Journal of Bioscience and Bioengineering
VOL. 127 No. 4, 430–440, 2019

www.elsevier.com/locate/jbiosc

Delta-9 fatty acid desaturase overexpression enhanced lipid production and oleic acid content in *Rhodosporidium toruloides* for preferable yeast lipid production

Yung-Yu Tsai,[1] Takao Ohashi,[1] Chih-Chan Wu,[1] Dolgormaa Bataa,[1] Ryo Misaki,[1] Savitree Limtong,[2] and Kazuhito Fujiyama[1,*]

International Center for Biotechnology, Osaka University, 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan[1] *and Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*[2]

Received 30 July 2018; accepted 10 September 2018
Available online 11 October 2018

The oil plants provide a sufficient source of renewable lipid production for alternative fuel and chemical supplies as an alternative to the depleting fossil source, but the environmental effect from these plants' cropping is a concern. The high oleic acid (OA; C18:1) content in plant-derived products provide advantages of multiple uses with improved oxidative stability and a wide range of applicable temperature. Here we used a promising lipid producer, the oleaginous yeast *Rhodosporidium toruloides*, to attempt to obtain an OA-enriched lipid. *Saccharomyces cerevisiae OLE1* (*ScOLE1*) gene encodes Δ9 fatty acid desaturase (Δ9FAD), which is generally known to synthesize palmitoleic acid (POA; C16:1) and OA, but the functions of putative *R. toruloides Δ9FAD* gene are not well understood. In a complementary test, the *RtΔ9FAD* gene rescued the survival of an OA-deficient *Scole1*Δ mutant, and we introduced the *RtΔ9FAD* gene into *R. toruloides* strains for the production of OA-enriched lipid. Increasing lipid production was observed in *ScOLE1* and genomic *RtΔ9FAD* gene-overexpressing *R. toruloides* strains. The *ScOLE1* transformant output fivefold more OA content in total amount, with >70% of total lipid. Different enhancing effects from the protein coding sequence and genomic sequence of *RtΔ9FAD* genes were also observed. Overall, this study resulted in *ScOLE1* and *RtΔ9FAD* gene overexpression in *R. toruloides* to obtain OA-enriched lipid as a candidate source of designed biodiesel and lipid-related chemicals.

© 2018, The Society for Biotechnology, Japan. All rights reserved.

[**Key words:** Biodiesel; Oleaginous yeast; Oleic acid; *Rhodosporidium toruloides*; Delta-9 desaturase]

In light of the well-established environmental pollution and resource depletion caused by the fossil fuel consumption, alternative sources of energy continue to be a focus of research. One of these alternative sources is oil plant-derived lipid, which generates less environment-disturbing effects than fossil sources. Such lipids are already being used as environmental friendly biofuels (1,2). In efforts to determine the precise relationship between the fatty acid composition of plant-derived lipids and their performance as biodiesel fuel, a focus of the research has been to identify the effects of different fatty acids (1–5). Monounsaturated fatty acids (MUFAs), especially oleic acid (OA, C18:1) rich in plant-derived lipid, offer better oxidative stability for application to lubricants or biodiesel than di- and tri-unsaturated fatty acids such as linoleic acid (C18:2) and linolenic acid (C18:3) (1–4). MUFAs show better cold flow ability compared to saturated fatty acids such as palmitic acid (PA; C16:0) and stearic acid (STA; C18:0). Moreover, a plant-derived biodiesel fuel was reported to produce less polluting products such as CO, NO_x, SO_x, and particulates after being used compared to fossil fuel (3–5). Plant-derived OA-enriched lipids have therefore become a preferable alternative as a renewable lipid source. However, the production of plant-derived lipids involves several issues regarding its impacts on the environment such as water consumption, and pesticide administration (resulting in contamination on land and in the water), plus questions regarding the limitation of field sizes, sun or artificial light requirements, and even the climate factors (6–8).

Oleaginous yeasts discovered from different living environments have the ability to use varied substrates including industrial waste water or biomass towards a lipid synthesis with less environmental-restricting factors compared to oil plants, and such yeasts may therefore serve as lipid-producing workhorses (9–16). In addition, flexible, controllable and trackable genetic tools will enable the oleaginous yeasts to become a feasible platform for OA-enriched lipid production for practical uses in the future (14,17–19). However, the OA content that has been produced from conventional or oleaginous yeasts thus far has been limited (approximately 40%–50% maximum) (20–23). The further enriched OA level (≥60%) in the plant-derived products was suggested to be the suitable material for biodiesel production (2,5). An attempt was thus done to produce OA level (85%) enriched soybean oil (24).

Yazawa et al. (25) achieved efficient OA accumulation with increased ethanol tolerance in *Saccharomyces cerevisiae* with an overexpression of rat elongase (*rELO*) gene, and the resultant proportion of OA is around 40% of the total lipid; however their system does not seem to provide a sufficient method of producing OA-enriched lipid. Several attempts to enhance the lipid production in the well-studied oleaginous yeast *Yarrowia lipolytica*

* Corresponding author. Tel.: +81 6 6879 7453; fax: +81 6 6879 7454.
E-mail address: fujiyama@icb.osaka-u.ac.jp (K. Fujiyama).

YT submitted this manuscript as partial fulfillment of the requirements for PhD at the Graduate School of Engineering, Osaka University.

1389-1723/$ – see front matter © 2018, The Society for Biotechnology, Japan. All rights reserved.
https://doi.org/10.1016/j.jbiosc.2018.09.005

resulted in limited levels of OA-enriched lipids (26–28). A very recent work that obtained an OA level over 90% in *Y. lipolytica* again emphasized the importance of OA-enriched lipid for the industrial purposes (5). Compared to *Y. lipolytica*, the oleaginous yeast *Rhodosporidium toruloides* is not yet understood well enough for the realization of its potential, due in part to the limitations of available investigative tools.

R. toruloides belongs to subphylum Pucciniomycotina in the phylum Basidiomycota (basidiomycotaetes). It is known as a producer of carotenoids or enzymes, and it is now being intensively studied and used for lipid production based on its promising lipid productivity (29–33). *R. toruloides* strains were shown to be able to accumulate high amount of lipids under the nitrogen-limited conditions and to naturally produce OA at a relatively higher ratio in total lipid compared to other yeasts.

In addition to providing better lipid properties for biodiesel and chemical uses, OA is also an important precursor for the further synthesis of valuable polyunsaturated fatty acids (PUFAs) in oleaginous yeasts (25,34–37). *OLE1* gene, with synonyms also known as stearoyl-CoA desaturase (*SCD*) or Δ9-fatty acid desaturase (*Δ9FAD*) gene, encodes Δ9-fatty acid desaturase (Δ9FAD), which catalyzes the double bond formation between carbons 9 and 10 of palmitic acid (PA; C16:0) and stearic acid (STA; C18:0) to synthesize palmitoleic acid (POA; C16: l) and OA (38–40). The majority of Δ9FAD-synthesized product (POA or OA) is highly host-dependent. Only a few *OLE1* homologues from oleaginous yeasts have been isolated and characterized for their lipid- or OA-related production capacity (5,27,28,41–43). Zhang et al. (42) demonstrated that the overexpression of the own *SCD* gene in *R. toruloides* resulted in enhanced lipid production, but the OA ratio is still relatively lower than the ratios of the currently used plant-derived products. Moreover, the exact function of *R. toruloides* Δ9FAD remains to be established.

In the present work, we performed a complementary test to examine the fatty acid POA and OA synthesizing function of *R. toruloides* Δ9FAD, which is from the genome-opened *R. toruloides* strain NP11 (NP11) (33). A newly isolated *R. toruloides* strain DMKU3-TK16 (TK16) which has promising lipid productivity was used as the expressing platform along with strain NP11 for a control model (44). We conducted a functional examination and attempted to enhance the OA production in *R. toruloides* by overexpressing *ScOLE1* and the homologue *RtΔ9FAD* genes. With the use of a previously established transformation system (45), *ScOLE1* and *RtΔ9FAD* genes were introduced individually in both the NP11 and TK16 strains for the production of OA-enriched lipid. We observed and discuss the effect on gene expression obtained by using the protein coding sequence (CDS) and genomic sequence of *RtΔ9FAD* gene.

MATERIALS AND METHODS

Microorganisms and medium *R. toruloides* DMKU3-TK16 was obtained from the Department of Microbiology, Faculty of Science, Kasetsart University, Thailand (44). *R. toruloides* ATCC 10657 and NP11 were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The *R. toruloides* strains were grown in YM broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% glucose) for cell preparation and on YM agar plate for routine maintenance. *R. toruloides* strains were cultivated in nitrogen-limited medium (0.075% yeast extract, 0.055% $(NH_4)_2SO_4$, 0.04% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$ and 7% glucose) for lipid production.

S. cerevisiae BY4389 (*MATa*, *ole1Δ::LEU2*, *ura3-52*, *his4*) strain was obtained from the National Bioresource Project-Yeast (NBRP-Yeast) at Osaka University, Japan. *S. cerevisiae* BY4389 strain was grown in YM broth or on the YM agar plates with a supply of 1% oleic acid (*v*/*v*) and 0.5% Tween 20 (*v*/*v*) for routine maintenance. *S. cerevisiae* transformants carrying pYES plasmid were grown in synthetic complete medium lacking uracil (SC-Ura) supplemented with 1% oleic acid and 2% glucose, or medium without oleic acid, 2% raffinose and 0.5% galactose was used for the induction of a target gene in the complementary experiment. *Escherichia coli* DH5α was used for all cloning procedures.

Plasmid construction The construction of an expression cassette for target gene overexpression was done as described (45). Target genes of the expression cassette were amplified by PCR using KOD-Plus-NEO DNA polymerase (Toyobo, Osaka, Japan). The fragment of *ScOLE1* gene for constructing the *R. toruloides* expression cassette was amplified with the primers ScOLE1-BamHI-F and ScOLE1-NdeI-R, and the ORF and genomic sequence of *RtΔ9FAD* gene from *R. toruloides* NP11 were also amplified with the primer pair NP11OLE1-BamHI-F and NP11OLE1-NdeI-R for cassette construction (Table 1).

The prepared fragments were then used for plasmid construction to obtain the expression cassette and resulted in plasmids that we named pP_{GPD}-Shble-P_{GPD}-ScOLE1-T_{GPD}, pP_{GPD}-Shble-P_{GPD}-RtΔ9FAD-T_{GPD} and pP_{GPD}-Shble-P_{GPD}-RtgΔ9FAD-T_{GPD} individually (Fig. S1A). Likewise, for the complementary experiment in *S. cerevisiae* BY4389, *ScOLE1* gene, CDS and the genomic sequence of *RtΔ9FAD* gene were amplified with the primer pairs ScOLE1-BamHI-F and ScOLE1-XbaI-R, and NP11OLE1-BamHI-F and NP11OLE1-XbaI-R, respectively (Table 1), by PCR using KOD-Plus-NEO DNA polymerase. They were then cloned into pYES2 vector under the control of *GAL1* promoter to obtain plasmids that we named pYES2-ScOLE1, pYES2-RtΔ9FAD and pYES2-RtgΔ9FAD individually (Fig. S1B).

Transformation of yeast The transformation of *R. toruloides* stains was conducted and modified by a lithium acetate-based method that has been developed and applied in *R. toruloides* strains or *Rhodosporidiobolus fluvialis* (21,45). Briefly, yeast cells were precultured for 16 h at 30°C in 5 mL of YM broth, and then inoculated into 25 mL of YM broth and cultivated at 30°C with agitation for 16 h. The grown yeast culture ($OD_{600\ nm}$ = 3.0) was harvested and resuspended in 100 μL of transformation mixture (35% PEG-4000, 100 mM lithium acetate, 10 mM Tris–HCl at pH 4.9, and 1 mM EDTA) with a linear DNA fragment and 10% DMSO (*v*/*v*). The transformation mixture was incubated without agitation at 30°C for 3 h and treated by heat shock, then recovered by the addition of YM broth. Finally, the cells were harvested and spread onto a YM agar plate containing 150 μg/mL of Zeocin and incubated at 30°C to obtain colonies.

The *S. cerevisiae* BY4389 was transformed by a lithium acetate-based method (46,47). All resulting transformants were named as shown in Table 2. The foreign gene insertion was verified by PCR with the colony and extracted genome. The primer pair used for confirming insertion of *ScOLE1* gene was ScOLE1-BamHI-F and ScOLE1-NdeI-R, and the inserted *Rt*NP11 *Δ9FAD* gene was amplified by NP11OLE1-BamHI-F and NP11OLE1-NdeI-R (Table 1).

Lipid staining Sudan IV (Wako, Osaka, Japan) stock solution was prepared at the concentration of 2 mg/mL in isopropanol and stored protected from light for later experimental use. Transformants were precultured in 5 mL of YM broth, and the preculture was further cultured in 25 mL of YM broth for 2 days. The grown cells were harvested and then resuspended in 25 mL of nitrogen-limited broth. After a 4-day culture, 1 mL of cells was collected from the nitrogen-limited medium, washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), and resuspended in 200 μL of Sudan IV staining mixture (Sudan IV stock solution:PBS:DMSO = 10:9:1).

The samples were incubated in the dark at room temperature for 30 min, and the cells collected by centrifugation were then washed twice with PBS. The washed cell pellet was resuspended in 100 μL of 10% formaldehyde aqueous solution for a 30-min sample fixation. The fixed cells were collected and washed with PBS again and then resuspended in 200 μL of PBS buffer. Four microliters of the cells were then placed on a glass slide for further microscopy observation. Microscopy images were obtained with an Axioskop 2 microscope (Zeiss, Oberkochen, Germany).

TABLE 1. Primers used in the present study.

Primer name	Sequence (5′-3′)	Feature
ScOLE1-BamHI-F	GTT TTG GAT CCA TGC CAA CTT CTG GAA CTA C	Plasmid construction
ScOLE1-NdeI-R	GTT TTC ATA TGT TAA AAG AAC TTA CCA GTT TCG	Plasmid construction
ScOLE1-XbaI-R	GTT TTT CTA GAT TAA AAG AAC TTA CCA GTT TCG	Plasmid construction
NP11OLE1-BamHI-F	GGA TCC ATG ACT GCC TCT TCG GCA C	Plasmid construction
NP11OLE1-NdeI-R	CAT ATG TTA CGC CTT GAC CTT CAG	Plasmid construction
NP11OLE1-XbaI-R	GTT TTC TAG ATT ACG CCT TGA CCT TCA G	Plasmid construction
RtNP11-URA3qPCR-F	ACC AAC CTC TGC GTT TCA GTC	Real-time PCR
RtNP11-URA3qPCR-R	CCT CCC AAA TCA GAA AAT CG	Real-time PCR
RtNP11-OLE1qPCR-F	CCG GTT TCA TCC ACG ATG TCA GC	Real-time PCR
RtNP11-OLE1qPCR-R	CTG AGA CCG CCC AAG AGG TTG GTT TC	Real-time PCR
RtTK16-URA3qPCR-F	ACG CAA TAA TGC TTG TGC AG	Real-time PCR
RtTK16-URA3qPCR-R	AGC GAT CTC TCT CCC TCT CC	Real-time PCR

TABLE 2. Strains used in the present study.

Strain	Genotype/feature	Source
BY4389	*Saccharomyces cerevisiae* strain NBRP ID BY4389 (*MATa*, *ole1Δ::LEU2*, *ura3-52*, *his4*)	NBRP-Yeast collection
BY4389 + vector	BY4389/pYES2 empty vector	This study
BY4389 + *ScOLE1*	BY4389/pYES2-ScOLE1 plasmid	This study
BY4389 + *RtΔ9FAD*	BY4389/pYES2-RtΔ9FAD plasmid	This study
BY4389 + *gRtΔ9FAD*	BY4389/pYES2-RtgΔ9FAD plasmid	This study
TK16	*Rhodosporidium toruloides* strain DMKU3-TK16	44
TK16 + *ScOLE1*	TK16/P_{GPD}-*Shble*-P_{GPD}-*ScOLE1*-T_{GPD}	This study
TK16 + *RtΔ9FAD*	TK16/P_{GPD}-*Shble*-P_{GPD}-[a]CDS *RtΔ9FAD*-T_{GPD}	This study
TK16 + *gRtΔ9FAD*	TK16/P_{GPD}-*Shble*-P_{GPD}-genomic *RtΔ9FAD*-T_{GPD}	This study
NP11	*Rhodosporidium toruloides* strain NP11	ATCC collection
NP11 + *ScOLE1*	NP11/P_{GPD}-*Shble*-P_{GPD}-*ScOLE1*-T_{GPD}	This study
NP11 + *RtΔ9FAD*	NP11/P_{GPD}-*Shble*-P_{GPD}-[a]CDS *RtΔ9FAD* -T_{GPD}	This study
NP11 + *gRtΔ9FAD*	NP11/P_{GPD}-*Shble*-P_{GPD}-genomic *RtΔ9FAD*-T_{GPD}	This study

[a] CDS, coding sequence.

Lipid sample preparation and analysis by gas chromatography Transformants were precultured in 5 mL of YM broth, and the preculture was cultivated in 25 mL of YM broth with shaking at 140 rpm for 2 days for preparing enough cell amount rapidly. The grown cells were harvested and resuspended in 25 mL of nitrogen-limited broth and cultured for ≥ 4 days. Grown yeast cells were then harvested, and total cellular lipids were extracted and used for the preparation of fatty acid methyl esters (FAMEs) by transmethylation by described modified methods (48). The resulted FAMEs samples were analyzed by gas chromatograph (GC 353B, GL Sciences, Tokyo, Japan) equipped with a TC-70 capillary column (0.25 mm ID × 60 m, film thickness 0.25 μm; GL Sciences) and a flame ionization detector (FID) method. We used a commercially available FAMEs mix standard (Supelco, F.A.M.E. Mix C8–C24, CRM18918, Sigma–Aldrich, St. Louis, MO, USA) and methyl heptadecanoate (C17; H0566, TCI, Tokyo, Japan) as the internal standard for the further FAME identification and quantification.

For the GC analysis, 1 μL of hexane recovered sample was applied for the injection. Helium was used as the carrier gas with the constant pressure at 20 kPa. The column temperature for analyzing was set to start at 120°C (2 min) then increased by 20°C/min up to 160°C (2 min), 6°C/min up to 190°C (1 min), and by 20°C/min until the temperature reached the final stage at 220°C (2 min). The temperature of the injector and the detector was 250°C. The quantitative analysis of the results was performed using the ImageJ program to determine the detected response signal, and all presented values are the means of three independent quantifications (49).

Real-time PCR analysis The RNA of the target transformants were extracted by the phenol/chloroform method (50,51) and applied for cDNA synthesis by a commercial kit (PrimeScript RT reagent Kit Perfect Real Time, Takara, Shiga, Japan). The real-time PCR was performed by a SYBR Green-based system (Thunderbird SYBR qPCR Mix, Toyobo) with the comparative C_T ($\Delta\Delta C_T$) method for the relative expression analysis of *RtΔ9FAD* gene. *URA3* gene was used as an endogenous control to normalize and the wild-type group was set as the reference for the NP11 transformants.

In the *R. toruloides* strain TK16, the *URA3* gene was used as an endogenous control (45) and the *RtΔ9FAD* group was set as the reference for the analysis of the expression level in the transformants. The NP11 *URA3* gene (XM_016415986) was amplified with the primer pair RtNP11-URA3qPCR-F and RtNP11-URA3qPCR-R as the endogenous control, and the TK16 *URA3* was amplified with the primer pair RtTK16-URA3qPCR-F and RtTK16-URA3qPCR-R for the same purpose. The expression of target *RtΔ9FAD* gene was defined with the primer pair RtNP11-OLE1qPCR-F and RtNP11-OLE1qPCR-R in all target samples (Table 1; Fig. S3).

RESULTS

Sequence analysis of *R. toruloides* NP11 Δ9-fatty acid desaturase The amino acid sequence of putative *R. toruloides* NP11 Δ9FAD (*Rt*Δ9FAD) (XP_016270987) was obtained from the NCBI protein database (33). The putative *Rt*Δ9FAD gene encodes a polypeptide of 545 amino acids with a predicted molecular mass of 60.8 kDa (52,53). A characterized Δ9FAD from *Cutaneotrichosporon curvatus* (CAA71449.1) was shown to share 64% similarity with *Rt*Δ9FAD (41). Herein, we analyzed the amino acid sequence of *Rt*Δ9FAD with several Δ9FAD homologous proteins, i.e., the characterized *Sc*Δ9FAD protein (AAA34826.1) (Figs. 1 and 2).

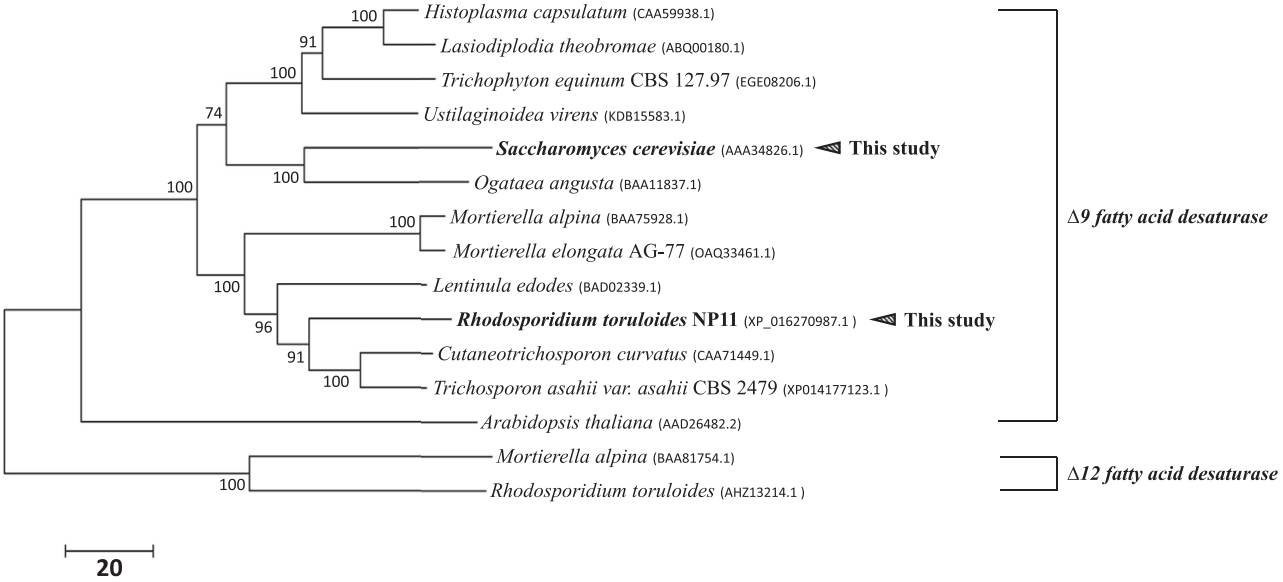

FIG. 1. Phylogenetic tree of the evolutionary relationships of Δ9 fatty acid desaturase protein orthologues. Molecular phylogeny shared by the characterized and deduced Δ9FAD protein homologues, with Δ12FADs (fatty acid desaturase) being an outgroup. The multiple alignment was performed and the phylogenetic tree was constructed using the neighbor-joining algorithm of MEGA7. Branch lengths are proportional to the phylogenetic distances, with the numbers representing the frequency which was replicated after 1000 bootstrap iterations.

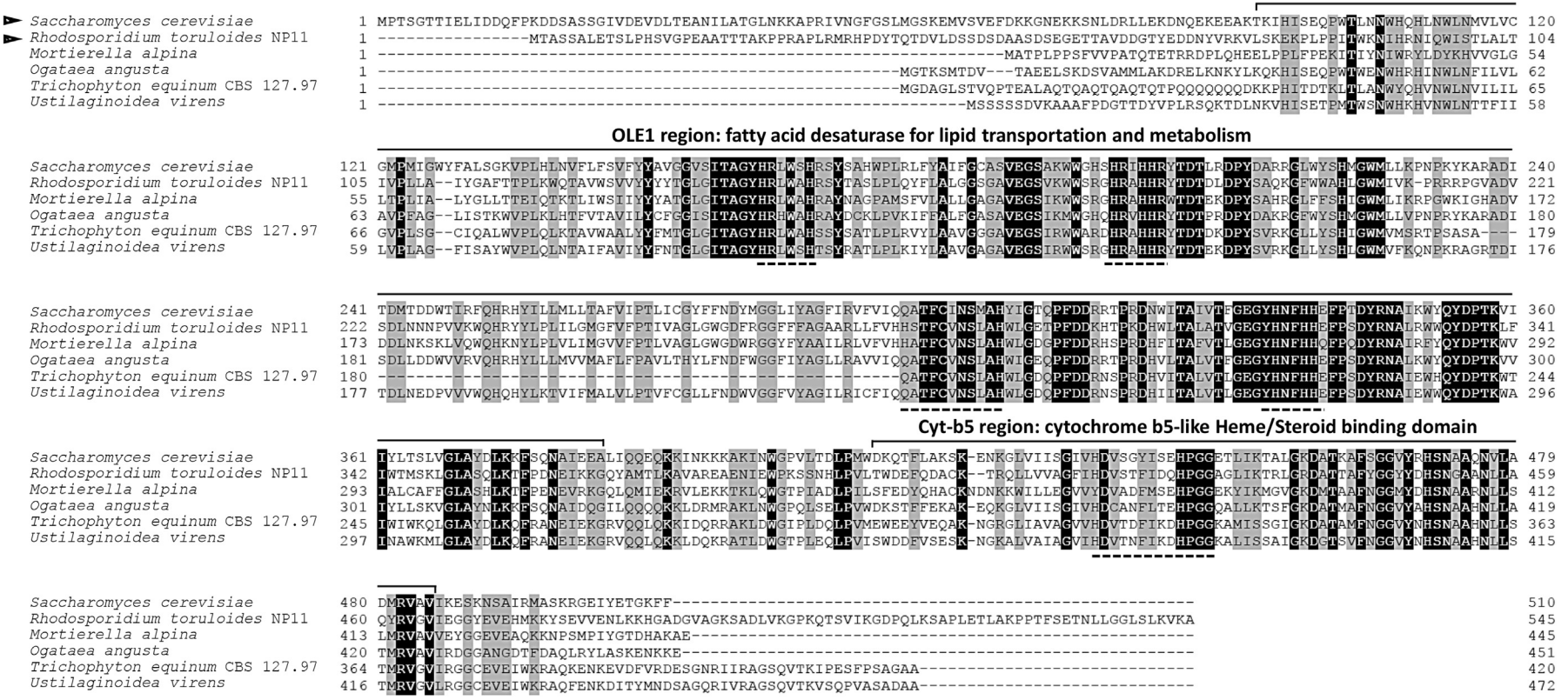

FIG. 2. Protein sequence alignment and analysis of *R. toruloides* NP11 Δ9FAD. Identical residues are boxed in black, and the conservative areas are shaded in gray. Two hypothetical regions (OLE1 and Cyt-b5 region) are briefly classified separately, with dashed underlining indicating the conservative histidine-involved domains.

The relatedness shown by the phylogenetic tree in Fig. 1 illustrates the clear distinctions among yeast, fungal, and plant Δ9 desaturases and the further distinction from the outgroup yeast Δ12 desaturases. In the phylogenetic tree, *Rt*Δ9FAD was relatively close to the characterized Δ9FAD from *C. curvatus* (CAA71449.1) (41). The *Sc*Δ9FAD was grouped with another characterized Δ9FAD from *Ogataea angusta* (BAA11837.1), a yeast from the subphylum Pezizomycotina (54). The alignment analysis compared FADs from *S. cerevisiae* (55), *Mortierella alpina* (56), *O. angusta* (54), *Trichophyton equinum*, and *Ustilaginoidea virens* (Fig. 2). The putative *Rt*Δ9FAD was divided into an OLE1 region (fatty acid desaturase region) and a Cyt-b5 region (cytochrome b5-like Heme/Steroid binding domain and nitrate reductase) according to previous studies (38,57,58). These Δ9FADs shared two highly conserved histidine sequences, HRXHHR and HNFHH (dashed underlining) in the hypothetical OLE1 region that would be expected to be responsible for the function of Δ9 fatty acid desaturase.

In addition, three domains with a conserved histidine residue (dashed underlining) were observed in both the OLE1 and Cyt-b5 regions; these domains might also serve as the active area of each region (59,60). The *Rt*Δ9FAD protein was also predicted to have four possible transmembrane domains (Fig. S2). *Rt*Δ9FAD was thus suggested to be a functional homologue of *Sc*Δ9FAD.

The functional complementation of *S. cerevisiae ole1* disruptant by *Rt⊿9FAD* gene The Δ9FAD is generally known to have the function of catalyzing the fatty acids PA (C16:0) and STA (C18:0) to POA (C16:1) and OA (C18:1) respectively in mammals, yeasts, and plants. To examine the function of *Rt*Δ9FAD, we introduced the *⊿9FAD* gene cloned from NP11 into the *S. cerevisiae ole1* disruptant BY4389 (*Scole1⊿*) for a complementary test (Fig. 3A). The *Scole1*Δ strain was incapable of surviving on the plate without OA supplement (Fig. 3A, upper left), but the cell survival was restored by the overexpression of *ScOLE1* gene as a reference strain under the galactose induction (Fig. 3A, upper right). *Rt⊿9FAD* gene was also overexpressed for complementation in *Scole1*Δ following the introduction of the coding sequence (CDS) or genomic sequence that was presented by *Rt⊿9FAD* and g*Rt⊿9FAD* respectively. The genomic *Rt⊿9FAD* sequence was unable to save the OA deficiency-induced cell death. Contrarily, the CDS *Rt⊿9FAD* expression restored the cell survival and showed an even better growth effect compared with the reference group (*ScOLE1*).

We also investigated the fatty acid composition of extracted lipid from each strain (Fig. 3B). Without OA, the empty vector pYES2-transformed *Scole1*Δ could not grow in YM broth (data not shown). As the reference, *Scole1*Δ carrying pYES2-ScOLE1 grew and showed the regained production of POA and OA. Likewise, the

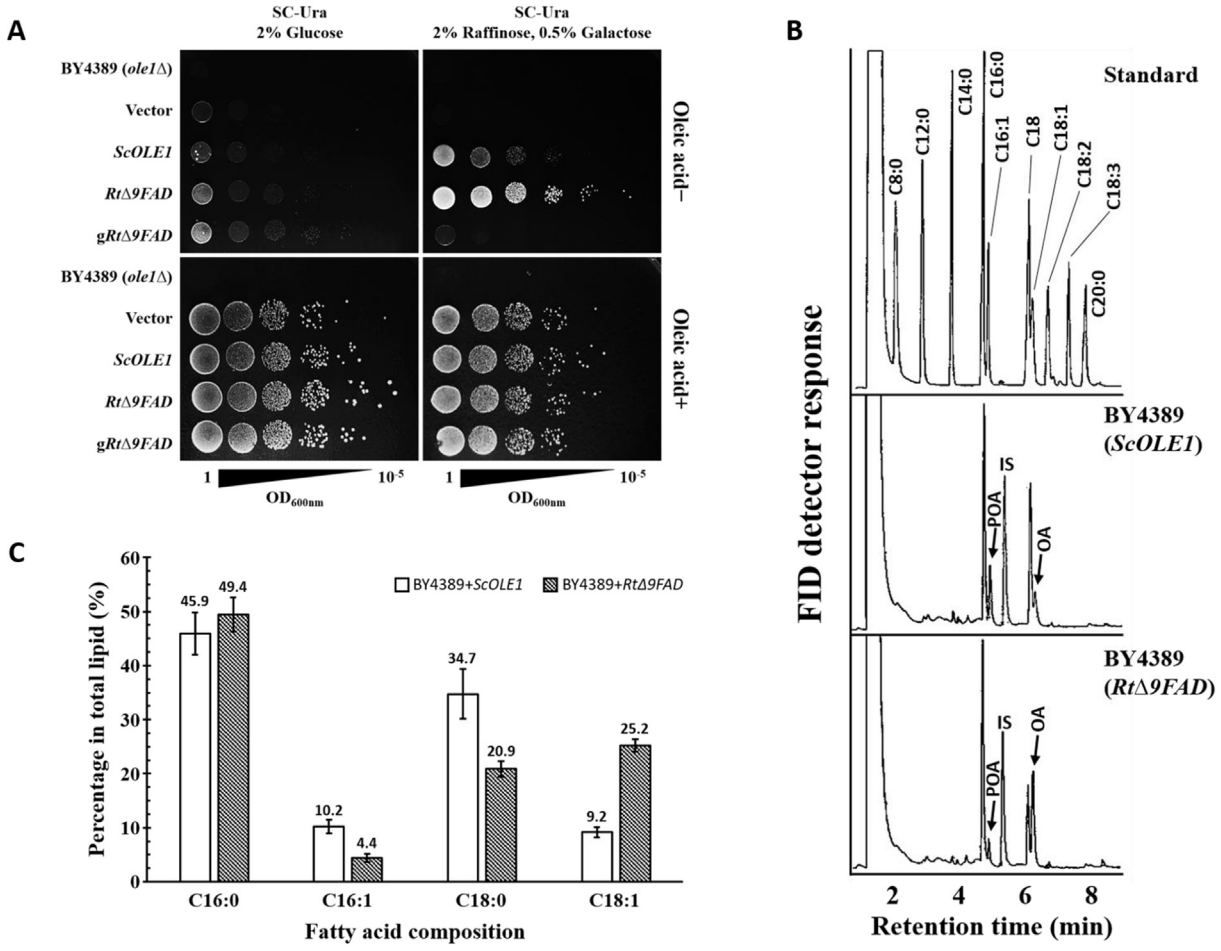

FIG. 3. *Rt*Δ9FAD restored the *S. cerevisiae ole1* disruptant's survival by the complementation of palmitoleic acid (POA; C16:1) and oleic acid (OA; C18:1) and oleic acid synthesis. (A) Spot assay of *Rt*Δ9FAD complementing cell survival. Live/dead assay among the background of *Scole1⊿* strain BY4389, comparing the function of overexpressing pYES2 empty vector, *ScOLE1*, *Rt⊿9FAD* (CDS) and g*Rt⊿9FAD* (genomic). The experiment was conducted on the SC-Ura medium with or without OA supplement. (B) Gas chromatography analysis of fatty acid composition in *ScOLE1* or *Rt⊿9FAD* overexpressing *Scole1⊿*. The peaks of POA and OA were identified by a comparison with the commercially available fatty acid methyl ester standard and are indicated by arrows. IS: internal standard, heptadecanoic acid methyl ester (C17). (C) Quantitative measurement of fatty acid profile in percent fraction. Numbers are the occupied ratio in total lipid of each indicated fatty acid. C16:0, palmitic acid, PA; C16:1, palmitoleic acid, POA; C18:0, stearic acid, STA; C18:1, oleic acid, OA. The values are the average of triplicate cultures. Error bar: SD.

Rt⊿9FAD expression also restored POA production and significantly enhanced the OA accumulation. Quantitatively, the production of PA was not significantly affected in the *Rt⊿9FAD* transformant, but the POA level (4.4%) was 2.3-fold less than the reference (10.2%) (Fig. 3C). The proportion of STA was 20.9% along with an OA ratio at 25.2%, which was greater than the OA level from the reference. These results demonstrated the PA and STA converting function of *Rt*Δ9FAD and also revealed the different substrate preference from each applied Δ9FAD in the *S. cerevisiae* host background.

Introduction of *ScOLE1* and *Rt⊿9FAD* gene increased lipid production in *R. toruloides* strains In order to produce an OA-enriched lipid with higher lipid productivity, we then introduced *ScOLE1* gene, and both the CDS and the genomic sequence of *Rt⊿9FAD* genes into *R. toruloides* strains NP11 and TK16. The growth effect of gene insertion was examined at the lipid accumulating condition, and most of the cell growth was not affected (Fig. 4A).

To identify the optimal period of desired lipid production, we inspected the lipid production in a time-dependent experiment (Fig. 4B). With both the TK16 and NP11 background, the *ScOLE1* and g*Rt⊿9FAD* transformants (TK16 + *ScOLE1*, TK16 + g*Rt⊿9FAD*, NP11 + *ScOLE1*, and NP11 + g*Rt⊿9FAD*) began to show increased lipid production since cultivation started in the nitrogen-limited environment for lipid accumulation after 24 h. We also observed that the stationary period of lipid accumulation started about at 96 h and reached the maximal amount at 120 h after the initiation of lipid accumulation in both *R. toruloides* backgrounds, and thereafter a slight decline of the lipid amount was also observed at 144 h.

Compared to the wild type, the TK16 + *ScOLE1*, TK16 + g*Rt⊿9FAD*, NP11 + *ScOLE1*, and NP11 + g*Rt⊿9FAD* showed approximately threefold lipid increases. The lipid productivity (μg/mg) at each time point was determined, and the probable period of lipid production was obtained with the highest efficiency at 96–120 h in transformants (Fig. 4C). All transformants were also directly observed by microscopy with lipid staining (Fig. 4D,E). Yeast cells were collected at 0 and 120 h, and apparent lipid droplets could be seen after 120 h of lipid accumulation. The TK16 + *ScOLE1*, TK16 + g*Rt⊿9FAD*, NP11 + *ScOLE1*, and NP11 + g*Rt⊿9FAD* displayed larger lipid droplets than the TK16, the NP11, TK16 + *Rt⊿9FAD*, and NP11 + *Rt⊿9FAD*, and we observed different appearances of the lipid droplets (separated small droplets distributed around the cell) from TK16 + *Rt⊿9FAD* and NP11 + *Rt⊿9FAD* compared to the others (condensed large droplets). Taking these results together, we chose 120-h cultivation for lipid accumulation in the subsequent experiments.

***ScOLE1* gene expression significantly increased the OA content in lipids** The ultimate goal of this study was the production of lipid with enhanced OA content, and we therefore examined the fatty acid composition of the transformants towards our purpose (Fig. 5A). The results of our fatty acid analysis showed that the lipid produced in the TK16 + *ScOLE1* contained OA at 72% of the total lipid, which was higher than the ratios produced in other yeast-originated lipids. The TK16 + g*Rt⊿9FAD* was able to produce lipid with the OA ratio of 62% of the total lipid, but a similar effect was not observed in the TK16 + *Rt⊿9FAD*. The *ScOLE1* gene expression was also determined from transformants by PCR (Fig. S4).

Compared to the wild-type TK16, the OA content was enhanced by 40% in the TK16 + *ScOLE1* and 20% in the TK16 + g*Rt⊿9FAD*. With the NP11 background, the NP11 + *ScOLE1* also produced lipid at 57% OA compared to wild type, reaching approximately 18% enhancement, but the effect was not significant in the NP11 + g*Rt⊿9FAD*. We also found that the TK16 + *Rt⊿9FAD* and NP11 + *Rt⊿9FAD* did not clearly enhance the produced lipid amount or OA content. Combining the enhanced lipid-producing ability and OA level, the *ScOLE1* and g*Rt⊿9FAD* transformants of both the NP11 and TK16 strains furnished higher lipid and OA production. Particularly, the TK16 + *ScOLE1* was able to produce lipid with a threefold total amount and fivefold increase in the OA content for the efficient production of OA-enriched lipid at the desired level (Fig. 5B).

The expression level of *Rt⊿9FAD* gene in the *R. toruloides* transformants Considering that using the CDS and the genomic sequence of *Rt⊿9FAD* gene resulted in notably different effects on lipid production, we conducted a qPCR to investigate whether the level of transcription contributed to these effects (Fig. 6). The genomic sequence conferred a higher transcriptional level among the wild-type and CDS transformants. A nearly threefold higher level was detected in the TK16 + g*Rt⊿9FAD* against the CDS-introduced group, and approximately treefold and twofold higher levels were detected in the NP11 transformants compared to the wild-type and CDS transformants. A similar result was obtained with a different primer pair at the 5' end of the target sequence (data not shown). These results indicated that the genomic sequence did provide a relatively higher transcriptional level, and that this might thence lead to the distinct enhancing effect on lipid production.

DISCUSSIONS

Renewable oil (or so-called lipid) sources are highly expected to replace the present oil mining system. Oleaginous yeast was reported to be able to obtain dry biomass by 100 g/L with 7-day culture which resulting about 65 g/L of lipid production that would meet an industrial requiring level (61). While the cost is still a considerable issue. The cost of lipid production from oleaginous yeasts was estimated to contain up to 80% by the using of glucose as a main substrate feedstock so far (62). Combining the manufacturing process and substrate supplement, the price was estimated to be around $8–9/kg, and the plant-derived lipid might cost less than $1.5–3/kg (63). A recent work conducted microbial lipid recovery by acid treatment has achieved estimated minimum fuel selling price (MFSP) about $5/gallon of gasoline equivalent (GGE) to meet a reduced and applicable price (64). Another trial produced an microbial biodiesel ($ 0.76/L) by means of a pilot scale fed-batch strategy with low-cost medium which is economically comparable to the plant-derived product ($ 0.81/L) (65,66). Utilization of cheaper substrates such like industrial by-product the crude glycerol or waste lignocellulose may further reduce the cost of producing process. These studies have shown the possibility to use oleaginous yeast platform for renewable lipid production in practice.

High OA plant-derived lipid have better applicability with preferable stability and less air pollutants, and the low melting point of OA methyl ester maintains its capability in the cold environments (1,2,4). An increased OA content was observed to enhance engine performance in soybean oil-derived biodiesel (3). The intensive cropping of oil plants has environmental influences, and lipid-rich algae are considered another conductive option that involves less pressure from the requirements of renewable oil sources, but the use of lipid-rich algae is also limited by light and water demands and the high cost of oil recovery (67–71). Oleaginous yeasts provide an effective platform without requiring similar restrictions on the process of production, and a feasible system of genetic engineering could provide designable products. We aimed to produce OA-enriched lipid from the oleaginous yeast *R. toruloides* by an overexpression of *ScOLE1* and *Rt⊿9FAD* genes.

Some studies of *Y. lipolytica* and *R. toruloides* have applied endogenous *⊿9FAD* homologues in the course of lipid production

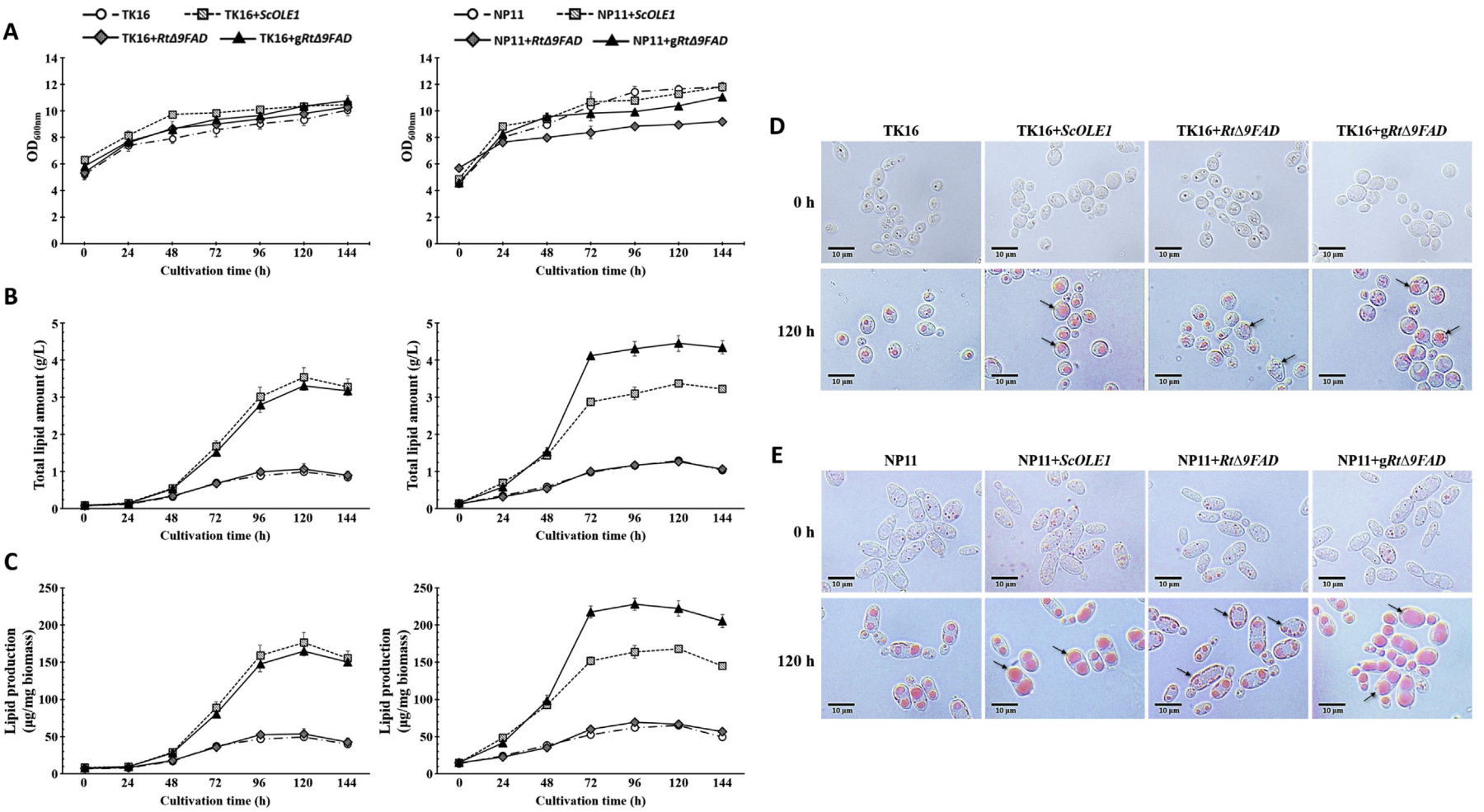

FIG. 4. Effects of *ScOLE1* and *RtΔ9FAD* gene overexpression on the growth of and lipid production by *R. toruloides*. (A) Growth effects of *ScOLE1* and *RtΔ9FAD* gene insertion on *R. toruloides* strains during lipid accumulating condition. The growth of the transformants compared with each wild-type strain was mostly equivalent. Cell density was determined by absorbance $OD_{600\ nm}$. (B) Time-dependent examination of lipid production and comparison among the wild type and transformants. (C) Corresponding lipid productivity at each observed time point. The presented values are the average of triplicate cultures. Error bars: SD. (D, E) Lipid droplet observation in wild-type TK16, NP11, and their transformants by microscopy. The lipid droplets were stained by Sudan IV, and the cells were observed at 0 and 122 h from a bright field. Scale bar: 10 μm. WT, wild type.

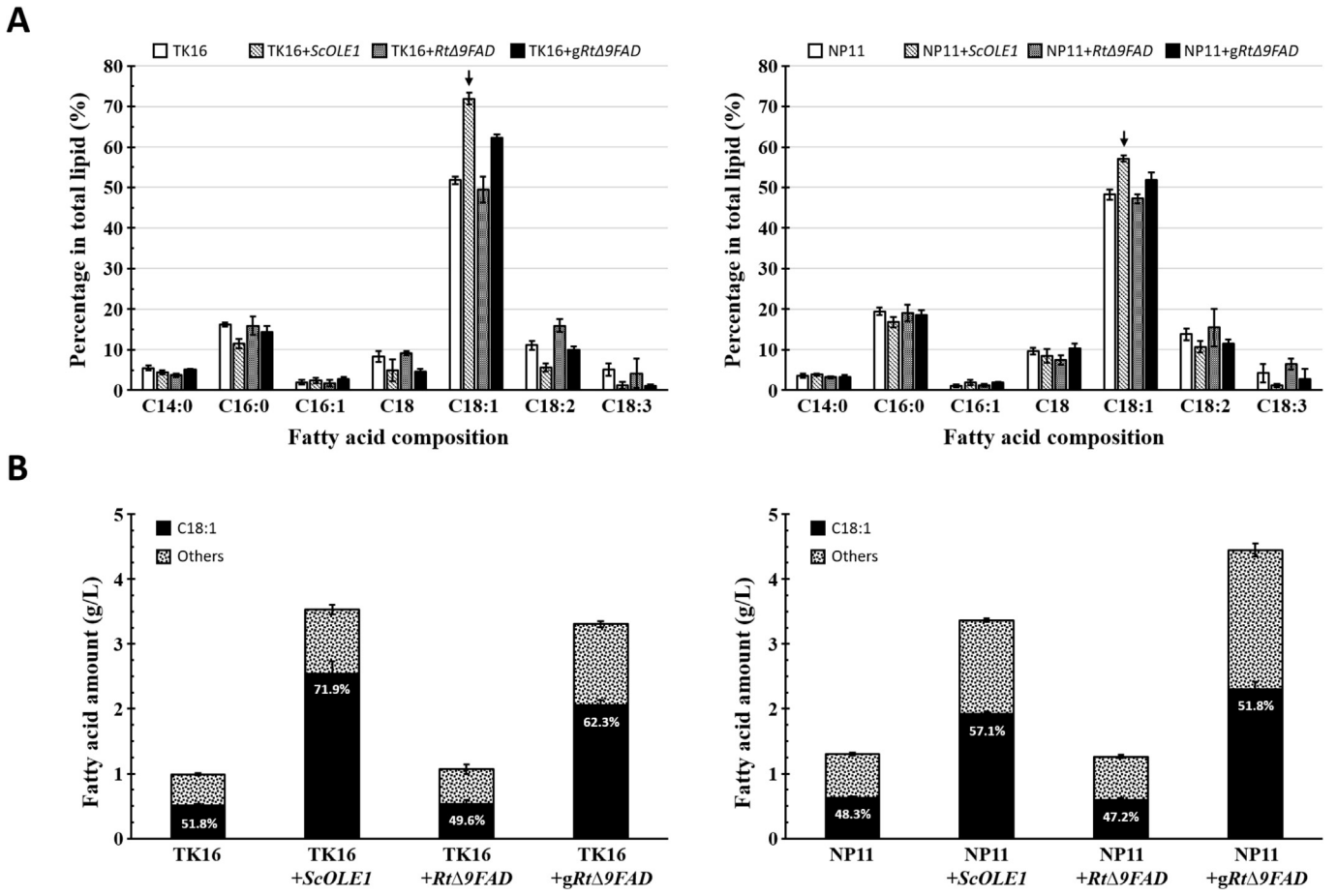

FIG. 5. *ScOLE1* and *RtΔ9FAD* gene overexpression in the *R. toruloides* resultant lipid and oleic acid production. (A) Fatty acid composition of all strains measured quantitatively from 122-h culture. The fatty acid profile is presented as the percent fraction for each indicated fatty acid. C14:0, myristic acid, MA; C16:0, palmitic acid, PA; C16:1, palmitoleic acid, POA; C18:0, stearic acid, STA; C18:1, oleic acid, OA; C18:2, linoleic acid, LA; C18:3, linolenic acid, LNA. (B) Lipid content with OA in the percent fraction from 122-h culture. Closed bars with numbers, the OA occupied ratio in the final output lipid. TK16, wild-type TK16. NP11, wild-type NP11. The presented values are the average of triplicate cultures. Error bars: SD.

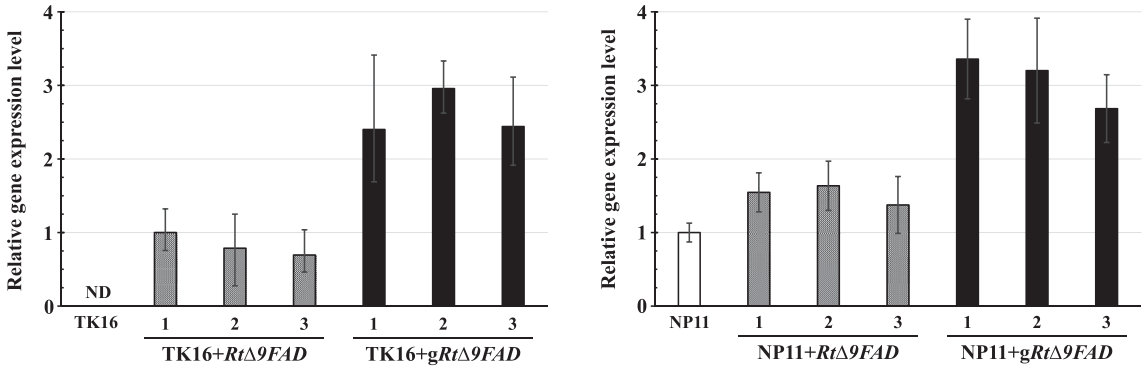

FIG. 6. Investigation of the *RtΔ9FAD* gene expression level in transformants. The mRNA expression level was normalized with *URA3* expression, which was used as the internal standard. In the TK16 strain, one of the *RtΔ9FAD* CDS transformants was selected for reference. With the NP11 background, the wild type strain (NP11) was selected for reference. Each number indicates an independent clone. ND, non-detected. The presented values are the average of triplicate cultures. Error bars: SD.

and enhanced the lipid amount. However, most of those effort did not significantly improve the OA level or further discuss the function of oleaginous *Δ9FAD* gene (27,28,42,43). Zhang et al. (42) overexpressed native *SCD* gene in *R. toruloides* IFO0880 to increase the lipid production by 28% of total lipid (g/L), which was described as a limited enhancement. Here, we analyzed the gene itself and used the CDS and the genomic sequence individually for the comparison. Consequently, threefold increased lipid production was obtained with enhanced OA content.

Several histidine domains were identified in the *Rt*Δ9FAD protein sequence and are speculated to be the active sites (Figs. 1 and 2). The *RtΔ9FAD* gene complemented the production of POA and OA and restored the survival of *Scole1Δ* without OA supplementation (Fig. 3A). Interestingly, the efficiency of OA production was higher than that of POA in *RtΔ9FAD* transformed *Scole1Δ*, which was found

to be different from the *ScOLE1* transformant (Fig. 3B,C). This result indicated that the substrate preference of Δ9FAD might be strongly related to the gene origin. Such phenomena of distinct substrate preferences among Δ9FADs were observed from Δ9FAD isomers in different models, i.e., the mouse SCD1-4 and *M. alpina* ole1p, ole2p and Δ9−3 (59,72−74).

After our functional examination, we then introduced *ScOLE1* and *RtΔ9FAD* genes into the *R. toruloides* TK16 and NP11 strains to obtain the production of OA-enriched lipid. Unexpectedly, the *ScOLE1* gene (which is rarely discussed when enhancing lipid production is considered) drove the *R. toruloides* transformants to give higher lipid amounts compared to the wild type (Fig. 4B). The genomic sequence of *RtΔ9FAD* gene also provided an enhancing effect on lipid production, but this effect was not clearly observed when a CDS fragment was used as the expression target (Fig. 4B). Although some Δ9FADs are known to be related to the obesity in mammal models or to lipid production in yeasts, the exact mechanism underlying their enhancement of yeast lipid production is still not fully understood (26−28,42,43,75,76).

In the present study's microscopic observations, the *ScOLE1* and g*RtΔ9FAD* gene transformants displayed larger lipid droplets (Fig. 4D,E). The phenomena of Δ9FAD overexpression in oleaginous yeasts thus increasing lipid accumulation have been observed and possible mechanisms have recently been proposed (29,31,33,42). As previous studies revealed, Δ9FAD has shown a strong relationship with stability of the cellular membrane by contributing the membrane fluidity (77−82). In addition, a research on human SCD revealed its role in the conversion of MUFAs toward the safe storage of fatty acids, thus protecting the cell from damage from saturated fatty acids (83). We propose that a probable mechanism is as follows: the enhanced membrane integrity and incorporation capability of a lipid droplet itself by Δ9FAD overexpression might also allow higher lipid accumulation beyond the natural limitation.

We also analyzed the lipid from the *ScOLE1* and g*RtΔ9FAD* gene transformants of the *R. toruloides* strains to increase the understanding of the fatty acid composition (Fig. 5A,B). The results of our analyses demonstrated that the *ScOLE1* transformants produced OA-enriched lipid with higher OA ratios than the other groups. Since we did not disrupt the fatty acid desaturase 2 (*FAD2*) gene which is responsible for double bond formation on Δ12 carbon, the linoleic acid (LA; C18:2) amount increased along with the OA reduction during longer cultivation periods, and the high-OA lipid could be obtained at the time point before LA was highly synthesized with the current culture conditions. We are continuing to investigate the function of *RtFAD2* gene and the effects on the host itself.

Interestingly, we also observed that the *ScOLE1* in the *R. toruloides* background seemed to have less effect on POA synthesis, which may indicate an effect of substrate abundance (Figs. 3B and 5A). Studies of *Sc*Ole1p identified the multiple factors that could regulate *OLE1* gene expression, revealing that the regulation of *OLE1* gene or Ole1p may not be easily assumed with a single factor (84). Accordingly, it may be suggested that the OA production in the g*RtΔ9FAD* transformants is regulated by an endogenous homeostasis system of fatty acid composition control, which has not been characterized well in *R. toruloides*. But this effect may less affected the heterologous *ScOLE1* gene. However, there is no clear evidence of this to date.

Our application of the CDS and the genomic sequence of *RtΔ9FAD* gene in *R. toruloides* led to a significant discrepancy in lipid production. Although we have determined the resulting influence on the transcriptional level, the reasons underlying such a difference between each sequence remain unclear (Fig. 6). In eukaryotic systems, intronic factors can dramatically affect the gene expression in various ways. An intron can mediate the gene expression by providing DNA accessibility, enhancing the initiation and activity of RNA polymerase II, or even regulating pre-mRNA processing, splicing, mRNA localization, and downstream RNA metabolism and alter the protein association in the post-splicing stage (85,86). The *Δ9FAD* gene of the industrial strain *M. alpina* was found to have one intron, but its intronic effect has not been described (56), and such factor has never been studied in *R. toruloides Δ9FAD*.

With the present *R. toruloides* background, we examined the region-specific mRNA stability by qRT-PCR with the 5′- and 3′-region specific primer pairs, but the results obtained were not clearly different. Therefore, the resulting change in the expression level seems not be directly derived from distinct mRNA stability. Therefore, an enzymatic assay may be also helpful to understand the effect that the intronic elements have dedicated on the protein activity. Due to the lack of similar studies, it is apparent that a more extensive understanding of how genomic and intronic elements are involved in gene expression is necessary for the further genetic manipulation of the *R. toruloides* platform in the future.

Despite the benefits microorganisms have provided, their genetically engineered strains perhaps will bring a distinct type of impact on our environment which should be sincerely concerned. Examples from genetically engineered microalgae revealed the potential risk of releasing such resultant genetically-varied organisms into the wild and effects will be barely estimated (87). Thus, the use of genetically modified microorganisms for a mass production of chemicals need to be carefully considered and treated.

In summary, we sought to obtain a high OA content lipid to produce preferable biodiesel and lipid-related chemicals. In this work, we performed an amino acid sequence analysis and examined the function of *RtΔ9FAD* gene as the candidate gene toward OA-enriched lipid production. By applying *ScOLE1* and *RtΔ9FAD* genes in the oleaginous yeast *R. toruloides*, we achieved increased lipid production with the desired OA content. We also observed an interesting expression effect by introducing a genomic sequence that could be an useful example for the future manipulation of endogenous genes in *R. toruloides*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.09.005.

ACKNOWLEDGMENTS

This research was conducted by the finance supplied by Osaka University basic budget expence. Thus any specific funders and grant agencies are not applicable to this study. The authors declare no conflict of interest.

References

1. **Knothe, G., Sharp, C. A., and Ryan, T. W.:** Exhaust emissions of biodiesel, petrodiesel, neat methyl esters, and alkanes in a new technology engine, Energy Fuels, **20**, 403−408 (2006).
2. **Knothe, G.:** "Designer" biodiesel: optimizing fatty ester composition to improve fuel properties, Energy Fuels, **22**, 1358−1364 (2008).
3. **Ferrari, R. A., Oliveira, V. S., and Scabio, A.:** Oxidative stability of biodiesel from soybean oil fatty acid ethyl esters, Sci. Agric., **62**, 291−295 (2005).
4. **Knothe, G.:** Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters, Fuel Process. Technol., **86**, 1059−1070 (2005).
5. **Tsakraklides, V., Kamineni, A., Consiglio, A. L., MacEwen, K., Friedlander, J., Blitzblau, H. G., Hamilton, M. A., Crabtree, D. V., Su, A., Afshar, J., and other 6 authors:** High-oleate yeast oil without polyunsaturated fatty acids, Biotechnol. Biofuels, **11**, 131 (2018).
6. **Hill, J., Nelson, E., Tilman, D., Polasky, S., and Tiffany, D.:** Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels, Proc. Natl. Acad. Sci. USA, **103**, 11206−11210 (2006).
7. **Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S. B., and Keasling, J. D.:** Microbial production of fatty-acid-derived fuels and chemicals from plant biomass, Nature, **463**, 559−562 (2010).
8. **Williams, P. R., Inman, D., Aden, A., and Heath, G. A.:** Environmental and sustainability factors associated with next-generation biofuels in the U.S.: what do we really know? Environ. Sci. Technol., **43**, 4763−4775 (2009).

9. **Ageitos, J. M., Vallejo, J. A., Veiga-Crespo, P., and Villa, T. G.:** Oily yeasts as oleaginous cell factories, Appl. Microbiol. Biotechnol., **90**, 1219–1227 (2011).
10. **Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J. L., Molina-Jouve, C., and Nicaud, J. M.:** *Yarrowia lipolytica* as a model for bio-oil production, Prog. Lipid Res., **48**, 375–387 (2009).
11. **Cho, H. U. and Park, J. M.:** Biodiesel production by various oleaginous microorganisms from organic wastes, Bioresour. Technol., **256**, 502–508 (2018).
12. **Kosa, M. and Ragauskas, A. J.:** Lipids from heterotrophic microbes: advances in metabolism research, Trends Biotechnol., **29**, 53–61 (2011).
13. **Li, Q., Du, W., and Liu, D.:** Perspectives of microbial oils for biodiesel production, Appl. Microbiol. Biotechnol., **80**, 749–756 (2008).
14. **Shi, S. and Zhao, H.:** Metabolic engineering of oleaginous yeasts for production of fuels and chemicals, Front. Microbiol., **8**, 2185 (2017).
15. **Sitepu, I. R., Garay, L. A., Sestric, R., Levin, D., Block, D. E., German, J. B., and Boundy-Mills, K. L.:** Oleaginous yeasts for biodiesel: current and future trends in biology and production, Biotechnol. Adv., **32**, 1336–1360 (2014).
16. **Tchakouteu, S. S., Kalantzi, O., Gardeli, C., Koutinas, A. A., Aggelis, G., and Papanikolaou, S.:** Lipid production by yeasts growing on biodiesel-derived crude glycerol: strain selection and impact of substrate concentration on the fermentation efficiency, J. Appl. Microbiol., **118**, 911–927 (2015).
17. **Adrio, J. L.:** Oleaginous yeasts: promising platforms for the production of oleochemicals and biofuels, Biotechnol. Bioeng., **114**, 1915–1920 (2017).
18. **Fillet, S. and Adrio, J. L.:** Microbial production of fatty alcohols, World J. Microbiol. Biotechnol., **32**, 152 (2016).
19. **Liang, M. H. and Jiang, J. G.:** Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology, Prog. Lipid Res., **52**, 395–408 (2013).
20. **Fillet, S., Ronchel, C., Callejo, C., Fajardo, M. J., Moralejo, H., and Adrio, J. L.:** Engineering *Rhodosporidium toruloides* for the production of very long-chain monounsaturated fatty acid-rich oils, Appl. Microbiol. Biotechnol., **101**, 7271–7280 (2017).
21. **Polburee, P., Ohashi, T., Tsai, Y. Y., Sumyai, T., Lertwattanasakul, N., Limtong, S., and Fujiyama, K.:** Molecular cloning and overexpression of DGA1, an acyl-CoA-dependent diacylglycerol acyltransferase, in the oleaginous yeast *Rhodosporidiobolus fluvialis* DMKU-RK253, Microbiology, **164**, 1–10 (2018).
22. **Uprety, B. K. and Rakshit, S. K.:** Compositional shift in fatty acid profiles of lipids obtained from oleaginous yeasts upon the addition of essential oil from *Citrus sinensis* L. Appl., Biochem. Biotechnol., **183**, 1158–1172 (2017).
23. **Yuzbasheva, E. Y., Mostova, E. B., Andreeva, N. I., Yuzbashev, T. V., Fedorov, A. S., Konova, I. A., and Sineoky, S. P.:** A metabolic engineering strategy for producing free fatty acids by the *Yarrowia lipolytica* yeast based on impairment of glycerol metabolism, Biotechnol. Bioeng., **115**, 433–443 (2018).
24. **Graef, G., LaVallee, B. J., Tenopir, P., Tat, M., Schweiger, B., Kinney, A. J., Van Gerpen, J. H., and Clemente, T. E.:** A high-oleic-acid and low-palmitic-acid soybean: agronomic performance and evaluation as a feedstock for biodiesel, Plant Biotechnol. J., **7**, 411–421 (2009).
25. **Yazawa, H., Kamisaka, Y., Kimura, K., Yamaoka, M., and Uemura, H.:** Efficient accumulation of oleic acid in *Saccharomyces cerevisiae* caused by expression of rat elongase 2 gene (*rELO2*) and its contribution to tolerance to alcohols, Appl. Microbiol. Biotechnol., **91**, 1593–1600 (2011).
26. **Gao, Q., Cao, X., Huang, Y. Y., Yang, J. L., Chen, J., Wei, L. J., and Hua, Q.:** Overproduction of fatty acid ethyl esters by the oleaginous yeast *Yarrowia lipolytica* through metabolic engineering and process optimization, ACS Synth. Biol., **7**, 1371–1380 (2018).
27. **Ledesma-Amaro, R. and Nicaud, J. M.:** *Yarrowia lipolytica* as a biotechnological chassis to produce usual and unusual fatty acids, Prog. Lipid Res., **61**, 40–50 (2016).
28. **Qiao, K., Abidi, S. H. I., Liu, H., Zhang, H., Chakraborty, S., Watson, N., Ajikumar, P. K., and Stephanopoulos, G.:** Engineering lipid overproduction in the oleaginous yeast *Yarrowia lipolytica*, Metab. Eng., **29**, 56–65 (2015).
29. **Coradetti, S. T., Pinel, D., Geiselman, G. M., Ito, M., Mondo, S. J., Reilly, M. C., Cheng, Y. F., Bauer, S., Grigoriev, I. V., Gladden, J. M., and other 4 authors:** Functional genomics of lipid metabolism in the oleaginous yeast *Rhodosporidium toruloides*, eLife, **7**, e32110 (2018).
30. **Park, Y. K., Nicaud, J. M., and Ledesma-Amaro, R.:** The Engineering potential of *Rhodosporidium toruloides* as a workhorse for biotechnological applications, Trends Biotechnol., **36**, 304–317 (2017).
31. **Sambles, C., Middelhaufe, S., Soanes, D., Kolak, D., Lux, T., Moore, K., Matoušková, P., Parker, D., Lee, R., Love, J., and Aves, S. J.:** Genome sequence of the oleaginous yeast *Rhodotorula toruloides* strain CGMCC 2.1609, Genom. Data, **13**, 1–2 (2017).
32. **Tai, M. and Stephanopoulos, G.:** Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production, Metab. Eng., **15**, 1–9 (2013).
33. **Zhu, Z., Zhang, S., Liu, H., Shen, H., Lin, X., Yang, F., Zhou, Y. J., Jin, G., Ye, M., Zou, H., and Zhao, Z. K.:** A multi-omic map of the lipid-producing yeast *Rhodosporidium toruloides*, Nat. Commun., **3**, 1112 (2012).
34. **Buček, A., Matoušková, P., Sychrová, H., Pichová, I., and Hrušková-Heidingsfeldová, O.:** Δ12-Fatty acid desaturase from *Candida parapsilosis* is a multifunctional desaturase producing a range of polyunsaturated and hydroxylated fatty acids, PLoS One, **9**, e93322 (2014).
35. **Sakuradani, E.:** Advances in the production of various polyunsaturated fatty acids through oleaginous fungus *Mortierella alpina* breeding, Biosci. Biotechnol. Biochem., **74**, 908–917 (2010).
36. **Uemura, H.:** Synthesis and production of unsaturated and polyunsaturated fatty acids in yeast: current state and perspectives, Appl. Microbiol. Biotechnol., **95**, 1–12 (2012).
37. **Wang, Y., Zhang, S., Pötter, M., Sun, W., Li, L., Yang, X., Jiao, X., and Zhao, Z. K.:** Overexpression of Δ12-fatty acid desaturase in the oleaginous yeast *Rhodosporidium toruloides* for production of linoleic acid-rich lipids, Appl. Biochem. Biotechnol., **180**, 1497–1507 (2016).
38. **Cook, H. W. and McMaster, C. R.:** Chapter 7 Fatty acid desaturation and chain elongation in eukaryotes, N. Compr. Biochem., **31**, 181–204 (2002).
39. **Stukey, J. E., McDonough, V. M., and Martin, C. E.:** Isolation and characterization of *OLE1*, a gene affecting fatty acid desaturation from *Saccharomyces cerevisiae*, J. Biol. Chem., **264**, 16537–16544 (1989).
40. **Xue, W. B., Liu, F., Sun, Z., and Zhou, Z. G.:** A Δ-9 fatty acid desaturase gene in the microalga *Myrmecia incisa* Reisigl: cloning and functional analysis, Int. J. Mol. Sci., **17**, 1143 (2016).
41. **Meesters, P. A. and Eggink, G.:** Isolation and characterization of a delta-9 fatty acid desaturase gene from the oleaginous yeast *Cryptococcus curvatus* CBS 570, Yeast, **12**, 723–730 (1996).
42. **Zhang, S., Ito, M., Skerker, J. M., Arkin, A. P., and Rao, C. V.:** Metabolic engineering of the oleaginous yeast *Rhodosporidium toruloides* IFO0880 for lipid overproduction during high-density fermentation, Appl. Microbiol. Biotechnol., **100**, 9393–9405 (2016).
43. **Díaz, T., Fillet, S., Campoy, S., Vázquez, R., Viña, J., Murillo, J., and Adrio, J. L.:** Combining evolutionary and metabolic engineering in *Rhodosporidium toruloides* for lipid production with non-detoxified wheat straw hydrolysates, Appl. Microbiol. Biotechnol., **102**, 3287–3300 (2018).
44. **Kraisintu, P., Yongmanitchai, W., and Limtong, S.:** Selection and optimization for lipid production of a newly isolated oleaginous yeast, *Rhodosporidium toruloides* DMKU3-TK16, Kasetsart J., **44**, 436–445 (2010).
45. **Tsai, Y. Y., Ohashi, T., Kanazawa, T., Polburee, P., Misaki, R., Limtong, S., and Fujiyama, K.:** Development of a sufficient and effective procedure for transformation of an oleaginous yeast, *Rhodosporidium toruloides* DMKU3-TK16, Curr. Genet., **63**, 359–371 (2017).
46. **Ito, H., Fukuda, Y., Murata, K., and Kimura, A.:** Transformation of intact yeast cells treated with alkali cations, J. Bacteriol., **153**, 163–168 (1983).
47. **Kawai, S., Hashimoto, W., and Murata, K.:** Transformation of *Saccharomyces cerevisiae* and other fungi: methods and possible underlying mechanism, Bioeng. Bugs., **1**, 395–403 (2010).
48. **Bligh, E. G. and Dyer, W. J.:** A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol., **37**, 911–917 (1959).
49. **Breuer, G., Evers, W. A., de Vree, J. H., Kleinegris, D. M., Martens, D. E., Wijffels, R. H., and Lamers, P. P.:** Analysis of fatty acid content and composition in microalgae, J. Vis. Exp., **80**, e50628 (2013).
50. **Collart, M. A. and Oliviero, S.:** Preparation of yeast RNA, Curr. Protoc. Mol. Biol., **23**, 13.12.1–13.12.5 (1993).
51. **Schmitt, M. E., Brown, T. A., and Trumpower, B. L.:** A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*, Nucleic Acids Res., **18**, 3091–3092 (1990).
52. **Artimo, P., Jonnalagedda, M., Arnold, K., Baratin, D., Csardi, G., de Castro, E., Duvaud, S., Flegel, V., Fortier, A., Gasteiger, E., and other 11 authors:** ExPASy: SIB bioinformatics resource portal, Nucleic Acids Res., **40**, W597–W603 (2012).
53. **Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., and Bairoch, A.:** ExPASy: the proteomics server for in-depth protein knowledge and analysis, Nucleic Acids Res., **31**, 3784–3788 (2003).
54. **Anamnart, S., Tomita, T., Fukui, F., Fujimori, K., Harashima, S., Yamada, Y., and Oshima, Y.:** The *P-OLE1* gene of *Pichia angusta* encodes a delta 9-fatty acid desaturase and complements the *ole1* mutation of *Saccharomyces cerevisiae*, Gene, **184**, 299–306 (1997).
55. **Stukey, J. E., McDonough, V. M., and Martin, C. E.:** The *OLE1* gene of *Saccharomyces cerevisiae* encodes the delta 9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene, J. Biol. Chem., **265**, 20144–20149 (1990).
56. **Sakuradani, E., Kobayashi, M., and Shimizu, S.:** Delta 9-fatty acid desaturase from arachidonic acid-producing fungus. Unique gene sequence and its heterologous expression in a fungus, *Aspergillus*, Eur. J. Biochem., **260**, 208–216 (1999).
57. **Bai, Y., McCoy, J. G., Levin, E. J., Sobrado, P., Rajashankar, K. R., Fox, B. G., and Zhou, M.:** X-ray structure of a mammalian stearoyl-CoA desaturase, Nature, **524**, 252–256 (2015).
58. **Lou, Y. and Shanklin, J.:** Evidence that the yeast desaturase Ole1p exists as a dimer *in vivo*, J. Biol. Chem., **285**, 19384–19390 (2010).
59. **MacKenzie, D. A., Carter, A. T., Wongwathanarat, P., Eagles, J., Salt, J., and Archer, D. B.:** A third fatty acid delta9-desaturase from *Mortierella alpina* with a different substrate specificity to ole1p and ole2p, Microbiology, **48**, 1725–1735 (2002).

60. **Shanklin, J., Whittle, E., and Fox, B. G.:** Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase, Biochemistry, **33**, 12787–12794 (1994).
61. **Santamauro, F., Whiffin, F. M., Scott, R. J., and Chuck, C. J.:** Low-cost lipid production by an oleaginous yeast cultured in non-sterile conditions using model waste resources, Biotechnol. Biofuels, **7**, 34 (2014).
62. **Fei, Q., Chang, H. N., Shang, L., Choi, J. D., Kim, N., and Kang, J.:** The effect of volatile fatty acids as a sole carbon source on lipid accumulation by *Cryptococcus albidus* for biodiesel production, Bioresour. Technol., **102**, 2695–2701 (2011).
63. **Lee, J. E., Vadlani, P. V., and Min, D.:** Sustainable production of microbial lipids from lignocellulosic biomass using oleaginous yeast cultures, J. Sustain. Bioenergy Syst., **7**, 36–50 (2017).
64. **Kruger, J. S., Cleveland, N. S., Yeap, R. Y., Dong, T., Ramirez, K. J., Nagle, N. J., Lowell, A. C., Beckham, G. T., McMillan, J. D., and Biddy, M. J.:** Recovery of fuel-precursor lipids from oleaginous yeast, ACS Sustain. Chem. Eng., **6**, 2921–2931 (2018).
65. **Koutinas, A. A., Chatzifragkou, A., Kopsahelis, N., Papanikolaou, S., and Kookos, I. K.:** Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production, Fuel, **116**, 566–577 (2014).
66. **Soccol, C. R., Dalmas Neto, C. J., Soccol, V. T., Sydney, E. B., da Costa, E. S. F., Medeiros, A. B. P., and Vandenberghe, L. P. S.:** Pilot scale biodiesel production from microbial oil of *Rhodosporidium toruloides* DEBB 5533 using sugarcane juice: performance in diesel engine and preliminary economic study, Bioresour. Technol., **223**, 259–268 (2017).
67. **Clarens, A. F., Nassau, H., Resurreccion, E. P., White, M. A., and Colosi, L. M.:** Environmental impacts of algae-derived biodiesel and bioelectricity for transportation, Environ. Sci. Technol., **45**, 7554–7560 (2011).
68. **Marrone, B. L., Lacey, R. E., Anderson, D. B., Bonner, J., Coons, J., Dale, T., Downes, C. M., Fernando, S., Fuller, C., Goodall, B., and other 9 authors:** Review of the harvesting and extraction program within the national alliance for advanced biofuels and bioproducts, Algal Res., **33**, 470–485 (2017).
69. **Richardson, J. W., Outlaw, J. L., and Allison, M.:** The economics of microalgae oil, AgBioforum, **13**, 119–130 (2010).
70. **Slade, R. and Bauen, A.:** Micro-algae cultivation for biofuels: cost, energy balance, environmental impacts and future prospects, Biomass Bioenergy, **53**, 29–38 (2013).
71. **Ullah, K., Ahmad, M., Sofia, Sharma, V. K., Lu, P., Harvey, A., Zafar, M., Sultana, S., and Anyanwu, C. N.:** Algal biomass as a global source of transport fuels: overview and development perspectives, Prog. Nat. Sci. Mater., **24**, 329–339 (2014).
72. **Miyazaki, M., Bruggink, S. M., and Ntambi, J. M.:** Identification of mouse palmitoyl-coenzyme A delta9-desaturase, J. Lipid Res., **47**, 700–704 (2006).
73. **Wongwathanarat, P., Michaelson, L. V., Carter, A. T., Lazarus, C. M., Griffiths, G., Stobart, A. K., Archer, D. B., and MacKenzie, D.:** Two fatty acid delta9-desaturase genes, *ole1* and *ole2*, from *Mortierella alpina* complement the yeast *ole1* mutation, Microbiology, **145**, 2939–2946 (1999).
74. **Zheng, Y., Prouty, S. M., Harmon, A., Sundberg, J. P., Stenn, K. S., and Parimoo, S.:** *Scd3*–a novel gene of the stearoyl-CoA desaturase family with restricted expression in skin, Genomics, **71**, 182–191 (2001).
75. **Dobrzyn, A. and Ntambi, J. M.:** Stearoyl-CoA desaturase as a new drug target for obesity treatment, Obes. Rev., **6**, 169–174 (2005).
76. **Jones, B. H., Maher, M. A., Banz, W. J., Zemel, M. B., Whelan, J., Smith, P. J., and Moustaïd, N.:** Adipose tissue stearoyl-CoA desaturase mRNA is increased by obesity and decreased by polyunsaturated fatty acids, Am. J. Physiol., **271**, E44–E49 (1996).
77. **Boutet, E., Mourabit, H. E. I., Prot, M., Nemani, M., Khallouf, E., Colard, O., Maurice, M., Durand-Schneider, A. M., Chrétien, Y., Grès, S., and other 4 authors:** Seipin deficiency alters fatty acid Delta9 desaturation and lipid droplet formation in Berardinelli-Seip congenital lipodystrophy, Biochimie, **91**, 796–803 (2009).
78. **Brock, T. J., Browse, J., and Watts, J. L.:** Fatty acid desaturation and the regulation of adiposity in *Caenorhabditis elegans*, Genetics, **176**, 865–875 (2007).
79. **Covino, R., Ballweg, S., Stordeur, C., Michaelis, J. B., Puth, K., Wernig, F., Bahrami, A., Ernst, A. M., Hummer, G., and Ernst, R.:** A eukaryotic sensor for membrane lipid saturation, Mol. Cell., **63**, 49–59 (2016).
80. **Fang, Z., Chen, Z., Wang, S., Shi, P., Shen, Y., Zhang, Y., Xiao, J., and Huang, Z.:** Overexpression of *OLE1* enhances cytoplasmic membrane stability and confers resistance to cadmium in *Saccharomyces cerevisiae*, Appl. Environ. Microbiol., **83**, e02319-16 (2016).
81. **Rohwedder, A., Zhang, Q., Rudge, S. A., and Wakelam, M. J.:** Lipid droplet formation in response to oleic acid in Huh-7 cells is mediated by the fatty acid receptor FFAR4, J. Cell Sci., **127**, 3104–3115 (2014).
82. **Shi, X., Li, J., Zou, X., Greggain, J., Rødkær, S. V., Færgeman, N. J., Liang, B., and Watts, J. L.:** Regulation of lipid droplet size and phospholipid composition by stearoyl-CoA desaturase, J. Lipid Res., **54**, 2504–2514 (2013).
83. **Li, Z. Z., Berk, M., McIntyre, T. M., and Feldstein, A. E.:** Hepatic lipid partitioning and liver damage in nonalcoholic fatty liver disease: role of stearoyl-CoA desaturase, J. Biol. Chem., **284**, 5637–5644 (2009).
84. **Martin, C. E., Oh, C. S., and Jiang, Y.:** Regulation of long chain unsaturated fatty acid synthesis in yeast, Biochim. Biophys. Acta, **1771**, 271–285 (2007).
85. **Le Hir, H., Nott, A., and Moore, M. J.:** How introns influence and enhance eukaryotic gene expression, Trends Biochem. Sci., **28**, 215–220 (2003).
86. **Rose, A. B.:** Intron-mediated regulation of gene expression, Curr. Top. Microbiol. Immunol., **326**, 277–290 (2008).
87. **Spicer, A. and Molnar, A.:** Gene editing of microalgae: scientific progress and regulatory challenges in Europe, Biology (Basel), **7**, E21 (2018).