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SHORT COMMUNICATION

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Cloning, characterization and application of a glyceraldehyde-3-phosphate dehydrogenase promoter from *Aspergillus terreus*

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Abstract It is important to develop native and highly efficient promoters for effective genetic engineering of filamentous fungi. Although *Aspergillus terreus* is an important industrial fungus for the production of itaconic acid and lovastatin, the available genetic toolbox for this microorganism is still rather limited. We have cloned the 5′ upstream region of the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*; 2,150 bp from the start codon) from *A. terreus* CICC 40205 and subsequently confirmed its promoter function using *sgfp* (synthetic green fluorescent protein) as the reporter. The sequence of the promoter *PgpdAt* was further analysed by systematic deletion to obtain an effective and compact functional promoter. Two truncated versions of *PgpdAt* (1,081 and 630 bp) were also able to drive *sgfp* expression in *A. terreus*. The activities of these three *PgpdAt* promoters of varying different lengths were further confirmed by fluorescence, western blot and transcription. The shortest one (630 bp) was successfully applied as a driver of *vgb* expression in the genetic engineering of *A. terreus*. The function of expressed haemoglobin was demonstrated by the CO (carbon monoxide)-difference spectrum and enhanced oxygen uptake rate, glucose consumption and itaconic acid titer. Our study was successful in developing and validating an efficient and compact native promoter for genetic engineering of *A. terreus*.

Keywords *Aspergillus terreus* · Glyceraldehyde-3-phosphate dehydrogenase promoter · Native promoter · Promoter function · *Vitreoscilla* haemoglobin

Introduction

Aspergillus is an extremely large and diverse genus of filamentous fungi, and its members have long been used in biotechnology applications as cell factories for the production of a wide range of high value-added bioproducts, such as organic acids, pharmaceuticals and enzymes [1]. Among these, *Aspergillus niger* has been widely utilized for the production of citric acid, gluconic acid, amylase, glucoamylase, among others [1]. By contrast, *A. terreus* is a rather less known but still valuable species which has been commercially developed as an excellent itaconic acid producer; it is also used to produce lovastatin (Mevacor; Merck & Co., Whitehouse Station, NJ) [2, 3]. Itaconic acid has been reported to be one of the most promising and flexible building blocks and is widely used as the monomer or co-monomer in the manufacturing of plastics, resins and other industrial processes [2]. Lovastatin is an efficient cholesterol-lowering agent [3].

Advances in genetic engineering tools have resulted in the development of increasingly powerful approaches for genetic and metabolic engineering for improving the productivity of the target metabolites of filamentous fungi [1, 4]. Improvement of the various components of genetic engineering, such as the promoters, the markers and the reporters, for a specific fungal host is a significant step towards developing the efficient and strong promoters required to achieve high-level expression of the target genes [5]. For fine-tuning of gene expression, some inducible promoters, including the glucoamylase

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promoter *PglaA* of *A. niger* [6], the Taka-amylase promoter *PamyB* of *A. oryzae*, among others, have been developed [7]. However, most of these usually require the strictly defined conditions for high- or low-level gene expression, respectively, and these are not suitable for industrial applications. Thus, the constitutive promoters, which are not dependent upon on the carbon or nitrogen source or the specific inducers, are preferred. For example, constitutive promoters, such as the *pkiA* promoter of the *A. niger* protein kinase A [8] and the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoters, have been successfully used to express the respective heterologous genes in filamentous fungi [9–12]. Recently, many promoters from *Aspergillus* species have been cloned, characterized and applied in the genetic engineering of these species [13–15]. *PgpdA* from *A. nidulans* is by far the most frequently used promoter in fungal genetic engineering [13, 16].

Native promoters are the preferred promoters for genetic engineering of fungi because they are more efficient in directing gene expression than heterologous ones in some species of fungi [9, 13, 17, 18]. Although *A. terreus* is an important producer of itaconic acid and lovastatin, the genetic tools for *A. terreus*, especially efficient native promoters, are lacking. *PgpdA* from *A. nidulans* is currently the most common promoter used for genetic engineering of *A. terreus* [19, 20]. However, in order to prevent squelching or titration of the specific transcription factors, it is preferable to utilize multiple distinct promoters, with one specific promoter for each gene to be expressed. Multiple native promoters are therefore needed for genetic engineering of *A. terreus*. To date, the only investigated native promoter from *A. terreus* is the *GlaA1* (glucoamylase) promoter; however, it failed to drive expression of rotavirus nucleocapsid protein (VP6) in *A. terreus* [21].

It has recently been reported that the transcription level of the *gpd* gene (ATEG_09817.1) from *A. terreus* increased 15.8-fold under the optimal itaconic acid production conditions, suggesting that the *gpd* gene is regulated by the constitutive and highly active promoter [22]. This finding indicates that the promoter of the *gpd* gene is a good candidate for developing a strong native one for *A. terreus*.

In the study reported here, we cloned the putative promoter region of the *gpd* gene from *A. terreus*. We then evaluated its promoter function and investigated the effects of promoter length on the promoter activity through the systematic deletion analysis. In order to demonstrate the potential of the *gpd* promoter as a useful tool for genetic engineering, it was used for heterologous expression of the *Vitreoscilla* haemoglobin gene (*vgb*) in *A. terreus*.

Materials and methods

Materials

Chemicals were obtained from Sigma (St. Louis, MO), oligonucleotides were synthesized by Shanghai Sangon Biotech Co. Ltd (Shanghai, China), and *Taq* and *Pfu* DNA polymerases and restriction endonucleases were from Fermentas/New England BioLabs (Ipswich, MA). The kits used for molecular cloning were from Omega Bio-tek Biotechnology (Norcross, GA). The vector pMD18-T-simple was obtained from Takara Biotechnology (Otsu, Japan), and the FastStart Universal SYBR Green Master ROX was obtained from Roche Applied Science (Penzberg, Germany). Trizol was from Invitrogen Life Technologies Corp. (Carlsbad, CA), hygromycin B was obtained from Solarbio Science Technology Co. Ltd (China), and mouse anti-GFP (green fluorescent protein) antibody and goat anti-mouse IgG-HRP (immunoglobulin G-horseradish peroxidase) were from TIANGEN Biotech (Beijing) Co. Ltd (Beijing, China).

Strains, plasmids, media and culture conditions

The plasmids pSGF957 and pWY1252, which were used for cloning the genes *sgfp*, *hph*, *trpC* and *vgb* from *Vitreoscilla*, were kindly provided by Professor Kim from Seoul National University and Professor Wen from China Agricultural University, respectively [10, 23]. *Escherichia coli* DH5α was used for routine DNA transformation and plasmid isolation and was grown in Luria–Bertani broth at 37 °C. *A. terreus* CICC 40205 was obtained from the China Center of Industrial Culture Collection and used in this study; it was grown on potato dextrose agar (PDA). Cultivation in shake flasks was carried out in itaconic acid production medium [IPM; g/L: glucose, 155; NH_4NO_3, 2; $(NH_4)_2HPO_4$, 0.2; $MgSO_4$, 0.4; $FeSO_4$, 0.02; $ZnSO_4$, 0.04; $CuSO_4$, 0.04; corn steep, 1; pH 3.5].

Cloning of the *gpd* gene and the *gpd* promoters and construction of the expression cassettes

The primers used in this study are listed in Electronic Supplementary Material (ESM) Table S1. General molecular biology techniques were carried out following standard procedures [24]. The primers gpd-F/gpd-R for the *gpd* gene of *A. terreus* CICC 40205 were designed based on the annotation for ATEG_09817 in the genome of *A. terreus* NIH 2624 (http://www.cadre-genomes.org.uk/Aspergillus_terreus/Info/Index). The coding DNA sequence (CDS) and the full-length fragment of the *gpd* gene (ATEG_09817) were amplified by PCR using the cDNA and genomic DNA of *A. terreus* CICC 40205 as the

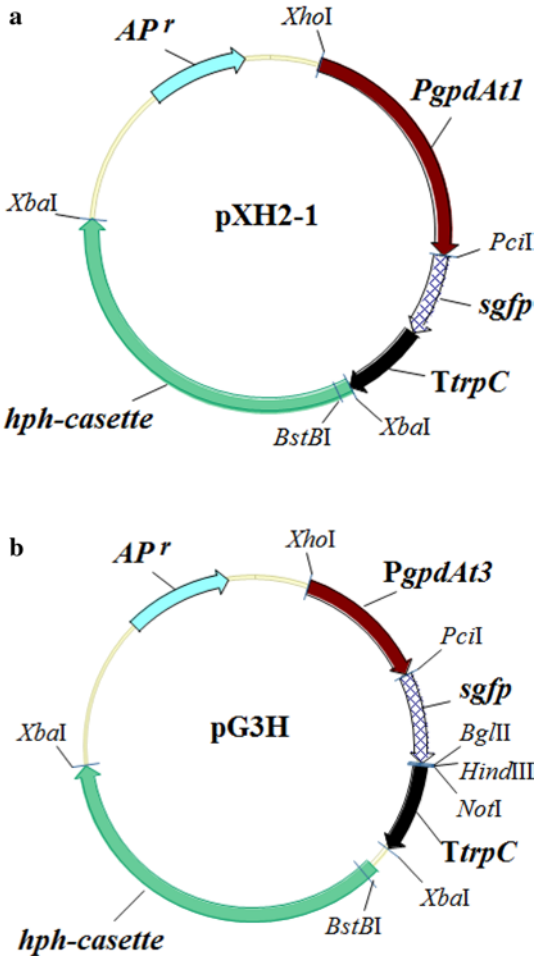

Fig. 1 Schematic structures of the expression vectors pXH2-1 (**a**) and pG3H (**b**). *hph-cassette* Selection marker for hygromycin B resistance, *Ttrpc Aspergillus nidulans trpC* terminator, *Pgpd* promoter of glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene

respective templates. The terminator *trpC* from *A. nidulans* was used as the terminator in the current study [5, 10].

In order to cover all possible regulatory elements of the promoter of the *gpd* gene (ATEG_09817), we amplified and subsequently sequenced the upstream region of *gpd* (2,234 bp from the start codon), starting from the stop codon (TAA) of the upstream gene (ATEG_09816) and extending to the start codon (ATG) of the *gpd* gene, by PCR using genomic DNA of *A. terreus* CICC 40205 as the template and the primers PgpdAt-F/PgpdAt-R. The promoter *PgpdAt1* (2,150 bp, full-length) was amplified by PCR using the primers PgpdAt1-F/PgpdAt-R1 and subsequently cloned into the vector pMD18-T-simple by TA cloning. The *Pci*I restriction site of the resulting vector was removed by site-directed mutagenesis using the primers mtpMD18-F/mtpMD18-R, thus generating the plasmid pMD18ms-PgpdAt1. The *sgfp-TtrpC* fragment was amplified by PCR using the plasmid pSGF957 as the template and the primers sgfp-F/TtrpC-R and then cloned into the vector pMD18ms-PgpdAt1 at the restriction sites of *Pci*I and *Xba*I, thus creating the plasmid pMD18ms-PgpdAt1-sgfp. The *hph* cassette, which was cut from the plasmid pSGF957 with *Xba*I, was inserted into the fragment pMD18ms-PgpdAt1-sgfp digested with *Xba*I, resulting in construction of the plasmid pXH2-1 (Fig. 1a). The promoters *PgpdAt2* (1,081 bp) and *PgpdAt3* (630 bp) were amplified by PCR using the primer pairs PgpdAt2-F/PgpdAt-R1 and PgpdAt3-F/PgpdAt-R1, respectively; these were then cloned into pXH2-1 at the restriction sites of *Xho*I and *Pci*I to give pXH2-2 and pXH2-3, respectively (Fig. 1a).

Construction of the *Vitreoscilla* haemoglobin expression cassette

The fragments *sgfp-A* and *TtrpC-B* were amplified by PCR from pXH2-1 using the primer pairs sgfp-F/sgfp-R1 and TtrpC-F1/TtrpC-R1 respectively, then fused into the fragment *sgfp-TtrpC* containing the restriction sites of *Bgl*II, *Hind*III, and *Not*I by overlap extension PCR. The fragment *sgfp-TtrpC-B* obtained by digestion with *Pci*I and *Bst*BI was inserted into pXH2-3, which was then cut with *Pci*I and *Bst*BI to generate the vector pG3H (Fig. 1b). The *vgb* fragment was amplified by PCR from the plasmid pWY1252 using the primers vgb-F/vgb-R and then cloned into pGH3 at the restriction sites of *Pci*I and *Bgl*II to give the construct for *vgb* expression. The *vgb* gene was expressed under the control of the P*gpdAt3* promoter.

Aspergillus terreus transformation

Mycelia from *A. terreus* CICC 40205 were grown in IPM at 30 °C for 16 h in shake flasks and then transformed using the protoplast-PEG method as previously described [13]. Transformants were selected on PDA-SH plates (PDA supplemented with 1.2 M sorbitol and 100 mg/L hygromycin B). The integrations of the *PgpdX::sgfp* and *vgb* cassettes into the genome of *A. terreus* were confirmed by genomic PCR using the primer pairs M13-47/sgfp-seqR1 and M13-47/vgb-R, respectively. Representative transformants were randomly selected for further analysis.

Evaluation of the *Pgpd* promoters

Quantitative real-time PCR (qRT-PCR) was performed using FastStart Universal SYBR Green Master ROX (Roche Applied Science) on a Light Cycler (Roche Applied Science) to estimate the transgene copy number of the selected transformants [5, 25]. The ATEG_02314 gene (a single copy in the genome of *A. terreus* NIH 2624) was used as the control.

The bright field and fluorescent images of the selected transformants at the stages of conidia, young hyphae, and mature hyphae were observed by fluorescence microscopy (Olympus model BX51; Olympus, Tokyo, Japan). Young and mature hyphae were obtained by cultivation in shake flasks at 37 °C for 11 and 36 h, respectively. The total proteins were extracted from mature hyphae ground with liquid nitrogen in solution (50 mM sodium phosphate, pH 7.2, 1 mM EDTA, 1 mM PMSF). The protein concentration was determined by the Bradford method using bovine serum albumin as a standard and diluted to approximately 0.5 mg/ml. The expressed GFP was also analysed by western blot following the standard protocol. Fluorescence intensity was measured using the Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT) at an excitation of 485 nm, emission at 516 nm, optics position at the top and sensitivity of 40.

The functions of the promoters were also investigated at the transcription level of *sgfp* by qRT-PCR [9]. The actin gene (ATEG_06973) was used as the endogenous control. qRT-PCR was performed according to the protocol provided by the manufacturer, and the data were analyzed as reported [26].

Analysis of the *vgb* transformants

The fermentation processes of the parental strain and the best itaconic acid producer V08 were investigated by cultivation in 500-ml shake flasks every 12 h. Each shake flask contained 55 ml IPM and following inoculation with 2.5×10^7 spores the flasks were incubated for 72 h at 37 °C, 200 rpm. The initial pH was 3.25 and fell to around 2.6 by the end of the fermentation. Three independent experiments were set for each transformant. All assays were done at least in duplicate.

The activity of the expressed VHb was demonstrated by the CO-difference spectra of the cell-free extracts of V08 and the parental strain [20]. Total proteins were extracted from mycelia as described above.

Cells were harvested by centrifugation, washed with 100 % oxygen-saturated IPM and resuspended in the same medium. The changes in dissolved oxygen were measured using the Oxylab Oxygen Monitoring System (Hansatech Instruments, Norfolk, UK).

Mycelial dry weight was determined as described previously [27].

Residual glucose was quantified using the biosensor (SBA-40C) from Biology Institute of Shandong Academy of Science (Jinan, China) following the standard protocol.

The itaconic acid produced was quantified by high-performance liquid chromatography using an Aminex HPX-87-H column (300 × 7.8 mm); detection was at 210 nm. The column was operated at 35 °C with a mobile phase of 4 mM H_2SO_4 at a flow rate of 0.6 ml/min. Authentic itaconic acid was used as a standard to calculate the final concentration of itaconic acid produced.

Results and discussion

Cloning and sequence analysis of the *gpd* gene from *A. terreus* CICC 40205

The *gpd* gene was amplified by PCR using the cDNA of *A. terreus* CICC 40205 as the template and the primers gpd-F/gpd-R. However, the sequencing results showed different open reading frames from the annotated *gpd* (ATEG_09817) gene in the genome sequence of *A. terreus* NIH 2624, including the translation initiation site, the numbers and positions of the exons and introns. Based on our experimental results and sequence alignment of the *gpd* genes from other Aspergilli, including *A. niger*, *A. nidulans*, *A. flavus*, *A. fumigatus* and *A. oryzae* (data not shown), we corrected the CDS of the *gpd* gene of *A. terreus* CICC 40205, which has been deposited in GenBank under accession no. KC213825.

Cloning, sequence analysis and functional characterization of *PgpdAt1*

A 2,234-bp upstream sequence from the start codon (ATG) of the *gpd* gene of *A. terreus* CICC 40205 was cloned and sequenced; this sequence showed 93.8 % identity with the annotated one for ATEG_09817 in the genome sequence of *A. terreus* NIH 2624. Sequence alignment of the upstream regions of the *gpd* genes from other Aspergilli was carried out (http://www.cadre-genomes.org.uk/index.html), and several conserved regions were observed (Fig. 2). The one located about −400 bp from the start codon (ATG, A as +1) has been referred to as the *gpd* box (Fig. 2) [11]. This *gpd* box is an important element in regulating transcription of the *gpd* gene, and its deletion was found to result in a 50 % decrease in the expression level of the reporter gene coding for β-glucuronidase (*gus*) [11, 17]. In contrast, introduction of the *gpd* box *A* into the upstream of the *amdS* gene led to a 30-fold increase in expression of the reporter gene [28].

The sequence analysis revealed that Intron-1 (−94 to −11 bp) was removed during the processing of mRNA (Fig. 2), which might not play a role or roles in the translation of the *gpd* gene. In an earlier study, deletion of this region in *A. nidulans* had no effect on the expression level of the reporter gene [11]. Therefore, we did not include Intron-1 in the amplification of the putative promoter *PgpdAt1*.

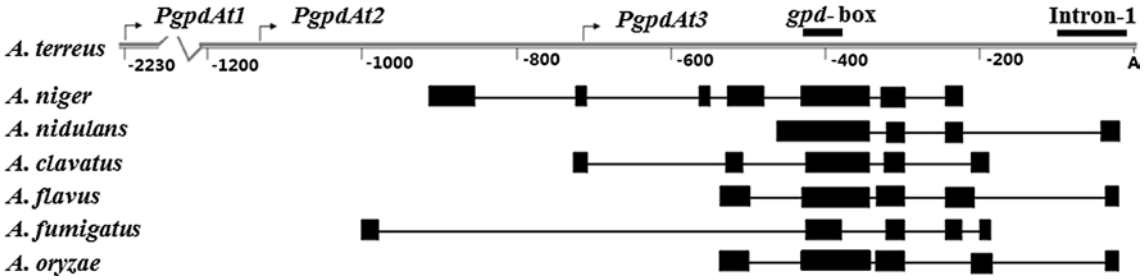

Fig. 2 Conserved regions in the putative promoters of the genes coding for the *gpd* gene from seven filamentous fungi. The GenBank accession numbers of the selected *gpd* genes are: *Aspergillus niger* CBS 513.88 (An16g01830); *A. nidulans* FGSC A4 (ANIA_08041); *A. flavus* NRRL 3357 (AFLA_025100); *A. fumigates* Af293 (AFUA_5G01970); *A. oryzae* RIB40 (AO090003001322); *A. clavatus* NRRL1(ACLA_003290)

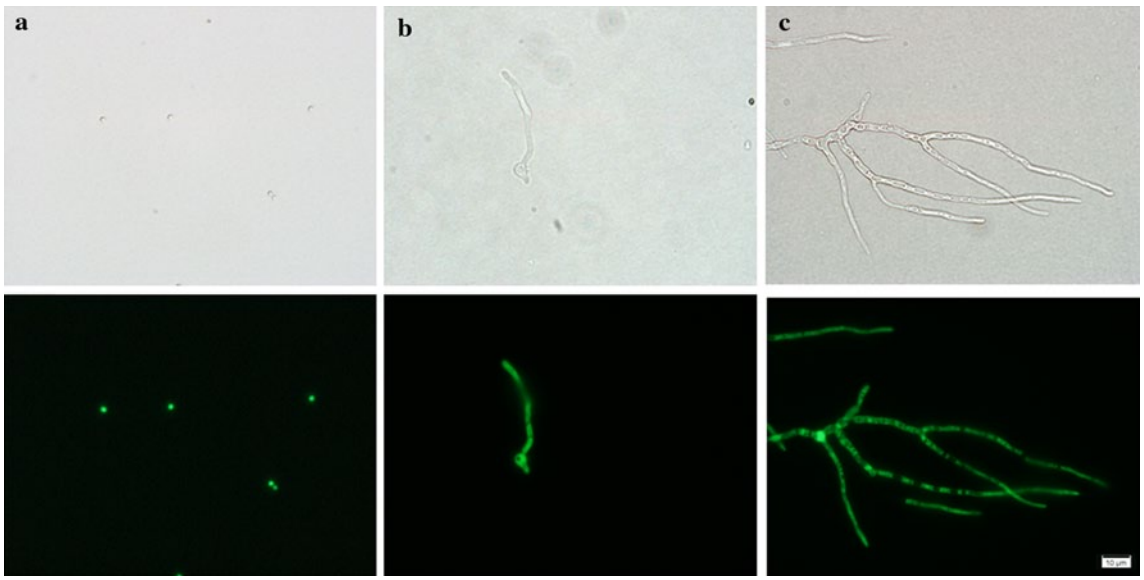

Fig. 3 Bright field and fluorescence images of *A. terreus* transformant 1-1 carrying the *PgpdAt1*::*sgfp* construct in different growth stages. **a** Conidia, **b** young hyphae, **c** mature hyphae. *sgfp* Synthetic green fluorescence protein. *Scale bar* 10 μm

The promoter function of the putative promoter region was evaluated using *sgfp* (synthetic GFP) as the reporter. The expression cassette pXH2-PgpdAt1 for the promoter *PgpdAt1* (2,150 bp, the full-length fragment of the *gpd* promoter) was constructed and expressed in *A. terreus* CICC 40205 (Fig. 1a). The integration of the *PgpdAt1::sgfp* cassette into the genome of *A. terreus* was confirmed by genomic PCR (ESM Fig. S1). The bright field and fluorescent images of randomly picked samples of *A. terreus* transformant 1-1 were taken at different stages: conidia, young hyphae and mature hyphae (Fig. 3). As a negative control, the parental strain *A. terreus* CICC 40205 did not show any visible GFP fluorescence at all three of these stages (data not shown). These results clearly demonstrate that *PgpdAt1* successfully drove *sgfp* expression in *A. terreus*.

Deletion analysis of *PgpdAt1*

In order to obtain the efficient and compact promoters which are desirable in genetic engineering, we designed two truncated promoters, namely, *PgpdAt2* (1,081 bp) and *PgpdAt3* (630 bp), which we amplified by PCR (Fig. 2). *PgpdAt3* contains the highly conserved regions, including the *gpd* box (Fig. 2). However, taking into account the possibility that *PgpdAt3* might not cover all regulatory features, we also designed *PgpdAt2*, which includes all conserved regions and is 451 bp longer than *PgpdAt3* (Fig. 2). The corresponding expression cassettes were constructed and expressed in *A. terreus* CICC 40205. Interestingly, the truncated promoters *PgpdAt2* and *PgpdAt3* were also able to drive *sgfp* expression in *A. terreus* at the conidia, young hyphae and mature hyphae stages (ESM Fig. S2 and Fig. S3).

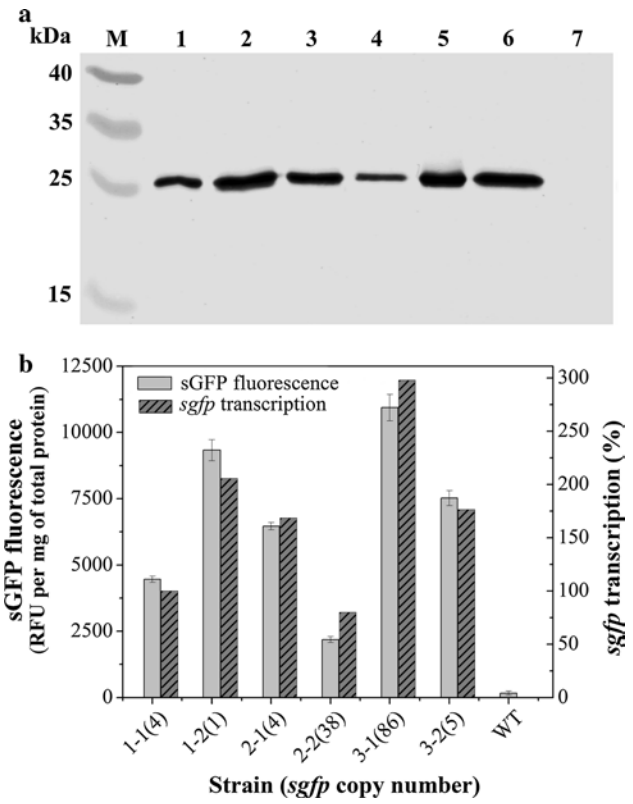

Fig. 4 Evaluation of the *Pgpd* promoters. **a** Western blot analysis of expressed GFP. *Lanes*: *M* Protein marker, *1*, *2* transformants 1-1 and 1-2, respectively, carrying the *PgpdAt1*::*sgfp* construct, *3*, *4* transformants 2-1 and 2-2, respectively, harboring the *PgpdAt2*::*sgfp* construct, *5*, *6* transformants 3-1 and 3-2, respectively, carrying the *PgpdAt3*::*sgfp* construct, *7 A. terreus* CICC 40205. **b** Fluorescence and transcription analysis of GFP (copy number in *parenthesis*). The transcription level of the *PgpdAt1* transformant 1-1 was set to 100 %

The promoter activities of these three *PgpdAt* promoters of different lengths were further demonstrated by western blot analysis (Fig. 4a), fluorescence studies (Fig. 4b), and transcription (Fig. 4b). These experiments gave consistent results and showed a similar trend (Fig. 4):

(1) The shortest promoter *PgpdAt3*, which includes the highly conserved regions, completely retained promoter activity.
(2) Deletion of the fragments −2150 bp to −1081 bp and −2150 bp to −630 bp, respectively, did not result in reduced promoter activity, implying that the absence of positive regulatory elements located between −2150 bp and −630 bp (Figs. 2, 4). The efficient and compact native *gpd* promoter *PgpdAt3* from *A. terreus* was identified. Some short and functional *gpd* promoters have been reported, including the 946-bp promoter from *Metarhizium acridum* [9], the 442-bp promoter from *Lentinus edodes* [29] and the 190-bp promoter from *Candida bombicola* [12]. It would appear that the *gpd* promoters from different microorganisms show different regulatory characteristics.
(3) Multicopy *sgfp* did not increase the translation/transcription level (Fig. 4b), possibly due to the titration of endogenous factors when multiple integrated copies are present [5]. However, it is still controversy on this possibility [5, 17].
(4) Two transformants for the same promoters exhibited different translation/transcription levels; for example, transformants 1-1 and 1-2 for *PgpdAt1*, transformants 2-1 and 2-2 for *PgpdAt2*. The difference might arise from the different integration sites, since there was no obvious relationship between copy number and translation/transcription, and the integrations were expected to be random in our study.

Application of *PgpdAt* in genetic engineering of *A. terreus*

Even a brief interruption of aeration during fermentation significantly decreases the itaconic acid titer [5]. *Vitreoscilla* haemoglobin (VHb) has been widely used to alleviate the adverse effects of hypoxic conditions and to improve growth, enzyme production and metabolism in various microorganisms [30, 31]. In order to demonstrate biotechnological application of *PgpdAt* as the promoters in genetic engineering of *A. terreus*, we expressed *vgb* in *A. terreus* CICC 40205 under the control of the efficient and compact promoter *PgpdAt3*.

All hygromycin-resistant transformants were grown in IPM. The best itaconic acid producer, V08, was chosen from 27 transformants for further analysis. The activity of expressed VHb in *A. terreus* was demonstrated by the CO (carbon monoxide)-difference spectrum (Fig. 5). The peak we observed at 422 nm for V08 resulted from CO binding to VHb, whereas a corresponding peak was not observed in

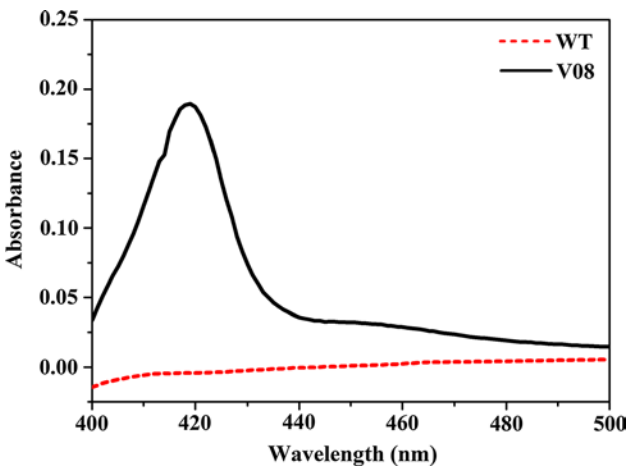

Fig. 5 Carbon monoxide difference spectra of cell-free extracts of transformant V08 and the wild type (*WT*)

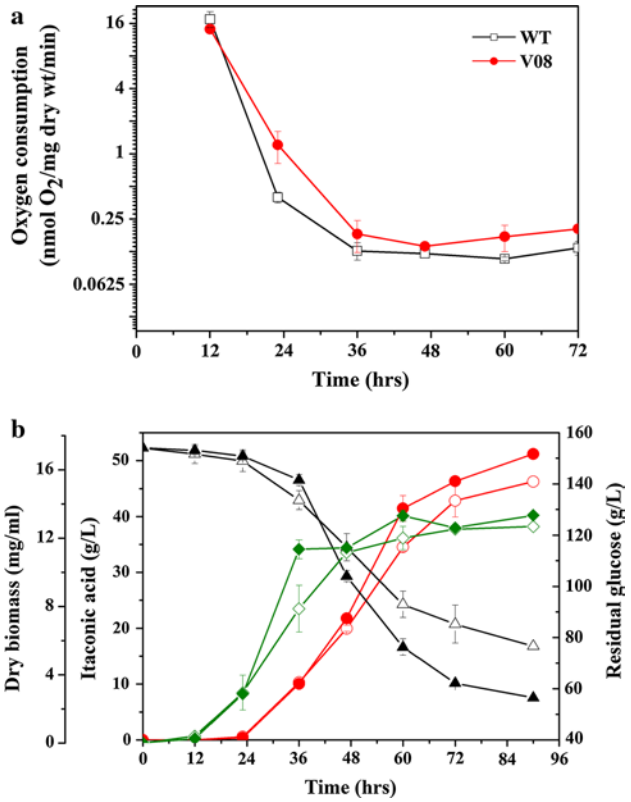

Fig. 6 **a** Oxygen uptake in transformant V08 and WT. **b** Time courses of dry biomass (*green diamond*), glucose concentration (*black triangle*), and itaconic acid production (*red circle*) in transformant V08 (*filled symbols*) and the WT (*open symbols*)

the wild-type (Fig. 5; [20]). Moreover, compared with the parental strain, V08 showed improved cell growth, glucose consumption and itaconic acid production (Fig. 6). The trend exhibited in our study is similar to those published by Lin et al., where VHb was expressed in *A. terreus* under the control of *PgpdA* [20]. Although the oxygen uptake rate was not that high as reported (Fig. 6a) by Lin et al. [20], the *vgb* transformant V08 showed faster glucose consumption and itaconic acid production when oxygen availability became limited (after 48 h) (Fig. 6b). The different results might result from the different initial glucose concentrations [160 (our study) vs. 100 g/L] or *A. terreus* hosts [*A. terreus* CICC 40205 (our study) vs. NRRL 1960] or promoters [*PgpdAt3* (our study) vs. *PgpdA*] or integration sites (the integration into the genome was expected to be random). As reported for *A. niger* harbouring the *vgb* gene [32], cell growth reflected by biomass was not greatly affected by the expressed VHb (Fig. 6b). These results prove that the *vgb* gene was successfully expressed in *A. terreus* under the control of *PgpdAt3* and that *PgpdAt* was suitable as the promoter for genetic engineering of *A. terreus*.

In summary, we have cloned and functionally characterized the promoter region of the *gpd* gene from *A. terreus*. The *PgpdAt* promoters with different lengths were able to successfully drive *sgfp* expression in *A. terreus*. The shortest one, *PgpdAt3* (630 bp), was also successfully applied in genetic engineering of *A. terreus* by driving *vgb* expression. We therefore have developed an effective and compact native promoter for engineering the important industrial microorganism—*A. terreus*.

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