Molecular cloning and overexpression of DGA1, an acyl-CoA-dependent diacylglycerol acyltransferase, in the oleaginous yeast Rhodosporidiobolus fluvialis DMKU-RK253

Pirapan Polburee,† Takao Ohashi,‡ Yung-Yu Tsai,‡ Thitinun Sumyai,† Noppon Lertwattanasakul,† Savitree Limtong,†,‡,§ and Kazuhito Fujiyama,†,*

Abstract

Triacylglycerol (TAG) is a major component of lipid storage in yeast. The acyl CoA: diacylglycerol acyltransferase (DGAT) that catalyzes the final and rate-limiting step in the production of TAG is rather interesting. Consequently, cloning and analysis of the gene-encoding TAG synthase, diacylglycerol acyltransferase gene (DGA1), of the oleaginous yeast Rhodosporidiobolus fluvialis DMKU-RK253 were undertaken. Analysis of the deduced amino acid sequence of DGA1 from R. fluvialis DMKU-RK253 (RIDGA1) showed similarity with the acyl-CoA:diacylglycerol acyltransferase 2 (DGAT2) from other organisms. The cDNA of RIDGA1 was cloned into the yeast expression vector pYES2 and heterologously overexpressed in Saccharomyces cerevisiae. One of the transformants showed a 1.6-fold increase in lipid content compared with the wild-type strain harbouring the pYES2 empty vector. Furthermore, DGA1 overexpression in R. fluvialis DMKU-RK253 resulted in a 2.5-fold increase in lipid content when compared with the wild-type strain, and no significant differences in fatty acid composition were observed between RIDGA1-overexpressed and wild-type strains. Taken together, our results supported our hypothesis that the RIDGA1 is a genetic factor that can be used for the development of a strain with improved lipid accumulation capabilities.

INTRODUCTION

Oleaginous yeasts that are able to accumulate cellular lipid in quantities higher than 20% of their biomass [1] represent a potential feedstock for biodiesel production due to the fact that the composition of their fatty acids is similar to that of vegetable oils [2]. Recently, the yeast Rhodosporidium fluviale DMKU-RK253 was proposed as a new oleaginous yeast strain that accumulates a large quantity of lipid when cultivated in crude glycerol [3]. Later, Rhodosporidium fluviale was transferred to the genus Rhodosporidiobolus and its name was changed to Rhodosporidiobolus fluvialis [4]. Therefore, information on the molecular basis of the triacylglycerol (TAG) metabolism in this oleaginous yeast is of interest.

The major neutral lipids occurring in oleaginous yeast are triacylglycerols (TAGs). Formally, TAG synthesis can be divided into two phases. The first phase comprises various reactions leading to diacylglycerol (DAG) formation and in the second phase DAG is converted to TAG [5]. Therefore, genes encoding enzymes involved in the second phase of TAG play a significant role in lipid accumulation.

Diacylglycerol acyltransferase (DGAT), which catalyzes acyl-CoA-dependent acylation of sn-1,2-diacylglycerol, is responsible for the terminal (and only dedicated) step in the formation of TAG [6]. Several research groups have demonstrated that DGAT is present in eukaryotic organisms, including plants, animals and fungi [7–9]. Previously, the DGAT family consisted of two main types, DGAT1 and DGAT2. DGAT1 was proposed to have high similarity with the sterol:acyl-CoA acyltransferase (ACAT), which is specific in sterol ester synthesis in plants and mammals [8]. DGAT2 plays a major role in TAG biosynthesis and lipid storage [5]. In Saccharomyces cerevisiae, acyl-CoA

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Author affiliations: 1Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, Thailand; 2International Center for Biotechnology, Osaka University, Osaka, Japan; 3The Royal Society of Thailand, Bangkok, Thailand.

*Correspondence: Savitree Limtong, fcsistl@ku.ac.th; Kazuhito Fujiyama, fujiyama@iicb.osaka-u.ac.jp

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Abbreviations: ACAT, sterol:acyl-CoA acyltransferase; DAG, diacylglycerol; DGA1, diacylglycerol acyltransferase gene; DGAT, acyl CoA: diacylglycerol acyltransferase; GPD, glyceraldehyde-3-phosphate dehydrogenase; KH2PO4, potassium dihydrogen phosphate; LB, Luria–Bertani medium; MgSO4·7H2O, magnesium sulfate heptahydrate; NaCl, sodium chloride; (NH4)2SO4, ammonium sulfate; NLSD, nitrogen-limited SD medium; RdDGA1, DGA1 gene from Rhodosporidium diobovatum; RIDGA1, DGA1 gene from Rhodosporidiobolus fluvialis; ScDGA1, DGA1 gene from Saccharomyces cerevisiae; SD, synthetic defined medium; Sh ble, zeocin-resistant gene; TAG, triacylglycerol; YM, yeast extract/malt extract medium.

†Present address: Rattanakosin College for Sustainable Environment and Energy (RC2EE), Rajamangala University of Technology Rattanakosin, Nakhon Pathom, Thailand.
diacylglycerol acyltransferase (Dga1p) is the homologue of mammalian DGAT2. Dga1p contributes the main activity for TAG synthesis in yeast, playing a similar role to that played by DGAT2 in mammals [10]. Dga1p localizes in lipid particles and the endoplasmic reticulum of the budding yeast [11]. Recently, a novel member of the DGAT family, DGAT3, has been isolated from Arabidopsis thaliana [12] and Rhodospiridium glutinis [13].

The DGAT2 members reported in several yeasts are homologues of DGA1, which accounts for 87% of DAG esterification activity in S. cerevisiae [14]. In the oleaginous yeast Yarrowia lipolytica, the gene YAL10E32769g (DGA1) encoded a major TGA synthase and the heterologous expression of DGA1 in a mutant of S. cerevisiae defective in TGA synthesis restored the formation of this neutral lipid [15]. The type-2 DGAT from Rhodospiridium diobovatum (RdDGAT), which catalyzed the final step of TAG synthesis, was cloned and functionally verified [16]. Furthermore, the amount of total lipid is significantly reduced in the R. glutinis strain lacking DGA1, indicating that none of the other genes can fully substitute for its role in TAG synthesis [13].

Although a number of previous reports have presented the results of lipid production by oleaginous yeast species, more molecular information on oleaginous yeast is still required to enrich our understanding of the molecular basis of lipid accumulation and improve the efficiency, robustness and economics of the microbial production of fatty acid derivatives. In this paper, we describe the molecular cloning of the gene YALI0E32769g (DGA1) from R. fluvialis (NBRP), Japan, was used as a host for the heterologous gene expression of DGA1 in its native strain to determine whether it may be possible to improve the efficiency of lipid accumulation.

**METHODS**

**Yeast strains**

*R. fluvialis* DMKU-RK253, an oleaginous yeast that accumulated a high quantity of lipid when cultivated in crude glycerol [3, 17], was used in this study. *S. cerevisiae* BY23849 (Mat a leu2Δ0 his3Δ1 ura3Δ0 met15Δ0), which was obtained from the National Bio-Resource Project (NBRP), Japan, was used as a host for the heterologous gene expression of DGA1 from *R. fluvialis*. The yeasts were maintained on yeast extract/malt extract (YM) agar slants (10 g glucose 1−1, 5 g peptone 1−1, 3 g yeast extract 1−1 and 3 g malt extract 1−1) at 4 °C. *Escherichia coli* DH5α, which was used as a host strain in all of the recombinant DNA experiments, was grown in Luria–Bertani (LB) medium (0.1 g tryptone 1−1, 0.05 g yeast extract 1−1 and 0.1 g NaCl 1−1). When needed, 100 mg ampicillin 1−1 was added to the medium.

**Cloning and analysis of the DGA1 gene**

Conventional recombinant DNA techniques were applied [18]. Yeast genomic DNA was prepared by the cell wall digest enzyme method using Dr GenTLE for yeast kit (Takara Bio, Inc., Shiga, Japan). Total RNA was isolated from exponential-phase cells grown in YM medium (24 h) using an RNeasy mini kit (Qiagen, Venlo, the Netherlands). The isolated RNA was then reverse-transcribed into a complementary DNA (cDNA) using a PrimeScript II 1st strand cDNA synthesis kit (Takara Bio, Inc.). The whole-genomic sequence of *R. fluvialis* DMKU-RK253 was sequenced by paired-end sequencing on an Illumina HiSeq 2500 sequencing system provided by the Hokkaido System Science Co., Ltd (Sapporo, Japan). The DGA1 gene from *R. fluvialis* DMKU-RK253 (*RfDGA1*) was cloned after PCR amplification using a specific primer set designed from a draft genome of this yeast strain (forward primer 5′-ATGTTGGCATCATGA TTGCGCAGGAGAATGG-3′ and reverse primer 5′-TCAAGCGGATAATGTGTAGCTCC-3′) and either cDNA or genomic DNA of *R. fluvialis* as a template. The DGA1 gene from *S. cerevisiae* (*ScDGA1*) was also cloned after PCR amplification using a specific primer set (forward primer 5′-ATGTAGGAACATTCA-3′ and reverse primer 5′-TTACCCAACACTTCTTCAATTCTCGATCCGGT-3′) and genomic DNA of *S. cerevisiae* as a template. These PCR products were purified and subsequently cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), resulting in pGEM-T Easy-ScDGA1 and pGEM-T Easy-ScDGA1, respectively. They were then subjected to nucleotide sequencing prior to transformation into *E. coli* competent cells. DNA sequences were determined by a BigDye Terminator version 3.1 cycle sequencing kit and a 3130XL genetic analyzer (Applied Biosystems, CA, USA), and examined via BLAST analysis [19].

**Overexpression of DGA1 gene in S. cerevisiae**

A non-oleaginous yeast *S. cerevisiae* was used as a host for the overexpression of *ScDGA1* and *RfDGA1*. The *RfDGA1* and *ScDGA1* were cloned into a pYES2 expression vector [20] that contained the galactose-inducible GAL1 promoter and URA3 (orotidine 5′-phosphate decarboxylase gene) as a selection marker. The *RfDGA1* gene was amplified using pGEM-T Easy-*RfDGA1* as a template by using a forward primer 5′-GTGGTGAATTCTACCAATCCATGA TTGCGCAGGAGAATGG-3′ and a reverse primer 5′-TTTGAATTCTCACGGGATTAATGTTGAGCTCC-3′ containing *BamHI* and *EcoRI* sites (underlined). The *ScDGA1* gene was amplified using a forward primer 5′-GTGGTGAATTCTACCAATCCATGA TTGCGCAGGAGAATGG-3′ and a reverse primer 5′-TGGTCCATCATGTCCAGGAAACATCATACTGTTTGCTCAGCGGTT-3′ containing *BamHI* and *EcoRI* sites (underlined), respectively, with pGEM-T Easy-*ScDGA1* as a template. These PCR products of *RfDGA1* and *ScDGA1* were digested with *BamHI* and *EcoRI*, and then ligated to pYES2, which was pre-digested with the corresponding restriction enzymes, to construct pYES2-*RfDGA1* and pYES2-*ScDGA1* vectors. The vectors were then introduced into the *S. cerevisiae* by the lithium acetate method [21].

The transformants harbouring the pYES2-*RfDGA1* vector, the pYES2-*ScDGA1* vector, and the control, pYES2 empty vector, were grown overnight in 20 ml of synthetic defined
(SD) medium at 30 °C. The SD medium contained 1.7 g Bacto-yeast nitrogen base l−1 without amino acids and (NH4)2SO4, 1 g glucose l−1, 20 g raffinose l−1, 5 g (NH4)2SO4 l−1 and the required nutrients. The grown cells (OD600 =20) were transferred to 50 ml of nitrogen-limited SD (NLSD) medium containing 1.7 g Bacto-yeast nitrogen base l−1 without amino acids and (NH4)2SO4, 50 g galactose l−1, 0.1 g (NH4)2SO4 l−1 and the required nutrients. Depending on the nutrient requirements of the strain, 0.1 g l−1 of each nutrient – adenine, arginine, cysteine, leucine, lysine, threonine and tryptophan – and 0.005 g l−1 of each nutrient – aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine and valine – were added. The cultivated cells were analysed for biomass, lipid production and lipid content after 72 h of cultivation.

**Overexpression of DGA1 gene in R. fluvialis**

To construct a recombinant RfDGA1 expression vector in *R. fluvialis* DMKU-RK253, the PCR product of RfDGA1 was amplified using a forward primer 5′-GTGTTGGATCCAT GATTGGCAGGAGA-3′ and a reverse primer 5′-GGATCCATGTCGAGGATATG-3′ containing BamHI and NdeI sites (underlined), respectively, with pGEM-T Easy-RfDGA1 being used as a template. The PCR product of RfDGA1 was digested with BamHI and NdeI, and then ligated to pPGP-Shble2 [22], which was pre-digested with the corresponding restriction enzymes, to construct the pPGP-Shble2-RfDGA1 vector. For transformation, the pPGP-Shble2-RfDGA1 vector was linearized by PCR amplification using a specific primer pair and then introduced into the 16 h-old *R. fluvialis* DMKU-RK253 cells by a slightly modified lithium acetate method [22]. The integration of the pPGP-Shble2-RfDGA1 fragment was subsequently confirmed by a colony PCR using a forward primer 5′-ATGATTGGCAGGAGAATGG-3′ and a reverse primer 5′-TCAGGGGATAATGGTGGAGTCC-3′.

The recombinants were inoculated to 50 ml crude glycerol-YM medium (30 g crude glycerol l−1, 3 g yeast extract l−1, 3 g malt extract l−1 and 5 g peptone l−1) in a 250 ml Erlenmeyer flask on a rotary shaker at 150 r.p.m. at 28 °C for 24 h to serve as an inoculum. The pre-culture was transferred to 50 ml nitrogen-limited medium (70 g crude glycerol l−1, 0.55 g (NH4)2SO4 l−1, 1 g monosodium glutamate l−1, 2 g MgSO4·7H2O l−1, 0.4 g KH2PO4 l−1 and pH 5.5) [3] in a 250 ml Erlenmeyer flask. The cells were analysed for biomass, lipid production and lipid content after 72 h of cultivation.

**Analytical methods**

Lipid was extracted according to a modified Bligh and Dyer [23] method. Derivation of methyl esters from fatty acids by transmethylation was performed according to the method of Holub and Skeaff [24] and the fatty acid methyl esters were then analysed with a gas chromatograph equipped with a flame ionization detector (Shimizu GC14-A, Japan) using a silica megabore capillary column (30 m × 0.52 mm × 1 μm; Durabond 225, J and W Scientific, USA) and helium as the carrier gas. Fatty acid methyl esters were identified and quantified by comparison of their retention times with authentic standards. The operating conditions were as follows: helium was used as the carrier gas at a flow rate of 10 ml min−1, the column temperature was 210 °C, and the injector and detector temperatures were both 250 °C [24]. Fatty acids were identified and quantified by comparison of their retention times and peak areas with those of standard mixtures of fatty acids. Pentadecanoic acid was used as an internal standard.

To analyse biomass, cells were harvested by a centrifuge (Hettich EBA 20, USA) at 5000 r.p.m. for 5 min, washed with distilled water and then dried at 100 °C until a constant weight was obtained. The biomass was determined gravimetrically.

For Southern blot analysis, 20 μg of genomic DNA isolated from each transformant of *R. fluvialis* DMKU-RK253 was double-digested with BamHI–HindIII, which have no cutting site on the expression cassette, and electrophoresed on 0.8 % agarose gel. The DNA was then transferred and fixed onto a positively charged nylon membrane (Roche, Germany). The blots were hybridized with a DIG-labelled fragment of Sh ble ORF as the probe, developed by chemiluminescence (DIG High Prime DNA Labeling and Detection Starter kit II, Roche, Germany), exposed to RX-U Medical X-ray film (Fuji Film, Japan) and visualized with an FPM100 medical processor (Fuji Film).

**RESULTS AND DISCUSSION**

**Cloning of the RfDGA1 gene in R. fluvialis**

For the cloning and characterization of RfDGA1, the gene was amplified from either genomic DNA or cDNA of *R. fluvialis*. Sequencing analysis revealed the existence of open reading frames (ORFs) of 1464 bp and 1050 bp, respectively, and of five introns and six exons in the RfDGA1 genomic DNA. This ORF showed nucleotide sequence identities of 73.7 % to BAH85840.1 of *Rhodosporidium toruloides*, 76.2 % to AIM47579.1 of *Rhodosporidium diobovatum* and 45.6 % to *S. cerevisiae* (data not shown). The deduced RfDga1p sequence consisted of 349 amino acids with AUG and UGA as the start and stop codons, respectively, and a calculated molecular mass of 39 659 daltons (BioEdit software version 7.0) was obtained. RfDga1p bears the lysophospholipid acyltransferases superfamily domain (cl17185) and the diaclylglycerol acyltransferase domain (pfam03982) of the DGAT family [25].

The RfDGA1 could be translated with a restricted set of preferred codons and the results showed that UUC for Phe, UUG for Leu, UCC for Thr, AUC for Ile, AUG for Met, GUC for Val, UCC for Ser, CCC for Pro, GCC and GCG for Asn, UAC for Tyr, CAC for His, CAG for Gln, AAC for Asn, AAG for Lys, GAC for Asp, GAG for Glu, UGG for Trp, CGC for Arg and GGC for Gly are predominantly used. It should be noted that only two amino
acids, Leu and Trp, frequently used UUG and UGG, respectively, as represented in the case of Dga1p from *S. cerevisiae*.

To obtain evidence regarding a structural relationship, the deduced amino acid sequence of RfDga1p was compared with the sequences of enzymes belonging to known DGAT families from other yeast species and organisms (Fig. 1). RfDga1p showed a very high identity score (83.1 %) to DGAT from *R. diobovatum*, which was identified to be type-2 of three DGAT enzyme families [16]. To identify DGAT2-conserved motifs, the deduced amino acid sequences from a wide range of organisms were aligned using the Clustal W algorithm (Fig. 2a). The results showed the identity as signature motifs within the DGAT2 subfamily proposed by Cao [26]: 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). The separation of the DGAT2 family seems to be reflected by differences in the amino acid sequence of a highly conserved motif. Guo *et al.* [27] indicated that the majority of DGAT2 is composed of a C-terminal cytoplasmic domain. The strongly conserved amino acid quadruple sequence of His within motif 1 as HPHG (His-Pro-His-Gly) plays a crucial role in the function of the enzyme and suggests a role for histidine as part of the active site. This HPHG motif is present in all of the proteins displayed in the DGAT2 clade [28]. The four putative transmembrane segments were predicted by TMPred software at the Eukaryotic Linear Motif resource (http://elm.eu.org/) (Fig. 2b). The four transmembrane segments were also proposed in the DGAT2 of *S. cerevisiae* [2] and *R. diobovatum* [16]. It is known that the DGAT families are integral microsomal membrane proteins. In *Y. lipolytica* and *S. cerevisiae*, Dga1p is integral endoplasmic reticulum proteins, and in *Y. lipolytica* Dga1p is one of the lipid body proteins identified by Athenstaedt *et al.* [29].

The results revealed that the amino acid sequence and hydrophobic regions pattern were similar to those of the RdDGAT2 of *R. diobovatum* described in the previous report by Chen *et al.* [16]. Therefore, it was assumed that RfDga1p belongs to type-2 DGAT, the *DGA1* homologue of *S. cerevisiae*.

**Fig. 1.** Phylogenetic relationships of the Dga1 protein of *R. fluvialis* DMKU-RK253 (RfDga1p) identified in this study and Dga1 proteins from the other yeasts in the GenBank database. The phylogenetic tree was constructed from Kimura's two-parameter correction, using the neighbour-joining method with MEGA software version 6.0. Numbers indicate percentages of bootstrap sampling derived from 1000 samples.
Fig. 2. (a) Sequence comparison of RfDga1p with those of DGAT2s from other organisms; RdDGAT (DGAT from R. diobovatum), RfDGAT2 (R. toruloides), Ur2B and Ur2A (Umbelopsis ramanniana), YIDGAT2 (Y. lipolytica), ScDga1p (S. cerevisiae), SpDGAT2 (Schizosaccharomyces pombe), AtDGAT2 (Arabidopsis thaliana). (b) Kyte–Doolittle hydropathy plot of RfDga1p. Hydrophobic regions are shown in black lines.
Overexpression of the DGA1 gene in S. cerevisiae

To verify the function of the RfDGA1 gene encoding a putative acyl-CoA diacylglycerol acyltransferase, pYES2-RfDGA1 and pYES2-ScDGA1 vectors were constructed and subsequently introduced into S. cerevisiae cells. The transformants were cultured in NLSD medium, and the biomass and total lipid production were then analysed. The biomass and lipid production by S. cerevisiae carrying the empty pYES2 vector were also subjected to analysis as a control.

As shown in Fig. 3, the transformant harbouring pYES2-ScDGA1 showed the highest lipid production and lipid content of 6.4 mg l\(^{-1}\) and 3.6 % of dry biomass, respectively, at 24 h of cultivation, while the control only produced 4.7 mg l\(^{-1}\) lipid and a lipid content of 2.3 %. At 48 h of cultivation, the lipid production and lipid content for the transformant harbouring pYES2-RfDGA1 were 6.3 mg l\(^{-1}\) and 3.4 % of dry biomass, respectively, similar values to those obtained for the transformant harbouring pYES2-ScDGA1. The biomass of the control was 2.4 g l\(^{-1}\) after 72 h of cultivation, while the biomass of the transformants harbouring pYES2-ScDGA1 and pYES2-RfDGA1 was 2.5 and 2.1 g l\(^{-1}\), respectively. The lipid production and lipid content of the transformant harbouring pYES2-RfDGA1 increased to 8.5 mg l\(^{-1}\) and 4.1 % of dry biomass, respectively, which was 1.6 and 1.5 times higher than those obtained for the control and the transformant harbouring pYES2-ScDGA1, respectively. This is the first report to show that when the DGA1 gene from R. fluvialis is cloned and heterologously expressed in S. cerevisiae the RfDGA1 gene results in a significant increase of lipid accumulation compared to that seen when a native DGA1 gene is introduced in S. cerevisiae.

The fatty acid composition and TAG produced by S. cerevisiae harbouring the pYES2-ScDGA1, pYES2-RfDGA1 and a negative control (pYES2) were determined and compared. The results showed that with overexpression of ScDGA1 and RfDGA1 the main components of the lipids were palmitic acid (C16 : 0) and stearic acid (C18 : 0), respectively, while in the negative control stearic acid (C18 : 0) and palmitoleic acid (C16 : 1) were the major fatty acids (Table 1); The saturated fatty acid composition of lipid produced from transformants harbouring pYES2-RfDGA1 (70 % of total lipid) was higher than for those produced by transformants harbouring pYES2-ScDGA1 (63 % of total lipid) and the negative control (54 % of total lipid), respectively. The

<table>
<thead>
<tr>
<th>Strain</th>
<th>C14 : 0</th>
<th>C16 : 0</th>
<th>C16 : 1</th>
<th>C18 : 0</th>
<th>C18 : 1</th>
<th>C18 : 2</th>
</tr>
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<tbody>
<tr>
<td>pYES2</td>
<td>2.55±0.5</td>
<td>19.88±3.0</td>
<td>26.97±4.6</td>
<td>31.27±4.3</td>
<td>18.06±2.1</td>
<td>6.05±0.3</td>
</tr>
<tr>
<td>pYES2/ScDGA1</td>
<td>1.43±0.0</td>
<td>35.18±1.6</td>
<td>13.61±4.5</td>
<td>26.46±1.2</td>
<td>21.97±5.0</td>
<td>7.43±1.3</td>
</tr>
<tr>
<td>pYES2/RfDGA1</td>
<td>6.92±0.3</td>
<td>32.75±0.6</td>
<td>16.13±2.8</td>
<td>29.92±0.8</td>
<td>12.92±2.9</td>
<td>8.48±3.5</td>
</tr>
</tbody>
</table>

*C14 : 0, myristic acid; C16 : 0, palmitic acid; C16 : 1, palmitoleic acid; C18 : 0, stearic acid; C18 : 1, oleic acid; C18 : 2, linoleic acid.

Table 1. Fatty acid composition of lipid produced from S. cerevisiae BY23849 transformed with pYES2 carrying ScDGA1, RfDGA1 and negative control (pYES2) in NLSD medium containing 5 % galactose.
results suggested that with overexpression of DGA1 in S. cerevisiae, saturated fatty acids were preferred to unsaturated fatty acids.

The fatty acid composition of lipid produced from S. cerevisiae revealed that the relative content of myristic acid (C14:0) and oleic acid (C18:1) from transformants harbouring pYES2-RfDGA1 was different from that of pYES2-ScDGA. However, the distinct preference for fatty acyl-CoA substrates of a specific chain length and desaturation in RfDga1p is still unclear. In vitro assays revealed that linoleic acid (C18:2), oleic acid (C18:1) and palmitic acid (C16:0), respectively, were the preferred substrates of DGAT from R. diobovatum, the related RfDga1 protein [16].

A significant increase in the lipid accumulation and DGAT activity of a strain of wild-type S. cerevisiae with Dga1p overexpression was also determined by Kamisaka et al. [30]. Recently, Friedlander et al. [31] reported a strain-engineering strategy centred on DGA gene overexpression that applied combinatorial screening of overexpression and deletion of genetic targets to construct a high lipid-producing yeast. In Y. lipolytica, overexpression of either a native Dga1 enzyme or a heterologous Dga1 enzyme from R. toruloides increased the lipid accumulation, and it was enhanced to an even greater extent for a combination of overexpression of a heterologous Dga1 enzyme from R. toruloides, a heterologous Dga2 enzyme from Claviceps purpurea, and deletion of the native Tgl3 lipase regulator. These three genetic modifications made it possible to achieve a lipid content of 77% and a yield of 0.21 g lipid per g glucose in batch fermentation.

The non-oleaginous yeast S. cerevisiae with RfDGA1 overexpression accumulated higher lipid than that of ScDGA1 overexpression. Due to a relatively low level of sequence homology and the differences in the hydrophobic profiles of certain regions observed between R. fluvialis and S. cerevisiae Dga1ps, it was assumed that the primary structure of RfDga1p should be different from ScDga1p, resulting in the unique functional feature of each Dga1p [2]. In addition, the functional and structural features might have resulted from evolutionary adjustments specific to the organisms [8]. Moreover, it has been reported that the expression of some DGAT genes had a correlation with nitrogen deprivation [32–34]. Therefore, it could be assumed that the evolution of the RfDGA1 gene from the oleaginous yeast R. fluvialis is the result of adaptation to its oligotrophic living environment under stress conditions.

Overexpression of the DGA1 gene in the yeast R. fluvialis DMKU-RK253

To continue developing a strain with improved lipid accumulation capabilities, RfDGA1 was inserted into the pPGP-Shble2 expression vector that contained a glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter and a zeocin-resistant gene (Sh ble) as a selective marker. The pPGP-Shble2-RfDGA1 vector and the control, pPGP-Shble2 empty vector, were linearized by PCR amplification and then R. fluvialis DMKU-RK253 was transformed with the linearized plasmids. Transformants harbouring transformed cassette(s) in their genome were confirmed by colony PCR using genomic DNA isolated from each transformant as a template. Six positive transformants, numbers 3, 4, 5, 6, 10 and 13 from sixteen selected colonies, were obtained. Next, the six transformants and the wild-type were cultivated in a nitrogen-limited medium containing 70 g l⁻¹ of crude glycerol, and incubated at 28 °C, 150 r.p.m., for 72 h. The biomass, lipid production and lipid content results for each transformant are shown in Fig. 4.

The results obtained from different transformants showed differences in lipid production and lipid content, but no significant difference in biomass production was observed. The

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**Fig. 4.** The biomass (■), lipid (■) and lipid content (□) of the overexpression of the DGA1 gene in each transformant (numbers 3, 4, 5, 6, 10 and 13) of R. fluvialis DMKU-RK253 and the control pPGP-Shble2 vector (Sh ble). Each strain was cultivated in 50 ml of nitrogen-limited medium containing 70 g crude glycerol l⁻¹ for 72 h. The average of three replicates is presented.
same biomass, lipid and lipid content were found in the wild-type and the control. The highest lipid production and lipid content of 1.2 g l$^{-1}$ and 18.5% of dry biomass, respectively, were derived from transformant no. 4, which accumulated up to 2.5 times more lipid than the wild-type.

Due to the random, non-targeted nature of the integration, fatty acid composition of lipid produced from each transformant was double-digested with BamHI–HindIII and Southern blot was carried out by using the Sh ble ORF as a probe.

Our results revealed that the overexpression of DGA1 in R. fluvialis effectively increased the lipid content in the medium containing 70 g l$^{-1}$ glycerol. The high lipid content of the transformants depended on glycerol concentration and nitrogen limitation. The increase in lipid content was achieved in conjunction with a substantial increase in total fatty acids without a decrease in cell biomass. This is the first report to indicate that R. fluvialis overexpressing the DGA1 gene shows a large increase in lipid production from glycerol.

In this study, we developed a method for obtaining RfDGA1-overexpressing transformants from R. fluvialis. The three transformants were obtained successfully to increase the lipid accumulation in R. fluvialis over that of the wild-type strain with an unknown integration site. The differences in lipid accumulation for each of the transformants were due to the random integration and the copy number of the expression cassette into the genome. Each transformant represented a different genetic event. The authors propose that overexpression obtained by using the molecular genetics method can be used for the development of lipid accumulation capabilities by R. fluvialis. This is the first step towards obtaining an understanding of the molecular basis of the TGA metabolism in this oleaginous yeast. Much information is required to improve the efficiency, robustness and economics of the microbial production.

**Conclusion**

Molecular cloning and characterization of the DGA1 gene in R. fluvialis were executed to investigate the molecular basis and its involvement in lipid biosynthesis. This is the random insertion of a foreign gene into the DMKU-RK253 genome occurred individually with different copy numbers.

The fatty acid composition of the RfDGA1-overexpressing transformant no. 4 and the wild-type is shown in Table 2. The major fatty acids were linoleic acid (C18:2) and oleic acid (C18:1). No significant differences in fatty acid composition were observed between the RfDGA1-overexpressing and wild-type strains. It is possible that RfDga1 has no fatty acid preference, but rather it simply outcompetes the elongases and desaturases for fatty acids modification. The alteration of the fatty acid profile pattern was also observed by Silverman et al. [35] when a native Dga2 enzyme was overexpressed in Y. lipolytica.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fatty acid (%)</th>
<th>Lipid content (% of dry biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C14 : 0</td>
<td>C16 : 0</td>
</tr>
<tr>
<td>pGPD-ShBle2</td>
<td>0.9±0.0</td>
<td>19.4±0.8</td>
</tr>
<tr>
<td>pGPD-ShBle2-RfDGA1</td>
<td>0.6±0.0</td>
<td>18.8±0.7</td>
</tr>
</tbody>
</table>

*C14 : 0, myristic acid; C16 : 0, palmitic acid; C18 : 0, stearic acid; C18 : 1, oleic acid; C18 : 2, linoleic acid; C18 : 3, linolenic acid.*
first report to show that when the DGA1 gene of *R. fluvialis* is cloned and heterologously expressed in *S. cerevisiae* the Rf/DGA1 gene results in a significant increase of lipid accumulation compared to that seen when a native DGA1 gene is introduced in *S. cerevisiae*. Overexpression of Rf/DGA1 was also performed. The result showed higher lipid accumulation in the Rf/DGA1-overexpressing transformant compared to the wild-type strain, with no significant differences in fatty acid composition. Thus, the overexpression of this gene has potential for the development of lipid accumulation capabilities in yeast strains.

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### Conflicts of interest
The authors declare that there are no conflicts of interest.

### References
15. Athenstaedt K, YALI0E32769g (DGA1) and YALI0E16797g (LRO1) encode major triacylglycerol synthases of the oleaginous yeast *Yarrowia lipolytica*. *Biochim Biophys Acta* 2011;1811:587–596.


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