Understanding and exploiting the fatty acid desaturation system in *Rhodotorula toruloides*

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**Abstract**

**Background:** *Rhodotorula toruloides* is a robust producer of triacylglycerol owing to its fast growth rate and strong metabolic flux under conditions of high cell density fermentation. However, the molecular basis of fatty acid biosynthesis, desaturation and regulation remains elusive.

**Results:** We present the molecular characterization of four fatty acid desaturase (FAD) genes in *R. toruloides*. Biosynthesis of oleic acid (OA) and palmitoleic acid (POA) was conferred by a single-copy ∆9 Fad (Ole1) as targeted deletion of which abolished the biosynthesis of all unsaturated fatty acids. Conversion of OA to linoleic acid (LA) and α-linolenic acid (ALA) was predominantly catalyzed by the bifunctional ∆12/∆15 Fad2. FAD4 was found to encode a trifunctional ∆9/∆12/∆15 FAD, playing important roles in lipid and biomass production as well as stress resistance. Furthermore, an abundantly transcribed *OLE1*-related gene, *OLE2* encoding a 149-aa protein, was shown to regulate Ole1 regioselectivity. Like other fungi, the transcription of FAD genes was controlled by nitrogen levels and fatty acids in the medium. A conserved DNA motif, (T/C)(G/A)TTG CAG A(T/C)CCCAG, was demonstrated to mediate the transcription of *OLE1* by POA/OA. The applications of these FAD genes were illustrated by engineering high-level production of OA and γ-linolenic acid (GLA).

**Conclusion:** Our work has gained novel insights on the transcriptional regulation of FAD genes, evolution of FAD enzymes and their roles in UFA biosynthesis, membrane stress resistance and cell mass and total fatty acid production. Our findings should illuminate fatty acid metabolic engineering in *R. toruloides* and beyond.

**Keywords:** Fatty acid desaturase, Lipid, γ-Linolenic acid, Palmitoleic acid, Regulation

Fatty acid desaturases (FADs) catalyze the sequential desaturation of fatty acids, leading to the production of MUFAs and PUFAs. FADs are classified into two groups, water-soluble acyl–acyl carrier protein (ACP) desaturases restricted in plant plastid [2, 3] and integral membrane type FADs, which share the highly conserved membrane-spanning motif, H(X)3-H(X)7-H(X)2-H(X)61-H(X)189-H(X)2-H(X)23H(X)61-189H(X)23H(X)4 [4]. FADs can also be functionally categorized as front-end and methyl-end desaturase, which introduces a double bond towards the carboxyl terminus and methyl-end of the fatty acyl chain, respectively [3]. The front-end desaturases, such as ∆4, ∆5, ∆6 and ∆8 FADs, contain a specific N-terminal cytochrome b5-like domain, and are generally found in animals and lower eukaryotic microorganisms [5, 6].

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The expression of FADs is usually regulated by fatty acids, nutrient and environmental cues. For example, *Saccharomyces cerevisiae* OLE1 expression is regulated at transcriptional and post-transcriptional (mRNA or protein stability) levels: the transcription is activated by unsaturated fatty acids through ubiquitin-mediated proteolytic processing of two membrane proteins, Mgα2p and Spt23p, triggering their nuclear targeting to become transcriptional co-activators. Mgα2p is believed to be a sensor for unsaturated fatty acid and it also regulates OLE1 transcripts stability by modulating exonuclease activity [7, 8]. These regulatory mechanisms appear to be quite conserved among different fungi [9].

Different fatty acids vary greatly in nutritional value and biological functions due to their unique fatty acid configuration in triacylglycerol, degree of desaturation and chain length. For example, γ-linolenic acid (GLA, 18:3Δ6,9,12) has anti-inflammation property and has applications in the treatment for atopic eczema, diabetes, heart disease, high blood pressure, arthritis, Alzheimer's disease, etc. [10, 11]. GLA is present in relatively low levels in oils extracted from a small number of plant seeds, such as those of evening primrose (*Oenothera biennis*) (8–10% of total fatty acid), blackcurrant (15–20%) and *Borago officinalis* (~20%) [12]. Although filamentous fungi such as *Cunninghamella echinulata* [13] and *Mortierella isabellina* [14] also produce GLA, they are not ideal hosts for industrial production due to slow growth, low lipid content and high viscosity during fermentation.

*R. toruloides* is an oleaginous yeast producing low levels of PUFAs, including linoleic acid (LA, 18:2Δ9,12) and α-linolenic acid (ALA, 18:3Δ9,12,15) [15]. Metabolic engineering offers an opportunity to drastically change its fatty acid composition and productivity [16–20]. As a highly robust oil producer, *R. toruloides* remains a challenging host to work with due to its highly GC-rich genome (~62%); unusual regulation of gene expression and limited engineering tools [25–27]. To date, two *Rhodotorula* FAD genes have been reported, a stearoyl-CoA desaturase (GenBank accession no. CAA96757, ScOLE1p), *Mortierella alpina* Δ12 FAD (ADE06660, MaFAD2), *M. alpina* Δ6 FAD (AAL73949, MaFAD6) and *Euglena gracilis* Δ8 FAD (ADD51570) as queries, we identified 3 homologous genes, which were tentatively named OLE1, FAD2 and FAD4. However, homolog of *M. alpina* Δ5 FAD (ACM9303), *Thraustochytrium sp.* Δ4 FAD (AAM09688) and *Saprolegnia diclina* Δ17 FAD (AY373823) was not found.

Gene organization of FADs, such as coding sequence (CDS), 5′ and 3′UTR (untranslated region), was determined by incorporating the sequences of 5′ and 3′ RACE (rapid amplification of cDNA ends), RT-PCR and whole transcriptome. OLE1, FAD2 and FAD4 contain 7, 4 and 4 exons, encoding 545, 451 and 609 aa, respectively (Table 1 and Additional file 1: Fig. S1a). All splicing junctions abide strictly to the canonical GU-AG rule. Notably, OLE1 and FAD2 transcripts have long 3′UTRs, 296 nt and 261 nt, respectively, while FAD4 has a long 5′UTR of 349 nt (Table 1 and Additional file 1: Fig. S1a).

Ole1 was predicted to contain two transmembrane helices while Fad2 and Fad4 have three (Additional file 1: Fig. S1b). All three FADs contain the pfam00487 membrane domain that is highly conserved in different

| Table 1 Gene annotations |
|--------------------------|
| **Gene** | **CDS length (nt)** | **Scaffold No** | **5′UTR (nt)** | **3′UTR (nt)** | **Exon** | **Protein (aa)** | **Best hit (identity)** |
|--------------------------|
| OLE1 | 2304 | 9 | 160 | 296 | 7 | 545 | XP_016270987.1 (97%) |
| FAD2 | 1703 | 24 | 21 | 261 | 4 | 451 | XP_016269356.1 (97%) |
| FAD4 | 1604 | 25 | 349 | 69 | 4 | 476 | XP_016270876.1 (94%) |
| OLE2 | 848 | 9 | NAa | NA | 5b | 149 | XP_016270986 (67%) |

a The GenBank accession numbers of the best hits in *R. toruloides* NP11. The number in parenthesis indicates the sequence identity of the encoded protein

b Not available. Exons and CDS were predicted according to the annotation of ATCC 204091
organisms [30], and other FAD-signature motifs, such as cd03505 (Δ9 FAD-like), cd03507 (Δ12 FAD-like) or cd03506 (Δ6 FAD-like) [31]. Surprisingly, only Ole1 contains the fungus-specific fused cytochrome b5 heme/steroid binding domain (pfam00173) at the carboxyl terminus (Additional file 1: Fig. S1b), suggesting Fad2 and Fad4 rely on the free form cytochrome b5 reductase to couple the fatty acid desaturation reaction. Like many reported membrane-bound FADs, all three FADs contain three conserved histidine boxes, H(X)_{3-4}H, H(X)_{2-3}HH and H/Q(X)_{2-3}HH (Additional file 1: Fig. S1b and Additional file 1: Fig. S2), which form the di-iron complex that is essential for the desaturation reaction. The positions of the first two histidine boxes are highly conserved, separated by 31–32 aa. The 3rd histidine box is located 130 aa from the 2nd one in Ole1 while the spacing in Fad2 and Fad4 is 185 aa and 193 aa, respectively (Additional file 1: Fig. S2). Notably, the 3rd histidine box of Fad4 has an imperfect sequence, QxxHH, which is often observed in the front-end desaturases [32].

Phylogenetic analysis of eukaryotic FADs showed that R. toruloides homologs fell into three distinct groups (Additional file 1: Fig. S3). Consistent with previous work [3], it is difficult to distinguish mono-functional Δ12 FAD and Δ15 FAD from bifunctional Δ12/Δ15 FAD based on the amino acid sequences. Bifunctional enzymes with both Δ12 and Δ15 regioselectivity are believed to derive from Δ12 FAD [3]. In nature, R. toruloides strains are usually haploids with two mating types (A1 and A2) [33]. Sequence comparison revealed no amino acid sequence difference in strains of the same mating type (mating type A1 strains ATCC 10657, ATCC 204091 and IFFO0880 or mating type A2 strains ATCC 10788, MTCC457 and CECT 1137) [34] while 94.3 – 97.2% identities were observed between different mating types (Additional file 1: Table S1). However, the nucleotide sequence identities were much lower, ranging from 87.1 to 88.9%.

Regulation of FAD gene transcription

Fatty acid biosynthesis and lipid accumulation are often regulated by environmental and nutrient cues [35]. Indeed, qRT-PCR analysis showed that OLE1, FAD2 and FAD4 mRNA levels were significantly increased under 6-h nitrogen starvation, and the level of OLE1, FAD2 and FAD4 transcripts in nitrogen-free YNB medium was 1.6, 5.9- and 2.1-fold higher than in YNB medium, respectively (Fig. 1a). These suggest the involvement of common nitrogen-regulated transcriptional factors.

Studies on the gene transcriptional effects of exogenous fatty acids were concentrated on Δ9 FAD. Strong repressive effects of UFAs were reported in several yeasts [36–38], however, different effects were also reported, with minor repression or no effect in other yeasts [39, 40]. With regard to FAD2 and FAD4, it is interesting to note that UFAs resulted in different regulatory patterns [41–43]. Thus, R. toruloides FADs were investigated on their responses to different fatty acids as the sole carbon source. The three FAD genes showed significantly different transcription levels (Fig. 1b). OLE1 mRNA was the most abundant, the transcription of which could be strongly induced by palmitoleic acid (POA, 16:1 09) and significantly depressed by most other fatty acids (Fig. 1b). FAD2 transcription was significantly induced by most fatty acids tested except 18:2. Like OLE1, POA was the
strongest inducer for FAD2. This regulatory pattern was
similar to the OA-inducible pattern of Yarrowia lipolytica Δ12 FAD [44]. FAD4 transcription, on the other hand, was significantly induced by POA, 18:2 and 18:3 although the overall mRNA level was the lowest among the three FAD transcripts. Taken together, the general regulatory network of FAD gene transcription is quite conserved among different fungi. Nevertheless, R. toruloides has evolved distinct regulatory controls, such as the strong induction of FAD gene expression by POA. It would be interesting to see how common this phenomenon is in other microbes although, as suggested previously [45], it could result from its unique evolutionary history and specific niche it resides. POA is a rare fatty acid present in the cells at very low levels in most systems. We speculate that supplying high levels of this may drastically change the membrane structure or fluidity, resulting in a stress response in the cells.

**Molecular basis of transcriptional control of FAD genes**

To investigate the transcriptional regulations, the upstream sequence of OLE1 (-843 to -1 from the 1st ATG codon) was cloned and analyzed by luciferase gene reporter assay. Time course study showed that the promoter was strongly induced by POA (peaked by 16.4-fold at 1 h) (Additional file 1: Fig. S4), which agrees well with the qRT-PCR results (Fig. 2b). The promoter was also induced by OA (peaked by 5.9-fold at 4 h). As POA is much more costly, OA was used as the inducer in later transcriptional studies.

To identify the common cis-acting elements involved in the transcriptional regulation by fatty acids, the upstream sequences of OLE1, FAD2 and FAD4 (Additional file 1) were analyzed using the MEME suite [46], leading to the identification of a 15-nt conserved DNA motif (Fig. 2a). In OLE1, two such motifs with 3-nt variations were found, at -638 and -434 from the translational start site. The motifs were tentatively named ORE1 and ORE2 (OLE1 Regulatory Element), respectively, Fig. 2b). To confirm its function, ORE1 and ORE2 were individually fused to the 5’ end of the basal GPD1-176 promoter (-176 to the 1st ATG codon) [25]. Neither ORE1 nor ORE2 significantly affected GPD1-176 activity when the reporter strains were cultured in YPD medium (Fig. 2c). In contrast, the ORE1-GPD1-176 promoter showed 1.5-fold higher activity than GPD1-176 when cultured in OA-supplemented medium, whereas ORE2 showed negligible effect. To determine which of the three substituted nucleotides was functionally important, an ORE1 mutant (ORE1m) was created by converting its first 2 nucleotides to the corresponding residues in ORE2 (Fig. 2b). Reporter assay revealed a complete loss of oleate-inducing effect after the sequence change (Fig. 2c). This suggests that ORE1, and possibly some related motifs, plays a significant role in regulating FAD gene transcription. The data also suggest the possibility to engineer a strong OA/POA-inducible gene expression system in R. toruloides by using the OLE1 promoter and ORE1 motif.

The transcripts of OLE1 and FAD2 have long 3’UTRs (Table 1 and Additional file 1: Fig. S1a). To investigate if the 3’UTR of OLE1 has any role in regulating OLE1 expression, the luciferase reporter construct was modified by replacing the terminator of Cauliflower mosaic virus (CaMV) 35S to that of OLE1, including the entire 296-bp 3’UTR and 32-bp downstream sequence (Additional file 1). This resulted in a significant drop in the luciferase activity (Fig. 2d). This explains the discrepancy between the results of qRT-PCR (Fig. 1b) and promoter reporter assay (Additional file 1: Fig. S4). Thus, OA and POA modulate OLE1 transcription via the cis-acting elements located in both the upstream and downstream regions of the gene.

**OLE1 is essential for cell viability and biosynthesis of oleic acid and palmitoleic acid**

We reported previously that gene deletion frequency could reach more than 95% when using the KU70 knock-out mutant [47]. However, no OLE1 deletion mutant was obtained after repeated attempts, regardless of OA supplementation to culture media. Subsequently, a true deletion mutant (ole1Δ) was generated in another strain, R. toruloides C3 (Additional file 1: Fig. S5a). Sequence analysis revealed that the single-copy OLE1 gene is highly conserved between C3 and ATCC 10657 strains, with only 7-nt substitutions that occurred in intron regions.

As expected, ole1Δ was unable to grow in medium with glucose or saturated fatty acids as the sole carbon source (Fig. 3a). In contrast, supplementation of any UFAs, such as 16:1, 18:1, 18:2 or 18:3, rescued the growth defect caused by the lack of OLE1 gene (Fig. 3a). Therefore, ole1Δ is an UFA-auxotrophic mutant, resembling its counterpart in S. cerevisiae [48]. ole1Δ was inactive in UFA biosynthesis (Fig. 3b). The small amount of 18:1 detected was probably derived from the inoculant cells that had been cultured in OA-supplemented medium (Fig. 3b). As expected, re-introduction of wild-type OLE1 gene into the ole1Δ genome completely restored the growth defects (Fig. 3a) and lipid biosynthesis (Fig. 3b). Furthermore, UFA, such as 16:1, 18:1, 18:2 and 18:3, partially restored the fatty acid profile of ole1Δ although fatty acid titer remained much lower due to the defect in cell growth (Fig. 3c). Supplement of 16:1 or 18:1, but not 16:0 or 18:0, was able to complement the growth and fatty acid biosynthesis defects of ole1Δ (Fig. 3a and c). These suggest that other FADS were functional in the absence of Ole1.
Overexpressing *OLE1* using the strong *GPD1* promoter resulted in a 5.3- and 1.3-fold increase in 16:1 and 18:1 level, respectively (Fig. 3d). Collectively, our results suggest that, similar to its homologs in *S. cerevisiae* and *Y. lipolytica* [49], Ole1 is a Δ9 desaturase with a strong substrate preference to stearoyl-CoA over palmitoyl-CoA. Our data also support the previous studies that oleic acid plays a central role in fungal growth and metabolism [4, 48, 50]. To date, Δ9 FAD null mutants
have been reported only in two ascomycetous yeasts, *S. cerevisiae* and *Candida parapsilosis* [4, 51]. To the best of our knowledge, this is the first report on the phenotypes of *OLE1* null mutant in basidiomycetous fungi and oleaginous yeasts.
# Table 2  Strains and plasmids used in this study

| Strain/plasmid  | Characteristics                                                                 | Source |
|-----------------|----------------------------------------------------------------------------------|--------|
| **Strains**     |                                                                                  |        |
| R. toruloides   | R. toruloides host                                                                 | ATCC   |
| C3              | A haploid strain isolated from Singapore                                         | This study |
| ole1Δ           | OLE1::Hyg in C3 background                                                        | This study |
| R. toruloides   | △ku70e, ku70Δ::loxP, marker free, designed as wild-type strain                    | [47]   |
| OLEY^{GPD1}     | ku70Δ::loxP carΔ::PGPD1-OLE1::Hyg^{GPD1}                                        | This report |
| fad2Δ           | ku70Δ::loxP fadΔ::Hyg^{GPD1}                                                     | This report |
| fad2e           | ku70Δ::loxP fadΔ::loxP                                                           | This report |
| fad2C           | ku70Δ::loxP fadΔ::loxP carΔ::P^{GPD1}-FAD2-Hyg^{GPD1} (alias fad2FAD2)            | This report |
| fad4Δ           | ku70Δ::loxP fad4Δ::Hyg^{GPD1}                                                    | This report |
| fad4e           | ku70Δ::loxP fad4Δ::loxP                                                          | This report |
| fad4FAD4        | ku70Δ::loxP fadΔ::loxP carΔ::P^{GPD1}-FAD4-Hyg^{GPD1} (alias fad4FAD4)            | This report |
| fad4FAD4a       | ku70Δ::loxP fad4Δ::loxP carΔ::FAD4 allele-Hyg^{GPD1}                              | This report |
| fad24Δ          | ku70Δ::loxP fad2Δ::loxP fadΔ::Hyg^{GPD1}                                         | This report |
| fad24e          | ku70Δ::loxP fad2Δ::loxP fadΔ::loxP                                              | This report |
| fad2FAD4        | ku70Δ::loxP fad2Δ::loxP carΔ::P^{GPD1}-FAD4-Hyg^{GPD1} (alias fad2FAD4)           | This report |
| fad24FAD2       | ku70Δ::loxP fad4Δ::loxP carΔ::P^{GPD1}-FAD2-Hyg^{GPD1} (alias fad24FAD2)          | This report |
| fad24FAD4       | ku70Δ::loxP fad4Δ::loxP carΔ::P^{GPD1}-FAD4-Hyg^{GPD1} (alias fad24FAD4)          | This report |
| fad24FAD4a      | ku70Δ::loxP fad4Δ::loxP carΔ::P^{GPD1}-FAD4 allele-Hyg^{GPD1}                    | This report |
| fad24FAD2       | ku70Δ::loxP fad4Δ::loxP carΔ::FAD2 allele-Hyg^{GPD1}                              | This report |
| fad24FAD4a      | ku70Δ::loxP fad4Δ::loxP carΔ::P^{GPD1}-FAD4 allele-Hyg^{GPD1}                    | This report |
| fad24FAD2       | ku70Δ::loxP fad4Δ::loxP carΔ::FAD2 allele-Hyg^{GPD1}                              | This report |
| fad24FAD4       | ku70Δ::loxP fad4Δ::loxP carΔ::P^{GPD1}-FAD4 allele-Hyg^{GPD1}                    | This report |
| fad24FAD2       | ku70Δ::loxP fad4Δ::loxP carΔ::P^{GPD1}-FAD4 allele-Hyg^{GPD1}                    | This report |
| fad24FAD2       | ku70Δ::loxP fad4Δ::loxP carΔ::P^{GPD1}-FAD4 allele-Hyg^{GPD1}                    | This report |
| A. tumefaciens   | recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac, A. tumefaciens host for ATMT    | [71]   |
| E. coli         | XL1-Blue                                                                         | Stratagene, USA |

| **Plasmids**    |                                                                                  |        |
|-----------------|----------------------------------------------------------------------------------|--------|
| pEX2            | Sp^{GPD1}, binary vector pZP200 derivative                                        | [69]   |
| pKC2            | Sp^{GPD1}, pEX2 derivative, CAR2L-P^{GPD1}-REGFP-Hyg^{GPD1}-CAR2R^{GPD1}, for promoter analysis, gene expression and CAR2 locus integration | [68]   |
| pKCL2           | Sp^{GPD1}, pKC2 derivative, CAR2L-P^{GPD1}-RtLUC2-Hyg^{GPD1}-CAR2R, for promoter analysis, gene expression and CAR2 locus integration | [68]   |
| pKCL25          | Sp^{GPD1}, pKC2 derivative, CAR2L-P^{GPD1}-RtLUC2-Hyg^{GPD1}-CAR2R, for promoter analysis, gene expression and CAR2 locus integration | This report |
| pKCL254         | Sp^{GPD1}, pKC2 derivative, CAR2L-P^{GPD1}-RtLUC2-Hyg^{GPD1}-CAR2R, for promoter analysis and CAR2 locus integration | This report |
| pKCL255         | Sp^{GPD1}, pKC2 derivative, CAR2L-P^{GPD1}-RtLUC2-Hyg^{GPD1}-CAR2R, for promoter analysis and CAR2 locus integration | This report |
| pKCL256         | Sp^{GPD1}, pKC2 derivative, CAR2L-P^{GPD1}-RtLUC2-Hyg^{GPD1}-CAR2R, for promoter analysis and CAR2 locus integration | This report |
| pKCLF66         | Sp^{GPD1}, pKC2 derivative, CAR2L-P^{GPD1}-RtLUC2-T^{GPD1}-Hyg^{GPD1}-CAR2R, for promoter analysis and CAR2 locus integration | This report |
| pKCLF661        | Sp^{GPD1}, pKC2 derivative, CAR2L-P^{GPD1}-RtLUC2-T^{GPD1}-Hyg^{GPD1}-CAR2R, for promoter analysis and CAR2 locus integration | This report |
| pKCLP4          | Sp^{GPD1}, pKC2 derivative, CAR2L-P^{GPD1}-RtLUC2-Hyg^{GPD1}-CAR2R, for promoter analysis and CAR2 locus integration | [26]   |
| pKOFAD2         | Sp^{GPD1}, pEX2 derivative, FAD2L-Hyg^{GPD1}-CAR2R, for deletion of FAD2           | This report |
| pKOFAD4         | Sp^{GPD1}, pEX2 derivative, FAD4L-Hyg^{GPD1}-CAR2R, for deletion of FAD4           | This report |
| pNE1OLE1ca      | Sp^{GPD1}, CAR2L-OLE1 allele-Hyg^{GPD1}-CAR2R, for complementation of ole1Δ        | This report |
| pKC2FAD4        | Sp^{GPD1}, CAR2L-P^{GPD1}-FAD4-Hyg^{GPD1}-CAR2R, for complementation of FAD4      | This report |
**Table 2 (continued)**

| Strain/plasmid | Characteristics | Source |
|----------------|-----------------|--------|
| pKC2FAD4a      | Sp<sup>+</sup>, pKC2 derivative, Hyg<sup>+</sup>-FAD4 allele, for complementation | This report |
| pKC2MF2        | Sp<sup>+</sup>, pKC2 derivative, CAR2L-P<sub>GPD1</sub>-MaFAD2-2-Hyg<sup>+</sup>-CAR2R, for overexpression of MaFAD2-2 | This report |
| pKC2LF3        | Sp<sup>+</sup>, pKC2 derivative, CAR2L-P<sub>GPD1</sub>-LuFAD3-2-Hyg<sup>+</sup>-CAR2R, for overexpression of LuFAD3-2 | This report |
| pKC2ML         | Sp<sup>+</sup>, pKC2 derivative, CAR2L-P<sub>GPD1</sub>-MaFAD2-2-P<sub>GPD1</sub>-LuFAD3-2-Hyg<sup>+</sup>-CAR2R, for overexpression of MaFAD2-2 and LuFAD3-2 | This report |
| pKP4OLE1       | Sp<sup>+</sup>, pKCPL4 derivative, P<sub>LDP1</sub>-RtLUC2-Hyg<sup>+</sup>, for overexpression of OLE1 | This report |
| pKP4MF2        | Sp<sup>+</sup>, pKCPL4 derivative, CAR2L-P<sub>LDP1</sub>-MaFAD2-2-Hyg<sup>+</sup>-CAR2R, for overexpression of MaFAD2-2 | This report |
| pKP4MF6        | Sp<sup>+</sup>, pKCPL4 derivative, CAR2L-P<sub>LDP1</sub>-MaFAD6-2-Hyg<sup>+</sup>-CAR2R, for overexpression of MaFAD6-2 | This report |
| pKP4MF6        | Sp<sup>+</sup>, pKCPL4 derivative, CAR2L-P<sub>LDP1</sub>-MaFAD2-2-P<sub>LDP1</sub>-MaFAD6-2-Hyg<sup>+</sup>, for overexpression of MtFAD2-2 and MtFAD6-2 | This report |

*<sup>a</sup> loxP (locus of X-over P1) used are the 34-bp lox77 and lox66 mutant Cre recombinase sites [72]  
*<sup>b</sup> Hyg<sup>+</sup> represents the hygromycin resistance gene cassette P<sub>GPD1</sub>-HPT-3-T<sub>SP</sub>, where P<sub>GPD1</sub>, HPT-3 and T<sub>SP</sub> is the glyceraldehyde 3-phosphate dehydrogenase promoter of *Rhodotorula gracilis* WP1 (UQ806386) [25], codon-optimized E. coli hygromycin phosphotransferase gene (UQ806387) [25] and the terminator of Simian virus 40 [73], respectively  
*<sup>c</sup> Sp<sup>+</sup> represents the spectinomycin resistant gene  
*<sup>d</sup> T-DNA regions of the binary plasmids

**FAD2 encodes a Δ12/Δ15 bifunctional fatty acid desaturase**  
The *FAD2* null mutant (*fad2Δ*, Table 2) was also generated (Additional file 1: Fig. S5b). Unlike *ole1Δ*, *fad2Δ* was able to grow normally without the presence of 18:2 in media (Fig. 6a, b), suggesting that Fad2 is dispensable for cell growth. The production of both 18:2 and 18:3 was abolished in *fad2Δ* while 18:1 was normal (Fig. 4a). These data suggest that Fad2 functions as both the Δ12 and Δ15 FAD to convert 18:1 to 18:2 and 18:3.

The enzymatic function of Fad2 was further investigated by complementation with certain substrates and genes. Addition of 18:2 did not restore the 18:3 level in *fad2Δ* (Fig. 4a), which is consistent with the lack of Δ15 FAD in *fad2Δ*. As *M. alpina* Δ12 FAD (MaFAD2) and *Linum usitatissimum* Δ15 FAD (LuFAD3) have been functionally confirmed [52, 53], the encoding genes were synthesized after codon optimization (MaFAD2-2 and LuFAD3-2, respectively) and used in the test. Introduction of *MaFAD2-2* (strain fad2MF2, Table 2) only restored the production of 18:2 (Fig. 4b). Introduction of *LuFAD3-2* (strain fad2LF3, Table 2) had little effect on fatty acid profile (Fig. 4b). 18:3 was produced only when *MaFAD2-2* and *LuFAD3-2* were co-expressed (strain fad2ML, Table 2) (Fig. 4b). As expected, re-introduction of the endogenous *FAD2* gene into *fad2Δ* (strain fad2FAD2, Table 2) restored the biosynthesis of UFAs (Fig. 4c). Collectively, Fad2 is a bifunctional FAD with Δ12 and Δ15 activities.

**FAD4 encodes a minor multi-functional desaturase with low regioselectivity**  
The primary structure of Fad4 is more related to Δ4, Δ5, Δ6 and Δ8 FADs (Additional file 1: Fig. S3). However, there was no Δ6 (e.g., γ-linolenic acid, GLA) or Δ8 fatty acid detected in *R. toruloides* oil. Fad4 and Fad2 share 20.5% identity at amino acid level and 44.9% identity at cDNA level, suggesting that *FAD4* may be derived from gene duplication or horizontal gene transfer from *FAD2*-related gene. Due to the presence of a di-proline motif at the N-terminus (P<sub>3</sub>-P<sub>4</sub>), Fad4 might be destabilized related gene. Due to the presence of a di-proline motif at the N-terminus (P<sub>3</sub>-P<sub>4</sub>), Fad4 might be destabilized.

To further investigate Fad4 function, *FAD2* and *FAD4* were overexpressed in *fad4Δ* (strain fad4FAD2 and fad4FAD4, respectively, Table 2) using the strong *GPD1* promoter. The increase of 18:3 level upon overexpression of either *FAD2* or *FAD4* strongly suggests both enzymes have the bifunctional Δ12/Δ15 FAD activity (Fig. 5a). The drop of 18:2 level was likely the result of substrate consumption by the Δ15 FAD activity. Notably, the lack of *FAD4* significantly enhanced lipid content and cell mass production (Fig. 5b), suggesting its role in suppressing lipid biosynthesis and cell growth.

These results prompted us to re-examine the fatty acid profile of *fad2Δ*. Indeed, trace amount of 18:2 (0.06 ± 0.01% TFA) was found, accounting for 0.7% of wild-type strain (Fig. 5c). Thus, we created a double mutant for *FAD2* and *FAD4* (*fad2Δ*, Table 2). As expected, 18:2 disappeared completely in *fad2Δ*
Overexpression of FAD2 in fad2Δ (strain fad24FAD2, Table 2) largely restored the fatty acid profile. Significantly, 18:2 and 18:3 levels were increased at the expense of their precursor 18:1 (Fig. 5d), reinforcing our conclusion for the Δ12/Δ15 FAD activity of Fad2. In contrast, FAD4 overexpression in fad2Δ background (strain fad24FAD4, Table 2) led to a slight increase of 18:1 (Fig. 5d), suggesting that Fad4 possessing a weak Δ9 FAD activity. To test this hypothesis, FAD4 was overexpressed in ole1Δ (strain ole1FAD4, Table 2). Although this strain remained defective in growth (Fig. 5e and Additional file 1: Fig. S6), 18:1, 18:2 and 18:3 levels were all increased (Fig. 5f). Notably, the increase in 18:2 and 18:3 levels were not accompanied by a drop of 18:1, a phenomenon observed in Δ12/Δ15 FAD overexpression. This further implied that ole1FAD4 strain contained a weak Ole1-like activity. Collectively, our results suggest that Fad4 is an unusual enzyme with Δ9/Δ12/Δ15 trifunctional FAD activity. To the best of our knowledge, only one similar case has been reported to date, a Δ6 FAD with Δ9 and Δ12 FAD activity [55].

Physiological roles of FAD

Being the major constituent, the number of double bonds in the fatty acids of phospholipids is critical for the physical property of cell membranes [56]. OLE1 deletion led
to almost complete halt of cell division (cell budding) (Fig. 3a), although cell morphology was little changed (Additional file 1: Fig. S7). It was puzzling that Δ9 MUFA (18:1), but not its saturated precursor (18:0) or further desaturation products (18:2 or 18:3), was critical for cell viability. Studies in animal cells show that OA is not simply a structural element of membranes; it plays complex signaling roles also [57].

FAD2 deletion also led to slower cell growth under most conditions, which appeared to enhance the sensitivity to thermal stress (37 °C and 24 °C) and osmotic stress (glycerol or sorbitol) (Fig. 6a). As expected, the growth defect of fad2Δ was relieved by genetic complementation with a heterologous Δ12 FAD (strain fad2MF2) or Δ12 + Δ15 FAD (strain fad2ML, Fig. 6a).
Although Fad4 displayed weak activity in fatty acid desaturation, deletion of the gene significantly enhanced cell sensitivity to PUFA (18:3 in Fig. 6b). It is believed that ethanol alters plasma membrane transport [58] and increases membrane fluidity [59]. This is consistent with the previous observation that high unsaturation of fatty acids correlates with high cytotoxicity [60]. Surprisingly, overexpression of FAD4 cDNA under the strong GPD1 promoter only slightly relieved 18:3 sensitivity (Fig. 6b). On the other hand, re-introduction of the genomic FAD4 allele to fad4 mutant (fad4FAD4a) successfully complemented the growth defect (Fig. 6c), suggesting the significant role of introns in regulating FAD4 expression. This is consistent with our earlier report on other genes in this host [26]. Taken together, Fad2 and Fad4 both play important roles in protecting cells from membrane stress. These findings open a new avenue to enhance fatty acids and terpenoid productivity in R. toruloides.

Fig. 6 Stress responses. a Effects of various stress stimuli on cell growth. b Effects of unsaturated fatty acids on cell growth. c Effects of 18:3 on cell growth of fad4Δ and fad4FAD4a. Cell cultured at exponential phase were water-washed twice and spotted in tenfold serial dilutions on YPD agar plates supplemented with the indicated UFAs (0.1%, w/v) or stress-inducing chemical, and incubated at different temperatures. Cells cultured at 30 °C on YPD agar in the absence of any supplement was used as the control. fad4FAD4, fad4Δ harboring FAD4 cDNA driven under GPD1 promoter; fad4FAD4a, fad4Δ harboring whole FAD4 allele; Supp., supplementation; Temp., temperature; CPL, β-caryophyllene. Concentrations used: glycerol, 2 M; sorbitol, 1 M; ethanol, 3% (w/v); CPL, 0.1% (w/v); NaCl, 0.8 M.
OLE2 encodes a weak regulator of Ole1

During the annotation of OLE1, another DNA fragment sharing high homology to OLE1 was found in the genome. The 240-nt sequence shares 86% identity with OLE1. We tentatively named the gene OLE2 (Table 1). The conservation of this gene in different isolates, such as ATCC 10,788, NP11 and C3 strains [34, 61], suggest that it is functionally important. qRT-PCR analysis showed the sequence was abundantly transcribed and the transcripts level was regulated by fatty acids (Fig. 7a). In strain ATCC 10657, the sequence is located ~13 kb from OLE1. Notably, the predicted 149-aa Ole2 protein exhibits high identity to the region around the 3rd histidine box of Ole1, a region believed to be crucial for regioselectivity of FADs (Fig. 7b). Targeted deletion of OLE2 (Additional file 1: Fig. S5d) showed that 16:1 level dropped by 46.5% ($p < 0.01$) while 16:0 level increased by 16% ($p < 0.05$) (Fig. 7c). The levels of most other fatty acid species were not changed significantly. Deletion of OLE2 did not appear to affect cell growth significantly. Thus, OLE2 encodes a weak regulator of fatty acid desaturation, modulating the regioselectivity of Ole1. The effects of OLE2 deletion were consistent with the overexpression of OLE1 as both preferentially affected 16:1 level. Considering the strong role of 16:1 in inducing FAD gene transcription, it is possible that Ole2 can also regulate the transcription of OLE1, FAD2 and FAD4 indirectly.

Metabolic engineering of fatty acids

As a proof of concept, we demonstrated how the FAD genes could be exploited for high-level production of OA and a novel fatty acid, γ-linolenic acid (GLA) (Fig. 8a). High OA oil has many applications, including food, cosmetics, textiles, adhesives and biofuels [62, 63]. Deletion of FAD2 led to little change in the volumetric productivity (titer) of OA, however, it significantly increased OA content, from 33.2 to 63.5% TFA (Fig. 8b). Further deletion of FAD4 resulted in a 1.3-fold improvement in OA titer, although OA content was slightly decreased (Fig. 8b). To further improve OA production, the endogenous OLE1 was overexpressed using the strong and lipogenic LDP1 promoter [26], resulted in a strain with two copies of OLE1 in the genome (mutant fad2OLE1 and fad24OLE1, Table 2 and Fig. 8a). This resulted in increased OA titer (15 ~ 17%, Fig. 8b). Surprisingly, OA content was reduced in fad2Δ, but not fad24Δ background (Fig. 8b). This difference may result from the interplay between cell growth mediated by the FAD4 gene and fatty acid imbalance conferred by OLE1 overexpression. Notably, increased expression of OLE1 was reported to be toxic to the cells in S. cerevisiae [48].

M. alpina is a natural GLA producer, and its Δ12 and Δ6 FAD catalyze the final two steps of GLA biosynthesis [64]. Overexpression of M. alpina MaFAD2-2 (synthetic Δ12 FAD) along with MaFAD6-2 (synthetic Δ6 FAD) (Fig. 8a) in fad2Δ and fad24Δ (mutant fad2MF26 and fad2MF26, Table 2) successfully turned R. toruloides into a GLA producer, resulting in a titer of 0.37 g/L (28.4% TFA) and 0.53 g/L (27.4% TFA), respectively (Fig. 8c). A preliminary 2-L-scale fed-batch fermentation showed that the maximal OA and GLA titer reached 3.5 and 2.6 g/L, representing 60.1% and 27.3% TFA, respectively (Fig. 8d).
Recently, an engineered *Y. lipolytica* strain has been reported to produce GLA to 4.6% of total fatty acids. The low yield could have resulted from the toxicity of GLA as lowering the culturing temperature increased GLA yield about 61% [65]. Notably, our GLA content has far exceeded the dominant commercial product, evening primrose oil [12]. We expect further improvement in OA and GLA yields when PUFA degradation and fatty acid selectivity in TAG synthesis can be manipulated. Thus, *R. toruloides* can be a strong platform for PUFA metabolic engineering and production.

**Conclusion**

*R. toruloides* genome encodes a single-copy highly conserved Δ9 FAD, which is essential for cell viability and biosynthesis of MUFAs and PUFAs. The mutant *ole1Δ* provided a rare genetic insight into the role of Δ9 FAD on cell growth, fatty acid desaturation and lipid accumulation. As a yeast that is phylogenetically distant to the popular yeast hosts, such as *S. cerevisiae* and *Y. lipolytica*, it was not surprising to see *R. toruloides* has evolved significantly in the control of fatty acid biosynthesis and FAD gene expression, which were exemplified by the regulation of gene transcription via the GC-rich ORE1 motif, preferential induction by palmitoleic acid and involvement of two similar multi-functional FADs for PUFA biosynthesis. Fad4 is particularly interesting, not only for its relaxed regioselectivity of fatty acid desaturation, but also its roles in stress tolerance and maintaining healthy biomass and lipid production. Our data should illuminate PUFA engineering beyond this host.

**Materials and methods**

**Strains, media, and culture conditions**

Strains used are listed in Table 2. *R. toruloides* strain ATCC 10657 was obtained from ATCC (USA), and strain C3 was isolated from a fish sample in Singapore. Both are haploid (mating type A1) and share high genome sequence homology to *R. toruloides* ATCC 204091 (GenBank No. AEVR02000000) [34, 66]. *R. toruloides* strain Δku70e, a *KU70* null mutant with high frequency of homologous recombination [47], is referred as the wild-type strain in this study. Yeast strains were maintained at 28–30 °C in YPD broth (1% yeast extract, 2% peptone, 2% glucose, w/v) or on potato-dextrose agar (PDA, Sigma-Aldrich, USA). YPDtO is YPD broth supplemented with 0.1% (w/v) oleic acid and 0.5% (w/v) Tergitol NP40 and was used for propagation of *ole1Δ*.

GJm3 is a lipid accumulation medium modified from the previous report [15]. It contains (per liter) 70 g glucose, 2.5 g yeast extract, 0.4 g KH₂PO₄, 1.5 g MgSO₄·7H₂O, 40 mg CaCl₂·2H₂O, 5.5 mg FeSO₄·7H₂O, 5.2 mg citric acid·H₂O, 1 mg ZnSO₄·7H₂O, 0.76 mg
MnSO$_4$$ \cdot $H$_2$O and pH was adjusted to 6.0 with sulfuric acid.

Yeast nitrogen base (without amino acid or ammonium sulfate) containing glucose (20 g/L) was used as nitrogen starvation medium (YNB-N$^-$) while nitrogen rich medium (YNB-N$^+$) was YNB-N$^-$ supplemented with 5 g/L ammonium sulfate. Cells were cultured until exponential phase; washed twice with water; inoculated to either YNB-N$^-$ or YNB-N$^+$ and cultured at 28 °C with 280 rpm agitation. In fatty acid supplementation experiments, R. toruloides cells were cultured in YNB broth containing 5 g/L ammonium sulfate, a fatty acid of interest or glucose (10 g/L) and Tergitol™ NP40 (1%, w/v).

**DNA constructs**

DNA constructs used are listed in Table 2. Oligonucleotide sequences are listed in Additional file 1: Table S2. DNA constructs were verified by restriction mapping and DNA sequencing using the BigDye method (ABI). Details for DNA vector construction are shown in Additional file 1: Fig. S8.

**Extraction of genomic DNA and total RNA**

Genomic DNA and total RNA extraction were performed as reported previously [25]. Nucleic acids were quantified using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA) and the quality was checked by agarose gel electrophoresis.

**Rapid amplification of cDNA ends (RACE)**

5′ RACE and 3′ RACE were done using BD SMARTer™ RACE cDNA Amplification Kit (BD CLONTECH Laboratories, Palo Alto, CA, USA) according to the manufacturer’s instructions. Oligo pair OLE1U1/OLE1L1, FAD2U1/FAD2L1 and FAD4U1/FAD4L1 (Additional file 1: Table S2) was used as the specific primer for 5′ and 3′ RACE of OLE1, FAD2 and FAD4, respectively.

**Gene annotation and phylogenetic analysis**

As the genome sequences of R. toruloides strain ATCC 10657, IFFO 0880 and ATCC 204091 [34, 66] are highly similar, the annotated genome database of R. glutinis ATCC 204091 was used as the reference. FAD genes were identified using tBLASTn at NCBI against the reference genome database using protein sequences of various types of well-characterized FADs as the queries. Full-length cDNA sequences were obtained by RT-PCR after the 5′ and 3′ ends were determined by RACES. The FAD orthologs of other Pucciniomycotina species were predicted by BLASTp program. Sequence alignment and phylogenetic analysis were performed with the MEGA 6 program (www.megasoftware.net) using the Neighbor-Joining algorithm [67]. The membrane configurations of the proteins were predicted at the transmembrane prediction server TMHMM-2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The consensus sequences were analyzed through the MEME suite server (http://meme-suite.org/) [46].

**Genetic manipulation**

Agrobacterium tumefaciens-mediated transformation (ATMT), targeted gene deletion and fungal colony PCR were performed as described previously [25, 47]. Oleic acid was supplemented to the media for co-culture and selection in order to obtain OLE1 the deletion mutant while LA was supplemented to culture media to facilitate the generation of FAD2 and FAD4 mutants.

Gene expression cassettes were usually site-specifically integrated to the CAR2 locus (encoding phytoene synthase/lycopene cyclase) to eliminate positional effects. Knock-in mutants were selected based on the albino phenotype [26, 47, 68]. At least 3 biological replicates were used in the assays.

**Southern blot analysis**

Genomic DNA (5 µg) was digested with a restriction enzyme and separated by electrophoresis in a 0.8% agarose gel. Southern blotting was performed as described using digoxigenin-labeled DNA as the probes [69]. The restriction enzymes and DNA probes used are shown in Additional file 1: Fig. S5a-d.

**Analyses of gene expression**

Total RNA preparation, cDNA synthesis, real-time PCR analysis and luciferase gene reporter assay were performed as reported previously [26]. Briefly, R. toruloides strains harboring different reporter cassettes were cultured in YPD broth until exponential phase. Cells were cultured for 8 h in fresh YPD broth, which may be supplemented with a fatty acid of interest at 0.1% (w/v).

**Quantification methods**

Quantification of cell biomass (dry cell weight), residual glucose and lipids were performed as previously reported [70]. Fatty acid profile was determined by gas chromatography–mass spectrometry (GCMS) after esterified to fatty acid methyl esters (FAMEs) as described previously [70]. The specific fatty acids were quantified by normalization against the internal standard (ISTD, 15:0) and the corresponding response factor against ISTD as calculated through a pre-run of
the standard FAME mixture (Supelco 37 Component FAME Mix, Sigma, USA).

GenBank accession numbers
Based on the codon preference of highly expressed genes in *R. toruloides*, MaFAD2-2, MaFAD6-2, LuFAD3-2 were synthesized by GenScript (USA) according to the protein sequence of *M. alpina* FAD2 (ADE06660), *M. alpina* FAD6 (AAL73949), *L. usitatissimum* omega-3 desaturase (AFN53677), respectively. The nucleotide sequences have been deposited to GenBank under the accession number MF152712 through MF152717.

Abbreviations
ATCC: American Type Culture Collection; CDS: Coding sequence; ER: Endoplasmic reticulum; 16:1/POA: Palmitoleic acid (16:1Δ9); 18:1/OA: Oleic acid (18:1Δ9); 18:2/LA: Linoleic acid (18:2Δ9,12); 18:3/ALA: α-Linolenic acid (18:3Δ9,12,15); GLA: γ-Linolenic acid (18:3Δ6,9,12); FAD: Fatty acid desaturase; Ole1: Δ9 Stearoyl-CoA amplification of cDNA ends; TFA: Total fatty acids; UTR: Untranslated region.

OLE1-Related gene; RACE: Rapid ole1∆ mutant. YL, CMJK, SAY and CL interpreted the data. YL drafted and LJ revised the manuscript. All authors read and approved the final manuscript.

Authors' contributions
LJ and YL conceived and designed the experiments. YL and CMJK performed sequence analysis, gene deletion, real-time PCR, Southern blotting, cell cultures, metabolite analysis and physiological studies. SAY contributed to plasmid construction, yeast transformation and fatty acid profiling. CL generated the ole1A mutant. YL, CMJK, SAY and CL interpreted the data. YL drafted and LJ revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The authors declare that all data supporting the findings of this study are available within the paper and its supplementary information files or from the corresponding author on request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests. Temasek Life Sciences Laboratory has interests in developing *Rhodotorula toruloides* as an industrial biotechnology platform.

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Additional file 1. Additional tables and figures.

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References
1. Sampath H, Ntambi JM. Polyunsaturated fatty acid regulation of genes of lipid metabolism. Annu Rev Nutr. 2005;25:317–40.
2. Shanklin J, Cahoon EB. Desaturation and related modifications of fatty acids. Annu Rev Plant Physiol Plant Mol Biol. 1998;49(1):611–41.
3. Sperling P, Ternes P, Zank TK, Heinz E. The evolution of desaturases. Prostaglandins Leukot Essent Fatty Acids. 2003;68(2):73–95.
4. Stukey JE, McDonough VM, Martin CE. The OLE1 gene of Saccharomyces cerevisiae encodes the Δ9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene. J Biol Chem. 1990;265(33):20144–9.
5. Los DA, Murata N. Structure and expression of fatty acid desaturases. Biochim Biophys Acta. 1998;1394(1):13–15.
6. Meesapyodsuk D, Qiu X. The front-end desaturase: structure, function, evolution and biotechnological use. Lipids. 2012;47(3):227–37.
7. Kandasamy P, Vemula M, Oh CS, Chellappa R, Martin CE. The endoplasmic reticulum membrane protein, Mga2p, a transcription Activator of the OLE1 gene, regulates the stability of the OLE1 mRNA through exosome-mediated mechanisms. J Biol Chem. 2004;279(35):36856–92.
8. Chellappa R, Kandasamy P, Oh CS, Jiang Y, Vemula M, Martin CE. The membrane proteins, Spt23p and Mga2p, play distinct roles in the activation of S. cerevisiae OLE1 gene expression. Fatty acid-mediated regulation of Mga2p activity is independent of its proteolytic processing into a soluble transcription activator. J Biol Chem. 2001;276(47):43548–56.
9. Oh C-S, Martin CE. Candida albicans Spt23p controls the expression of the OLE1p Δ9 fatty acid desaturase and regulates unsaturated fatty acid biosynthesis. J Biol Chem. 2006;281(11):7030–9.
10. Kapoor R, Huang YS. Gamma linolenic acid: an antiinflammatory omega-6 fatty acid. Curr Pharm Biotechnol. 2006;7(6):531–4.
11. Horrobin D.F. Clinical applications of n-6 essential fatty acids: atopic eczema and inflammation, diabetic neuropathy and retinopathy, breast pain and viral infections. In: Sinclair A, Gibson R editors. Essential fatty acids and eicosanoids. Champaign: American Oil Chemists’ Society; 1992. p. 367–372.
12. Huang YS, Ziboh VA. Chapter 1. Gamma linolenic acid: an introduction. In: Huang Y-S, Ziboh VA editors. Gamma linolenic acid: recent advances in biotechnology and clinical applications. Champaign: American Oil Chemists’ Society, 2001. p. 1–5.
13. Lu S, Yue Q, Zhou S, Yan J, Zhang X, Ma F. Trehalose contributes to gamma-linolenic acid accumulation in *Cunninghamella echinulata* based on de novo transcriptomic and lipidomic analyses. Front Microbiol. 2018;9(1296):1296.
14. Papanikolaou S, Sarantou S, Komaitis M, Aggelis G. Retention of free fatty acid turnover in *Cunninghamella echinulata* and *Mortierella isabellina* cultivated in multiple-limited media. J Appl Microbiol. 2004;97(4):867–75.
15. Jin G, Zhang Y, Shen H, Yang X, Xie H, Zhao ZK. Fatty acid ethyl esters production in aqueous phase by the oleaginous yeast *Rhodospirillum toruloides*. Bioresour Technol. 2013;150:266–70.
16. Qiao K, Wasylenko TM, Zhou K, Xu P, Stephanopoulos G. Lipid production in *Yarrowia lipolytica* is maximized by engineering cytosolic redox metabolism. Nat Biotechnol. 2017;35:173.
17. Yu T, Zhou YJ, Huang M, Liu Q, Pereira R, David F, Nielsen J. Reprogramming yeast metabolism from alcoholic fermentation to lipogenesis. Cell. 2018;174(6):1549-1558.e1514.
26. Liu Y, Yap SA, Koh CM, Ji L. Developing a set of strong intronic promoters.

25. Liu Y, Koh CM, Sun L, Hlaing MM, Du M, Peng N, Ji L. Characterization and metabolic engineering of oleaginous yeasts for production of fuels and chemicals. Front Microbiol. 2017;8:2185.

24. Park YK, Nicaud JM, Ledesma-Amaro R. The engineering potential of Rhodosporidium toruloides—a potential red yeast chassis for lipids and beyond. FEBS Yeast Res. 2020.

23. Coradetti ST, Pinel D, Geiselman GM, Ito M, Mondo SJ, Reilly MC, Cheng et al. Biotechnol Biofuels (2021) 14:73

22. Wen Z, Zhang S, Oodoh CK, Jin M, Zhao ZK. Rhodosporidium toruloides—a potential red yeast chassis for lipids and beyond. FEBS Yeast Res. 2020.

21. Wen Z, Zhang S, Oodoh CK, Jin M, Zhao ZK. Rhodosporidium toruloides—a potential red yeast chassis for lipids and beyond. FEBS Yeast Res. 2020.

20. Ledesma-Amaro R, Dulermo R, Niehus X, Nicaud JM. Combining meta-

19. Kim HM, Chae TU, Choi SY, Kim WJ, Lee SY. Engineering of an oleaginous bacterium for the production of fatty acids and fuels. Nat Chem Biol. 2019;15(7):721–9.

18. Shi S, Zhao H. Metabolic engineering of oleaginous yeasts for production of fuels and chemicals. Front Microbiol. 2017;8:2185.

17. Liu Y, Koh CM, Sun L, Hlaing MM, Du M, Peng N, Ji L. Characterizing metabolic engineering of oleaginous yeast Rhodosporidium toruloides. Elife. 2018;7:e32110.

16. Coradetti ST, Pinel D, Geiselman GM, Ito M, Mondo SJ, Reilly MC, Cheng et al. Biotechnol Biofuels (2021) 14:73

15. Luo SF, Tolstorukov MI, Anamnart S, Kaneko Y, Harashima S. Cloning, sequencing, and functional analysis of H-OLE1 gene encoding delta9 fatty acid desaturase in Hansenula polymorpha. Appl Microbiol Biotechnol. 2000;54(4):499–509.

14. Kajiwara S. Molecular cloning and characterization of the Δ9 fatty acid desaturase gene and its promoter region from Saccharomyces cerevisiae. FEMS Yeast Res. 2002;2(3):333–9.

13. Watanabe K, Oura T, Sakai H, Kajiwara S. Yeast Delta 12 fatty acid desaturase gene cloning, expression, and function. BioSci Biotechnol Biochem. 2004;68(3):721–7.

12. Oura T, Kajiwara S. Saccharomyces cerevisiae FAD3 encodes an omega3 fatty acid desaturase. Microbiology. 2004;150(Pt 6):1983–90.

11. Sangvallek J, Kaneko Y, Tsukamoto T, Marui M, Sugiyama M, Ono H, Bamba T, Fukusaki E, Harashima S. Cloning and functional analysis of HpFAD2 and HpFAD3 genes encoding Delta12- and Delta15-fatty acid desaturases in Hansenula polymorpha. Gene. 2014;533(1):110–8.

10. Tezaki S, Iwama R, Kobayashi S, Shioya Y, Yoshikawa H, Ohta A, Horiuichi H, Fukuda R. Delta12-fatty acid desaturase is involved in growth at low temperature in yeast. Yarrowia lipolytica. Biochem Biophys Res Commun. 2017;488(1):165–70.

9. Santomartino R, Riego-Ruiz L, Bianchi MM. Three, two, one yeast fatty acid desaturases: regulation and function. World J Microbiol Biotechnol. 2017;33(5):89.

8. Bailey TL, Boden M, Buske FA, Firth M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res. 2009;37:202–21.

7. Park YK, Nicaud JM, Ledesma-Amaro R. The engineering potential of Rhodosporidium toruloides as a workhorse for biotechnological applications. Trends Biotechnol. 2018;36(3):304–17.

6. Liu Y, Koh CM, Sun L, Hlaing MM, Du M, Peng N, Ji L. Characterization of glyceraldehyde-3-phosphate dehydrogenase gene RGDPI5 and development of a genetic transformation method by dominant selection in oleaginous yeast Rhodosporidium toruloides. Appl Microbiol Biotechnol. 2013;97(2):719–29.

5. Liu Y, Yap SA, Koh CM, Ji L. Developing a set of strong intronic promoters for robust metabolic engineering in oleaginous Rhodotorula (Rhodo-

4. Liu Y, Koh CM, Sun L, Hlaing MM, Du M, Peng N, Ji L. Characterization of glyceraldehyde-3-phosphate dehydrogenase gene RGDPI5 and development of a genetic transformation method by dominant selection in oleaginous yeast Rhodosporidium toruloides. Appl Microbiol Biotechnol. 2013;97(2):719–29.

3. Liu Y, Koh CM, Sun L, Hlaing MM, Du M, Peng N, Ji L. Characterization of glyceraldehyde-3-phosphate dehydrogenase gene RGDPI5 and development of a genetic transformation method by dominant selection in oleaginous yeast Rhodosporidium toruloides. Appl Microbiol Biotechnol. 2013;97(2):719–29.

2. Liu Y, Koh CM, Sun L, Hlaing MM, Du M, Peng N, Ji L. Characterization of glyceraldehyde-3-phosphate dehydrogenase gene RGDPI5 and development of a genetic transformation method by dominant selection in oleaginous yeast Rhodosporidium toruloides. Appl Microbiol Biotechnol. 2013;97(2):719–29.
61. Zhu Z, Zhang S, Liu H, Shen H, Lin X, Yang F, Zhou YJ, Jin G, Ye M, Zou H, et al. A multi-oomic map of the lipid-producing yeast *Rhodosporidium toruloides*. Nat Commun. 2012;3:1112.

62. Cosgrove JP, Hayden JG, Robison PL. Process for making high-purity oleic acid. 1993, U.S. Patent No. 5,194,640.

63. Liu Q, Singh S, Green A. High-oleic and high-stearic cottonseed oils: nutritionally improved cooking oils developed using gene silencing. J Am Coll Nutr. 2002;21(3 Suppl):205S-211S.

64. Huang YS, Chaudhary S, Thurmond JM, Bobik EG Jr, Yuan L, Chan GM, Kirchner SJ, Mukerji P, Knutson DS. Cloning of delta 12- and delta6-desaturases from *Mortierella alpina* and recombinant production of gamma-linolenic acid in Saccharomyces cerevisiae. Lipids. 1999;34(7):649–59.

65. Sun M-L, Madzak C, Liu H-H, Song P, Ren L-J, Huang H, Ji X-J. Engineering *Yarrowia lipolytica* for efficient γ-linolenic acid production. Biochem Eng J. 2017;117:172–80.

66. Paul D, Magbanua Z, Arick M 2nd, French T, Bridges SM, Burgess SC, Lawrence ML. Genome sequence of the oleaginous yeast *Rhodotorula glutinis* ATCC 204091. Genome Announc. 2014. https://doi.org/10.1128/genomeA.00046-14.

67. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30(12):2725–9.

68. Liu Y, Koh CM, Ngoh ST, Ji L. Engineering an efficient and tight D-amino acid-inducible gene expression system in *Rhodosporidium/Rhodotorula* species. Microb Cell Fact. 2015;14(1):170.

69. Liu Y, Koh CM, Sun L, Ji L. Tartronate semialdehyde reductase defines a novel rate-limiting step in assimilation and bioconversion of glycerol in *Ustilago maydis*. PLoS ONE. 2011;6(1):e16438.

70. Liu Y, Koh CM, Yap SA, Du M, Hlaing MM, Ji L. Identification of novel genes in the carotenogenic and oleaginous yeast *Rhodotorula toruloides* through genome-wide insertional mutagenesis. BMC Microbiol. 2018;18(1):14.

71. Lazo GR, Stein PA, Ludwig RA. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. Biotechnology (N Y). 1991;9(10):963–7.

72. Araki K, Okada Y, Araki M, Yamamura K. Comparative analysis of right element mutant lox sites on recombination efficiency in embryonic stem cells. BMC Biotechnol. 2010;10:29.

73. Wickens M, Stephenson P. Role of the conserved AAUAAA sequence: four AAUAAA point mutants prevent messenger RNA 3’ end formation. Science. 1984;226(4678):1045–51.

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