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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Modulation of gluconeogenesis and lipid production in an engineered oleaginous *Saccharomyces cerevisiae* transformant

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Abstract We previously created an oleaginous *Saccharomyces cerevisiae* transformant as a *dga1* mutant overexpressing Dga1p lacking 29 amino acids at the N-terminal (Dga1ΔNp). Because we have already shown that *dga1* disruption decreases the expression of *ESA1*, which encodes histone acetyltransferase, the present study was aimed at exploring how Esa1p was involved in lipid accumulation. We based our work on the previous observation that Esa1p acetylates and activates phosphoenolpyruvate carboxykinase (PEPCK) encoded by *PCK1*, a rate-limiting enzyme in gluconeogenesis, and subsequently evaluated the activation of Pck1p by yeast growth with non-fermentable carbon sources, thus dependent on gluconeogenesis. This assay revealed that the Δ*dga1* mutant overexpressing Dga1ΔNp had much lower growth in a glycerol-lactate (GL) medium than the wild-type strain overexpressing Dga1ΔNp. Moreover, overexpression of Esa1p or Pck1p in mutants improved the growth, indicating that the Δ*dga1* mutant overexpressing Dga1ΔNp had lower activities of Pck1p and gluconeogenesis due to lower expression of *ESA1*. In vitro PEPCK assay showed the same trend in the culture of the Δ*dga1* mutant overexpressing Dga1ΔNp with 10 % glucose medium, indicating that Pck1p-mediated gluconeogenesis decreased in this oleaginous transformant under the lipid-accumulating conditions introduced by the glucose medium. The growth of the Δ*dga1* mutant overexpressing Dga1ΔNp in the GL medium was also improved by overexpression of acetyl-CoA synthetase, Acs1p or Acs2p, indicating that supply of acetyl-CoA was crucial for Pck1p acetylation by Esa1p. In addition, the Δ*dga1* mutant without Dga1ΔNp also showed better growth in the GL medium, indicating that decreased lipid accumulation was enhancing Pck1p-mediated gluconeogenesis. Finally, we found that overexpression of Ole1p, a fatty acid Δ9-desaturase, in the Δ*dga1* mutant overexpressing Dga1ΔNp improved its growth in the GL medium. Although the exact mechanisms leading to the effects of Ole1p were not clearly defined, changes of palmitoleic and oleic acid contents appeared to be critical. This observation was supported by experiments using exogenous palmitoleic and oleic acids or overexpression of elongases. Our findings provide new insights on lipid accumulation mechanisms and metabolic engineering approaches for lipid production.

Keywords Acetyl-CoA · *ESA1* · *OLE1* · Palmitoleic acid · *PCK1* · *Saccharomyces cerevisiae*

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Introduction

Microbial oils are new sources of sustainable oils as alternatives to fossil fuels or variety of nutraceuticals (Kosa and Ragauskas 2011; Beopoulos et al. 2011; Uemura 2012; Gong et al. 2014; Sitepu et al. 2014). Recent advances in metabolic engineering of oil production in microbes allow us to design oils more promptly in order to meet demands in terms of quantity and quality (Keasling 2010; Tai and Stephanopoulos 2013; Pfleger et al. 2015). In order to fully and efficiently use the potential of metabolic engineering, it is

essential to elucidate the mechanisms that allow microbes to accumulate oils in response to nutrient conditions. Although numerous biosynthetic enzymes involved in oil production have been identified and utilized for engineering purposes, regulatory processes involved in oil production are not sufficiently understood.

In oleaginous fungi and yeast, ATP citrate lyase and malic enzyme were described as being active enough to supply excess levels of cytoplasmic acetyl-CoA and NADPH for active oil production (Ratledge and Wynn 2002). This indicates that the regulation of the primary metabolism, involving processses such as glycolysis and citrate cycle, plays an important role in oil production. Currently, the increment of cytoplasmic acetyl-CoA is a major target for metabolic engineering of oil production as well as other biotechnological products (Krivoruchko et al. 2015; Sheng and Feng 2015). Supply of NADPH has also been investigated regarding the improvement of fatty acid production yields, due to its role as a cofactor (Ratledge 2014; Wasylenko et al. 2015). The production of these compounds by the primary metabolism is tightly regulated to ensure proper lipid homeostasis for the organisms. Therefore, these regulatory mechanisms need to be understood to enable their use toward microbial oil production engineering, a process that, by definition, is driven against the natural lipid homeostasis behavior of the organisms.

Yeast *Saccharomyces cerevisiae* is extensively studied as a model organism for basic and applied studies (Sherman 2002; Rajakumari et al. 2008; Henry et al. 2012; Nielsen et al. 2013). Oil production capability of this yeast has been improved by metabolic engineering of various processes such as fatty acid synthesis (Runguphan and Keasling 2014; Li et al. 2014), triacylglycerol (TAG) production (Kamisaka et al. 2007; Kamisaka et al. 2013; Greer et al. 2015), β-oxidation (Valle-Rodriguez et al. 2014), glycerol assimilation (Yu et al. 2013), acyl-CoA metabolisms (Chen et al. 2014; Thompson and Trinh 2014; Leber et al. 2015), and ethanol metabolisms (de Jong et al. 2014). Diacylglycerol acyltransferase (DGAT) encoded by *DGA1* in this yeast is one of the important targets for oil production. Overexpression of this enzyme has been found to be more effective in increasing lipid content when combined with *snf2* (Kamisaka et al. 2007) or *dga1* disruption (Kamisaka et al. 2013). The overexpression combined with *dga1* disruption generated a transformant with a high lipid content of about 45 %, while association with *dga1* disruption affected expression of *ESA1* encoding histone acetyltransferase, which we found to be involved in lipid accumulation (Kamisaka et al. 2013). The present study was aimed at exploring how Esa1p is involved in lipid accumulation in the generated oleaginous transformant, providing a valuable insight for oil production engineering in this yeast. While investigating the growth of the transformant in non-fermentable carbon sources, we found that the Δ*dga1* oleaginous transformant had repressed gluconeogenesis due to decreased activity of phosphoenolpyruvate carboxykinase (PEPCK) encoded by *PCK1*, which catalyzes the rate-limiting step in gluconeogenesis via regulation by Esa1p (Lin et al. 2009). Further studies revealed that the growth of the Δ*dga1* oleaginous transformant in non-fermentable carbon sources was dependent on cytoplasmic acetyl-CoA levels, and influenced by its fatty acid profiles, in particular its content of palmitoleic acid (POA) and oleic acid (OA).

Materials and methods

Materials

Heptadecanoic acid methylester, and POA were purchased from Sigma (St. Louis, MO, USA). Sodium ampicillin, LB medium, ADP, NADH, and potassium phosphoenolpyruvate were purchased from Wako Pure Chemical Industries (Osaka, Japan). OA was purchased from Nippon Oils & Fats Ltd. (Tokyo, Japan). Yeast malate dehydrogenase was purchased from Oriental Yeast Co Ltd. (Tokyo, Japan). All other reagents were of analytical grade.

Strains and cultures

S. cerevisiae BY4741 wild-type strain (*Mat a leu2Δ0 his3Δ1 ura3Δ0 met15Δ0*) and the disruptants of this genetic background (the Δ*dga1* mutant (*dga1*::*kanMX4*), Δ*pck1* mutant (*pck1*::*kanMX4*)) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The yeast cells were transformed by the lithium acetate method (Ito et al. 1983) using a transformation kit (Invitrogen Life Technologies). Plasmid pL1091–5 and pL1177–2 with *URA3* and *LEU2* respective selection markers, incorporating a constitutive *ADH1* promoter of *S. cerevisiae*, were used for transformation (Kainou et al. 2006). The transformed yeast cells were aerobically grown at 30 °C for the indicated time in (i) nitrogen-limited SD medium containing 0.17 % Bacto-yeast nitrogen base without amino acids and ammonium sulfate, 10 % glucose, 5 g/l ammonium sulfate, 20 μg/ml histidine, and 20 μg/ml methionine or (ii) glycerol-lactate (GL) medium containing 0.17 % Bacto-yeast nitrogen base without amino acids and ammonium sulfate, 2 % glycerol, 2 % sodium lactate, 5 g/l ammonium sulfate, 20 μg/ml histidine, and 20 μg/ml methionine.

Escherichia coli JM109 competent cells were purchased from Nippon Gene (Tokyo, Japan) and cultured in the LB medium containing 50 μg/ml ampicillin.

Plasmid constructions

pL1091–5/DGA1ΔN (*DGA1ΔN* encodes Dga1p with N-terminal 29 amino acids deleted) and pL1177–2/ESA1 were

constructed as described previously (Kamisaka et al. 2013), and pL1177–2/OLE1 was constructed as described (Kamisaka et al. 2015). Rat fatty acid elongase 1 and 2 genes (*rELO1*, *rELO2*) (kindly provided by Tsunehiro Aki, Hiroshima University) (Inagaki et al. 2002) were subcloned into the *Hin*dIII/*Xba*I site of pL1177–2. *PCK1*, *ACS1*, and *ACS2* were amplified with a high-fidelity DNA polymerase (KOD plus; Toyobo, Osaka, Japan) using *S. cerevisiae* genomic DNA as a template. The forward primer of *PCK1* was 5′-GCAAGCTTATGTCCCCTTCTAAAATG-3′ containing a *Hin*dIII site (underlined) and the reverse primer was 5′-ATGAGCTCTTACTCGAATTGAGGACC-3′ containing a *Sac*I site (underlined). The forward primer of *ACS1* was 5′-GCAAGCTTATGTCGCCCTCTGCCGTA-3′ containing a *Hin*dIII site (underlined) and the reverse primer was 5′-ATGCGGCCGCATCATTACAACTTGACCG-3′ containing a *Not*I site (underlined). The forward primer of *ACS2* was 5′-GCAAGCTTATGACAATCAAGGAACAT-3′ containing a *Hin*dIII site (underlined) and the reverse primer was 5′-ATGCGGCCGCGAAAACAGAAAAGGAGCG-3′ containing a *Not*I site (underlined). These amplified products were excised as *Hin*dIII-*Sac*I or *Hin*dIII-*Not*I fragments and used to construct pL1177–2/PCK1, pL1177–2/ACS1, and pL1177–2/ACS2, respectively. Correctness of all PCR amplification products was verified by DNA sequencing.

Lipid analysis

The direct transmethylation of all fatty acid residues in yeast cells was carried out using methanolic 10 % (*v*/*v*) HCl and methylene chloride, and the resultant fatty acid methyl esters were loaded into a gas chromatograph as described previously (Kamisaka et al. 2007). Total fatty acids were quantified by using heptadecanoic acid methyl ester as the internal standard. The lipid content (%) of the yeast cells was expressed as the total fatty acid amount (mg) per dry cell weight (mg) × 100.

Other analytical methods

Yeast cells were homogenized with a Braun homogenizer (Melsungen, Germany) as described previously (Kamisaka et al. 2007) and the homogenized cell suspensions were centrifuged at 1500×*g* for 5 min to remove unbroken cells and nuclei. The resultant supernatant (homogenate) was then centrifuged at 100,000×*g* for 1 h. The obtained supernatant served as the soluble fraction. Protein was measured by using Coomassie blue dye reagent (Bio-Rad Laboratories) (Bradford 1976) in the presence of 0.05 M NaOH. PEPCK activity was assayed spectrophotometrically at 340 nm as described previously (Lin et al. 2009). The reaction mixture (500 μl) contained 80 mM Tris-HCl (pH 8.8), 2 mM $MnCl_2$, 10 mM potassium phosphoenolpyruvate, 10 mM $NaHCO_3$, 12 mM sodium aspartate, 1 mM ADP, 0.15 mM NADH, 14 units malate dehydrogenase, and the soluble fraction (10 μg protein). NADH consumption rate related to malate dehydrogenase reduction of oxaloacetate, produced from phosphoenolpyruvate by PEPCK, was monitored at 340 nm.

Results

Effects of ESA1 or PCK1 overexpression on the growth and lipid profiles of the Δdga1 oleaginous transformant

Previously, we found that the Δ*dga1* mutant overexpressing Dga1ΔNp, which showed high lipid content, had lower expression of *ESA1*, which is crucial for lipid accumulation in this transformant (Kamisaka et al. 2013). To verify that the acetylation by Esa1p was modulated in the Δ*dga1* mutant, we based our work on a previous observation wherein Esa1p acetylated and activated PEPCK encoded by *PCK1*, the rate-limiting enzyme in *S. cerevisiae* gluconeogenesis (Lin et al. 2009). The authors evaluated the activation of Pck1p by yeast growth with non-fermentable carbon sources, thus dependent on gluconeogenesis. Therefore, we tested whether the Δ*dga1* mutant overexpressing Dga1ΔNp could grow in the 2 % GL medium, containing non-fermentable carbon sources. The growth of the Δ*dga1* mutant overexpressing Dga1ΔNp in the GL medium as determined by the dry cell weight was lower than that of the wild-type strain overexpressing Dga1ΔNp. However, overexpression of *ESA1* or *PCK1* in the Δ*dga1* mutant suppressed this decreasing effect (Fig. 1a), indicating that the Δ*dga1* mutant overexpressing Dga1ΔNp had diminished activities of Pck1p and gluconeogenesis due to reduced expression of *ESA1*. We confirmed that the Δ*pck1* mutant overexpressing Dga1ΔNp did not grow in the GL medium, and that overexpression of *PCK1*, but not *ESA1*, could induce a recovery of the growth (Fig. 1a). The growth of these transformants monitored by OD600 (Fig. 1b) was consistent with the dry cell weight data. The Δ*dga1* mutant overexpressing Dga1ΔNp showed a delayed growth pattern compared with the wild-type control, with lower overall growth rate and maximum cell number. Additionally, the effects of Esa1p and Pck1p on the growth delay and those of Pck1p on the maximum cell number were partial. The fatty acid composition of the Δ*dga1* mutant overexpressing Dga1ΔNp cultured in the GL medium showed higher POA content than for the wild-type control. Furthermore, overexpression of *ESA1* or *PCK1* in the Δ*dga1* mutant lead to a decrease in POA content along with an increased OA content (Table 1), similarly to those cultured in the 10 % glucose medium (Kamisaka et al. 2013, some data are presented in Table 2).

We then cultured these transformants in the 10 % glucose medium and investigated whether the activation of Pck1p was consequently also hampered. For that purpose, we measured in vitro PEPCK activity in the soluble fraction of these

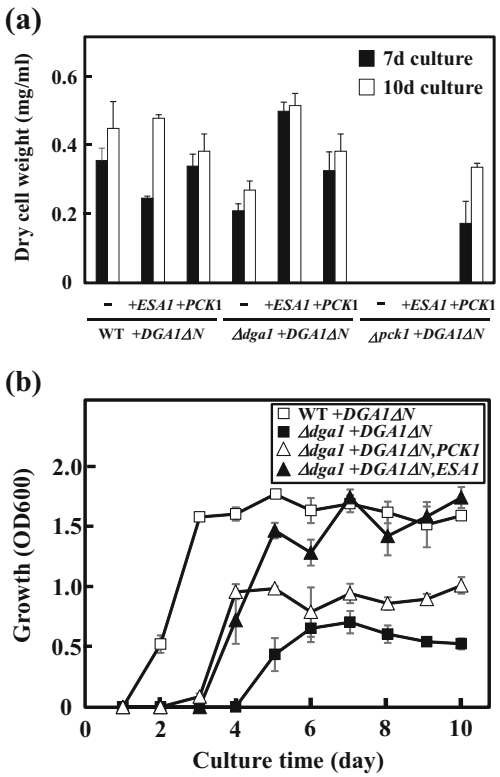

Fig. 1 Effects of Esa1p and Pck1p overexpression on the growth of the BY4741 transformants in the GL medium. **a** The BY4741 transformants with or without overexpression of Esa1p or Pck1p were cultured at 30 °C for 7 or 10 days in 50 ml 2 % GL medium. **b** The growth of these transformants was spectrophotometrically monitored at OD600. Results are shown as the mean ± SD of three different cultures

transformants and found that it was greatly reduced in the Δ*dga1* mutant overexpressing Dga1ΔNp compared with the wild-type control. Moreover, overexpression of *ESA1* or *PCK1* in the Δ*dga1* mutant suppressed this reduction effect (Fig. 2). The results suggested that the Δ*dga1* mutant overexpressing Dga1ΔNp had lower activities of Pck1p and gluconeogenesis due to reduced expression of *ESA1* in the glucose culture as in the culture of the GL medium. The Δ*pck1* mutant overexpressing Dga1ΔNp, however, had low, but significant, PEPCK activity, indicating the existence of PEPCK activity underived from Pck1p.

Because Pck1p activity and gluconeogenesis were lower in the Δ*dga1* mutant overexpressing Dga1ΔN, we tested whether the Δ*pck1* mutant overexpressing Dga1ΔNp would have an increased lipid accumulation in the 10 % glucose medium (Table 2). The results depicted that Δ*pck1* mutant overexpressing Dga1ΔNp showed the same lipid content as the wild-type control, indicating that a complete blockage of gluconeogenesis itself did not trigger lipid accumulation. Nevertheless, overexpression of *PCK1* in the Δ*dga1* mutant overexpressing Dga1ΔNp induced a decrease in lipid accumulation, similar to the one obtained for *ESA1* (Table 2), confirming that Pck1p-mediated gluconeogenesis affected lipid accumulation in the Δ*dga1* mutant overexpressing Dga1ΔNp. In addition, overexpression of *PCK1* in the Δ*dga1* mutant overexpressing Dga1ΔNp generated a drop in POA content and an enhanced OA content, as also observed for *ESA1*, resulting in the same fatty acid composition as in the wild-type control (Table 2). These results confirmed that the effect of *ESA1* in the Δ*dga1* mutant was mediated by the activation of Pck1p probably via its acetylation.

Effects of acetyl-CoA supply modulation on the growth and lipid profiles of the Δdga1 oleaginous transformant

We investigated whether the supply of acetyl-CoA toward the acetylation of Pck1p by Esa1p would affect the growth and lipid profiles of the Δ*dga1* oleaginous transformant. It had been previously reported that levels of cytoplasmic acetyl-CoA influenced protein acetylation for cell growth (Cai et al. 2011), and that acetyl-CoA for nucleocytosolic protein acetylation was derived from acetyl-CoA synthetase encoded by *ACS1* and *ACS2* in *S. cerevisiae* (Takahashi et al. 2006). Therefore, we tested whether the overexpression of *ACS1* or *ACS2* in the Δ*dga1* mutant overexpressing Dga1ΔNp affected

Table 1 Lipid content and fatty acid composition of the BY4741 transformants cultured in 2 % GL medium

Strains	Lipid content (%)	Fatty acid composition (%)				
		16:0	16:1	18:0	18:1	Others
WT + *DGA1ΔN*	13.8 ± 0.2	26.1 ± 1.1	40.8 ± 1.7	5.7 ± 0.5	22.2 ± 2.0	5.1 ± 0.5
Δ*dga1* + *DGA1ΔN*	15.8 ± 1.5	24.0 ± 0.4	47.9 ± 1.4	2.2 ± 0.2	20.7 ± 2.0	5.1 ± 0.2
Δ*dga1* + *DGA1ΔN*, *ESA1*	11.5 ± 0.3	21.8 ± 0.3	40.1 ± 0.4	6.7 ± 0.3	27.8 ± 0.6	3.6 ± 0.4
Δ*dga1* + *DGA1ΔN*, *PCK1*	15.1 ± 1.2	20.4 ± 0.9	39.3 ± 0.8	7.4 ± 0.1	30.1 ± 2.0	2.6 ± 0.7
Δ*dga1* + *DGA1ΔN*, *ACS1*	9.4 ± 0.6	16.1 ± 0.2	32.8 ± 0.4	9.8 ± 0.2	34.5 ± 0.2	6.8 ± 0.7
Δ*dga1* + *DGA1ΔN*, *ACS2*	12.3 ± 0.5	18.4 ± 0.4	38.9 ± 0.3	9.2 ± 0.3	27.9 ± 0.5	5.6 ± 0.8
Δ*dga1* -*DGA1ΔN*	2.7 ± 0.4	16.5 ± 1.9	48.3 ± 2.1	6.9 ± 1.5	26.6 ± 1.4	1.6 ± 2.8

The BY4741 transformants were cultured at 30 °C for 7 days in 50 ml 2 % GL medium. Results are shown as the means ± SD ($n = 3$) of three different cultures

Table 2 Growth and lipid content of the BY4741 transformants cultured in 10 % glucose medium

Strains	Dry cell weight (mg/ml)	Total fatty acids (mg/ml)		Lipid content (%)	
WT + *DGA1ΔN*	2.17 ± 0.19	0.65 ± 0.05	(1.0)	30.0 ± 1.1	(1.0)
Δ*dga1* + *DGA1ΔN*	2.22 ± 0.10	1.07 ± 0.05	(1.6)	48.3 ± 4.0	(1.6)
Δ*dga1* + *DGA1ΔN*, *ESA1*	2.23 ± 0.06	0.62 ± 0.01	(1.0)	28.0 ± 0.9	(0.9)
Δ*dga1* + *DGA1ΔN*, *PCK1*	2.03 ± 0.10	0.62 ± 0.02	(1.0)	30.3 ± 2.1	(1.0)
Δ*dga1* + *DGA1ΔN*, *ACS1*	1.85 ± 0.05	0.46 ± 0.01	(0.7)	24.7 ± 0.4	(0.8)
Δ*dga1* + *DGA1ΔN*, *ACS2*	2.02 ± 0.08	0.66 ± 0.01	(1.0)	32.9 ± 1.3	(1.1)
Δ*dga1* -*DGA1ΔN*	1.83 ± 0.03	0.26 ± 0.01	(0.4)	13.9 ± 0.5	(0.5)
Δ*pck1* + *DGA1ΔN*	2.07 ± 0.03	0.62 ± 0.01	(1.0)	30.0 ± 0.5	(1.0)
Δ*pck1* + *DGA1ΔN*, *ESA1*	2.20 ± 0.13	0.55 ± 0.02	(0.8)	25.0 ± 1.8	(0.8)
Δ*pck1* + *DGA1ΔN*, *PCK1*	2.07 ± 0.15	0.61 ± 0.03	(0.9)	29.6 ± 2.1	(1.0)
Δ*pck1* + *DGA1ΔN*, *ACS1*	1.73 ± 0.03	0.43 ± 0.01	(0.7)	24.7 ± 0.9	(0.8)
Δ*pck1* + *DGA1ΔN*, *ACS2*	1.97 ± 0.03	0.59 ± 0.01	(0.9)	30.0 ± 1.0	(1.0)
Δ*pck1* -*DGA1ΔN*	1.82 ± 0.08	0.19 ± 0.01	(0.3)	10.4 ± 0.8	(0.3)

The BY4741 transformants were cultured at 30 °C for 7 days in 50 ml 10 % glucose SD medium. Values in parentheses represent the ratio to that in the wild-type strain overexpressing Dga1ΔNp. Results are shown as the means ± SD (n = 3) of three different cultures

its growth in the GL medium. As a result, we observed that overexpression of *ACS1* or *ACS2* greatly improved the growth (Fig. 3), suggesting that increased supply of acetyl-CoA by Acs1p or Acs2p stimulated the acetylation of Pck1p by Esa1p. Overexpression of *ACS1* or *ACS2* also modulated the fatty acid composition with decreased POA content and increased OA content (Table 1). On the other hand, overexpression of *ACS1* or *ACS2* in the Δ*pck1* overexpressing Dga1ΔNp did not improve growth in the GL medium, indicating that Pck1p was required to enable the effects produced by Acs1p and Acs2p.

The culture of these transformants overexpressing *ACS1* or *ACS2* in the 10 % glucose medium revealed an attenuated lipid content for the Δ*dga1* mutant (Table 2), similar to those obtained when overexpressing *ESA1* or *PCK1*. Overexpression of *ACS1* yielded a more dramatic decrease in lipid content, which may result from functional differences between *ACS1* and *ACS2* (Van den Berg et al. 1996). These results suggest that the improvement of gluconeogenesis in the Δ*dga1* mutant was associated with a drop in lipid content as seen when overexpressing *ESA1* or *PCK1*. Overexpression of *ACS1* or *ACS2* in the Δ*pck1* overexpressing Dga1ΔNp did not affect the lipid content to the same extent even though the lipid content of the transformant overexpressing *ACS1* reached similar levels as for the Δ*dga1* mutant overexpressing *ACS1*. These results may relate to the observations describing how overexpression of *ACS1* or *ACS2* in the Δ*pck1* mutant did not affect the growth in the GL medium.

Cytoplasmic acetyl-CoA pools are also modulated by fatty acid production, during which acetyl-CoA carboxylase consumes acetyl-CoA for malonyl-CoA synthesis (Galdieri et al. 2014). Overexpression of Dga1ΔNp in the Δ*dga1* mutant increased lipid accumulation, where acetyl-CoA is supposed to be actively utilized for de novo fatty acid synthesis. Although

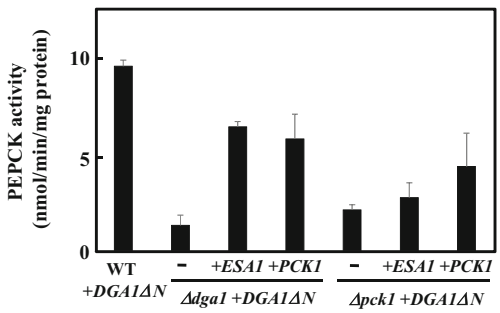

Fig. 2 PEPCK activity in the soluble fraction of the BY4741 transformants overexpressing Esa1p or Pck1p cultured in the glucose medium. The BY4741 transformants with or without overexpression of Esa1p or Pck1p were cultured at 30 °C for 7 days in 50 ml 10 % glucose SD medium. Homogenization of yeasts, preparation of the soluble fraction, and PEPCK assay are described in the "Materials and methods" section. Results are shown as the means ± SD of three different biological samples

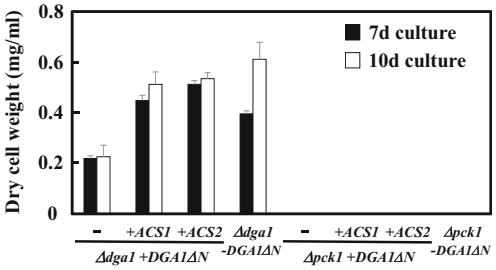

Fig. 3 Effects of Acs1p and Acs2p overexpression on the growth of the BY4741 transformants in the GL medium. The BY4741 transformants with or without overexpression of Acs1p or Acs2p or without overexpression of Dga1ΔNp were cultured at 30 °C for 7 or 10 days in 50 ml 2 % GL medium. Results are shown as the mean ± SD of three different cultures

the levels of lipid accumulation were much lower in the 2 % GL medium than in the 10 % glucose medium, overexpression of Dga1ΔNp also induced an increase in lipid content in the 2 % GL medium (Table 1). We subsequently tested whether lipid accumulation due to overexpression of Dga1ΔNp would affect the growth in the GL medium. As a result, we discovered that the Δ*dga1* mutant without overexpression of Dga1ΔNp grew better than the overexpressing one (Fig. 3), suggesting that the decrease in fatty acid production in the Δ*dga1* mutant without overexpression of Dga1ΔNp (Table 1) results in enhanced acetyl-CoA levels and consequently, in an improved gluconeogenesis. On the other hand, the Δ*pck1* mutant without overexpression of Dga1ΔNp did not grow in the GL medium, suggesting that the increased acetyl-CoA supply related to fatty acid production decrease was used for Pck1p acetylation with regard to improving gluconeogenesis.

Effects of POA and OA contents variations on the growth and lipid profiles of the Δdga1 oleaginous transformant

To explore other factors involved in lipid accumulation of the Δ*dga1* mutant overexpressing Dga1ΔNp, we further evaluated the growth of Δ*dga1* transformants in the GL medium. We found that overexpression of *OLE1*, encoding a fatty acid Δ9-desaturase, greatly contributed in improving the growth (Fig. 4). Furthermore, overexpression of *OLE1* in the Δ*pck1* mutant overexpressing Dga1ΔNp resulted in partially restoring its growing ability. In fact, this transformant did not grow for 7 days, but then started, indicating that the effects of Ole1p were not likely to be dependent on Pck1p. Although Ole1p produces both POA and OA, overexpression of Ole1p only yielded an increased OA content, while the POA content dropped (Table 3). This effect might be due to the dramatic decrease in palmitic acid content. This trend was also observed when overexpressing Ole1p in the wild-type strain overexpressing Dga1ΔNp. It should also be noted that, in the GL culture, POA content was higher in the Δ*dga1* mutant overexpressing Dga1ΔNp than in the wild-type control, a characteristic which has also been observed previously in the glucose culture (Kamisaka et al. 2013). Considering that this change in fatty acid composition was also observed when overexpressing *ACS1* and *ACS2* (Table 1), we assume that the content of POA and OA may affect growth in the GL medium.

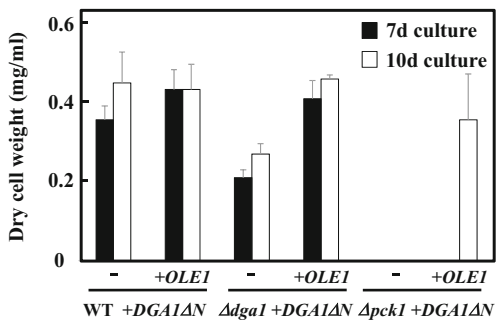

Fig. 4 Effects of Ole1p overexpression on the growth of the BY4741 transformants in the GL medium. The BY4741 transformants with or without overexpression of Ole1p were cultured at 30 °C for 7 or 10 days in 50 ml 2 % GL medium. Results are shown as the mean ± SD of three different cultures

To determine whether this variation in fatty acid composition induced an improvement of the growth in the GL medium, we investigated the effects of exogenously added POA and OA on the Δ*dga1* mutant overexpressing Dga1ΔNp in the GL medium (Fig. 5). When the contents of POA or OA in yeast cells were increased through this treatment (Table 4), the growth was differently affected. Growth rates and maximum cell numbers were improved by both POA and OA, while the delay in growth initiation was rather enhanced by POA, but not by OA. These results suggest that exogenous unsaturated fatty acids generally improved the growth in the GL medium, but that POA had a specific negative effect on the initiation of growth. The variation between POA and OA supported our assumption that an increase in OA content associated with a decrease in POA content is preferable for the growth in the GL medium. These results also suggest that the contents of POA and OA are critical in the early stage of the growth. Nevertheless, the addition of OA, which induced a stronger increase in intracellular OA content than overexpression of Ole1p itself, only partly generated the effects of Ole1p, suggesting that other factors may play a role in reaching the characteristics obtained with Ole1p.

Alternatively, we modulated the fatty acid composition of the Δ*dga1* mutant overexpressing Dga1ΔNp by overexpression of a fatty acid elongase. Rat fatty acid elongases 1 and 2 (rElo1p and rElo2p) have been reported to have different substrate specificities; overexpression of rElo1p in *S. cerevisiae* increased *cis*-vaccenic acid (C18:1(n-7)) from POA, while with rElo2p, an increase in OA concomitant with a decrease in C16 fatty acids was observed (Inagaki et al. 2002; Yazawa et al. 2011). Overexpression of *rELO1* or *rELO2* in the Δ*dga1* mutant overexpressing Dga1ΔNp, increasing *cis*-vaccenic acid or OA, respectively (Table 4), caused different effects on the growth in the GL medium (Fig. 5). While the rElo1p overexpression did not affected the growth, the rElo2p overexpression diminished the growth initiation delay. This indicates that the increase in OA content is crucial for the growth contrary to n-7 fatty acids. Nevertheless, the dry cell weight and OD600 of the transformant overexpressing rElo2p did not reach the levels of the one overexpressing Ole1p, indicating that rElo2p only partly reproduces the effects of Ole1p. It was interesting to note the difference between the effects produced by either exogenous OA or rElo2p, even if they both similarly provoked an increase in OA content. This difference might be due to the fact that rElo2p consumed malonyl-CoA to enable

Table 3 Effects of Ole1p overexpression on lipid content and fatty acid composition of the BY4741 transformants cultured in 2 % GL medium

Strains	Lipid content (%)	Fatty acid composition (%)				
		16:0	16:1	18:0	18:1	Others
WT + *DGA1ΔN*	13.8 ± 0.2	26.1 ± 1.1	40.8 ± 1.7	5.7 ± 0.5	22.2 ± 2.0	5.1 ± 0.5
WT + *DGA1ΔN*, *OLE1*	14.9 ± 0.8	5.8 ± 0.2	37.3 ± 2.2	5.1 ± 0.3	49.1 ± 1.9	2.8 ± 0.3
Δ*dga1* + *DGA1ΔN*	15.8 ± 1.5	24.0 ± 0.4	47.9 ± 1.4	2.2 ± 0.2	20.7 ± 2.0	5.1 ± 0.2
Δ*dga1* + *DGA1ΔN*, *OLE1*	13.8 ± 0.7	8.2 ± 0.4	40.7 ± 0.2	5.3 ± 0.1	43.0 ± 0.3	2.8 ± 0.1
Δ*pck1* + *DGA1ΔN*, *OLE1* (10 days)	9.5 ± 0.8	10.0 ± 0.6	38.0 ± 4.1	4.9 ± 1.0	41.8 ± 2.3	5.3 ± 3.6

The BY4741 transformants except for the Δ*pck1* overexpressing Dga1ΔNp and Ole1p were cultured at 30 °C for 7 days in 50 ml 2 % GL medium. Because the Δ*pck1* overexpressing Dga1ΔNp and Ole1p did not grow at 7 days, but grew at 10 days, the data at 10 days were presented. Results are shown as the means ± SD (n = 3) of three different cultures

the elongase reaction, decreasing acetyl-CoA concentrations down to levels which were insufficient to activate Pck1p and gluconeogenesis.

We have already shown using transformants with three plasmids including a plasmid with a *HIS3* selection marker (pL2137–26) that overexpression of *OLE1* in the Δ*dga1* mutant overexpressing Dga1ΔNp yielded a decrease in lipid content down to the same level as the wild-type control when cultured in the 10 % glucose medium, while that in the wild-type strain overexpressing Dga1ΔNp did not vary its lipid content (Kamisaka et al. 2015). Similar results were obtained using the transformants without pL2137–26 (data not shown), suggesting that the overexpression of *OLE1* in the Δ*dga1* mutant influenced gluconeogenesis and lipid production in the same way as that of *ESA1*, *PCK1*, *ACS1*, or *ACS2*. Overexpression of *rELO1* or *rELO2* in the Δ*dga1* mutant overexpressing Dga1ΔNp generated lipid contents of 40.0 ± 0.4 % (n = 3) or 36.3 ± 1.9 % (n = 3), respectively, when these transformants were cultured for 7 days in the 10 % glucose medium. The overexpression of rElo1p did not significantly impact the lipid content, while overexpressing rElo2p induces a decrease in lipid content, but not to the level observed with the wild-type control (Table 2).

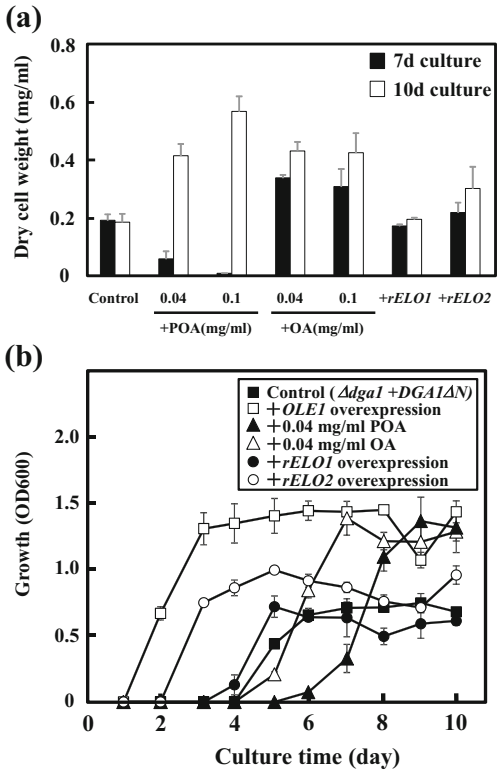

Fig. 5 Effects of fatty acid profile variations on the growth of the BY4741 transformants in the GL medium. **a** The BY4741 Δ*dga1* overexpressing Dga1ΔNp was cultured in the presence of OA or POA at 30 °C for 7 or 10 days in 50 ml 2 % GL medium. The BY4741 Δ*dga1* overexpressing Dga1ΔNp and rElo1p or Dga1ΔNp and rElo2p were cultured at 30 °C for 7 or 10 days in 50 ml 2 % GL medium. **b** The growth of these transformants was monitored spectrophotometrically at OD600. Results are shown as the mean ± SD of three different cultures

Discussion

We previously found that *ESA1* expression decreased in the Δ*dga1* mutant overexpressing Dga1ΔNp and that overexpression of *ESA1* in this mutant yielded a lower lipid content, suggesting that Esa1p is involved in the oleagenicity of the transformant (Kamisaka et al. 2013). The present study revealed that the acetylation of Pck1p by Esa1p modulated gluconeogenesis and lipid production in the oleaginous transformant (Fig. 6). Blockage of gluconeogenesis by disruption of *PCK1*, however, did not induce lipid accumulation, suggesting that factors other than gluconeogenesis were also required for this process. For example, histone acetylation by Esa1p, which causes various effects on gene regulation (Sapountzi and C té 2011), might be involved in lipid accumulation mechanisms.

The effects of Esa1p and Pck1p on gluconeogenesis and lipid production were consistent with the effects of Acs1p and Acs2p, indicating the importance of acetyl-CoA supply for

Table 4 Lipid content and fatty acid composition of the BY4741 Δ*dga1* transformants cultured in 2 % GL medium

Strains	Lipid content (%)	Fatty acid composition (%)					
		16:0	16:1	18:0	18:1	18:1 (n-7)	Others
Control	16.0 ± 1.0	19.7 ± 0.8	45.7 ± 3.5	4.8 ± 0.5	24.4 ± 4.0	0.0 ± 0.0	5.4 ± 0.5
+0.04 mg/ml POA	13.3 ± 0.4	23.1 ± 1.4	51.5 ± 5.1	6.6 ± 0.5	14.6 ± 1.7	0.0 ± 0.0	4.2 ± 1.4
+0.1 mg/ml POA	16.9 ± 1.0	18.3 ± 0.2	61.8 ± 1.0	8.6 ± 0.5	9.9 ± 0.8	0.0 ± 0.0	1.4 ± 0.4
+0.04 mg/ml OA	13.1 ± 1.1	20.8 ± 0.6	25.5 ± 1.6	3.6 ± 0.2	47.0 ± 3.6	0.0 ± 0.0	3.0 ± 2.6
+0.1 mg/ml OA	18.4 ± 2.4	15.6 ± 1.0	8.3 ± 2.4	3.6 ± 0.2	70.0 ± 3.6	0.0 ± 0.0	2.6 ± 0.2
+rElo1p overexpression	12.7 ± 1.1	15.5 ± 0.5	26.6 ± 0.5	6.0 ± 0.2	27.7 ± 1.8	20.0 ± 1.2	4.2 ± 2.6
+rElo2p overexpression	12.7 ± 2.1	7.0 ± 0.4	17.0 ± 1.4	11.6 ± 1.0	59.9 ± 1.4	0.0 ± 0.0	4.5 ± 0.8

The BY4741 Δ*dga1* overexpressing Dga1ΔNp was cultured in the presence of OA or POA at 30 °C for 10 days in 50 ml 2 % GL medium. The BY4741 Δ*dga1* overexpressing Dga1ΔNp and rElo1p or Dga1ΔNp and rElo2p were cultured at 30 °C for 10 days in 50 ml 2 % GL medium. Because yeast did not grow at 7 days under some conditions, data at 10 days were presented. Results are shown as the means ± SD (n = 3) of three different cultures

gluconeogenesis in the Δ*dga1* mutant overexpressing Dga1ΔNp (Fig. 6). Acetyl-CoA supply is also regulated by acetyl-CoA carboxylase, which utilizes acetyl-CoA to produce malonyl-CoA (Galdieri and Vancura 2012). We found that low lipid accumulation in transformants without overexpression of Dga1ΔNp improved gluconeogenesis, which we assumed occurred because acetyl-CoA was less actively utilized by acetyl-CoA carboxylase for fatty acid production. It appeared that overexpression of Dga1ΔNp generated TAG vigorously retrieving long-chain fatty acyl-CoAs, which may alleviate inhibition of acetyl-CoA carboxylase by long-chain fatty acyl-CoAs (Kamiryo et al. 1976; Neess et al. 2015). Acetyl-CoA carboxylase may also play a crucial role in modulating the effects of exogenous OA, which increased lipid accumulation while improving gluconeogenesis, considering the previous observations describing how exogenously added fatty acids could generate long-chain acyl-CoAs, directly

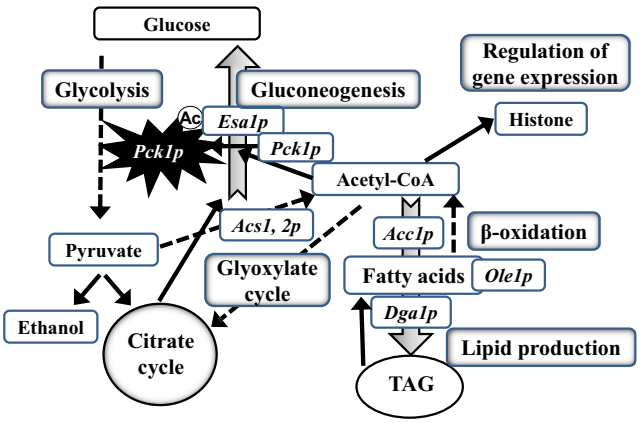

Fig. 6 Schematic view of gluconeogenesis and lipid production in *S. cerevisiae*. The Δ*dga1* mutant overexpressing Dga1ΔNp highly accumulates TAG in 10 % glucose medium, which is closely related to decreased gluconeogenesis due to decreased acetylation of Pck1p by Esa1p. Cytoplasmic acetyl-CoA appears to be a key intermediate between gluconeogenesis and lipid production

inhibiting acetyl-CoA carboxylase in *S. cerevisiae* (Kamiryo et al. 1976). On the other hand, addition of exogenous OA generates acetyl-CoA by β-oxidation in peroxisomes (Hiltunen et al. 2003), which may contribute to the activation of Pck1p and gluconeogenesis. Acetyl-CoA supply is one of the most important targets in metabolic engineering of microbial oil production (Krivoruchko et al. 2015; Sheng and Feng 2015). Our observations provide a valuable insight on the pathway leading to acetyl-CoA supply, with a potential to speed up fatty acid production.

In *S. cerevisiae*, the storage of carbohydrates such as glycogen and trehalose formed via gluconeogenesis is favored to the storage of lipids (Quain and Haslam 1979; François and Parrou 2001). In addition, it has been reported that gluconeogenesis was enhanced with aging (Lin et al. 2001), and that repressed gluconeogenesis contributed in extending life span (Hachinohe et al. 2013). These observations may suggest that gluconeogenesis is a suitable target for metabolic engineering when aiming to increase lipid instead of carbohydrate storages, which are produced in the late stationary phase, i.e., in aged cells (Ratledge and Wynn 2002). This assumption is supported by our findings showing a negative correlation between lipid production and gluconeogenesis in the oleaginous Δ*dga1*transformants. Our results also highlight acetyl-CoA as an important factor for gluconeogenesis via Pck1p acetylation. Supplied acetyl-CoA has been shown to enter the glyoxylate cycle, increasing gluconeogenesis through the production of gluconeogenesis precursors (Kunze et al. 2006; Chen et al. 2012). Therefore, we speculate that acetyl-CoA is a key intermediate between lipid production and gluconeogenesis through its important role as a precursor of fatty acid production.

The positive effects obtained through overexpression of Ole1p on the growth of the Δ*dga1* mutant overexpressing Dga1ΔNp in the GL medium may be partly explained by the regulatory roles of acetyl-CoA carboxylase in the acetyl-CoA

supply process, which is inhibited by long-chain acyl-CoAs (Kamiryo et al. 1976). Since Ole1p converts saturated acyl-CoAs into oleoyl-CoA and palmitoleoyl-CoA (Los and Murata 1998) and preferably produced OA under the conditions of this study (Table 3), oleoyl-CoA levels may increase temporarily prior to its reacylation into TAG or phospholipids, inducing similar effects to the ones obtained with the addition of exogenous OA. Similarly, rElo2p utilizes acyl-CoA as a substrate in the elongation reaction (Tehlivets et al. 2007), suggesting a temporal increase in oleoyl-CoA induced the effects observed when overexpressing Ole1p. However, rElo2p consumed malonyl-CoA and hence showed decreased levels of acetyl-CoA, which may moderate the effects of rElo2p on the growth in the GL medium. In addition to the increased OA content, a decrease in POA content in the transformant overexpressing Ole1p (Table 3) may contribute in improving the growth in the GL medium, as the addition of exogenous POA lead in an increased delay of growth initiation (Fig. 5b). Palmitoleoyl-CoA has been reported to inhibit rat liver acetyl-CoA carboxylase significantly less effectively than oleoyl-CoA (Nikawa et al. 1979), which might explain the difference observed between POA and OA.

It has been shown that addition of free fatty acids causes lipotoxicity in *S. cerevisiae*, especially when TAG synthesis is blocked (Eisenberg and Büttner 2014). The addition of POA was also reported to impair growth of a disruptant of *PAH1* encoding phosphatidate phosphatase more severely than OA (Fakas et al. 2011). Moreover, it was reported that the effect of POA on yeast cell death was more apparent in mutants with defects in maintaining a functional mitochondria (Sheibani et al. 2014). Finally, *cis*-vaccenic acid, an elongation product of POA, is thought to have a protecting effect against fatty acid-induced lipotoxicity (See et al. 2015). Altogether, these observations describing differential effects of POA and OA on yeast growth in the glucose medium provide indications that may help understand the influence of POA and OA on the growth in the GL medium and thus, the differences in POA and OA contents observed in the transformants cultured in the 10 % glucose medium. As an example, the effects observed might be related to mitochondria dysfunctions in the Δ*dga1* mutant overexpressing Dga1ΔNp, because the negative effects of exogenous POA on the growth in the GL medium were not seen in the wild-type strain overexpressing Dga1ΔNp (data not shown). This explanation may also be applicable to the effects of Ole1p. In fact, Ole1p mRNA levels were induced by non-fermentable carbon sources and Ole1p may play a role in increasing functional mitochondria during respiratory growth (Martin et al. 2007). The potential roles of Ole1p in maintaining functional mitochondria may help understanding the effects of Ole1p overexpression on growth in the GL medium, which were partly independent of Pck1p (Fig. 4).

It has been shown that fine-tuning of gene expression is a key factor for successful metabolic engineering of microbes rather than strong overexpression or repression, which often disturbs homeostasis of the cells (Nevoigt 2008; Kim et al. 2012; Kondo et al. 2013). The present observations revealed that modulation of gluconeogenesis in the Δ*dga1* transformants had positive effects on lipid accumulation, while complete shutdown of gluconeogenesis in the Δ*pck1* transformants did not, suggesting that the repression of gluconeogenesis have to be fine-tuned to promote lipid accumulation. Such partial activation/repression of metabolic pathways may be beneficial to establish optimal production conditions. The metabolic fine-tuning may be also achieved by modulation of post-translational modifications, which are involved in regulating metabolisms harmoniously in response to environmental and intracellular conditions in yeast (Oliveira and Sauer 2012). Among these modifications, protein acetylation is particularly supposed to regulate central carbon and energy metabolism (Guan and Xiong 2011), which is consistent with our present observation of the effect of Esa1p acetyltransferase on lipid accumulation. Because protein acetylation appears to respond to cellular energy status, Esa1p and other protein acetyltransferases can be engineered for efficient oil production with maintaining vigorous growth. Further studies on physiological roles of Esa1p and other protein acetyltransferases in yeast metabolisms may enable these protein acetylation reactions to be useful targets for fine-tuning of oil production.

In conclusion, we found that gluconeogenesis, estimated through yeast growth in the 2 % GL medium, was modulated via Pck1p acetylation by Esa1p in the oleaginous Δ*dga1* transformant. Furthermore, the genetic modifications of the Δ*dga1* transformant aiming at improving gluconeogenesis lead to a decreased lipid production in the 10 % glucose medium. Although this negative relationship between gluconeogenesis and lipid production was effective in Δ*dga1* transformants, but not in Δ*pck1* transformants, our findings provide new insight on lipid accumulation mechanisms and metabolic engineering approaches for lipid production. In particular, acetyl-CoA, which has emerged as an important intermediate in the anabolic and catabolic metabolisms (Galdieri et al. 2014; Krivoruchko et al. 2015; Sheng and Feng 2015), appeared to play an important role in regulating lipid production and gluconeogenesis. Moreover, fatty acid profiles, especially POA and OA contents, also influenced gluconeogenesis independently of Pck1p. Even though the exact mechanisms underlying the effect of fatty acid profiles were not clear, the differential effects observed with POA and OA may provide us with a strategy to increase POA production. Further studies on the regulatory roles of fatty acids and fatty acid precursors such as acetyl-CoA in the central carbon metabolism may reveal a metabolic state suitable for lipid accumulation and bring to light new metabolic engineering targets for lipid production.

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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