

Research Article

A strong hybrid fatty acid inducible transcriptional sensor built from *Yarrowia lipolytica* upstream activating and regulatory sequences.†

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Abbreviations: **ARA**, arachidonic acid; **EPA**, eicosapentaenoic acid; **LA**, linoleic acid; **LIP2**, lipase 2; **OA**, oleic acid; **POT1**, peroxisomal 3-ketoacyl-thiolase; **POX2**, peroxisomal acyl CoA oxidase 2; **SLIC**, sequence and ligation independent cloning; **TEF1- α** , translation elongation factor 1- α ; **UAS**, upstream activating sequence; **URS**, upstream regulatory sequence.

Abstract

The engineering of *Yarrowia lipolytica* to accumulate lipids with high titers and productivities has been enabled with a handful of constitutive promoters for pathway engineering. However, the development of promoters that are both strong and lipid responsive could greatly benefit the bioproduction efficiency of lipid-derived oleochemicals in oleaginous yeast. In this study, we sought to engineer a fatty acid regulated hybrid promoter for use in *Y. lipolytica*. We identified a 200 bp upstream regulatory sequence in the peroxisomal acyl CoA oxidase 2 (POX2) promoter. Further analysis of the promoter sequence revealed a regulatory sequence, that when used in tandem repeats, led to a 48-fold induction of gene expression relative to glucose and 4-fold higher than the native POX2 promoter. To date, this is the strongest inducible promoter reported in *Y. lipolytica*. Taken together, our results show that it is possible to engineer strong promoters that retain strong inducibility. These types of promoters will be useful in controlling metabolism and as fatty acid sensors.

1 Introduction

Metabolic engineering of microorganisms for bioproduction greatly benefits from transcriptional control of native or heterologous genes [1, 2]. Precision control of gene expression enables rapid pathway optimization [3]. To that end, significant work has been put into developing libraries of promoters with predictable strength for a number of microorganisms [1, 4, 5]; however, in even well-characterized eukaryotes, such as *Saccharomyces cerevisiae*, the tool kit of promoters remains small compared to bacteria. Given the benefits of metabolic engineering in yeast [6, 7], additional focus on expanding the yeast promoter tool kit is warranted. Random mutagenesis of promoters has resulted in only modest improvements in promoter strength [1]. Similarly, it has been quite simple to identify loss-of-strength mutations in promoters [1, 4]. Rational approaches to increasing promoter strength have focused on hybrid promoters, where heuristically identified, well-defined DNA elements called upstream activating sequences (UASs) are placed in front of a core promoter sequence and can be used in a modular fashion to tune transcription strength [8-12]. While improving the strength of yeast promoters has been successful, the toolkit is currently lacking many options for inducible promoters.

Libraries of constitutive promoters with different strength can be used to tune gene expression; however, the expression level is statically set. Inducible promoters are useful genetic tools for metabolic engineering because in addition to the benefits of being able to tune the transcriptional output, expression level can be changed dynamically by metabolites or inducers. Inducible promoters can be especially important to improve the carbon flux efficiencies in a metabolic process by separating the growth phase from the production phase of a cell [13, 14]. Additionally, if the excess production of an enzyme or a product is toxic to the cell, it would be beneficial to have production switched off so cell growth would not be inhibited until the desired biomass is made [15, 16]. Inducible promoters used in metabolic engineering of yeast have largely been endogenous promoters responsive to small molecules such as copper, methionine, tryptophan, and phosphate [17-20]. Unfortunately, these promoters exhibit complex regulation patterns and are of modest strength at best. One notable exception is the Gal promoter from *S. cerevisiae*, which is both strongly repressed by glucose and strongly activated by galactose [17]. An upstream regulatory sequence (URS) from the GAL1-10 promoter has been used with a strong endogenous promoter, TDH, to confer galactose inducible control of the TDH promoter to create an inducible chimeric promoter [21]. Recently, a tryptophan inducible TDH promoter

was engineered, using a mutant ARO80 transcription factor and tandem repeats of the URS_{ARO9}, resulting in promoters that are both strong and induced by tryptophan [22].

In recent years, there has been an increase in genetic tools for metabolic engineering in the oleaginous yeast, *Y. lipolytica*. The advancement of genetic tools [8, 12, 23, 24] has enabled metabolic engineering in this microorganism to produce a significant amount of FAs [25] and lipids [26-28] from different substrates [29-31]. Given the relative weakness of endogenous *Y. lipolytica* promoters, hybrid promoters have had great success in heterologous gene expression [8]. This success stems from the ability to tune expression strengths in hybrid systems. Whereas high expression is often desired [26], the option to tune transcriptional strength to lower levels can be critical to attain optimal heterologous protein production [32]. An important set of hybrid promoters developed in *Y. lipolytica* contain the UAS1B element originally derived from the nitrogen and pH regulated XPR2 promoter [11, 33]. When used in tandem, the UAS1B elements provide enhancement in expression independent of nitrogen level and pH [8, 9, 11]. Another hybrid promoter used for metabolic engineering contains UAS_{TEF} from the constitutive promoter of the translation elongation factor 1- α (TEF1- α) gene of *Y. lipolytica* [9]. Strong expression of genes is enabled by hybrid promoters; however, the current set of strong hybrid promoters is limited. Although tunable in expression strength, hybrid promoters sometimes elicit carbon source dependent regulation from the UAS, as was previously demonstrated using the POX2 UAS1B [12]. While this property might be used to create regulated promoters, the inducibility is neither rational nor tightly regulated. There has only been one report of a rationally designed inducible hybrid promoter in *Y. lipolytica*. This alkane inducible promoter was constructed from repeats of an alkane response element, but confers relatively weak expression when grown on alkanes [34-37]. Therefore, development of hybrid promoters that are inducible to a biomolecule that is both readily metabolized in *Y. lipolytica* and acts as a precursor molecule for the synthesis of several other important biomolecules is desirable. Fatty acids are ideally suited for this task.

The native promoters from the peroxisomal acyl-CoA oxidase 2 (POX2), peroxisomal 3-ketoacyl-thiolase (POT1), and lipase 2 (LIP2) genes are commonly used as fatty acid inducible systems for metabolic engineering [38-44]. While the expression strength of the inducible promoter of POX2 has previously been characterized as strong [43, 45, 46], we show that the POX2 promoter is relatively weak compared to the beta actin promoter and engineered hybrid promoters. In this work, we performed a series of

truncations to identify fatty acid responsive UASs and URSs that were combined to construct a library of hybrid promoters. A URS, called R1, was initially found to be critically important for fatty acid responsiveness and promoter strength. The amplifying effect of the R1 sequence was shown to be synergistic with an UAS, called A1. Tandem repeats of the R1 sequences from the POX2 promoter were used to create a strong fatty acid inducible system. This new hybrid promoter was approximately four-times the strength of the native POX2 promoter and smaller in size. This promoter had a 48-fold oleic acid induction of expression relative to glucose. The promoter was also induced by other fatty acids and lipids, but remained strongly repressed in glycerol and glucose. Stronger inducible promoters were engineered by placing tandem R1 sequences upstream of either the TEF1- α promoter minimal promoter or the TEF1- α intron promoter, achieving expression levels greater than 10-fold higher than the native POX2 promoter. The hybrid promoter described here is the first engineered and strongest fatty acid inducible promoter system for *Y. lipolytica*.

2 Materials and methods

2.1 Chemicals & enzymes

Chemicals used in this study were obtained from Sigma Aldrich unless otherwise stated. All enzymes were purchased from New England Biolabs (NEB). Plasmid mini-preps were performed using the Zyppy™ Plasmid Miniprep Kit (Zymo Research). PCR clean-ups were performed using the DNA Clean & Concentrator™ (Zymo Research). RNA extractions were done using the E.Z.N.A® Yeast RNA Kit (Omega Biotek). Real-time PCR experiments were done using the qScript™ One Step SYBR® Green qRT-PCR Kit (Quanta Biosciences).

2.2 Strains & culture conditions

Plasmid propagation was performed using *Escherichia coli* DH10 β competent cells (NEB). Transformations in *E. coli* were performed using standard methods [47]. *Y. lipolytica* strain PO1f (ATCC MYA-2613; *MATa leu2-270 ura3-302 xpr2-322 axp*) was purchased from ATCC. *Y. lipolytica* transformations were done using the lithium acetate method as previously described with a minor modification for cell propagation after the transformation [12]. Briefly, following the heat shock step, the cells were mixed with 800 μ L of 0.1 M lithium acetate, spun down at 6,000 \times g for 2 minutes (4°C) and re-suspended in 100 μ L of 0.1 M LiAc buffer prior to inoculating 2 mL cultures that were grown in 14 mL culture tubes. All *Y. lipolytica* cultures were grown at 215 rpm and 28 °C. Transformations and growth were performed in biological triplicates. *Y. lipolytica* was grown in Yeast

Synthetic Complete media without leucine (YSC-LEU) comprised of 6.7 g/L yeast nitrogen base (YNB) without amino acids (Difco) and 0.69 g/L CSM-LEU (Sunrise Science Products). Carbon substrates used for characterization during the construction of the hybrid promoter were 2% (v/v) oleic acid (EMD Millipore) emulsified in 0.05% (v/v) Tween 80, and 2% (w/v) D-glucose. Other substrates that were used to characterize the substrate responsiveness of the hybrid promoter were all added at 30 mM, an equimolar concentration equivalent to 2% (v/v) oleic acid. These substrates include, glycerol, linoleic acid (Alfa Aesar), triolein (Tokyo Chemical Industry), chicken fat (Animal Coproducts Research and Education Center, Clemson University) and n-decane. In cases where two carbon substrates were used, each was added at a final concentration of 15 mM. The hydrophobic substrates in the dual carbon experiments were emulsified with Tween 80 at a final concentration of 0.05% (v/v).

2.3 Promoter and plasmid design

All primers used to create the hybrid promoters designed in this study are presented in Supplementary Table 1. Unless otherwise stated, sequence and ligation independent cloning (SLIC) [48] was used for cloning, and all PCRs were performed from *Y. lipolytica* genomic DNA. The base vector used for cloning was pSL16-CEN1-UAS1B8-POX2(100 bp)-hrGFP [12]. Our previous work identified a large region of the POX2 promoter that was important for POX2 promoter fatty acid responsiveness (-1590 bp to -513 bp) [12]. Within this region, we made periodic truncations using the primer pairs described in Section 1 of Table 1. The truncated POX2 promoter PCR fragments was inserted between the SphI/Ascl restriction sites using SLIC. Hybrid promoter systems containing UAS1_{POX2} and UAS2_{POX2} (A1A2), UAS2_{POX2} and UAS3_{POX2} (A2A3) and UAS1_{POX2} and UAS3_{POX2} (A1A3) were constructed by performing a three-piece SLIC with each UAS and the base vector as described in Section 2 of Table 1. To construct A1A2A3, the A2 fragment was PCR amplified and inserted in the A1A3 vector at the MfeI site, as shown in Section 3 of Table 1. Inserts A1R1 and A1R1A2R2 were PCR amplified from genomic DNA and inserted in the base vector A1A3 digested with AvrII / SphI. Promoters containing URS1_{POX2} (R1) and URS2_{POX2} (R2) were constructed by PCR and cloned in between the SphI and Ascl sites of the base vector (Section 4 of Table 1). The R1 sequence was inserted between into A2A3 and A1A3 respectively using the MfeI site (Sections 5 and 6 of Table 1). Additional R1 sequences were inserted to make A1(R1x2)A3 and A1(R1x3)A3 (Section 7 and 8 of Table 1). Construction of the R1A3 plasmid required the R1A3 insert be PCR amplified from the

A1R1A3 vector and inserted into the A1R1A3 base vector digested with AvrII / SphI. A1R1A1R1A3 was created by PCR amplifying the A1R1 sequence from gDNA and inserting it into the A1R1A3 vector digested with MfeI. To create the fatty acid inducible hybrid promoter containing the TEF(136) (Section 9 of Table 1), TEF(136)-hrGFP was PCRed from a previous constructed vector [8] and A3 fragment was PCRed from the POX2 promoter. These fragments were inserted into a MfeI / AscI digested hybrid fatty acid vector (Section 8 of Table 1). Construction of the fatty acid inducible promoter containing the TEF-intron utilized the same strategy instead in this case, the TEF-intron-hrGFP vector had to first be constructed as described in Sections 10 and 11 of Table 1. The vector constructed in Section 11 of Table 1 was then used as the template to PCR the TEF-intron-hrGFP.

2.4 RNA extraction and quantitative PCR

RNA extractions were performed on cell cultures that were grown until mid-exponential phase. Prior to RNA extraction, all cell cultures were normalized to an OD₆₀₀ of 10 and 1 ml was used for the extraction using the Omega Biotek RNA Extraction Kit with the optional DNaseI digestion step. qPCR was done using the CFX Manager real-time machine from Bio-Rad. In accordance with MIQE guidelines, standard curves for quantification of hrGFP and beta actin were created and the efficiency of each primer pair was calculated. Two housekeeping genes, beta actin and TEF1- α were used as reference genes to initially validate analysis method. Equal mass of RNA from each of the samples was loaded. Protocols for qPCR conditions are described in protocols provided by qPCR kit supplier. A relative quantification method was used to determine GFP expression. Standard curves were used to calculate copy numbers of for the above-mentioned genes taking into account priming efficiency. The ratio of GFP mRNA copy number to beta actin mRNA copy number was used to quantify changes in expression strength for the different POX2 promoter truncations. qPCR was used to measure GFP expression instead of flow cytometry because of its higher sensitivity.

2.5 GFP fluorescence analysis

During the development of the POX2 promoter, fluorescence spectroscopy with the Biotek Synergy MX fluorescence spectrophotometer was the method of choice for characterization of promoter strength. Cells grown in glucose were spun down at 6000 x g (4 °C) for 2 minutes and re-suspended in 0.1 M Phosphate Buffered Saline (PBS) (pH 7) while cell cultures from oleic acid were spun down, washed once with 0.1 M PBS containing 5% (v/v) Tween 80 and re-suspended in 0.1 M PBS. All cell cultures were grown for 36

hours, until mid to late exponential phase, where OD600 values across the samples were similar. The harvested cells were placed in 96 well plates and serial dilutions of the cells were performed to obtain an average fluorescence (Ex. 485 nm and Em. 510 nm). Serial dilutions were performed to obtain a fluorescence value in the linear range of detection without changing the gain for each experiment. The same gain was used for all measurements. The BD Accuri® C6 Flow Cytometer was used for promoter characterization with different carbon sources. In all flow cytometry measurements, 20,000 single cell events were counted and fluorescence was measured using the GFP channel. VirtualGain® was used to normalize the gain across all samples post-analysis. Fluorescent cell populations were gated and the same gate was used across all samples analyzed in each day. To obtain the specific mean fluorescence, the mean fluorescence of the non-fluorescent cells was subtracted from the mean fluorescence of the gated fluorescent cells.

3 Results

3.1 Identification of fatty acid responsive upstream sequences in the POX2 promoter

The most common approach for identifying UASs and URSs by measuring expression strength determined by a reporter gene from truncated promoters [9, 10, 49-51]. Such a description of the promoter architecture provides information about important DNA sequences in the promoter, but leaves unanswered questions about the nature of these sequences and how they contribute to gene regulation patterns. Nevertheless, in systems such as *Y. lipolytica*, our lack of understanding of gene regulation prevents a more informed promoter engineering strategy *a priori*.

We previously made truncations to the POX2 promoter and identified a large region upstream (1590 bp) of the POX2 gene that is required for measurable transcription in glucose-free oleic acid media [12]. As a result, we chose to make truncations from the 5' end of the POX2(1590 bp) promoter at 200 bp intervals (Figure 1A). Our rationale for choosing these particular truncations was based on the identification of putative Por1p binding sites (Figure 1A). Por1p (YALI0D12628p) is a *Y. lipolytica* homolog of the fatty acid responsive FarA transcriptional factor in *Aspergillus nidulans* [52, 53]. Homologs for *S. cerevisiae* fatty acid responsive transcription factor Oaf1p do not exist in *Y. lipolytica* or other oleaginous yeast. Therefore, the well-studied *S. cerevisiae* regulatory system does not inform our work in *Y. lipolytica*.

Quantitative PCR was used to detect changes in transcriptional profiles resulting from POX2 truncations (Figure 1B). In YSC-LEU media with oleic acid, there is a general decrease in expression strength with truncations moving towards to the core promoter. A four-fold decrease in mRNA copy number was observed between POX2 (1590 bp) and POX2 (1190 bp), suggesting the presence of an activating sequence we call $\text{UAS}_{1\text{POX2}}$ or A1. Another significant change in mRNA transcript was observed between POX2 (990 bp) and POX2 (540 bp); therefore, we call this region $\text{UAS}_{2\text{POX2}}$ or A2. A smaller drop in expression was observed between POX2 (438 bp) and the core promoter, POX2 (100 bp), defining a third activating sequence, $\text{UAS}_{3\text{POX2}}$ or A3. This truncation strategy also enabled us to identify regions in the native POX2 promoter where a single 200 bp truncation lead to a three-fold increase of transcriptional activity, as seen between the POX2 (1190 bp) and POX2 (990 bp) promoters. We call this upstream regulatory sequence $\text{URS}_{1\text{POX2}}$ or R1. A similar repressor sequence was observed for the truncation between POX2 (540 bp) and POX2 (513bp) and statistically similar POX2 (438 bp), albeit to a weaker extent than R1, that we call $\text{URS}_{2\text{POX2}}$ or R2. Using this truncation strategy, we were able to map sequences in the native POX2 promoter that were potential fatty acid responsive activating sequences and other sequences that appeared to behave as repressor sequences, although further investigation was required.

3.2 Discovery of a fatty acid inducible upstream regulatory sequence from POX2

By performing promoter truncations, 3 UASs and 2 URSs were identified in the native POX2 promoter. Our initial hypothesis was that by removing URSs, equivalent to combining the 3 UASs, would increase the fatty acid inducible expression. Furthermore, we reasoned loss of UASs unimportant for fatty acid regulated transcription would not greatly impact transcription. To test these hypotheses, we constructed promoters with various combinations of UAS in tandem (Figure 2A), expecting to obtain a promoter more strongly induced by oleic acid. When all three UASs were combined (A1A2A3), expression in YSC-LEU with oleic acid was diminished compared to the full native POX2 promoter (Figure 2B), suggesting the URSs might have a more complex role than could be predicted by the truncation experiments. This idea will be explored in section 3.3. When A3 was removed, resulting in promoter A1A2, transcription was further decreased, suggesting an important role for A3 sequence. By restoring the A3 sequence and removing the A1 to create promoter A2A3, expression strength recovered to a value closer to that of the native POX2 promoter,

further validating the importance of A3. When A2 was removed, resulting in promoter A1A3, gene expression was now comparable to the native POX2 promoter, suggesting that the A2 is dispensable and actually inhibitory in the context of these hybrid promoters. The A1A2 hybrid promoter that lacks the A3 sequences confers the weakest expression while A1A3, which is half the size of the native promoter, confers the strongest expression. This results suggests that the A1 and A3 sequence combine to provide essential function for A1A3 promoter. It should be noted that two tandem copies of the either A1 or A3 were tested and expression was significantly weaker than the native POX2 promoter (Supplementary Figure 1). Furthermore, these UAS sequences elicited a positional dependence as switching the order of A1A3 to A3A1 resulted in a significant drop in fluorescence (Supplementary Figure 1), suggesting again that substantial complexity exists in these systems. All promoters showed almost no expression when cells were cultured in YSC-LEU with glucose (Figure 2B).

From the initial 5' truncation data, removal of either the R1 or R2 sequences conferred an increase in transcriptional activation suggesting their role as repressor sequences. However, because of the unexpected results from 5' truncations, we created a series of 3' truncations (Figure 2C). Promoter A1R1A2R2 has oleic acid induced transcription similar to but lower than POX2 (Figure 2D), indicating the importance of A3. Further truncation to create A1R1A2 resulted in increased transcription, producing GFP similar to the POX2 promoter. This result is consistent with our previous findings that R2 is regulatory sequence that acts as a repressor. The relative unimportance of the A2 sequence is further confirmed by oleic acid induced expression from A1R1, which is similar to A1R1A2.

By comparing the results in Figure 2B and D, it became clear R1 also exhibited complex behaviors not predicted by the original truncation experiment. These data suggest that R1 can act as an activator when placed after the A1 sequence, contrary to the repression observed when the A1 sequence was removed from the native POX2 promoter during our truncation experiments (Figure 1). The same amplifying effect of the R1 sequence was not observed when the R2 sequence was placed in its natural position between the A2 and A3 sequences in promoter A2R2A3 (Supplementary Figure 1). The R1 sequence, when placed between the A2 and A3 sequences (A2R1A3) also did not confer strong activation of the promoter (Figure 2D), suggesting that there is a synergistic effect between the A1 and R1 sequence. The R1A3 promoter shows lower expression than A1R1, however the difference is not statistically significant. The results here demonstrated the importance of the R1

sequence as an enhancer element when paired with the A1 and perhaps A3 sequence. This is also suggested by the A1R1 data in Figure 2D. To support this hypothesis, we created promoter A1R1A3, which combines the synergistic effects of A1R1 and the A3 sequence. This promoter was nearly three-fold stronger than the native POX2 promoter, and serves as the foundation for building even stronger fatty acid inducible promoters.

3.3 Engineering a strong fatty acid inducible hybrid promoter

Including the R1 in between the A1 and A3 sequences to form hybrid promoter A1R1A3 resulted in strong oleic acid activation compared to the native POX2 promoter, and was a logical starting point to design a stronger fatty inducible promoter. Based on previous hybrid promoter work [8, 9, 54], we hypothesized that we could increase the oleic acid inducible transcriptional activation using tandem repeats of the R1 sequence. Therefore, hybrid promoters containing 0-3 copies of the R1 sequences were created (Figure 3A) and compared to the native POX2 promoter. The addition of each copy of the R1 sequence increased gene expression induced by oleic acid while the expression in glucose remained significantly and equally repressed, demonstrating the oleic acid inducible nature of the R1 sequence (Figure 3B). Addition of the first R1 sequence created a 2-fold increase in expression while subsequent additions of R1 sequences lead to about a 4-fold improvement in expression strength compared to the native POX2 promoter. Furthermore, we were able to improve the fold induction in oleic acid to 48-fold in the A1(R1x3)A3 hybrid promoter compared to the 19-fold induction in the native POX2 promoter (Figure 3C). Another promoter, (A1R1)x2A3 was created and had similar expression levels in oleic acid media as well as similar fold induction (Figure 3B and 3C). Given its larger size compared to A1(R1x3)A3, we chose to move forward with smaller promoter. This new inducible hybrid promoter demonstrates the ability to engineer a tightly regulated oleic acid inducible switch and to tune the transcriptional output of the activated promoter.

The A1(R1x3)A3 promoter is already comparable in strength to strong hybrid promoters containing UAS1B8 and the POX2 core promoter (Supplementary Figure 2). We have previously shown that the strength of a hybrid promoter can be tuned by manipulating the modular elements of the promoter [12]. We demonstrate additional improvements to the A1(R1x3)A3 promoter by replacing the weaker POX2 core promoter with the stronger TEF(136) and TEF-intron core promoter (Supplementary Figure 2A), resulting in an additional two-fold and a three-fold increase in expression, respectively (Supplementary

Figure 2B). Engineering the modular core promoter element allows us to tune the induction strength over a 10-fold range of expression; however, the increased expression in glucose led to a reduction in the fold induction (Supplementary Figure 2C). The core promoter is likely to exhibit some level of regulation mediated by regulatory transcription factors that bridge URS and the core promoter [55].

3.4 The A1(R1x3)A3 hybrid promoter is a fatty acid sensor

To better understand how different substrates affect transcription from the A1(R1x3)A3 hybrid promoter, we used flow cytometry to measure hrGFP expression controlled by A1(R1x3)A3 and compared it to the native POX2 promoter. Figure 4A shows that while both promoters were activated by various fatty acids (oleic and linoleic acid) and lipids (triolein and chicken fat), the inducible hybrid promoter A1(R1x3)A3 is consistently stronger than the native POX2 promoter. Linoleic acid elicited the highest transcriptional response for both promoters (Figure 4A). In all fatty acid and lipid substrates, the A1(R1x3)A3 promoter is two-to-four-fold stronger than the POX2 promoter (Figure 4B). Tween 80 was used as an emulsifier for fatty acid media and on its own does not elicit strong transcriptional activation.

When glucose, glycerol, or n-decane are used as the sole carbon source, the hybrid promoter remains strongly repressed, with only basal transcriptional activity similar to the native POX2 promoter. In media containing both glucose and oleic acid, the native POX2 promoter remained repressed, while the A1(R1x3)A3 hybrid promoter was strongly activated, suggesting the hybrid promoter is not catabolite repressed. Interestingly, in media containing both glycerol and oleic acid, both the native POX2 promoter and the A1(R1x3)A3 hybrid promoter were more strongly activated than oleic acid alone. The mechanism underlying this synergy remains unclear, however a similar behavior was reported for the Lip2 promoter [44].

3.5 Fatty acid induction of the A1(R1x3)A3 hybrid promoter

In order to use the A1(R1x3)A3 promoter as an fatty acid inducible promoter, we grew cells to stationary phase in YSC-LEU glucose media and then induced the A1(R1x3)A3 promoter by titrating oleic acid into the media, at concentrations ranging from 0.25% (v/v) to 8%(v/v). The induction was measured using fluorescence spectroscopy. At all concentrations tested within this range, we observe nearly identical induction profiles of

the hybrid promoter. This suggests the A1(R1x3)A3 promoter can be induced at oleic acid concentrations as low as 0.25% (v/v) during the stationary phase (Supplementary Figure 3).

Given the high sensitivity of the hybrid promoter, we determined if other fatty acids would similarly induce the hybrid promoter at this low concentration. We used 0.25% (v/v) of oleic (OA), linoleic (LA), arachidonic (ARA), and eicosapentaenoic (EPA) acids in YSC-LEU to induce the hybrid promoter in the stationary phase. The hybrid promoter is strongly and similarly induced by the different fatty acids (Figure 5A-D) while glucose did not induce GFP expression (Figure 5E).

4 Discussion

Our work has resulted in the development of a fatty acid inducible hybrid promoter for *Y. lipolytica*. UAS and URS sequences were initially identified using a rational truncation strategy; however, our subsequent experiments resulted in different conclusions about the roles of the A1 and R1 sequences when tested in isolation of the native POX2 promoter. Therefore, the truncation approach is useful in identifying functionally important sequences of promoters, but in this case, fails to correctly identify how these sequences will work in different contexts. This analysis showed that the R1 sequence contains a fatty acid response element and that it acts synergistically with the A1 sequence. Hybrid promoters with tandem repeats of the R1 sequence lead to increased transcriptional strength in oleic acid media (four-fold stronger than native POX2) while maintaining tight repression in glucose media (48-fold induced by oleic acid). These hybrid promoters are strongly activated by a variety of long chain fatty acid and lipids. Interestingly, the engineered hybrid promoter is not catabolite repressed in contrast to the native POX2 promoter and carbon metabolism promoters [20, 56-58].

The only comparable work in *Y. lipolytica* focuses on alkane responsive elements (AREs). Prior studies have identified a URS in the ALK1 gene (responsible for alkane oxidation in alkane metabolism) that contains an ARE that binds the Yas1p/Yas2p transcription factors [35]. A hybrid promoter containing three copies of the ARE1 sequence were placed upstream of the LEU2 core promoter, resulting in 6-fold activation on n-decane compared to glucose. By comparison, the A1(R1x3)A3 promoter is 48-fold activated by fatty acids compared to glucose. While differences in assay methods prevent a direct comparison of the strength of these two promoters, we have shown alkane promoters are significantly

weaker than the beta-oxidation promoters (Supplementary Figure 4). This finding opens opportunities to engineer additional responsive hybrid promoter tools for *Y. lipolytica*.

Enabled by a deeper understanding of its genetics, there has been more work engineering strong inducible promoters in *S. cerevisiae*. For example, the most well studied inducible yeast promoter is the Gal1-Gal10 system. It exhibits remarkably low basal transcription in glucose containing media and activated up to four orders of magnitude by galactose. These levels of regulation are determined by the combination of six repressing operator sites that overlap four Gal4p binding sites [59]. Analogously, further improvements to our fatty acid responsive hybrid promoter may be possible once the transcription factors that bind to the R1 sequence of POX2 are identified.

We demonstrated that combining the hybrid promoter containing R1 repeats with other core promoters, including the TEF(136) and TEF-intron core promoters increased the both the basal level expression and the induced expression. This result was expected in light of work combining different *S. cerevisiae* UAS sequences. For example, combining a constitutive UAS from CYC1 with the Gal1 or Gal10 promoter elements resulted in galactose regulated expression [21]. Stronger hybrid promoters were engineered by placing the UAS_{gal} upstream of weak core promoters (pLEU and pCYC) leading to glucose repression and galactose activation; however, when UAS_{gal} was placed upstream of strong core promoters (pTEF and pTDH3), a higher level of basal expression in glucose was observed [60]. This study also directly used individual Gal4p binding sites to further tune and enhance promoter regulation. More recently, ultra-strong and tryptophan regulated promoters were created by placing 5 UAS_{aro} sequences upstream of the TDH core promoter [22]. These promoters were 1.7-fold stronger than the TDH promoter and had 14-fold induction by tryptophan. Future efforts to increase promoter strength and maintain strong inducibility would benefit from additional focus on upstream sequences, transcription factor binding sites, and less emphasis on strong core promoters.

UASs and URSs from native promoters have traditionally been identified through truncation studies [9, 33, 49]. While this method proves to be a solid foundation for identifying the parts needed for hybrid promoters, it fails to always capture the complexity of eukaryotic transcription regulation, which is controlled by the association of multiple transcriptional factors to their cognate binding sites [61, 62]. Because of the heuristic way

in which UASs and URSs are identified, they are inherently subject to context dependent behaviors. Our work demonstrates the difficulty encountered as a result of this disconnect. The UAS/URS sequences contain multiple and often overlapping transcription factor binding sites enabling higher strength transcription or regulation of transcription [21]. Unlike *S. cerevisiae*, there are few studies on *Y. lipolytica* transcription factors or transcription factor binding site motifs, and at least with respect to fatty acid metabolism, transcription factors in *S. cerevisiae* do not always have homologs in *Y. lipolytica*. To date, there has been only one transcription factor associated with fatty acid regulation in *Y. lipolytica*. Deletion of the POR1 gene causes some growth defect on oleic acid and a reduction in POX2 mRNA expression [53]. As POR1p is a homolog of FarA from *A. nidulans*, POR1p may bind a similar DNA sequence. Putative POR1p binding sites were found in the R1 region, suggesting a significant role of POR1 in regulating the A1(R1x3)A3 promoter. A better understanding of *Y. lipolytica* transcription factors and their binding sites may lead to a more direct identification UAS/URS sequences and more rapid design of regulated hybrid promoters.

The A1(R1x3)A3 promoter is induced by a several different long chain fatty acids, including OA, LA, ARA, and EPA. Interestingly, stationary phase induction (Figure 5) of this promoter was nearly identical for OA, LA and ARA, whereas when cells were grown on the fatty acids (Figure 4), LA more strongly induced the promoter. EPA caused the greatest induction of the hybrid promoter, which was unexpected since EPA is not synthesized by *Y. lipolytica*. When measuring induction during growth, the hybrid promoter was not repressed by glucose or glycerol when co-fed oleic acid, consistent with recent observations for the native Lip2 promoter; however, the PO1f strain used in our study does not co-utilize glucose and oleic, so the results shown by Sassi et al. [44] showing Lip2 promoter induction was strongest with a 40/60 mixture of glucose and oleic acid (w/w), are not likely to work in all strains. These works do suggest a potential strategy for fatty acid inducible gene expression when using glucose or glycerol as a substrate.

The fatty acid regulated hybrid promoter created in this study represents a significant advance in the toolkit for engineering *Y. lipolytica*. We demonstrated the identification of a URS, R1, and its construction into a fatty acid regulated promoter significantly stronger than the native POX2 promoter. We also showed that the hybrid promoter design can lead to tuning of both the transcriptional output as well as the

inducibility of the promoter. This promoter system is one of the strongest identified, and is the strongest inducible promoter for *Y. lipolytica*. This regulated promoter has great promise for use as a sensor for strain engineering applications, for dynamic regulation of heterologous gene expression, or as an inducible promoter for toxic genes. We anticipate further development of regulated and strong promoters to expand the genetic engineering tools available for *Y. lipolytica*.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

5 References

- [1] Alper, H., Fischer, C., Nevoigt, E., Stephanopoulos, G., Tuning genetic control through promoter engineering (vol 102, pg 12678, 2005). *P Natl Acad Sci USA* 2006, 103, 3006-3006.
- [2] Shabbir Hussain, M., Rodriguez, G., D., G., M., S., *et al.*, Recent Advances in Metabolic Engineering of *Yarrowia lipolytica*. *AIMS Bioengineering* 2016, 3, 493-514.
- [3] Blazeck, J., Alper, H. S., Promoter engineering: Recent advances in controlling transcription at the most fundamental level. *Biotechnology Journal* 2013, 8, 46-+.
- [4] Qin, X. L., Qian, J. C., Yao, G. F., Zhuang, Y. P., *et al.*, GAP Promoter Library for Fine-Tuning of Gene Expression in *Pichia pastoris*. *Appl Environ Microb* 2011, 77, 3600-3608.
- [5] De Mey, M., Maertens, J., Lequeux, G. J., Soetaert, W. K., Vandamme, E. J., Construction and model-based analysis of a promoter library for *E. coli*: an indispensable tool for metabolic engineering. *Bmc Biotechnol* 2007, 7.
- [6] Nielsen, J., Larsson, C., van Maris, A., Pronk, J., Metabolic engineering of yeast for production of fuels and chemicals. *Curr Opin Biotech* 2013, 24, 398-404.
- [7] Da Silva, N. A., Srikrishnan, S., Introduction and expression of genes for metabolic engineering applications in *Saccharomyces cerevisiae*. *Fems Yeast Res* 2012, 12, 197-214.
- [8] Blazeck, J., Liu, L. Q., Redden, H., Alper, H., Tuning Gene Expression in *Yarrowia lipolytica* by a Hybrid Promoter Approach. *Appl Environ Microb* 2011, 77, 7905-7914.
- [9] Blazeck, J., Reed, B., Garg, R., Gerstner, R., *et al.*, Generalizing a hybrid synthetic promoter approach in *Yarrowia lipolytica*. *Appl Microbiol Biot* 2013, 97, 3037-3052.
- [10] Guarente, L., Yocum, R. R., Gifford, P., A Gal10-Cyc1 Hybrid Yeast Promoter Identifies the Gal4 Regulatory Region as an Upstream Site. *P Natl Acad Sci-Biol* 1982, 79, 7410-7414.
- [11] Madzak, C., Treton, 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 Microb Biotech* 2000, 2, 207-216.
- [12] Shabbir Hussain, M., Gambill, L., Smith, S., Blenner, M. A., Engineering Promoter Architecture in Oleaginous Yeast *Yarrowia lipolytica*. *Acs Synth Biol* 2016, 5, 213-223.
- [13] Zaslaver, A., Mayo, A. E., Rosenberg, R., Bashkin, P., *et al.*, Just-in-time transcription program in metabolic pathways. *Nat Genet* 2004, 36, 486-491.
- [14] Holtz, W. J., Keasling, J. D., Engineering Static and Dynamic Control of Synthetic Pathways. *Cell* 2010, 140, 19-23.
- [15] Xu, P., Li, L., Zhang, F., Stephanopoulos, G., Koffas, M., Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. *Proc Natl Acad Sci U S A* 2014, 111, 11299-11304.
- [16] Zhang, F. Z., Carothers, J. M., Keasling, J. D., Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nature Biotechnology* 2012, 30, 354-U166.
- [17] Weinhandl, K., Winkler, M., Glieder, A., Camattari, A., Carbon source dependent promoters in yeasts. *Microb Cell Fact* 2014, 13.

- [18] Wimalarathna, R. N., Pan, P. Y., Shen, C. H., Co-dependent recruitment of Ino80p and Snf2p is required for yeast CUP1 activation. *Biochem Cell Biol* 2014, 92, 69-75.
- [19] Korber, P., Barbaric, S., The yeast PHO5 promoter: from single locus to systems biology of a paradigm for gene regulation through chromatin. *Nucleic Acids Research* 2014, 42, 10888-10902.
- [20] Solow, S. P., Sengbusch, J., Laird, M. W., Heterologous protein production from the inducible MET25 promoter in *Saccharomyces cerevisiae*. *Biotechnol Prog* 2005, 21, 617-620.
- [21] West, R. W., Chen, S. M., Putz, H., Butler, G., Banerjee, M., Gal1-Gal10 Divergent Promoter Region of *Saccharomyces-Cerevisiae* Contains Negative Control Elements in Addition to Functionally Separate and Possibly Overlapping Upstream Activating Sequences. *Gene Dev* 1987, 1, 1118-1131.
- [22] Leavitt, J. M., Tong, A., Tong, J., Pattie, J., Alper, H. S., Coordinated transcription factor and promoter engineering to establish strong expression elements in *Saccharomyces cerevisiae*. *Biotechnology Journal* 2016, 11, 866-876.
- [23] Schwartz, C. M., Hussain, M. S., Blenner, M., Wheeldon, I., Synthetic RNA Polymerase III Promoters Facilitate High-Efficiency CRISPR-Cas9-Mediated Genome Editing in *Yarrowia lipolytica*. *Acs Synth Biol* 2016, 5, 356-359.
- [24] Schwartz, C., Shabbir-Hussain, M., Frogue, K., Blenner, M., Wheeldon, I., Standardized Markerless Gene Integration for Pathway Engineering in *Yarrowia lipolytica*. *Acs Synth Biol* 2017, 6, 402-409.
- [25] Ledesma-Amaro, R., Dulermo, R., Niehus, X., Nicaud, J. M., Combining metabolic engineering and process optimization to improve production and secretion of fatty acids. *Metab Eng* 2016, 38, 38-46.
- [26] Blazeck, J., Hill, A., Liu, L. Q., Knight, R., *et al.*, Harnessing *Yarrowia lipolytica* lipogenesis to create a platform for lipid and biofuel production. *Nat Commun* 2014, 5.
- [27] Qiao, K., Abidi, S. H. I., Liu, H. J., Zhang, H. R., *et al.*, Engineering lipid overproduction in the oleaginous yeast *Yarrowia lipolytica*. *Metab Eng* 2015, 29, 56-65.
- [28] Friedlander, J., Tsakraklides, V., Kamineni, A., Greenhagen, E. H., *et al.*, Engineering of a high lipid producing *Yarrowia lipolytica* strain. *Biotechnol Biofuels* 2016, 9.
- [29] Hu, P., Chakraborty, S., Kumar, A., Woolston, B., *et al.*, Integrated bioprocess for conversion of gaseous substrates to liquids. *P Natl Acad Sci USA* 2016, 113, 3773-3778.
- [30] Rodriguez, G. M., Hussain, M. S., Gambill, L., Gao, D., *et al.*, Engineering xylose utilization in *Yarrowia lipolytica* by understanding its cryptic xylose pathway. *Biotechnol Biofuels* 2016, 9, 149.
- [31] Shaw, A. J., Lam, F. H., Hamilton, M., Consiglio, A., *et al.*, Metabolic engineering of microbial competitive advantage for industrial fermentation processes. *Science* 2016, 353, 583-586.
- [32] Dulermo, R., Brunel, F., Dulermo, T., Ledesma-Amaro, R., *et al.*, Using a vector pool containing variable-strength promoters to optimize protein production in *Yarrowia lipolytica*. *Microb Cell Fact* 2017, 16, 31.

- [33] Madzak, C., Blanchin-Roland, S., Otero, R. R. C., Gaillardin, C., Functional analysis of upstream regulating regions from the *Yarrowia lipolytica* XPR2 promoter. *Microbiol-Uk* 1999, *145*, 75-87.
- [34] Sumita, T., Iida, T., Yamagami, S., Horiuchi, H., *et al.*, YlALK1 encoding the cytochrome P450ALK1 in *Yarrowia lipolytica* is transcriptionally induced by n-alkane through two distinct cis-elements on its promoter. *Biochem Bioph Res Co* 2002, *294*, 1071-1078.
- [35] Yamagami, S., Morioka, D., Fukuda, R., Ohta, A., A basic helix-loop-helix transcription factor essential for cytochrome P450 induction in response to alkanes in yeast *Yarrowia lipolytica*. *J Biol Chem* 2004, *279*, 22183-22189.
- [36] Mori, K., Iwama, R., Kobayashi, S., Horiuchi, H., *et al.*, Transcriptional repression by glycerol of genes involved in the assimilation of n-alkanes and fatty acids in yeast *Yarrowia lipolytica*. *Fems Yeast Res* 2013, *13*, 233-240.
- [37] Thevenieau, F., Le Dall, M. T., Nthangeni, B., Mauersberger, S., *et al.*, Characterization of *Yarrowia lipolytica* mutants affected in hydrophobic substrate utilization. *Fungal Genet Biol* 2007, *44*, 531-542.
- [38] Nicaud, J. M., Madzak, C., van den Broek, P., Gysler, C., *et al.*, Protein expression and secretion in the yeast *Yarrowia lipolytica*. *Fems Yeast Res* 2002, *2*, 371-379.
- [39] Nthangeni, M. B., Urban, P., Pompon, D., Smit, M. S., Nicaud, J. M., The use of *Yarrowia lipolytica* for the expression of human cytochrome P450CYP1A1. *Yeast* 2004, *21*, 583-592.
- [40] Garofano, A., Eschemann, A., Brandt, U., Kerscher, S., Substrate-inducible versions of internal alternative NADH: ubiquinone oxidoreductase from *Yarrowia lipolytica*. *Yeast* 2006, *23*, 1129-1136.
- [41] Gasmi, N., Ayed, A., Ammar, B. B. H., Zrigui, R., *et al.*, Development of a cultivation process for the enhancement of human interferon alpha 2b production in the oleaginous yeast, *Yarrowia lipolytica*. *Microb Cell Fact* 2011, *10*.
- [42] Hong, S. P., Seip, J., Walters-Pollak, D., Rupert, R., *et al.*, Engineering *Yarrowia lipolytica* to express secretory invertase with strong FBA1IN promoter. *Yeast* 2012, *29*, 59-72.
- [43] Pignede, G., Wang, H. J., Fudalej, F., Seman, M., *et al.*, Autoclone and amplification of LIP2 in *Yarrowia lipolytica*. *Appl Environ Microbiol* 2000, *66*, 3283-3289.
- [44] Sassi, H., Delvigne, F., Kar, T., Nicaud, J. M., *et al.*, Deciphering how LIP2 and POX2 promoters can optimally regulate recombinant protein production in the yeast *Yarrowia lipolytica*. *Microb Cell Fact* 2016, *15*.
- [45] Juretzek, T., Wang, H.J., Nicaud, J.M., Mauersberger, S., Barth, G., Comparison of Promoters Suitable for Regulated Overexpression of Beta-Galactosidase in the Alkane-Utilizing Yeast *Yarrowia lipolytica*. *Biotechnology & Bioprocess Engineering* 2000, *5*, 320-326.
- [46] De Pourcq, K., Vervecken, W., Dewerte, I., Valevska, A., *et al.*, Engineering the yeast *Yarrowia lipolytica* for the production of therapeutic proteins homogeneously glycosylated with Man(8)GlcNAc(2) and Man(5)GlcNAc(2). *Microb Cell Fact* 2012, *11*, 53.
- [47] Green, M. R., Sambrook, J., Sambrook, J., *Molecular cloning : a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 2012.

- [48] Jeong, J. Y., Yim, H. S., Ryu, J. Y., Lee, H. S., *et al.*, One-step sequence- and ligation-independent cloning as a rapid and versatile cloning method for functional genomics studies. *Appl Environ Microbiol* 2012, *78*, 5440-5443.
- [49] Ding, W. T., Zhang, G. C., Liu, J. J., 3' Truncation of the GPD1 Promoter in *Saccharomyces cerevisiae* for Improved Ethanol Yield and Productivity. *Appl Environ Microb* 2013, *79*, 3273-3281.
- [50] Eckert, S. E., Muhlschlegel, F. A., Promoter regulation in *Candida albicans* and related species. *Fems Yeast Res* 2009, *9*, 2-15.
- [51] Lopez-Estrano, C., Gopalakrishnan, A. M., Semblat, J. P., Fergus, M. R., *et al.*, An enhancer-like region regulates *hrp3* promoter stage-specific gene expression in the human malaria parasite *Plasmodium falciparum*. *Bba-Gene Struct Expr* 2007, *1769*, 506-513.
- [52] Hynes, M. J., Murray, S. L., Duncan, A., Khew, G. S., Davis, M. A., Regulatory genes controlling fatty acid catabolism and peroxisomal functions in the filamentous fungus *Aspergillus nidulans*. *Eukaryot Cell* 2006, *5*, 794-805.
- [53] Poopanitpan, N., Kobayashi, S., Fukuda, R., Honuchi, H., Ohta, A., An ortholog of *farA* of *Aspergillus nidulans* is implicated in the transcriptional activation of genes involved in fatty acid utilization in the yeast *Yarrowia lipolytica*. *Biochem Bioph Res Co* 2010, *402*, 731-735.
- [54] Li, R., Stimulation of DNA replication in *Saccharomyces cerevisiae* by a glutamine- and proline-rich transcriptional activation domain. *J Biol Chem* 1999, *274*, 30310-30314.
- [55] Butler, J. E., Kadonaga, J. T., The RNA polymerase II core promoter: a key component in the regulation of gene expression. *Genes Dev* 2002, *16*, 2583-2592.
- [56] Koller, A., Valesco, J., Subramani, S., The CUP1 promoter of *Saccharomyces cerevisiae* is inducible by copper in *Pichia pastoris*. *Yeast* 2000, *16*, 651-656.
- [57] de Winde, J. H., Grivell, L. A., Global regulation of mitochondrial biogenesis in *Saccharomyces cerevisiae*. *Prog Nucleic Acid Res Mol Biol* 1993, *46*, 51-91.
- [58] Pinkham, J. L., Guarente, L., Cloning and molecular analysis of the HAP2 locus: a global regulator of respiratory genes in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1985, *5*, 3410-3416.
- [59] Finley, R. L., Jr., Chen, S., Ma, J., Byrne, P., West, R. W., Jr., Opposing regulatory functions of positive and negative elements in UASG control transcription of the yeast GAL genes. *Mol Cell Biol* 1990, *10*, 5663-5670.
- [60] Blazeck, J., Garg, R., Reed, B., Alper, H. S., Controlling promoter strength and regulation in *Saccharomyces cerevisiae* using synthetic hybrid promoters. *Biotechnol Bioeng* 2012, *109*, 2884-2895.
- [61] Barrett, L. W., Fletcher, S., Wilton, S. D., Regulation of eukaryotic gene expression by the untranslated gene regions and other non-coding elements. *Cell Mol Life Sci* 2012, *69*, 3613-3634.
- [62] Struhl, K., Fundamentally different logic of gene regulation in eukaryotes and prokaryotes. *Cell* 1999, *98*, 1-4.

Table 1. Detailed list of vectors and primers used to construct hybrid promoters tested in this study.

	Starting Vector	Restriction Enzymes	Primer Pair(s)	Final Vector(s)
1	pSL16-UAS1B8-POX2(100 bp)-hrGFP	SphI / AscI	F1 / R1 F2 / R1 F3 / R1 F4 / R1 F5 / R1 F6 / R1 F7 / R1 F8 / R1 F9 / R1	POX2(1590 bp)-hrGFP POX2(1390 bp)-hrGFP POX2(1190 bp)-hrGFP POX2(990 bp)-hrGFP POX2(790 bp)-hrGFP POX2(590 bp)-hrGFP POX2(513 bp)-hrGFP POX2(430 bp)-hrGFP POX2(100 bp)-hrGFP
2	pSL16-UAS1B8-POX2(100 bp)-hrGFP	XmaI / SphI	F10 / R2 F11 / R3 F12 / R4 F13 / R5 F10 / R6 F11 / R5	A1A2-POX2(100bp)-hrGFP or A1A2 A2A3-POX2(100bp)-hrGFP or A2A3 A1A3-POX2(100bp)-hrGFP or A1A3
3	A1A3	MfeI	F11 / R4	A1A2A3-POX2(100bp)-hrGFP or A1A2A3
4	A1A3	AvrII / SphI	F10 / R11	A1R1-POX2(100bp)-hrGFP or A1R1
5	A1A3	AvrII / SphI	F10 / R12	A1R1A2R2-POX2(100bp)-hrGFP or A1R1A2R2
6	pSL16-UAS1B8-POX2(100bp)-hrGFP	SphI / AscI	F10 / R3 F12 / R5	A1R1A2-POX2(100bp)-hrGFP or A1R1A2 A2R2A3-POX2(100bp)-hrGFP or A2R2A3
7	A2A3	MfeI	F15/ R7	A2R1A3-POX2(100bp)-hrGFP or A2R1A3
8	A1A3	MfeI	F16 / R7	A1R1A3-POX2(100bp)-hrGFP or A1R1A3
9	A1A3	MfeI	F22 / R13	A1R1A1R1A3-Pox2(100 bp)-hrGFP or A1R1A1R1A3
9	A1R1A3	MfeI	F17 / R7	A1(R1x2)A3-POX2(100bp)-hrGFP or A1(R1x2)A3
10	A1R1A3	AvrII / SphI	F21/ R5	R1A3-POX2(100bp)-hrGFP or R1A3
11	A1(R1x2)A3	MfeI	F17 / R7	A1(R1x3)A3-POX2(100bp)-hrGFP or A1(R1x3)A3
12	A1(R1x3)A3	MfeI / AscI	F18 / R8 F19 / R9	A1(R1x3)A3-TEF(136bp)-hrGFP
13	pSL16-UAS1B8-TEF(136)-hrGFP	PstI / NheI	gBlock®	pSL16-UAS1B8-TEF-intron
14	pSL16-UAS1B8-TEF-intron	BtgZI	F20 / R10	pSL16-UAS1B8-TEF-intron-hrGFP

15	A1(R1x3)A3- POX2(100bp)-hrGFP or A1(R1x3)A3	MfeI / NheI	F18 / R8 F19 / R9	A1(R1x3)A3-TEF-intron-hrGFP
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Figure legends

Figure 1. Identification of activating and regulatory sequences in the POX2 promoter. (A) Identification of hypothetical POR1p binding sites in the POX2 native promoter that were used to guide rational 5' truncations. (B) Schematic of systematic truncations were made to the native POX2 promoter to identify DNA fragments containing hypothetical fatty acid responsive transcription factor binding sites. (C) Changes in truncated promoter strength were monitored with real-time PCR measurements of GFP mRNA relative to control beta actin. Activating sequences (A1, A2, A3) were defined as those resulting in loss of transcriptional activity when truncated. Regulatory sequences (R1, R2) were defined as those resulting in either gain or constant transcriptional activity when truncated. The data are the average of mRNA copy number relative to beta actin determined from biological triplicates. The error bars are standard deviation of the biological triplicates.

Figure 2. Discovery of the R1 UAS from the POX2 promoter. (A) New promoters were designed by combining parts of the POX2 promoter upstream of the POX2 core promoter. Activating (A1, A2, and A3) sequences were previously identified by 5' truncations. (B) Promoter strength is determined by expression of hrGFP and measured as mean fluorescence of an equal number of transformed cells grown in YSC-LEU with either glucose or oleic acid. Glucose samples are shown on the left and oleic acid on the right. A1 and A3 are important for oleic acid responsiveness, while A2 appears dispensable. (C) New promoters were designed by combining 5' and 3' truncations of the POX2 promoter upstream of the POX2 core promoter. Activating (A1, A2, and A3) sequences previously identified by 5' truncations. Regulatory (R1 and R2) sequences were previously defined by 5' truncations. (D) Promoter strength quantified by expression of hrGFP shows the R1 sequence results in strong expression in oleic acid media compared to glucose media. Glucose samples are shown on the left and oleic acid on the right. The R1 sequence appears

to work synergistically with the A1 sequence. The data are the average of mean fluorescence measurements from biological triplicates. The error bars are standard deviation of the biological triplicates.

Figure 3. Engineering a strong fatty acid inducible hybrid promoter. (A) Schematic of hybrid promoters constructed with tandem repeats of the POX2 R1 sequence and A1R1 sequence. (B) Promoter strength is determined by expression of hrGFP and measured as mean fluorescence of an equal number of transformed cells. Glucose samples are shown on the left and oleic acid on the right. (C) Promoter induction using oleic acid as the carbon source relative to glucose as carbon source. In (B) and (C) the data are the average of mean fluorescence measurements from biological triplicates. The error bars are standard deviation of the biological triplicates.

Figure 4. Substrate responsive induction of the A1(R1x3)A3 hybrid promoter. (A) Promoter strength was measured by hrGFP expression using flow cytometry. The A1(R1x3)A3 hybrid promoter is most strongly induced by linoleic acid and is strongly repressed by glucose and glycerol. Interestingly a combination of glycerol and oleic acid synergistically activated the hybrid promoter. (B) Fatty acids and mixtures of fatty acids with other carbon sources more strongly activate the hybrid promoter A1(R1x3)A3 compared to the native POX2 promoter. In (A) and (B), the data are the average of mean fluorescence measurements from biological triplicates. The error bars are standard deviation of the biological triplicates.

Figure 5. Inducibility of the A1(R1x3)A3 hybrid promoter by different fatty acids. Cells were grown to stationary phase with glucose and induced by the addition of 0.25% (v/v) of fatty acid. Induction was monitored over 20 hours using a fluorescence plate reader. Fatty

acids used in this experiment include: (A) oleic acid (OA, 18:1), (B) linoleic acid (LA, 18:2), (C) arachidonic acid (ARA, 20:4 ω -6), (D) eicosapentaenoic acid (EPA, 20:5 ω -3) and (E) Glucose as a control shows no induction. The data are the average of mean fluorescence measurements from biological triplicates. The error bars are standard deviation of the biological triplicates.

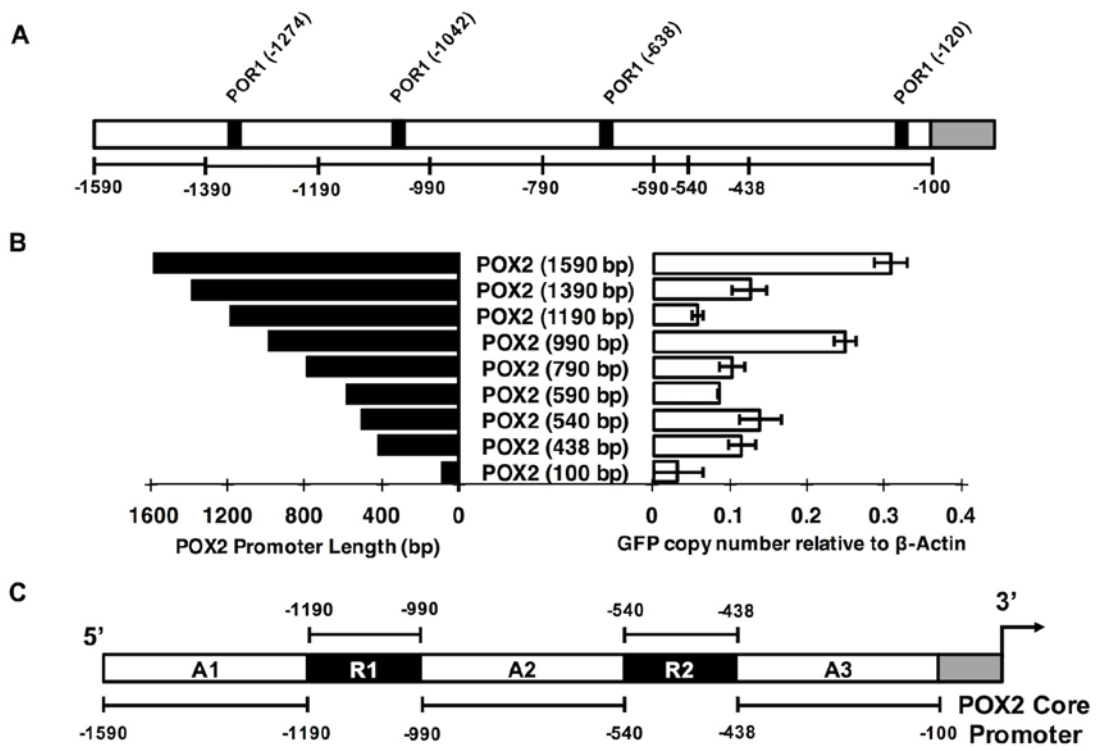


Figure 1.

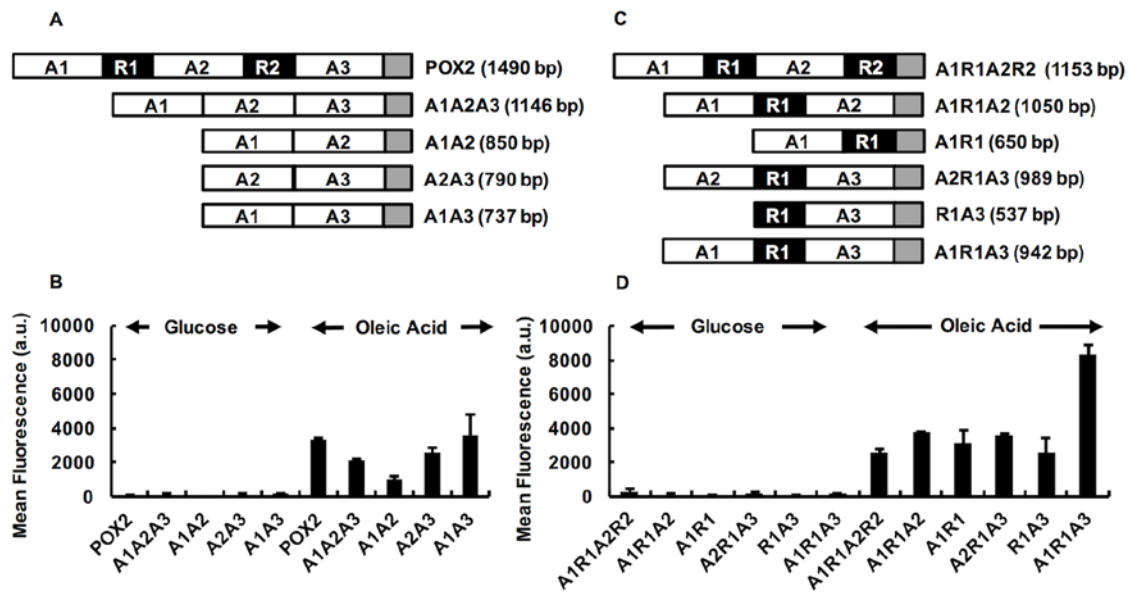


Figure 2.

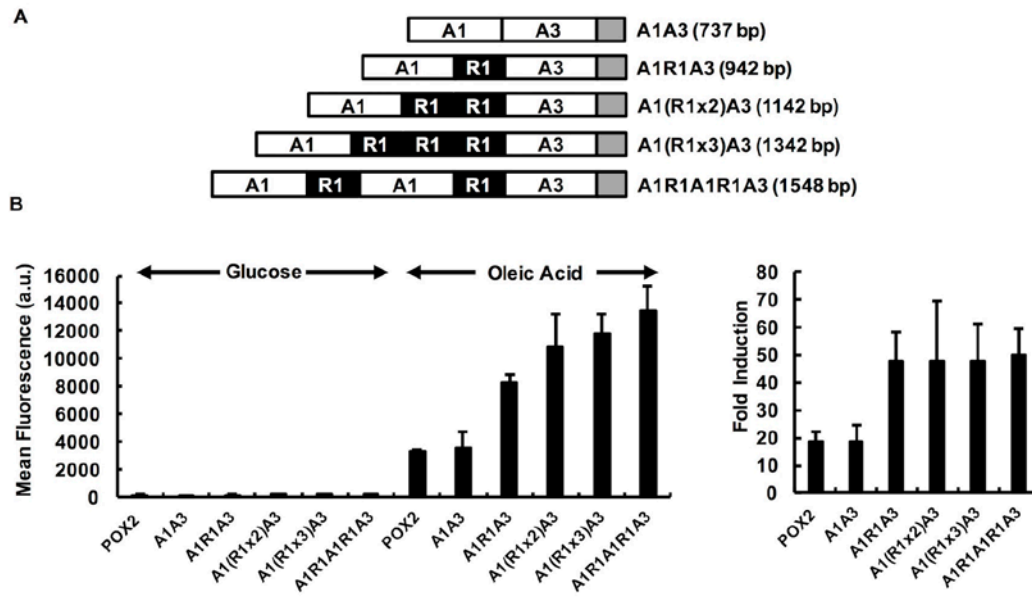


Figure 3.

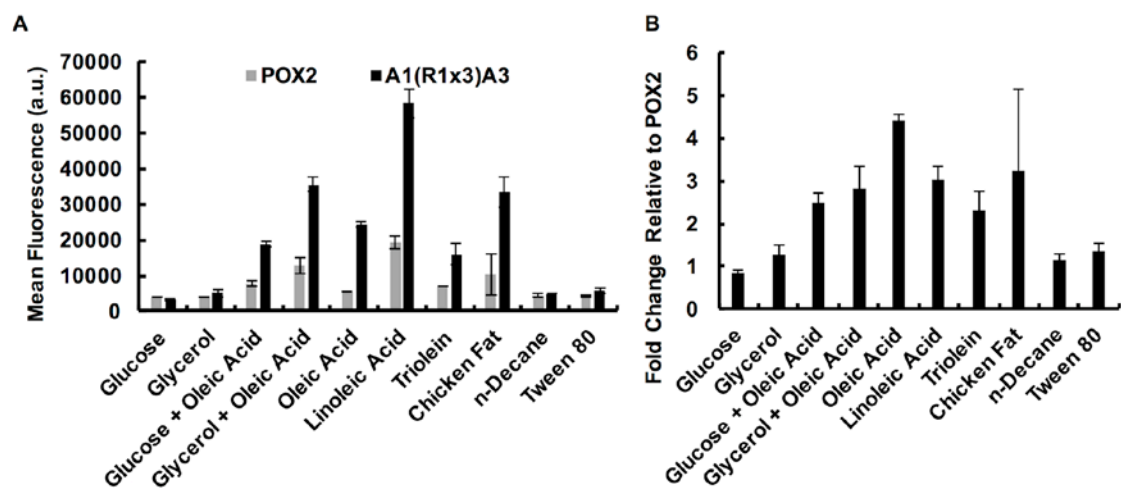


Figure 4.

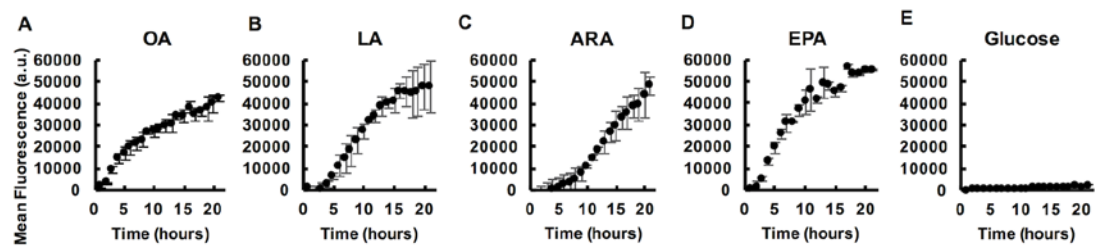


Figure 5.