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Enhancing Cellulase Production in Thermophilic Fungus *Myceliophthora thermophila* ATCC42464 by RNA Interference of *cre1* Gene Expression

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The role of CRE1 in a thermophilic fungus, *Myceliophthora thermophila* ATCC42464, was studied using RNA interference. In the *cre1*-silenced strain C88, the filter paper hydrolyzing activity and β-1,4-endoglucanase activity were 3.76-, and 1.31-fold higher, respectively, than those in the parental strain when the strains were cultured in inducing medium for 6 days. The activities of β-1,4-exoglucanase and cellobiase were 2.64-, and 5.59-fold higher, respectively, than those in the parental strain when the strains were cultured for 5 days. Quantitative reverse-transcription polymerase chain reaction showed that the gene expression of *egl3*, *cbh1*, and *cbh2* was significantly increased in transformant C88 compared with the wild-type strain. Therefore, our findings suggest the feasibility of improving cellulase production by modifying the regulator expression, and an attractive approach to increasing the total cellulase productivity in thermophilic fungi.

Keywords: *Myceliophthora thermophila*, thermophilic fungi, RNA interference, cellulase, CRE1

Introduction

Recently, thermostable enzymes from thermophilic fungi have become a significant focus of research [19]. Extracellular enzymes produced by thermophilic fungi might be more active and stable under extreme conditions [14]. Enzymes with thermal stability have advantages in the production of chemicals and biomass-based fuels. *Myceliophthora thermophila* ATCC42464 is a thermophilic fungus that can be cultured at 45°C. Its genome has been analyzed from telomere to telomere [3]. The fungus is capable of hydrolyzing all major polysaccharides found in biomass, thereby representing a potential reservoir of thermostable enzymes [3].

Carbon catabolite repression (CCR) is an important mechanism for controlling metabolic processes in prokaryotes and eukaryotic microorganisms. The expression of genes necessary for the use of alternative carbon sources is repressed in the presence of easily metabolized carbon sources such as glucose [27]. In *Trichoderma reesei* and other fungi, the key regulator of CCR is the Cys_2His_2-type transcription factor CRE1 [1]. CRE1-related transcriptional regulators exist widely in mesophilic fungi, such as *Neurospora crassa* [6, 29], *T. reesei* [9, 27], *Aspergillus niger* [7], and *Fusarium oxysporum* [10]. It is orthologous to CreA from *Aspergillus* sp. [7, 11, 28]. Additionally, the function of Mig1 in *Saccharomyces cerevisiae* is similar to that of CRE1 [22]. Mig1 represses the transcription of about 90 genes associated with the use of alternative carbon sources, including sucrose, galactose, and maltose, when glucose is sufficient [25].

Compared with studies in mesophilic fungi, research on the regulation of cellulase gene expression in thermophilic fungi is relatively limited [15]. Nevertheless, consensus sequences of a potential regulatory element have been identified in the 5′ upstream region of thermophilic fungal cellulase genes [5, 12, 26, 30], and the *cre1* gene from two

thermophilic fungi (*Talaromyces emersonii* and *Thermoascus aurantiacus*) was cloned (GenBank AF440004.4 and AY604200.1, respectively). However, the function of these regulators has not yet been reported.

In this study, the *cre1* homologous gene *Mtcre1* was identified in the genome of *M. thermophila* ATCC42464. Compared with cellulase-related transcriptional regulators in *Humicola grisea* var. *thermoidea*, *N. crassa* OR74A, *F. oxysporum* FOSC 3-a, *T. reesei* QM6a, and *A. niger* CBS 513.88, the amino acid sequence (GenBank AEO60646.1) of MtCRE1 shares 76%, 67%, 61%, 60%, and 54% homology, respectively. However, the role of MtCRE1 in the regulation of the major cellulolytic genes in *M. thermophila* is unknown. The objective of this work was to validate the role of MtCRE1 in *M. thermophila* using RNA interference (RNAi) and to determine whether cellulase production could be improved. We constructed *cre1*-silenced recombinant strains and detected cellulase activities under inducing conditions. The zymograms between the tranformant and wild-type strain were analyzed. The different proteins were identified through mass spectrometry. The results indicated that MtCRE1 acts as a repressor for cellulase expression, and silencing of *cre1* effectively improves cellulase production in *M. thermophila*.

Materials and Methods

Strains, Plasmids, and Culture Conditions

Escherichia coli JM107 (Fermentas, Vilnius, Lithuania) was used for plasmid manipulations. The *M. thermophila* strain (ATCC42464) was purchased from the American Type Culture Collection (ATCC) and used as the parent strain throughout this study. *E. coli* was maintained on Luria-Bertani (LB) medium, in which ampicillin (100 μg/ml) was supplemented as necessary. *M. thermophila* was maintained on potato dextrose agar (PDA). The spores were washed with a 2% Tween-20 solution before spore suspension.

For enzyme production, a total of 1×10^7 *M. thermophila* spores that were collected from cultures grown on PDA plates were inoculated into 30 ml of basal medium supplemented with 5% corncob powder and 3% wheat bran in a 250 ml conical flask and grown at 45°C with shaking at 250 rpm. The basal medium contained 0.3% peptone, 0.2% $(NH_4)_2SO_4$, 0.05% yeast extract, 0.4% KH_2PO_4, 0.03% $CaCl_2{\cdot}2H_2O$, 0.03% $MgSO_4{\cdot}7H_2O$, and 0.02% Tween-20. The pUC-19-*pdc* plasmid contains the promoter and terminator of the pyruvate decarboxylase gene of *M. thermophila* (P*pdc* and T*pdc*, respectively) and was previously constructed in our laboratory. Plasmid pAN7-1 was used in the co-transformation of *M. thermophila*. PDA medium supplemented with 50 μg/ml hygromycin B was used to select transformants.

Construction of the RNAi Vector

The *cre1* gene sequence (XM_003665843.1) was acquired from the National Center for Biotechnology Information (NCBI). A small interfering RNA (siRNA) design program (http://rnaidesigner.lifetechnologies.com/rnaiexpress/) was used to design siRNA sequences of the target gene. The oligonuleotide sequences are shown in Table 1. The stable stem-loop structure 5′-TTCAAGAGA-3′ connected the complementary oligonucleotides (underlined and double underlined in Table 1), which can produce a hairpin siRNA during transcription [4]. The double-stranded siRNA-*cre1* can be gained from polymerase chain reaction (PCR) with a reaction system consisting of 40% nuclease-free water, 20% annealing buffer for DNA oligos (5×), 20% DNA oligo S (50 μM), and 20% DNA oligoA (50 μM). The PCR protocol consisted of initial denaturation at 95°C for 2 min and then falling 0.1°C per second until the temperature was 25°C. Finally, the double-stranded siRNA-*cre1* with a cohesive terminus was inserted into the pUC-19-*pdc* plasmid. Sequence analysis was performed (Sangon, Shanghai, China) to verify the insertion of the siRNA-*cre1* in the recombinant vector.

Fungal Transformations and Isolation of Single-Copy Transformants

The protoplast preparation and transformation of *M. thermophila* have not been reported previously. We referenced the method of *T. reesei* RUT-C30 described previously [21] with the following modifications. Lysing enzymes (10 mg/ml) from *T. harzianum* (Sigma-Aldrich, Brondby, Denmark) in 1 M $MgSO_4$ was used for protoplast formation. The heat shock temperature was 60°C for 2 min. PDA medium supplemented with 20 mg/ml hygromycin B was used to select transformants. The single colonies were screened from hygromycin B-containing selection plates. Then, the colonies were screened using PCR analysis of genomic DNA. Three primers (Table 2) were designed to verify the integration of the siRNA-*cre1* in the genome. Upstream primers siRNA-*cre1*-F and siRNA-P*pdc*-F were paired with downstream primer siRNA-T*pdc*-R to selecting *cre1*-silenced transformants.

Table 1. siRNA oligonuleotide sequences of the target gene *cre1*.

Primer	Sequence (5′-3′)
$cre1_{sense}$	*GGCC*GCAACAGCAGAAGCAGCAA TTCAAGAGA TTGCTGCTTCTGCTGTTGC
$cre1_{anti}$	*CTAG*GCAACAGCAGAAGCAGCAATCTCTTGAA TTGCTGCTTCTGCTGTTGC

Note: The 5′ end of the sense and antisense siRNA sequences are *Not*I and *Xba*I restriction sites, respectively (italics). Underlined and double underlined sequences are complementary oligonucleotides. The shaded sequences are stem-loop structures.

Table 2. Primer sequences used for verifying the recombinant vector and qRT-PCR.

Primers	Sequence (5′-3′)
siRNA-*cre1*-F	TGCTTCTGCTGTTGCCTAG
siRNA-P*pdc*-F	CCCAAGCTTCCGAGTGTACTCCGTAAGGA
siRNA-T*pdc*-R	CCGGAATTCGGATTACAGCGCAGTGCACG
RTq-*tub*-F	AGGGTATGGATGAGATGGAG
RTq-*tub*-R	AGAAGCAAGCCCTGGAAC
RTq-*cre1*-F	GAATCTTCCCCCGCCGCTCAG
RTq-*cre1*-R	CTCGCCAACATTGCCACATCC
RTq-*xyr1*-F	CCAGATCTTCGGGGGCAGC
RTq-*xyr1*-R	GTACTCGCAGCCCAGTTGGAAC
RTq-*cbh1*-F	GATGGTCCTTCTTGCGCCTCC
RTq-*cbh1*-R	TTACCGCTCGTGGTGATGC
RTq-*cbh2*-F	TTCTCGGGCGTCCGGCTCTTC
RTq-*cbh2*-R	GGAATGGCGAGATTGTGGA
RTq-*egl1*-F	TCGGGCAACGGTTACGAGG
RTq-*egl1*-R	AGCTCTTGACCTGGTTCTGG
RTq-*egl3*-F	TCCGCCACCAGCACCGCCCCTC
RTq-*egl3*-R	TTGCTGCCGAGCCACTTC

Nucleic Acid Isolation

Fungal genomic DNA was extracted from frozen mycelia using a fungal DNA extraction kit (Sangon). Total RNA was isolated from frozen mycelia by Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quantity and quality were measured with a NanoDrop ND-1000 spectrometer, and RNA integrity was assessed by standard denaturing agarose gel electrophoresis.

Quantitative Reverse-Transcription PCR (qRT-PCR)

Up to 1 μg of total RNA and RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) were prepared for the reverse-transcription reaction on ice. According to the SYBR Green qPCR (Takara) manufacturer's instructions, the reverse-transcription reaction solution is to be introduced to a real-time PCR system using an ABI Prism 7300 System (Applied Biosystems, Carlsbad, CA, USA). The PCR protocol consisted of 30 sec of initial denaturation at 95°C and then 40 cycles of 5 sec at 95°C and 30 sec at 60°C; melting curve analysis followed with 1 cycle of 15 sec at 95°C, 30 sec at 60°C, and 15 sec at 95°C. Gene expression levels in the different samples were determined using the ΔΔCT method [17]. The β-tubulin (*tub*) gene (GenBank AEO58945.1) of *M. thermophila* was used as an endogenous control. All samples were carried out in triplicate within a plate. The means ± standard deviations of the replicates are shown in the figures. Primers (Table 2) were designed so that the size of the amplicon was 80–200 bp, and complementary sequences within primers and mismatches were avoided.

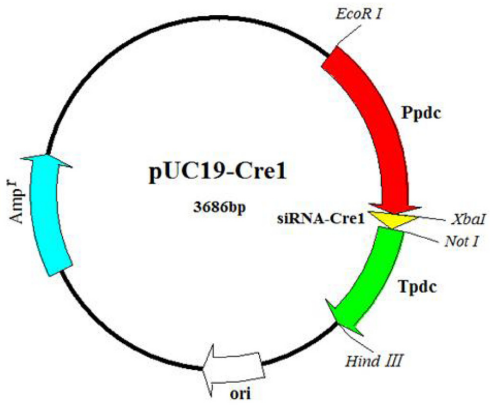

Fig. 1. Schematic representation of the RNA interference vector. P*pdc*, *pdc* promoter of *Myceliophthora thermophila*; siRNA-*cre1*, small interfering RNA sequences including the *cre1* sense sequence, stem-loop, and *cre1* antisense sequence; T*pdc*, *pdc* terminator of *M. thermophila*.

Enzyme Activity Assays

Filter paper hydrolyzing activity (FPA), β-1,4-exoglucanase (CBH) activity, β-1,4-endoglucanase (EG) activity, and cellobiase (BG) activity were measured according to the method of Eveleigh *et al*. [8]. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol product per minute. Each experiment was performed in triplicate.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Identification of Different Proteins

The fermented supernatant of the original strain and the transformant were analyzed by SDS-PAGE on a 12% polyacrylamide gel as described by Laemmli [13]. The protein bands were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA). The protein bands that differed between the original strain and the transformant were excised, transferred to a PCR tube with 100 μl of ultrapure water, washed, and destained on a temperature-controlled board at 25°C. Pretreatment used the in-gel digestion method of Li *et al*. [16]. The proteins were identified using matrix-assisted laser desorption ionization time-of-flight/time-of-flight (MALDI TOF/TOF) mass spectrometry (Applied Biosystems), according to the method of Rauscher *et al*. [23]. To identify the protein sequences, a homology search was performed using Mascot software (ver. 2.2, Matrix Science). The partial amino acid sequences were used to identify analogous proteins through a Basic Local Alignment Search Tool search of the NCBI protein sequence database.

Stability Analysis of *cre1*-Silenced Strains

To test the stability of cellulase production in the *cre1*-silenced transformant, the transformant strains were subcultured for four rounds (one round lasted 5 days) on solid medium containing hygromycin B, and the cellulase activity with filter paper as the substrate was analyzed.

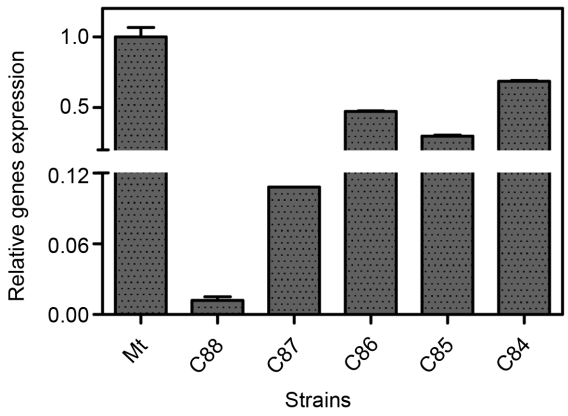

Fig. 2. Quantitative reverse transcription polymerase chain reaction analysis of the *cre1* mRNA levels in the *M. thermophila* transformants under inducing conditions.

Results

Construction of *M. thermophila cre1*-Silenced Strains

To silence the expression of the *cre1* gene in *M. thermophila*, a recombinant RNAi plasmid was transformed into *M. thermophila* ATCC42464 with the aid of plasmid pAN7-1 through co-transformation. More than 20 single colonies were screened from hygromycin B-containing selection plates. Then, the colonies were verified by PCR analysis of genomic DNA. In total, five colonies contained the *cre1*-interference sequence. The transformants were grown on a cellulase-inducing medium that contained corncob powder and wheat bran, and the expression level of *cre1* was analyzed by qRT-PCR (Fig. 2). The mRNA level of *cre1* in the five transformants (C84, C85, C86, C87, and C88) was 68.6%, 29.9%, 47.3%, 10.8%, and 1.2%, respectively, of that of the original strain (100%). Finally, transformant C88, with the best RNAi efficiency, was selected for subsequent studies. The above results indicated that the silencing of *cre1* in strain C88 was achieved through RNAi under inducing conditions.

Silencing of *cre1*-Enhanced Cellulase Production in *M. thermophila*

The parental strain and RNAi-mediated *cre1*-silencing strain C88 were grown in basal medium with corncob powder and wheat bran. The FPA, CBH, EG, and BG activities of the two strains were measured. In the *cre1*-silenced strain C88, FPA and EG activity was 3.76-, and 1.31-fold higher, respectively, than that in the parental strain when the strains were cultured in inducing medium

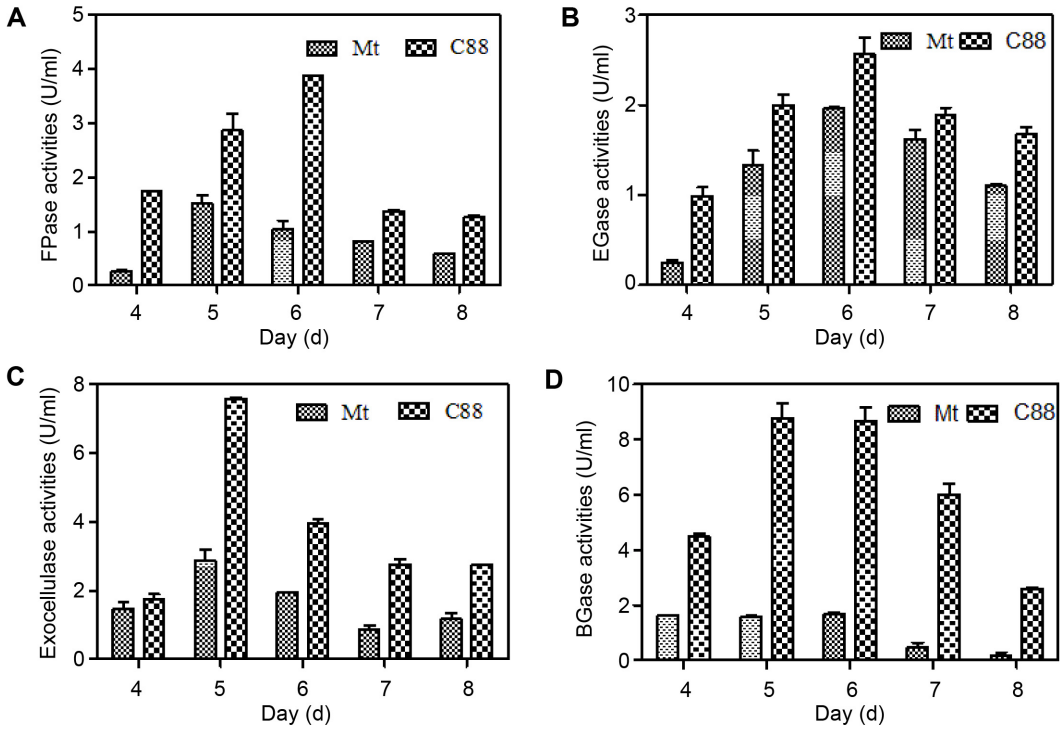

Fig. 3. Cellulase activities of the original strain and tranformants.
(**A**), (**B**), (**C**), and (**D**) are the filter paper (FP), β-1,4-endoglucanase (EG), β-1,4-exoglucanase (CBH; exocellulase), and cellobiase (BG) activities, respectively.

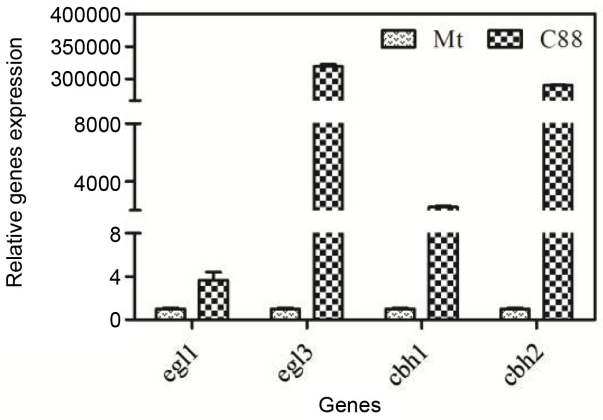

Fig. 4. Expression of the main cellulase genes in the *cre1*-silenced transformant under inducing conditions.

Table 3. Total protein production of the transformant and original strain under inducing conditions.

Strain	Total amount of secreted protein (μg/ml)	
	5 days	6 days
Mt (Original strain)	203 ± 1.3	214 ± 0.5
C88 (Transformed strain)	266 ± 4.6	262 ± 1.7

for 6 days (Figs. 3A and 3B). The activities of CBH and BG were 2.64-, and 5.59-fold higher, respectively, than that in the parental strain when the strains were cultured for 5 days (Figs. 3C and 3D). The FPA and EG activities peaked at 6 days. The CBH and BG activities peaked at 5 days. The qRT-PCR results showed that the gene expression of *egl3*, *cbh1*, and *cbh2* was significantly increased in the transformant compared with the wild-type strain (Fig. 4).

The total protein production of C88 was 1.31- and 1.22-fold higher than that of the parent strain under inducing conditions after culture for 5 and 6 days, respectively (Table 3).

This result indicated that cellulase activity and total protein production were enhanced in the transformant.

Mass Spectrometry of Proteins That Differed Between the Parent Strain and the Transformant

Proteins secreted into the culture medium were analyzed by SDS-PAGE (Fig. 5). The bands that differed between the parent and transformant strains were excised and identified using MALDI TOF/TOF mass spectrometry. The MS data showed that three endoglucanases and five cellobiohydrolases were differentially produced in the transformant. These

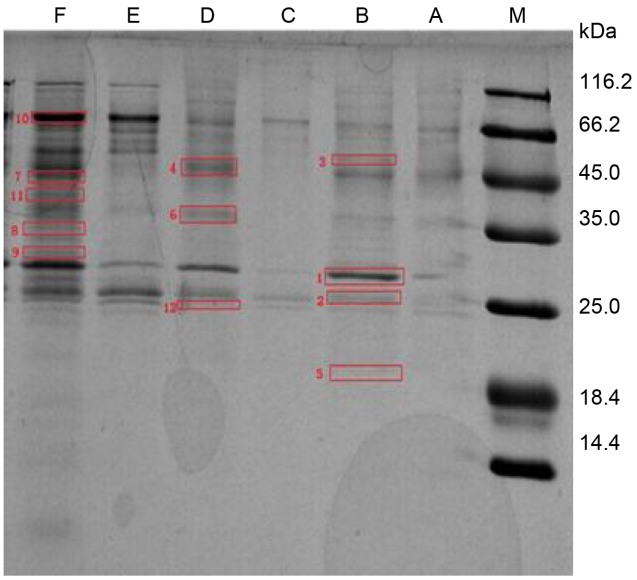

Fig. 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of the transformant and original strain.
M: protein ladder 1; A–F: Culture filtrate of the parent strain (Mt) and transformant (C88) cultured for 4–6 days. A, C, and E are Mt cultured for 4, 5, and 6 days, respectively. B, D, and F are C88 cultured for 4, 5, and 6 days, respectively. Bands 1–12 in red boxes were excised and identified by matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry.

Table 4. Identification of different proteins between the transformant and original strain using MS.

Band number	NCBI number	Glycoside hydrolase family	Protein name
1	GI:164598122	7	Exocellobiohydrolase
2	GI:310801037	7	A protein contains CBH and EG regions
3	GI:367023008	7	Cellulose 1,4-beta-Cellobiosidase
4	GI:367034450	7	Cellobiohydrolase; Exoglucanase
5	GI:367026566	25	Lysozyme
6	GI:367029733	16	Cell wall glucanase-like protein; Cell wall glucanosyl transferase
7	GI:367019458	5	Endoglucanase 3 precursor
8	GI:367023495	6	Cellobiohydrolase II
9	No data	No data	No data
10	GI:367035092	15	Glucan 1,4-alpha-glucosidase
11	GI:367033165	Unkown	Extracellular dioxygenase
12	GI:367032989	12	Endoglucanase1

bands included various glucoside hydrolases belonging to the GH 5, 6, 7, 16, and 25 families (Table 4).

Discussion

In this study, the effects of *cre1* on cellulase expression, cellulase activity, and the amount of extracellular protein produced in the *cre1*-silenced transformant (C88) were investigated. To the best of our knowledge, this is the first application of RNAi technology in *M. thermophila*. The hairpin-type RNAi construct could be useful for fungal silencing genes as reported for *ace1* and *cre1* in *T. koningii* [31, 32]. In this study, the interference produced a satisfactory effect. The mRNA level of *cre1* in transformant C88 was only 1.2% of that in the original strain. The cellulase activity was similar between the C88 after four rounds of subculture and the primary strain during cultivation. Importantly, the interference sequence could be detected in the genome of the subcultured C88 strain by PCR, suggesting that the gene silencing in *M. thermophila* was rather stable.

CRE1 directly suppresses *cbh1* transcription by binding to two closely spaced 5′-CCCCAC-3′ motifs in the *cbh1* promoter region in *Hypocrea jecorina* [18]. Deletion or mutation of *cre1* caused an increase in the *cbh1* transcript level under repressing conditions [24]. CRE1 is involved indirectly in the control of *cbh2* expression by regulating the main inducer XYR1 [18]. About 250 genes are under the regulatory influence of CRE1. These genes encode enzymes involved in plant cell wall degradation, nitrogen uptake, developmental processes, components of the transcriptional mediator complex, and chromatin remodeling, supporting a role for CRE1 as a cell-wide regulator [22]. In this study, we also observed differences in the transcript levels of four cellulase genes, *egl1*, *egl3*, *cbh1*, and *cbh2*. The relative transcript ratio of the last three genes was significantly increased in the transformant relative to the wild-type strain. However, the enzyme activities did not increase so dramatically. Recent research suggests that a substantial fraction of regulatory genetic variants influence gene expression at all levels from mRNA to steady-state protein abundance; a number of effects have a specific impact on particular expression phenotypes [2]. Therefore, the expression level of RNA is not related to the protein abundance at any time, which can explain the contradiction between the enzyme activity and transcript level of *egl3*, *cbh1*, and *cbh2* in C88.

The downregulation of *cre1* plays an important role in enhancing enzyme production in *T. reesei* [20]. In our previous study, the silenceing of *cre1* also improves cellulase and xylanase expression, indicating that *cre1* represses the basal expression level of *xyr1* in *T. koningii* [31]. In the present study, the role of CRE1 was verified in the thermophilic fungus *M. thermophila* ATCC42464 through RNAi. Therefore, our findings suggest the feasibility of improving cellulase production by modifying the expression of regulators in thermophilic fungi. Thus, mechanisms of cellulase gene regulation in thermophilic fungi may share certain similarities with those in mesophilic fungi.

Acknowledgments

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