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ORIGINAL PAPER

A Synthetic Hybrid Promoter for Xylose-Regulated Control of Gene Expression in *Saccharomyces* Yeasts

Ronald E. Hector[1] · **Jeffrey A. Mertens**[1]

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Abstract Metabolism of non-glucose carbon sources is often highly regulated at the transcriptional and post-translational levels. This level of regulation is lacking in *Saccharomyces cerevisiae* strains engineered to metabolize xylose. To better control transcription in *S. cerevisiae*, the xylose-dependent, DNA-binding repressor (XylR) from *Caulobacter crescentus* was used to block transcription from synthetic promoters based on the constitutive *Ashbya gossypii TEF* promoter. The new hybrid promoters were repressed in the absence of xylose and showed up to a 25-fold increase in the presence of xylose. Activation of the promoter was highly sensitive to xylose with activity seen at concentrations below 2 μM xylose. These new xylose-inducible promoters allow improved control of gene expression for engineered strains of *Saccharomyces* yeasts.

Keywords Xylose inducible · Promoter · *Saccharomyces* · *Caulobacter crescentus* · Gene regulation

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✉ Ronald E. Hector
ronald.hector@ars.usda.gov

Jeffrey A. Mertens
jeffrey.mertens@ars.usda.gov

[1] Bioenergy Research Unit, National Center for Agricultural Utilization Research, USDA-Agricultural Research Service, 1815 N. University Street, Peoria, IL 61604, USA

Introduction

Economical production of bio-renewable fuels and chemicals will require the utilization of all of the sugars available in lignocellulosic materials. While glucose is the most abundant and is readily metabolized by industrial organisms like *Saccharomyces cerevisiae*, xylose can make up a significant percentage of the available sugar [1]. Natural xylose utilizing organisms coordinately regulate the expression of genes required for xylose metabolism. The *XYL1* and *XYL2* genes, encoding xylose reductase and xylitol dehydrogenase, from five xylose utilizing yeasts are highly induced by xylose, indicating the presence of a xylose-specific transcription factor in yeasts like *Scheffersomyces stipitis* [2]. These transcription factors are lacking in *S. cerevisiae* as expression of the *S. stipitis XYL1* and *XYL2* genes from their native promoters in *S. cerevisiae* resulted in low, constitutive expression of the genes [3, 4].

There is increasing interest in generating a variety of promoters for enhanced control of gene expression in *S. cerevisiae*. Much of this effort has focused on constitutive expression, and there are now a wide variety of options for constitutive expression from low to high levels [5, 6]. Inducible promoters are available for several conditions including the presence of galactose, copper, oxygen depletion, and glucose concentration [6, 7]. Xylose-dependent gene regulation has been lacking for *S. cerevisiae* until recently, where XylR repressors from six different bacteria were investigated for their ability to repress transcription from promoters modified to contain the repressor binding sites [8, 9].

Strains of *S. cerevisiae* engineered for xylose fermentation have been described with up to eight genes over expressed [10]. Constitutive, high level, expression of all the required genes is not necessarily beneficial to the cell.

Even when one gene is over expressed, it can be a waste of resources to express the gene when it is not needed. In certain cases, constitutive expression has been shown to be detrimental. Constitutive expression of *XYL1*, *XYL2*, and *TAL1*, while beneficial for xylose metabolism, decreased specific growth rate and ethanol production from glucose [11]. Additionally, other genes such as *ZWF1* (glucose-6-P dehydrogenase) and *PGI1* (phosphoglucose isomerase) are induced in xylose utilizing yeasts, but have decreased levels in *S. cerevisiae* grown on xylose medium [12–14]. The availability of a xylose-inducible expression system for *S. cerevisiae* would allow fine-tuning the expression of genes required for xylose metabolism, while minimizing the impact of expressing numerous genes.

One possible approach to developing a xylose-specific promoter for *S. cerevisiae* would be to utilize xylose-inducible promoters from fungi. Transcriptional activation of xylanases in *Aspergillus niger* is mediated by the transcription factor XlnR, a zinc binuclear cluster-type DNA-binding protein similar to Gal4p in *S. cerevisiae* [15]. Similarly, Xyr1 from *Trichoderma reesei*, a homolog of XlnR, has been shown to bind to xylanase promoters [16]. XlnR is highly phosphorylated when it is activated by the presence of xylose [17]. However, the identity of the kinase responsible for the xylose-induced phosphorylation is not known. Without knowledge of the protein/signal responsible for activation, the expression system cannot be reconstituted in *S. cerevisiae*.

Transcriptional regulation has also been well-studied in bacteria, and two different types of xylose-regulated transcription have been found. These include promoters based on either transcriptional activation or repression, the latter due to DNA-binding protein interference [18–20]. Activation-type systems potentially have the same problem as using a transcription factor from fungi, which is, not having the proper activation signal in the heterologous organism. Repressor-based transcriptional control can be readily transferred to another organism and typically requires only the modified promoter sequence and expression of the repressor in the target organism [21]. In this study, we expressed the sequence-specific DNA-binding repressor protein XylR from *Caulobacter crescentus* in *S. cerevisiae*. Strains expressing the XylR protein were then used to identify synthetic hybrid promoters containing the XylR binding site sequence that also showed xylose-dependent regulation.

Materials and Methods

Strains, Media, and General Methods

Escherichia coli strain NEB10β (NEB; Beverly, MA, USA) was used for routine maintenance and preparation of plasmids, and cells were grown in LB medium [22]. Yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Plasmid DNA was transformed into yeast cells using a standard lithium acetate method [23]. Synthetic complete medium (SC) consisted of 6.7 g/l Difco yeast nitrogen base (YNB) (United States Biological; Marblehead, MA, USA) and was supplemented with amino acids [24]. For maintenance of plasmids, media was made without tryptophan or leucine as necessary. Synthetic medium was filter sterilized, and sterile carbon sources were added separately. For integration of the repressor and promoter::*lacZ* fusion cassettes into the genome, *Not*I-linearized pRH561 (P_{TEF}) and *Not*I-linearized pRH564 ($P_{TEF\text{-}xylO2\text{-}1}$) plasmids were transformed into *S. cerevisiae* strain CEN.PK2-1C. After a 4-h recovery in YPD, cells that had integrated the plasmid were selected on YPD plates containing G418 (200 mg/L).

Transcriptional Activity Assays

The Beta-Glo Assay system (Promega; Madison, WI, USA) was used to determine the level of transcriptional activity from promoter::*lacZ* fusions, essentially as reported in [25]. Cells from cultures in log-phase (0.2–0.5 OD_{660}) grown in SC medium were diluted in fresh medium to a final $OD_{660} = 0.004$. Unless specified, carbon sources used were either 2% sucrose or 2% each sucrose and xylose. Each assay was initiated using the same amount of cell mass to minimize variation due to differing cell concentrations. Assays were started by adding 50 µL of diluted cells to 50 µL of Beta-Glo reagent, mixed thoroughly, and incubated at room temperature. Using these conditions, activity measurements were stable from 60 to 120 min. All assays were performed in 96-well, opaque (white), flat-bottomed microtiter plates. During 60–90 min, the samples were read using the luminescence mode of a SpectraMax M5 microplate reader (Molecular Devices; Sunnyvale, CA, USA). Promoter activity (i.e., β-galactosidase activities) reported in figures is based on the Relative Light Units (RLU in millions), normalized to starting cell mass. Unless specified, promoter activity assays were performed using centromere-based plasmids to express both the repressor and *lacZ* marker. Assays were performed at least in triplicate.

Construction of XylR Repressor Variants and Wild-Type and Xylose-Regulated Promoters

DNA fragments for cloning were amplified using Phusion High-fidelity DNA polymerase (NEB). The codon-optimized XylR gene from *Caulobacter crescentus* (accession # AAK25027) was synthesized (DNA2.0; Menlo Park, CA, USA) and cloned into vectors for expression in *S.*

Table 1 Microorganisms used in this study

Strain	Genotype (description)	Source
CEN.PK2-1C[a]	*S. cerevisiae MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8^C SUC2*	Euroscarf
YRH1054	CEN.PK[a] [pRH511 (P_{TEF} − *lacZ* − T_{ADH1}) + pRS414[b]]	This work
YRH1055	CEN.PK [pRH511 (P_{TEF} − *lacZ* − T_{ADH1}) + pRH483 (P_{HXT7} − NLS-XylR-*SSN6* − T_{HXT7})]	This work
YRH1056	CEN.PK [pRH512 ($P_{TEF\text{-}xylO1}$ − *lacZ* − T_{ADH1}) + pRS414]	This work
YRH1057	CEN.PK [pRH512 ($P_{TEF\text{-}xylO1}$ − l*acZ* − T_{ADH1}) + pRH483 (P_{HXT7} − NLS-XylR-*SSN6* − T_{HXT7})]	This work
YRH1058	CEN.PK [pRH513 ($P_{TEF\text{-}\ xylO2\text{-}2}$ − *lacZ* − T_{ADH1}) + pRS414]	This work
YRH1059	CEN.PK [pRH513 ($P_{TEF\text{-}\ xylO2\text{-}2}$ − l*acZ* − T_{ADH1}) + pRH483 (P_{HXT7} –NLS-XylR-*SSN6* − T_{HXT7})]	This work
YRH1060	CEN.PK [pRH514 ($P_{TEF\text{-}\ xylO2\text{-}1}$ − *lacZ* − T_{ADH1}) + pRS414]	This work
YRH1061	CEN.PK [pRH514 ($P_{TEF\text{-}\ xylO2\text{-}1}$ − l*acZ* − T_{ADH1}) + pRH483 (P_{HXT7} − NLS-XylR-*SSN6* − T_{HXT7})]	This work
YRH1156	CEN.PK [pRH546 ($P_{TEF\text{-}UAS\text{-}\ xylO1}$ − *lacZ* − T_{ADH1}) + pRS414]	This work
YRH1157	CEN.PK [pRH546 ($P_{TEF\text{-}UAS\text{-}\ xylO1}$ − *lacZ* − T_{ADH1}) + pRH483 (P_{HXT7} –NLS-XylR-*SSN6* − T_{HXT7})]	This work
YRH1158	CEN.PK [pRH547 ($P_{TEF\text{-}UAS\text{-}\ xylO2}$ − *lacZ* − T_{ADH1}) + pRS414]	This work
YRH1159	CEN.PK [pRH547 ($P_{TEF\text{-}UAS\text{-}\ xylO2}$ − *lacZ* − T_{ADH1}) + pRH483 (P_{HXT7} –NLS-XylR-*SSN6* − T_{HXT7})]	This work
YRH1160	CEN.PK [pRH548 ($P_{TEF\text{-}\ xylO3}$ − *lacZ* − T_{ADH1}) + pRS414]	This work
YRH1161	CEN.PK [pRH548 ($P_{TEF\text{-}\ xylO3}$ − *lacZ* − T_{ADH1}) + pRH483 (P_{HXT7} − NLS-XylR-*SSN6* − T_{HXT7})]	This work
YRH1162	CEN.PK [pRH549 ($P_{TEF\text{-}\ xylO4}$ − *lacZ* − T_{ADH1}) + pRS414]	This work
YRH1163	CEN.PK [pRH549 ($P_{TEF\text{-}\ xylO4}$ − *lacZ* − T_{ADH1}) + pRH483 (P_{HXT7} –NLS-XylR-*SSN6* − T_{HXT7})]	This work
YRH1208	CEN.PK *hoΔ*::P_{HXT7} − NLS-XylR-*SSN6* − T_{HXT7} ::P_{TEF} − *lacZ* − T_{ADH1} :: *KanMX*	This work
YRH1211	CEN.PK *hoΔ*::P_{HXT7} − NLS-XylR-*SSN6* − T_{HXT7} ::$P_{TEF\text{-}\ xylO2\text{-}1}$ − *lacZ* − T_{ADH1} :: *KanMX*	This work
YRH1216	CEN.PK [pRH483] *hoΔ*::P_{HXT7} − NLS-XylR-*SSN6* − T_{HXT7} ::P_{TEF} − *lacZ* − T_{ADH1} :: *KanMX*	This work
YRH1217	CEN.PK [pRH483] *hoΔ*::P_{HXT7} − NLS-XylR-*SSN6* − T_{HXT7} ::$P_{TEF\text{-}\ xylO2\text{-}1}$ − *lacZ* − T_{ADH1} :: *KanMX*	This work
YRH1227	CEN.PK [pRH514 ($P_{TEF\text{-}\ xylO2\text{-}1}$ − *lacZ* − T_{ADH1}) + pRH467 (P_{HXT7} − NLS − XylR − T_{HXT7})]	This work
YRH1239	CEN.PK [pRH568 (P_{PGK1} − *lacZ* − T_{ADH1})]	This work
YRH1240	CEN.PK [pRH569 (P_{ADH1} − *lacZ* − T_{ADH1})]	This work
YRH1241	CEN.PK [pRH570 (P_{PDC1} − *lacZ* − T_{ADH1})]	This work
YRH1242	CEN.PK [pRH571 (P_{HXT7} − *lacZ* − T_{ADH1})]	This work
YRH1276	CEN.PK [pRH514 ($P_{TEF\text{-}\ xylO2\text{-}1}$ − *lacZ* − T_{ADH1}) + pRH467 (P_{HXT7} − XylR − T_{HXT7})]	This work

[a] CEN.PK strain designation refers to CEN.PK2-1C

[b] Plasmid pRS414 was included as an empty vector control

cerevisiae using restriction endonuclease sites added to the ends of the gene. To facilitate entry into the nucleus, the SV40 large T-antigen nuclear localization signal (PKKKRKV) was added to the amino terminus of the XylR gene by PCR amplification of the XylR gene using primers *Spe*I-NLS-XylR-F (5′-GGACTAGTGCATGCCCAAGAAGAAAAGGAAAGTTAATCAACCAGTAGAAAGACAGCGTAGG-3′) and *Sal*I-XylR-R (5′-GCGTCGACTTATTAGACTCCAGCAGGGCCTGAGG-3′) which contained the NLS sequence. The *Spe*I-*Sal*I fragment-containing NLS-XylR was cloned into *Spe*I-*Sal*I digested expression vector pRH164 to create pRH467. Fusion of the NLS-XylR repressor to the *S. cerevisiae SSN6* gene was done by overlap PCR using primers *Spe*I-NLS-XylR-F (5′-GGACTAGTGCATGCCCAAGAAGAAAAGG-3′), XylR-linker-R (5′- GAACCTCCACCTCCGGAACCGACTCCAGCAGGGCCTGAG-3′), linker-*SSN6*-F (5′- GGTTCCGGAGGTGGAGGTTCTATGAATCCGGGCGGTGAAC-3′), and *SSN6*-SalI-R (5′-GGTCGACTTAGTCGTCGTAGTTTTCATCTTCTTCCACTTG-3′). A short flexible linker peptide (GSGGGGS) was used to separate the XylR and *SSN6* coding regions. The fusion product from the overlap PCR was ligated to pCR2.1, and the plasmid (pRH481) was sequenced to confirm that no mutations occurred during PCR amplification of the DNA fragments. The *Spe*I-*Sal*I fragment from pRH481, containing NLS-XylR-*SSN6,* was then sub-cloned into *Spe*I-*Sal*I digested pRH164 to create pRH483, which was used as the main repressor throughout this study.

The *Ashbya gossypii TEF* promoter (Accession # X73978) and derivatives of the promoter containing varying numbers of the operator sequence (*xylO*) for binding the XylR repressor were synthesized (GenScript; Piscataway, NJ, USA). Promoters were sub-cloned into vectors for expression in *S. cerevisiae* using *Sac*I/*Spe*I restriction endonuclease sites added to the ends of the synthesized DNA fragments. To create expression vectors pRH511 − pRH514 and pRH546 − pRH549 containing

Table 2 Plasmids used in this study

Plasmid	Description	Reference
pRS414	pBluescript II SK + , *TRP1, CEN6, ARSH4*	[41]
pRS415	pBluescript II SK + , *LEU2, CEN6, ARSH4*	[41]
pUC57	Gene synthesis vector	(GenScript)
pJ201	Gene synthesis vector	(DNA2.0)
YEp353	*lacZ* fusion vector	[42]
pRH164[a]	pRS414 + P_{HXT7} − MCS − T_{HXT7}	[43]
HO-Poly-KanMX4-*HO*	Vector for targeted integration at the *HO* locus	[44]
pRH457	pJ201 + *C. crescentus* XylR[b]	(DNA2.0)
pRH463	pRS414 + P_{HXT7} − XylR − T_{HXT7}	This work
pRH467	pRS414 + P_{HXT7} − NLS-XylR − T_{HXT7}	This work
pRH481	pCR2.1 + NLS[c] − XylR − *SSN6*	This work
pRH483	pRS414 + P_{HXT7} − NLS − XylR − *SSN6* − T_{HXT7}	This work
pRH497	pRS415 + *HIS3*	This work
pRH498	pRS415 + his3::*lacZ*	This work
pRH499	pUC57 + *Ashbya gossypii TEF* promoter (P_{TEF})	(GenScript)
pRH500	pUC57 + *TEF* promoter + 1 xylO ($P_{TEF\text{-}xylO1}$)	(GenScript)
pRH501	pUC57 + *TEF* promoter + 2 xylO ($P_{TEF\text{-}xylO2\text{-}1}$)	(GenScript)
pRH502	pUC57 + *TEF* promoter + 2 xylO ($P_{TEF\text{-}xylO2\text{-}2}$)	(GenScript)
pRH511	pRS415 + P_{TEF} − *lacZ* − T_{ADH1}	This work
pRH512	pRS415 + $P_{TEF\text{-}xylO1}$ − *lacZ* − T_{ADH1}	This work
pRH513	pRS415 + $P_{TEF\text{-}xylO2\text{-}2}$ − *lacZ* − T_{ADH1}	This work
pRH514	pRS415 + $P_{TEF\text{-}xylO2\text{-}1}$ − *lacZ* − T_{ADH1}	This work
pRH531	pUC57 + *TEF* promoter + 1 xylO at UAS ($P_{TEF\text{-}UAS\text{-}xylO1}$)	(GenScript)
pRH532	pUC57 + *TEF* promoter + 2 xylO at UAS ($P_{TEF\text{-}UAS\text{-}xylO2}$)	(GenScript)
pRH533	pUC57 + *TEF* promoter + 3 xylO ($P_{TEF\text{-}xylO3}$)	(GenScript)
pRH534	pUC57 + *TEF* promoter + 4 xylO ($P_{TEF\text{-}xylO4}$)	(GenScript)
pRH546	pRS415 + $P_{TEF\text{-}UAS\text{-}xylO1}$ − *lacZ* − T_{ADH1}	This work
pRH547	pRS415 + $P_{TEF\text{-}UAS\text{-}xylO2}$ − *lacZ* − T_{ADH1}	This work
pRH548	pRS415 + $P_{TEF\text{-}xylO3}$ − *lacZ* − T_{ADH1}	This work
pRH549	pRS415 + $P_{TEF\text{-}xylO4}$ − *lacZ* − T_{ADH1}	This work
pRH561	*HO* − Poly- KanMX4- *HO* + P_{HXT7} − NLS-XylR-*SSN6* − T_{HXT7} ::P_{TEF} − *lacZ* − T_{ADH1}	This work
pRH564	*HO*-Poly-KanMX4-*HO* + P_{HXT7} − NLS-XylR-*SSN6* − T_{HXT7} ::$P_{TEF\text{-}xylO2\text{-}1}$ -*lacZ*-T_{ADH1}	This work
pRH568	pRS415 + P_{PGK1} − *lacZ* − T_{ADH1}	This work
pRH569	pRS415 + P_{ADH1} − *lacZ* − T_{ADH1}	This work
pRH570	pRS415 + P_{PDC1} − *lacZ* − T_{ADH1}	This work
pRH571	pRS415 + P_{HXT7} − *lacZ* − T_{ADH1}	This work

[a] The *HXT7* promoter (P_{HXT7}) used in this work refers to the truncated, constitutive promoter, containing 390 nucleotides 5′ of the *HXT7* ORF [45]

[b] The *C. crescentus* XylR gene used throughout this study was codon optimized for expression in *S. cerevisiae*

[c] The NLS used in this study was from SV40 large T-antigen, and the sequence (MPKKKRKV) was fused to the amino terminus of XylR

the various promoter::*lacZ* fusions, a DNA fragment containing a *his3-lacZ* fusion from pRH498 was sub-cloned into plasmids containing the various promoters using *Spe*I and *Sal*I restriction sites. Plasmid pRH498 was created by replacing the *Hind*III-*Sal*I fragment of *HIS3* with a *Hind*III-*Sal*I *lacZ* fragment that was PCR amplified from the *lacZ* fusion vector YEp353 using primers *Hind*III-*lacZ*-F (5′-CGACCTGCAGCCAAGCTTGCGATCC-3′) and *Sal*I-*lacZ*-R (5′-GCGTCGACCCTGCCCGGTTATTATTATTTTTGACAC-3′). This latter cloning step generated an in-frame fusion of the *lacZ* gene to a short N-terminal fragment of the *HIS3* gene and served as the *lacZ* marker for

transcription activity assays. Nucleotide sequences for all promoter and repressor variants are available in supplemental Table S1.

Statistical Analyses

For experiments with three or greater biological replicates, probability analyses were performed using the Student's *t* test with a two-tailed distribution and compared to the appropriate control strain. Values with $P < 0.05$ were considered significant for this study. Statistical analysis was performed using Microsoft Excel.

Results and Discussion

Comparison of Xylose-Dependent Regulation from Hybrid Promoters

The *Ashbya gossypii TEF* promoter is a constitutive promoter that works well in *S. cerevisiae* [26]. The *A. gossypii TEF* promoter has two TATA sites with transcription most likely initiating from the TATA at position -102, relative to the *A. gossypii TEF* ATG codon [27]. In this study, the second TATA sequence present at position -63 was mutated to reduce the possibility of transcription initiation at this location under conditions where the main site is inhibited. This type of regulation is observed with the *CYC1* promoter, where inhibition of the main TATA sites leads to increased use of downstream sites [28]. Activity of the *TEF* promoter containing the mutated TATA site was comparable to constitutive *S. cerevisiae* promoters, indicating that mutation of the TATA at position -63 had minimal effect on promoter activity (supplemental Fig. S1).

Our initial efforts using this promoter focused on inhibiting assembly of the RNA polymerase complex by placing *xylO* operator sites for binding XylR around the TATA element (Fig. 1). A twenty-base pair *xylO* sequence, 5′-ACA*TGTTAGCGCTACCA*AGT-3′, encompassing the XylR binding site from the promoter region of the *Caulobacter crescentus xylX* gene was incorporated at various positions in the *TEF* promoter. In the absence of xylose, the XylR repressor protein from *C. crescentus* binds to a 14 bp DNA sequence in the *xylO* operator. When xylose is present, XylR was shown to dissociate from DNA containing this sequence [20], indicating that this operator sequence could be used to conditionally inhibit transcription from a synthetic promoter (Fig. 1). A similar strategy was used with $tetO_2$ operator sites that bind to the TetR repressor protein in the absence of tetracycline to create a Tet de-repressible promoter based on the *GAL1* promoter from *S. cerevisiae* [29]. This approach was also used more recently to create xylose-regulated gene expression in *S. cerevisiae* [8, 9].

The repressor used to assay promoter activity from hybrid promoters containing the *C. crescentus xylO* sites was modified to contain an amino-terminal nuclear localization signal (NLS) and a C-terminal yeast chromatin remodeling protein, Ssn6p. The nuclear localization tag was added to the N-terminus of the *C. crescentus* XylR repressor (37.4 kDa) to facilitate transport through the nuclear pore, which has a 20-40 kDa diffusion limit, and into the yeast nucleus [30, 31]. Fusion of XylR to the *S. cerevisiae* protein Ssn6p (i.e., NLS-XylR-Ssn6p) was done to enhance repression when bound to the hybrid promoters. Ssn6p interacts with Tup1p and the Ssn6p/Tup1p complex is a general repressor of transcription which is thought to modify the chromatin structure around the TATA element and transcription initiation site [32]. Fusing Ssn6p to the DNA-binding protein lexA and targeting it to a hybrid promoter-containing lexA binding sites has been shown to repress transcription [33].

It was possible that replacing DNA sequence around the TATA element with *xylO* sequence could result in a non-functional promoter. To determine if incorporating *xylO* sites into the *TEF* promoter had a negative effect on promoter activity, strains with and without expression of the repressor were analyzed (Fig. 2). In cells without the repressor, addition of 1 *xylO* site immediately downstream of the TATA element (i.e., promoter $P_{TEF\text{-}xylO1}$) resulted in decreased promoter activity. When assayed in cells expressing repressor, activity from promoter $P_{TEF\text{-}xylO1}$ was significantly repressed relative to cells not expressing the repressor. Adding xylose to the medium reversed the repression. Promoter $P_{TEF\text{-}xylO2\text{-}2}$ contained 2 *xylO* sites downstream of the TATA element. This promoter showed even less activity compared to the parent promoter but was still regulated by xylose. The best performing promoter, $P_{TEF\text{-}xylO2\text{-}1}$, contained 2 *xylO* sites flanking the TATA element. This promoter had activity comparable to the strong constitutive *S. cerevisiae* promoters (Fig. 2 and supplemental Fig. S1). It also showed the most repression of transcription in the absence of xylose. An 8.4-fold induction (or de-repression) was observed when xylose was added to the medium (Fig. 2, strain 1061, + repressor). Induction with this promoter/repressor system was slightly higher than that obtained with other xylose-regulated expression systems which led to 1.8- to 8-fold induction [8, 9].

Disrupting the interaction of enhancers at UAS sites has also been an effective approach for regulating promoters. The *A. gossypii TEF* promoter has two UAS_{rpg} sites, and removal of these sites results in a non-functional promoter [27]. The yeast protein Rap1p has been shown to bind UAS_{rpg} sites, and these sites are able to activate

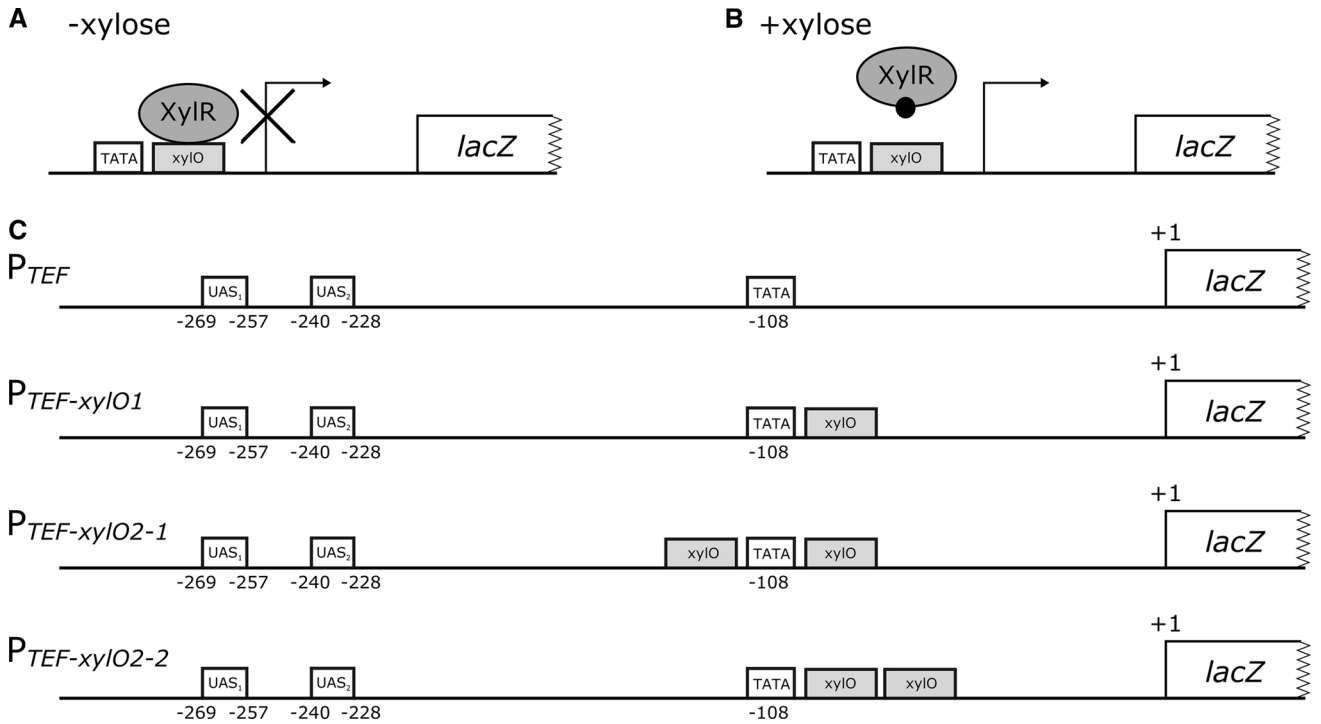

Fig. 1 Diagram showing the synthetic promoters. **a** Cartoon depiction showing XylR binding the sequence-specific *xylO* operator in the absence of xylose. **b** In the presence of xylose, XylR dissociates from the DNA allowing transcription to occur. **c** Using the *Ashbya gossypii TEF* promoter, hybrid promoters were generated using *xylO* sequence to replace DNA around the TATA element. Positions shown are relative to the *lacZ* start codon

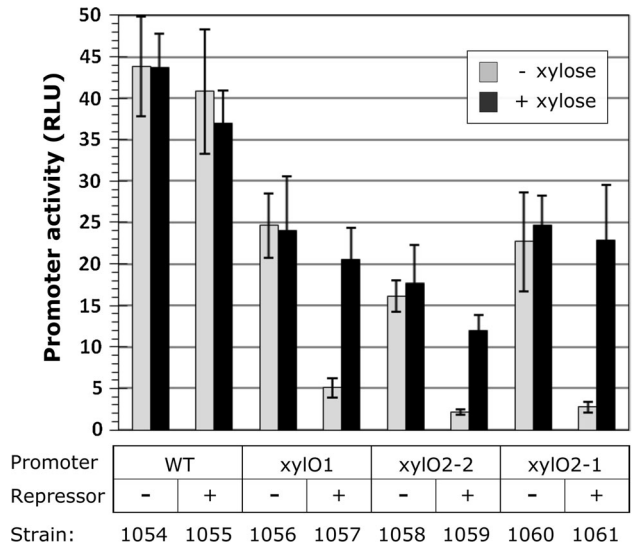

Fig. 2 Promoter activity of hybrid *TEF* promoters containing varying numbers of *xylO* sites. Promoter activity was measured as beta-galactosidase activity from expression of the *lacZ* marker gene fused to each promoter. Both the repressor and promoter::*lacZ* fusions were maintained on low-copy plasmids. Activity measurements are in relative light units (RLU, millions) and are normalized to cell mass. Activity from each promoter was measured at least three times. *Error bars* represent the standard deviation

transcription when added to minimal promoters [34]. To interfere with Rap1p binding and further increase repression, *xylO* sequence was used to replace sequence at various positions around the UAS_{rpg} activation sites. With the exception of $P_{TEF\text{-}xylO3}$, increased repression and/or xylose-dependent gene expression were not observed, and this approach was not pursued further (supplemental Fig. S2).

Integration of the P_{TEF} and $P_{TEF\text{-}xylO2\text{-}1}$ Promoters into the Genome

Plasmid stability in cells is rarely near 100% in *S. cerevisiae*. Selective pressure was maintained for plasmids used in this study to determine promoter activity; however, even under these conditions, 20–30% of the cells may have lost the plasmid [35]. Cells without the repressor plasmid would be able to activate the hybrid promoter, leading to elevated promoter activity under repressive conditions. To ensure that every cell expressed the repressor, both the repressor and promoter::*lacZ* fusion were integrated into the genome. When grown on medium lacking xylose, transcription was repressed in strains with the hybrid promoter compared to strains with the WT promoter (Fig. 3). As seen previously with plasmid-based promoters, activity

increased when xylose was present in the medium (Fig. 3, strain 1211). Compared to promoter activity with plasmids (Fig. 2, strain 1061), transcriptional repression was not significantly increased when the repressor was integrated into genome, and some promoter activity was still seen in the absence of xylose. To determine if the low level of promoter activity seen in strain 1211 was due to the repressor not fully occupying the *xylO* sites, additional repressor was expressed from the low-copy plasmid pRH483 (strains 1216 and 1217). A larger increase in repression on was observed in cells with extra repressor, resulting in a 25-fold induction in the presence of xylose (Fig. 3, strain YRH1217). This latter result suggested that repressor expression may be limiting and improvement in repression can be made by increasing the amount of repressor available in the cell.

Effect of XylR Modifications on Repression of the $P_{TEF\text{-}xylO2\text{-}1}$ Promoter

As mentioned previously, the repressor used in these previous experiments was modified to contain an NLS and a C-terminal fusion to the yeast chromatin remodeling protein Ssn6p. To determine the impact of the added elements, we tested different combinations lacking the modifications (e.g., NLS-XylR and XylR). When xylose was added to the medium, activity from the $P_{TEF\text{-}xylO2\text{-}1}$ promoter was similar for all repressor variants (Fig. 4). In the absence of xylose, the highest level of repression was observed with the NLS-XylR-Ssn6p repressor (Fig. 4, strain YRH1061). Removing the Ssn6p fusion decreased repression slightly, but the difference was not statistically significant (Fig. 4, strain 1227). Strain 1276, expressing just the native XylR, lacking both the NLS and Ssn6 fusion, further decreased the repression. These data suggested that while the NLS was beneficial, it was not absolutely required. The *C. crescentus* XylR (37.4 kDa) is at the top range of the passive diffusion limit through nuclear pore complexes and would not be expected to enter the nucleus efficiently in the absence of an NLS. Further inspection of the XylR sequence showed a highly charged amino terminus with a run of basic amino acids (e.g., RQRRR). Many NLS sequences contain a short cluster of basic amino acids which have been shown to contribute to NLS function [36]. It is possible that the N-terminal region of the *C. crescentus* XylR protein (MNQPVERQRRR) provides some function as a NLS in *S. cerevisiae*.

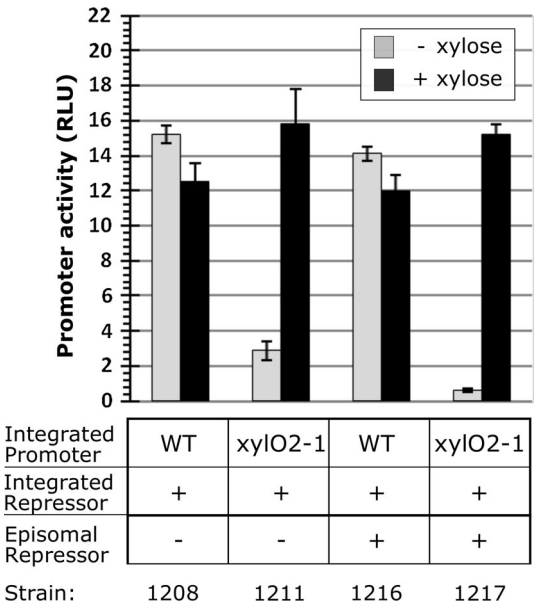

Fig. 3 Promoter activity of the chromosomally integrated xylose-regulated promoter $P_{TEF\text{-}xylO2\text{-}1}$. Promoter activity was measured as beta-galactosidase activity from expression of the *lacZ* marker gene fused to each promoter. In all the strains, repressor was also integrated into the genome. For strains with additional repressor, extra repressor was plasmid-based (i.e., episomal repressor). Activity measurements are in relative light units (RLU, millions) and are normalized to cell mass. Activity from each promoter was measured at least three times. *Error bars* represent the standard deviation

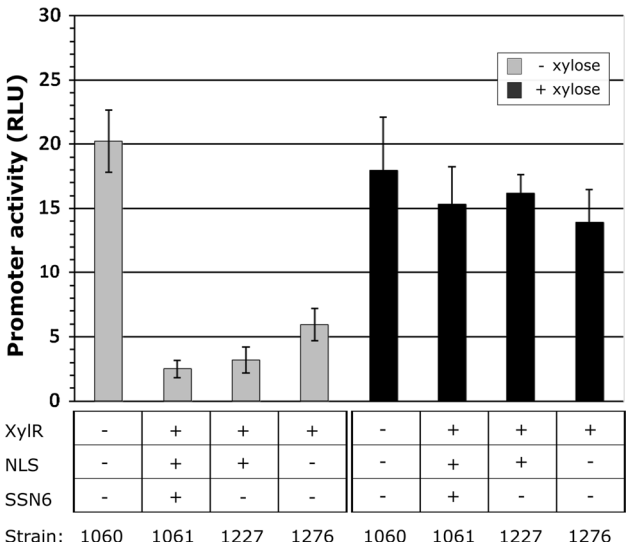

Fig. 4 Promoter activity of strains expressing varying XylR proteins. The effect of modifying the XylR repressor was determined by measuring promoter activity from strains expressing the fully modified repressor (NLS-XylR-Ssn6p) in comparison to variants of the repressor lacking modification (e.g., NLS-XylR and XylR). Promoter activity was measured as beta-galactosidase activity from expression of the *lacZ* marker gene fused to the $P_{TEF\text{-}xylO2\text{-}1}$ promoter. Both the repressor and $P_{TEF\text{-}xylO2\text{-}1}$::*lacZ* fusion were maintained on low-copy plasmids. Activity measurements are in relative light units (RLU, millions) and are normalized to cell mass. Activity from each promoter was measured at least three times. *Error bars* represent the standard deviation

Promoter Sensitivity

In order for de-repression of transcription to occur, xylose has to be present in the cell at a high enough concentration to bind to XylR. The *C. crescentus* XylR protein has an estimated dissociation constant of 20–50 μM xylose [20]. However, xylose enters *S. cerevisiae* through hexose transporters, and their affinity for xylose is very poor ($K_m \sim$ 190 mM to 1.5 M) [37]. Additionally, glucose is a competitive inhibitor of xylose and is preferentially transported. To determine how sensitive the hybrid promoter was to xylose, and if glucose was inhibitory to de-repression, activity of the $P_{TEF\text{-}xylO2\text{-}1}$ promoter was measured at low xylose concentration in medium that contained either sucrose or glucose as a carbon source. Sucrose enters the cell through a sucrose-permease, and this disaccharide would not be expected to compete with xylose for uptake. In the absence of xylose, as expected, cells lacking repressor showed high promoter activity compared to cells expressing the repressor (Fig. 5). De-repression was observed at very low levels of xylose. When sucrose was used as the carbon source, 0.2 mg/L xylose (1.33 μM) resulted in a 3.3-fold increase in promoter activity. A six-fold increase in promoter activity was seen with 2 mg/L xylose (13.3 μM). When cells were grown in medium containing glucose, xylose also increased promoter activity at low concentrations. However, after 2-h exposure to xylose, promoter activity was approximately 1.8-fold less than what was observed with sucrose. After 4-h exposure to 2 mg/L xylose, the difference in promoter activity between cells grown in glucose vs. sucrose was much less, suggesting that any inhibitory effect due to glucose occurs only at very low xylose concentration and early time points after induction.

Our observation of induction with 2 mg/L xylose suggests that this hybrid promoter is as sensitive to induction as the Tet- inducible promoter is in yeast with doxycycline [38]. It is also considerably more sensitive than the endogenous galactose-inducible promoter which has been shown to require between 1 and 3 g/L galactose for optimum activation [39]. Additionally, the $P_{TEF\text{-}xylO2\text{-}1}$ promoter is rapidly induced, compared again to galactose induction which can take more than 20 h to reach maximal expression [39]. The $P_{TEF\text{-}xylO2\text{-}1}$ promoter is also functional in the presence of glucose, making this promoter an excellent candidate for protein expression in situations where glucose is the carbon source of choice. The $P_{TEF\text{-}xylO2\text{-}1}$ promoter can also be used with strains not engineered for xylose consumption. In this scenario, xylose would be an inexpensive, stable inducer that would not be significantly degraded or consumed. When used at such low concentrations, the effect of xylose on cellular metabolism would also be minimal.

Conclusions

Metabolic engineering of *S. cerevisiae* to convert biomass-derived xylose to fuels and chemicals will require the expression of numerous genes. Expressing genes at high levels can be detrimental to the cell, resulting in decreased cell growth and productivity. Thus, for optimum yield and productivity, it is important to control the level of expression. Correct timing of expression is also important.

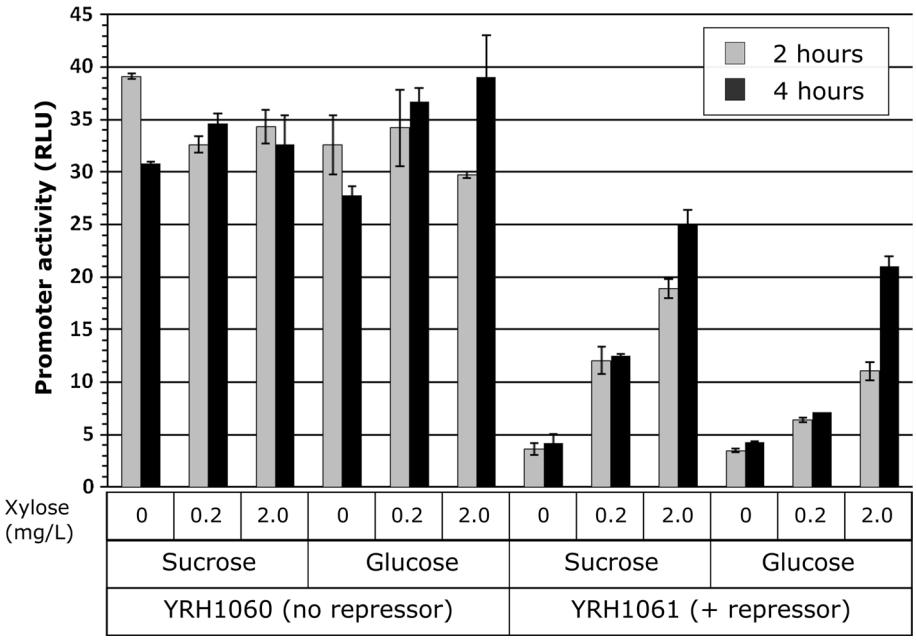

Fig. 5 Promoter activity for strains expressing $P_{TEF\text{-}xylO2\text{-}1}$ in the presence of low xylose concentration. Promoter activity was measured as beta-galactosidase activity from expression of the *lacZ* marker gene fused to the $P_{TEF\text{-}xylO2\text{-}1}$ promoter. Both the repressor and $P_{TEF\text{-}xylO2\text{-}1}$::*lacZ* fusion were maintained on low-copy plasmids. Activity measurements are in relative light units (RLU, millions) and are normalized to cell mass. Activity from each promoter was measured at least three times. *Error bars* represent the standard deviation

For example, use of a constitutive promoter for expression of the 2-PS gene for production of triacetic acid lactone resulted in 4.5-fold less product compared to using the late phase *ADH2* promoter [40]. In the case of xylose metabolism, it would not be beneficial to express all of the required genes constitutively at elevated levels. To address this issue, hybrid, xylose-regulated synthetic promoters were developed. When xylose was present, promoter $P_{TEF\text{-}xylO2\text{-}1}$ had activity comparable to *S. cerevisiae* promoters such as P_{PGK1}, P_{ADH1}, or P_{HXT7} and showed up to a 25-fold difference in activity compared to when xylose was not available. These new promoters provide improved control of gene expression for engineered *Saccharomyces* strains and are a starting point for generating new promoters with additional regulation (e.g., glucose repression), increased XylR repression, and more tunable expression levels.

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Compliance with Ethical Standards

Conflict of interest Ronald Hector is an inventor on U.S. Patent No. 9,506,097 B1, which pertains to the xylose-regulated promoters used in this study. Jeffrey Mertens declares that he has no competing interests.

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