

RESEARCH ARTICLE

Promoters for lipogenesis-specific downregulation in *Yarrowia lipolytica*

Annapurna Kamineni, Shuyan Chen, Gamuchirai Chifamba and Vasiliki Tsakraklides^{*,†}

Novogy, Inc., 85 Bolton Street, Cambridge MA 02140, USA

*Corresponding author: Novogy, Inc., 85 Bolton Street, Cambridge MA 02140, USA. Tel: +857-242-5207; E-mail: vtsakraklides@novogyinc.com

One sentence summary: Identification of promoters with a novel expression pattern to specifically reduce targeted activities in the lipid accumulation phase of the industrially important oleaginous yeast *Yarrowia lipolytica*.

Editor: Hyun Ah Kang

[†]Vasiliki Tsakraklides, <http://orcid.org/0000-0003-1998-1075>

ABSTRACT

Yarrowia lipolytica is a non-conventional yeast with potential applications in the biofuel and biochemical industries. It is an oleaginous yeast that accumulates lipids when it encounters nutrient limitation in the presence of excess carbon. Its molecular toolbox includes promoters for robust constitutive expression, regulated expression through the addition of media components and inducible expression during lipid accumulation. To date, no promoters have been identified that lead to downregulation at the transition from growth to lipid accumulation. We identified four native *Y. lipolytica* promoters that downregulate the expression of genes at this natural transition. Using the fatty acid desaturase genes *FAD2* and *OLE1* as reporter genes for these promoters, we correlated repression of desaturase transcript levels with a reduction of desaturated fatty acids at the transition to lipid accumulation. These promoters can restrict to the growth phase an essential or favorable activity that is undesirable during lipid accumulation under traditional fermentation conditions without media additions. This expression pattern results in lipogenesis phase-specific changes that could be useful in applications relating to optimizing lipid yield and composition.

Keywords: *Yarrowia lipolytica*; regulated promoters; expression; repression; lipid

INTRODUCTION

Yarrowia lipolytica is an attractive host organism for industrial production of biofuels and chemicals (Nicaud 2012; Ganesan et al. 2019; Miller and Alper 2019). It is a non-conventional oleaginous yeast that is Generally Regarded as Safe (GRAS) and is amenable to metabolic engineering thanks to the availability of its genome sequence and wide array of genetic tools.

Numerous promoters have been identified and developed for metabolic pathway engineering efforts in *Y. lipolytica* (reviewed in (Madzak 2015; Larroude et al. 2018; Ganesan et al. 2019)). The promoter from the *Y. lipolytica* alkaline extracellular

protease *XPR2* gene, was one of the first promoters identified and is still widely used (Blanchin-Roland, Cordero Otero and Gailardin 1994). While p*XPR2* is capable of strong expression of heterologous proteins, it is repressed at pH < 5.5 and requires rich media for full induction (Ogrydziak, Demain and Tannenbaum 1977). This type of complex regulation system is not compatible with many industrial processes. Strong promoters were also identified from translation elongation factor-1 α (*TEF1*), ribosomal protein S7 (*RPS7*) (Müller et al. 1998), isocitrate lyase (*ICL1*), 3-oxo-acyl-CoA thiolase (*POT1*) and acyl-CoA oxidases (*POX2*) (Juretzek et al. 2000). While p*TEF1*, p*RPS7* and p*ICL1* are used

Received: 20 April 2020; Accepted: 11 June 2020

© The Author(s) 2020. Published by Oxford University Press on behalf of FEMS. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

as constitutive promoters, pPOT1 and pPOX2 are repressed by glucose and active only in the presence of fatty acids, their derivatives, or alkanes (Müller et al. 1998; Juretzek et al. 2000). These types of preferences limit their usage in bioprocess applications. The EYK1 promoter is induced using erythrose and erythrose (Trassaert et al. 2017). This promoter can enable tight modulation of protein levels but requires a process change (addition of an inducer), limiting the culture media that can be used and adding to fermentation cost. The YAT1 promoter is induced during lipid accumulation and can be used to specifically turn on an activity during this phase of *Y. lipolytica* fermentation (Xue and Zhu 2006). Hybrid promoters fusing multiple copies of upstream activating sequences (UAS) to a core promoter region have also been developed resulting in improved promoter performance (Madzak, Tréton and Blanchin-Roland 2000; Blazek et al. 2011, 2013; Shabbir Hussain et al. 2016; Trassaert et al. 2017). Lacking from this toolbox are promoters that are naturally downregulated during lipid accumulation in order to specifically affect this phase during an industrial lipid production process.

Accumulation of lipid in *Y. lipolytica* is triggered by nutrient limitation in the presence of excess carbon. Nitrogen limitation is commonly used for lipid accumulation studies because it is an easily controlled parameter (Beopoulos et al. 2009; Ochoa-Estropier and Guillouet 2014; Friedlander et al. 2016). A typical fermentation for the production of lipids has two phases: a growth phase in which there is enough nitrogen present for the cells to divide and produce catalytic biomass, and a lipid production phase in which nitrogen depletion causes a decrease in growth rate and activation of lipid synthesis and storage. The process involves a high carbon to nitrogen ratio (C:N) such as 50:1 (G1) or 100:1 (Qiao et al. 2015) and the initial nitrogen concentration dictates the amount of lipid-free biomass. At the transition from growth phase to lipid accumulation, some pathways are repressed (nucleic acid and protein synthesis) and others are activated (fatty acid and triacylglycerol (TAG) synthesis) (Beopoulos et al. 2009; Morin et al. 2011; Ochoa-Estropier and Guillouet 2014).

In this study, we describe promoters which reduce gene expression at the natural transition of *Y. lipolytica* from biomass production to lipid accumulation. Use of these promoters ties regulation of gene expression into established methods of lipid production without changing the fermentation process. By linking the expression of two desaturase genes to these promoters, we show that we can change the cellular lipid profile in the lipid accumulation phase without the need for culture additives.

MATERIALS AND METHODS

Strains and media

Wild-type *Y. lipolytica* strain YB-392 was obtained from the ARS Culture Collection (NRRL). *Y. lipolytica* fatty acid desaturase deletion strains $\Delta ole1$ and $\Delta fad2$ were previously described and each contains a single gene replacement with the hygromycin-resistance gene in the YB-392 background (Tsakraklides et al. 2018) (Table 1). Strains were cultured in YPD (10 g/L yeast extract, 20 g/L bacto peptone and 20 g/L glucose) at 30°C. 20 g/L agar was added to prepare solid media. $\Delta ole1$ strains were grown in YPD supplemented with 1% Tween 80 and 0.01% oleic acid. Antibiotic selection was achieved with the addition of hygromycin B (300 µg/mL) or nourseothricin (500 µg/mL) as appropriate (Tsakraklides et al. 2018). Strains were characterized in sterilized lipid production media (0.5 g/L urea, 1.5 g/L yeast extract, 0.85 g/L casamino acids, 1.7 g/L Yeast Nitrogen Base without

Table 1. Strains used in this study.

Strain	Genotype	Reference
YB-392	<i>Y. lipolytica</i> wild-type strain	ARS Culture Collection (NRRL)
$\Delta fad2$	FAD2::hyg in YB-392	(Tsakraklides et al. 2018)
p1-FAD2	p1-FAD2 in $\Delta fad2$	This work
p2-FAD2	p2-FAD2 in $\Delta fad2$	This work
p3-FAD2	p3-FAD2 in $\Delta fad2$	This work
p4-FAD2	p4-FAD2 in $\Delta fad2$	This work
p5-FAD2	p5-FAD2 in $\Delta fad2$	This work
$\Delta ole1$	OLE1::hyg in YB-392	(Tsakraklides et al. 2018)
p1-OLE1	p1-OLE1 in $\Delta ole1$	This work
p4-OLE1	p4-OLE1 in $\Delta ole1$	This work
p5-OLE1	p5-OLE1 in $\Delta ole1$	This work

amino acids and ammonium sulfate, 100 g/L glucose and 5.11 g/L potassium hydrogen phthalate) in 96-well (300 µL per well) or 24-well (1.5 mL per well) deep-well plates, or in 250 mL flasks (25 mL per culture) (Friedlander et al. 2016).

Gene overexpression cassettes

Linear expression constructs were prepared using standard molecular biology techniques (all sequences in Additional File 1). Each expression construct contained an expression cassette for the desaturase gene of interest (OLE1 or FAD2) driven by the promoters described in this study, in tandem with an expression cassette for the nourseothricin selectable marker. Promoters and desaturase gene sequences were amplified from YB-392 genomic DNA and all constructs were confirmed by sequencing of the expression cassettes.

Transformation

Log-phase *Y. lipolytica* cells were washed with water and resuspended in a volume of water equal to the wet cell pellet. 50 µL was aliquoted per transformation reaction. 18 µL of desired DNA and 92 µL of transformation mix (80 µL 60% PEG4000, 5 µL 2 M DTT, 5 µL 2 M lithium acetate pH 6 and 2 µL 10 mg/mL single stranded salmon sperm DNA) were added to the cell pellet. The transformation reaction was mixed by vortexing and heat shocked at 39°C for 1 h (Chen, Beckerich and Gailardin 1997; Friedlander et al. 2016). Cells were centrifuged, the supernatant was discarded, cells were resuspended in 1 mL of non-selective medium (YPD for $\Delta fad2$ or supplemented YPD as described above for $\Delta ole1$), transferred to culture tubes and cultured overnight at 30°C before plating 100 µL on selective media.

Relative fatty acid composition measurement

To obtain a relative fatty acid composition profile, a plate transesterification procedure was used to extract and convert lipids to fatty acid methyl esters (FAMES). 1-mL samples from shake flasks were washed with water and transferred to a 96-well deep-well plate. Cells were resuspended in a small amount of water (~50 µL) and frozen at -80°C for 30 min before placing the entire plate in a lyophilizer overnight. To each well 0.25 mL 0.5 M sodium methoxide in methanol (Acros Organics) was added and the plate was sealed closed and incubated at 50°C for 30 min with mixing by vortexing at 15 min. 0.25 mL of 0.6 N hydrochloric acid in water and 1 mL isoctane were then added to each well

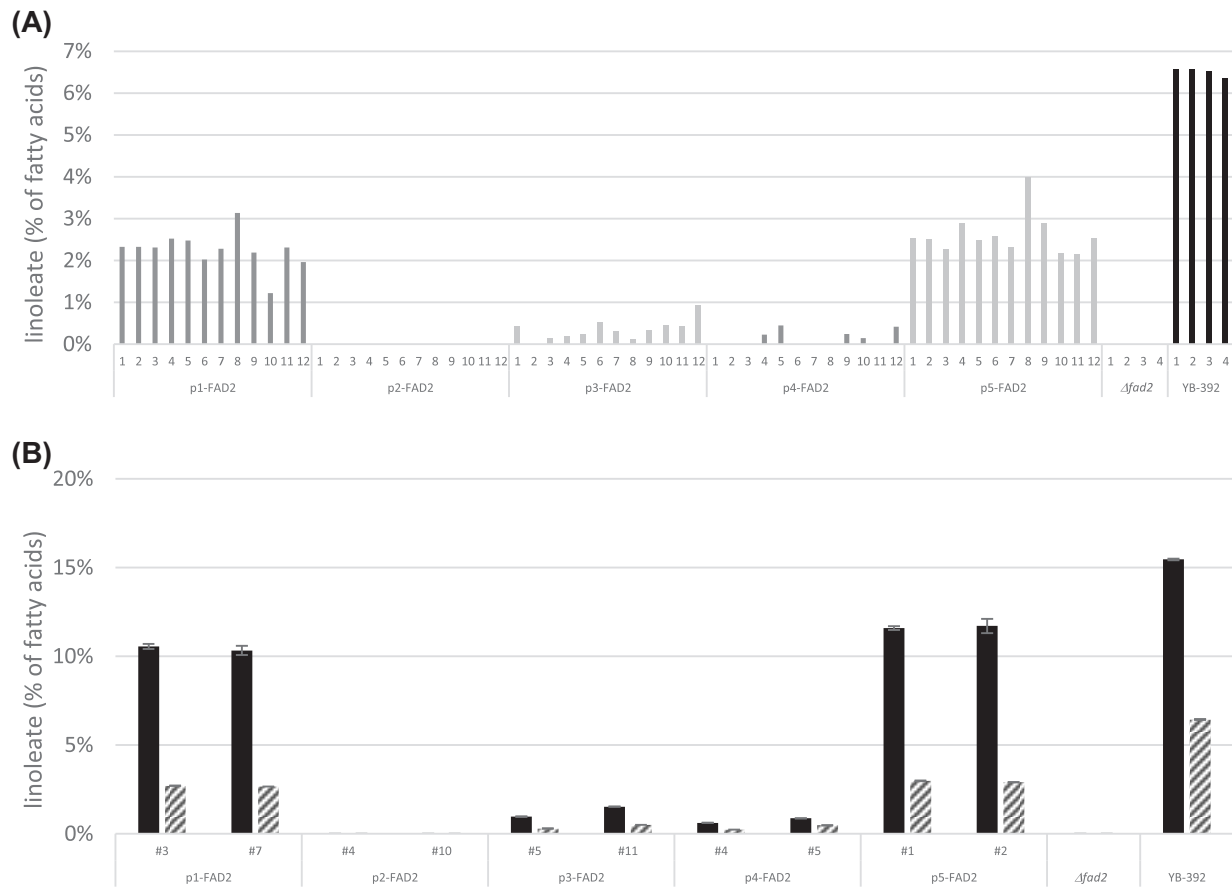


Figure 1. Linoleate content of FAD2 strains. (A) Wild-type strain YB-392, desaturase deletion strain Δ fad2 and 12 transformants of Δ fad2 with each of the FAD2 constructs were grown in 96-well plates for 6 days. Relative fatty acid composition was determined by GC. The fraction of linoleate is shown as percentage of total fatty acids by GC peak area. (B) Two isolates from each of the transformant sets analyzed in (A) were grown in duplicate in 96-well plates and analyzed after 24 h (solid bars) and 96 h (hatched bars). Linoleate content was determined as in (A) and the average and standard deviation of duplicate cultures is shown.

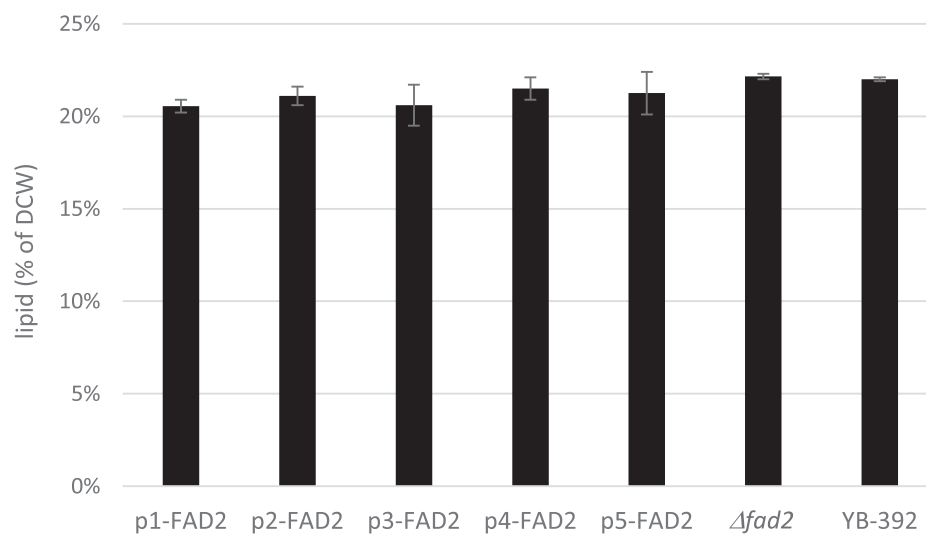


Figure 2. Lipid content of FAD2 strains. An isolate from each of the FAD2 strains tested in Fig. 1, desaturase deletion strain Δ fad2 and wild-type strain YB-392 were grown in duplicate in 24-well plates for 4 days. Lipid content was determined by quantitative GC as percentage of total dry cell weight (DCW). The average and standard deviation of duplicate cultures is shown.

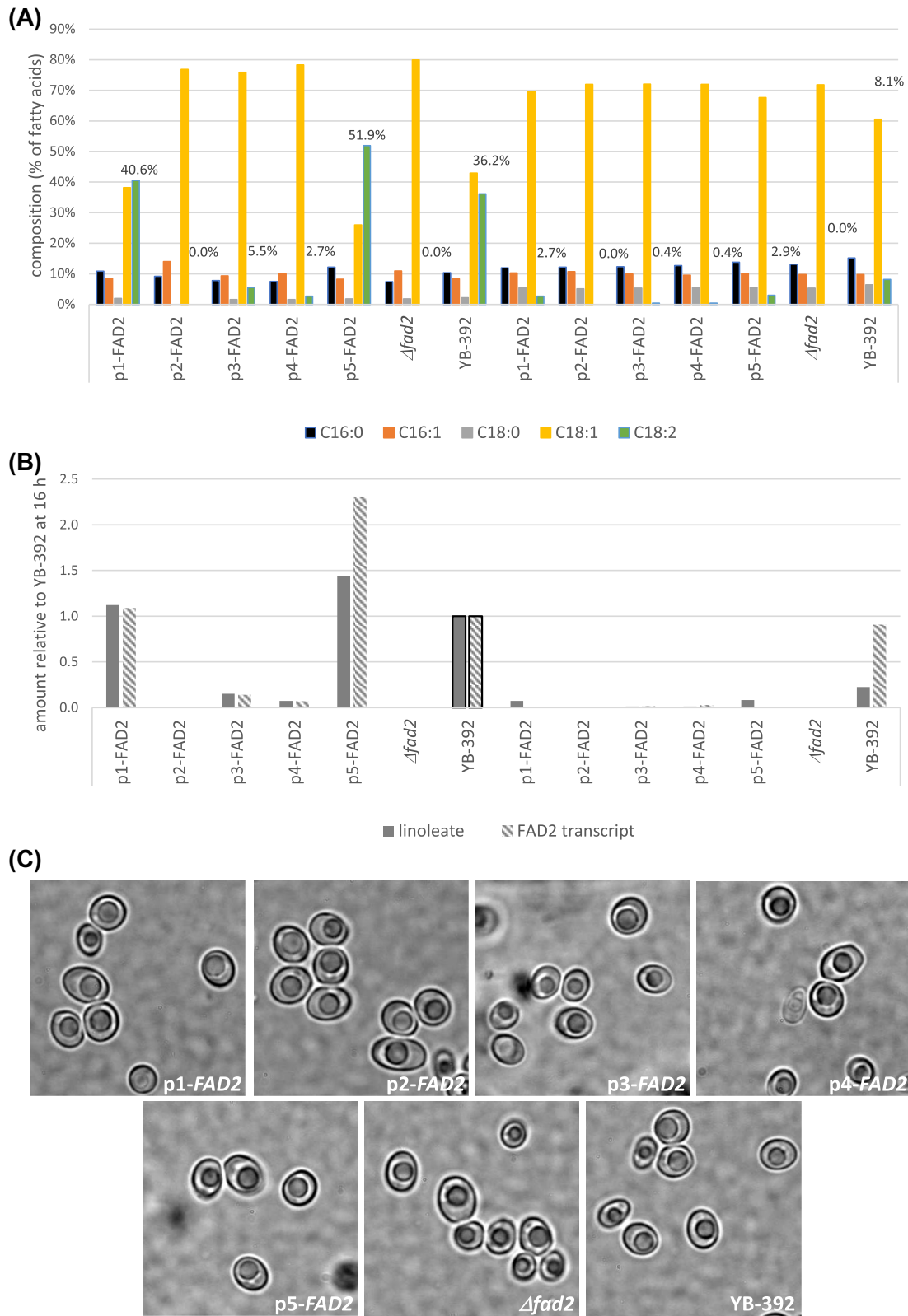


Figure 3. Fatty acid composition and FAD2 transcript levels in FAD2 strains. The selected strains were grown in 50-mL shake flasks and sampled at 16 h and 96 h. **(A)** Relative fatty acid composition was determined by GC. The fraction of each fatty acid is shown as percentage of total fatty acids by GC peak area. Data labels denote linoleate levels. **(B)** Linoleate composition (solid bars) and FAD2 transcript levels (striped bars) normalized to the wild-type strain YB-392 at 16 h. **(C)** Microscope images of each strain at 96 h.

Table 2. Promoters used in this study.

Promoter	Gene	Location relative to ATG	Gene annotation (NCBI Gene Database (Gene 2019))	Transcript reduction at high C:N (GEO 2020a,b,c)
p1	YALIOB16522g	-1000 to -1	Similar to uniprot P19145 <i>Saccharomyces cerevisiae</i> YKR039w GAP1 general amino acid permease	51
p2	YALIOC01411g	-1000 to -1	Similar to uniprot Q9UVK9 <i>Yarrowia lipolytica</i> Fks1p	34
p3	YALIOA04631g	-370 to -1	Weakly similar to uniprot Q6C9E1 <i>Yarrowia lipolytica</i> YALIOD11924g	32
p4	YALIOA12815g	-1000 to -1	No similarity	28
p5	YALIOB19800g	-1000 to -1	Similar to uniprot P19145 <i>Saccharomyces cerevisiae</i> YKR039W GAP1 General amino acid permease	20

Table 3. Comparison of FAD2 transcription and linoleate composition between growth and lipid accumulation phases. FAD2 transcript levels were measured by qPCR and fold-change between timepoints was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Ratios of percent linoleate composition were calculated by dividing percent linoleate values at 16 h by those at 96 h using the data presented in Fig. 1a. ND: not detected.

Strain	Ratio of FAD2 transcript at 16 h / 96 h	Ratio of linoleate composition at 16 h / 96 h
p1-FAD2	178	15
p2-FAD2	0.3	ND
p3-FAD2	8.8	14
p4-FAD2	2.6	7
p5-FAD2	1855	18
$\Delta fad2$	ND	ND
YB-392 (wt)	1.1	4.5

and mixed by pipetting. The plate was centrifuged at 3500 rpm at 22°C to separate the organic and aqueous layers. A sample of the FAME-containing isooctane layer (top layer) was analyzed by gas chromatography (GC) equipped with a flame ionization detector (Agilent Technologies 7890B GC) and VF-23 ms capillary column (20 m × 0.15 mm × 0.15 μm, Agilent Technologies). The fatty acid composition was determined as % of total peak area (sum of C16:0, C16:1, C18:0, C18:1 and C18:2) for each FAME species (Christie and Esterification 1993). Because the dry cell weight in each of the 96 wells is not measured, this method yields relative compositional analysis by comparing peak areas within each sample, but not absolute fatty acid levels (Tsakraklides et al. 2018).

Total lipid quantitation

To obtain the total lipid content and lipid composition from a sample, a quantitative GC procedure was used. The samples were processed and lyophilized as above in the fatty acid composition measurement except that each sample was lyophilized in separate vials. The dried biomass was weighed and subjected to acid-catalyzed transesterification along with an internal standard (C13:0) using 1.25 M hydrochloric acid in methanol (Sigma) at 85°C for 90 min. After the transesterification, the lipid-soluble components of the reaction mixture were separated from the water-soluble components using a two-phase liquid extraction by adding water and isooctane, and subsequently analyzed by

Table 4. Comparison of OLE1 transcription and $\Delta 9$ desaturated fatty acid composition between growth and lipid accumulation phases. OLE1 transcript levels were measured by qPCR and fold-change between timepoints was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Ratios of percent desaturated fatty acid composition were calculated by dividing percent fatty acid values at 16 h by those at 96 h using the data presented in Fig. 2a.

Strain	Ratio of OLE1 transcript at 16 h / 96 h	Ratio of $\Delta 9$ product composition at 16 h / 96 h
p1-OLE1	63	1.8
p4-OLE1	63	1.8
p5-OLE1	688	1.8
YB-392 (wt)	1.2	1.1

GC. The internal standard was used to correct for transesterification reaction efficiency. An external standard mix of FAMES (NHI-D, Supelco Analytical, Bellefonte, PA) at various concentrations was used to quantify the methyl-ester products. The % lipid content is calculated as the sum of FAMES divided by the weight of the dried biomass.

RNA extraction and transcript quantification

A total of 20 OD₆₀₀ units of culture was removed from shake flask fermentations at 16 h and 96 h, washed with water and stored at -80°C. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad CA). Genomic DNA was removed using DNase (Qiagen, Germantown MD) followed by RNA cleanup using the RNeasy Mini Kit (Qiagen). A total of 500 ng of clean RNA was used for cDNA synthesis using M-MuLV Reverse Transcriptase enzyme (NEB). PCR quantification was performed by Wyzer Biosciences (Cambridge, MA) using PerfeCTa SYBR qPCR mix with High ROX (QuantaBio, Beverly MA) as a reference dye. The reactions were run in triplicate in PCR-96-LP-AB-C plates (Corning) on a StepOnePlus real-time PCR system (Applied Biosystems, Foster City CA). Software analysis was performed with StepOne v.2.3 software (Applied Biosystems). Gene expression levels were standardized using the *Y. lipolytica* actin gene ACT1 as a reference (ΔCT method). For comparisons to YB-392 at 16 h, the $2^{-\Delta\Delta CT}$ method was used to calculate fold change. The $2^{-\Delta\Delta CT}$ method was also used for measuring fold repression for each strain, between the 16 h timepoint and 96 h timepoint (Livak and Schmittgen 2001). The qPCR primers used were OLE1 F: 5'-CACAACTGCTTGCCACCATG-3', OLE1 R: 5'-CAGCT

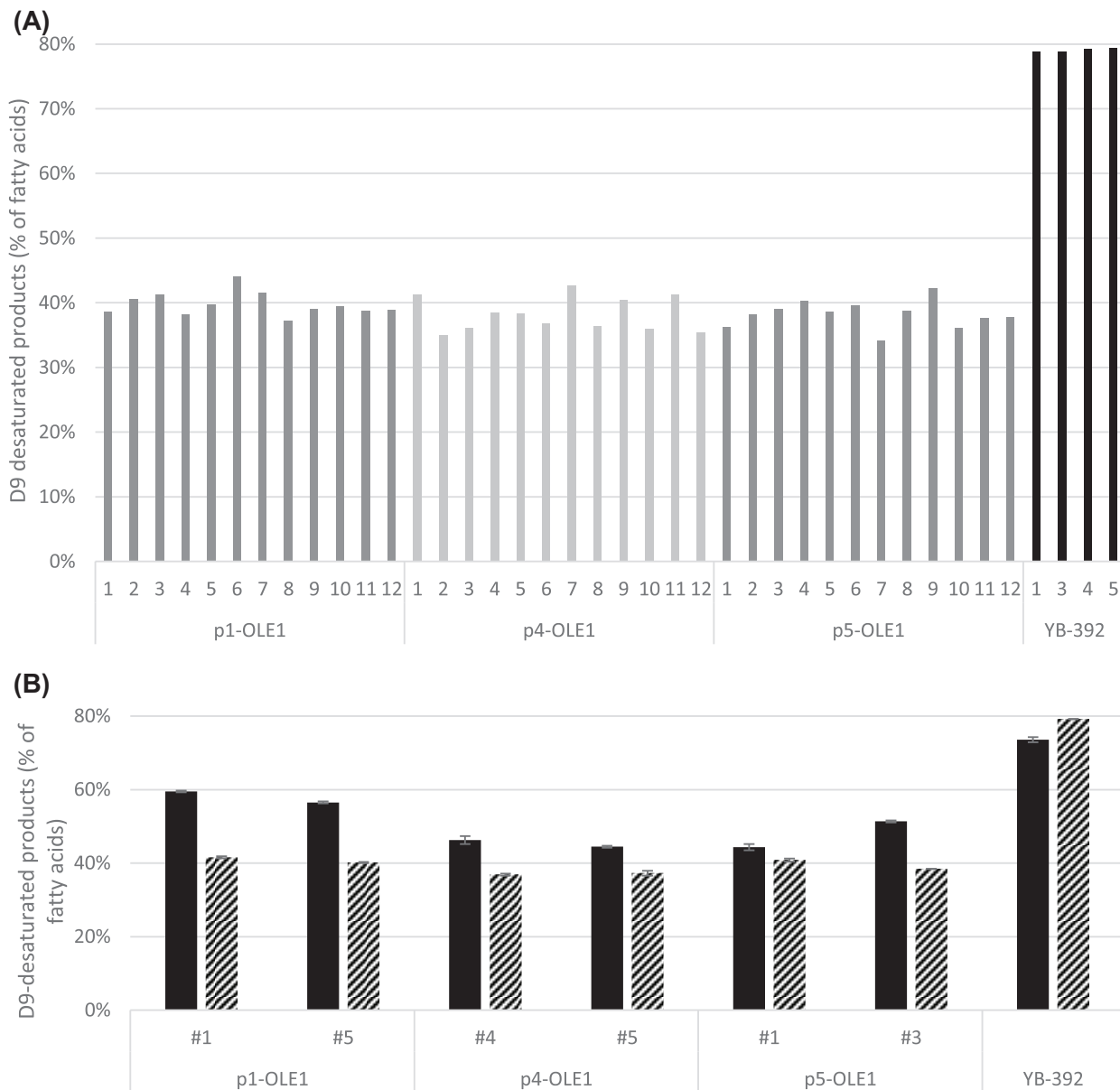


Figure 4. $\Delta 9$ -desaturated fatty-acid content of *OLE1* strains. **(A)** Wild-type strain YB-392 and 12 transformants from each of the *OLE1* constructs in $\Delta ole1$ were grown in 96-well plates for 4 days. Relative fatty acid composition was determined by GC. The fraction of $\Delta 9$ -desaturated fatty acids is shown as the sum of palmitoleate (C16:1), oleate (C18:1) and linoleate (C18:2) as a percentage of total fatty acids. **(B)** Two isolates from each of the transformant sets analyzed in (A) were grown in duplicate in 96-well plates and analyzed after 24 h (solid bars) and 96 h (hatched bars). $\Delta 9$ -desaturated fatty acid content was determined as in (A) and the average and standard deviation of duplicate cultures is shown.

CGGTGGATTTCGGTTTC-3', FAD2 F: 5'-CCATGACATCATCGAGACC CACG-3', FAD2 R: 5'-GTTGGTGTGTCGTGTCGGTAG-3', ACT1 F: 5'-GCTGCTGGTATCCACGAGACTAC-3', ACT1 R: 5'-GCGGAGATC TCCTTGTGCATTTCG-3'.

RESULTS

Identification of candidate promoters for reduced expression during lipid accumulation

To identify promoters that could be used to mediate distinct lipid phenotypes in the growth and lipogenesis phases through reduced gene expression during lipid accumulation, we analyzed a published *Y. lipolytica* transcriptomic dataset (GEO

2020a,b,c) acquired in Ochoa-Estopier and Guillouet (2014) and analyzed in Trébulle *et al.* (2017). In that experiment, *Y. lipolytica* W29 was cultivated under chemostat and decelerostat (D-stat) conditions. During chemostat growth, cells were supplied with enough nutrients to maintain continuous growth. During D-stat cultivation, the C:N ratio was adjusted by a linear decrease of the nitrogen feed. We compared transcript levels between chemostat growth (reference sample in GEO (2020a,b,c)) and D-stat cultivation at a C:N molar ratio of 31 (sample 47, 0.0323 N:C molar ratio at 166 h in GEO (2020a,b,c)). This timepoint was selected because the culture reached a maximal respiratory quotient (RQ) value at this time, indicating potentially high lipid production (Ochoa-Estopier and Guillouet 2014). We identified transcripts with more than 95% reduction between the two

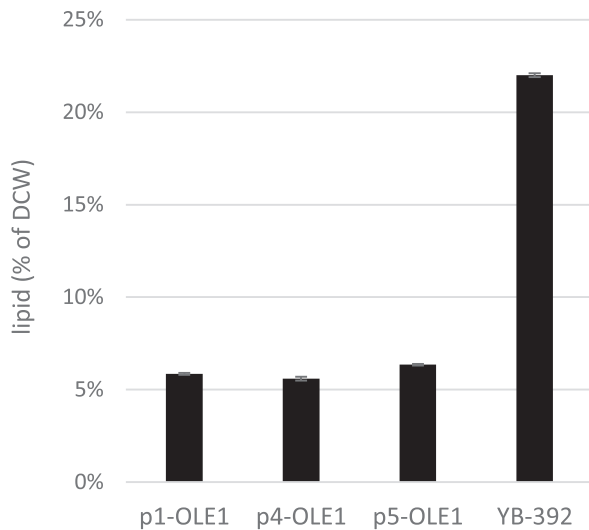


Figure 5. Lipid content of *OLE1* strains. An isolate from each of the *OLE1* strains tested in Fig. 4 and wild-type strain YB-392 were grown in duplicate in 24-well plates for 4 days. Lipid content was determined by quantitative GC as percentage of total dry cell weight (DCW). The average and standard deviation of duplicate cultures is shown.

conditions and selected the promoters from five of these transcripts with a range of reduction of 20- to 51-fold for further study (p1–p5, Table 2). The *Y. lipolytica* genes transcribed by these promoters have similarity to amino acid permeases (p1, p5), a gene involved in cell wall synthesis and maintenance (p2), or proteins of unknown function (p3, p4).

To assay the activity of these promoters, we used the 1000 bp (or 370 bp for p3 where the intergenic region is shorter) upstream of each transcription start site to drive transcription of two lipid-related reporter genes: $\Delta 12$ fatty acid desaturase (*FAD2*) and $\Delta 9$ fatty acid desaturase (*OLE1*). The promoter sequence length chosen was based on the observation that most promoters used in *Y. lipolytica* strain engineering extend 300–1000 bp upstream from the transcription start site (Blazek et al. 2011). The desaturase genes were chosen as reporter genes because we could track desaturase activity through changes in fatty acid composition (Tsakraklides et al. 2018). As opposed to the D-stat experiment in Ochoa-Estropier and Guillouet (2014), all characterizations in the present study were performed in batch fermentation in lipid production media for up to 6 days (starting C:N molar ratio of 100). These conditions allow for growth during the first day while nitrogen is available to produce biomass, and subsequent lipid accumulation through the following days when nitrogen is depleted (Friedlander et al. 2016). Our goal was to identify promoters that result in changes in fatty acid composition specific to the lipid accumulation phase.

Control of $\Delta 12$ fatty acid desaturase expression

Yarrowia lipolytica $\Delta 12$ fatty acid desaturase (*FAD2*, YALIOB10153) adds a double bond in the $\Delta 12$ position of oleyl phospholipid (C18:1) to produce linoleyl phospholipid (C18:2). Linoleate can subsequently be incorporated into storage lipids through fatty acid exchange between phospholipid and TAG synthesis pathways (Yuan and Bloch 1961; Ferrante and Kates 1983; Dahlqvist et al. 2000). It was previously demonstrated that deleting the *FAD2* gene leads to elimination of all detectable linoleate (Tezaki et al. 2017; Tsakraklides et al. 2018). Therefore, the $\Delta fad2$ background was chosen to characterize linoleate composition in

strains expressing *FAD2* under the control of p1–p5 without interference from the endogenous locus.

We examined linoleate content in strains carrying p1–p5 constructs. Linear cassettes driving *FAD2* expression from each of the promoters under study, paired with an antibiotic resistance constitutive expression cassette (Additional file 1) were transformed into a $\Delta fad2$ strain. This $\Delta fad2$ strain was previously created by deletion of the endogenous *FAD2* gene in the wild-type haploid strain YB-392 (Tsakraklides et al. 2018). Random integration of linear constructs into the genome leads to a range of phenotype expressivity. To select representative strains rather than outliers, we compared linoleate levels at the end of the lipid accumulation phase between the native *FAD2*-expressing wild-type strain YB-392 and 12 transformants from each construct (Fig. 1a; complete lipid profiles can be found in Additional File 2). We observed the expected heterogeneity within each set of twelve transformants due to integration site variation but saw clear differences in the level of linoleate present in each group. Linoleate levels were lower than YB-392 in all cases. p5-*FAD2*, closely followed by p1-*FAD2* gave the highest linoleate levels among the engineered strains. p3- and p4-*FAD2* strains had very low levels of the $\Delta 12$ -desaturated fatty acid. In p2-*FAD2* strains, linoleate was undetectable, similar to the $\Delta fad2$ strain. To verify that linoleate absence was not due to failure to introduce the expression cassette, colony PCR was used to confirm the presence of the *FAD2* construct in all transformants (data not shown). These results indicate that p1–p5 all lead to lower-than-wild-type $\Delta 12$ fatty acid desaturase activity during lipid accumulation.

To determine whether $\Delta 12$ fatty acid desaturase is down-regulated under our fermentation conditions, we compared linoleate composition between growth and lipid accumulation phases in two isolates of average performance from each promoter set (Fig. 1b; complete lipid profiles can be found in Additional File 2). Average performers represent promoter strength more accurately than outliers that likely reflect a particularly favorable or unfavorable integration event. YB-392 exhibited a reduction of linoleate from 24 to 96 h. Lipid accumulation involves increased fatty acyl-CoA storage into TAG leading to a final lipid content that is predominantly composed of storage lipids and a small fraction of phospholipids (Athenstaedt et al. 2006). As the substrate and product of $\Delta 12$ desaturase are phospholipids (Ferrante and Kates 1983), we hypothesize that the linoleate reduction in YB-392 is a result of the natural dilution of linoleate in storage TAG during lipid accumulation and will return to this question in a subsequent experiment (Fig. 3b). By comparison, p2-*FAD2* strains failed to produce linoleate at 24 h as well as 96 h, suggesting very low activity. p1 and p3–p5 strains maintained the relative levels of linoleate seen in Fig. 1a and showed a decrease in linoleate levels at 96 h compared to 24 h. This decrease was generally greater than that seen with YB-392, suggesting downregulation of *FAD2* in addition to dilution due to lipid accumulation.

Given the consistent results between the chosen average performers, the first of these two isolates for each promoter (from Fig. 1b) was used as a representative strain for further characterization. Total lipid content at the end of lipid accumulation was similar in YB-392, $\Delta fad2$ and the p1–p5 strains (Fig. 2), indicating that changes in lipid accumulation do not account for the decreased linoleate levels observed. *FAD2* gene copy number was determined by qPCR and it was found that all strains contained 1 (YB-392 and p1, p2 and p3 strains) or 2 (p4 and p5 strains) copies of the *FAD2* gene (data not shown). Copy number would affect the absolute level of expression but should not

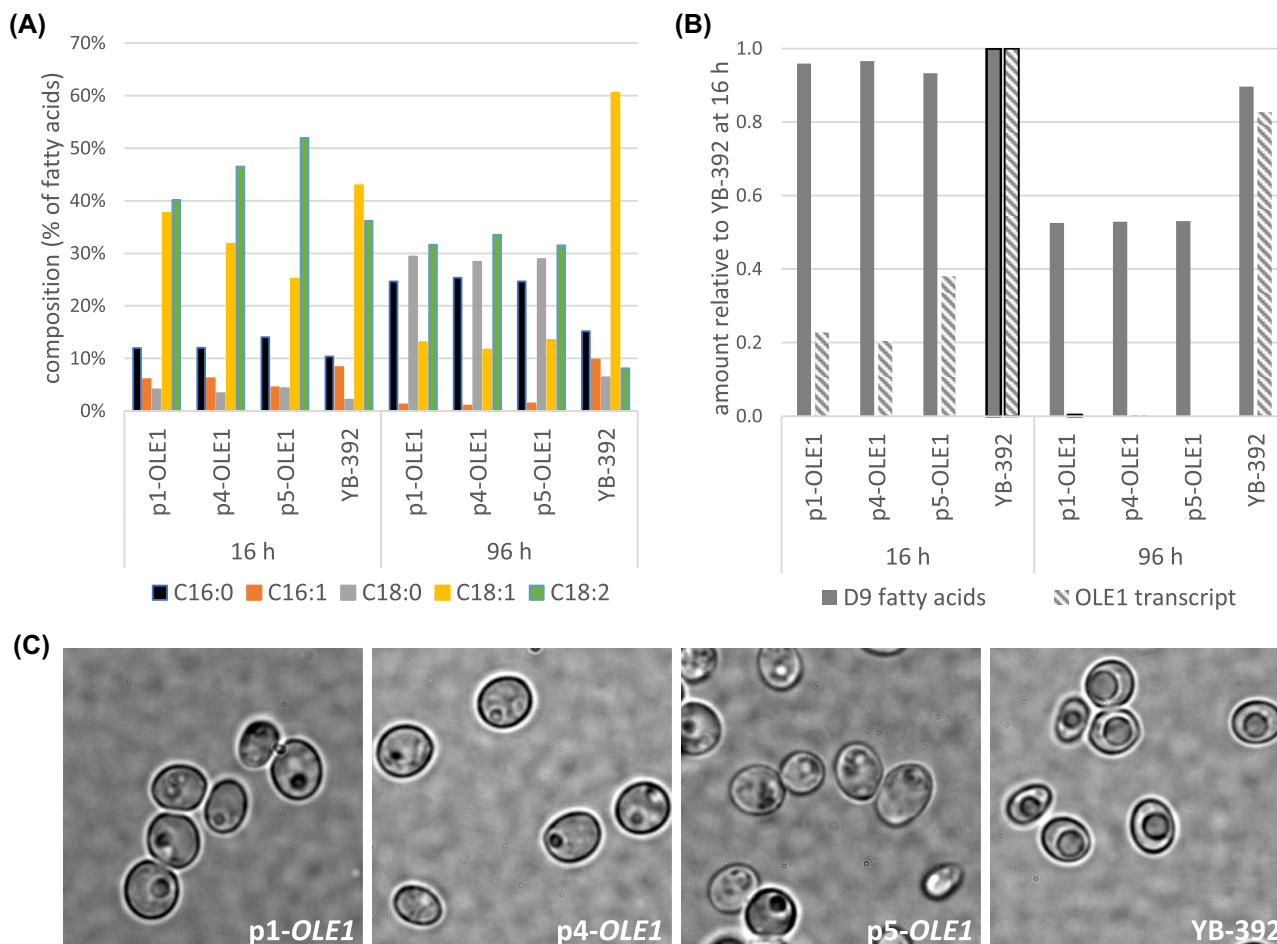


Figure 6. Fatty acid composition and *OLE1* transcript levels in *OLE1* strains. **(A)** Relative fatty acid composition was determined by GC. The fraction of each fatty acid is shown as percentage of total fatty acids by GC peak area. **(B)** $\Delta 9$ fatty acid composition (solid bars) and *OLE1* transcript levels (striped bars) normalized to the wild-type strain YB-392 at 16 h. **(C)** Microscope images of each strain at 96 h.

affect the evaluation of phase-specific relative changes in fatty acid composition and transcription.

Growth samples collected at 24 h contained lower than wild-type levels of linoleate (Fig. 1b). Although this could be due to lower starting activity of p1–p5 compared to the native *FAD2* promoter, we speculated that p1–p5-mediated desaturase repression may occur earlier in the fermentation. To evaluate linoleate composition at an earlier timepoint, we collected growth samples from the selected isolates at 16 h and 96 h (Fig. 3a). Relative fatty acid composition of p1 and p3–p5 strains showed higher linoleate values at 16 h than previously seen at 24 h and relative to YB-392 suggesting that desaturase activity under the control of these promoters may indeed already be diminished by 24 h. As previously seen, p5-*FAD2* and p1-*FAD2* strains exhibited the highest linoleate content during growth phase, followed by p3-*FAD2* and p4-*FAD2*. Observed linoleate levels for p4-*FAD2* and p5-*FAD2* strains are the result of $\Delta 12$ fatty acid desaturase expression from two copies of the expression cassette. It is expected that expression from a single copy would yield proportionately lower linoleate at both timepoints. Linoleate was again undetectable in the p2-*FAD2* strain even at 16 h.

cDNA from the same samples was analyzed by qPCR to characterize promoter activity and relate transcription to lipid composition. For ease of comparison, the fraction of linoleate and the *FAD2* transcript levels of each sample were normalized to

wild-type levels during growth (Fig. 3b). The ratio of transcript levels and the ratio of % linoleate composition at 16 h to those at 96 h were also calculated to quantify changes between growth and lipid accumulation phases (Table 3). In YB-392, *FAD2* expression from the native promoter is relatively stable between 16 h and 96 h but % linoleate composition is reduced 4.5-fold. This supports the hypothesis that linoleate reduction in YB-392 is a result of the natural dilution of linoleate during lipid accumulation rather than transcriptional changes. Promoters p1, p3, p4 and p5 show transcript reduction ranging from 2.6- to > 1800-fold between growth and lipid accumulation. This transcriptional repression correlates with the sharper decrease in linoleate composition for these four promoters between the two timepoints (ranging from 7- to 18-fold) when compared to YB-392 (4.5-fold). Despite altered fatty acid composition, microscopic observation of the transformants at 96 h revealed no discernible defects in growth or lipid production and all strains appeared similar to the control strains (Fig. 3c).

Control of $\Delta 9$ fatty acid desaturase expression

To investigate the potential of these promoters to control an essential gene, we extended our study to the expression of $\Delta 9$ fatty acid desaturase. *Y. lipolytica* $\Delta 9$ desaturase (*OLE1*, YALI0C05951) acts on saturated palmitic (C16:0) and stearic

(C18:0) fatty acids to produce the corresponding unsaturated palmitoleic (C16:1) and oleic (C18:1) fatty acids. *OLE1* is an essential gene. Its deletion leads to auxotrophy for monounsaturated fatty acids; reintroduction of an active $\Delta 9$ desaturase rescues growth on unsupplemented media (Tsakraklides et al. 2018).

The promoters under study were evaluated in a $\Delta 9$ desaturase null strain. *OLE1* was previously deleted by targeted integration in wild-type strain YB-392 (Tsakraklides et al. 2018). $\Delta ole1$ was cultured in media supplemented with oleic acid to maintain viability and transformed with expression cassettes for *OLE1* driven by p1–p5. Transformants were selected through antibiotic resistance on supplemented plates and then tested for their ability to grow on unsupplemented media. Expression of *OLE1* from promoters p1, p4 and p5 enabled growth on media without supplementation, indicating that sufficient $\Delta 9$ desaturase activity was produced during growth to supply essential monounsaturated fatty acids. p2–p3 strains and the parental $\Delta ole1$ strain, could not grow without supplementation suggesting that *OLE1* expression was below levels necessary for growth. Therefore, these strains were excluded from subsequent analysis as they cannot grow in the lipid production media which does not contain monounsaturated fatty acids.

Transformants of p1-, p4- and p5-*OLE1* were grown, sampled and analyzed as for the *FAD2* experiment. To better visualize the effect of *OLE1* expression, $\Delta 9$ desaturase products (C16:1, C18:1 and C18:2, a $\Delta 12$ desaturation product of C18:1) were combined. In the 12-transformant screen, all transformants showed a similar reduction in $\Delta 9$ -desaturated fatty acid content at the end of lipid accumulation compared to the wild-type strain (Fig. 4a; complete lipid profiles can be found in Additional File 2). When 2 representative isolates were re-examined at 24 h and 96 h, the engineered strains each showed higher $\Delta 9$ -desaturated fatty acid content during the growth phase than the lipid accumulation phase and all showed lower $\Delta 9$ -desaturated fatty acid content than YB-392 at both timepoints (Fig. 4b; complete lipid profiles can be found in Additional File 2). For each promoter, the first of these two isolates was used as a representative strain for further characterization. These three chosen strains each contained a single copy of the *OLE1* gene as determined by qPCR (data not shown). Total lipid content during lipid accumulation was significantly lower than YB-392 (Fig. 5). *OLE1* is known to be an important gene in the lipid production pathway (Dobrzyn and Ntambi 2005; Qiao et al. 2015), and low $\Delta 9$ desaturase levels could explain this phenotype.

We next evaluated fatty acid composition and *FAD2* transcription in the selected strains at 16 h and 96 h. Composition measurements revealed varying lipid profiles for p1, p4 and p5 transformants compared to each other and compared to the wild-type strain (Fig. 6a). $\Delta 9$ -desaturated fatty-acids and *OLE1* transcript levels were normalized to the 16 h timepoint for YB-392 (Fig. 6b) and the ratios of $\Delta 9$ -desaturated fatty-acids and *OLE1* transcript levels between growth and lipid accumulation were calculated (Table 4). p1-, p4- and p5-*OLE1* strains all exhibited a reduction of $\Delta 9$ desaturated fatty acids by 96 h but this was more modest (1.8-fold) than the change in transcript levels (63- to 688-fold) between the two timepoints (Table 4). Low lipid level (Fig. 5) is likely responsible for the retention of a significant fraction of $\Delta 9$ -desaturated fatty acids in the total lipid composition despite the loss of $\Delta 9$ desaturase transcript at 96 h. Microscopic examination revealed a reduction in the size of lipid bodies in all three strains when compared to wild-type YB-392, confirming a defect in lipid accumulation (Fig. 6c). In the absence of new lipid production, the desaturated fatty acids produced during growth remain a significant portion of total lipid content. It is

important to keep in mind that the wild-type strain accumulates approximately 3.7-fold more lipid (~22% of its dry cell weight as opposed to ~6% in the engineered strains; Figs 5 and 6c). Therefore, at the end of lipid accumulation, the higher $\Delta 9$ -desaturated fatty acid composition of YB-392 compared to the lower-lipid engineered strains (Fig. 6b) in fact translates to an even higher absolute quantity of accumulated $\Delta 9$ -desaturated fatty acids in YB-392.

DISCUSSION

In this study, we set out to expand the *Y. lipolytica* metabolic engineering toolbox through the identification of promoters that are downregulated during the switch to lipogenesis. We identified four native promoters capable of mediating distinct phenotypes between the growth and lipid accumulation phases of a typical nitrogen-limited *Y. lipolytica* fermentation. The desaturase genes *FAD2* and *OLE1* were chosen to illustrate the ability to modify fatty acid profiles. These enzymes are directly involved in lipid synthesis and, therefore, could help translate transcriptional effects to lipid phenotypes. Lipid composition and transcript level data were collected to characterize the promoters and demonstrate the range of phenotypes attainable. Overall, we found agreement between reduced transcript levels and reduced product for the targeted activity during lipid accumulation in strains expressing desaturases driven by promoters p1, p3, p4 and p5.

p2 was included in our study because it was reported to be active (Ochoa-Estopier and Guillouet 2014; Trébulle et al. 2017). However, p2-driven *OLE1* failed to complement $\Delta ole1$ and led to very low or undetectable linoleate and *FAD2* transcript levels. We speculate that the differences between our results are related to the differences between the two experimental setups. We reasoned that the region 1000 bp upstream of the transcription start site may contain the elements required to replicate promoter behavior in a heterologous construct but it remains possible that regulatory elements beyond this sequence are required for proper p2 function. Furthermore, we linked these promoters to reporter genes and tested them in strain YB-392, whereas Ochoa-Estopier and Guillouet 2014 measured native gene transcription in the W29 *Y. lipolytica* wild-type strain. These native gene transcripts could differ from *FAD2* and *OLE1* in transcript stability. Finally, we cultivated our strains in nitrogen-limited, excess glucose batch fermentation as opposed to the chemostat and D-stat cultivation system used in Ochoa-Estopier and Guillouet 2014. It is possible that p2 responds differently to these different process conditions for lipid production than the other promoters in our study.

Our work did not probe the mechanism through which promoter activity is regulated. Our initial promoter selection was based on transcriptional response to the C:N ratio in the media and our desaturase expression experiments were carried out in a high C:N batch fermentation where the cells transition from growth to lipid accumulation when the nitrogen is exhausted. In addition, the two most active promoters in our experiments, p1 and p5, are associated with genes predicted to encode amino acid permeases (Table 2). Therefore, we speculate that low nitrogen levels or downstream signals may regulate promoter activity.

Promoters with reduced activity during the lipid accumulation phase are particularly interesting when applied to the expression of an essential gene. Strains carrying *OLE1* under the control of p1, p4 or p5 supplied enough $\Delta 9$ desaturase activity to maintain growth but restricted this activity to growth phase,

leading to a defect in lipid accumulation upon nitrogen limitation. Low $\Delta 9$ desaturase activity uniformly led to a severe reduction in lipid body size and reduced $\Delta 9$ desaturase products, demonstrating the power of this method to specifically affect the lipid accumulation phase. Genes that are essential or favorable for growth can be supplied under the control of these promoters to specifically reduce their activities during lipid production for such purposes as altering lipid composition, improving yield through alternate pathways or eliminating side-products. This regulation can be achieved without changing the fermentation process because promoter activity is tied to the established process of lipid production in *Y. lipolytica*.

Although p1, p3–p5 have in common the ability to restrict activity to the growth phase, these promoters exhibit a range of phenotypes illustrated by the range of fatty acid compositions observed during growth (Figs 3a and 6a). These differences are likely due to differences in promoter strength, copy number, the timing of transcriptional repression and may also be affected by translational efficiency. The rates at which each promoter transitions from maximum to minimum expression as well as the level of expression at these timepoints are possibly key determinants of enzyme levels during these fermentations. The optimum level and timing of activity for a targeted enzyme will depend on the pathway, properties (enzyme kinetics, protein half-life) and experimental objectives. The availability of a set of promoters capable of turning off a targeted activity with a range of expression patterns and without the necessity of additional fermentation control mechanisms will provide a valuable tool for fine-tuning activity levels.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSYR](https://www.femsyr.com) online.

AUTHOR CONTRIBUTIONS

VT identified the promoters studied in this work. AK and VT designed the project plan. AK constructed desaturase expression vectors and strains, performed qPCR experiments and analysis. VT performed copy number analysis and assisted in strain verification, culture and sampling. SC and GC performed all analytical chemistry. AK and VT prepared the manuscript with contributions from SC. All authors read and approved the final manuscript.

ETHICAL APPROVAL

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

ACKNOWLEDGMENTS

The authors are grateful to Lev Kotler (Wyzer Biosciences) and Hannah Blitzblau for advice designing qPCR experiments, John Sullivan for laboratory support, Hannah Blitzblau and Jean-Marc Nicaud for careful reading of the manuscript, Joe Shaw for helpful comments and the Novogy team for insightful discussions throughout the course of this work.

FUNDING

This study was wholly funded by Novogy Inc.

Conflict of interest. The authors are employees of Novogy, Inc., which has a commercial interest in the strains described in this study. The authors declare no other non-financial competing interests.

REFERENCES

- Abghari A, Madzak C, Chen S. Combinatorial engineering of *Yarrowia lipolytica* as a promising cell biorefinery platform for the de novo production of multi-purpose long chain dicarboxylic acids. *Fermentation* 2017;3:40.
- Athenstaedt K, Jolivet P, Boulard C et al. Lipid particle composition of the yeast *Yarrowia lipolytica* depends on the carbon source. *Proteomics* 2006;6:1450–9.
- Beopoulos A, Cescut J, Haddouche R et al. *Yarrowia lipolytica* as a model for bio-oil production. *Prog Lipid Res* 2009;48:375–87.
- Blanchin-Roland S, Cordero Otero RR, Gaillardin C. Two upstream activation sequences control the expression of the XPR2 gene in the yeast *Yarrowia lipolytica*. *Mol Cell Biol* 1994;14:327–38.
- Blazeck J, Liu L, Redden H et al. Tuning gene expression in *Yarrowia lipolytica* by a hybrid promoter approach. *Appl Environ Microbiol* 2011;77:7905–14.
- Blazeck J, Liu L, Redden H et al. Tuning gene expression in *Yarrowia lipolytica* by a hybrid promoter approach. *Appl Environ Microbiol* 2011;77:7905–14.
- Blazeck J, Reed B, Garg R et al. Generalizing a hybrid synthetic promoter approach in *Yarrowia lipolytica*. *Appl Microbiol Biotechnol* 2013;97:3037–52.
- Chen D-C, Beckerich J-M, Gaillardin C. One-step transformation of the dimorphic yeast *Yarrowia lipolytica*. *Appl Microbiol Biotechnol* 1997;48:232–5.
- Christie WW, Esterification BA. Preparation of ester derivatives of fatty acids for chromatographic analysis. In: *Advances in Lipid Methodology*, 2nd edn, The Oily Press LTD. 1993.
- Dahlqvist A, Ståhl U, Lenman M et al. Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proc Natl Acad Sci* 2000;97:6487–92.
- Dobrzyn A, Ntambi JM. The role of stearoyl-CoA desaturase in the control of metabolism. *Prostaglandins Leukot Essent Fatty Acids* 2005;73:35–41.
- Ferrante G, Kates M. Pathways for desaturation of oleoyl chains in *Candida lipolytica*. *Can J Biochem Cell Biol* 1983;61:1191–6.
- Friedlander J, Tsakraklides V, Kamineneni A et al. Engineering of a high lipid producing *Yarrowia lipolytica* strain. *Biotechnol Biofuels* 2016;9:77.
- Ganesan V, Spagnuolo M, Agrawal A et al. Advances and opportunities in gene editing and gene regulation technology for *Yarrowia lipolytica*. *Microb Cell Fact* 2019;18:208.
- Gene [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004 – [cited 2019 08 14]. Available from: <https://www.ncbi.nlm.nih.gov/gene/>.
- GEO Accession viewer [Internet]. [cited 2020a Feb 25]. Available from: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35445>
- GEO Accession viewer [Internet]. [cited 2020b Feb 25]. Available from: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35446>

- GEO Accession viewer [Internet]. [cited 2020c Feb 25]. Available from: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35447>
- Juretzek T, Wang H-J, Nicaud J-M et al. Comparison of promoters suitable for regulated overexpression of β -galactosidase in the alkane-utilizing yeast *Yarrowia lipolytica*. *Biotechnol Bio-process Eng* 2000;5:7.
- Larroude M, Rossignol T, Nicaud J-M et al. Synthetic biology tools for engineering *Yarrowia lipolytica*. *Biotechnol Adv* 2018;36:2150–64.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001;25:402–8.
- Madzak C, Tréton B, Blanchin-Roland S. Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. *J Mol Microbiol Biotechnol* 2000;2: 207–16.
- Madzak C. *Yarrowia lipolytica*: recent achievements in heterologous protein expression and pathway engineering. *Appl Microbiol Biotechnol* 2015;99:4559–77.
- Miller KK, Alper HS. *Yarrowia lipolytica*: more than an oleaginous workhorse. *Appl Microbiol Biotechnol* 2019;103:9251–62.
- Morin N, Cescut J, Beopoulos A et al. Transcriptomic analyses during the transition from biomass production to lipid accumulation in the oleaginous yeast *Yarrowia lipolytica*. *Polymenis M, ed. PLoS One* 2011;6:e27966.
- Müller S, Sandal T, Kamp-Hansen P et al. Comparison of expression systems in the yeasts *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Cloning of two novel promoters from *Yarrowia lipolytica*. *Yeast* 1998;14:1267–83.
- Nicaud J-M. *Yarrowia lipolytica*. *Yeast* 2012;29:409–18.
- Ochoa-Estopier A, Guillouet SE. D-stat culture for studying the metabolic shifts from oxidative metabolism to lipid accumulation and citric acid production in *Yarrowia lipolytica*. *J Biotechnol* 2014;170:35–41.
- Ogrydziak DM, Demain AL, Tannenbaum SR. Regulation of extracellular protease production in *Candida lipolytica*. *Biochim Biophys Acta* 1977;497:525–38.
- Qiao K, Imam Abidi SH, Liu H et al. Engineering lipid overproduction in the oleaginous yeast *Yarrowia lipolytica*. *Metab Eng* 2015;29:56–65.
- Shabbir Hussain M, Gambill L, Smith S et al. Engineering promoter architecture in oleaginous yeast *Yarrowia lipolytica*. *ACS Synthetic Biology* 2016;5:213–23.
- Tezaki S, Iwama R, Kobayashi S et al. $\Delta 12$ -fatty acid desaturase is involved in growth at low temperature in yeast *Yarrowia lipolytica*. *Biochem Biophys Res Commun* 2017;488: 165–70.
- Trassaert M, Vandermies M, Carly F et al. New inducible promoter for gene expression and synthetic biology in *Yarrowia lipolytica*. *Microb Cell Fact* 2017;16:141.
- Trébulle P, Nicaud J-M, Leplat C et al. Inference and interrogation of a coregulatory network in the context of lipid accumulation in *Yarrowia lipolytica*. *NPJ Syst Biol Appl* 2017; 3:21.
- Tsakraklides V, Kamineneni A, Consiglio AL et al. High-oleate yeast oil without polyunsaturated fatty acids. *Biotechnol Biofuels* 2018;11:131.
- Xue Z, Zhu Q. Ammonium transporter promoter for gene expression in oleaginous yeast [Internet]. US20060094102A1, 2006 [cited 2019 Apr 30]. Available from: <https://patents.google.com/patent/US20060094102A1/en>
- Yuan C, Bloch KS. Conversion of oleic acid to linoleic acid. *J Biol Chem* 1961;236:1277–9.