American Journal of Biochemistry and Biotechnology

Original Research Paper

Identification and Characterization of Diacylglycerol Acyltransferase in Oleaginous Yeast *Rhodosporidium toruloides*

[1]Zhen Wang, [2]Huaiyuan Zhang, [1]Lina Zhao and [1,2]Yuanda Song

[1]*School of Food Science and Technology, Jiangnan University, Wuxi 214122, Jiangsu, People's Republic of China*
[2]*Colin Ratledge Center for Microbial Lipids, School of Agricultural Engineering and Food Science, Shandong University of Technology, Zibo, 255049, Shandong, People's Republic of China*

Article history
Received: 30-03-2016
Revised: 23-06-2016
Accepted: 15-10-2016

Corresponding Author:
Yuanda Song
School of Food Science and Technology, Jiangnan University, Wuxi 214122, Jiangsu, People's Republic of China
Colin Ratledge Center for Microbial Lipids, School of Agricultural Engineering and Food Science, Shandong University of Technology, Zibo, 255049, Shandong, People's Republic of China
Tel: 86-510-85197130
Email: ysong@jiangnan.edu.cn

Abstract: Diacylglycerol acyltransferase (DGAT), which catalyzes TAG formation from DAG and acyl-CoA, has been considered to play a vital role in TAG accumulation in oleaginous microorganisms. The genome of oleaginous yeast *Rhodosporidium toruloides* contains two putative DGAT genes, RtDGATa and RtDGATb, which shared little conserved amino acid coding sequence with each other. Phylogeny tree analysis showed that RtDGATa belonged to DGAT1 family and RtDGATb belonged to DGAT2 family. For functional identification of the DGATs, RtDGATa and RtDGATb were individually expressed in *Saccharomyces cerevisiae* TAG-deficient quadruple mutant (H1246). RtDGATa had obvious preference for monounsaturated fatty acids, however the expression of RtDGATa did not alter the TAG content in *S. cerevisiae* H1246 and it had non-involvement in TAG accumulation according to its mRNA expression level in *R. toruloides*. The expression of RtDGATb could completely resume TAG biosynthesis in *S. cerevisiae* H1246. Substrate preference experiments revealed that RtDGATb preferred unsaturated fatty acids over saturated fatty acids, but not C18:3. Only the expression pattern of RtDGATb was related to the process of fatty acid biosynthesis, suggesting that RtDGATb plays an important role in lipid accumulation in *R. toruloides*.

Keywords: Diacylglycerol Acyltransferase, Fatty Acid, Oleaginous, *Rhodosporidium toruloides*, Triglyceride

Introduction

In recent years, alternative sources of fuels attracted more and more interests because of petroleum crisis and accumulation of greenhouse gases worldwide. Biodiesel, esterified from vegetable oils or animal fats with lower alcohol by now, acts as an alternative of fossil fueland biodiesel production, which is commercially available in Europe, the United States and other countries (Li *et al*., 2008; Sitepu *et al*., 2014). The main drawback of this technology is that it conflicts with food resources. Alternative to plant oil and animal fat, oils produced from oleaginous microorganisms can also be used to produce biodiesel, which has several advantages, such as high lipid yield and growth rate over current biodiesel production technologies (Liang and Jiang, 2013). Almost all microbes are intrinsically capable of synthesizing fatty acids as cell membrane components and energy storage materials. However only a few microbes could accumulate intracellular lipids over 20% of their Dry Cell Weight (DCW) and these microorganisms are regarded as oleaginous microorganisms (Ageitos *et al*., 2011; Ratledge and Wynn, 2002).

TAG synthesis, from glycerol-3-phosphate to form TAG, commonly known as the Kennedy pathway, is catalyzed by a series of acyltransferases which contains glycerol-3-phosphate acyltransferase (GPAT; EC 2.3.1.15), lyso-phosphatidic acid acyltransferase (LPAAT; EC 2.3.1.51) and diacylglycerolacyl transferase (DGAT; EC 3.2.1.20) (Gong *et al*., 2013). DGAT catalyzes the terminal step in TAG synthesis and has been considered to play a vital role in storage lipid accumulation. There are three major families of DGATs, namedmembrane-bound type 1,type 2 and a soluble cytosolic type 3 which had rarely been found (Liu *et al*., 2012). DGAT1 and DGAT2 exhibit no sequence homologies to each other. It is

suggested that DGAT2 is the most important enzyme in lipid accumulation and had been utilized to improve the lipid content in several microorganisms in some recent researches. Over expression of DGAT2 in *Y. lipolytica* led to 3-fold increase in lipid accumulation and its coupling with *Acetyl-CoA* Carboxylase (ACC) over expression simultaneously improved the lipid content from 11.7 to 61.7% of their biomass in 2-literbaffled stirred-tank bioreactor (Tai and Stephanopoulos, 2013). After genetic transformation of *Colletotrichum* strain with the CtDGAT2b over expression, total lipid titer in the transformants (73% of DCW) was found to be 1.7-fold more than the wild type (38% of DCW) when grown under standard condition without imposition of any nutrient-stress (Dey *et al*., 2014). DGAT2 over expression in marine diatom *Phaeodactylum tricornutum* stimulated more oil bodies and the neutral lipid content increased by 35% and the fatty acid composition showed a significant increase in the proportion of polyunsaturated fatty acids, especially Eicosapentaenoic Acid (EPA) (Niu *et al*., 2013).

The red yeast *R. toruloides* has a high capability of growth and lipid biosynthesis on a wide range of carbon sources from glucose, fructose and xylose to glycerol. It can accumulate lipids up to 70% of its biomass under certain conditions and the content of neutral lipids (mainly TAG and DAG) was up to over 90% of total lipids (Jin *et al*., 2013). However, the molecular mechanism of TAG biosynthesis in *R. toruloides* had barely been studies. Recently a DGAT2 gene from another red yeast *Rhodosporidium diobovatum* has been cloned and its function has been confirmed in vivo by expression in the *S. cerevisiae* TAG-deficient quadruple mutant (Chen *et al*., 2014). However, not all DGAT genes have been identified in *R. diobovatum* because of the absence of its genome information. Recently the genome information of *R. toruloides* has been published (Zhu *et al*., 2012), so, in this study, we investigated the identification and functional analysis of all putative DGAT genes from *R. toruloides* and explored the role of DGATs in TAG synthesis in this oleaginous yeast.

Materials and Methods

Microorganism Strains and Culture Medium

The *R. toruloides*strain CGMCC 2.1389 was bought from China General Microbiological Culture Collection Center (CGMCC) and *R. diobovatum* CICC 31994 was from China Center of Industrial Culture Collection (CICC). *S. cerevisiae* mutantstrain H1246 (*dga1*Δ *lro1*Δ *are1*Δ *are2*Δ) kindly provided by prof. Stymne from Swedish University of Agricultural Sciences was used for functional complementation of putative DGAT genes in *R. toruloides* and *R. diobovatum*. *E.coli* Top10 was kept in our lab and used for plasmid construction.

For lipid accumulation of *R. toruloides* CGMCC 2.1389 in a ferment or, the nitrogen limited medium contained (g/L): Glucose 60, $(NH_4)_2SO_4$ 0.1, yeast extract 0.75, KH_2PO_4 0.4 and $MgSO_4{\cdot}7H_2O$ 1.5. After the medium was sterilized at 121°C for 30 min, it was supplemented with 1% (v/v) trace element solution contained (g/L): $CaCl_2{\cdot}2H_2O$ 4.0, $FeSO_4{\cdot}7H_2O$ 0.55, citric acid·H_2O 0.52, $ZnSO_4{\cdot}7H_2O$ 0.10, $MnSO_4{\cdot}H_2O$ 0.076 and 18 M H_2SO_4 100 uL (Wu *et al*., 2011). All chemical reagents was from Sinopharm Group CO. LTD (Beijing, China) and belonged to analytic grade.

For functional expression in *S. cerevisiae*H1246, transformants harboring each plasmid were grown at 30°C in synthetic minimal medium containing 2% raffinose, 0.67% yeast nitrogen base (with ammonium sulfate) and appropriate amino acid contents according to the protocol (Invitrogen).

Construction of Plasmids and S. cerevisiae Mutant Strains

Standard molecular genetic techniques were employed throughout this study. Total RNA of *R. toruloides* CGMCC 2.1389 and *R. diobovatum* CICC 31994 was isolated using TRIzol Reagent (TaKaRa, Dalian, China) according to the manufacturer's instruction and reverse transcribed into cDNA. DGAT gene fragments were obtained by PCR using their cDNA as template with the corresponding primers (Table 1) and then these DNA fragments were cloned into expression vector pYES2/NT C using restriction endonuclease *Bam* HI and *Eco* RI, the plasmids used in this study were showed in Table 1.

The resulting plasmids pYES2/NT C, pYES2-DGA1, pYES2-RdDGAT2, pYES2-RtDGATa and pYES2-RtDGATb were transformed into *S. cerevisiae* H1246 (TAG-deficient quadruple mutant) using the PEG/lithium acetate method (Elble, 1992). *S. cerevisiae* H1246 harboring empty vector pYES2/NT C was the negative control and *S. cerevisiae* H1246 harboring pYES2-DGA1 was the positive control strains. Transformants were selected by plating on complex synthetic minimal medium agar lacking uracil and grown at 30°C for 2-3 days. The expression of DGAT cDNA and DGA1 was induced under transcriptional control of the *GAL1* promoter. For protein expression, transformants were inoculated in synthetic minimal medium until OD_{600} reached to 0.4 and then induced by supplementing 2% (w/v) galactose and cultivated for a further 90 h at 28°C. The growth curve of mutant strains was monitored by measuring their OD_{600}. To determine the fatty acid substrate preference of RtDGATb isozyme compared to the endogenous ScDGA1 of *S. cerevisiae*cells, two different free fatty acids likelinoleic acid (C18:2, Sigma, 99%) and a-linolenic acid (C18:3, Sigma, 99%), were supplementedin culture media. Yeast cells growth curve was also determined, simultaneously. After culture end and cells were harvested by centrifugation, washed three times with double-distilled water and used for total lipids analysis.

Table 1. Plasmids and microorganism strains used in this study

Plasmids and strains	Use, relevant characteristic (s) and source
Plasmids	
pYES2/NT C	Ampicillin resistance, transformation vector for *S. cerevisiae*, stored in our lab
pYES2-DGA1	Ampicillin resistance, *S. cerevisiaeDGA1* genecloned into *Bam*HI/*Eco*RI-cut pYES2/NT C for positive control
pYES2-RdDGAT2	Ampicillin resistance, *R. diobovatumRdDGAT2* gene cloned to *Bam*HI/*Eco*RI-cut pYES2/NT C for another positive control
pYES2-RtDGATa	Ampicillin resistance, *R. toruloides* putative *RtDGATa* gene cloned to *Bam*HI/*Eco*RI-cut pYES2/NT C
pYES2-RtDGATb	Ampicillin resistance, *R. toruloides* putative *RtDGATb* gene cloned to *Bam*HI/*Eco*RI-cut pYES2/NT C
Strains	
E. coli Top10	*E. coli* host for DNA manipulations
R. toruloides CGMCC 2.1389	From China General Microbiological Culture Collection Center
R. diobovatum CICC 31994	From China Center of Industrial Culture Collection
H1246	*Saccharomyces cerevisiae* H1246 (MATα*are1*-Δ::*HIS3 are2*-Δ::*LEU2 dga1*-Δ::*KanMX4 lro1*-Δ::*TRP1 ADE2*)
HY1	*S. cerevisiae* H1246 harboring empty plasmid pYES2/NT C
HY2	*S. cerevisiae* H1246 harboring plasmid pYES2-DGA1
HY3	*S. cerevisiae* H1246 harboring plasmid pYES2-RdDGAT2
HY4	*S. cerevisiae* H1246 harboring plasmid pYES2-RtDGATa
HY5	*S. cerevisiae* H1246 harboring plasmid pYES2-RtDGATb

Lipid Extraction and Fatty Acid Analysis

Total lipids were extracted from 100mg of lyophilized biomass with a solvent mixture of chloroform: Methanol (2:1, v/v) according to the modified method (Bligh and Dyer, 1959). TAGs were separated from total lipids by thin layer chromatography (TLC) using a solvent system of hexane: Diethyl ether: Acetic acid (70:30:1, v/v/v). Individual lipid spots were visualized by exposing the silica gel plates (Qingdao, China) to iodine vapor and TAGs were scraped off the plates, eluted with 1 mL chloroform for three times and then submitted to methyl esterification. Total Fatty Acid (TFA) isolation and fatty acid composition were analyzed as their methyl esters as described by (Zhang *et al*., 2013a) using C15:0 as internal standard[17]. Each sample was performed in triplicate.

R. toruloides CGMCC 2.1389 Fermentation and DGAT Expression Pattern

For analyzing lipid accumulation and expression pattern of putative DGAT genes in oleaginous yeast *R. toruloides*, bioreactor scale fermentation was carried out in a 2.8L ferment or contained 2L nitrogen limited medium (0.5 vvm aeration, pH 5.6, 28^{o}C, 250 rpm agitation). Culture samples were collected at different time for biomass and lipid analysis and total RNAs were also extracted from yeast cells according to the manufacturer's instruction. Genomic DNA was removed from total RNAs with gDNA Eraser (TaKaRa) at 37^{o}C for 15 min. After total RNAs were reverse transcribed into cDNA using the PrimeScript® RT reagent Kit (TaKaRa), 2 uLcDNAs was used for quantitative Real Time-PCR amplification using the SYBR Green I master mix Kit (TaKaRa). Reactions were run in a Mini Opticon Thermal Cycler (Bio-Rad). DGAT cDNA fragments were amplified with primer pairs listed in S1 Table and 18S cDNA was used as an internal standard. Gene expression analyses were performed with the previous method (Livak and Schmittgen, 2001). Each sample was performed in triplicate. Nile Red fluorescence assays of yeast cells were carried out according to the method described previously (Dey *et al*., 2014).

Results

Identification and Protein Sequence Analysis of R. toruloides DGATs

According to annotated genome of oleaginous yeast *R. toruloides*, two deduced DGAT sequences, RtDGATa (protein ID: 726) and RtDGATb (protein ID: 1957), were found from the published genome database (http://genome.jgi.doe.gov/Rhoto1/Rhoto1.home.html). RtDGATa and RtDGATb are 698 and 349 amino acid residuals in length respectively, sharing different domains and subfamilies. The analytical results using the conserved domain database of National Center for Biotechnology Information (NCBI) showed that RtDGATb possessed a lysophospholipidacyl transferase (LPLAT) super family domain. The putative DGAT amino acid sequences from *R. toruloides* and other DGATs from animals, plants, fungi and microorganisms, cover three DGAT families (DGAT1, DGAT2 and cytosolic DGAT3), were used for construction of phylogenetic tree. All 36DGAT protein sequences were clearly classified to DGAT1, DGAT2 and DGAT3 families with extremely strong bootstrap support

(Fig. 1). As showed in Fig. 1, RtDGATa was clustered within DGAT1 family and had substantial similarity with Acyl-CoA: Cholesterol acyltransferase from *S. cervisiae* (ScARE). However RtDGATb was distinctly separated into DGAT2 family and had the largest similarity with RdDGAT2 from *R. diobovatum*, which had been proved to belong to DGAT2 family (Chen *et al*., 2014). Multiple sequence alignment of DGAT2 from seven organisms was performed (Fig. 2). RtDGATb contained six highly conserved motifs that were identified as signature motifs within the DGAT2 family by (Cao, 2011), namely Motif 1 (PH Block), Motif 2 (PR Block), Motif 3 (GGE Block), Motif 4 (RGFA Block), Motif 5 (VPFG Block) and Motif 6 (G Block).

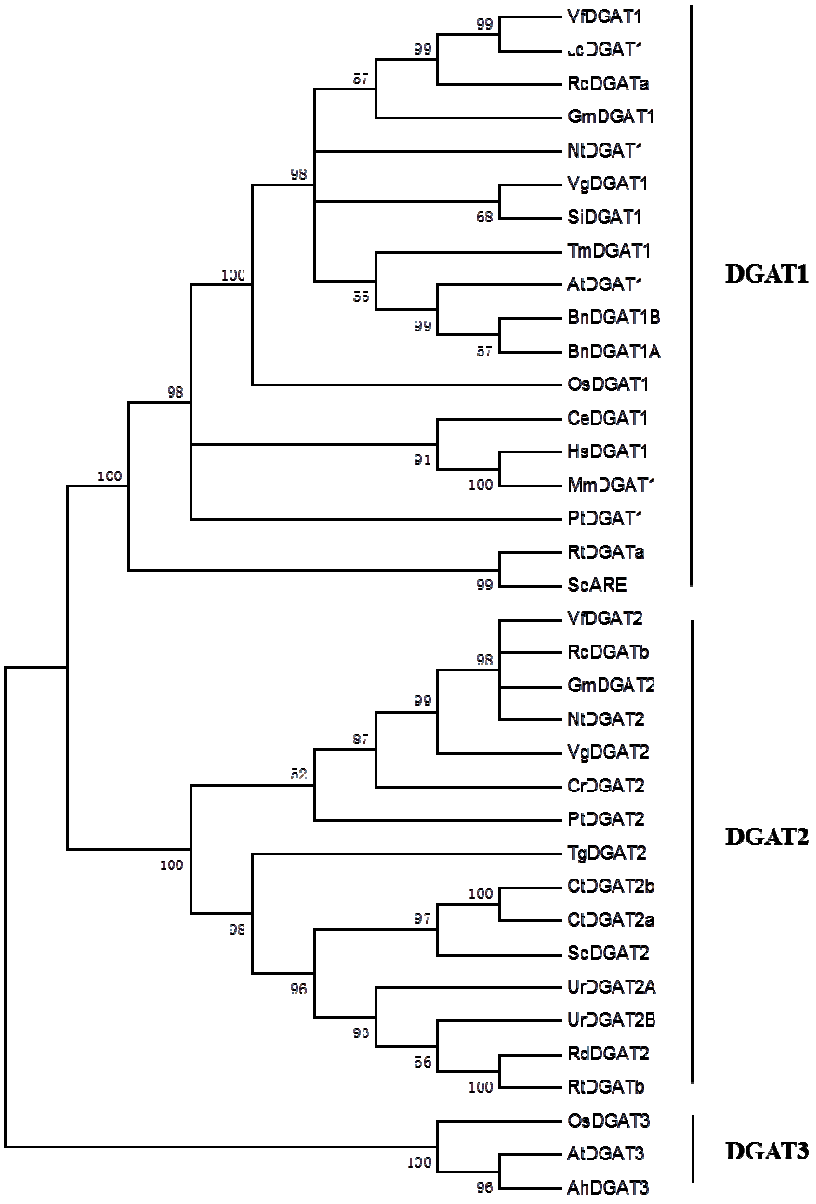

Fig. 1. Phylogenetic analysis of homolog sequences of RtDGAT from plant, fungi and microorganisms. Multiple sequences were aligned by Clusta lX 2.1 multiple alignment software. The phylogenetic tree was generated using MEGA 6.06 software. Bootstrap values are expressed in the percentages of 1000 replicates on the nodes. *Arabidopsis thaliana* (At), *Brassica napus* (Bn) var. napus; *Caenorhabditis elegans* (Ce), *Chlamydomonas reinhardtii* (Cr), *Candida tropicalis* (Ct), *Glycine max* (Gm), *Homo sapiens* (Hs), *Jatropha curcas* (Jc), *Musmusculus* (Mm), *Nicotianatabacum* (Nt), *Oryza sativa Japonica* (Os), *Phaeodactylum tricornutum* (Pt), *Ricinus communis* (Rc), *Rhodosporidium diobovatum* (Rd), *Rhodosporidium toruloides* (Rt) *Saccharomyces cerevisiae* (Sc), *Sesamum indicum* (Si), *Trypanosomagrayi* (Tg), *Tropaeolum majus* (Tm), *Umbelopsis ramanniana* (Ur), *Vernicia fordii* (Vf), *Vernonia galamensis* (Vg) Sc ARE, *S. cerevisiae*acy l-CoA: Sterol acyltransferase

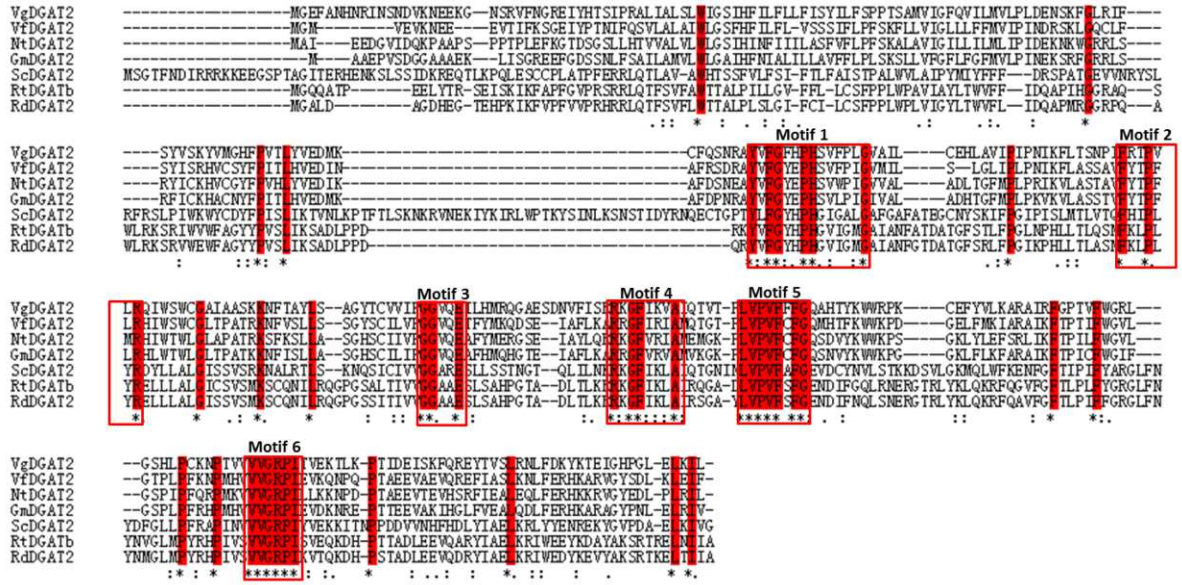

Fig. 2. Protein sequence alignment of RtDGATb with DGAT2s from six organisms. All protein sequences were obtained from GenBank or *R. toruloides* genome database (http://genome.jgi.doe.gov/Rhoto1/Rhoto1.home.html). *Vernicia fordii* (Vf), *Vernonia galamensis* (Vg), *Nicotiana tabacum* (Nt), *Glycine max* (Gm) *Saccharomyces cerevisiae* (Sc), *Rhodosporidium diobovatum* (Rd), *Rhodosporidium toruloides* (Rt)

R. toruloides DGATs Recovered TAG Synthesis in S. cervisiae Mutant

Four genes, *DGA1* (DGAT2 homolog), *LRO1* (encoding phospholipid: Diacylglycerol acyltransferase), *ARE1* and *ARE2* (both involved insteryl ester synthesis) were found in the yeast *S. cervisiae* which contributing to TAG biosynthesis (Sandager *et al*., 2002). A TAG-deficient quadruple mutant of these four genes in *S. cervisiae* (*S. cervisiae* H1246) is an impeccable platform for identification of DGAT activity from various organisms (Liu *et al*., 2012). To affirm whether RtDGATa and RtDGATb indeed encoded proteins with DGAT activity, RtDGATa and RtDGATb were expressed individually in *S. cervisiae* H1246 to obtain mutant strains HY4 and HY5. In addition, empty vector and pYES2-DGA1 harboring DGA1 from *S. cervisiae* were transformed into *S. cervisiae* H1246 as negative (HY1) and positive controls (HY2) respectively (Table 1). Meanwhile, A *RdDGAT2*from other red yeast *R. diobovatum* which had been discussed in previous study (Chen *et al*., 2014) was also considered as another positive control (HY3). The results of Western blot analysis showed that all proteins were expressed in *S. cervisiae* H1246 and their expression levels were undifferentiated (Fig. 1). After cultivation for 96 h in synthetic minimal medium, yeast cells were harvested for lipid isolation and analysis. Total lipid was separated by TLC and the results showed that TAG was at undetectable level in *S. cervisiae* H1246 with empty vector and with pYES2-RtDGATa, whereas expression of RtDGATb recovered TAG biosynthesis as did the expression of DGA1 and RdDGAT2 (Fig. 3A). However the TAG content in *S. cervisiae* strainHY5 was up to 3.6% of cell dry weight and was lower than mutant strain HY2 (5.8%) and HY3 (7.7%).The trend of TFA content in respective strains was similar with TAG content, TFA biosynthesis in HY5 (8.9%) was also less than HY2 and HY3 (Fig. 3A).

Acyl-CoA Preference Analysis of R. toruloides DGATs

To determine the possible substrate preference of RtDGATa and RtDGATb for those fatty acids which are naturally present in *R. toruloides*, *S. cervisiae* mutant strains were inoculated in synthetic minimal medium with different fatty acids supplement (C18:2 orC18:3) and added 2% (w/v) galactose as the inducer for protein expression. The results of growth curve and lipid biosynthesis were shown in Fig. 3 and 4. α-linolenic acid (C18:3) supplement did not alter the growth trend of all *S. cervisiae* mutant strains compared to cultivation mode devoid of supplement, however the growth situation with linoleic acid (C18:2) supplement had significant difference between DGAT active strains and DGAT inactive strains. In the early period of cultivation, addition of linoleic acid suppressed the yeast cell proliferation in DGAT inactive strains, but it also surprisely promoted the biomass upsurge at the later period of the culture cycle and final OD_{600} reached about 24.0 in DGAT inactive strains (Fig. 4).

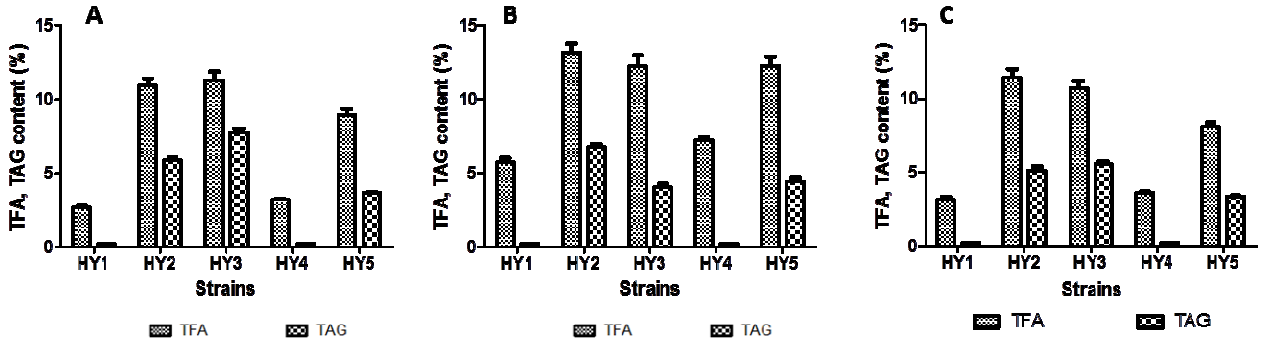

Fig. 3. TFA and TAG synthesis of *S. cervisiae* mutant cultivated in synthetic minimal medium with fatty acid supplement to final concentration of 1.0 mM. The sample was harvested after 96 h culture, total lipid was separated by TLC and fatty acid methyl ester was determined by GC-MS.A, no supplement; B, C18:2 supplement; B, C18:3 supplement

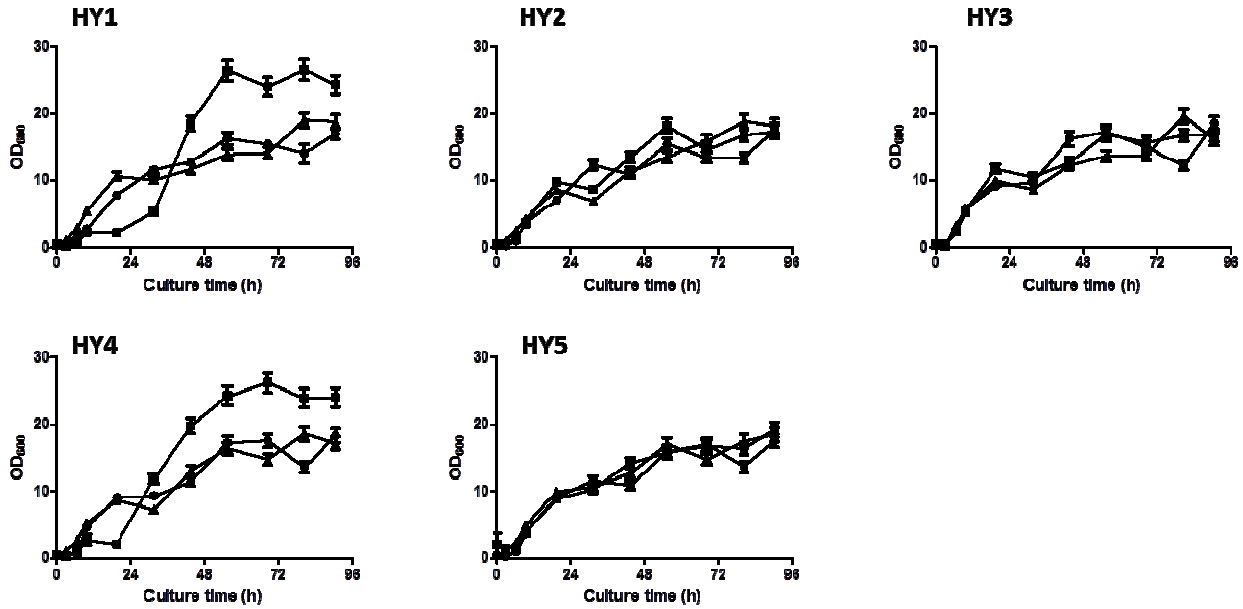

Fig. 4.Growth curve of *S. cervisiae* mutant cultivated in synthetic minimal medium with fatty acid supplement to final concentration of 1.0 mM (●, no supplement; ■, added C18:2; ▲, added C18:3). Data are mean±S.D. from triple biological replicates

At the end of cultivation with fatty acid supplement, intracellular lipid was isolated from freeze-dried yeast cells and TAG was separated from total lipids by TLC.C18:2 supplementation apparently increased the TFA content in all mutant strains, although, addition of C18:3 had little influence on TFA and TAG contents (Fig. 3B and 3C). However fatty acid supplement obviously changed the fatty acid composition of TFA (Fig. 2) and TAG (Table 2) in *S. cervisiae* mutant strains. Compared with fatty acid profile of TAG and TFA in HY2 and HY3, RtDGATb prefered monounsatruated fatty acid (C16:1 and C18:1) to satruated fatty acid (C16:0 and C18:0). For these addition experiments of fatty acids, C18:2 could be accumulated over 20% of total fatty acids in all strains, expression of RdDGAT2 led to the highest content of C18:2 of TAG and the lowest content was found in strain HY2 with DGA1 expresssion. There was very low C18:3 content in TAG (less than 2%), supplementation of C18:3 had little effect on fatty acid composition of TAG (Table 2). For fatty acid profile of TFA, mutant strains with an active DGAT also had tremendously low content of C18:3, however, C18:3 was accumulated more than 10% of TFA (Fig. 2) in DGAT inactive mutants. RtDGATb prefered monounsaturated fatty acid to saturated fatty acid and had no preferenceforC18:3.The expression of RtDGATa also alter the fatty acid profile in *S. cervisiae* mutant strains (Fig. 2), RtDGATa had significant preference for C16:1 and C18:1 and had no preference for C16:0, C18:0, C18:2 andC18:3.

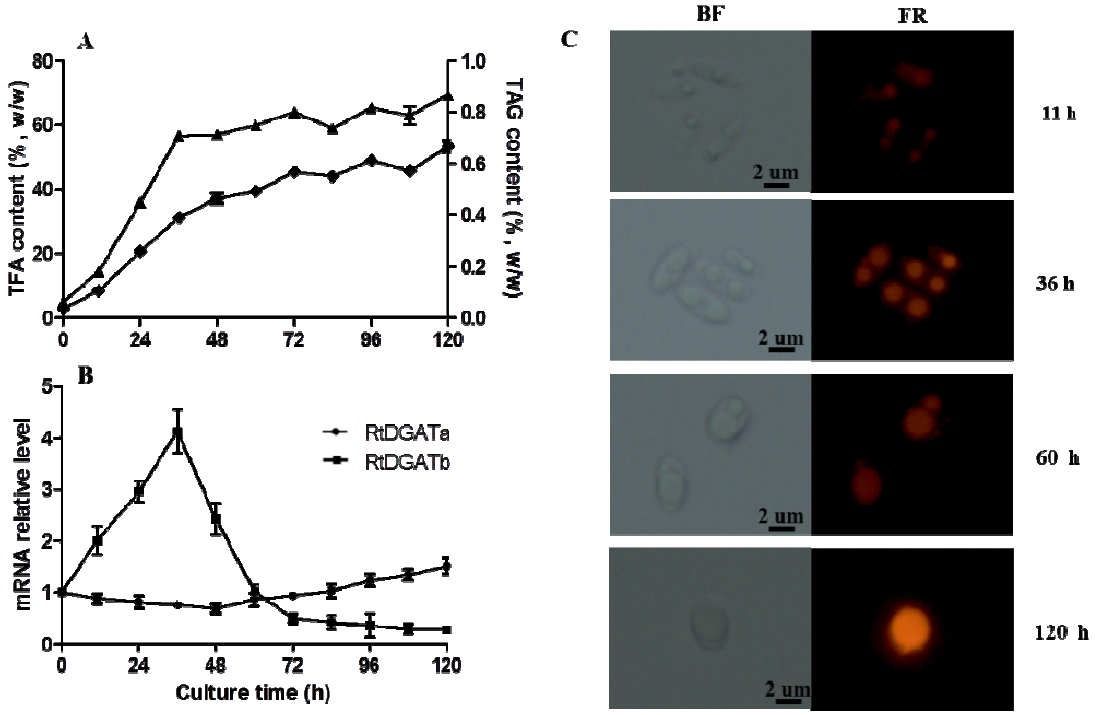

Fig. 5. The lipid accumulation and DGATs gene expression pattern in *R. toruloides*. (A) TFA (▲) and TAG (◆) content of *R. toruloides* cultivated in a fermentor. (B) DGATs gene expression in *R. toruloides*. (C) Lipid bodies where neutral lipid accumulate were visualized in yeast cells with the fluorescent dye Nile Red.BF, Bright-field image; FR, image of Nile Red fluorescence. Data are mean ± S.D. from triple biological replicates

Table 2. Fatty acid profile of TAG in *S. cervisiae* H1246 mutant strains harboring each plasmid cultivated with different fatty acid supplement

		Fatty acid composition (%)					
Strains		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
No supplement	HY2	22.40±0.89	36.25±1.23	10.34±1.03	31.00±1.56	-	-
	HY3	19.44±0.96	38.88±1.02	7.02±0.23	36.65±1.35	-	-
	HY5	15.44±0.86	36.88±0.95	9.02±0.15	38.65±2.04	-	-
Added C18:2	HY2	14.13±0.35	27.32±0.65	9.66±0.24	24.10±1.86	24.73±1.21	-
	HY3	11.01±0.13	28.62±0.43	7.03±0.09	26.12±1.07	27.25±0.95	-
	HY5	9.73±0.46	29.10±1.32	7.25±0.39	29.11±0.99	25.64±0.43	-
Added C18:3	HY2	21.01±0.23	38.10±2.58	11.02±0.49	29.20±0.68	-	0.88±0.52
	HY3	16.14±0.86	40.88±3.21	7.32±0.36	34.25±0.48	-	1.48±0.79
	HY5	14.64±1.03	39.88±1.09	8.82±0.08	35.45±1.57	-	1.29±0.45

Data are mean±S.D. from triple biological replicates.-, not detected

Table 3. Fatty acid composition of TAG in *R. toruloides* CGMCC 2.1389cultivated in fermentor with nitrogen limited medium

	Fatty acid composition (%)					
Culture time (h)	C16:0	C18:0	C18:1	C18:2	C18:3	other
11	30.92±1.23	14.80±0.37	35.50±1.02	15.88±0.12	1.61±0.01	1.29±0.02
24	29.89±2.14	12.41±0.57	42.94±1.15	8.81±0.31	2.58±0.02	1.29±0.01
36	30.80±1.45	13.83±0.29	42.54±2.01	9.91±0.04	1.68±0.02	1.25±0.04
48	28.74 ±1.25	11.91±0.10	49.48±1.89	5.71±0.09	2.89±0.00	1.27±0.13
60	28.40 ±1.86	11.78±0.25	49.96±1.81	5.54±0.11	2.99±0.01	1.32±0.02
72	28.88 ±1.49	11.83±0.43	49.82±1.67	5.58±0.12	2.64±0.02	1.24±0.10
84	30.06 ±2.04	12.35±0.37	48.17±1.59	5.96±0.08	2.18±0.02	1.29±0.07
96	28.05 ±2.18	12.64±0.26	47.60±1.09	8.52±0.06	2.08±0.03	1.11±0.09
108	27.53 ±1.84	12.46±0.20	50.19±1.35	6.40±0.12	2.27±0.00	1.15±0.11
120	27.35 ±1.76	11.63±0.19	49.65±0.98	7.85±0.13	2.43±0.03	1.09±0.06

The Relationship between DGATs Gene Expression Pattern and Lipid Accumulation in R. toruloides

To explore the role of DGATs in the process of lipid accumulation in *R. toruloides*, this yeast was cultivated in a fermentor with nitrogen limited medium and the lipid biosynthesis and DGATs gene expression pattern was determined. Following the consumption of glucose in the medium, biomass increased continuously until glucose was exhausted and final biomass was up to 23 g L^{-1} (Fig. 3). TFA and TAG increased sharply at cultivation time from 11 h to 48 h and at the later period of cell growth TFA and TAG contents increased very slowly. The final TFA content was near 70% of DCW and TFA production was up to 16 g L^{-1} (Fig. 5A). Lipid bodies in yeast cells were visualized with the fluorescent Nile Red and results were shown in Fig. 5C. There were only 1-2 small lipid bodies inside each spindle yeast cell at the earlier period of fermentation and lipid bodies grew bigger in size by continuous TAG biosynthesis later on. At the end of fermentation, a large lipid body was almost the size of whole yeast cell with TAG content up to 53% of DCW. The fatty acid composition of TAG and TFA was analyzed by GC and the results were showed in Table 3 and 2. Only C18:1 content in TAG and TFA was gradually increased and up to about 50% of total fatty acid. In addition, fluorogenic quantitative PCR was used to determine the mRNA expression level of DGTA genes in *R. toruloides*. The transcript level of RtDGATa was almost unchanged during the process of fermentation but the trend of RtDGATb mRNA was related to the rate of lipid accumulation (Fig. 5B). The expression level of RtDGATb increased from inoculation to 36 h, peaked at 36 h and then decreased to a low level in the following cultivations.

Discussion

The lipid content in red yeast *R. toruloides* was up to 70% of its biomass under certain conditions and total lipids mainly composed with neutral lipids (mainly TAG and DAG) (Jin *et al*., 2013). DGAT, which catalyzes TAG formation from DAG and fatty acyl-CoA, is the terminal step for lipid accumulation. This oleaginous yeast cell contains two putative DGAT proteins, RtDGATa and RtDGATb, which shared little conserved amino acid with each other. Phylogeny tree analysis showed that RtDGATa belonged to DGAT1 family and RtDGATb belonged to DGAT2 family. To confirm whether these two proteins showed the DGAT activity RtDGATa and RtDGATb from this yeast were expressed in *S. cerevisiae* TAG-deficient quadruple mutant (H1246). The results showed that RtDGATa did not have DGAT activity and did not enhance the sterol ester biosynthesis capacity of the complemented *S. cervisiae* HY4, although RtDGATa belonged to DGAT1 family and shared substantial similarity with ScARE. Furthermore the level of sterol ester in *R. toruloides* wild type was extremely low (Jin *et al*., 2013) and these results were consistent with mRNA expression level of RtDGATa in *R. toruloides*.

However, RtDGATb of DGAT2 family could completely resume TAG biosynthesisin *S. cerevisiae* H1246 and the lipid content in *S. cerevisiae* HY5 was lower than mutant strains HY2 and HY3. This may due to the higher DGAT activity of RdDGAT2 than RtDGATb because the subtle differences of RdDGAT2 and RtDGATb amino acid residues, although the sequences of RdDGAT2 and RtDGATb are highly similar. Knockout of DGATs, especially DGAT2,in microbial cells obviously resulted in diminution of TAG synthesis. In *Y. lipolytica* ATCC 20362 strain, the total lipids of DCW in DGAT2 mutant decreased to 36% of the wild-type strain (Zhang *et al*., 2012). The atf2-disrupted *Rhodococcus opacus* mutant exhibited a decrease in TAG accumulation (from 40-60% to 25-30%, w/w) and approximately ten-fold increase in glycogen formation in comparison with the wild-type strain (Hernández *et al*., 2013). DGAT mutant experiments suggested that the reaction catalyzed by DGAT is an important regulatory factor in lipid biosynthesis. In recent years, it has been considered that the genetic engineering of DGAT of lipid biosynthesis pathway could be a promising approach to increase the storage lipid content in plants, microalgae and fungi including yeasts for economic production of lipid feedstock (Table 4). Over expression of DGAT2 in *Y. lipolytica* led to total lipid production increase from 8.7% of DCW to 33.8% (Tai and Stephanopoulos, 2013). In oleaginous bacteria *R. opacus* PD 630mutant strain with atf2-overexpression (WS/DGAT, wax ester synthase/acyl-CoA: diacylglycerol acyltransferase), TAG accumulation was promoted about 10% (Hernández *et al*., 2013). Total lipid titer by the transformed *Colletotrichum* (lipid content,73% of DCW) was found to be 1.7-fold morethan the wild type (lipid content, 38% of DCW) due to functional activity of DGAT2 from oleaginous yeast *Candida troplicalis* SY005 when grown understandard condition without imposition of any nutrient-stress (Dey *et al*., 2014). As showed in Table 4, many DGAT2 family members have been used for improving lipid accumulation. In this study, RtDGATa did not alter the TAG content in *S. cerevisiae* mutant and it could not be the metabolic target for improving the TAG biosynthesis in *R. toruloides*. However RtDGATb could restore the TAG synthesis in *S. cerevisiae* TAG-deficient quadruple mutant. Furthermore the mRNA expression level of RtDGATb was associated with lipid accumulation in *R. toruloides* and RtDGATb was up regulated before the onset of TAG accumulation and might functionally contribute to the accumulation of large amount of TAGs, suggesting that RtDGATb may participate in lipid biosynthesis and plays a key impact for TAG accumulation in oleaginous yeast.

Table 4. Genetic engineering of DGAT for increasing lipid accumulation in various organisms

Host	DGAT source	DGAT family	Lipid change	Reference
Y. lipolytica	*Y. lipolytica*	DGAT2	From 8.7% of DCW to 33.8%	Tai and Stephanopoulos (2013)
P. tricornutum	*P. tricornutum*	DGAT2	Neutral lipid increased by 35%	Niu *et al*. (2013)
Rhodotorula glutinis	*Vernicia fordii*	DGAT2	6.24-fold increase	Chen *et al*. (2015)
Chlamydomonas reinhardtii	Chlamydomonas	DGAT2	2.5-fold increase	Iwai *et al*. (2014)
Rhodococcus opacus	*Rhodococcus opacus*	WS/DGAT	Approximately 10%increase of TAG	Hernández *et al*. (2013)
S. cerevisiae	*Arabidopsis thaliana*	DGAT2	3 to 9-fold increase of TAGs	Bouvier-Nave *et al*. (2000)
Colletotrichum sp.	*Candida troplicalis*SY0	DGAT2	From 38% of DCW to 73%	Dey *et al*. (2014)
Chlamydomonas reinhardtii	Brassica napus	DGAT2	From 12.33% to 18.76%	Ahmad *et al*. (2015)
Petunia	*Vernonia galamensis*	DGAT1	From 1.85% of DCW to 2.5% in leaves	Li *et al*. (2010)
		DGAT2	From 1.85% of DCW to 2.2% in leaves	
Arabidopsis thaliana	*Jatrophacurcas*	DGAT1	30-41% increase	Misra *et al*. (2013)
Arabidopsis thaliana	*Thraustochytriumaureum*	DGAT2	Nearly 2-fold increase of oleic acid	Zhang *et al*. (2013b)
Soybean	*U. ramanniana*	DGAT2	From 25 to 26.5%	Lardizabal *et al*. (2008)
Tobacco	*Arabidopsis thaliana*	DGAT2	Lipid in leaves increased by 20-fold	Andrianov *et al*. (2010)
Maize	Maize	DGAT1	Seed oil increased by 41%	Zheng *et al*. (2008)

Similar results were also found in other oleaginous diatom *P. tricornutum* (Gong *et al*., 2013). Further researches are required to investigate whether over expression of DGAT genes alters lipid accumulation in *R. toruloides*.

Conclusion

Two putative DGAT genes from oleaginous yeast *R. toruloides*, RtDGATa and RtDGATb, were individually expressed in *S. cerevisiae* TAG-deficient quadruple mutant (H1246) and the results showed that RtDGATa had no DGAT activity, but RtDGATb could completely resume TAG biosynthesis in *S. cerevisiae* H1246. Substrate preference experiments revealed that RtDGATb preferred unsaturated fatty acids over saturated fatty acids but had no preference forC18:3. The expression pattern of RtDGATb was associated with the process of fatty acid biosynthesis and it suggested that RtDGATb may play as a rate-limiting step in lipid accumulation in *R. toruloides*.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (31271812), the National High Technology Research and Development Program of China (863 Program 2012AA02 2105C).

Author Contributions

Yuanda Song: Conceived and designed the experiments and wrote the paper.

Zhen Wang and Huaiyuan Zhang: Performed the experiments, analyzed the data, contributed reagents/materials/analysis tools and wrote the paper.

Lina Zhao: Analyzed the data, contributed reagents/materials/analysis tools and wrote the paper.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all the other authors have read and approved the manuscript and no ethical issues involved.

References

Ageitos, J.M., J.A. Vallejo, P. Veiga-Crespo and T.G. Villa, 2011. Oily yeasts as oleaginous cell factories. Applied Microbiol. Biotechnol., 90: 1219-27. DOI: 10.1007/s00253-011-3200-z

Ahmad, I., A.K. Sharma, H. Daniell and S. Kumar, 2015. Altered lipid composition and enhanced lipid production in green microalga by introduction of brassica diacylglycerol acyltransferase 2. Plant Biotechnol. J., 13: 540-550. DOI: 10.1111/pbi.12278

Andrianov, V., N. Borisjuk, N. Pogrebnyak, A. Brinker and J. Dixon *et al*., 2010. Tobacco as a production platform for biofuel: Over expression of *Arabidopsis* DGAT and LEC2 genes increases accumulation and shifts the composition of lipids in green biomass. Plant Biotechnol. J., 8: 277-87. DOI: 10.1111/j.1467-7652.2009.00458.x

Bligh, E.G. and W.J. Dyer, 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 37: 911-917. DOI: 10.1139/o59-099

Bouvier-Nave, P., P. Benveniste, P. Oelkers, S.L. Sturley and H. Schaller, 2000. Expression in yeast and tobacco of plant cDNAs encoding acyl CoA:diacylglycerol acyltransferase. Eur. J. Biochem., 267: 85-96. DOI: 10.1046/j.1432-1327.2000.00961.x

Cao, H., 2011. Structure-Function Analysis of Diacylglycerol Acyltransferase Sequences from 70 Organisms. BMC Res. Notes, 4: 249-249. DOI: 10.1186/1756-0500-4-249

Chen, Y., Q. Cui, Y. Xu, S. Yang and M. Gao, 2015. Effects of tung oilseed *FAD*2 and *DGAT*2 genes on unsaturated fatty acid accumulation in *Rhodotorula glutinis* and *Arabidopsis thaliana*. Molecular Genet. Genom., 290: 1605-1613. DOI: 10.1007/s00438-015-1011-0

Chen Z, Liu P, Liu Y, Tang H, Chen Y, Zhang L (2014) Identification and characterization of a type-2 diacylglycerol acyltransferase (DGAT2) from *Rhodosporidium diobovatum*. Antonie Van Leeuwenhoek, 106: 1127-1137. DOI: 10.1007/s10482-014-0282-5

Dey, P., N. Mall, A. Chattopadhyay, M. Chakraborty and M.K. Maiti, 2014. Enhancement of lipid productivity in oleaginous *Colletotrichum* fungus through genetic transformation using the yeast CtDGAT2b gene under model-optimized growth condition. PLoS One, 9: e111253-e111253. DOI: 10.1371/journal.pone.0111253

Elble, R., 1992. A simple and efficient procedure for transformation of yeasts. Bio Techniques, 13: 18-20. PMID: 1503765

Gong, Y., J. Zhang, X. Guo, X. Wan and Z. Liang *et al*., 2013. Identification and characterization of PtDGAT2B, an acyltransferase of the DGAT2 acyl-Coenzyme A: Diacylglycerol acyltransferase family in the diatom *Phaeodactylum tricornutum*. FEBS Lett., 587: 481-487. DOI: 10.1016/j.febslet.2013.01.015

Hernández, M.A., A. Arabolaza, E. Rodríguez, H. Gramajo and H.M. Alvarez, 2013. The *atf*2 gene is involved in triacylglycerol biosynthesis and accumulation in the oleaginous *Rhodococcus opacus* PD630. Applied Microbiol. Biotechnol., 97: 2119-2130. DOI: 10.1007/s00253-012-4360-1

Iwai, M., K. Ikeda, M. Shimojima and H. Ohta, 2014. Enhancement of extraplastidic oil synthesis in *Chlamydomonas reinhardtii* using a type-2 diacylglycerol acyltransferase with a phosphorus starvation–inducible promoter. Plant Biotechnol. J., 12: 808-819. DOI: 10.1111/pbi.12210

Jin, G., Y. Zhang, H. Shen, X. Yang and H. Xie *et al*., 2013. Fatty acid ethyl esters production in aqueous phase by the oleaginous yeast *Rhodosporidium toruloides*. Bioresour Technol., 150: 266-270. DOI: 10.1016/j.biortech.2013.10.023

Lardizabal, K., R. Effertz, C. Levering, J. Mai and M.C. Pedroso *et al*., 2008. Expression of *Umbelopsis ramanniana DGAT*2A in seed increases oil in soybean. Plant Physiol., 148: 89-96. DOI: 10.1104/pp.108.123042

Li, Q., W. Du and D. Liu, 2008. Perspectives of microbial oils for biodiesel production. Applied Microbiol. Biotechnol., 80: 749-756. DOI: 10.1007/s00253-008-1625-9

Li, R., K. Yu, T. Hatanaka and D.F. Hildebrand, 2010. *Vernonia* DGATs increase accumulation of epoxy fatty acids in oil. Plant Biotechnol. J., 8: 184-195. DOI: 10.1111/j.1467-7652.2009.00476.x

Liang, M.H. and J.G. Jiang, 2013. Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. Prog. Lipid Res., 52: 395-408. DOI: 10.1016/j.plipres.2013.05.002

Liu, Q., R.M. Siloto, R. Lehner, S.J. Stone and R.J. Weselake, 2012. Acyl-CoA:diacylglycerol acyltransferase: Molecular biology, biochemistry and biotechnology. Prog. Lipid Res., 51: 350-377. DOI: 10.1016/j.plipres.2012.06.001

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, 25: 402-408. DOI: 10.1006/meth.2001.1262

Misra, A., K. Khan, A. Niranjan, P. Nath and V.A. Sane, 2013. Over-expression of JcDGAT1 from *Jatropha curcas* increases seed oil levels and alters oil quality in transgenic *Arabidopsis thaliana*. Phytochemistry, 96: 37-45. DOI: 10.1016/j.phytochem.2013.09.020

Niu, Y.F., M.H. Zhang, D.W. Li, W.D. Yang and J.S. Liu *et al*., 2013. Improvement of neutral lipid and polyunsaturated fatty acid biosynthesis by overexpressing a type 2 diacylglycerol acyltransferase in marine diatom *Phaeodactylum tricornutum*. Mar Drugs., 11: 4558-4569. DOI: 10.3390/md11114558

Ratledge, C. and J.P. Wynn, 2002. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv. Applied Microbiol., 51: 1-51. DOI: 10.1016/S0065-2164(02)51000-5

Sandager, L., M.H. Gustavsson, U. Stahl, A. Dahlqvist and E. Wiberg *et al*., 2002. Storage lipid synthesis is non-essential in yeast. J. Biol. Chem., 277: 6478-6482. DOI: 10.1074/jbc.M109109200

Sitepu, I.R., L.A. Garay, R. Sestric, D. Levin and D.E. Block *et al*., 2014. Oleaginous yeasts for biodiesel: Current and future trends in biology and production. Biotechnol. Adv., 32: 1336-1360. DOI: 10.1016/j.biotechadv.2014.08.003

Tai, M. and G. Stephanopoulos, 2013. Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production. Metab. Eng., 15: 1-9. DOI: 10.1016/j.ymben.2012.08.007

Wu, S., X. Zhao, H. Shen, Q. Wang and Z.K. Zhao, 2011. Microbial lipid production by *Rhodosporidium toruloides* under sulfate-limited conditions. Bioresour, Technol., 102: 1803-1807. DOI: 10.1016/j.biortech.2010.09.033

Zhang, C., U. Iskandarov, E.T. Klotz, R.L. Stevens and R.E. Cahoon *et al*., 2013a. A thraustochytrid diacylglycerol acyltransferase 2 with broad substrate specificity strongly increases oleic acid content in engineered *Arabidopsis thaliana* seeds. J. Exp. Bot., 64: 3189-3200. DOI: 10.1093/jxb/ert156

Zhang, H., H.G. Damude and N.S. Yadav, 2012. Three diacylglycerol acyltransferases contribute to oil biosynthesis and normal growth in *Yarrowia lipolytica*. Yeast, 29: 25-38. DOI: 10.1002/yea.1914

Zhang, H., L. Zhang, H. Chen, Y.Q. Chen and C. Ratledge *et al*., 2013b. Regulatory properties of malic enzyme in the oleaginous yeast, *Yarrowia lipolytica* and its non-involvement in lipid accumulation. Biotechnol. Lett., 35: 2091-2098. DOI: 10.1007/s10529-013-1302-7

Zheng, P., W.B. Allen, K. Roesler, M.E. Williams and S. Zhang *et al*., 2008. A phenylalanine in DGAT is a key determinant of oil content and composition in maize. Nat Genet., 40: 367-372. DOI: 10.1038/ng.85

Zhu, Z., S. Zhang, H. Liu, H. Shen and X. Lin *et al*., 2012. A multi-omic map of the lipid-producing yeast *Rhodosporidium toruloides*. Nat Commun., 3: 1112-1112. DOI: 10.1038/ncomms2112