ACE3 as a Master Transcriptional Factor Regulates Cellulase and Xylanase Production in *Trichoderma orientalis* EU7-22

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As an efficient fungus in secreting cellulase, *Trichoderma orientalis* EU7-22 has an important value in the degradation of biomass. Compared with other filamentous fungi, it has an important, unique nature, and it deserves intensive study. Therefore, the function of positive transcriptional regulator ACE3 was investigated for cellulase and hemicellulase production in the strain. As the *ace3* knockout strain, the Δ*ace3* mutant was constructed by homologous recombination, so that the enzyme activities (FPA, CMC, CBH, BG, XYN) and the extracellular protein concentration in the mutant strain decreased to 17.8%, 8.3%, 0.14%, 1.8%, 6.8%, and 19.2% of the parent strain, respectively. The transcription level of cellulase and hemicellulase genes also decreased significantly. The result of SDS-PAGE demonstrated that the Δace3 mutant only clearly showed a protein band, which was characterized by protein profiling with LC-MS/MS and identified as the GH10 family of xylanases. It was proposed that ACE3 is a main up-regulation transcriptional factor of *T. orientalis* EU7-22 and expected to be applicable to further genetic modification.

Keywords: Trichoderma orientalis EU7-22; Transcriptional factor ACE3; Cellulase; Xylanase

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INTRODUCTION

Lignocellulose is the most abundant renewable resource in the world, and microbial hydrolytic enzymes, especially those from different species of fungi, play the main role in the degradation of lignocellulosic biomass. However, currently only limited strains such as *Trichoderma reesei* and *Aspergillus* spp. of ascomycetes have been developed for industrial application (Pariza and Johnson 2001). *Trichoderma orientalis* EU7-22 was obtained by mutation screening. Some articles report that compared with *T. reesei*, *T. koningii*, *A. niger*, and *P. decumbens*, *T. orientalis* EU7-22 shows a higher activity of cellulase (exo-glucanase, endo-glucanase, and β-glucosidase) and xylanase, and the corresponding genes have also been cloned and classified (Long *et al.* 2012, 2013). Compared with other filamentous fungi, it has an important unique nature, and it deserves intensive study (Long *et al.* 2012, 2013; Li *et al.* 2014). This is meaningful for exploiting the mechanism of enzyme production in EU7-22.

Transcription factors play a crucial role in the regulation of cellulase and xylanase gene expression in filamentous fungi. Cre1/A acts as a repressor for mediating carbon catabolite repression (CCR), and is conserved through the fungal kingdom (Todd *et al.* 2014). XlnR in *Aspergillus niger* encodes a binuclear DNA cluster binding protein that promotes the expression of cellulase and xylanase genes (Hasper *et al.* 2004). Its orthologs have also been identified in *Talaromyces cellulolyticus*, *Aspergillus oryzae* (*xlnR*), and *T.*

reesei (*xyr1*) (Stricker *et al.* 2006; Noguchi *et al.* 2011; Fujii *et al.* 2014). Recently, Häkkinen *et al.* (2014) conducted a preliminary study on ACE3 acting as a novel positive regulator in *T. reesei*, but further extending research is necessary. Through phylogenetic analysis (Fig. 1), this transcription factor spreads throughout the whole fungi kingdom, and its function has not been characterized in other species (Benocci *et al.* 2017). In this study, the transcription factor ACE3 was cloned and sequenced in the *Trichoderma orientalis* EU7-22. The cellulase and xylanase activity, transcription level, and protein production in the Δ*ace3* mutant are all investigated to compare with the parent strain, which contributes to identifying the function of ACE3 in this strain, and further demonstrating the role of ACE3 factor during the degradation of cellulase and hemicellulase in filamentous fungi.

EXPERIMENTAL

Strain and Culture Conditions

Trichoderma orientalis EU7-22 was preserved by the authors' laboratory and used as the parent strain in this study. The strain was cultivated on potato dextrose agar (PDA) for 4 days to 6 days, and then the spores were collected and diluted to 1×10^5 spores/mL with sterile water. For DNA extraction and enzyme production, the spores were inoculated in minimum medium (2 g/L glucose, 15 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.6 g/L $CaCl_2$, 0.6 g/L $MgSO_4$, and pH 5.5) for 18 h at 30 ºC with 180 rpm agitation on a rotary shaker, then inoculated as 10% inoculum to inducing medium (1% Avicel®, 1% wheat bran, 0.05% $CaCl_2$, 0.5% peptone, 0.25% KH_2PO_4, 0.05% $MgSO_4$, and 0.4% Tween-80).

Cloning of ACE3, Upstream and Downstream Sequences

According to *ace3* of *Trichoderma virens* (GenBank accession No. XM014102116.1), *T. reesei* (GenBank accession No. CP016233.1), and *Purpureocillium lilacinum* (GenBank accession No. XM018319611.1), degenerate primers ACE3-F1 and ACE3-R2 were designed in the conserved sequence and used for amplifying the homologous *ace3* gene of *T. orientalis* EU7-22. The sequences were procured from the NCBI database (https://www.ncbi.nlm.nih.gov/genbank/). After purification, the product was linked to the vector of pMD 19-T. *Escherichia coli* DH5α was used as the host of vector propagation. The gene was sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). In the same way, the primers (listed in the supplementary material) used for amplifying the upstream and downstream of *ace3* were designed according to conserved sequences. After a total of three times of PCR using EU7-22 genome DNA as the template, the products were sequenced and then spliced by DNAMAN. Finally, the authors got the *ace3* gene, including the flanking region. The intron was analyzed by DNAMAN, and the exon was translated into amino acid sequences using Primer Premier 5.0. A phylogenetic tree was constructed based on MEGA 6.06 software by neighbor-joining (NJ) method. BlASTP in NCBI was used to analyze the amino acid sequences and orthologs found in different species of filamentous fungi family.

Construction of the Δ*ACE3* Mutant

The *ace3* gene was knocked out by homologous recombination, and the selective marker *hph* was used for isolating the transformants. As the sketch map showed in Fig. 2a, the deletion cassette Δ*ace3::hph* was constructed using primers P2 and P5 by fusion PCR with three fragments (5' ace3 flanking region, the hygromycin B resistance gene hph

expression cassette, and 3' ace3 flanking region) (Szewczyk *et al*. 2006). The Δ*ace3::hph* cassette was amplified and transformed into the wild-type protoplasts to obtain the Δ*ace3* mutant. The transformants were isolated using minimum medium plates supplemented with 1% hygromycin B. The primers used for the construction were listed in the Table 1.

Enzymatic and Protein Concentration Assays

For the enzyme assays, the spores were incubated the same way as in the previous research (Long *et al.* 2018). Filter paper activity (FPA) and endoglucanase activity (CMC) were measured using the reported method (Ghose 1987). pNPC and pNPG (SIGMA, St. Louis, MO, USA) were used as the substrates to determine cellobiohydrolase activity (CBH) and β-glucosidase activity (BG), respectively (Saha 2004). Xylanase activity (XYN) was examined with the substrate of xylan (extracted from sugarcane fibre) (Bailey and Jeger 1992). The protein concentration was measured by the Bradford protein assay kit (Sangon, China). Under the assayed conditions, the amount of enzyme required to release 1 μmol of product (glucose/xylose/p-nitrophenyl) from the substrate per min was defined as one unit of enzyme activity.

Phenotypic Analysis

The spores were diluted to 1×10^7 spores/mL and spotted on the plates, and then were cultivated at 30 °C for 4 days. The colony diameter was measured every 12 h.

RNA Preparation and Quantitative Real-Time Reverse Transcription PCR

Based on the above results, a quantitative PCR was carried out for the analysis of gene transcription (*cbh1*, *cbh2*, *eg1*, *eg2*, *bgl1*, *xyn1*, and *xyn2*) (Long *et al.* 2012). The protocol for RNA extraction and RT-qPCR was shown in the previous research (Long *et al.* 2018). The transcription levels of target genes of *T. orientalis* and Δ*ace3* mutant were normalized against that of the 18s gene.

SDS-PAGE and LC-MS/MS Analysis

The supernatant was analyzed with SDS-PAGE on 15% polyacrylamide by the method of Laemmli (1970) and stained with Coomassie Brilliant Blue R-250. The mixture of 15 μL culture supernatant and 3 μL 5× loading buffer was placed in boiling water for 10 min and was loaded into the gel. The protein band from the Δ*ace3* mutant was excised from SDS-PAGE gels for in-gel trypsin digestion. The tryptic peptides were extracted with aqueous solution containing 67% acetonitrile/0.15% formic acid and subjected to freeze-drying. The dried peptides were dissolved in aqueous solution containing 2% acetonitrile/ 0.1% formic acid, and then separated by a fused silica capillary emitter packed with C18 resin and analyzed in an AB SCIEX TripleTOF 5600 system (Applied Biosystems, Foster City, CA, USA). Proteins were identified by the Swiss-Prot database using the Mascot program.

RESULTS AND DISCUSSION

Sequence Analysis

The sequence of *ace3* was a fragment of 6241 bp, including a putative gene coding region of 2024 bp with one intron (155 bp), encoding 628 amino acids.

According to the alignment result of protein sequence from DNAMAN, the putative ACE3 in *T. orientalis* showed 95.55% of identity with ACE3 protein in *T. reesei* (XP006964947.1), and it belonged to the Zn_2Cys_6 class. The nucleotides and amino acids of *T. orientalis* EU7-22 were deposited in the GenBank with the accession No. MG720022. Through phylogenetic analysis (Fig. 1), *ace3* was widely spread in filamentous fungi. Based on the conserved sequences of *ace3* gene in filamentous fungi, the primers were successfully applied to clone and sequence *ace3* gene, including flanking sequences in *T. orientalis* EU7-22.

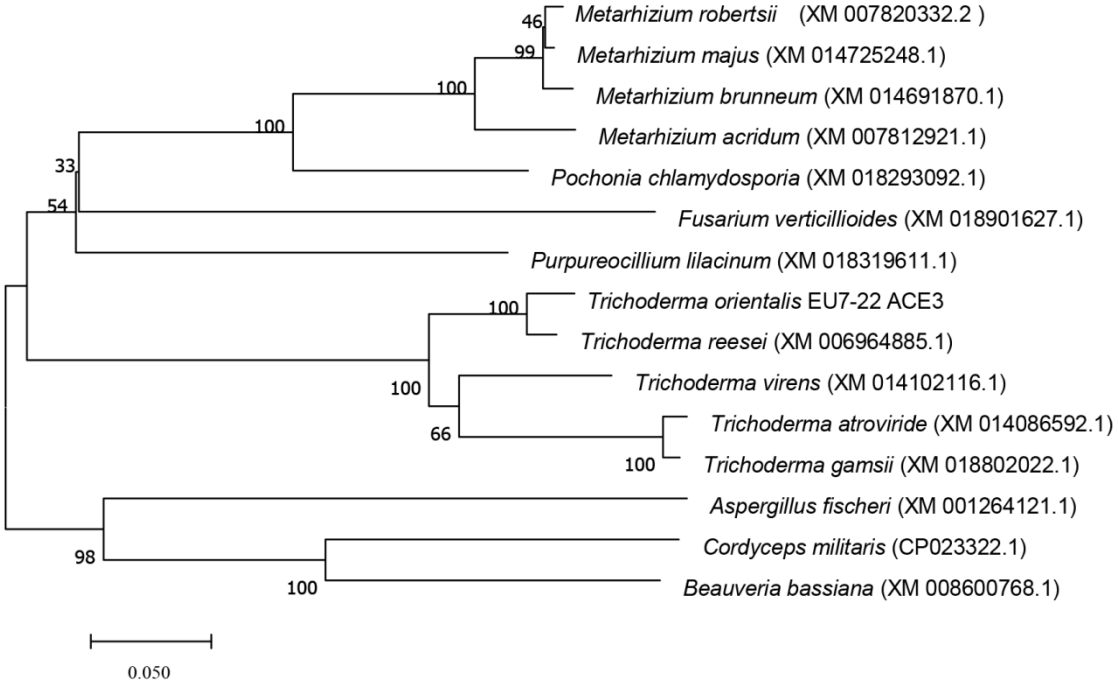

Fig. 1. Phylogenetic tree analysis of *ace3* gene homological degrees in filamentous fungi

Obtaining the Mutant

The Δ*ace3*::*hph* cassette was amplified and transformed into the protoplasts of EU7-22 to obtain the mutant. The transformants were isolated using minimum medium plates supplemented with 1% hygromycin B. After streak cultivation for three generations, only one transformant was obtained. From the PCR analysis with primers hph-F and hph-R, it was confirmed that the deletion cassette Δ*ace3*::*hph* was translated to the mutant (Fig. 2b).

Primers Cace3u-F and Cace3d-R were used for the analysis of *ace3* gene, and they showed that the target gene did not exist in the mutant, but it was positive in the parent strain (Fig. 2c). Primers P1 and PgpdA-yz, as well as P6 and TrpC-yz, were used to confirm the homologous recombination location of transformant by PCR. As shown in Fig. 2d and 2e, those two fragments were not present in the parent strain, but were present in the mutant. From the above experiments, the authors got the *ace3* deletion strain, named Δ*ace3* mutant. The primers used for identifying the Δ*ace3* mutant are listed in Table 1.

Table 1. Primers in this Study for PCR

Primer	Sequence（5'→3'）	Application
Cace3u-F	ATGCTGCGCTACTCCCCCGTCTTAC	Clone *ace3* gene
Cace3d-R	TTAGCCAACAACGGTATTAGACGTA	
Ace3-F1	TYTGCGCAGAGGCAGCATTYATG	Clone *ace3* gene and *ace3* flanking region
Ace3-R2	AYGGGTCTGCCGAGTCCAAGCAGT	
ADu–F	ATGTGACACGACAYATGCAGCTTCACC	
ADu -R	GTGGCAGGTCGAAAGTGCACTGCA	
ADd–F	CCTGTTTGTTGTCTGGATGCTGCAGG	
ADd –R	GGCATCTCKCTCAAYGGYGACTACT	
P1	CGACGATTATGTGACACGACACTA	Using for fusion PCR and identify the Δ*ace3* mutant
P2	CCGTCTGAGGTCGCATTCT	
P3	CGAAAGATCCCTGCAGGAAAAGGGCTGGGAGAAGATGTATG	
P4	GGTAATCCTTCTTAAGCTTGGGCGATGTAAATATGGCAAGGATGT	
P5	AGCGACGGATGGAGTTTCAC	
P6	GCATCTCGCTCAATGGTGACTA	
Hph-F	CGACAGCGTCTCCGACCTGA	
Hph-R	CGCCCAAGCTGCATCATCGAA	
PgpdA-yz	TTCCTGCAGGGATCTTTCGAC	
TrpC-yz	GATTACCGACTTCACAACTCTAGATAT	

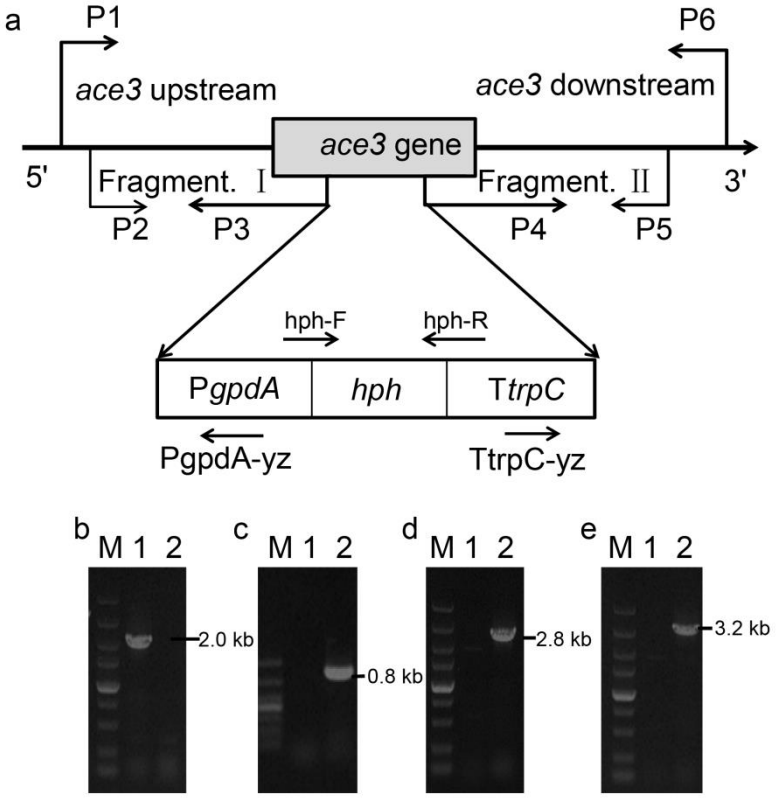

Fig. 2. Schematic map of constructing Δ*ace3::hph* deletion cassette and identifying the Δ*ace3* mutant. (M) maker; (1) the parent strain EU7-22; (2) the Δ*ace3* mutant; (a) schematic map of the primers used for constructing and identifying Δ*ace3* mutant; (b) PCR analysis of genomic DNA, EU7-22 and Δ*ace3*. Primers hph-F and hph-R test for *hph* gene; (c) primers Cace3u-F and Cace3u-R test for *ace3* gene; (d) primers P1 and PgpdA-yz test for homologous recombination location; (e) primers TrpC-yz and P6 test for homologous recombination location

Enzyme Analysis

The parent strain *T. orientalis* EU7-22 and the Δ*ace3* mutant were cultivated for enzyme activity analysis. According to Fig. 3, when *ace3* gene was disrupted, the activities of cellulase and xylanase were almost lost. Comparing with EU7-22, the FPA, CMC, and XYN activities of Δ*ace3* mutant were reduced to 17.8%, 8.3%, and 6.8%, respectively. Regarding the lack of ACE3, the CBH and BG activities were completely lost. Meanwhile, the intracellular protein concentrations were reduced to 19.2%. As shown in Fig. 3, the CMC and XYN activities still existed in the Δ*ace3* mutant, but the CMC and XYN activities were kept at a relatively low level. It was observed that ACE3 deletion seriously repressed cellulase and xylanase activities in *Trichoderma orientalis* EU7-22, and the extracellular protein was also produced at a low level.

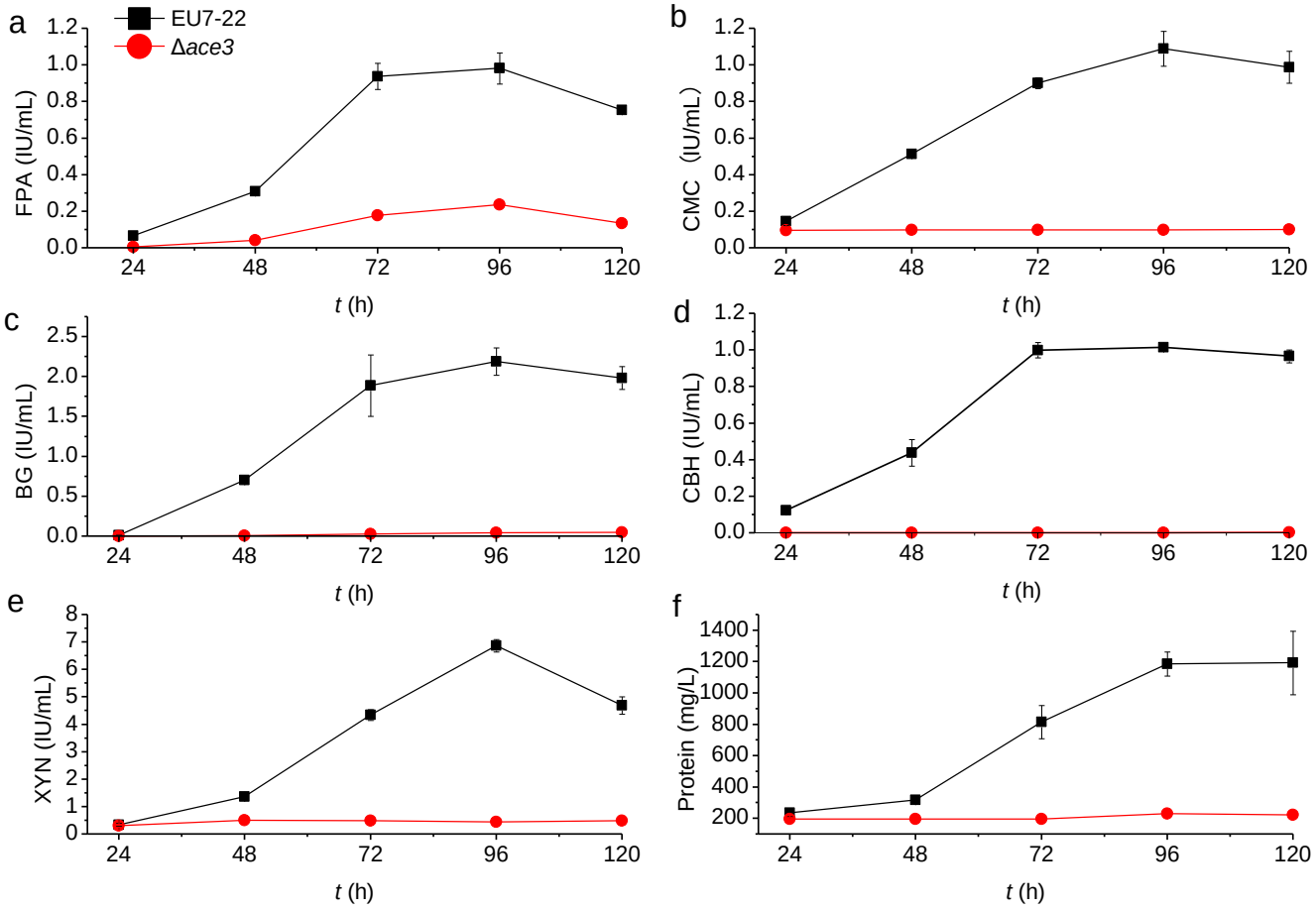

Fig. 3. Production of cellulase, xylanase activity, and protein secretion by the Δ*ace3* mutant; (a) filter paper activity; (b) endoglucanase activity; (c) β-glucosidase activity; (d) exoglucanase activity; (e) xylanase activity; (f) protein concentration

Quantitative PCR

Based on the above results, a quantitative PCR was carried out for the analysis of cellulase and xylanase gene transcription (*cbh1*, *cbh2*, *eg1*, *eg2*, *bgl1*, *xyn1*, and *xyn2*). As shown in Fig. 4, after cultivating the Δ*ace3* mutant for 48 h, the expression of cellobiohydrolase gene *cbh1*, cellobiohydrolase gene *cbh2*, endoglucanase gene *eg1*, and endoglucanase gene *eg2* were all almost undetectable, and the expressions of β-glucosidase gene *bgl1* and xylanase genes *xyn1* and *xyn2* were reduced to 0.06-fold, 0.02-fold, and

0.15-fold of the parent strain, respectively (Fig. 4a). In 72 h, similar results were also observed in that the transcriptions of *cbh1*, *cbh2*, *eg1*, and *eg2* were still kept at a very low level, and those of *bgl1*, *xyn1*, and *xyn2* were reduced to 0.17-fold, 0.05-fold, and 0.25-fold of the parent strain, respectively (Fig. 4b). Compared with those of 48 h, the expression levels of *bgl1*, *xyn1*, and *xyn2* increased insignificantly. Eventually, the results showed that the cellulase and xylanase activities decreased rapidly as the *ace3* gene was disrupted in EU7-22, and ACE3 regulated the transcription of cellulase and xylanase genes in *T. orientalis* EU7-22 as a major transcription factor.

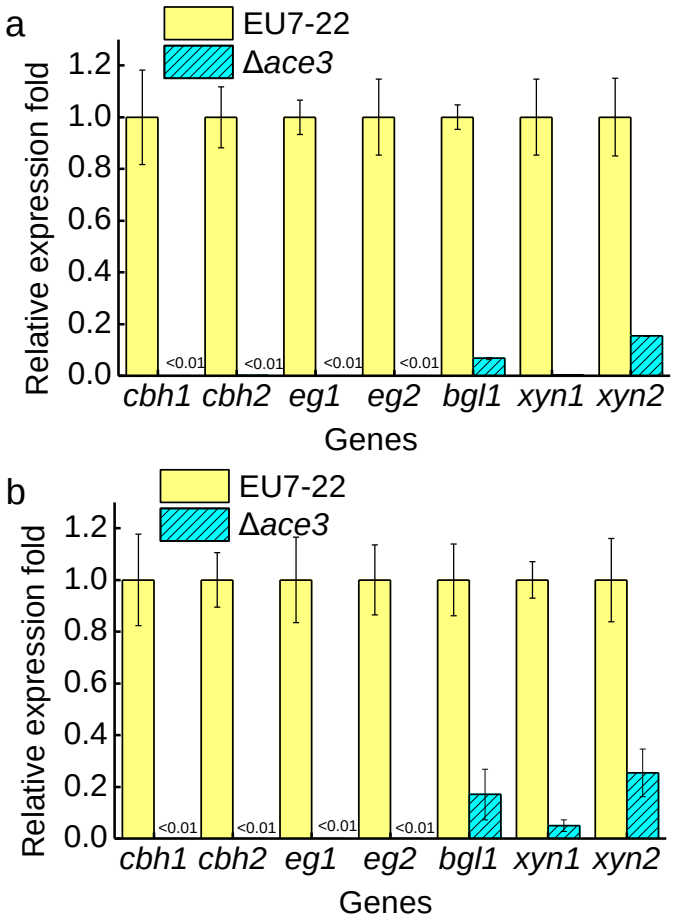

Fig. 4. Analysis of the cellulase and xylanase gene expression of EU7-22 and Δ*ace3* mutant by quantitative PCR; (a) cultivated for 48 h; (b) cultivated for 72 h

pH Value of the Supernatant

In the cultivation, pH value of supernatant from the parent strain decreased to a minimum in 24 h, but the pH value of the mutant strain showed higher pH value and increased over time. This was attributed to the disruption of ACE3, which caused the decrease of organic acid content. The color of the supernatant also became lighter after knockout (Fig. 6). The authors speculated that the transcription factor ACE3 through mediating the ambient pH, and then affected the efficiency of permeases and extracellular enzymes through mediating the ambient pH, which provides the potential application of gene regulation by acidic intermediate (Peñalva and Arst Jr. 2004).

Table 2. Primers Used for Quantitative Real Time RT-PCR

Primer	Sequence（5'→3'）
18S-YGF	AGGCGCGCAAATTACCCAATCC
18S-YGR	GCCCTCCAATTGTTCCTCGTTAAG
cbh I-YGF	ATCGGCTTCGTCACGCAATC
cbh I-YGR	GACAAACCTCGGCACTCC
cbh II-YGF	GACAAACCTCGGCACTCC
cbh II-YGR	GACCAGCGTCCAGATACATT
eg I-YGF	CAGGGCTTCTGCTGTAATGAG
eg I-YGR	TTGAACTGGGTGATGATGGTG
eg II-YGF	GCTCCGCCAGAATAACCG
eg II-YGR	CAGCCAACATAGCCAAGATAGAC
bgl 1-YGF	ATCACCTACCCGCCTTCA
bgl 1-YGR	TCTCGTCGTCGGATGTTG
xyn I-YGF	CGTCAACACGGCGAACCA
xyn I-YGR	CGGTGATGGAAGCAGAGCC
xyn II-YGF	TTTGTCGTGGGAGTTGGCTG
xyn II-YGR	TGTGCTGGGTAGTTGTGGTTG

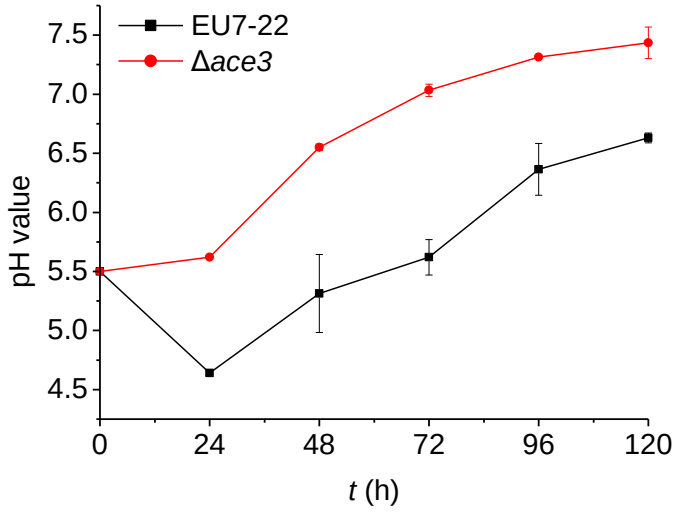

Fig. 5. pH value of *T. orientalis* EU7-22 and the Δ*ace3* mutant

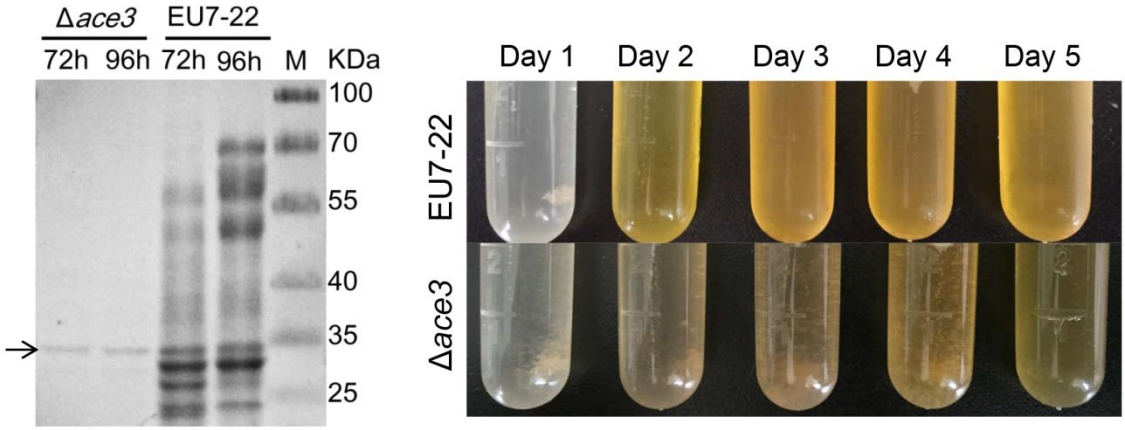

Fig. 6. SDS-PAGE analysis and phenotype observations of the supernatants of EU7-22 and the Δ*ace3* mutant

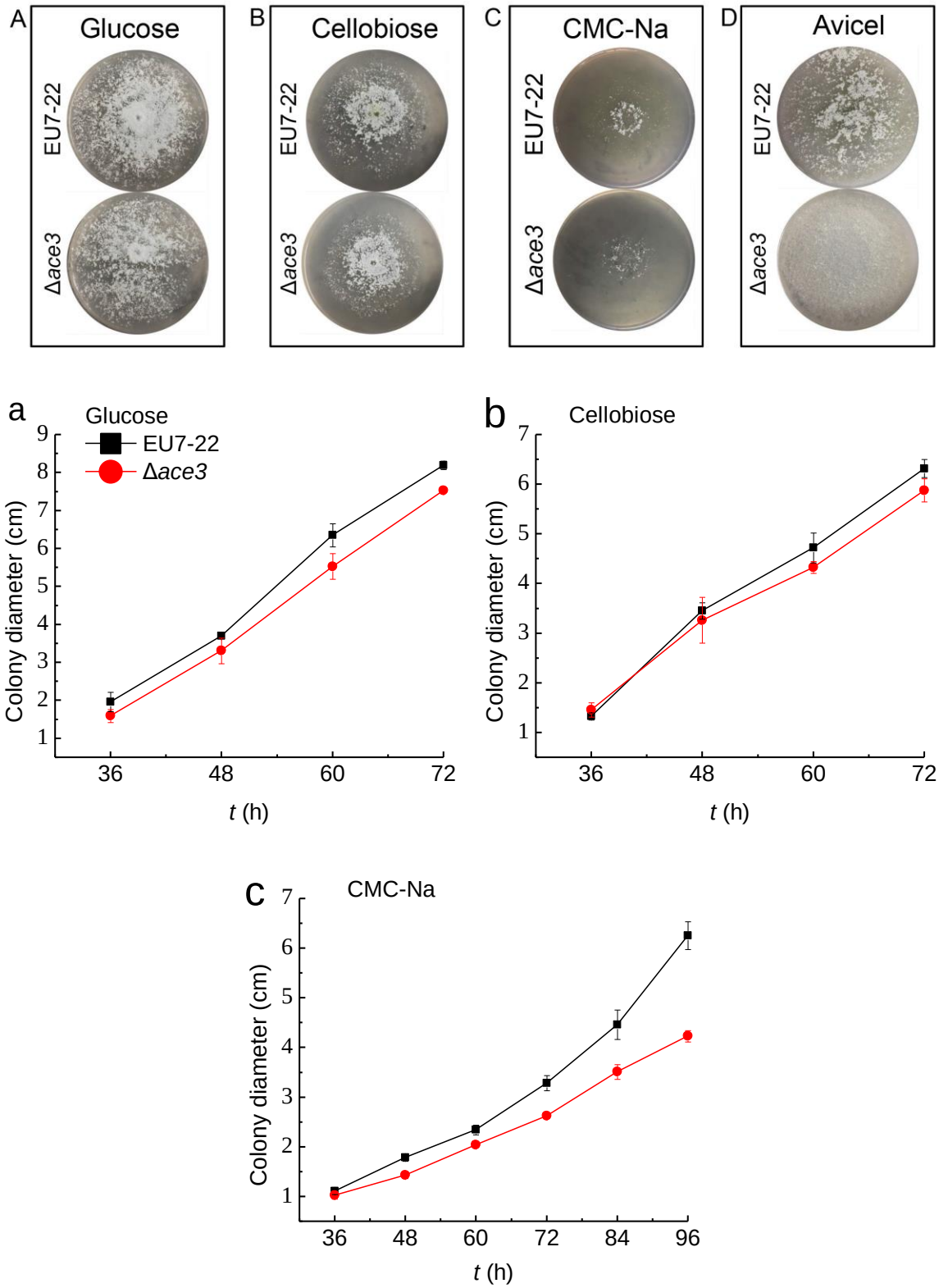

Fig. 7. Phenotype observations and colony size measurements of EU7-22 and the Δ*ace3* mutant on the different carbon plates. (A and a) 2% glucose plate; (B and b) 1% cellobiose plate; (C and c) 1% CMC-Na plate, (d) 1% Avicel® plate

SDS-PAGE and LC-MS/MS Analysis

Compared to the multiple kinds of protein in the parent strain, only one obvious protein band was detected in the SDS-PAGE of Δ*ace3* mutant (Fig. 6). The protein was identified as part of the xylanase GH10 family (Accession No. G0RA32), but the xylanase activity was still at a very low level compared with the parent strain. The lack of ACE3 seriously influenced the secretion of proteins.

Growth Analysis of the Δ*ace3* mutant

Cultivated in the plates using sodium carboxymethylcellulose (CMC-Na) as the sole carbon source, the phenotype and colony diameter of the Δ*ace3* mutant significantly declined compared with the parent strain (Fig. 7C). When the carbon source was Avicel®, the Δ*ace3* mutant produced very few spores (Fig. 7D), but a non-significant difference was found in those of glucose and cellobiose (Figs. 7A and 7B). As shown in Fig. 7c, with CMC-Na as the sole carbon resource, the colonies of Δ*ace3* mutant were smaller than those of the parent strain. The deletion of *ace3* clearly affected the colony diameter and the morphology of *T. orientalis*. The colonies were smaller, and they produced fewer spores and aerial hyphae than those of the parental strain in both Avicel®, and CMC-Na plates.

CONCLUSIONS

1. The function of transcriptional regulator ACE3 in *Trichoderma orientalis* EU7-22 was identified by means of sequence analysis, enzyme analysis, phenotypic analysis, protein analysis, and quantitative PCR. ACE3 deletion seriously repressed cellulase and xylanase activities in *Trichoderma orientalis*, and extracellular protein was also produced at a low level. Compared with parental strains, the transcription level of the cellulase and xylanase genes (*cbh1*, *cbh2*, *eg1*, *eg2*, *bgl1*, *xyn1*, and *xyn2*) in the Δ*ace3* mutant also reduced dramatically. Meanwhile, the change was related to the ambient pH of Δ*ace3* mutant.
2. SDS-PAGE coupled with LC-MS/MS was a powerful tool that was able to identify the difference in protein secretion. The only protein band in the Δ*ace3* mutant and the same protein band in EU7-22 were characterized as part of the GH10 family of xylanases, while the xylanase activity of the Δ*ace3* mutant was still at a low level.
3. The ACE3 expression influenced the enzymatic degradation of CMC-Na and microcrystalline cellulose. Since only a few such kinds of transcription factors exist extensively, and the majority of them are limited in the subgroups of fungal species, the function investigation of ACE3 provided the reference of regulating gene expression in other fungi.

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