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ORIGINAL ARTICLE

A MADS-box gene, *EgMADS21*, negatively regulates *EgDGAT2* expression and decreases polyunsaturated fatty acid accumulation in oil palm (*Elaeis guineensis* Jacq.)

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Abstract

Key message **EgMADS21 regulates PUFA accumulation in oil palm.**

Abstract Oil palm (*Elaeis guineensis* Jacq.) is the most productive world oil crop, accounting for 36% of world plant oil production. However, the molecular mechanism of the transcriptional regulation of fatty acid accumulation and lipid synthesis in the mesocarp of oil palm by up- or downregulating the expression of genes involved in related pathways remains largely unknown. Here, an oil palm MADS-box gene, *EgMADS21*, was screened in a yeast one-hybrid assay using the *EgDGAT2* promoter sequence as bait. *EgMADS21* is preferentially expressed in early mesocarp developmental stages in oil palm fruit and presents a negative correlation with *EgDGAT2* expression. The direct binding of *EgMADS21* to the *EgDGAT2* promoter was confirmed by electrophoretic mobility shift assay. Subsequently, transient expression of *EgMADS21* in oil palm protoplasts revealed that *EgMADS21* not only binds to the *EgDGAT2* promoter but also negatively regulates the expression of *EgDGAT2*. Furthermore, *EgMADS21* was stably overexpressed in transgenic oil palm embryoids by *Agrobacterium*-mediated transformation. In three independent transgenic lines, *EgDGAT2* expression was significantly suppressed by the expression of *EgMADS21*. The content of linoleic acid (C18:2) in the three transgenic embryoids was significantly decreased, while that of oleic acid (C18:1) was significantly increased. Combined with the substrate preference of *EgDGAT2* identified in previous research, the results demonstrate the molecular mechanism by which *EgMADS21* regulates *EgDGAT2* expression and ultimately affects fatty acid accumulation in the mesocarp of oil palm.

Keywords Oil palm · MADS-box gene · Diacylglycerol acyltransferases · PUFA

Introduction

The African oil palm (*Elaeis guineensis* Jacq.) is a perennial monocotyledonous plant belonging to the Arecaceae palm family and is the most productive oil-bearing crop in the world. Palm fruits have two storage tissues, the mesocarp and kernel, that can produce two oils of major economic importance, commonly referred to as palm oil and palm kernel oil (Teh et al. 2019). Palm oil, mainly referring to oil derived from the mesocarp of oil palm fruit, is in high demand due to its nutritional attributes and competitive price compared to other vegetable oils (Parveez et al. 2015a) The cultivation of oil palm has expanded greatly in recent years, such that it has now surpassed soybean oil as the most widely produced vegetable oil in the world (Loganathan et al.2017). Compared to other plant sources of oil, the major fatty acids (FAs) of palm oil are palmitic acid (16:0) and oleic acid (18:1) predominated (each about 40%), while only 8% of the FAs are polyunsaturated fatty acids (PUFAs) (Dussert et al. 2013). Although the role of palmitic acid as a potential cause of cardiovascular diseases is still a matter of debate, the production of less saturated oil with higher oleic and lower palmitic contents by genetic improvement remains

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one of the important objectives in the breeding of oil palm (Parveez et al. 2015b).

Diacylglycerol acyltransferases (DGATs) are a key group of enzymes that catalyze the final step in acyl-CoA-dependent triacylglycerol (TAG) biosynthesis, which has demonstrated it to be the important rate-limiting step of triacylglycerol production in plants (Routaboul et al. 1999; Zou et al. 1999). The first plant DGAT gene was isolated from *Arabidopsis thaliana* in 1999 (Zou et al. 1999), and then different types of DGATs have been identified from several kinds of species, including castor bean (*Ricinus communis*) (He et al. 2004), peanut (*Arachis hypogea*) (Zheng et al. 2017), and tung tree (*Vernicia fordii*) (Shockey et al. 2006). Current research works showed that there are four divergent types of DGAT (DGAT1, DGAT2, DGAT3 and WS/DGAT) in higher plants, sharing little homology with each other. For example, DGAT1 is the main enzyme responsible for TAG synthesis during seed development, while DGAT2s come from plant species that produce large amounts of unusual fatty acids (Iskandarov et al. 2017). In *Arabidopsis,* AtDGAT2 showed a different acyl-CoA substrate preference than AtDGAT1 (Zhou et al. 2013). Moreover, the DGAT3 was considered to be an enzyme participate in a cytosolic pathway of TAG biosynthesis (Hernandez et al. 2012), and WS/DGAT is believed to be responsible for formation of surface wax esters (Kalscheuer and Steinbuchel 2003).

In oil palm, four distinct functional families of DGAT enzymes have been identified, including DGAT1, DGAT2, DGAT3 and WS/DGAT (Rosli et al. 2018). Although metabolic control analysis to the Kennedy pathway for triacylglycerol formation in callus of oil palm indicated that DGAT is unlikely to affect oil accumulation in oil palm (Ramli et al. 2005), the evaluation of the gene expression of the four DGAT families in different tissues and developmental stages suggests that the four DGAT groups play distinct physiological roles in mesocarp and endosperm tissues (Rosli et al. 2018). In addition, one type of DGAT2 (XM_010933834) that is highly expressed in the mesocarp of oil palm was characterized for its in vivo activity. Seed-specific overexpression of *EgDGAT2* in *Arabidopsis thaliana* increased the content of polyunsaturated 18:2 and 18:3 TAGs in the seeds compared to that in wild-type *Arabidopsis*. The results indicated that *EgDGAT2* prefers to use polyunsaturated FAs as substrates in both yeast and plants (Jin et al. 2017). However, despite some progress in the gene cloning and functional analysis of the DGATs of oil palm, a more detailed understanding of the transcriptional regulation of DGATs in the mesocarp of oil palm is currently unavailable.

In plants, the MADS-box gene family is one of the best-studied gene families and plays important roles during plant development, especially in floral organ development (Airoldi and Davies 2012; Shan et al. 2009). The regulation of MADS-box genes contributes the majority of components in the classical ABC and expanded ABCE models of floral development (Cheng et al. 2017). Moreover, MADS-box genes have been found to be expressed in the endosperm, ovule, embryo and pericarp of fruit, suggesting their diverse roles in plant development (Kumar et al. 2016; Smaczniak et al. 2012). In oil palm, diverse MADs box transcripts were found to show expression associated with the different phases of mesocarp development in previous research. Comparative transcriptome analysis data indicate the involvement of MADS box genes, particularly AGAMOUS-like genes, during the maturation and ripening of the oil palm mesocarp (Tranbarger et al. 2011). However, it is still unknown whether there is a relationship between oil accumulation and transcriptional activity and whether these MADS box gene products play regulatory roles in fatty acid synthesis or TAG assembly during mesocarp ripening.

Here, we isolated *EgMADS21*, an AGAMOUS-like (AGL) MADS-box transcription factor (TF) that is highly expressed in the mesocarp of oil palm by yeast one-hybrid assay, and characterized its function in TAG biosynthesis by directly regulating the expression of the *EgDGAT2* gene. In addition, the negative regulation of *EgDGAT2* expression and reduced unsaturated fatty acid levels in transgenic oil palm cells were demonstrated. Finally, the potential for use of *EgMADS21* in the genetic manipulation of oil accumulation in oil crops was discussed. In summary, we reveal a novel regulatory mechanism of *EgMADS21*-mediated TAG biosynthesis and oil accumulation in the mesocarp of oil palm.

Materials and methods

Plant materials and growth conditions

Oil palm (*Elaeis guineensis* Jacq.) fruits at the developmental stages of 30–60 days after pollination (DAP; Phase 1), 60–100 DAP (Phase 2), 100–120 DAP (Phase 3), 120–140 DAP (Phase 4) and 140–160 DAP (Phase 5) were harvested from the Coconut Research Institute, Chinese Agricultural Academy of Tropical Crops, Hainan, China (Zheng et al. 2019). The calli used in this study were induced from the young leaves of oil palm (*Elaeis guineensis* cv. Tenera). They were grown on MS medium supplemented with 5 mg/L 2, 4-D, 10 mg/L TDZ and 2% charcoal at 28 °C in the dark. The calli were transferred to woody plant medium (WPM) containing 0.1 mg/L 2, 4-D and 0.2% coconut water for secondary embryo induction and embryoid proliferation (Zou et al. 2019). The obtained embryoids were subcultured at 1-month intervals and used for genetic transformation and other assays.

DNA and RNA extraction and gene isolation

Genomic DNA was extracted based on the CTAB (cetyltrimethylammonium bromide) method. Total RNA was isolated by the CTAB method, and first-strand cDNA was synthesized from total RNA using TIANScript One Step RT-PCR Kits (Tiangen, Beijing, China). The primers for the *EgDAGT2* promoter are listed in Supplemental Table 2. The PCR conditions were as follows: 98 °C for 30 s, 30 cycles of 98 °C for 10 s, 58.5 °C for 30 s, and 72 °C for 30 s, and a final step at 72 °C for 2 min.

Yeast one-hybrid assay

The Matchmaker™ One-Hybrid Library Screening system was used to identify proteins that bind to the *EgDGAT2* promoter. The sequence of the *EgDGAT2* promoter was cloned into the pHis yeast expression vector, which was then integrated into an oil palm one-hybrid library constructed with the pGADT7 AD vector in the Y187 yeast strain. The background histidine expression of the Y187 pHIS-*EgDGAT2*-promoter strain was tested and restrained with 3-amino-1, 2, 4-triazole (3-AT) at the appropriate concentration. Furthermore, the full length of each transcription factor obtained was cloned separately into the pGADT7 AD vector to confirm the screening results. The relationship between *EgMADS21* and the EgDGAT2 promoter was examined individually.

Gene cloning and bioinformatics analysis

The open reading frame (ORF) cDNA sequence of *EgMADS21* was cloned from oil palm mesocarp based on sequencing analysis using PCR. The products and original EST fragments were aligned and assembled into the full-length *EgMADS21* cDNA (XM_010932108.2) using the DNAMAN program. The ORF Finder of NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to identify the ORF of *EgMADS21*.

The full-length of ORF nucleotide and amino acid sequence analyses were performed with the BLAST program from the NCBI website. Amino acid alignment of EgMADS21 was carried out with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), and a phylogenetic tree was constructed using the neighbor-joining method in MEGA 5.0 software (Tamura et al. 2007).

Real-time quantitative pcr (qRT-PCR) analysis

The cDNAs from oil palm mesocarp were diluted and mixed with primers and SYBR® Premix Ex Taq™II (TaKaRa, Dalian, China). qRT-PCR reactions were carried out in triplicate using SYBR® Premix Ex Taq™ II (TaKaRa, Tokyo, Japan) and were monitored with the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA) according to the manufacturer's instructions. The Egβ-actin housekeeping genes from oil palm were used as internal control genes. RT-PCR primers are listed in Supplemental Table 2. Expression levels were quantified as comparative cycle threshold (Ct) values using the $2^{-\Delta\Delta Ct}$ method.

Subcellular localization analysis

The full-length cDNA of the *EgMADS21* transcription factor was cloned into the p35MK1 vector. The construct was expressed transiently in tobacco (*Nicotiana benthamiana*) leaves by *Agrobacterium tumefaciens*-mediated infiltration (strain EHA105). The transiently infected leaves were imaged on day 2 after infiltration using a Leica (TCC-SP8) laser scanning confocal microscope. The 4′, 6-diamidino-2-phenylindole (DAPI) was used as a marker for nuclear localization. The excitation wavelength for GFP fluorescence was 488 nm, and fluorescence was detected at 490 to 520 nm.

Electrophoretic mobility shift assay (EMSA)

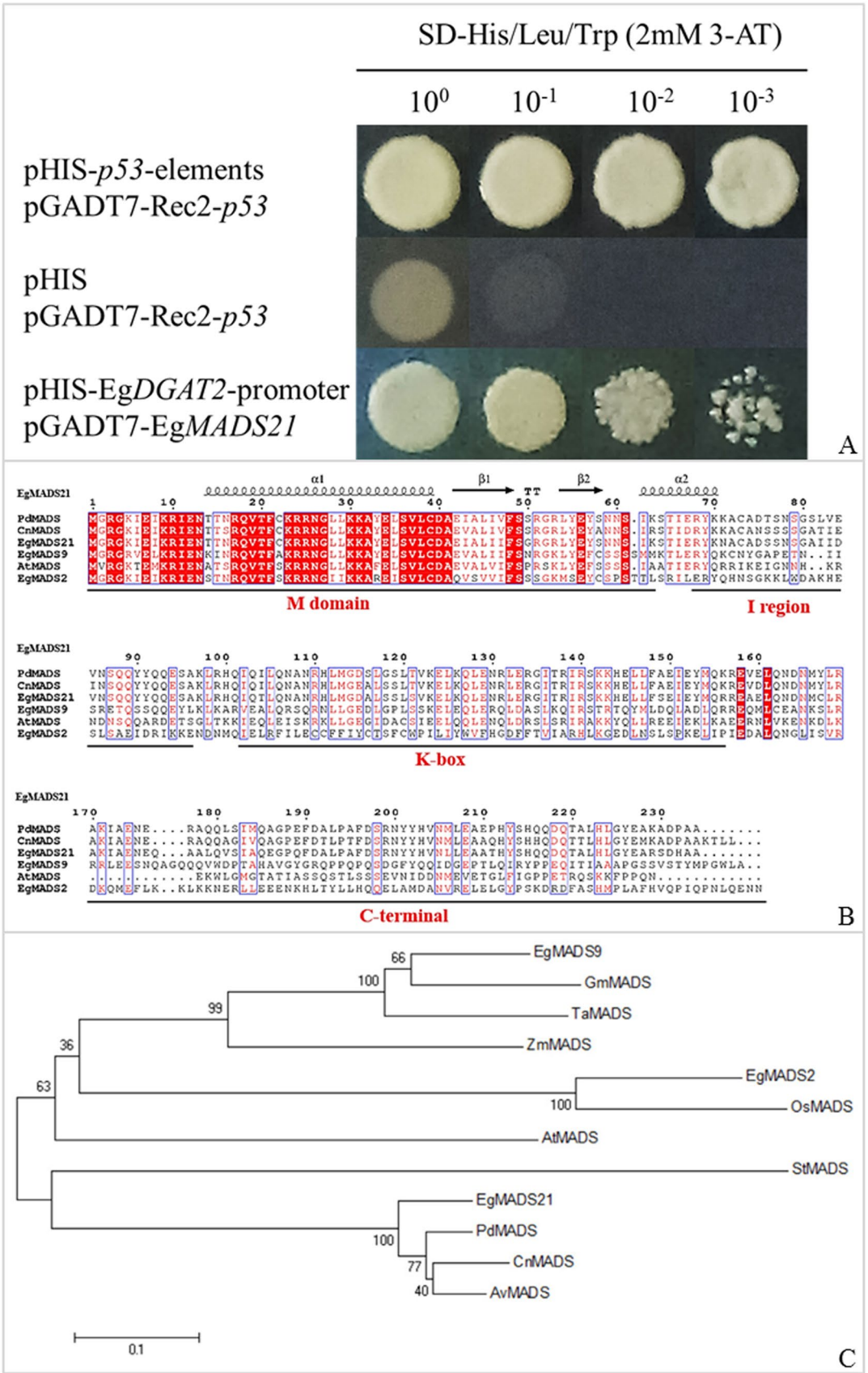
The protein expression vector pCold-*EgMADS21* was constructed and transferred into the *E. coli* protein expression strain BL21. Mycoprotein production was induced by IPTG, and the product was obtained by ultrasonic processing. The recombinant protein was purified via Ni-chelating affinity chromatography, and the size and purity of the protein were verified by SDS-PAGE and Western blot analyses. Double-stranded segments were obtained by PCR, and a biotin label was added with a Biotin 3′ End DNA Labeling Kit (Thermo). The LightShift® Chemiluminescent EMSA Kit (Thermo) was used to perform EMSA experiments via the manufacturer's instructions.

Vector construction for plant transformation

For transient and stable expression in oil palm, the ORF of *EgMADS21* was amplified from cDNA using the 1300 s-*EgMADS21* F/R primers and ligated into the binary expression vector pCAMBIA1300s under the control of the 35S promoter to generate the plant expression vector pCAMBIA1300-*EgMADS21*.

Transient expression of *EgMADS21* in protoplasts

PEG-mediated transfection and analysis was performed as previously described (Jung et al. 2015) with minor modifications. Fresh oil palm leaves from young seedling were cut into 1 mm-wide threadlets and placed in a hydrolysate enzyme solution (containing cellulase, lyase, mannitol,


SD-His/Leu/Trp (2mM 3-AT)
10^0
10^-1
10^-2
10^-3
pHIS-p53-elements
pGADT7-Rec2-p53
pHIS
pGADT7-Rec2-p53
pHIS-EgDGAT2-promoter
pGADT7-EgMADS21
A
EgMADS21
PdMADS
CnMADS
EgMADS21
EgMADS9
AtMADS
EgMADS2
M domain
I region
K-box
C-terminal
B
EgMADS9
GmMADS
TaMADS
ZmMADS
EgMADS2
OsMADS
AtMADS
StMADS
EgMADS21
PdMADS
CnMADS
AvMADS
0.1
C

◂**Fig. 1** Screening of proteins interacting with the *EgDGAT2* promoter and sequence analysis. **a** Yeast one-hybrid analysis of the interaction of EgMADS21 and the *EgDGAT2* promoter. pHIS-p53-elements and pGADT7-Rec2-p53 were used as the positive control, and pHIS and pGADT7-Rec2-p53 were used as the negative control. SD-His/Leu/Trp, SD medium without His, Leu, Trp, or Leu supplemented with 3-AT at a concentration of 2 mM. **b** Multiple sequence alignment of EgMADS21 and related proteins from *Arabidopsis* and palm plants. The horizontal lines mark the four conserved domains. **c** Phylogenetic analysis of *EgMADS21* and related proteins from other plant species. The scale bar represents 0.1 substitutions per site. The protein sequences include *EgMADS21* (XP_010930410), EgMADS2 (XP_010911265) and EgMADS9 (NP_001306837) from *Elaeis guineensis*, AtMADS (CAA19810.1) from *Arabidopsis thaliana*, PdMADS (XP_008781978) from *Phoenix dactylifera*, CnMADS (AIK66813) from *Cocos nucifera*, OsMADS (AAK17066) from *Oryza sativa*, TaMADS (AAQ11687) from *Triticum aestivum*, GmMADS (NP_001236390) from *Glycine max*, ZmMADS (NP_001105692) from *Zea mays*, StMADS (NP_001274754) from *Solanum tuberosum* and AvMADS (BAD83772) from *Asparagus virgatus*

KCl and MES) for 3 to 4 h. The protoplasts were collected by centrifugation at 100 g for 5 min, washed twice with washing solution and suspended in suspension buffer; the protoplasts could be stored temporarily at 4 °C. Then, the constructed vector was transferred to protoplasts via the PEG method. After the transfection, the protoplasts are collected by centrifugation for RNA extraction and RT-PCR. The expression levels of *EgMADS21* and *EgDGAT2* were analyzed by qRT-PCR as described above.

Stable expression of *EgMADS21* in transgenic oil palm embryoids

The oil palm embryoids were subcultured under aseptic conditions and expanded by tissue culture. The MADS21-1300 s plasmid was electroporated into *Agrobacterium* EHA105, and the positive bacteria were screened by PCR. The embryoid was transformed by *Agrobacterium* infection according to the procedure described for *Citrus sinensis* callus (Li et al. 2003) with several modifications. The infected embryoids were grown on screening medium containing 30 mg/L hygromycin B (Sigma) to select positive transgenic embryoids. After 5 months of selection and subculture, the obtained transgenic embryoids were expanded and used for subsequent experiments (qRT-PCR and FAs analysis).

Total lipid extraction and fatty acid analysis

A chloroform–methanol (2:1 by volume) extraction solvent system was used for lipid extraction from the WT and transgenic embryoids of oil palm (Yuan et al. 2014). Dried samples were dissolved in 1 mL of n-hexane, and 1 mL of 14% BF3-methanol solvent was added for methyl esterification (Liang et al. 2014). The GC analysis of fatty acid methyl esters was performed on a Hewlett-Packard Ultra-GC instrument (Sun et al. 2017). An HP-FFAP capillary column (30 m × 0.25 mm, 0.25 μm thickness) was used to separate the fatty acid methyl esters.

Statistical analysis

Every sample was verified to show reproducibility using three biological replicates. Significant differences were determined by SPSS Statistics 19.0. Figures were prepared with GraphPad Prism 5. Student's *t* test ($*P < 0.05$; $**P < 0.01$) was used to determine significant differences between two groups in this study.

Results

Screening of proteins interacting with the *EgDGAT2* promoter

Firstly, based on genome blast, only one copy of *EgDGAT2* was found on *Elaeis guineensis* genome (Chromosome 10). To identify transcription factors that regulate the lipid metabolic pathway by affecting the expression of *EgDGAT2*, a yeast one-hybrid library (Y187) screening system was applied in which the *EgDGAT2* promoter was used as bait to screen an oil palm cDNA library. After screening, 54 different genes belonging to *Elaeis guineensis* were obtained based on sequence BLAST analysis (Supplemental Table 1). Among these genes, one clone encoding a MADS-box family protein (designated *EgMADS21*) was selected for analysis considering the important function of such genes in fruit development.

A yeast one-hybrid assay was performed to verify the interaction between *EgMADS21* and the *EgDGAT2* promoter. Cotransformation of fusion vectors (pHIS-p53-elements and pGADT7-Rec2-p53) that could combine in yeast Y187 was performed as a positive control. pGADT7-Rec2-p53 and pHIS without binding elements were used as negative controls. Bait yeast cells cotransformed with the fusion vector pGADT7-*EgMADS21* could survive on the auxotrophic medium with 2 mM 3-AT (3-Amino-1, 2, 4-triazole). The growth of these colonies indicated that the *EgMADS21* protein could interact with the *EgDGAT2* promoter in the yeast system (Fig. 1a).

Gene cloning, sequence analysis and expression of *EgMADS21*

The open reading frame (ORF) cDNA sequence (708 bp) of *EgMADS21* was cloned from oil palm mesocarp based on sequencing analysis using PCR. Bioinformatics analysis indicated that EgMADS21 contains 708 nucleotides, which

encode 235 amino acids containing four typical domains: a MADS-box domain, a variable I domain, a conserved K domain, and a C-terminal region (Fig. 1b). Phylogenetic analysis showed that EgMADS21 indeed belongs to the AGAMOUS (AG)-like group. In addition, three typical MADS sequences from oil palm (EgMADS2, EgMADS21 and EgMADS9) were grouped into two subclades; EgMADS21 was closely linked to PdMADS, CnMADS and ApMADS, whereas EgMADS2 and EgMADS9 were grouped with plants that do not belong to Palmae, such as *Arabidopsis* and *Oryza sativa* (Fig. 1c).

Expression pattern of *EgMADS21* and its subcellular localization

To understand the dynamic relationship between the *EgMADS21* and *EgDGAT2* genes during fruit development in oil palm, qRT-PCR was applied to analyze the expression levels of the two genes in five different stages of fruit development. The results indicated differences in their expression levels at five developmental stages (Fig. 2a); in the first three stages of oil palm fruit development, *EgDGAT2* expression was very stable. However, the expression level increased significantly during the fourth and fifth stages of fruit ripening. In contrast, *EgMADS21* exhibited higher expression levels in the first three stages of fruit development and lower expression levels in the last two stages of fruit ripening. The expression level of *EgDGAT2* was negatively correlated with that of *EgMADS21*, suggesting that *EgMADS21* may negatively regulate the expression of *EgDGAT2* during the development of oil palm fruits. At the same time, according to the dynamic change of fatty acids in the mesocarp of oil palm in the previous references (Dussert et al. 2013; Zheng et al. 2019), the expression of EgMADS21 is also negatively correlated with the increase of unsaturated fatty acids in this process.

To elucidate the subcellular localization pattern of *EgMADS21* in plant cells, the CDS region (in which stop codon was deleted) was inserted into the p35MK1 vector to construct an *EgMADS21*-GFP fusion gene, which was then transiently expressed by infiltration in tobacco leaves. As shown in Fig. 2b, the localization results showed that *EgMADS21* localized to the nucleus.

Verification of the interaction of EgMADS21 and the *EgDGAT2* promoter

To confirm the binding of *EgMADS21* at the *EgDGAT2* promoter, EMSA was performed using prokaryon-expressed and purified *EgMADS21*-His fusion proteins. The binding site of the *EgDGAT2* promoter was predicted and designed with AliBaba2.1 (http://gene-regulation.com/pub/programs/alibaba2/index.html) and 5 potential binding sites were predicted (Supplemental Fig. 1). The 50 bp fragments containing the binding site was amplified by PCR; the biotin tag was added as a probe; and a fragment without a biotin tag was added as a competitive sequence. After EMSA verification, only one site was proved can bind with EgMADS21. In the

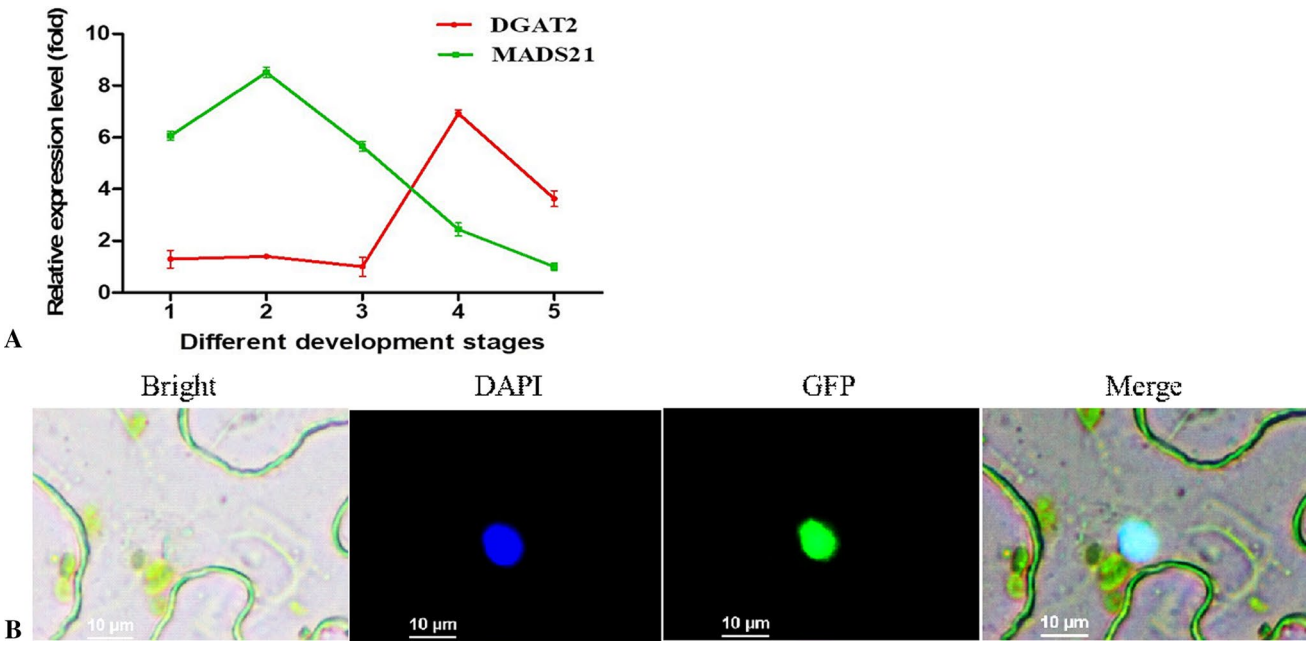


Fig. 2 Expression pattern and subcellular localization of *EgMADS21*. **a** Expression levels of *EgMADS21* and *EgDGAT2* in five developmental stages of oil palm fruit. **b** Bright-field, GFP, DAPI and merged images of the *EgMADS21* protein fused with GFP in *N. benthamiana* leaves

presence of the inhibitor, it can be seen from the EMSA results that the protein expressed from pCOLD without a label did not bind to the promoter fragment, whereas the expressed EgMADS21 protein showed a band corresponding to binding with the probe, confirming that EgMADS21 can bind to the element in this fragment of the *EgDGAT2* promoter (Fig. 3a).

Transient expression of *EgMADS21* in protoplasts

Protoplasts were extracted from wild-type oil palm leaves, and the pCAMBIA1300s (as blank) and pCAMBIA1300-*EgMADS21* plant expression vectors were transferred to fresh oil palm protoplasts via the PEG induction method, from which RNA was extracted after overnight transformation. According to the results of fluorescence quantification (Fig. 3b), the expression level of *EgMADS21* was significantly increased, indicating that the pCAMBIA1300-*EgMADS21* plasmid was successfully transferred, while the expression of *EgDGAT2* was significantly decreased. Combined with the EMSA results, it was shown that *EgMADS21* not only binds to the *EgDGAT2* promoter but also negatively regulates *EgDGAT2* expression.

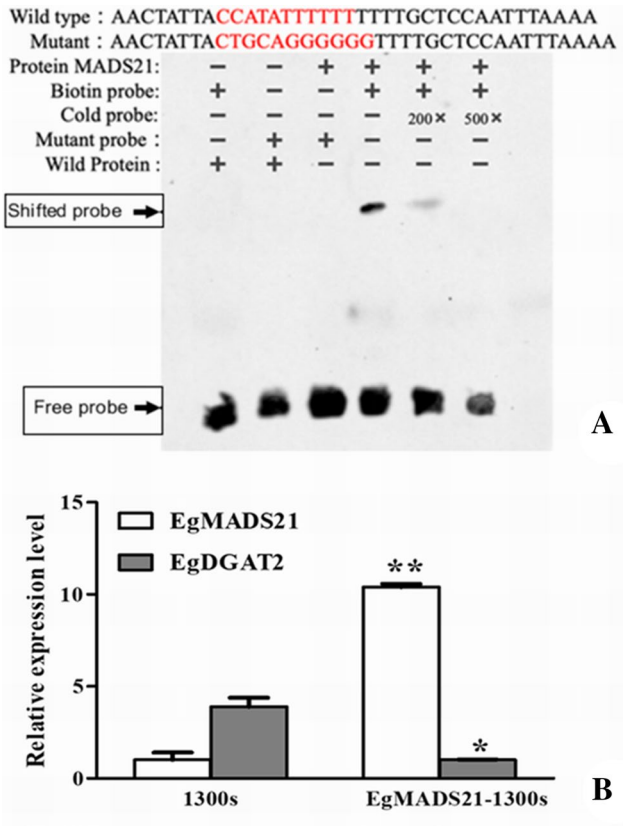


Fig. 3 EMSA and transient expression of *EgMADS21* in the protoplasts of oil palm. **a** Binding of *EgMADS21* to the motif in the promoter of the *EgDGAT2* gene. Wild protein means the protein expressed from plasmid pCOLD without EgMADS21. **b** Transient overexpression of *EgMADS21* in the protoplasts of oil palm. 1300S: Empty vector (control)

Overexpression of *EgMADS21* in Oil palm embryoids

To further verify the influence of *EgMADS21* on *EgDGAT2*, *EgMASD21* was stably overexpressed in the embryoids of *Elaeis guineensis*. After three rounds of hygromycin screening and PCR identification, three positive transgenic lines of embryoids were selected for further analysis (Fig. 4a). The relative expression levels of *EgMADS21* and *EgDGAT2* in wild and transgenic embryoids were quantified by qRT-PCR, and it was found that *EgMADS21* transcript expression in the embryoids varied up to sixfold and 33-fold, respectively, compared to that in wild embryoids (Supplement Table 3). In contrast, the expression of *EgDGAT2* declined remarkably in the *EgMADS21*-2 line, and *EgDGAT2* expression was suppressed substantially (Fig. 4b). In conclusion, *EgDGAT2* expression was seriously inhibited, while the expression of *EgMADS21* was increased.

Fatty acid analysis

To investigate the effects of the overexpression of *EgMADS21* in transgenic embryoids, we extracted the total lipids from three individual transformation-positive lines and wild-type embryoids and analyzed the contents of different fatty acids by GC. The results showed that the contents of C18:1, C20:0, and C20:1 in the three transgenic callus lines were increased significantly compared with the wild-type calli. More specifically, the *EgMADS21* transgenic embryoids contained 23.1 mol % C18:1 FA on average, while the content was only 15.53 mol % in WT embryoids. In addition, the content of 18:2 FA in the transgenic embryoids of *EgMADS21*-overexpressing lines was decreased from 32.5 to 23.6 mol % compared with that in WT. However, compared with the WT, the contents of linoleic acid (C18:2) in the three types of transgenic embryoids were obviously decreased. Moreover, the contents of long-chain fatty acids, including C20:0, C20:1, C22:1 and C24:0, were increased significantly (Fig. 4c). Therefore, combined with the substrate preference of *EgDGAT2* identified in earlier research (Jin et al. 2017), the results of fatty acid analysis further demonstrated that *EgMADS21* inhibits the expression of the *EgDGAT2* gene by directly interacting with the DGAT2 promoter and ultimately decreases polyunsaturated fatty acid accumulation during TAG synthesis in oil palm.

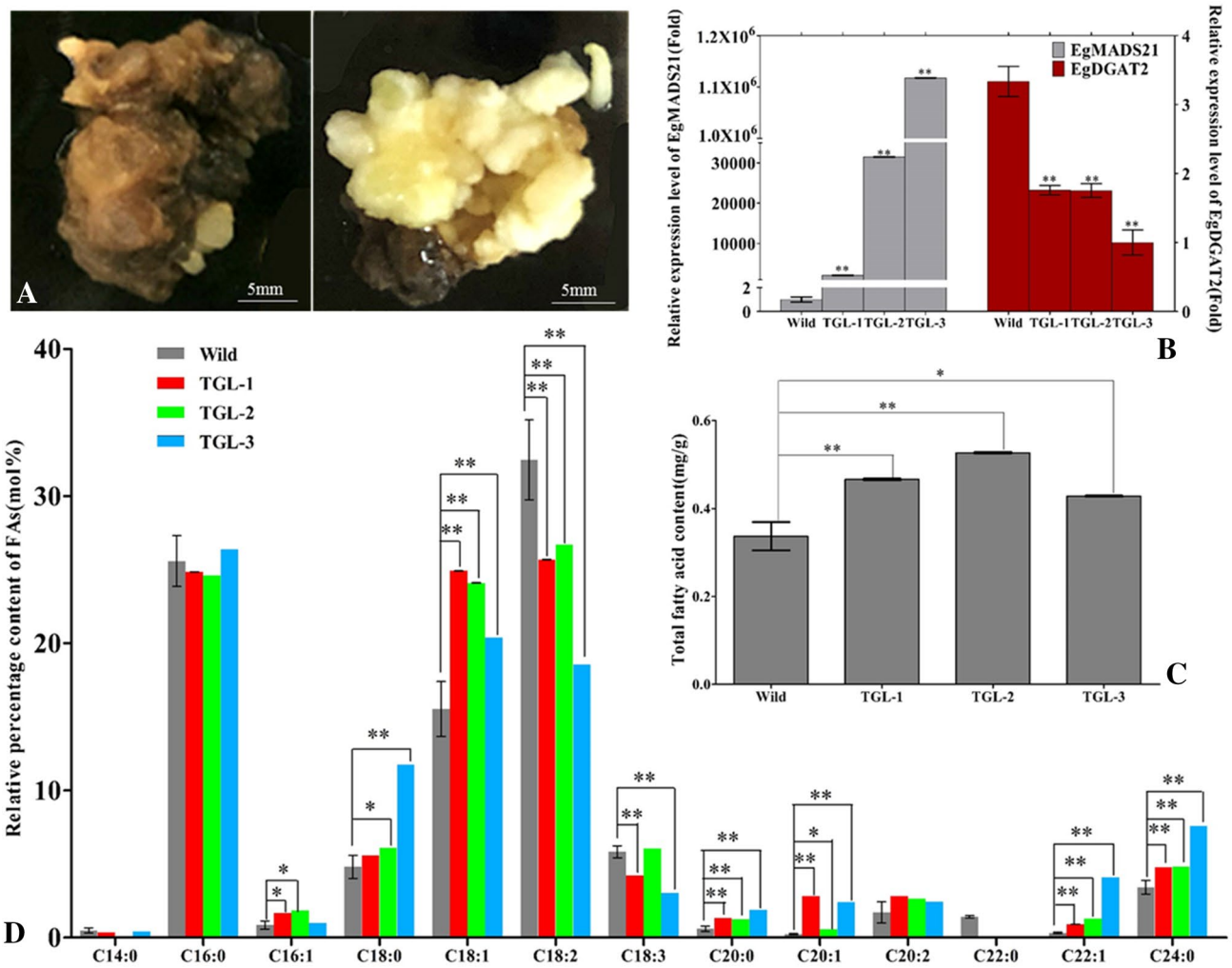


Fig. 4 Stable overexpression of *EgMADS21* in the embryoids of oil palm and fatty acid analysis. **a** Wild-type and transgenic oil palm embryoids after selection. **b** Expression levels of *EgMADS21* in different transgenic lines. **c** Total FA content in different transgenic lines. **d** FA profiling of different transgenic lines

Discussion

In this study, we described an AGAMOUS-like MADS box TF, *EgMADS21*, that modulates unsaturated FAs during TAG accumulation via the regulation of key metabolic gene. *EgMADS21* was identified as an inhibitory factor of the *EgDGAT2* gene promoter, which is involved in a key step in TAG synthesis during mesocarp maturation. In *Arabidopsis thaliana*, members of the AGAMOUS subfamily of MADS-box genes have been demonstrated to control ovule development and were subsequently revealed to participate in the transition to flowering (Hugouvieux et al. 2018; Koo et al. 2010; Zheng et al. 2009). Furthermore, Sl-AGL11 from tomato is involved in the conversion of sepals into fleshy organs under ethylene-dependent ripening and starch and sugar accumulation during fruit ripening (Huang et al. 2017). *MaMADS7* from banana (*Musa acuminata*) plays an important role in initiating endogenous ethylene biosynthesis and fruit ripening (Liu et al. 2015). In the mesocarp of oil palm, the expression of the *EgMADS21* gene is high in the early fruit stages (60–100 DAP) and decreases gradually with fruit ripening. In contrast, the expression level of *EgDGAT2* does not change during the early developmental stages and increases significantly with fruit ripening (Jin et al. 2017). Correlated with changes in *EgDGAT2* expression, the polyunsaturated fatty acid (PUFA) content dramatically declines during this period. Considering that *EgDGAT2* preferentially uses PUFAs (especially C18:3 and C18:3 PUFAs) as substrates in transgenic plants, *EgMADS21* may modulate TAG assembly and PUFA accumulation by suppressing *EgDGAT2* expression during the ripening of oil palm fruit (Fig. 5).

In higher plants, the complicated networks of seed oil accumulation are precisely controlled by intricate multilevel

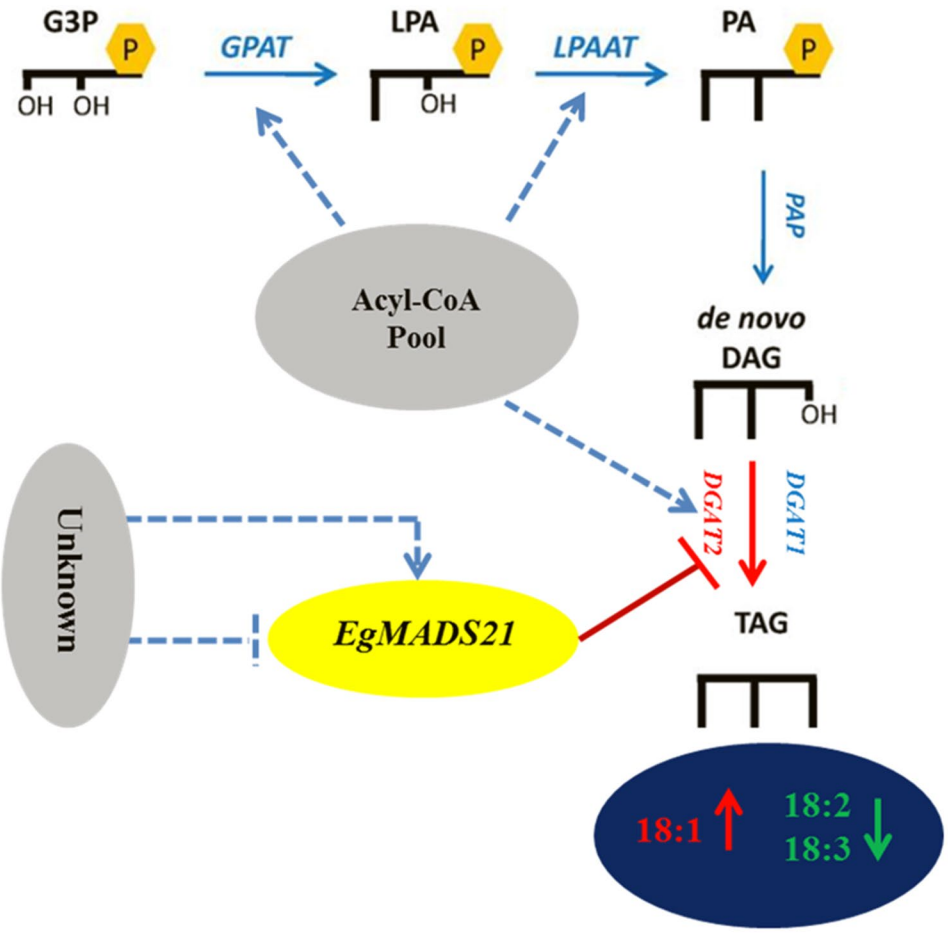


Fig. 5 Simplified model shows that *EgMADS21* directly and indirectly regulates the expression of the *EgDGAT2* gene, which controls the accumulation of fatty acids in oil palm. *DAG* diacylglycerol, *G3P* glycerol 3-phosphate, *GPAT* glycerol-3-phosphate acyltransferase, *LPA* lysophosphatidic acid, *LPAAT* lysophosphatidic acid acyltransferase, *PA* phosphatidic acid, *TAG* Triacylglycerols

regulatory networks, among which multiple routes are regulated in a coordinated fashion, including carbon partitioning, FA synthesis, and lipid assembly (Tan et al. 2019). Previous transcriptomic profiling analyses of different kinds of plant species also indicated that transcriptional regulation plays an important role in the lipid biosynthesis process (Chen et al. 2012; Liang et al. 2014; Lu et al. 2018). Several kinds of transcription factors (TFs), including WRINKLED1 (WRI1), LEAFY COTYLEDON1 (LEC1), LEC2, FUSCA3 (FUS3), and an R2R3-MYB (MYB89) transcription factor, were identified in these analyses, and the regulatory network of the TFs controlling seed oil accumulation was revealed (Baud et al. 2007; Kagaya et al. 2005; Roscoe et al. 2018). Among these TFs, WRI1, belonging to the APETALA2-ethylene-responsive element-binding protein family, was demonstrated to be a central regulator in seed oil accumulation by interacting directly with an AWbox sequence, [CnTnG(n)7CG], in the upstream regions of several FA biosynthesis genes during seed maturation (Maeo et al. 2009). In addition, in the seeds of *Arabidopsis thaliana*, WRI1 and FUS3 are positively regulated by LEC1 and LEC2 and jointly control the expression of genes related to the accumulation of seed oil (Baud et al. 2007; Kong and Ma 2018; Maeo et al. 2009). In oil palm, WRI1-like transcript levels are high in oil palm mesocarp and continuously increase during ripening (Dussert et al. 2013). However, in contrast to what is observed in *Arabidopsis thaliana*, EgWRI1-1 in the mesocarp of oil palm is activated by three transcription factors, EgNF-YA3, EgNF-YC2 and EgABI5, as no homologs of LEC1 and 2 and FUS3 were identified in oil palm mesocarp (Yeap et al. 2017). The results suggest that TFs are likely to control oil accumulation and FA synthesis in the mesocarp of oil palm by different regulatory networks, which are independent of the upstream factors that participate in seed development.

Most TFs related to oil synthesis are derived from oil crop seeds in which TAGs accumulate during maturation (Baud et al. 2010; Baud and Lepiniec 2010; Li et al. 2015). In contrast, little is known about the molecular basis of the regulation of lipid metabolism by TFs in fleshy fruits that accumulate high levels of TAGs in their mesocarp, such as those of oil palm and avocado (*Persea americana*). The fruit-specific regulatory network appears to be controlled by ethylene-dependent and abscisic acid (ABA)-dependent pathways and other independent pathways (Kourmpetli and Drea 2014). The members of the MADS box family of

transcription factors (TFs), including the class D MADS-box gene Sl-AGL11 from tomato (Huang et al. 2017), the MdAGL24-like gene in red-fleshed apple (*Malus sieversii* f. *niedzwetzkyana*) (Su et al. 2018), and the TM6 MADS-box gene from strawberry (Fragaria × ananassa) (Martin-Pizarro et al. 2019), have been identified as common regulatory elements during fruit ripening. However, possibly due to a lack of data, only a few MADS box family TFs have been identified as being involved in FA biosynthesis during maturation and ripening of oil palm fruit, which presents an especially abundant oil content in its mesocarp (Yeap et al. 2017). During the maturation and ripening of the oil palm mesocarp, the expression of certain MADS box transcripts coincides with oil accumulation and FA synthesis in a positive and negative manner, respectively (Tranbarger et al. 2011). These results support the hypothesis that some MADS box proteins play regulatory roles either upstream or downstream of oil accumulation or FA synthesis during mesocarp ripening.

Although a large number of contigs with differential representation have been revealed by comparative transcriptome and metabolite analysis of the oil palm mesocarp, very few studies have focused on the MADS-box TFs involved in the regulatory mechanism underlying FA synthesis and TAG assembly during mesocarp development (Dussert et al. 2013). The selection of a suitable model plant for the functional analysis of MADS-box TFs derived from oil palm has caused a dilemma for researchers. Although knowledge obtained from the model species *Arabidopsis* has advanced enough to characterize oil accumulation-related TFs from nonmodel plant species (Yeap et al. 2017), whether the functions of these TFs from mesocarp (nonseed tissues) are conserved and play a role in seed oil synthesis in *Arabidopsis* remains unclear. In this kind of tissue, the ripening process is always closely controlled by both ethylene-dependent and ethylene-independent pathways (Gapper et al. 2014; Giovannoni 2007). While tomato is a good model plant for examining fleshy fruit development and metabolite accumulation (Dong et al. 2013), its low content or lack of triacylglycerols (TAGs) means that it is not an optimal model for studying the transcriptional regulation of downstream pathways of FAs and TAG synthesis (Tranbarger et al. 2011). Moreover, the elucidation of MADS box-mediated regulation might be particularly challenging in the fruit of oil palm, because MADS-box genes are known to have redundant functions, and TFs may be involved in multiple pathways; hence, their ectopic expression may result in pleiotropic phenotypes (Zhang 2003). Therefore, functional characterization of the components involved in oil synthesis via embryogenic callus transformation combined with virus-induced gene silencing (VIGS) (Wang and Fu 2018) in fleshly fruit will be an alternative method for future research.

Based on the above results and discussion, *EgMADS21* was characterized as a regulator of TAG metabolism in this study. As shown in Fig. 5, the underlying molecular pathway responsible for the effects of *EgMADS21* involves inhibition of *EgDGAT2* expression and a decrease in PUFAs assembled at the 3-n of DAG, finally leading to decreased PUFA accumulation in the mesocarp of oil palm. To our knowledge, this is the first report to clearly demonstrate the mechanism by which a MADS-box family gene of oil palm negatively regulates fatty acid accumulation during fruit development. The results also provide new insight into the potential functions played by MADS-box family genes in transcriptional regulatory mechanisms, such as the qualitative and quantitative control of oil palm fruit development and metabolism.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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