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M. Matthes · R. Singh · S.-C. Cheah · A. Karp

Variation in oil palm (*Elaeis guineensis* Jacq.) tissue culture-derived regenerants revealed by AFLPs with methylation-sensitive enzymes

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Abstract Tissue culture-derived plants of oil palm (*Elaeis guineensis* Jacq.) can develop abnormal flowers in which stamen primordia are converted into carpel-like tissues (mantled fruit). This abnormality can be heritable; individual palms may show variation in mantling and reversion to the normal phenotype over time has been observed. Four sets of ortets (mother plant used as tissue source) and ramets (regenerated plants) were compared using standard amplified fragment length polymorphism (AFLP) analysis and AFLPs using methylation-sensitive enzymes. No polymorphisms were found when standard AFLPs were produced with ten different primer combinations. In contrast, when methylation-sensitive AFLPs were used, polymorphisms were detectable. Polymorphisms appeared as new bands in the ramets, suggesting that a reduction in methylation had occurred during tissue culture. The highest number of polymorphic bands (0.3%) was obtained when *Hpa*II was used as the restriction endonuclease, indicating that the loss of methylation had occurred most frequently at the internal C within the *Hpa*II recognition sequence 5'-CCGG-3'. Conversion of nine of the polymorphic bands into probes for Southern analysis confirmed that these were not due to partial digestion of the AFLP templates and showed that the majority were single-copy sequences. The exceptions were fragments showing homology to 25S ribosomal RNA genes and the chalcone synthase gene family. Examination of the Southern blots suggested that most of the single-copy sequences were partially de-methylated, and one example was found in which de-methylation affected only one allele. No polymorphism was consistently different between normal and abnormal clones in all the sets. This suggests that, whilst this method is an effective way of detecting variation in tissue culture-derived plants, different approaches will be required to identify the causal basis of the mantled fruit abnormality.

Keywords Oil palm · Tissue culture · Regeneration · Somaclonal variation · Methylation · AFLP

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M. Matthes · A. Karp (✉)
ACR-Long Ashton Research Station,
Department of Agricultural Sciences,
University of Bristol, Long Ashton, Bristol, BS 41 9AF, UK
e-mail: angela.karp@bbrc.ac.uk
Fax: +44 1275 394007

R. Singh · S.-C. Cheah
Palm Oil Research Institute of Malaysia, 6, Persiaran Institusi,
Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia

Introduction

Somaclonal variation is the general term used to describe ''variation displayed among plants derived from tissue cultures'' (Larkin and Scowcroft 1981). Over the past two decades, this phenomenon has been extensively investigated using cytological, biochemical and molecular techniques, and the basis and uses of the variation have been discussed in numerous reviews (e.g. Karp and Bright 1985; Peshke and Phillips 1992; Karp 1995). The factors that have been shown to influence somaclonal variation include:departure from organized meristematic growth, the genetic constitution (genotype, ploidy) of the source material, the choice and concentration of growth regulators in the medium and the tissue source (Karp 1991). Investigation of these factors has provided a useful experimental framework, but attempts to eliminate somaclonal variation are made difficult by the numerous influencing variables involved and the complex way they interact (Karp 1989, 1995).

The scaling-up of clonal plant production of oil palm (*Elaeis guineensis* Jacq.) is currently hindered by the occurrence of somaclonal variants (approx. 5%) among populations of tissue culture-derived plants (Jaligot et al. 2000). In particular, some of the clones derived from tissue culture develop abnormal flowers in which a feminization of the male parts of the flower has occurred (Corley et al. 1986). In male inflorescences no pollen is formed, whereas in female inflorescences a ring of supplementary carpels surrounds the gynoecium, thereby inhibiting the ripening of the fruit (mantled fruit). The

severity of the ''mantled'' fruit phenotype can show heterogeneity between different clonal lines, between palms of the same clonal line and even between different flowers of the same individual and can also differ in the extent of reversion.

Investigations into the basis of somaclonal variation in oil palm, particularly into the causes of abnormal flower production, have not, as yet, led to any conclusive results. Regeneration of oil palm plantlets is achieved through somatic embryogenesis, which can be initiated from two types of calli:primary nodular calli and fast-growing friable calli that can be derived from the nodular callus type. Earlier reports suggested that palms with abnormal flowers derive from only the fast-growing friable calli (Marmey et al. 1991), but this has since been questioned by more recent data (Jones et al. 1995). Investigations of cytokinin content indicated that higher levels are found in nodular callus and zygotic embryos than in friable calli (Besse et al. 1992). However, this may be a reflection of the open structure of the friable callus compared with the small densely cytoplasmic cells of nodular calli and zygotic embryos (Jones et al. 1995). Analysis of two cell lines of a single oil palm genotype, which produced clones with only normal flowers or only abnormal flowers, respectively, revealed that cytokinin levels were significantly lower in embryoids and shoots (or roots) of the ramets produced by the line with abnormal flowers. Furthermore, the abnormal inflorescences had higher concentrations of biologically active zeatin riboside and dihydrozeatin-9-glucoside and less zeatin than the normal inflorescence at a comparable stage of development. In all other cases, however, differences in cytokinin levels between clones exceeded those found between abnormal and normal flowers (Jones et al. 1995).

Previous analyses of normal and abnormal clones using biochemical and cytogenetic markers have also not helped to reveal any simple basis to the production of abnormal clones (Shah and Ahmed-Parveez 1995). DNA profiling techniques, such as randomly amplifed polymorphic DNAs (RAPDs), whilst effective in detecting polymorphisms between different lines, were not able to distinguish clones differing in floral morphology (Shah et al. 1994; Rival et al. 1998). The observation of the variable expression of the mantled phenotype suggests that the underlying cause responsible might be epigenetic. Shah and Ahmed-Parveez (1995) have shown that levels of 5-methylcytosine, measured by high pressure liquid chromatography, were significantly higher in regenerants with abnormalities than in normal regenerated clones. There are also many reports indicating that hypomethylation occurs at high frequency in tissue culture (Kaeppler 1992). In most studies, however, the identity of the affected DNA is unknown, making direct correlations between methylation and specific phenotypic changes difficult. Where changes in the methylation of specific DNA sequences have been monitored, they are often highly repetitive non-coding DNA (Smulders et al. 1995; Bogani et al. 1995), again making correlations with phenotypic changes of an associative nature rather than a causal one.

In the study reported here, we investigated variation in the methylation status of genomic DNA of tissue culture-derived oil palm regenerants. Leaves were used as source material for two reasons:(1) inflorescences are difficult to obtain as they reside deep inside the heart of the palm, and their dissection invariably results in death of the adult palm; (2) an ideal marker would be one that could be used to assay leaves of young palms. We applied the amplified fragment length polymorphism (AFLP) method using restriction enzymes displaying sensitivity to cytosine methylation. This approach, previously used by Xiong et al. (1999), has the advantage that any polymorphisms detected can be cloned and identified.

Materials and methods

Tissue culture-derived material

The oil palm material used in the study was supplied by the Palm Oil Research Institute of Malaysia (PORIM). This comprised four sets of ortets (mother plant used as the source of tissue for regeneration) and ramets (tissue culture-derived clonal lines) as shown in Table 1. All four sets consisted of both normal and abnormal ramets, whilst in two of the sets revertant ramets were also provided. A normal ramet is classified as having 100% normal flowers and fruits. The appearance of one abnormal flower is sufficient for a ramet to be classified as being abnormal. In the case of the material supplied for this study, the ramets were all classified as being severely mantled, such that almost all fruits in the bunch were mantled and all the bunches were abortive. A revertant is classified as a palm that has previously been scored as abnormal but has now reverted to bearing 100% normal flowers and fruits.

DNA extraction

DNA for AFLP analysis was extracted from young leaves of adult oil palms using a modified form of the method of Doyle and Doyle (1990) in which 2% (w/v) polyvinylpyrolidone (PVP-40) (Sigma) was added to the original extraction buffer [2% (w/v) CTAB, 1.4 *M* NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 m*M* EDTA and 100 m*M* Tris-HCl (pH 8.0)].

Table 1 Oil palm clones used for AFLP and Southern blot analysis

	DNA sample no.	Type
Set 1	A-1185/12.53	Ortet
	A-567/23.43	Normal ramet
	A-566/23.43	Abnormal ramet
Set 2	B-528/12.53	Ortet
	B-680/23.43	Normal ramet
	B-686/23.43	Abnormal ramet
Set 3	CDJ60-FC1292	Ortet
	C1302	Normal ramet
	C1303	Abnormal ramet
	C166/3/64	Reverted ramet
Set 4	CDI48-FC1298	Ortet
	D1304	Normal ramet
	D1305	Abnormal ramet
	D298/1/74	Reverted ramet

Table 2 Sequences (5'-3') of adapters and primers used for AFLP analysis

Name	Enzyme	Type	Sequence (5'-3')[a]
EcoA+	*Eco*RI	Adapter(+)	*bio*-CTCGTAGACTGCGTACC
EcoA–	*Eco*RI	Adapter(–)	AATTGGTACGCAGTC
PstA+	*Pst*I	Adapter(+)	*bio*-CTCGTAGACTGCGTACATGCA
PstA–	*Pst*I	Adapter(–)	TGTACGCAGTCTAC
MseA+	*Mse*I	Adapter(+)	GACGATGAGTCCTGAG
MseIa–	*Mse*I	Adapter(–)	TACTCAGGACTCAT
Msp/HpaA+	*Msp*I/*Hpa*II	Adapter(+)	GACGATGAGTCCTGAT
Msp/HpaA–	*Msp*I/*Hpa*II	Adapter(–)	CGATCAGGACTCAT
E47	*Eco*RI	Primer +3	CTGCGTACCAATTC*caa*
E35	*Eco*RI	Primer +3	CTGCGTACCAATTC*aca*
E32	*Eco*RI	Primer +3	CTGCGTACCAATTC*aac*
E49	*Eco*RI	Primer +3	CTGCGTACCAATTC*cag*
E39	*Eco*RI	Primer +3	CTGCGTACCAATTC*aga*
E40	*Eco*RI	Primer +3	CTGCGTACCAATTC*agc*
E43	*Eco*RI	Primer +3	CTGCGTACCAATTC*ata*
P1	*Pst*I	Primer +2	GACTGCGTACATGCAG*ac*
P2	*Pst*I	Primer +2	GACTGCGTACATGCAG*aa*
P3	*Pst*I	Primer +2	GACTGCGTACATGCAG*ca*
P4	*Pst*I	Primer +2	GACTGCGTACATGCAG*cc*
MseI1	*Mse*I	Primer +3	GATGAGTCCTGAGTAA*gaa*
MseI2	*Mse*I	Primer +3	GATGAGTCCTGAGTAA*aca*
MseI3	*Mse*I	Primer +3	GATGAGTCCTGAGTAA*acc*
MseI4	*Mse*I	Primer +3	GATGAGTCCTGAGTAA*ccg*
MseI5	*Mse*I	Primer +3	GATGAGTCCTGAGTAA*ggc*
MseI6	*Mse*I	Primer +3	GATGAGTCCTGAGTAA*ttg*
Msp/Hpa1	*Msp*I+*Hpa*II	Primer +3	GATGAGTCCTGATCGG*gaa*
Msp/Hpa2	*Msp*I+*Hpa*II	Primer +3	GATGAGTCCTGATCGG*aca*
Msp/Hpa4	*Msp*I+*Hpa*II	Primer +3	GATGAGTCCTGATCGG*ccg*
Msp/Hpa7	*Msp*I+*Hpa*II	Primer +3	GATGAGTCCTGATCGG*cgc*
Msp/Hpa8	*Msp*I+*Hpa*II	Primer +3	GATGAGTCCTGATCGG*tcg*
Msp/Hpa10	*Msp*I+*Hpa*II	Primer +3	GATGAGTCCTGATCGG*gag*

[a] Selective nucleotides are in lowercase italics

AFLP analysis

The primer and adapter sequences were synthesised and desalted by Genosys Biotechnologies. Unless otherwise stated, all enzymes and reagents were obtained from Pharmacia. AFLP analysis was performed essentially as described in the original method by Zabeau and Vos (1993). For selective polymerase chain reaction (PCR), ten primer combinations were used for each enzyme combination (see Table 2).

Elution and re-amplification of AFLP fragments of interest

The autoradiogram was aligned with the gel using Glogos Autorad Markers (Stratagene), and bands were cut from the gel using a sharp scalpel. The pieces of acrylamide were placed into 40 µl of polyacrylamide gel extraction buffer (0.5 *M* NH_4OAc, 10 m*M* MgOAc, 1 m*M* EDTA and 0.1% SDS) and left overnight at 37°C. The tubes were centrifuged at 13,000 rpm for 5 min and the supernatant transferred into a fresh tube. The pellet was re-suspended in 40 µl of extraction buffer, centrifuged as above and the supernatant pooled with the supernatant obtained after the first centrifugation. The pooled supernatants were purified over a glasswool column by centrifugation at 1,200 rpm for 5 min. To precipitate the DNA, we added 80 µl of 100% ethanol to the tube, left it for 20 min on ice and centrifuged it at 13,000 rpm for 20 min. The pellet was washed once with 70% ethanol, dried in a Speedivac and re-suspended in 10 µl of $TE_{1.0}$ (10 m*M* TRIS-HCl, 1.0 m*M* EDTA, pH 8). For re-amplification 1 µl of the purified supernatant was used with *Pfu* DNA polymerase (Stratagene) as described by the manufacturer.

Cloning and sequencing

The PCR products of the re-amplified AFLP fragments were purified through 0.8% low-melting-point agarose (NuSieve GTG, FMC BioProducts) gels and isolated following the phenol extraction protocol according to Sambrook et al. (1989). Aliquots of the purified fragments were then cloned into the modified pBluescript II SK(+) plasmid provided with the pCR-Script SK(+) Cloning System (Stratagene) and transformed into XL1-blue supercompetent cells (Stratagene). Plasmid DNA sequencing of cloned AFLP fragments with either (–)20 or reverse primer was carried out using the Sequenase kit from Amersham Life Sciences. Homology search was performed on the NCBI BLAST server at both the nucleotide and amino acid levels.

Preparation of DNA probes

DNA probes were produced by PCR amplification (Saiki et al. 1988) from plasmids containing the cloned AFLP fragments. Inserts were amplified using 25-bp that were designed to include the universal and reverse sequencing priming sites. PCR products were purified through 0.8% low-melting-point agarose (NuSieve GTG, FMC BioProducts) gels, and the excised DNA was labelled with γ-[^{32}P] dCTP (Amersham) via random priming (Feinberg and Vogelstein 1983).

DNA restriction and Southern blot analysis

Oil palm DNA was digested using 5 U of enzyme/µg according to the manufacturer's instructions (New England Biolabs or Pharma-

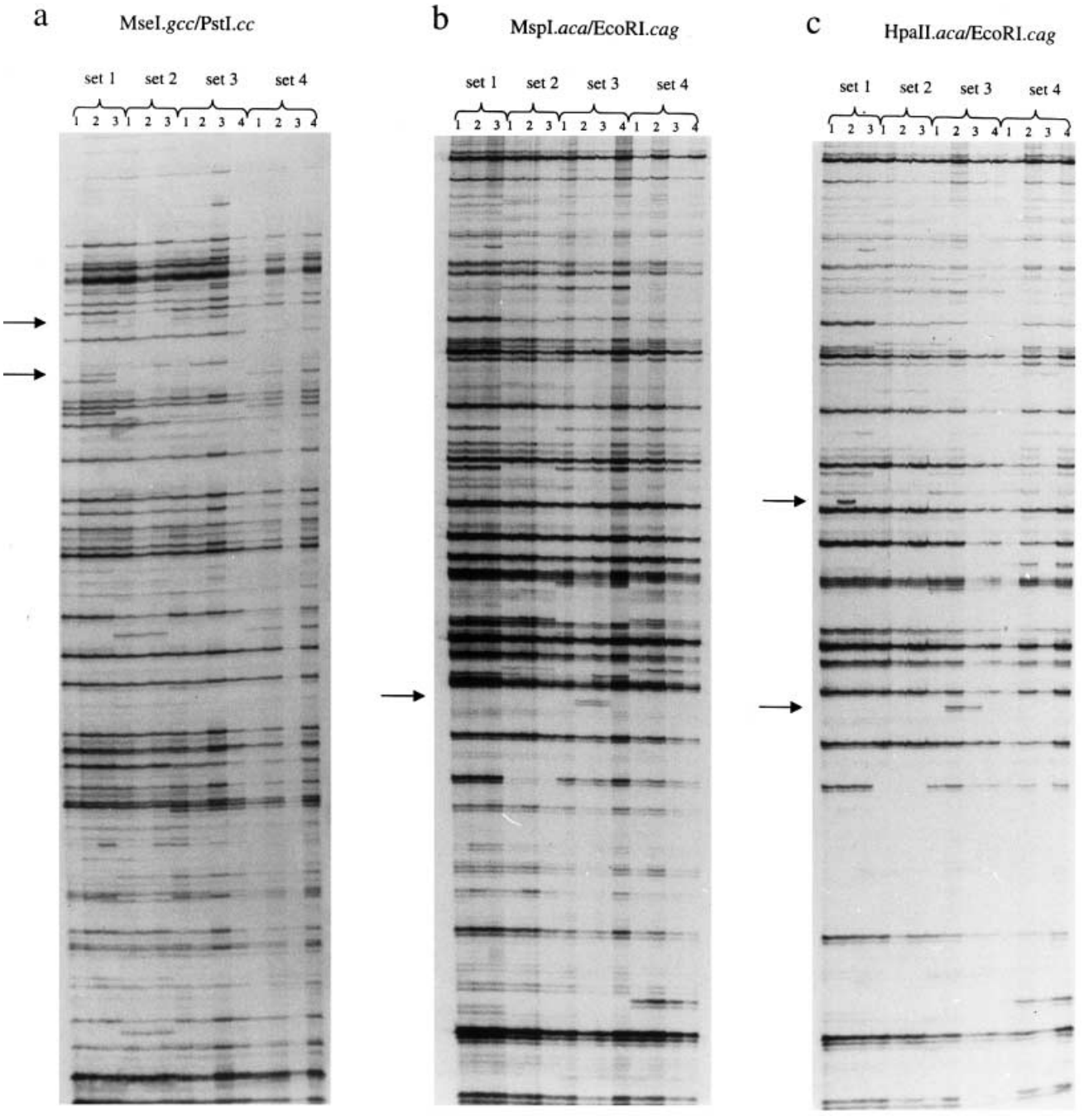

Fig. 1 AFLP profiles of the four different oil palm sets (see Table 1) using three different enzyme combinations: **a** *Mse*I and *Pst*I, **b** *Msp*I and *Eco*RI, **c** *Hpa*II and *Eco*RI. The selective nucleotides used for amplification are indicated in *italics*. Polymorphisms are marked by *arrows* and are found: **a** in the normal and abnormal ramets of set 1 (A-567/23.43 and A-566/23.43), **b** in the normal and abnormal ramets of set 3 (C1302 and C1303), **c** in the normal ramet of set 1 (A-567/23.43) and in the normal and abnormal ramets of set 3 (C1302 and C1303). Note that in all sets the ortet is *track 1*, the normal ramet *track 2*, the abnormal ramet *track 3*; in sets 3 and 4 *track 4* represents the revertet ramet

cia). Eight milligrams of DNA per lane was loaded onto 0.8% TBE-agarose (Gibco BRL) slab gels, and electrophoresis was performed overnight at 3 V/cm. The DNA was blotted onto Hybond-NX membrane (Amersham) and hybridizations were carried out overnight using standard conditions (Sambrook et al. 1989). Unbound probe was removed by two 30-min washes in 2 × SSC, 0.1% SDS at 60°C. Filters were exposed to Kodak Biomax MR-2 film with two intensifying screens at –80°C.

Results

Analysis of four oil palm ortet/ramet sets using methylation-insensitive enzymes *Eco*RI and *Mse*I for the template preparation did not show any polymorphisms between the ortets and their tissue culture-derived regene-

Table 3 Summary of polymorphism obtained using the different primer combinations. AFLP templates generated with four different enzyme combinations were amplified with ten different primer pairs, and the number of polymorphic bands was compared. Note that in the case of the templates prepared with *Msp*I or *Hpa*II and *Eco*RI, primer pairs with the same selective nucleotides were used for comparison

Enzyme combination	Mean number of bands per enzyme combination and per sample	Total no. of bands[a]	Total no. of polymorphic bands[b]	Percentage of polymorphism
*Eco*RI/*Mse*I	95	13,300	0	0%
*Pst*I/*Mse*I	82	11,480	5	0.04%
*Msp*I/*Eco*RI	73	10,220	5	0.04%
*Hpa*II/*Eco*RI	64	8,960	28	0.3%

[a] The total number of bands was calculated by taking into consideration that, in total, 14 samples (see the four sets) were analysed with ten different primer combinations

[b] The same polymorphic bands present in 2 or more regenerants were considered as 2 or more polymorphisms in the calculation of the percentage of polymorphism

Table 4 Nine polymorphic AFLP fragments obtained with methylation-sensitive enzymes were investigated for their copy number on Southern blots of *Eco*RI-digested genomic DNA. A search for homologies was performed on the NCBI BLAST server

Isolated fragment	Derived from	Homology to	Number of bands in Southern blot
*Mse***ggc**/*Pst***ccA**	A-567/23.43 A-566/23.43	Not found	3
*Mse***ggc**/*Pst***ccB**	A-567/23.43 A-566/23.43	Not found	3
*Msp/Hpa***aca**/*Eco***aac**	C1302 C1303	Chalcone synthase	6
*Msp/Hpa***aca**/*Eco***aac**	A-567/23.43	Not found	1
*Msp/Hpa***aca**/*Eco***aca**	C166/3/64	Not found	1
*Msp/Hpa***gaa**/*Eco***caa**	A-567/23.43 A-566/23.43	Not found	1
*Msp/Hpa***cgc**/*Eco***agc**	Found in all ramets	25S ribosomal DNA	Many
*Msp/Hpa***cgc**/*Eco***caa**	B-670/23.43 B-680/23.43	Not found	2
*Msp/Hpa***cgc**/*Eco***caa**	A-566/23.43	Zn-finger	3

rants (ramets). A total of ten different primer combinations were used for amplification, each sample giving a mean number of 95 bands per primer combination. A modification of the AFLP procedure was then introduced by substituting the standard enzymes with enzymes that are methylation-sensitive. Two substitutions were introduced. Firstly, the 6-bp cutter, *Eco*RI, was replaced with the methylation-sensitive enzyme *Pst*I and, secondly, the 4-bp cutter, *Mse*I, was replaced with the methylation-sensitive isoschizomers *Msp*I or *Hpa*II. The methylation-sensitive enzymes *Msp*I and *Hpa*II cleave the same sequence (5'-CCGG-3') but differ in their sensitivity to cytosine methylation (Butkus et al. 1987; McClelland et al. 1994). *Msp*I cleaves if the internal C is methylated but is unable to cut if the external C is methylated; *Hpa*II does not cut if the inner C is methylated.

Substitution of *Eco*RI with *Pst*I in the digest and amplification of the template with ten different primer combinations resulted in AFLP profiles with an average number of 82 bands per primer combination and per sample. Primer pair *Mse*I.*ggc*/*Pst*I.*cc* showed 2 polymorphic bands, both of which were present in samples A-567/23.43 and A-66/23.43, the normal and abnormal ramets, respectively, of set 1 (Fig. 1a). An additional polymorphic band was detected by primer pair *Mse*I.*ggc*/*Pst*I.*aa* for D1305, the abnormal ramet of set 4 (not shown). The percentage of polymorphism found was 0.04%.

Amplification of the templates, prepared with *Eco*RI and either *Msp*I or *Hpa*II as restriction enzymes, with ten primer pairs containing the same selective nucleotides revealed a low level of polymorphism (0.04%) between ortet and ramets for templates generated with *Msp*I/*Eco*RI. For each sample an average number of 73 bands per primer pair was amplified and in total 5 polymorphic bands could be identified. As an example, see Fig. 1b showing a polymorphism for ramets C1302 and C1303 (set 3) detected with the primer pair *Msp*I.*aca*/*Eco*RI.*cag*.

An increased number of polymorphic bands was observed when the *Hpa*II/*Eco*RI-generated template was used for amplification (Fig. 1c). Per primer pair, an average of 62 bands was amplified per sample, and in total 28 fragments could be identified which were polymorphic between the mother oil palm plants and the respec-

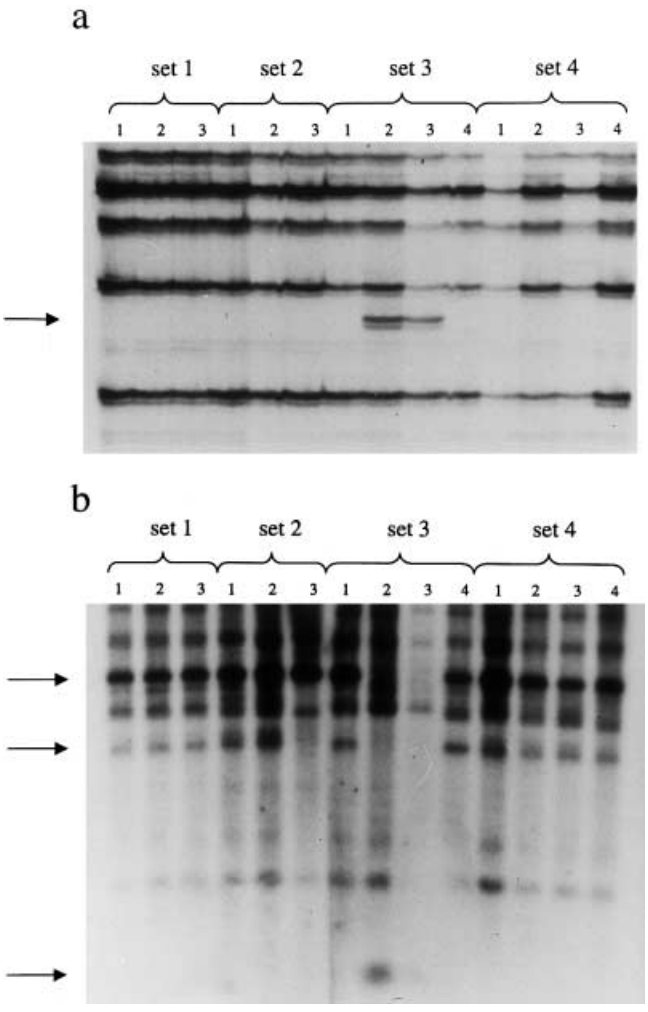

Fig. 2 a Portion of the AFLP profile obtained after amplification with *Hpa*II.*aca* and *Eco*RI.*cag*. The *arrow* indicates the polymorphic band present in the normal and abnormal ramet of set 3 (C1302 and C1303), which was isolated, cloned and sequenced. BLAST analysis revealed a high homology of this fragment to chalcone synthase. **b** Southern blot of *Hpa*II-digested genomic DNA of the four oil palm sets. Polymorphic bands are indicated with an *arrow*

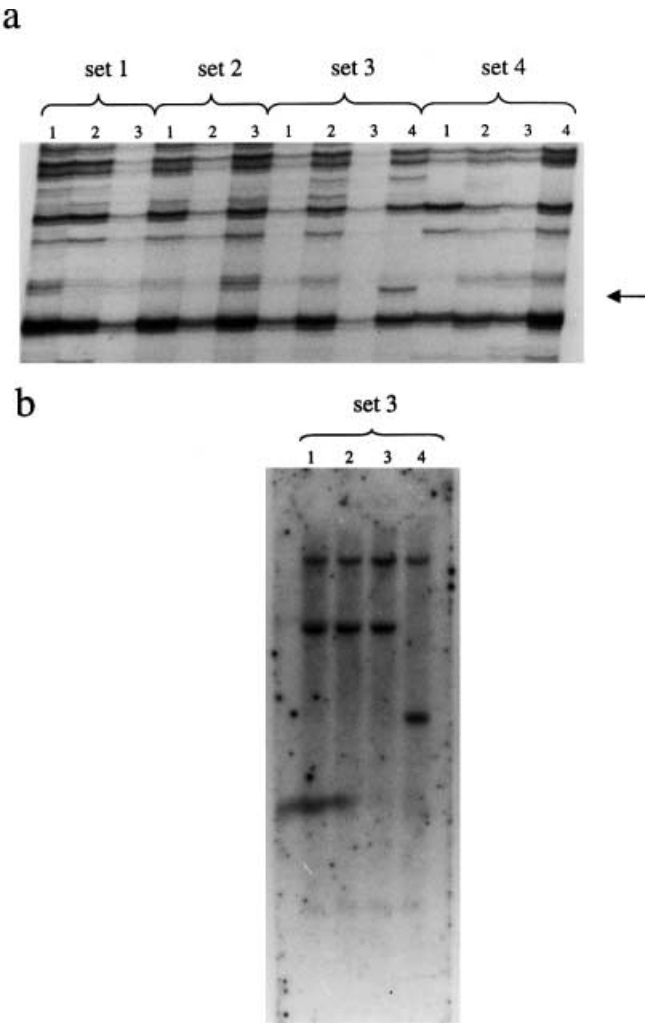

Fig. 3 a Portion of an AFLP profile obtained with the primer combination *Hpa*II.*aca*/EcoRI.*aca*. The polymorphic band used for further analysis was found in the revertet ramet of set 3 (C166/3/64) and is indicated by the *arrow*. **b** Southern blot of genomic DNA of oil palm set 3 digested with *Hind*III and *Hpa*II. This enzyme combination makes the distinction of the two alleles possible and shows that in the reverted ramet of set 3 loss of methylation has affected one allele only. Digestion with *Hind*III alone revealed no polymorphism (not shown)

tive tissue culture-derived regenerants (% of polymorphism:0.3%). None of these polymorphic bands was found to be common to all the ramets. Even ramets belonging to the same set displayed different polymorphic bands. Out of the 28 fragments, only 2 were in common with the *Msp*I polymorphisms. The results obtained with all the different primer combinations are summarized in Table 3.

To exclude the possibility that the detected polymorphisms were due to partial digestion of the DNA during AFLP template preparation, we isolated the first 9 polymorphic fragments and used these as probes in Southern blots (Table 4).

In order to confirm the polymorphisms obtained with the primer combination *Mse*I.***gcc***/*Pst*I.***cc***, the genomic DNA of the four sets under investigation was digested with *Pst*I and *Mse*I for Southern blot analysis. A polymorphism was detected between the ortet (A-185/12.53) and the normal and abnormal ramets (A-67/23.43 and A-66/23.43) of set 1 (data not shown) using fragment *Mse***ggc**/*Pst***cc*A** as a probe, whereas no variation was present in the other three sets. No polymorphism could be identified with DNA digested with *Mse*I alone (data not shown).

The polymorphisms revealed by the two fragments (*Msp/Hpa***aca**/*Eco***aac** and *Msp/Hpa***cgc**/*Eco***caa**) showing homology to multiple-copy sequences (chalcone synthase and 25S ribosomal RNA respectively) were confirmed using *Hpa*II-digested genomic DNA for the Southern blot analysis. In each case, additional smaller

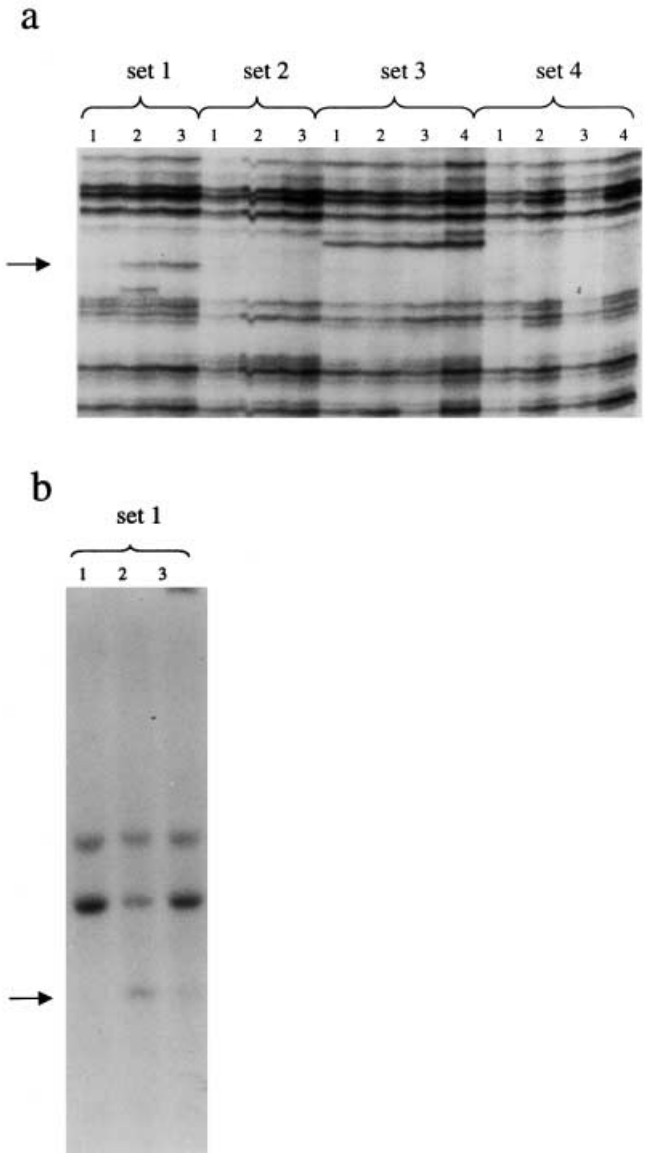

Fig. 4 a Portion of the AFLP profile obtained with the primer combinations *Hpa*II.gaa and *Eco*RI.caa. The normal and abnormal ramets of set1 (A-567/23.43 and A-566/23.43) show an additional band (fragment *Hpa***gaa**/*Eco***caa**), indicated by the *arrow*, which was used for further analysis. **b** Fragment *Hpa***gaa**/*Eco***caa** was used as a probe in a Southern blot of genomic DNA of set 1 digested with *Hind*III and *Hpa*II. Both alleles can be distinguished by this primer combination, and an additional band is revealed for the normal and abnormal ramets (*arrow*). This suggests that the methylation status of one allele is not uniform among the cells. Digestion with *Hind*III alone revealed no polymorphism (not shown)

bands could be identified in the same samples that originally had shown the AFLP polymorphism (Fig. 2).

The polymorphism of the single/low-copy sequences could not be confirmed by digests of the genomic DNA with *Hpa*II alone. Due to extensive methylation of the genomic DNA many fragments generated by digestion with *Hpa*II are still part of the high-molecular-weight fraction. In order to reduce the fragment size double digests were performed, choosing the second enzyme so that in several cases it became possible to distinguish the two different alleles of the single-copy sequences. Two different patterns were found. In one case, complete loss of methylation was revealed for one allele, whereas the other remained unaffected (Fig. 3). In the other case, only a partial loss of methylation of one allele could be detected, whereas again, the other allele remained unaffected (Fig. 4).

Discussion

The standard AFLP technique (using *Eco*RI and *Mse*I for the preparation of the AFLP template) was used for the analysis of four ortet and ramet sets of oil palm, comprising mother (tissue source) plants and regenerated clones that produced either normal or abnormal flowers. Ten different primer combinations were tested, displaying an average of 95 bands per sample, but no polymorphisms could be detected. This agrees with a previous study using RAPDs as a profiling technique. Rival et al. (1998) could not detect polymorphisms between normal and abnormal palms, although a total of 8,900 markers were scored. These results suggest that major genomic rearrangments do not occur at a high frequency among tissue culture-derived oil palm regenerated plants and that standard AFLPs and RAPDs are not the appropriate techniques for detecting differences between ortets and normal or abnormal ramets showing the ''mantled'' fruit phenotype.

Subsequent to the development of the AFLP procedure by Vos et al. (1995), numerous modifications of the technique have been described (e.g. Ellis et al. 1998; Witsenboer et al. 1997). The modification adopted here was the substitution of the standard methylation-insensitive enzymes (*Eco*RI and *Mse*I) with methylation-sensitive enzymes (*Pst*I and *Mse*I, *Msp*I and *Eco*RI, *Hpa*II and *Eco*RI). This approach was used by Xiong et al. (1999) to investigate the methylation status of hybrid rice plants in comparison with their parental lines, where it was given the name of methylation-sensitive amplified polymorphism (MSAP). The rationale for using MSAP to study oil palm regenerants was:(1) previous studies had shown that methylation differences exist between normal and abnormal tissue culture-derived clones (Shah and Ahmed-Parveez 1995; Jaligot et al. 2000); (2) the heterogeneity of expression of the phenotypic abnormality and its reversible nature strongly suggested an underlying epigenetic mechanism; (3) although the nature of the phenotypic abnormality pointed to changes in the expression of floral determining genes, the location of these genes in the oil palm genome was unknown. MSAP would permit a wide sampling of the genome whilst targeting the detection of polymorphisms specifically to those resulting from changes in methylation status. Furthermore, any polymorphic fragments could be excised from the gel, cloned, sequenced and characterized.

MSAP proved to be an effective method for detecting polymorphisms in tissue culture-derived oil palm regenerants. Although only few polymorphisms were identified

when AFLP templates prepared with *Pst*I/*Mse*I or *Msp*I/*Eco*RI were amplified with ten different primer combinations (5 polymorphic bands were obtained in total for each enzyme combination), a higher frequency of polymorphic fragments was obtained using the template generated with *Hpa*II/*Eco*RI (28 in total) (Table 3). In all cases, the polymorphisms appeared in the ramets as additional bands in the AFLP profile. Together these findings suggest that methylation is lost most frequently at the internal C within the recognition sequence 5'-CCGG-3'. It might be argued that a polymorphism which appears as the loss of a band in the ramet compared with the ortet would be infrequent because this occurrence would necessitate heterozygosity in the ortet for the locus concerned. However, a common feature of somaclonal variation compared to classical mutation is the high frequency with which changes affecting both alleles occur (Karp 1991), and homozygous methylation changes in regenerated plants have been reported previously (Kaeppler and Phillips 1993a).

Our findings of hypomethylation of DNA sequences in oil palm regenerants do not agree with the results of Shah and Ahmed-Parveez (1995), although the methods of detecting methylation changes used by these authors (high pressure liquid chromatography) were quite different. Our observation of loss of methylation during oil palm tissue culture is in agreement, however, with Jaligot et al. (2000), who described a trend towards hypomethylation in abnormal plant material, although our results also show that there is a tendency for hypomethylation in normal regenerants. Decreases in methylation status during tissue culture have also been reported previously for progeny of regenerated A188 maize plants (Kaeppler and Philipps 1993a,b) or for suspension cultures of soybean (Quemada et al. 1987). Of related interest is that *Arabidopsis thaliana* plants transformed with an antisense construct of a methyltransferase cDNA and showing a decrease in cytosine methylation exhibit a variety of aberrant floral homeotic phenotypes (Finnegan et al. 1996) as does the *ddm1* DNA hypomethylation mutant of *A. thaliana* (Kakutani 1997). However, tissue culture clearly does not always result in decreased methylation since Smulders et al. (1995) detected increased methylation in tomato callus compared with leaves. Similarly, tissue culture-derived regenerants of the pea (*Pisum sativum* L.) cv. Dolce Provenza showed evidence of hypermethylation, whilst regeneration from an experimental line of pea (5075) was not associated with any obvious difference in methylation levels (Cecchini et al. 1992).

Conversion of the polymorphic bands into probes for Southern analysis confirmed that the observed polymorphisms were not due to partial digestion of the AFLP templates and also enabled copy number determination of the polymorphic sequences. The majority of the polymorphisms were found in single or low-copy sequences, with the exceptions of the highly repetitive 25 S ribosomal RNA genes and the intermediate repetitive chalcone synthase genes. Changes in the methylation pattern of repetitive sequences among tissue culture-derived plants is a well-known phenomenon and rDNA genes, in particular, have frequently been reported to be affected (eg. Quemada et al. 1987; Anderson et al. 1990; Vyskot et al. 1993; Koukalova et al. 1994).

For most of the single or low-copy sequences, Southern blots revealed only a partial de-methylation at the corresponding loci, whereas one example was found in which one allele was affected by a loss of methylation whilst the other allele remained unmodified. These findings could be explained if cells within the tissue are not epigenetically uniform, such that intercellular variation of different methylation states of the same sequence exists. There is evidence that regenerated plants can arise from a multicellular origin. If methylation differences were present in the original cultured cells, and these were stably propagated, mosaicism would result (Karp 1991). Conversely, it could be that instability occurs in the methylation patterns during regeneration, which could be more consistent with the occurrence of reversion in some of the abnormal palms. It is not possible from our results to distinguish these two possibilities, both of which are equally plausible. In preliminary studies of different leaf portions of ortet leaves using three different primer combinations with methylation-sensitive enzymes we did not observe any differences in the AFLP profiles produced, suggesting that mosaicism is not obvious in the ortet. Mosaicism has been observed in somaclonal variants, however, being particularly evident in variegated regenerants (Karp 1991). Epigenetic mosaicism involving methylation differences has also previously been reported by Matzke and Matzke (1996) in leaves of tobacco plants transformed with a transgene encoding hygromycin resistance.

None of the polymorphisms identified was consistently different between normal or abnormal clones in all sets. This suggests that the tissue culture process induces changes in methylation in oil palm regenerants at many sites within the genome, although the possibility that certain sequences are more predisposed than others to alterations of this kind cannot be excluded.

The use of AFLPs with methylation-sensitive enzymes has made possible the identification of some tissue culture-induced polymorphisms in oil palm regenerants that were not detected by other methods. Although the approach appears sensitive enough to detect even slight changes in the methylation status, such as a partial loss of methylation of a specific sequence, no single polymorphism could be identified which was consistently different between normal and abnormal clones within all four sets of ortets and ramets. This suggests that different approaches will be required to identify the causal basis of the mantled fruit abnormality.

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