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Screening of Yeast Diauxic Promoters for Production of Foreign Proteins

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Abstract This study explored yeast diauxic promoters using a green fluorescent protein (GFP) reporter to screen growth phase-controlled promoters applicable for foreign protein production. Twenty-five diauxic promoters were inserted into a yeast 2-micron vector in front of the reporter GFP gene. The expressed GFP signal intensity measurements showed that 23 out of the 25 promoters produced a significant fluorescent signal when the cells were in the diauxic growth phase. Among the two strongest promoters pYDL204W and pYLR258W, the former remained constantly active after its activation at the diauxic shift, whereas the latter was only transiently activated right after the deprivation of the medium glucose.

Key words: Diauxic promoter, glucose, yeast, GFP reporter, DNA microarray

Saccharomyces cerevisiae has already been exploited as an ideal host for the production of foreign proteins, such as enzymes and antigenic proteins, because of its well-defined molecular biology and classification as a Generally Recognized As Safe (GRAS) strain [7, 8, 12]. However, despite its advantageous features, the use of *S. cerevisiae* in protein production has been restricted because of a low level of protein expression. The identification of efficient promoters to address this problem has already been attempted by several research groups using promoters from foreign origins [7, 12]. However, in the present study, the diauxic phase of yeast cell growth was examined to identify strong promoters for the binding of RNA polymerase that would eventually lead to the over production of the mRNA of the target gene. The use of a promoter from the diauxic phase may be advantageous over an inducible promoter, as the expression of a gene under the control of the former can be spontaneously induced upon depletion of the medium glucose, thereby excluding the need for inducer molecules. Furthermore, the overall cellular protein concentration in the diauxic phase is relatively lower than that in the active growth phase, which may ease the purification procedure.

S. cerevisiae metabolizes glucose preferentially through fermentation to produce ATP and ethanol. When the medium glucose is exhausted, the cells then switch the metabolic pathway into oxidative respiration, catabolizing the accumulated ethanol via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation in the mitochondria [3]. This phenomenon is known as the "diauxic shift," which is a complex biochemical process that requires drastic changes in the cellular transcriptional activity for numerous yeast genes, including the genes for heat-shock proteins, metabolic enzymes, and the proteins related to control of the cellular reduction potential [2]. Evidence has shown that protein kinases, such as cyclin-dependent protein kinases Pho85, PKA, and Snf1, play regulatory roles in this complex process through mediating the binding of their downstream components onto the specific diauxic promoters [4, 11]. However, the detailed mechanisms of the regulation of most diauxic gene expression are largely unknown.

Accordingly, to identify yeast diauxic promoters suitable for the production of foreign proteins, the transcriptional activity of 25 yeast promoters was investigated using a green fluorescent protein (GFP) as the reporter. The 25 promoters, all of which had been shown to exhibit a more than 9-fold increase in mRNA expression upon the exhaustion of glucose, were selected from previously reported DNA microarray results [2]. The transcriptional activity of each promoter was examined by measuring the GFP fluorescence intensity under a fluorescent microscope and by immunoblot analysis.

The *S. cerevisiae* W303 strain (*leu*2-3, 112 *ura*3-52 *ade*2-1 *his*3-11 *trp*1-1) was used as the host for the promoter assay. To generate a plasmid vector containing the yeast

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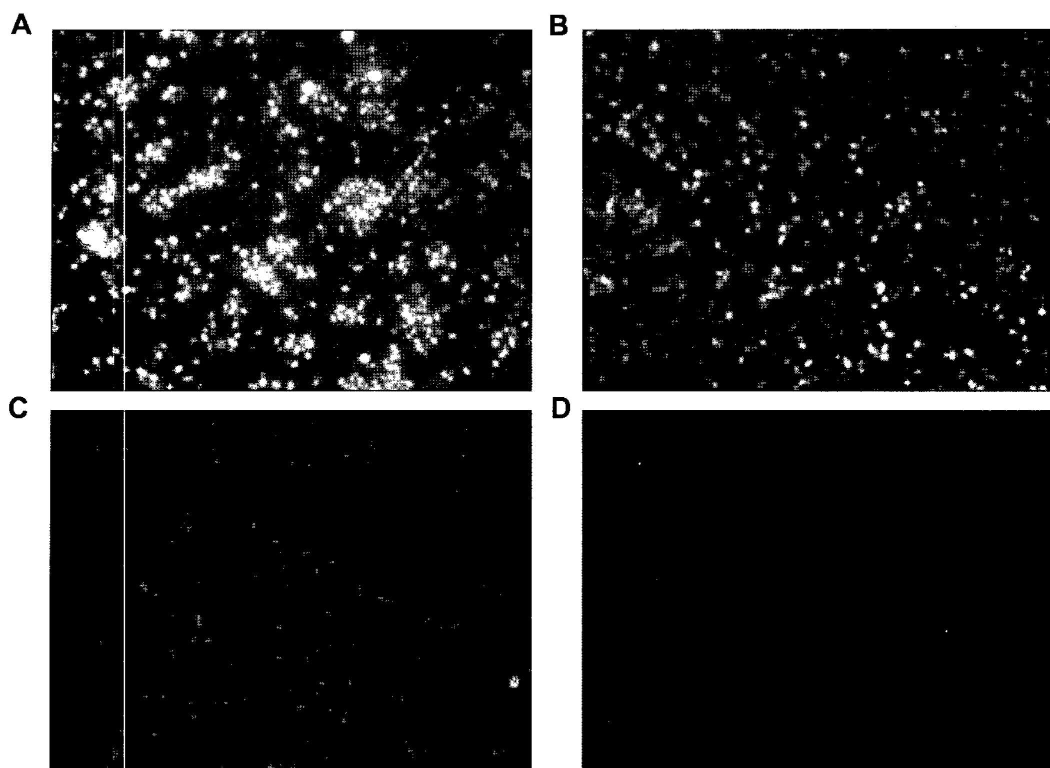

Fig. 1. Fluorescent microscopic detection of GFP expression controlled by the following promoters: pYDL204W (**A**), pYLR258W (**B**), pYNR001C (**C**), and empty vector, pRS425 (**D**).
Cells harboring the pRS425-promoter-GFP were grown until the medium glucose was exhausted.

diauxic promoter and reporter GFP genes, the GFP gene was PCR-amplified from the plasmid pFA6a-GFP-KanMX [9] using a primer set containing a BamHI site at the 5'-end and SacI site at the 3'-end. The PCR fragment was then purified and ligated into the BamHI/SacI sites of a 2-micron multicopy vector, pRS425, that contained *LEU2* as a selective marker, generating pRS425-GFP. To insert the yeast diauxic promoters just in front of the GFP gene, the promoter regions, 500 bp upstream from the start codon of the diauxic genes, were PCR-amplified from a yeast chromosome using primer sets containing specific restriction enzyme sequences. The resulting DNA fragments were then digested with restriction enzymes and ligated into the ApaI/XhoI sites of pRS425-GFP, generating the plasmid constructs pRS425-promoter-GFP. Each pRS425-promoter-GFP was introduced to the yeast cells using lithium acetate transformation protocols [5]. The recombinant *S. cerevisiae* strain carrying the vector was then grown in minimal media (SM), containing a yeast nitrogen base (6.7 g/l), amino acid mixture except for leucine (0.72 g/l, CSM-LEU, Qbiogene, U.S.A.), and glucose (20 g/l) at 30°C.

To investigate the transcriptional activity of the diauxic promoters, the yeast cells harboring the pRS425-promoter-GFP plasmids were grown in an SM-glucose medium until the medium glucose was exhausted. In the presence of a high concentration of glucose, no GFP signal was detected (data not shown). Upon depletion of the medium glucose, as confirmed by a dinitrosalicylate (DNS) assay [10], the cells were harvested for analysis of the GFP expression. The expressed GFP fluorescence was analyzed by a fluorescence microscope (Olympus AX80 model) using a 40× objective with a GFP filter. As shown in Fig. 1, the yeast cells exhibited different levels of GFP fluorescence depending on the strength of the promoters, whereas the empty plasmid vector pRS425 did not show any significant fluorescence signal (Fig. 1D). The promoters for the open reading frames YDL204W (pYDL204W) and YLR258W (pYLR258W) were mostly active in transcriptional activity, exhibiting the strongest fluorescence signal upon excitation of the GFP (Figs. 1A and 1B). Conversely, the pYNR001C, pYLR327C, and pYNL194C promoters exhibited the minimum fluorescence signal (Figs. 1C and Fig. 2), suggesting weak promoters as regards expressing their target genes.

The observed strength of the 25 diauxic promoters using the GFP reporter was analyzed with image analysis software (Scion Image, Scion Corp., U.S.A.) and the results summarized in Fig. 2. Twenty-three out of the 25 promoters expressed a significant GFP signal. Contrary to the expectation that the cellular mRNA level based on a DNA microarray analysis, as summarized in Table 1, might correlate with

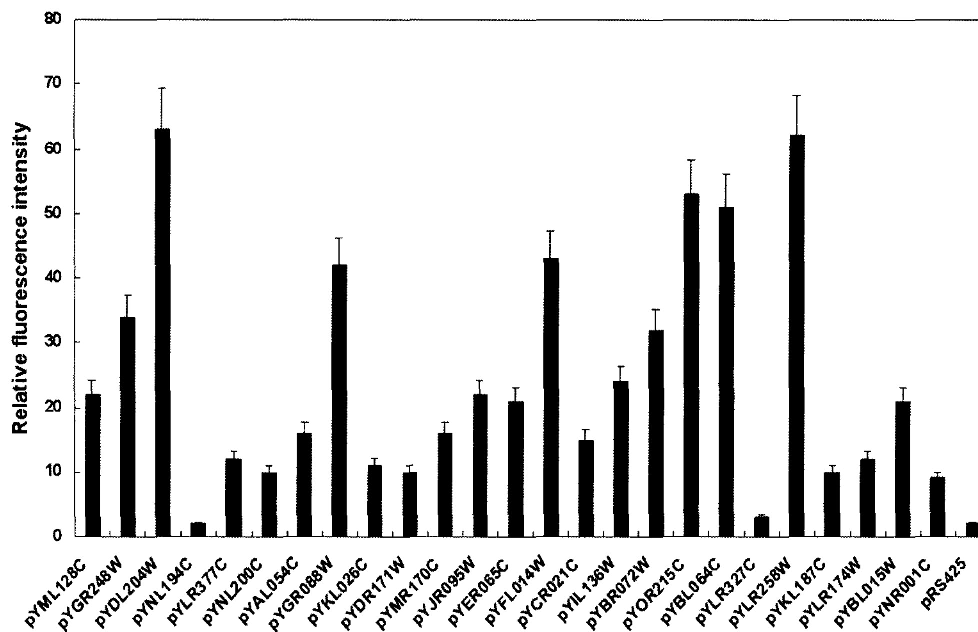

Fig. 2. Transcriptional activity of various promoters. The cell image taken under a fluorescent microscope was analyzed using image analysis software.

Table 1. Characteristics of diauxic genes and transcriptional activation upon diauxic shift derived from DNA microarray analysis.

ORF name	Gene name	Fold increase*	Biochemical function
YML128C	MSC1	20	Meiotic Recombination
YDL204W	RTN2	16.7	Unknown
YGR248W	SOL4	16.7	6-Phosphogluconolactonase
YNL200C		14.3	Unknown
YNL194C		14.3	Unknown
YLR377C	FBP1	14.3	Fructose-bisphosphatase
YKL026C	GPX1	12.5	Glutathione peroxidase
YER065C	ICL1	12.5	Isocitrate lyase
YDR171W	HSP42	12.5	Unfolded protein binding
YCR021C	HSP30	12.5	Unknown
YGR088W	CTT1	12.5	Catalase
YAL054C	ACS1	12.5	Acetate-CoA ligase
YMR170C	ALD2	12.5	Aldehyde dehydrogenase
YJR095W	SFC1	12.5	Succinate:fumarate antiporter
YFL014W	HSP12	12.5	Response to oxidative stress
YBR072W	HSP26	11.1	Unfolded protein binding
YIL136W	OM45	11.1	Unknown
YBL064C	PRX1	11.1	Thioredoxin peroxidase
YOR215C		11.1	Unknown
YLR258W	GSY2	10	Glycogen (starch) synthase
YLR174W	IDP2	10	Isocitrate dehydrogenase
YLR327C	RBF9	10	Unknown
YKL187C		10	Unknown
YBL015W	ACH1	10	Acetyl-CoA hydrolase
YNR001C	CIT1	9.1	Citrate (Si)-synthase

*Adopted from previously reported DNA microarray results [2].

the amount of protein expression, the GFP signal intensity did not directly reflect the DNA microarray data. For example, pYML128C, estimated as the most active diauxic promoter in the DNA microarray results, only exhibited 1/3 of the pYDL204W-controlled GFP signal. More strikingly, pYNL194C, one of the most active promoters in the DNA microarray results, showed virtually no GFP expression. The large discrepancy between the GFP signal intensity described in this study and the transcriptional activity observed in the DNA microarray analysis (Table 1) may reflect the differences in the experimental conditions applied. The expression vector constructed in this study was based on a yeast 2-micron plasmid, and the copy number of the vector was about 20 per haploid yeast cell, meaning that the number of copies of the promoters in this study was 20 times higher than that used in the DNA microarray analysis. Although the copy number of a 2-micron plasmid is generally known to remain stable throughout cell growth, the stability of the plasmid may have been challenged under diauxic conditions. Thus, in response to such a challenge, the copy number of the plasmid may have been affected by the characteristics of each promoter in the plasmid, thereby causing inconsistency between the DNA microarray data [2] and the GFP expression level in this study. Furthermore, various reports have also shown a poor correlation between the concentration of mRNA and the expressed protein level, which can be partially explained by the stability of the mRNA [1, 6].

To analyze the dynamics of the transcriptional activity of the diauxic promoters, the GFP expression was examined

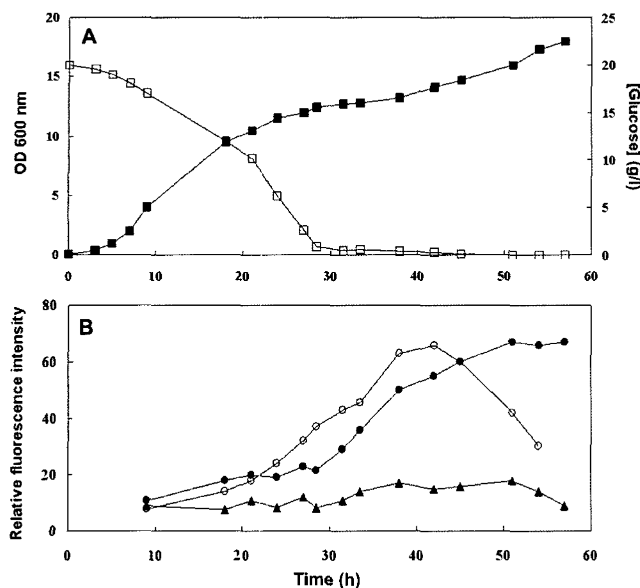

Fig. 3. Dynamics of cell growth and transcriptional activity of diauxic promoters: **A**. Aliquots of the medium were taken to measure the cell growth (OD 600 nm, ■) and concentration of the medium glucose (□). **B**. Expressed GFP signal intensity under the control of the promoters pYDL204W (●), pYLR258W (○), and pYNR001C (▲).

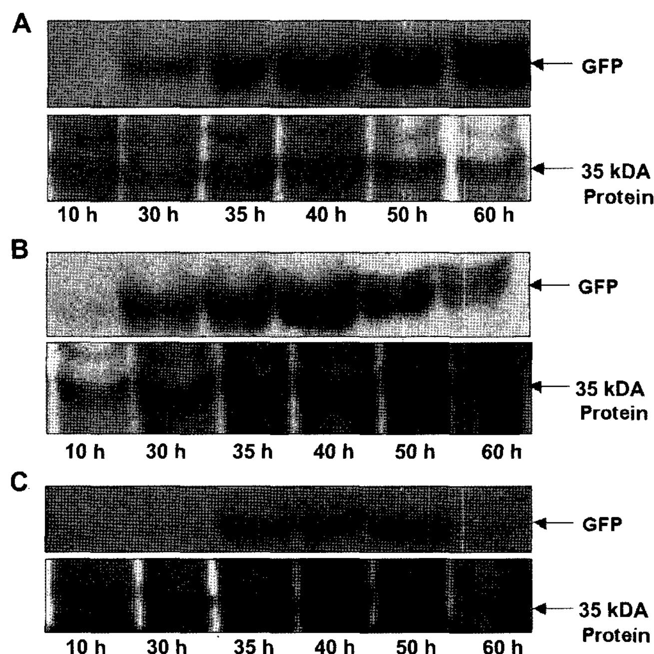

Fig. 4. Immunoblot analysis of expressed GFP protein using anti-GFP antibody.

Expression of GFP under control of pYDL204W (**A**), pYLR258W (**B**), and pYNR001C (**C**). The immunoblot analysis was carried out using the same samples taken for Fig. 3. The Coomassie-stained 35-kDa protein is shown as the loading control. The numbers under the lanes represent the protein samples from the samples taken at the given time.

under the control of three selected promoters (pYLR258W, pYDL204W, and pYNR001C) with time-dependent sampling. The medium glucose was exhausted 28 h after the initiation of the culture, reaching 12 as the optical density at 600 nm (OD 600 nm) in the culture broth (Fig. 3A). The cell growth then apparently stopped and entered a stationary phase until 10 h later. Yet, further incubation of the culture broth showed a gradual increase in the OD 600 nm, indicating that the cells started to metabolize the medium ethanol that had accumulated in the fermentative growth on glucose. While confirming the presence of the "diauxic shift," the GFP expression within the period of diauxic growth was examined using a fluorescence microscope (Fig. 3B) and immunoblot analyses with an anti-GFP antibody (Fig. 4). The pYNR001C promoter expressed the minimum GFP signal throughout the culture period (Figs. 3B and 4C), whereas the pYDL204W and pYLR258W promoters showed a significantly higher GFP expression after the exhaustion of glucose. Under the control of pYDL204W, the expression of the GFP was induced when the cells entered the diauxic period (Figs. 3B and 4A), and continued to gradually increase even after the diauxic period. In contrast, the transcriptional activity of pYLR258W was activated after the exhaustion of glucose and showed a peak at 40 h of cultivation (Figs. 3B and 4B). Further cultivation of the cells essentially diluted the expressed GFP, suggesting that the transcriptional activity of the pYLR258W promoter was turned off after the activation. Consequently, the present results demonstrated that the yeast diauxic promoter pYDL204W may be applicable for the production of foreign proteins, whereas the other strong promoter pYLR258W is only suited for transient protein expression.

Acknowledgments

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