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Methylation-sensitive RFLPs: characterisation of two oil palm markers showing somaclonal variation-associated polymorphism

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Abstract The occurrence of "mantled" somaclonal variants (approx. 5%), which display alterations in floral organ structure, among populations of somatic embryo-derived oil palms (*Elaeis guineensis* Jacq.) currently hampers any scaling-up of clonal plant micropropagation. As a first step towards the identification of abnormality-discriminating markers, we have screened a set of 27 oil palm cDNA probes for methylation-sensitive restriction fragment length polymorphisms (RFLPs) using callus genomic DNA digested with the isoschizomeric enzymes *Msp*I and *Hpa*II. Only two probes (CPHO62 and -63) were found to differentiate reproducibly in two different genotypic backgrounds between nodular compact calli (NCC) and fast-growing calli (FGC), which generate 5% and 100% "mantled" plantlets, respectively. Comparative analyses were then conducted on DNA from inflorescences and leaves of normal and abnormal adult regenerants. With both probes, the observed methylation patterns were strongly clone-dependent and monomorphic with respect to the phenotype of the regenerants, except for the type-specific banding pattern obtained with the CPHO62 probe on material from the LMC3 clonal offspring. The results presented here mirror the higher difference in genomic DNA methylation observed between normal and abnormal embryogenic calli when compared to more differentiated plant material. Moreover, they reinforce the paramount interest of NCC and FGC callus lines as a material of choice in the search for early epigenetic markers of the "mantled" somaclonal variation. The potential use of methylation-sensitive RFLPs for the early detection of somaclonal variation at early stages of the micropropagation process is discussed.

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Introduction

Evidence has been accumulating over the past few years that DNA methylation at the genome level plays a key role in regulating plant development (Finnegan et al. 2000). Changes in DNA methylation on deoxycytosine (dC) residues have been shown to be involved in the transcriptional regulation of gene expression. Naturally occurring as well as induced differentiation/dedifferentiation processes and various environmental stresses are likely to initiate perturbations in the level and distribution of DNA methylation (Demeulemeester et al. 1999; LoSchiavo et al. 1989; Oakeley et al. 1997). These same conditions favouring epigenetic instability occur during micropropagation processes and often result in disruptions of clonal fidelity in the regenerant offspring. Since a wide range of variant phenotypes is observed, multiple genetic and epigenetic causal mechanisms are likely to be involved (Kaeppler et al. 2000).

In oil palm (*Elaeis guineensis* Jacq.), an average of 5% of the somatic embryo-derived adult palms exhibit an aberrant floral structure, with male parts being transformed into carpel-like structures in flowers of both sexes. Following fertilisation, the pseudocarpels develop into fleshy structures surrounding the true-fruit, hence the name "*mantled*" for this phenotype (Rival 2000). Flower sterility increases with the increasing severity of this developmental abnormality, with a resulting decrease in fruit set and, consequently, oil yields. Embryogenic fast-growing Calli (FGC) are a source of abnormal regenerants, as nearly 100% of their clonal offspring exhibit the "*mantled*" phenotype, compared to 5% of nodular compact calli (NCC)-derived plantlets (Rival 2000).

Neither flow cytometric nor random amplified polymorphic DNA (RAPD) analyses have detected any major defects in genomic structure in variant plant material

(Rival et al. 1997, 1998a, b). These results, taken together with the characteristics of the "mantled" phenotype (spatial and temporal heterogeneity, reversion to normal phenotype and non-Mendelian segregation), have led to the hypothesis of an epigenetic origin for this somaclonal variant phenotype.

We first evaluated DNA methylation rate using two complementary quantification methods directed to the whole genome. High-performance liquid chromatography (HPLC) estimation of genomic 5-methyl-deoxycytosine (5mdC) amounts demonstrated the occurrence of a significant hypomethylation in FGC (–4.5%; $P<10^{-5}$) and leaves from "mantled" regenerants (–1.2%; $P<10^{-5}$), compared with their normal counterparts. This result was confirmed by the *Sss*I-methylase-accepting assay, since after treatment with a CG-methyltransferase, DNA from abnormal plant material incorporated 60% more tritiated methyl groups (Jaligot et al. 2000). A striking epigenetic phenomenon, involving the modification of the base content of the whole genome, was thus found to be at work in the variant plant material. However, because of appreciable inter-individual and inter-clonal variability, this exploratory study did not allow us to define a single "methylation threshold" as a tool for the detection of variants. Moreover, despite the importance of DNA hypomethylation, it is likely that very few of the affected cytosines do play a direct biological role in either the onset or the maintenance of the somaclonal variation. As a matter of fact, a number of developmental aberrations have been hypothesised to result from global DNA hypomethylation induced by genetic or epigenetic defects in single sequences – in plants as well as in animals (Baylin and Herman 2000; Finnegan et al. 1996).

Therefore, we found it necessary to target more precisely sequences which, when disturbed in their methylation status, could account for the "mantled" phenotype or at least exhibit a tight association with somaclonal variation events so that they could be used in a prognosis purpose. To this end, we have carried out methylation-sensitive restriction fragment length polymorphism (RFLP) studies involving the use of the isoschizomeric enzymes *Msp*I and *Hpa*II, since this method has been widely used for monitoring the epigenetic stability of plant tissue cultures (Kaeppler and Phillips 1993; Kingham et al. 1998; Kovarik et al. 1994; Smulders et al. 1995; Vergara et al. 1990; Vyskot et al. 1995).

Materials and methods

Plant material

Plant material used for this work was sampled at CNRA research station in LaMé (Ivory Coast) from hybrid *pisifera* palms (Deli × LaMé) obtained through in vitro somatic embryogenesis (Pannetier et al. 1981). The genetic origin of the clonal lines studied is given in Table 1.

Extraction of genomic DNA

DNA was isolated from oil palm leaves of F_{+1} order as described by Rival et al. (1998a). It was extracted from embryogenic calli and from immature inflorescences according to Dolezel et al. (1989).

Table 1 Genetic origins of the plant material analysed

Clonal lines	Parental cross[a]	Sampled material
LMC3	L16T×D3D	Immature inflorescences Leaves
LMC51	L2T×D8D	Immature inflorescences Leaves
LMC52	D3D×L2T	Immature inflorescences
LMC63	L2T×L269D	Leaves
LMC209	D115D×L2T	Immature inflorescences
LMC458	(D5D×D5D)×(L5T×L5T)	Calli (NCC+FGC)
LMC464	(D5D×D5D)×(L5T×L5T)	Calli (NCC+FGC)

[a] Each cross is written as female parent × male parent

Isoschizomeric RFLP-Southern blot analyses

Southern analysis were performed according to Hoisington (1992), using 50U of either *Msp*I or *Hpa*II (Roche Molecular Biochemicals, Indianapolis, Ind.) on 10 µg of oil palm genomic DNA. These enzymes share the same restriction site (CCGG) but differ in their sensitivity to cytosine methylation. Both are unable to cut $^{m}C^{m}CGG$, but while cutting by *Hpa*II is inhibited whichever cytosine is methylated, *Msp*I activity is blocked by methylation only at the outer cytosine (Fulnecek et al. 1998; Jeddeloh and Richards 1996).

Digestion products were separated overnight on a 0.8% agarose gel in TBE buffer, at 25 V. DNA was first depurinated in 0.25 *M* HCl for 20 min, and then submitted to denaturation in 0.4 *M* NaOH for 15 min, before being transfered onto a Hybond N+ membrane (Amersham, Amersham, UK) and cross-linked by incubation for 2 h at 80 °C. Completion of the digestion, migration and transfer steps were systematically checked on the ethidium bromide-stained gel under UV light with a camera-print monitor system (Appligene Oncor).

Probe selection and synthesis

Both nodular callus and immature inflorescence oil palm cDNA libraries were constructed using the Lambda ZAPII kit (Stratagene, La Jolla, Calif.). A representative portion of each library was converted into phagemid form (pBluescript) in order to obtain individual cDNA clones directionally inserted between the *Eco*RI and *Xho*I restriction sites. The probes used in our study were selected at random for their ability to give clear hybridisation patterns with oil palm genomic DNA digests.

Labeling with α-[^{32}P]dCTP was performed by the random priming method (Feinberg and Vogelstein 1983) on 20 ng of oil palm cDNA using the Megaprime kit (Amersham) following the supplier's instructions. Membranes were incubated for 5 h at 65 °C in pre-hybridisation buffer: 50 m*M* Tris, 10 m*M* EDTA, 5 × SSC, 1 × Denhardt, 0.2% SDS, 0.1 mg/ml herring sperm DNA. The solution was supplemented with 20% dextran sulfate upon addition of the radiolabeled probe, and hybridisation was carried out overnight at 65 °C. The filters were washed twice at low stringency with room-temperature 2 × SSC, 0.1% SDS, and followed by two high-stringency washes at 65 °C in 0.5 × SSC, 0.1% SDS. The hybridisation signal was detected by autoradiography using Kodak Biomax MS film after 24–72 h of exposure at –80 °C (depending on the strength of the signal).

Results

Methylation-dependent markers of embryogenic callus lines

This preliminary analysis was undertaken on DNA extracts from NCC and FGC originating from two different

Table 2 Summary of methylation-sensitive restriction polymorphisms detected in two embryogenic callus lines using homologous cDNA probes

Number of polymorphic probes 22 (81.5%)[a]	
Probes displaying only enzyme-dependent polymorphism 16 (59%)	Probes displaying type-dependent polymorphism 6 (22%)

[a] Numbers in parenthesis represent the proportion of that class of probes as a percentage of the total number of probes tested

genotypes. A total of 27 cDNAs from oil palm libraries were used as probes.

Only 18.5% of the probes tested produced a monomorphic, methylation-independent CCGG pattern on the whole set of DNA samples and with both enzymes. Nevertheless, the majority (22 out of 27) allowed the detection of a restriction polymorphism that was dependent on the methylation sensitivity of the enzyme used (Table 2). The differences in banding patterns between the *Hpa*II and *Msp*I digests of the same DNA sample indicate that methylation of the inner C of the common restriction site occurs frequently in these two callus genotypes. The occurrence of the C^{m}CGG sites revealed by these probes is mostly independent of the callus phenotype, as 73% of the probes did not differentiate between FGC and NCC for the CCGG methylation status irrespective of which enzyme was used. Among the remaining six type-dependent polymorphic probes, four displayed a type-differential banding pattern in both clonal lines with at least one enzyme. Two of these patterns did not prove to be reproducible, whereas the other two, CPHO62 and CPHO63, were found to be polymorphic respectively for *Hpa*II and *Msp*I digestions or for *Msp*I digestion only.

Complete nucleotide sequences were obtained for the CPHO62 and CPHO63 cDNAs. Database searches using the BLASTX program (Altschul et al. 1997) were performed in order to identify related sequences. Both cDNAs were found to contain full-length coding regions. The putative product of the CPHO62 cDNA (989 bp) was found to be a protein elongation factor of the 1B class (66% identity with the corresponding proteins from *Arabidopsis thaliana* and rice; Hericourt and Jupin 1999). More specifically, the open reading frame (ORF) detected between positions 70 and 612 (corresponding to a polypeptide of 180 amino acids) is predicted to encode the GDP-GTP nucleotide-exchange subunit of the translation elongation complex. The 1,096-bp CPHO63 sequence was found to contain an ORF between positions 375 and 887. Its deduced polypeptide sequence of 170 amino acids shows strong similarities to zinc-finger domain proteins, notably LIM metal-binding protein family members found in *Nicotiana tabacum* (86% identity; Eliasson et al. 2000).

With both probes, the polymorphism between NCC and FGC involved a banding shift towards lower molecular weights in FGC calli for the same restriction enzyme. In contrast, the pattern was similar from one genotype to another for a given callus type and with the same enzyme. When using the CPHO62 probe (Fig. 1a), we observed this type-specific band shift in both digestion products, indicating that methylation at both cytosine residues (^{m}C^{m}CGG) is reduced in FGC compared to NCC with respect to the hybridising fragments observed. With the CPHO63 probe (Fig. 1b), the differences in methylation patterns were only observed between *Msp*I digestions, thus reflecting a decrease in methylation which is limited to the external cytosine (^{m}CCGG).

Overall, these results are in agreement with our previous results which showed differences in genomic methylation rates between NCC and FGC embryogenic calli (Jaligot et al. 2000). The observed band shifts as well as the global hypersensitivity of FGC DNA to restriction by both *Hpa*II and *Msp*I enzymes (Fig. 1c) indicate that hypomethylation occurs on at least one of the two cytosines from the CCGG motif in the abnormal callus type.

Detection of RFLP polymorphisms by probes CPHO62 and CPHO63 in adult clonal palms

Blotting of DNA from immature inflorescences

Inflorescences are the only organs in regenerant plants that are affected by the "*mantled*" phenotype. It is interesting to note, however, that they display an intermediate genomic hypomethylation (–2% of normal levels; our unpublished data) relative to calli and leaves (Jaligot et al. 2000). Moreover, the existence in several clones of a gradation in the severity of the variant phenotype (classified separately from one to three for flowers of each sex with increasing severity of the floral phenotype; see legend of Fig. 2) gives us the opportunity to investigate a possible correlation with the restriction polymorphisms revealed with CPHO62 and CPHO63.

Differences in the *Msp*I and *Hpa*II hybridisation patterns of CPHO62 reveal the high degree of differential methylation of C^{m}CGG sites in these organs (Fig. 2a). *Hpa*II generates fewer products, which are of a much higher molecular weight, than *Msp*I. The hybridisation pattern of abnormal inflorescences for the LMC3 clone shows the progressive appearance of one low-molecular-weight supplementary band that is barely visible in 2–2 (slightly "*mantled*") regenerants but clearly distinguishable in those with the 2–3 (severely "*mantled*") phenotype.

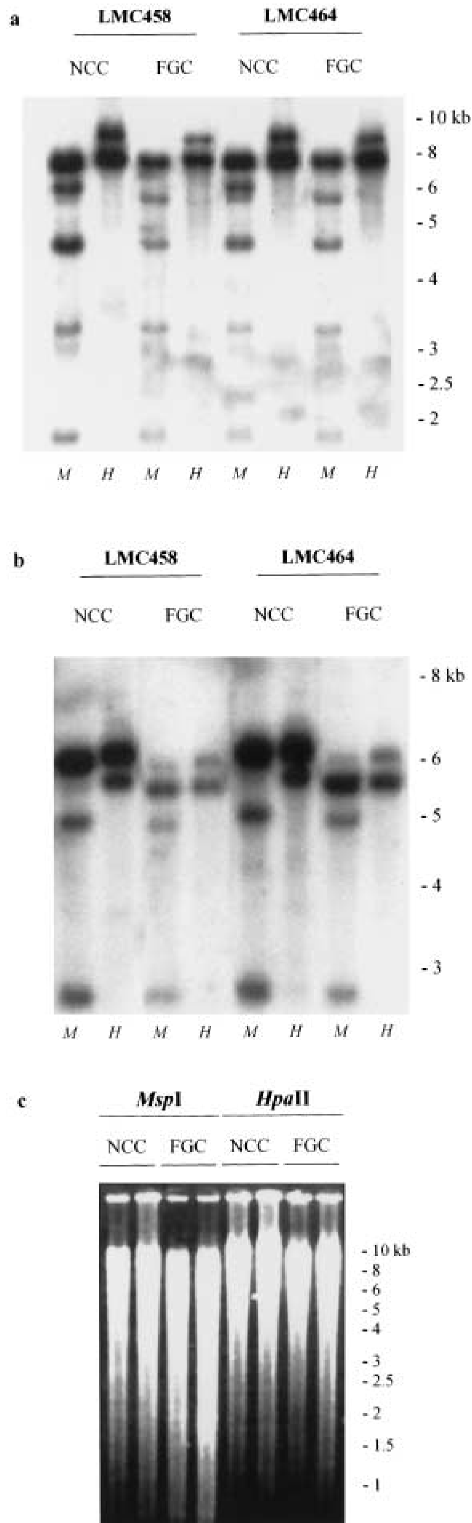

Fig. 1 Southern blot hydridisation of probes CPHO62 (**a**) and CPHO63 (**b**) on embryogenic calli DNA (NCC and FGC types) from two different clonal lines, digested with *Msp*I (*M*) or *Hpa*II (*H*). **c** *Msp*I and *Hpa*II restriction products visualised by ethidium bromide staining before membrane transfer. The *two lanes* represented for each type of callus correspond to the LMC458 and LMC464 clonal lines (from *left* to *right*)

None of the other three clones was found to display a polymorphic pattern correlated with the abnormal phenotype. A slight shift towards lower molecular weights in the *Hpa*II banding pattern might nevertheless be present between normal and reverting abnormal inflorescences.

The CPHO63 probe (Fig. 2b) detected enzyme-specific differences in methylation pattern, but only for some of the clonal progenies studied: we observed the disappearance of a 4.3-kb *Msp*I band in clones LMC51 and LMC3 and, in LMC3, the concomittant appearance of a 5.2-kb band in the *Hpa*II lane. Clones LMC52 and LMC209 exhibited a single band with a methylation-independent pattern, indicative of the occurrence of a demethylated CCGG site near or within the genomic sequence to which the probe hybridises.

Blotting of leaf DNA

In an attempt to further evaluate the discriminating power of the CPHO62 and CPHO63 probes on adult regenerant palms known to bear the “*mantled*” phenotype, we assayed these markers on restricted leaf genomic DNA from four normal/abnormal clonal pairs from different lines.

The banding pattern displayed by CPHO62 (Fig. 3a) involved broadly in all four clones the substitution of the six *Msp*I bands by one high-molecular-weight signal and a few faint, low-molecular-weight bands in the *Hpa*II digestion products. This observation confirmed the frequent methylation of the inner cytosine in CCGG sites. No type-specific polymorphism was seen, except for the LMC3 clone, where a 5-kb digestion product, which was absent in samples from true-to-type regenerants, appeared in both *Msp*I and *Hpa*II restrictions of leaf DNA from “mantled” regenerants.

In contrast, CPHO63 (Fig. 3b), produced no marked differences in banding pattern between either different phenotypes or with different restriction enzymes. Compared to the other clonal lines examined, the LMC3 clone showed one extra hybridising band of approximately 2 kb in the *Msp*I lane for both types of regenerants. The alteration of a ^{m}C^{m}CGG site to a C^{m}CGG site, within or near the genomic region hybridising to the probe, has therefore occurred in this genotype.

With both CPHO62 and CPHO63, the hybridisation patterns detected for leaves were mostly identical between different clonal progenies and monomorphic between types with respect to methylation at CCGG sites. We thus decided to further examine the differential banding pattern generated by CPHO62 on a wider population of LMC3 adult regenerants.

The autoradiographs (Fig. 4a,b) show the robustness of the differential, abnormal-specific signals observed for both the intermediate-weight band in the *Msp*I pattern and for the lower weight band in the *Hpa*II products.

Discussion

The present paper reports our investigation of methylation changes in individual sequences in a genomic con-

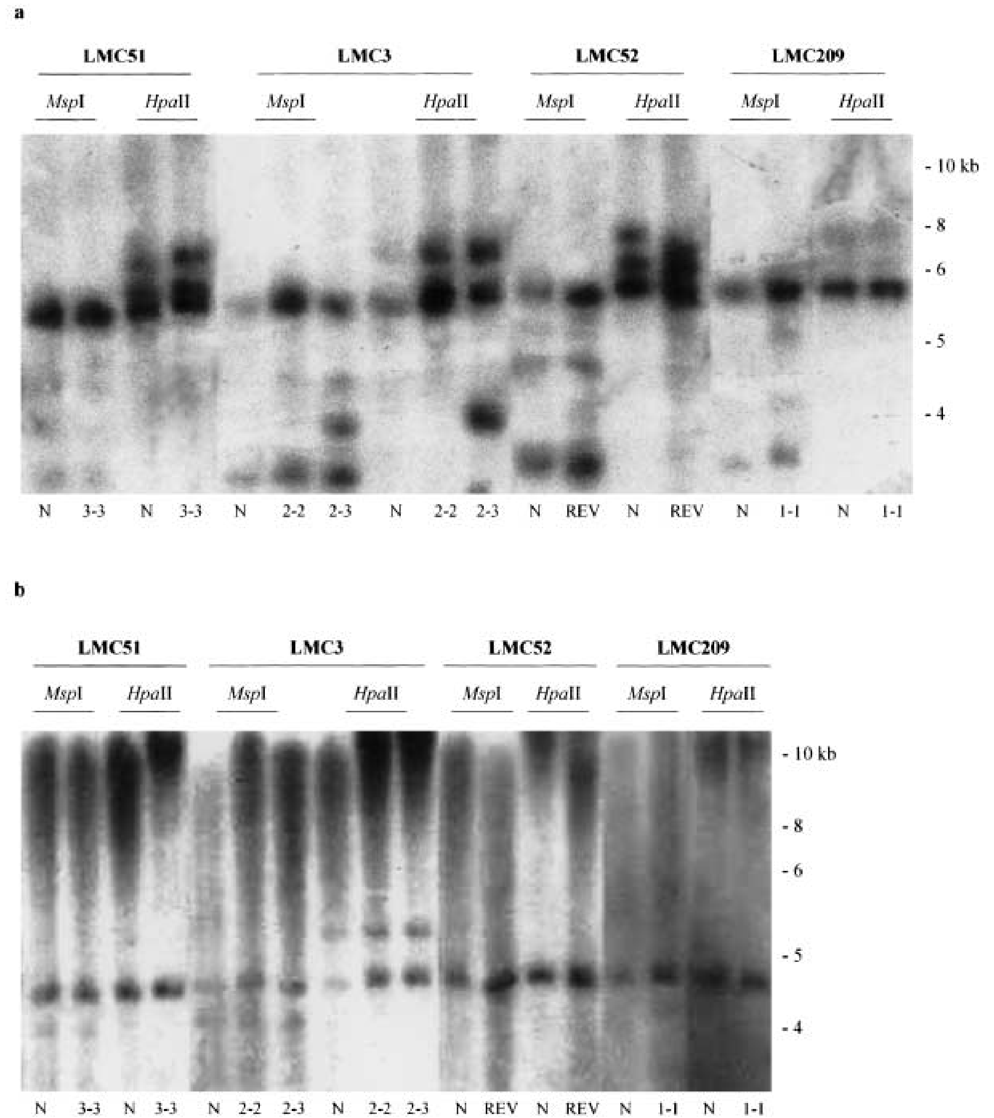

Fig. 2a, b Southern blot hydridisation of probes CPHO62 (**a**) and CPHO63 (**b**) on DNA from immature inflorescences (normal and mantled-type) from four different clonal lines digested with *Msp*I or *Hpa*II. *N* True-to-type regenerant. Regenerants affected by the "mantled" abnormality are designated by two numbers representing the degree of mantledness of their female and male flowers, respectively. *REV* Inflorescences showing partial reversion (from stage 3–3 to 2–3)

text already known to be subject to considerable epigenetic disturbance, as illustrated by the important hypomethylation detected in variant oil palm material (Jaligot et al. 2000).

All of the observed type-dependent changes in CCGG methylation reported here involved decreased methylation in the abnormal plant material, thus confirming the deficit in genomic methylation previously characterised. One interesting aspect of our data is that relatively few methylation polymorphisms correlated to the abnormality were identified when using *Msp*I/*Hpa*II isoschizomeric enzymes. This is the case both for RFLPs (this work) and methylation-sensitive amplification polymorphisms (MSAPs) (our unpublished results; Matthes et al. 2001). This result is somewhat surprising given the considerable differences in global methylation rates previously measured in the same material and may be attributable to a weak contribution of CCGG sites to type-dependent variations in methylation patterns, especially in leaf DNA. In contrast, when comparing NCC and FGC calli, we measured a three fold greater decrease in global methylation rates (Jaligot et al. 2000). Such discrepancies in the extent of type-dependent methylation differences between tissues support the hypothesis that the major epigenetic perturbations occurring at the callus stage, which might affect virtually any methylatable site, may progressively regress throughout development (Kaeppler et al. 2000). Thus, only a subset of these sites may remain hypomethylated and be responsible for the methylation differences detected in leaves from regenerants at the adult age and, in their floral organs, for the expression of the variant phenotype. Changes in methylation patterns are known to take place during organ differentiation and throughout the ageing process of a given tissue (Finnegan et al. 1998). These naturally occurring changes might mask some of the somaclonal variation-linked methylation differences in our adult plant material, among which those of the genomic sequences corresponding to the CPHO62 and CPHO63 probes.

Other type-specific probes are being isolated in our laboratory on the basis of differential gene expression using the dideoxy-reverse transcriptase polymerse chain

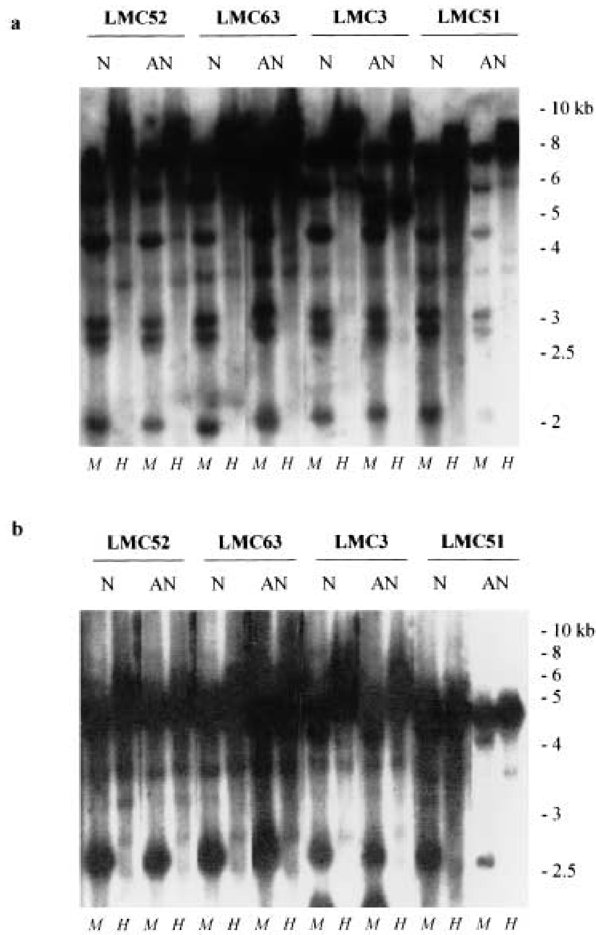

Fig. 3a, b Southern blot hydridisation of probes CPHO62 (**a**) and CPHO63 (**b**) on DNA of adult regenerant leaves (normal and mantled type) from four different clonal lines, digested with *Msp*I (*M*) or *Hpa*II (*H*). *N* True-to-type regenerant, *AN* abnormal regenerant. Leaf DNA was extracted from eight or nine regenerants, depending on the availability of the plant material

reaction (ddRT-PCR) approach (Rival et al. 1998b; Tregear et al. 2002). In addition, the MSAP approach will provide us with the opportunity to simultaneously compare large samples of normal/abnormal individual pairs from different genotypes on the basis of methylation status in a subset of CCGG sites evenly distributed in the genome (Matthes et al. 2001; Xiong et al. 1999). These two approaches have so far yielded only clone-dependent markers that must be considered for further studies in the aim of getting a hint on the mechanisms affected in the context of the "*mantled*" abnormality.

Amongst the 27 probes tested from our oil palm cDNA library, promising results were obtained with two, namely CPHO62 and CPHO63. Their identification as coding sequences for a translation elongation factor and for a LIM-domain protein gene, respectively, raises the question of the roles that such proteins could play in the emergence of the “mantled” somaclonal variation.

It has been proposed that cell-cycle-dependent regulation of protein synthesis could be achieved through phosphorylation of the protein elongation factor 1B (Monnier et al. 2001). LIM proteins have been hypothesised to be involved in phragmoplast assembly (Mundel et al. 2000). Thus, the differential methylation pattern seen with probes CPHO62 and CPHO63 in embryogenic callus lines might be indicative of global perturbations of cell division in FGC calli.

It is not clear, however, if the changes in methylation at CCGG sites observed with probes CPHO62 and CPHO63 between normal and abnormal plant material reflect specific modifications in gene activity associated with mantledness, or whether they might be a “by-product” of the sizeable global differences in 5mdC amounts in the whole genome. The abilities of these probes to discriminate between the two callus types should first be assessed on different genotypes. Our results are encouraging in that they constitute a first step in an extensive search for early markers of the somaclonal variation.

It will now be of great interest to continue this study by characterising the expression pattern of the genes cor-

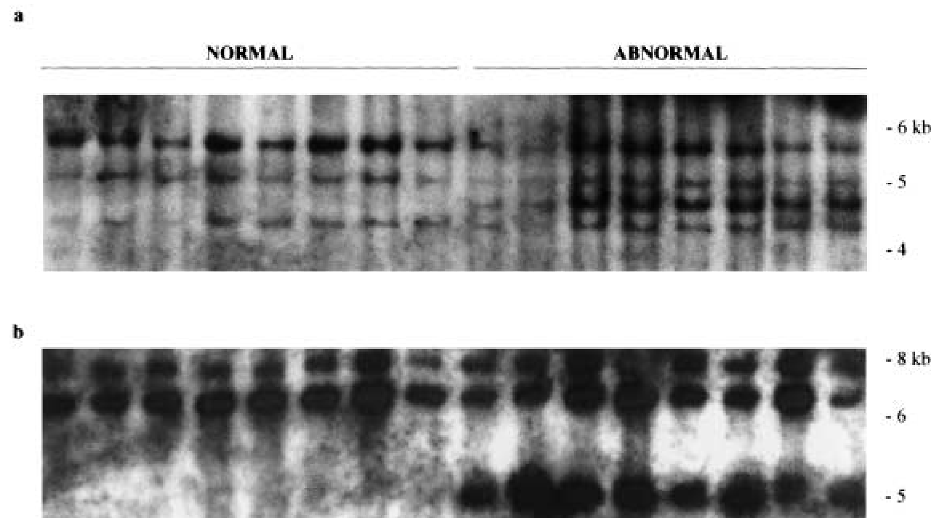

Fig. 4a, b Southern blot hydridisations of probe CPHO62 on a population of LMC3 adult regenerants leaf DNA digested with *Msp*I (**a**) or *Hpa*II (**b**). The membranes were loaded with DNA from eight normal and eight abnormal individuals

responding to CPHO62 and CPHO63 probes in Northern blotting experiments. Moreover, as some of the methylation polymorphisms observed here might reside within genomic regions not addressed by the probes, the isolation of the full genomic coding and regulatory sequences is necessary. This would allow us to determine which of the RFLP-polymorphic sites might act to generate differences in transcriptional activity of the CPHO62 and CPHO63 genes. In the event that a methylation-based regulation could be identified, a fine mapping of methylation at individual cytosines using the bisulfite sequencing method (under way in our group) could be undertaken.

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References

Altschul SF, Stephen F, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Baylin SB, Herman JG (2000) DNA hypermethylation in tumorigenesis. Epigenetics joins genetics. Trends Genet 16:168–174

Demeulemeester MAC, van Stallen N, De Proft MP (1999) Degree of DNA methylation in chicory (*Cichorium intybus* L.): influence of plant age and vernalization. Plant Sci 142:101–108

Dolezel J, Binarova P, Lucretti S (1989) Analysis of nuclear DNA content in plant cells by flow cytometry. Biol Plant 31: 113–120

Eliasson A, Gass N, Mundel C, Baltz R, Krauter R, Evrard J-L, Steinmetz A (2000) Molecular and expression analysis of a LIM protein gene family from flowering plants. Mol Gen Genet 264:257–267

Feinberg AP, Vogelstein B (1983) A technique of radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Finnegan EJ, Peacock WJ, Dennis ES (1996) Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. Proc Natl Acad Sci USA 93:8449–8454

Finnegan EJ, Genger RK, Peacock WJ, Dennis ES (1998) DNA methylation in plants. Annu Rev Plant Physiol Plant Mol Biol 49:223–247

Finnegan EJ, Peacock WJ, Dennis ES (2000) DNA methylation, a key regulator of plant development and other processes. Curr Opin Genet Dev 10:217–223

Fulnecek J, Matyàsek K, Kovarik A, Bezdek M (1998) Mapping of 5-methylcytosine residues in *Nicotiana tabacum* 5S rRNA genes by genomic sequencing. Mol Gen Genet 259:133–141

Hericourt F, Jupin I (1999) Molecular cloning and characterization of the *Arabidopsis thaliana* alpha-subunit of elongation factor 1B. FEBS Lett 464:148–152

Hoisington D (1992) Laboratory protocols. Applied Molecular Genetics Laboratory, CIMMYT, Mexico

Jaligot E, Rival A, BeuléT, Dussert S, Verdeil J-L (2000) Somaclonal variation in oil palm (*Elaeis guineensis* Jacq.): the DNA methylation hypothesis. Plant Cell Rep 19:684–690

Jeddeloh JA, Richards EJ (1996) ^{m}CCG methylation in angiosperms. Plant J 9:579–586

Kaeppler SM, Phillips RL (1993) Tissue culture-induced DNA methylation variation in maize. Proc Natl Acad Sci USA 90:8773–8776

Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. Plant Mol Biol 43:179–188

Kingham KI, Duckett JG, Gazdova B, Kovarik A, Bezdek M, Leitch AR (1998) The role of DNA methylation on nuclear and cell differentiation in the filamentous caulomena of the moss *Funaria hygrometrica*. New Phytol 138:567–577

Kovarik A, Koukalová B, Holý A, Bezdek M (1994) Sequence-specific hypomethylation of the tobacco genome induced with dihydroxypropyladenine, ethionine and 5-azacytidine. FEBS Lett 353:309–311

LoSchiavo F, Pitto L, Giuliano G, Torti G, Nuti-Ronchi V, Marazziti D, Vergara R, Orselli S, Terzi M (1989) DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. Theor Appl Genet 77:325–331

Matthes M, Singh R, Cheah S-C, Karp C (2001) Variation in oil palm (*Elaeis guineensis* Jacq.) tissue culture-derived regenerants revealed by AFLPs with methylation-sensitive enzymes. Theor Appl Genet 102:971–979

Monnier A, BelléR, Morales J, Cormier P, Boulben S, Mulner-Morillon O (2001) Evidence for regulation of protein synthesis at the elongation step by CDK1/cyclin B phosphorylation. Nucleic Acids Res 29:1453–1457

Mundel C, Baltz R, Eliasson A, Bronner R, Grass N, Kräuter R, Evrard J-L, Steinmetz A (2000) A LIM-domain protein from sunflower is localized to the cytoplasm and/or nucleus in a wide variety of tissues and is associated with the phragmoplast in dividing cells. Plant Mol Biol 42:291–302

Oakeley EJ, PodestàA, Jost J-P (1997) Developmental changes in DNA methylation of the two tobacco pollen nuclei during maturation. Proc Natl Acad Sci USA 94:11721–11725

Pannetier C, Arthuis P, Lievoux D (1981) Néoformation de jeunes plantes d'*Elaeis guineensis* àpartir de cals primaires obtenus sur fragments foliaires cultivés in vitro. Oléagineux 36:119–122

Rival A (2000) Somatic embryogenesis in oil palm. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants. Kluwer, Dordrecht, pp 249–290

Rival A, BeuléT, Barre P, Hamon S, Duval Y, Noirot M (1997) Comparative flow cytometric estimation of nuclear DNA content in oil palm (*Elaeis guineensis* Jacq.) tissue cultures and seed-derived plants. Plant Cell Rep 16:884–887

Rival A, Bertrand L, BeuléT, Combes M-C, Trouslot P, Lashermes P (1998a) Suitability of RAPD analysis for the detection of somaclonal variants in oil palm (*Elaeis guineensis* Jacq.). Plant Breed 117:73–76

Rival A, Tregear J, Verdeil J-L, Richaud F, Beulé T, Hartman C, Rode A, Duval Y (1998b) Molecular search for mRNA and genomic markers of the oil palm "mantled" somaclonal variation. Acta Hortic 461:165–171

Smulders MJM, Rus-Kurtekaas W, Vosman B (1995) Tissue-culture induced DNA methylation polymorphisms in repetitive DNA of tomato calli and regenerated plants. Theor Appl Genet 91:1257–1264

Tregear JW, Morcillo F, Richaud F, Berger A, Singh R, Cheah SC, Hartmann C, Rival A, Duval Y (2002) Characterisation of a defensin gene expressed in oil palm inflorescence: induction during tissue culture and possible association with epigenetic somaclonal variation events. J Exp Bot (in press)

Vergara R, Verde F, Pitto L, Lo Schiavo F, Terzi M (1990) Reversible variations in the methylation pattern of carrot DNA during somatic embryogenesis. Plant Cell Rep 8:697–700

Vyskot B, KoukalováB, Kovarik A, Sachambula L, Reynolds D, Bezdek M (1995) Meiotic transmission of a hypomethylated repetitive DNA family in tobacco. Theor Appl Genet 91:659–664

Xiong LZ, Xu CG, Saghai-Maroof MA, Zhang Q (1999) Patterns of cytosine methylation in an elite rice hybrid and its parental lines, detected by a methylation-sensitive amplification polymorphism technique. Mol Gen Genet 261:439–446