CSIRO PUBLISHING

Functional Plant Biology, 2014, **41**, 321–329
http://dx.doi.org/10.1071/FP12388

Characterisation of *DGAT1* and *DGAT2* from *Jatropha curcas* and their functions in storage lipid biosynthesis

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Abstract. Diacylglycerol acyltransferases (DGATs) catalyse the final step of triacylglycerol (TAG) biosynthesis of the Kennedy pathway, and play a critical role during TAG accumulation in developing oleaginous seeds. In this study, the molecular cloning and characterisation of two DGAT genes, *JcDGAT1* and *JcDGAT2*, from jatropha (*Jatropha curcas* L., a potential biodiesel plant) is presented. Using heterogonous overexpression techniques, both *JcDGAT1* and *JcDGAT2* were able to restore TAG biosynthesis in a yeast mutant H1246 strain, and enhance the quantity of TAG biosynthesis by 16.6 and 14.3%, respectively, in strain INVSc1. In transgenic tobacco, overexpression of *JcDGAT1* and *JcDGAT2* resulted in an increase in seed oil content of, respectively, 32.8 and 31.8%. Further, the functional divergence of *JcDGAT1* and *JcDGAT2* in TAG biosynthesis was demonstrated by comparing the fatty acid compositions in both the transgenic yeast and tobacco systems. In particular, *JcDGAT2* incorporated a 2.5-fold higher linoleic acid content into TAG than *JcDGAT1* in transgenic yeast and exhibited a significant linoleic acid substrate preference in both yeast and tobacco. This study provides new insights in understanding the molecular mechanisms of DGAT genes underlying the biosynthesis of linoleic acids and TAG in plants.

Additional keywords: diacylglycerol acyltransferase, fatty acid composition, oil content, triacylglycerols.

Received 7 January 2013, accepted 1 October 2013, published online 19 November 2013

Introduction

Jatropha (*Jatropha curcas* L., Euphorbiaceae), which is commonly known as purging nut or physic nut, is an oilseed-bearing perennial shrub native to Mexico and Central America. Owing to its biological properties such as drought hardiness, rapid growth, easy propagation, high oil content, short gestation period and wide adaptation to soil conditions, jatropha has created tremendous interest for the use of its seed oil (storage lipids) as a commercial source of biodiesel (Abdulla *et al.* 2011). However, worldwide introduction of jatropha as a biodiesel plant has had limited success because of unreliable oil yields and low economic returns. There is, therefore, an immediate need to enhance seed oil yields by genetic improvement of jatropha, which requires a comprehensive understanding and identification of the genes encoding the rate-limiting enzymes responsible for lipid biosynthesis in the developing seeds of in this species.

The biosynthesis of seed oils (in the form of triacylglycerols, TAGs) involves *de novo* fatty acid (FA) synthesis and TAG assembly. The process of *de novo* FA synthesis produces cytosolic acyl-CoAs in plasmids. The TAG assembly consumes acyl-CoAs using substrate glycerol 3-phosphate with diverse enzymes that sequentially transfer acyl-CoAs to sn-1, -2 and -3 positions in glycerol 3-phosphatein the endoplasmic reticulum (Ohlrogge and Browse 1995). There, acyl-CoA: diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) transfers an acyl-CoA to the sn-3 position of sn-1, 2-diacylglycerol (DAG) and plays an essential role in controlling TAG assembly (Shockey *et al.* 2006). At least two classes of genes, *DGAT1* and *DGAT2*, encoding the DGAT enzymes have been identified in diverse plants (Zou *et al.* 1999; Giannoulia *et al.* 2000; Lardizabal *et al.* 2001; Nykiforuk *et al.* 2002; He *et al.* 2004; Yu *et al.* 2008; Li *et al.* 2010*a*). Results suggest that *DGAT1* and *DGAT2* are ubiquitous in plants. In *Arabidopsis*, *AtDGAT1* controls TAG biosynthesis in developing seeds, whereas *AtDGAT2* seems to have no obvious phenotype functionally (Shockey *et al.* 2006; Zhang *et al.* 2009). Similarly, the *DGAT1*s significantly regulate TAG accumulation in maize (Zheng *et al.* 2008), *Tropaeolum majus* L. (Xu *et al.* 2008) and soybean (Li *et al.* 2010*b*). Further, studies have shown that the *DGAT2*s play a critical role in the accumulation of unusual FAs (such as epoxy and hydroxy fatty acids) in castor bean (Kroon *et al.* 2006), tung tree (Shockey *et al.* 2006) and *Vernonia* (Li *et al.* 2010*a*). In addition, temporal and spatial expression patterns of *DGAT1s* and *DGAT2s* among organs or in different stages of seed development often display obvious

differences (Li *et al.* 2010*b*; Banilas *et al.* 2011; Xu *et al.* 2011). However, little is known about the functional similarity or divergence of *DGAT1* and *DGAT2* during the pathway of storage lipid biosynthesis *in planta*.

In this study, we isolated and characterised *DGAT*1 and *DGAT*2 from jatropha, and demonstrated their functions in storage lipid biosynthesis by heterologous expression in yeast (*Saccharomyces cerevisiae*) and tobacco systems. Results obtained from this study add new insights into our understanding of the molecular mechanisms of the oil content or fatty acid composition of TAG in seed oils of plants, and provide a step towards identifying the genes encoding the rate-limiting enzymes responsible for lipid biosynthesis in the developing seeds of jatropha.

Materials and methods

Plant material and yeast strain

Jatropha curcas L. was grown at the field station of Xishuangbanna Tropical Botanical Garden (21°56′N, 101°15′E, 600 m above sea level), Chinese Academy of Sciences, Yunnan, SW China under natural conditions. Leaves, roots and seeds at different developmental stages were sampled as described in our previous study (Xu *et al.* 2011). Tobacco (*Nicotiana tabacum* L. cv. Honghua da jin yuan) was used as plant material for heterologous gene transformation. Both wild-type tobacco and its transgenic derivatives were grown in a greenhouse at 22°C with a 16-h photoperiod (16 h of light 120 $\mu E\, m^{-2}\, s^{-1}$) and 8 h of darkness.

We used a yeast (*Saccharomyces cerevisiae*) quadruple mutant strain H1246 (*Matα dga1*::*KanMX4 lro1*::*TRP1 are1*::*HIS3 are2*::*LEU2 ADE2 ura3*) for complementary experiments (provided by Dr Sten Stymne, Swedish University of Agricultural Sciences, Uppsala). The H1246 strain lacks the *DGA1* and *LRO1* genes encoding yeast DGAT and PDAT enzymes responsible for the TAG synthesis, and *ARE1* and *ARE2* encoding ASAT (acyl-CoA: sterol acyltransferase) that are responsible for the synthesis of steryl esters (SE). Due to their absence, this mutant is devoid of the synthesis of TAG and SE, and does not form lipid droplets (LDs) (Sandager *et al.* 2002; Sorger *et al.* 2004). Therefore, this mutant is very useful for studying the enzymes of TAG synthesis, especially for identifying functional DGATs (Siloto *et al.* 2009). A wild-type yeast *S. cerevisiae* strain INVSc1 (Invitrogen, Guangzhou, China) was used as a control.

Cloning of DGAT1 *and* DGAT2 *genes from* Jatropha curcas

To isolate the *DGAT* genes from jatropha, total RNA was extracted from developing seeds as previously described (Xu *et al.* 2011). Complementary DNA was synthesised from 1μg of total RNA using a PrimeScript RT–PCR Kit (Takara Bio Co., Dalian, China). For the cloning of the *DGAT1*, specific primers, D1-*Hind*III and D1-Xho I (see Table 1) covering the open reading frame (ORF)), were designed based on an available jatropha *DGAT* sequence (DQ278448). The full length ORF of *JcDGAT1* was amplified using high fidelity PCR TransStart FastPfu DNA Polymerase (Transgen, Beijing, China). The PCR product was cloned into a pEASY-Blunt Cloning Vector (Transgen) and the generated plasmid was designated pEasy-JcDGAT1.

For the cloning of *DGAT2*, the 5′ and 3′ RACE (rapid amplification of cDNA ends) methods were applied by using the Smart-RACE cDNA Amplification kit (Clontech Takara Biomedical Tech. Co., Beijing, China) according to the manufacturer's instructions. Gene specific primers, D2–51 and D2–31 (Table 1), were designed based on a partial sequence of jatropha *DGAT* (EU395774). The RACE-PCR products were analysed on a 1.2% (w/v) agarose gel and the purified DNA was cloned into pEASY-T1 Cloning Vector (Transgen). Selected clones were purified and sequenced. After obtaining the full-length cDNA of *JcDGAT2*, the ORF was amplified with the primers D2-*Hind*III and D2-Xho I (see Table 1), and cloned into pEASY-Blunt Cloning Vector as described above. The generated plasmid was designated pEasy-JcDGAT2 and sequenced.

To investigate the structural characters of *JcDGAT1* and *JcDGAT2* in the jatropha genome, DNA was extracted from jatropha leaves using the CTAB method and genomic PCR was performed using ORF primer pairs as described above. The reaction mixture (50 μL) contained 100 ng of genomic DNA template, 5 μL 10 × PCR buffer, 1 μM of each primer, 0.25 mM of dNTPs, and 2.5U of Transstart HiFi DNA polymerase (Transgen). The following PCR conditions were used: initial denaturation at 94°Cfor 3 min, and 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 5 min. The PCR products were purified using silica column (Tiangen, Beijing, China) and sequenced. All sequencing was performed at Sangon Laboratories (Shanghai, China).

Table 1. Oligonucleotide primers used in this work
Restriction sites are underlined

Primer	Sequence
JcDGAT1-S-*Hind*III	5′-GCGAAGCTTACCATGACGATTTTGGAGACCACT-3′
JcDGAT1-A-XhoI	5′-GCGCTCGAGTCATCTTAATTCAGCATTGCCTTTCCGA-3′
JcDGAT2-51	5′-GACCACACCAAGGGGGAAAACTGAAT-3′
JcDGAT2-31	5′-ATTCAGTTTTCCCCCTTGGTGTGGTC-3′
JcDGAT2-S-*Hind*III	5′-GCGAAGCTTACCATGGTAGGCGGAGATGGCAAT-3′
JcDGAT2-A-XhoI	5′-GCGCTCGAG TCAAAGGATTTCAAGTTTAAGGT-3′
NtActin-F	5′-AAGGGATGCGAGGATGGA-3′
NtActin-R	5′-CAAGGAAATCACCGCTTTGG-3′

Bioinformatical analysis

Nucleotide sequences and amino acid sequences were primarily analysed using Vector NTI advanced software (ver. 11, Invitrogen Corporation, Carlsbad, CA, USA). Sequence homology searches in GenBank were conducted using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 4 October 2011). AlignX was used to align DGAT amino acid sequences with the default settings, and further refined by visual inspection. Phylogenetic analysis was conducted using the neighbour-joining criteria in MEGA (ver. 5.0; Tamura *et al*. 2011). Branch support of the phylogenetic tree was estimated on the basis of 1000 bootstrap replicates of the data. Transmembrane domains of proteins were predicted using the TMHMM Server ver. 2.0 (http://www.cbs.d.tu.dk/services/TMHMM/, accessed 11 September 2011) and subcellular localisation of the deduced polypeptide was estimated using PSORT (http://psort.ims.u-tokyo.ac.jp/form.html, accessed 10 September 2012). The genomic structure of both *JcDGAT1* and *JcDGAT2* were identified using the Spidey server (http://www.ncbi.nlm.nih.gov/spidey/, accessed 5 September 2011) and schematised using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/index.php, accessed 5 September 2011).

Heterologous expression and fatty acid substrate feeding in yeast

For expression of *JcDGAT1* and *JcDGAT2* in yeast, pEasy-JcDGAT1 and pEasy-JcDGAT2 were digested with *Hind*III and Xho I, and the ORFs of *JcDGAT1* and *JcDGAT2* were respectively cloned into the p426-GAL vector with a GAL1 promoter (Mumberg *et al*. 1995), generating vectors p426-JcDGAT1 and p426-JcDGAT2. The cloned sequences were verified by sequencing. Subsequently, p426-JcDGAT1 and

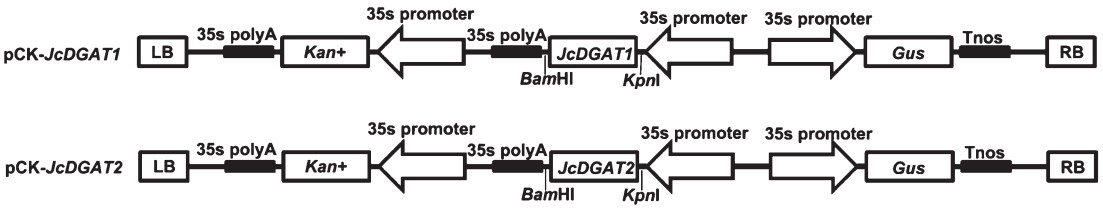

Fig. 1. Schematic structures of the T-DNA regions of pCK-DGAT1 and pCK-DGAT2 vectors for overexpression of *JcDGAT1* and *JcDGAT2* in tobacco.

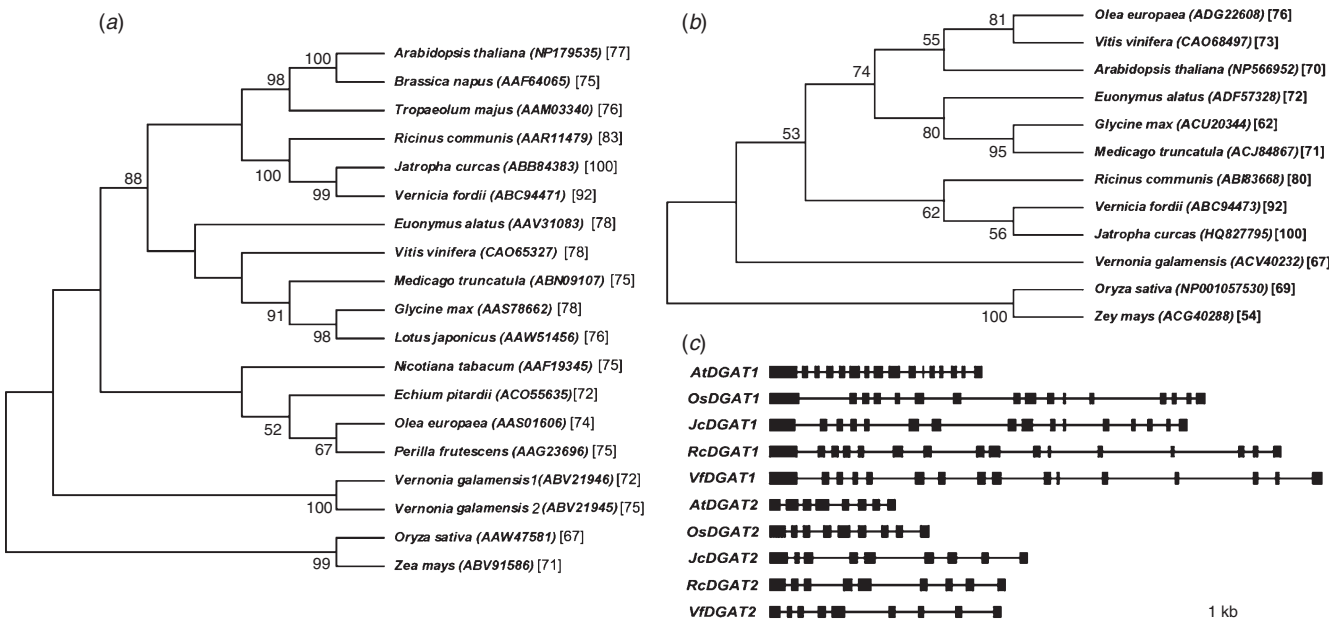

Fig. 2. Phylogenetic relationships and their genomic structures of plant *DGAT1* and *DGAT2* genes. The trees were reconstructed with the neighbour-joining criteria. GenBank accession numbers are given in parentheses. Numbers above branch nodes are bootstrap values from 1000 replicates. The 0.1 scale represent 10% divergence, calculated as estimate numbers of replacement. Only values ≥50% are present. Numbers within brackets correspond to % homologies between jatropha and other species within each group. (*a*) Phylogenetic tree of DGAT1; (*b*) phylogenetic tree of DGAT2; (*c*) genomic structure of plant DGAT1 (*AtDGAT1*, NM_127503, *OsDGAT1*, NC_008398, *JcDGAT1*, JQ3198121, *RcDGAT1*, NW_002994288, *VfDGAT1*, DQ356679) and DGAT2 (*AtDGAT2*, NC_003074, *OsDGAT2*, NC_008399, *JcDGAT2*, JQ3198123, *RcDGAT2*, NW_002994600, *VfDGAT2*, DQ356681) genes. The filled boxes indicate exons and the lines indicate introns.

p426-JcDGAT2 were transformed into H1246 and INVSc1, respectively, using the S. ~EasyComp Transformation Kit (Invitrogen). Yeast strains H1246 and INVSc1 harboured the p426-GAL empty vector and were used as negative and positive controls. Transformants were selected and expression of the recombinant genes in yeast was induced as previously described (Xu *et al.* 2008). After induction, cultures were grown at 30°C, 250 rpm.

To test whether the DGATs encoded by *JcDGAT1* and *JcDGAT2* exhibit substrate preference in yeast cells, the transgenic yeast cells were fed on linoleic acid (18C:2) by exogenously adding linoleic acid into media as acyl substrate of DGAT action. Specifically, the transgenic yeast cell culture was induced at an OD600 of 0.4 by supplementing galactose to 2% (w/v). Linoleic acid was subsequently added into the media to a final concentration of 200 mmol L^{-1} with 1% (w/v) Tergitol NP-40 (Sigma-Aldrich, Guangzhou, China) for making linoleic acid dissolve completely. After a 24 h incubation period, yeast cells (20 mL) were collected by centrifugation, washed with 0.1 M $NaHCO_3$ and stored at −20°C for further lipid analysis.

Yeast lipid analysis

Total lipid (TL) content of yeast cells was spectrophotometrically assayed using sulfophospho-vanillin methods (Izard and Limberger 2003; Wang *et al.* 2009). A total of 50 µL of yeast cells at stationary phase were used for vanillin assay, and 1950 µL of yeast cells were used for dry weight quantisation. Standard curves for converting absorbency readings into quantities of lipids were obtained from two replicate series of experiments, in which the absorbency of known concentrations of each reserve was measured.

To analyse the lipid composition of the yeast, TLs were extracted using a hexane/isopropanol (3 : 2, v/v) method, as previously described (Xu *et al.* 2011), from 20 mL of different yeast strain cells at the stationary growth phase. The TL was then dissolved in 500 µL of hexane and the neutral lipids (NLs) were separated by one-dimensional TLC using silica gel 60 TLC plates (Haiyang, Qingdao, China). Plates were activated in an oven at 120°C for 1 h before use. The sample spots were developed with hexane/diethyl ether/acetic acid (80 : 20 : 1, v/v/v) and NL classes were visually determined using iodine staining and identified using aco-chromatograph with authentic standards. Fatty acid methyl esters (FAMEs) were prepared from the TAG spot and analysed as previously described (Xu *et al.* 2011).

Nile red staining and microscopy

Intracellular lipid bodies as an indicator for TAG formation were visualised by fluorescent microscopy using Nile red (Invitrogen) staining as described previously (Wagner *et al.* 2010). Aliquots of yeast cells (500 µL) grown to stationary phase were harvested, washed twice with 1 × PBS, dissolved in 500 µL of 1 × PBS, stained with 10 µL Nile red dye (2 mg mL^{-1} in DMSO), and then incubated in a dark room for 15 min at room temperature. The stained cells were washed twice with 1 × PBS and resuspended in 200 µL distilled water, and analysed using fluorescence microscopy (Olympus BH2–UMA, Optical Co., LTD, Tokyo, Japan).

Plant transformation

The ORFs of *JcDGAT1* and *JcDGAT2* were subcloned into the *Kpn*I-*Bam*HI sites of a pCambia 2301–101 binary vector with the 35S cauliflower mosaic virus promoter, and named pCK-*JcDGAT1* and pCK-*JcDGAT2* (see Fig. 1). These vectors also carry the nptII gene coding for neomycine phosphotransferase conferring kanamycin resistance and the β-glucuronidase (GUS) gene. The recombinant plasmids (35S/*JcDGAT1*/nos, 35S/*JcDGAT2*/nos) were transformed into wild-type tobacco by *Agrobacterium tumefaciens* (strain GV3101) mediated transformation using the modified leaf-disk method (Horsch *et al.* 1985). Transgenic plants (T_0) were selected and analysed as previously described (Worrall 1998). DNA and RNA were extracted from 100 mg of tobacco leaf material. Successful transformation and the presence of the targeted genes were confirmed using GUS histochemistry analysis and PCR amplification of genomic DNA with primers specific to *JcDGAT1* and *JcDGAT2*.

GUS histochemistry

Seedlings of different transgenic lines and the wild-type tobacco were incubated in a solution of 100 mM potassium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mM 5-broma-4-chloro-3-indolyl-β-D-glucuronide for 24 h at 37°C. The tissues were decolourised with 70% ethanol and photographed with a digital camera (DMC-FZ100GK, Panasonic, Japan).

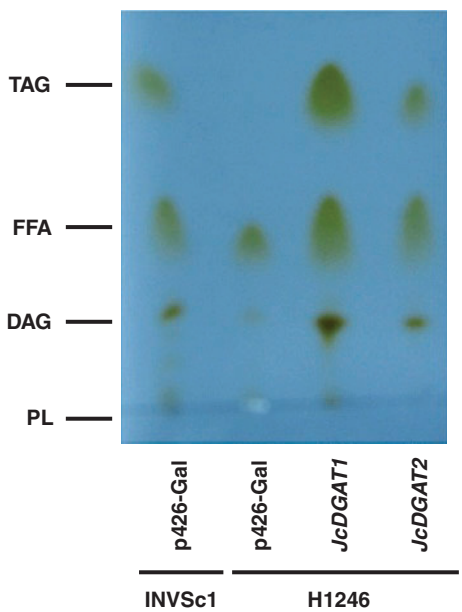

Fig. 3. TLC separation of neutral lipids collected from different yeast strains. The transgenic H1246 strains with the *JcDGAT1* and *JcDGAT2* restore triacylglycerol (TAG) synthesis, compared with controls. The amount of diacylglycerol (DAG), free fatty acid (FFA), phosphatidic lipid (PL) and TAG were visualised by iodine staining. Presented is a representative result of three independent experiments.

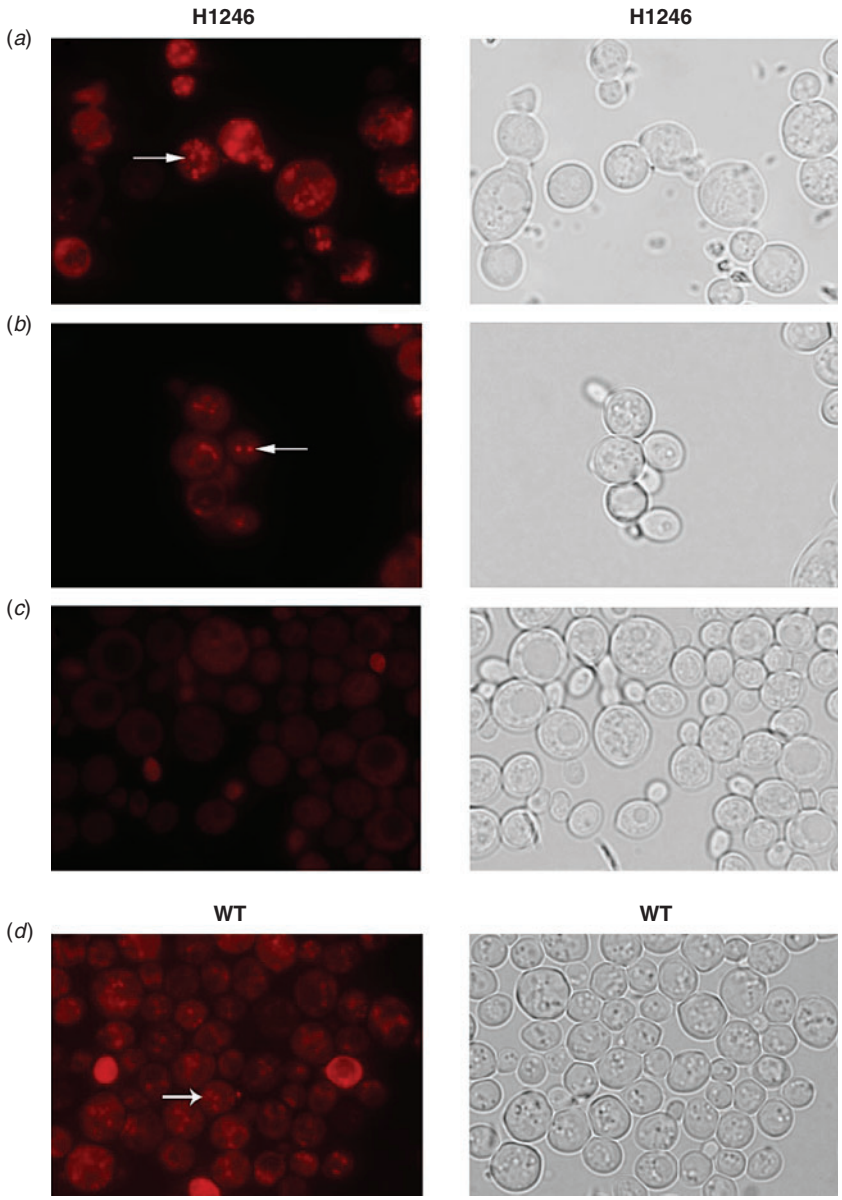

Fig. 4. Formation of lipid bodies is restored upon expression of *JcDGAT1* and *JcDGAT2*. Triacylglycerol (TAG) accumulation in lipid bodies was visualised in yeast cells using the fluorescent dye Nile Red as described in Materials and methods. The TAG-deficient quadruple mutant strain H1246 was expressing either *JcDGAT1* (*a*), or *JcDGAT2* (*b*). As negative control the mutant strain H1246 harbouring the empty plasmid was analysed (*c*). As positive control the wild-type strain INVSc1 was analysed (*d*).The left panel images showed the yeast cells under the fluorescence microscopes and the right panel images showed the yeast cells under optical microscope. Presented is a representative result of three independent experiments.

Seed weight determination and lipid analysis

To determine average seed weight, four replicates of 50 tobacco seeds randomly counted from each line were weighed. These seeds were dried in open tubes in desiccators for 24 h before weighing and counting (Maisonneuve *et al.* 2010). For lipid analysis of transgenic tobacco, four aliquots of 50 tobacco seeds from each line were homogenised three times (15 000 rpm, 30 s each) in 800 µL hexane/isopropanol (3 : 2, v/v, 0.01% BHT) mixture with a superfine homogenizer (Fluko Equipment Shanghai Co., Ltd, Shanghai, China). After dispersion, the homogenate was subjected to direct transmethylation as described by Maisonneuve *et al.* (2010). This method involves adding 2 mL of methanol containing 2.5% H_2SO_4 (v/v) to each sample and heating at 90°C for 90 min. In addition, we used a heptadecanoic acid internal standard at a final concentration of 5 ng µL^{-1} for quantification.

After cooling, 500 µL of hexane and 2.5 mL of 500 mM Na_2SO4 were added. FAMEs were extracted into the hexane phase by vigorous shaking followed by centrifugation at 1500*g* for 5 min. GC analysis of FAMEs were carried out as previously described (Xu *et al.* 2011). Individual methyl esters were identified by comparison with standards (Sigma-Aldrich). Fatty acid mehyl esters and total lipids were calculated by comparing with the heptadecanoic acid methyl ester standard.

Results

Cloning, gene structure and phylogenetic analysis of JcDGAT1 *and* JcDGAT2

The full length ORFs of the *JcDGAT1* and *JcDGAT2* (HQ827795) contain 1563 and 1373 nucleotides, encoding 521 and 352 amino acids respectively. The amino acid sequences of JcDGAT1 and JcDGAT2 are 67 and 56% similar to the corresponding orthologues from *Arabidopsis* (At2 g19450 and At3 g51520, see Stone *et al.* 2006; Liu *et al.* 2011).

Genomic structure analysis showed that *JcDGAT1* (JQ319812) contains 16 exons and 15 introns, while *JcDGAT2* (JQ319813) contains nine exons and eight introns. Further, we compared the genomic structures of *JcDGAT1* and *JcDGAT2* with *DGAT1s* and *DGAT2s* identified from other plants, and found that the number of introns/exons is same or similar (Fig. 2*c*), suggesting that splicing patterns of *DGAT1* and *DGAT2* are conserved in plants. Predictions of transmembrane domain (TMD) and subcellular localisation showed that both JcDGAT1 and JcDGAT2 were located in the endoplasmic reticulum. A blast search against the jatropha genomes released (http://www.kazusa.or.jp/jatropha/, accessed 3 March 2012) revealed that both *JcDGAT1* and *JcDGAT2* were represented by a single copy in jatropha genomes. Phylogenetic analysis showed both *JcDGAT1* (see Fig. 2*a*) and *JcDGAT2* (see Fig. 2*b*) are closely related to their allies cloned from other members (such as castor bean and tung tree) in Euphorbiaceae, reflecting their evolutionary homology.

Functional analyses of JcDGAT1 *or* JcDGAT2 *in yeast*

After *JcDGAT1* and *JcDGA2* were heterologously expressed in the yeast quadruple mutant strain H1246, The TLC fraction analysis showed that both *JcDGAT1* and *JcDGAT2* transgenic H1246 strains presented TAG spots (see Fig. 3), indicating that the DGAT enzymes encoded by *JcDGAT1* and *JcDGAT2* were able to restore TAG biosynthesis in yeast. As shown in Fig. 4, the subcellular oil bodies were visualised in both *JcDGAT1* and *JcDGAT2* transgenic cells under a fluorescent microscope. Moreover, the number of oil bodies visualised within a single *JcDGAT1* transgenic cell is more than that within a single *JcDGAT2* transgenic cell, implying that the *JcDGAT1* transgenic cells produced more TAG than the *JcDGAT2* transgenic cells.

The oil content of cell dry weight (including TLs and NLs) in the transgenic H1246 and wild-type INVSc1 lines was shown in Fig. 5. Within the H1246 strain, the oil content of TLs and NLs in both *JcDGAT1* (13.2 and 9.1%) and *JcDGAT2* (10.6 and 7.1%) transgenic H1246 cells were significantly higher ($P < 0.01$) than the control. Within the INVSc1 strain, the oil content of TLs and NLs in both *JcDGAT1* and *JcDGAT2* overexpressed cells were significantly higher ($P < 0.05$) than the control. In particular, within both H1246 and INVSc1 transgenic strains, *JcDGAT1* produced significantly higher oil content of both TLs and NLs than *JcDGAT2*. These results clearly indicate that DGATs

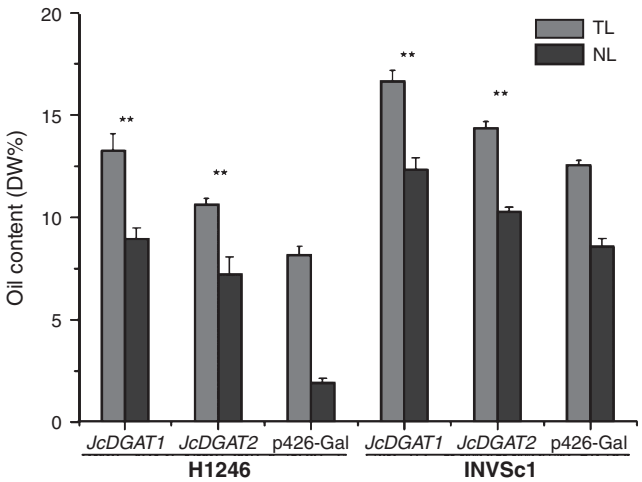

Fig. 5. Oil content analyses of strains H1246 and INVSc1 transformed with *JcDGAT1*, *JcDGAT2* and the empty vector p426-gal respectively. The extraction of total lipids (TLs) from different yeast strains, the isolation of neutral lipids (NLs), and their estimation were described in 'Materials and methods'. Presented here is a representative result of three independent experiments with standard deviation as indicated by the error bars: *P*-values are: *, $P < 0.05$;**, $P < 0.01$.

Table 2. Comparison of fatty acid composition of TAGs accumulated from transgenic yeast H1246 strains of *JcDGAT1* and *JcDGAT2*, and their culture fed with linoleic acid (JcDGAT1-C18:2 and JcDGAT2-C18:2 respectively)

Results are representative of three independent experiments. FAs, fatty acids

	JcDGAT1	JcDGAT1-C18:2	JcDGAT2	JcDGAT2-C18:2
C16:0	10.9% ± 0.9%	11.9% ± 1.2%	6.4% ± 1.0%	7.5% ± 0.9%
C16:1	27.7% ± 1.2%	25.2% ± 2.4%	29.8% ± 5.8%	24.7% ± 0.7%
C18:0	9.9% ± 0.6%	10.1% ± 0.7%	7.4% ± 0.2%	7.2% ± 0.5%
C18:1	51.5% ± 1.8%	42.2% ± 0.9%	56.4% ± 4.7%	35.7% ± 1.5%
C18:2	–	10.6% ± 0.8%	–	25% ± 2.6%
Total saturated FAs	20.8%	22%	13.8%	14.7%
Total unsaturated FAs	79.2%	78%	86.2%	85.4%

encoded by *JcDGAT1* and *JcDGAT2* were able to restore TAG biosynthesis, and that *JcDGAT1* produced higher oil content within transgenic yeast cells than *JcDGAT2*.

As Table 2 shows, comparisons of fatty acid compositions of TAG accumulated within transgenic H1246 cells revealed that the *JcDGAT1* transgenic cells produced significantly higher ($P < 0.01$) saturated fatty acids (20.8%), including 16C:0 and 18C:0, than the *JcDGAT2* transgenic cells (13.8%). Since yeast TAG lacks linoleic acid (Sandager *et al.* 2002), the substrate preference of DGATs encoded by *JcDGAT1*and *JcDGAT2* could be determined by feeding external linoleic acid. Linoleic acid incorporated within the *JcDGAT2* transgenic H1246 cells (25%) was 2.5-fold greater than in *JcDGAT1* transgenic strains (10.6%), suggesting the JcDGAT2 has a linoleic acid substrate preference than the JcDGAT1. The lower oleic acid content in *JcDGAT2* transgenic cells (35.7%) than in *JcDGAT1* transgenic cells (42.2%) might be due to substrate competition resulting in the reduction of oleic acid during TAG biosynthesis.

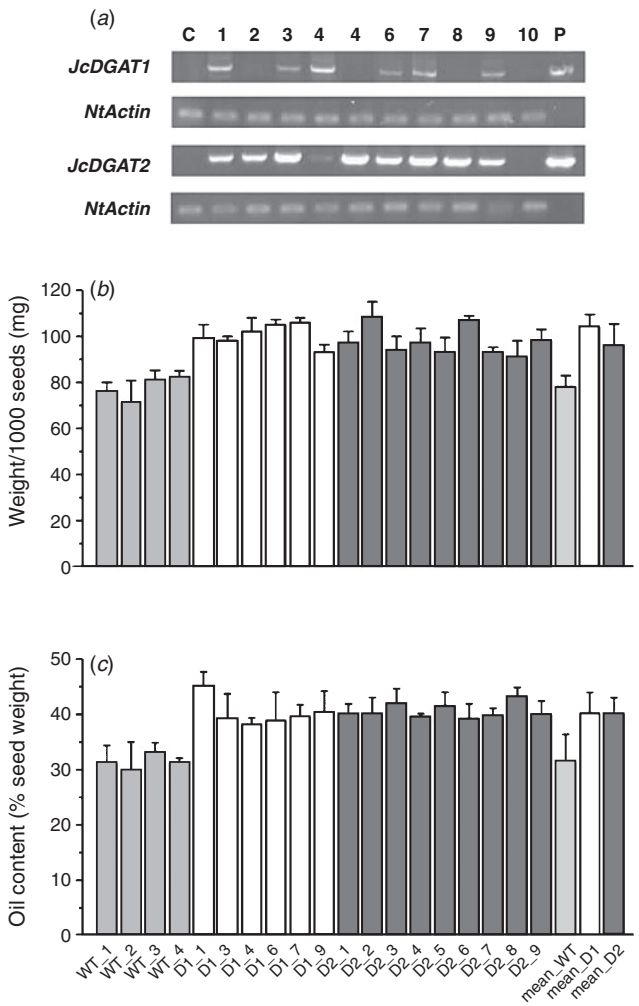

Fig. 6. RT–PCR analysis and comparison of seed weight and oil content among transgenic tobacco lines and the controls. (*a*) RT–PCR analyses of 10 *JcDGAT1* or *JcDGAT2* transformants in transgenic lines and controls (C and P denote the negative and positive control, respectively, the actin gene of tobacco was used as a housekeeping control). Based on the presence of the targeted genes in RT–PCR analyses (*a*), the six and nine successful transformants of *JcDGAT1* and *JcDGAT2* were sorted out respectively. The seed weight (*b*) and oil content (*c*) of *JcDGAT1*and *JcDGAT2* transformants and four wild types were compared with their mean, mean_D1, mean_D2, mean_WT respectively.

Functional analyses of JcDGAT1 *or* JcDGAT2 in planta

After the transformants were determined by GUS analysis and RT–PCR amplification, six and nine independent transgenic lines of *JcDGAT1* and *JcDGAT2* were identified and cultivated in the greenhouse. Five non-transformed wild-type plants were cultivated as controls. Their seeds (T_0) were harvested to analyse. Seed oil content and weight were compared between different transgenic plants and the control (see Fig. 6). Both *JcDGAT1* and *JcDGAT2* transgenic plants showed that ~32% increased oil content, and seed weight increased by 33 and 23% respectively. However, there was no significant difference between seed oil content and weight of *JcDGAT1* and *JcDGAT2* transgenic plants. The total saturated/unsaturated FA content did not make a significant difference among the *JcDGAT1*, *JcDGAT2* transformants and the controls (Table 3). But, the content of the 18C:2 FA in the *JcDGAT2* transformants (73.85%) increased significantly compared with the *JcDGAT1* transformants (66.60%) and the control (67.12%), correspondingly, the 18C:1 FA (11.08%) significantly decreased. This suggests that *JcDGAT1* and *JcDGAT2* make different contributions to the accumulation of 18C:2 FA in tobacco seeds.

Discussion

DGAT is the only enzyme in the Kennedy pathway that is thought to be exclusively committed to TAG biosynthesis, and thus it is considered a key enzyme in this reaction (Lung and Weselake 2006). In addition to *DGAT1* and *DGAT2* (which have been extensively identified in plants), the third class *DGAT3* gene was identified from *Arabidopsis* (Hernández *et al.* 2012) and peanut (Saha *et al.* 2006), but little is known about the *DGAT3* gene in other plants. Studies have shown that the *DGAT1* and *DGAT2* represent different gene families with its own functional motifs in gene structure. Why the *DGAT1* and *DGAT2* present similar

Table 3. Comparison of fatty acid composition of seed oils accumulated from the transformants of *JcDGAT1* and *JcDGAT2* in tobacco

Presented here is the mean of four individual wild types (WT), six individual *JcDGAT1* transformants and nine individual *JcDGAT2* transfomrants. FAs, fatty acids. *P*-values are: *, $P < 0.05$;**, $P < 0.01$

	WT (%)	JcDGAT1 (%)	JcDGAT2 (%)
C14:0	0.3 ± 0.1	0.3	0.5 ± 0.2
C16:0	10.4 ± 1.2	10.1 ± 1.7	9.6 ± 0.7
C18:0	5.4 ± 1.1	3.9 ± 1.2	3.6 ± 0.8
C16:1	0.2 ± 0.1	0.2 ± 0.1	0.2
C18:1	15.1 ± 1.4	16.8 ± 1.8	11.1 ± 1.3*
C18:2	67.1 ± 1.3	66.6 ± 1.2	73.9 ± 1.5**
Total saturated FAs	16.1	15.3	13.7
Total unsaturated FAs	82.4	83.6	85.1
others	1.5 ± 0.5	1.1 ± 0.3	1.2 ± 1.2

function in biosynthesis of TAG in plants is, however, not well understood (Li *et al.* 2010*b*; Turchetto-Zolet *et al*. 2011).

Our results show that both *JcDGAT1* and *JcDGAT2* were able to restore TAG synthesis in the yeast cell and tobacco, reflecting the functional similarity of *JcDGAT1* and *JcDGAT2* in TAG biosynthesis. However, the oil contents of *JcDGAT1* and *JcDGAT2* transgenic yeast cells made significant differences, implying *JcDGAT1* has a greater contribution to regulation of TAG quantity during the pathway of TAG biosynthesis. However, the seed oil content and weight of *JcDGAT1* and *JcDGAT2* transformants in tobacco did not present a significant difference in our current study. This may be because we omitted screening the homozygous transgenic seeds for each transgenic line, meaning that results obtained were the average values from different transgenic genotypes such as homozygous, hemizygous, or non-transgenic seeds. The non-transgenic and hemizygous seeds could decrease the difference between the oil content of *JcDGAT1* or *JcDGAT2* transgenic lines. The oil content and weight of homozygous transgentic seeds might be higher than our current average values.

In addition, we demonstrated that JcDGAT2 had an obvious substrate preference to linoleic acid in both yeast and tobacco systems, compared with JcDGAT1. To our knowledge, this is the first report on the substrate preference of DGAT2 to linoleic acid in plants. Previous studies also showed that *RcDGAT2*, *VgDGAT2* and *VfDGAT2* played a major role in TAG assembly of hydroxy or epoxy fatty acids in developing seeds (Shockey *et al.* 2006; Burgal *et al.* 2008; Li *et al.* 2010*b*). It seems that *DGAT2*s have a greater contribution to accumulation of specific fatty acid compositions of TAG *in planta* than *DGAT1*s. The major function of *DGAT2*s may be for regulating or controlling the fatty acid composition of TAG by selectively incorporating different acyls in plants. However, it is uncertain whether the substrate preference of DGAT2 to linoleic acid is species dependent or ubiquitous in plants. If it is jatropha-species dependent, *JcDGAT2* could be used as a gene resource for altering fatty acid composition of TAG by genetic engineering. Further investigation on overexpression or silence of *JcDGAT1* and *JcDGAT2* is necessary to determine their functions in regulating TAG biosynthesis during jatropha seed development.

Conclusions

Our data characterised the structure and function of *JcDGAT1* and *JcDGAT2*, and demonstrated their functions in regulating the quantity of TAG and fatty acid composition of TAG during TAG biosynthesis in both yeast and tobacco systems. These results provide new insights into understanding the molecular mechanisms underlying the extensive difference of oil content or fatty acid composition of TAG in seed oils.

Acknowledgements

We thank Dr Sten Stymne (Swedish University of Agricultural Sciences, Uppsala) for kindly providing the H1246strain. This research was jointly supported by NSFC (Grant No.30871548 and 31300569), and the ‘Hundred Talents Program of the Chinese Academy of Sciences’ to AL.

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