DGA1 (diacylglycerol acyltransferase gene) overexpression and leucine biosynthesis significantly increase lipid accumulation in the *Δsnf2* disruptant of *Saccharomyces cerevisiae*

Yasushi KAMISAKA[1], Nao TOMITA, Kazuyoshi KIMURA, Kumiko KAINOU and Hiroshi UEMURA

Lipid Engineering Research Group, Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan

We previously found that *SNF2*, a gene encoding a transcription factor forming part of the SWI/SNF (switching/sucrose non-fermenting) chromatin-remodelling complex, is involved in lipid accumulation, because the Δ*snf2* disruptant of *Saccharomyces cerevisiae* has a higher lipid content. The present study was conducted to identify other factors that might further increase lipid accumulation in the Δ*snf2* disruptant. First, expression of *LEU2* (a gene encoding β-isopropylmalate dehydrogenase), which was used to select transformed strains by complementation of the leucine axotroph, unexpectedly increased both growth and lipid accumulation, especially in the Δ*snf2* disruptant. The effect of *LEU2* expression on growth and lipid accumulation could be reproduced by adding large amounts of leucine to the culture medium, indicating that the effect was not due to Leu2p (β-isopropylmalate dehydrogenase) itself, but rather to leucine biosynthesis. To increase lipid accumulation further, genes encoding the triacylglycerol biosynthetic enzymes diacylglycerol acyltransferase (*DGA1*) and phospholipid:diacylglycerol acyltransferase (*LRO1*) were overexpressed in the Δ*snf2* disruptant. Overexpression of *DGA1* significantly increased lipid accumulation, especially in the Δ*snf2* disruptant, whereas *LRO1* overexpression decreased lipid accumulation in the Δ*snf2* disruptant. Furthermore, the effect of overexpression of acyl-CoA synthase genes (*FAA1*, *FAA2*, *FAA3* and *FAA4*), which each supply a substrate for Dga1p (diacylglycerol acyltransferase), was investigated. Overexpression of *FAA3*, together with that of *DGA1*, did not further increase lipid accumulation in the Δ*snf2* disruptant, but did enhance lipid accumulation in the presence of exogenous fatty acids. Lastly, the total lipid content in the Δ*snf2* disruptant transformed with *DGA1* and *FAA3* overexpression vectors reached approx. 30 %, of which triacylglycerol was the most abundant lipid. Diacylglycerol acyltransferase activity was significantly increased in the Δ*snf2* disruptant strain overexpressing *DGA1* as compared with the wild-type strain overexpressing *DGA1*; this higher activity may account for the prominent increase in lipid accumulation in the Δ*snf2* disruptant with *DGA1* overexpression. The strains obtained have a lipid content that is high enough to act as a model of oleaginous yeast and they may be useful for the metabolic engineering of lipid production in yeast.

Key words: *DGA1* (diacylglycerol acyltransferase gene), *FAA3* (acyl-CoA synthase gene), leucine biosynthesis, lipid accumulation in *Saccharomyces cerevisiae* (baker's yeast), *LRO1* (phospholipid:diacylglycerol acyltransferase gene), *SNF2* (transcription factor snf2 gene).

INTRODUCTION

Baker's yeast (*Saccharomyces cerevisiae*) is a model organism for eukaryotes, and its lipid metabolism has been studied extensively [1,2]. Previously, several important genes encoding storage-lipid biosynthetic enzymes have been identified [3–6], and proteins associated with lipid particles have been elucidated [7]. Knowledge about storage-lipid biosynthesis in *S. cerevisiae* would make it possible to elucidate the molecular mechanisms of lipid accumulation. Nevertheless, *S. cerevisiae* does not accumulate storage lipids because only 5–7 % (per dry cell weight) of lipids are produced, whereas some oleaginous yeasts and fungi have 30–50 % lipids. It is thus important to obtain *S. cerevisiae* strains with a higher lipid content that could be used as a model for studying lipid accumulation. Studies in oleaginous yeasts and fungi have revealed that several factors, such as ATP:citrate lyase and malic enzyme in the energy-production system [8,9], TAG (triacylglycerol) biosynthetic enzymes [10,11], and modulators of lipid body biogenesis [7,12], play important roles in lipid accumulation. Thus it could be also evaluated whether these factors affect lipid accumulation in *S. cerevisiae*.

With the aim of increasing lipid accumulation in *S. cerevisiae*, we recently identified genes that affect lipid accumulation by using transposon mutagenesis and found that the disruption of these genes did increase lipid content in this yeast [13]. The lipid content in these disruptants, however, was not high enough to represent a model of oleaginous yeasts, suggesting that other factors may be rate-limiting for further lipid accumulation. TAG-biosynthetic enzymes are possible candidates for such factors, and overexpression of these enzymes, such as DGAT (diacylglycerol acyltransferase) and phospholipid:diacyglycerol acyltransferase, has previously been reported to increase the lipid content in plants and yeasts [3,14–16]. Therefore, to increase further the lipid content in *S. cerevisiae*, we have, in the present study, overexpressed TAG-biosynthetic enzymes in the Δ*snf2* disruptant, which has the highest lipid content among the previously characterized disruptants [13]. The *snf2* mutant was initially isolated as a suppressor of sucrose fermentation [17], and it has been shown that the *SNF2* gene encodes a DNA-dependent ATPase that forms part of the SWI/SNF (switching/sucrose non-fermenting) chromatin-remodelling complex [18]. Although extensive studies have focused on transcriptional regulation by the

Abbreviations used: DGAT, diacylglycerol acyltransferase; LB, Luria–Bertani; NLSD, nitrogen-limited synthetic defined; ORF, open reading frame; SD, synthetic defined; SWI/SNF, switching/sucrose non-fermenting; TAG, triacylglycerol.

[1] To whom correspondence should be addressed (email kamisaka.y@aist.go.jp).

SNF2 gene product, little is known about the effect of *SNF2* gene expression on lipid accumulation. The results of the present study indicated that overexpression of *DGA1* encoding yeast DGAT in the *Δsnf2* disruptant induced greater lipid accumulation than its overexpression in the wild-type. In addition, leucine biosynthesis was unexpectedly found to increase lipid accumulation, especially in the *Δsnf2* disruptant. Using these conditions, we constructed a yeast strain with a lipid content of approx. 30 %, which is high enough to represent a model of oleaginous yeasts.

EXPERIMENTAL

Materials

Oleoyl-CoA, *sn*-1,2-dioleoylglycerol and Tergitol NP-40 were purchased from Sigma (St Louis, MO, U.S.A). [1-^{14}C]Oleoyl-CoA (58 mCi/mmol) was purchased from PerkinElmer Japan (Tokyo, Japan). Triton X-100 was purchased from Nacalai Tesque (Kyoto, Japan). Sodium ampicillin and LB (Luria–Bertani) medium were purchased from Wako Pure Chemical Industries (Osaka, Japan). Silica-gel 60 TLC plates were purchased from Merck (Darmstadt, Germany). Mouse anti-histidine monoclonal antibody and peroxidase-labelled sheep anti-mouse antibody were purchased from GE Healthcare UK Ltd. (Little Chalfont, Amersham, Bucks., U.K.). All other reagents were of analytical grade.

Strains and cultures

The *S. cerevisiae* BY4741 wild-type (*Mat a leu2Δ0 his3Δ1 ura3Δ0 met15Δ0*) and *Δsnf2* disruptant (*Mat a leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 SNF2::kanMX*) strains were purchased from Invitrogen (Carlsbad, CA, U.S.A.). The yeast cells were transformed by the lithium acetate method [19] using a transformation kit (Invitrogen). The yeast cells were aerobically grown at 30 °C in a synthetic medium as follows. The SD (synthetic defined) medium contained 0.17 % Bacto-yeast nitrogen base without amino acids and ammonium sulfate, 2 % (w/v) glucose, the required nutrients and 5 g/l ammonium sulfate. NLSD (nitrogen-limited SD) medium contained 0.17 % Bacto-yeast nitrogen base without amino acids and ammonium sulfate, 2 % (w/v) glucose, the required nutrients and 1 g/l ammonium sulfate. Depending on the nutrient requirement of the strain, 20 μg/ml uracil, 60 μg/ml L-leucine, 20 μg/ml L-histidine and/or 20 μg/ml L-methionine were added as described in [20]. When the yeast cells were cultured with exogenous fatty acids, 0.25 % Tergitol NP-40 was added to the medium. *Escherichia coli* JM109 competent cells were purchased from Nippon Gene (Tokyo, Japan) and cultured in LB medium containing 50 μg/ml of ampicillin.

Cloning of TAG-biosynthetic-enzyme genes in yeast expression vectors

Genes encoding TAG biosynthetic enzymes in *S. cerevisiae* such as DGAT (*DGA1*), phospholipid:diacylglycerol acyltransferase (*LRO1*) and acyl-CoA synthase (*FAA1*, *FAA2*, *FAA3*, *FAA4*) were cloned by PCR into plasmid pL1091-5 or pL1177-2. PCR amplification was carried out by using *S. cerevisiae* genomic DNA as a template with KOD plus (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The pL1091-5 vector contains the 2 μm replication origin, an *ADH1* (alcohol dehydrogenase gene) promoter and a *URA3* (orotidine 5′-phosphate decarboxylase gene) selection marker; and pL1177-2 is a *LEU2* (β-isopropylmalate dehydrogenase gene) version of pL1091-5 [21]. To construct pL1091-5/DGA1-6 × His, *DGA1* was initially cloned into pYES2/NT-C (Invitrogen) and the ORF (open reading frame) for *DGA1* with a 6 × His coding region at the 5′ end was excised as an HindIII–XbaI fragment for insertion into pL1091-5. The ORFs were then amplified with restriction sites to facilitate cloning into expression vectors. *DGA1* was amplified by using the forward primer 5′-TAGAGCTCCGCAGAGGCATA-3′ and the reverse primer 5′-AGTCTAGACAGCCCAAACAC-3′ containing SacI and XbaI sites (underlined) respectively. To construct pL1091-5/DGA1-6 × His, another forward primer, 5′-GCGAATTCATGTCAGGAACA-3′, containing an EcoRI site, was used to avoid amplification of stop codons at the 5′ end non-coding region of *DGA1* by the former primer. The other TAG genes were cloned in the same way using the following primers: *LRO1* forward, 5′-TAGGATCCATGGGCACACTGTTTC-3′, and reverse, 5′-GCTCTAGATTACATTGGGAAGGGC-3′ (BamHI and XbaI sites respectively); *FAA1* forward, 5′-GCGAGCTCATGGTTGCTCAATATACCG-3′, and reverse, 5′-ATGCGGCCGCGATCCATTAAGACGAAC-3′ (SacI and NotI sites respectively); *FAA2* forward, 5′-CGTCTAGAATGGCCGCTCCAGATTA-3′, and reverse, 5′-TAGCGGCCGCTATGGATGTGCATAGGG-3′ (XbaI and NotI sites respectively); *FAA3* forward, 5′-TAGAGCTCCAATGTCCGAACAACAC-3′, and reverse, 5′-GCTCTAGAGGGAAGGTAATAAGCTTC-3′ (SacI and XbaI sites respectively); *FAA4* forward, 5′-CGTCTAGACGCATCAAAATGACCGA-3′, and reverse, 5′-TAGCGGCCGCGTGTTTATGAAGGGCAG-3′ (XbaI and NotI sites respectively). The PCR amplification products were verified by DNA sequencing.

Microscopy

A laser scanning confocal microscope (LSM 410; Carl Zeiss, Jena, Germany) with a 63× oil-immersion plan-apochromat objective lens (numerical aperture 1.4; Zeiss) was used for the imaging of Nile Red-stained yeast cells as described elsewhere [13,22].

Lipid analysis

The direct transmethylation of all fatty acid residues, including non-esterified ('free') fatty acids and esterified fatty acids in yeast cells was conducted using methanolic 10 % (v/v) HCl and methylene chloride, and the resultant fatty acid methyl esters were applied to a gas chromatograph (GC-17-A; Shimadzu, Kyoto, Japan) equipped with a TC-70 capillary column (GL Science, Tokyo, Japan) under temperature programming (180–220 °C at 4 °C/min increments) as described elsewhere [13,23]. Total fatty acids were quantified by using heptadecanoic acid methyl esters as the internal standards. The lipid content (%) of the yeast cells was usually expressed as the total fatty acid amount (mg) per dry cell weight (mg) × 100.

The total lipids were extracted by homogenizing the yeast cells with glass beads in chloroform/methanol (2:1, v/v) as described for the total lipid extraction of fungal cells [24]. The content of each lipid class, except for free sterol, was also expressed as the fatty acid amount included in the lipid class as described in [13]. Free sterol was quantified by densitometry of the TLC spots as described in [13].

Immunoblotting

The homogenate from the yeast cells was subjected to SDS/12.5%-(w/v)-PAGE and blotted on to a PVDF membrane (Hybond-P; GE Healthcare). The membrane was blocked and incubated with a mouse anti-histidine monoclonal antibody (1:2000). After washing, the membrane was incubated with peroxidase-labelled sheep anti-mouse antibody (1:2000), and immune complexes were visualized using an enhanced-chemiluminescence kit (ECL®; GE healthcare). The image was

Table 1 Effects of vector transformation on the growth and lipid content of the BY4741 wild-type

Each strain was cultured at 30 °C in 50 ml of NLSD or SD medium, which was supplemented with required nutrients such as 20 μg/ml of histidine, 20 μg/ml of methionine, 20 μg/ml of uraci, and/or 60 μg/ml of leucine. Results are means ± S.D. ($n = 3$) for a 50 ml sample of culture. Values in parentheses represent the ratio to that in the wild-type with no vector transformation. Lipid content is expressed as total fatty acids (mg) per dry cell weight (mg) × 100.

Strain	Medium	Dry cell weight (mg)	Total fatty acids (mg)	Lipid content (%)
4-Day culture				
No vector	NLSD	27.7 ± 0.8	1.39 ± 0.12 (1.0)	5.0 ± 0.3 (1.0)
+pL1091-5	NLSD	25.0 ± 0.0	1.71 ± 0.16 (1.2)	6.8 ± 0.6 (1.4)
+pL1177-2	NLSD	55.3 ± 0.6	4.03 ± 0.14 (2.9)	7.3 ± 0.3 (1.5)
+pL1091-5, pL1177-2	NLSD	51.0 ± 2.6	4.54 ± 0.15 (3.3)	8.9 ± 0.5 (1.8)
+pL1091-5, pL1177-2	SD	54.3 ± 1.5	3.97 ± 0.06 (2.9)	7.3 ± 0.3 (1.5)
7-Day culture				
No vector	NLSD	27.2 ± 0.3	1.97 ± 0.08 (1.0)	7.2 ± 0.2 (1.0)
+pL1091-5	NLSD	26.0 ± 1.0	2.03 ± 0.14 (1.0)	7.8 ± 0.8 (1.1)
+pL1177-2	NLSD	51.0 ± 1.0	4.21 ± 0.38 (2.1)	8.2 ± 0.6 (1.1)
+pL1091-5, pL1177-2	NLSD	55.0 ± 8.7	4.74 ± 0.46 (2.4)	8.8 ± 2.0 (1.2)
+pL1091-5, pL1177-2	SD	53.0 ± 2.0	4.23 ± 0.03 (2.1)	8.0 ± 0.3 (1.1)

Table 2 Effects of vector transformation on growth and lipid content of the BY4741 *Δsnf2* disruptant

Each strain was cultured at 30 °C in 50 ml of NLSD or SD medium, which was supplemented with required nutrients such as 20 μg/ml of histidine, 20 μg/ml of methionine, 20 μg/ml of uracil, and/or 60 μg/ml of leucine. Results are means ± S.D. ($n = 3$) for a 50 ml sample of culture. Values in parentheses represent the ratio to that in the *Δsnf2* disruptant with no vector transformation. Lipid content is expressed as total fatty acids (mg) per dry cell weight (mg) × 100.

Strain	Medium	Dry cell weight (mg)	Total fatty acids (mg)	Lipid content (%)
4-Day culture				
No vector	NLSD	25.7 ± 0.8	1.41 ± 0.06 (1.0)	5.5 ± 0.4 (1.0)
+pL1091-5	NLSD	28.7 ± 0.6	1.94 ± 0.15 (1.2)	6.8 ± 0.6 (1.2)
+pL1177-2	NLSD	105.0 ± 1.7	7.55 ± 0.39 (5.4)	7.2 ± 0.3 (1.3)
+pL1091-5, pL1177-2	NLSD	105.3 ± 4.7	8.07 ± 0.50 (5.8)	7.7 ± 0.2 (1.4)
+pL1091-5, pL1177-2	SD	84.0 ± 9.8	4.31 ± 0.37 (3.1)	5.2 ± 1.1 (0.9)
7-Day culture				
No vector	NLSD	23.5 ± 0.9	2.17 ± 0.03 (1.0)	9.2 ± 0.3 (1.0)
+pL1091-5	NLSD	23.7 ± 0.6	2.16 ± 0.04 (1.0)	9.1 ± 0.2 (1.0)
+pL1177-2	NLSD	100.0 ± 1.7	12.28 ± 0.56 (5.7)	12.3 ± 0.7 (1.3)
+pL1091-5, pL1177-2	NLSD	108.7 ± 2.1	12.64 ± 2.14 (5.8)	11.6 ± 1.8 (1.3)
+pL1091-5, pL1177-2	SD	91.0 ± 10.6	5.85 ± 0.61 (2.7)	6.5 ± 0.6 (0.7)

obtained with an image analyser (LAS-1000 plus; Fuji Photo Film Co., Tokyo, Japan).

Other analytical methods

Yeast cells suspended in Tris/HCl buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA, were homogenized with a Braun (Melsungen, Germany) homogenizer together with glass beads (diameter 0.45–0.50 mm) as described for the homogenization of fungal cells [25]. The homogenized cell suspensions were then centrifuged at 1500 ***g*** for 5 min to remove unbroken cells and nuclei, and the resultant supernatant was used as the homogenate. The DGAT assay was conducted in a reaction mixture containing 10 mM phosphate buffer, pH 7.0, 150 mM KCl, 3.4 μM (0.2 μCi/ml) [1-^{14}C]oleoyl-CoA, 1 mM 1,2-dioleoylglycerol, 0.1 % Triton X-100 and appropriate amounts of the homogenate as described elsewhere [25]. Protein was measured using Coomassie Blue dye reagent (Bio-Rad Laboratories, Herculus, CA, U.S.A.) [26] in the presence of 0.05 M NaOH.

RESULTS

Effects of *LEU2* expression and addition of higher concentrations of leucine on lipid accumulation in the *Δsnf2* disruptant

We intended to investigate whether vectors expressing the TAG-biosynthetic-enzyme genes could increase lipid accumulation in the *Δsnf2* disruptant of *S. cerevisiae*. During the course of the experiment we found that transformation with pL1177-2 carrying no insert gene significantly increased dry cell weight and total fatty acids in the wild-type and *Δsnf2* disruptant (Tables 1 and 2). The increase in dry cell weight and total fatty acids caused by transformation with pL1177-2 was much greater in the *Δsnf2* disruptant than in the wild-type. In addition, the increase in total fatty acids in the *Δsnf2* disruptant was significantly affected by the culture conditions. Culture in NLSD medium caused a greater increase in total fatty acids than culture in SD medium, and culture for 7 days accumulated more lipids than culture for 4 days. Transformation with pL1091-5, which contained *URA3* as a selection marker instead of *LEU2*, did not affect dry cell

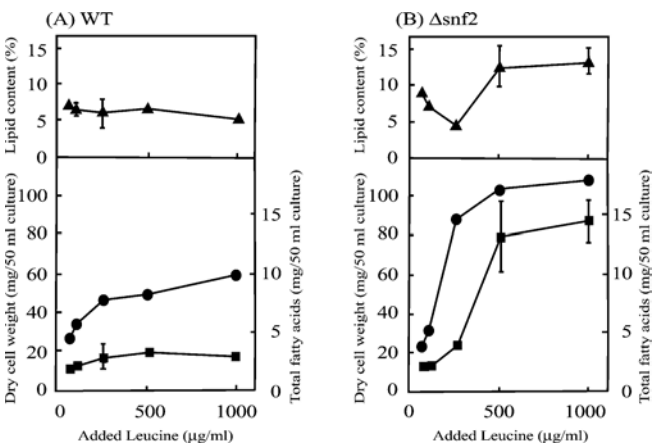

Figure 1 Effect of leucine addition on growth and lipid content of the wild-type and *Δsnf2* disruptant

The BY4741 wild-type strain (**A**) and *Δsnf2* disruptant (**B**) were cultured at 30 °C in 50 ml of NLSD medium, to which required nutrients such as 20 μg/ml of histidine, 20 μg/ml of methionine or 20 μg/ml of uracil and the indicated concentration of leucine were added. Dry cell weight (●) and total fatty acids (■) were measured as described in the Experimental section. Lipid content (▲) is expressed as total fatty acids (mg) per dry cell weight (mg) × 100. Results are means ± S.D. ($n = 3$) for a 50 ml sample of the culture.

weight or total fatty acids, indicating that expression of *LEU2* was responsible for the increase in dry cell weight and total fatty acids.

To investigate further the effect of leucine metabolism on lipid accumulation, the wild-type and *Δsnf2* disruptant were cultured in the presence of increasing concentrations of leucine. Concentrations of leucine higher than 250 μg/ml increased dry cell weight in the wild-type and *Δsnf2* disruptant (Figure 1), and this increase was basically the same as that observed for the expression of *LEU2* by pL1177-2. In addition, higher concentrations of leucine increased total fatty acids and lipid content in the *Δsnf2* disruptant, whereas the increase in total fatty acids with higher concentrations of leucine was not significant in the wild-type. The increase in total fatty acids was induced at

Table 3 Effects of the overexpression of *DGA1* or *LRO1* on the growth and lipid content of the wild-type and *Δsnf2* disruptant

BY4741 wild-type and *Δsnf2* disruptant strains were transformed with pL1091-5 and pL1177-2 and cultured at 30 °C for 7 days in 50 ml of NLSD medium containing 20 μg/ml of histidine, and 20 μg/ml of methionine. *DGA1* or *LRO1* was overexpressed from the pL1091-5 vector, together with an empty pL1177-2 vector. Results are means ± S.D. ($n = 3$) for a 50 ml sample of culture. Values in parentheses represent the ratio to that of the vector control. Lipid content is expressed as total fatty acids (mg) per dry cell weight (mg) × 100.

Strain	Dry cell weight (mg)	Total fatty acids (mg)	Lipid content (%)
Wild-type			
Vector control	55.0 ± 8.7	4.74 ± 0.46 (1.0)	8.8 ± 2.0 (1.0)
+ *DGA1*	59.0 ± 5.3	8.76 ± 0.55 (1.8)	15.0 ± 2.2 (1.7)
+ *LRO1*	51.7 ± 1.5	5.17 ± 1.60 (1.1)	10.0 ± 2.9 (1.4)
Δsnf2			
Vector control	108.7 ± 2.1	12.64 ± 2.14 (1.0)	11.6 ± 1.8 (1.0)
+ *DGA1*	112.0 ± 6.0	30.34 ± 2.32 (2.4)	27.1 ± 2.1 (2.3)
+ *LRO1*	118.3 ± 2.3	6.41 ± 0.41 (0.5)	5.4 ± 0.2 (0.5)

higher concentrations of leucine as compared with the increase in dry cell weight of the *Δsnf2* disruptant, indicating that the exogenous leucine initially acted on cell growth and then increased lipid accumulation. These results indicated that leucine biosynthesis, rather than the presence of Leu2p, was crucial for the increase in cell growth and lipid accumulation.

The addition of larger amounts of amino acids, including other required nutrients such as histidine, methionine or uracil, did not have such an effect (results not shown). The addition of 500 μg/ml phenylalanine, valine, isoleucine or tyrosine increased dry cell weight, but did not affect lipid accumulation (results not shown). These results suggest that the effects of leucine biosynthesis arise from mechanisms specific to leucine in which Snf2p is involved. On the basis of these observations, yeast transformation with pL1091-5 and pL1177-2 and yeast culture in NLSD medium for 7 days were subsequently used to investigate the effects of overexpression of the TAG-biosynthetic enzymes.

Effects of overexpression of TAG-biosynthetic enzymes on lipid accumulation in the *Δsnf2* disruptant

DGA1 or *LRO1*, both encoding enzymes crucial for TAG biosynthesis in yeast [27–29], were overexpressed to increase lipid accumulation in the wild-type and the *Δsnf2* disruptant. Table 3 shows that overexpression of *DGA1* using a multicopy vector, pL1091-5, with a strong *ADH1* promoter, had a much greater effect on total fatty acids and lipid content in the *Δsnf2* disruptant than in the wild-type. These results indicate that DGAT is rate-limiting for the biosynthesis of storage lipids, especially in the disruptant. Overexpression of *LRO1*, by contrast, rather decreased total fatty acids and lipid content in the *Δsnf2* disruptant, whereas a slight increase in total fatty acids and lipid content was observed on *LRO1* overexpression in the wild-type.

As a next step to increase lipid accumulation in the *Δsnf2* disruptant, we investigated the effect of overexpression of an acyl-CoA synthase gene, which would increase levels of one of the DGAT substrates, acyl-CoA. *FAA1*, *FAA2*, *FAA3*, and *FAA4* encode acyl-CoA synthases with different substrate specificities [30] and Faa1p and Faa4p are involved in the fatty-acid-transport system [31]. It is not known, however, which gene products are involved in lipid biosynthesis, unlike those in the oleaginous yeast *Candida lipolytica* [32]. We found that overexpression of *FAA1*, *FAA2*, or *FAA4* together with *DGA1* decreased total fatty acids and lipid content in both wild-type and the *Δsnf2* disruptant,

Table 4 Effects of overexpression of *DGA1* and a gene encoding acyl-CoA synthase on growth and lipid content of the wild-type and *Δsnf2* disruptant

The BY4741 wild-type or *Δsnf2* disruptant strain transformed with pL1091-5 and pL1177-2 was cultured at 30 °C for 7 days in 50 ml of NLSD medium containing 20 μg/ml of histidine and 20 μg/ml of methionine. *DGA1* was overexpressed from the pL1091-5 vector. *FAA1*, *FAA2*, *FAA3* or *FAA4* was overexpressed from the pL1177-2 vector. Results are means ± S.D. ($n = 3$) for a 50 ml sample of culture. Values in parentheses represent the ratio to that in the wild-type or *Δsnf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2. Lipid content is expressed as total fatty acids (mg) per dry cell weight (mg) × 100.

Strain	Dry cell weight (mg)	Total fatty acids (mg)	Lipid content (%)
Wild-type			
+ *DGA1*, pL1177-2	59.0 ± 5.3	8.76 ± 0.55 (1.0)	15.0 ± 2.2 (1.0)
+ *DGA1*, *FAA1*	56.7 ± 0.6	4.21 ± 0.91 (0.5)	7.4 ± 1.7 (0.5)
+ *DGA1*, *FAA2*	54.7 ± 0.6	4.11 ± 0.36 (0.5)	7.5 ± 0.7 (0.5)
+ *DGA1*, *FAA3*	56.0 ± 1.0	9.95 ± 1.00 (1.1)	17.8 ± 2.0 (1.2)
+ *DGA1*, *FAA4*	58.0 ± 1.0	3.84 ± 0.20 (0.4)	6.6 ± 0.2 (0.4)
Δsnf2			
+ *DGA1*, pL1177-2	112.0 ± 6.0	30.34 ± 2.32 (1.0)	27.1 ± 2.1 (1.0)
+ *DGA1*, *FAA1*	116.0 ± 3.5	23.28 ± 0.09 (0.8)	20.1 ± 0.6 (0.7)
+ *DGA1*, *FAA2*	119.3 ± 2.3	13.26 ± 0.72 (0.4)	11.1 ± 0.8 (0.4)
+ *DGA1*, *FAA3*	119.3 ± 2.3	29.12 ± 0.58 (1.0)	24.4 ± 0.6 (0.9)
+ *DGA1*, *FAA4*	124.0 ± 2.0	14.85 ± 0.10 (0.5)	12.0 ± 0.1 (0.4)

whereas overexpression of *FAA3* together with *DGA1* increased or maintained the lipid content (Table 4). On the basis of these results, it seems most likely that Faa3p is involved in lipid biosynthesis, but Faa1p, Faa2p and Faa4p are not. The limited effect of *FAA3* overexpression on total fatty acid and lipid content suggests that the supply of acyl-CoA is not rate-limiting for TAG biosynthesis, especially in the *Δsnf2* disruptant.

To confirm that the increase in growth and total fatty acids in the *Δsnf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2/FAA3 was due to the transformed vectors, we obtained colonies from the transformed strain that had subsequently lost these vectors. Loss of pL1177-2/FAA3 decreased the dry cell weight, but a high lipid content was kept, whereas loss of pL1091-5/DGA1 kept a high dry cell weight but resulted in a decreased lipid content (Table 5). Loss of both vectors altered the dry cell weight and lipid content to the levels seen in the *Δsnf2* disruptant. These results confirmed that the increase in growth was due to *LEU2* expression, and the increase in lipid content was due to *DGA1* overexpression.

Characterization of the *Δsnf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2/FAA3

Because the *Δsnf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2/FAA3 was much more oleaginous than the wild-type, as judged by total fatty acid content, we conducted a more detailed characterization of this strain. On the basis of the total extracted lipids, including sterols, the lipid content reached nearly 30 % after the 7-day culture (Table 6). The composition of the extracted lipids indicated that TAG accounted for a major lipid class (Table 7), apparently due to overexpression of *DGA1*. Different sizes of lipid bodies were observed in the strain, but large lipid bodies with a diameter of approx. 2 μm, which were not observed in the untransformed *Δsnf2* disruptant [13], were detected (Figure 2).

In addition to the accumulation of endogenous lipids, the *Δsnf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2/FAA3 had the capacity to incorporate exogenous fatty acids into storage lipids (Table 8). The strain accumulated almost four times

Table 5 Effects of vector loss from the *Δsnf2* disruptant transformed with *DGA1* and *FAA3* overexpression vectors on the growth and lipid content

The BY4741 *Δsnf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2/FAA3 was plated on YPD (yeast extract/ peptone/dextrose) agar medium containing 10 g/l yeast extract, 20 g/l polypeptone, 20 g/l glucose and 20 g/l Bacto Agar®. The procedure was repeated twice, and the colonies obtained were plated on SD agar medium with different nutrients to determine their nutrient requirement. Colonies requiring uracil and/or leucine were regarded as those with the loss of pL1091-5/DGA1 and/or pL1177-2/FAA3. These strains were cultured at 30 °C for 7 days in 50 ml of NLSD medium containing 20 μg/ml histidine, and 20 μg/ml methionine. When required, 20 μg/ml uracil and/or 60 μg/ml leucine was added to the medium. The vectors that were lost are underlined. Results are means ± S.D. ($n = 3$) for a 50 ml sample of culture. Values in parentheses represent the ratio to that in the *Δsnf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2/FAA3. Lipid content is expressed as total fatty acids (mg) per dry cell weight (mg) × 100.

Strain	Dry cell weight (mg)	Total fatty acids (mg)	Lipid content (%)
+pL1091-5/DGA1, pL1177-2/FAA3	119.3 ± 2.3	29.12 ± 0.58 (1.00)	24.4 ± 0.6 (1.00)
+pL1091-5/DGA1, pL1177-2/FAA3	26.0 ± 0.0	6.25 ± 0.13 (0.21)	24.0 ± 0.5 (0.98)
+pL1091-5/DGA1, pL1177-2/FAA3	98.3 ± 4.2	10.41 ± 4.30 (0.36)	10.7 ± 4.7 (0.44)
+pL1091-5/DGA1, pL1177-2/FAA3	22.0 ± 0.9	1.89 ± 0.02 (0.06)	8.6 ± 0.3 (0.35)

Table 6 Growth and total lipid content of the *Δsnf2* disruptant transformed with *DGA1* and *FAA3* overexpression vectors

The BY4741 *Δsnf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2/FAA3 was cultured at 30 °C for 4 or 7 days in 250 ml of NLSD medium containing 20 μg/ml histidine and 20 μg/ml methionine. Lipids were extracted as described in the Experimental section. Results are means ± S.D. ($n = 3$) for a 250 ml sample of culture. Lipid content is expressed as total extracted lipids(mg) per dry cell weight (mg) × 100.

Culture time (days)	Dry cell weight (mg)	Total fatty acids (mg)	Total extracted lipids (mg)	Lipid content (%)
4	574 ± 14	98 ± 5	113 ± 12	19.7 ± 1.7
7	563 ± 48	132 ± 12	167 ± 13	29.8 ± 3.5

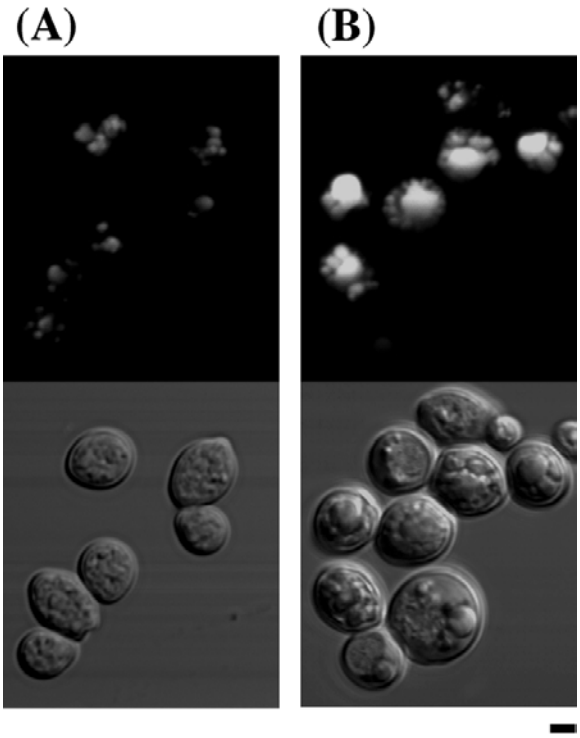

Figure 2 Nile Red staining of the *Δsnf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2/FAA3

The BY4741 *Δsnf2* disruptant (**A**) and *Δsnf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2/FAA3 (**B**) cultured in NLSD medium for 7 days were stained with Nile Red. The upper panels show a fluorescent image; lower panels show a differential-interference-contrast image. The bar represents 2 μm.

more fatty acids in the presence of exogenous fatty acids than did the wild-type. Because its dry cell weight was also significantly increased, however, its percentage lipid content was not changed from that of the wild-type. Incorporation of exogenous fatty acids was estimated by linoleic acid incorporation, because linoleic acid is not synthesized in *S. cerevisiae*. Incorporation of linoleic acid was significantly increased in the *Δsnf2* disruptant with *DGA1* overexpression, suggesting that the *Δsnf2* disruption coupled with *DGA1* overexpression caused efficient incorporation of exogenous fatty acids into storage lipids. Additional overexpression of *FAA3* further increased linoleic acid incorporation, suggesting that Faa3p is also involved in the incorporation of exogenous fatty acids into storage lipids. In addition to oleic acid and linoleic acid, we investigated the incorporation of α-linolenic acid into each strain, which was almost the same as that for linoleic acid (results not shown).

To confirm that *DGA1* overexpression induced an increase in DGAT activity, we measured DGAT activity in the homogenate of each strain (Table 9). It was evident that DGAT activity in the homogenate of the *Δsnf2* disruptants transformed with pL1091-5/DGA1 was significantly increased, a finding that may explain the greater lipid accumulation in these strains. DGAT activity, however, remained very low in the homogenate of the wild-type with *DGA1* overexpression. In addition, the homogenate of *Δsnf2*

Table 7 Major lipid classes in the *Δsnf2* disruptant transformed with *DGA1* and *FAA3* overexpression vectors

The BY4741 *Δsnf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2/FAA3 was cultured at 30 °C for 4 or 7 days in 250 ml of NLSD medium containing 20 μg/ml of histidine, and 20 μg/ml of methionine. Lipids were extracted as described in the Experimental section. Major lipid classes were separated by TLC on silica-gel 60. Each lipid class except for free sterol is expressed as the amount of fatty acid present. Values in parentheses represent the percentage of total fatty acids. Free sterol was quantified by the densitometry of TLC spots as described in the Experimental section. Results are means ± S.D. ($n = 3$) for a 250 ml sample of culture.

	Amount (mg)					
Culture time (days)	TAG	Non-esterified fatty acids	Polar lipids	Diacylglycerol	Sterol ester	Non-esterified sterol
4	82.0 ± 5.7 (83.6 %)	0.72 ± 0.16 (0.7 %)	2.14 ± 0.38 (2.2 %)	1.67 ± 0.22 (1.7 %)	1.87 ± 0.33 (1.9 %)	4.43 ± 0.78 (–)
7	101.3 ± 10.5 (76.8 %)	2.82 ± 0.80 (2.1 %)	3.44 ± 0.46 (2.6 %)	1.76 ± 0.27 (1.3 %)	1.88 ± 0.21 (1.4 %)	6.92 ± 0.20 (–)

Table 8 Growth and lipid content of BY4741 yeast transformed with *DGA1* and/or *FAA3* overexpression vectors cultured in the presence of exogenous fatty acids

Each strain was cultured at 30 °C for 7 days in 50 ml of NLSD medium containing the required nutrients, 0.25 % Tergitol NP-40 and 2 g/l exogenous fatty acid, which was suspended in 200 μl of ethanol. When required, 20 μg/ml histidine, 20 μg/ml methionine, 20 μg/ml uracil and 60 μg/ml leucine were included in the medium. Incorporation of linoleic acid was calculated by dividing the amount of linoleic acid incorporated by the amount of linoleic acid added. Results are means ± S.D. ($n = 3$) for a 50 ml sample of culture. Values in parentheses represent the ratio to that in the wild-type (WT). Lipid content is expressed as total fatty acids (mg) per dry cell weight (mg) × 100.

Strain	Dry cell weight (mg)	Incorporation of linoleic acid (%)	Total fatty acids (mg)	Lipid content (%)
Plus 100 mg of oleic acid				
WT	46.3 ± 1.5	–	20.4 ± 2.5 (1.0)	44.1 ± 6.1 (1.0)
Δsnf2	39.3 ± 1.5	–	21.5 ± 0.4 (1.1)	54.7 ± 1.1 (1.2)
WT (pL1091-5/DGA1, pL1177-2/FAA3)	95.6 ± 5.4	–	49.9 ± 1.6 (2.4)	52.3 ± 3.9 (1.2)
Δsnf2 (pL1091-5/DGA1, pL1177-2)	154.4 ± 10.2	–	73.0 ± 2.8 (3.6)	47.4 ± 2.8 (1.1)
Δsnf2 (pL1091-5/DGA1, pL1177-2/FAA3)	182.7 ± 9.0	–	82.6 ± 6.4 (4.0)	45.2 ± 1.6 (1.0)
Plus 100 mg of linoleic acid				
WT	55.3 ± 3.2	26.5 ± 0.9	31.2 ± 1.1 (1.0)	56.4 ± 1.6 (1.0)
Δsnf2	48.3 ± 1.5	24.4 ± 2.2	28.6 ± 2.2 (0.9)	59.2 ± 3.6 (1.0)
WT (pL1091-5/DGA1, pL1177-2/FAA3)	106.7 ± 5.0	24.1 ± 0.2	37.8 ± 0.5 (1.2)	35.4 ± 1.3 (0.6)
Δsnf2 (pL1091-5/DGA1, pL1177-2)	177.8 ± 5.1	51.7 ± 3.6	78.3 ± 5.5 (2.5)	44.0 ± 3.0 (0.8)
Δsnf2 (pL1091-5/DGA1, pL1177-2/FAA3)	215.3 ± 7.0	78.3 ± 2.0	113.4 ± 2.7 (3.6)	52.7 ± 0.7 (0.9)

Table 9 DGAT activity in the homogenate of BY4741 yeast with the overexpression of *DGA1* and/or *FAA3*

The homogenate from each strain, each of which was cultured at 30 °C for 4 or 7 days in 50 ml of NLSD medium containing required nutrients such as 20 μg/ml histidine, 20 μg/ml methionine, 20 μg/ml uracil and 60 μg/ml leucine, was obtained as described in the Experimental section. The DGAT activity in the homogenate (3–10 μg of protein) of each strain was assayed as described in the Experimental section. Results are means ± S.D. ($n = 3$).

Strain	Homogenate...	DGAT activity (pmol/min per mg of protein) 4 days	7 days
WT		3 ± 1	1 ± 0
Δsnf2		6 ± 0	2 ± 0
WT (pL1091-5, pL1177-2)		1 ± 1	1 ± 0
Δsnf2 (pL1091-5, pL1177-2)		21 ± 8	16 ± 1
WT (pL1091-5/DGA1, pL1177-2)		1 ± 0	1 ± 1
Δsnf2 (pL1091-5/DGA1, pL1177-2)		569 ± 12	755 ± 134
WT (pL1091-5/DGA1, pL1177-2/FAA3)		1 ± 0	1 ± 1
Δsnf2 (pL1091-5/DGA1, pL1177-2/FAA3)		705 ± 151	676 ± 147

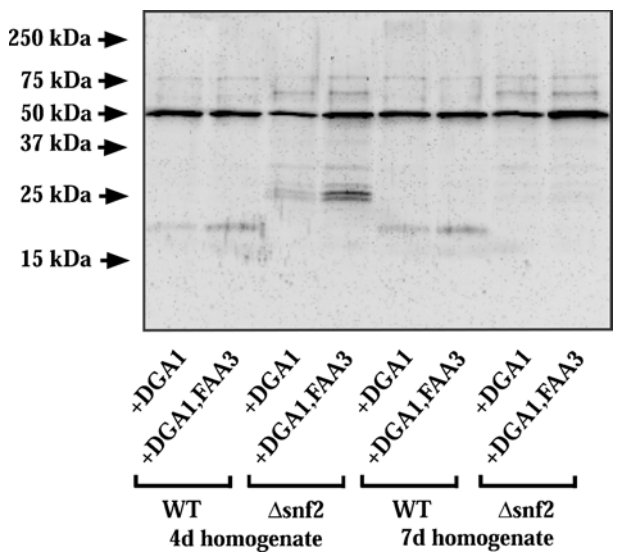

Figure 3 Immunoblotting of Dga1p in the homogenate of yeast transformed with pL1091-5/DGA1-6 × His

The cell homogenate was prepared from the wild-type (WT) and *Δsnf2* disruptant transformed with pL1091-5/DGA1-6 × His and pL1177-2 (or pL1177-2/FAA3) that had been cultured for 4 (4d) or 7 days (7d). Each fraction (2 μg of protein) was subjected to SDS/12.5%-(w/v)-PAGE under reducing conditions, followed by immunoblotting with anti-hexahistidine monoclonal antibody as detailed in the Experimental section. Arrows indicate molecular-mass standards (Bio-Rad). DGAT activity in the homogenate of the *Δsnf2* disruptant transformed with pL1091-5/DGA1-6xHis and pL1177-2 was 799 (4 days) or 354 (7 day) pmol/min per mg protein, whereas DGAT activity in the homogenate of the *Δsnf2* disruptant transformed with pL1091-5/DGA1-6 × His and pL1177-2/FAA3 was 936 (4 days) or 388 (7 days) pmol/min per mg of protein. DGAT activity in the homogenate of the wild-type transformed with pL1091-5/DGA1-6xHis and pL1177-2 (or pL1177-2/FAA3) was less than 2 pmol/min per mg of protein.

disruptants transformed with empty vectors had significantly higher DGAT activity than that of the wild-type, suggesting that DGAT was also activated by expression of *LEU2* in the *Δsnf2* disruptant. These results suggest that there are mechanisms to regulate the increase in DGAT activity, which may be abrogated in the *Δsnf2* disruptant.

To determine whether the increase in DGAT activity in the *Δsnf2* disruptants with *DGA1* overexpression was due to increased levels of Dga1p, we expressed Dga1p with a hexahistidine (6 × His) tag by using pL1091-5/DGA1-6 × His for transformation of the *Δsnf2* disruptant. The DGAT activity in the homogenate of the *Δsnf2* disruptant transformed with pL1091-5/DGA1-6 × His was as high as that in the *Δsnf2* disruptant transformed with pL1091-5/DGA1, whereas the DGAT activity remained very low in the homogenate of the wild-type. These results indicated that the attachment of hexahistidine did not affect the differential increase in DGAT activity between the *Δsnf2* disruptant and the wild-type. Immunoblotting with anti-hexahistidine revealed that the homogenate of the wild-type and *Δsnf2* disruptant transformed with pL1091-5/DGA1-6 × His had similar amounts of Dga1p with a molecular mass of 50 kDa (Figure 3). The differential in the amount of Dga1p was too low to explain the observed increase in DGAT activity, indicating that the increase in DGAT activity in the *Δsnf2* disruptant with *DGA1* overexpression was not due to greater amounts of Dga1p. Instead, Dga1p may be activated by some modification of Dga1p or by cofactors that interact with Dga1p.

DISCUSSION

Although the *SNF2* gene, which encodes a subunit of the SWI/SNF chromatin-remodelling complex [18], has been studied extensively from the aspect of transcriptional regulation, there have been very limited studies on its involvement in lipid metabolism. Kodaki et al. [33] previously reported that *SNF2* is involved in the regulation of phospholipid synthesis enzymes that are repressed by inositol and choline in *S. cerevisiae*. Considering this with our previous observation that the *Δsnf2* disruptant has a higher TAG content [13], we assumed that *SNF2* might regulate the biosynthesis of phospholipids and storage lipids such as TAG. The present study further demonstrated that overexpression of *DGA1*, a gene encoding one of the TAG-biosynthetic enzymes, significantly increased total fatty acids and lipid content in the *Δsnf2* disruptant. Overexpression of *DGA1* in the *Δsnf2* disruptant significantly increased DGAT activity, which may contribute to the increase in lipid accumulation. Furthermore, we revealed that the higher DGAT activity in the *Δsnf2* disruptant with *DGA1* overexpression was not due to larger amounts of Dga1p. The observation suggests that DGAT is activated by post-translational modifications, as suggested previously [34,35]. Alternatively, DGAT may be activated by other factors such as an increase in activator(s) or a decrease in inhibitor(s). Further studies using *DGA1* overexpression in the *Δsnf2* disruptant will address how the *Δsnf2* disruption induces such a high increase in DGAT activity, which is not observed in the wild-type, and may reveal regulatory factors of DGAT.

The present study demonstrates that a non-oleaginous *S. cerevisiae* strain with 5–7 % lipid content can be changed to an oleaginous yeast with approx. 30 % lipid content by genetic modification. Although overexpression of a DGAT gene has previously been reported to increase the content of storage lipids [14–16], such a high lipid content as presently obtained (30 %) had not been achieved. The *Δsnf2* disruption together with specific culture conditions, such as leucine biosynthesis and nitrogen limitation, helped to increase lipid accumulation due to *DGA1* overexpression.

Overexpression of *LRO1*, a gene encoding another important TAG-biosynthetic enzyme, somewhat decreased storage lipid content in the *Δsnf2* disruptant, but slightly increased storage lipids in the wild-type. Because the *Δsnf2* disruption is known to markedly decrease the expression of genes encoding the phospholipid-biosynthetic enzymes regulated by inositol and choline [33], the phospholipid substrates of Lro1p may not be sufficient in the *Δsnf2* disruptant. Alternatively, Lro1p may catalyse the reverse reaction to hydrolyse TAG in the *Δsnf2* disruptant. Transacylation reactions of TAG have been observed in plants [36,37], and Lro1p may function to compensate for the decrease in phospholipids in the *Δsnf2* disruptant [13].

Two types of acyl-CoA synthase have been reported on the basis of work in *C. lipolytica*: one is involved in the synthesis of cellular lipids and the other is involved in the degradation of fatty acids [32]. Although it is known that *S. cerevisiae* has four acyl-CoA synthase genes whose gene products have different substrate specificities [30], the physiological roles of these genes are not well understood. Among the four acyl-CoA synthase genes, overexpression of *FAA2* or *FAA4* significantly decreased lipid accumulation in the wild-type and *Δsnf2* disruptant, and *FAA1* overexpression decreased lipid accumulation, especially in the wild-type. The results are consistent with previous observations that Faa1p and Faa4p are involved in the import of exogenous fatty acids and their degradation [31]. Overexpression of *FAA3* together with *DGA1* significantly increased lipid accumulation in the wild-type and slightly decreased lipid accumulation in the *Δsnf2* disruptant. In addition, overexpression of *FAA3* increased lipid accumulation in the presence of exogenous fatty acids in the *Δsnf2* disruptant. These results suggest that Faa3p plays the most important role in the biosynthesis of storage lipids from endogenous and exogenous fatty acids among the four different acyl-CoA synthases, although the possibility that Faa3p is also involved in degradation of the fatty acids cannot be excluded.

The present study revealed that expression of *LEU2* enhanced growth and lipid accumulation, especially in the *Δsnf2* disruptant. The addition of larger amounts of leucine induced similar effects on growth and lipid accumulation, indicating that leucine biosynthesis due to *LEU2* expression was important in this effect rather than Leu2p itself. Because the addition of larger amounts of other amino acids did not have such an effect, we assume that leucine biosynthesis has a specific role in lipid accumulation in the *Δsnf2* disruptant. It has been reported that leucine affects protein synthesis and autophagic proteolysis via the cell-growth-regulating kinase TOR (target of rapamycin) [38], which might also be involved in the effect of leucine on lipid accumulation. Although the nutritional regulation of leucine biosynthesis has been studied extensively [39], its relationship to the synthesis of storage lipids has not been elucidated in yeast.

In conclusion, we found that factors such as *DGA1* overexpression and leucine biosynthesis increased lipid accumulation especially in the *Δsnf2* disruptant of *S. cerevisiae*. The total lipid content in the *Δsnf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2/FAA3 reached approx. 30 %; as a result, this strain can be used as a model of oleaginous yeasts. Studies using these strains may provide new insights into the mechanisms of lipid accumulation. In addition, these yeasts can be useful host organisms for the production of valuable lipids [40]. Further studies on genes involved in other glycerolipid syntheses [1,2], lipid degradation [41], nutrient signalling [42,43] and energy-production systems [8,9,44], in concert with the *Δsnf2* disruption, may reveal other factors that regulate storage lipid biosynthesis.

We thank Professor Kohei Hosaka (Department of Basic Sciences for Medicine, Gunma University School of Health Sciences, Maebashi, Japan) for his critical review of this manuscript before its submission.

REFERENCES

1 Daum, G., Lees, N. D., Bard, M. and Dickson, R. (1998) Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. Yeast **14**, 1471–1510
2 Carman, G. M. and Henry, S. A. (1999) Phospholipid biosynthesis in the yeast *Saccharomyces cerevisiae* and interrelationship with other metabolic processes. Prog. Lipid Res. **38**, 361–399
3 Dahlqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H. and Stymne, S. (2000) Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc. Natl. Acad. Sci. U.S.A. **97**, 6487–6492
4 Oelkers, P., Tinkelenberg, A., Erdeniz, N., Cromley, D., Billheimer, J. T. and Sturley, S. L. (2000) A lecithin cholesterol acyltransferase-like gene mediates diacylglycerol esterification in yeast. J. Biol. Chem. **275**, 15609–15612
5 Lardizabal, K. D., Mai, J. T., Wagner, N. W., Wyrick, A., Voelker, T. and Hawkins, D. J. (2001) DGAT2 is a new diacylglycerol acyltransferase gene family. J. Biol. Chem. **276**, 38862–38869
6 Han, G.-S., Wu, W.-I. and Carman, G. M. (2006) The *Saccharomyces cerevisiae* lipin homolog is a Mg^{2+}-dependent phosphatidate phosphatase enzyme. J. Biol. Chem. **281**, 9210–9218
7 Zweytick, D., Athenstaedt, K. and Daum, G. (2000) Intracellular lipid particles of eukaryotic cells. Biochim. Biophys. Acta **1469**, 101–120
8 Ratledge, C. (1989) Biotechnology of oils and fats. In Microbial Lipids (Ratledge, C. and Wilkinson, S. G., eds.), vol. 2, pp. 567–668, Academic Press, London
9 Ratledge, C. and Wynn, J. P. (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv. Appl. Microbiol. **51**, 1–51

10 Tijburg, L. B. M., Geelen, M. J. H. and and van Golde, L. M. G. (1989) Regulation of the biosynthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine in the liver. Biochim. Biophys. Acta **1004**, 1–19
11 Lehner, R. and Kuksis, A. (1996) Biosynthesis of triacylglycerols. Prog. Lipid Res. **35**, 169–201
12 Murphy, D. J. (2001) The biogenesis and functions of lipid bodies in animals, plants and microorganisms. Prog. Lipid Res. **40**, 325–438
13 Kamisaka, Y., Noda, N., Tomita, N., Kimura, K., Kodaki, T. and Hosaka, K. (2006) Identification of genes affecting lipid content using transposon mutagenesis in *Saccharomyces cerevisiae*. Biosci. Biotechnol. Biochem. **70**, 646–653
14 Hobbs, D. H., Lu, C. and Hills, M. J. (1999) Cloning of a cDNA encoding diacylglycerol acyltransferase from *Arabidopsis thaliana* and its functional expression. FEBS Lett. **452**, 145–149
15 Bouvier-Nave, P., Benveniste, P., Oelkers, P., Sturley, S. L. and Schaller, H. (2000) Expression in yeast and tobacco of plant cDNAs encoding acyl-CoA:diacylglycerol acyltransferase. Eur. J. Biochem. **267**, 85–96
16 Jako, C., Kumar, A., Wei, Y., Zou, Y., Barton, D. L., Giblin, E. M., Covello, P. S. and Taylor, D. C. (2001) Seed-specific over-expression of an *Arabidopsis* cDNA encoding a diacylglycerol acyltransferase enhances seed oil content and seed weight. Plant Physiol. **126**, 861–874
17 Neigeborn, L. and Carlson, M. (1984) Genes affecting the regulation of *SUC2* gene expression by glucose repression in *Saccharomyces cerevisiae*. Genetics **108**, 845–858
18 Peterson, C. L. and Tamkun, J. W. (1995) The SWI–SNF complex: a chromatin remodeling machine? Trends Biochem. Sci. **20**, 143–146
19 Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**, 163–168
20 Sherman, F., Fink, G. R. and Hicks, J. B. (1986) Methods in Yeast Genetics. pp. 163–167, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21 Kainou, K., Kamisaka, Y., Kimura, K. and Uemura, H. (2006) Isolation of Δ^{12} and ω3-fatty acid desaturase genes from the yeast *Kluyveromyces lactis* and their heterologous expression to produce linoleic and α-linolenic acids in *Saccharomyces cerevisiae*. Yeast **23**, 605–612
22 Kamisaka, Y., Noda, N., Sakai, T. and Kawasaki, K. (1999) Lipid bodies and lipid body formation in an oleaginous fungus, *Mortierella ramanniana* var. *angulispora*. Biochim. Biophys. Acta **1438**, 185–198
23 Kimura, K., Yamaoka, M. and Kamisaka, Y. (2004) Rapid estimation of lipids in oleaginous fungi and yeasts using Nile Red fluorescence. J. Microbiol. Methods **56**, 331–338
24 Kamisaka, Y., Kikutsugi, H., Yokochi, T., Nakahara, T. and Suzuki, O. (1988) Studies on production of lipids in fungi. XX. Effect of metal ions in cultural media and temperature on fungal lipids. J. Jpn. Oil Chem. Soc. **37**, 344–348
25 Kamisaka, Y., Yokochi, T., Nakahara, T. and Suzuki, O. (1993) Characterization of the diacylglycerol acyltransferase activity in the membrane fraction from a fungus. Lipids **28**, 583–587
26 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254
27 Sorger, D. and Daum, G. (2002) Synthesis of triacylglycerol by the acyl-coenzyme A:diacylglycerol acyltransferase Dga1p in lipid particles of the yeast *Saccharomyces cerevisiae*. J. Bacteriol. **184**, 519–524
28 Sandager, L., Gustavsson, M. H., Stahl, U., Dahlqvist, A., Wiberg, E., Banas, A., Lenman, M., Ronne, H. and Stymne, S. (2002) Storage lipid synthesis is non-essential in yeast. J. Biol. Chem. **277**, 6478–6482
29 Oelkers, P., Cromley, D., Padamsee, M., Billheimer, J. T. and Sturley, S. L. (2002) The DGA1 gene determines a second triglyceride synthetic pathway in yeast. J. Biol. Chem. **277**, 8877–8881
30 Knoll, L. J., Johnson, D. J. and Gordon,, J. L. (1994) Biochemical studies of three *Saccharomyces cerevisiae* acyl-CoA synthases, FAA1p, FAA2p, and FAA3p. J. Biol. Chem. **269**, 16348–16356
31 Faergeman, N. J., Black, P. N., Zhao, X. D., Knudsen, J. and DiRusso, C. C. (2001) The acyl-CoA synthetases encoded within *FAA1* and *FAA4* in *Saccharomyces cerevisiae* function as components of the fatty acid transport system linking import, activation, and intracellular utilization. J. Biol. Chem. **276**, 37051–37059
32 Kamiryo, T., Nishikawa, Y., Mishina, M., Terao, M. and Numa, S. (1979) Involvement of long-chain acyl coenzyme A for lipid synthesis in repression of acetyl-coenzyme A carboxylase in *Candida lipolytica*. Proc. Natl. Acad. Sci. U.S.A. **76**, 4390–4394
33 Kodaki, T., Hosaka, K., Nikawa, J. and Yamashita, S. (1995) The *SNF2/SWI2/GAM1/TYE3/RIC1* gene is involved in the coordinate regulation of phospholipid synthesis in *Saccharomyces cerevisiae*. J. Biochem. (Tokyo) **117**, 362–368
34 Coleman, R. A. and Lee, D. P. (2004) Enzymes of triacylglycerol synthesis and their regulation. Prog. Lipid Res. **43**, 134–176
35 Lung, S.-C. and Weselake, R. J. (2006) Diacylglycerol acyltransferase: a key mediator of plant triacylglycerol synthesis. Lipids **41**, 1073–1088
36 Mancha, M. and Stymne, S. (1997) Remodeling of triacylglycerols in microsomal preparations from developing castor bean (*Ricinus communis* L.) endosperm. Planta **203**, 51–57
37 Stobart, K., Mancha, M., Lenman, M., Dahlqvist, A. and Stymne, S. (1997) Triacylglycerols are synthesized and utilized by transacylation reactions in microsomal preparations of developing safflower (*Carthamus tinctorius* L.) seeds. Planta **203**, 58–66
38 Meijer, A. J. and Dubbelhuis, P. F. (2004) Amino acid signalling and the integration of metabolism. Biochem. Biophys. Res. Commun. **313**, 397–403
39 Kohlhaw, G. B. (2003) Leucine biosynthesis in fungi: entering metabolism through the back door. Microbiol. Mol. Biol. Rev. **67**, 1–15
40 Veen, M. and Lang, C. (2004) Production of lipid compounds in the yeast *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **63**, 635–646
41 Wagner, A. and Daum, G. (2005) Formation and mobilization of neutral lipids in the yeast *Saccharomyces cerevisiae*. Biochem. Soc. Trans. **33**, 1174–1177
42 Wilson, W. A. and Roach, P. J. (2002) Nutrient-regulated protein kinases in budding yeast. Cell **111**, 155–158
43 Kamisaka, Y., Noda, N. and Yamaoka, M. (2004) Appearance of smaller lipid bodies and protein kinase activation in the lipid body fraction are induced by an increase in the nitrogen source in the *Mortierella* fungus. J. Biochem. (Tokyo) **135**, 269–276
44 Wynn, J. P., Hamid, A. A., Li, Y. and Ratledge, C. (2001) Biochemical events leading to the diversion of carbon into storage lipids in the oleaginous fungi *Mucor circinelloides* and *Mortierella alpina*. Microbiology **147**, 2857–2864

Received 2 April 2007/27 July 2007; accepted 10 August 2007
Published as BJ Immediate Publication 10 August 2007, doi:10.1042/BJ20070449