

Shuffling of Promoters for Multiple Genes To Optimize Xylose Fermentation in an Engineered *Saccharomyces cerevisiae* Strain^{∇†}

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We describe here a useful metabolic engineering tool, multiple-gene-promoter shuffling (MGPS), to optimize expression levels for multiple genes. This method approaches an optimized gene overexpression level by fusing promoters of various strengths to genes of interest for a particular pathway. Selection of these promoters is based on the expression levels of the native genes under the same physiological conditions intended for the application. MGPS was implemented in a yeast xylose fermentation mixture by shuffling the promoters for *GND2* and *HXK2* with the genes for transaldolase (*TALI*), transketolase (*TKL1*), and pyruvate kinase (*PYK1*) in the *Saccharomyces cerevisiae* strain FPL-YSX3. This host strain has integrated xylose-metabolizing genes, including xylose reductase, xylitol dehydrogenase, and xylulose kinase. The optimal expression levels for *TALI*, *TKL1*, and *PYK1* were identified by analysis of volumetric ethanol production by transformed cells. We found the optimal combination for ethanol production to be *GND2-TALI-HXK2-TKL1-HXK2-PYK1*. The MGPS method could easily be adapted for other eukaryotic and prokaryotic organisms to optimize expression of genes for industrial fermentation.

Strain improvement strategies often involve engineering a de novo pathway or eliminating rate-limiting steps in a microorganism by overexpressing rate-limiting genes or by deleting genes that draw metabolites away from the desired product. These changes greatly modify enzymatic activity. In the present study, we concentrated on fine-tuning the expression levels of multiple genes that interact to enable metabolite flux.

Manipulation of promoter strength is extensively used to tune transcript levels (1, 14, 24), but changes in gene expression levels do not always result in corresponding changes in enzymatic activities, due to translational, posttranslational, feedback, and other regulatory mechanisms. Moreover, changes in the expression level of one gene can affect the optimal expression levels of other genes. Because most biological processes are regulated at the level of transcription (33), modification of gene expression and alteration of protein kinetic properties are the most widely adopted strategies to improve a strain for useful chemical production (5, 41).

A typical genetic engineering approach to overexpress certain genes usually adopts strong promoters and results in radical changes in target gene transcript levels. While useful, the optimal expression level does not necessarily equate to strong expression (14). Strong overexpression of target genes can cause growth inhibition. For example, a very high expression level of xylulokinase (*XKS1*) is toxic to *Saccharomyces cerevisiae* when grown on xylose (18), xylanase driven by strong promoters such as *PGK1* and *ADH2* slows down growth of *S. cerevisiae* (10), and a high expression level of the gluconeogen-

esis gene *pck* in *Escherichia coli* can exert a growth burden on the cell (4). To avoid these undesirable effects, regulated promoters (29, 36) such as *T7 phi 10* (*E. coli*) and *GALI* (*S. cerevisiae*) are often used to achieve optimal expression levels of desired genes. Although they are efficient in tuning expression levels in laboratory scale experiments, inducible promoters are not economical for industrial-scale fermentation (14, 35).

Alternative approaches create libraries of constitutive promoters with various strengths that allow tuning expression to particular levels. One method randomizes a strong promoter, using error-prone PCR (1, 24). The other randomizes the spacer-flanking –15 and –35 regions (14, 35). Alper et al. (1) created a promoter library for *S. cerevisiae* by randomizing the strong *TEF1* promoter and screening for differential expression, using three metrics: green fluorescent protein fluorescence, expression levels monitored by real-time PCR, and the degree of chloramphenicol resistance. Solem and Jensen (35) created a promoter library for *Lactococcus lactis* by PCR amplification of the target gene, using oligonucleotides incorporated with a synthetic promoter, which is generated by randomizing the spacer-flanking –15 and –35 consensus regions.

These approaches are labor intensive and depend on creating artificial promoters that could respond in unpredictable ways to multiple transcriptional effectors. Most eukaryotic promoters consist of multiple *cis*-acting control elements, and random mutagenesis could alter them to increase expression of some, while decreasing that of others. Instead of making a promoter library, we chose promoters with various strengths or profiles based on the levels of transcripts obtained from global expression analyses.

The ultimate goal of genetic engineering for strain improvement is not to change the expression level, protein level, or even enzyme activity level per se but to change the metabolite flux distribution and thereby optimize product formation. Ma-

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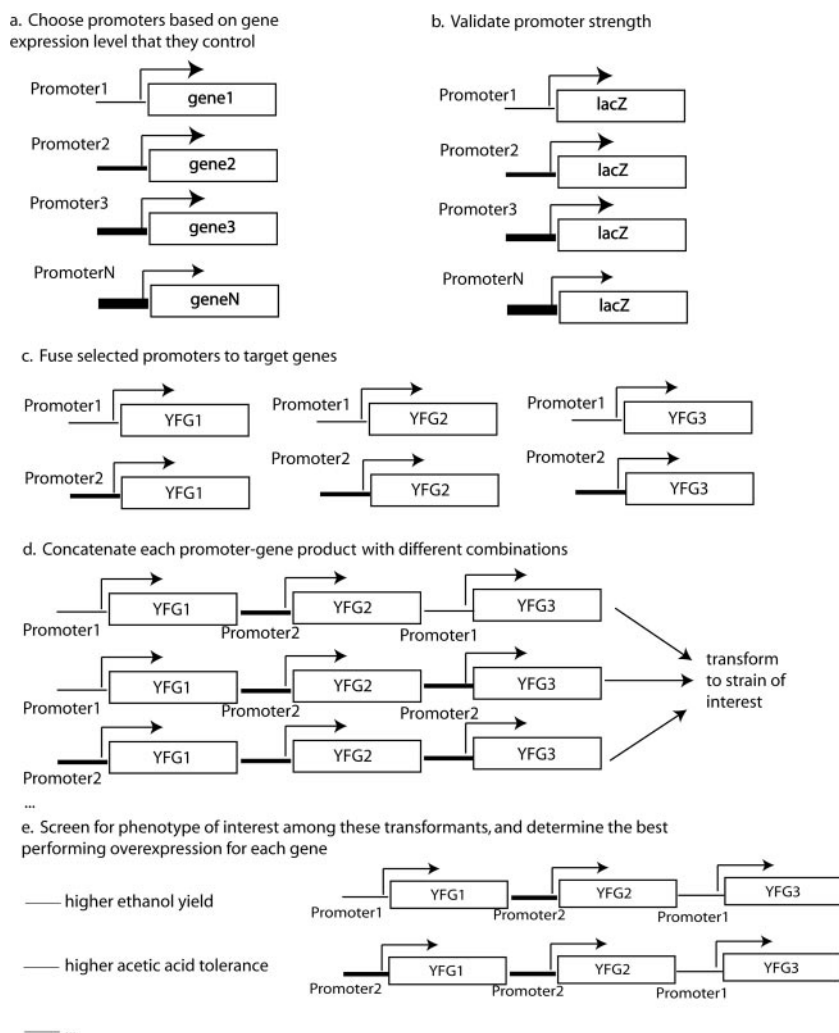


FIG. 1. A schematic representation of MGPS. (a) Appropriate promoters are chosen on the basis of the gene expression levels that they control. (b) The candidate promoters are validated by fusion with *lacZ* and subjected to β -Gal assays. (c) The validated promoters are fused with target genes. (d) Each gene is concatenated with different combinations of promoters and transformed to strains of interest. (For multiple DNA assembly details, see Lu et al. [23]). (e) Finally, each transformant is screened for the desired phenotype and the best combination of promoters is chosen.

nipulating a single gene usually has little effect on metabolite flux because each individual enzyme has only partial control over a pathway (7, 39). Altering expression of multiple genes could overcome this and form the basis for strain improvement (2), as has been the case with lysine production (22) and xylose fermentation (19, 21). In the present study, we developed a multiple-gene-promoter-shuffling (MGPS) method that can achieve optimal levels of overexpression for several genes at a time. This technique improved xylose fermentation in recombinant *S. cerevisiae* (11).

MATERIALS AND METHODS

Strains, media, and growth conditions. The strains and plasmids used in this study are listed in Table S1 in the supplemental material (18, 25, 34, 38). We used the following protocol to name our transformants. After FPL, the first t/T stands for *TALI* under the control of a weaker (lowercase t) or a stronger (uppercase T) promoter. The second t/T stands for *TKL1*, and the p/P stands for *PYK1*. For example, FPLtTP is a transformant that has weaker-promoter-controlled *TALI* but stronger promoters controlling *TKL1* and *PYK1*. *E. coli* DH5 α (Gibco BRL, Gaithersburg, MD) was routinely used for cloning. *S. cerevisiae* YXS3 (*MAT* α

leu2::LEU2-XYL1 ura3::URA3-XYL2 Ty3::NEO-XYL3) was grown in YP medium as described by Jin et al. (17). Yeast and bacterial strains were maintained in 15% glycerol at -70°C . *E. coli* was grown in Luria-Bertani medium. Fifty micrograms of ampicillin/ml was added to the medium when required. Yeast batch cultures were grown under xylose in limited-oxygen conditions as described by Jin et al. (17) in three replicated 125-ml Erlenmeyer flasks with 50 ml YNB defined medium containing 50 g/liter xylose at a shaking speed of 200 rpm. Heavy inoculation (≈ 1.7 g/liter) was used to achieve oxygen limitation. We established from earlier studies (18) in our lab that one unit at an optical density of 600 nm is equivalent to 0.17 g of cells/liter for *S. cerevisiae*.

DNA manipulation. Transformations, cloning procedures, and DNA isolation were performed using standard protocols (30). For yeast transformation, the lithium acetate method described by Ito et al. (12) was used with minor modifications. PCR amplifications were performed in 50- μl volumes containing primers (0.5 mM each) custom-made by the manufacturer (Invitrogen, Carlsbad, CA), deoxynucleotide triphosphates (10 mM each), chromosomal DNA (0.5 mg), and *Taq* polymerase (1 U) in the buffer recommended by the manufacturer (Promega, Madison, WI). Temperature cycling was performed by a programmable thermocycler (PTC-200 thermal cycler; MJ Research, Inc., Watertown, MA) following standard protocols, with minor modifications based on specific primers and amplification results. All PCR products were sequenced prior to further manipulation.

Construction of *lacZ* fusion vectors. The vector pCT was constructed by inserting a CYC terminator PCR product into pRS314 via the BamHI and SpeI sites. pLC was constructed by inserting a 3.0-kb *E. coli lacZ* PCR product into pCT via the Sall and PstI sites. Promoter-*lacZ* fusion products were constructed by inserting the promoter PCR product into pLC via the ApaI and Sall sites 1.0 kb upstream of the corresponding gene (*GND2*, *HXX2*, *PFK1*, *TDH1*, *PGK1*, and *TDH3*). Each plasmid was further transformed to FPLYX3 by using standard lithium acetate methods. The plasmids and primers used are listed in Tables S1 and S2 in the supplemental material.

Construction of a promoter gene library. The individual promoter-gene cassette was constructed by inserting the gene (*TAL1*, *TKL1*, or *PYK1*) PCR product into pRS314 via the XhoI and BamHI sites (Fig. 1). Then the promoter (*GND2* or *HXX2*) PCR product was fused to each of these genes via the ApaI and Sall sites. All six promoter-gene fusion products were constructed by this method. To create each of the library's individual cassettes (i.e., *GND2-TAL1-HXX2-TKL1-HXX2-PYK1*), three fusion products containing the above three genes were concatenated by using modified SfiI cleavage methods (23, 37). Briefly, degenerate SfiI restriction sites were attached to each fusion product, and three base sticky ends were generated by SfiI cleavage of each construct and further assembled together into pSfi-314 in a one-step ligation procedure (23). Each assembled vector was transformed to parental strain FPLYX3 by using the standard lithium acetate method, and the control strain, FPL314, was just FPLYX3-transformed with empty vector pSfi-314. All eight transformants, plasmids, primers, and adaptors used are listed in Tables S1 and S2 in the supplemental material.

Preparation of crude cell extracts and enzyme assays. Yeast cells from two independent cultures were grown on xylose under limited-oxygen conditions (17) and used for each assay. Preparation of the crude cell extract for enzyme assays was performed using the protocol described by Jin and Jeffries (16). Protein concentrations were measured by using a protein assay kit (Bio-Rad, Hercules, CA). Reporter β -galactosidase (β -Gal) activity was determined as described by Cho and Jeffries (6). Endogenous *TAL1* and *TKL1* activities were determined as described by Karhumaa et al. (21), and *PYK1* activity was determined as described by Bergmeyer (3). All enzymatic assays were performed on an 8452A photodiode array spectrophotometer (Hewlett Packard, Wilmington, DE).

Transcriptional analysis. Cells from three independent cultures were used for each condition. cDNA preparation and real-time PCR analysis were performed as described by Jin et al. (17). SYBR green (Applied Biosystems, Foster City, CA) was used as a fluorescent dye to quantify double-stranded DNA formation on an ABI PRISM 7000 sequence detection system (Applied Biosystems). All expression levels were normalized to that for the actin gene *ACT1*.

Analytical methods. Glucose, xylose, and xylitol concentrations were quantified following separation on an HPLC (Hewlett Packard) ION 300 column (Interaction Chromatography, San Jose, CA) and detection by refractive index. Ethanol concentrations were determined by gas chromatography. Cell growth was monitored by measuring the turbidity at OD₆₀₀ and correlating it with dry weight (1.0 unit of OD \approx 0.17 g/liter).

Statistical analysis. Friedman's test (9) was used to analyze results from the fermentation mixtures with multiple gene-promoter combinations. In this method, the ethanol production rank was summed for each time point. The sum of the rank is assumed to follow a chi-square distribution, and a significance threshold was calculated. Values with different letters were significantly different at the 90% confidence level.

RESULTS

Selection of promoters. The promoters for this study were chosen on the basis of the native gene expression that they control. The 1-kb DNA fragments upstream from genes that had relatively constitutive expression when grown on glucose or xylose under aerobic or limited-oxygen conditions (17) were chosen for further analysis. We validated these promoter strengths by using *lacZ* expression. β -Gal assays were performed, using cells grown on xylose under limited-oxygen conditions. A very high correlation was found between β -Gal activity and the native gene expression level that these promoters control when the latter was determined by gene chip expression studies (Fig. 2). Based on either β -Gal assays or transcript expression levels, these promoters span roughly two orders of

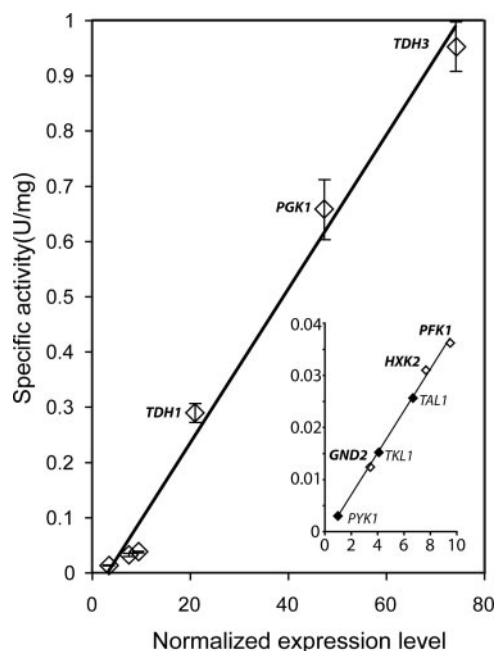


FIG. 2. Correlation between expression array data and β -Gal activities resulting from promoter strengths of selected genes. The gene expression data was adapted from Jin et al. (16). The promoters of these genes were further fused with *lacZ* and subjected to β -Gal assays. In the inset plot in the lower right corner, in addition to the experimental data (open symbols), predicted β -Gal activities for *PYK1*, *TKL1*, and *TAL1* (solid symbols) were also plotted, based on their expression data.

magnitude in strength. Weaker promoters (Fig. 2, from the lower left corner) were plotted along with predicted *PYK1*, *TKL1*, and *TAL1* β -Gal activities (Fig. 2, inset). The transcript levels for the three genes of interest (*PYK1*, *TKL1*, and *TAL1*) were all within an order of magnitude, and all were lower than the transcript level for *HXX2*, from which we derived one of the two promoters. The transcript level for *GND2*, from which we derived the other promoter, was severalfold higher than that for *PYK1* and about the same level as that for *TKL1* (Fig. 2, inset).

Case study: xylose fermentation. MGPS was applied to the xylose fermentation mixture, using a two-promoter level, three-gene minilibrary, which consisted of eight combinations of promoter *GND2* or *HXX2* combined with three endogenous genes: *TAL1* (transaldolase), *TKL1* (transketolase), and *PYK1* (pyruvate kinase). Weak promoters (*GND2* and *HXX2*) were chosen to avoid saturating the system, which would have distorted the relationship between transcript level and enzyme activity. The activities from *TAL1* and *TKL1* connect the pentose phosphate pathway (PPP) to the glycolytic pathway via two reactions that convert xylulose-5-phosphate to glucose 6-phosphate and glyceraldehyde 3-phosphate. These are considered rate-limiting steps (40). Located in the lower glycolytic pathway, *PYK1* determines the glycolytic rate and direction (27). Vectors consisting of eight promoter and gene combinations (see Table S1 in the supplemental material) were constructed using the method based on the SfiI degenerate recognition sequence (23). Enzyme assays and quantitative PCR

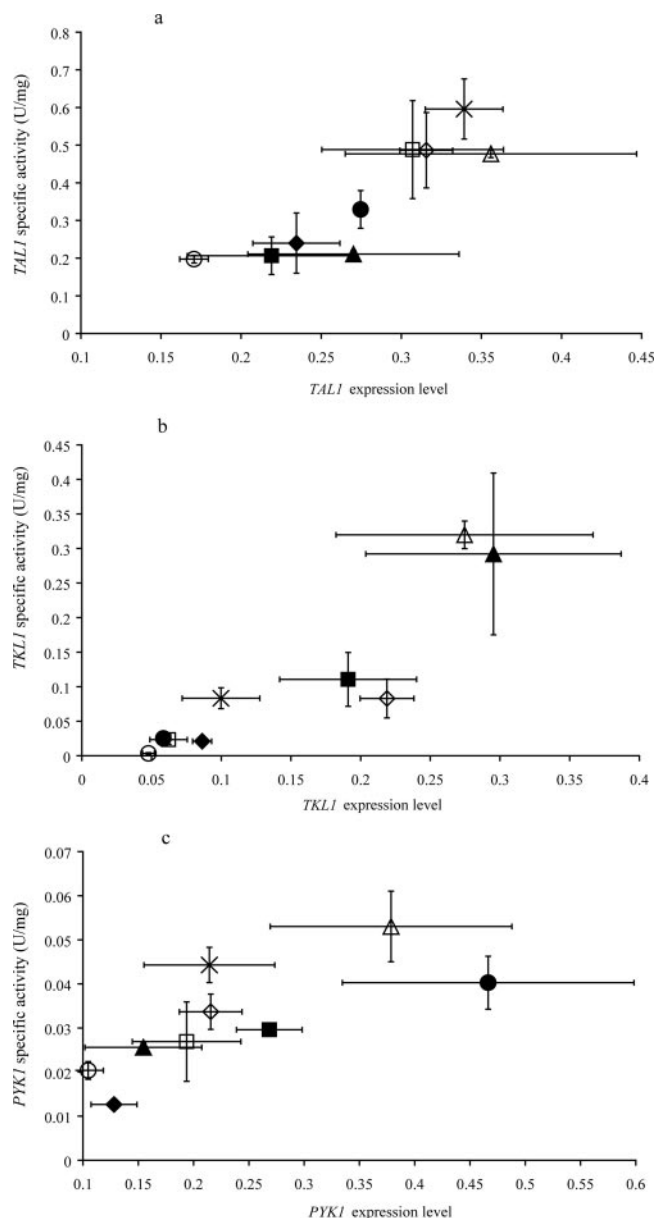


FIG. 3. Transcript expression profile, as determined by quantitative PCR (normalized to that of *ACT1*), correlated with corresponding enzyme activities in two-level, three-gene transformants: (a) *TALI*, (b) *TKL1*, and (c) *PYK1*. Symbols: ○, control strain FPL314 carrying an empty vector; ◆, FPLtP; ●, FPLtP; ▲, FPLtP; ■, FPLtP; □, FPLtP; ×, FPLtP; ◇, FPLTTP; △, FPLTTP. For a description of the strains, see Table S1 in the supplemental material.

were performed to monitor the correlation between expression level and enzyme activity in these eight transformants.

Genes fused with the stronger *HXX2* had higher expression levels than those fused with *GND2*, and all the transformants had higher expression levels than the parental strain (Fig. 3). Expression of *TKL1* and *PYK1* under control of the same promoter was more heterogeneous than expression of *TALI*. The specific activity of these genes increased with increasing transcript expression levels (Fig. 3), indicating that our approach successfully adjusted enzyme activity by tuning the pro-

TABLE 1. Ranking of ethanol volumetric yield for promoter-shuffled transformants as determined by Friedman's test^a

Strain ^b	Sum of ranks	Group
FPLtTP	57	A
FPLtTp	50	A
FPLttP	45	AB
FPLTTP	35	BC
FPLTtp	33	BC
FPLTtP	31	BC
FPLTTp	30	C
FPLttp	27	C
FPL314 (control)	7	D

^a Analysis was performed using Friedman's test (9); rankings with the same group letter(s) are not significantly different at a confidence level of 0.9.

^b A lowercase letter indicates that the weaker *GND2* promoter was used; an uppercase letter indicates that the stronger *HXX2* promoter was used.

moter strength. Although the transcript expression level was roughly proportional to the enzyme level, similar transcript expression levels resulted in very different enzyme activities with different genes. Even though the transcript expression levels of all three genes were of the same magnitude, the enzyme activity of *PYK1*, for example, was significantly lower than for the other two genes (Fig. 3c).

Batch fermentation was carried out for the transformants (FPLtP~FPLTTP) to characterize the ethanol volumetric yield as a function of changing overexpression levels of *TALI*, *TKL1*, and *PYK1*. The rank of transformant ethanol yield for each time point was summed and subjected to Friedman's test (32). The ranking profile shown in Table 1. showed that a higher ethanol yield favors moderate overexpression of *TALI* and a higher level of overexpression of *PYK1*. As shown in Table 1 and Table 2), the top three rankings all had *TALI* controlled by the weaker *GND2* promoter. Similar results were observed in the fermentation profile (see Fig. S1a in the supplemental material). Interactive effects were also evident. We could observe a clear effect of the stronger promoter on *PYK1* when both *TALI* and *TKL1* were driven by the weaker promoter, but when both *TALI* and *TKL1* were driven by the stronger promoter, no additional effect could be observed with the stronger promoter on *PYK1*. All of the combinations were clearly better than the control.

TABLE 2. Specific ethanol production rates for promoter-shuffled transformants

Strain ^a	Fermentation rate (μmol · g ⁻¹ · h ⁻¹)
FPLtTP	0.12 ± 0.009
FPLtTp	0.11 ± 0.009
FPLttP	0.09 ± 0.004
FPLTTP	0.10 ± 0.02
FPLTtp	0.09 ± 0.01
FPLTtP	0.07 ± 0.01
FPLTTp	0.08 ± 0.009
FPLttp	0.11 ± 0.01
FPL314 (control)	0.06 ± 0.009

^a A lowercase letter indicates that the weaker *GND2* promoter was used; an uppercase letter indicates that the stronger *HXX2* promoter was used.

DISCUSSION

Here we presented a method to find optimal expression levels for multiple genes (outlined in Fig. 1). This was achieved by fusing several promoters of various strengths to target genes, joining them together in a one-step ligation procedure, and screening for the desired phenotype. Selection of promoters was based on microarray data obtained under the desired target conditions. Compared to other promoter library construction methods such as using randomized spacers (14, 35) or randomized promoter sequences (1), this method requires less-extensive screening for various promoter strengths and avoids problems that might arise through mutation of native eukaryotic promoters that have multiple *cis*-acting sequences.

The β -Gal assay (Fig. 2) showed a high correlation between native gene expression data and β -Gal activity, indicating that the downstream gene context plays little role in the promoters tested. Because the promoter selections were based on microarray data under target conditions, these promoter strengths could be conditional; i.e., they may be valid only under the same or similar conditions as those of the microarray experiments. Native gene expression under the control of selected promoters showed relatively little variation between cells grown on glucose or xylose under aerobic or limited-oxygen conditions, but promoters with variable profiles could have been selected as well. Enzyme activity also increased with increasing transcript abundance, but this correlation was not always linear and varied with the gene. For example, the enzymatic activity for *PYK1* tended to stay constant as transcript levels increased to the maximum values tested. In contrast, the activity for *TAL1* did not continue to decrease with decreasing transcript levels (Fig. 3). Transcript abundances reflected activities of the endogenous native genes as well as those of the exogenous genes driven by the stronger promoters, but we were still able to observe a correlation between transcript abundance and measured enzyme activity.

A single enzyme rarely controls metabolic flux. For example, in *S. cerevisiae* no single enzyme significantly affected metabolic fluxes of glycolysis (31). Metabolic control is usually distributed over multiple reactions, so successful metabolic engineering requires eliminating multiple rate-limiting steps (8, 14). Previous metabolic engineering strategies have involved overexpression of multiple genes (22, 26). These methods have integrated one gene at a time and have required extensive vector construction. If two reactions are interdependent, changing either one without the other could result in suboptimal performance; therefore, they are unsuitable for tuning the expression of multiple genes simultaneously. Our development of MGPS is designed to tune overexpression of multiple genes with less-intensive labor and greater flexibility.

We selected a number of genes to demonstrate the feasibility of MGPS. They were chosen on the basis of a previous gene chip study with engineered *S. cerevisiae* grown on glucose and xylose (17), which indicated that transcripts for transaldolase (*TAL1*) were 2.5 times more abundant than those for principal transketolase (*TKL1*) and that abundance did not change in cells grown on these two carbon sources. By comparison, our preliminary studies on *TAL1* and *TKL1* expression in *Pichia stipitis* showed that the transketolase transcript was strongly induced on xylose, present in threefold-greater abundance

than that for *TAL1* (13). Moreover, the topology of the metabolic network and independent studies from other research groups indicate that these two enzymes could be rate-limiting steps (21, 40). Transaldolase (*TAL1*) and transketolase (*TKL1*) are key enzymes in the nonoxidative PPP. These steps connect xylose assimilation to the glycolytic pathway and have been routinely engineered in xylose-metabolizing *S. cerevisiae* fermentation (19, 20, 40). By overexpressing *TAL1* and *TKL1*, we hoped to increase the flux from the PPP into the glycolytic pathway. Pyruvate kinase (*PYK1*), which is in the lower part of the glycolytic pathway, was previously reported to determine the glycolytic rate (27). Again, our previous gene chip studies on engineered *S. cerevisiae* (17) showed that the *PYK1* transcript was induced at levels of roughly threefold when cells were grown on glucose as opposed to xylose, and we knew that the cells were more fermentative when cultivated on glucose.

Although all strains had similar growth rates initially, the transformant growth rates of the test strains were less robust than that of the control strain (FPL-314) after 24 h. This was possibly attributable to the larger sizes of the test vectors (14 kb) in comparison to the control vector (5 kb), but the production of extra protein might also have exerted some burden on the cells (36). Alternatively, growth might have been compromised by increased carbon flux through the glycolytic pathway. We expect that further adaptive evolution could restore growth characteristics due to metabolite imbalance. As determined by Friedman's test (Table 1), for three out of the top four ethanol-producing transformants, *TAL1* was under the control of *GND2* and *PYK1* was under the control of *HXK2*.

Ethanol yield was sensitive to the overexpression of *TAL1* and *PYK1* but less sensitive to that of *TKL1* in the transformants (see Fig. S1a, b, and c in the supplemental material). Several laboratories have found that *TAL1* is rate-limiting for xylose fermentation in recombinant *S. cerevisiae* (15, 20), which was also the case in this study. We found that only moderate *TAL1* expression was required to increase ethanol yield. Higher levels of *PYK1* expression, though not as dominant as *TAL1*, also contributed to higher ethanol yields. Despite its elevated expression level, the enzyme activity of *PYK1* was still rather low. It is well known that *PYK1* is a key glycolytic enzyme and under tight regulation through phosphorylation by the protein kinase A cascade (28). Furthermore, interaction between these genes could play a role in the fermentation results; after all, the metabolic pathway does behave highly dynamically and synergistically. Despite these possible explanations, we cannot rule out the possibility that differences in ethanol yield are influenced by respiration.

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