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Characterization of oil palm MADS box genes in relation to the mantled flower abnormality

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Abstract

In vitro propagation of oil palm (*Elaeis guineensis* Jacq.) frequently induces a somaclonal variant called 'mantled' abnormality, in which the stamens of both male and female flowers are transformed into carpels. This leads to a reduced yield or complete loss of the harvest of palm oil. The high frequency of the abnormality in independent lines and the high reversal rate suggest that it is due to an epigenetic change. The type of morphological changes suggest that it involves homeotic MADS box genes that regulate the identity of the flower whorls. We have isolated a number of MADS box genes from oil palm inflorescences by a MADS box-directed mRNA display approach. The isolated partial cDNAs included genes that were likely to function at the initial stages of flowering as well as genes that may function in determination of the inflorescence and the identity of the flower whorls. For four genes that were homologous to genes known to affect the reproductive parts of the flower, full length cDNAs were isolated. These were a B-type MADS box gene which may function in the determination of stamen formation, a C-type gene expected to be involved in stamen and carpel formation, and two putative SEP genes which act in concert with the A-, B- and C-type MADS box gene in determining flower whorl formation. The B-type gene EgMADS16 was functionally characterized as a PISTILLATA orthologue; it was able to complement an *Arabidopsis thaliana pi* mutant. Whether EgMADS16, or any of the other EgMADS genes, are functionally involved in the mantled condition remains to be established.

Abbreviations: SEP – SEPALLATA; EgMADS – *Elaeis guineensis* Jacq. MADS box gene; AG – AGAMOUS; PI – PISTILLATA

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a perennial monocot with male and female flowers occurring separately in distinct male and female inflorescences. A succession of several inflorescences of one sex is produced, followed by a succession of the other in alternating cycles (Van Heel et al., 1987). As the breeding cycle of the oil palm is very long, and the progeny of crosses shows large variation in yield, the palm oil industry has invested in the development of new techniques for multiplying superior parents for seed production and elite progeny palms for commercial planting (Basri et al., 2004). In the 1970s, clonal propagation of oil palm via tissue culture was

developed (Jones, 1974; Rabéchault and Martin, 1976). It was successfully adopted resulting in the production of elite palms that are uniform with desirable traits and optimum yield. However, several forms of somaclonal variation, particularly in the form of flower architecture mutants, were regularly observed in the micropropagated palms (Corley et al., 1986). A particularly common flowering abnormality, still occurring in ca. 5% of the tissue cultured plants with optimized protocols, is the 'mantled' phenotype. In affected plants the male productive organs are feminized, with carpelloid structures appearing at the position where normally stamens are formed. This transformation results in loss of productivity of the female inflorescences as the fruits that are formed are usually aborted during development (Eeuwens et al., 2002). A better understanding of the mechanisms underlying the architecture of normal flowers in oil palm will be instrumental to identify the molecular causes for the mantled phenotype. This knowledge may be used to circumvent or solve this particular problem associated with *in vitro* propagation of oil palm.

Extensive studies on flower organogenesis in eudicots have established that the architecture of flowers is largely determined by the activity of a number of homeotic genes containing a conserved MADS box domain. These genes were originally studied in *Antirrhinum majus* and *Arabidopsis thaliana*, which has led to the development of the ABC model of floral organ identity (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). In this classic model, three classes of homeotic gene activities were proposed, called A, B, and C. Expression of A alone specifies sepal formation, combination of AB specifies petal development and the combination of BC specifies stamen formation. Expression of C alone leads to carpel development. More recent studies have included D-function genes that specify development of ovules (Colombo et al., 1995; Angenent and Colombo, 1996) and E-function genes (the SEPALLATA (SEP) genes in Arabidopsis) that are necessary for the specification of the organ identity of petals, stamens and carpels in concert with the ABC genes (Pelaz et al., 2000).

MADS box genes with similarity to the Arabidopsis and Antirrhinum genes have been identified in a large number of dicotyledonous species (reviewed by Purugganan et al., 1995; Theissen et al., 2000; Theissen, 2001). In monocotyledonous plants, MADS box genes have been extensively studied, e.g. in maize (Schmidt et al., 1993; Fischer et al., 1995; Mena et al., 1995; Theissen et al., 1995), rice (Chung et al., 1994, 1995; Greco et al., 1997), orchid (Lu et al., 1993), hyacinth (Li et al., 2001, 2002), and wheat (Murai et al., 1997), but not in monocot trees. Palms represent about half of the subclass *Arecidae* of monocot trees with over 2600 species in the world.

Based on the phenotype of mantled flowers in micropropagated oil palm, with formation of carpelloid structures where normally stamens develop, it is not unlikely that changes in the expression of the MADS box genes specifying the affected flower whorls underlie the mantled phenotype. As a first step in elucidating the molecular mechanisms underlying oil palm flower development and the 'mantled' phenotype, we have isolated 14 MADS box genes from oil palm. Phylogenetic and expression analysis showed that the genes were assigned to several different clades and are likely to perform diverse functions. We have obtained the full length coding sequences of four genes that are likely to be involved in stamen and carpel formation, and functionally characterized the B-type gene EgMADS16 as a PISTILLATA analogue.

Materials and methods

Plant material

Selected adult palms (ortets) were cultured on Murashige and Skoog (MS) media incorporated with 2,4-dichlorophenoxyacetic acid and α-naphthalene acetic acid (NAA) giving rise to 20% calluses at 12 months from inoculation at 3–5% embryogenesis rate (Wong et al., 1997). The embryoids were subcultured on MS media + NAA at reduced concentration to obviate the occurrence of somaclonal variation. Rates of embryoid proliferation and germination varied among the embryoid lines within and between clones. Floral census of 52,000 ramets at the fruiting stage showed 3.3% palms with mantled fruit bunches, coming from 38 out of 71 clones. Fruit mantling therefore occurred sporadically within the embryoid lines of a clone and among clones. Material of oil palm inflorescences from all stages of development and of vegetative shoots were obtained from ortets and

from either phenotypically normal or mantled ramets. The ramets had flowered for a number of years before sampling. Tissues were frozen in liquid nitrogen and stored at −80 °C.

Arabidopsis thaliana plants (ecotype *Col-0*) and *pi-1* mutants (ecotype *Ler*) (Bowman et al., 1989) were grown under normal greenhouse conditions (22 °C, 16-h light/8-h dark cycles). Plants for the complementation experiment were derived from crosses.

MADS domain-directed differential display

Total RNA was isolated according to Schultz et al. (1994) from normal oil palm inflorescence tissue. Several steps in the MADS box directed differential display have been described previously (van der Linden et al., 2002). A MADS box-specific degenerate primer (MADS5′) complementary to the 5′-end of the MADS box consensus sequence (the LIKRIEN protein motif; sequence: 5′-ACCTCRGCRTCRCARAGSAC-3′) was used to synthesize second strand cDNA in a linear amplification procedure at 95 °C for 3 min followed by thirty cycles of denaturation for 30 s at 94 °C, annealing for 90 s at 55 °C and extension for 2 min at 68 °C. Immediately following the linear amplification, PCR was performed using the MADS5′ and an anchored poly-dT primer with non-specific 5′-tail. After two initial PCR cycles (94 °C for 30 s, 40 °C for 2 min and 68 °C for 3 min) 10 pmol of MADS5′ primer in 2.5 μl was added to the PCR mix, and cycling was immediately continued for 30 cycles at 55 °C annealing temperature using the linear amplification cycling conditions described above. One microliter of this PCR product was used in a second PCR with identical conditions, but with a nested degenerate primer (MADS3′: sequence 5′-GTKCTYTGY-GAYGCYGAGGT-3′) instead of the MADS5′ primer. PCR products were separated on a 4% polyacrylamide sequencing gel. Bands of interest were excised, eluted and reamplified with conditions identical to the MADS3′ to poly-dT primer PCR described above. Re-amplified fragments were purified and sequenced directly on an ABI 3700 automatic sequencer using the MADS3′ primer. In addition, the multiple products from the nested PCRs were cloned directly into the T/A cloning vector pCR®2.1-TOPO® vector (Invitrogen, CA), and a number of clones were sequenced on an ABI 3700 automatic sequencer. The nucleotide sequences obtained were analyzed using the Lasergene software package (DNAstar, Madison, USA) and compared to the entries in Genbank using blastx and blastn programs (Altschul et al., 1990). The 5′ missing parts of the cDNA sequences of four MADS box genes (EgMADS16, EgMADS5, EgMADS8, and EgMADS12) were obtained using the 5′/3′ (Rapid Amplification of cDNA Ends) kit of Roche Diagnostics and were analyzed by sequencing. The deduced protein sequences were aligned with the ClustalX program (latest version at: ftp://www.ftp-igbmc.u-strasbg.fr/pub/ClustalX, website: http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX) and the tree was compiled with the neighbour-joining method.

Reverse transcriptase (RT)-PCR

One microgram of total RNA isolated from a range of tissues was used for first strand cDNA synthesis with the cDNA-for-PCR kit of Clontech. The cDNA was synthesized from a poly-dT primer. This cDNA served as a template in PCR using gene-specific primers for most of the EgMADS genes isolated in this study, with 0.5 μl 50× Advantage™ cDNA Polymerase Mix (Clontech Laboratories, Inc.), 1.5 U Advantage™ cDNA Polymerase and 20 μM dNTPs. PCR conditions were 35 cycles of 30 s 95 °C, 45 s 60 °C, 90 s 72°C, preceded by 3 min 95 °C, and followed by 10 min at 72 °C. Control PCR reactions contained the RNA that was used as a template in the cDNA synthesis. Products were analyzed on 1.5% agarose gels.

EgMADS16 functional analysis

Total RNA was isolated from oil palm male inflorescences (23 cm in length). First strand cDNAs (synthesised with the cDNA for PCR kit, Clontech) of RNA samples from several tissues were used as a template for a PCR reaction with EgMADS16-specific primers located in the 5′- and 3′-noncoding regions of the EgMADS16 mRNA. The PCR product was excised from an agarose gel, and subcloned into the PCR21.TOPO vector (Invitrogen). EgMADS16 full length cDNA inserts were confirmed by sequencing.

Subsequently, the *EgMADS16* cDNA was cloned as a *Hin*dIII–*Xba*I fragment under the control of the double 35S enhancer of the

Cauliflower mosaic virus (CaMV) in the the binary pBINPLUS (Van Engelen et al., 1995) based vector pGD121 (derived from pGD120) (Immink et al., 2002). The resulting binary construct (pGD710) was used to transform *Agrobacterium tumefaciens* GV3101, followed by Arabidopsis transformation of wild-type plants using the floral dip method described by Clough and Bent (1998).

Results

Isolation of oil palm MADS box genes

A MADS domain-directed differential display approach was used to isolate oil palm MADS box genes from mRNA of various stages of flowering in both normal and mantled ramets. The three-step PCR procedure includes an asymmetric PCR with the degenerate MADS5′-primer (Van der Linden et al., 2002), and two exponential amplification steps towards the polyA tail with MADS5′ and the nested MADS3′R primer respectively, as described before (Van der Linden et al., 2002). PCR products were either cloned directly, or radioactively labeled and separated on a 4% polyacrylamide gel. In the direct cloning approach, 48 clones were picked randomly and sequenced, yielding eight different MADS box gene-like sequences (see Table 1). The approach using polyacrylamide gel electrophoresis displayed multiple discrete fragments ranging in size from 500–1600 bp. Thirty bands were excised, re-amplified and either sequenced directly or subcloned and sequenced. Fifty percent of the directly sequenced re-amplified bands produced a readable sequence. Nearly all of the obtained sequences turned out to be highly similar to MADS box sequences in blastx and blastn analyses (Table 1). These included 6 of the 8 putative MADS box genes produced by the direct cloning procedure, as well as 6 new MADS box gene fragments. We named the genes EgMADS, for *Eleais guineensis* MADS box gene.

A tree of EgMADS translated protein sequences was constructed to determine in which clades the oil palm MADS box genes were classified (Figure 1). According to the cladogram, the oil palm MADS box genes belong to various clades and subclades. In model dicots and monocots, these clades were shown to perform distinct roles in the flowering processes (Theissen et al., 2000). Several of the EgMADS genes were highly homologous or even identical to recently released

Table 1. Overview and annotation of isolated oil palm MADS box genes

Gene	Genebank accession number	Homology to	Putative function	Homology to oil palm genes
EgMADS2	AJ581467	AP1	Floral initiation, sepal/petal identity	Similar to PAS3 and EgMADS10
EgMADS3	AJ581468	AP1	Floral initiation, sepal/petal identity	Identical to PAS1
EgMADS4	AJ581469	AGL6/13	Unknown	
EgMADS5	AJ581470	SEP1 (AGL2)	Flower identity	Identical to PAD4
EgMADS6	AJ581471	AGL33	Unknown	
EgMADS7	AJ581460	AGL33	Unknown	
EgMADS8	AJ581461	SEP3 (AGL9)	Petal/stamen/carpel identity	highly similar to PAD1
EgMADS9	AJ581462	AGL20 (SOC1)	Floral initiation	Similar to OpMADS1
EgMADS10	AJ581464	AP1	Floral initiation, sepal/petal identity	Similar to PAS3
EgMADS11	AJ581465	AGL20 (SOC1)	Floral initiation	Similar to OpMADS1
EgMADS12	AJ581463	AG	Stamen/carpel identity	
EgMADS13		AGL20 (SOC1)	Floral initiation	Identical to OpMADS1 (AF207699)
EgMADS14	AJ581466	AP1	Floral initiation, sepal/petal identity	Identical to PAS3
EgMADS16		PISTILLATA	Petal/stamen identity	Identical to FEG1 (Acc. Nr AF227195)

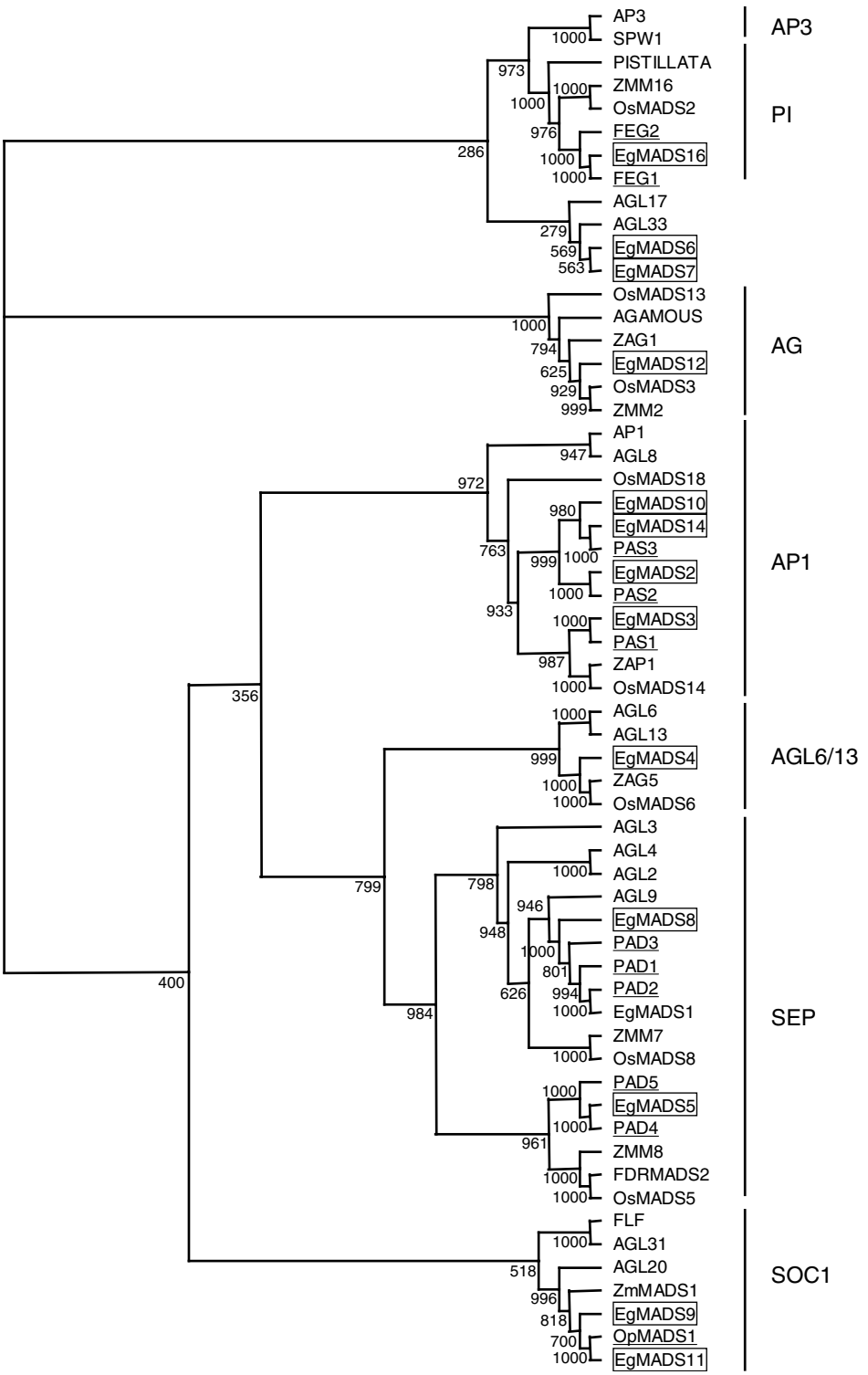

Figure 1. Phylogenetic tree (Neighbour-Joining) based on ClustalW alignment of deduced EgMADS protein fragments and Arabidopsis, Rice and Maize MADS box gene fragments. The sequences from the 3′-part of the MADS box (the binding site of the MADS3′R primer) until the 3′-end of the (putative) coding regions of all the gene products (IKC domains) were translated and used for all genes in the alignment. Bootstrap values are indicated at the nodes.

accessions of oil palm MADS box genes present in Genbank (see Table 1).

Description of EgMADS genes

SOC1-like genes

EgMADS13 is one of three EgMADS genes (EgMADS9, -11 and -13) that were classified in the SOC1 subgroup, whose members are expressed in a wide variety of tissues. The subgroup includes *Arabidopsis* SOC1 which is expressed in leaves and flowers (Borner et al., 2000), TM3 from tomato, TOBMADS1 from tobacco and maize ZmMADS1 which are expressed in both vegetative and floral tissues (Pnueli et al., 1994; Mandel et al., 1994; Heuer et al., 2001). EgMADS13 is identical to OpMADS1 (Acc. Nr. AF207699). The 860 nt EgMADS9 (Acc. Nr. AJ581462) fragment was 56% identical (in 160 amino acids (aa)) to *SOC1* suggesting that it may be a putative oil palm *SOC1* homologue and as such be involved in the switch from vegetative to reproductive meristem by activating the meristem identity genes. The EgMADS11 fragment (Acc. Nr. AJ581465) was highly similar (80% identity in 170 aa) to EgMADS13/OpMADS1. EgMADS9 clearly differed from the other two EgMADS SOC1-like genes.

AP1-like genes

Four EgMADS genes were placed in the AP1 group, namely EgMADS2, -3, -10, and -14. The AP1 group contains the meristem identity genes that control the transition from vegetative to reproductive meristem. AP1 also functions as an A type gene that specifies sepals and petals (Gustafson-Brown et al., 1994; Mandel and Yanofsky, 1995; Mandel et al., 1992). The 950 nt EgMADS3 fragment (Acc. Nr. AJ581468) was 70% identical to OsMADS14 at the protein level. EgMADS3 is identical to PAS1 (Acc. Nr. AF411840) submitted to the Genbank nucleotide databases by Adam et al. (2006). EgMADS3a was identical to EgMADS3 except for a deletion of 42 bp at 300 nt downstream of the 3′-end of the MADS3′ primer annealing site, resulting in a 14 amino-acid deletion in the protein. EgMADS14 (Acc. Nr. AJ581466) is identical to PAS3 (Acc. Nr. AF411842). EgMADS2 and EgMADS10 cDNA fragments (806 and 504 nt; Acc. Nrs. AJ581467 and AJ581464, respectively) were identical in the first 300 bp, but diverged at the 3′-ends of the fragments. The EgMADS2 translated protein sequence was 55% identical (in 200 aa) to the rice AP1-like genes OsMADS28 and OsMADS18. EgMADS10 is highly similar to the oil palm PAS2 gene (Acc. Nr. AF411841)

Other classes

Only a few genes that act downstream of the floral identity genes have been identified to date. One of these is AGL6 that has carpel-specific activity in *Arabidopsis* (Rounsley et al., 1995). The EgMADS4 fragment (772 bp, Acc. Nr. AJ581469) is a putative homologue of AGL6 in oil palm

Two EgMADS genes (EgMADS6 and -7, Acc. Nrs. AJ581471 and AJ581460) do not fall into any of the existing groups of the MADS box genes. They do not have a high degree of similarity to any known MADS box genes outside of the MADS domain. These may be genes that form a new class. EgMADS6 cDNA has a 177 amino acid protein sequence in frame with the MADS box-like sequence, which indicates that this is an expressed gene with possibly unknown function. EgMADS7 does not have a large continuous sequence in frame with the MADS box sequence.

Floral structure identity genes

Four additional EgMADS gene fragments were identified as genes that may affect third whorl formation, the whorl that is affected in 'mantled' oil palms.

EgMADS5 (Acc. Nr. AJ581470) full-length cDNA sequence was 1057 bp long, encoding a 250 aa protein. It was close to identical to the oil palm gene PAD4 submitted by Adam et al. (2006; Acc. Nr. AF411846) It was most similar to the other PAD genes cloned by Adam et al. (2006) and to EgMADS8. It was close to 80% similar to other SEP1 homologues. The SEP 1/2/3 genes are thought to form active protein complexes with meristem identity and floral identity genes (Pelaz et al. 2000), and as such play a part in specifying the stamens and carpels.

The full-length cDNA sequence obtained for EgMADS8 (Acc. Nr. AJ581461) was 992 bp long encoding a 207 amino acid protein. The translated protein sequence was identical to the oil palm gene PAD1 (Acc. Nr. AF411843), and had 74% full protein sequence identity with *Arabidopsis* SEP3.

EgMADS12 (Acc. Nr. AJ581463) full length coding sequence was 1116 bp long encoding a 224

amino acid MADS box protein. EgMADS12 had 66% full protein sequence identity to the *Arabidopsis* C-type gene AGAMOUS (AG), and more than 80% protein sequence identity with AG homologues from other monocots. Unlike AG and ZAG1 from maize, the MADS domain is not preceded by an N-terminal region. This sequence pattern is similar to OsMADS3, the rice homologue for AG (Kyozuka et al., 2000).

EgMADS16 full length coding sequence is 1062 bp and the longest open reading frame is 208 amino acids long. The full length coding sequence is completely identical to the oil palm gene FEG1 (Acc. Nr. AF227195), and it has more than 80% protein sequence identity to several PI homologues from other monocots. It is therefore a putative PI analogue.

mRNA expression of EgMADS genes

The expression levels of EgMADS cDNAs were analysed by RT-PCR in shoot apices and inflorescences at different developmental stages of both normal and mantled oil palms (Figure 2). In general, the expression pattern of EgMADS genes was consistent with their putative homologues in dicots and monocots. All genes clustering in the same subgroup were found to have similar expression pattern suggesting a functional conservation (Ng and Yanofsky, 2001).

SOC1-like genes EgMADS9 and EgMADS11 were expressed in shoot apices and the early stages of flowering, in line with a function at the onset of flowering. AP1-like genes EgMADS2, -3, -10 and -14 were expressed almost equally in the shoots and throughout flower development, in line with putative functions of these genes in determination of meristem identity and organ identity. No expression differences between normal and mantled samples were detected.

EgMADS4 was hardly expressed in vegetative shoots, but present in all inflorescences tested. It is the putative homologue of AGL13, which is expressed in ovules. However, EgMADS4 has an almost equal expression level in all flowering stages of both mantled and normal oil palm.

EgMADS6 primers failed to produce a distinct PCR product.

EgMADS7 was detected in all samples, with markedly lower levels in shoots compared to

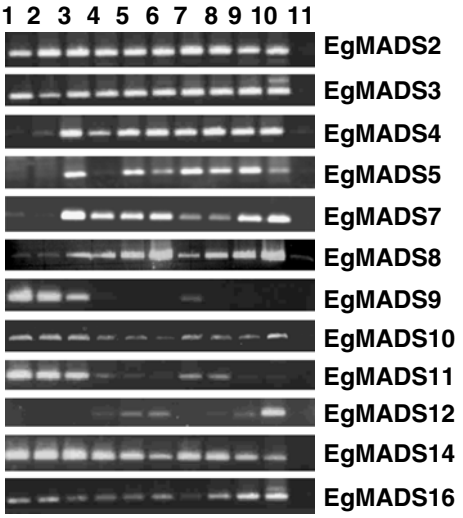

Figure 2. RT-PCR expression analysis of EgMADS genes. RNA was isolated and converted into cDNA from shoot apices, and female and male inflorescences at several developmental stages from both normal and mantled (abnormal) oil palms. Lanes1/2: Shoot apex normal/abnormal. Lanes 3–10: Inflorescences of: 2.5 cm/6 cm/10 cm female normal (lanes 3/4/5), 13 cm male normal (6), 2.5 cm/6 cm/10 cm female abnormal (7/8/9), 28 cm male normal (10). Lane 11: water control (no cDNA added).

inflorescences. EgMADS7 is one of the MADS box genes isolated that does not fit any of the existing MADS subgroups. Its expression increased with the early stages of flower development suggesting that it maybe involved in determination of organ identity.

Levels of EgMADS5 expression appeared to be more or less equal in inflorescences of different sizes from both normal and abnormal oil palms. Expression was not detected in shoots. Similarly, other orthologues of SEP1 such as VvMADS2 are also expressed primarily in floral tissues and are not detected in shoots and vegetative tissues (Flanagan and Ma, 1994; Boss et al., 2002).

EgMADS8 expression was present in all samples tested. Its expression level was very low in shoots and increased in flowers along with progress through development. The expression pattern is consistent with this gene being a functional homologue of SEP3.

EgMADS12 expression was consistent with a C-type MADS box gene. Expression was not detectable in shoots and in 2.5 cm inflorescences but visible in 6 cm female inflorescences and increased with the developmental stages of the inflorescences. Expression was detected in normal

as well as abnormal inflorescences, and in female as well as male inflorescences. Highest expression was detected in mature male inflorescences.

Expression of EgMADS16 was found in all tissues examined, including shoot apices, with slightly higher levels in the mature inflorescences. No clear differences in expression between normal and mantled were detected.

Functional analysis of EgMADS16

Based on sequence homology, EgMADS16 is the likely oil palm orthologue of the Arabidopsis gene PI. PI is a B-type gene, specifying the second and third whorl. Other studies in monocots (notably maize) have shown that this B-type function is to a large extent conserved in monocots. Since an impaired B-type function would result in a phenotype much like the mantled abnormality, any B-type gene is a candidate for involvement in the mechanism underlying the mantled phenotype. Therefore, we wanted to positively confirm that EgMADS16 is functionally involved in petal and stamen identity in oil palm.

For the functional characterization of EgMADS16 as the oil palm PI orthologue, the following two strategies were used:

1) Over-expression of EgMADS16 in wild type *Arabidopsis* by fusion of the EgMADS16 gene to a 35S promoter and subsequent transformation. Both AP3 and PI are needed to activate the B function. Nevertheless, partial transformation of sepals into petals can be expected in the transgene plant upon EgMADS16 overexpression, because expression of AP3 is 'leaky' in the first whorl.
2) Complementation of the *Arabidopsis pi* mutant with 35S::EgMADS16. The *Arabidopsis* PI gene and other homologues can partly complement the *Arabidopsis pi* mutant. Similarly, the oil palm ortholog of PI may be able to complement this phenotype, at least partly, as was shown by other heterologous PI-like genes, including a PI homologue from maize (Whipple et al., 2004).

In the first strategy wild type *Arabidopsis* plants (ecotype *Col*) were transformed with a binary construct containing full length EgMADS16 driven by the 35S promoter. Transformants (48) were obtained, from which approximately 30% showed an aberrant phenotype. Aberrations were observed in the first whorl, which showed chimaeric organs of petalloid–sepalloid identity (Figure 3). Also, the positions of these first whorl organs were changed compared to wild type sepals. This demonstrates that the EgMADS16 gene is functional to determine petal identity. Whether it is also able to specify stamen identity can not be concluded from this experiment. However, since the B function is responsible for both petals and stamens, these results indicate that EgMADS16 is the oil palm ortholog of PI.

For the complementation of the *Arabidopsis pi* mutant, this mutant was crossed with two lines showing the over-expressor phenotype. F1 progeny plants from this cross segregated for the over-expression phenotype and all plants were hemizygous for the pi allele. In the F2 of these crosses, a (partial) complementation of the *pi* phenotype is expected, which would demonstrate that EgMADS16 is the true orthologue of PI. For both crosses, 54 plants were analyzed in the F2. Figure 4 shows the results of the complementation of the *pi* mutant. Figure 4D–F show the floral phenotype obtained by the partial complementation of the *pi* mutant by EgMADS16 over-expression. The over-expression phenotype of EgMADS16 is still visible in the first whorl, which causes a change of sepals into petalloid structures. Changes in the second and third whorl show the partial complementation of the *pi* phenotype. In the second whorl, sepals are converted into petals and in the third whorl a conversion of carpelloid structures into stamenoid structures is visible. In other flowers the conversion into stamens is even stronger, resulting in the production of pollen and seed.

These results demonstrate that EgMADS16 is able to complement the *pi* mutant; i.e. it specifies the identity of petals and stamens. Therefore, it can be concluded that EgMADS16 represents a class B function gene with a function that is identical to the *Arabidopsis* homeotic gene PI.

Discussion

The mantled flower abnormality that occurs in some micropropagated oil palm affects flower morphology in such a way that oil production from mantled trees is severely impaired. The molecular mechanism underlying this somaclonal variation is

Figure 3. Flowers of a wild type *Arabidopsis thaliana* (*Col*-0 ecotype) (left upper picture) and of some of the overexpressor lines (wild type *Arabidopsis thaliana* (*Col-1* ecotype) transformed with a 35S::EgMADS16 construct). Arrows indicate partial conversion of sepals into petals in the first whorl.

as yet unclear, but aberrant DNA methylation during the *in vitro* procedure (Kaeppler and Phillips, 1993; Smulders et al., 1995) that may affect gene expression (Kaeppler et al., 2000) is a possible explanation (Matthes et al., 2001; Jaligot et al., 2002; Kubis et al., 2003). The involvement of this epigenetic mechanism is consistent with the fact that the same abnormality occurs in many ramets, and that reversion of the mantled condition has been observed.

The architecture of the mantled flowers has led us to hypothesize that changes in transcriptional activity of MADS box genes specifying the stamen and carpel structures may be causal or at least instrumental in the formation of the mantled phenotype. Particularly the transition of stamens into supernumerary carpels in Antirhinum major as the result of a B-type mutation (described by Zachgo et al., 1995) resembles this phenotype.

As a first step towards elucidating gene expression in mantled oil palm, we cloned a number of MADS box genes from oil palm. Six out of the fourteen EgMADS gene sequences were identical to oil palm gene sequences that were recently made available in the nucleotide databases, including EgMADS16 (which is identical to FEG1). However, characterization of these genes has not been published. In this study, we presented partial sequences of an additional eight new oil palm MADS box genes, and we also have functionally characterized one of the genes. Based on its putative B-type function, EgMADS16 was selected for detailed analysis and functional characterization. Our results clearly show that EgMADS16 is capable of complementing the B-type function in a *pi* Arabidopsis mutant. This is an example of the remarkable conservation of the B-class floral homeotic function between dicots and monocots, even though flower morphology is clearly different. In another example occasional conversion of stamens into carpels was observed when the transcript level of the rice AP3 homologue OsMADS16 was reduced by RNAi (Xiao et al., 2003). In a study by Whipple et al. (2004), both PI and AP3 homologues from maize were shown to be able to complement the Arabidopsis *pi* and *ap3* mutants, respectively. The partial conversion of sepals into petals as observed in our study was also

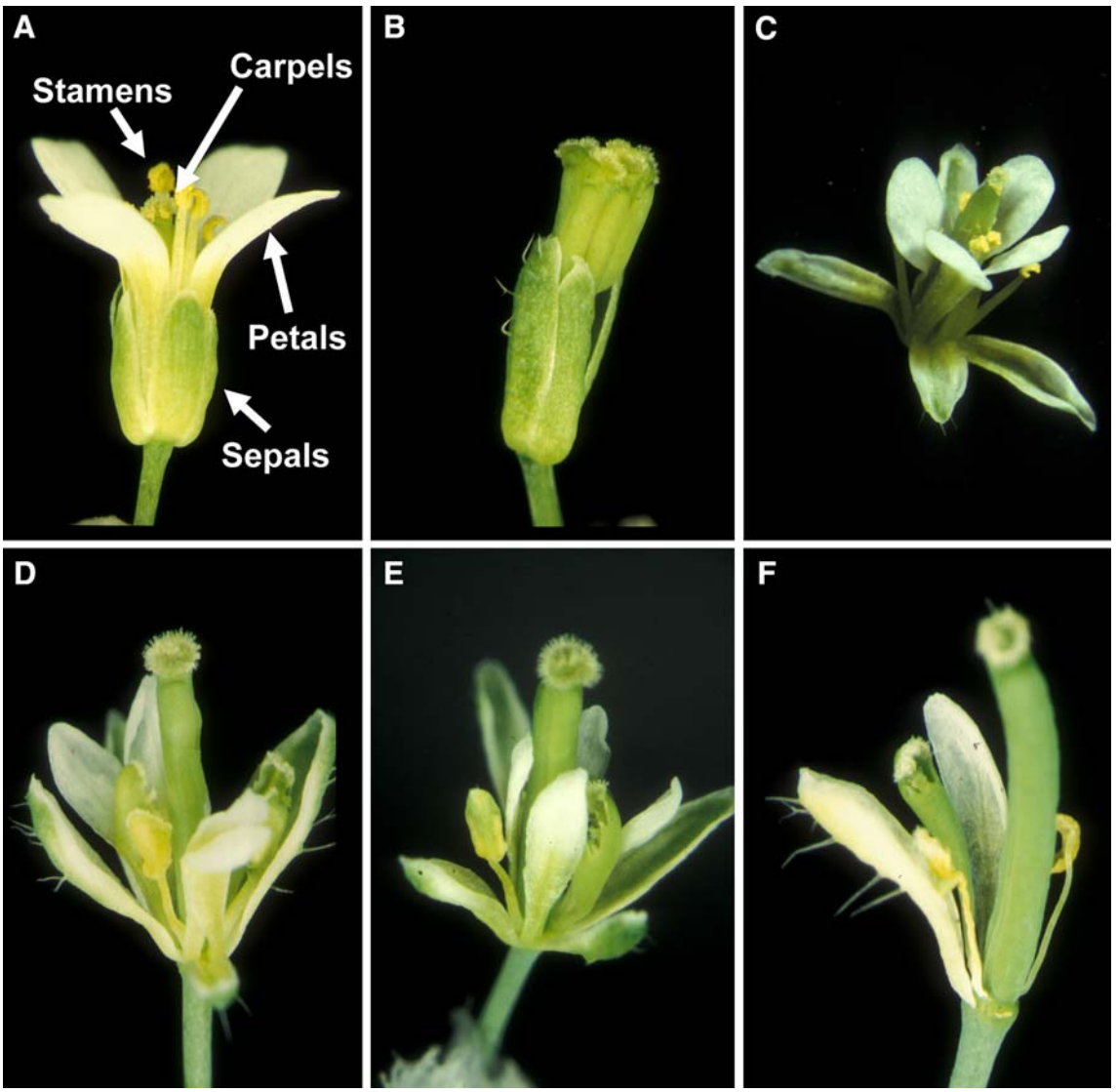

Figure 4. Flowers of wild type *Arabidopsis thaliana* (*Col-0* ecotype) (A), *pi* mutant (B), a wild type *Arabidopsis thaliana* overexpressing EgMADS16 (C) and F2 lines of the (*pi* mutant × EgMADS16 over-expressor line) cross (D–F). The F2 lines showed partial complementation of the *pi* phenotype. The partially complemented flowers have petalloid whorl 1 organs derived from the overexpression phenotype, restored petals in whorl 2, stamen/carpel structures in whorl 3 and an unaffected pistil in whorl 4.

observed with the maize PI transgene, which may result from a low level of AP3 expression in sepals; ectopic expression of a PI homologue can thus induce petalloid structures in the first whorl.

The functional determination of EgMADS16 qualifies this gene as a likely candidate for an involvement in the mantled phenotype. However, the B-type function is determined by an interplay between at least two B-type MADS box genes (PI and AP3 in Arabidopsis), and it is very likely that this is also the case in oil palm. In fact, more than two B-type MADS box genes have been identified in a number of monocots. For instance, in maize at least three AP3-like genes have been identified (Münster et al., 2001), and two in rice (Chung et al., 1995). In oil palm, another B-type gene has been identified in addition to EgMADS16: FEG2 (Acc. Nr. AF411848) has 87% amino acid similarity with FEG1 and EgMADS16, and most of the variation is present at the C-terminal part. The

motif (QPNLQ) typical for PI-homologues at the C-terminal end (Kramer et al., 1998) is present in both FEG1 and FEG2. Whether these genes have redundant functions or whether they have distinct roles in specifying flower architecture remains to be established. Each of the known and unknown B-type genes may be affected in the mantled oil palms, resulting in the conversion of stamens into carpelloid structures.

In addition to B-type MADS box genes, it is also possible that a C-type gene may play a role in the determination of the mantled phenotype. The AG homologue of *Hyacinthus orentalis* (HAG1) *wa*s regulated *in vitro* by plant hormones (Li et al., 2002). Several hormones were found to influence the expression of MADS box genes. These include gibberellins that control meristem identity in the *Arabidopsis* flower mutants *apetala2* and *apetala1*, and cytokinin or gibberellins that induce flower formation by activating SaMADSA in *Sinapis* (Okamuro et al., 1997; Bonhomme et al., 2000). In oil palm, a correlation was found between lowered endogenous cytokinin levels in *in vitro* cultured plants and the appearance of abnormal inflorescences at a later stage (Duval et al., 1995). Eeuwens et al. (2002) demonstrated that a relatively long transfer interval (8 weeks) and a high auxin-to-cytokinin ratio resulted in the lowest incidence of mantled flowering across all types of material. Reducing the transfer interval down to 2 or 4 weeks, and/or using media with relatively high concentrations of kinetin (0.25 mg l^{-1}), and low concentrations of NAA (0 or 0.1 mg l^{-1}), resulted in a high incidence of mantled flowering (above 30%), even with low/medium risk clones. Exclusion of plant growth regulators from the embryoid multiplication medium did not prevent some mantled flowering (12%) of new embryoid lines of low/medium risk clones following embryoid multiplication on a 4 week transfer interval, and a much higher incidence of mantling (above 90%) of lines from high risk clones. It remains to be determined whether changes in hormone concentration affect the mantled condition through regulation of MADS box gene expression.

EgMADS12 falls into the AG subgroup whose members include the *Arabidopsis* AG, *Antirrhinum* PLENA and maize ZAG1 and ZMM2 loci that are involved in stamen and carpel development. The expression pattern of EgMADS12 is consistent with a function in stamen and carpel development. Additional support for the C-type function of EgMADS12 is given by preliminary *in situ* hybridization experiments, in which EgMADS12 expression is restricted to the cells giving rise to the reproductive organs early in flower development, while at later developmental stages expression is confined to the stamens and carpels. In the mantled phenotype, two whorls of carpel (carpel and supernumerary carpel) develop in the flowers of the abnormal female inflorescence and stamens are converted to carpel-like structures in the flowers of the male inflorescences. Whether expression of EgMADS12 is the main trigger for ectopic carpel development, or the reaction on another trigger, remains to be determined.

In recent years, a third group of genes (the E-type or SEP MADS box genes) have been identified that interacts with the B- and C-type MADS box genes. EgMADS5 and -8 are categorized in the subgroup AGL2 and were expressed throughout flower development in both normal and abnormal palms but not the shoot apices. Members of the AGL2 subfamily act as mediators between the floral meristem and the floral organ identity genes (Pnueli et al., 1994; Savidge et al., 1995). AGL2-like genes in maize and rice are involved in meristem determination. EgMADS5 has similarity with ZMM8 and FDRMADS2 (a putative SEP1 homologue) whereas EgMADS8 is more similar to AGL9 (SEP3). The notion that EgMADS8 may be a SEP3 orthologue is supported by the fact that EgMADS8 was expressed in the flower primordia as well as in pollen and carpels (not shown). The gene was also expressed in the carpel-like structures in abnormal male flowers, similarly to expression in carpels in Arabidopsis (Mandel and Yanofsky, 1998). SEP functions have been found to be conserved in distantly related eudicots, and possibly also in monocots (Becker and Theissen, 2003; Malcomber and Kellog, 2004). In Petunia, the SEP3 analogue FBP2 was shown to interact with the B-type MADS box protein heterodimer, indicating a role for SEP3 in the B-type functionality (Ferrario et al., 2003). Nevertheless, designating EgMADS8 as a functional orthologue of SEP3 should be done with caution, as SEP genes in the monocots have complicated and heterogeneous expression patterns, and often can not be designated orthologues of eudicot SEP genes (Malcomber and Kellog, 2004). As in Arabidopsis, the onset of EgMADS8

expression occurred after the onset of EgMADS genes that belong to the AP1 subgroup and before that of EgMADS12, which is the probable homologue of AG. Therefore EgMADS8 may have a role in mediating the interactions of the meristem and organ identity genes. EgMADS8 was found to be expressed in the pollen grains of oil palm whereas in *Arabidopsis* this was not observed (not shown). When comparing EgMADS8 expression pattern with OsMADS8 of rice, a difference is that OsMADS8 is only expressed in reproductive organs. This suggests a divergence in the general function of SEP3-type genes in the monocots. This also reflects the broad variety of flower developmental biology among the monocots.

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