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Enhancing xylanase production in the thermophilic fungus *Myceliophthora thermophila* by homologous overexpression of *Mtxyr1*

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Abstract The xylanase regulator 1 protein in *Myceliophthora thermophila* ATCC42464 (*MtXyr1*) is 60 % homologous with that of *Trichoderma reesei*. However, *MtXyr1*'s regulatory role on cellulolytic and xylanolytic genes in *M. thermophila* is unknown. Herein, *MtXyr1* was overexpressed under the control of the *MtPpdc* (pyruvate decarboxylase) promoter. Compared with the wild type, the extracellular xylanase activities of the transformant cultured in non-inducing and inducing media for 120 h were 25.19- and 9.04-fold higher, respectively. The *Mtxyr1* mRNA level was 300-fold higher than in the wild type in corncob-containing medium. However, the filter paper activity and endoglucanase activities were unchanged in corncob-containing medium and glucose-containing medium. The different zymograms between the transformant and the wild type were analyzed and identified by mass spectrometry as three xylanases of the glycoside hydrolase (GH) family 11. Thus, overexpression of *xyr1* resulted in enhanced xylanase activity in *M. thermophila*. Xylanase production could be improved by overexpressing *Mtxyr1* in *M. thermophila*.

Keywords Thermophilic fungi · Xylanase · *Myceliophthora thermophila* · *xyr1* · Homologous expression

Introduction

Thermophilic fungi are extremophiles that grow at high temperatures. Their extracellular enzymes might be more active and stable under extreme conditions and may provide economic advantages in the production of chemicals and biomass-based fuels [17, 23]. *Myceliophthora thermophila* is a mild thermophilic fungus which can grow in temperatures between 25 and 55 °C. The genome of *M. thermophila* has been analyzed [6]. Genomic data analysis reveals that this fungus has the potential to produce more than 200 kinds of glucoside hydrolases and a large number of accessory proteins related with cellulase and hemicellulase degradation and has fine application prospects in the biorefinery industry [12, 14].

Available carbon sources regulate the major cellulase and hemicellulase genes in mesophilic fungi in a coordinated manner [11, 22]. Cellulose, xylan and other materials induce the expression of cellulase and hemicellulase genes, while glucose acts as a repressing carbon source [40]. Most of these genes are controlled by a repressor/inducer system. Some regulators, such as the carbon catabolite repressor *Cre1* [34], the repressor *Ace1* [31], the activator *Ace2* [2] and the activator *Xyr1* [29] have been isolated. The mechanism and binding sites on the upstream region of target genes of the above regulators have been identified.

Xyr1 (xylanolytic transcriptional regulator), a homolog to *XlnR* of *Aspergillus niger* [40], is a member of the zinc binuclear cluster protein family and belongs to the Zn_2Cys_6 type. *Xyr1* was cloned and shown to act as a

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transactivator, mediating both xylose- and xylan-dependent induction [29]. *Xyr1* is a general and essential transcription factor controlling the expression of the major cellulolytic and xylanolytic genes, regardless of the inducer (xylose, xylobiose, sophorose and lactose) and mode of expression (basal, derepression and induction) [27, 35]. *Xyr1* is currently considered to be one of the main regulators of the cellulase and hemicellulase system of *T. reesei*. The target genes of *Xyr1* are hydrolase-encoding genes, such as *xyn1, xyn2, bxl1, cbh1, cbh2, egl1* and *bgl1* [1, 21, 33, 35]. *Xyl1* and its homologous proteins exist widely in mesophilic fungi, such as *Neurospora crassa* [38], *Trichoderma reesei* [21, 33], and *A. niger* [41]. However, compared with studies in mesophilic fungi, researches on the regulation of cellulase and hemicellulase gene expressions in thermophilic fungi are relatively few. The full repertoire of transcription factors influencing cellulase and xylanase gene expression in thermophilic fungi has not been fully described to date [18]. In particular, xylanolytic transcriptional regulators have not yet been reported in thermophilic fungi.

In the present study, the *xyr1* homologous gene, *Mtxyr1*, was identified in the genome of M. *thermophila* ATCC42464. Compared with xylanolytic transcriptional regulators in *N. crassa* OR74A, *Pseudallescheria apiosperma*, *T. reesei* QM6a and *A. niger* CBS 513.88, the amino acid sequence (GenBank: AEO60673.1) of *MtXyr1* shares 80, 76, 59 and 50 % homology, respectively.

However, the role of *MtXyr1* on the regulation of the major cellulolytic and xylanolytic genes in *M. thermophila* is unknown. The objective of this work was to validate the role of *MtXyr1* in *M. thermophila* through overexpression and to determine whether enzyme production could be improved. We constructed *Mtxyr1*-overexpression recombinant strains and detected cellulase and xylanase activities under inducing and non-inducing conditions. We also identified the different bands on SDS-PAGE between the transformant and the parent strain. The results indicated that *MtXyr1* acted as a xylanolytic transcriptional activator for xylanase expression in *M. thermophila*. Overexpressing *Mtxyr1* effectively improved xylanase production in *M. thermophila*.

Materials and methods

Strains, plasmids and culture conditions

Escherichia coli JM107 (Fermentas, Canada) was used as the host strain for recombinant plasmid construction. *M. thermophila* (ATCC 42464) was purchased from the American Type Culture Collection center and used as the parent strain throughout this study. The *E. coli* strain was cultivated in LB medium supplemented with 100 μg/ml ampicillin (Applied Biosystems, Carlsbad, CA) when necessary. *M. thermophila* was maintained on potato dextrose agar (PDA). The spores were washed with a 2 % Tween-20 solution to prepare the spore suspension. For liquid culture, the strain was grown in Mandel's medium with 2 % glucose [24] at 45 °C for 120 h in 250-ml Erlenmeyer flasks. *M. thermophila* produced cellulase and xylanase when 5 g/l corncob powder and 3 g/l wheat bran were used as inducers.

Recombinant *M. thermophila* strains were plated on PDA agar supplemented with hygromycin B (50 μg/ml). *E. coli* was routinely cultured at 37 °C, whereas *M. thermophila* was cultured at 45 °C. Plasmid pUC19 was used for the construction of *Mtxyr1* overexpression cassette. Plasmid pAN7-1, which contained the hygromycin B-resistance cassette, was used as an assisting plasmid for the transformation of *M. thermophila* [28].

RNA extraction and cDNA synthesis

About 2×10^7 *M. thermophila* spores collected from a PDA plate grown for 5 days were inoculated into a 1-l flask containing 200 ml of Mandel's medium with glucose at a final concentration of 20 g/l. They were then grown at 45 °C and 250 rotations/min. Samples were taken at 24, 48, 72, 96 and 120 h. Mycelia were harvested by centrifugation, washed twice with DEPC water, snap frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction. The glucose concentration in the samples was measured using the 3,5-dinitrosalicylic acid (DNS) method [26].

The total RNA of the samples was extracted using a Universal Plant Total RNA Extraction Kit (BioTeke Corporation, China). To remove the genomic DNA, the RNA preparations were treated with DNase I (TaKaRa, Japan). Total RNA was quantified using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE). A PrimeScript reagent kit (TaKaRa) was used to synthesize the complementary DNA (cDNA) using 1.0 μg of the total RNA per reaction (20 μl).

Quantitative real-time reverse transcription PCR

The primers used for qRT-PCR were designed with the aid of the software Prime 5.0 (Table 1). The PCR protocol has been described previously [20]. The expression levels of the genes in the glucose metabolism were normalized with an endogenous control, beta-tubulin, as previously described [4]. The means ± standard deviations of replicates are shown in the figures.

Table 1 Sequences of primers used in qRT-PCR

Name	Sequence (5′–3′)	Amplification target
β-tubulin-F	AGGGTATGGATGAGATGGAG	*β-tubulin* cDNA
β-tubulin-R	AGAAGCAAGCCCTGGAAC	
pdc-F	CGAGATAGCCGACCAGAT	*pdc* cDNA
pdc-R	GTTGGCGGGTTCTGTGAG	
gpd-F	TCCTGGACCTCCTCGCCTA	*gpd* cDNA
gpd-R	GCTTCCCATTCCAAGACTAA	
eno-F	ACAGCGACCCGAGCAAGT	*eno* cDNA
eno-R	CGCTTAGGGTTGGTGACAG	
fba-F	CCCTCAACCGAGAATCAG	*fba* cDNA
fba-R	CGAGGTTCCCACCAAAGG	

Cloning of *Mtxyr1*

The genomic DNA of *M. thermophila* was isolated by a Wizard Genomic DNA Purification kit (Promega, Madison, WI), following the manufacturer's protocols and used as the template. The primers *Mtxyr1*-F and *Mtxyr1*-R were used to amplify *Mtxyr1* (GenBank accession number: XM 003665870.1); *Not*I and *Xba*I sites were added to the 5′ and 3′ ends, respectively (Table 2).

Construction of the overexpression plasmid

The primers *MtPpdc*-F and *MtPpdc*-R were used to amplify the *MtPpdc* (pyruvate decarboxylase gene) promoter (a 587-bp upstream fragment starting from the start codon of *pdc*). *Hin*dIII was added to the 5′ ends, and *Bam*HI and *Not*I sites were added to the 3′ ends. The primers *MtTpdc*-F and *MtTpdc*-R were used to amplify the *MtTpdc* terminator (a 357-bp downstream fragment starting from the stop codon of *pdc*). *Bam*HI and *Xba*I were added to the 5′ ends and an *Eco*RI site was added to the 3′ ends.

The *MtPpdc* was inserted into plasmid pUC19 after digestion by *Hin*dIII and *Bam*HI, which generated the plasmid pUC19–*MtPpdc*. The *MtTpdc* was then inserted into pUC19–*MtPpdc* after digestion by *Bam*HI and *Eco*RI, which generated the recombinant plasmid pUC19–*MtPpdc*–*MtTpdc*. Finally, *Mtxyr1* was inserted into the recombinant plasmid using the *Not*I and *Xba*I sites, which generated plasmid pUC19–*MtPpdc*–*MtXyr1*–*MtTpdc*. This plasmid was used for the transformation of *M. thermophila* and the recombinant expression of *MtXyr1* (for the schematic diagram of the plasmid, see supplementary Fig. S2). The primers are listed in Table 2. The restriction enzymes, DNA polymerase and T4 DNA ligase were purchased from Takara.

Protoplast preparation and transformation of *M. thermophila*

About 1×10^8 *M. thermophila* spores in suspension were inoculated into 30 ml of Mandel's medium and cultured at 45 °C and 250 rpm for 12 h. The germlings were centrifuged at 6026×*g* for 10 min. The pellet was washed with 1 mol/l $MgSO_4$ twice and then resuspended in 10 ml of 1 mol/l $MgSO_4$ containing 100 mg lysing enzymes (Sigma-Aldrich). 20 ml of STC solution (containing 1.2 mol/l sorbitol, 10 mmol/l pH 7.5 Tris–Cl, 50 mmol/l $CaCl_2$) was added to the suspension, which was mixed and centrifuged at 6026×*g* for 10 min. The pellet was washed with STC solution twice and resuspended in STC solution. 200 μl of the protoplast STC solution was mixed gently with 10 μg of the recombinant plasmid and 10 μg of pAN7-1. The mixture was heated at 60 °C for 2 min before 50 μl 60 % PEG 4000 (containing 50 mmol/l $CaCl_2$ and 10 mmol/l pH 7.5 Tris–Cl buffer) was added. The mixture was left at room temperature for 20 min. The solution was then

Table 2 Sequences of primers used for amplification of *Mtxyr1* and construction of overexpressing vector

Name	Sequence (5′–3′)[a]	Description	Length of target genes (bp)
Mtxyr1-F	ATAAGAATGCGGCCGCATGTTGTCTAACCCGCTTCA	Amplification of full length of *Mtxyr1*	3030
Mtxyr -R	GCTCTAGACTACAGCGCCAGACCGGTA		
MtPpdc-F	CCCAAGCTTCCGAGTGTACTCCGTAAGGA	Amplification of the *Mtpdc* promoter, 587 bp upstream from the start codon of *pdc*	587
MtPpdc-R	CGCGGATCCGCGTTGTTTAAATAAGAATGCGGCCGCGTCTGTTGTTGGCGATGTTTGTG		
MtTpdc-F	CGCGGATCCGCGGTATACAGCCGCTCTAGAGATGGTGGCTTGGTGGCTGA	Amplification of the *Mtpdc* terminator, 357 bp downstream from the stop codon	357
MtTpdc-R	CCGGAATTCGGATTACAGCGCAGTGCACG		
Vpc-F[b]	CCTGGGTATCGTGCCTGTG	Part of *MtPpdc* and part of *Mtxyr1*	440
Vpc-R[b]	TGTTGCCTATGCTTCGGT		

[a] Restriction sites are underlined by single lines

[b] Vpc-F and Vpc-R mean the upstream and downstream primers for verification of positive clones

transferred to a 50-ml centrifuge tube to which 2 mL of 60 % PEG4000 was added and mixed gently. The solution was left at room temperature for 50 min and then 20 ml of STC solution was added and mixed. The mixture was centrifuged at 6026×*g* for 15 min. The pellet was resuspended in 1 ml of STC solution. The suspension was added to protoplast regeneration medium and cultured at 30 °C, 70 rpm for 13 h. The cells were then cultured at 35 °C, 70 rpm for 27 h and 20 ml of STC solution was added. The mixture was centrifuged at 6026×*g* for 10 min after mixing gently. The pellet was resuspended in 1.0 ml of STC solution and plated on PDA solid medium containing 50 μg/ml hygromycin. The transformants were selected according to the resistance to hygromycin. The conidia of the candidate transformants were further spread onto hygromycin B selection plates to form single colonies. The single colonies were screened using genomic DNA PCR analysis to verify that the recombinant expression cassette was integrated into the genome of the transformants. The primers Vpc-F and Vpc-R (listed in Table 2) were used for PCR verification.

Enzyme activity assays

Xylanase activity was assayed as described in Bailey et al. [3] using 1 % birchwood xylan as the substrate. The amount of released sugar was determined by the dinitrosalicylic acid (DNS) method, as described by Miller et al. [26]. The filter paper activity (FPA) and endoglucanase (EG) activities were measured according to the method provided by Ghose [10], using filter paper (Whatman No. 1, Springfield Mill, UK) and sodium carboxymethyl cellulose (CMC-Na) as substrates, respectively. Enzymatic analysis was carried out in triplicate. One unit of enzyme activity (IU) was defined as the amount of enzyme that released 1 μmol reducing sugar per minute at 50 °C.

SDS-PAGE and identification of different proteins

When the parent strain and MX11 had been cultivated in corncob-containing medium for 5 days (45 °C, 250 rotations/min), proteins secreted into the culture media were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12 % polyacrylamide gel, as described by Laemmli et al. [16]. The fermentation liquor was centrifuged at 6026×*g* for 10 min. 10μl of supernatant was loaded in each well.

The protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA). The different protein bands between the parent strain and the transformant were excised, transferred to a PCR tube with 100-μl ultrapure water, washed and destained on a temperature-controlled board at 25 °C. Pretreatment used the in-gel digestion method of Li et al. [19]. The proteins were identified using matrix assisted laser desorption ionization time-of-flight/time-of-flight (MALDI TOF/TOF) mass spectrometry (Applied Biosystems, Foster City, CA), according to the method of Ravalason et al. [30]. To identify the protein sequences, a homology search was performed using Mascot software (Version 2.2, Matrix Science). The parameters were set as follows: max missed cleavages: 1; fixed modifications: carboxymethyl (C); variable modifications: oxidation (M); mass values: MH+ , monoisotopic; peptide mass tolerance: ±0.2 Da. The partial amino acid sequences were used to identify analogous proteins through a BLAST search of the NCBI protein sequence database.

Results

Selection of a strong promoter from *M. thermophila*

Four critical genes that participate in glucose metabolism [8, 20] were selected and their transcriptional profiles were analyzed quantitatively using qRT-PCR. These genes were glyceraldehyde-3-phosphate dehydrogenase (*gpd*), pyruvate decarboxylase (*pdc*), enolase (*eno*) and aldolase (*fba*), which participate in glucose metabolism. The genes that are responsible for glucose use are usually regarded as housekeeping genes, and constitutive promoters drive their expression in *T. reesei* [20]. The four genes all exist as single copies in the genome of *M. thermophila*. Thus, the expression level of the genes is proportional to the strength of their promoters. In the present study, we aimed to select a strong promoter and use it to construct an overexpression plasmid for *Mtxyr1*.

The genomic regions of *M. thermophila* containing the four genes were acquired from the website (http://www.ncbi.nlm.nih.gov/genome/?term=Myceliophthora+thermophila). Mt*Eno* (MYCTH_2295989) is located at 3,716,046–3,718,626 bp in scaffold 1; Mt*Fba* (MYCTH_2300033) is located at 137,539–140265 bp in scaffold 2. Mt*Pdc* (MYCTH_112121) is located at 3,335,044–3,337,250 bp in scaffold 5; and Mt*Gpd* (MYCTH_2311855) is located at 1,538,035–1,540,796 bp in scaffold. Beta-tubulin (MYCTH_2306723) was used as the reference gene. It is located at 2,379,225–2,380,120 bp in scaffold 4.

Myceliophthora. thermophila was cultured and samples were taken at 24, 48, 72, 96 and 120 h. The residual concentrations of glucose in the samples were 18.88, 12.94, 6.84, 0.37 and 0.18 g/l, respectively (Supplementary Fig. S1). Hyphae were taken at 24, 72 and 120 h of cultivation, because the relative expression levels of the genes were affected by different concentrations of glucose. Total RNA was extracted and the relative

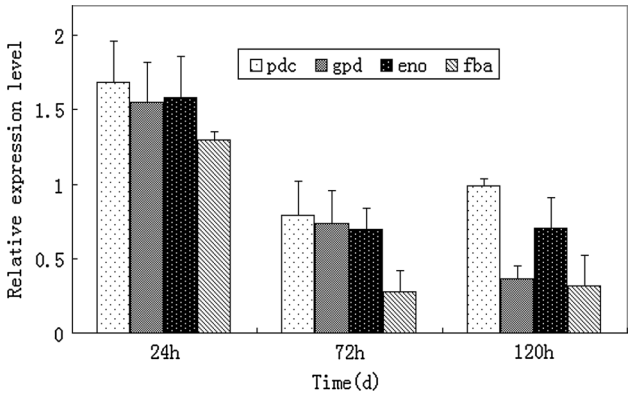

Fig. 1 The transcriptional profiles of four key genes related to glucose metabolism during cultivation of *M. thermophila*. *Error bars* represent standard deviations. The relative expression levels of the four genes are shown by *white dotted bars* (*pdc*), *gray bars* (*gpd*), *black dotted bars (eno*) and *slash bars* (*fba*), respectively

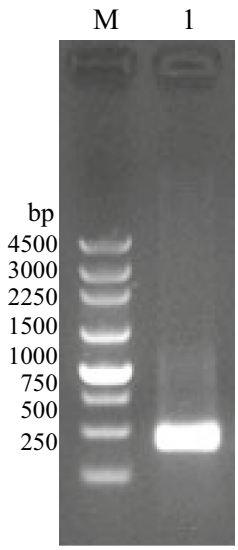

Fig. 2 The PCR products amplified using primers Vpc-F and Vpc-R. *M* 250-bp marker, *1* part of the cassette *MtPpdc–MtXyr1–MtTpdc*

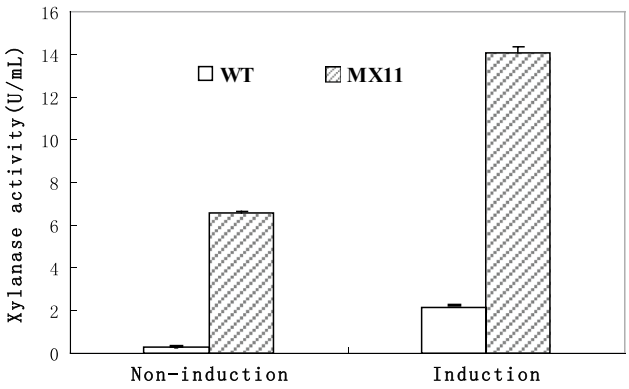

Fig. 3 The xylanase activity of the original strain and the transformed strain MX11

expression levels of four genes in *M. thermophila* at 24, 72 and 120 h are shown in Fig. 1. The expression levels of all four genes were the highest under high glucose concentration and decreased at low glucose concentrations. Thus, the expression levels of the four genes are related to glucose consumption. In all conditions, the transcription efficiency of *pdc* was the highest among the four genes, indicating that the promoter of *pdc* was the strongest. Therefore, the promoter of *pdc* in *M. thermophila* was selected to construct the recombinant expression cassette.

Construction of a thermophilic strain overexpressing *Mtxyr1*

To produce the regulator *Xyr1* in *M. thermophila* under the control of the promoter *MtPpdc*, the cassette *MtPpdc*–Mt*Xyr1–MtTpdc* was constructed by linking the coding region of the *xyr1* gene to 587 bp of the *pdc* promoter and 357 bp of the *pdc* gene transcription terminator. The expression cassette was co-transformed into *M. thermophila* with plasmid pAN7-1. Upon screening of 25 randomly selected transformants by PCR using primers Vpc-F and Vpc-R, 12 showed a 450-bp product, indicating successful integration of the *MtXyr1* expression cassette (Fig. 2). The sequencing result agreed completely with the PCR results. According to qPCR analysis, the *Xyr1* expression levels in three randomly selected transformants carrying the *xyr1* expression cassette were much higher than those in the original strain (see supplementary Fig. S3). One of them, the transformant MX11, which showed the highest *Xyr1* expression levels at 300-fold higher than that of the parent strain with glucose as the carbon source, was selected for subsequent studies.

Increased xylanase production by *Mtxyr1* overexpression

There were pronounced differences in the enzyme activity levels between the parent strain and the MX11 strain. In glucose-containing medium (non-inducing condition), the xylanase activities of the parent strain and MX11 were 0.26 ± 0.03 IU/ml and 6.55 ± 0.09, respectively, after 5 days of cultivation. In corncob-containing medium (inducing condition), the xylanase activities of the two strains after 5 days of cultivation were 2.14 ± 0.09 IU/ml and 14.10 ± 0.27, respectively (Fig. 3). The xylanase activities of *xyr1*-overexpressed transformant MX11 were 25.19- and 6.59-fold higher compared with those of the parent strain in glucose-containing medium and corncob-containing medium, respectively. The total protein concentrations of

Table 3 Total protein production of *xyr1*-overexpressed transformant under inducing and non-inducing conditions (5 days)

Strains	Total amount of secreted proteins (μg/ml)	
	Non-induced medium	Induced medium
Mt (original strain)	78.5 ± 1.5	203.2 ± 13.3
MX11 (transformed strain)	242.7 ± 14.6	298.5 ± 17.2

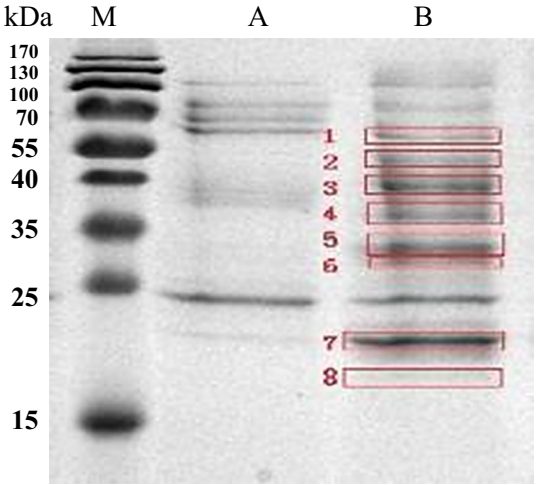

Fig. 4 SDS-PAGE analysis of the transformant and parent strain. A 10 μl sample was loaded in each well. *M* PageRuler™ prestained protein ladder SM0671, *A* culture filtrate from the parent strain *M. thermophila* ATCC42464, *B* culture filtrate from positive transformant MX11. The bands in *red boxes* were excised and identified by MALDI TOF/TOF mass spectrometry

MX11 were 3.09- and 1.47-fold higher compared with those of the parent with glucose as the carbon source and with corncob with the carbon source, respectively (Table 3). The results showed that xylanase activity and total protein production were significantly enhanced in the transformant.

The FPA and EG activities were measured to examine the influence of other cellulose degradation enzymes by the transcription of *Mtxyr1*. However, the enzyme activities did not show obvious changes in the parent strain and MX11, both in glucose-containing medium and corncob-containing medium (see supplementary Table S1). Taken together, the results showed that overexpression of *xyr1* mainly resulted in enhanced xylanase activity in *M. thermophila*.

Mass spectrometry analysis of protein differential expression between the parent strain and the transformant

Proteins in culture media of parent strain and MX11 were analyzed by SDS-PAGE (Fig. 4). The different bands between the parent and transformant strains were excised and identified using MALDI TOF/TOF mass spectrometry. The results showed that three kinds of xylanase were present in band no. 8, and they all belonged to the glycoside hydrolase (GH) family 11. The accession numbers in NCBI (http://www.ncbi.nlm.nih.gov/) are gi: 367020944, gi: 367022650 and gi: 367031002, respectively. Various glucoside hydrolases belonging to GH family 16, 17, 25, 47, 55, 72 and 125 were identified in bands no. 1–7, but no cellulase was identified, which was consistent with the enzyme activity results. However, whether *MtXyr1* regulates the above glucoside hydrolases remains to be proven.

Discussion

The genes encoding cellulolytic and xylanolytic enzymes are usually regulated by a repressor/inducer system in fungi. However, xylanolytic transcriptional regulators have not yet been reported in thermophilic fungi. In the present study, we screened a strong promoter, *MtPpdc*, and used to it to overexpress *Mtxyr1* homologously. We also improved the *M. thermophila* transformation method. The results are very important for the research on the regulation of *Xyr1* in *M. thermophila* and provide a solid technical support for studies of other regulatory factors in *M. thermophila*.

The choice of the promoter used for expression is vital for the final expression efficiency. Several different promoters have been used to express *xyr1* in various hosts. For instance, the *nag1* promoter used by Kubicek et al. [15] was not much stronger than the native *xyr1* promoter, and thus the final enzyme activity in that strain was not significantly enhanced. Seiboth et al. [32] overexpressed the *xyr1* gene using the constitutive *pki1* promoter in *T. reesei* QM9414, which resulted in increased cellulase formation. In our previous study, the *pdc* promoter was identified as a strong promoter in *T. reesei* and was used to generate very high expression levels of recombinant xylanase II [20]. Additionally, we expressed endogenous and exogenous proteins successfully using the *pdc* promoter in *T. reesei* [37–39]. In this study, we also showed that the *pdc* promoter was a strong promoter in *M. thermophila* when cultured in higher or lower concentrations of glucose. The quantitative data showed that the expression level of *Mtxyr1* mRNA was significantly higher in the MX11 strain than in the parent strain, which confirmed the strength of the *pdc* promoter. Therefore, the *pdc* promoter should receive more attention in fungal research.

XlnR homologs are found commonly in filamentous ascomycetes and are often assumed to have the same function in different fungi. However, Klaubauf et al. [13] compared the wild-type and *xlnR*/*xlr1*/*xyr1* mutants of five fungi (*Fusarium graminearum, M. oryzae, Trichoderma reesei,*

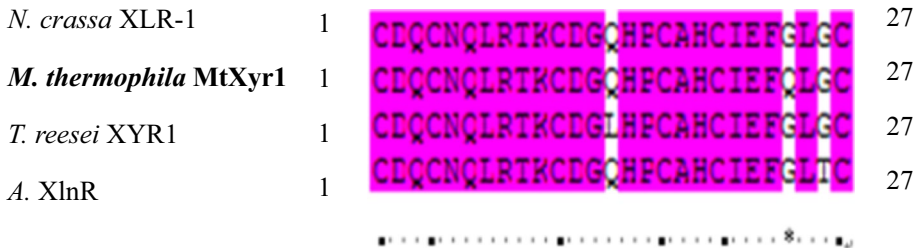

Fig. 5 The amino acid sequence alignment of Zn_2C_6 dinuclear cluster from four types of filamentous fungi. *Black shaded squares* indicate six cysteines of the zinc finger

A. niger and A. nidulans). Large differences were found in the set of genes controlled by *XlnR* (*Xlr1*/*Xyr1*) in the different species. In the head blight fungus *F. graminearum*, *Xyr1* regulates xylanase but not cellulase formation [7]. In *Neurospora crassa, XlnR* is not essential for use of cellulose and only modulates the expression of some cellulolytic genes [35]. However, the ortholog of *XlnR* in *Magnaporthe oryzae* (*Xlr1*) does not regulate its xylanolytic system, but appears to be involved only in D-xylose catabolism [5]. The regulatory role of *MtXyr1* is similar to that of the *F. graminearum*.

The sequences of *XlnR* (*Xlr1*/*Xyr1*) binding sites have been reported: the *XlnR* binding site is GGCTRA in *Aspergillus oryzae* [25]. The functional binding sequences of *Xyr1* can interact not only with the 5′-GGCTAA-3′ motif, but also with several 5′-GGC(A/T)(3)-3′ motifs in *T. reesei* [9]. In the present study, the genes encoding two of the three different kinds of xylanase present in band no. 8 were found to have potential binding sites in the promoter regions. There are two potential binding sites in the upstream region (−1008 to −1003 bp, −1334 to −1329 bp) of gi: 367022650. There is a single potential binding site located in −403 to −398 bp upstream of gi: 367031002. We also found more than eight 5′-GGC(A/T)(3)-3′ motifs in the promoter regions of the three kinds of xylanase genes. Nonetheless, compared with Xln R and *Xyr1*, *MtXyr1* has slight differences in the amino acid sequence alignment of the Zn_2C_6 dinuclear cluster (Fig. 5), which may explain the differences in the binding sites. Therefore, DNase I footprinting analysis and electrophoretic mobility shift assays need to be performed to identify the precise binding sites.

Xyr1 is permanently available in the cell. The corresponding xylanolytic enzyme activities were clearly elevated in a *xyr1*-expressing strain, emphasizing this factor as a good target for genetically engineered strain improvement in *Hypocrea jecorina* [21]. The positive regulator *Xyr1* was successfully expressed in *T. reesei* RUT C30, resulting in significant increases in cellulase activity and levels of corresponding mRNAs [40]. The results in this study demonstrated that *MtXyr1* is a positive regulatory factor for xylanase gene expression. It is feasible to improve xylanase production by overexpressing *Mtxyr1* in *M. thermophila*, which represents an effective approach to increase total xylanase productivity in *M. thermophila*.

Conclusions

In this study, we improved the method of protoplast preparation and transformation in *M. thermophila*. Under the control of a strong promoter, *MtPpdc*, *MtXyr1* was overexpressed. Compared with the parent strain, the extracellular xylanase activities of the transformant cultured in non-inducing and inducing conditions for 120 h were 25.19- and 9.04-fold higher, respectively. The results also showed that overexpression of *xyr1* altered the xylanolytic gene expression profile in *M. thermophila*. Thus, it is feasible to improve xylanase production by overexpressing *xyr1* in this thermophilic fungus.

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Conflict of interest The authors declare that they have no competing interests.

References

1. Akel E, Metz B, Seiboth B, Kubicek CP (2009) Molecular regulation of arabinan and L-arabinose metabolism in *Hypocrea jecorina* (*Trichoderma reesei*). Eukaryot Cell 8:1837–1844
2. Aro N, Saloheimo A, Ilme´n M, Penttila¨ M (2001) ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. J Biol Chem 276(26):24309–24314
3. Bailey MJ, Biely P, Poutanen KJ (1992) Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 23:257–270
4. Barker B, Kroll K, Vödisch M, Mazurie A, Kniemeyer O, Cramer RA (2012) Transcriptomic and proteomic analyses of the *Aspergillus fumigatus* hypoxia response using an oxygen-controlled fermenter. BMC Genome 13:62
5. Battaglia E, Klaubauf S, Vallet J, Ribot C, Lebrun MH, de Vries RP (2013) *Xlr1* is involved in the transcriptional control of the pentose catabolic pathway, but not hemi-cellulolytic enzymes in *Magnaporthe oryzae*. Fungal Genet Biol 57:76–84
6. Berka RM, Grigoriev IV, Otillar R, Salamov A, Grimwood J, Reid I, Ishmael N, John T, Darmond C, Moisan MC, Henrissat B, Coutinho PM, Lombard V, Natvig DO, Lindquist E, Schmutz J, Lucas S, Harris P, Powlowski J, Bellemare A, Taylor D, Butler G, de Vries RP, Allijn IE, van den Brink J, Ushinsky S, Storms R, Powell AJ, Paulsen IT, Elbourne LD, Baker SE, Magnuson J, Laboissiere S, Clutterbuck AJ, Martinez D, Wogulis M, de Leon AL, Rey MW, Tsang A (2011) Comparative genomic

analysis of the thermophilic biomass-degrading fungi *Myceliophthora thermophila* and *Thielavia terrestris*. Nat Biotechnol 29(10):922–927
7. Brunner K, Lichtenauer AM, Kratochwill K, Delic M, Mach RL (2007) *Xyr1* regulates xylanase but not cellulase formation in the head blight fungus *Fusarium graminearum*. Curr Genet 52(5–6):213–220
8. Chambergo FS, Bonaccorsi ED, Ferreira AJ, Ramos AS, Ferreira JR, Abrahao-Neto J, Farah JP, El-Dorry H (2002) Elucidation of the metabolic fate of glucose in the filamentous fungus *Trichoderma reesei* using expressed sequence tag (EST) analysis. J Biol Chem 277(16):13983–13988
9. Furukawa T, Shida Y, Kitagami N, Mori K, Kato M, Kobayashi T, Okada H, Ogasawara W, Morikawa Y (2009) Identification of specific binding sites for *Xyr1*, a transcriptional activator of cellulolytic and xylanolytic genes in *Trichoderma reesei*. Fungal Genet Biol 46(8):564–574
10. Ghose T (1987) Measurement of cellulase activities. Pure Appl Chem 59:257–268
11. Ilmén M, Saloheimo A, Onnela ML, Penttilä ME (1997) Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. Appl Environ Microbiol 63(4):1298–1306
12. Karnaouri A, Topakas E, Antonopoulou I, Christakopoulos P (2014) Genomic insights into the fungal lignocellulolytic system of *Myceliophthora thermophila*. Front Microbiol 5:281
13. Klaubauf S, Narang HM, Post H, Zhou M, Brunner K, Mach-Aigner AR, Mach RL, Heck AJ, Altelaar AF, de Vries RP (2014) Similar is not the same: differences in the function of the (hemi-) cellulolytic regulator *XlnR* (*Xlr1*/*Xyr1*) in filamentous fungi. Fungal Genet Biol S1087–1845(14):00130–00133. doi:10.1016/j.fgb.2014.07.007
14. Kolbusz MA, Di Falco M, Ishmael N, Marqueteau S, Moisan MC, Baptista CS, Powlowski J, Tsang A (2014) Transcriptome and exoproteome analysis of utilization of plant-derived biomass by *Myceliophthora thermophila*. Fungal Genet Biol 72:10–20
15. Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B (2009) Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. Biotechnol Biofuels 2(19):1–14
16. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
17. Li AN, Xie C, Zhang J, Zhang J, Li DC (2011) Cloning, expression, and characterization of serine protease from thermophilic fungus *Thermoascus aurantiacus* var. *levisporus*. J Microbiol 49(1):121–129
18. Li DC, Li AN, Papageorgiou AC (2011) Cellulases from thermophilic fungi: recent insights and biotechnological potential. Enzyme Res. doi:10.4061/2011/308730
19. Li JK, Feng M, Zhang L, Zhang ZH, Pan YH (2008) Proteomics analysis of major royal jelly protein changes under different storage conditions. J Proteome Res 7(8):3339–3353
20. Li JX, Wang J, Wang SW, Xing M, Yu SW, Liu G (2012) Achieving efficient protein expression in *Trichoderma reesei* by using strong constitutive promoters. Microb Cell Fact 11:84–93
21. Mach-Aigner AR, Pucher ME, Steiger MG, Bauer GE, Preis SJ, Mach RL (2008) Transcriptional regulation of *Xyr1*, encoding the main regulator of the xylanolytic and cellulolytic enzyme system in *Hypocrea jecorina*. Appl Environ Microbiol 74(21):6554–6662
22. Mach RL, Zeilinger S (2003) Regulation of gene expression in industrial fungi: *Trichoderma*. Appl Microbiol Biotechnol 60(5):515–522
23. Maheshwari R, Bharadwaj G, Bhat MK (2000) Thermophilic Fungi: their physiology and enzymes. Microbiol Mol Biol Rev 3:461–488
24. Mandels M, Weber J (1969) The production of cellulases. Adv Chem 95:391–414
25. Marui J, Tanaka A, Mimura S, de Graaff LH, Visser J, Kitamoto N, Kato M, Kobayashi T, Tsukagoshi N (2002) A transcriptional activator, Ao*XlnR*, controls the expression of genes encoding xylanolytic enzymes in *Aspergillus oryzae*. Fungal Genet Biol 35(2):157–169
26. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3):426–428
27. Nemoto T, Maruyama J-I, Kitamoto K (2009) Improvement of heterologous protein production in *Aspergillus oryzae* by RNA interference with alpha-amylase genes. Biosci Biotechnol Biochem 73(11):2370–2373
28. Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, Van den Hondel CA (1987) Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 56:117–124
29. Rauscher R, Würleitner E, Wacenovsky C, Aro N, Stricker AR, Zeilinger S, Kubicek CP, Penttilä M, Mach RL (2006) Transcriptional regulation of *xyn1*, encoding xylanase I Hypocrea jecorina. Eukaryot Cell 5(3):447–456
30. Rauscher R, Würleitner E, Wacenovsky C, Aro N, Stricker AR, Zeilinger S, Kubicek CP, Ravalason H, Jan G, Mollé D, Pasco M, Coutinho PM, Lapierre C, Pollet B, Bertaud F, Petit-Conil M, Grisel S, Sigoillot JC, Asther M, Herpoël-Gimbert I (2008) Secretome analysis of *Phanerochaete chrysosporium* strain CIRM-BRFM41 grown on softwood. Appl Microbiol Biotechnol 80(4):719–733
31. Saloheimo A, Aro N, Ilmén M, Penttilä M (2000) Isolation of the *ace1* gene encoding a Cys(2)-His(2) transcription factor involved in regulation of activity of the cellulase promoter *cbh1* of *Trichoderma reesei*. J Biol Chem 275(8):5817–5825
32. Seiboth B, Karimi RA, Phatale PA, Linke R, Hartl L, Sauer DG, Smith KM, Baker SE, Freitag M, Kubicek CP (2012) The putative protein methyltransferase LAE1 controls cellulase gene expression in *Trichoderma reesei*. Mol Microbiol 84(6):1150–1164
33. Silva-Rocha R, Castro LdS, Antoniêto ACC, Guazzaroni M-E, Persinoti GF, Silva RN (2014) Deciphering the Cis-regulatory elements for *Xyr1* and *CRE1* regulators in *Trichoderma reesei*. PLoS One 9:e99366
34. Strauss J, Mach RL, Zeilinger S, Hartler G, Stöffler G, Wolschek M, Kubicek CP (1995) *Cre1*, the carbon catabolite repressor protein from *Trichoderma reesei*. FEBS Lett 376(1–2):103–107
35. Stricker AR, Grosstessner-Hain K, Würleitner E, Mach RL (2006) *Xyr1* (xylanase regulator 1) regulates both the hydrolytic enzyme system and D-xylose metabolism in *Hypocrea jecorina*. Eukaryot Cell 5(12):2128–2137
36. Sun J, Tian C, Diamond S, Glass NL (2012) Deciphering transcriptional regulatory mechanisms associated with hemicellulose degradation in *Neurospora crassa*. Eukaryot Cell 11:482–493
37. Wang J, Mai GQ, Liu G, Yu SW (2013) Molecular cloning and heterologous expression of an acid-stable endoxylanase gene from *Penicillium oxalicum* in *Trichoderma reesei*. J Microbiol Biotechnol 23(2):251–259
38. Wang J, Zeng DS, Liu G, Wang SW, Yu SW (2014) Truncation of a mannanase from *Trichoderma harzianum* improves its enzymatic properties and expression efficiency in *Trichoderma reesei*. J Ind Microbiol Biotechnol 41:125–133

39. Wang J, Zeng DS, Mai GQ, Liu G, Yu SW (2014) Homologous constitutive expression of *Xyn*III in *Trichoderma reesei* QM9414 and its characterization. Folia Microbiol 59:229–233
40. Wang S, Liu G, Wang J, Yu J, Huang B, Xing M (2013) Enhancing cellulase production in *Trichoderma reesei* RUT C30 through combined manipulation of activating and repressing genes. J Ind Microbiol Biotechnol 40(6):633–641
41. Zeilinger S, Schmoll M, Pail M, Mach RL, Kubicek CP (2003) Nucleosome transactions on the *Hypocrea jecorina* (*Trichoderma reesei*) cellulase promoter *cbh2* associated with cellulase induction. Mol Genet Genomics 270(1):46–55