ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Cloning and Characterization of a Panel of Constitutive Promoters for Applications in Pathway Engineering in *Saccharomyces cerevisiae*

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ABSTRACT: *Saccharomyces cerevisiae* is an important platform organism for synthesis of chemicals and fuels. However, the promoters used in most pathway engineering studies in *S. cerevisiae* have not been characterized and compared in parallel under multiple conditions that are routinely operated in laboratory and the number of known promoters is rather limited for the construction of large biochemical pathways. Here a total of 14 constitutive promoters from *S. cerevisiae* were cloned and characterized using a green fluorescent protein (GFP) as a reporter in a 2 μ vector pRS426, under varying glucose and oxygen concentrations. The strengths of these promoters varied no more than sixfold in the mean fluorescence intensity of GFP, with promoter *TEF1p* being the strongest and promoter *PGI1p* the weakest. As an example of application for these promoters in metabolic engineering, the genes involved in xylan degradation and zeaxanthin biosynthesis were subsequently cloned under the control of promoters with medium to high strength and assembled into a single pathway. The corresponding construct was transformed to a *S. cerevisiae* strain integrated with a D-xylose utilizing pathway. The resulting strain produced zeaxanthin with a titer of 0.74 ± 0.02 mg/L directly from birchwood xylan.

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Introduction

Saccharomyces cerevisiae is one of the most widely used platform organisms due to its well-characterized physiology and genetics, fast cell growth rate, and availability of abundant genetic tools. Its potential has been demonstrated not only in the production of a wide variety of important high-value compounds such as steroid hydrocortisone (Szczebara et al., 2003), alkaloids (Hawkins and Smolke, 2008; Minami et al., 2008), terpenoids artemisinic acids (Liu et al., 2006; Ro et al., 2006), taxadiene (Engels et al., 2008), and non-ribosomal peptide antibiotic precursor δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (Siewers et al., 2009), but also in addressing global energy-related issues such as biofuel production and biomass conversion (Den Haan et al., 2007b; Fernandes and Murray, 2010; Ha et al., 2011; Hahn-Hagerdal et al., 2007; Kotter and Ciriacy, 1993; Krahulec et al., 2010; Li et al., 2010; Runquist et al., 2009; Steen et al., 2010; Tsai et al., 2010; Wen et al., 2010). In these studies, multiple genes were cloned under either constitutive promoters or inducible promoters and functionally expressed in *S. cerevisiae*.

Most pathway engineering studies have focused on the selection of proper enzymes because enzyme homologs

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cloned from various organisms could behave differently in terms of expression and activity in a heterologous host. However, selection of appropriate promoters is also important especially for yield and productivity optimization. This is not only because the protein transcription level is determined by the promoter strength, but also because promoters could be regulated and behave differently under different growth conditions. For example, in order to improve taxadiene production, Stephanopoulos and co-workers systematically tested promoters of different strengths and plasmid copy-numbers, which maximized the taxadiene production to approximately 1 g/L with minimal accumulation of any toxic intermediate (Ajikumar et al., 2010). Therefore, evaluating the behaviors of a panel of promoters under different growth conditions is highly desirable.

A number of *S. cerevisiae* constitutive promoters have been described and shown to be functional for expression of heterologous genes (Bitter and Egan, 1984; Denis et al., 1983; Diderich et al., 1999; Gatignol et al., 1990; Hauf et al., 2000; Holland and Holland, 1978; Ogden et al., 1986; Reifenberger et al., 1997). The strengths of different promoters were evaluated using well-characterized reporter genes such as β-galactosidase (LacZ), GFP, and β-lactamase (Cartwright et al., 1994; Mumberg et al., 1995; Partow et al., 2010; West et al., 1987). Recently, Partow et al. (2010) characterized 7 constitutive promoters and 2 inducible promoters using LacZ as a reporter in both batch and continuous cultivation. Promoter *TEF1p* showed the most constant activity during fermentation and promoter *HXT7p* represented the strongest one in continuous cultivation. Moreover, other interesting promoter studies included creating libraries of artificial promoters to fine-tune gene expression (Hammer et al., 2006; Hartner et al., 2008; Jensen and Hammer, 1998), engineering synthetic gene networks with desired functions (Ellis et al., 2009), and maximizing product yield and quality (Lu and Jeffries, 2007).

Here we report the cloning of 14 constitutive promoters from the *S. cerevisiae* genome and the comparison of their strengths under various culture conditions that are routinely used in the laboratory for production of value-added compounds in *S. cerevisiae*. The availability of these promoters has greatly enabled the use of our recently developed DNA assembler method (Shao et al., 2009) for pathway engineering in *S. cerevisiae*, which allows the rapid assembly of multi-gene pathways either on a plasmid or on a chromosome. As an example, we chose the promoters with relatively high strength and cloned the xylan degradation pathway (Dodd and Cann, 2009; Saha, 2003) and zeaxanthin biosynthesis pathway (Chemler et al., 2006; Misawa et al., 1990; Misawa and Shimada, 1997) under these promoters, and transformed the resulting constructs to the strain capable of utilizing xylose as a carbon source. The resulting strain synthesized zeaxanthin directly from birchwood xylan, and therefore can be utilized as a consolidated bioprocessing (CBP) organism (Lynd et al., 2005) for biomass utilization and conversion.

Materials and Methods

Strains, Vectors, Media, and Reagents

S. cerevisiae YSG50 (*MATα*, *ade*2–1, *ade*3Δ22, *ura*3–1, *his*3–11, 15, *trp*1–1, *leu*2–3, 112, and *can*1–100) was obtained as a gift from Professor Peter Orlean and used in the promoter characterization experiments. Synthetic complete drop-out medium lacking uracil (SC-Ura) was used to select transformants containing the assembled pathways. *S. cerevisiae* EBY100 (Invitrogen, Carlsbad, CA) and *Escherichia coli* DH5α (Cell Media Facility, University of Illinois at Urbana-Champaign, Urbana, IL) were used for recombinant DNA manipulation. *Trichoderma reesei* DSM769 and *Aspergillus niger* DSM821 were purchased from DSMZ (Braunschweig, Germany). *T. reesei* and *A. niger* were grown on YPAX media (1% yeast extract, 2% peptone, 2% birchwood xylan, 0.01% adenine hemisulfate) to form spores. The cDNA was synthesized from mRNA using the first-strand cDNA synthesis kit (Roche, Indianapolis, IN). *S. cerevisiae* L2612 (*MATα*, *leu*2-3, *leu*2-112, *ura*3-52, *trp*l-298, *can*1, *cyn*l, and *gal*+) was a gift from Professor Yong-su Jin (Jin et al., 2003) and used for integration of the D-xylose utilizing pathway. Plasmids pRS426 and pRS416 and all restriction enzymes were obtained from New England Biolabs (Ipswich, MA). The primers and the universal probes used for real-time PCR were purchased from Integrated DNA Technologies (Coralville, IA) and Roche, respectively.

Plasmid Construction

The 14 promoters and terminators were amplified from the genomic DNA of *S. cerevisiae*. Their sequences were obtained from the promoter database of *S. cerevisiae* (http://rulai.cshl.edu/cgi-bin/SCPD/getgenelist). Overlaps of 40 bp were designed between adjacent fragments (Table S1). The amplified promoters, green fluorescent protein (GFP) gene, and terminators were gel-purified and co-transformed into YSG50 with the 2 μ plasmid pRS426 linearized with *Kpn*I and *Eco*RI. Through homologous recombination, a GFP expression cassette composed of a promoter, a *gfp* gene, and a terminator was successfully assembled into pRS426.

Two plasmids containing xylanases and a five-gene zeaxanthin biosynthetic pathway, pC414-Xyn2-XlnD and pRS425-ZeaX, were individually constructed. Plasmid pC414-Xyn2-XlnD was constructed using a conventional homologous recombination method (Ma et al., 1987). The xylanase II (Xyn2) and β-xylosidase D (XlnD) genes were PCR-amplified from the cDNA of *T. reesei* and *A. niger*, and subcloned to plasmids pRS425-EGII-ctrl and pRS425-BGL1-ctrl (Wen et al., 2010) by replacement of the EGII and BGL1 genes. Two templates from previous work (Wen et al., 2010), pYD1-CipA3 and pYD1-CipA3-CBH2, were used to replace the inducible promoters *GAL1/10* with the constitutive promoters *TEF1/2*, resulting in the plasmid

pRS414-CipA3-alpha-CBH2. Plasmid pC414-CipA3-Xyn2 was obtained by yeast homologous recombination of His-Xyn2-HR-L with *Xho*I/*BstE*II-linearized pRS414-CipA3-alpha-CBH2. Plasmid pC425-XlnD was obtained by co-transforming C-XlnD-HR1, C-XlnD-HR2, and C-XlnD-HR3. Plasmid pC414-Xyn2-XlnD was constructed by co-transforming 414-Xd-HR with *EcoRI*/*Spe*I digested pC414-CipA3-Xyn2. See Tables S2 and S3 in the supplementary materials for more details.

Plasmid pRS425-ZeaX was constructed using the DNA assembler method as described elsewhere (Shao et al., 2009). The vector pRS416m was created by incorporating a *hisG* sequence and a δ2 sequence in the empty plasmid pRS416, and then linearized with *Bam*HI (Shao et al., 2009). The five zeaxanthin biosynthetic pathway genes *CrtE*, *CrtB*, *CrtI*, *CrtY*, and *CrtZ* from *Erwinia uredovora* were PCR amplified from the plasmid pCAR-ΔCrtX (kindly provided by E. T. Wurtzel at the City University of New York). Individual gene expression cassettes, *PDC1p-crtE-PDC1t*, *TPI1p-crtB-TPI1t*, *GPM1p-crtI-GPM1t*, *GPDp-crtY-GPDt*, and *FBA1p-crtZ-FBA1t*, were assembled by overlap extension-PCR (OE-PCR) (Ho et al., 1989), purified by agarose gel electrophoresis and mixed with linearized vector pRS416m. Plasmid pRS425-ZeaX was obtained by homologous recombination of the PCR product leu-HR with *Apa*I/*Ahd*I digested pRS416-ZeaX in *S. cerevisiae* YSG50. See Tables S4 and S5 in the supplementary materials for details.

The zeaxanthin-producing strain was obtained by co-transforming the plasmids pC414-Xyn2-XlnD and pRS425-ZeaX using the lithium acetate method into *S. cerevisiae* L2612 (Daniel Gietz and Woods, 2002). All the plasmids were purified from *S. cerevisiae* and then transformed into DH5α, recovered, and confirmed by DNA sequencing.

Glucose-Abundant Cultivation in Shake-Flasks

The 14 recombinant yeast strains harboring the 14 pairs of promoters and terminators were characterized in shake-flasks under the oxygen-abundant, glucose-abundant (oxy^+–glu^+), or oxygen-limited, glucose-abundant (oxy^-–glu^+) conditions. Each recombinant strain was first grown on a SC-Ura plate for 3–4 days. A single colony was grown to saturation in 3 mL SC-Ura media in 15 mL culture tubes (BD Biosciences, Canada) at 30°C, 250 rpm overnight. For the oxy^+–glu^+ condition, 30 μL of the overnight cultures were inoculated into a fresh 3 mL media and grown for approximately 9 h till OD_{600} reached ~1. For the oxy^-–glu^+ condition, 30 μL of the overnight cultures were transferred into an anaerobic tube with the fresh 3 mL media and sealed with septum stopper and aluminum seal cap (BelloGlass, Vineland, NJ). The anaerobic tubes were degassed in vacuum for 20 min and purged with nitrogen for 20 s. The cultures were grown for 12 h to an OD_{600} of ~1. GFP intensities of these cells were subsequently determined by flow cytometry. All experiments were performed in triplicate.

RNA Extraction and Quantitative PCR

Yeast cells were harvested during the exponential growth phase. Approximately 1×10^7 cells were used for the total RNA extraction using RNeasy Mini Kit (QIAGEN, Valencia, CA). Genomic DNA contamination was eliminated by DNase I treatment. RNA concentration was quantified by measuring the absorbance at 260 nm using NanoDrop 2000c (Thermo Scientific, Waltham, MA). All RNA samples were diluted to the same final concentration of 10 ng/μL and stored at −80°C.

The Universal Probe Library probes and the gene-specific primers for *gfp* and actin (*act1*, internal reference gene) were designed using the ProbeFinder (https://www.roche-applied-science.com) (Table S6). The resulting PCR amplicons were about 60 bp. Both probes were labeled with fluorescein (fluorescein amidite, FAM) at the 5′-end and a dark quencher dye near the 3′-end. Quantitative PCR analysis was performed using LightCycler 480 real-time machine with the LightCycler RNA Master Hydrolysis Probes kit (Roche) according to the manufacturer's instructions. Each 20 μL reaction contained 10 ng total RNA, 7.4 μL 2.7× RNA Master Hydrolysis Probes mix, 2.0 μL 10× Primer/Probe mix (with final concentrations of 0.5 μM and 0.25 μM, respectively), 1.3 μL 10× Activator, and DEPC-Treated water (Applied Biosystems/Ambion, Austin, TX). Thermal cycling conditions were set as follows: reverse transcription, 1 cycle of 63°C for 3 min; denaturation, 1 cycle of 95°C for 30 s; amplification, 45 cycles of 95°C for 10 s and 60°C for 30 s; cooling, 1 cycle of 40°C for 10 s. The relative quantification for the mRNA levels was calculated using both the standard curve and the second derivative maximum method (Schefe et al., 2006). All assays were performed in triplicate, and the reaction without reverse transcriptase was used as a negative control.

Glucose-limited Fed-Batch Fermentation in Bioreactors

A single colony was inoculated into 3 mL SC-Ura, and the culture was grown with shaking at 30°C for 24 h. An aliquot of the resulting culture was used to inoculate 20 mL SC-Ura media with an initial $OD_{600} \sim 0.1$. The culture was grown with shaking at 30°C for 24 h until an OD_{600} of 8 was achieved. Five percent of the resulting culture was used as seed culture for studies in the MULTIFORS bioreactor (INFORS-HT, Laurel, MD). The initial OD_{600} was ~0.4 with a working volume of 340 mL. Before inoculation, the vessels were autoclaved with half volume of ddH_2O. In order to reduce contamination, the autoclaved bioreactors were allowed to cool down in a biological hood for several hours. All the following installations were carried out in a biological hood, including filling with the other half volume of 2× SC-Ura media, installing the pH sensor and peristaltic pumps. Temperature, pH, and glucose feeding were controlled by proportional-integral-derivative (PID) control loops. The parameters were set to 0 for the derivative of the error, 0 (minimum control action) for the integration of the error,

and 5 for the proportional response to an error. The temperature was maintained at 30°C and pH at 5.5. Dissolved oxygen was measured using an O_2 sensor (Mettler Toledo, Columbus, OH) supplied with a fitted membrane. All the sensors were calibrated using standard buffers.

In total, there were three stages used to maintain pO_2 and glucose concentration at a desired set-point throughout the whole fermentation. With the air flow at an initial setting of 0.8 L/min, pO_2 was maintained at the maximum value (100%) under the impeller speed of 250 rpm. This phase lasted for approximately 24 h. For the oxygen-limited condition (oxy^-–glu^-), the air flow rate was then set to 0.6 L/min for the next 48 h during which the accumulated ethanol was consumed. The pO_2 level dropped to around 30%. After 72 h, the glucose solution (60 g/L) was fed automatically when the residual ethanol was consumed. The maximum pump speed for the glucose feed was limited to 3 mL/h. The pO_2 level was maintained at 5% via oxygen sensor-controlled flow rate and glucose dosage cascade. For the oxygen-abundant condition (oxy^+–glu^-), after maintaining the starting condition for 72 h, pO_2 was maintained at 30% via oxygen sensor-controlled flow rate and glucose dosage cascade. Samples (3 mL) were collected at 24 h intervals.

Measurement of GFP Fluorescence Intensity

The GFP fluorescence intensity of the cells harvested from shake flasks and bioreactors was measured using flow cytometry. Briefly, cells were first stored for at least 1 h at 4°C, and resuspended with phosphate-buffered saline (PBS) to an optimal density between 0.1 and 0.2 at 600 nm, and transferred to a flow tube. Flow cytometry analysis was performed using Becton Dickinson FACScan (BD Falcon, Franklin Lakes, NJ), and the mean fluorescence intensity distribution of each clonal population was calculated.

Determination of Ethanol and Glucose Concentrations

The cultures were harvested and filtered with 0.22 μm filters. The supernatant was diluted 5-fold for ethanol and glucose concentration measurement using commercial kits from r-Biopharm (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO), respectively, according to the manufacturer's instructions.

Functional Analysis of the Artificial Ten-Gene Pathway

The recombinant yeast strain L2612 containing the artificial ten-gene pathway capable of converting xylan to zeaxanthin was cultivated in 300 mL SC-Leu-Trp-Ura medium, supplemented with 2% birchwood xylan and 0.2% glucose, starting with an OD_{600} of 0.2. Cells were grown at 30°C for 20 days and centrifuged to remove the supernatant. Zeaxanthin was extracted with 5 mL of acetone from the cell pellets of either the positive strain or the negative control (only with xylan-utilizing and D-xylose utilizing pathways). The resuspended cells were passed through French press several times at 10,000 psi. Supernatants were collected after centrifugation, evaporated to dryness and resuspended in 200 μL methanol. 100 μL of the sample was loaded onto the Agilent ZORBAX SB-C18 column and analyzed using Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA). The HPLC program was set as a flow rate of 0.4 mL/min: 0–3 min, 60% CH_3OH; 3–15 min, linear gradient from 60% CH_3OH to 100% CH_3OH; 15–25 min, 100% CH_3OH; 25–28 min, linear gradient from 100% CH_3OH to 60% CH_3OH; 28–35 min, 60% CH_3OH. Authentic zeaxanthin from Sigma was used as a standard and monitored by HPLC at 450 nm.

Results

Cloning of 14 Constitutive Promoters

A set of 14 constitutive promoters and their corresponding terminators were cloned from the genomic DNA of *S. cerevisiae*. The constitutive promoters *ADH1p*, *TEF1p*, *TEF2p*, and *GPDp* (also known as *TDH3p* in literature) are well characterized (Mumberg et al., 1995; Ruohonen et al., 1995; Schirmaier and Philippsen, 1984). The others including *PDC1p*, *FBA1p*, *PGK1p*, *PGI1p*, *TDH2p*, *PYK1p*, *ENO2p*, *GPM1p*, *TPI1p*, and *HXT7p* were selected from the glucose utilization pathway including glycolysis (Hauf et al., 2000). For each individual promoter, an expression cassette was constructed, including a promoter, a *gfp* gene, and a terminator, each of which was PCR-amplified and assembled using OE-PCR. The multicopy vector pRS426 used to construct promoter-GFP-terminator cassettes contains a 2 μ origin and is a widely used vector. In total, 14 cassettes, *PDC1p-GFP-PDC1t*, *FBA1p-GFP-FBA1t*, *TEF2p-GFP-TEF2t*, *PGK1p-GFP-PGK1t*, *PGI1p-GFP-PGI1t*, *ADH1p-GFP-ADH1t*, *TDH2p-GFP-TDH2t*, *PYK1p-GFP-PYK1t*, *ENO2p-GFP-ENO2t*, *GPDp-GFP-GPDt*, *GPM1p-GFP-GPM1t*, *TPI1p-GFP-TPI1t*, *TEF1p-GFP-TEF1t*, and *HXT7p-GFP-HXT7t* were obtained.

Characterization of the 14 Constitutive Promoters Under Glucose-Abundant Conditions

The strengths of the 14 promoters were first evaluated under four different growth conditions that are routinely utilized in the laboratory for production of value-added compounds in *S. cerevisiae*. The oxygen-limited and glucose-abundant (oxy^-–glu^+) and oxygen-abundant and glucose-abundant (oxy^+–glu^+) conditions were evaluated in shake-flasks. For the oxy^+–glu^+ condition, cells were collected at $OD_{600} \sim 1$, when the glucose was still at half of the initial concentration (data not shown), which was considered as glucose-abundant. The oxy^-–glu^+ condition was created by placing the culture

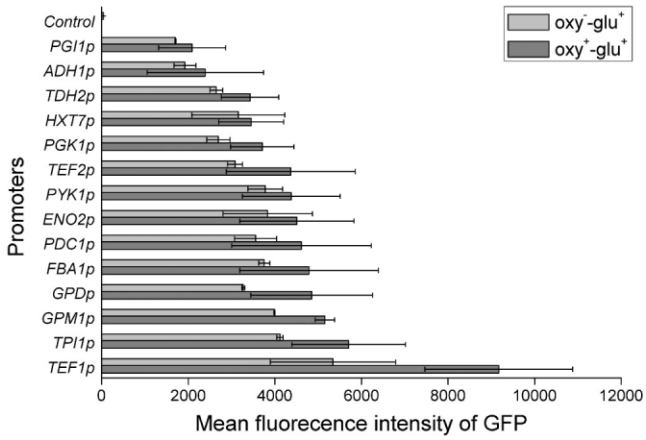

Figure 1. Comparison of the 14 promoters in the glucose-abundant condition. *X*-axis shows the mean fluorescence intensity of GFP while *Y*-axis lists the names of the promoters. The experiments were carried out in shake-flasks. The light gray columns show the results from the oxygen-limited and glucose-abundant condition, while the dark gray columns show the results from the oxygen-abundant and glucose-abundant condition. Error bars represent the standard deviations of three independent experiments.

tubes under vacuum followed by injection with sterile nitrogen gas. Samples were collected when OD_{600} reached ~1. Figure 1 illustrates the strengths of the 14 promoters under oxy^{-}–glu^{+} and oxy^{+}–glu^{+} conditions in shake flasks. The mean fluorescence intensity of GFP was always higher in the oxygen-abundant condition than the oxygen-limited condition. Promoter *TEF1p* exhibited the highest strength in both conditions, whereas promoters *PGI1p* and *ADH1p* showed the lowest strengths, representing about 23% and 26% of the strength of the promoter *TEF1p* in the oxy^{+}–glu^{+} condition, and 32% and 36% of the strength of the promoter *TEF1p* in the oxy^{-}–glu^{+} conditions, respectively (Fig. 1). All the other promoters varied from 37% to 77% in strengths in comparison to the *TEF1p* promoter.

In parallel, the strengths of these 14 promoters were evaluated by measuring the relative mRNA level of *gfp* through quantitative PCR (qPCR). The housekeeping gene *act1* was used as a reference gene. The relative levels of target mRNAs from oxy^{-}–glu^{+} and oxy^{+}–glu^{+} conditions were calculated based on a standard curve. Figure 2 compared the mean fluorescence intensity and relative mRNA level of *gfp* in the oxy^{+}–glu^{+} and oxy^{-}–glu^{+} conditions. The individual promoters were labeled with the same color under the two conditions. Although the abundance of transcript did not match exactly with the fluorescence intensity, the promoters with higher GFP intensity generally exhibited higher mRNA levels, and vice versa. Considering the possible expression variation of *act1* when environment changes (Teste et al., 2009), another housekeeping gene *alg9* was used in parallel. As shown in Figure S1, for the higher strength promoters, including *TEF1p*, *TPI1p*, *GPM1p*, *GPDp* (*TDH3p*), *FBA1p*, *PDC1p*, *ENO2p*, *PYK1p*, and *TEF2p*, the order from high to low strength was slightly altered. Noticeably, the transcriptional levels of *gfp* can be retained within approximately ±10% in the two culture conditions.

Characterization of Select Promoters Under Glucose-Limited Conditions

The strongest promoter *TEF1p* and the weakest promoter *PGI1p* were also evaluated under the oxy^{-}–glu^{-} and oxy^{+}–

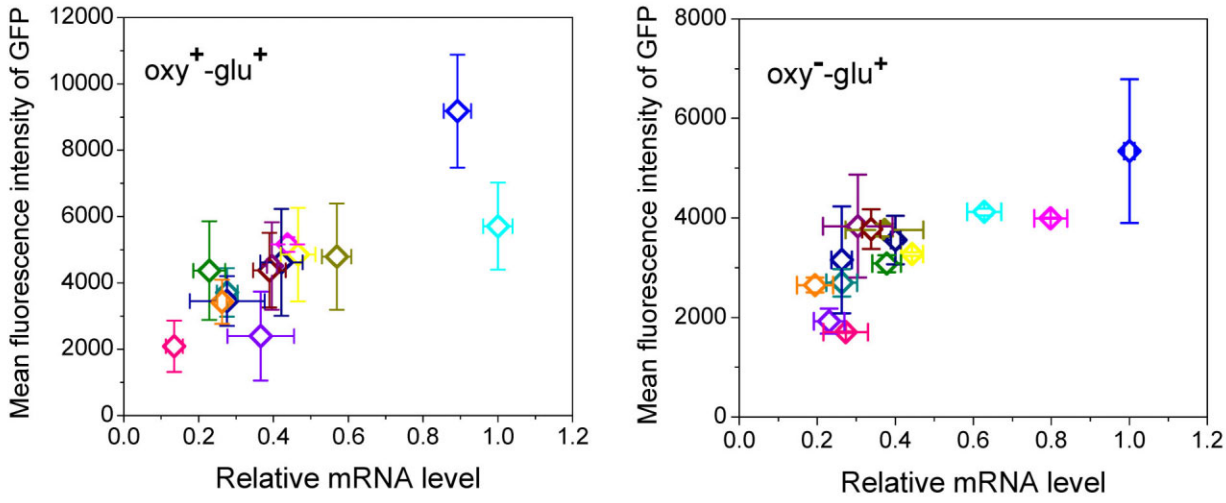

Figure 2. Characterization of promoter strengths at the translational and transcriptional levels. *X* and *Y* axes represent the relative mRNA level and the mean fluorescence intensity of GFP, respectively. The results in the two conditions, oxy^{+}–glu^{+} and oxy^{-}–glu^{+}, were shown side by side. The same promoter under the two conditions was marked with the same color. All the experiments were done in triplicate.

glu$^-$ conditions in a bioreactor. The glucose-limited condition was generated by continuously feeding glucose to maintain glucose at a minimal level. Figure S2 shows the results of cell density, glucose concentration and ethanol concentration profiles during the 144 h fermentation. Glucose was consumed quickly in the first 24 h, while ethanol was consumed as the second stage carbon source between 24 and 72 h. The glucose-limited condition was then controlled by feeding a glucose solution only sufficient for cell multiplication, while the oxygen concentration was monitored. During this phase, it was clearly observed that the strong promoter *TEF1p* exhibited higher GFP fluorescence intensity than the weak promoter *PGI1p* under oxy$^-$–glu$^-$ and oxy$^+$–glu$^-$, by five- and sixfold, respectively (Fig. 3).

Assembly of an Artificial Ten-Gene Pathway by DNA Assembler

We sought to design a multi-gene biochemical pathway using the DNA assembler method, in which each individual gene contains a different promoter in the expression cassette. Based on the data from Figure 1, the 14 promoters were classified into three groups, including the high strength group—*TEF1p* and *TPI1p*; the medium strength group—*GPM1p*, *GPDp* (*TDH3p*), *FBA1p*, *PDC1p*, *ENO2p*, *PYK1p*, and *TEF2p*; the low strength group—*PGK1p*, *HXT7p*, *TDH2p*, *ADH1p*, and *PGI1p*. To demonstrate the utility of these 14 characterized promoters in DNA assembler based pathway engineering, we attempted to use this information to create a CBP organism carrying an artificial ten-gene pathway that converts xylan to a high-value compound zeaxanthin (Fig. 4). Considerable research effort has been dedicated to enabling microbes to utilize lignocellulose in CBP (Lynd et al., 2005). In CBP, enzyme synthesis, cellulose hydrolysis, monosaccharide fermentation, and production of value-added compounds are performed by a single organism. In this study, *TEF1p* and *TEF2p* were used in constructing plasmid pC414-Xyn2-XlnD (Fig. 5A). For expression of xylanases, Xyn2 and XlnD were individually assembled with a promoter, a secretion signal peptide (ss1 or ss2), and a terminator, and the corresponding constructs were named as *TEF2p*-(prepro signal peptide)-*Xyn2-ADH1t* and *TEF1p*-(prepro signal peptide)-*XlnD-ADH2t*. With secretion signals, the cells will secrete xylanases extracellularly, resulting in the degradation of xylan to D-xylose. We used the highest strength promoter *TEF1p* to express XlnD because XlnD was shown to be the rate-limiting step in xylan degradation (Dekker, 1983; Poutanen and Puls, 1988; van Peij et al., 1997). The two expression cassettes were individually prepared and transformed into *S. cerevisiae* using the DNA assembler method (Shao et al., 2009). The enzyme Xyn2 showed activities towards birchwood xylan and arabinoxylan to release reducing sugars. Enzyme XlnD could hydrolyze xylobiose, xylotriose, xylotetraose, and even xylopentose to xylose with high catalytic efficiency (data not shown).

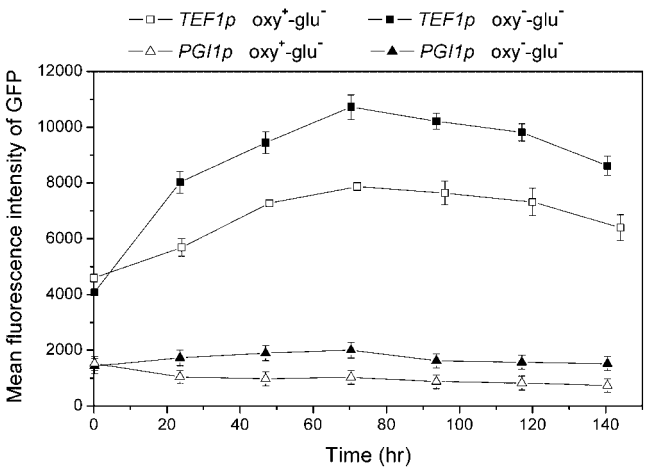

Figure 3. The time-course of the mean fluorescence intensities of GFP during cell cultivation in a bioreactor. The samples were collected at 0, 24, 48, 72, 96, 120, 144 h. The error bars were calculated from three different batches of fermentation. (□) promoter TEF1p in oxy$^+$–glu$^-$; (■) promoter TEF1p in oxy$^-$–glu$^-$; (△) promoter PGIp in oxy$^+$–glu$^-$; (▲) promoter PGI1p in oxy$^-$–glu$^-$.

The zeaxanthin biosynthetic pathway carrying *CrtE/B/I/Y/Z* was separately constructed with medium and high strength promoters *PDC1p*, *TPI1p*, *GPM1p*, *GPDp* (*TDH3p*), and *FBA1p* (Fig. 5B). Before assembling the five-gene pathway, the individual cassettes *PDC1p-CrtE-PDC1t*, *TPI1p-CrtB-TPI1t*, *GPM1p-CrtI-GPM1t*, *GPDp-CrtY-GPDt*, and *FBA1p-CrtZ-FBA1t* were prepared by OE-PCR. Then the entire five-gene pathway was successfully obtained via DNA assembler (Shao et al., 2009). The correct plasmid was confirmed by restriction digestion.

To enable *S. cerevisiae* to utilize D-xylose, the known D-xylose utilization pathway consisting of xylose reductase (XR), xylitol dehydrogenase (XDH), and D-xylulokinase (XKS) from *Scheffersomyces stipitis* was integrated into the *S. cerevisiae* L2612 genome (Fig. 5C). Individual cassettes *hisG-ADH1p-XR-ADH1t*, *PGK1p-XDH-CYC1t*, and *PYK1p-XKS-ADH2t-δ2*, were assembled by OE-PCR, purified by agarose gel electrophoresis, and mixed with *Bam*HI/*Xho*I digested integration fragment *δ1-hisG-ura3-hisG*, carrying a *ura3* selection marker and δ sequence which shares sequence identity to the partial sequence of δ sites on the yeast chromosome (Shao et al., 2009). The function of the D-xylose utilization pathway was confirmed by rapid growth of cells harboring the pathway in the D-xylose medium (data not shown). Notably, the parent *S. cerevisiae* cannot utilize D-xylose (Li et al., 2010). The plasmids pC414-Xyn2-XlnD and pRS425-ZeaX were subsequently transformed to the same strain.

Zeaxanthin Synthesis From Xylan

The engineered yeast strain was first demonstrated to produce zeaxanthin from glucose or D-xylose, both on solid medium and in liquid medium. After being grown on a SC-

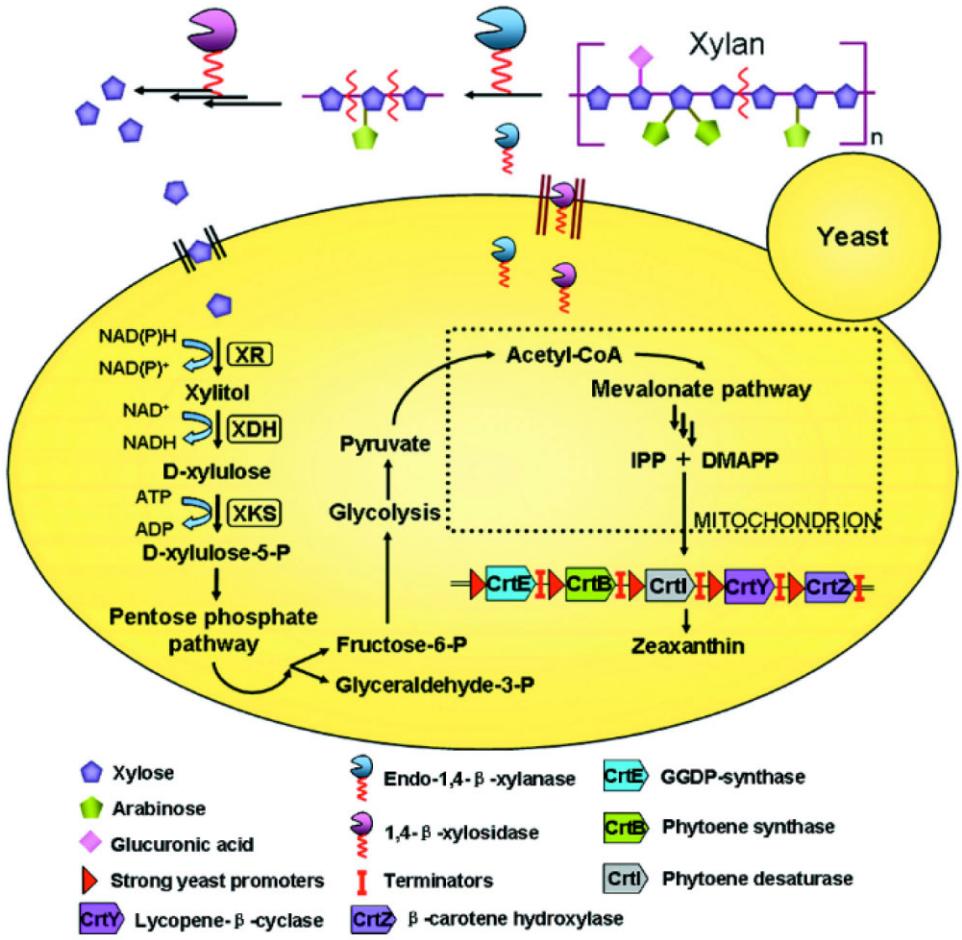

Figure 4. Scheme for engineering *S. cerevisiae* as a CBP organism for production of zeaxanthin from hemicellulose. The engineered yeast strain secretes out extracellular xylanases under strong promoters *TEF1p* and *TEF2p*. Xylose is produced from the xylan and enters the yeast cell. The integrated D-xylose utilizing pathway will enable cell growth and synthesis of zeaxanthin.

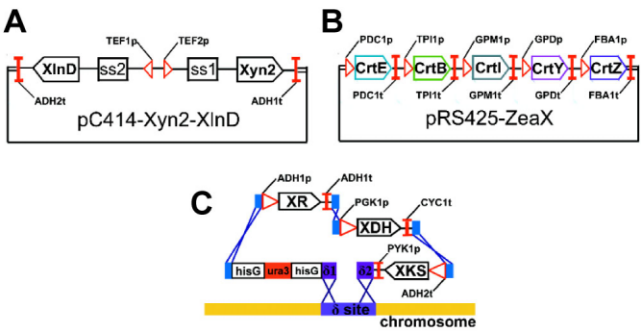

Figure 5. Construction of a combined xylan utilization and zeaxanthin synthesis pathway in *S. cerevisiae*, consisting of the xylan degradation pathway (**A**), the zeaxanthin synthesis pathway (**B**), and the integrated D-xylose utilizing pathway (**C**).

Leu-Trp-Ura plus 2% glucose or D-xylose plate for 4–5 days, the color of the cells changed from white to yellow, indicating the D-xylose utilizing pathway is active and the yellowish compound zeaxanthin was produced.

To demonstrate zeaxanthin synthesis from xylan, a small amount of glucose (0.2%) was supplemented in the xylan medium (2%) to increase the cell growth rate. The glucose was completely consumed in 24 h. Zeaxanthin production was observed in the medium with 2% xylan and 0.2% glucose, with a titer of 0.74 ± 0.02 mg/L (Fig. 6). Zeaxanthin was only detected in the presence of birchwood xylan. No zeaxanthin peak was found in the negative control in which the medium only contained 0.2% glucose, excluding the effect of glucose in product synthesis.

Discussion

A total of 14 pairs of constitutive promoters and terminators were cloned from *S. cerevisiae* and their strengths were

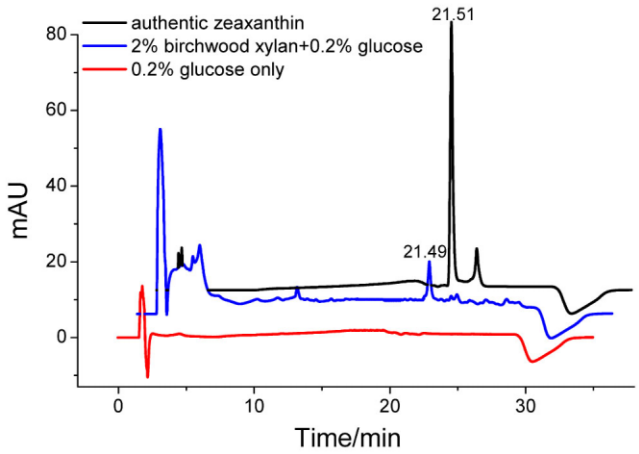

Figure 6. HPLC profile of zeaxanthin synthesis from 2% birchwood xylan plus 0.2% glucose. Authentic zeaxanthin is shown in black. The blue line and red line represent 2% birchwood xylan plus 0.2% glucose and 0.2% glucose, respectively. The retention times for authentic zeaxanthin and the zeaxanthin produced by the positive strain were 21.51 min and 21.49 min, respectively. All experiments were performed in triplicate.

evaluated using GFP as a reporter in a widely used 2 μ vector pRS426. A combined xylan utilization and zeaxanthin synthesis pathway was constructed in the *S. cerevisiae* strain L2612 using the strong promoters, *TEF1p*, *PDC1p*, *TPI1p*, *GPM1p*, *GPDp* (*TDH3p*), *FBA1p*, and *TEF2p*. The resulting strain could produce zeaxanthin from xylan with a titer of 0.74 ± 0.02 mg/L.

Most organisms transcribe genes differentially according to the changes in the environment. Such processes are very important for cell fitness and survival. Genomic studies in *S. cerevisiae* have revealed a variety of environmental stresses, including temperature shock, osmotic stress, oxidative stress, low pH, glucose or nitrogen starvation, and DNA-damaging agents, will trigger the expression of a large set of genes differentially (Costa and Moradas-Ferreira, 2001; Gasch et al., 2000; Lai et al., 2005; Moskvina et al., 1998). Recently, the effect of oxygen availability in yeast was analyzed (Kwast et al., 2002; Lai et al., 2005). *S. cerevisiae* is one of the few yeast species that can grow in the complete absence of oxygen. Complete repression of a promoter-regulated gene would only occur in the absence of oxygen (Martens et al., 2001). Comprehensive transcription analysis indicated that nearly one-sixth of the *S. cerevisiae* genome were differentially expressed with respect to oxygen availability, the majority (>65%) being down-regulated under anaerobic condition (Kwast et al., 2002). In order to make sure the promoters we cloned are still active in anaerobic condition, in which *S. cerevisiae* is often used as a fermentable host, we examined the performance of these promoters in the oxygen-limited condition and compared the result with that evaluated under the oxygen-abundant condition (Fig. 1). Our work provides a guideline when multiple promoters of different strengths are needed. The levels of oxygen limitation used in this study did not affect the cell growth. As a result, the relative strengths of these 14 promoters were independent of the oxygen concentration. The downstream gene *gfp* in this study did not seem to be repressed under oxygen-limited condition, but activated in varying levels. Moreover, although the absolute values of the promoter strength dropped slightly in the oxygen-limited condition, their relative strengths are roughly maintained, indicating the decreasing might be due to common physiological changes, rather than being promoter-dependent.

It should be noted that the promoters were evaluated using a 2 μ plasmid vector. However, both copy number and plasmid stability may be affected by promoter strength. Partow et al. (2010) used a *lacZ* reporter gene to characterize the strengths of promoters *TPI1p*, *ADH1p*, *TEF1p*, *PGK1p*, *GPDp* (*TDH3p*), *PYK1p*, and *HXT7p* that were integrated into the genome of *S. cerevisiae*. Under shake-flask cultivation after 8 h (considered as the oxy^+–glu^+ condition in this study), *PGK1p* and *GPDp* had the same activity as *TEF1p*. *TPI1p* and *PYK1p* showed 60% of the activity of *TEF1p*, while *ADH1p* and *HXT7p* was 20% and 10% of the activity of *TEF1p*, respectively. When switched to a different reporter GFP on a 2 μ vector, *TEF1p* also exhibited the highest activity under the oxy^+–glu^+ condition, and *PGK1p*, *GPDp*, *TPI1p*, *PYK1p*, *ADH1p*, and *HXT7p* represents 40%, 53%, 62%, 48%, 26%, and 38% of the activity of *TEF1p* (Fig. 1). Furthermore, we used *lacZ* as a second reporter gene and compared the results between two reporters. Changing the reporter did not significantly alter the order of the promoters in Figure 1 as the promoter with relatively high GFP fluorescence intensity also retained relative high LacZ activity. The promoters in the high and medium strength groups (*TEF1p*, *TPI1p*, *GPM1p*, *GPDp*, *FBA1p*, *PDC1p*, and *ENO2p*) still showed higher activities than those in the low strength group (*PGK1p*, *TDH2p*, *ADH1p*, and *PGI1p*). Therefore, the ranking of the promoters based on the 2 μ vector was generally reliable in this study. However, different results may be found with integrated genes. Thus, a systematic comparison of promoter strengths should be performed with integrated genes where both copy number and plasmid stability are controlled.

Next, the correlation between mRNA levels and protein expression levels was shown in Figure 2, which suggests that the varying levels of GFP synthesis under 14 promoters have similar trends in both translational and transcriptional levels. Since the promoters evaluated and used here were cloned largely from the glycolytic pathways, very low glucose concentrations may alter the promoter strengths. To test the performance of promoters in the glucose-limited condition, one strong promoter (*TEF1p*) and one weak promoter (*PGI1p*) were selected and evaluated in bioreactors. For both of them, the mean fluorescence intensity of GFP was higher in oxy^-–glu^- than oxy^+–glu^- (*TEF1p*, ~10000 in oxy^-–glu^-, ~6,000 in oxy^+–glu^-; *PGI1p*, ~2000 in oxy^-–glu^-, ~1,000 in oxy^+–glu^-) (Fig. 3). Despite these differences, *TEF1p* promoter strength was still higher than that of *PGI1p*

in the glucose-limited conditions, consistent with the results in the glucose-abundant conditions.

Notably, classification and strength measurement of 100 promoters in *Escherichia coli* were reported (Shimada et al., 2004), however, no such a wide range of yeast promoters has been studied. Fine-tuning gene expression has been demonstrated to be very useful for pathway optimization in *S. cerevisiae* (Alper et al., 2005; Nevoigt et al., 2006). Our strategy provides the basis for (i) analysis of yeast promoters in response to culture conditions (e.g., glucose concentration and oxygen concentration), (ii) optimization of exogenous gene expression for a multi-step pathway, and (iii) rewiring of the endogenous metabolic network by replacing native promoter(s) with ones of desired strength.

As an example of application in the DNA assembler method based pathway engineering, these characterized constitutive promoters were successfully engineered into a *S. cerevisiae* strain capable of synthesizing zeaxanthin from a renewable feedstock, birchwood xylan, demonstrating the possibility to achieve CBP through a single step of cultivation. Hemicellulose is the second most abundant component of lignocellulosic biomass, which accounts for 20–40% of biomass (Saha, 2003). Most of the hemicelluloses contain a β-1,4-xylosidic linked xylose backbone with other residues such as arabinose and glucuronic acid forming side-chains, also known as xylan. As shown in Figure 4, xylan deconstruction usually requires a minimum of two xylanases, endo-1,4-β-xylanase (EC 3.2.1.8), and 1,4-β-xylosidase (EC 3.2.1.37). Endo-1,4-β-xylanase is required for depolymerizing xylan's β-1,4-glycosidic linkages between xylose residues in the backbone, and smaller xylo-oligosaccharides are subsequently broken down into xylose monomers by 1,4-β-xylosidase (Dodd and Cann, 2009; Saha, 2003). Unlike the glucose decomposed from cellulose which can be utilized directly, the pentoses contained within the hydrolysates of hemicelluloses cannot be directly utilized by the majority of industrial microorganisms including *S. cerevisiae*. In order to overcome this hurdle, xylose and arabinose utilization pathways were introduced into yeast, converting pentoses into D-xylulose-5-phosphate, which can be further metabolized through the pentose phosphate pathway (Hahn-Hagerdal et al., 2007; Kotter and Ciriacy, 1993). Therefore, if an organism could both decompose hemicelluloses and utilize the resulting xylose and arabinose as the carbon sources, it would have great potential to be utilized as a microbial factory for synthesis of value-added products.

CBP is considered to have the potential of lower cost and higher efficiency than processes featuring dedicated cellulase production (Lynd et al., 2005; Xu et al., 2009). Development of superior CBP yeasts has been pursued to combine saccharification and fermentation to achieve high product yields (van Zyl et al., 2007). Successful examples include secreting free enzymes extracellularly to act on lignocelluloses (Den Haan et al., 2007a) and displaying enzymes on the surface (Fujita et al., 2004; Wen et al., 2010), mimicking the naturally occurring complexed cellulase system. Nonetheless, most studies were targeted at producing biofuels and few studies were carried out to develop an engineered CBP yeast strain to synthesize other value-added compounds from lignocelluloses (Steen et al., 2010). Here, we used zeaxanthin as a proof of concept to show that a CBP yeast can be engineered to produce a carotenoid. More importantly, the promoters characterized in this study should be highly useful in engineering recombinant yeast strains for production of fuels and chemicals from renewable feedstocks.

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