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ORIGINAL PAPER

Enhancing Cellulase and Hemicellulase Production in *Trichoderma orientalis* EU7-22 via Knockout of the *creA*

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Abstract

The role of the transcription factor *creA*-mediating carbon catabolite repression in *Trichoderma orientalis* EU7-22 was investigated for cellulase and hemicellulase production. The binary vector pUR5750G/*creA*::*hph* was constructed to knock out *creA* by homologous integration, generating the Δ*creA* mutant *Trichoderma orientalis* CF1D. For strain CF1D, the filter paper activities (FPA), endoglucanase activities (CMC), cellobiohydrolase activity(CBH), β-glucosidase activity (BG), xylanase activity (XYN), and extracellular protein concentration were 1.45-, 1.15-, 1.71-, 2.51-, 2.72, and 1.95-fold higher in inducing medium and were 6.41-, 7.50-, 10.27-, 11.79-, 9.25-, and 3.77-fold higher in glucose repressing medium, respectively, than those in the parent strain after 4 days. SDS–PAGE demonstrated that the extracellular proteins were largely secreted in the mutant CF1D. Quantitative reverse-transcription polymerase chain reaction indicated that the expressions of *cbh1*, *cbh2*, *eg1*, *eg2*, *bgl1*, *xyn1*, and *xyn2* were significantly increasing for the mutant CF1D not only in the inducing medium but also in the repressing medium. Those results indicated that *creA* was a valid target gene in strain engineering for improved enzyme production in *T. orientalis*.

Keywords *Trichoderma orientalis* · *creA* · Cellulase · Hemicellulase · Extracellular protein

Introduction

Lignocellulosic biomass is a renewable resource, which can be used to produce environmental-friendly biofuels, chemicals, polymers, and materials [1]. Conversion of lignocellulosic biomass into fermentable sugars mainly depends on the degradation of cellulolytic enzymes such as cellulase and hemicellulase, which are produced by many filamentous fungi including *Trichoderma*, *Aspergillus*, *Penicillium*, and *Acremonium* [2–4]. The cellulases include three major groups of enzymes: endoglucanases, which randomly attack internal glycosidic linkages; cellobiohydrolases, which produce cellobiose from the reducing and non-reducing ends of the cellulose chain; and β-glucosidases, which convert cellobiose into glucose [3, 4]. In addition to cellulases, a number of hemicellulases like xylanases, β-xylosidases, α-arabinosidases, α-glucuronidases, α-galactosidases, ferulic acid esterases, and acetyl xylan esterases are needed for a complete hydrolysis of lignocellulosic substrates. Xylanases is the most important enzyme among the hemicellulases, which randomly cleave the β-1,4 backbone of the complex plant cell wall polysaccharide xylan [4].

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However, carbon catabolite repression (CCR) is an important mechanism for controlling metabolic processes in prokaryotic and eukaryotic microorganisms. Filamentous fungi yield large amounts of biomass-degradation enzymes that are regulated by CCR to mainly control carbon assimilation [5]. The Cys_2His_2-type transcription factor *creI*/*creA* has been shown to act as a repressor for mediating CCR, which binds to the promoter region of target genes via the consensus motif 5′-SYGGRG-3′, whose function in vivo has been shown in *Trichoderma reesei* [5, 6], *Aspergillus nidulans* [7], *Aspergillus niger* [8], and *Neurospora crassa* [9]. Production of cellulolytic enzymes could be induced by cellulose, but are strongly repressed by hydrolysate-glucose, the major end-product.

In this study, the transcription factor *creA* of the *Trichoderma orientalis* EU7-22 was investigated to evaluate the production of cellulase and hemicellulase not only in cellulose-inducing medium but also in glucose repressing medium.

Materials and Methods

Strains

Trichoderma orientalis EU7-22, which was isolated and preserved in this laboratory, was cultivated on potato dextrose agar (PDA) for 5 days. Spores were collected in sterile water and then filtered. The number of spores were counted using a hemocytometer, inoculated in the potato dextrose liquid medium (1×10^5 total spores per mL), incubated at 30 °C for 34 h with 180 rpm agitation on a rotary shaker, and then transferred to submerged fermentation medium with 10% inoculum for enzyme production. *Escherichia coli* DH5a was used for vector construction. *Agrobacterium tumefaciens* AGL1 was used to mediate the transformation.

Plasmid Construction

The gene *creA* (GenBank no. JQ238603) from the parent strain *T. orientalis* EU7-22 was knocked out for enzyme and protein analyses. The T-DNA binary vector pUR5750G/*creA*::*hph* was constructed on the backbone of pUR5750G (Fig. 1a). The first homologous arm sequence (fragment I = 1942 bp) of *creA* upstream was amplified by PCR with the primers CreA-QC1F (containing *Kpn* I) and CreA-QC1R (containing *Sac* I), and then, it was inserted into the *Kpn* I/*Sac* I sites of pUR5750G. Then, the second homologous arm sequence (fragment II = 1487 bp) of *creA* downstream was amplified by PCR with the primers CreA-QC2F (containing *Xba*I) and CreA-QC2R (containing *Hin*dIII), and then, it was also inserted to the *Xba*I/*Hin*dIII sites of pUR5750G. The primer sequences used in this study are listed in Table 1, and the schematic maps of the primer locus for the knockout of *creA* are shown in Fig. 1b.

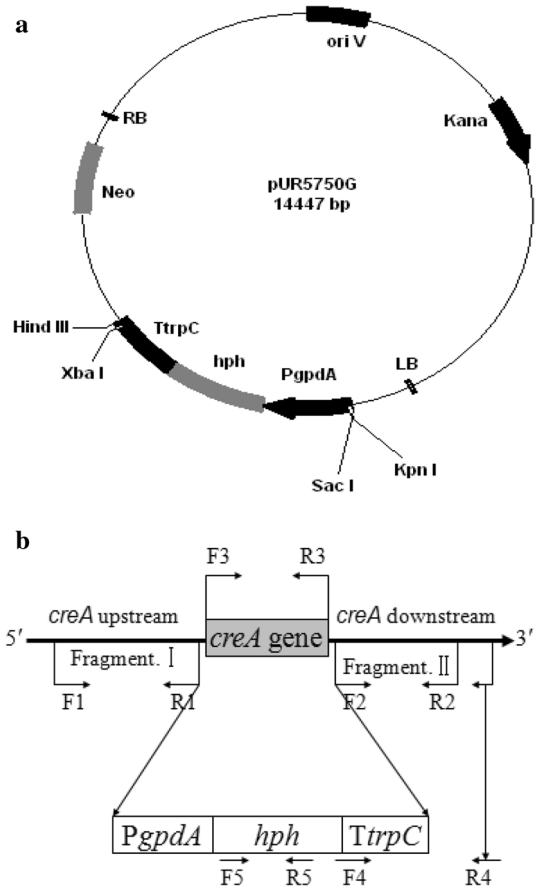

Fig. 1 Schematic map of the vector and the primer locus for knocking out *creA* (**a** pUR5750G; **b** F1, primer CreA-QC1F; R1, primer CreA-QC1R. F2, primer CreA-QC2F; R2, primer CreA-QC2R. F3, primer CreA-F; R3, primer CreA-R.F4, primer HtrpC-F; R4, primer CreA-YZR.F5, primer Hph-F; R5, primer Hph-R)

Obtaining the Δ*creA* Transformants

The binary vector pUR5750G/*creA*::*hph* was transferred into the parental strain *T. orientalis* EU7-22 via *Agrobacterium tumefaciens*-mediated transformation (ATMT) [10]. The putative visible transformants were first picked and transferred to a PDA agar plate containing hygromycin B. The transformants were then cultured on PDA agar plates without hygromycin B for three times. Then, the monoconidial cultures were transferred to PDA plates containing 100 μg/mL hygromycin B for determining the stability of the transformants. Genomic DNA of the transformants was extracted from all available mycelia according to the method of Penttilä et al. [11]. The gene fragments were analyzed by PCR amplification using

Table 1 Primers used in this study for PCR

Primer name	Primer sequence (5′–3′)	Description
Hph-F	CGACAGCGTCTCCGACCTGA	For detection 811 bp *hph* gene fragment
Hph-R	CGCCCAAGCTGCATCATCGAA	
CreA-F	ATGCAACGAGCACAGTCTG	For amplification of 1209 bp *creA* gene fragment
CreA-R	CTACATCCGATCCATGAGGTC	
CreA-QC1F	CGG**GGTACC**AGTCGTGCCCAGAAGCAAAACC	For amplification of 1942 bp fragment I
CreA-QC1R	CC**GAGCTC**GATCGAGCGGCAGTCAAAAGG	
CreA-QC2F	GC**TCTAGA**CCTCGAATGATTTACGACTTTGG	For amplification of 1487 bp fragment II
CreA-QC2R	CCC**AAGCTT**GTAGGTGTGTCGAACATGGATGG	
HtrpC-YZF	AGGAATAGAGTAGATGCCGACC	For detection 2691 bp knockout fragment
CreA-YZR	GTTACAGCACCATGATAACAACC	
18S-YGF	AGGCGCGCAAATTACCCAATCC	For qRT-PCR analysis of 18S rRNA gene
18S-YGR	GCCCTCCAATTGTTCCTCGTTAAG	
cbh I-YGF	ATCGGCTTCGTCACGCAATC	For qRT-PCR analysis of *cbh1* gene
cbh I-YGR	ACGCCACCATCCGCATCCA	
cbh II-YGF	GACAAACCTCGGCACTCC	For qRT-PCR analysis of *cbh2* gene
cbh II-YGR	GACCAGCGTCCAGATACATT	
eg I-YGF	CAGGGCTTCTGCTGTAATGAG	For qRT-PCR analysis of *eg1* gene
eg I-YGR	TTGAACTGGGTGATGATGGTG	
eg II-YGF	GCTCCGCCAGAATAACCG	For qRT-PCR analysis of *eg2* gene
eg II-YGR	CAGCCAACATAGCCAAGATAGAC	
bgl 1-YGF	ATCACCTACCCGCCTTCA	For qRT-PCR analysis of *bgl1* gene
bgl 1-YGR	TCTCGTCGTCGGATGTTG	
xyn I-YGF	CGTCAACACGGCGAACCA	For qRT-PCR analysis of *xyn1* gene
xyn I-YGR	CGGTGATGGAAGCAGAGCC	
xyn II-YGF	TTTGTCGTGGGAGTTGGCTG	For qRT-PCR analysis of *xyn2*
xyn II-YGR	TGTGCTGGGTAGTTGTGGTTG	

the primer CreA-F&-R(1209 bp), hph-F&-R (811 bp), and HtrpC-YZF and CreA-YZR (2691 bp). The positive mutant strain was analyzed by PCR (Fig. 2).

Cellulase and Xylanase Preparation

Experiments were conducted in 250-mL Erlenmeyer flasks. The inducing medium (IM, pH 5.2) was 50 mL, containing Avicel (PH101, 50 μm particle size, FMC Corporation) inducer substrate (2%, w/v), wheat bran (1%, w/v), 0.5%

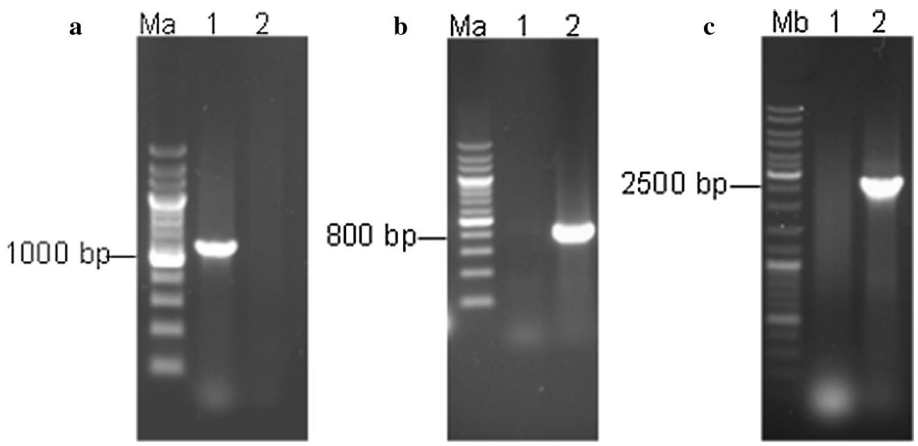

Fig. 2 Identifying *T. orientalis* CF1D transformants. (**a** primers CreA-F and CreA-R test; **b** primers Hph-F and Hph-R test; **c** primers HtrpC-F and CreA-YZRtest; lanes 1 and 2 represent the genomic DNA as the template from the parental strain *T. orientalis* EU7-22 and the *T. orientalis* CF1D transformant, respectively. Ma: 200 bp DNA Ladder Marker; Mb: GeneRuler™ Mix Marker)

peptone, 0.05% $CaCl_2$, 0.05% $MgSO_4$, 0.4% Tween-80, and 0.25% KH_2PO_4.

The repressing medium (RM) was based on the IM supplemented with 3.0% glucose. The submerged fermentation conditions were 30 °C for 4 days with 180 rpm agitation on a rotary shaker.

Enzyme Activities Assay and SDS–PAGE Analysis

Crude enzyme was first centrifuged (6000 rpm for 10 min) to remove the cells and solid material. The enzyme activity of the supernatant was then determined. The filter paper activity (FPA) and endoglucanase activities (CMC) were measured as described by Ghose [12], and a standard curve of D-glucose was used as a reference. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of reducing sugar per minute, and was expressed as U/mL. The cellobiohydrolase activity (CBH) and β-glucosidase activity (BG) were assayed as described by Saha [13], and a standard curve of *p*-nitrophenol (*p*NP) was used as a reference. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol *p*NP per minute in the reaction and expressed as U/mL. Xylanase activity (XYN) was assayed according to the method of Bailey et al. [14] by measuring the total reducing sugars released from 1% (w/v) beechwood xylan (Sigma, St. Louis, USA, X4252) (1.0 mL) in citrate buffer (50 mM, pH 4.8). One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol xylose per minute and was expressed as U/mL.

The protein concentration of crude enzyme was determined with a Bradford protein assay kit (Sangon Biotech Co. Ltd., Shanghai, China). The supernatant was denaturized with boiling water for 10 min and directly analyzed with SDS–PAGE on a 13% polyacrylamide gel stained with Coomassie brilliant blue. The protein marker (SM0431) was purchased from the company Fermentas. Every lane contained an equal volume of the protein samples.

Transcription Analysis

Approximately 200 mg of the fermentation sample was grounded to a fine powder under liquid nitrogen and then transferred to a 50-mL Corning tube on ice. RNA was extracted from the parental strain sample and Δ*creA* strain with Trizol reagent (Takara, Japan). The quality of the extracted total RNA was identified by agarose gel electrophoresis, and the concentration of mRNA was measured by spectroscopy (Nanodrop 2000, Thermo Fisher Scientific). Reverse transcription was carried out using the PrimeScript® RT reagent kit (Takara, Japan). Relative expression levels of cellobiohydrolase-encoding gene *cbh1* (JQ238604) and *cbh2* (JQ238605), endoglucanase-encoding gene *eg1* (JQ238606) and *eg2* (JQ238607), β-glucosidase-encoding gene *bgl1* (JQ904600), xylanase-encoding gene *xyn1* (JQ238610) and *xyn2* (JQ238611) were calculated in comparison with the expression of the 18S rRNA gene by real-time PCR (ABI Step One Plus). The primers are listed in Table 1.

Results

Obtaining the Δ*creA* Mutant Strain

After *A. tumefaciens* AGL1-mediated transformation of pUR5750G/*creA*::*hph* into *T. orientalis* EU7-22, three transformants were selected and verified to retain their mitotic stability. Genomic DNA from the three transformants were tested by PCR analysis. However, only one of the transformants was positive. Firstly, the primers CreA-F&-R were used to detect *creA*, which was present in the parental strain but was absent in the Δ*creA* mutant (Figs. 1b, 3a). At the same time, the primers Hph-F&-R were used to detect *hph*, which was absent in the parental strain but was present in the Δ*creA* mutant (Figs. 1b, 2b). Then, the primers HtrpC-F and CreA-YZR were used to verify that the *hph* cassettes were successfully inserted into the *creA* locus in the Δ*creA* mutant (Figs. 1b, 3c). Therefore, the mutant was named *T.*

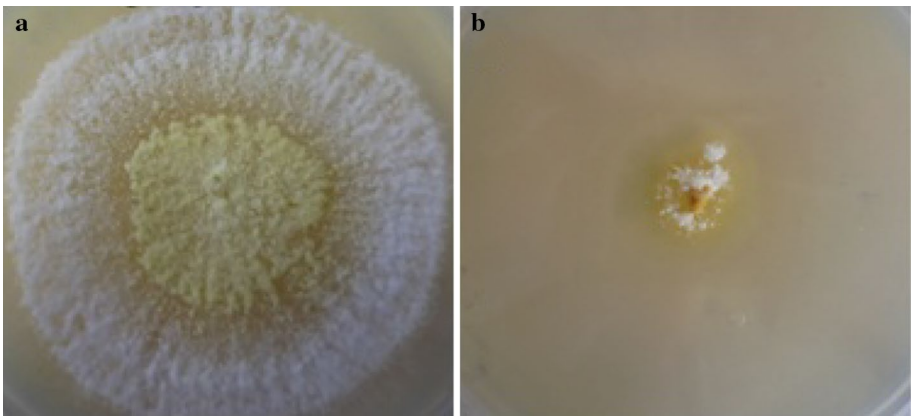

Fig. 3 Phenotype of wild-type and Δ*creA* strains grown on PDA for 3 days (**a** *T. orientalis* EU7-22, **b** *T. orientalis* CF1D)

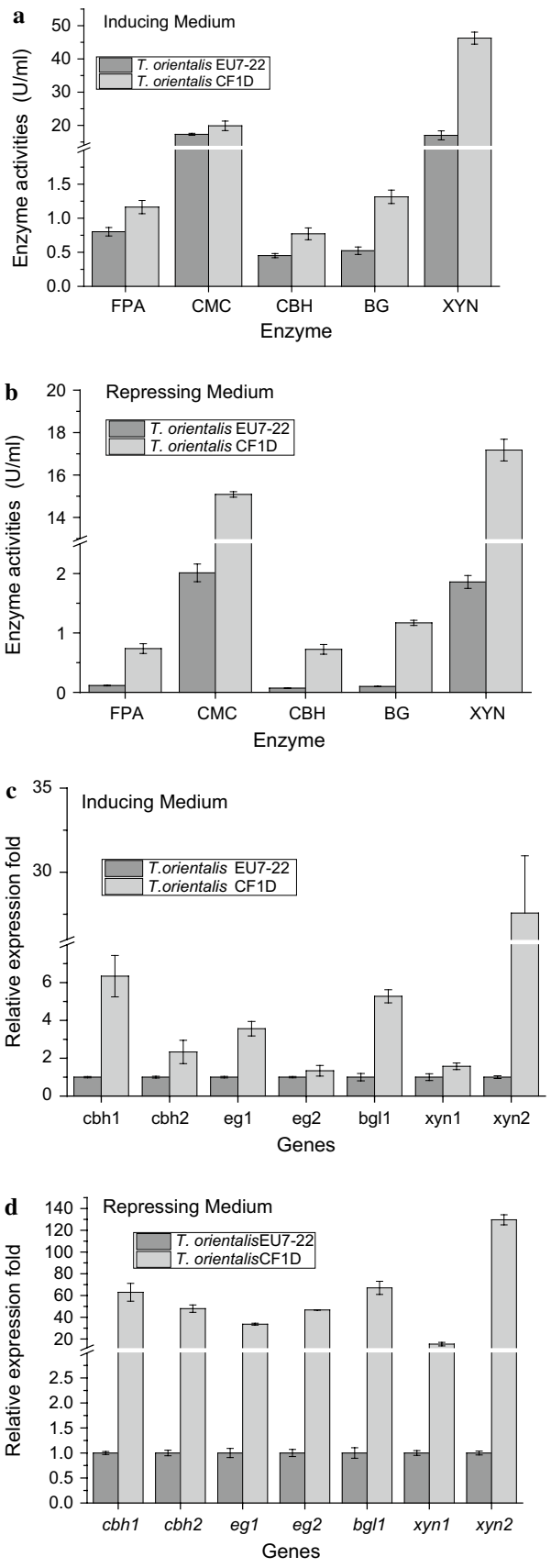

Fig. 4 Relative expression analysis of enzyme activities and genes (**a** enzyme activities for *T. orientalis* EU7-22 and *T. orientalis* CF1D after 4 days of cultivation in inducing medium. **b** Enzyme activities for *T. orientalis* EU7-22 and *T. orientalis* CF1D after 4 days of cultivation in repressing medium. **c** Gene relative expression fold analysis for *T. orientalis* EU7-22 and *T. orientalis* CF1D cultivated in inducing medium. **d** Gene relative expression fold analysis for *T. orientalis* EU7-22 and *T. orientalis* CF1D cultivated in repressing medium. Inducing medium: 2% Avicel with 1% wheat bran. Repressing medium: 2% Avicel with 1% wheat bran and 3% glucose)

orientalis CF1D. The phenotype of the parental strain and *T. orientalis* CF1D were grown on PDA for 3 days and are shown in Fig. 3. The Δ*creA* mutant grew slower and denser than the parental strain.

Deletion of *creA* Increased Cellulolytic Enzyme Production

The parental strain *T. orientalis* EU7-22 and mutant *T. orientalis* CF1D were cultivated for cellulase and xylanase analysis. In *T. orientalis* CF1D, the FPA, CMC, CBH, BG, and XYN activities were 1.45-, 1.15-, 1.71-, 2.51-, and 2.72-fold higher, respectively, than that of the parental stain when cultured in IM for 4 days (Fig. 4a). However, when both strains were cultured in RM for 4 days, the cellulase and hemicellulase activities were higher in *T. orientalis* CF1D (Fig. 4b). The FPA, CMC, CBH, BG, and XYN activities were 6.41-, 7.50-, 10.27-, 11.79-, and 9.25-fold higher, respectively, than that in the parental strain. Furthermore, 98.58% of the glucose was utilized by the parental strain, while only 71.58% glucose was utilized by *T. orientalis* CF1D in the RM.

Compared to the IM, the FPA, CMC, CBH, BG, and XYN activities of the parental strain decreased by 85.67, 88.38, 84.39, 81.04, and 89.07% in the RM, respectively. The FPA, CMC, CBH, BG, and XYN activities of *T. orientalis* CF1D decreased by 36.71, 24.08, 0.06, 0.11, and 62.87% in the RM, respectively. However, the FPA, CMC, CBH, BG, and XYN activities of *T. orientalis* CF1D in RM were higher than that of the parental strain in the IM. With the Avicel inducer in the RM, production of cellulase and hemicellulase was repressed in the parental strain but was derepressed in *T. orientalis* CF1D.

Increased Expression Levels of Cellulolytic Enzymes Correlated with Increased Enzymatic Activity in the Δ*creA* Strain

To determine whether increased cellulolytic enzyme activities in the *T. orientalis* CF1D were due to a higher level of cellulolytic genes, the major genes were analyzed by qRT-PCR. As predicted, the expression levels of *cbh1*, *cbh2*, *eg1*, *eg2*, *bgl1*, *xyn1*, and *xyn2* were significantly higher in the Δ*creA* mutant than in the parental strain not only when

cultured in the IM but also in the RM. The *cbh1*, *cbh2*, *eg1*, *eg2*, *bgl1*, *xyn1*, and *xyn2* genes were 6.34-, 2.33-, 3.56-, 1.33-, 5.27-, 1.57-, and 27.57-fold higher in IM culture, respectively (Fig. 4c),whereas they were 62.98-, 47.94-, 33.64-, 46.71-, 67.04-, 15.30-, and 129.63-fold higher in the RM culture, respectively, than that in the parental stain (Fig. 4d).

Effect of Δ*creA* on Extracellular Protein Secretion

Extracellular proteins secreted into the culture medium were analyzed by SDS–PAGE. When grown in IM, *T. orientalis* CF1D secreted 1.95-fold more extracellular protein than that of the parent strain (Fig. 5a, c), which also contributed to the increase in cellulase and hemicellulase. When grown on RM, *T. orientalis* CF1D secreted 3.77-fold more extracellular protein than that of the parent strain (Fig. 5b, c), which also contributed to the increase in cellulase and hemicellulase. Compared to IM, the protein concentration for the parent strain and *T. orientalis* CF1D showed a 62.67 and 27.94% decrease, respectively. However, *T. orientalis* CF1D secreted 1.41-fold more extracellular protein in RM than that for the parental strain in IM.

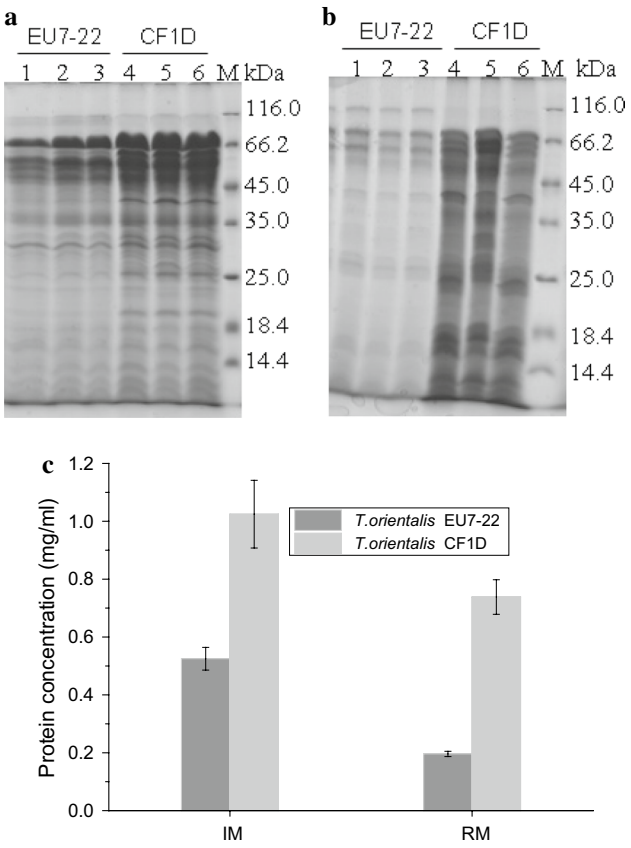

Fig. 5 Secreted protein of *T. orientalis* EU7-22 and *T. orientalis* CF1D after 4 days of cultivation. (**a** *T. orientalis* EU7-22 and CF1D in inducing medium. **b** *T. orientalis* EU7-22 and CF1D in repressing medium. **c** Secreted protein concentration for *T. orientalis* EU7-22 and CF1D in inducing or repressing medium)

Discussion

The effects of *creA* on cellulase and hemicellulase activity as well as the amount of extracellular protein produced in the mutant strain were investigated. Portnoy et al. [5] elucidated the *cre1* regulatory range in the fungus *T. reesei* (anamorph of *Hypocrea jecorina*) by profiling transcription in wild-type and Δ*cre1* mutant strains. Analysis by genome-wide microarrays revealed 2.8% of the transcripts whose expression was regulated in at least by one of the four experimental conditions: 47.3% of which were repressed by *cre1*, whereas 29.0% were actually induced by *cre1*, and 17.2% were only affected by the growth rate but were also *cre1* independent.

It was observed in this study that *creA* deletion has clear effects on the colony morphology of *T. orientalis*. The colonies were smaller and produced fewer aerial hyphae and spores than colonies of the parental strain. The same effect could be detected similar to the phenotypes of *A. niger* [8], *T. reesei* [9], *N. crassa* [15] *creA*/*cre1* mutants because *cre1* could affect the growth rate of fungi [5]. Higher cellulase and xylanase activities (Fig. 4a, b) and amounts of corresponding mRNAs (Fig. 4c, d), which were observed both for the IM and RM, were detected in the strain CF1D-based the Δ*creA* transformant than those in the parental strain. In the RM, this was an expected result, since loss of *creA* released the strain from carbon catabolite repression. The *cre1* directly suppressed *cbh1* transcription by binding to two closely spaced 5′-CCCCAC-3′ motifs in the *cbh1* promoter region in *H. jecorina* [6, 16]. Deletion of *cre1* caused an increase in the *cbh1* transcript levels under repressing conditions [17]. The *cre1* involved indirectly in the control of *cbh2* expression by regulating the main inducer XYR1 [16]. We also observed differences in the transcript levels of genes encoding cellulase and xylanase such as *cbh1*, *cbh2*, *egl1*, *egl2*, *bgl1*, *xyn1*, and *xyn2*. The relative transcript ratio of the *cbh1*, *egl1*, *bgl1*, and *xyn2* genes was significantly increased in the transformant relative to the wild-type strain. Mach-Aigner et al. [16] reported that the xylanase and cellulase activator gene *xyr1* is under the control of the *cre1* repressor. This could provide an explanation for our results since the *creA* gene deletion also had a strong effect in a medium inducing the cellulase and hemicellulase genes.

However, it was also noteworthy that even though the deletion of *creA* led to increased production of proteins both in the IM and RM (Fig. 5). The production levels of cellulase and xylanase in glucose-based RM were still lower than the levels obtained from the CF1D strain in the IM (Figs. 4, 5). The large effect on protein production detected in this

work suggested that *creA* also has a role in modulating the levels of gene expression and then regulating protein secretion [5]. In any case, this result shows that deletion *creA* is a relevant strategy for improving even highly developed industrial strains.

Conclusions

This study focused on the C2H2-type transcription factor *creA* from *T. orientalis* EU7-22 to study cellulase and hemicellulase production. The homologous integration vector pUR5750G/*creA*::*hph* was constructed to knockout *creA* gene. And we obtained the mutant strain *T. orientalis* CF1D, which exhibited higher cellulase and hemicellulase activity with largely extracellular secretion protein not only in cellulose-inducing medium but also in glucose repressing medium. The qRT-PCR showed that the expression levels of *cbh1*, *cbh2*, *eg1*, *eg2*, *bgl1*, *xyn1*, and *xyn2* were significantly higher in the Δ*creA* mutant than in the parent strain. Therefore, deletion of *creA* is a relevant strategy for improving industrial strains.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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