

Increasing seed oil content and altering oil quality of *Brassica napus* L. by over-expression of diacylglycerol acyltransferase 1 (*SsDGAT1*) from *Sapium sebiferum* (L.) Roxb.

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Received: 5 December 2015 / Accepted: 1 August 2016 / Published online: 17 September 2016
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Abstract *Sapium sebiferum* (L.) Roxb. [*S. sebiferum*] is one of the most important woody oil trees and traditional herbal medicines in China. Diacylglycerol acyltransferases (DGATs) esterify *sn*-1,2-diacylglycerol with a long-chain fatty acyl-CoA acting as a key enzyme at the last and rate-limiting step of triacylglycerol (TAG) biosynthesis in eukaryotic organisms. Although at least 61 DGAT1 sequences from 48 organisms have been identified, until now there have been almost no reports on the *DGAT1* gene in *S. sebiferum* (*SsDGAT1*). In an attempt to clarify *SsDGAT1*'s function, we cloned the CDS (coding sequence) of *SsDGAT1* by RACE (rapid amplification of cDNA ends) technology. The full-length CDS of *SsDGAT1* contains 1524 bp, encoding a protein of 507 amino acids. Even recombinant *SsDGAT1* was able to

restore TAG biosynthesis in the yeast strain *S. cerevisiae* H1246 TAG-deficient mutant. Moreover, when *SsDGAT1* was placed under the control of 35S promoter in *Brassica napus* L. *W10*, the total oil content of transgenic rape plants showed an increase of 12.3–14.7 % compared with the wild type. In addition, the transgenic rapeseed with heterologous expression of *SsDGAT1* showed an alteration in seed oil composition overall: a significant decrease in oleic acid levels but a tendency towards an increase in linolenic acid levels. The result confirmed that *SsDGAT1* may be involved in flux control of oil biosynthesis and could be used specifically to manipulate and improve oil content and composition in plants. These experimental findings suggest that we

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might be able to develop a plant high in industrial oils by over-expression of *SsDGAT1* in *S. sebiferum*.

Keywords *Sapium sebiferum* L. · *SsDGAT1* · Clone · Function · *Brassica napus* L. · Oleic acid content

Abbreviations

qPCR	Quantitative real-time polymerase chain reaction
RT-PCR	Reverse-transcription polymerase chain reaction
RACE	Rapid amplification of cDNA ends
GFP	Green fluorescent protein
cDNA	Complementary DNA
CTAB	Hexadecyltrimethylammonium bromide
TLC	Thin-layer chromatography
GC–MS	Gas chromatography–mass spectrometry
FA	Fatty acids

Introduction

At present, as the conflict between ever-increasing consumption of fossil fuels and rapidly dwindling resources worsens every year, there is panic about the scarcity of fuel and therefore more and more calls to seek other alternative sources of fuel in the future. Triacylglycerols (TAGs) occur largely in oil-rich plants as oils, so the TAGs of oil-rich plants are being considered as a possible renewable source of fuels in the form of biodiesel (Cahoon et al. 2007; Durrett et al. 2008; Yang et al. 2015). The added advantage of biofuels is less emission of greenhouse gases (Yang et al. 2015). However, traditional TAG-oil-rich plants for biodiesel such as soybean, rapeseed and sunflower are also food oil crops (Yang et al. 2015). Owing to the importance and economics of food oil, the ideal crop source for biodiesel products should be non-edible and grown on waste-agricultural lands. Therefore, oil-rich plants such as *Sapium sebiferum* (L.) Roxb. (Euphorbiaceae) [*S. sebiferum*] seem to be a better source for biodiesel products.

S. sebiferum is one of the most important perennial woody trees. In China, *S. sebiferum* has been cultivated for over 1400 years for seed oil and traditional herbal medicines (Peng et al. 2008). Moreover, Yang et al. (2007) reported that the seeds of *S. sebiferum* contain many kinds of fatty acids, such as oleic acid (15.12 %),

linoleic acid (31.60 %), caproic acid (2.70 %), linolenic acid (44.19 %) and palmitic acid (6.38 %). The parameters of *S. sebiferum* oil such as the cetane number, kinematics of viscosity and exhaust emission meet the requirements of biodiesel production (Divi et al. 2016). A previous study showed that chemical component of caproic acid is 2t,4c-decadienoic acid (10:2Δ^{2t}, 4c, stilingic acid), which belongs to the conjugated fatty acids (Yang et al. 2015).

The terminal enzyme of the Kennedy pathway, diacylglycerol acyltransferase (DGAT; EC 3.2.1.20), is one of the rate-limiting steps in storage lipid accumulation in plants and plays a central role in diverting fatty acid flux towards the formation of TAGs (He et al. 2004; Jako et al. 2001; Lung and Weselake 2006; Yu et al. 2006, 2008). DGATs are generally localized in the endoplasmic reticulum, although a cytosolic form has been identified in peanuts (Saha et al. 2006). At least two different types of *DGAT* gene families (*DGAT1* and *DGAT2*) that differ considerably in sequence have been reported, and play non-redundant roles in TAG biosynthesis (Kroon et al. 2006; Shockey et al. 2006). These two forms of the DGAT enzyme have species-specific preferences for TAG production and accumulation during seed development. He et al.'s results indicated that *DGAT1* plays a dominant role in ricinoleic acid production in castor bean (2004), whereas the data of Kroon et al. (2006) suggested that *DGAT2* is the major contributor to TAG formation in castor seed oil. On the other hand, Shockey et al.'s study (2006) indicated that *DGAT2* rather than *DGAT1* plays a key role in TAG accumulation in developing seeds of the tung tree. Moreover, *DGAT3* was also identified in some plants and fungi, such as peanut (*Arachis hypogaea*) (Saha et al. 2006), *Arabidopsis* (Bouvier et al. 2000; Hernández et al. 2012), *Vernicia fordii* (Cao et al. 2013) and *Phaeodactylum tricornutum* (Cui et al. 2013). There was even a study suggesting that *DGAT3* could play a role in an alternative pathway for TAG synthesis during *pxa1* seedling establishment (Hernández et al. 2012). Analysis of publicly available Affymetrix data revealed that *DGAT3* expressed throughout seed development and seedling establishment in *Arabidopsis* (Hernández et al. 2012).

Much previous research has indicated that manipulation of *DGAT1* has a considerable effect on plant oil development and its composition. On the one hand,

there was deficient DGAT activity, less oil accumulated and decreased TAG/DAG ratio in *Arabidopsis* lines lacking *DGAT1* (Katavic et al. 1995; Routaboul et al. 1999; Zou et al. 1999), but decreased seed oil content and increased protein and carbohydrate in RNAi-mediated silencing of *DGAT1* in tobacco (Zhang et al. 2005). On the other hand, the oil levels of *Arabidopsis*, tobacco and *Brassica napus* L. were increased in *DGAT1* over-expression lines (Jako et al. 2001; Weselake et al. 2008; Andrianov et al. 2010).

PDAT1 (phospholipid:diacylglycerol acyltransferase) is another key enzyme which makes TAGs independent of the Kennedy pathway and is involved in TAG biosynthesis in plants (Zhang et al. 2009). Furthermore, PDAT1 may have overlapping functions with DGAT1 for normal viable pollen and seed development in *Arabidopsis* (Zhang et al. 2009). However, a more recent study suggested that DGAT2 and PDAT play critical roles in epoxy and hydroxy fatty acid accumulation in seed oils of source plants, while DGAT1 appears to be a key enzyme for oil accumulation in all plants that have been studied, especially in *Arabidopsis* and soybean (Li et al. 2010). At the same time, the accumulation of total hydroxy fatty acids was significantly enhanced up to 25 % in castor-like oil in transgenic *Arabidopsis* seeds heterologously expressing *PDAT1-2* of castor bean (Kim et al. 2011).

Although the function of DGAT1 has recently been well studied in some species, there has been no reported investigation of *SsDGAT1* in *S. sebiferum* until now. In this paper, the annotation of gene structure for *SsDGAT1* from *S. sebiferum* has been carried out based on clone data. In addition, gene function analysis of *SsDGAT1* will provide an excellent reference for the molecular characterization of *SsDGAT*.

Materials and methods

Plant material

Developing seeds of *S. sebiferum* were taken from the tree grown in the fields of Central South University of Forestry and Technology and collected at ~15-day intervals from the day after pollination (DAP) to seed fully matured, and all stages of these seeds were used for RNA isolation. For investigating the expression of

SsDGAT1, tissues such as the root, stem and leaf were harvested from 30-day-old seedlings and flower materials were acquired from adult plant. All tissues mentioned above were frozen in liquid nitrogen and stored at -80°C until further experiment.

RNA preparation and reverse transcription

RNA was extracted from various samples using a RNA purification kit (InvitrogenTM, USA) according to the manufacturer's protocol. To synthesize complementary DNA (cDNA) from isolated RNA, reverse transcription was performed using a reverse transcription kit (MBI, Lithuania) following the manufacturer's instructions.

Isolation of the conserved region and full-length cDNA of *SsDGAT1*

For amplifying homologous region sequences of *SsDGAT1*, the second-step PCR amplification was performed using primers *SsDGAT1*-Fc [5'-AGTCATGC(A/G)GGTCT(C/G)TT(T/C)AA(C/T)TGTATA-3', forward]/*SsDGAT1*-Rc [5'-GCC(C/T)GTTCA(C/T)A(G/A)TGGATGGT(C/T)CGCGAT-3', reverse], which were designed based on multiple amino acid sequence alignment of *DGAT1* homologs from plants of interest (Fig. 1b), such as *Vernicia fordii* (GenBank accession No. DQ356680.1), *Jatropha curcas* (GenBank accession No. ABB84383.1), *Ricinus communis* (GenBank accession No. XP_002514132.1) and *Camellia oleifera* (GenBank accession No. JF264900). The PCR cycling parameters were 35 cycles with denaturation for 30 s at 94°C , annealing for 30 s at 60°C , and extension for 5 min at 72°C . The PCR reaction mix comprised of $10\times$ PCR buffer, first-strand cDNA, 10 μmol of each primer pair, 20 mM dNTP mix and *Taq* polymerase (Takara, Dalian, China) in a total volume of 20 μL . The PCR product was sub-cloned into pGEM[®]-T Easy vector (Promega, USA) and confirmed by DNA sequencing. In order to obtain 5' and 3' residue sequences of *SsDGAT1* from *S. sebiferum*, the RACE clone was adopted with four pairs of degenerate primers (F1: 5'-ACTTCATGGTTGCTCCCACCCTATGT-3', R1: 5'-CTAATA CGACTCACTATAGGGCAAG CAGTGGTATCAACGCAGAGT-3', F2: 5'-CGAA TGGATGCA GTGCGAGGATAAC-3', R2: 5'-CTAA

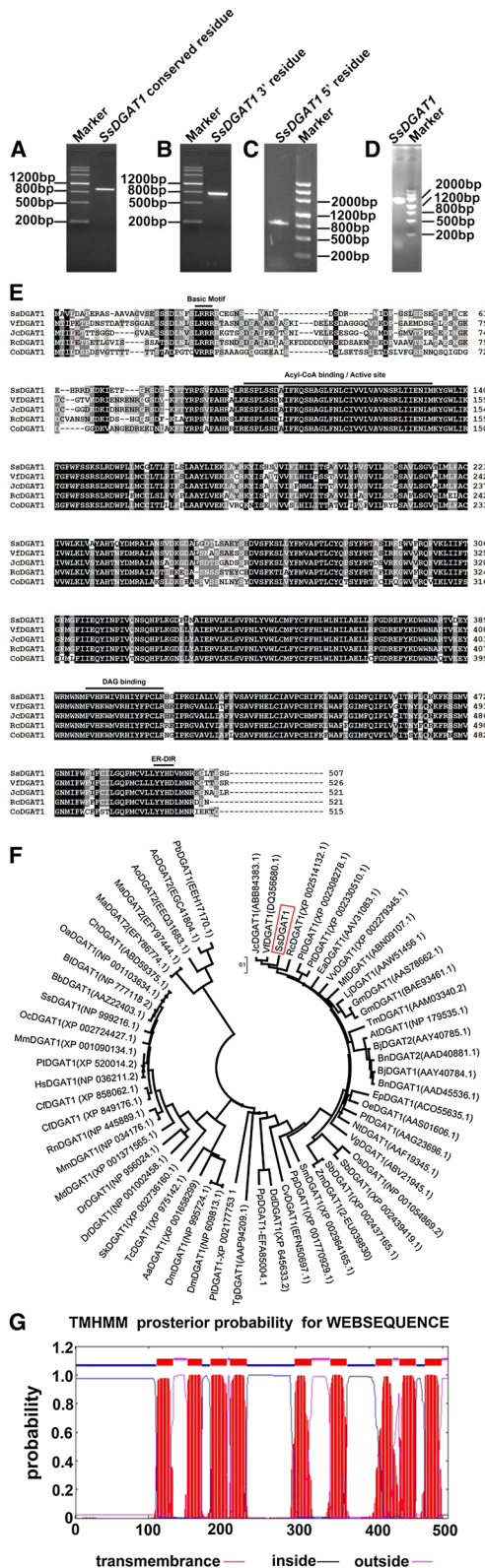


Fig. 1 Cloning of *SsDGAT1* from *S. sebiferum* seed cDNA. **a** Amplification of the conserved region of *SsDGAT1* by RT-PCR; **b** amplification of the 3' terminus of *SsDGAT1* by 3' RACE; **c** amplification of the 5' terminus of *SsDGAT1* by 5' RACE; **d** amplification of the full length of *SsDGAT1* by RT-PCR; **e** amino acid sequence alignment of DGAT1 homologs from various plant species; **f** phylogenetic analysis of DGAT1s; **g** predicted transmembrane domain of *SsDGAT1*. The evolutionary relationships among the 64 DGAT1 from 49 organisms were subjected to phylogenetic analysis based on the neighbor-joining method of Tamura et al. (2007). The *framed section* indicates the *SsDGAT1* protein studied here. Genes are labeled with their GenBank accession numbers, followed in parentheses by the initials of the organism. The calculated distance values are indicated before the gene labels, and reflect the degree of divergence between all pairs of *SsDGAT1* sequences analyzed. Abbreviations: Aa in XP_001658299, *Aedes aegypti*; Ac in EGC41804.1, *Ajellomyces capsulatus* H88; Ao in EEQ 31683.1, *Arthroderma otae* CBS 113480; At in NP_179535.1, *Arabidopsis thaliana*; Bb in AAZ22403.1, *Bubalus bubalis*; Bn in AAD45536.1, in AAD40881.1, *Brassica napus*; Bj in AAY40784.1, in AAY40785.1, *Brassica juncea*; Bt in NP_777118.2, *Bos taurus*; Ca in ABD59375.1, *Capra hircus*; Cf in XP_849176.1 and XP_858062.1, *Canis familiaris*; Cv in EFN50697.1, *Chlorella variabilis*; Dd in XP_645633.2, *Dicτυostelium discoideum* AX4; Dm in NP_609813.1 and NP_995724.1, *Drosophila melanogaster*; Dr in NP_001002458.1 and NP_956024.1, *Danio rerio*; Ea in AAV31083.1, *Euonymus alatus*; Ep in ACO55635.1, *Echium pitardii*; Gm in AAS78662.1 and BAE93461.1, *Glycine max*; Hs in NP_036211.2, *Homo sapiens*; Jc in ABB84383.1, *Jatropha curcas*; Lj in AAW51456.1, *Lotus japonicas*; Ma in EFY86774.1, *Metarhizium acridum* CQMa 102; Ma in EFY97444.1, *Metarhizium anisopliae* ARSEF 23; Md in XP_001371565.1, *Monodelphis domestica*; Mm in NP_034176.1, *Mus musculus*; Mt in ABN09107.1, *Medicago truncatula*; Nt in AAF19345.1, *Nicotiana tabacum*; Oa in NP_001103634.1, *Ovis aries*; Oc in XP_002724427.1, *Oryctolagus cuniculus*; Oe in AAS01606.1, *Olea europaea*; Os in NP_001054869.2, *Oryza sativa japonica*; Pb in EEH17170.1, *Paracoccidioides brasiliensis* Pb03; Pf in AAG23696.1, *Perilla frutescens*; Pp in XP_001770929.1, *Physcomitrella patens*; Pp in EFA85004.1, *Polysphondylium pallidum* PN500; Pt in XP_520014.2, *Pan troglodytes*; Pt in XP_002177753.1, *Phaeodactylum tricornerum* CCAP 1055/I; Pt in XP_002308278.1 and XP_002330510.1, *Populus trichocarpa*; Rc in XP_002514132.1, *Ricinus communis*; Rn in NP_445889.1, *Rattus norvegicus*; Sb in XP_002439419.1 and XP_002437165.1, *Sorghum bicolor*; Sk in XP_002736160.1, *Saccoglossus kowalevskii*; Sm in XP_002964165.1, *Selaginella moellendorffii*; Ss in NP_999216.1, *Sus scrofa*; Tc in XP_975142.1, *Tribolium castaneum*; Tg in AAP94209.1, *Toxoplasma gondii*; Tm in AAM03340.2, *Tropaeolum majus*; Vg in DQ356680.1, *Vernicia fordii*; Vg in ABV21945.1, *Vernonia galamensis*; Vv in XP_002279345.1, *Vitis vinifera*; Zm in 2-EU039830, *Zea mays*

TACGACTCACTATAGGGC-3', F3: 5'-AGCCAAG TTATCCTCGCACTGCATCCA-3', R3: 5'-ACTA TAGGGCAAGCAGTGG TATCAACGCAGA GT-3', F4: 5'-CTTCATGGTTGCTCCCACCTATGTTA-3', R4: 5'-CTAATACGACTACTA TAGGGC-3'). Primers (F1, R1 and F2, R2) were used for the first and second step clones of the 5' sequence of *SsDGATI*, respectively. For the 3' RACE clone, primers (F3, R3 and F4, R4) were respectively utilized in the first and second steps. Gene-specific primers F0 (5'-ATG GCGGTTCTTGACGC-3') and R0 (5'-TCAACCT GATTCAGTTAGCCCTTTTCG-3') were designed to obtain the complete open reading frame (ORF) by RT-PCR. This ORF fragment was cloned and sequenced.

Phylogenetic analysis

The phylogenetic analysis was performed in accordance with the method of Wang et al. (2015).

Putative transmembrane domains

We predicted the transmembrane domain of *SsDGATI* using <http://www.cbs.dtu.dk/services/TMHMM/> online software.

Semi-quantitative RT-PCR and quantitative real-time PCR (qPCR)

Semi-quantitative RT-PCR and/or qPCR were performed to detect the expression of *SsDGATI* in each organ and in seeds at different growth phases. *Ss18S* (GenBank accession No. DQ864377.1) fragment, encoding a 240-bp region of *Triadica sebifera* 18S ribosomal RNA, was used as internal reference. Primer sets used for semi-quantitative RT-PCR and qPCR were as follows: *SsDGATI*-specific 5' (5'-TT ACCAGCCAAGTTATCCTCG-3') and 3' (5'-AGC AGTAGAACATGCAGAGCC-3'), internal control's *Ss18S*-F (5'-ATGGGATAACATCATAGGATTTTCG G-3') and *Ss18S*-R (5'-TGGTCGGCATCGTTTA TGGTT-3'). The thermal cycling program of semi-quantitative RT-PCR consisted of 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. qPCR was performed using a CFX-96 (Bio-Rad, USA) Real-Time system. Samples were run in triplicate, with starting quantity estimated from critical thresholds using the standard curve of amplification.

Yeast transformation and selection

The *SsDGATI* gene was introduced into the pYES2 vector by using *XhoI* and *XbaI* digestion sites and then the *pYES2-SsDGATI* construct was transferred into *S. cerevisiae* H1246 (W303; MAT α *are1*- Δ ::HIS3 *are2*- Δ ::LEU2 *dgal1*::KanMX4 *lro1*- Δ ::TRP1 ADE2 met ura3) (Sandager et al. 2002) using the polyethylene glycol (PEG)/lithium acetate method (Gietz and Woods 1998). Yeast cells harboring the empty pYES2 vector were used as the negative control and *S. cerevisiae* W3013A was used as the wild-type (WT) yeast strain in this experiment. Selection of positive transformants was carried out as described by Wang et al. (2015).

Lipid extraction and analysis

Extraction and analysis of lipids from yeast cell was performed as described previously (Wang et al. 2015). TAGs were separated from total lipids by thin-layer chromatography (TLC) using a solvent system of hexane/ether/acetic acid (70/30/1, v/v/v) (Xu et al. 2013). Individual lipid spots were visualized by exposing the silica gel plates (Shanghai, China) to iodine vapor.

Analysis of seed oil, protein, glucosinolate and glucose content in *B. napus*

The oil, protein, glucosinolate and glucose content of mature *B. napus* seed were determined by the method of Lock et al. (2009).

Construction and identification of transgenic *B. napus* plants

The *SsDGATI* gene from *S. sebiferum* cDNA was amplified using the primers 5'-TCCCCCGGGAT GGCGGTTCTTGACGC-3' and 5'-CGCGGATCC ACCTGATTCAGTTAGCCCTTTTCG-3' (restriction sites *SmaI* 'CCCGGG' and *EcoRI* 'GGATCC' are shown in boldface, and the sequence corresponding to *SsDGATI* is underlined). The amplified product was sub-cloned into the *SmaI*-*XhoI* cloning site of the pGEM[®]-T Easy vector (Promega, USA). After the cloned intact *SsDGATI* was confirmed correct by sequencing, the *SmaI*-*XhoI* DNA fragments of

SsDGAT1 were excised from pGEM[®]-T Easy vector by *Sma*I and *Xho*I digestion and inserted into the binary vector pEGAD (Fig. 4a). The construct *35S::GFP-SsDGAT1* was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation using a Gene Pulser II system (Bio-Rad, Hercules, CA, USA) and the stabilized positive constructs selected with 100 mg L⁻¹ rifampicin and 50 mg L⁻¹ kanamycin were confirmed by PCR, and then *B. napus* plants were transformed by the *Agrobacterium tumefaciens*-mediated floral dip transformation method (Zhou et al. 2012). In detail, the grown healthy flora inflorescences at the top of *B. napus* plants which have many immature flower clusters and only few fertilized siliques were selected as transformation material, while the siliques, opened flowers found on the flora inflorescences and all other branches and flora inflorescences that could be immersed were removed with pruning scissors before gene transformation. The pruned inflorescences were swathed and immersed in the plastic valve bag with *Agrobacterium* containing *35S::GFP-SsDGAT1* inoculum (OD = 0.6) for 30 s with gentle agitation. The dipped inflorescences were removed from the solution and the treated inflorescences drained for 3–5 s. A film of liquid should be observed coating the inflorescences. The bacteria were distributed to all inflorescences including very young flower shoots by gently pressing the outside of the plastic bag with the hands, taking care not to break the *B. napus* plants and inflorescences. The transformation procedure was repeated twice every 3 days after the first transformation. The *35S::GFP-SsDGAT1* construct and its transgenic daughter lines were selected for further study. T1 generation seeds were selected using the herbicide Basta (v/v, 1:1000). T2 lines were used for seed oil analysis.

DNA of 7-day-old seedlings was extracted from the WT and Basta-resistant transgenic lines using the CTAB method (Murray and Thompson 1980). The Basta resistance *bar* gene, *GFP* gene and *GFP-SsDGAT1* gene were determined by PCR analysis with primers (*bar*-F: 5'-CTACATCGAGACAAGCACG GT-3' and *bar*-R: 5'-CTGAAGTCCAGCTGCCA GAA-3', *GFP*-F: 5'-GACCCTGAAGTTCATCTG CACCACC-3' and *GFP*-R: 5'-CCGATGGGGGTG TTCTGCTG-3', *G + S*-F: 5'-TAAACGGCCACAA GTTCAGCG-3', *G + S*-R: 5'-CGTCTACTGTTCTT GCATTCCACC-3'). PCR was performed at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 58 °C

for 30 s and 72 °C for 30 s, and then 72 °C for 5 min. PCR products were analyzed using 1.0 % agarose gel electrophoresis. Total RNA was extracted from 100 mg of seedlings of 7-day-old *B. napus* using Trizol reagent (Invitrogen, USA) and treated with RNase-free DNase I (Promega Biotech Co., USA). The DNase-digested RNA samples were used for reverse transcription by Superscript III reverse transcriptase (Invitrogen). Samples serving as cDNA stocks for PCR analysis were stored at -80 °C. Gene expression was analyzed by semiquantitative RT-PCR using rTaq DNA polymerase (Takara, Dalian, China) and a programmable thermal cycler CFX-96 (Bio-Rad, USA) in a 20-μL solution containing 1 μL cDNA stock. The PCR amplification consisted of an initial denaturation step at 94 °C for 5 min followed by 22 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 20 s. The sense and antisense primers of *SsDGAT1* were 5'-TAGTTCGCCATATCTACTTTCCA-3' and 5'-AACCTGATTCAGTTAGCCCTTTT-3', respectively. *BnACTIN7* was used an internal control; its sense primer (5'-TGTTGCTATCCAGGCTGTTCT-3') and anti-sense primer (5'-TTAATGTCACGGAC GATTTC-3') were designed according to the Genbank Accession Number AF111812.

DNA gel blotting

Genomic DNA was isolated from leaves of individual seedlings using the Plant DNA extraction kit (Takara) according to the manufacturer's instructions. After endonuclease digestion with *Eco*RI and electrophoresis on a 1 % agarose gel, DNA fragments were transferred to a positively charged nylon membrane (Roche). Probes were prepared using a PCR DIG probe synthesis kit (Roche). Hybridization was performed according to the DIG Application Manual (Roche). Hybridization was carried out at 42 °C and washing was performed under high-stringency conditions at 68 °C. *GFP* gene was used as probe in this study; the primers of *GFP* were *GFP*-F: 5'-CAC AAGTTCAGCGTGTCCG-3', *GFP*-R: 5'-AGTTCA CCTTGATGCC GT TC-3'.

Immunoblot analysis

Total protein was isolated from *B. napus* seeds after flowering for 60 days according to the procedure described by Zhou et al. (2012). The enhanced

chemiluminescence immunoblot analysis was performed following the method of Wang et al. (2015). In this study, the immunoblot was probed with appropriate primary antibodies (anti-GFP antibody) and secondary antibody conjugated with horseradish peroxidase (Beyotime Institute of Biotechnology, China). Histone1 was used to verify equal loading. All other chemicals were acquired from Sigma (St. Louis, MO, USA).

Oil analysis by GC–MS

The *B. napus* seed lipids were extracted from 10 mg dried seeds of control and transgenic lines (Li et al. 2006). The oil analysis was carried out by GC–MS according to the method of Misra et al. (2013).

Results

Cloning of the *SsDGAT1* gene from *S. sebiferum*

An 837-bp conserved nucleotide sequence corresponding to the *SsDGAT1* gene (Fig. 1a) was obtained by PCR amplification, homologous region cloning and sequencing, then a 3' terminal fragment of the *SsDGAT1* gene was successfully amplified based on this nucleotide sequence (Fig. 1b). Furthermore, a 5' terminal fragment of the *SsDGAT1* gene was produced by nested PCR after 5' RACE (Fig. 1c). Sequencing results showed that the 3' terminal fragment and 5' terminal fragment were 783 and 812 bp in length, respectively. The *SsDGAT1* full-length cDNA with 1524 bp (Fig. 1d) was determined by the overlapping sequences of the 5' and 3' ends and then amplified by RT-PCR, which encoded a protein with 507 amino acids (Fig. 1e). We have submitted the sequence to National Center for Biotechnology Information (NCBI); the BankIt No. is 1926735 and the accession number is KX372341. The transmembrane structure analysis result indicated that SsDGAT1 encoded a peptide consisting of nine putative transmembrane domains (Fig. 1g). A phylogenetic tree was generated from an alignment of the amino acid sequences between SsDGAT1 and four DGAT1 homologs from diverse species, and three highly conserved motifs, the Basic motif acyl-CoA binding signature, DAG-binding and putative ER retrieval motifs (ER-DIR), were contained in all the five DGAT1 proteins (Fig. 1e). The dendrogram revealed that SsDGAT1 was most closely related to RcDGAT1, JcDGAT1 and

VfDGAT1, which together formed a separate cluster from other DGAT1 branches (Fig. 1f).

Investigation of the expression pattern of *SsDGAT1*

To clarify the spatio-temporal expression pattern of *SsDGAT1* in *S. sebiferum*, semi-quantitative RT-PCR and qPCR were carried out using cDNA extracted from various organs. As shown in semi-quantitative RT-PCR analysis, the *SsDGAT1* gene had a high expression level in seed, leaf and stem, whereas a low transcript level of *SsDGAT1* was detected in flower and root (Fig. 2a). This semi-quantitative RT-PCR result was coincident with qPCR (Fig. 2b). During developmental stages of seeds, the relative expression levels of *SsDGAT1* peaked at 120 days after pollination (DAP) (Fig. 2c), which indicated that the oil might have formed at a point during this time. Overall, *SsDGAT1* was observed at high levels of expression in the seed, leaf, and stem but low levels in the flower and root; during the entire development of seeds, the highest *SsDGAT1* expression was found at 120 DAP.

Evaluation of SsDGAT1 as a functional DGAT enzyme

To investigate the function of SsDGAT1, its complete coding sequence was expressed in the TAG-deficient *S. cerevisiae* strain H1246, which contains disruptions of four acyltransferase genes contributing to TAG synthesis: *are1*, *are2*, *dgal* and *lro1* (Sandager et al. 2002). In this series of assays, *SsDGAT1* was driven by the galactose-inducible *GAL1* promoter in pYES2 to generate the plasmid *pYES2-SsDGAT1* and the wild-type *S. cerevisiae* strain W3013A was used as a positive control. TLC analysis of extracted lipids revealed marked accumulation of TAG in cells of H1246 with *pYES2-SsDGAT1* construct as well as W3013A, but absent in cells of H1246 transformed with only the empty vector pYES2 (Fig. 3), which suggested that SsDGAT1 possessed sufficient DGAT activity to restore TAG synthesis in *S. cerevisiae* H1246.

Molecular characterization of *SsDGAT1* transgenic *B. napus*

An expression cassette fused the green fluorescent protein (GFP) gene, the *SsDGAT1* gene and the

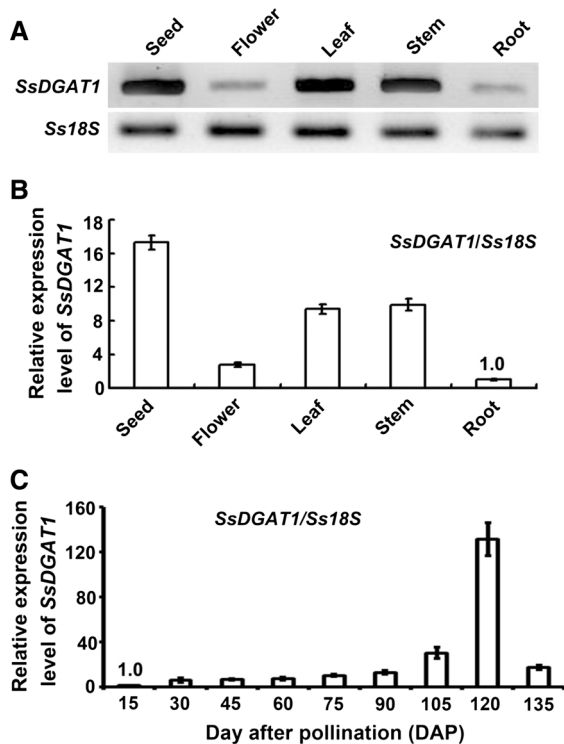


Fig. 2 Investigation of the expression pattern of *SsDGAT1*. **a** Semi-quantitative RT-PCR to investigate the expression level of *SsDGAT1* in various organs; **b** qPCR to investigate the expression level of *SsDGAT1* in various organs; **c** qPCR to investigate the expression level of *SsDGAT1* at different developmental stages of *S. sebiferum* seeds. Samples were collected at 15-day intervals from the day after pollination (DAP) to seed fully matured

herbicide Basta-resistant selectable marker gene was constructed, in which *SsDGAT1* and *GFP* was driven by the CaMV35S promoter, and expressed in *B. napus* plants (Fig. 4a). Twenty-nine Basta-resistant independent *B. napus* lines were obtained after the first treatment with the Basta herbicide (Fig. 4b). Three Basta-resistant independent lines (*35S::GFP-SsDGAT1-4*, *35S::GFP-SsDGAT1-7* and *35S::GFP-SsDGAT1-10*) with 3:1 segregation ratio of Basta-resistant:Basta-sensitive T1 seedlings were selected for further studies. The *bar* genes (Fig. 4c), *GFP* genes (Fig. 4d) and *GFP-SsDGAT1* genes (Fig. 4e) were detected in these three Basta-resistant lines but not in WT plants, showing that the expression cassette was successfully integrated into the *B. napus* genomic DNA, importantly, which was as a single copy insert

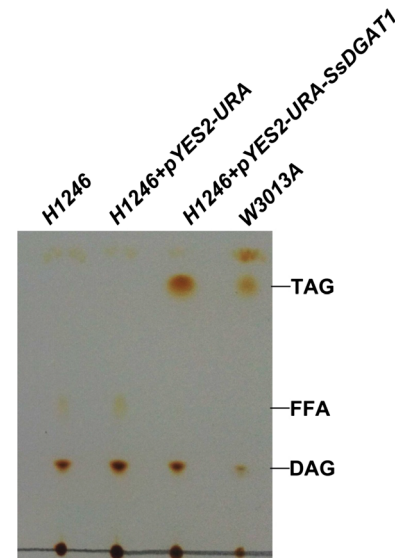


Fig. 3 TLC analysis of neutral lipids from yeast transformants. *FFA* free fatty acid

in transgenic lines genomic DNA by DNA gel blot using *GFP* gene as a probe (Fig. 4f). In addition, the high transcription signal of *SsDGAT1* by semi-quantitative RT-PCR was clearly observed in these three Basta-resistant *B. napus* lines but was not detected in WT seedlings (Fig. 4g), which further confirmed that the *SsDGAT1* gene was truly expressed in transgenic plants. Moreover, an immunoblot assay also showed that the GFP-*SsDGAT1* fusion protein was accumulated in transgenic *B. napus* plants but not in the WT (Fig. 4h). Taken together, all the results demonstrated that *SsDGAT1* was successfully over-expressed in *B. napus*.

Enhancement of seed oil content in over-expressing transgenic *B. napus*

To determine whether the increased *SsDGAT1* expression was accompanied by a rise in oil content in matured seeds, we measured the latter in the over-expressing *SsDGAT1* seeds as well as in WT seeds. Notably, over-expressing *SsDGAT1* led to about 13 % higher oil content in transgenic seeds than WT seeds (Fig. 5a). Interestingly, other metabolite pools, such as glucosinolates, glucose and protein, all showed an trend of increase in transgenic seeds compared with WT seeds (Fig. 5b–d).

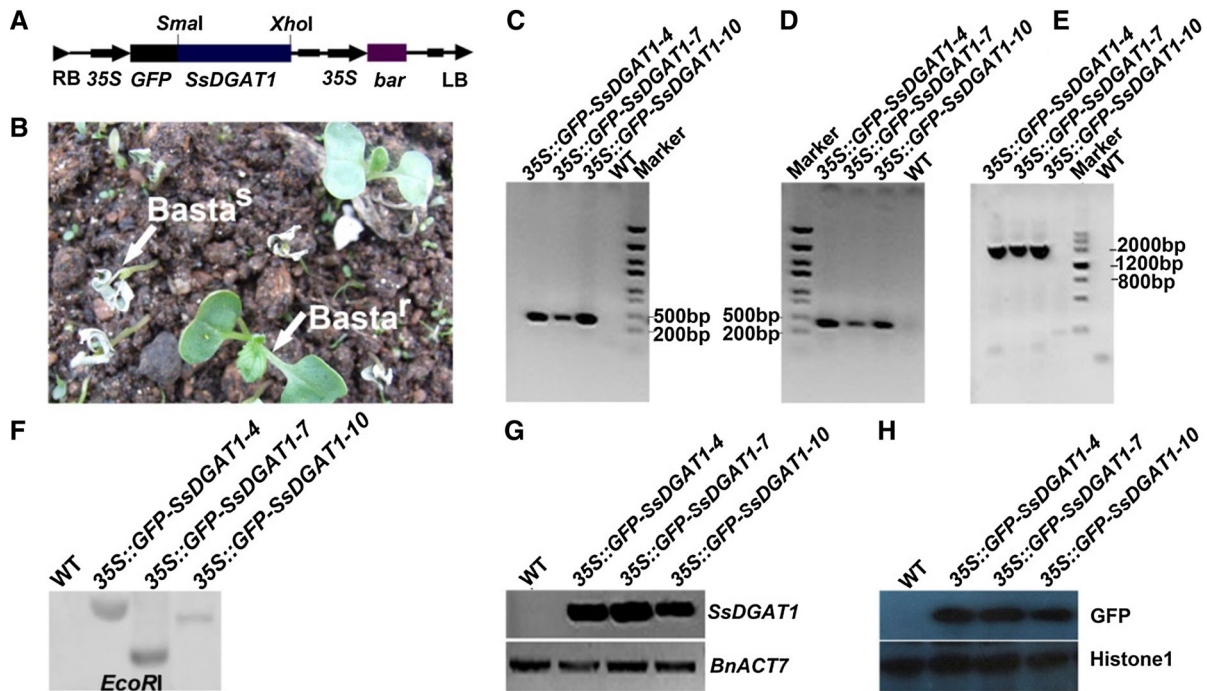


Fig. 4 Transformation and molecular characterization of transgenic *B. napus* plants. **a** Construct of *SsDGAT1* expression cassette; **b** screening of representative Basta-resistant *B. napus* seedling; **c** PCR analysis for *bar* gene from 7-day-old transgenic seedlings (35S::GFP-*SsDGAT1*-4, 35S::GFP-*SsDGAT1*-7, 35S::GFP-*SsDGAT1*-10); **d** PCR analysis for *GFP* gene from 7-day-old transgenic seedlings (35S::GFP-*SsDGAT1*-4, 35S::GFP-*SsDGAT1*-7, 35S::GFP-*SsDGAT1*-10); **e** PCR analysis for *GFP-SsDGAT1* gene from 7-day-old transgenic seedlings (35S::GFP-*SsDGAT1*-4, 35S::GFP-*SsDGAT1*-7,

35S::GFP-*SsDGAT1*-10); **f** DNA gel blot analysis using *GFP* gene as a probe for 7-day-old transgenic lines (35S::GFP-*SsDGAT1*-4, 35S::GFP-*SsDGAT1*-7, 35S::GFP-*SsDGAT1*-10); **g** semi-quantitative RT-PCR of 7-day-old transgenic lines (35S::GFP-*SsDGAT1*-4, 35S::GFP-*SsDGAT1*-7, 35S::GFP-*SsDGAT1*-10); the housekeeping gene *Ss18S* was used as a control; **h** immunoblots of protein from seeds of transgenic and WT plants. *GFP-SsDGAT1* was detected using an anti-GFP antibody

Alteration of the seed weight and fatty acid composition in *B. napus* plants over-expressing *SsDGAT1*

To test if the increased *SsDGAT1* expression was associated with the mass of seeds, we determined the 1000-seed weight. As shown in Fig. 5e, seeds of *B. napus* over-expressing *SsDGAT1* showed 21 % greater 1000-seed weight than the WT seeds. In addition, the fatty acid composition was analyzed to examine the effect of increased *SsDGAT1* expression on the proportion of individual fatty acids. Notably, plants over-expressing *SsDGAT1* showed amounts of oleic acid (18:1) decreased by 13.3–18.3 %, whereas linoleic acid (18:2) and linolenic acid (18:3) content were observed to increase by 14.5–17.8 % and

7.4–11.2 % respectively, compared with the WT (Fig. 5f).

Discussion

In oil-rich seeds, the most important form of energy storage is triacylglycerols (TAGs), especially in woody oil tree seeds. DGAT is believed to be a key enzyme synthesizing TAGs from DAGs in the major flux control step in oil biosynthesis. Previous studies indicated that TAGs are synthesized by four independent family genes, *DGAT1*, *DGAT2*, *DGAT3* and *PDAT*, each encoding unique proteins (Shockey et al. 2006; Zhang et al. 2009; Cao et al. 2013). In plants, a member of the *DGAT1* family plays a major role in

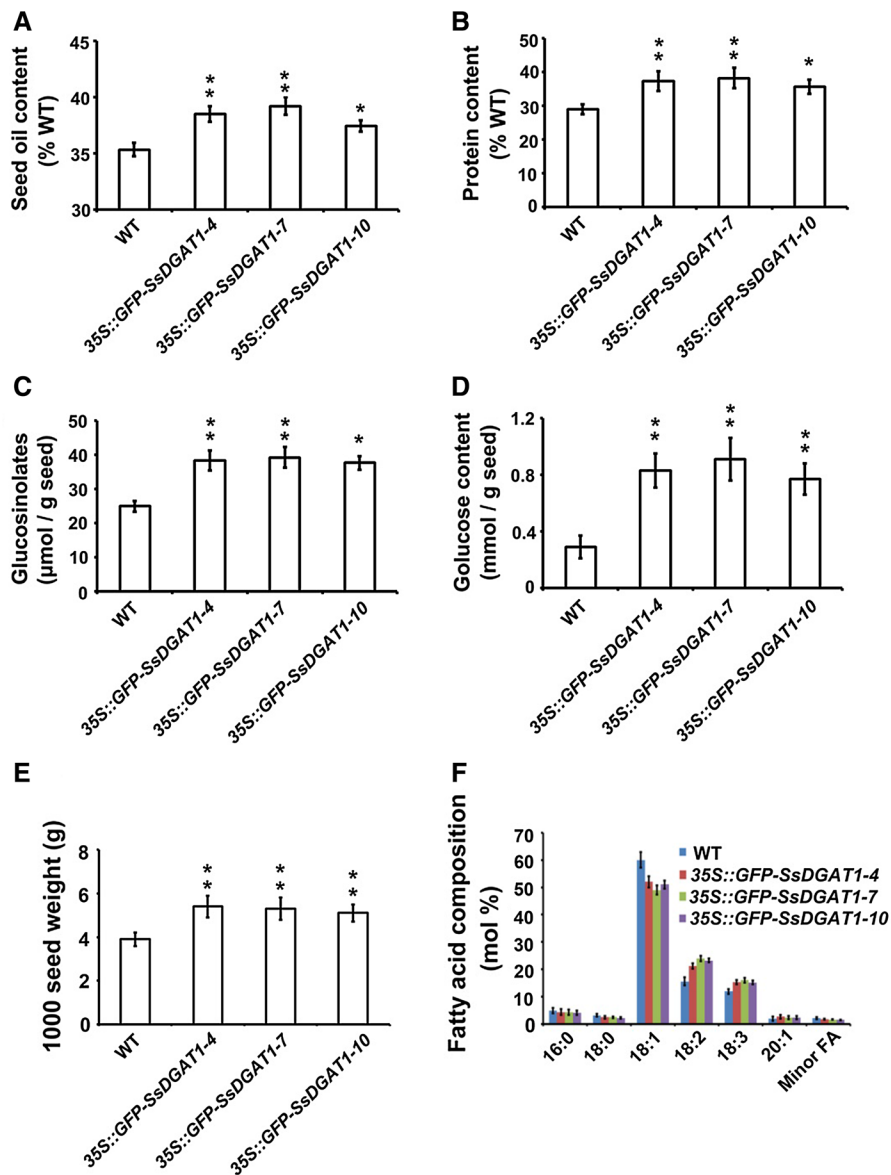


Fig. 5 Analysis of the seed oil, protein, glucosinolate and glucose content, 1000-seed weight and fatty acid composition in WT and transgenic *B. napus*. Values were calculated on a dry weight basis. Bars indicate mean \pm standard error. Statistical analysis was carried out by Student's *t* test and Fisher's least squares difference/ $P = 0.001$ for a–f). * $P < 0.05$, ** $P < 0.01$. **a** Investigation of the seed oil content in WT and transgenic *B.*

napus. **b** Investigation of the seed protein content in WT and transgenic *B. napus*. **c** Investigation of the seed glucosinolate content in WT and transgenic *B. napus*. **d** Investigation of the seed glucose content in WT and transgenic *B. napus*. **e** 1000-seed weight of seeds in WT and transgenic *B. napus*. **f** Fatty acid composition of seeds in WT and transgenic *B. napus*

TAG accumulation in several tissues including seeds while members of the *DGAT2* and *PDAT* families appear to be active mainly in seeds, especially in the accumulation of unusual fatty acids (FAs) in seed oils

such as castor (Kroon et al. 2006; Kim et al. 2011) and tung tree (Shockey et al. 2006). At the same time, previous research suggested that *DGAT3* is expressed throughout seed development and seedling

establishment in *Arabidopsis*, and could play a role in an alternative pathway for TAG synthesis during *pxa1* seedling establishment (Hernández et al. 2012). In our study, we characterized a homolog of *DGAT1* from *S. sebiferum*, a seed-oil-rich woody tree, showed its expression pattern and assessed its role in *B. napus* for the first time, which led to an increase in oil accumulation and alteration of FA composition in transgenic rapeseed oil.

The *SsDGAT1* gene encoded a peptide consisting of nine putative transmembrane domains and a relatively hydrophilic N-terminal segment, like other DGAT1 proteins reported previously (Lung and Weselake 2006; Misra et al. 2013), besides an YYHDL motif at the C-terminus that has been reported to be required for ER localization (Shockey et al. 2006). Transcript accumulation of the *SsDGAT1* mRNA in seeds revealed a specific increase during the later stages of seed development with a maximum level at 120 days after pollination, which matched the results reported by Xu et al. (2011) and showed a pattern similar to other oil-seed crops such as *B. napus*, *Ricinus communis*, *Euphorbia lagascae* and *Vernonia galamensis* (Li et al. 2010). Of course, the relative expression of *SsDGAT1* was detected in other tissues, particularly high in the stem but still much lower than in seeds.

In addition to transcriptional characterization of *SsDGAT1*, functional validation of *SsDGAT1* was demonstrated by the effect of its expression on a yeast mutant and *B. napus*. In the complementation analysis, when *SsDGAT1* was transformed into yeast mutant cell H1246, a quadruple-deficient strain in lipid biosynthesis, a compensating TAG accumulation was able to be regenerated. Importantly, expression of *SsDGAT1* under the CaMV35S promoter in *B. napus* did indeed improve rapeseed oil biosynthesis, increasing oil content by 12.3–14.7 % over that of the control. Considering that the DAG pool in seeds is small, the expression of *SsDGAT1* brought about a considerable increase in seed oil biosynthesis, which indicated that it must in some way influence the biosynthesis and/or the flux of FAs that enter the TAG pathway and their subsequent incorporation in seed TAGs. Indeed, changes in expression of *DGAT* in *Arabidopsis*, *B. napus* and *Nicotiana tabacum* have been shown to affect not only oil content but also seed size and shape and a large number of developmental events (Jako et al. 2001; Weselake et al. 2007; Sharma

et al. 2008; Misra et al. 2013). Additionally, many genes were found to be differentially regulated in *DGAT* over-expressing *Arabidopsis* lines and hormone levels, such as those of auxin, cytokinins and abscisic acid, were unusually influenced by *DGAT* expression in plants (Sharma et al. 2008). This may partly explain the changes that go beyond utilization of the unused DAG pool.

Interestingly, the oil content of over-expressed seeds increased by 13.0 % and the level of linolenic acid was higher at 11.2 % compared with control. This study suggested that over-expressing heterologous *DGAT1* in plants can not only increase oil content but also alter oil composition, which was mirrored similarly by the result of Wang et al. (2014), who reported that over-expression of *Sesamum indicum* L. *DGAT1* (*SiDGAT1*) in *Arabidopsis thaliana* ‘Col 0’ resulted in an increase in oil content and seed weight, and altered the oil composition. As well-known, there is a double bond in linolenic acid which can store up more energy when the carbon skeleton is unchanged. Thus, increased unsaturated fatty linolenic acid content, meaning an increase in double bonds, can meet the requirements of biodiesel production. Nevertheless, there was no change in the levels of saturated FAs such as palmitic acid and stearic acid. It has been speculated that variety in oil composition between different plants may be partly due to the specificities of DGATs for various fatty acyl-CoAs (saturated 16:0, 18:0 or mono- and polyunsaturated FAs), especially in seeds such as those from tung, cocoa, rapeseed and castor where there is often a higher incorporation of many unusual FAs in seeds (Burgal et al. 2008; Griffiths and Harwood, 1991; Kroon et al. 2006; Shockey et al. 2006). Other studies suggested that the differences in oil composition reflected differences in acyl-CoA pools and not necessarily FA specificities of DGAT: e.g. the manipulation of the tobacco FA pool by expression of an epoxygenase gene from *E. lagascae* (Cahoon et al. 2002), and higher incorporation of the modified FAs into the oil, although with no manipulation of the DGAT but only by the co-expression of a Δ 12-epoxygenase and a Δ 12-desaturase from *C. palaestina* in *Arabidopsis* (Zhou et al. 2006). In our results, a visible increase in linolenic acid and a specific decrease in oleic acid from *SsDGAT1* expression would suggest a higher affinity for linolenic acids and a lower one for oleic acid.

Our study shows that the seed oil was markedly increased and glucosinolates and glucose were significantly decreased by over-expression of *SsDGATI* in *B. napus*. Further studies indicated that the increased seed oil content was largely compensated by decreases in other metabolite pools, such as glucosinolates and glucose, but most predominantly protein. Indeed, this relationship has been negatively demonstrated in tobacco, where both protein and carbohydrate increased as seed oil decreased in *DGATI*-silenced plants (Zhang et al. 2005); at the same time, this relationship has been negatively demonstrated in *B. napus* (Lock et al. 2009).

Conclusions

In this research, *S. sebiferum SsDGATI* transcription showed enhancement during seed development and that *SsDGATI* heterologous expression in yeast H1246 TAG-deficient mutant and in *B. napus* can lead to increased TAG biosynthesis. Furthermore, our results showed not only increased oil content but also alterations of oil composition: an increase in linolenic acids levels, a decrease in oleic acid levels and no changes in saturated fatty acid levels in *SsDGATI* over-expressing *B. napus*. Thus, *SsDGATI* could be considered as a candidate gene for increasing oil content and improving oil quality.

Acknowledgments This work was supported by the National Natural Science Foundation of China (31200516), the Outstanding Youth Scientific Research Foundation held by the Hunan Education Department (13B150), The Hunan Province Outstanding Youth Teacher Cultivation Project (2014gg04), the Youth Scientific Research Foundation of Central South University of Forestry & Technology (QJ2011046B), and the Foundation of the Project for Talents Introduction of the Central South University of Forestry & Technology (104-0182). We also thank Professor Aizhong Liu (Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences) for providing the yeast strain H1246 and the corresponding parental line for this study.

Author contributions Idea and study design: DP and BZ; data collection and analysis: DP, BZ, LZ, DY and TX with the support of XL; writing of the manuscript: DP and BZ.

Compliance with ethical standards

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

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