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Fine-tuning of xylose metabolism in genetically engineered *Saccharomyces cerevisiae* by scattered integration of xylose assimilation genes

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ABSTRACT

Manipulation of multiple genes is a common experience in metabolic engineering and synthetic biology studies. chromosome integration of multiple genes in one single position is always performed, however, there is so far no study on the integration of multiple genes separately in various positions (here in after referred to as "scattered integration") and its effect on fine-tuning of cellular metabolism. In this study, scattered integration of the xylose assimilation genes *PsXR*, *PsXDH* and *ScXK* was investigated in *Saccharomyces cerevisiae*, and transcription analysis of these genes as well as their enzyme activities were compared with those observed when the genes were integrated into one single site (defined as "tandem integration" here). Not only notable differences in transcription levels and enzyme activities were observed when the genes were integrated by the two strategies, but also change of the cofactor preference of *PsXR* gene was validated. Xylose fermentation was further studied with the strains developed with these strategies, and elevated xylose utilization rate was obtained in the scattered integration strain. These results proved that by positioning multiple genes on different chromosomes, fine-tuning of cellular metabolism could be achieved in recombinant *S. cerevisiae*.

1. Introduction

Proper enzyme activities are critical in metabolic engineering to control metabolic flux distribution, and adjusting expression levels of individual genes through varying promoter strength and copy numbers have been explored [1–4]. However, chromosome integration of genes is desirable for engineering industrial microbial strains to obtain stable phenotype without a necessity for selective pressure that is not practical for industrial applications. And the selection of integration sites has great impact on gene expression. For example, about a 8.7-fold variation of β-galactosidase activity was observed in the budding yeast *Saccharomyces cerevisiae* when *lacZ* gene was integrated into different positions along chromosome, suggesting that epigenetic effects associated with the chromosome integration site exert control on the gene expression [2]. In metabolic engineering and synthetic biology studies, delicate manipulation of multiple genes is often required. We assume that separated integration of multiple genes into different positions of chromosomes, which is defined as scattered integration in this study, may have different influences comparing with that of the general design, which integrates all genes in one site (referred to as tandem integration here). However, such an integration strategy and its effect on the fine-tuning of cellular metabolism have not been explored so far.

Xylose is abundant in lignocellulosic biomass, and there is considerable interest in utilization of xylose by metabolic engineering of *S. cerevisiae* that natively is not capable of consuming xylose. Xylose can be reduced to xylitol by xylose reductase (XR), after which xylitol is oxidized to xylulose by xylitol dehydrogenase (XDH). Subsequently, xylulose is converted to xylulose 5-phosphate that can then enter the pentose phosphate pathway and glycolysis by xylulokinase (XK) [5]. Integration of genes *xyl1*, *xyl2* and *xks1* encoding XR, XDH and XK, respectively into the chromosome sites of *URA3*, *AUR1-C* or delta region was achieved [6–8], and we are interested in comparing the difference in the scattered integration and tandem integration of these genes.

In the present study, *xyl1* and *xyl2* genes from *Pichia stipitis* (*PsXR*, *PsXDH*) and *xks1* gene from *S. cerevisiae* (*ScXK*) were employed to manipulate xylose utilization pathway in *S. cerevisiae*. Gene expression was investigated from aspects of transcription levels, enzyme activities and co-fermentation performance of

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xylose and glucose. The results indicated that through integration of multiple foreign genes via the scatted integration strategy, fine-tuning of cellular metabolism could be achieved.

2. Materials and methods

2.1. Cloning PsXR, PsXDH, ScXK and construction of expression plasmids

Routine molecular techniques were employed for gene cloning and plasmid construction [9]. *xyl1* and *xyl2* genes were amplified from *P. stipitis* JCM 10742 (purchased from RIKEN BioResource Center, Japan). *xks1*, *PGK1* promoter, *ADH1* promoter, *CYC1* terminator were amplified from *S. cerevisiae* model strain S288c (kindly provided by Prof. Dr. Jens Nielson, Chalmers University, Sweden). All the primers used in this study were listed in Table S1. Design strategy of scattered integration and tandem integration was depicted in Fig. 1. The three positions for integration were cited from the previous work [2]. Six homologous arms were amplified from industrial yeast strain *S. cerevisiae* 6525 (provided by Dr. Jana Otrubo, Department of Chemical Engineering, University of Waterloo, Canada). Plasmid pAUR135 purchased from Takara Bio, Kyoto, Japan was used for cloning of xylose assimilation genes. And all the fragments of constructs were sub-cloned into pUC18 for ligation. The correct integration of various constructs was verified by PCR and subsequent sequencing of the PCR products. The constructed plasmids pAUR-XR-XDH-XK, pAUR-XR, pAUR-XDH and pAUR-XK were transformed into *S. cerevisiae* 6525 after linearization with *Spe*I. Detailed descriptions of expression plasmid construction were summarized in Supplementary data (Fig. S1–S4).

2.2. Medium composition and co-fermentation of glucose and xylose

LB broth containing of 50 mg L^{-1} ampicillin and YPD were used to culture *E. coli* and *S. cerevisiae*, respectively. Oxygen-limited sugar mixture fermentation was performed as described by Kim et al. [10], and YP medium (peptone 2%, yeast extract 1%) containing 50 g L^{-1} glucose and 25 g L^{-1} xylose was used as fermentation medium. Fermentation data is average values of triplicate experiments. All the fermentation experiments were repeated more than three times.

2.3. Yeast transformation and screening

Electroporation was used for yeast transformation [11]. Two steps were used to screen positive clones in YPD-Aba^{+} medium (YPD plate with addition of 2.5 μg mL^{-1} of aureobasidin A) for primary screening and YPGal medium (1% yeast extract, 2% peptone, 2% galactose, 2% agar) for secondary screening, and the correct clones were finally verified by PCR and sequencing.

2.4. mRNA preparation and semi-qRT-PCR

Hot acidic phenol method was applied on extraction of mRNA [12]. cDNA synthesis protocol was followed as recommended by the manufacturer (PrimeScript RT-PCR Kit, Taraka Bio, Kyoto, Japan). Housekeeping gene *ACT1* was used as internal standard in quantification of expression levels of *xyl1*, *xyl2* and *xks1* [13]. RT-PCR primers used for RNA analysis were listed in Table S2.

2.5. Enzyme activities of XR, XDH and XK genes

Enzyme assay was measured according to the previous work [5]. Each assay was repeated three times, and the average values were presented.

2.6. HPLC analyses

Fermentation products were analyzed by HPLC. A Waters Breeze system (Waters Corp., MA, USA) equipped with an organic acid column, Aminex HPX-87H (Bio-rad, California, USA) was used along with Waters 2414 refractive index detector, with a mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}, and the HPLC system was operated at 50 °C.

3. Results and discussion

3.1. Construction of strains carrying tandem and scattered chromosomal integrations

Plasmid pAUR135 contains a growth inhibitory gene *GIN11M86* which is directed by *GAL10* promoter. On galactose-containing plates, homologous recombined clones are selected, and the plasmid can then be repeatedly used for transformation of foreign

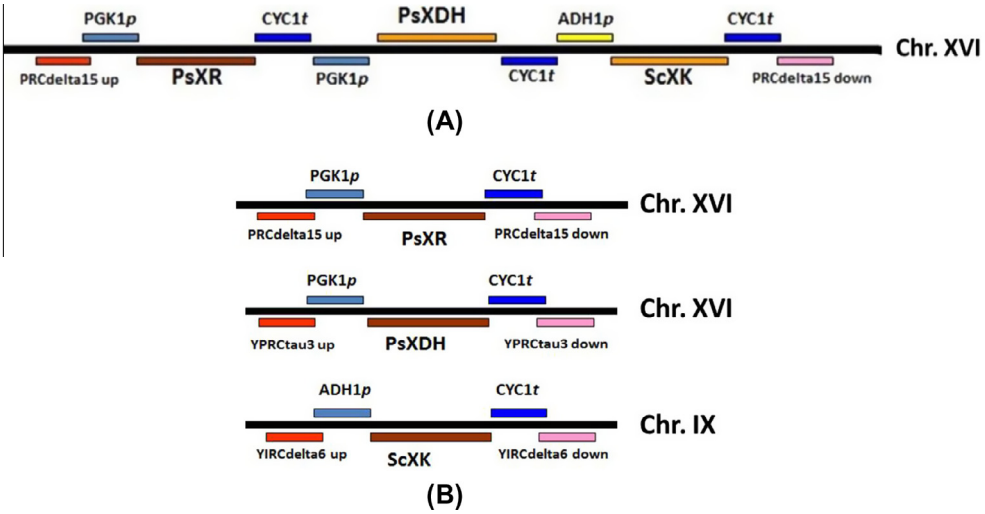

Fig. 1. Design of the tandem and scattered integration strategies. (A) Tandem integration of the xylose assimilation genes in the YPRCdelta15 position of chromosome XVI; (B) scattered integration with *PsXR* inserted in the YPRCdelta15 position of chromosome XVI, *PsXDH* inserted in the YPRCtau3 region of chromosome XVI, and *ScXK* inserted in the YIRCdelta6 site of chromosome IX.

genes without leaving any selection marker in the chromosome. The description of two integration methods was illustrated in Fig. 1. In tandem integration method, the three gene fragments were arranged in a head-to-tail way inserting in the locus of *YPRCdelta15* on chromosome XVI (Fig. 1A), and resultant strain was designated as ZQt. In scattered integration method, individual cassettes harboring *PsXR* gene, *PsXDH* gene and *ScXK* gene were inserted in the positions of *YPRCdelta15* on chromosome XVI, *YPRCtau 3* on chromosome XVI, *YIRCdelta6* on chromosome IX, respectively (Fig. 1B), and the resultant strain was registered as ZQs. Genetically engineered strains via scatted integration (strain ZQs) and tandem integration (strain ZQt) showed equal growth rate in YPD medium, which is also similar to that of the wild-type strain (data not shown), demonstrating no halt to cell growth by the expression of three xylose assimilation genes in the two strains.

3.2. Comparison of expression levels of xylose pathway genes with two integration strategies

In reconstruction of xylose metabolism pathway in *S. cerevisiae*, the common strategy is integrating three xylose assimilation genes in one single position of chromosome. To our knowledge, no effort has ever been made to integrate these genes in a scatted integration strategy. In this study, different positions were designed to accommodate three genes and intended to detect whether expression level varies in the whole xylose pathway under such arrangement. The gene expression variation was first measured at mRNA level between ZQt and ZQs. In the recombinant strain ZQs, the expression levels of *PsXR*, *PsXDH* and *ScXK* are 1.67, 1.5 and 5.7-fold of those of the corresponding genes in ZQt (Fig. 2). We propose that elevated mRNA abundance resulted from the accelerated transcription rate via positioning the same gene in different positions of the chromosome. Enzyme assays were further performed to investigate the correlation with mRNA change. In contrast to ZQt, corresponding enzyme activities of ZQs were 66.7% increase in PsXR, 50% increase in PsXDH, albeit the same enzyme activity was observed in ScXK (Table 1). Interestingly, the cofactor preference of PsXR was also altered. As shown in Table 1, the enzyme activity of PsXR in ZQs using NADH as cofactor was 3-fold of that of ZQt, whereas only slight increase was observed when NADPH was tested as cofactor. The mechanism of such change in differential regulation of enzyme activity and cofactor preference still needs further investigation.

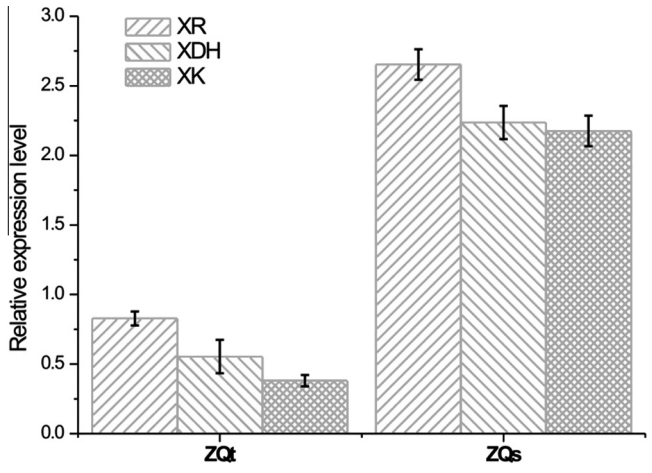

Fig. 2. Transcription analysis of *PsXR*, *PsXDH* and *ScXK* in the tandem integration strain ZQt and scattered integration strain ZQs. Graph represents average ± SD of the triplicate experiments.

Table 1
Enzyme activities of PsXR, PsXDH and ScXK in ZQt and ZQs.[a]

Strain	PsXR-NADPH (U/mg of protein)	PsXR-NADH (U/mg of protein)	PsXDH-NAD (U/mg of protein)	ScXK-NADH (U/mg of protein)
ZQt	0.12 ± 0.02	0.03 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
ZQs	0.16 ± 0.04	0.09 ± 0.01	0.09 ± 0.02	0.07 ± 0.03

[a] Values are the averages of triplicate experiments ± SD.

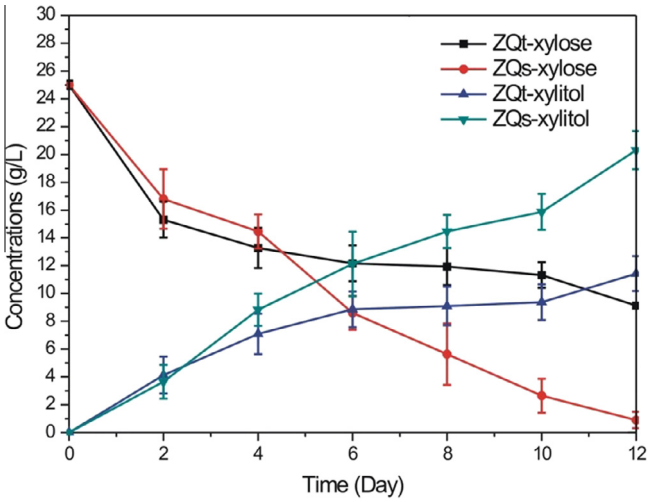

Fig. 3. Batch fermentation using 50 g L^{-1} glucose and 25 g L^{-1} xylose. Symbols: black square-xylose consumption of ZQt, red circle-xylose consumption of ZQs, blue triangle-xylitol production of ZQt, aquamarine triangle-xylitol production of ZQs. The results shown were the mean values of the triplicate experiments. (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

After verification of expression change caused by position change on molecular basis, fermentation experiments were also performed to evaluate xylose utilization of the two recombinant strains. Significant improvement of xylose utilization was observed when scattered integration was performed. Strain ZQs was capable to completely consume all the xylose, whereas ZQt only consumed about 60% xylose (15.9 ± 1.2 g L^{-1}) in the medium (Fig. 3). It is worth noting that all the results reported here were obtained using original recombinant strains without any adaptation in xylose. By strain adaptation, the evolved ZQs strain was able to consume 11 g L^{-1} xylose completely within 72 h, and further improvement of its xylose utilization performance is now under investigation (unpublished data). The consistent results of mRNA, enzyme activity and sugar mixture fermentation demonstrated that scattered integration strategy benefits xylose utilization in genetically engineered *S. cerevisiae* strain.

Our results agree with the previous observations that position effect exerts influence when one gene was integrated into different positions on the same chromosome or on different chromosomes [14–17]. We also demonstrated that when a heterologous pathway containing multiple genes are required to be manipulated, scattered integration offers an alternative way to adjust gene expression to achieve improved functional performance. We believe that such integration design will also arouse the interest in researchers in the field of metabolic engineering of other microorganisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.046.

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