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Effects of Gene Orientation and Use of Multiple Promoters on the Expression of *XYL1* and *XYL2* in *Saccharomyces cerevisiae*

Ju Yun Bae · José Laplaza · Thomas W. Jeffries

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Abstract Orientation of adjacent genes has been reported to affect their expression in eukaryotic systems, and metabolic engineering also often makes repeated use of a few promoters to obtain high expression. To improve transcriptional control in heterologous expression, we examined how these factors affect gene expression and enzymatic activity in *Saccharomyces cerevisiae*. We assembled D-xylose reductase (*XYL1*) and D-xylitol dehydrogenase (*XYL2*) in four ways. Each pair of genes was placed in two different tandem (1→2→ or ←1←2), convergent (1→←2), and divergent (←1 2→) orientations in autonomous plasmids. The *TEF1* promoter was used to drive *XYL1* and the *TDH3* promoter to drive *XYL2* in each of the constructs. The effects of gene orientation on growth, transcription, and enzyme activity were analyzed. The transcription level as measured by quantitative PCR (q-PCR) correlated with enzyme activities, but our data did not show a significant effect of gene orientation. To test the possible dilution of promoter strength due to multiple use of the same promoter, we examined the level of expression of *XYL1* driven by either the *TEF1* or *TDH3* promoter when carried on a single copy plasmid. We then co-expressed *XYL2* from either a single or multicopy plasmid, which was also driven by the same promoter. *XYL2* transcript and enzyme expression increased with plasmid copy number, while the expression of *XYL1* was constant regardless of the number of other *TEF1* or *TDH3* promoters present in the cell. According to our data, there is no significant effect of gene orientation or multiple promoter use on gene transcription and translation when genes are expressed from plasmids; however, other factors could affect expression of adjacent genes in chromosomes.

Keywords Yeast · Metabolic engineering · Promoter saturation · Gene orientation · *S. cerevisiae* · Gene expression · Enzyme activities

J. Y. Bae · T. W. Jeffries
Molecular and Environmental Toxicology Center, University of Wisconsin, Madison, WI 53705, USA

J. Laplaza · T. W. Jeffries (✉)
USDA Forest Service, Forest Products Laboratory, 1 Gifford Pinchot Drive, Madison, WI 53726, USA
e-mail: twjeffri@wisc.edu

Present address:
J. Laplaza
Cargill, Minneapolis, MN, USA

Introduction

Yeasts have become important hosts for heterologous gene expression [1]. For optimal metabolic engineering, it is necessary to control gene expression quantitatively. Genome structure and function are known to correlate, and Williams et al. found co-expression of neighboring genes in *Arabidopsis* [2]. This pattern has been observed for many eukaryotic genomes [3–5]. Gene orientation has been suggested as one of the mechanisms for co-expression [2, 6]. For example, effects of divergent gene orientation or tandem orientation can be found in co-expressed genes in plants [7, 8]. Prescott et al. [9] identified transcriptional repression of convergently arranged genes in yeast, which was explained by transcriptional collision. These findings imply that intergenic regions between adjacent genes can have important roles in transcription. Deletion of regions between the two ORFs caused decreased transcription in yeast [10, 11]. In *Saccharomyces cerevisiae* the intergenic region has regulatory function [12, 13] and different base compositions according to the orientation of neighboring ORFs [14].

In the present study, we have manipulated the orientation of the D-xylose reductase (*XYL1*) and D-xylitol dehydrogenase (*XYL2*) genes to model the effect of two different tandem (1→2→ or ←1←2), convergent (1→←2), and divergent (←1 2→) orientations. Two strong promoters were used—translation elongation factor promoter (*TEF1*p) and glyceraldehyde 3-phosphate dehydrogenase promoter (*TDH3*p). *XYL1* and *XYL2* used their own terminator sequences. The effect of gene orientation on growth, transcription, and enzyme activities were analyzed in *S. cerevisiae*.

We also investigated the promoter effectiveness in the condition of multiple use of the same promoter. To test this, we examined the expression level of *XYL1* with co-expression of single or multiple copies of *XYL2*. Both *TEF1*p and *TDH3*p were tested separately. We found increased *XYL2* transcription and enzyme activities of multiple copies of *XYL2* with constant expression and activities of *XYL1*. Both *TEF1*p and *TDH3*p showed the same pattern.

Materials and Methods

Strains, Media, and Cultivation Conditions *S. cerevisiae* strain L2612 (MATα leu2–3 leu2–112 ura3–52 trp1–298 can1 cyn1 gal^{+}) was used for transformation of all the constructed gene plasmids. Enzymatic manipulation and cloning of DNA were performed as described in Sambrook and Russell [15]. Yeast transformants with *URA3* or *TRP1* selectable markers were cultivated in yeast synthetic complete (YSC) medium containing 6.7 g of yeast nitrogen base/l without amino acids plus 20 g of glucose/l, 20 g of agar/l, and an appropriate mixture of nucleotides and amino acids. Yeast cells were cultured in a 125-ml flask with 50 ml medium at 30°C and 200 rpm.

Plasmid Construction to Test Gene Orientation Polymerase chain reaction (PCR) was used to amplify and create in-frame fusions of D-xylose reductase (*XYL1*) and D-xylitol dehydrogenase (*XYL2*) with the *TEF1* and *TDH3* promoters, respectively. Gene 1 (*TEF1*+*XYL1*) and 2 (*TDH3*+*XYL2*) were assembled into two different tandem (1→2→ or 1←2←), convergent (1→←2), and divergent (←1 2→) orientations in pRS316 (*URA3* CEN/ARS) plasmid (Fig. 1). Each construct was sequenced to confirm the structure.

Plasmid Construction to Examine Promoter Dilution *XYL1* was amplified and fused with the *TEF1* or *TDH3* promoter by PCR as above. This fragment was then inserted into pRS316

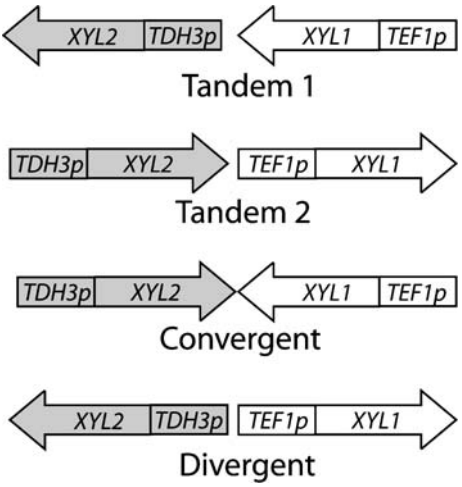

Fig. 1 Four constructs with gene 1 (*TEF1* promoter+*XYL1*) and gene 2 (*TDH3* promoter+*XYL2*). The genes for xylose reductase (*TEF1*+*XYL1*) and xylitol dehydrogenase (*TDH3*+*XYL2*) were assembled into two different tandem (1→2→ or 1←2←), convergent (1→←2), and divergent (←1 2→) orientations in pRS316 (*URA3* CEN/ARS) plasmid. *URA3* was the selectable marker for all constructs

plasmid. *XYL2* was amplified and fused with *TEF1* or *TDH3* promoter by PCR. This fragment was inserted into pRS314 (*TRP1* CEN/ARS) or pRS424 (*TRP1* 2 μm origin; Fig. 5).

Yeast Transformation All yeast transformations were performed as described in Gietz and Woods [16]. Transformants of gene orientation test were selected on yeast synthetic complete (YSC) dropout medium (Ura^-) containing 20 g of glucose/l. Transformants of promoter dilution examination were selected on Ura^-, Trp^- YSC dropout medium containing 20 g of glucose/l.

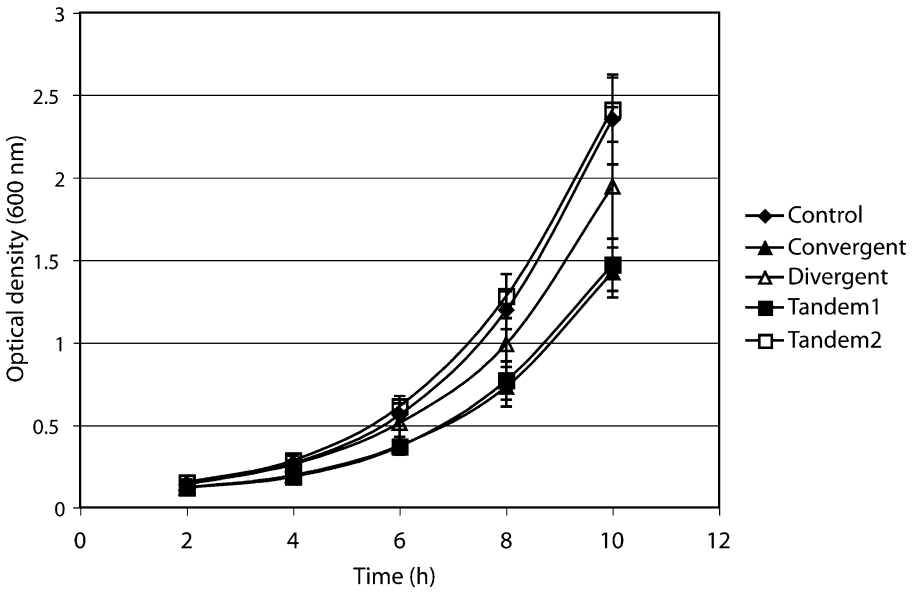

Fig. 2 Growth of strains with different gene orientations. Each stain was cultured in a 125-ml flask with 50 ml medium at 30°C and 200 rpm. OD_{600} value was the mean of three replicates, and *error bar* represents SD

Table 1 Growth rate of each strain of different gene orientations.

Strains	Doubling time[a] (h)
Control	2.7
Tandem1	2.68
Convergent	3.07
Divergent	2.88
Tandem2	2.61

[a] Doubling time (Td)=$(t_2-t_1)/\mu$; $\mu=\ln(cd_2/cd_1)$ (cd_1 Cell density at time t_1, cd_2 cell density at time t_2) cd_1 was OD_{600} value at 4 h (t_1) and cd_2 at 8 h (t_2).

Cell Growth Experiments For the growth rate measurement, overnight cultures were diluted to the initial OD_{600} of 0.1. Three replicates of each strain were performed. Initial cell growth (OD_{600}, <2) was used for calculation of the specific growth rate.

Preparation of RNA Total RNA was prepared from each transformant as described by Rose et al. [17].

Relative Quantitative Reverse Transcriptase-Polymerase Chain Reaction For each RT reaction, 5 μg of total RNA was reverse-transcribed with avian myeloblastosis virus-reverse transcriptase (Promega, Madison, WI). Relative quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) primers were designed to XYL1 (5′-GATACCTTCGT CAATGGCCTTCT-3′ and 5′-TTCGAC GGTGCCGAAGA-3′), XYL2 (5′-CAA GACCGGTGGTTCTGAAGA-3′ and 5′- CCAGTA CATTCCAAAACGACGTT-3′), and *ACT1* (5′-TGGATTCCGGTGATGGTGTT-3′ and 5′- TCAAAATGGCGTGAGGTA GAGA-3′) using Primer Express software (Applied Biosystems). We used SYBR green PCR master mix (Applied Biosystems) and an ABI PRISM 7000 sequence detection system (Applied Biosystems) to perform RT-PCR. PCR conditions were as recommended by the

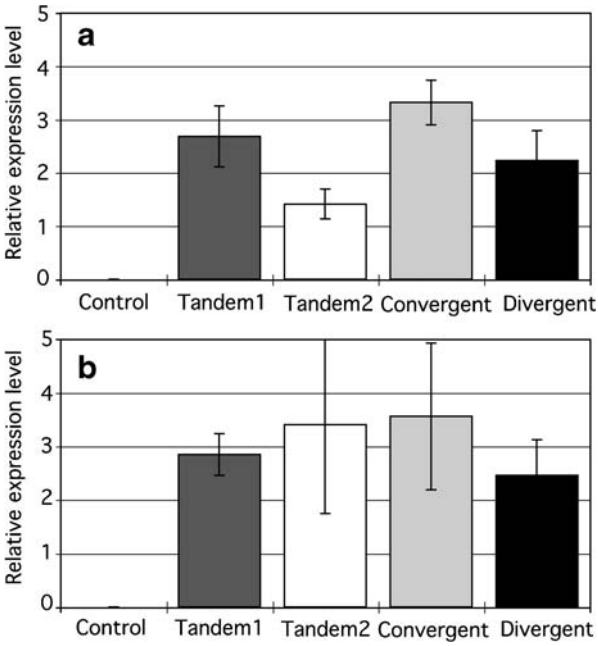

Fig. 3 *XYL1* (**a**) and *XYL2* (**b**) expression in each strain with different gene orientation. Graph represents the average±SD of three replicates

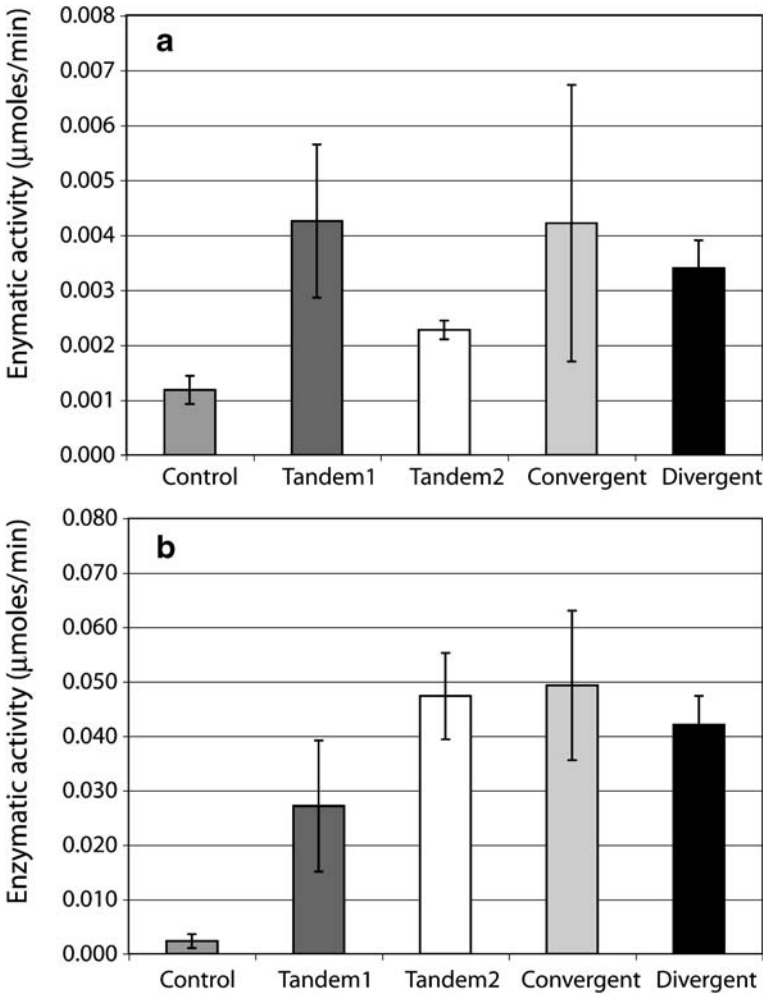

Fig. 4 *XYL1* (**a**) and *2* (**b**) enzyme activities in each strain of different gene orientation. Graph represents the average±SD of three replicates

manufacturer except that one-half of the reaction volume was used: 7.5 pmol of each primer was used in cycles of 50°C for 2 min and 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantification for copy number of *XYL1* and *XYL2* was performed using both the standard curve method and the comparative C_T method [18]. The C_T value of serial dilution of *Pichia stipitis* genomic DNA (30, 3, 0.3, 0.003, 0.0003, and 0.00003 ng) with *ACT1* primers was used to construct a standard curve. Then, the relative copy numbers of the *XYL1* and *XYL2* were calculated by comparison with *ACT1*. All reactions were performed in triplicate.

Enzymatic Assay Cells were harvested by centrifugation at 10,000×*g* for 10 min. The pellet was washed and suspended in buffer (100 mM phosphate buffer, 1 mM EDTA, 5 mM β-mercaptoethanol [pH 7.0]). The suspended cells were mixed with glass beads (Sigma), vortexed at maximum rate in bursts of 30 to 120 s, and then cooled on ice. The cell debris

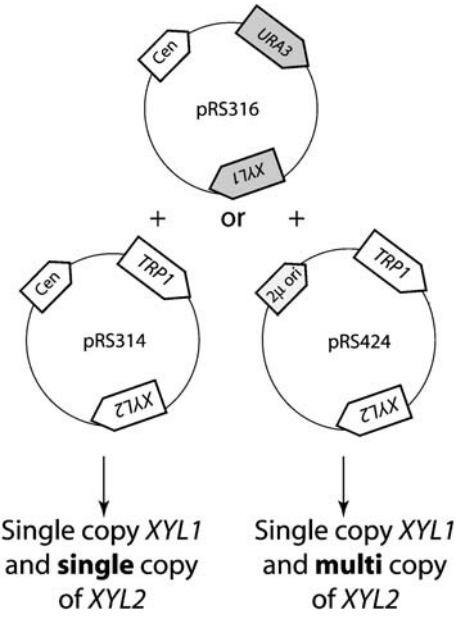

Fig. 5 Experimental strategy to identify the effect of multiple use of same promoter. Each stain has a single copy of *XYL1* with variation of *XYL2* expression. The construct with 2μ *ori* had multicopy gene expression. *TEF1* and *TDH3* promoter were examined separately

and glass beads were separated by centrifugation for 10 min at 15,000×*g*. In vitro activities of xylose reductase (with NADPH and NADH) and xylitol dehydrogenase (with NADH) in the supernatant were determined by using a previously described method [19]. All reactions were performed in triplicate. The unit was μmol min^{-1} mg^{-1}.

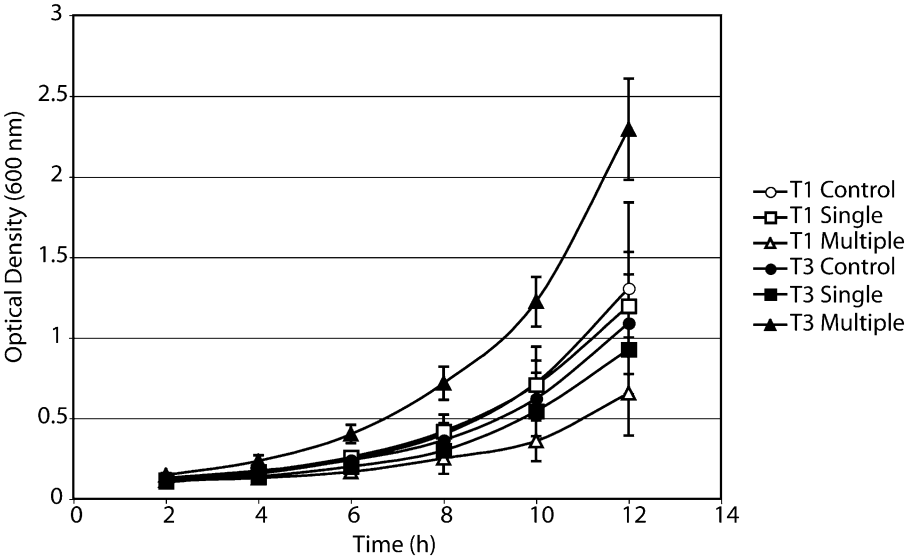

Fig. 6 Growth of each strain with variation of *XYL2* expression. Each stain was cultured in a 125-ml flask with 50 ml medium at 30°C and 200 rpm. OD_{600} value was the mean of three replicates, and the *error bar* represents SD. Every construct had a single copy of *XYL1* with either *TEF1* promoter (T1) or TDH3 promoter (T3). *T1 Control* No XYL2, *T1 single* single copy of XYL2, *T1 Multiple* multicopy of XYL2, *T3 Con* no XYL2, *T3 single* single copy of XYL2, *T3 Multiple* multicopy of XYL2

Table 2 Growth rate of each strain with variation of *XYL2* expression.

Strains	Doubling time[a] (h)
T1Control	4.28
T1Single	4.43
T1Multiple	6.04
T3Control	5.45
T3Single	5.17
T3Multiple	3.6

[a] Doubling time (Td)=$(t_2-t_1)/\mu$; $\mu=\ln(cd_2/cd_1)$ (cd_1 Cell density at time t_1, cd_2 cell density at time t_2) cd_1 was OD_{600} value at 8 h (t_1) and cd_2 at 4 h (t_2)

Results and Discussion

Effect of Gene Orientation on Cell Growth, Transcription, and Translation We constructed single copy expression vectors with two genes inserted in four different orientations (Fig. 1). Each of these was transformed into the *S. cerevisiae* host. The control consisted of the host strain carrying the parental vector without any gene inserts. Yeast strains bearing

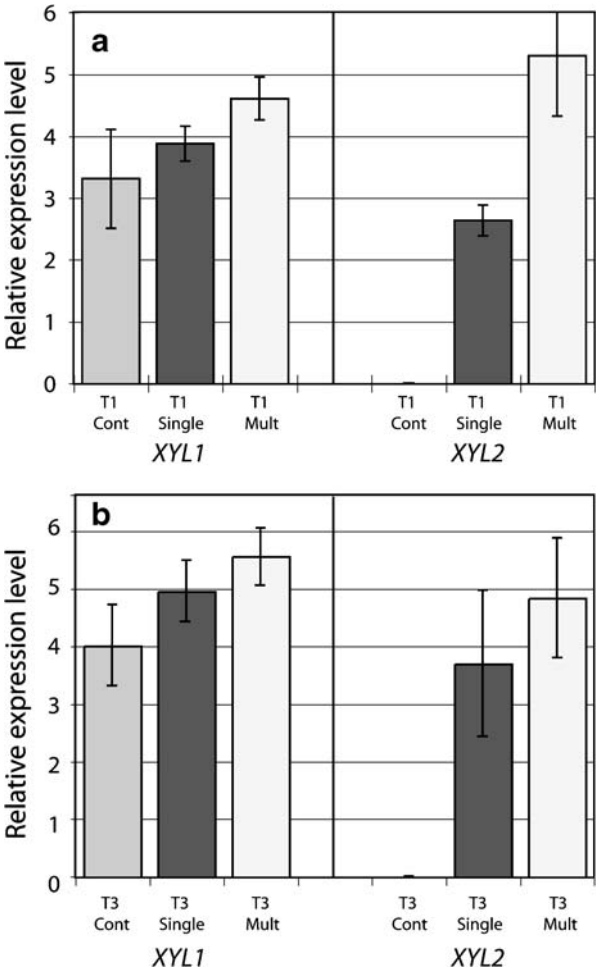

Fig. 7 *XYL1* (**a**) and *2* (**b**) expression in each strain with variation of *XYL2* expression. Graph represents the average±SD of three replicates. Every construct had a single copy of *XYL1* with either *TEF1* promoter (T1) or TDH3 promoter (T3). *T1 Cont* No XYL2, *T1 Single* single copy of XYL2, *T1 Mult* multicopy of XYL2, *T3 Cont* no XYL2, *T3 Single* single copy of XYL2, *T3 Mult* multicopy of XYL2

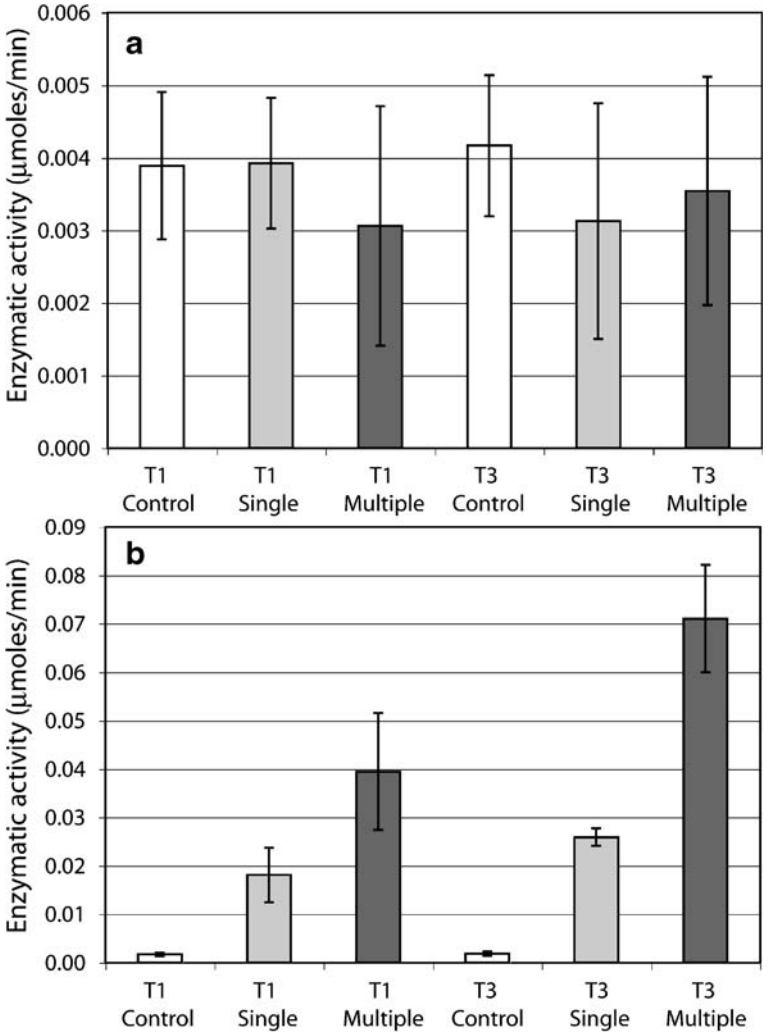

Fig. 8 *XYL1* (**a**) and *2* (**b**) enzyme activities in each strain with variation of *XYL2* expression. Graph represents the average ± SD of three replicates. Every construct had a single copy of *XYL1* with either *TEF1* promoter (T1) or TDH3 promoter (T3). *T1 Control* No XYL2, *T1 Single* single copy of XYL2, *T1 Multiple* multicopy of XYL2, *T3 Control* no XYL2, *T3 Single* single copy of XYL2, *T3 Multiple* multicopy of XYL2

vectors with the divergent (←1 2→) and tandem2 (1←2←) gene orientations showed similar growth rates with the control (Fig. 2). Yeast strains bearing vectors with the convergent (1→←2) and tandem1 orientations (1→2→) showed slightly retarded growth rates compared with the control. Strains with the tandem2 orientation showed the fastest growth rates (doubling time (Td)=2.61 h), and strains with the convergent orientation were the slowest (Td=3.07 h) (Table 1).

We could not detect significant differences in the gene expression profiles of *XYL1* and *XYL2* among these various strains (Fig. 3). *XYL2* enzyme activities were higher than *XYL1*

overall (Fig. 4), but no significant difference could be detected in enzymatic activities among the various orientations.

Effect of Multiple Use of Same Promoter We examined promoter dilution effect using *TEF1* and *TDH3* promoter, separately. Control was carrying single copy of *XYL1* expression vector and no *XYL2* expression vector (Fig. 5). Other stains contained single or multiple copies of the *XYL2* expression vector with single copy of *XYL1* expression vector. Therefore, we could identify if there was any change of *XYL1* expression due to *XYL2* overexpression. Figure 6 and Table 2 show the growth rates of each strain. The strain bearing the *TDH3* promoter with multiple copies of *XYL2* (T3 multiple) showed the highest growth rate (Td=3.6 h). The strain with the *TDH3* promoter with a single copy of *XYL2* (T3 single) had a slow growth rate compared to the T3 multiple strain (Td=5.17 h). The stain bearing the *TEF1* promoter with multicopy vector of *XYL2* (T1 multiple) showed the slowest growth rate (Td=6.04 h). In the case of *TEF1*, the single copy of *XYL2* (T1 single) had faster growth rate compared to T1 multiple (Td=4.43 h). According to these data, overexpression of *XYL2* had different growth effects with each promoter. To determine whether this difference is from the multiple use of the same promoter or other factors, however, will require more studies.

Transcription of *XYL1* and *2* showed an increase in *XYL2* with a multicopy vector bearing *XYL2*, and there was no effect on *XYL1* expression due to *XYL2* overexpression (Fig. 7).

Enzyme activities showed correlation with the transcript expression level for each construct, and *XYL1* had no influence due to co-expression of *XYL2* (Fig. 8).

According to our data, there was no significant effect of gene orientation or multiple promoter use on gene transcription and translation. However, our data were based on plasmid expression. The expression of adjacent genes in chromosomes can be affected by several other factors in addition to gene orientation. The base composition of intergenic regions and chromatin structure can have influence on gene expression [14]. It has been reported that distance between genes, gene length, and number of exons are also important parameters for expression level [20]. Moreover, genes with similar promoter regions show significantly higher correlations in expression profiles even though the actual expression level depends on the genes [21]. There is a complex relationship between regulatory elements and gene expression [22]. Therefore, we need to consider several critical factors that can influence expression in host genome.

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