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RESEARCH PAPER

Isolation and expression analysis of genes encoding MET, CMT, and DRM methyltransferases in oil palm (*Elaeis guineensis* Jacq.) in relation to the 'mantled' somaclonal variation

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Abstract

In oil palm (*Elaeis guineensis* Jacq.), ~5% of somatic embryo-derived regenerants show homeotic changes during floral development, involving an apparent feminization of male parts in flowers of both sexes, called the 'mantled' phenotype. This variant phenotype is associated with a reduction in the level of global DNA methylation. To explore possible relationships between DNA methylation level and accumulation of DNA-(cytosine-5) methyltransferase (DNMT) transcripts, the full-length coding sequences corresponding to three different DNMT families in oil palm, namely the MET, CMT, and DRM classes, have been isolated and characterized. The corresponding genes were designated as *EgMET1*, *EgCMT1*, and *EgDRM1*, and encode predicted polypeptides of 1543, 925, and 591 amino acid residues, respectively. Expression of oil palm DNMTs was compared between normal and variant calli and inflorescence tissues using quantitative reverse-transcription PCR. A consistent increase in transcript levels of *EgMET1* and *EgCMT1* was found in variant fast-growing calli relative to nodular-compact calli. Nodular-compact calli give rise to about 5% of abnormal regenerants whereas fast-growing calli generate 95% of 'mantled' palms in their clonal offspring and were previously demonstrated as having markedly hypomethylated DNA. In immature abnormal inflorescences only *EgMET1* transcript levels were increased, while no changes in relative abundance of the *EgCMT1* or *EgDRM1* transcripts were observed. Therefore, the genome-wide hypomethylation previously described in 'mantled' material cannot be explained by a decrease in expression levels of the *de novo* or maintenance DNMTs, a paradox which has been previously reported in tumour cells, where there is evidence for global hypomethylation of DNA.

Key words: Developmental regulation, DNA methylation, *Elaeis guineensis* Jacq., epigenetics, stability.

Introduction

Methylation of cytosine residues within DNA plays an important role in many biological processes including the silencing and inactivation of transposons and in imprinted gene expression in both plants and animals (Chan *et al*., 2006; Gehring and Henikoff, 2007). Cytosine methylation is mediated by a suite of DNA methyltransferases (DNMTs) which establish and maintain DNA methylation patterns (Goll and Bestor, 2005). In many higher eukaryotes, DNA methylation is essential for normal development; null mutations of the DNMTs that maintain CpG methylation are embryo lethal in mammals and plants (Li *et al*., 1992; Saze *et al*., 2003; Xiao *et al*., 2006). Aberrant DNA methylation is associated with developmental abnormalities in plants and with disease in humans. There is increasing evidence that tumour development is associated with global hypomethylation and local hypermethylation resulting in the misregulation of a large number of genes (Feinberg and Tycko, 2004). In

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plants, developmental abnormalities such as the change from bilateral to radial symmetry in flowers of *Linaria vulgaris* and increased stamen number in *Arabidopsis* flowers are associated with hypermethylation and silencing of genes involved in floral development (Jacobsen and Meyerowitz, 1997; Cubas *et al*., 1999). Changes in DNA methylation have been observed during plant tissue culture, and have been correlated with the formation of somaclonal variants, the phenotypic variants among clonally propagated plants from a single donor genotype (Larkin and Scowcroft, 1983), in several species (reviewed in Kaeppler *et al*., 2000). This loss of phenotypic fidelity is a major impediment to the development of large-scale propagation of plants through *in vitro* processes such as somatic embryogenesis.

In oil palm (*Elaeis guineensis* Jacq), ~5% of somatic embryo-derived palms show an abnormality that is called the 'mantled' phenotype (Corley *et al*., 1986). This somaclonal variation results in a conversion of the male floral organs (stamens in male flowers and staminoids in female flowers) into supernumerary carpels (Adam *et al*., 2005) and may lead to partial or complete flower sterility, depending on the severity of the abnormality. While fast-growing calli yielding 95% of 'mantled' palms are discarded early in the regeneration process, nodular compact calli still produce on average 5% of variant trees. The frequency of 'mantled' palms varies greatly depending on the genotype, demonstrating the need for a diagnostic test to identify abnormal regenerants well before flowering (Rival, 2007). An epigenetic origin for the 'mantled' abnormality was proposed, based on the spatial and temporal instability of this phenotype and the absence of any detectable genetic alteration (Rival, 2000). Both genome-wide and sequence-specific DNA hypomethylation have subsequently been demonstrated in variant tissues compared with their normal counterparts (Jaligot *et al*., 2000, 2002; Matthes *et al*., 2001). Kubis *et al*. (2003) have addressed the possible role of transposable elements and shown that neither their genomic organization nor their methylation status was altered in relationship with the 'mantled' phenotype. To date it has been impossible to establish a molecular-based screen for material carrying the abnormality because there is considerable variability in the DNA methylation level between independent samples of abnormal material. In addition, differences in the patterns of DNA methylation between materials with normal or abnormal phenotype appear to be dependent on the genotype (Jaligot *et al*., 2000, 2004).

Plant genomes encode three well-characterized classes of DNMTs (Finnegan and Kovac, 2000; Goll and Bestor, 2005; Pavlopoulou and Kossida, 2007). The METI methyltransferase gene family was isolated by virtue of its extended similarity with the catalytical domain of bacterial and mammalian proteins (Finnegan and Dennis, 1993). In *Arabidopsis*, AtMETI is the main maintenance methyltransferase targeting symmetric CpG dinucleotides (Kishimoto *et al*., 2001); it has also been shown to have some *de novo* methylation activity on silenced transgenes (Chan *et al*., 2006). *Arabidopsis* antisense-*METI* plants are viable, but display a number of developmental abnormalities that become progressively more severe during successive generations of inbreeding (Finnegan *et al*., 1996; Ronemus *et al*., 1996). AtMETI activity is essential for embryogenesis and the development of viable seed (Xiao *et al*., 2006). The chromomethylase (CMT) family, which is characterized by the presence of a chromatin-associated domain (chromodomain) embedded within the catalytic domain of the protein, is unique to the plant kingdom (Henikoff and Comai, 1998). This family of enzymes maintain methylation at symmetrical CpHpG sequences, where H is either A, G, or T (Lindroth *et al*., 2001; Papa *et al*., 2001) and play a role in the maintenance of RNA-directed methylation of cytosines in an asymmetric CpHpH sequence context (Bender, 2004). *In vitro*, AtCMT3 interacts with the amino-terminal tail of histone H3 marked with both trimethyl-lysine 9 and trimethyl-lysine 27 (Lindroth *et al*., 2004) suggesting that histone modifications may regulate the activity of CMT3. The third class of plant DNMT, the domain-rearranged (DRM) family, has homology to the Dnmt3 *de novo* methyltransferases found in mammals, but the conserved motifs that comprise the catalytic domain of DRM have undergone a circular permutation with respect to other DNMTs (Cao *et al*., 2000). Small RNA molecules target DRM activity to DNA where DRMs establish methylation at previously unmethylated sites in all sequence contexts (Cao and Jacobsen, 2002*a*; Chan *et al*., 2004, 2006). The DRM and CMT methyltransferases in *Arabidopsis* are partially redundant as single mutants have no phenotype, whereas the *drm1 drm2 cmt3* triple mutant loses non-CpG methylation and shows a range of developmental abnormalities (Chan *et al*., 2006).

To determine whether the decrease in DNA methylation that is associated with the occurrence of the 'mantled' phenotype (Jaligot *et al*., 2000, 2002, 2004) can be explained by down-regulation of DNA methyltransferase expression, cDNAs representing the MET, CMT, and DRM methyltransferase families from oil palm were isolated. The expression of each gene in normal and abnormal callus material was compared. The expression of both *EgMET1* and *EgCMT1* increased in abnormal calli where there was a decrease in DNA methylation, indicating that there is no simple correlation between the level of gene expression and DNA methylation in this material.

Materials and methods

Plant material

Oil palm (*Elaeis guineensis* Jacq.) embryogenic calli (nodular-compact calli and fast-growing calli) were obtained from CNRA La

Mé Research Station (Côte d'Ivoire), FELDA (Malaysia), and United Plantations (Malaysia) through the *in vitro* culture of leaf explants, according to Pannetier *et al.* (1981). Immature inflorescences (size of external spathe ranging from 10 cm to 13 cm) from adult regenerants of oil palm obtained through *in vitro* somatic embryogenesis (according to the protocol described by Pannetier *et al.*, 1981) were sampled at FELDA Agricultural Services plantations in Malaysia.

Clonal lines LMC458 and LMC464 were investigated during a previous study on global DNA methylation rates (Jaligot *et al.*, 2000). The X1/X2/X3 triplet has been used in previous work aimed at characterizing an oil palm defensin gene as a putative early marker of the 'mantled' somaclonal variation (Tregear *et al.*, 2002). This series of nodular-compact calli were derived from a normal seed-derived palm (X1), a normal regenerant palm previously cloned from this seed-derived palm (X2), and a 'mantled' regenerant palm cloned from the same seed-derived palm (X3).

Isolation of full-length cDNAs

Total RNA was isolated from inflorescences and callus cultures as described by Morcillo *et al.* (2006). Two complementary homology-based approaches were used: a PCR-based strategy using degenerate primers and screening of oil palm cDNA libraries. Isolation of partial cDNA sequences for oil palm DNMT genes was undertaken through the screening of cDNA libraries with a partial *EgMET1* cDNA clone [obtained by reverse-transcription (RT)-PCR using degenerate primers corresponding to conserved motifs IX and X] and through the data mining of the oil palm EST database of Jouannic *et al.* (2005). The nodular-compact callus oil palm cDNA library was constructed using the Lambda ZAPII kit (Stratagene, La Jolla, CA, USA), and screened as described in Adam *et al.* (2006).

To extend these partial cDNA clones, the CODEHOP (Consensus Degenerate Hybrid Oligonucleotide Primers) approach (Rose *et al.*, 2003) was used for the design of degenerate primers anchored to conserved structural motifs for each of the three DNMT families. These primers were used to amplify cDNA generated by the reverse transcription of total RNA from callus or immature oil palm inflorescences through the ImProm II™ reverse transcription system (Promega, Madison, WI, USA). The 5′ and 3′ ends of the transcripts were recovered through the RACE (rapid amplification of cDNA ends), using the SMART™ RACE cDNA amplification kit and the Advantage®2 PCR enzyme system (both from Clontech, Mountain View, CA, USA). Amplified fragments were cloned into the pGEM®-T easy vector (Promega) and sequenced (Cogenics, Meylan, France). The complete coding region for each gene was assembled and then verified by overlapping RT-PCR and sequencing. The primers used in this study are listed in Table S1 in Supplementary data available at *JXB* online.

To determine whether there are alternate splice products produced by the three DNMT genes, a tiling PCR approach was used. Overlapping fragments, ranging in size from 405 bp to 1356 bp, were amplified from cDNA prepared for total RNA isolated from nodular-compact and fast-growing calli, respectively, using the high-fidelity Accuprime Taq polymerase (Invitrogen) according to the manufacturer's specifications. The length of fragments amplified from nodular-compact and fast-growing calli cDNAs were compared by separation on 1% agarose and sequencing the products. Primers used for this analysis are presented in Table S1 in Supplementary data available at *JXB* online.

The nucleotide sequences reported in this paper have been submitted to EMBL/Genbank data libraries under accession numbers EU117216, EU117217, and EU117218.

Sequence analysis

Sequence alignments of the deduced protein products were performed using CLUSTALW (Thompson *et al.*, 1994) available at EBI (http://www.ebi.ac.uk/Tools/clustalw/) and the alignment was adjusted manually. Comparisons with sequence databases were performed with BLASTX (Altschul *et al.*, 1997) accessible through the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Identification of the characteristic structural motifs within the protein sequences was conducted using both InterProScan (http://www.ebi.ac.uk/InterProScan/index.html) and Motif Scan (http://hits.isb-sib.ch/cgi-bin/PFSCAN) (Falquet *et al.*, 2002).

Southern blot analysis

The probes used to determine the copy number were amplified from genomic oil palm DNA. For each DNMT family, the primers (Table S2 in Supplementary data available at *JXB* online) targeted highly conserved motifs to maximize the likelihood of detecting other members of the each gene family. The identity of the three genomic fragments was verified by cloning and sequencing.

Oil palm genomic DNA was extracted from immature inflorescences according to Rival *et al.* (1998) and 15 μg DNA was digested with either *Eco*RI, *Hin*dIII, *Bam*HI (Fermentas) according to the supplier's recommendations. DNA fragments were separated on 0.7% agarose and transferred to Hybond-N^+ membrane (Amersham) according to the manufacturer's specifications. High stringency hybridization was performed at 65 °C in buffer containing 1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, and 7% SDS. The filters were stripped and reprobed at 55 °C to identify closely related genes in a low stringency approach. Standard washing conditions were used for high stringency hybridization (two washes, each of 10 min in 2× SSC, 0.1% SDS at room temperature, two washes, each of 10 min in 0.1× SSC, 0.1% SDS at room temperature, followed by one wash 0.1× SSC, 0.1% SDS at 65 °C). For low stringency hybridization the washing conditions were two washes, each of 10 min in 2× SSC, 0.1% SDS at room temperature, and two washes, each of 10 min in 1× SSC, 0.1% SDS at room temperature. The filters were exposed and then washed again in 0.1× SSC, 0.1% SDS at room temperature. There was no change in the hybridization pattern after this final wash.

Quantitative RT (qRT)-PCR

For each DNMT, gene-specific oligonucleotides were designed (Table S2 in Supplementary data available at *JXB* online). Amplification mixtures (20 μl per reaction) contained 1× Platinum *Taq* buffer (Invitrogen), 3.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 16 pmol of each primer, 2.5× SYBR Green I (Molecular Probes, Eugene, OH, USA), 0.8 U of Platinum *Taq* DNA polymerase (Invitrogen), and an oil palm cDNA aliquot corresponding to 25 ng of total RNA. Reactions were run on a Rotor-Gene 2000 real-time cycler (Corbett Research, Sydney, Australia). Cycling conditions were as follows: 5 min at 94 °C, 40 cycles of 94 °C/15 s; 60 °C/15 s; 72 °C/20 s. Triplicate reactions were performed for each cDNA template with each primer pair. A 'no-template' control was included to monitor the formation of non-specific products. The oil palm elongation factor EgEF1-α1 (GenBank accession no. AY550990) was used as an internal control for each analysis. The primers used for these analyses are listed in Table S3 in Supplementary data available at *JXB* online. The relative amount of cDNA in each sample was determined by comparative quantitation as described in Finnegan *et al.* (2005), to generate a relative concentration. The data are presented as a ratio of a ratio; for example, $(EgMET1/EgEF\text{-}\alpha1)^{\text{fast-growing callus}}/(EgMET1/EgEF\text{-}\alpha1)^{\text{nodular-compact callus}}$. The identity of the qRT-PCR products was verified by sequencing.

Statistical analysis

Log_{10}-transformed data for each gene were averaged over the technical replicates of each clonal-line/tissue-type combination, adjusted for housekeeping gene level, then analysed as a randomized block design with tissue type as treatment, and clonal lines as blocks. Differences between the resulting means were then back-transformed to ratios of (geometric) means on the original scale, for plotting in Fig. 2B. The SED bars shown in this figure are approximations only.

Results

Isolation of full-length cDNAs encoding oil palm DNMTs homologues and structural analysis of the deduced protein product

Methyltransferase1 (EgMET1): A short (135 bp) fragment of a *MET*-like gene was isolated by RT-PCR of oil palm callus RNA, using degenerate primers corresponding to conserved motifs IX and X of *MET*-like genes from other plants. Using this fragment as a probe to screen an oil palm callus cDNA library, a partial cDNA about 2 kb in length that encompassed the 3' end of a *METI* orthologue was isolated. The remainder of the cDNA sequence was isolated by PCR using primers based on CODEHOP design principles (Rose *et al*., 2003) on a mixed cDNA template, followed by 5' RACE. A full-length *EgMET1* cDNA of 5306 bp (GenBank accession number EU117216), encoding a predicted protein of 1543 amino acid residues with a calculated mass of 174.3 kDa (BioEdit Software v7.0.9; Ibis Biosciences), was assembled. The comparison of the deduced amino acid sequence with DNMTs from other plants revealed very high overall sequence identity with DNMTs of the METI family, particularly those from the monocotyledonous plants, maize (67%) and rice (65%) (Table 1). The sequence identity with MET-like proteins from dicotyledonous plants, tobacco (62%) and *Arabidopsis* (57%), was somewhat lower. The predicted product of this cDNA includes the protein motifs characterizing this family (Fig. S1 in Supplementary data available at *JXB* online), and therefore the gene product was designated as *Elaeis guineensis* MET1 (EgMET1).

Chromomethylase (EgCMT): When searching the oil palm EST database of Jouannic *et al*. (2005) a 260 bp sequence was identified, the putative translation product of which displayed high sequence identity (53–58%) to CMT-type proteins found in *Arabidopsis*, rice, and maize. This fragment was used to isolate the corresponding full-length cDNA sequence through the use of CODEHOP degenerate primers and 5′- and 3′-RACE. A complete cDNA of 3197 bp was obtained (GenBank accession number EU117217), which encoded a predicted protein of 925 amino acid residues with a calculated mass of 103.5 kDa. Comparison of the deduced amino acid sequence with other higher plant CMTs (Table 1) revealed the highest identity (64%) to the chromomethylase ZmCMT1/Zmet2a of *Zea mays* (Papa *et al*., 2001) and strong overall similarity to other plant CMTs. All the domains characteristic of the CMT family, including the signature chromodomain between motifs II and IV of the catalytic domain, were identified (Fig. S1 in Supplementary data available at *JXB* online) and so the gene product was designated as *Elaeis guineensis* CMT1 (EgCMT1).

Table 1. *Main structural similarities between the isolated oil palm sequences and higher plant DNMT sequences available in public databases*

This table displays the highest scores obtained through the BLASTX program.

	Sequences		
	METI	CMT	DRM
Oryza sativa Accession no.	AAP44671.1	AAN60988.1	ABF93591.1
% Identity	65	62	55
% Similarity	79	75	70
Zea mays Accession no.	AAC16389.1	AAK11516.1	AAF68437.1
% Identity	67	64	54
% Similarity	81	76	68
Arabidopsis thaliana Accession no.	AAA32829.1	AAK69756.1	AAF66129.1
% Identity	57	55	53
% Similarity	71	70	69
Nicotiana tabaccum Accession no.	BAF36443.1	BAC53936.1	BAC67060.1
% Identity	62	59	59
% Similarity	76	73	74

Domain-rearranged methyltransferase (EgDRM): In the absence of any cDNA sequence for an oil palm DRM-type gene, the cloning of this DNMT family was initiated by directly implementing a CODEHOP-based PCR on oil palm cDNA. This strategy, followed by 5′- and 3′-RACE gave a full-length cDNA of 2477 bp (GenBank accession number EU117218), encoding a predicted protein of 591 amino acid residues and an estimated mass of 66.9 kDa. The sequence was most similar to NtDRM1 from tobacco (59% identity) (Wada *et al*., 2003), whereas its similarity with the rice and maize sequences (55% and 54% of identity, respectively), was substantially lower than was seen for METI-type and CMT-type sequences (Table 1). The predicted protein has the different domains characteristic of plant DRMs (Fig. S1 in Supplementary data available at *JXB* online), including two ubiquitin-associated domains as for maize Zmet3. By contrast, there are three ubiquitin-associated domains in DRM2 from *Arabidopsis* (Cao *et al*., 2000). The gene product was designated as *Elaeis guineensis* DRM1 (EgDRM1).

The oil palm genome encodes small gene families of MET and DRM methyltransferases

Southern blot experiments were used to estimate the number of genes encoding each DNMT gene family in oil palm. Figure 1A shows that genomic DNA probes for the genes *EgMET1* and *EgCMT1* each hybridized to a unique fragment, independent of the restriction enzyme used. The *EgDRM1* probe also hybridized to a unique fragment when genomic DNA was cleaved with either *Eco*RI or *Bam*HI, but when DNA was digested with *Hin*dIII, this probe hybridized with two fragments consistent with the fact that the probe contains one *Hin*dIII cleavage site. These data indicate that the oil palm genome encodes a single copy each of the *EgMET1*, *EgCMT1*, and *EgDRM1* genes.

When reprobed at lower stringency, a more complex hybridization pattern was observed for *EgDRM1* with all enzymes used and for *EgMET1* when genomic DNA was cut with *Hin*dIII and *Bam*HI, but not for *Eco*RI-cut DNA (Fig. 1B). The increased number of hybridizing fragments suggests that these genes belong to small multigene families, each with at least two members. By contrast, even after prolonged exposure of the membrane, no additional DNA fragments were detected with the *EgCMT1* probe. The probe fragment for *EgCMT1* covers the distal end of the chromodomain and conserved motifs IV–VIII and was therefore expected to identify other members of this gene family.

These three DNMTs have been compared against the 17 009 oil palm ESTs that are deposited in the GenBank (Jouannic *et al*., 2005; Ho *et al*., 2007) to look for other representatives of the three DNMT families. While no match with EgMET1 could be found, EgCMT1 matched the shoot apex EST that was originally detected in the database of Jouannic *et al*. (2005) (accession CN599427.1). EgDRM1 (nucleotides 1094–1300) matched only the first 200 bp of accession EL691809.1 (597 bp) isolated from root tissues by Ho *et al*. (2007).

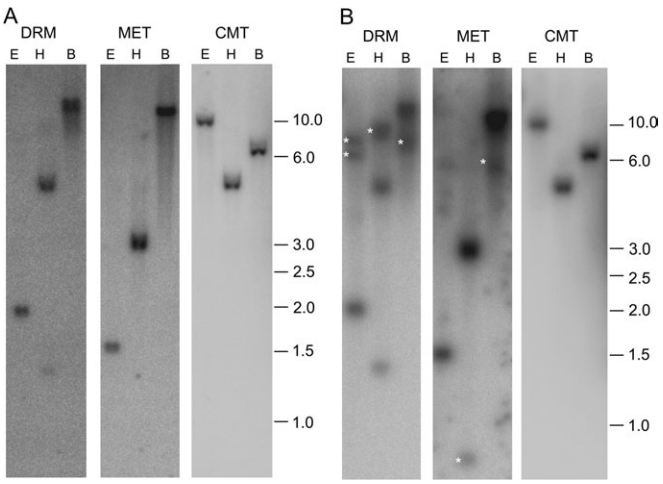

Fig. 1. Copy number determination of oil palm DNMTs by Southern blot analysis. (A) High-stringency hybridization; (B) low-stringency hybridization. Oil palm genomic DNA was digested with *Eco*RI (E) *Hin*dIII (H), or *Bam*HI (B). The membranes were probed with genomic DNA fragments corresponding to *EgDRM1* (DRM), *EgMET1* (MET), or *EgCMT1* (CMT), respectively.

The expression of EgMET1 and EgCMT1 *is increased in fast-growing calli versus nodular-compact calli*

The expression of the three DNMTs has been investigated at the embryogenic callus stage of the micropropagation process (Pannetier *et al*., 1981) for two different callus types isolated from three different clonal lines, LMC458, LMC464, and FC2063. For all three clonal lines, both *EgMET1* and *EgCMT1* genes showed higher transcript levels in fast-growing callus compared with nodular-compact callus (Fig. 2A). The increased transcript abundance ranged from 1.7- to 5.6-fold for *EgMET1* and from 3.3- to 4.9-fold for *EgCMT1*. The relative expression of *EgDRM1* in the two types of callus differed between the clonal lines (Fig. 2A). Transcripts of *EgDRM1* were more abundant in fast-growing calli from two of the lines (2.0-fold in LMC458 and 2.9-fold in LMC464) but less abundant in the fast-growing callus of line FC2063 (0.6-fold).

To determine whether the differences in transcript abundance relate to callus type, transcript levels were compared in fast-growing callus and nodular-compact

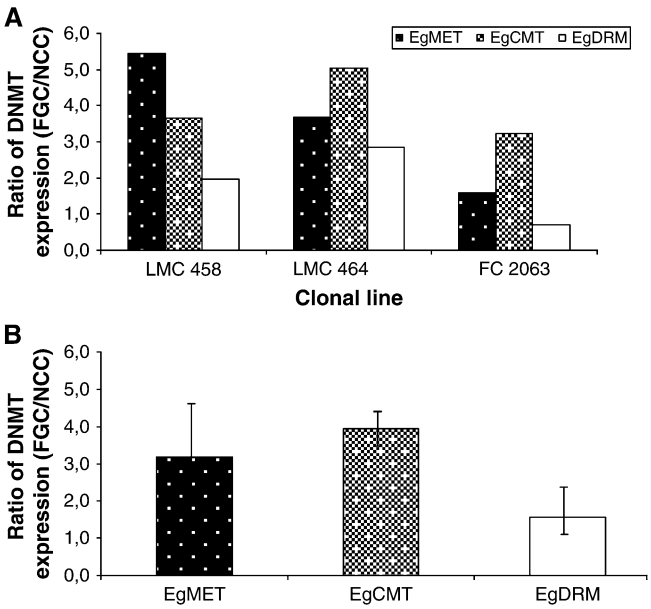

Fig. 2. Comparative *EgMET1*, *EgCMT1*, and *EgDRM1* expression in nodular-compact callus (NCC) and fast-growing callus (FGC). (A) Relative abundance of transcripts for each DNMT gene in the individual clonal lines. (B) Geometric mean of transcript abundance ±standard error. The elongation factor *EgEF1-α1* gene (GenBank accession no. AY550990) was selected as an internal control for each sample. The results are expressed as mean values of the ratio of normalized DNMT expression in fast-growing calli versus nodular-compact calli.

callus across the three clonal lines as it was not possible to obtain sufficient material to allow biological replication within each clonal line (Fig. 2B). A statistical analysis of these data (see Materials and methods) demonstrated that the abundance of *EgCMT1* transcripts was significantly higher in fast-growing callus compared with nodular-compact callus (P=0.008). Even though the level of *EgMET1* transcripts in fast-growing callus from each of the clonal lines was higher than in their normal counterparts, there was considerable variability in the relative transcript levels across clonal lines. When the average of *EgMET1* transcript levels was taken across the three clonal lines, there was no significant difference between *EgMET1* transcript levels in the two callus types (P=0.08). A larger sample size would be required to demonstrate whether or not *EgMET1* is elevated in all fast-growing calli. Similarly, there was no significant difference in the abundance of *EgDRM* transcripts between callus types (P=0.14).

Lines LMC458 and LMC464 are closely related genetically and share a similar *in vitro* history as they were produced simultaneously in the same laboratory at CNRA in Côte d'Ivoire (Table 2). FC2603, which was produced at the UP Laboratories in Malaysia, differs from the other two in both genetic origin and tissue-culture background (Table 2). Thus the diversity in the genetic background of these lines and/or the different culture regimes used might account for the variability in the expression of *EgMET1* and the different behaviour of *EgDRM1*.

There is no difference in DNMT expression in nodular-compact calli originating from normal versus 'mantled' somaclones

The increase in *EgMET1* and *EgCMT1* transcript levels in fast-growing calli (Fig. 2) could relate to the altered physiology of the calli, or the capacity to give rise to abnormal plantlets. To distinguish between these possibilities the transcript abundance of the three DNMT genes was measured in nodular-compact calli that were derived from a normal seed-derived palm (X1), a normal regenerant palm previously cloned from this seed-derived palm (X2), and a 'mantled' regenerant palm cloned from the same seed-derived palm (X3). In this case, the calli differed only in their 'mantled' status, with X3 callus giving rise to a high level of 'mantled' regenerants. There was no difference in anatomy or physiology between these calli (Besse *et al.*, 1992). In terms of abnormality, the X2 culture represents an intermediate situation; it was initiated from a normal palm, but has undergone an extra cycle of somatic embryogenesis-based tissue culture when compared with X1 callus (Tregear *et al.*, 2002). To eliminate any effect of culture age, cultures X1, X2, and X3 were initiated at the same time.

The relative transcript levels for all three DNMT genes were comparable across these three different callus lines (Fig. 3), independent of the phenotype of the plant from which the callus was derived.

EgMET1 *expression is elevated in immature inflorescences originating from 'mantled' somaclones compared with their normal counterparts*

DNMT expression was compared in immature inflorescences originating from either normal or 'mantled' palms regenerated from each of two clonal lines, FC1406 and FC1726. In both clonal lines the level of *EgMET1* transcripts was increased >2-fold in 'mantled' material compared with the normal inflorescences (Fig. 4). In another line, FC166, for which there are regenerants displaying normal, 'mantled', and a normal-revertant floral phenotype, there was a small increase in *EgMET1* transcripts in the 'mantled' inflorescences (1.38) when compared with normal inflorescences (Fig. 5), whereas essentially no difference was found in the normal-revertant (0.83). A statistical analysis of the relative expression of *EgMET1* across these three clonal lines indicated that there was no significant difference between 'mantled' and normal tissues (P=0.137). A larger sample size would be required to determine whether *EgMET1* expression is increased in 'mantled' inflorescence tissues.

Table 2. *Genetic origin of the plant material analysed*

Conventionally, each cross is given in the form: male parent×female parent.

Clonal line	Origin	Organ	Male parent	Female parent
LMC 458*	CNRA, Ivory Coast	Embryogenic calli	DA 5 D×DA 5 D	LM 5 T×LM 5 T
LMC 464*	CNRA, Ivory Coast	Embryogenic calli	DA 5 D×DA 5 D	LM 5 T×LM 5 T
X1, X2, X3	UP, Malaysia	Embryogenic calli	DD12	L718T×L322P
FC2063	FELDA, Malaysia	Embryogenic calli	(UR434/10×UR419/2)	(L5T×L312P)
FC1406	FELDA, Malaysia	Immature inflorescences	(D10D×D115D)	(L718T×L322P)
FC1726	FELDA, Malaysia	Immature inflorescences	(L404D×L270D)	1928
FC166	FELDA, Malaysia	Immature inflorescences	D3D	L238T

* LMC458 and LMC464 were obtained from two different mother palms originating from the same cross. Consequently, while they are related they must be considered as different genotypes.

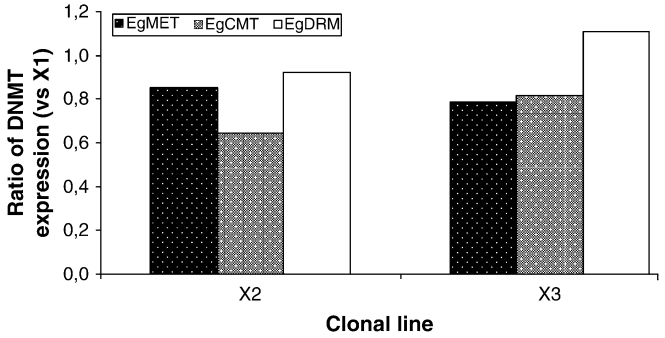

Fig. 3. Comparative *EgMET1*, *EgCMT1*, and *EgDRM1* expression in three different origins of NCC oil palm embryogenic calli: X1 originated from the cloning of a seed-derived mother palm, X2 from the cloning of a normal regenerant of this mother palm, and X3 from a 'mantled' regenerant of the same mother palm. The results are expressed as mean values of the ratio of normalized DNMT expression (relative to *EgEF1-α1*) in X2 and X3 callus lines versus the normal X1 line.

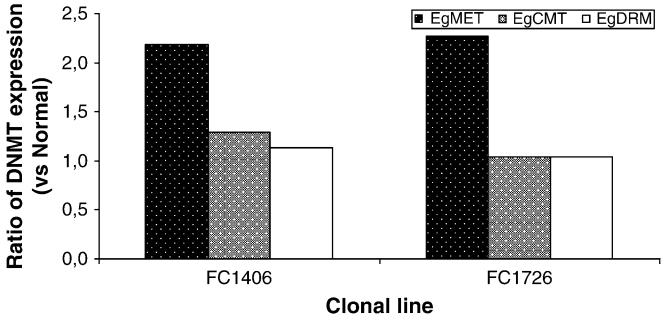

Fig. 4. Comparative *EgMET1*, *EgCMT1*, and *EgDRM1* mRNA accumulation in immature inflorescences of normal and 'mantled' oil palm somaclones from clonal lines FC1406 and FC1726. The results are expressed as mean values of the ratio of normalized DNMT expression (relative to *EgEF1-α1*) in 'mantled' versus normal material.

The variability of *EgMET1* transcript abundance between samples of 'mantled' inflorescence tissue may relate to genetic differences between plant lines (Table 2). Alternatively, the length of time since plant regeneration may contribute to the differences observed in *EgMET1* transcript abundance as the 'mantled' phenotype gradually reverts towards normality (Rival, 2007). Plants of clonal line FC166, which were planted in 1989 and sampled in 2002 (12 years in the field), show a very small increase in *EgMET1* expression, whereas the younger clonal lines FC1406 and FC1726 from which palms were planted in 2002 and sampled in 2004 (2 years in the field), have higher *EgMET1* expression in the abnormal inflorescences.

There was no difference in the transcript abundance of *EgCMT1* (P=0.123) and *EgDRM1* (P=0.144) between normal and 'mantled' or normal-revertant materials from any of the three genetically distinct, clonal lines (Figs 4, 5; Table 2).

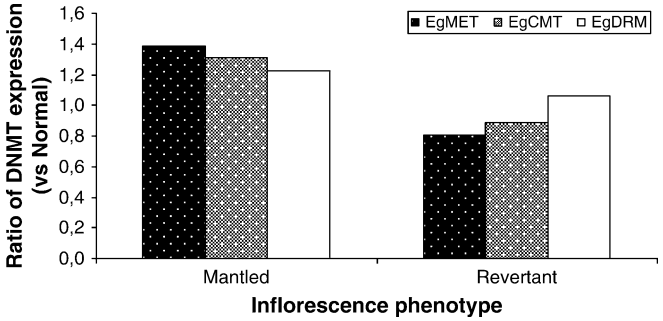

Fig. 5. Comparative *EgMET1*, *EgCMT1*, and *EgDRM1* mRNA accumulation in immature inflorescences of normal, 'mantled', and revertant oil palm somaclones from clonal line FC166. The results are expressed as mean values of the ratio of normalized DNMT (relative to *EgEF1-α1*) in 'mantled' versus normal and normal-revertant versus normal.

Hypomethylation of DNA in fast-growing callus is not associated with alternate splicing of DNMT transcripts

The real-time PCR analysis of DNMT transcripts described above measured the abundance of transcripts covering the catalytic domain of the protein, suggesting that these encode functional methyltransferases. The entire transcript for each of the DNMTs was compared in nodular-compact callus and fast-growing callus using a tiling PCR strategy (see Materials and methods) to determine whether there was any evidence for alternately spliced transcripts that might be associated with hypomethylation of DNA in fast-growing callus. The transcripts for *EgMET1*, *EgCMT1*, and *EgDRM1* in fast-growing LMC464 callus were identical to those found in nodular-compact callus of the same clonal line (data not shown).

A similar comparison of the RT-PCR products from normal and 'mantled' inflorescence indicated that the there was no change in the transcripts between normal and abnormal inflorescences. This suggests that the decrease in DNA methylation in abnormal callus or inflorescence tissues cannot be explained by the production of non-functional DNMT proteins.

Discussion

Representatives of the three plant DNA methyltransferase gene families have been isolated from oil palm, using a homology-based strategy. The predicted proteins of the three genes identified in this study, *EgMET1*, *EgCMT1*, and *EgDRM1*, show high similarity to the DNMTs identified in other plant species (Goll and Bestor, 2005). As expected, the oil palm MET1 and CMT1 are more closely related to the corresponding methyltransferases from monocotyledonous plants than to those of dicotyledonous species. By contrast, the predicted EgDRM1 protein is most similar to NtDRM1 of tobacco, a dicotyledonous plant.

Just as in other plants, *EgMET1* and *EgDRM1* are members of small multigene families (Bernacchia *et al*., 1998; Genger *et al*., 1999; Cao *et al*., 2000; Teerawanichpan *et al*., 2004; Pavlopoulou and Kossida, 2007). Although in some plant species CMTs are also part of a multigene family (Papa *et al*., 2001; Wada, 2005; Pavlopoulou and Kossida, 2007), Southern hybridization data suggest that the oil palm genome, like that of *Brassica rapa* (Fujimoto *et al*., 2006), encodes a single CMT. Where there are multigene families encoding DNMTs, it seems that frequently one gene of the family is dominant. *AtMETI*, which is expressed at a much higher level than the other members of this gene family, is the only family member that has been shown to have methyltransferase activity (Genger *et al*., 1999). Both the carrot and rice genomes encode two METI-like proteins; in both these species, one gene is more highly expressed than the other, suggesting that it plays a dominant role in maintaining DNA methylation (Bernacchia *et al*., 1998; Teerawanichpan *et al*., 2004). Similarly, *AtDRM2* is expressed at a much higher level than *AtDRM1* (Cao *et al*., 2000), and the loss of asymmetric methylation in *Atdrm2* but not *Atdrm1* mutants suggests that AtDRM2 is the more active *de novo* methyltransferase (Cao and Jacobsen, 2002*a*, *b*).

In the case of oil palm, *EgMET1* is probably the more highly expressed member of this family as it was the only gene isolated through cDNA screening. The CODEHOP strategy used to isolate *EgDRM1* also yielded a unique product from a cDNA template, suggesting that it too may be the dominant family member. Given that sequences corresponding to the other members of the MET and DRM gene families could not be isolated from either callus or inflorescence cDNAs, it is unlikely that they play a significant role in methylating DNA, at least in these tissues. Therefore the analyses of gene expression in calli and inflorescences focused on the genes identified through cDNA sequences.

Global hypomethylation is not associated with decreased expression of genes encoding DNMTs

In previous studies, fast-growing oil palm calli and immature 'mantled' inflorescences were found to show significant hypomethylation of genomic DNA when compared with their normal counterparts (Jaligot *et al*., 2000, 2002, 2004). The average decrease in methylation level was 19.3% in fast-growing calli compared with nodular-compact calli from lines LMC458 and LMC464 (Jaligot *et al*., 2000), whereas in 'mantled' inflorescences DNA methylation was on average 7.4% lower than in normal inflorescences (E Jaligot and A Rival, unpublished data). A comparison of the transcript abundance of each of the DNMT genes was made to determine whether the decrease in DNA methylation in abnormal callus or inflorescence tissues was associated with decreased abundance of any of the DNMT transcripts identified in this study. Contrary to expectation, the abundance of *EgCMT1* transcripts was found to increase significantly in fast-growing callus compared with the nodular-compact callus from which it arose. Similarly, *EgMET1* transcripts were increased in all three fast-growing callus lines, but this increase was not statistically significant. In immature 'mantled' inflorescences where the decrease in DNA methylation is less marked, transcript levels of *EgMET1*, but not *EgCMT1*, were elevated in the three clonal lines examined. The increase in abundance was smaller than in fast-growing callus and was not statistically significant, perhaps due to the small number of samples available for analysis. These data indicate that the reduced levels of DNA methylation observed in fast-growing calli and 'mantled' palms is not a consequence of decreased expression of either the *de novo* or maintenance DNA methyltransferases.

These observations parallel a major unresolved paradox in cancer biology (Laird and Jaenisch, 1994) where a global decrease in DNA methylation in many tumour cell genomes occurs despite evidence for increased levels of transcripts for both *de novo* and maintenance DNA methyltransferases (reviewed in Clark and Melki, 2002). Overexpression of a novel splice variant of Dnmt3b has been associated with hypomethylation in tumour cells (Kanai *et al*., 2004; Klinck *et al*., 2008). However, no evidence for alternate splicing of DNMT transcripts was found in either fast-growing callus or immature 'mantled' inflorescences. In addition to global hypomethylation, there is extensive hypermethylation of CpG islands that results in the inactivation of many genes, including tumour suppressor genes (Clark and Melki, 2002; Clark, 2007). If 'mantledness' is associated with hypermethylation of genes that regulate flower development this might further extend the parallel with the epigenetic changes seen in cancer cells.

Conclusions

The molecular basis for the 'mantled' phenotype remains unknown, but the instability of the phenotype and the absence of any genetic change strongly point to an aberration in epigenetic regulation (Rival *et al*., 1998). While initiation of the 'mantled' status may be associated with either global hypomethylation of DNA or elevated levels of *EgMET1* and/or *EgCMT1* transcripts in calli, maintenance of the phenotype does not depend on altered methyltransferase expression. The level of DNMT transcripts was not changed in nodular-compact calli derived from 'mantled' palms that show a high propensity to regenerate 'mantled' palms compared with calli that give rise to predominantly normal palms. This suggests that elevated expression of *EgMET1* and *EgCMT1* in fast-growing calli probably reflects differences in physiology of this callus type rather than being directly associated

with 'mantledness'. Consistent with this, previous studies have demonstrated significant differences in metabolic traits between the nodular-compact and fast-growing oil palm calli. These include protein patterns and content (Marmey *et al*., 1991) as well as the histological structure and accumulation of endogenous growth regulators (Besse *et al*., 1992).

The link, if any, between genome-wide hypomethylation and the elevated expression of *EgMET1* and *EgCMT1* in fast-growing callus is not yet clear. Perhaps there is some feedback between genome methylation and methyltransferase expression, or perhaps the transcription of these genes is directly modulated by DNA methylation leading to increased expression in a hypomethylated genome. It appears that there is a dynamic interaction between methylation of a genome and the activity of the enzymes, DNA methyltransferases and demethylases, that together give rise to the final genome-wide pattern of DNA methylation. For example, it has been shown that the gene encoding the *Arabidopsis* DNA demethylase, *ROS1*, is transcriptionally down-regulated in plants that have mutations in the RNA-directed DNA methylation pathway or that have decreased CpG methylation due to mutation of *METI* (Huettel *et al*., 2006; Penterman *et al*., 2007). In these plants, loci demethylated by ROS1 accumulate DNA methylation, suggesting a mechanism whereby genome-wide hypomethylation could result in local hypermethylation and gene silencing.

Research is now focusing on DNA methylation around candidate marker genes, in relation to the 'mantled' somaclonal variation. Orthologues of the MADS-box genes involved in the formation of floral organs have been recently identified in the genome of oil palm (Adam *et al*., 2006; Syed Alwee *et al*., 2006), and research has shown that oil palm B-type MADS-box genes display differential transcript levels between normal and abnormal inflorescence tissues (Adam *et al*., 2007). A range of genes with altered expression in abnormal tissues has been recently identified through the use of subtractive PCR (SSH) and subsequent macroarray hybridization (T Beulé, unpublished data). Investigating methylation patterns of these target genes will pave the way for understanding the epigenetic mechanisms underlying the induction and maintenance of somaclonal variation in the plant system of the present study.

Supplementary data

Supplementary Table 1: Primers used for the identification of the full-length cDNA sequences of EgMET1, EgCMT1 and EgDRM1, through CODEHOP-PCR, traditional PCR or RACE.

Supplementary Table 2: Primers and probes used for the Southern blot determination of gene copy number.

Supplementary Table 3: Specific oil palm DNMTs primers designed for Real-Time Quantitative PCR analysis.

Supplementary Figure 1: Alignment of DNMTs of the METI (A), CMT (B) and DRM (C) class.

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