Biotechnology Reports 33 (2022) e00695

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Novel pentose-regulated promoter of *Aspergillus oryzae* with application in controlling heterologous gene expression

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ARTICLE INFO

Keywords:
Aspergillus oryzae
Gene expression
Inducible promoter
Pentose
Xylose reductase

ABSTRACT

The potent promoter and its transcriptional control make a significant contribution to strain optimization. Using transcriptome-based approach, a novel pentose-regulated promoter of the xylose reductase gene (*PxyrA*) of *Aspergillus oryzae* was identified. The promoter analysis showed that the *PxyrA* was tightly regulated by pentose sugars, which xylose and xylan were favorable inducers. The *PxyrA* function was highly efficient as compared with the maltose-inducible promoters of *A. oryzae*. It also exhibited the efficient transcription induction even though certain amounts of glucose and sucrose existed in the cultures. The expression control of *PxyrA* was dependent on xylose consumption capacity for fungal growth. The control mode of *PxyrA* offers a simple operation in simultaneous gene expression and cultivation optimization in Aspergilli. This study provides a prospective development of fungal production platform using cellulosic sugars by the xylose-utilizing strains for sustainable growing in circular economy.

1. Introduction

The significance of fungal production system for industrial applications has been renowned. Among the industrial strains of filamentous fungi with generally recognized as safe (GRAS) status, *Aspergillus oryzae* has been attracted as a cell factory for production of diverse primary and secondary metabolites with commercial interest [1, 2]. Comparative genome analysis of 11 *Aspergillus* strains revealed that *A. oryzae* harbors several enzyme-coding genes with the involvement in degrading complex raw materials (i.e., agricultural biomass and agro-industrial residues) more than *Aspergillus niger* that is one of the most important enzyme producers [3]. The potent genetic toolboxes for rational strain improvement of *A. oryzae* have been developed, such as gene expression, precise gene editing and marker recycling systems [4–6]. Similar to other organisms, the efficient expression cassette in filamentous fungi consists of essential elements of promoter and terminator as well as gene of interest. Typically, the promoters can be categorized into constitutive and inducible promoters based on their function modes. The choice of a suitable promoter for driving gene expression depends on the type of targeted products and the relevant metabolic fluxes in the cells. Although the constitutive promoters offer a simplicity without the requirement of certain inducer, they are not suited for the production of some products harmful to cell growth and survival, and thus the process operation should be criticized to acquire high production yield. Alternatively, the inducible promoters are of interest because the transcriptional regulations can be fine-tuned, and also overcome such limitation in growth perturbation by the product generated.

With the accumulative knowledge on promoter structure and function, a number of inducible and constitutive promoters have been identified and developed for *A. oryzae*. For inducible promoters, their regulatory modes are mostly dependent on carbon sources, such as the sorbitol-sensitive (*Psor*) [7], taka-amylase A (*PamyB*) [8, 9] and glucoamylase (*PglaA*) promoters [10]. Although *Psor* promoter was strictly controllable, its ability in inducing gene expression is rather low as illustrated by green fluorescent protein (GFP) reporter. Two maltose/malto-oligosaccharides-inducible promoters, *PamyB* and *PglaA*, have frequently been used for fungal systems [11–13]. However, they were not tightly related to the inducible conditions, in which some enzyme expressions under the promoter controls were still detected in the non-induction or the glucose repression conditions [7, 8, 10]. Moreover, it is not practical and feasible, particularly in the industrial

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https://doi.org/10.1016/j.btre.2021.e00695
Received 26 July 2021; Received in revised form 12 December 2021; Accepted 15 December 2021
Available online 21 December 2021

Abbreviations

*P*amyB	taka-amylase A promoter
DEGs	differentially expressed genes
FPKM	fragments per kilobases of exon region per million mapped reads
PglaA	glucoamylase promoter
GUS	β-glucuronidase
pyrG	orotidine 5′-phosphate decarboxylase
PxyrA	xylose reductase promoter
Qs	volumetric carbon consumption rate
TLS	translation start site
TSS	transcription start site

application that requires an additional step in the production process, such as cell washing to eliminate the remained glucose repressor before expression induction [11–13]. Further, the development of thiamine-dependent promoter (*PthiA*) of *A. oryzae* [14] has been reported, however its function was inactive under alkaline growth condition. Apart from the control mode of promoters, the promoter strength is also taken into consideration according to several criteria, such as strain background, tailored-made cultivation, product of target and production cost.

Regarding to the ability of filamentous fungi in utilization of xylose and lignocellulosic feedstocks [15], the xylose/xylan-inducible promoters of endoxylanase genes were developed for many industrial fungal strains, such as *Aspergillus awamori* [16], *Penicillium chrysogenum* [17], *Acremonium chrysogenum* [18] and *Trichoderma reesei* [19]. These promoters displayed strong control in the gene expressions by induction with xylose or xylan, but were tightly repressed by glucose and sucrose. Comparative analysis in controlling the gene expression between the xylose-inducible *PexlA* promoter of *A. awamori* [16] and maltose-inducible *PglaA* promoter of *A. niger* [20] showed that the β-glucuronidase (GUS) activity under the control of *PexlA* promoter was 3-fold increase as compared with that using *PglaA* promoter.

Based on the empowering omics technology, we aimed to identify the pentose-regulated promoter of *A. oryzae* BCC7051 through comparative transcriptome analysis of the xylose- and glucose-grown cultures. By bioinformatics tools and experimental analysis, the functional characterization of the selected promoters was implemented. The strength of the pentose-regulated promoters of the strain BCC7051 for controlling GUS expression was also investigated in comparison to the known inducible promoters (*PamyB* and *PglaA*). Additionally, the expression control of the potent promoter in *A. oryzae* as well as the cultivation condition were established to acquire a practice guideline useful for overexpressing the genes of interest in filamentous fungi. Not only for addressing the gene function or biological queries arisen, this potent promoter is also highly beneficial for rational strain optimization through metabolic engineering and gene editing technologies for industrial purposes.

2. Materials and methods

2.1. Microorganisms and cultivations

A. oryzae strain BCC7051 was employed as a DNA source for promoter sequence analysis. The *pyrG* auxotrophic strain of *A. oryzae* (Δ*pyrG*) was used as a recipient strain for fungal transformation. Spore inoculum was prepared by growing the fungal strains on polished rice grains at 30 °C for 120 h, and then the spore suspension was carried out using 0.05% (v/v) Tween 80 solution. For submerged cultivation, the fungal cultures were routinely grown in 250-ml Erlenmeyer flasks containing 50 ml of semi-synthetic medium (SM) [21] at 30 °C with shaking at 250 rpm.

Saccharomyces cerevisiae strain INVSCI (*MATα, his3-Δ1, leu2, trp1-289, ura3-52, MAT, his3-Δ1, leu2, trp1-289, ura3-52*) was used as a recipient for DNA assembly. Yeast cells were routinely grown following the Invitrogen's instruction manual (USA).

Escherichia coli strain DH5α (*supE44, ΔlacU169*, (*Φ80, lacZΔM15*), *hsdR17, recA1, endA1, gyrA96, thi1, relA1*) was used for plasmid propagation. Bacterial cells were cultured according to the standard method [22].

2.2. Transcriptome-based identification of the xylose-regulated genes of *A. oryzae*

To identify the regulatory promoter for controlling the gene expression, the transcriptome data of the xylose- and glucose-grown cultures of *A. oryzae* BCC7051 at active growth phase (C5_t1 and C6_t1) and late logarithmic phase (C5_t2 and C6_t2), which have been deposited at NCBI Sequence Read Archive (SRA) database under the accession number SRR14929132−SRR14929135 [23], were subjected to the analyses using the HTSeq software [24] and NovoFinder program (www.novogene.com). The resulting transcript levels were represented as value of fragments per kilobases of exon region per million mapped reads (FPKM). Using the $\log_2$ FoldChange > 5, q-values < 0.001 and FPKM values > 1000 as cut-off values, the differentially expressed genes (DEGs) between the C5 and C6 cultures at both growth phases were grouped. The genes with high expression levels only in the xylose culture were chosen for further analysis of their promoter sequences and functions.

2.3. Promoter element analysis

The coding region and the translation start site (TLS) of two xylose-regulated DEGs, xylose reductase (*xyrA*) and xylitol dehydrogenase (*xdhA*) of *A. oryzae* BCC7051 were predicted based on the sequence identity using NCBI database. Using Standalone BLAST software package (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the identified coding regions were then subjected to BLAST analysis against the *A. oryzae* BCC7051 genome [25] for localizing their upstream sequences and 5′-untranslated regions (5′-UTR). Accordingly, the putative promoter regions of individual genes with approximate 1,500-nt length (nt −1,000 to + 500 relative to TLS) were subjected to identify the putative transcription start site (TSS), core regulatory elements (i.e., TATA box and CAAT box) and transcription factor binding sites using the PromPredict (http://nucleix.mbu.iisc.ac.in/prompredict/prompredict.html) and GPMiner programs (http://gpminer.mbc.nctu.edu.tw/index.php).

2.4. RT-qPCR analysis of the xylose-regulated genes in the wild type of *A. oryzae*

RT-qPCR technique was employed for verifying the expression of targeted DEGs in the wild type of *A. oryzae* BCC7051. The fungal cultures were grown in SM broth containing 2% (w/v) glucose for 24 h, then added with xylose at different concentrations (1, 2 and 4%. w/v) and further incubated for 24 h. For investigating the induction time of xylose, the glucose cultures grown for 24 h were added with 4% (w/v) xylose, and subsequently incubated at different time intervals (12, 24 and 48 h). The cultures before and after xylose addition were harvested for total RNA extraction, and measuring residual sugar concentration and dry cell weight (DCW). Total RNA was extracted using RNeasy mini kit (Qiagen, Germany), and then reverse-transcribed into cDNA using oligo-dT primer and SuperScript II first-strand synthesis system (Invitrogen, USA) according to the manufacturer's instruction. RT-qPCR analysis was carried out according to the previous method [26]. 18S rRNA was used for normalization of the targeted transcript ratios.

2.5. Construction of expression cassettes and fungal transformation

The β-glucuronidase (*uidA*) of *E. coli* [27] was used as a reporter gene for functional characterization of the candidate promoters. It was synthesized by using a service of GenScript (Piscataway, USA). A series of promoter-reporter expression cassettes individually containing the *A. oryzae* promoters (*PxyrA, PamyB* and *PglaA*) upstream to *uidA* DNA fragment, and *trpC* terminator were designed. To facilitate the specific integration of the expression cassettes into the *pyrG* locus on *A. oryzae* genome, the individual cassettes were flanked upstream and downstream with portions of 5′- and 3′-UTR sequences of the *pyrG* gene (*pyrG-LF* and *pyrG-RF*), respectively. All DNA fragments were amplified by PCR using Platinum® *Taq* Hi-fidelity DNA polymerase, and then the amplified fragments were fused with the backbone plasmid (YEp356) by DNA assembly in *S. cerevisiae* cells [28]. The control plasmid without promoter fragment (pGus) was also constructed. Restriction enzyme analysis and DNA sequencing were conducted to verify the constructed expression plasmids.

Fungal protoplast transformation was carried out using the PEG-mediated method [29]. Transformed cells were selected by growing them on Czapek's-Dox agar at 30 °C for 5−7 days [30, 31]. Single colony isolation of the transformants was then performed. The precise DNA integration into the chromosome was verified by PCR using a pair of specific oligonucleotide primers designed from the sequences of the constructed expression cassettes. All primers used in this study are listed in Supplementary Table S1. In addition, the selected transformant clones of individual constructs were subjected to determine the copy number of integrated expression cassettes. Genomic DNAs of the transformants were extracted [32], and digested with *Eco*RI for Southern blot analysis using the Gene Images Alkphos Direct Labelling and Detection System (GE Healthcare, UK), and the 0.8-kb *uidA* fragment as a probe. The signal generation and detection were carried out using CDP-Star™ chemiluminescent detection reagent with the Alliance Q9 Advanced Chemiluminescence Imaging System (Uvitec, UK).

2.6. Functional characterization of the xylose-inducible PxyrA in A. oryzae

To investigate the *PxyrA* function, the constructed *A. oryzae* transformant carrying the pPxyrA expression cassette was grown in SM broth containing 2% (w/v) glucose for 24 h. Then, the expression induction was carried out by adding with 4% (w/v) of individual C5 sugars, which were ribose, arabinose and xylose, and C5 derivative (xylan). In addition, various xylose concentrations (2, 4, 6 and 8%, w/v), and induction times (24−96 h) were investigated for optimizing the expression induction condition in the pPxyrA transformant.

The influence of glucose and sucrose on the *PxyrA* function under xylose induction was also studied. The pPxyrA transformant was grown in 2% (w/v) glucose or sucrose for 24 h. Then, glucose or sucrose at various concentrations (2−6%, w/v), and xylose inducer (4%, w/v) were simultaneously added into the fungal culture. The induction was carried out for 24 h.

For comparative study of the promoter strength, the fungal transformants carrying single copy number of individual expression cassettes (pPxyrA, pPamyB and pPglaA) and pGUS plasmid were cultivated in SM broth containing 2% (w/v) glucose for 24. Then, appropriate xylose or maltose at 4% (w/v) concentration were used for the expression induction for 24 h.

2.7. Analysis of gene expression under the PxyrA control

To further investigate the controlling mode of *PxyrA*, the pPxyrA transformant of *A. oryzae* was grown in SM media containing 4% (w/v) carbon sources, which there was no inducer addition along the cultivations for 48. The assimilated carbon sources for fungal growth were tested, including C3 (glycerol), C5 (arabinose, ribose andxylose) and C5 derivative (xylan), C6 (galactose, fructose and glucose) and C12 (maltose andsucrose) saccharides. Time-course analysis of xylose-regulated expression of the reporter gene under the control of *PxyrA* was also implemented.

2.8. β-Glucuronidase assay

The harvested mycelia were subjected to prepare the extracts as previously described [8]. Protein concentration was measured according to the Bradford method [33] using Bio-Rad protein assay kit (Hercules, USA), and bovine serum albumin (BSA) as a standard. Using *p*-nitrophenyl glucuronide as a substrate, the GUS activity assay was performed by spectrophotometry, in which one unit is defined as the amount of enzyme producing 1 nmol *p*-nitrophenol/min at 37°C [27].

2.9. Biomass and sugar measurements

Mycelial cells were harvested by filtration using Miracloth (EMD Chemicals, USA), and then were hot-air dried at 60 °C until the constant weight was obtained. The fungal biomass titer was represented as DCW/L.

Residual sugars in the fermented broths of the *A. oryzae* cultures were quantified by high-pressure liquid chromatography (HPLC, Ultimate 3000, Thermo, USA) equipped with a refractive index detector (RID), and Aminex HPX-87H ion exclusion column (Bio-Rad Laboratories, Hercules, USA). The column was run in an isocratic mode using 5 mM H_2SO_4 as a mobile phase as previously described [34]. All data are shown as the mean of values derived from independently triplicate experiments. The statistical analysis of data was conducted using the Statistical Package for the Windows®, and the data were considered statistically significant at $p < 0.05$.

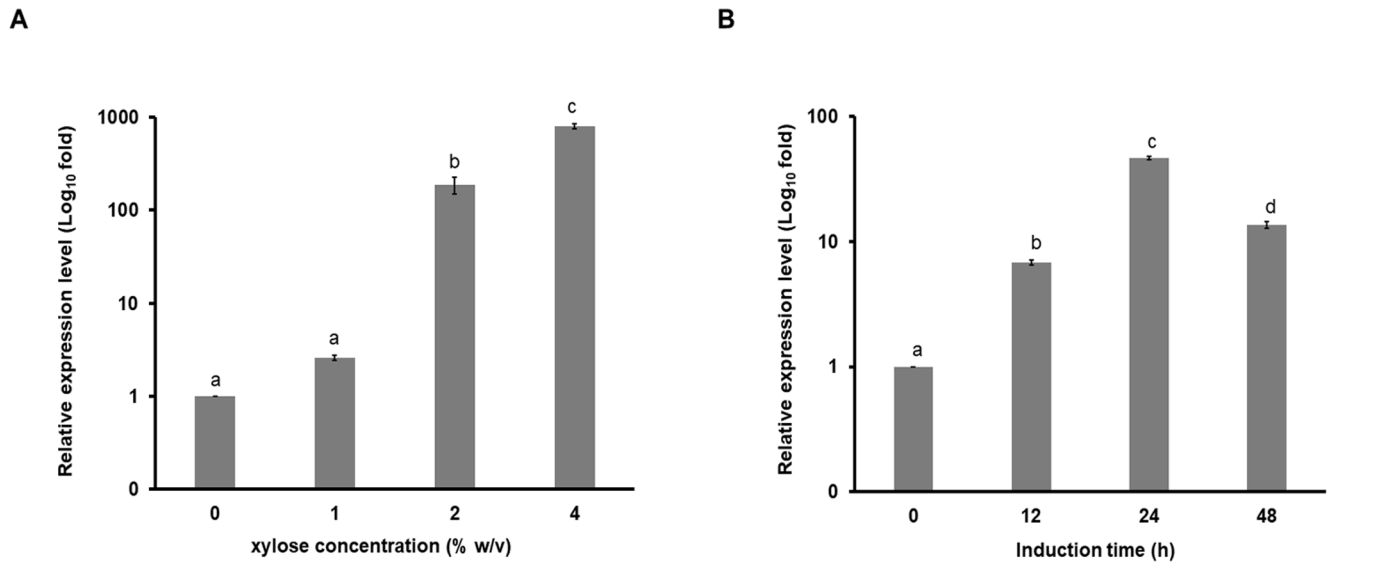
3. Results

3.1. Putative xylose-regulated DEGs of A. oryzae identified through comparative transcriptome analysis

To identify the regulatory promoter controlling the gene expression, the comparative transcriptome analysis of the C5 and C6 cultures of *A. oryzae* grown at active growth and late logarithmic phases was performed. A set of DEGs were identified based on FPKM values using cut-off of $\log_2$ FoldChange > 5. The result of volcano plots revealed that 162 and 439 DEGs were up-regulated in the active growth and late logarithmic phases of the C5 cultures, respectively, as compared to those of the C6 cultures. Among them, two xylose-regulated genes of *A. oryzae, xyrA* and *xdhA*, were highly expressed (FPKM > 1,000) at both growth stages (Supplementary Fig. S1). However, the *xyrA* gene exhibited higher expression level than the *xdhA* gene (Supplementary Fig. S1). The expressions of both xylose-regulated genes in *A. oryzae* were further verified by RT-qPCR, showing the similar results to the transcriptome data. The *xyrA* gene was strongly expressed in the xylose-grown cultures (Supplementary Fig. S2), and also remained high expression level even though the fungal culture entered the late logarithmic phase. Accordingly, the 5′ upstream sequence of *xyrA* was chosen to further functional characterization in controlling the gene expression in *A. oryzae*.

3.2. Xylose-induced expression of the xyrA gene in the wild type of A. oryzae

The *xyrA* gene expression of *A. oryzae* in response to xylose induction was investigated by RT-qPCR analysis. As shown in Fig. 1A, it was found that the *xyrA* expression was markedly up-regulated by adding xylose into the 24 h-grown glucose cultures, wherein glucose was almost completely consumed (0.14 g/L of residual glucose). However, the expression levels of *xyrA* in *A. oryzae* were attributed by xylose concentration, in which 4% xylose induction exhibited the highest level of



	Residual sugar (g/L)			
Glucose	0.15±0.03	0.14±0.00	0.15±0.02	0.14±0.00
Xylose	0	0.11±0.00	3.16±0.17	22.78±0.29

	Residual sugar (g/L)			
Glucose	0.19±0.00	0.16±0.01	0.14±0.00	0.12±0.02
Xylose	41.16±1.93	27.0.8±0.24	23.48±1.44	7.64±1.16

Fig. 1. Relative expression levels of *xyrA* gene in the wild type of *A. oryzae* strain BCC7051 quantified by RT-qPCR. The fungal cultures were grown in the SM broth containing 2% (w/v) glucose for 24 h, and then added with xylose. (a) The gene expression levels before (0% xylose) and after adding xylose at different concentrations for 24 h. (b) Induction of *xyrA* expression using 4 % (w/v) xylose at different time intervals. 18S rRNA was used as an internal control. Residual sugar concentrations (glucose or xylose) in the cultures are shown. All data are presented as mean values with standard deviation (SD). Superscript letters above the bars indicate statistical significance ($p < 0.05$) of the *xyrA* expression levels across all cultures.

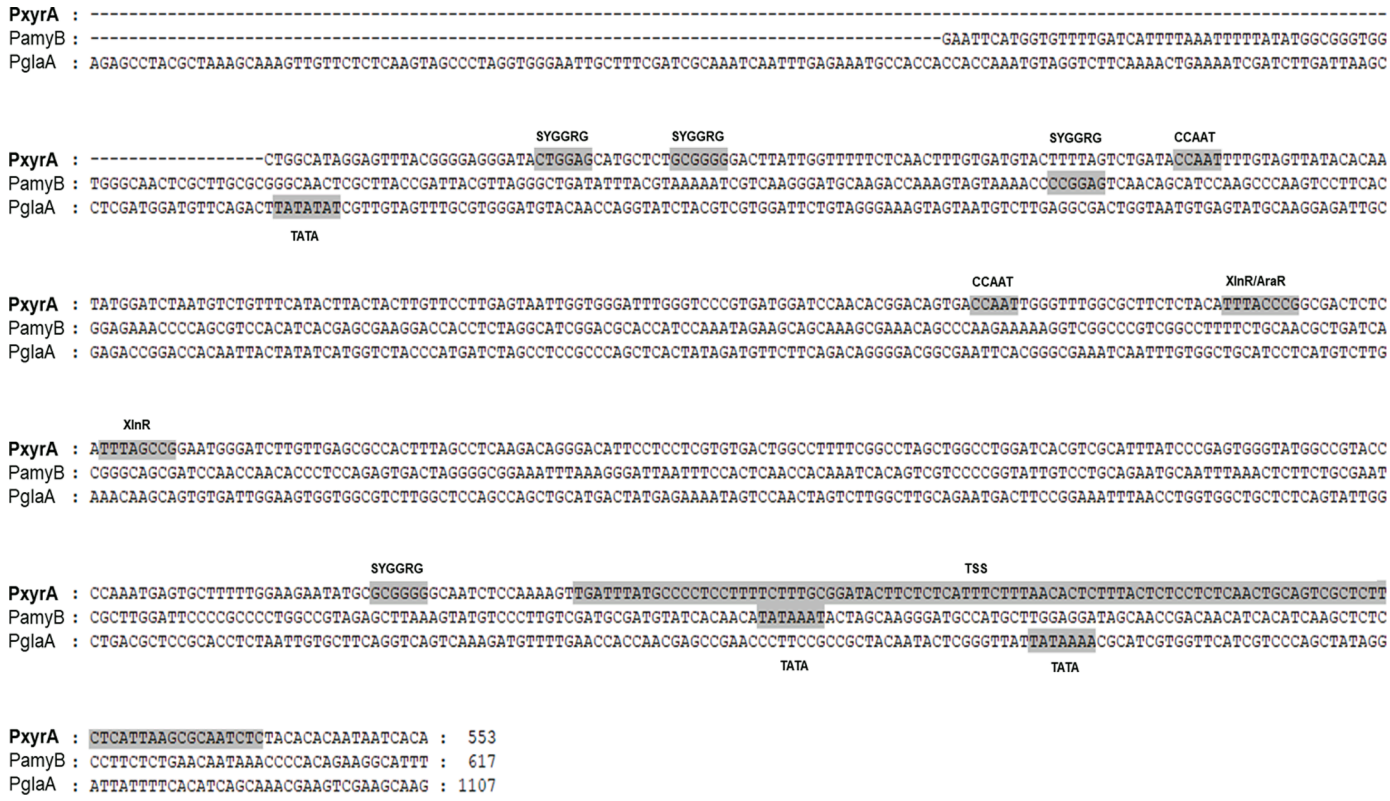


Fig. 2. Alignment of nucleotide sequences of *PxyrA* promoter of *A. oryzae* BCC7051 and other inducible promoters of *A. oryzae, PamyB* and *PglaA*. The putative TSS, upstream promoter elements (TATA, CCAAT and SYGGRG boxes), and binding sequences of XlnR and AraR transcription factors are illustrated.

xyrA expression. In addition to the xylose concentration, the induction time also affected the *xyrA* expression, in which the maximal transcript level was observed after the induction with 4% xylose for 24 h (Fig. 1B).

3.3. Characterization and functional verification of PxyrA promoter in *A. oryzae*

Using PromPredict program and available genome sequence of *A. oryzae* BCC7051 [25], the promoter region of *xyrA* gene, designated as *PxyrA*, was identified. The TSS having a low average free energy was predicted at nucleotide (nt) −18 to −119, wherein nt −1 was counted from TLS. Two putative CCAAT boxes were located in the *PxyrA* sequence at nt −346 and −459 (Fig. 2). Sequence alignment of three inducible promoter sequences of *A. oryzae, PxyrA, PamyB* and *PglaA*, revealed that there was no TATA-box in the upstream sequence of *xyrA* gene in contrast to the maltose-inducing promoters. Similar to the regulatory promoters of pentose catabolic genes [17, 35], two binding motifs for XlnR (CGGNTAAW), and a binding motif for AraR (CGGDTAAW) transcription factors were predicted in the *PxyrA* sequence as shown in Fig. 2. These transcription factors are positive regulator involved in degradation of cellulose, hemicellulose and catabolism of pentose [36, 37]. In addition, three binding motifs (SYGGRG box) specific for CREA transcription factor, which is a negative regulator mediated in carbon catabolism repression [38, 39], were also detected in the *PxyrA* sequence. These motifs were thus used as reference sites for functional characterization of the *PxyrA* of *A. oryzae* BCC7051.

Three expression plasmids, pPxyrA, pPamyB and pPglaA, which individually contained *PxyrA, PamyB* and *PglaA* promoters of *A. oryzae*, respectively, were constructed by flanking upstream to the *uidA* reporter gene. After transformed each plasmid into the *pyrG* auxotrophic strain of *A. oryzae,* the correct integration site (*pyrG*) of expression cassette in all transformants was acquired as verified by PCR analysis, which approximately 2.6- and 2.0-kb fragments corresponding to the 5′- and 3′-regions of the targeted *pyrG* homologous integration, respectively, were amplified. By Southern blot analysis, three transformant clones, which each contained a single copy number of respective expression cassette, were selected for GUS assay. The results showed that the *PxyrA* promoter was able to control the *uidA* gene expression in *A. oryzae* as clearly indicated by the specific GUS activity, whereas the enzyme activity was not detected in the transformant carrying pGUS backbone plasmid. As illustrated in Table 1, the expression induction of the pPxyrA transformant was achieved by addition of 4% xylose as an inducer, which over 400-fold increase of GUS activity was found in the pPxyrA transformant after the induction for 24 h as compared with the control without the xylose addition.

Table 1
GUS activities of the pPxyrA, pPamyB and pPglaA transformants grown under carbon induction conditions.

Transformant *	Inducer	Specific GUS activity (unit/mg protein)**		Induction ratio (fold)***
		Control	Induction condition	
pGUS	Xylose	0	0	0
pPxyrA	Xylose	0.95 ± 0.27	407.85 ± 17.95	429.32
pPamyB	Maltose	9.72 ± 1.54	64.22 ± 5.42	6.61
pPglaA	Maltose	6.81 ± 0.45	30.02 ± 4.71	4.41

* Three transformant clones containing each expression cassette were subjected to expression induction using respective inducers for 24 h.

** Control indicates the specific GUS activities of the transformant cultures without sugar inducer

*** Ratio of specific GUS activities of the fungal cultures grown under induction versus control conditions.

By 4% maltose induction for 24 h, 6.61- and 4.41-fold increases of GUS activities were obtained in the pPamyB and pPglaA transformants, respectively, as compared to the non-added maltose cultures (Table 1). Considering the promoter strength, the GUS activity of the transformants under the control of *PxyrA* promoter was significantly higher than those controlled by *PamyB* and *PglaA* promoters for 6.35 and 13.58 folds, respectively (Table 1).

3.4. Transcriptional control of PxyrA using pentose sugars as inducers

To investigate the *PxyrA* function, various pentose sugars were tested for their roles in the expression induction. As shown in Fig. 3, all 5-carbon sugars tested and xylan were able to induce the *uidA* gene expression of the *A. oryzae* transformant under the *PxyrA* control, in which xylose and its polymer (xylan) were favorable as indicated by GUS activities. Obviously, the ribose and arabinose were poor inducers for the expression.

Due to xylose was the best inducer for *PxyrA* function, the effect of xylose concentration on the promoter efficiency was then investigated. The results showed that the highest GUS activity of the pPxyrA transformant was obtained in the culture after induction with 4% xylose for 24 h (Fig. 4A) similar to the result of *xyrA* expression of the wild-type strain of *A. oryzae* (Fig. 1). The extension of induction period over 24 h did not promote the GUS activities of the *PxyrA* cultures using various concentrations of xylose inducer excepted for the 8% xylose induction, in which the prolonged induction time (48 h) was required to derive the high expression level. Notably, the *A. oryzae* transformants could utilize the xylose as a carbon source along the induction period as indicated by residual xylose concentration. The maximal xylose consumption rates were correlated with the highest GUS activities, which were found in all cultures (Fig. 4B). However, the efficiency in the gene induction decreased when using xylose over the optimal concentration, which the GUS activity markedly decreased in the cultures added with 6 and 8% xylose even though the C5 sugar highly remained in the culture system (Fig. 4C).

In addition, the repression effect of other carbon sources on the xylose-induced *PxyrA* function was also investigated. In the presence of glucose or sucrose with various concentrations (2−6%, w/v), the expression induction by 4% xylose for 24 h exhibited that the GUS activities of the cultures with low amount of glucose or sucrose (2%, w/v) were not significantly different ($p > 0.05$) to the control (no addition of C6 and C12 sugars) as shown in Fig. 5. However, high concentrations of both glucose and sucrose (4 and 6%, w/v) in the cultures displayed

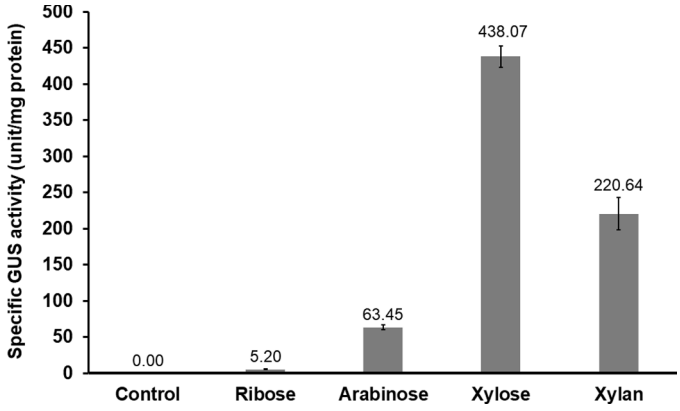


Fig. 3. Expression induction analysis of the pPxyrA transformants of *A. oryzae* using various pentose sugars, and xylan as inducers. The fungal cultures were grown in the 2% glucose-containing medium for 24 h, and added with individual inducers, including ribose, arabinose, xylose and xylan, at 4% final concentration. The culture without inducer was used as a control. The β-glucuronidase (*uidA*) of *E. coli* was used as a reporter gene for evaluating *PxyrA* function by measuring GUS activity.

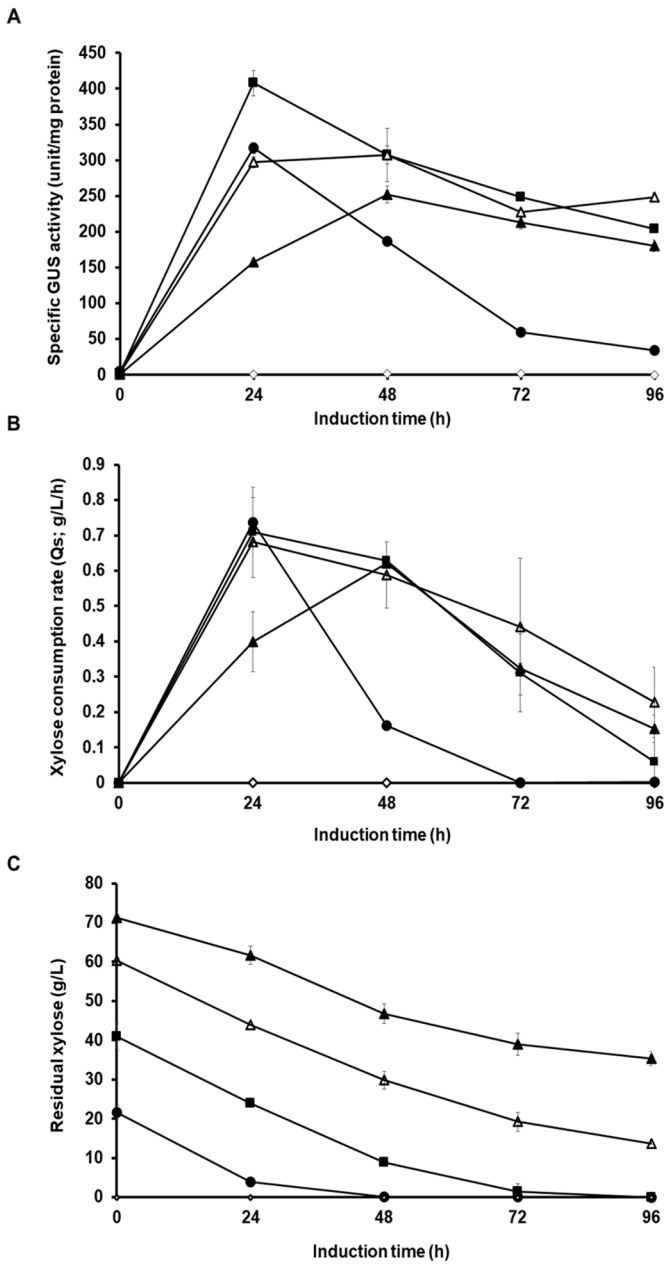


Fig. 4. Time-course expression induction of the pPxyrA transformants of *A. oryzae* using various xylose concentrations. The GUS activities (a), carbon (xylose) consumption rate; Qs (b) and residual xylose concentrations (c) are shown. The fungal cultures were grown in the 2% glucose-containing medium for 24 h, and added with xylose at various concentrations, 2% (●), 4% (■), 6% (Δ) and 8% (▲) w/v. The cultures without xylose addition (◊) were used as controls.

negative effect on the *PxyrA* function during the xylose induction. Observably, sucrose was negative attribute to the xylose-induced expression rather than glucose.

3.5. Heterologous gene expression in A. oryzae under the PxyrA control

To define a potential of *PxyrA* promoter in controlling gene expression, the 48-h cultivations of pPxyrA transformant were conducted in the media containing various sugars that could be utilized as carbon sources for fungal growth (Fig. 6). The results showed that although C3, C6 and C12 sugars were assimilated for the biomass production of pPxyrA transformant, the GUS activities of the cultures were very low

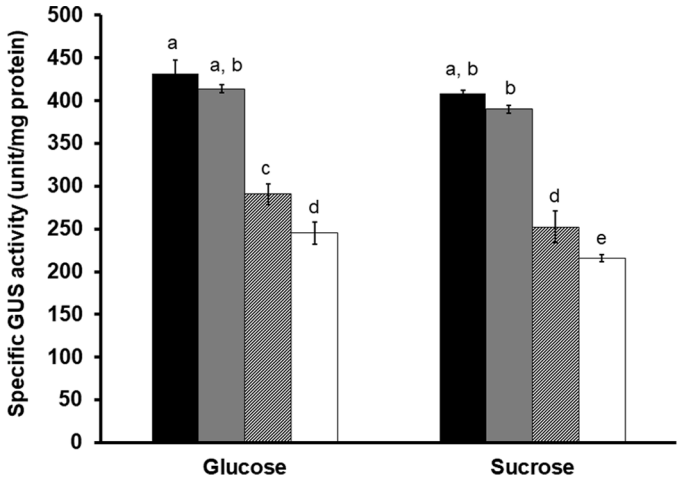


Fig. 5. Effect of remaining glucose and sucrose in the pPxyrA transformant cultures on the xylose induction under the *PxyrA* control. Different sugar concentrations, including 2% (grey bar), 4% (upward diagonal bar) and 6% (white bar), were added into the 24-h glucose-grown cultures before the expression induction. The 4% xylose induction was carried out for 24 h. The black bar indicates the 4% xylose-induced cultures without the sucrose/glucose addition as a control. Superscript letters above bars indicate statistical significance ($p < 0.05$) of the GUS activities across all cultures.

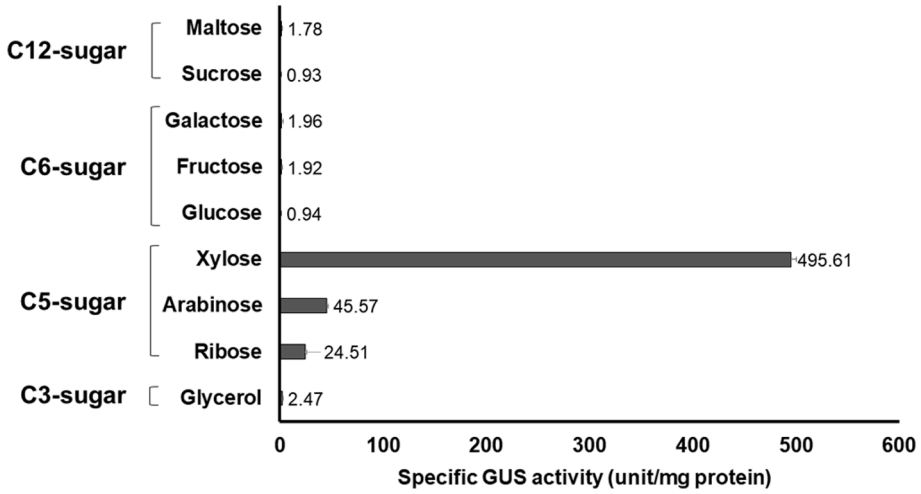
(<3 units/mg protein). In contrast, the GUS activities were clearly detected in the cultures using C5 sugars (arabinose, ribose and xylose). However, the xylose culture exhibited higher GUS activity than the arabinose and ribose cultures. Under the *PxyrA* control, it was demonstrated that the fungal cells efficiently utilized xylose for both cell growth and gene expression induction as indicated by residual sugar, biomass titer and GUS activity (Fig. 6).

More focusing on the time-course expression under the *PxyrA* control, the GUS analysis of the pPxyrA transformant grown in the medium containing 4% (w/v) xylose at different cultivation times was performed. The results revealed that the GUS activity of the xylose cultures increased with the increase of biomass corresponding to the maximal consumption rate of xylose, in which the highest enzyme activity was found at the end of logarithmic phase or the initial stationary phase of the xylose growth (48-h cultivation). Afterwards, the GUS activity of the xylose culture sharply decreased (72-h and 96-h cultures), wherein xylose was completely consumed (Fig. 7).

4. Discussion

Heterologous gene expression is an empowering tool for microbial production of non-natural compounds or target bio-products. With the progress in multi-omics tools and technologies, a seek for promising promoter with specific regulatory mode can be acquired using an integrated approach. To address the industrial purposes, the selected promoter for driving the gene/protein expression in filamentous fungi also relies on considerable aspects, such as products of target, practical use and induction cost or operating cost in addition to the promoter strength. For regulatory promoters, their functional features are essential prerequisite for optimizing fermentation process to attain the maximal production yield. Considerable attention has been given on the efficient promoters for expression control to meet metabolic demands during fungal growth and production of target metabolite. The xylose-inducible expression system was initially developed from the endoxylanase genes of xylose-degrading fungi, such as *A. awamori* [16] and *P. chrysogenum* [17]. Using transcriptome and growth phenotypic analyses, in this work we focused on the identification and functional characterization of pentose-regulated promoter of

A. oryzae. Expectedly, a set of enzyme-coding genes in the xylose



	DCW (g/L)	Qs (g/L/h)	Residual sugar (g/L)
Maltose	15.30±1.68	0.83±0.00	0
Sucrose	16.31±0.98	0.80±0.04	1.48±1.81
Galactose	13.79±0.78	0.52±0.02	14.87±0.91
Fructose	15.41±0.79	0.74±0.02	4.60±1.01
Glucose	16.70±0.90	0.83±0.00	0
Xylose	16.14±0.85	0.75±0.01	3.87±0.28
Arabinose	16.01±0.50	0.61±0.02	10.59±0.80
Ribose	13.84±0.31	0.61±0.02	10.73±0.99
Glycerol	12.0±0.69	0.48±0.01	16.88±0.65

Fig. 6. Specific GUS activity of the pPxyrA transformants of *A. oryzae* grown on different carbon sources. The 48-h cultivations were performed using SM broth containing 4% (w/v) of carbon sources for fungal growth. The average value of specific GUS activity was derived from three transformant clones, which each was analyzed in triplicate. Cell growth (DCW), carbon consumption rate (Qs) and residual sugar concentrations are shown.

assimilation pathway were highly expressed in the fungal cultures using xylose as a carbon source, which were similar to the previous reports [40, 41]. Of them, the novel *PxyrA* promoter identified from the gene sequence involved in the xylose reductase pathway was intensively characterized, showing that it contained two putative HAP complex-binding CCAAT boxes, which are enhancer binding site commonly found in TATA-less eukaryotic promoters [42]. It has been proposed that this regulatory element is required for efficient gene regulation in Aspergilli [43, 44]. In addition, the identified consensus binding motifs, CGGNTAAW and CGGDTAAW, in the *PxyrA* sequence could refer to its function that might be regulated by XlnR and AraR transcription factors via xylose and arabinose induction, respectively, as previously described [17, 35, 36]. In addition, the presence of SYGGRG motifs in the *PxyrA* sequence of *A. oryzae* indicated that the expression control might participate in the CREA negative regulator-mediated carbon catabolism repression, as observed in several carbon-inducible promoters, such as endoxylanase [16, 17], sorbitol-induced [7] and taka-amylase A promoters [8, 9].

Functional characterization of the *PxyrA* promoter in *A. oryzae* revealed that pentose sugars (xylose, arabinose and ribose) and xylan could act as specific inducers for the promoter function. Of them, xylose was the best inducer for the *PxyrA* function (Fig. 3) that probably due to its simple molecule facilitating the uptake and assimilation processes by the fungal cells rather than xylan polysaccharide. However, the induction with high xylose concentration (8%, w/v) showed negative attribute to the expression that might be explained by either poor xylose transport or catalytic repression as a result of low xylose uptake/consumption rate during the induction (Fig. 4), thus leading to low GUS activity. The previous study demonstrated that the xylose transport system of the xylose-utilizing yeast, *Candida tropicalis,* grown under the high osmotic pressure (100 g/L xylose) was interfered, yielding low xylitol production [45]. Interestingly, the promoter strength of *PxyrA* was higher than the *PamyB* and *PglaA* promoters for inducible regulation mode that provides an alternative and efficient tool for fungal expression system. Not similar to the maltose-inducible promoters, the *PxyrA* was not fully repressed by glucose or sucrose, particularly at certain concentrations ($\leq$ 2% w/v) (Fig. 5). This finding indicated that *PxyrA* was strongly regulated by pentose even though small amount of other sugars, which are commonly used for fungal cultivations, remained in the induction conditions that was similar to the *PexlA* promoter of *A. awamori* [16]. This experiment refers to a simple practice in expression induction without the steps of fermented broth removal and mycelial washing. It is noteworthy that this could not be adopted in the presence of high concentrations of glucose or sucrose (4−6%, w/v) during expression induction under the *PxyrA* control. This evidence is similar to the previous reports for the sorbitol- and xylose-inducible promoters of *A. oryzae* [7] and *Caulobacter crescentus* [46]. In addition to the inducer concentration, the *PxyrA* efficiency was also dependent on the induction time or in turn the xylose consumption rate of *A. oryzae* cultures (Fig. 4B). This finding is useful for the experimental design in controlling the gene expression, particularly in different cultivation modes, such as batch and fed-batch fermentations. Somewhat surprisingly, the *PxyrA* could regulate the gene expression along the fungal cultivation using xylose as a sole carbon source for growth. However, it seems likely that the GUS expression level was associated with xylose availability in the culture system or in turn the growth stage as the finding of sharp decrease in GUS activity after 48 h of cultivation of the pPxyrA transformant. It has been documented that the GUS protein was degraded by intracellular protease that was highly produced during stationary growth phase of microorganisms [42]. Accordingly, the xylose concentration in the culture system should be optimized and monitored for ensuring the expression level of the gene of interest. The control mode of *PxyrA* by the C5 sugar is beneficial for further developing the heterologous expression system of choice in filamentous fungi.

Glucose and sucrose are usually exploited as a sole carbon source for cell growth and production of an array of products by microorganisms [47, 48]. However, some exotic strains of filamentous fungi and recombinant microbes are able to utilize xylose and other C5-sugars [48–50]. With the prospect in growing circular economy, the renewable feedstocks, especially in the lignocellulosic biomass, are being interested in the microbial bioconversion for production of value-added products. Actually, xylose is abundant in the cellulosic feedstocks, which is non-food carbon sources. Thus, it is highly possible that the xylose-regulated expression system under the *PxyrA* control is promising for biotechnological production by the fungal system through fine-tuning the pentose/hexose-assimilating pathways, thereby the mixed C5 and C6 sugars derived from such cheap feedstocks can be efficiently utilized for the production process with economic feasibility. For further application of the *PxyrA* promoter, it is noteworthy that the culture medium and fermentation process should be rationally designed and optimized in relevant to the transcriptional control mode of choice by leveraging the metabolic demands for cell growth and targeted

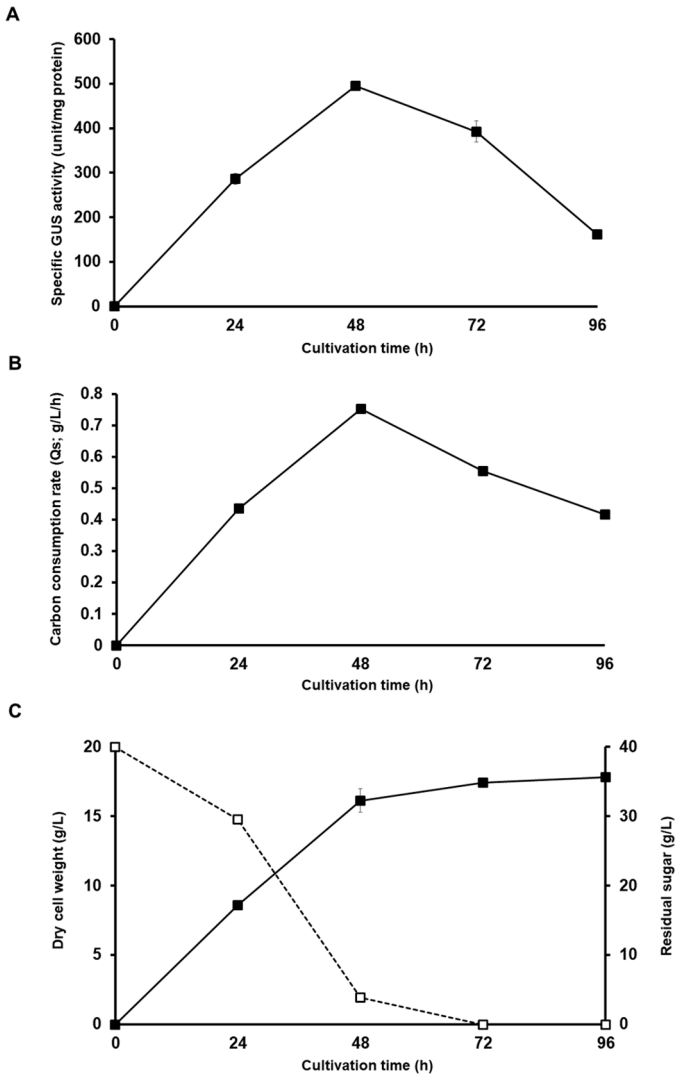


Fig. 7. Time-course expression of the 4% xylose-grown culture of pPxyrA transformant. The GUS activity (a), carbon consumption rate (b), and cell growth (c) were analyzed. The residual sugar concentrations in the culture are shown by dash line.

metabolite production.

5. Conclusions

The identified *PxyrA* promoter renders the inducible mode controlling gene expression in *A. oryzae*, which was tightly regulated with pentose sugars, particularly xylose. The inducible transcription control in the filamentous fungus was dependent on two main factors, including xylose inducer concentration and induction time. The *PxyrA* function on xylose also provided an efficient expression system, which is applicable in the xylose growth of *A. oryzae.* In addition, the concentration of non-pentose sugars during the expression condition should be taken into considerable practice. The potent function of *PxyrA* promoter of *A. oryzae* is beneficial in the development of tailored-made expression systems with desired purposes, such as gene function study and cost-effective production process development.

Author contributions

S.J. conceived and designed research, carried out experiments, interpreted the data and wrote the manuscript. J.A. and S.P. conducted experiments and analyzed the data. C.C. suggested on the manuscript and experimental design. K.L. conceived and supervised the research, interpreted the data, wrote and revised the manuscript. All authors read and approved the manuscript.

Funding

This work was supported by Targeted Research Grant of Functional Ingredients and Food Innovation, National Science and Technology Development Agency (NSTDA), Thailand (Project No. P19-52263).

Declaration of Competing Interest

The authors declare no conflict of interest

Acknowledgements

We are grateful to Dr. Chinae Thammarongtham and Mr. Tanawut Srisuk, Biochemical Engineering and Systems Biology Research Group (BIOTEC), for valuable suggestions on transcriptome analysis. We also thank Dr. Wattana Jeamton, King Mongkut's University of Technology Thonburi, for providing Southern hybridization analysis kit and valuable comments.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2021.e00695.

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