ACS SyntheticBiology

Letter

pubs.acs.org/synthbio

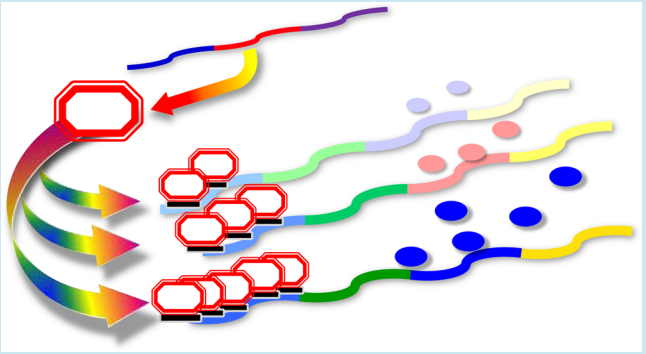
A Highly Tunable System for the Simultaneous Expression of Multiple Enzymes in *Saccharomyces cerevisiae*

Yoichiro Ito,*[,†,§] Mamoru Yamanishi,[†] Akinori Ikeuchi,[‡] and Takashi Matsuyama*[,†]

[†]Matsuyama Research Group and [‡]Biotechnology Laboratory, Toyota Central Research and Development Laboratories, Inc., 41-1 Yokomichi, Nagakute, Aichi 480-1192, Japan

Supporting Information

ABSTRACT: Control of the expression levels of multiple enzymes in transgenic yeasts is essential for the effective production of complex molecules through fermentation. Here, we propose a tunable strategy for the control of expression levels based on the design of terminator regions and other gene-expression control elements in *Saccharomyces cerevisiae*. Our genome-integrated system, which is capable of producing high expression levels over a wide dynamic range, will broadly enable metabolic engineering and synthetic biology. We demonstrated that the activities of multiple cellulases and the production of ethanol were doubled in a transgenic yeast constructed with our system compared with those achieved with a standard expression system.



With the view of realizing a truly sustainable society based not on petroleum but on renewable biomass resources, attention is increasingly being paid to bioprocesses that can be used to inexpensively produce valuable pharmaceutical and industrial products.[1−3] Recent technological developments have facilitated the creation of complex bioprocesses comprising a series of enzyme-mediated reactions that have been used to efficiently produce products, such as drug precursors, that are difficult to synthesize chemically.[4−6] When designing a complex bioprocess, the greatest challenge is to enhance and optimize the expression levels of the individual enzymes within the metabolic pathway. However, while the majority of efforts have focused on the development of regulatory tools in bacteria, fewer studies have focused on the development of synthetic regulatory tools in eukaryotic organisms such as *Saccharomyces cerevisiae*.[7] The tools currently available for modulating enzyme expression in *S. cerevisiae* include developing new promoters[8,9] and controlling mRNA stability via synthetic terminator regions.[10] Although these tools are useful, further improvements in expression level and dynamic range are needed. In a recent comprehensive analysis, we found that yeast terminator region design strongly influenced enzyme expression,[11] thereby opening the possibility of a novel strategy for the broad control of expression levels.[11−13] Combination of these yeast terminator and promoter technologies may enable the broad regulation of protein expression in yeast cells. Here, we propose this new approach that allows control of the expression levels of multiple enzymes over a broad dynamic range in yeast cells.

Our tunable protein-expression system used one construct expressing an artificial transcriptional activator and expression cassettes containing the target transgenes (Figure 1a). The target transgenes were all regulated by the same transcriptional activator. The reconstructed transcriptional activator (LNV1) used in this study was composed of three well-characterized components: the DNA binding site from the lexA protein,[14] a nuclear transfer signal,[15] and the transcriptional activation domain VP16[16] (Supporting Information Figure 1). Since not all of the components of LNV1 were derived from yeasts, transgene expression was expected to be independent from endogenous protein expression. The expression of the *LNV1* gene was controlled under the hybrid promoter and the *CYC1* terminator (Supporting Information Figure 2). In a transgenic yeast strain harboring both the LNV1-expressing construct and an expression cassette containing five LNV1-binding sites, fluorescence intensity was observed to be roughly stable under various carbon source and growth conditions (Supporting Information Figure 3). These empirical results show that the target transgenes were stably expressed; however, optimization of the promoter and terminator of the LNV1-expressing construct could further improve the control of transgene expression.

Transgene expression level is dependent on the number of transcription activator binding sites,[17] the type of core promoter (CP),[18] and the terminator region[11] used in the expression cassette. The expression of green fluorescent protein (GFP) was used as an index of intracellular protein expression. Fluorescence intensity was measured by means of flow cytometry (Figure 1b). The highest fluorescence intensity was observed in the L5TR strain and was approximately 8-fold

Special Issue: SEED 2014

Received: March 3, 2014
Published: May 1, 2014

DOI: 10.1021/sb500096y
ACS Synth. Biol. 2015, 4, 12−16

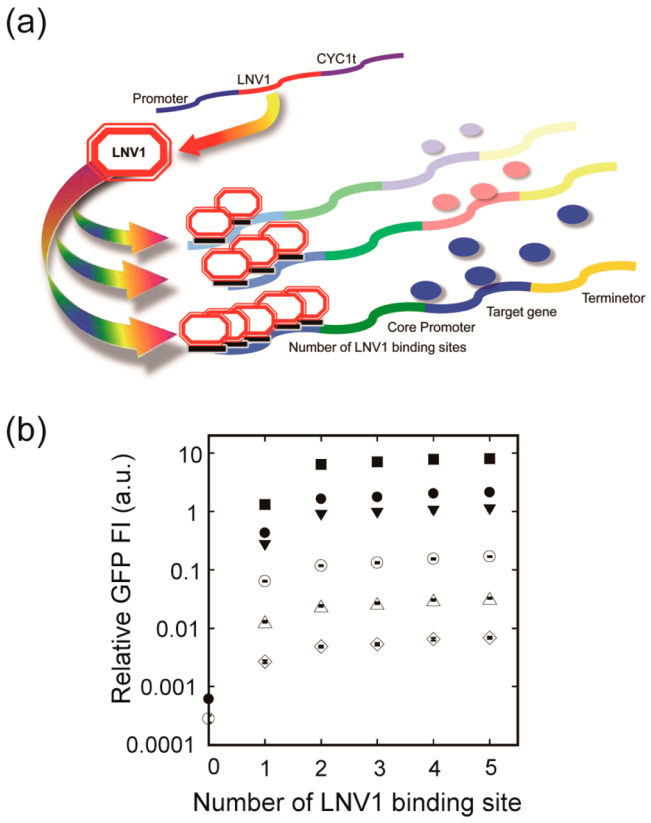


Figure 1. Overview of the genome-integrated multiple protein-expression system. (a) LNV1 expression construct (upper) and target transgene expression cassette (lower). Protein expression is regulated by three factors: the number of LNV1 binding sites, the type of core promoter, and the type of terminator region. The expression cassettes used in this study were named LxAB, where x is the number of LNV1 binding sites, A is the type of core promoter ("T" or "C" indicating CP_{TDH3} or CP_{CYC1}, respectively), and B is the initial letter of the terminator region. (b) Expression of GFP with a library of the expression cassettes. Shaded and empty symbols indicate strains carrying the *TDH3* or *CYC1* core promoter, respectively. Squares, circles, inverted triangles, triangles, and diamonds indicate strains carrying *RPL41Bt* (highest activity),[11] *CYC1t*, *MFA2t*, *STD1t*, and *GIC1t* (lowest activity),[11] respectively. Fluorescence intensity (FI) was normalized to that of the standard construct (*TDH3pro+CYC1t*).

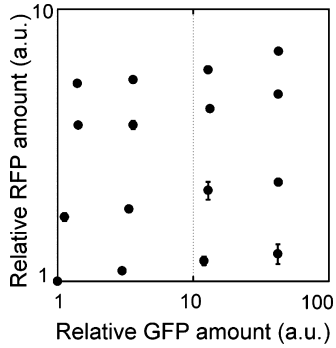


Figure 2. Simultaneous expression of GFP and RFP. Combinations of the expression cassettes L5TC, L1TC, L5CC, and L1CC were used for the dual expression of GFP and RFP (Supporting Information Figure 4). Fluorescence intensity was used to calculate protein amount, and each protein amount was normalized to that of a transformant carrying two L1CC constructs.

greater than that observed in the standard *TDH3pro+CYC1t* strain (Figure 1b). One of the most effective promoters in *S. cerevisiae* so far produced by promoter engineering is a *TEF1pro* variant.[9] The promoter activity of this variant is approximately 2.3-fold greater than that of wild-type *TEF1pro*, which has the same activity as *TDH3pro*.[19] In contrast, the strain containing expression cassette L0CC, which contained the lowest performing components, had a fluorescence intensity that was approximately 0.03% that of the standard strain (Figure 1b). Therefore, the dynamic range of our system, measured as the difference in fluorescence intensity of transformants containing L5TR or L0CC, was approximately 30 000.

When tested with GFP and RFP the different expression levels all worked as expected (Figure 2 and Supporting Information Figure 4) and allow for two or more different proteins to be coexpressed in a cell at a variety of levels, regardless of the site of integration (Supporting Information Figure 5).

To confirm the levels of enzymes produced in our expression system, we examined the expression of three secretory cellulases: cellobiohydrolase 1 (CBH1),[20] a thermostable mutant of cellobiohydrolase 2 (CBH2),[21] and endoglucanase 2 (EG2).[22] Secretory signal peptides[23] were added to the 5′ end of each cellulase gene (Supporting Information Figure 6a). Transformants containing the same cellulase genes inserted into a construct containing *TDH3pro+CYC1t* were used as standards. The levels of secreted cellulase of the L5TR strains ranged from 1.5- to 4-fold greater than those of the standard construct strains (Figure 3a–c). When the cellulase activities of aliquots of the culture supernatants were assessed by using phosphoric acid–swollen cellulose (PASC) (Figure 3d–f), the cellulase activities of the L5TR transformant supernatants ranged from 1.5- to 2.5-fold greater than those of the standard construct supernatants. For each cellulase, the activity of the transformant increased with increasing performance of the expression cassette it contained, which was comparable to the results of the characterization by using GFP (Figure 1b); the only exception was for expression cassette L1CC containing the gene encoding CBH1 (Figure 3a). We are currently unable to explain this exception but the results were reproducible. Together, these data indicated that by using our expression system we were able to control enzyme production in yeast cells.

With the crystalline cellulose Avicel as the substrate instead of PASC, the cellulase activity of the yeasts simultaneously secreting cellulases was measured (Figure 3g). The activities of the secreted cellulases were observed to additively increase for both the L5TR and standard constructs (Figure 3g). In all transformants, the L5TR construct resulted in higher cellulase activity than the standard construct, and the cellulase activity of the strain with all three L5TR cellulase constructs (HR strain) was approximately 1.7-fold greater than that of the standard (SW strain) (Figure 3g and Supporting Information Figure 7a).

Furthermore, by using these strains, we demonstrated that Avicel was converted to soluble sugars by the secreted cellulases and β-glucosidase, which was introduced externally, and then to ethanol by the innate glycolysis enzymes produced by the yeast strain. The amount of ethanol produced by the HR strain was approximately 1.4-fold greater than that produced by the SW strain (Supporting Information Figure 7b). Together, these results show that our system can be used to produce yeast cells

DOI: 10.1021/sb500096y
ACS Synth. Biol. 2015, 4, 12–16

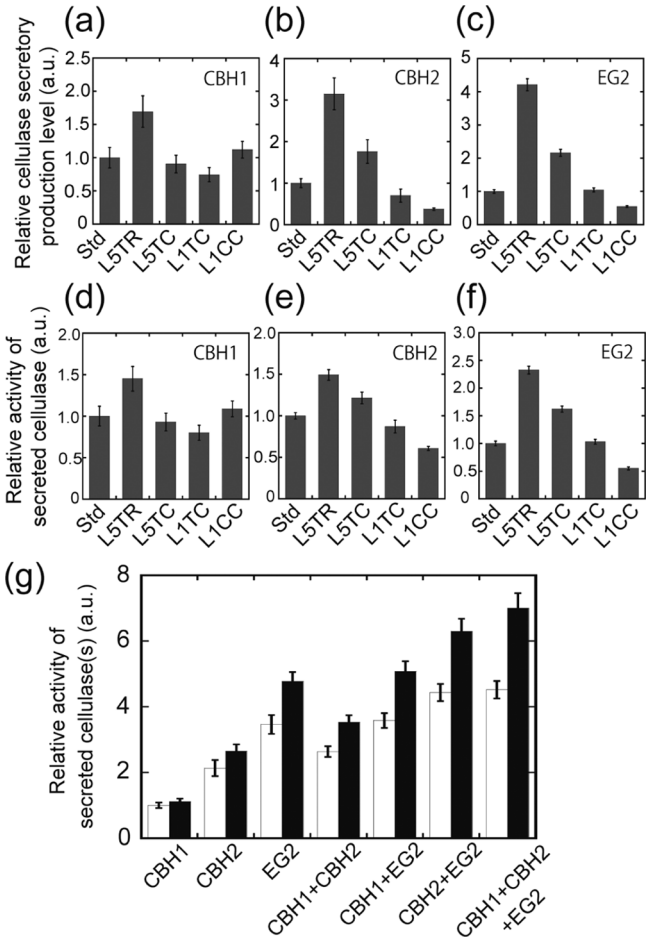


Figure 3. Activity of cellulases secreted from transgenic yeasts. Production of the secretory cellulases cellobiohydrolase 1 (CBH1) (a), CBH2 (b), and endoglucanase 2 (EG2) (c). Activity of cellulases secreted by transformants carrying the indicated constructs for the expression of CBH1 (d), CBH2 (e), and EG2 (f). Cellulase activities were measured by using phosphoric acid−swollen cellulose (PASC) as the substrate. Cellulase production levels were calculated from cellulase activity (see Supporting Information Figure 6) and normalized to the cellulase production level of the standard strain (Std). (g) Cellulase activities on crystalline cellulose (Avicel) of supernatants containing the indicated secreted cellulases. White bar, transformants carrying the standard construct (*TDH3pro+CYC1t*); black bar, transformants carrying the L5TR construct.

simultaneously expressing one or more cellulases and that this system can be applied to the production of ethanol.

Our tunable system utilizes modifiable elements that can be selected to specifically control the expression levels of multiple enzymes. In our recent study, the activity of the *DIT1* terminator region (*DIT1t*) showed the greatest activity in the EG2 secretory expression.[24] By replacing the *RPL41Bt* terminator element of the HR strain with *DIT1t*, we constructed a strain (HD strain) that was expected to produce more ethanol than the HR strain. The protease-deficient strain BJ5465, which is generally suitable for the production of secreted products,[25] was used as the host. A BJ5465 strain carrying standard constructs for the three cellulases was also created (SB strain). By using these strains, we again investigated ethanol fermentation (Figure 4). The HD strain showed greater ethanol production than the HR strain, which was double that of the SB strain. Ethanol production over 4 g/L was achieved at

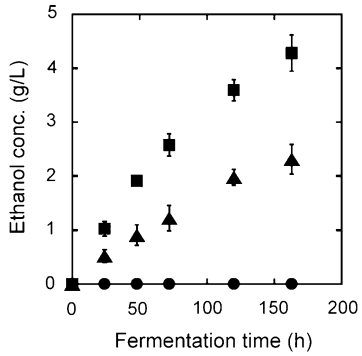


Figure 4. Ethanol fermentation of transgenic yeasts simultaneously secreting three cellulases. Ethanol fermentation of the HD strain (squares), SB strain (triangles), and reference strain (circles) with Avicel cellulose as the substrate.

168 h of fermentation, which corresponded to approximately 40% of the theoretical maximum yield.

In the present study, we constructed a packaged system that allowed the fine-tuning of the expression level of intracellular and secreted proteins. The expression cassettes with various performance levels produce simultaneously extensive range of multiple gene expressions as appropriate. The combination of gene expression control elements in our system allowed tuning of protein-expression levels for multiple transgenes that will broadly enable metabolic engineering and synthetic biology.[26]

METHODS

Host Strain and Media. *S. cerevisiae* strain W303-1a (*MAT*a *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) and BJ5465 (*MAT*a *ura3-52 trp1 leu2D1 his3D200 pep4::HIS3 prb1D1.6R can1 GAL*) were used as the host strain for genetic manipulations. Yeast transformants were cultured in synthetic complete medium containing 0.67% Yeast Nitrogen Base without amino acids (Difco, Detroit, MI); 0.082% single, double, or triple drop-out Complete Supplement Mixture (without leucine, tryptophan, and/or uracil; ForMedium, Norfolk, U.K.); adenine (40 mg/L); and 2% glucose. Cellulase-expressing transformants were cultured in synthetic complete medium supplemented with 1% (w/v) casamino acids.

Modular Construction of Plasmids. DNA fragments (see Supporting Information Table 1 for nucleotide sequences) were designed encoding artificial transcription factor LNV1, operator sequences with 1−5 LNV1 binding sites, core promoters derived from *TDH3pro* or *CYC1pro*, secretory signal peptides from *Rhizopus oryzae glycoamylase*,[23] and the codon-optimized cellulases EG2, which is derived from *Trichoderma reesei* (GenBank AAA34213),[22] and CBH1, which is derived from *Talaromyces emersonii* (GenBank AAL89553).[20] All synthetic DNA fragments were purchased from Genscript, Inc. (Piscataway, NJ).

Genes expressing codon-optimized GFP (opGFP),[27] mKO2 (Amalgaam, Tokyo, Japan), and a thermostable mutant of CBH2 derived from *Phanerochaete chrysosporium* (Mall4)[21] were amplified by using PCR. PCR primers were purchased from Operon Biotechnologies (Tokyo, Japan).

All genetic constructs were cloned into the pSP73 vector (Promega, Madison, WI) by means of standard restricted enzyme digestion and ligation techniques or by using an In-Fusion Advantage PCR cloning kit (Clontech, Mountain View,

DOI: 10.1021/sb500096y
ACS Synth. Biol. 2015, 4, 12−16

CA). The standard construct (*TDH3pro+CYC1t*) and representative expression cassettes are shown in Supporting Information Figure 8.

Yeast Transformation. The LNV1 expression construct was integrated into the yeast genome at the *PDC5* locus. Cassettes for the fluorescent proteins or cellulases were inserted into the locus on the yeast genome by means of recombination with homology at the 5′ and 3′ ends and single-crossover integration, respectively.[28] The seven loci (*PDC5*, *PDC6*, upstream of *HXT3*, *GPD1*, *TRP1*, *URA3*, and *LEU2*) into which the expression constructs were integrated do not severely affect phenotype when deleted. Linear DNA fragments were digested by using the appropriate restriction enzymes and transformants were created by using a Frozen-EZ Yeast Transformation II kit (Zymo Research, Irvine, CA). Transformants were grown on suitable selection media, and integration of the DNA fragment was confirmed by means of colony PCR and/or quantitative-PCR. Genotypes of the yeast strains used in this study are summarized in Supporting Information Table S2.

Flow Cytometry and Data Processing. Flow cytometric analysis and data processing were conducted as described previously with slight modification.[27,29] Briefly, transformants expressing GFP or mKO2 were grown in SD medium (i.e., synthetic complete medium containing 2% glucose) at 30 °C in a test tube shaken at 70 rpm. Overnight cultures were diluted with 5 mL of fresh SD medium in an L-shaped tube to an OD_{660} of approximately 0.1. The diluted cells were then cultured at 30 °C with the use of a rocking incubator (TN-1506; ADVANTEC, Tokyo, Japan) for about 6 h until an OD_{660} of approximately 0.7 was reached. Fluorescent intensities of approximately 10 000 transformants expressing GFP or mKO2 were measured by using a Cell Lab Quanta MPL flow cytometer (Beckman-Coulter, Brea, CA) equipped with a 488 nm laser and the appropriate filter sets for GFP (510/10) or mKO2 (570/15). The data were analyzed by using IGOR Pro software version 6.1 (Wavemetrics, Inc., OR). Briefly, the peak value of histograms of fluorescence intensity/side-scatter intensity (SS) fitted to a log-normal distribution was used as the fluorescence intensity of each transformant.

Secreted Cellulase Activity. Three secretory cellulases were used in this study: cellobiohydrolase 1 (CBH1; derived from *Talaromyces emersonii*),[20] a thermostable mutant of cellobiohydrolase 2 derived from *Phanerochaete chrysosporium* (CBH2),[21] and endoglucanase 2 (EG2; derived from *Trichoderma reesei*).[22] Cellulase activity was measured as described previously with slight medication.[21] Briefly, colonies of cellulase-expressing transformants were transferred into 500 μL of the appropriate SD medium (without leucine, tryptophan, and/or uracil) supplemented with 1% casamino acids in a deep 96-well plate and grown at 30 °C with shaking at 1800 rpm for 24 h in a specialized shaker for deep-well plates (MBR-022UP; Taitec, Aichi, Japan). Next, a 100-μL aliquot of each culture was inoculated into 500 μL of SD medium supplemented with 1% casamino acids and the appropriate Complete Supplement Mixture and again grown at 30 °C with shaking at 1800 rpm for 24 h. After centrifugation at 3000 rpm for 10 min, the cellulase activity of 5-μL (10-μL for CBH1) aliquots of supernatant was determined by measuring the concentration of reducing sugar with 0.5% (w/v) PASC or 2% (w/v) Avicel cellulose as the substrate, as described previously.[21,24] Cellulase reactions were conducted at 50 °C for 1.5 h (EG2 or CBH2 with PASC as the substrate) or 4 h (CBH1 with PASC as the substrate; EG2, CBH1, or CBH2 with Avicel cellulose as the substrate). All measurements were performed in quadruplicate.

Fermentation of Avicel to Ethanol. Standard strains (SW and SB), HR and HD strains, and reference strains expressing no cellulase were cultured in 50 mL of yeast extract-peptone-dextrose (YPD) medium supplemented with 40 mg/L of adenine in baffled, 200 mL shake flasks incubated at 30 °C on a rotary shaker at 130 rpm for 3 days. Seventeen milliliters of each culture was then added to a 10 mL shake flask containing 0.38 g of Avicel PH-105 (Sigma-Aldrich, St Louis, MO), 1.9 mL of 0.5 M citrate buffer (pH 5.0), and 100 μL of β-glucosidase (Novozyme 188, Sigma-Aldrich) (final Avicel concentration, 20 g/L). The flask was sealed with a rubber bung and a check valve to maintain anaerobic conditions and stirred for 7 days. Samples were taken on days 0, 1, 2, 3, 5, and 7 and the glucose and ethanol content was analyzed with a high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) containing a Biorad HPX 87H column (Biorad, Hercules, CA). The column was eluted at 60 °C with 0.5 g/L H_2SO_4 at a flow rate of 0.8 mL/min. A refractive index detector (model RID-10A, Shimadzu) was used for detection.

■ ASSOCIATED CONTENT

Supporting Information

Figure 1. Amino acid sequence of the artificial transcriptional activator LNV1. Figure 2. Optimizing LNV1 expression. Figure 3. Fluorescent intensity of the L5TR strain under various conditions. Figure 4. Coexpression of GFP and RFP. Figure 5. GFP expression of transformants containing various constructs. Figure 6. Activity of secreted cellulases. Figure 7. Properties of the L5TR strains containing the three cellulases. Figure 8. Expression cassettes. Table 1. Nucleotide sequences of genes and module parts used in this study. Table 2. Strains used and constructed in this study. This material is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*Tel: +81-78-803-6633. Fax: +81-78-803-6192. Email: yito@dragon.kobe-u.ac.jp.

*Tel: +81-561-71-7424. Fax: +81-561-63-6498. Email: e1215@mosk.tytlabs.co.jp.

Present Address

[§]Technology Research Association of Highly Efficient Gene Design 7-1-48 Minatojimaminami, Chuo-ku, Kobe city, Hyogo, Japan, 650-0047.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Takao Tani for his helpful discussions and Sayo Takei for her help in preparing the artwork for Figure 1a. We also thank Mami Kanemitsu, Mieko Horikiri, Takako Nakamura, Sayo Takei, and Risa Nagura for their technical assistance.

■ ABBREVIATIONS

CP: core promoter; GFP: green fluorescent protein; mKO2: monomeric Kusabira-Orange 2; CBH1: cellobiohydrolase 1; CBH2: cellobiohydrolase 2; EG2: endoglucanase 2; PASC: phosphoric acid−swollen cellulose

DOI: 10.1021/sb500096y
ACS Synth. Biol. 2015, 4, 12−16

REFERENCES

(1) Peralta-Yahya, P. P., Zhang, F. Z., del Cardayre, S. B., and Keasling, J. D. (2012) Microbial engineering for the production of advanced biofuels. *Nature 488*, 320−328.

(2) Kung, Y., Runguphan, W., and Keasling, J. D. (2012) From fields to fuels: Recent advances in the microbial production of biofuels. *ACS Synth. Biol. 1*, 498−513.

(3) Siddiqui, M. S., Thodey, K., Trenchard, I., and Smolke, C. D. (2012) Advancing secondary metabolite biosynthesis in yeast with synthetic biology tools. *FEMS Yeast Res. 12*, 144−170.

(4) Ajikumar, P. K., Xiao, W. H., Tyo, K. E., Wang, Y., Simeon, F., Leonard, E., Mucha, O., Phon, T. H., Pfeifer, B., and Stephanopoulos, G. (2010) Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. *Science 330*, 70−74.

(5) Ro, D. K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C. Y., Withers, S. T., Shiba, Y., Sarpong, R., and Keasling, J. D. (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature 440*, 940−943.

(6) Hawkins, K. M., and Smolke, C. D. (2008) Production of benzylisoquinoline alkaloids in *Saccharomyces cerevisiae*. *Nat. Chem. Biol. 4*, 564−573.

(7) Blount, B. A., Weenink, T., and Ellis, T. (2012) Construction of synthetic regulatory networks in yeast. *FEBS Lett. 586*, 2112−2121.

(8) Blazeck, J., and Alper, H. S. (2013) Promoter engineering: Recent advances in controlling transcription at the most fundamental level. *Biotechnol. J. 8*, 46−58.

(9) Alper, H., Fischer, C., Nevoigt, E., and Stephanopoulos, G. (2005) Tuning genetic control through promoter engineering. *Proc. Natl. Acad. Sci. U.S.A. 102*, 12678−12683.

(10) Babiskin, A. H., and Smolke, C. D. (2011) A synthetic library of RNA control modules for predictable tuning of gene expression in yeast. *Mol. Syst. Biol. 7*, 1−15.

(11) Yamanishi, M., Ito, Y., Kintaka, R., Imamura, C., Katahira, S., Ikeuchi, A., Moriya, H., and Matsuyama, T. (2013) A genome-wide activity assessment of terminator regions in *Saccharomyces cerevisiae* provides a "Terminatome" Toolbox. *ACS Synth. Biol. 2*, 337−347.

(12) Curran, K. A., Karim, A. S., Gupta, A., and Alper, H. S. (2013) Use of expression-enhancing terminators in *Saccharomyces cerevisiae* to increase mRNA half-life and improve gene expression control for metabolic engineering applications. *Metab. Eng. 19C*, 88−97.

(13) Yamanishi, M., Katahira, S., and Matsuyama, T. (2011) *TPS1* terminator increases mRNA and protein yield in a *Saccharomyces cerevisiae* expression system. *Biosci. Biotechnol. Biochem. 75*, 2234−2236.

(14) Brent, R., and Ptashne, M. (1985) A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell. 43*, 729−736.

(15) Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984) A short amino-acid sequence able to specify nuclear location. *Cell. 39*, 499−509.

(16) Triezenberg, S. J., Kingsbury, R. C., and Mcknight, S. L. (1988) Functional dissection of Vp16, the trans-activator of herpes-simplex virus immediate early gene-expression. *Genes Dev. 2*, 718−729.

(17) Khalil, A. S., Lu, T. K., Bashor, C. J., Ramirez, C. L., Pyenson, N. C., Joung, J. K., and Collins, J. J. (2012) A synthetic biology framework for programming eukaryotic transcription functions. *Cell 150*, 647−658.

(18) Vogl, T., Ruth, C., Pitzer, J., Kickenweiz, T., and Glieder, A. (2014) Synthetic core promoters for *Pichia pastoris*. *ACS Synth. Biol. 3*, 188−191.

(19) Partow, S., Siewers, V., Bjørn, S., Nielsen, J., and Maury, J. (2010) Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*. *Yeast. 27*, 955−964.

(20) Ilmen, M., den Haan, R., Brevnova, E., McBride, J., Wiswall, E., Froehlich, A., Koivula, A., Voutilainen, S. P., Siika-Aho, M., la Grange, D. C., Thorngren, N., Ahlgren, S., Mellon, M., Deleault, K., Rajgarhia, V., van Zyl, W. H., and Penttila, M. (2011) High level secretion of cellobiohydrolases by *Saccharomyces cerevisiae*. *Biotechnol. Biofuels 4*, 30.

(21) Ito, Y., Ikeuchi, A., and Imamura, C. (2013) Advanced evolutionary molecular engineering to produce thermostable cellulase by using a small but efficient library. *Protein Eng. Des. Sel. 26*, 73−79.

(22) Saloheimo, M., Lehtovaara, P., Penttila, M., Teeri, T. T., Stahlberg, J., Johansson, G., Pettersson, G., Claeyssens, M., Tomme, P., and Knowles, J. K. C. (1988) EGIII, a new endoglucanase from *Trichoderma reesei*: The characterization of both gene and enzyme. *Gene 63*, 11−21.

(23) Yanase, S., Yamada, R., Kaneko, S., Noda, H., Hasunuma, T., Tanaka, T., Ogino, C., Fukuda, H., and Kondo, A. (2010) Ethanol production from cellulosic materials using cellulase-expressing yeast. *Biotechnol. J. 5*, 449−455.

(24) Ito, Y., Yamanishi, M., Ikeuchi, A., Imamura, C., Tokuhiro, K., Kitagawa, T., and Matsuyama, T. (2013) Characterization of five terminator regions that increase the protein yield of a transgene in *Saccharomyces cerevisiae*. *J. Biotechnol. 168*, 486−492.

(25) Morawski, B., Lin, Z., Cirino, P., Joo, H., Bandara, G., and Arnold, F. H. (2000) Functional expression of horseradish peroxidase in *Saccharomyces cerevisiae* and *Pichia pastoris*. *Protein engineering 13*, 377−384.

(26) Cobb, R. E., Si, T., and Zhao, H. (2012) Directed evolution: An evolving and enabling synthetic biology tool. *Curr. Opin. Chem. Biol. 16*, 285−291.

(27) Yamanishi, M., and Matsuyama, T. (2012) A modified Cre-*lox* genetic switch to dynamically control metabolic flow in transgenic *Saccharomyces cerevisiae*. *ACS Synth. Biol. 1*, 172−180.

(28) Da Silva, N. A., and Srikrishnan, S. (2012) Introduction and expression of genes for metabolic engineering applications in *Saccharomyces cerevisiae*. *FEMS Yeast Res. 12*, 197−214.

(29) Matsuyama, T., Yamanishi, M., and Takahashi, H. (2011) Improvement of galactose induction system in *Saccharomyces cerevisiae*. *J. Biosci. Bioeng. 111*, 175−177.

DOI: 10.1021/sb500096y
ACS Synth. Biol. 2015, 4, 12−16