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
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 has been 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 (chromodomains) 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., 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, 2002a; 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 *EgMETI* 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 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/PSSCAN) (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 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 EcoRI, HindIII, BamHI (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 NaHPO4, 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 2x SSC, 0.1% SDS at room temperature, two washes, each of 10 min in 0.1x SSC, 0.1% SDS at room temperature, followed by one wash 0.1x 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 MgCl2, 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 EgEF1α (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/EgEF1α)nodular-compact callus/(EgMET1/EgEF1α)fast-growing callus.
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 (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).

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 NidRM1 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).

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

<table>
<thead>
<tr>
<th>Sequences</th>
<th>METI</th>
<th>CMT</th>
<th>DRM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Oryza sativa</em></td>
<td>AAP44671.1</td>
<td>AAN60988.1</td>
<td>ABP93591.1</td>
</tr>
<tr>
<td>Accession no.</td>
<td>% Identity</td>
<td>% Similarity</td>
<td>% Identity</td>
</tr>
<tr>
<td>65</td>
<td>79</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>AAC16389.1</td>
<td>AAK11516.1</td>
<td>AAF68437.1</td>
</tr>
<tr>
<td>Accession no.</td>
<td>% Identity</td>
<td>% Similarity</td>
<td>% Identity</td>
</tr>
<tr>
<td>67</td>
<td>71</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>AAA32829.1</td>
<td>AAK69756.1</td>
<td>AAF66129.1</td>
</tr>
<tr>
<td>Accession no.</td>
<td>% Identity</td>
<td>% Similarity</td>
<td>% Identity</td>
</tr>
<tr>
<td>57</td>
<td>71</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>BAF36443.1</td>
<td>BAC53936.1</td>
<td>BAC67060.1</td>
</tr>
<tr>
<td>Accession no.</td>
<td>% Identity</td>
<td>% Similarity</td>
<td>% Identity</td>
</tr>
<tr>
<td>62</td>
<td>76</td>
<td>74</td>
<td></td>
</tr>
</tbody>
</table>
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 EcoRI or BamHI, but when DNA was digested with HindIII, this probe hybridized with two fragments consistent with the fact that the probe contains one HindIII 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 HindIII and BamHI, but not for EcoRI-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).

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 callus...
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 EgDRM1 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 callus, 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.

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

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

<table>
<thead>
<tr>
<th>Clonal line</th>
<th>Origin</th>
<th>Organ</th>
<th>Male parent</th>
<th>Female parent</th>
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</thead>
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<tr>
<td>LMC 458*</td>
<td>CNRA, Ivory Coast</td>
<td>Embryogenic calli</td>
<td>DA 5 × DA 5 D</td>
<td>LM 5 T × LM 5 T</td>
</tr>
<tr>
<td>LMC 464*</td>
<td>CNRA, Ivory Coast</td>
<td>Embryogenic calli</td>
<td>DA 5 × DA 5 D</td>
<td>LM 5 T × LM 5 T</td>
</tr>
<tr>
<td>X1, X2, X3</td>
<td>UP, Malaysia</td>
<td>Embryogenic calli</td>
<td>DD12</td>
<td>L718T × L322P</td>
</tr>
<tr>
<td>FC2063</td>
<td>FELDA, Malaysia</td>
<td>Embryogenic calli</td>
<td>(UR434/10 × UR419/2)</td>
<td>(L5T × L312P)</td>
</tr>
<tr>
<td>FC1406</td>
<td>FELDA, Malaysia</td>
<td>Immature inflorescences</td>
<td>(D10D × D15D)</td>
<td>(L718T × L322P)</td>
</tr>
<tr>
<td>FC1726</td>
<td>FELDA, Malaysia</td>
<td>Immature inflorescences</td>
<td>(L404D × L270D)</td>
<td>1928</td>
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<tr>
<td>FC166</td>
<td>FELDA, Malaysia</td>
<td>Immature inflorescences</td>
<td>D3D</td>
<td>L238T</td>
</tr>
</tbody>
</table>

* 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.
The variability of \(\text{EgMET1}\) 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 \(\text{EgMET1}\) 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 \(\text{EgMET1}\) 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 \(\text{EgMET1}\) expression in the abnormal inflorescences.

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

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 \(\text{EgMET1}\), \(\text{EgCMT1}\), and \(\text{EgDRM1}\) 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, \(\text{EgMET1}\), \(\text{EgCMT1}\), and \(\text{EgDRM1}\), 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. AtMET1, 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, 2002a, 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 MET1 (Huet 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 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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