A synthetic promoter system for well-controlled protein expression with different carbon sources in *Saccharomyces cerevisiae*

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Abstract

**Background:** *Saccharomyces cerevisiae* is an important synthetic biology chassis for microbial production of valuable molecules. Promoter engineering has been frequently applied to generate more synthetic promoters with a variety of defined characteristics in order to achieve a well-regulated genetic network for high production efficiency. Galactose-inducible (GAL) expression systems, composed of GAL promoters and multiple GAL regulators, have been widely used for protein overexpression and pathway construction in *S. cerevisiae*. However, the function of each element in synthetic promoters and how they interact with GAL regulators are not well known.

**Results:** Here, a library of synthetic GAL promoters demonstrate that upstream activating sequences (UASs) and core promoters have a synergistic relationship that determines the performance of each promoter under different carbon sources. We found that the strengths of synthetic GAL promoters could be fine-tuned by manipulating the sequence, number, and substitution of UASs. Core promoter replacement generated synthetic promoters with a twofold strength improvement compared with the GAL1 promoter under multiple different carbon sources in a strain with GAL1 and GAL80 engineering. These results represent an expansion of the classic GAL expression system with an increased dynamic range and a good tolerance of different carbon sources.

**Conclusions:** In this study, the effect of each element on synthetic GAL promoters has been evaluated and a series of well-controlled synthetic promoters are constructed. By studying the interaction of synthetic promoters and GAL regulators, synthetic promoters with an increased dynamic range under different carbon sources are created.

**Keywords:** Synthetic promoter, Carbon sources, Protein expression, *Saccharomyces cerevisiae*

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regulatory elements, including promoters, activators and suppressors, as well as other regulators, which have been extensively engineered for pathway optimizations [3, 4].

In S. cerevisiae, a set of native promoters has been well-characterized, including constitutive promoters, which sustain stable expression levels across different growth conditions, and inducible promoters which vary their strengths in response to internal or external stimuli. Strong constitutive promoters are frequently used for driving protein overexpression, such as PTDH3, PTEF1, PGK1, PTP1, PENO2 [5, 6]. Commonly used inducible promoters include galactose-inducible promoters, such as PGAL1, PGAL2, PGAL7, and PGAL10. These promoters have been broadly applied in metabolic engineering [7, 8], thus their regulation has been extensively studied and engineered.

The genes involved in the GAL network are divided into two categories, one is related to galactose metabolism and includes GAL1, GAL2, GAL7 and GAL10, and the other is responsible for the regulation of the former including GAL4, GAL80, GAL3. Gal4p is the transcriptional activator, Gal80p is a repressor of Gal4p. In general, Gal4p docks on the GAL promoters. When galactose is added, Gal3p is a transcriptional regulator that forms a complex with Gal80p to relieve Gal80p inhibition of GAL promoters. When galactose is added, Gal80p has been deleted to inhibit Gal80p inhibition of Gal4p and activates the activity of the GAL promoters. However, this activation could be hindered by glucose repression [9]. GAL1, a gene encoding a galactokinase to covert galactose into galactose-1-phosphate, has also be knocked out to inhibit galactose metabolism so that galactose became a gratuitous inducer for galactose-inducible promoters [10, 11]. Gal80p has been deleted to allow all GAL promoters to be functional under diverse carbon sources because it is not economically feasible to use galactose as a sole carbon source for the fermentation of many products [12, 13].

More recently, promoter engineering has been utilized to construct synthetic promoters for various applications. It has been found that synthetic promoters could be created by combining of core promoters, which directly interact with RNA polymerase II (Pol-II) and other general transcription factors, and upstream activating sequences (UASs), which improve promoter activity and increase protein production. The UASs include UASCLB from the CLB2 promoter, UASGIT from the CIT1 promoter, UASENO from the ENO2 promoter, and UASGAL from the GAL1 promoter [14, 15]. However, few studies have been performed to systematically characterize the synergy between UASs and core promoters or the role of other endogenous regulatory elements on the resulting synthetic promoters. In this study, a library of synthetic GAL promoters using different UASs and core promoters was built to investigate their interaction. Furthermore, a synthetic promoter system was developed by engineering endogenous GAL regulators into synthetic promoters, and this promoter system could control protein production with improved dynamic range in S. cerevisiae grown in different carbon sources.

**Results and discussion**

**Engineering the upstream activating sequences of GAL1 promoter**

Herein, we divided the GAL1 promoter (P_{GAL1}) into an UAS region (UAS_{GAL1}) and a core promoter (cP_{GAL1}) for study, as shown in Fig. 1A. UAS_{GAL1} is the region containing four UASs designated as U1, U2, U3, U4, while cP_{GAL1} was used to represent the sequence between UAS region and the start codon. In a previous study, it was shown that UASs of GAL1 promoter had different activities [16], however, their individual roles in promoter strength have not yet been elucidated. In this study, to understand how UASs work on GAL1 promoter, we firstly analyzed several UASs (Fig. 1B), including four UASs from GAL1 promoter, the most widely used GAL promoter, one from GAL7 promoter, which contains one single strong UAS (U8), and three (U5, U6 and U7) from Saccharomyces kudriavzevii GAL2 promoter (SkPGAL2), the strongest GAL promoter in the literature [17]. In addition, the non-conserved CGC triplet at the 5’ terminus of U4 was mutated to the conserved CGG triplet and named U4g (Fig. 1B). These UASs were fused directly to the 5’-end of cPGAL1 (Fig. 1C) and cPCYC1, the core promoter of CYC1 (Fig. 1D) [14] to obtain a series of synthetic promoters. The gene encoding green fluorescent protein (eGFP) was then placed under the control of these synthetic promoters as a sensor to monitor their activities [18].

As shown in Fig. 1C, core promoter cPGAL1 showed low basal activity in synthetic complete (SC) medium containing 2% galactose, while the addition of every single UAS improved the promoter activities under the same condition. Addition of U2 and U3 resulted in the highest activities which were 36.5-fold and 37.6-fold higher than cPGAL1, respectively. The fusion of U1 and U4 also had a positive effect on the promoter strength, about 10.9-fold and 4.5-fold higher compared with cPGAL1 respectively, although these activities were significantly lower than that of the U2-U3 fusion. The activity of U4g-cPGAL1 was 4.6-fold greater than U4-cPGAL1, indicating the conserved CGG triplet was important for UAS activity. Moderate activities were observed for the synthetic promoters with U8 and heterologous U5, U6, and U7. When cPCYC1 was used as the core promoter, the effect of each UAS showed a similar pattern as in the case of cPGAL1 (Fig. 1D). UAS engineering did not alter the basal
activities of the synthetic promoters when cells were grown in SC medium with 2% glucose as compared to cPGAL1 and cPCYC1 (Additional file 1: Fig S1A and S1B). These results illustrated that individual UASs could be used to fine-tune the promoter activity.

To analyze the synergistic effect of UASs, we used P_{GAL1} as a model to create single and multiple UAS-negative constructs with minimal perturbation of the promoter sequences by mutating the conserved 5′ terminal CGG triplet to AGG, which resulted in a sharp reduction in promoter activity for one or more UASs (Additional file 1: Fig S1C). As shown in Additional file 1: Fig S1D, mutation of a single UAS led to 10% to 18% reduction of the promoter strength while the synthetic promoter with mutations in all four UASs, U4m, had 50% of the activity of PGAL1. Mutations in U4m was recovered individually to study the individual role of each UAS in the complex. The results showed that recovery of U2 or U3 did not have a significant change in the promoter activity, while U1 and U4 led to a significantly higher activity. These results were not in line with single UAS fusions, indicating that U1 and U4 play essential roles in GAL1 promoter. We further investigated the UAS region of the GAL1 promoter by replacing U1 and U4, two weak UASs in the
original construct, with U7, U4g, and/or U8, stronger UASs, to afford a few synthetic promoters. However, similar or lower activities were observed for these synthetic promoters. We also replaced all four UASs with U4, the weakest UAS in PGAL1. The resulting promoter showed low basal activity. Partially replacement of U4s with U2 generated stronger promoters, while the activity did not correlate to the number of U2s. These results indicated there was no direct linkage between individual UASs and the resulting promoter activity (Fig. 1C). A previous study showed that U1 and U4 may be not involved in promoter activation but take part in a larger regulatory mechanism that regulated equal expression of Gal1p and Gal10p [19]. Thus, substitutions of U1 and U4 would probably disrupt this large regulatory mechanism and result in a decrease of PGAL1 activity. By engineering the sequence, number, and substitution of UASs, we created a series of galactose-inducible promoters with different strengths which could be used for future engineering work (Fig. 1C) and these promoters displayed low basal expression under glucose growth condition (Additional file 1: Fig S1A). These results demonstrated that a single UAS was important but not determinant to PGAL1 activity, indicating that the GAL1 UASs may have subtle synergistic effects and be involved in other positive regulation mechanisms which would be destroyed when engineered.

The function of core promoter on GAL1 promoter

We then tried to replace the core promoter region to further explore the interplay of UASs and core promoters. Several constitutive promoters with a dynamic range of activity were used to replace cPGAL1; their sequences can be found in Additional file 1: Table S1 [5, 18]. The activities of the selected promoters were similar as previously reported in the presence of 2% glucose, and no significant activity difference could be observed when galactose was used as the sole carbon source (Fig. 2A). As shown in Fig. 2B, when cPGAL1 was switched with the core regions of several constitutive promoters, more than half of the resulting synthetic promoters became galactose-inducible as expected. The activities of PUAS-TDH3 and PUAS-TEF1, in which strong constitutive promoter PTDH3 and PTEF1 was used, were 30% and 68% higher than PGAL1 under 2% galactose, respectively, whereas the activity of PUAS-CYC1 and PUAS-CIT1 were comparable to PGAL1 in the same condition. However, some of the core promoter substitutions did not respond to galactose induction, such as PUAS-TPI1, PUAS-HHF1, PUAS-POX1, and PUAS-STE5, despite the fact that PTP1 and PHHF1 showed stronger activity than cPGAL1 under 2% galactose, indicating that the upstream activating sequence region may have some specificity for the core promoter. When glucose was used as the sole carbon source, most of the synthetic promoters maintained the same expression levels as their corresponding.

Fig. 2 Characterization of the synthetic promoters by core promoter replacement. The normalized fluorescence for constructs with only the core promoter (a) and with USGAL1 fusion (b). The activities of all synthetic promoters were tested after cultivation with 2% glucose (grey) or galactose (orange) for 24 h. Data are mean ± SD from three biological replicates
core promoters, indicating that combinatorial interaction between UAS\textsubscript{GAL1} and core promoter did not drastically affect the native properties of core promoters. These results show that galactose-inducible promoters stronger than P\textsubscript{GAL1} can be created by fusing UASs to the core promoters, which significantly expanded the dynamic range of galactose inducible promoters by more than 50% over previously reported maximum activity [14].

**GAL80 deletion increased synthetic promoter activity under galactose and glucose growth condition**

To analyze how the synthetic promoters interact with the endogenous regulator, Gal80p, the constructs with eGFP driven by P\textsubscript{GAL1}, P\textsubscript{UAS-TDH3} and P\textsubscript{UAS-TEF1} were evaluated in both the wildtype and the GAL80 deletion strains in the presence of 2% galactose or glucose (Fig. 3A). Cell density and fluorescence were continuously monitored for 36 h to accurately characterize the host growth rate as well as the promoter strength in the presence of these carbon sources. As shown in Fig. 3B, deletion of GAL80 further increased the activity of P\textsubscript{GAL1}, P\textsubscript{UAS-TDH3} and P\textsubscript{UAS-TEF1} in the presence of 2% galactose. The maximal activity of P\textsubscript{GAL1} in the GAL80 deletion strain was improved by 23% compared to that of a wildtype strain, whereas the activities of P\textsubscript{UAS-TEF1} and P\textsubscript{UAS-TDH3} increased by 23% and 11%, respectively, while no change in the growth rate was observed (Additional file 1: Fig S2A). Thus, we speculated that, even in the presence of 2% galactose, apo-Gal80p still presented in a concentration that could interact with Gal4p to suppress its activity, whereas deletion of Gal80p released all Gal4p to act as activator, leading to the improvement of the corresponding promoters’ activity.

We then compared the activities of these promoters in strains with or without Gal80p in the presence of 2% glucose. As shown in Fig. 3C, P\textsubscript{GAL1} showed a dramatic increase of activity in the GAL80 knockout strain as expected, while the maximal activities of P\textsubscript{UAS-TDH3} and P\textsubscript{UAS-TEF1} were increased by 32% and 120% in the GAL80 deletion strain compared to those in the wildtype strain. In addition, GAL80 deletion did not affect host growth on glucose (Additional file 1: Fig S2B). It is interesting to notice that all tested promoters in the GAL80 deletion strains had lower activities in the presence of 2% glucose compared with that in the presence of 2% galactose, especially for P\textsubscript{GAL1} where a 54% decrease was observed (Fig. 3B, C). The dramatic decrease in P\textsubscript{GAL1} activity even in GAL80 knockout strain is presumably because of glucose inhibition through the repressor Mig1p binding sites within the core promoter cP\textsubscript{GAL1} (Fig. 3A). Mig1p is a transcriptional repressor that responds to glucose and binds to a consensus sequence 5′-SYGGGG-3′ [20]. P\textsubscript{GAL1} contains two Mig1p binding sites [21]. According to sequence analysis, the synthetic promoters P\textsubscript{UAS-TDH3} and P\textsubscript{UAS-TEF1} do not contain Mig1p binding sites and thus should escape glucose repression. By testing these two promoters in glucose concentrations between 0 and 2%, we found that P\textsubscript{GAL1} activity in the GAL80 deletion strain decreased when the glucose concentration was higher than 0.0625%, which was not observed in the case of P\textsubscript{UAS-TDH3} and P\textsubscript{UAS-TEF1}. It confirmed that two synthetic promoters did not suffer from glucose repression (Fig. 3D).

We examined the activity of GAL and core promoters in the presence of different galactose concentrations with flow cytometry and measured the mean fluorescence intensity as well as the cell population with active transcription (ON cell). The results revealed that the percentage of ON cells for P\textsubscript{GAL1} in the wildtype strain increased with increasing galactose concentration which was not observed in the GAL80 deletion strain (Additional file 1: Fig S3A). The percentage of ON cells in the populations for P\textsubscript{UAS-TDH3} and P\textsubscript{UAS-TEF1} were similar in both the wildtype and GAL80 deletion strain and not affected by different galactose concentration (Additional file 1: Fig S3B), indicating that the higher activity of promoters in the presence of galactose in the GAL80 deletion strains were not due to the activation of more cells, but rather the increase of mean transcriptional level. Further analysis of the mean fluorescence intensity revealed that the expression levels driven by P\textsubscript{GAL1}, P\textsubscript{UAS-TDH3} and P\textsubscript{UAS-TEF1} were positively correlated with the galactose concentrations in both wildtype strain and GAL80 deletion strains, but showed no such correlation with constitutive promoters P\textsubscript{TDH3} and P\textsubscript{TEF1} (Additional file 1: Fig S3C and Fig. 3E), demonstrating that galactose may have an additional role in increasing the activity of galactose-inducible promoters even in the GAL80 deletion strain.

**Double deletion of GAL80 and GAL1 acquired high promoter activity under different carbon sources**

We then deleted GAL1 in GAL80 deletion strain to block galactose metabolism so that galactose would be a gratuitous inducer (Fig. 4A). Unanticipatedly, we observed that P\textsubscript{UAS-TDH3} and P\textsubscript{UAS-TEF1} in the GAL1/GAL80 double deletion strain had 35% higher activity than in the GAL80 single deletion strain under 2% glucose condition (Fig. 4B). The same effects can also be observed with other carbon sources, including fructose and raffinose, whereas the constitutive promoters such as P\textsubscript{TEF1} did not have any improvement (Fig. 4C and Additional file 1: Fig S4A). However, their activities were no longer affected
by galactose in the GAL1/GAL80 double deletion strains (Additional file 1: Fig S4B). The galactose inducible profile showed that the percentage of ON cells and the expression levels driven by all promoters were not affected by additional galactose in the double deletion strains (Additional file 1: Fig S3D and 4D). These results indicate that double deletion of GAL80 and GAL1 could completely relieve the galactose dependence of galactose-inducible
promoters, thereby allowing them to have maximal activities on multiple carbon sources. In all, compared to \( P_{\text{GAL1}} \) in the wildtype strain, the maximal activity of \( P_{\text{UAS-TDH3}} \) and \( P_{\text{UAS-TEF1}} \) in the double deletion strains increased by about 100% under both galactose and glucose growth conditions, with an expanded dynamic range of promoter activity under other carbon sources as well (Fig. 4E).
The efficient promoter system enhanced β-glucosidase expression

To test the utility of the synthetic promoters, secretory and surface-displayed expression of β-glucosidase (BGL1) and a cellulase responsible for degrading cellulose into glucose were used as examples. As shown in Fig. 5A, compared to the secretion of BGL1 driven by PGAL1 in wildtype strain, BGL1 under control of PUAS-TEF1 in the GAL80 deletion strain was more than two-folder higher in the presence of galactose, and its secretion driven by PUAS-TDH3 or PUAS-TEF1 in the GAL80 and GAL1 double deletion strains was more than two-folder higher under glucose growth condition. The surface-display of BGL1 exhibited nearly identical expression pattern as in secretion (Fig. 5B). These results illustrate that the synthetic promoters could increase intracellular expression and extracellular secretion of recombinant proteins both under glucose and galactose growth condition.

Conclusions

In summary, our results demonstrate that engineering PGAL1 enabled creation of synthetic galactose-inducible promoters with an expanded dynamic range, and it is the first reported synthetic promoter in S. cerevisiae with a twofold higher activity than PGAL1 under a variety of different carbon sources. The function of each element of these promoters was analyzed; we found that the upstream activating sequences UAS_{GAL1} are important to the inducibility of synthetic promoters and subtle synergistic effects within the UAS region are destroyed when the UASs are perturbed. When considering the core promoter region, stronger core promoters tend to produce stronger synthetic promoters. Furthermore, a system was developed for well-controlled protein expression in S. cerevisiae under different carbon sources. We found that deletion of GAL80 could further strengthen galactose-inducible promoter activities under galactose growth conditions, and double deletion of GAL80 and GAL1 could completely relieve the galactose dependence of these synthetic promoters derived from GAL1 to thereby unleash their maximal activities on different carbon sources.

Materials and methods

Strains and media

*Escherichia coli* Trans5a was used for plasmid construction and propagation, and its culture medium was LB (10 g/L tryptone, 5 g/L Yeast extract, 10 g/L NaCl) with or without ampicillin. *S. cerevisiae* CEN. PK2-1C was used as the host for testing promoters’ activities and expressing recombinant proteins. CEN. PK2-1C was grown in YPD (20 g/L peptone, 10 g/L yeast extract and 20 g/L glucose). The eGFP expressing strains were cultivated in SC medium, and the BGL1 expressing strains...
were cultivated in SC-SCAA medium [21], 2% of glucose or galactose was added according to the experimental requirements.

**Plasmid construction**

The primers used in this study were shown in Additional file 1: Table S2. The yeast centromeric plasmid pPOT2 containing the *URA3* gene as a marker was used as the backbone. The *GAL1* promoter amplified from the commercial plasmid pYD1 (Invitrogen), the eGFP gene and the *ADH1* terminator were inserted into pPOT2 by Gibson assembly and the recombinant plasmid named *P* _GAL1-*eGFP*. The promoter *cPGAL1* and *cPCYC1* fused with or without UASs were cloned to *P* _GAL1-*eGFP* though *P* _GAL1_ replacement. Point mutation of UASs were constructed from *P* _GAL1-*eGFP* by QuickChange mutagenesis. Replacement of UAS sites was achieved by Gibson assembly of mutant fragment and corresponding backbone amplified from *P* _GAL1-*eGFP*. All constitutive promoters were amplified from CEN.PK2-1C genomic DNA and then were used to replace the *cPGAL1* of *P* _GAL1-*eGFP*, respectively. The coding gene of BGL1 was amplified from the previously study’s plasmid [22]. The recombinant plasmids for expression of BGL1 controlled by *P* _GAL1*, *P* _UAS-TEF1_ and *P* _UAS-TDH3_ were constructed by eGFP replacement.

**GAL1 and GAL80 deletion**

In order to knock out the *GAL1* and *GAL80*, the pCUT plasmid containing Cas9 gene was used in this study as previously described [5]. The guide RNA and homologous fragments were designed by Yeaststriction (http://yeastrstriction.tnw.tudelft.nl/#!/ and SGD (https://www.yeastgenome.org/), respectively (Additional file 1: Table S3). Linearized pCUT plasmid, guide RNA and homologous fragment were transformed into CEN.PK2-1C for *GAL1* and *GAL80* deletion, respectively.

**Fluorescence measurement**

Three clones of each strain were placed in 300 μL SC-URA (2% glucose) medium for 24 h (96-well plate, 800 rpm), and then transferred into SC-URA (2% glucose or galactose) with initial OD600 at 0.2. After 24 h of culture, the GFP fluorescence were measured by microplate readers (Tecan, Infinite® 200 PRO), the excitation at 488 nm and the emission at 520 nm. Continuous monitoring of fluorescence and growth was cultured in an enzyme-labeled instrument for 36 h.

**Galactose or glucose response measurements**

In this experiment, 2% raffinose was used as a background carbon source. Colonies were cultured in SC medium (2% raffinose). After the overnight cultivation, cells were inoculated into in 2% raffinose and galactose (or glucose) with concentration from 0 to 2% to induce for 5 h with initial OD600 at 0.1. After induction, cells were collected and resuspended with PBS, and then the fluorescence distribution and the mean fluorescence intensity of 30,000 cells in each sample was recorded by flow cytometry (CytoFLEX S, Beckman Coulter). According to the fluorescence distribution, cells were divided into two populations of active transcription (ON cell) and inactive transcription (OFF cell) [23].

**Enzyme assays**

The strains expressing BGL1 were inoculated into SC-SCAA (2% glucose or galactose) medium and grown for 24 h. BGL1 activity was detected using *p*-nitrophenyl-β-D-glycopyranoside pNPG as the substrate, as described previously [22]. Enzymes were incubated in 50 mM citrate buffer (pH 5.0) with 5 mM pNPG at 50 °C for 30 min. Sodium carbonate (10%, w/v) was added to stop the reaction, and the absorbance was measured at 405 nm. One unit of the BGL1 activity was defined as the amount of enzyme that released 1 μmol of pNP from the substrate per minute at 50 °C.

**Supplementary Information**

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Authors' contributions
HT, XL and JDK designed experiments. JD, YW, ZZ and NC carried out the experiments. HT, JD, XL and JDK wrote and edited the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its Additional file 1.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
X.L. has a financial interest in Demetrix. J.D.K. has a financial interest in Amyris, Genomatica, Lycos, Demetrix, Napigen, Maple Bio, Apertor Labs, Zero Acre Farms, Berkeley Yeast, and Ansa Biotechnology.

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