its parental types. Also of interest is the higher methylation in *tenera* when compared with the other species (*E. oleifera*) of oil palm.

CONCLUSION

The *tenera* form of the oil palm *E. guineensis* and *E. oleifera* were observed to have a higher degree of DNA methylation than the *dura* dan *pisifera* form of *E. guineensis*. Nuclear DNA from *tenera* was digested with *Pst*I restriction enzyme. Fragments (0.4 - 1.0kb) were cloned in pUC19 vector and transformed in DH5α as host. Seventy six percent of the recombinant clones obtained were found to represent single or low copy sequences. Work is being carried out to obtain probe/enzyme combinations that are capable of detecting RFLP in oil palm.

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REFERENCES

BELTZ, G; JACOB, K; EICHBUCH, T; CHEERBAS, RR and KAFATOS, F (1981). Isolation of multigene families and determination of homologies by filter hybridization methods. *Methods Enzymol, 100,* 266-285.

BIRNBOIM, H C and DOLY, J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res., 7,* 1513-1523.

BONIERBALE, M W; PLAISTED, R L and TANKSLEY, S D (1988). RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. *Genetics, 120,* 1095-1103.

BOTSTEIN, O; WHITE, R L; SKOLNICK, M and DAVIS, R M (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet., 32,* 314-331.

BURR, B; BURR, R A; THOMPSON, K H; ALBERSTRON, M C and STUBER, C W (1988). Gene mapping with recombinant inbreds in maize. *Genetics, 118,* 519-526.

DOEFLER, W (1983). DNA methylation and gene activity. *Ann. Rev Biochem., 52,* 93-124.

FISHER, R L and GOLDBERG, R B (1982). Structure and flanking regions of soybean seed protein genes. *Cell, 29,* 651-660.

FLAVELL, R B (1989). Variation in structure and expression of ribosomal DNA loci in wheat. *Genome, 31,* 963-968.

GEBHARDT, C; RITTER, E; DEBENER, T; SCHACHTSCHABEL, U; WALKEMEIER, B; UHRIG, H and SALAMINI, F (1989). RFLP analysis and linkage mapping in *Solanum tuberosum. Theor. Appl. Genet., 78,* 65-75.

GRUNSTEIN, M and HOGNESS, D S (1975). Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci.*, USA, *72,* 3961-3965.

HELENTJARIS, T; KING, G; SLOCUM, M; SIDENSTRANG, C and WEGMAN, S (1985). Restriction fragment length polymorphisms as probe for plant diversity and their development as tools for applied plant breeding. *Pl. Mol. Biol., 5,* 109-119.

LANDRY, B S and MICHELMORE, R W (1985). Selection of probes for Restriction Fragment Length analysis from plant genomic clones. *Plt. Mole. Biol. Reporter, 3,* 174-179.

LANDRY, B S; KESSELI, R; HEI LEUNG and MICHELMORE, R W (1988). Comparison of restriction endonucleases and sources of probes for their efficiency in detecting restriction fragment length polymorphisms in lettuce (*Lactuca sativa* L.). *Theor. Appl. Genet., 74,* 646-653.

LANDRY, B S; RIVARD, S R; CAPPADOCIA, M and VINCENT, G (1989). Restriction fragment length polymorphism (RFLP) analyses of plant produced by *in vitro* anther culture of *Solanum charconse*. Bitt. *Theor. Appl. Genet., 78,* 49-56.

MANIATIS, T; FRITSCH, E F and SAMBROOK, J (1982). Molecular Cloning - A Laboratory Manual. Cold Spring Harbor Laboratory, U.S.A.

NELSON, M and McCLELLAND, M (1987). The effect of site specific methylation on restriction-modification enzymes. *Nucleic Acid Res., 15 Suppl.* 219-130.

PERBAL, B (1984). *A Practical Guide To Molecular Cloning.* John Wiley & Sons, New York.

TANKSLEY, S D; MILLER, J; PETERSON, A and BERNATZKY, R (1987). Molecular mapping of plant chromosomes. In: Gustafson J P, and Appels, R A (eds). *Chromosome structure and function* Plenum Press, New York.

TANKSLEY, S D; McCOUCH, S R; KOCHERT, G; YU, Z H; WANG, Z Y; KHUSH, G S S and COFFMAN, W R (1988). Molecular mapping of rice chromosomes. *Theor. Appl. Genet., 76,* 815-829.

TANKSLEY, S; YOUNG, N D; PATERSON, A H and BONIERBALE, M W (1989). RFLP mapping in plant breeding: New tools for an old science *Biotechnology ,7,* 257-264.

TIMBERLAKE, W E (1978). Low repetitive DNA content in *Aspergillus nidulans. Science, 202,* 973-975.