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Promoters inducible by aromatic amino acids and γ-aminobutyrate (GABA) for metabolic engineering applications in *Saccharomyces cerevisiae*

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Abstract A wide range of promoters with different strengths and regulatory mechanisms are valuable tools in metabolic engineering and synthetic biology. While there are many constitutive promoters available, the number of inducible promoters is still limited for pathway engineering in *Saccharomyces cerevisiae*. Here, we constructed aromatic amino-acid-inducible promoters based on the binding sites of Aro80 transcription factor, which is involved in the catabolism of aromatic amino acids through transcriptional activation of *ARO9* and *ARO10* genes in response to aromatic amino acids. A dynamic range of tryptophan-inducible promoter strengths can be obtained by modulating the number of Aro80 binding sites, plasmid copy numbers, and tryptophan concentrations. Using low and high copy number plasmid vectors and different tryptophan concentrations, a 29-fold range of fluorescence intensities of enhanced green fluorescent protein (EGFP) reporter could be achieved from a synthetic U_4C_{ARO9} promoter, which is composed of four repeats of Aro80 binding half site (CCG) and *ARO9* core promoter element. The U_4C_{ARO9} promoter was applied to express *alsS* and *alsD* genes from *Bacillus subtilis* for acetoin production in *S. cerevisiae*, resulting in a gradual increase in acetoin titers depending on tryptophan concentrations. Furthermore, we demonstrated that γ-aminobutyrate (GABA)-inducible *UGA4* promoter, regulated by Uga3, can also be used in metabolic engineering as a dose-dependent inducible promoter. The wide range of controllable expression levels provided by these tryptophan- and GABA-inducible promoters might contribute to fine-tuning gene expression levels and timing for the optimization of pathways in metabolic engineering.

Keywords Acetoin · Aro80 · Aromatic amino acid · GABA · Inducible promoter · *Saccharomyces cerevisiae*

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Introduction

Controlling the expression of genes in metabolic pathways or in regulatory networks is an essential component in metabolic engineering and synthetic biology (Andrianantoandro et al. 2006; Keasling 2010). Although gene expression can be regulated at multiple points, promoter-driven transcriptional initiation is a key regulatory step in determining gene expression levels and timing (Blazeck and Alper 2013). Successful pathway engineering requires diverse range of constitutive and inducible promoters, which allow sophisticated transcriptional regulation of each gene participating in the pathway (Blazeck and Alper 2013; Da Silva and Srikrishnan 2012). Therefore, numerous efforts have been made to isolate native promoters (Mumberg et al. 1995; Sun et al. 2012) or to develop synthetic promoters suitable for genetic engineering (Alper et al. 2005; Blazeck et al. 2013; Blount et al. 2012; Hartner et al. 2008).

Saccharomyces cerevisiae is a well-studied eukaryotic model system with great potential as microbial cell factories for the production of fuels and chemicals (Nevoigt 2008; Nielsen et al. 2013). Strong constitutive promoters in the glycolytic pathway, P_{TDH3} (also known as P_{GPD}), P_{PGK1}, P_{TPI1}, and P_{PDC1}, and the promoter of translation elongation factor (P_{TEF1}) have been widely used for gene expression in *S. cerevisiae*, along with other weaker constitutive promoters

such as P_{CYC1} and P_{ADH1} (Cartwright et al. 1994; Da Silva and Srikrishnan 2012; Mumberg et al. 1995; Sun et al. 2012). Although constitutive promoters are convenient to maintain gene expression without additional manipulation, they are not suitable for the metabolic pathway containing toxic intermediates or for the expression of target genes at a specific time point (Da Silva and Srikrishnan 2012). Inducible or regulated promoters can complement these problems. The galactose-inducible P_{GAL1} and P_{GAL10} promoters have been mostly used in metabolic engineering applications, although other inducible promoters such as P_{CUP1}, P_{PHO5}, and P_{MET25} are also available in *S. cerevisiae* (Hottiger et al. 1995; Macreadie et al. 1991; Mumberg et al. 1994; Rudolph and Hinnen 1987). The *GAL* promoters are tightly repressed in the presence of glucose, resulting in about 1000-fold induction by galactose (Adams 1972; Blazeck et al. 2012). Recently, a series of synthetic galactose-inducible promoters with higher basal activity and dynamic range of galactose-induced expression levels have been generated by combining various upstream activation sequences (UASs) and core promoter elements (Blazeck et al. 2012). However, the *GAL* promoters have several disadvantages. Because of the glucose repression effect, *GAL* promoters cannot be induced by direct addition of galactose if the culture medium contains glucose (Johnston 1987; Lohr et al. 1995). Therefore, complete medium exchange is necessary for cells grown in glucose. In addition, galactose is used not only as an inducer but also as a carbon source. Since galactose is a less preferred carbon source than glucose, shifting the glucose-grown cells into galactose medium reduces cell growth rate, and the galactose-induced expression levels decrease as galactose is consumed during growth (Lee and DaSilva 2005).

The *GAL* promoters are activated by Gal4 transcription factor, which belongs to the Zn_2Cys_6 family of fungal-specific transcription factors (MacPherson et al. 2006; Todd and Andrianopoulos 1997). These transcription factors form homodimers and each Zn_2Cys_6 domain binds to CGG half sites aligned in various orientations (inverted repeat, $CGGN_xCCG$; everted repeat, $CCGN_xCGG$; direct repeat, $CGGN_xCGG$; and reverse direct repeat, $CCGN_xCCG$) and spacing (MacPherson et al. 2006; Todd and Andrianopoulos 1997). Although galactose-dependent activation of Gal4 is mediated by relieving the Gal80-dependent repression of Gal4, other Zn_2Cys_6 transcription factors are activated by direct binding of specific inducers (MacPherson et al. 2006). For example, proline directly binds and activates Put3 transcription factor, involved in proline utilization (Des Etages et al. 1996), and Leu3, involved in branched chain amino acids biosynthesis, is activated by binding of α-isopropylmalate, a pathway-specific intermediate (Hahn and Young 2011; Sze et al. 1992). Therefore, the promoters regulated by other Zn_2Cys_6 family proteins might have the potential to be developed as inducible promoters for genetic engineering applications. When selecting inducible promoters for biotechnological purposes, several factors have to be considered, which include the basal activity and induction fold of the promoter, and the cost and side effects of the inducer (Nevoigt et al. 2007).

Aro80, a member of Zn_2Cys_6 family, is involved in the utilization of aromatic amino acids as nitrogen sources through transcriptional activation of *ARO9* and *ARO10* in the presence of aromatic amino acids (Iraqui et al. 1999; Lee and Hahn 2013). Aro9 and Aro10 act as transaminase and decarboxylase, respectively, in the degradation of aromatic amino acids through Ehrlich pathway (Hazelwood et al. 2008). *ARO9* and *ARO10* genes are also regulated by nitrogen catabolite repression (NCR), thus activated by GATA transcription activators (Gat1 and Gln3) in the absence of good nitrogen sources (Lee and Hahn 2013). However, the availability of aromatic amino acids is the major determinant for the transcriptional activation of these genes (Lee and Hahn 2013). Aro80 is believed to act as a dimer like other Zn_2Cyc_6 family of transcription factors (MacPherson et al. 2006). Aro80 binding site consists of two CCG direct repeats separated by 7 base pairs ($CCGN_7CCG$), and the binding sites are repeated twice in the promoters of *ARO9* and *ARO10*, allowing binding of up to two Aro80 dimers. *S. cerevisiae* genome contains two additional genes containing Aro80 binding sites; *ARO80* itself and *ESBP6* encoding a protein homologous to a monocarboxylate permease (Eden et al. 2007). However, these genes, containing only one copy of the $CCGN_7CCG$ element in their promoters, are largely insensitive to the induction by aromatic amino acids (Lee and Hahn 2013).

In this study, we used Aro80 binding site to design promoters inducible by aromatic amino acids. A wide range of tryptophan-induced expression levels could be achieved by modulating the number of Aro80 binding sites, plasmid copy numbers, and the concentrations of inducer. The effectiveness of the tryptophan-inducible promoters was demonstrated by applying this promoter system to express heterologous genes for the production of acetoin in *S. cerevisiae*. In addition, we demonstrated the possibility of using γ-aminobutyrate (GABA)-inducible *UGA4* promoter for metabolic engineering. The *UGA4* promoter is regulated by Uga3, another member of the Zn_2Cyc_6 family of transcription factors.

Materials and methods

Strains and media

Escherichia coli strain DH5α [F^- *Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA*] was used for genetic manipulations. *S. cerevisiae* BY4741 (*MAT*a *his3Δ1 leu2Δ0 met15Δ0*

ura3Δ0) and *bdh1Δ* (BY4741, *bdh1Δ*::*KanMX6*) were obtained from EUROSCARF. Yeast cells were cultured in YPD medium (20 g/L glucose, 10 g/L yeast extract, and 20 g/L bacto-peptone) or synthetic defined (SD) medium (20 or 50 g/L glucose, 6.7 g/L yeast nitrogen base without amino acids) supplemented with auxotrophic requirements (120 μg/mL Leu and 50 μg/mL each of His, Met, and Ura).

Plasmid construction

The DNA fragment encoding EGFP was prepared by PCR amplification and cloned into p416GPD and p416ADH, resulting p416GPD-EGFP and p416ADH-EGFP, respectively. To construct the synthetic promoters, PCR amplifications were performed using the primers containing additional Aro80 binding sites, F_U_nC_{ARO80}/R_C_{ARO80} for *ARO80* core element (−129 to −1) and F_U_nC_{ARO9}/R_C_{ARO9} for *ARO9* core element (−132 to −1), generating [U_nC_{ARO80}] and [U_nC_{ARO9}] (n=2, 3, 4). The *ARO9* promoter (P_{ARO9}) was amplified by PCR using the primers, F_P_{ARO9}/R_C_{ARO9}. The *TDH3* promoter of p416GPD-EGFP was removed by cutting the plasmid with *Sac*I and *Bam*HI, and replaced with the DNA fragments [U_nC_{ARO80}], [U_nC_{ARO9}], or P_{ARO9} resulting in p416[U_nC_{ARO80}]-EGFP, p416[U_nC_{ARO9}]-EGFP, and p416[P_{ARO9}]-EGFP, respectively. The selected tryptophan-inducible promoter, [U_4C_{ARO9}], was replaced the *TDH3* promoter of p413GPD, p423GPD, p416GPD, and p426GPD, generating p413[U_4C_{ARO9}], p423[U_4C_{ARO9}], p416[U_4C_{ARO9}], and p426[U_4C_{ARO9}], respectively. The *alsS* gene from *Bacillus subtilis* was amplified by PCR using genomic DNA and cloned into p413[U_4C_{ARO9}] and p423[U_4C_{ARO9}], generating p413[U_4C_{ARO9}]-*alsS* and p423[U_4C_{ARO9}]-*alsS*. The *alsD* gene from *B. subtilis* was amplified by PCR using genomic DNA and cloned into p416[U_4C_{ARO9}] and p426[U_4C_{ARO9}], generating p416[U_4C_{ARO9}]-*alsD* and p426[U_4C_{ARO9}]-*alsD*. To construct GABA-inducible acetoin producing pathway, *UGA4* promoter sequence (−460 to −1) was prepared by PCR amplification and replaced the [U_4C_{ARO9}] promoter of p413[U_4C_{ARO9}]-*alsS* and p426[U_4C_{ARO9}]-*alsD*, creating p413[P_{UGA4}]-*alsS* and p426[P_{UGA4}]-*alsD*. Plasmids and primers used in this study are listed in Tables 1 and 2.

Culture conditions

For the experiments investigating the promoter inducibility by aromatic amino acids, overnight culture cells were diluted to OD_{600} of 0.45, incubated for 6 h in SD-Ura containing 20 g/L glucose, and then induced with 200 μg/mL tryptophan, phenylalanine, or tyrosine. Tryptophan and phenylalanine were dissolved in distilled water at a concentration of 10 mg/mL and used as stock solution. For tyrosine induction, instead of treatment of tyrosine using stock solution, the culture medium was exchanged with SD-Ura containing 200 μg/mL tyrosine because of the low solubility of tyrosine in water.

Yeast cells harboring *alsS* and *alsD* genes in various plasmids were precultured in SD-His-Ura containing 20 g/L glucose and then inoculated to OD_{600} of 0.2 in SD-His-Ura containing 50 g/L glucose with indicated concentrations of tryptophan or GABA for acetoin production.

RNA preparation and quantitative reverse transcription PCR

One milliliter of cells was harvested and frozen at −80 °C in 300 μL of lysis buffer [10 mM Tris–HCl (pH 7.4), 10 mM EDTA, 0.5 % SDS]. Acidic phenol (300 μL) was added to each sample and incubated at 65 °C for 20 min with occasional vortexing. Prior to chloroform extraction, the solution was chilled on ice for 10 min. After centrifugation and ethanol precipitation, the resulting RNA pellets were dissolved in RNase-free water. The relative amount of messenger RNA (mRNA) was determined by quantitative reverse transcription PCR (qRT-PCR) as previously described (Lee and Hahn 2013). Reverse transcription (RT) of 2 μg of total RNA was carried out with 0.1 μg of oligo-(dT) for 1 h at 42 °C using M-MLV reverse transcriptase (M-biotech, Inc., Korea), followed by heat inactivation for 10 min at 75 °C. PCR mixture containing 1 of 20 μL RT reaction solution, 1× SYBR master mix (Roche Diagnostics), and gene-specific primers was subjected to qPCR reaction with 45 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s using Roche LightCycler 480 real-time PCR system (Roche Diagnostics). Primer sequences used for qRT-PCR are shown in Table 2.

Measurement of EGFP fluorescence intensity

To measure the EGFP fluorescence intensity, cells were harvested at appropriate point and resuspended with phosphate-buffered saline (PBS). The EGFP fluorescence intensity was measured by a TECAN GeNios Pro microplate reader (TECAN, USA) at excitation wavelength of 485 nm and emission wavelength of 535 nm. Cell density was measured using the microplate reader Multiskan GO (Thermo Scientific, USA) at 600 nm.

High-performance liquid chromatography

To quantify the concentration of acetoin, 1 mL of culture supernatants was collected and filtered through a 0.22-μm syringe filter. High-performance liquid chromatography (HPLC) analysis was performed in UltiMate 3000 HPLC system (Thermo fishers scientific) equipped with a BioRad Aminex HPX-87H column (300×7.8 mm, 5 μm) at 60 °C with 5 mM H_2SO_4 as a flow rate of 0.6 mL/min and refractive index (RI) detector.

Table 1 Strains and plasmids used in this study

Name	Description	Reference
Strains		
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
bdh1Δ	BY4741 *bdh1Δ::KanMX6*	EUROSCARF
Plasmids		
p413GPD	CEN/ARS plasmid, P_{TDH3}, T_{CYC1}, *HIS3* marker	(Mumberg et al. 1995)
p416ADH	CEN/ARS plasmid, P_{ADH1}, T_{CYC1}, *URA3* marker	(Mumberg et al. 1995)
p416GPD	CEN/ARS plasmid, P_{TDH3}, T_{CYC1}, *URA3* marker	(Mumberg et al. 1995)
p423GPD	2 μ plasmid, P_{TDH3}, T_{CYC1}, *HIS3* marker	(Mumberg et al. 1995)
p426GPD	2 μ plasmid, P_{TDH3}, T_{CYC1}, *URA3* marker	(Mumberg et al. 1995)
p416ADH-EGFP	p416ADH harboring EGFP	This study
p416GPD-EGFP	p416GPD harboring EGFP	This study
p416[U_nC_{ARO80}]-EGFP (n=2, 3, 4)	$(UAS_{ARO80})_n$-$Core_{ARO80}$ cloned into p416GPD-EGFP, replacing the *TDH3* promoter	This study
p416[U_nC_{ARO9}]-EGFP (n=2, 3, 4)	$(UAS_{ARO80})_n$-$Core_{ARO9}$ cloned into p416GPD-EGFP, replacing the *TDH3* promoter	This study
p416[P_{ARO9}]-EGFP	*ARO9* promoter (P_{ARO9}) cloned into p416GPD-EGFP, replacing the *TDH3* promoter	This study
p413[U_4C_{ARO9}]	$(UAS_{ARO80})_4$-$Core_{ARO9}$ cloned into p413GPD, replacing the *TDH3* promoter	This study
p423[U_4C_{ARO9}]	$(UAS_{ARO80})_4$-$Core_{ARO9}$ cloned into p423GPD, replacing the *TDH3* promoter	This study
p416[U_4C_{ARO9}]	$(UAS_{ARO80})_4$-$Core_{ARO9}$ cloned into p416GPD, replacing the *TDH3* promoter	This study
p426[U_4C_{ARO9}]	$(UAS_{ARO80})_4$-$Core_{ARO9}$ cloned into p426GPD, replacing the *TDH3* promoter	This study
p413[U_4C_{ARO9}]-*alsS*	*alsS* gene from *B. subtilis* cloned into p413[U_4C_{ARO9}]	This study
p423[U_4C_{ARO9}]-*alsS*	*alsS* gene from *B. subtilis* cloned into p423[U_4C_{ARO9}]	This study
p416[U_4C_{ARO9}]-*alsD*	*alsD* gene from *B. subtilis* cloned into p416[U_4C_{ARO9}]	This study
p426[U_4C_{ARO9}]-*alsD*	*alsD* gene from *B. subtilis* cloned into p426[U_4C_{ARO9}]	This study
p413[P_{UGA4}]-*alsS*	*UGA4* promoter (P_{UGA4}) cloned into p413[U_4C_{ARO9}]-*alsS*, replacing the [U_4C_{ARO9}] promoter	This study
p426[P_{UGA4}]-*alsD*	*UGA4* promoter (P_{UGA4}) cloned into p426[U_4C_{ARO9}]-*alsD*, replacing the [U_4C_{ARO9}] promoter	This study

Results

Construction of aromatic amino-acid-inducible synthetic promoters

We investigated the possibility of using Aro80 binding site to design synthetic promoters inducible by aromatic amino acids. *ARO80* and *ARO9* promoters contain two and four CCG repeats, respectively, and the binding of Aro80 to these promoters has been confirmed by chromatin immunoprecipitation assays (Lee and Hahn 2013). However, aromatic amino acids induce transcription of only *ARO9*, but not *ARO80* (Lee and Hahn 2013). Therefore, we hypothesized that the number of Aro80 binding site in the promoter might affect the inducibility by aromatic amino acids. To prove this, we fused one or two CCG half sites to the native *ARO80* promoter consisting of UAS_{ARO80} (U_2) and the core promoter (C_{ARO80}), generating U_3C_{ARO80} and U_4C_{ARO80} (Fig. 1a). The synthetic promoters were fused to an EGFP reporter gene, and then cloned into p416, a CEN/ARS-based low copy number plasmid vector containing *CYC1* terminator, to test tryptophan-dependent transcriptional induction. However, the addition of more Aro80 binding sites to the *ARO80* promoter failed to induce transcriptional activation in the presence of tryptophan (Fig. 1b). On the contrary, when the same Aro80 binding sites were fused to the *ARO9* core promoter (C_{ARO9}), the transcription of EGFP was induced by tryptophan depending on the increasing number of Aro80 binding sites, without affecting the basal expression levels (Fig. 1b). The U_4C_{ARO9} promoter showed about 20-fold induction after the treatment of 200 μg/mL tryptophan for 1 h. These results suggest that tryptophan-inducible synthetic promoters with different induction folds can be constructed by controlling the number of CCG repeating units, but the core promoter region also plays an important role for the transcriptional activation by Aro80. It needs further studies to elucidate which features of the *ARO80* core promoter prevent the Aro80-dependent activation.

Next, we compared the effects of different aromatic amino acids on the induction of the U_4C_{ARO9} promoter by measuring

Table 2 Primers used in this study (restriction enzyme sites are underlined)

Primer	Sequence
F_U_2C_{ARO80}	CGCGAGCTCTTGCCGATGATAACCGAGATAAATG
F_U_3C_{ARO80}	CGCGAGCTCTTGCCGATACTATCCGATGATAACCGAGATAAATG
F_U_4C_{ARO80}	CGCGAGCTCTTGCCGATGCTTACCGATACTATCCGATGATAACCGAGATAAATG
R_C_{ARO80}	CGCGGATCCAGAGGATAAAGCAGTGCTTAATG
F_U_2C_{ARO9}	CGCGAGCTCTTGCCGATGATAACCGAACCATCATTGGGGTAGGAAAC
F_U_3C_{ARO9}	CGCGAGCTCTTGCCGATACTATCCGATGATAACCGAACCATCATTGGGGTAGGAAAC
F_U_4C_{ARO9}	CGCGAGCTCTTGCCGATGCTTACCGATACTATCCGATGATAACCGAACCATCATTGGGGTAGGAAAC
F_P_{ARO9}	CGCGAGCTCCATTGCCGATGCTTACCGAGATTTGCCGCG
R_C_{ARO9}	CGCGGATCCTGAGTCGATGAGAGAGTGTAATTGTGG
F_EGFP	GAGGGATCCATGTCTAAAGGTGAAGAATTATTCAC
R_EGFP	GAGGAATTCTTATTTGTACAATTCATCCATACCATG
F_*alsS*	CTGAGGATCCATGACAAAAGCAACAAAAGAAC
R_*alsS*	CTGACTCGAGCTAGAGAGCTTTCGTTTTCA
F_*alsD*	CTGAGGATCCATGAAACGAGAAAGCAACAT
R_*alsD*	CTGACTCGAGTTATTCAGGGCTTCCTTCAG
F_P_{UGA4}	CGCGAGCTCCTAGGCATCTTACTAAGGTAC
R_P_{UGA4}	CGCGGATCCTGTTAGTAATAATAAATTATAAG
qF_EGFP	GTTCTGTTCAATTAGCTGAC
qR_EGFP	TTATTTGTACAATTCATC
qF_*ENO1*	CTATCGAAAAGAAGGCTGCC
qR_*ENO1*	CGTGGTGGAAGTTTTCACCAGC

EGFP fluorescence intensities. Although all three aromatic amino acids induced EGFP expression, tyrosine was less effective than tryptophan and phenylalanine (Fig. 2a). Moreover, because of its low solubility (<0.5 g/L), tyrosine might not be suitable as a practical inducer of the U_4C_{ARO9} promoter. The EGFP expression levels decreased after 13 h of induction, which might reflect the degradation or utilization of the inducers. Although tryptophan exerted a little lower induction fold (8.1-fold) than did phenylalanine (9.1-fold) up to 4 h, tryptophan served as a better inducer than phenylalanine after 13 h. Note that since we used 200 μg/mL amino acids, the molar concentration of phenylalanine (1.21 mM) is slightly higher than that of tryptophan (0.98 mM). In the case of tryptophan, its degradation product tryptophol is also known as an activator of Aro80, which might be in part responsible for the longer induction period in the presence of tryptophan. Because the U_4C_{ARO9} promoter sequence is very similar to that of the native *ARO9* promoter (P_{ARO9}) (Fig. 1a), we also investigated the inducibility of P_{ARO9} by aromatic amino acids. As expected, the aromatic amino-acid-dependent induction pattern of the P_{ARO9} promoter was comparable to that of the U_4C_{ARO9} promoter (Fig. 2b).

We also tested whether further addition of Aro80 binding sites to the native *ARO9* promoter can enhance the tryptophan-dependent induction levels. However, fusion of additional two or four half sites to the upstream of USA_{ARO9} rather reduced transcription levels in the presence of tryptophan (data not shown). Therefore, for Aro80-dependent activation, most efficient activation seems to be achieved by binding of two Aro80 dimers.

Regulation of tryptophan-induced expression levels by plasmid copy numbers and tryptophan concentrations

Next, we examined tryptophan-induced expression levels from the U_4C_{ARO9} promoter depending on various tryptophan concentrations and plasmid copy numbers. Cells harboring low copy number plasmid, p416[U_4C_{ARO9}]-EGFP, or high copy number plasmid, p426[U_4C_{ARO9}]-EGFP, were treated with 50–800 μg/mL tryptophan, and transcription and protein expression levels were determined by qRT-PCR and fluorescence detection, respectively. The EGFP mRNA levels increased gradually depending on tryptophan concentrations, resulting in up to 20- and 15-fold induction levels in low and high copy number plasmids, respectively, compared with each uninduced control (Fig. 3a). Because of the leaky basal expression from the U_4C_{ARO9} promoter, both uninduced and induced EGFP mRNA levels expressed from high copy number plasmid were about 10- to 15-fold higher than those expressed from low copy number plasmid. The activities of commonly used constitutive promoters, P_{ADH1} (in p416ADH-EGFP) and P_{TDH3} (in p416GPD-EGFP), were not affected by

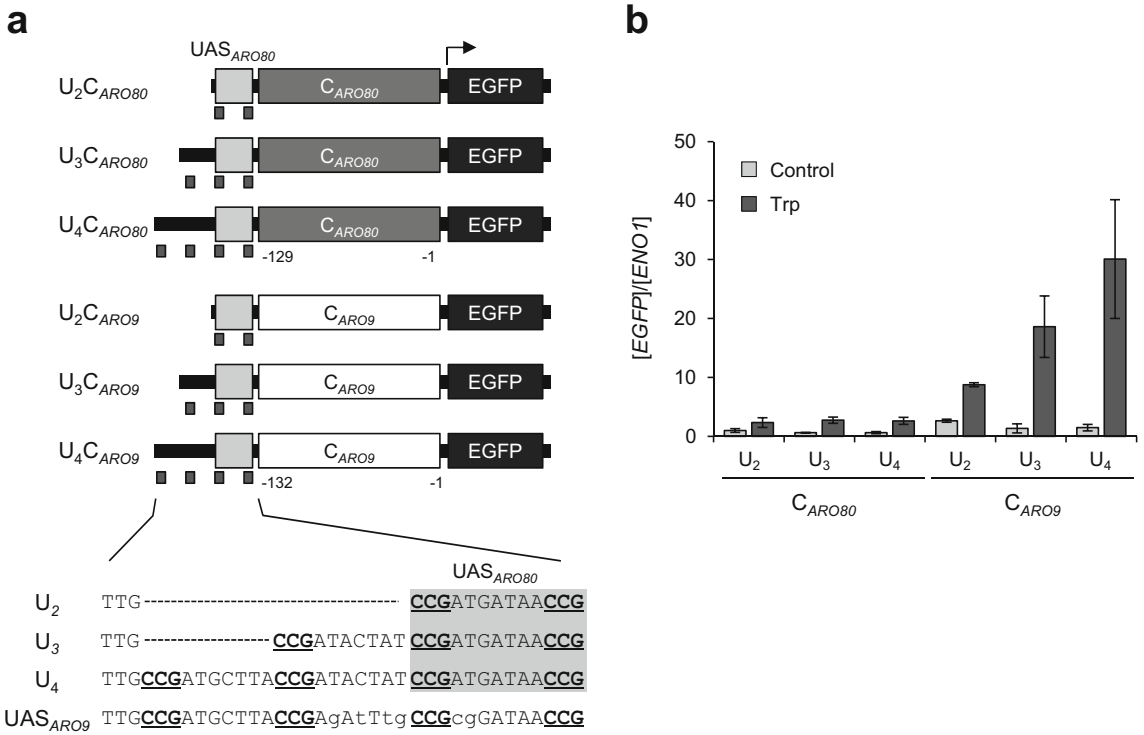

Fig. 1 Construction of promoters inducible by aromatic amino acids. **a** Schematic representation of the promoter constructs containing core promoter region and additional upstream activating sequences (*UAS*). The EGFP gene was used as a reporter. The sequence details of UAS elements are shown below. CCG triplets, the binding sites of Aro80, are *underlined*. UAS$_{ARO9}$ is upstream activating sequence of native *ARO9* promoter and the sequences different from the synthetic UAS are represented in *lowercase letters*. **b** Tryptophan-dependent induction of synthetic promoters. Cells harboring p416-based plasmid expressing EGFP from the indicated promoter elements were treated with 200 μg/mL tryptophan for 1 h, and the EGFP mRNA levels were determined by qRT-PCR normalized to *ENO1*. *Error bars* represent standard deviation from triplicates

tryptophan. The U$_4$C$_{ARO9}$-controlled tryptophan-induced expression levels were comparable to the P$_{ADH1}$-driven expression levels when expressed from low copy number plasmid.

The EGFP protein expression levels detected by fluorescence intensities reflected the transcription induction patterns (Fig. 3b). Cells harboring p416[U$_4$C$_{ARO9}$]-EGFP plasmid showed a gradual increase in fluorescence intensities as increasing tryptophan concentrations from 50 to 800 μg/mL with a maximum induction fold of 6 (Fig. 3b). Expression of [U$_4$C$_{ARO9}$]-EGFP from high copy number plasmid resulted in about 5-fold higher uninduced and induced expression levels, while keeping the tryptophan concentration-dependent induction profile (Fig. 3b). Using these two vectors and different tryptophan concentrations, a 29-fold range of expression

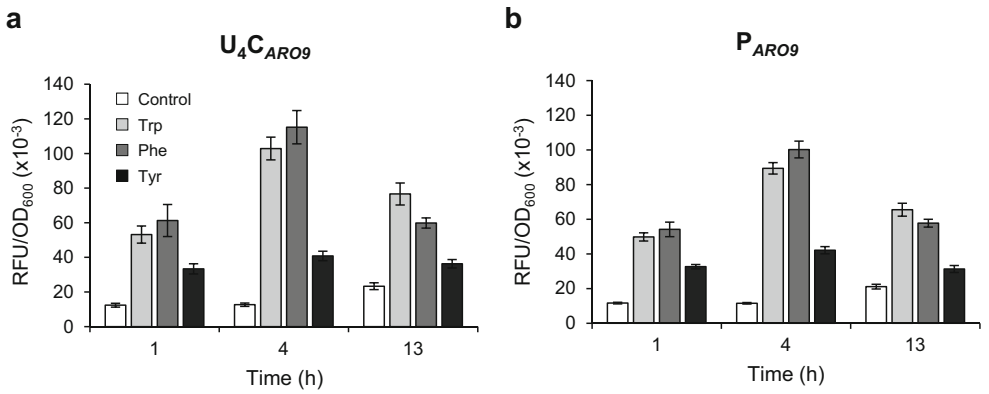

Fig. 2 Effect of different aromatic amino acids on EGFP expression levels from the U$_4$C$_{ARO9}$ promoter (**a**) and the native *ARO9* promoter (P$_{ARO9}$) (**b**). Cells harboring p416[U$_4$C$_{ARO9}$]-EGFP or p416[P$_{ARO9}$]-EGFP were treated with 200 μg/mL tryptophan (Trp), phenylalanine (Phe), or tyrosine (Tyr) for the indicated time period, and the fluorescence intensities (RFU) were detected and normalized to the cell densities (OD$_{600}$)

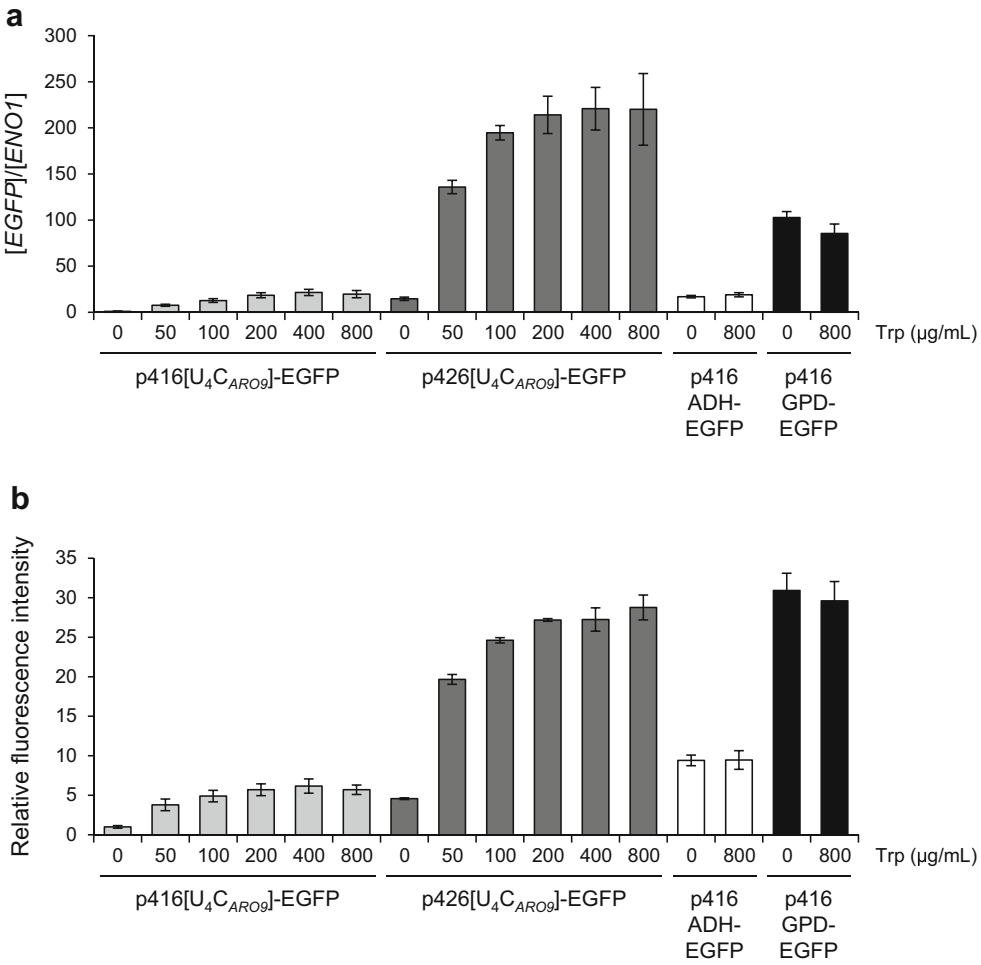

Fig. 3 Effect of plasmid copy numbers and tryptophan concentrations on EGFP expression levels from the U_4C_{ARO9} promoter. Cells harboring the indicated plasmids were treated with the indicated concentrations of tryptophan for 1 h, and transcription and protein levels of EGFP were investigated. The constitutive promoters, P_{ADH1} and P_{TDH3}, were used as controls. **a** EGFP mRNA levels were determined by qRT-PCR normalized to *ENO1*. *Error bars* represent standard deviation from triplicates. **b** The RFU/OD_{600} value was normalized to that of untreated cells harboring p416[U_4C_{ARO9}]-EGFP, and represented as relative fluorescence intensity

levels could be achieved from the U_4C_{ARO9} promoter. Taken together, a wide range of tryptophan-inducible promoter strengths can be obtained by modulating the number of Aro80 binding sites, plasmid copy numbers, and tryptophan concentrations, thereby enabling the fine tuning of transcription levels for metabolic engineering applications.

Acetoin production using the U_4C_{ARO9} promoter

To verify the effectiveness of the tryptophan-inducible promoters in metabolic engineering, we applied this system to the biosynthetic pathway of acetoin, a potential high-value platform chemical for a broad range of applications such as food, flavor, and pharmaceutical industries (Xiao and Lu 2014). For effective production of acetoin from pyruvate, a heterologous pathway consisting of acetolactate synthase (AlsS) and acetolactate decarboxylase (AlsD) from *B. subtilis* were introduced into *S. cerevisiae*. In addition, endogenous *BDH1* gene, encoding 2,3-butanediol dehydrogenase, was deleted to prevent the formation of 2,3-butanediol from acetoin (Fig. 4a).

The *alsS* and *alsD* genes were expressed under the control of U_4C_{ARO9}, and the required balance between AlsS and AlsD was simply tested by cloning the genes into both low copy number (p413 or p416) and high copy number (p423 or p426) plasmids, and examining acetoin production levels in cells harboring four different combinations of plasmid types (Fig. 4b). In the presence of 200 μg/mL tryptophan, cells expressing *alsS* from low copy number plasmid (p413) and *alsD* from high copy number plasmid (p426) produced 2.5 g/L acetoin after 48 h, the highest concentration among the four combinations. Expressing both *alsS* and *alsD* from high copy number plasmids did not give the best result, exemplifying the importance of regulating and balancing gene expression levels in pathway engineering. Because of the basal activity of the U_4C_{ARO9} promoter, lower levels of acetoin production were also observed even in the absence of tryptophan (Fig. 4b). The acetoin titers increased gradually as increasing tryptophan concentrations, faithfully reflecting the tryptophan concentration-dependent increase in U_4C_{ARO9} promoter activity (Fig. 4c). As a result, up to 3.4 g/L acetoin was produced in the presence of 800 μg/mL tryptophan. Although promoters stronger than the U_4C_{ARO9} promoter could be more effective in maximizing acetoin production levels, these results demonstrate the usefulness of the tryptophan-inducible promoter

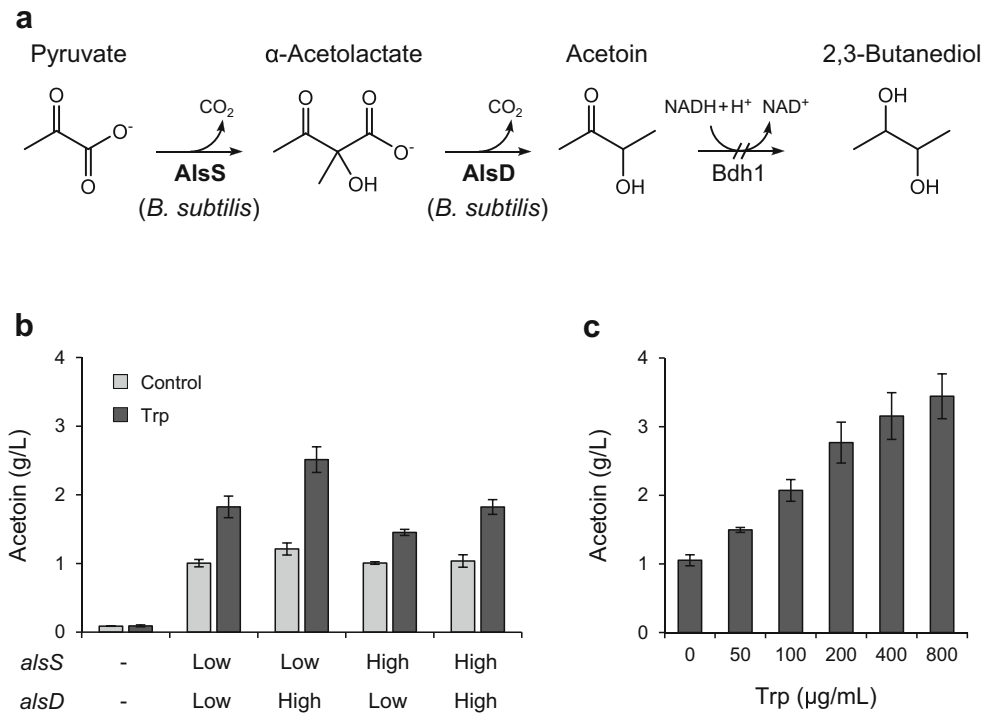

Fig. 4 Application of the U_4C_{ARO9} promoter to metabolic engineering for acetoin production. **a** Metabolic pathway for acetoin production. Two molecules of pyruvate are converted α-acetolactate by acetolactate synthase (AlsS), and then acetolactate decarboxylase (AlsD) converts α-acetolactate to acetoin. To block the 2,3-butanediol production from acetoin, *BDH1* gene encoding 2,3-butanediol dehydrogenase was deleted. **b** Cells carrying *alsS* expression vector, p413[U_4C_{ARO9}]-*alsS* (Low) or p423[U_4C_{ARO9}]-*alsS* (high), and *alsD* expression vector, p416[U_4C_{ARO9}]-*alsD* (low) or p426[U_4C_{ARO9}]-*alsD* (high), in four different combinations were grown for 48 h in the absence or presence of 200 μg/mL tryptophan, and acetoin production levels were monitored. **c** Acetoin production depending on tryptophan concentrations in cells harboring p413[U_4C_{ARO9}]-*alsS* and p426[U_4C_{ARO9}]-*alsD*

in modulating metabolic flux simply by changing the concentrations of tryptophan.

Application of the GABA-inducible *UGA4* promoter to metabolic engineering

Since we demonstrated that Aro80-dependent transcriptional regulation can be successfully used to design novel inducible promoters, we searched for other Zn_2Cys_6 family member of transcription factors that are regulated by inducers suitable for genetic engineering. Uga3 transcription factor is involved in the utilization of GABA as a nitrogen source by activating transcription of *UGA1*, *UGA2*, and *UGA4* genes in response to GABA (Andre 1990; Cardillo et al. 2011; Idicula et al. 2002; Talibi et al. 1995). In the *UGA4* promoter, the region from −404 to −386 was identified as UAS_{GABA} (Idicula et al. 2002; Talibi et al. 1995), where CGG half sites are aligned in everted orientation ($CCGN_4CGG$) (Fig. 5a). It has been known that Uga3-dependent activation of UAS_{GABA} requires Uga35, another Zn_2Cys_6 protein with a pleiotropic function (Garcia et al. 2000).

We investigated whether the GABA-inducible *UGA4* promoter can be applied to metabolic engineering for acetoin production. The *alsS* and *alsD* genes were expressed under the control of *UGA4* promoter (−460 to −1) from low and high copy number plasmids, respectively. Cells harboring the two plasmids produced 0.1 g/L acetoin in the absence of GABA (Fig. 5b), which is about 10-fold lower than that produced in cells expressing *alsS* and *alsD* from the U_4C_{ARO9} promoter (Fig. 4c). Therefore, the *UGA4* promoter might have a lower basal activity than that of U_4C_{ARO9} under our experimental conditions. However, acetoin production increased in correlation to GABA concentrations, resulting up to 5-fold increase in acetoin titer in the presence of 800 μg/mL GABA. These results demonstrate that GABA can be used as a dose-dependent modulator of the *UGA4* promoter activity in metabolic engineering.

Discussion

Promoters, the key determinants of transcriptional initiation, are essential components for controlling gene expression in metabolic engineering and synthetic biology (Blazeck and Alper 2013; Nevoigt et al. 2007). In this study, we demonstrated that promoters regulated by Aro80 transcription factor can be used as tryptophan-inducible promoters for pathway

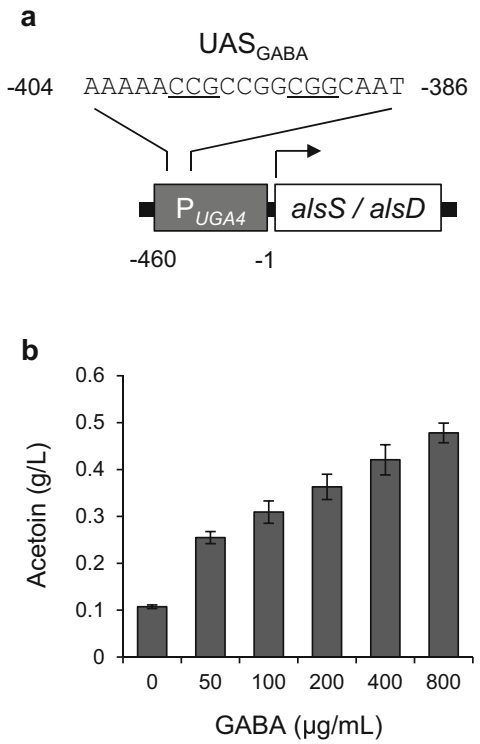

Fig. 5 Application of GABA-inducible *UGA4* promoter for acetoin production. **a** Construction of GABA-inducible system for acetoin production. **b** Cells harboring p413[P_{UGA4}]-*alsS* and p426[P_{UGA4}]-*alsD* were tested for acetoin production depending on GABA concentrations

engineering in *S. cerevisiae*. The tryptophan-induced expression levels can be modulated by changing the number of Aro80 binding sites, plasmid copy numbers, and the concentrations of inducer, providing a dynamic range of promoter strengths available for fine-tuning gene expression levels for pathway optimization. Furthermore, we showed that GABA-inducible *UGA4* promoter, regulated by Uga3, can also be used in metabolic engineering.

The tryptophan- or GABA-inducible promoters have advantages in that their promoter strengths can be easily modulated by adding different concentrations of inducers directly into the culture medium. Therefore, these promoters might be useful in regulating gene expression levels at specific time points during the growth. Among the inducible promoters available in *S. cerevisiae*, the *CUP1* promoter can also be regulated by Cu^{2+} concentration-dependent manner, but the toxicity of Cu^{2+} can be a problem when using high concentrations of Cu^{2+} (Hottiger et al. 1995). In the case of *GAL* promoters, complete medium exchange is necessary to prevent glucose repression effect (Johnston 1987; Lohr et al. 1995). In addition, because galactose is used as a carbon source, it is not convenient to modulate galactose concentrations as a way to regulate expression levels. Other regulated promoters such as P_{ADH2}, P_{PHO5}, and P_{MET25} are repressed in the presence of glucose (Price et al. 1990), inorganic phosphate (Rudolph and Hinnen 1987), and methionine (Mumberg et al. 1994), respectively. Therefore, these promoters are useful for inducing gene expression when such nutrients or metabolite are depleted during the cultivation, but not appropriate for dose-dependent regulation by the regulating chemicals. As dose-dependent inducible systems, synthetic transcription factors, constructed by fusing DNA binding domains with transcription activating domains, have been developed, which are regulated by tetracycline analog doxycycline or hormones such as β-estradiol (Belli et al. 1998; Liang et al. 2013; McIsaac et al. 2013). The tryptophan-induced expression levels form the U_4C_{ARO9} promoter was within a similar range of the P_{ADH1}-deriven expression levels. Since P_{ADH1} promoter is weaker than other widely used strong promoters such as P_{TDH3} and P_{TEF1} (Mumberg et al. 1995; Sun et al. 2012), the tryptophan-inducible promoters might be suitable for genes requiring low- to intermediate-level expression in the metabolic pathway. The activity of tryptophan- or GABA-inducible promoters could be further enhanced by various promoter engineering strategies, which include combining the UAS with different core promoters and terminators (Blazeck et al. 2012; Curran et al. 2013), or introducing poly(dA:dT) tracks that disfavor nucleosome assembly (Raveh-Sadka et al. 2012).

Taken together, we newly introduced tryptophan- and GABA-inducible promoters as useful tools for metabolic engineering in *S. cerevisiae*. The wide range of controllable expression levels of these promoter systems might contribute to fine-tuning gene expression levels and timing for pathway optimization.

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