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Analysis of Heat Shock Promoters in *Hansenula polymorpha:* The *TPS1* Promoter, a Novel Element for Heterologous Gene Expression

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Abstract The strength and regulatory characteristics of the heat-inducible *HSA1*, *HSA2* and *TPS1* promoters were compared with those of the well-established, carbon source-regulated *FMD* promoter in a *Hansenula polymorpha*-based host system *in vivo*. In addition, the *Saccharomyces cerevisiae*-derived *ADH1* promoter was analysed. While *ADH1* promoter showed to be of poor activity in the foreign host, the strength of the heat shock *TPS1* promoter was found to exceed that of the *FMD* promoter, which at present is considered to be the strongest promoter for driving heterologous gene expression in *H. polymorpha*.

Keywords: *Hansenula polymorpha*, reporter genes, promoter test system, heat-inducible promoter, *FMD* promoter.

INTRODUCTION

The increasing recognition of the methylotrophic yeast *Hansenula polymorpha* as host for heterologous protein production is primarily due to the availability of the two strong *FMD* and *MOX* promoters which are derived from methanol metabolism genes. Both promoters are well characterized and have found successful industrial application in the high level expression of foreign genes [1,2]. *MOX* and *FMD* are co-regulated with respect to carbon source. Repression takes place on glucose, derepression on glycerol and induction on methanol [3]. However, high-yield heterologous gene expression is not restricted to conditions of methanol induction, but can also be obtained using glycerol derepression or even glucose starvation conditions [4]. To further extend the potential of the *H. polymorpha* system, strong, differently regulated promoter elements are desirable which are not derived from methanol pathway genes. Recently, *PMA1* promoter has been found to provide strong constitutive expression of foreign genes [5]. In addition, three promoters of nitrate assimilation pathway genes have been characterized, one of which is both strong and to some extent regulatable by appropriate nitrate supplementation [6]. However, in none of these cases has a direct comparison with established promoter elements in the expression of a defined gene under defined conditions been performed. This compromises the accurate evaluation of their benefits.

H. polymorpha is a thermophilic yeast species which can tolerate temperatures of more than 50°C [7]. Anticipating that this tolerance is associated with high expression of heat-induced genes, identification of strong heat-inducible promoters with favourable characteristics for heterologous gene expression seems feasible. However, only three *H. polymorpha* genes have been characterized to date as being inducible under conditions of increased temperature. While *HSA1* and *HSA2* are integral to heat and stress response [8,9], *TPS1* (trehalose-6-phosphate synthase) is involved in the heat-induced high-level synthesis and accumulation of trehalose which is considered to be a protective compound at elevated temperatures [10]. Recently, *TPS1* has been cloned and characterized [7]. The present study is aimed at assessing the regulation and strength of these heat-inducible promoters for heterologous gene expression in comparison to the established *FMD*- and the *Saccharomyces cerevisiae*-derived *ADH1* promoter.

For comparative promoter studies, it is necessary to employ recombinant strains of an otherwise identical genetic and genomic background. Unfortunately, it is very difficult to generate recombinant *H. polymorpha* strains that meet these requirements. Stable non-integrative vectors that provide comparable copy numbers are not available for the *H. polymorpha* system. The presence of a *S. cerevisiae*. 2 μm fragment has been found to provide an incomplete episomal fate for the vector; the reduction of genomic integration furthermore has appeared to be strain-dependent [11]. The generation of identical strains by homologous recombination has also turned out to be difficult. In *H. polymorpha*, only three examples of homologous integration at acceptable frequencies have been reported: targeting of the foreign DNA to subtelomeric *HARS* loci [12,13], to the *leu2* locus (use of vectors containing a *S.*

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cerevisiae. 2 μm sequence) [6], and to the *MOX/TRP3* locus by a disruption/replacement approach [14]. In the latter case, a double selection system had to be used to screen out the majority of transfomants caused by non-homologous integration events.

The test system presented here is based on the simple generation of strain mixtures using *HARS1* vectors for transformation. *H. polymorpha* transformants generated with these vectors differ in copy number and integration site [15]. Therefore, several hundred individual recombinant strains were pooled to form representative strain mixtures from which promoter-dependent gene activity could be measured and compared.

We present data indicating that the *TPS1* promoter has a high potential for biotechnological applications in *H. polymorpha*. Additionally, *lacZ* was found to be of superior reporter gene characteristics when compared with *GFP* under the conditions tested.

MATERIALS AND METHODS

Microorganisms

Hansenula polymorpha RB11 (*odc1*) [16] was used for promoter studies. *Escherichia coli* DH5αF′ (F′ (ϕ80 d*lacZ*ΔM15)Δ(*lacZYA-argF*) U196 *recA1 endA1 hsdR17* r_k- m_k+ *supE44λ thy-1 gyrA relA*; Gibco BRL) was used for all cloning steps.

Plasmid Constructions

Plasmid M1 (Fig. 1(a)) was generated from pFPMT121 [1] by replacing a 1.223 kb *Hin*dIII/*Bam*HI fragment with a synthetic 85 bp double stranded DNA sequence harbouring a set of restriction recognition sites in the following order: *Hin*dIII, *Afl*II, *Bgl*II, *Xba*I, *Spe*I, *Bsa*AI, *Bcl*I, *Age*I, *Pme*I, *Eco*RI, *Nru*I, *Sac*I, *Not*I, *Nhe*I, *Bss*HII, *Sph*I, *Bln*I, and *Bam*HI. The 5.8 kbp *Eco*RI/*Bam*HI fragment of pM1 was ligated with PCR-generated DNA fragments with *Eco*RI and *Bam*HI cohesive ends harbouring the entire reading frames of *yGFP* (0.7 kbp) [21] or *lacZ* (3.2 kbp). This resulted in the promoterless reporter gene plasmids pC10 and pC11, respectively (Fig. 1(b) and (c)). PCR amplificates harbouring the various promoters to be tested (*FMD, TPS1, HSA1, HSA2*, or *S.c. ADH1*) were then cloned between remaining polylinker sites still present in pC10 and pC11. This led to two sets of integrative reporter plasmids which differed only in the reporter gene and/or in the promoter controling it. The sizes of the promoter fragments and the cloning sites used were in particular: P_{FMD} (1.2 kbp, *Eco*RI/*Hin*dIII); P_{TPS1} (0.58 kbp, *Eco*RI/*Spe*I); P_{HSA1} (0.3 kbp, *Hin*dIII/*Xba*I); P_{HSA2} (0.3 kbp, *Hin*dIII/*Spe*I); P_{ADH2} (1.5 kbp, *Eco*RI/*Hin*dIII).

The sequence of the *H. polymorpha HSA2* gene has been deposited under the GenBank accession number U49932.

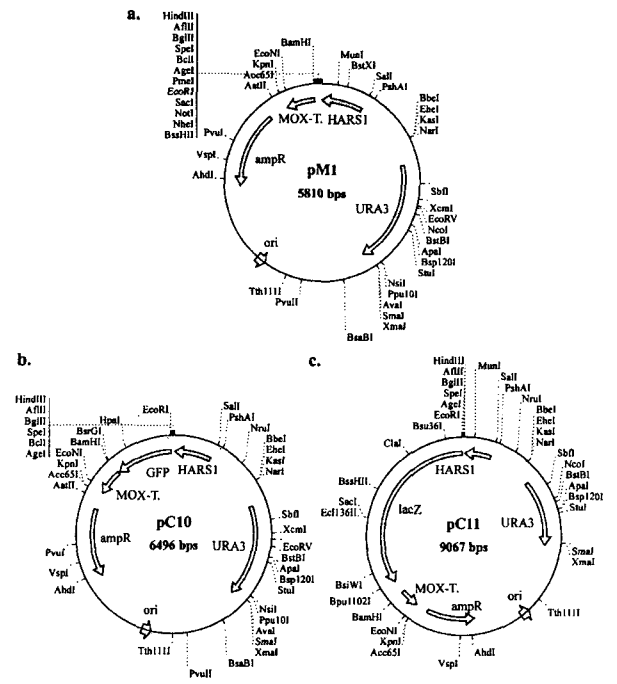

Fig. 1. Basic vectors used for comparative promoter analyses in *H. polymorpha*. pM1 (a) without promoter and reporter gene, harbours a polylinker between *HARS1* and *MOX* terminator. pC10, (b) and pC11, (c) contain *yGFP* or *lacZ* as a reporter gene, repectively. The various promoters to be tested were inserted between *HARS1* and the reporter ORF of pC10 and pC11. For more details, see text.

Generation of Reporter Strain-Mixtures

H. polymorpha RB11 was transformed to Ura^+ with either pM1, pC10, pC11 or their derivatives equipped with the different promoters. 800 colonies of the various transformants were grown three times on fresh selective plates, once on rich medium plates, and once again on selective plates (in this sequence). This passaging/stabilizing procedure has led to mitotically stable strains [17]. 300 individual strains derived from the various collections were pooled and kept as glycerol stocks at -70°C. From these stocks, cultures were inoculated for DNA isolation and subsequent Southern blot analysis and for the determination of promoter strength under various conditions.

Determination of β-Galactosidase Activities

For qualitative detection, 10 μL of the glycerol stocks described above harbouring the *lacZ* reporter constructs were spotted on selective plates (with the appropriate carbon source) and incubated overnight at the temperatures to be tested. Subsequently, X-gal overlay assays were performed as described by Suckow and Hollenberg [18]. After 24 h at 37°C, the reactions were completed, and the relative intensities of the blue colour of the various cell spots were estimated.

For determination of specific β-galactosidase activities, 2 mL of test media were inoculated with 10 μL aliquots of the various stock suspensions and cultured to stationary phase at defined test temperatures. Aliquots from these pre-cultures were used to inoculate 10ml of the various test media to OD_{600} = 0.1 densities. Culturing was continued under the pre-culturing conditions to OD_{600} = 5 densities. After harvesting, cell extracts were prepared and analyzed for total protein content [19] and β-galactosidase activities [20]. All values were normalized to specific β-galactosidase activities.

Determination of GFP Activities

Pre-culturing and culturing of strain collections containing *GFP* constructs were performed as described for the *lacZ* constructs. GFP activities were analysed qualitatively by fluororescent microscopy. Precise GFP-determinations were performed by FACS analyses in a Cell Sorter (Becton & Dickinson) according to Niedenthal *et al.* [21]; the fluorescent shifts of 10,000 individual cells per strain mixture/test condition were determined.

RESULTS AND DISCUSSION

The Use of *lacZ* and *GFP* as Reporter Genes in *H. polymorpha*

The use of *lacZ* as reporter gene in *H. polymorpha* has been reported recently [6]; however, use of *GFP* in this yeast has not yet been described. Since there are several variants of *lacZ* and *GFP*, we first analyzed in a pilot experiment whether the particular *lacZ* and *yGFP* [21] reading frames chosen for this study were functional in *H. polymorpha*. Strain RB11 (*odc1*) was transformed to Ura^+ using plasmids pC10-TPS1 (P_{TPS1}-*GFP*), pC11-TPS1 (P_{TPS1}-*lacZ*), or pM1 (without any reporter gene inserted), and twenty stabilized transformants were generated for each plasmid. Reporter gene expression was then determined qualitatively via X-gal overlay assay (*lacZ*) or fluorescence microscopy (*GFP*) under standard conditions defined for *TPS1* promoter analysis (YNB; 2% glucose; 37°C). All twenty strains containing *lacZ* constructs showed weak up to very strong levels of β-galactosidase activity, but none of the strains harbouring pM1 (no reporter gene) or pC10-TPS1 (P_{TPS1}-*GFP*) yielded any signal in this test (data not shown). Similarly, green fluorescence was observed in all twenty strains harbouring pC10-TPS1 (P_{TPS1}-*GFP*; see Fig. 2(a)) but in none of the pM1- (no reporter gene) or pC11-TPS1-strains (P_{TPS1}-*lacZ*; data not shown). These results indicated that both the *lacZ* and the *GFP* genes are functional in the examined *H. polymorpha* strain.

Prior to comparative promoter analyses, it was also necessary to confirm the functional persistance of both gene products, LacZ and GFP, at high temperatures. For this purpose, individual strains of high productivity

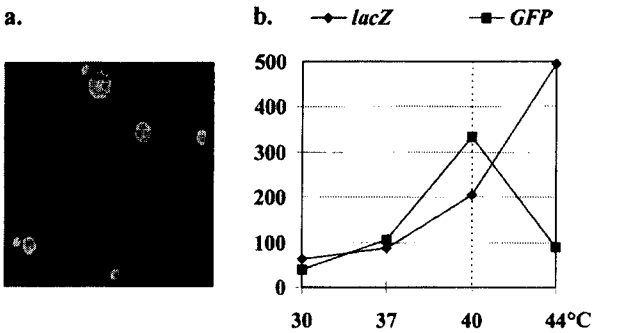

Fig. 2. Functional analysis of the *lacZ* and *GFP* reporter genes in *H. polymorpha*. (a) Green-fluorescent cells expressing *yGFP* under control of *TPS1* promoter, grown on glucose at 37°C. Cells harbouring the negative control plasmid pC10 did not show any green fluorescence (data not shown). (b) Specific β-galactosidase activities (mU/mg total protein) or green-fluorescent shifts (1000 cells measured) of cells harbouring pC10-TPS1 or pC11-TPS1, respectively, grown on glucose at different temperatures. For further details, see Materials and Methods section.

from both the P_{TPS1}-*lacZ* or P_{TPS1}-*GFP* collections were cultured in YNB/2% glucose at four different test temperatures (30°C, 37°C, 40°C and 44°C). As shown in Fig. 2(b), specific β-galactosidase activity was positively correlated with temperature, as would be expected for the heat-inducible *TPS1* promoter. A similar positive correlation could be observed for GFP signal intensities for temperatures up to 40°C. However, at 44°C, a decrease in fluorescence occurred (Fig. 2(b)) independent of the promoter used for controlling *GFP* (data not shown). This observation corroborates similar findings of Lim *et al.* [22] that the GFP-fluorophore may not adopt its functional conformation in *S. cerevisiae* at higher temperatures. Together, these data suggest that the problems encountered with the use of GFP at higher temperatures (beyond 40°C) may be generally independent of the host organism. As this study focuses on heat-inducible promoters, we consequently favoured *lacZ* over *GFP* for promoter comparison.

A Promoter Test System Based on Representative Strain Mixtures

Having demonstrated the suitability of *lacZ* in *H. polymorpha*, the set of pC11-derivatives harbouring *lacZ* under control of the different promoters was chosen for transformation. Taking into account that transformation with such *HARS1* plasmids results in collections of individual strains which differ significantly in copy number and integration loci, and since these differences can influence expression levels independent of promoter characteristics (see Introduction) [15], it was necessary to ensure the representative character of the strain mixtures chosen for comparison.

Indeed, initial determination of β-galactosidase activities in individual pC11-TPS1 transformants revealed

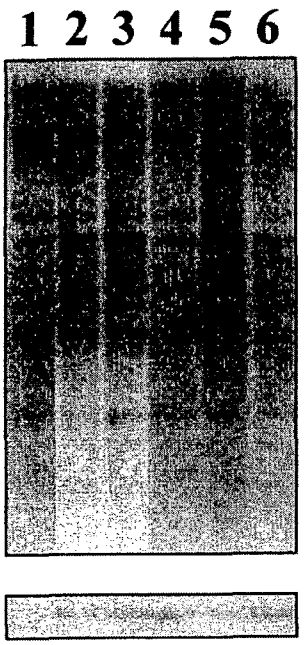

Fig. 3. Comparison of the relative copy numbers of integrated plasmids within the various strain mixtures tested. Each 1 μg of genomic DNA of strain RB11 or its derived strain mixtures harbouring *lacZ* expression cassettes were cleaved with *Eco*RI/*Bam*HI and separated on a 1% agarose gel (upper panel). After Southern blotting, the nylon membrane was developed using the 3.2 kb *Eco*RI/*Bam*HI-*lacZ*-fragment of pC11 as a probe (lower panel). While with RB11 (lane 1) no signals were obtained, with all strain mixtures a signal at 3.2kb was observed (lanes 2-6). The strain mixtures tested were in particular: pC11-FMD (lane 2); pC11-TPS1 (lane 3); pC11-HSA1 (lane 4); pC11-HSA2 (lane 5); pC11-ADH1 (lane 6). Except for lane 5, all lanes show very similar signal strengths.

high standard deviations (data not shown). However, when pools of 300 individual transformants were compared, variability was on a very low level (data not shown), comparable to the variability observed in recombinant *S. cerevisiae* strains when using *CEN* vectors for transformation [23]. In Southern Blot analyses we furthermore found the heterologous expression cassettes to be present in similar gene dosages when probing the genomic DNA isolated from such pools with a *lacZ* probe (Fig. 3). As the only exception, the gene dosage observed in the pC11-HSA2-derived mixture was only about half of the average (see Fig. 3). Therefore, we concluded that mixtures of at least 300 strains provide representative strain spectra for conclusive comparative promoter studies.

The Relative Activities of *H.p. FMD, HSA1, HSA2,* and *TPS1* Promoter, and *S.c. ADH1* Promoter in *H. polymorpha*

Representative strain mixtures differing in the promoter to be tested were cultivated under identical conditions and inspected for *lacZ* expression. The expression levels were analyzed in cultures cultivated at different temperatures and using media supplemented with different carbon sources. For control, a representative strain mixture was generated by transformation with plasmid pC11 (without any promoter controling *lacZ*), cultivated and analysed under identical conditions. In these controls, no LacZ activity could be observed (data not shown), indicating that vector sequences upstream of *lacZ* cannot function as a promoter. Thus, all enzyme activities found in strain mixtures containing functional expression cassettes can be attributed to the promoter present upstream of *lacZ*.

FMD promoter was chosen for comparison in this study since its strength and regulative properties have been characterized in detail. On glucose as a sole carbon source the activity of *FMD* promoter is repressed; on glycerol or methanol derepression or induction takes place, resulting in high expression rates of *FMD* or a foreign gene controlled by this promoter (see Introduction) [24,25]. In the *lacZ* test system *FMD* promoter performed as expected. On glucose the β-galactosidase levels were low at all three temperatures tested (30°C, 37°C, and 44°C) indicating repression (Fig. 4(a)). However, on glycerol or methanol β-galactosidase activities increased up to 400-fold, with the highest value measured on methanol at 37°C (Fig. 4(a)). This can be explained by the derepressed or induced status of the *FMD* promoter. This profile thus is in agreement with previously described observations [1,2,24], further suggesting a proper functioning of the *lacZ* reporter system used.

The only non-*H. polymorpha*-promoter tested was that of the glycolytic alcohol dehydrogenase gene (*ADH1*) from *S. cerevisiae*. In the original host *ADH1* promoter provides strong and constitutive expression on glucose, with decreased activities on glycerol as a sole carbon source [26]. In *H. polymorpha*, *ADH1* promoter appeared to be weak and constitutive on methanol; only a slight temperature-dependent increase of specific β-galactosidase activities was observed on glucose or glycerol (Fig. 4(b)). These results may indicate a sub-optimal interaction between the *S.c. ADH1* promoter and the *H. polymorpha* transcription machinery. Nevertheless, *ADH1* promoter-driven heterologous gene expression in *H. polymorpha* may be useful in cases where low expression rates are needed.

The heat shock proteins Hsa1p and Hsa2p of *H. polymorpha* have been described as homologues of the *S. cerevisiae* Hsp70 proteins Ssa1p and Ssa2p, respectively [8,9]. For both *HSA1* and *HSA2* an increase in mRNA levels has been observed in response to higher temperatures, indicating that the corresponding promoters are heat-inducible [8,9]. This effect, however, is much more pronounced in the case of *HSA1* [9]. With the *lacZ* test system these data were further confirmed (Fig. 4(c) and (d)). With *HSA1* promoter, a clear positive correlation between β-galactosidase levels and increasing temperatures was observed on glucose or glycerol as sole carbon source, but not on methanol (Fig. 4(c)). Thus, on all

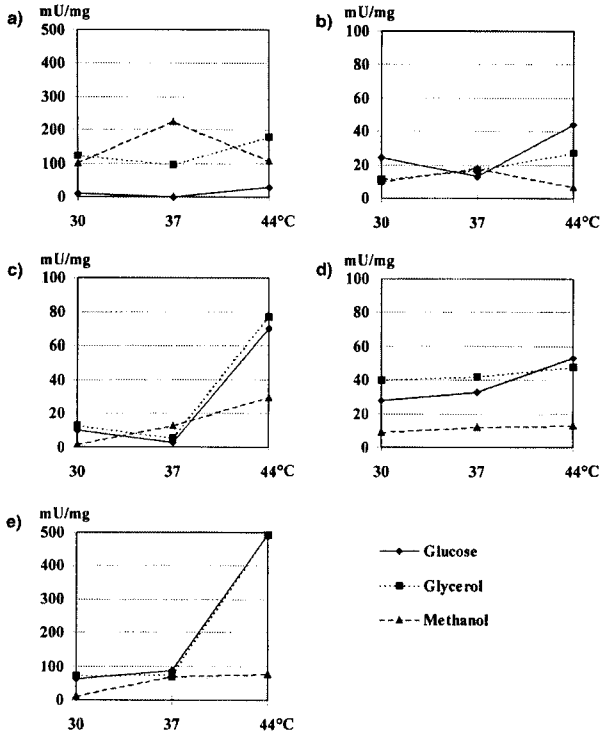

Fig. 4. Specific β-galactosidase activities in pools of 300 individual *H. polymorpha* strains containing derivatives of pC11 with *lacZ* controlled by various promoters. Cell extracts were prepared from cells grown under the conditions indicated and specific β-galactosidase activities (mU/mg total protein) were determined, as described in the MATERIALS AND METHODS section. (a) pC11-FMD; (b) pC11-ADH1; (c) pC11-HSA1; (d) pC11-HSA2; (e) pC11-TPS1. All promoters tested originate from *H. polymorpha* genes except for the *S. cerevisiae*-derived *ADH1* promoter. Please note that scaling of (a) and (e) differs from that of (b)-(d) by a factor of 5.

three different carbon sources tested the regulatory characteristics of the *HSA1* promoter qualitatively resemble those of *TPS1* promoter (Fig. 4(e)), indicating a possible co-regulation of *HSA1* and *TPS1*. However, the values obtained for *HSA1* promoter were in all situations about a factor of 6 below those of *TPS1* promoter (Fig. 4(c) and (e)).

The performance of *HSA2* promoter was found to differ from that of the *HSA1* promoter. On methanol or glycerol the specific β-galactosidase activities were nearly constant over the range of temperatures tested, with stronger expression rates obtained with glycerol (about a factor of 5; Fig. 4(d)). A moderate positive correlation of specific β-galactosidase activities with increasing temperatures was observed only with glucose as sole carbon source (less than a factor of 2; Fig. 4(d)). Thus, *HSA2* promoter activity is regulated differently with respect to carbon source and temperature in comparison to *HSA1* and *TPS1* promoter (Fig. 4(c), (d), and (e)). As shown above, the strain mixture harbouring P_{HSA2}-*lacZ* contained only half as many plasmid copies as all other strain collections tested (see Fig. 3). For a direct comparison, all values obtained with the P_{HSA2}-*lacZ* construct should therefore be multiplied by this factor. This adjustment would put activities on a level comparable to those of *HSA1* promoter.

Recently, the *TPS1* (trehalose-6-phosphate synthase) gene of *H. polymorpha* has been cloned and characterized [7]. The authors found that the level of Tps1p activity immediately increases upon temperature stress and is followed by an accumulation of intracellular trehalose which has been considered a protective compound at elevated temperatures [10]. This significant increase in the activity of the *TPS1* promoter at higher temperatures was likewise observed using the *lacZ* reporter system (Fig. 4(e)). At 30°C, the specific β-galactosidase activities were minimal on all three carbon sources tested. Compared with the values obtained at 30°C, the *lacZ* expression increased slightly at 37°C, again independent of the carbon source used (Fig. 4(e)). The values observed at these temperatures were comparable to those for *FMD* promoter on glycerol or methanol at 30°C (Fig. 4(a)). However, at 44°C a fivefold increase in promoter strength was observed on glucose or glycerol as sole carbon sources, but not on methanol (Fig. 4(e)). Here, two observations are of interest. The hitherto unobserved lack of heat induction on methanol (Fig. 4(e)) may indicate a more complicated regulation of *TPS1* than previously assumed. The data rather suggest that not only temperature but the carbon source also can significantly influence *TPS1* promoter activity. Secondly, the specific β-galactosidase activities measured for *TPS1* promoter-driven gene expression on glycerol or glucose at 44°C significantly exceed the best values obtained with *FMD* promoter by more than a factor of 2 (Fig. 4(a), (e)). This is a striking result since the promoters of genes involved in methanol metabolism are generally considered to drive heterologous gene expression at upper limit rates [25]. Thus, *TPS1* promoter may constitute an important novel control element for heterologous protein production in forthcoming developments.

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