

Characterizing and engineering promoters for metabolic engineering of *Ogataea polymorpha*

Chunxiao Yan^{a,b}, Wei Yu^{b,d,e}, Xiaoxin Zhai^{b,d}, Lun Yao^{b,d}, Xiaoyu Guo^{a,**}, Jiaoqi Gao^{b,c,d,*}, Yongjin J. Zhou^{b,c,d}

^a School of Biological Engineering, Dalian Polytechnic University, Dalian, 116034, PR China

^b Division of Biotechnology, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian, 116023, PR China

^c CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, 116023, PR China

^d Dalian Key Laboratory of Energy Biotechnology, Dalian Institute of Chemical Physics, CAS, 457 Zhongshan Road, Dalian, 116023, PR China

^e University of Chinese Academy of Sciences, Beijing, 100049, China

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ABSTRACT

Bio-manufacturing via microbial cell factory requires large promoter library for fine-tuned metabolic engineering. *Ogataea polymorpha*, one of the methylotrophic yeasts, possesses advantages in broad substrate spectrum, thermal-tolerance, and capacity to achieve high-density fermentation. However, a limited number of available promoters hinders the engineering of *O. polymorpha* for bio-productions. Here, we systematically characterized native promoters in *O. polymorpha* by both GFP fluorescence and fatty alcohol biosynthesis. Ten constitutive promoters (P_{PDH} , P_{PYK} , P_{FBA} , P_{PGM} , P_{GLK} , P_{TRL} , P_{GPI} , P_{ADHI} , P_{TEF1} and P_{GCW14}) were obtained with the activity range of 13%–130% of the common promoter P_{GAP} (the promoter of glyceraldehyde-3-phosphate dehydrogenase), among which P_{PDH} and P_{GCW14} were further verified by biosynthesis of fatty alcohol. Furthermore, the inducible promoters, including ethanol-induced P_{ICL1} , rhamnase-induced P_{LRA3} and P_{LRA4} , and a bidirectional promoter ($P_{Mal-P_{Per}}$) that is strongly induced by sucrose, further expanded the promoter toolbox in *O. polymorpha*. Finally, a series of hybrid promoters were constructed via engineering upstream activation sequence (UAS), which increased the activity of native promoter P_{LRA3} by 4.7–10.4 times without obvious leakage expression. Therefore, this study provided a group of constitutive, inducible, and hybrid promoters for metabolic engineering of *O. polymorpha*, and also a feasible strategy for rationally regulating the promoter strength.

1. Introduction

Bio-manufacturing represents for a promising approach for sustainable supplying of chemicals with mild reaction conditions, low energy consumption [1]. Microbial cell factories with extensive metabolic engineering have been applied for productions of bulk chemicals [2] and natural products [3,4]. Construction of biosynthetic pathways requires expression of multiple genes, which is normally realized by different promoters with various strengths. Besides, fine-tuning metabolic flux, including overexpression and down-regulation of key genes [5],

directed evolution of enzymes [6], cofactor engineering [7], and so on, reduce the toxic intermediates and enhance the production of target products. Consequently, the convenient and commonly used transcriptional regulation via a large promoter library guarantees a superior microbial cell factory for efficient productions [8–10].

Ogataea polymorpha (hereafter *O. polymorpha*), a methylotrophic yeast, has a broad spectrum of substrates like glucose, xylose, glycerol, methanol, and high thermo-tolerance [11]. For example, high temperature (45 °C) fermentation enabled efficient ethanol production from xylose in *O. polymorpha* [11]. In addition, the characteristics of

; GFP, Green Fluorescent Protein; UAS, Upstream Activation Sequence; EMP, Embden-Meyerhof-Parnas pathway.

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* Corresponding author. Division of Biotechnology, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian, 116023, PR China.

** Corresponding author.

E-mail addresses: gxycw0102@dipu.edu.cn (X. Guo), jqgao@dicp.ac.cn (J. Gao).

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O. polymorpha in post-translational modification and high-density fermentation make it a promising candidate to produce heterologous proteins [12]. However, there is limited reports on chemicals production in *O. polymorpha*, which may be partially attributed to the poor genetic manipulation tools and promoter library [13].

Recently, CRISPR/Cas9 based genome editing system was established for *O. polymorpha* [14–16]. While the promoter lack situation is still remaining and seriously hinders the extensive metabolic engineering of *O. polymorpha* [17]. Generally, promoters are classified into constitutive and inducible promoters. Constitutive promoters possess basically stable activities among different fermentative conditions. The strength of inducible, or repressive promoters are dynamic regulated by specific inducers or repressors. Promoters commonly used for gene expression in *O. polymorpha* includes the strong methanol-induced promoter P_{AOXI} (the promoter of alcohol oxidase I gene) and promoter P_{FMD} (the promoter of formate dehydrogenase gene) [18,19], and the strong constitutive promoter P_{GAP} (the promoter of glyceraldehyde 3-phosphate dehydrogenase gene) [20]. Obviously, these limited tools are far from enough for extensive metabolic engineering. Therefore, further screening other available promoters in *O. polymorpha* is essential, which should promote its potential as a chassis host in protein and chemicals production.

In this study, green fluorescent protein (GFP) was used to characterize the promoter strength (Fig. S1). A total of ten constitutive promoters were characterized, among which the promoters P_{GCW14} and P_{PDH} were further verified by production of fatty alcohol. Additionally, multiple inducible promoters were evaluated, and the regulation of promoter activity was achieved by constructing tandem repeats of upstream activation sequence (UAS), generating hybrid promoters with greatly enhanced activities [21–23]. Overall, our results offered a promoter toolbox with distinguished activities, and a feasible strategy to control promoter activities in *O. polymorpha*, which will pave the way to adopt this superior host for extensive metabolic engineering in both fundamental and industrial applications.

2. Materials and methods

2.1. Strains and media

All strains used in this study are listed in Supplementary Table S1. YPD medium contains 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose. LB medium was composed of 5 g/L yeast extract, 10 g/L NaCl and 10 g/L tryptone. 100 mg/mL ampicillin or 50 µg/mL chloramphenicol was added to LB medium depending on the resistance of the plasmids. Synthetic Dropout (SD) medium containing 20 g/L glucose and 6.7 g/L amino acid-free yeast nitrogen source (YNB) were used for strain screening during transformation, and 60 mg/L L-leucine was added into SD medium when necessary. Basic salt (Delft) medium was adopted to cultivate *O. polymorpha* strains [24], containing 2.5 g/L $(\text{NH}_4)_2\text{SO}_4$, 14.4 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mL/L trace metals, 1 mL/L vitamin solution and one of the following carbon sources, 20 g/L glucose (Delft-Glu), 20 g/L L-rhamnose (Delft-Rha), 10 g/L xylose (Delft-Xyl), 10 g/L methanol (Delft-MeOH), 30 g/L ethanol (Delft-EtOH), 10 g/L glycerol (Delft-Gly), or 20 g/L sucrose (Delft-Suc). 20 mg/L uracil, or/and 60 mg/L L-leucine was supplemented when necessary. For solid plates, 20 g/L agar was used.

2.2. Genetic engineering

Plasmids and primers used in this study were listed in Table S2 and Table S3, respectively. Genetic manipulation was achieved by using our previously reported CRISPR/Cas9 system in *O. polymorpha* [15]. Gene expression cassettes were constructed by overlap extension PCR and integrated into neutral sites of *O. polymorpha* [25]. For screening of constitutive promoters from glycolysis pathway (EMP) and inducible promoters, purified donor DNA including *GFPuv* gene, promoter,

terminator (T_{CYC}), upstream and downstream homologous arms, was introduced into NS2 site of strain JQCr03. For characterization of P_{GCW14} and construction of the hybrid promoters, strain Yan01 was used, with in situ complementation of *OpLEU2* gene in strain JQCr03. To minimize the detection errors, *eGFP* expression cassette was integrated into NS3 site in Yan01 strain.

2.3. DNA transformation of *O. polymorpha*

Electroporation was carried out for transformation of *O. polymorpha* according to previous procedures with slight modifications [26]. Briefly, cell culture with OD_{600} of 0.8–1.0 was transferred into a 15 mL tube, and centrifuged at 3000 g for 5 min. Cell pellets was resuspended in 4 mL sterile 50 mM PBS buffer (pH 7.5) supplemented with 25 mM dithiothreitol (DTT). After incubation at 37 °C for 15 min, cells were washed twice with ice-cold STM buffer (270 mM sucrose, 10 mM Tris-HCl pH 7.5 and 1 mM MgCl_2). Cells were resuspended in cold STM solution to obtain competent cells. 50 µL competent cells were mixed with 500 ng donor DNA and 500 ng gRNA plasmid, and transferred into a prechilled 2-mm electroporation cuvette, and electroporated in MicroPulser (Bio-rad) under “PIC” model. Cells were incubated at 37 °C for 1 h, washed with ddH₂O, plated on the selective SD plates, and then incubated at 37 °C for 3–4 days.

2.4. Fluorescence assay

GFP expression strains were cultured in Delft medium containing different carbon sources at 37 °C, 220 rpm. Samples were taken at 24 h, 48 h and 72 h and then diluted to optical density at 600 nm (OD_{600}) of 0.2–0.8 to measure biomass and fluorescence intensity. Fluorescence values were measured by TECAN spark (TECAN, Switzerland). The excitation wavelengths of *GFPuv* and *eGFP* were 396 nm and 485 nm and the emission wavelengths were 510 nm and 525 nm, respectively.

2.5. Production and detection of fatty alcohol

As shown in Fig. 3A, a modular engineering strategy was used to construct fatty alcohol biosynthetic pathway [27]. To promote accumulation of fatty acids and fatty aldehydes, hexadecenal dehydrogenase (encoded by *HFD1*) and fatty acyl-CoA synthase (encoded by *FAA1*) were both disrupted in strain Yan01, obtaining strain Yan03. For fatty alcohol production, *CAR* and its co-factor *NpgA*, and *ADH5* were codon optimized and heterologously expressed in *O. polymorpha*, of which *ADH5* and *NpgA* were integrated at NS2 site, obtaining strain Yan04. To evaluate the effect of promoters on fatty alcohol production, *CAR* was expressed under the control of P_{GAP} , P_{GCW14} or P_{PDH} , and were integrated at NS3 site in strain Yan04. All strains were pre-cultivated in YPD medium for 24 h, and then transferred into Delft medium containing 20 g/L glucose and cultivated at 37 °C, 220 rpm. Fatty alcohols were measured according to previous methods after 120 h cultivation [25–27].

2.6. Determine the promoter upstream activation sequence

Core promoter region and binding sites of were transcription factors were analyzed online (http://www.fruitfly.org/seq_tools/promoter.html) [28] and (<http://gene-regulation.com/pub/programs/alibab2/index.html>) [29]. To determine the region of upstream activation sequence (UAS), promoters were truncated according to the transcription factor binding sites (Fig. S2). The truncated promoters were characterized by the fluorescence intensity of *eGFP*.

2.7. Construction of hybrid promoters

Hybrid promoters were constructed by Golden-gate assembly using BsaI-HF®v2 kit (New England Biolabs, USA). Fragments of P_{LRA3} and UAS- P_{LRA3} were amplified using primers in Table S3, containing the

recognition sites of *Bsa*I and four additional bases. The fragments were ligated together and transformed into *Escherichia coli* competent cells, which were screened on plates of LB + Chl (chloramphenicol) for 12–16 h. The correct colonies were verified by PCR and Sanger sequencing. The hybrid promoters were amplified and inserted into plasmid Hp01-eGFP and integrated into NS3 site of *O. polymorpha* for further characterization.

3. Results

3.1. Characterization of native constitutive promoters of *O. polymorpha*

In yeasts, the commonly used constitutive promoters are P_{GAP} , the promoter of alcohol dehydrogenase (P_{ADH1}), the promoter of translation elongation factor (P_{TEF1}), and so on [30]. To test our characterization system, these promoters were first quantified in glucose and methanol media by the normalized fluorescence intensity. As expected, the promoter P_{GAP} was the strongest constitutive promoter, which was 1.7-fold, and 4.3-fold higher than P_{ADH1} and P_{TEF1} , respectively (Fig. 1A). In methanol medium, the strength of P_{ADH1} and P_{TEF1} was 37% and 26% of P_{AOX1} (Fig. 1B), a strong methanol induced promoter [31].

To further explore constitutive promoters in *O. polymorpha*, we characterized the promoters of genes from the glycolysis pathway (Embden-Meyerhof-Parnas pathway, EMP). EMP is a common pathway in most organisms to assimilate hexose for the generation of intermediates and energy, in which most genes are constitutively expressed for cell growth [32]. Therefore, a total of 7 native gene promoters from EMP was characterized. Generally, statistical analysis indicated that there was a significant difference in fluorescence intensity between the control strain and the engineered strains, which demonstrated that all these promoters possessed the transcriptional activities (Fig. 1C and D). In glucose culture, the promoter activity ranged from

13% to 42% of P_{GAP} with a strength order of $P_{PDH} > P_{PYK} > P_{FBA} > P_{PGM} > P_{GLK} > P_{TRI} > P_{GPI}$ (Fig. 1C). While in methanol culture, the promoter strength were 8%–42% of P_{AOX1} with an order of $P_{TRI} > P_{FBA} > P_{PDH} > P_{PYK} > P_{PGM} > P_{GPI} > P_{GLK}$ (Fig. 1D). Most promoters from EMP like P_{FBA} , P_{TRI} , P_{PYK} , and P_{PDH} had the comparable strength to P_{ADH1} and P_{TEF1} in either glucose or methanol culture, which demonstrated the availability of these promoters for gene expression. Interestingly, the relative activities of constitutive promoters varied among different conditions (comparing Fig. 1A and B, Fig. 1C and D). These promoters with distinguished activities might expand biological elements for the precise regulation of metabolic engineering in *O. polymorpha*.

In addition to the promoters of glycolysis, the promoter of a predicted glycosylphosphatidylinositol (GPI) anchored protein (encoded by gene *GCW14*) was proved to be a strong constitutive promoter in *Pichia pastoris* (hereafter *P. pastoris*) [33,34]. Here, a promoter with the length of 882 bp was identified as P_{GCW14} by sequence similarity in *O. polymorpha*, and subsequently characterized to evaluate activities under various conditions. The enhanced green fluorescent protein (eGFP) was used here instead of GFPuv due to its limited intensity. Promoter P_{GCW14} from *O. polymorpha* and *P. pastoris* were both tested by using the promoters P_{GAP} and P_{AOX1} as the positive control. The native promoter P_{GCW14} had very high activity, which was 1.3 times higher than P_{GAP} in glucose and 1.1 times higher than P_{AOX1} in methanol (Fig. 2A and B). Surprisingly, the promoter P_{pGCW14} from *P. pastoris* was also functional in *O. polymorpha* with about half of the activity of P_{GCW14} . Subsequently, activities of promoter P_{GCW14} was characterized in media containing different carbon sources including glucose, xylose, methanol, ethanol, and glycerol. Similar strengths were achieved under these cultivation conditions, which further proved that P_{GCW14} was a strong constitutive promoter (Fig. 2C). A decreased activity of P_{GCW14} with time, especially in glucose and glycerol, was observed, which might be related to cell viability (Fig. 2C).

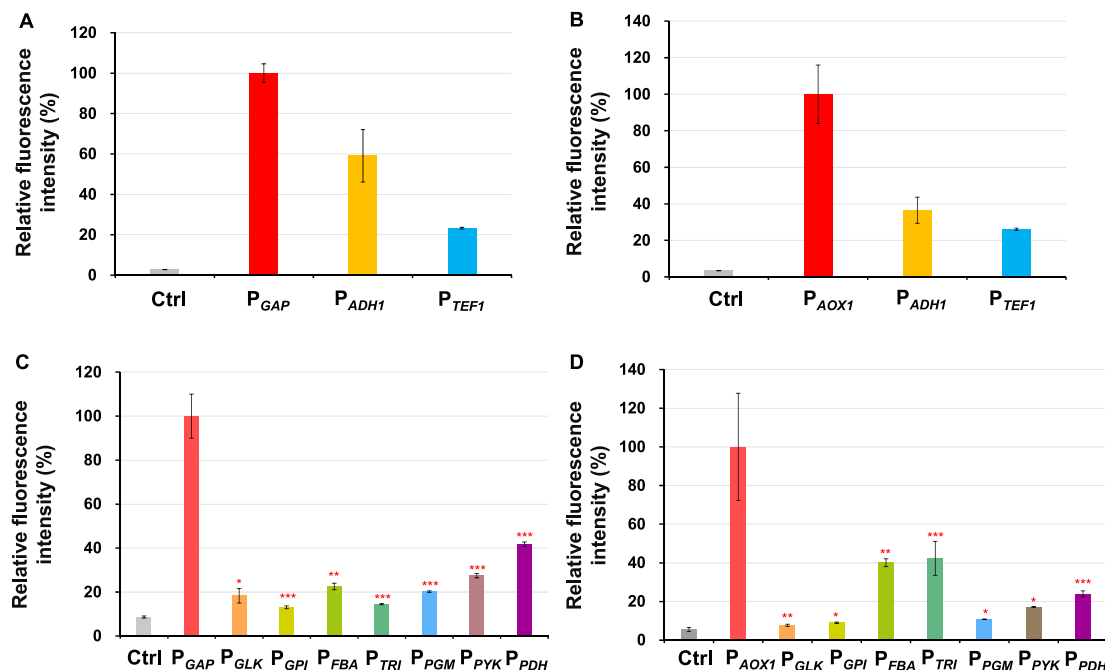


Fig. 1. Characterization of constitutive promoters in glucose and methanol cultures. eGFP was used as the characterization protein and the expression box was integrated at the neutral site (NS3). Engineered strains were cultivated in both 20 g/L glucose for 48 h (A), and 10 g/L methanol for 72 h (B). On this basis, seven promoters from glycolysis pathway were selected to test their activities. GFPuv under control of different promoters was integrated at neutral site (NS2). Engineered strains were cultivated in both 20 g/L glucose for 48 h (C), and 10 g/L methanol for 72 h (D). The starting strain without promoter integration was taken as negative control (Ctrl), and native promoter P_{GAP} and P_{AOX1} from *O. polymorpha* are positive controls (100%) to normalize the fluorescence values of other promoters. All data was represented as the mean \pm s.d. of three clone samples. Abbreviations of $P_{Gene X}$ means the promoter of a specific gene, including glyceraldehyde 3-phosphate dehydrogenase (GAP), alcohol oxidase (AOX1), translation elongation factor (TEF1), alcohol dehydrogenase (ADH1), hexokinase (GLK), glucose 6-phosphate isomerase (GPI), fructose 1,6-bisphosphate aldolase (FBA), triosephosphate isomerase (TRI), phosphoglycerate mutase (PGM), pyruvate kinase (PYK), and pyruvate dehydrogenase (PDH). Red asterisks indicate statistical significance as determined using paired *t*-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

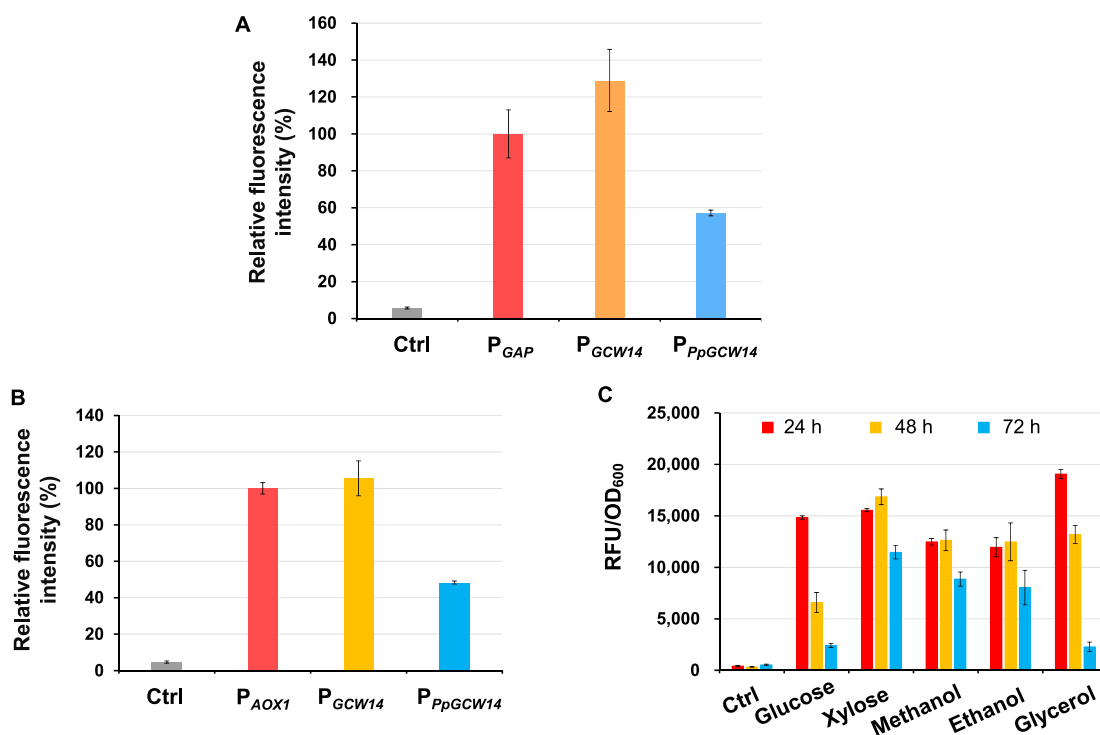


Fig. 2. Characterization of promoter P_{GCW14} under different carbon sources. Promoter of gene $GCW14$ (predicted GPI anchored protein) from both *O. polymorpha* (P_{GCW14}) and *P. pastoris* ($P_{PpGCW14}$) were selected to drive $eGFP$ expression at neutral site NS3. Engineered strains were cultivated with 20 g/L glucose for 48 h (A) or 10 g/L methanol for 72 h (B). The original strain without promoter integration was taken as negative control (Ctrl), and native promoter P_{GAP} and P_{AOX1} from *O. polymorpha* are positive controls (100%) to normalize the fluorescence values of other promoters. (C) Promoter P_{GCW14} was evaluated under 10 g/L of multiple carbon sources, and samples were taken at 24 h, 48 h, and 72 h, respectively, to measure fluorescence values. All data was represented as the mean \pm s.d. of three clones.

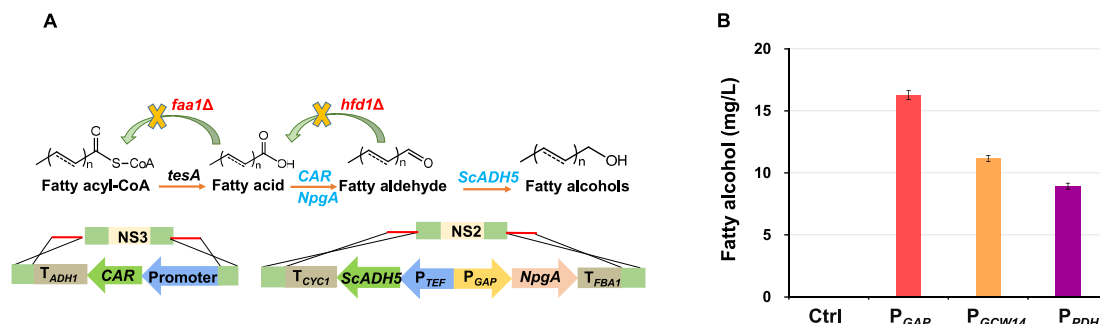


Fig. 3. Biosynthesis of fatty alcohol under the control of promoter P_{GCW14} and P_{PDH} . (A) To promote accumulation of fatty acids and fatty aldehydes, hexadecanal dehydrogenase (encoded by $HFD1$) and fatty acylCoA synthase (encoded by $FAA1$) were disrupted, and codon-optimized $ADH5$ and $NpgA$ were integrated into NS2 site. CAR gene under control of various promoters (P_{GAP} , P_{GCW14} or P_{PDH}) were integrated into NS3 site. All strains were pre-cultured in YPD medium for 24 h, and then transferred into Delft medium containing 20 g/L glucose and cultivated at 37 °C, 220 rpm for 120 h. Production of fatty alcohols were measured after 120 h cultivation (B). All data was presented as the mean \pm s.d. of three clones.

3.2. Construction of fatty alcohol biosynthetic pathway using constitutive promoters

Most promoters were characterized based on fluorescence intensity of $eGFP$ [35]. However, when applied in metabolic engineering, the biosynthetic efficiency may be not well correlated with fluorescence intensity driven by the same promoter. In order to better characterize the applicability of the above promoters, promoter P_{PDH} with a medium strength from EMP pathway and the strong constitutive promoter P_{GCW14} were selected for construction of biosynthetic pathway of fatty alcohol, which is a bulk chemical widely-used for production of lubricants, skin care products and plastics [36]. The pathway for fatty alcohol synthesis is illustrated in Fig. 3A. Deletions of fatty acyl-CoA synthetase

(encoded by $FAA1$) and fatty aldehyde dehydrogenase (encoded by $HFD1$) promoted the accumulation of fatty aldehydes and fatty acids, which provided sufficient precursors for fatty alcohol production. The carboxylic acid reductase gene CAR was expressed with different promoters, since fatty acid reduction was showed to be the limited step in fatty alcohol synthesis [27]. In detail, CAR was drove by P_{PDH} , P_{GCW14} or P_{GAP} , respectively, and $NpgA$ and $ScADH5$ was expressed under the control of P_{GAP} and P_{TEF1} with genome-integrated at neutral site NS2 [25]. The fatty alcohol titers were 8.9 mg/L and 11.2 mg/L with CAR overexpression driven by P_{PDH} and P_{GCW14} , respectively, which was 55% and 69% of the titer in strain containing gene CAR driven by P_{GAP} (Fig. 3B). These results showed that promoters were functional for an efficient biosynthesis and metabolic regulation of target products.

3.3. Characterizing inducible promoters from *O. polymorpha*

Fine-tuned metabolic engineering like time-sequential regulation and toxicity decrease requires extensive inducible promoters, such as methanol-induced P_{AXO1} [37]. Hence, to further enrich the limited inducible promoter in *O. polymorpha*, promoter P_{ICL1} (the promoter of isocitrate lyase gene, 680 bp) [38], P_{LRA3} (the promoter of L-rhamnonate dehydratase gene, 210 bp) and P_{LRA4} (the promoter of L-2-keto-3-deoxyrhamnonate (L-KDR) aldolase gene, 210 bp) [39], and P_{Mal} (the promoter of maltase, 1434 bp) [40], were evaluated. All these promoters demonstrated distinguished performances while cultivating in different carbon sources (Fig. 4). Interestingly, promoter P_{ICL1} was strongly activated by both ethanol and methanol with up to twice activity of P_{GAP} (Fig. 4A), which demonstrated glyoxylate bypass that gene *ICL1* mainly functions in may play a vital role in the assimilation of short-chain alcohols. Similarly, promoter P_{LRA3} and P_{LRA4} were both induced by rhamnose, whose activity was 44% and 28% of P_{GAP} , respectively (Fig. 4B). As reported in *Ogataea thermomethanolica* [40], the bidirectional promoter $P_{Mal-P_{Per}}$ in *O. polymorpha* was also strictly repressed by both glucose and methanol, and achieved 70% and 24% of the strength of P_{GAP} , respectively, in sucrose (Fig. 4C). Consequently, all these inducible promoters with a diverse strength were suitable for metabolic rewiring at different levels.

3.4. Construction of hybrid inducible promoter

Construction of hybrid promoters represents a feasible solution to obtain a preferred promoter based on practical demands [41,42]. Here, we tried to construction of an artificial hybrid promoter based on promoter P_{LRA3} . Promoters usually consist of upstream activation sequence (UAS) regions and core promoter regions. UAS determines the transcription efficiency, which is usually adopted to enhance the promoter strength [35]. To determine the UAS region of promoters P_{GAP} and P_{ADH1} , stepwise truncation strategy was used based on sequence prediction (Fig. S2A). To avoid the disruption of transcriptional binding

sites and core promoter region, P_{GAP} and P_{ADH1} were cut to 3 and 4 truncated promoters, respectively. UAS regions of P_{GAP} and P_{ADH1} were defined as the region of -336~-110 bp, and -125~-680 bp, respectively, according to GFP fluorescence (Fig. S3A). Subsequently, the hybrid promoter was constructed by combining promoter P_{LRA3} with the UAS regions of P_{GAP} and P_{ADH1} , respectively. As shown in Fig. S3B, the hybrid promoter $UAS_{PADH1} + P_{LRA3}$ seemed to promote the strength with no influence on inductivity. However, the correspondingly increased activity in glucose demonstrated that a direct combination of core promoter with UAS region from another promoter resulted in expression leakage (Fig. S3B).

It has been reported that a tandem UAS contributed to an enhanced promoter strength [43]. Therefore, another hybrid inducible promoter was constructed by combining P_{LRA3} with its own UAS. Firstly, as shown in Fig. 5A and Fig. S2B, promoter P_{LRA3} was truncated according to the predicted core sequence and transcriptional binding sites, and 90 bp UAS region (-210 bp~-120 bp) of P_{LRA3} was identified (Fig. 5B). Then, 1–3 UAS regions were placed upstream of the promoter P_{LRA3} [21,22]. Excitingly, the hybrid promoters had dramatically higher strength and the hybrid promoter $P_{LRA3+UAS^*3}$ with three copies of UAS had maximum fluorescence intensity, 10.4 times higher compared with the original promoter P_{LRA3} at 96 h (Fig. 5C). The activity of $P_{LRA3+UAS^*3}$ was twice higher than that of P_{GAP} and the promoter activities were positively correlated with the numbers of UAS regions ($R^2 = 0.98$, Fig. 5D), which demonstrated great application potential of the tandem UAS in promoter regulation.

4. Discussion

Availability of promoters is very essential for extensive metabolic engineering. This study identified and characterized three different types of native promoters (constitutive, inducible and hybrid) in *O. polymorpha* for further constructions of cell factory, and also provides a feasible strategy for promoter mining and control.

We characterized several endogenous constitutive promoters from

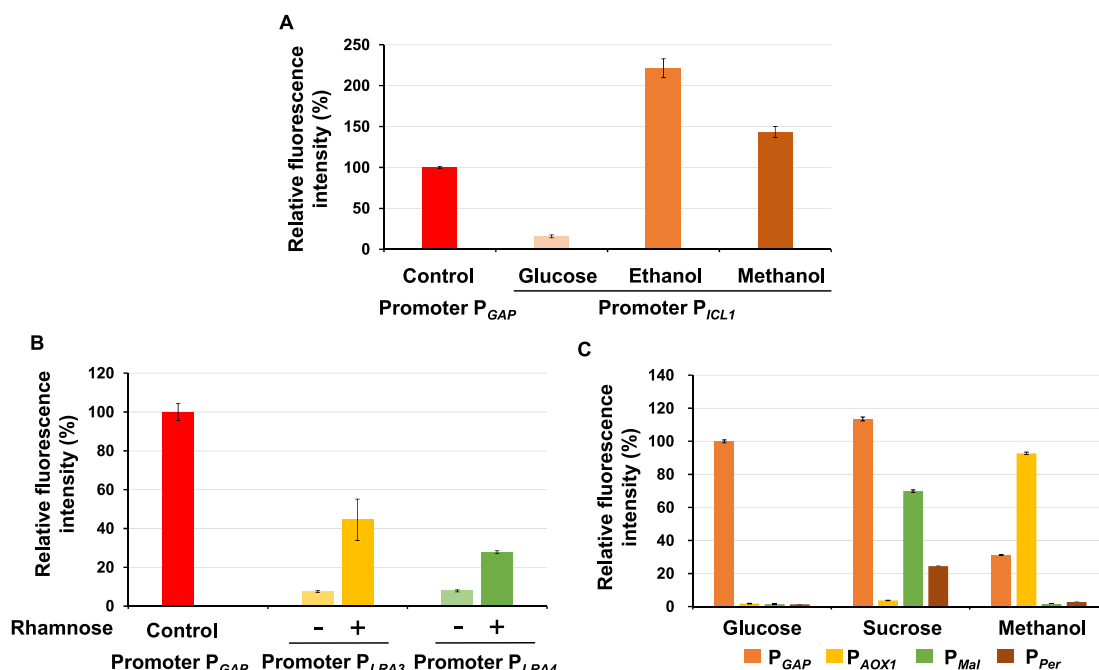


Fig. 4. Characterization of inducible promoters by eGFP fluorescence intensities in *O. polymorpha*. (A) Inducible promoter P_{ICL1} were characterized in Delft medium containing 20 g/L glucose, or 30 g/L ethanol, or 10 g/L methanol. (B) Inducible promoters P_{LRA3} and P_{LRA4} were characterized under inducible (20 g/L rhamnose) and non-inducible (20 g/L glucose) conditions. (C) Bidirectional promoter ($P_{Mal-P_{Per}}$) was characterized in Delft medium containing 20 g/L glucose, 20 g/L sucrose or 10 g/L methanol, respectively. Strains were cultivated at 37 °C, 220 rpm, and samples were taken at 48 h to measure the biomass and fluorescence. Results were normalized by fluorescence intensity of P_{GAP} cultivated with 20 g/L glucose (100%). All data was represented as the mean \pm s.d. of three clones.

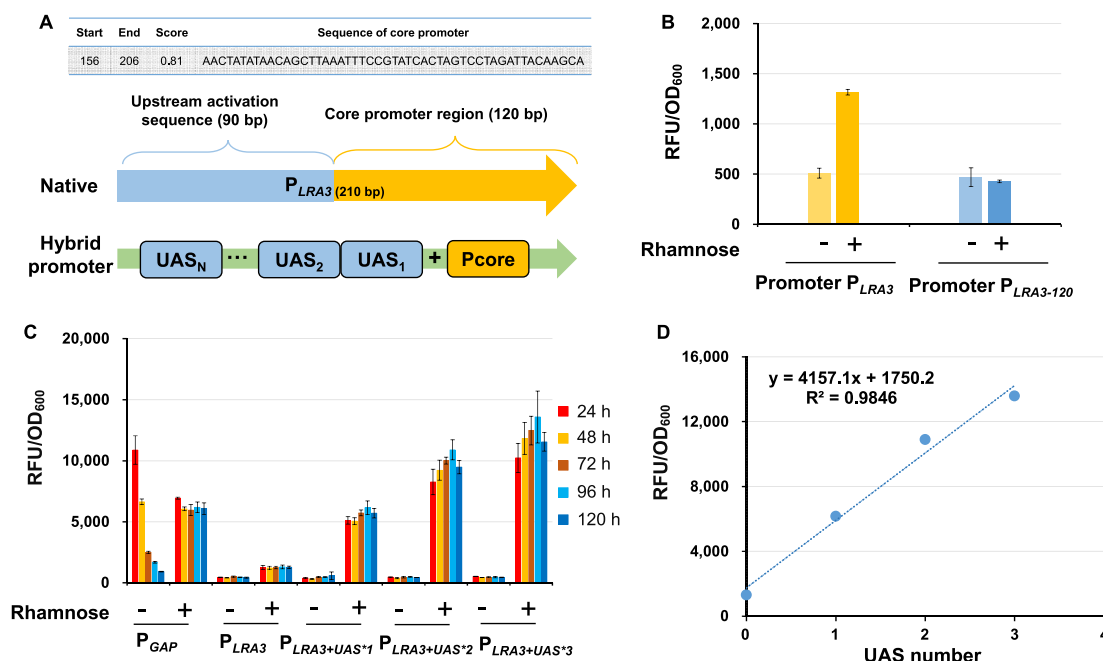


Fig. 5. Fluorescence characterization of hybrid promoters. (A) Determination of upstream activation sequence (UAS) and core region of promoter P_{LRA3} by stepwise truncation strategy, and the construction of hybrid promoters by UAS tandem strategy. (B) Characterization of P_{LRA3} and truncated P_{LRA3} under inducible (20 g/L rhamnose) and non-inducible (20 g/L glucose) conditions. (C) Characterization of P_{LRA3} and hybrid promoters P_{LRA3+UAS*1}, P_{LRA3+UAS*2}, P_{LRA3+UAS*3}. All promoters were evaluated by fluorescence values under inducible (20 g/L rhamnose) and non-inducible (20 g/L glucose) conditions at 24 h, 48 h, 72 h, 96 h, and 120 h. Promoter P_{GAP} was used as the positive control. (D) Correlation between promoter strength and numbers of UAS series. All data was represented as the mean ± s.d. of three clones.

central metabolic pathways, which however was much weaker compared with the strong constitutive promoter P_{GAP} (10%–60%). We here found the promoter strength was varied between glucose and methanol, which demonstrated that these promoters are not strictly constitutive, and may be related to cell growth status under various fermentative conditions. In addition, a strong constitutive promoter P_{GCW14} of a potential glycosylphosphatidylinositol (GPI) anchored protein, which is not related to specific pathway, was characterized to be a relatively constitutive promoter under different culture conditions. Interestingly, a homologous promoter P_{ppGCW14} from *P. pastoris* was also functional in *O. polymorpha*. This universal expression regulation among different yeasts may provide a reference strategy for further mining similar promoters with different intensities [33].

To further expand the promoter library in *O. polymorpha*, the inducible promoters were excavated, including ethanol-induced promoter P_{ICL1}, rhamnose-induced promoters P_{LRA3} and P_{LRA4}, and a bidirectional promoter P_{Mal-P_{Per}}. These inducible promoters demonstrated strict glucose repression, and even though in a mixture of glucose and inducers, the activity remained an extremely low level. Just like GAL system in *S. cerevisiae* [44], these inducible promoters may be regulated by other transcriptional factors, and their regulatory mechanisms are worthy of further exploration by a more refined stepwise truncation strategy [45]. We also found that the strength of the inducible promoter such as P_{LRA3} was relatively low. A UAS-tandem strategy was developed to increase the promoter strength of P_{LRA3} by 4.7–10.4 times without influencing the inducible feature. Compared with the site-directed mutagenesis approach [46], this rational design may directly obtain the hybrid promoter with a predictable manner [35,47]. We can expect that our hybrid promoter strategy can be applied to other large number of inducible promoters with low strengths.

We also used various promoters P_{PDH} and P_{GCW14} for regulating the biosynthesis of fatty alcohols. The positive relation between promoter strength and fatty alcohol production verified the practicability of promoter based pathway regulation. Larger number of promoters with various strengths can help to regulate the metabolic pathways with a precise manner, which should be helpful for optimization of metabolic

network in cell factory construction [48].

In summary, we identified and evaluated three different types of promoters for providing sound biological elements in metabolic engineering of *O. polymorpha*, as well as provided feasible strategies for promoters mining and engineering.

Availability of data and material

Data that supports the finding of this study are available in the main text and the supplementary materials.

Ethical approval

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

Consent for publication

All listed authors have approved the manuscript before submission, including the names and order of authors.

CRediT authorship contribution statement

Chunxiao Yan: Methodology, Investigation, Writing – original draft. **Wei Yu:** Methodology, Resources. **Xiaoxin Zhai:** Methodology, Resources. **Lun Yao:** Methodology, Resources. **Xiaoyu Guo:** Writing – review & editing. **Jiaoqi Gao:** Methodology, Resources, Writing – review & editing. **Yongjin J. Zhou:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that there is no conflict of interests.

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Appendix A. Supplementary data

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