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Vectors for Glucose-Dependent Protein Expression in *Saccharomyces cerevisiae*

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Abstract Based on the p426 series of expression vectors developed by Mumberg et al. (Gene 156, 119–122, 1995), we have generated a set of plasmids that allow the glucose-dependent expression of target genes in the yeast, *Saccharomyces cerevisiae*. The *ADH1* promoter in plasmid p426-ADH1 was replaced by the 1-kb 5′-region from either of the following genes: *HXK1*, *YGR243*, *HXT4* and *HXT7*. Expression mediated by the respective 5′-regions was monitored with EGFP, yEGFP3-CLN2pest and TurboGFP as marker genes. Fluorescence is induced 2.7-fold using the *HXK1*, 2.3-fold using the *YGR243*-, 5-fold using the *HXT7*- and 12.6-fold using the *HXT4* 5′-regions upon depletion of glucose to a concentration of <0.5 g/l.

Keywords *Saccharomyces cerevisiae* · 5′-Regulatory region · Glucose limitation · Gene expression · Vector construction

Introduction

Glucose is an important signalling factor of regulatory pathways in yeast. The budding yeast *Saccharomyces cerevisiae* preferentially ferments glucose even when oxygen is available [1]. *S. cerevisiae* is adapted to varying glucose concentrations by the regulation of membrane transport systems and proteins involved in glucose metabolism. As a consequence, the expression of many genes is induced or repressed by glucose. In order to extend the spectrum of vectors available for the controlled expression of target genes in the yeast *S. cerevisiae*, we applied glucose-sensitive 5′-regulatory regions to express recombinant genes in response to glucose limitation.

Hexokinase 1 (Hxk1p), encoded by the *HXK1* gene, is one of three proteins in *S. cerevisiae* which are able to catalyze the phosphorylation of glucose [2, 3]. *HXK1* is strongly expressed in media lacking glucose as the carbon source or by amino acid starvation [4]. In the presence of glucose, expression is repressed in an Hxk2p-dependent manner [5, 6].

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The function of the protein encoded by the *YGR243* (*FMP43*) gene is unknown. Ygr243p is exclusively detected in highly purified mitochondria [7]. The expression of *YGR243* is regulated by osmotic and alkaline stress [8–10], in response to phosphate, ammonium or sulphate starvation [11], and specifically induced by glucose limitation [12]. Transcriptome analysis of chemostat cultures by Tai et al. [13] identified both *HXK1* and *YGR243* as up-regulated signature transcripts for glucose-limited growth in 25 g/l glucose.

An important group of glucose-controlled genes encode hexose transporters (*HXT*). Seventeen members of this protein family, all anchored in the plasma membrane, are known to be engaged in glucose uptake. Given the wide range of sugar concentrations that can be used by *S. cerevisiae*, the presence of multiple hexose transporters with differing affinities for glucose is reasonable [14]. Here, we use 5′-regulatory regions of two well-characterized hexose transporter genes: *HXT4* encodes a high-affinity glucose transporter whose expression is increased approximately 5- to 20-fold in low glucose concentrations (lower than 0.1%, *w*/*v*) [15]. The DNA sequence ranging from position −770 to −460 of the 5′-regulatory region of *HXT4* functions as an upstream activating sequence element and mediates the increased transcription of a reporter gene under conditions of low glucose in a Snf3p-dependent manner [16]. The plasma membrane protein Snf3p serves as a sensor of low levels of glucose and induces a signalling pathway for hexose transporters [17]. During glucose-limited growth of chemostat cultures in 25 g/l glucose, *HXT4* was identified as a down-regulated signature transcript [13].

Hxt7p—like the almost identical Hxt6p [18]—is a high-affinity transporter that is strongly expressed in glucose-depleted cultures [19, 20]. Lai et al. [21] suggested that Hxt7p could provide a powerful tool for protein expression systems.

Mumberg et al. [22] established a set of valuable expression vectors for *S. cerevisiae* composed of DNA cassettes containing promoters of different strength, a multiple cloning site (MCS) and the terminator region of the *CYC1* gene [23]. These cassettes were introduced into low (*CEN/ARS*) or high copy number (2 μm) plasmids carrying one of the four different auxotrophic selection markers of the pRS series of vectors [24, 25].

On the basis of these expression vectors, we constructed a set of new vectors that allow glucose-dependent expression of recombinant proteins on different levels. These vectors may offer the opportunity for the self-induction of recombinant protein expression in a fermentation process when low concentrations of glucose limit a further biomass production.

Materials and Methods

Yeast Strains and Media

S. cerevisiae strain BY4741 (*MATa his3Δ1 leu2Δ0*, *met15Δ0*, *ura*Δ*30*; EUROSCARF) was grown at 30 °C on rich medium (yeast extract–peptone–dextrose, YPD) or mineral medium (mineral medium, MM). YPD contained 1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone and 2% (*w*/*v*) glucose. Defined MM composition supplemented with appropriate amino acids was as described by Verduyn et al. [26]. Glucose limitation medium contained (per litre): 5.0 g $(NH4)_2SO_4$, 3.0 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$ and 0.5 g glucose. Control medium contained (per litre) 59 g glucose.

Plasmid Construction and Transformation of Yeast

Oligonucleotides were synthesized by Eurofins MWG Operon (Germany). Restriction enzymes were obtained from New England Biolabs (Germany).

One-kilobase fragments of the 5′-regions of *HXK1*, *YGR243*, *HXT4* and *HXT7* encompassing the putative promoter regions and upstream regulatory sequences of the respective genes were PCR-amplified from genomic DNA of strain BY4741 as a template and the primer sets listed in Table 1. The primers introduced suitable restriction sites (*Sac*I/*Spe*I) at the ends of the PCR fragments. The *ADH1* promoter of vector p426-ADH1 [22] was removed by restriction with *Sac*I and *Spe*I. The cleaved vector and the *Sac*I/*Spe*I restricted 5′-regulatory region fragments were ligated using T4 ligase (Promega, Germany) and transformed into *Escherichia coli* TOP10 F′ (Invitrogen, Germany) by electroporation. The sequence of the cloned fragments in recombinant clones was verified by DNA sequencing.

Subsequently, the reporter genes EGFP (Invitrogen), yEGFP3-CLN2pest [27] and TurboGFP (BioCat, Germany) were cloned under the control of the four selected 5′-regions using the *Eco*RI/*Sal*I and *Spe*I/*Xho*I restriction sites in the MCS. The open reading frames of the reporter genes were PCR-amplified with the primer combinations and templates listed in Table 1. As a control, the reporter genes were cloned under the control of the constitutive *ADH1* promoter.

The resulting plasmids were sequenced and correct plasmids were transformed into *S. cerevisiae* strain BY4741 cells using the lithium acetate method [28].

Fluorescence Measurement and Glucose Assay

Fluorescence of the cells was measured in 96-well plates in a TECAN infinite M200 plate reader with a gain of 100 at λ_{ex}=487 nm and λ_{em}=520 nm. Cells were grown overnight in 20 ml control medium at 30 °C, incubated for 2 h in fresh control medium and transferred to either 100 ml limitation medium or 100 ml control medium. Samples (10 ml) were taken every hour and cells were harvested by centrifugation, resuspended and adjusted to

Table 1 Primers for PCR amplification

Primer	Restriction site	Sequence 5′–3′
F1	*Sac*I	TATTAT GAGCTC **TGGCGTGGGGTGGGGTGATTATCTAGACCATG**
R1	*Spe*I	TATTAT ACTAGT **CTTATTTTTTCAGTATTCTAATTGAGTTGTTTGGGTG**
F2	*Sac*I	TATTAT GAGCTC **ATGGGAATAGTGCCTTAGCGTGACATCTTTTTC**
R2	*Spe*I	TATTAT ACTAGT **TAAAAATATAGGTTTGTGTTTCTATGTGTCTTGAATG**
F3	*Sac*I	TATTAT GAGCTC **TCAACGATGTTGCCAAATAGTCGTACCTG**
R3	*Spe*I	TATTAT ACTAGT **TTTTGGCAGATTTTATTGTAAAAGTGTTTC**
F4	*Sac*I	TATTAT GAGCTC **AATAGTACTCTCATCGCTAAGATCATTTGG**
R4	*Spe*I	TATTAT ACTAGT **TTTTTGATTAAAATTAAAAAAACTTTTTGTTTTTG**
F5	*Eco*RI	TATTAT GAATTC **ATGGTGAGCAAGGGCGAGGAG**
R5	*Sal*I	TATTAT GTCGAC **TTACTTGTACAGCTCGTCCATGCCG**
F6	*Spe*I	TATTAT ACTAGT **ATGTCTAAAGGTGAAGAATTATTCACTGGT**
R6	*Xho*I	TATTAT CTCGAG **CTATATTACTTGGGTATTGCCCATACC**
F7	*Spe*I	TATTAT ACTAGT **ATGGAGAGCGACGAGAG**
R7	*Xho*I	TATTAT CTCGAG **TTATTCTTCACCGGCATC**

Sequences given in bold letters are complementary to the templates used. Restriction sites are underlined

$OD_{600\ nm}$=1 in distilled water. Of the respective suspensions, 100 µl was used for the fluorescence measurement.

Glucose concentration of the medium was determined with the dinitrosalicylic acid assay [29]. Absorption at 540 nm was used to calculate glucose concentration.

Fluorescence Microscopy

Cells were grown overnight in limitation or control medium, harvested, resuspended in distilled H_2O, embedded in 0.5% agarose and analysed by fluorescence microscopy using a Zeiss Axio Imager A1 fluorescence microscope.

SDS-PAGE and Western Blot Analysis

For SDS-PAGE, cells (1 unit of OD_{600nm}) were collected by centrifugation, resuspended in 15 µl sample buffer [30], including 100 mM DTT, and boiled for 5 min. Protein samples were separated by SDS-PAGE according to Laemmli [30] and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Germany). PVDF membranes were incubated with primary antibodies directed against GFP (Roche Applied Science, Germany) or TurboGFP (BioCat) and detected with horseradish peroxidase-conjugated secondary antibodies using the ECL-Plus Kit (GE Healthcare, Germany).

Results

Construction of the Vectors

On the basis of the p426-ADH1 vector [22], we developed a set of plasmids with different glucose-sensitive 5′-regulatory regions (Fig. 1), offering a glucose-inducible protein expression system in *S. cerevisiae*. This high copy *E. coli*/yeast shuttle vector harbours

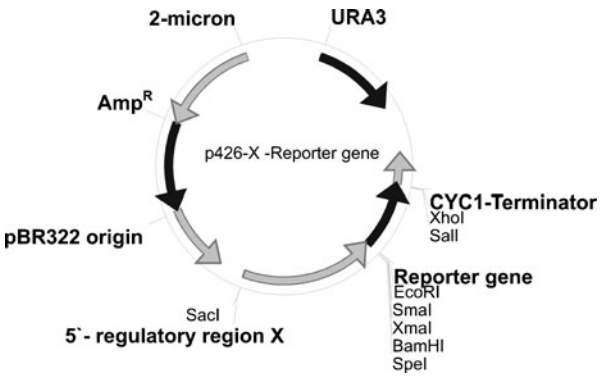

Fig. 1 Map of vectors. The nomenclature is based on the vectors described by Mumberg et al. [22]. The pBR322 and the 2-µm plasmid origins of replication mediate replication of the high copy vectors in *E. coli* and yeast, respectively. The *URA3* gene and the Amp^R gene are used for selection. The MCS with its restriction sites is shown between the 5′-regulatory regions X and the reporter gene. As 5′-regulatory regions, we used the 1,000-bp upstream region of the ORFs *HXK1*, *YGR243*, *HXT4* and *HXT7*, respectively. Termination of transcription in yeast is mediated by the terminator element of the *CYC1* gene. Reporter genes were *EGFP*, *yEGFP3-CLN2pest* or *TurboGFP*

the 2-μm replication origin, *URA3* as selection marker and the *CYC1* transcription terminator element. We replaced the approx. 1,500-bp fragment containing the *ADH1* promoter and its 5′-flanking sequences in plasmid p426-ADH1 [22] by the 1-kb 5′-regulatory regions of the genes *HXK1*, *YGR243*, *HXT4* and *HXT7* encompassing the respective promoter and upstream (regulatory) sequences. The original MCS of the vector p426-ADH1 was preserved. EGFP, yEGFP3-CLN2pest or TurboGFP was used as a marker gene to monitor the expression driven by the cloned 5′-regulatory sequences. The vector p426-ADH1 that allows constitutive expression of marker genes [22] with or without the cloned reporter genes served as controls.

Expression Analysis

Glucose dependency of EGFP expression was analysed by fluorescence measurements. Cells were grown in control medium and limitation medium as described in "Materials and Methods". Samples were taken at the indicated time points and used to determine the fluorescence of the cells adjusted to OD_{600nm}=1 (Figs. 2 and 3, part a) and the glucose concentration of the medium (Figs. 2 and 3, part b). Figures 2 and 3 (part c) show the differences of fluorescence of cells cultured in control or limitation medium in relation to the glucose concentration.

The expression analysis of EGFP under the control of glucose-sensitive 5′-regulatory regions of *HXK1* and *YGR243* is shown in Fig. 2. Cells harbouring the empty vector (negative control) exhibit only a low and almost constant fluorescence, which is most likely due to autofluorescence. Cells expressing EGFP under the control of the *ADH1* regulatory sequences (positive control) display a nearly constant fluorescence of about 600–700 arbitrary units (AU). When cultured in control medium, yeast transformants harbouring the *HXK1-EGFP* construct show a low fluorescence similar to the negative control. As a result of glucose depletion—glucose is exhausted after 3–4 h—the fluorescence increases from 150 up to 400 AU and reaches a stable plateau after 4 h. These results are in line with published data according to which *HXK1* expression is repressed in the presence of glucose and induced due to glucose limitation [4–6].

The *YGR243-EGFP* construct exhibited a similar characteristic, although the basal fluorescence of the transformants grown in control medium of about 300 AU is higher than in *HXK1-EGFP* harbouring transformants. We observed a pronounced induction up to 700 AU using the *YGR243-EGFP* vector in limitation medium. This is in line with the report that *YGR243* expression is induced under carbon limitation [13].

Figure 3 focuses on the fluorescence measurements of cells expressing *EGFP* under the control of the 5′-regulatory regions of *HXT7* and *HXT4*. Curve progression is similar as with the constructs shown in Fig. 2, albeit the induction rate differs considerably. The fluorescence of cells harbouring the construct *HXT7-EGFP* increases from 200 up to 1,000 AU, that of cells harbouring the construct *HXT4-EGFP* from 500 up to 6300 AU. Therefore, this additional pair of constructs offers a strong induction of gene expression under conditions of glucose limitation and provides an ideal complementation to the moderately inducible *HXK1-EGFP* and *YGR243-EGFP* constructs.

In conclusion, the new constructs offer the possibility of expressing recombinant proteins in *S. cerevisiae* in a glucose-dependent manner. The compatibility of the MCS to other expression plasmids offers a simple shuttle of target genes from or to other well-established expression vectors. The novel vectors containing the 5′-regulatory region of *HXK1* and *YGR243* may preferentially be used for moderate protein expression, whilst the

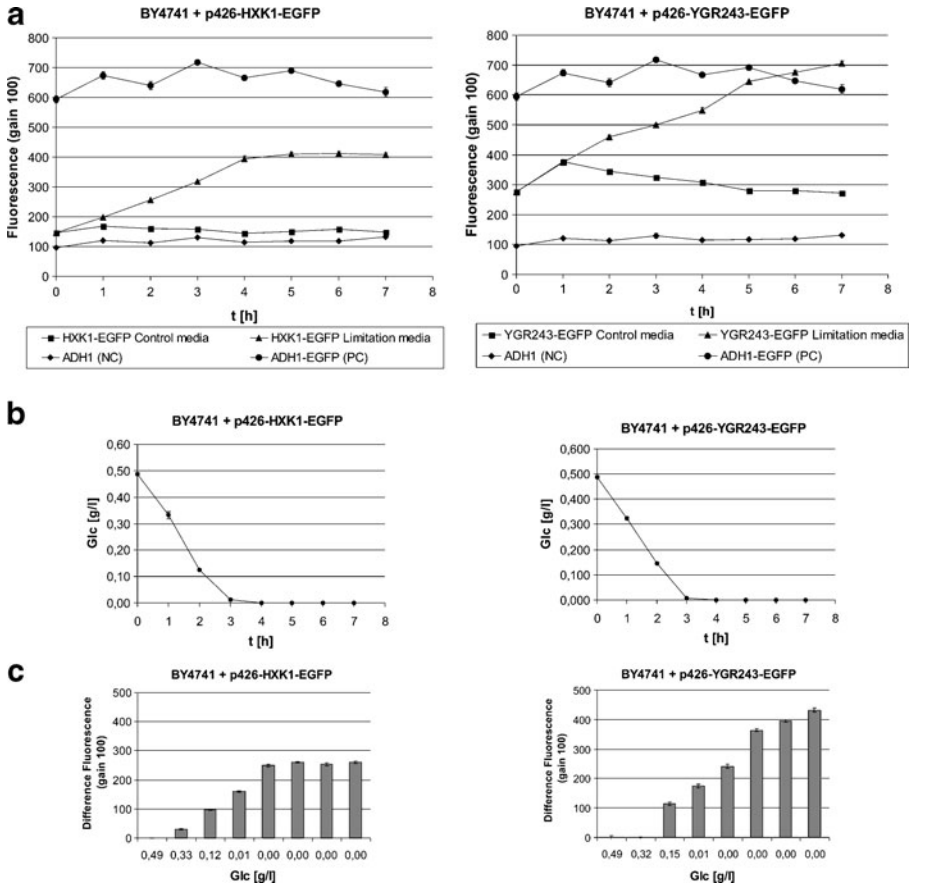

Fig. 2 Expression of EGFP under the control of glucose-sensitive 5′-regulatory regions *HXK1* and *YGR243*. **a** *S. cerevisiae* BY4741 cells expressing EGFP under the control of the glucose-sensitive 5′-regulatory regions of the genes *HXK1* or *YGR243* were grown overnight in control medium (0 h) and shifted to fresh control or limitation medium. At indicated time points, samples were taken and fluorescence of cells was measured. Cells harbouring vectors with EGFP under the control of the *ADH1* regulatory sequence served as positive control (*PC*); cells with the empty vector were used as negative control (*NC*). **b** Glucose concentrations of limitation medium at indicated time points. **c** The difference in fluorescence of cells cultured in limitation or control medium is shown as a function of glucose concentration. **a**–**c** Mean of three measurements. Standard deviation is indicated by *error bars*

plasmids with the 5′-regulatory region of *HXT4* and *HXT7* are suitable for the stronger expression of recombinant protein.

Suitable Fluorescent Proteins for Monitoring

One challenge of studying promoters is the direct measurement of transcription activity. Measuring the fluorescence of EGFP, which requires a long maturation time and is extraordinarily stable within the cells, is inappropriate to follow directly alterations in transcription [31]. We compared a destabilized version of EGFP (yEGFP3-CLN2pest) consisting of the yeast codon optimized enhanced GFP and the PEST-rich sequence of G1

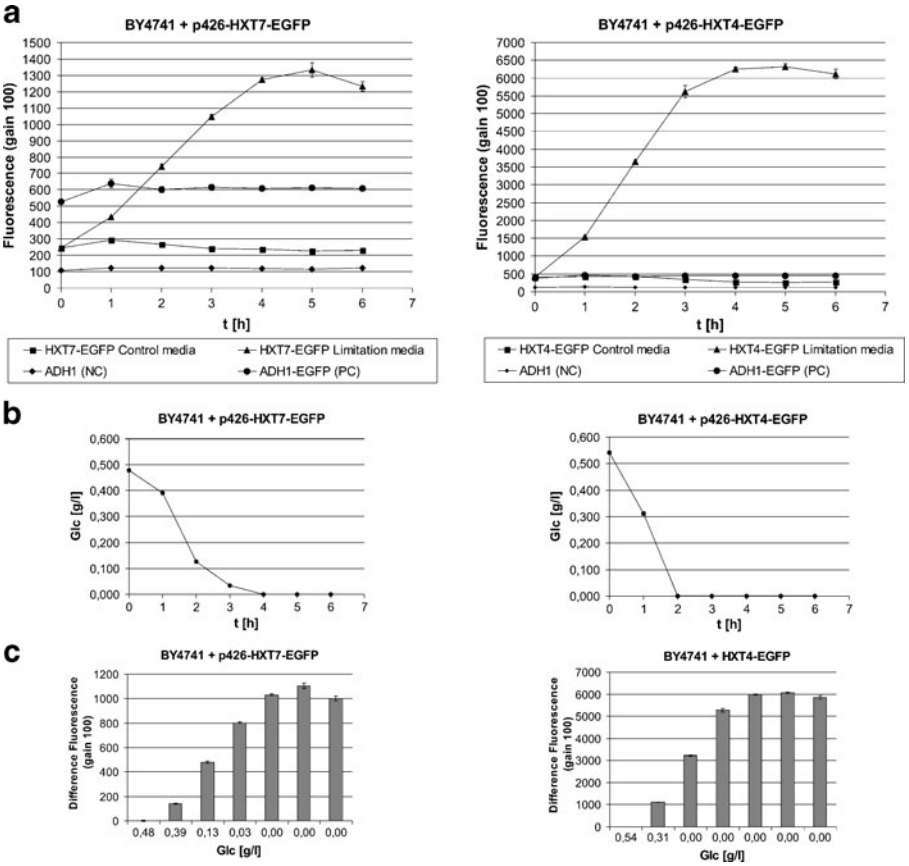

Fig. 3 Expression of EGFP under the control of glucose-sensitive 5′-regulatory regions *HXT7* and *HXT4*. **a** Fluorescence of *S. cerevisiae* BY4741 cells expressing EGFP under the control of the glucose-sensitive 5′-regulatory regions of the genes *HXT7* and *HXT4* was determined as described in Fig. 2. **b** Glucose concentrations of limitation medium at indicated time points. **c** Differences in fluorescence of cells grown in limitation or control medium are shown as a function of glucose concentration. **a**–**c** Mean of three measurements. Standard deviation is indicated by *error bars*

cyclin Cln2p and the fast maturing TurboGFP (a GFP from copepod *Pontellina plumata*) to assess these questions.

Figure 4 shows the expression of the reporter genes EGFP, yEGFP3-CLN2pest and TurboGFP under the control of the glucose-sensitive *HXK1* 5′-regulatory region as an example. For comparison, two methods were applied: fluorescence microscopy (Fig. 4a) and Western blot analysis (Fig. 4b). Samples for positive control (PC) and negative control (NC) were taken from an overnight culture in control medium. As expected, no fluorescence was observed in the negative control, whereas cells of the positive control showed a distinct fluorescence (Fig. 4a). In the control medium, EGFP and its derivate yEGFP3-CLN2pest displayed no fluorescence. In contrast, cells expressing TurboGFP exhibit fluorescence even under non-limiting conditions. In the limitation medium, the induction of protein expression results in higher fluorescence. These results are in line with the respective Western blot analysis (Fig. 4b). In the PC, a clear signal is detectable

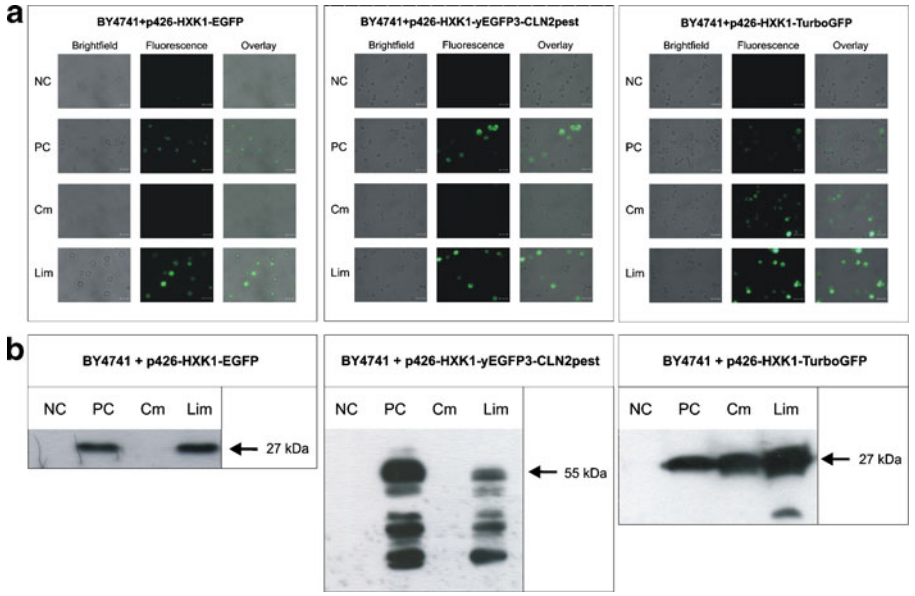

Fig. 4 Expression of the fluorescent reporter genes under the control of the glucose-sensitive *HXK1* 5′-regulatory region. **a** Fluorescence microscopy of cells grown overnight in limitation (*Lim*) and control medium (*Cm*). Controls were as in Fig. 2. Exposure times: EGFP 2,000 ms, yEGFP3-CLN2pest 1,000 ms, TurboGFP 200 ms. **b** Protein extracts of cells harvested after overnight culture in limitation and control medium were subjected to SDS-PAGE and Western blot analysis with GFP or TurboGFP antibodies. Samples for positive control (*PC*) and negative control (*NC*) were taken from an overnight culture in control medium

compared to the NC. Furthermore, as in the fluorescence microscopy analysis, no signal can be detected under non-limiting conditions (Cm) with EGFP and yEGFP3-CLN2pest, whilst a strong signal is visible with TurboGFP. A clear increase of the signal is apparent under limiting conditions (Lim) for all three proteins.

Upon shifting the cells to non-limiting conditions, the transcription should reach again the initial level (regeneration). To follow the induction of expression and the regeneration by shifting cells from non-limiting to limiting conditions and vice versa, expression of the three marker genes under the control of the *HXK1* 5′-regulatory region was analysed (Fig. 5). Cells were grown overnight in control medium and then induced by shifting to the limitation medium. Conversely, protein expression was induced by incubating cells overnight in the limitation medium and then shifted to the control medium to analyse the kinetics of regeneration. Samples were taken at the indicated time points and subjected to SDS-PAGE and Western blot analysis. As expected, EGFP is detectable in the limitation medium for the first time 2 h after induction, with only a weak increase thereafter. Due to its high stability, EGFP is still visible after 6 h, although transcription should be markedly reduced. yEGFP3-CLN2pest is detectable 2 h after induction and increases in concentration up to 4 h before decreasing again. Multiple bands of lower molecular weight most likely reflect the strong degradation of the protein due to the presence of the PEST sequence. During regeneration, the concentration of the marker protein declines very fast (no signal detectable after 4 h), indicating that yEGFP3-CLN2pest is more suitable than EGFP to follow a decline in transcription. TurboGFP shows a basal expression level, even under

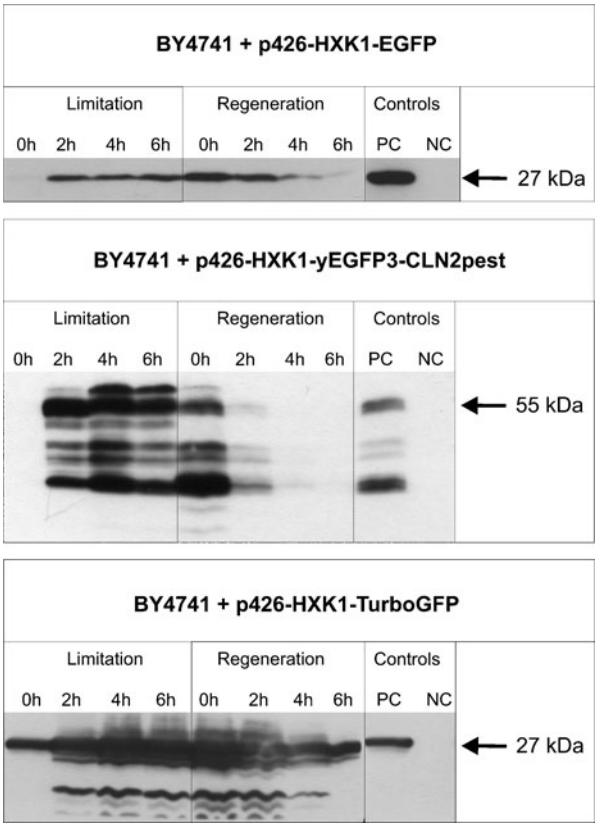

Fig. 5 Time course of EGFP-, yEGFP3-CLN2pest- and TurboGFP-mediated fluorescence as a function of glucose limitation. Cells were grown overnight in control medium and then shifted to limitation medium. For regeneration, cells were grown overnight in limitation medium and then shifted to control medium. Samples were taken at indicated time points and subjected to SDS-PAGE and Western blot analysis. Samples for positive control (*PC*) and negative control (*NC*) were taken from an overnight culture in control medium

non-limiting conditions, and increases considerably after induction. Bands of low molecular mass indicate protein degradation during the strong accumulation of TurboGFP. The persistent signals during regeneration show that TurboGFP is very stable and therefore not suitable for a direct monitoring of small variations in the expression level.

Discussion

The new constructs described here offer the possibility to express recombinant proteins in *S. cerevisiae* on different levels in a glucose-dependent manner. The MCS allows easy cloning of target genes from well-established expression vectors.

With our new constructs, expression is induced when limiting glucose concentrations are reached. Therefore, after biomass production and entry into the stationary phase, expression of a gene of interest is induced and the respective protein can be produced at moderate or high levels. This is of special interest for the production of proteins that are toxic for the cells. When biomass has been produced and glucose is exhausted, the toxic protein can be expressed at high levels. When combined with a suitable secretion signal, it will be secreted, thus offering an easy way for purification. By resuspending the cells in fresh medium with non-limiting glucose concentrations, expression of the target protein will stop, allowing the cells to regenerate and to initiate a further round of protein production if

glucose is exhausted again. Using this strategy, the biomass could be maintained and protein production be performed in multiple consecutive induction steps.

Due to its short half-life, yEGFP3-CLN2pest proved to be the best choice to follow the transcriptional activity and can therefore also be used for advanced process control. In contrast, determination of the fluorescence of the highly stable TurboGFP does not allow monitoring small variations in the expression level.

A major challenge for the specific induction of protein expression in biological systems is the cross-reactivity of signalling cascades and control circuits. Glucose sensing by Snf3p is mediated by direct binding of sugar molecules, which leads to the induction of a signalling pathway for the expression of hexose transporters [17]. Binding of Snf3p is not exclusively restricted to glucose, but encompasses a variety of other sugars. Recently, the amino acid residues involved in binding other sugar molecules besides glucose were identified by site-directed mutagenesis [32]. Alterations of these amino acids partially decreased the binding of competing sugars, whilst the binding of glucose was not affected. These data open up the possibility to increase the specificity of Snf3p for glucose and hence to limit the expression of recombinant proteins by the novel vectors strictly to glucose-limiting conditions.

In summary, we constructed novel vectors to achieve a moderate glucose-inducible expression via the 5′-regulatory regions of *HXK1* or *YGR243* and a stronger glucose-inducible expression via the 5′-regulatory regions of *HXT4* or *HXT7*.

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