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Somaclonal variation in oil palm (*Elaeis guineensis* Jacq.): the DNA methylation hypothesis

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Abstract The occurrence of somaclonal variants (ca 5%) among populations of somatic embryo-derived oil palms (*Elaeis guineensis* Jacq.) currently hampers the scaling-up of clonal plant production. In order to investigate the relationship between the "mantled" somaclonal variant and possible alterations in genomic DNA methylation rate, two complementary approaches have been used. HPLC quantification of relative amounts of 5-methyl-deoxycytidine has shown that global methylation in leaf DNA of abnormal regenerants is 0.5–2.5% lower than in their normal counterparts (20.8% vs 22%, respectively). When comparing nodular compact calli and fast growing calli, yielding respectively 5% and 100% of "mantled" plantlets, this decrease was up to 4.5% (from 23.2 to 18.7%). An alternative method, the *Sss*I-methylase accepting assay, based on the enzymatic saturation of CG sites with methyl groups, gave convergent results. This work demonstrates that a correlation exists between DNA hypomethylation and the "mantled" somaclonal variation in oil palm.

Key words DNA methylation · Epigenetics · Genetic stability · Somatic embryogenesis · Somaclonal variation

Abbreviations *dC*: Deoxycytidine · *5mdC*: 5-Methyl-deoxycytidine · *FGC*: Fast-growing callus · *GAs*: Gibberellins · *HPLC*: High-performance liquid chromatography · *NCC*: Nodular compact callus · *NMI*: Normalised methylation index · *RAPD*: Random amplified polymorphic DNA · *([3H]-)SAM*: (Tritiated) S-adenosyl-methionine · *SssI-MAA*: *Sss*I-Methylase accepting assay

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Introduction

The large-scale development of in vitro clonal propagation of oil palm (*Elaeis guineensis* Jacq.) by somatic embryogenesis has resulted in the identification of a novel variant phenotype among adult regenerant palms (Rival et al. 1997a). Indeed, approximately 5% of somatic embryo-derived palms show abnormalities in their floral development, involving an apparent feminisation of male parts in flowers of both sexes, called the "mantled" phenotype by Corley et al. 1986 (see Fig 1). This somaclonal variant can exhibit a marked heterogeneity in its occurrence and intensity between different clonal lines, between palms of the same clonal line, and between different flowers of the same individual variant palm. It may result in partial or complete flower sterility, thus directly affecting oil production, depending on the severity of the abnormality. Interestingly, reversions to the normal phenotype over time have been found to occur, leading to a complete recovery of the normal phenotype for 100% of the slightly "mantled" individuals, and for 50% of the severely "mantled" ones after 9 years in the field (Rival et al. 1998b).

The phenotypic fidelity of regenerants depends on the nature of embryogenic callus lines used in the micropropagation process. Whereas nodular compact calli (NCC) have been found to produce on average 5% variant palms, this rate reaches 100% in plantlets derived from fast growing calli (FGC), thus demonstrating the importance of the callus stage in the determination of trueness-to-type of regenerants.

Previous studies did not allow us to point out any major alterations in genomic DNA structure that could be linked with the "mantled" phenotype. Flow cytometric analyses performed on NCC and FGC, and on plantlets originating either from seeds or from

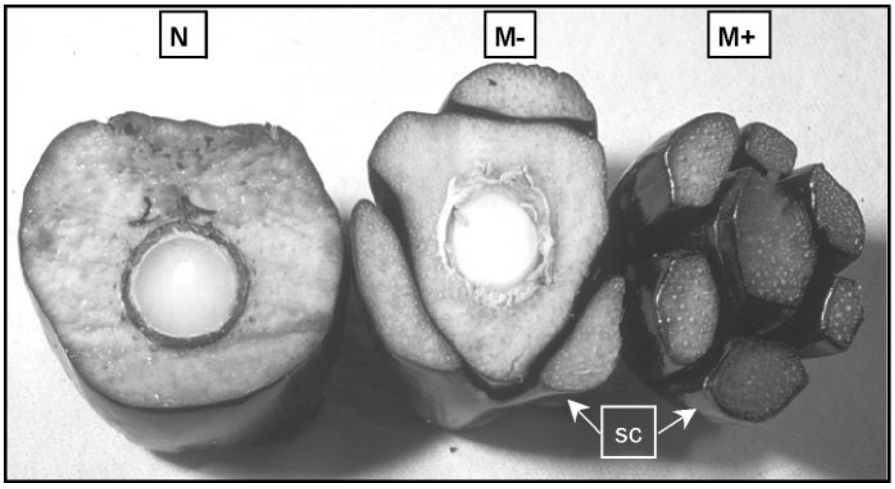

Fig. 1 Cross-sections of oil palm fruits originating from somaclones. From *left* to *right*: fruits from normal (*N*), slightly mantled (*M*–) and severely mantled (*M*+) somatic embryo-derived oil palm. sc = Supernumerary carpel

somatic embryos, demonstrated a uniform 2C ploidy level (Rival et al. 1997b). Extensive random amplified polymorphic DNA (RAPD) experiments, involving the examination of 8900 markers, also failed to show banding patterns discriminating either the mother palm genome from its clonal offspring, or true-to-type from variant clonal regenerants (Rival et al. 1998a).

On the basis of observed characteristic features of the "mantled" somaclonal variation (spatial heterogeneity, temporal instability, absence of any detectable defect in DNA organisation) the hypothesis of an epigenetic alteration of genome expression has been proposed to explain the origin of the variant plants.

Fluctuations of DNA methylation on deoxycytidine (dC) residues have attracted our attention, since they have been shown to be involved in gene regulation at the transcriptional level, particularly during the differentiation/dedifferentiation processes and as a response to a variety of environmental stresses (Finnegan et al. 1993, 1998; Phillips et al. 1994). The in vitro somatic embryogenesis protocol used for oil palm, which includes growth regulator treatments, may be seen as a point of interaction between these two distinct phenomena which might affect the level of DNA methylation. Similar conclusions have been supported by the work of Cavallini et al. (1996) on pea, LoSchiavo et al. (1989) and Vergara et al. (1990) on carrot, Sabbah et al. (1995) on potato, Schmitt et al. (1997) on tobacco, and Smulders et al. (1995) on tomato.

Moreover, evidence is currently accumulating for an involvement of DNA methylation dysfunctions in the emergence of developmental abnormalities in animals as well as in plants (Burn et al. 1993; Chen et al. 1998; Finnegan et al. 1996; Laird and Jaenisch 1997; Richards 1997; Ushijima et al. 1997).

Hence, a global approach for the investigation of the rate of DNA methylation has been chosen (Rival et al. 1998b), aiming to reveal significant differences between normal and variant plant material.

In this study we used two complementary methods to evaluate global methylation rates: (1) direct determination of 5-methyl-deoxycytidine (5mdC) amounts in genomic DNA by HPLC separation and quantification of nucleosides, and (2) a reverse dosage method (*Sss*I-MAA) involving the in vitro saturation of all unmethylated CG sites with labelled methyl residues using *Sss*I methyltransferase, thus allowing quantification of the proportion of sites which were methylated or unmethylated at the outset by measurements of incorporated methyl groups. The methylation status of leaf DNA has been examined, since it has been observed that the re-cloning of variant regenerants via leaf explants gave rise to increased percentages of severely affected offspring (Durand-Gasselin, personal communication), presumably reflecting the existence of a latent "abnormality message" in leaves. DNA methylation rates have also been investigated in embryogenic calli, because of the likely key role of this plant material in the generation of somaclonal variants.

Materials and methods

Plant material

Mother palms and adult regenerants of oil palm obtained through in vitro somatic embryogenesis (according to the protocol described by Pannetier et al. 1981) were sampled at SOCFINDO plantations, Indonesia (clonal lines SOC2104 and SOC2804), and at the CNRA La Mé Research Station, Côte d'Ivoire (clonal lines LMC3 and LMC51).

Calli (NCC and FGC from clonal lines LMC458 and LMC464) were obtained at CNRA through in vitro culture of leaf explants, according to Pannetier et al. (1981).

As a complement to the genetic origin of the studied plant material given in Table 1, it should be noted that clonal lines LMC458 and LMC464 were obtained from two different mother palms originating from the same cross. Consequently, they must be considered as bearing different genotypes.

Table 1 Genetic origin of the analysed plant material. Conventionally, each cross is given in the form: male parent×female parent

Clonal line	Plant material	
	Leaves	Calli
SOC2104	(D115D×D115D) ×(L2T×L2T)	–
SOC2804	(L404D×D10D) ×(L2T×L2T)	–
LMC3	L16T×D3D	–
LMC51	L2T×D8D	–
LMC458	–	(D5D×D5D) ×(L5T×L5T)
LMC464	–	(D5D×D5D) ×(L5T×L5T)

Genomic DNA extraction

Genomic DNA was isolated from F_{+1} order oil palm leaves as described by Rival et al. (1998a). The procedure for DNA extraction from embryogenic calli was essentially the same, except that the lysis buffer of Dolezel et al. (1989) was used for the isolation of nuclei from freeze-dried material.

Enzymatic hydrolysis of DNA

The method employed for DNA enzymatic hydrolysis was adapted from Gehrke et al. (1984), and Palmgren et al. (1990). DNA samples (20 µg each, as estimated by fluorimetry, using the PicoGreen assay; Molecular Probes P-7581, Leiden, The Netherlands) were added to 10 µl of a 0.5 U/µl solution of nuclease P1 and 35 µl of a 0.017 U/µl solution of alkaline phosphatase (Sigma N8630 and P4252, respectively) and the reaction volume adjusted to 200 µl with the digestion buffer (30 mM $NaCH_3$, 0.1 mM $ZnCl_2$, pH 5.3). Hydrolysis of DNA to nucleosides was performed in triplicate, for 3 h at 37 °C. The reaction was stopped by the addition of 490 µl absolute ethanol; then the samples were centrifuged at 11,000 *g* for 15 min. The supernatant was transferred to a new tube, vacuum-dried, and nucleosides were resuspended in 1 ml of sterile water. The extracts were then filtered (0.2 µm) prior to HPLC analysis.

HPLC analysis of nucleosides

An isocratic elution protocol was followed, using a modified version of the buffer described by Gehrke et al. (1984): 50 mM KH_2PO_4, 8% [v/v] methanol, pH 3.5 (instead of 4.4), on a Supelcosil LC-18S reverse-phase column (SUPELCO Inc., Bellefonte, Pa., USA; 25 cm×4.6 mm; particles diameter: 5 µm), with a flow rate of 0.8 ml/min and a run time of 30 min. The effluent was monitored at a wavelength of 285 nm with a photodiode array detector (Waters 996; Waters, Milford, Mass., USA). The percentage of 5mdC was calculated using the formula:

$$100\times[5mdC]/([dC]+[5mdC]) \quad (1)$$

where [5mdC] and [dC] are the respective concentrations of the two forms of dC. The latter were calculated by integrating the differences in molar extinction coefficient for dC and 5mdC, as deduced from the calibration curves for external standards of known concentrations, monitored simultaneously with the samples. The improved conditions used allowed highly efficient separation of the peaks corresponding to dC and uracil (compounds identified by their respective retention time and UV spectra), thereby eliminating a common problem encountered in our preliminary studies (Rival et al. 1998c).

For each DNA extract (seven from leaves of each clonal line and type, three to five from calli), the products of three independent hydrolyses were analysed.

*Sss*I methylase accepting assay

The protocol was essentially as described by Schmitt et al. (1997), modified as follows, according to Oakeley (personal communication): each assay was performed in triplicate on 150 ng DNA; the reaction buffer was composed of 20 mM Tris, 5 mM EDTA, 0.5 mM DTT, pH 7.5, and the isotopic dilution of [^{3}H]-SAM was such as to yield a specific activity of 30 mCi/µmol.

Normalised values of DNA methylation at CG sites were obtained by comparison with a unique standard (leaf DNA from seed-derived adult oil palm). The equation used for the calculation of the normalised methylation index (NMI) is:

$$NMI=(Q_{METH}{}^{sample}/[DNA]^{sample})/(Q_{METH}{}^{standard}/[DNA]^{standard}) \quad (2)$$

where Q_{METH} represents the amount of [^{3}H]-methyl groups incorporated in sample or in standard DNA (as determined by the *Sss*I-MAA method), and [DNA] the corresponding concentration of DNA. According to this formula, an NMI of less than 1 corresponds to an increase of CG methylation of the sample relative to the standard.

The same individual palms were studied using both HPLC and *Sss*I-MAA techniques, except that *Sss*I-MAA was used on bulks of leaf genomic DNA samples. These bulks were composed of equimolar concentrations of DNA extracted from seven different palms for each clone and type. The DNA extracts from calli were the same in HPLC and *Sss*I-MAA analyses.

Statistical analyses

A hierarchical three-way analysis of variance was applied to test clone, type and palm-tree effects on the global methylation rate in leaf DNA, where clone and type were fixed-effect factors and palm-tree was subordinated to the type factor (seven palm-trees per type). A two-way analysis of variance was performed to test clone- and type-effects on the global methylation rate in callus DNA and the NMI in callus and leaf DNAs. Clone and type were fixed-effect factors. In all experiments, Newman's (1939) and Keuls' (1952) tests were used for multiple comparison of categorial means.

Results and discussion

DNA methylation in leaves

Chromatographic estimation of global DNA methylation rates

As deduced from HPLC analyses on genomic DNA samples from leaves collected on mother palms, the global methylation rate averaged 20.39%±1.23 in adult oil palms. This result is consistent with the range of DNA methylation rates found amongst plant species, using the same method: 33% in rye (Finnegan et al. 1993), 26.5, 25.7 and 27.2% in pea, barley and corn shoots, respectively (Klaas and Amasino 1989) and 25% in tomato mature leaves (Smulders et al. 1995).

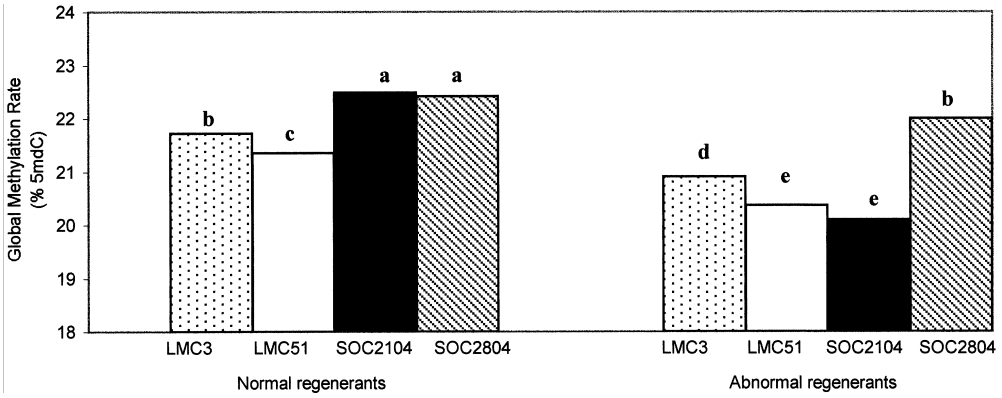

Fig. 2 Methylation rates in genomic DNA from leaves of adult regenerant palms. Each value is the mean of three independent measurements performed on seven different palms (i.e. 21 measurements). Data followed by the same letter are not significatively different at the 1‰ level

Among the regenerants, global DNA methylation rates were found to be significantly ($F_{(3,113)} = 35.40$; $P<10^{-5}$) lower in abnormal palms (20.8% vs 22%): the percentages of 5mdC thus decreased from 0.5% to 2.5% compared with true-to-type levels, depending on the clone (Fig. 2). An obvious effect attributable to the type of regenerant (mantled or normal) was observed on the DNA methylation rate, yet this effect was variable in intensity from one genotype to another. These average methylation rates enabled us to distinguish, within each group of clonal progeny studied, the variant regenerants from the normal ones. Nevertheless, the methylation rate data did not always allow discrimination between normal regenerants of one clonal group and abnormal regenerants of another. Furthermore, DNA methylation levels were found to be lower in seed-derived palms from various different origins (20.4%) than in abnormal regenerants taken as a whole (20.8%). This inherent genotype-dependent variability associated with global DNA methylation rates makes the demonstration of a direct cause-effect relationship between DNA methylation and the mantled flowering abnormality difficult. Measurements of adult palm leaf global DNA methylation rates do not therefore provide a predictive individual test for the early detection of the mantled abnormality. We sought to determine if distinction between normal and abnormal regenerants could be made by investigating DNA methylation at CG sites instead of the whole genome, using a different approach: the *Sss*I-MAA method.

Evaluation of CG site methylation status by SssI-MAA

Using the *Sss*I-MAA technique, we detected an overall, highly significative, hypomethylation within the CG sites of bulked DNA from leaves of abnormal oil palms (Table 2; $F_{(3,8)} = 14.42$; $P<10^{-2}$).

As for the data obtained from HPLC analysis, a greater overall NMI (+60 to +85%) was recorded in all clonal lines examined for regenerants of normal type compared with abnormal ones (0.90 and 1.22, respectively). These results thus reflect the lower frequency of 5mdCG modified dinucleotides in mantled palm DNA, compared with true-to-type DNA. The only exception to this trend was LMC51, in which normal regenerants showed the greater NMI; it should be noted, however, that intra-treatment variance was particularly high in this case. Confirming the HPLC results, we noticed a very highly significant ($F_{(3,8)} = 16.22$; $P<10^{-3}$) clone-effect on NMI values, irrespective of the regenerant type. The 'clone' and 'type' factors were also shown to significant interact with each other ($F_{(3,8)} = 7.02$; $P<5.10^{-2}$), thereby indicating that both the trend (towards hypo- or hypermethylation) and the extent of the influence exerted by the type of regenerant on NMI depend on the clonal line considered.

On the whole, it seems that a variation towards DNA hypomethylation is successfully detected by HPLC analysis in leaves from adult mantled regenerants, and it is unambiguously confirmed for CG sites with the *Sss*I-MAA method for three out of four clonal lines investigated.

Table 2 Normalised methylation index (*NMI*) calculated from *Sss*I-MAA analysis of DNA extracted from leaves of adult somatic embryo-derived palms. Each NMI, representing the relative saturation in methyl groups of CG sites using *Sss*I methyltransferase, was calculated from Eq. (2), as an average of three independent measurements. Data followed by the same letter(s) are not significantly different at the 5% level

Clonal line	Regenerant type	NMI±SD
LMC3	Normal	0.67 ± 0.12^{cd}
	Mantled	1.11 ± 0.04^{bc}
LMC51	Normal	1.50 ± 0.28^{ab}
	Mantled	1.19 ± 0.27^{bc}
SOC2104	Normal	0.95 ± 0.01^{cd}
	Mantled	1.71 ± 0.18^{a}
SOC2804	Normal	0.46 ± 0.10^{d}
	Mantled	0.85 ± 0.00^{cd}

DNA methylation in embryogenic calli

Chromatographic estimation of global DNA methylation rates

As shown in Fig. 3, the difference between types was even more marked within a population of calli sharing the same genotype than for leaves of adult regenerants. The hypomethylation of FGC DNA represented an average reduction of 5mdC amounts of 4.5% compared with NCC DNA methylation (%5mdC: 18.7% vs 23.2%), and this difference was highly significant ($F_{(1,11)} = 58.19$; $P<10^{-5}$). No clonal effect was detected, and there was no interaction between clone and type.

Besse et al. (1992) have shown on other oil palm genotypes that these two types of calli display differences in their endogenous auxin/cytokinin ratio: whereas indoleacetic acid levels were found to be the same in NCC and in FGC, the latter exhibited generally lower cytokinin concentrations. Such results may be connected with the defective DNA methylation observed in FGC, according to the hypothesis developed by Hare and van Staden (1997). These authors proposed that some interactions may exist in plants between cytokinin signal transduction (especially long-term effects involving modulation of gene expression) and methyl-transfer reactions, mediated through a hormonal alteration of SAM metabolism. In this putative mechanism, a low endogenous cytokinin level would be correlated with global DNA hypomethylation. Nevertheless, which of these decreases is responsive to the other is still unknown. It is worth noting, however, that *Arabidopsis thaliana* plants carrying the putative methyltransferase *METI*-antisense transgene were shown to exhibit a variety of aberrant floral phenotypes, the severity of which tightly paralleled both the extent of the reduction in cytosine methylation and the ectopic expression of floral homeotic genes (Finnegan et al. 1996). These effects were found to be similar to those observed in tobacco plants expressing the bacterial ipt gene and led to the hypothesis that levels of endogenous cytokinins and expression of genes governing floral identity could be inversely correlated (Hare and van Staden 1997). Furthermore, several groups have recently demonstrated that DNA methylation and gibberelins (GAs) were involved in the vernalization-dependant control of flowering in *Thlaspi arvense* (Hazebroek et al. 1993) and *Arabidopsis thaliana* (Koornneef et al. 1998; Sheldon et al. 1999). Koornneef et al. (1998) suggest that the inductive action of GAs on flowering may be blocked by the product of genes (like FLF, for flowering locus F) controlled by the level of DNA methylation, which has been shown to be modulated by the vernalization process.

Evaluation of CG sites methylation status by SssI-MAA

In both clones subjected to *Sss*I-MAA analysis, DNA from FGC-type calli appeared to be significantly hypomethylated ($F_{(1,8)} = 9.46$; $P<5.10^{-2}$) compared with NCC. We observed an overall 60% increase of NMI from normal to abnormal calli DNA (0.67 vs 1.08), which accounted for a lesser initial modification of CG dinucleotides in FGC.

Contrasting with the results obtained with leaf DNA from adult regenerants, no clonal effect on the extent of methylation at CG sites could be demonstrated. This, together with the absence of clonal effect in the HPLC data, is in accordance with the genetic origin of the two clonal lines, which belong to the same cross (Table 3). Moreover, there was no significant interaction between clone and type.

Whether or not this sole deficit of methylation at CG sites can fully explain the lower amount of 5mdC in the whole genomic DNA remains to be determined. In contrast with animal DNA, methylation of plant DNA also occurs at CNG trinucleotides, N standing for any base (Diéguez et al. 1998; Finnegan et al. 1993; Jeddeloh and Richards 1996; Richards 1997). As the location of methylatable sites critical for the onset and maintenance of the mantled somaclonal variation is currently unknown, it is still difficult to speculate on the proportion of crucial CNG sites, whose methyla-

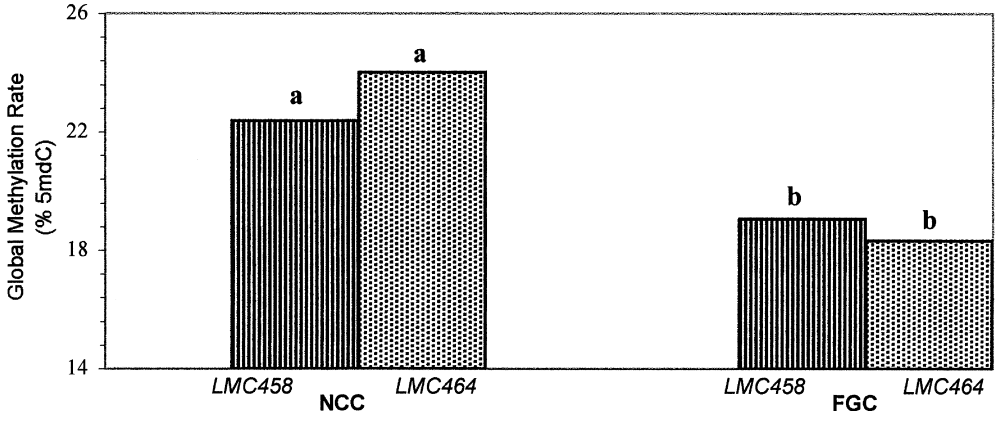

Fig. 3 Methylation rates in genomic DNA from embryogenic calli. Each value is the mean of three independent measurements performed on at least three different palms (i.e. at least nine measurements) . Data followed by the same letter are not significatively different at the 1‰ level

Table 3 NMI calculated from *Sss*I-MAA analysis of DNA extracted from embryogenic calli. Each NMI, representing the relative saturation in methyl groups of CG sites using *Sss*I methyltransferase, was calculated from Eq. (2), as an average of three independent measurements. Data followed by the same letter are not significantly different at the 5% level

Clonal line	Callus type	NMI±SD
LMC458	NCC	0.60±0.16[a]
	FGC	0.99±0.04[b]
LMC464	NCC	0.73±0.09[a]
	FGC	1.17±0.43[b]

tion status has escaped investigation by the *Sss*I-MAA method.

Conclusions and perspectives

In the present study, we have established a relationship between genomic DNA hypomethylation and mantled somaclonal variation in oil palm, on two different plant materials (calli and leaves) originating from various genotypes and following two complementary approaches (HPLC quantification of nucleosides and *Sss*I-mediated reverse-dosage of CG-specific methylation).

Even though detectable with both methods, the trend towards hypomethylation observed in abnormal plant material proved to be more subtle in leaves, where the accumulation of inter-individual and inter-clonal variations can in some cases mask it. This is not surprising, given that leaves are not the organs where the abnormality is phenotypically expressed, although its determinant factor is likely to be present. Moreover, the considerable genomic hypomethylation seen in abnormal calli may be, if not erased, at least hidden under stage- and organ-specific methylation patterns during the development of the regenerants.

Many studies of variations in the amount of 5mdC during plant development have been reported (see for a review Finnegan et al. 1998; Richards 1997). For this reason, we considered only comparable leaves (sampled at the same leaf order – F_{+1} – on regenerants of the same age). The possibility of a difference in palm or in leaf age as the cause for this levelling of variations in methylation rates between types can thus be discarded.

Hypomethylation of DNA in FGC compared with NCC was detected with both methods (more readily with HPLC), highlighting the marked expression of this molecular feature which may well contribute to the high level of somaclonal variation observed for FGC-derived vitroplants. Interestingly, the two types of calli used are of the same genotype and have arisen from the same cultural conditions, so the expression of the abnormal callus type must reflect the parameters of a presumably epigenetic nature. In support of this idea, protein patterns and hormonal levels have been shown to diverge from the normal state in FGC (Marmey et al. 1991; Besse et al. 1992). Moreover, flow cytometric analysis of these two callus types (Rival et al. 1997b) did not reveal any variation in their nuclear DNA content, thus eliminating the possibility of an alteration of the ploidy level as the source of somaclonal variation. Taken together, these previous studies strongly suggest that an epigenetic mechanism, caused or at least mediated by a hormonal imbalance, is at work in FGC and that it may give rise to the mantled phenotype in their regenerants. The results presented here constitute a further line of evidence and demonstrate that NCC/FGC calli are a very promising starting point as a means of identifying early molecular markers of the mantled abnormality. We are currently monitoring the methylation status of DNA from NCC/FGC-derived plantlets throughout the micropropagation process, in order to assess whether or not the marked difference in methylation rate observed at the callus stage is maintained from the somatic embryo to the adult palm.

The question of the evolution of DNA methylation in reverting individuals should also be addressed, since it constitutes the opportunity to test the reciprocal of our hypothesis, that is whether the DNA methylation rate increases as the normal phenotype is recovered. Reversion naturally occurs for abnormal adult palms, and transition from NGC- to FGC-type calli can been obtained with a modification of hormonal supplies, thus potentially offering two different points of view on this phenomenon.

In a related study, we are currently investigating the DNA methylation status of flowers, encouraging preliminary results (by HPLC as well as by *Sss*I-MAA) having demonstrated a trend towards hypomethylation in abnormal flowers.

Ultimately, we wish to target more precisely the genomic sequences responsible for the abnormality, using Southern analysis involving isoschizomeric restriction enzymes (the *Msp*I/*Hpa*II pair, as described by Diéguez et al. 1998; Jeddeloh and Richards 1996; Smulders et al. 1995; Vergara et al. 1990). Complementary studies are currently being performed in our laboratory (Tregear, personal communication) to identify gene expression changes associated with the mantled abnormality. Clearly it will be of great interest to determine whether gene-specific methylation differences can be correlated with the differential expression patterns observed.

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