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Improving Cellulase Production in *Trichoderma koningii* Through RNA Interference on *ace1* Gene Expression

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Ribonucleic acid interference (RNAi) inhibits the expression of target genes in a sequence-specific manner, and shows potential for gene knockdown in filamentous fungi, in which the locus-specific gene knockout occurs in low frequency. In this study, the function of the repressor of cellulase expression I (ACEI) was verified in *Trichoderma koningii* (*T. koningii*) YC01 through RNAi, and *ace1*-silenced strains with improved cellulase productivity were obtained. An expression cassette that transcribed the interfering double-stranded RNA (dsRNA) of *ace1* was constructed and transformed into *T. koningii*, and the transformants, in which the expression of *ace1* was successfully silenced, were selected. As a result of the *ace1* gene silencing, the expression levels of the main cellulase and xylanase genes were elevated, and the enhanced production of total proteins, cellulase, and xylanase was observed in the cultivation. In addition, the down-regulation of *ace1* resulted in an increasing expression of *xyr1,* but no clear variation in the expression of *cre1*, which suggested that ACEI acted as a repressor of the *xyr1* transcription, but was not involved in the regulation of the *cre1* expression. The results of this work indicate that *ace1* is a valid target gene for enhancing enzyme production in *T. koningii*, and RNAi is an appropriate tool for improving the properties of industrial fungi.

Keywords: *Trichoderma koningii*, RNA interference, cellulase, ACEI

RNA interference (RNAi), which is triggered by endogenous or exogenous double-stranded RNAs (dsRNAs), diminishes or abolishes gene expression through degradation of specific messenger RNAs (mRNAs) [8, 38]. RNAi has been proven to be effective in silencing single genes in various fungi, with synthetic exogenous dsRNAs, or endogenously transcribed dsRNAs [14, 23, 37], and has been also utilized for simultaneous silencing of homologous genes or even heterologous genes by targeting a conserved sequence of a gene family or constructing a chimeric sequence derived from different genes [6, 9, 24]. The increasing application of RNAi for manipulation of fungal gene expression is partly driven by the relatively low efficiency of homologous recombination in some fungal systems [22], which is a major drawback of the conventional gene disruption method. When RNAi was used in *Mortierella alpina*, an oleaginous fungus used commercially for arachidonic acid production, the types and relative amounts of fatty acids were altered without a dependency on mutagenesis or other permanent changes in the genetic background of the producing strains [36]. Recently, Qin *et al*. [27] demonstrated that RNAi-mediated gene silencing could effectively suppress *T. reesei cbh1* gene expression, and the reduction of CBHI could result in obvious improvement of heterologous lipase production in *T. reesei*.

Several fungal species of the genus *Trichoderma* have been extensively studied because they produce large amounts of extracellular hydrolytic enzymes, and *T. reesei* is one of the most important fungi for cellulase production [12, 25]. The major cellulase and hemicellulase genes in *T. reesei* are regulated in a coordinate manner by the carbon source available [3, 11]. Cellulose and other plant materials, and other substances (*e.g*., lactose) induce the expression of the cellulase and hemicellulase genes, whereas glucose acts as a repressing carbon source. Several genes coding for regulators of cellulase and hemicellulase expression have been isolated. These include the carbon catabolite repressor CREI [33], the repressor ACEI [29], the activator ACEII [4], the CCAAT binding complex Hap2/3/5 [3, 18, 30], and the activator XYRI [34]. ACEI contains three Cys_2His_2-type zinc fingers and was shown to bind *in vitro* to eight sites containing the core sequence 5'-AGGCA scattered along the 1.15 kb *cbh1* promoter [29]. The

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repressive role of ACEI in *T. reesei* has been demonstrated. The deletion of *ace1* results in an increase in the expression of all the main cellulase and hemicellulase genes in sophorose- and cellulose-induced cultures [2]. ACEI also represses *xyr1* expression during growth on D-xylose [17]. It is currently believed that a dimer of XYRI is the basis for the induction-specific complex for *xyn1* expression, and ACEI is a competitor of XYRI for one of the two binding elements in the *xyn1* promoter [28]. The functional ACEI repressor complex consists of a single ACEI, XYRI, and ACEI-interacting protein [35].

The objective of this work was to validate the role of ACEI in *T. koningii* YC01, a cellulase-hyperproducing strain with genetic similarity to *T. reesei*, through RNAi, and address whether enzyme production could be further improved by silencing the *ace1* gene. We have constructed *ace1*-silenced *T. koningii* recombinant strains, and analyzed the effect of *ace1* silencing on the expression of the main cellulase and xylanase genes under inducing conditions. The results indicate that ACEI indeed acts as a repressor for cellulase and xylanase expression in *T. koningii*, and silencing of *ace1* effectively improves cellulase and xylanase production in *T. koningii*.

Materials and Methods

Strains, Plasmids, and Culture Conditions

Escherichia coli Top10F' (Invitrogen, USA) was used for plasmid construction and maintenance. The *E. coli* strain was cultivated in LB medium, in which ampicillin (100 μg/ml; Invitrogen) was supplemented when necessary. The genomic DNA of *T. reesei* QM9414 (ATCC 26921) was used as the template for polymerase chain reaction (PCR) amplification of intron 2 of the *eg2* gene, which was used for construction of the RNAi vector. The *T. reesei* strain was maintained on potato dextrose agar (PDA), and for liquid cultivation, grown in Mandel's medium that contained 2% glucose [19]. *T. koningii* YC01 is a cellulase-hyperproducing mutant obtained through fast neutron mutation in this laboratory. It produced cellulase with filter paper activity of 40.4 FPU per gram dried substrate in a solid-state cultivation process with corncob powder and wheat bran as the main substrate (unpublished data). For conidia formation, it was cultivated on corn meal agar (CMA) plates at 28°C for 5 days. For enzyme production, a total of 1×10^7 *T. koningii* spores, collected from cultures grown on CMA plates, was inoculated into 30 ml of the basal medium supplemented with 5% corncob powder and 3% bran mixture in a 250 ml conical flask and grown at 28°C with shaking at 200 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-80. For the selection of *T. koningii* transformants, PDA medium supplemented with 20 μg/ml of hygromycin B was used. Plasmid pGPD, a pUC19-derived plasmid that contains the promoter of the *T. reesei gpd* gene (encoding glyceraldehyde-3-phosphate dehydrogenase; GenBank ID: EF043568) and the *cbh1* gene terminator, was previously constructed in this laboratory. Plasmid pAN7.1, a commonly used plasmid for fungus transformation, was applied in the co-transformation of *T. koningii* YC01.

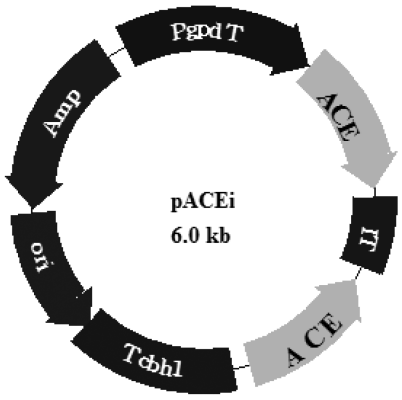


Fig. 1. Schematic diagram of the structure of the RNAi vector for *ace1* gene silencing (pACEi).
PgpdT: the *T. reesei gpd* promoter; Tcbh1: the *T. reesei cbh1* terminator; IT: intron 2 of the *T. reesei eg2* gene; ACE (clockwise): 425 bp coding sequences of *ace1*; ACE (anti-clockwise): antisense strand of the 425 bp coding sequences of *ace1*.

Construction of the RNAi Vector

The RNAi vector for silencing the *T. koningii ace1* gene mainly contained an expression cassette for an intron-harboring hairpin RNA (ihpRNA) (Fig. 1). Two 425 bp *ace1* fragments, $ace1_{sense}$ and $ace1_{anti}$, were amplified from the *T. koningii* genomic DNA by using primer pairs Acesf/Acesr and Aceaf/Acear (Table 1), respectively. Next, a 209 bp spacer fragment that included intron 2 of the *T. reesei eg2* gene was amplified from the *T. reesei* genomic DNA by using primers Aitf and Aitr. Then, fragments $ace1_{sense}$ and a spacer were connected by overlap extension PCR by using primers Acesf and Aitr, which contained overlapping sequences as indicated in Table 1. The above overlap-connected fragment and $ace1_{anti}$ were inserted into the *Bam*HI/*Xba*I and *Xba*I/*Hin*dIII sites of the pGPD. The resulting RNAi vector was designated as pACEi.

Fungal Transformations and Analysis of the Transformants

The protoplast transformation of *T. koningii* was performed as previously described [26] with the following modifications. Ten milligrams per milliliter lysing enzymes from *T. harzianum* (Sigma-Aldrich, Brondby, Denmark, Cat. No. L1412) in 1 mol/l $MgSO_4$ was used for protoplast formation. For transformation, 10 μl (20 μg, including 10 μg pACEi and 10 μg pAN7.1) of plasmid DNA and 200 μl of PEG buffer (60% PEG 4000; 50 mmol/l $CaCl_2$; 10 mmol/l Tris-HCl, pH 7.5) were used. Plasmid pAN7.1 was used as an assisting plasmid to render hygromycin B resistance to the transformants. The transformants were selected on PDA plates that contained 20 μg/ml hygromycin B. The conidia of the candidate transformants were further spread onto hygromycin B selection plates to form single colonies. The single colonies were screened through a genomic DNA PCR analysis to verify that the ihpRNA expression cassette was integrated into the genome of the transformants. The chromosomal site for integration of the RNAi acting sequence would be 1,179–1,604 bp of the *ace1* open reading frame.

Table 1. Sequences of oligonuleotides used in this work for the construction of the RNAi vector.

Primers	Sequences (5'–3')[a]	Specificity[b]
Acesf	ACGCGGATCCTATGGCTACTGGAAAGATTGTCG	*ace1* (positions 1179 to 1201), includes *Bam*HI site
Acesr	CTCACTCTGTGGTACAGCCAACATAGGTCCAGCCGTGAGC	*ace1* (positions 1585 to 1603)
Aitf	GCTCACGGCTGGACCTATGTTGGCTGTACCACAGAGTGAG	*egl2* intron2
Aitr	CTAGTCTAGATAACGCAAGTGCCACTGTAAC	*egl2* intron2 + *Xba*I
Aceaf	CTAGTCTAGACATAGGTCCAGCCGTGAGC	*ace1* (positions 1585 to 1603), includes *Xba*I site
Acear	ACCCAAGCTTTATGGCTACTGGAAAGATTGTCG	*ace1* (positions 1179 to 1201), includes *Hin*dIII site

[a]Restriction sites are underlined; overlapping regions in Acesr and Aitf are double underlined.
[b]The nucleotide positions are the positions in the *T. koningii ace1* gene open reading frame.

Nucleic Acid Isolation

Mycelia in a liquid culture were harvested by centrifugation, frozen in liquid nitrogen, and stored at −86°C. The genomic DNA was extracted as previously described [31]. The total RNA was isolated from the frozen mycelia by using a Universal Plant Total RNA Extraction Kit (BioTeke, Beijing, China) with the protocol specified by the manufacturer. To remove the genomic DNA, RNA preparations were treated with DNase I (Fermentas, Vilnius, Lithuania). The quantity and quality of the extracted RNA were assessed on a GeneQuant 1300 spectrophotometer (Biochrom Ltd., England) and by agrose gel electrophoresis.

Reverse Transcription and Quantitative PCR

About 500 ng of the total RNA were subjected to reverse transcription by using an RT-PCR kit (TaKaRa, Dalian, China), which contained a blend of oligo(dT) and random hexamer primers. The quantitative PCRs (qPCRs) were performed in an ABI Prism 7300 System (Applied Biosystems, CA, USA). Each reaction mixture contained 2 μl of the template [1:60 dilution of the reverse transcriptase (RT) reaction product], 10 μl of SYBR Premix Ex Taq 2× (TaKaRa), 300 nmol/l forward and reverse primers (Table 2), and nuclease-free water for a final volume of 20 μl. The PCR protocol consisted of 30 s of initial denaturation at 95°C, followed by 40 cycles of 5 s at 95°C and 31 s at 60°C. A melting curve was performed after each run to check the PCR product specificity. Primers are designed so that the size of the amplicon is 100~200 bp, and complementary sequences within primers and mismatches are avoided. The primer are also designed so that half hybridizes to the 3' end of one exon and the other half to the 5' end of the adjacent exon. Alternatively, primers are designed to flank a region that contains at least one intron.

Table 2. qPCR oligonuleotides used in this work.

Primers	Sequences (5'–3')
Sar1-F	TGGATCGTCAACTGGTTCTACGA
Sar1-R	GCATGTGTAGCAACGTGGTCTTT
Ace1f	TGCGAGACCTTGAGAAGACCCT
Ace1r	ATCGGACAGACGTGAGGCAGAA
Xyr1f	CCCATTCGGCGGAGGATCAG
Xyr1r	CGAATTCTATACAATGGGCACATGGG
Cre1f	CACAAGCGACCGAGACTGCC
Cre1r	GCCCGTATGGGTGCGAATG
Cbh1f	CTTGGCAACGAGTTCTCTT
Cbh1r	TGTTGGTGGGATACTTGCT
Cbh2f	GCTGGATTCGTTTGTCTGGGTC
Cbh2r	CAGTGGGAGTCAAATCGTGGC
Egl1f	CGGCATGGTCCTCATCTTCA
Egl1f	CGGCATGGTCCTCATCTTCA
Egl2f	CAGCTACAGCGGAGATGTCAAG
Egl2r	TGAAGGGCTCAGTGCCAAAC
Xyn2f	CCGTCAACTGGTCCAACT
Xyn2r	ACACGGAGAGGTAGCTGTT

All PCRs were carried out in triplicate within a plate. The data obtained by using an ABI Prism 7300 Sequence Detection System were analyzed according to the "standard curve method for relative quantization" (as described in *Applied Biosystems User Bulletin #2*). The expression levels of the genes in the different samples were determined from the standard curve and normalized with an endogenous control, *sar1*, as previously described [32]. The means ± standard deviations of the replicates are shown in the figures.

Enzyme Activity Assays

A concentration of total soluble proteins in the culture supernatant was assayed by using a Bradford reagent (Sangon Biotech, Shanghai, China). Xylanase activity was measured with birch xylan (Sigma-Aldrich, Brondby, Denmark) as the substrate as previously described [5]. β-1,4-Exoglucanase activity against *p*-nitrophenyl-β-D-cellobioside (*p*NPC; Sigma-Aldrich) was measured as previously described [7]. Filter paper activity and β-1,4-endo-glucanase activity were measured with a method provided by Ghose [10]. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of product per minute.

RESULTS

Construction of *T. koningii ace1*-Silenced Strains

To silence the expression of the *ace1* gene in *T. koningii*, plasmid pACEi was transformed into *T. koningii* YC01 with the aid of plasmid pAN7.1 through co-transformation.

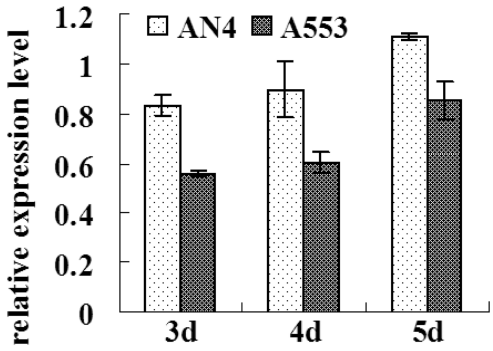
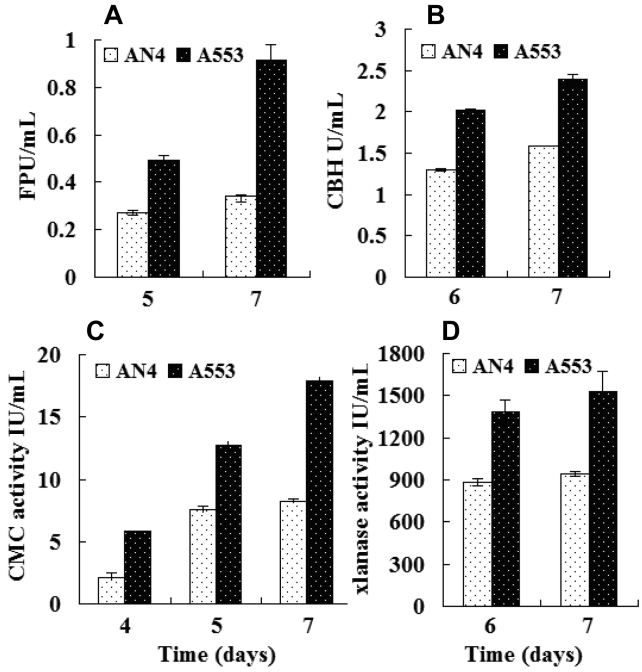


Fig. 2. Quantitative RT-PCR analysis of the *ace1* mRNA levels in the *T. koningii* transformants under inducing condition.
The total RNAs were extracted after 3, 4, and 5 days of cultivation on a medium that contained bran and corncob powder, and then the expression of the *ace1* gene was analyzed by RT-qPCR. The expression levels of *ace1* were normalized with the *sar1* reference gene.

A genomic DNA PCR analysis was applied to verify that the hpRNA expression cassette was integrated into the genome of the transformants. To avoid the effect of a stem loop structure on PCR, two fragments of the expression cassette were respectively amplified, one from 270 bp upstream of the start codon to the spacer region, and the other from the spacer region to the *cbh1* terminator. The results indicated that the expression cassette had been successfully integrated into some of the transformants (data not shown). One of them, transformant A553, was selected for the following studies and named as *T. koningii* A553. *T. koningii* AN4, a transformant obtained by the transforming of *T. koningii* YC01 with only plasmid pAN7.1, was selected as the control strain. Strains A553 and AN4 were grown on a cellulase-inducing medium that contained corncob powder and bran mixture, and the expression level of *ace1* was analyzed through RT-qPCR. As shown in Fig. 2, the mRNA level of *ace1* in strain A553 is lower than that in the control strain AN4, which indicates that partial silencing of *ace1* is achieved through dsRNA expression under inducing condition.

Silencing of *ace1* Enhanced Cellulase and Xylanase Production in *T. koningii*

The cellulase and xylanase activities produced by the *ace1*-silenced transformant A553 and the control strain were measured after 4, 5, 6, and 7 days of growth on a medium that contained bran and corncob powder; filter paper, carboxymethylcellulose (CMC), *p*NPC, and birchwood xylan were used as the substrates, respectively (Fig. 3). Moreover, the total proteins in the culture supernatants were measured. The difference in the production levels of the total secreted proteins between the control strain and the *ace1*-silenced A553 strain was obvious. Larger amounts of total proteins (about 21% higher in amount) were secreted into the culture medium by the A553 strain (Fig. 3E).



E

Strain	Total amount of secreted protein（mg/mL）	
	6 days	7 days
AN4	1.54	2.34 ± 0.02
A553	2.35 ± 0.01	2.82 ± 0.09

Fig. 3. Comparison between the enzyme and total protein production of strain A553 and the control strain AN4 under inducing condition.
Strain A553 and the control strain were both cultivated on a medium that contained bran and a corncob powder mixture. The FPA, CBH, EG, and xylanase activity were measured by using filter paper, *p*NPC, sodium carboxymethyl cellulose (CMC-Na), and birchwood xylan as the substrates, respectively. **A**: FPA; **B**: CBH; **C**: EG; **D**: xylanase activity; **E**: Total protein production of *ace1*-silenced transformant under inducing condition. Averages from the two parallel cultivations of each strain and standard deviations of the results are shown. AN4: the control strain; A553: the *ace1*-silenced transformant.

The differences in the enzyme activity levels between the A553 strain and the control strain were more pronounced. Production of endoglucanase (EG) was induced earlier and to a higher level in the *ace1*-silenced transformant A553 than in the control strain (Fig. 3C). After 7 days of cultivation, the filter paper activity (FPA), cellobiohydrolase (CBH), EG, and xylanase activities in the culture supernatants of strain A553 were 1.2, 0.5, 1.7, and 0.6 times higher than those of the control strain, respectively. Taken together, the results showed that silencing of *ace1* resulted in enhanced enzyme productivity of *T. koningii*.

The expression levels of the major cellulase genes (*cbh1*, *cbh2*, *egl1*, and *egl2*) and one xylanase gene (*xyn2*) under inducing condition were analyzed by RT-qPCR. The results indicated that the transcription of all of the above

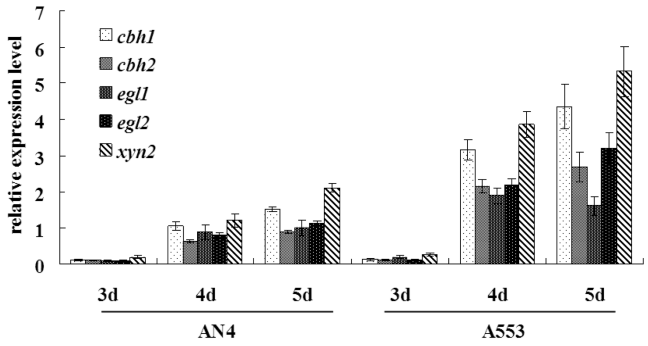


Fig. 4. Expression of the main cellulase and xylanase genes in the *ace1*-silenced transformant under inducing condition.
The total RNAs were extracted after 3, 4, and 5 days of cultivation on a medium that contained bran and corncob powder, and then the expression of all the genes was analyzed by RT-qPCR. The expression levels of the main cellulase and xylanase genes were normalized with the *sar1* reference gene. *cbh1*: cellobiohydrolase I; *cbh2*: cellobiohydrolase II; *egl1*: endoglucanase I; *egl2*: endoglucanase II; *xyn2*: xylanase II.

genes was induced earlier and to a higher level in strain A553 than in the control strain (Fig. 4). On the third day of cultivation, strain A553 expressed slightly more cellulase and xylanase genes than the control strain. However, on the fourth day, the difference in cellulase and xylanase transcript levels became profound. After 5 days of cultivation, the expression levels of *cbh1*, *cbh2*, *egl1*, *egl2*, and *xyn2* in strain A553 were 1.9, 2.0, 0.6, 1.8, and 1.5 times higher, respectively, than the control strain (Fig. 4). These results were in good agreement with the enzyme activity results.

Effect of *ace1* Silencing on Expression of Other Cellulase-Regulating Genes

To examine if the transcriptions of *cre1* and *xyr1* were subject to the regulation of ACEI in *T. koningii*, the expression levels of these two genes were analyzed by RT-qPCR. As indicated in Fig. 5, the expression of *cre1* was unaffected by the silencing of the *ace1* gene in strain A553, which suggests that *cre1* expression is not regulated by ACEI under inducing condition. On the other hand, the expression of the *xyr1* gene was higher in strain A553 than the control strain, which indicates that ACEI acts as a repressor for the transcription of the *xyr1* gene.

Stability Analysis of *ace1*-Silenced Strains

To test the stability of cellulase production in the *ace1*-silenced transformant, strain A553 was subcultured for four rounds (one round lasted for about 5 days) on a solid medium that contained hygromycin B, and the cellulase activity with filter paper as the substrate was analyzed. As shown in Fig. 6, the cellulase activity was similar between the strains after four rounds of subculture and the primary strain during cultivation, which suggests that the gene silencing in *T. koningii* was rather stable.

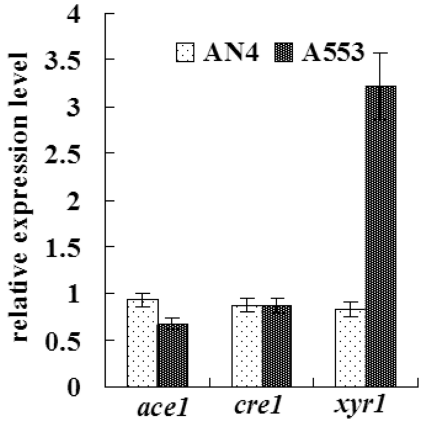


Fig. 5. Expressions of the cellulase and xylanase regulator-encoding genes under inducing condition.
The total RNAs were extracted after 4 days of cultivation on a medium that contained bran and corncob powder, and then the expressions of *ace1*, *cre1*, and *xyr1* were analyzed by RT-qPCR. The expression levels of *ace1*, *cre1*, and *xyr1* were normalized with the *sar1* reference gene. *cre1*: carbon catabolite repressor I; *ace1*: activator I of cellulase; *xyr1*: xylanase regulator I.

DISCUSSION

Gene Silencing of *ace1* Gene Improves Cellulase Production in *T. koningii*

The *T. reesei ace1* gene that encodes a DNA-binding protein with zinc fingers of the C_2H_2 type has been previously cloned [29]. Deletion of the *ace1* gene in *T. reesei* resulted in an increase in the expression of all the main cellulase and hemicellulase genes in sophorose- and cellulose-induced cultures, which indicates that ACEI acts as a repressor of cellulase and xylanase expression [2]. The deduced ACEI protein of *T. koningii* YC01 (GenBank Accession No. AFD62912), a cellulase-hyperproducing mutant, was 97% identical to *T. reesei* ACEI. Therefore, we propose to test whether ACEI also represses cellulase and xylanase expressions in *T. koningii* and enzyme

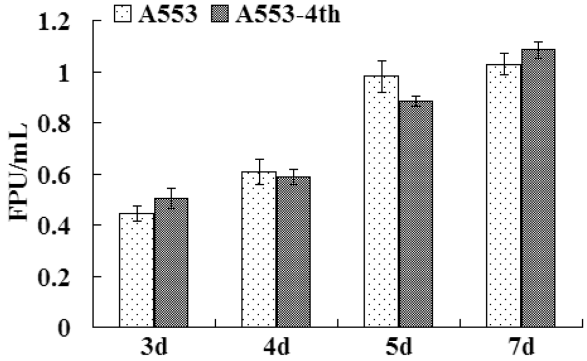


Fig. 6. Filter paper activities in inducing culture supernatants of *ace1*-silenced strains after four rounds of subculture of A553-4th and its primary strain A553 after 3, 4, 5, and 7 days of growth.

production can be improved by silencing the *ace1* gene in this mutant producing high levels of cellulase and xylanase.

It was observed in this work that the silencing of *ace1* really affects the cellulase and xylanase production of *T. koningii* under inducing condition. More cellulase and xylanase accumulated in the culture supernatant of the *ace1*-silenced transformant in comparison with the control strain. This observation is in accordance with the RT-qPCR analysis of the cellulase and xylanase genes expressions in the same inducing condition, which showed that the transcription of all the major cellulase and xylanase genes, *cbh1*, *cbh2*, *egl1*, *egl2*, and *xyn2*, were induced earlier and to a higher level in the *ace1*-silenced transformant than the control strain (Fig. 5). The increases in the amounts of the individual cellulase and xylanase mRNAs varied from 0.6- to 2.0-folds after 5 days of cultivation. These results suggest that *T. koningii* ACEI also plays a role in regulating the expression levels of the hydrolase genes under inducing condition. The data presented in this paper clearly show that ACEI negatively regulates, either directly or indirectly, the expressions of cellulase and xylanase genes in *T. koningii*. In addition, our results demonstrate that down-regulation of *ace1* is one of the strategies to improve the level of cellulase and hemicellulase production in the *Trichoderma* strain.

Inactivation of genes in filamentous fungi is commonly accomplished using homology-driven site-directed gene replacement. However, according to our previous experiments, transformed DNA in *T. koningii* with site-specific recombination occurs at low frequencies. We applied RNAi as an alternative tool to knock down the expression of the *ace1* gene, and obtained partial silencing of the *ace1* gene expression. Variable efficiency of gene silencing has been found in some fungi, including *Cryptococcus neoformans*, *Magnaporthe oryzae*, and *Aspergillus fumigatus* [13, 15, 20]. The variability of the silencing level may depend on the type of construct, transgene copy number, site of integration, and the target gene. It is reported that the gene-silencing efficiency of siRNA is strongly dependent on the local structure of mRNA at the targeted region [16]. We speculate that there are some effects specific to the site of transgene integration that affects expression levels of the *ace1*-silencing hairpin RNA, or the *T. reesei gpd* promoter is not strong enough to drive high efficiency of the gene silencing of *ace1*.

Effect of Silencing of the *ace1* Gene on *cre1* and *xyr1* Gene Expression

CREI mediates carbon catabolite repression in *T. reesei*. Although the *cre1* promoter contains three copies of the core-binding sequence for ACEI, 5'AGGCA, deletion of the *ace1* gene does not affect the expression of *cre1* during growth under inducing condition [2], which suggests that ACEI is not involved in the regulation of the *cre1* expression.

In this work, the RT-qPCR analysis shows that the expression of *cre1* in the *ace1*-silenced strain is similar to that in the control strain, which suggests that ACEI did not regulate the expression of *cre1* under the inducing condition studied here. Based on the expression data here and in previous studies, it seems that *cre1* is not repressed by ACEI in *T. koningii*; it may regulate its own transcription, and/or by phosphorylation and dephosphorylation in the post-translation level.

XYRI is currently considered to be the main regulator of the cellulase and hemicellulase system of *T. reesei*, because deletion of the *xyr1* gene results in the loss of the formation of all cellulases, xylanases, and two arabinofuranosidases [1, 34]. Regulation of the above genes in *T. reesei via* XYRI is not affected by the substances that mediate inducing (xylose, xylobiose, and sophorose) and is indispensable for all modes of gene expression (basal, derepressed, and induced) [34]. It is shown that the cellulase repressor ACEI of *T. reesei* is proven to directly antagonize the XYRI function by competing for one of its binding sites in the *xyn1* promoter [28]. Our quantitative data showed that in the *ace1*-silenced transformant, the level of the *xyr1* mRNA was 2.2-folds higher than the level in the control strain under inducing condition, which suggests that ACEI acted as a repressor of the *xyr1* gene transcription in *T. koningii*. This could provide an explanation for our finding in which *ace1* gene silencing also has a strong effect on *xyn2* expression.

Recently, Nakari-Setälä *et al*. [21] showed that deletion of *cre1* leads to derepressing, and significantly elevates the levels of cellulase and hemicellulase production, which suggests that *cre1* is also a potential target gene in strain improving to enhance the enzyme production of *Trichoderma*. Therefore, further improvements might be obtained in which simultaneous silencing two repressor genes, such as *ace1* and *cre1*, or silencing the repressor gene *ace1* at the same time, up-regulates the activator gene *xyr1* in *Trichoderma*.

The truncated *T. reesei* ACEI that lacked 242 amino acids from the N-terminus was expressed in yeast and functioned as an activator of the *cbh1* promoter [29], whereas the full-length protein was demonstrated to mediate repression of cellulase and xylanase in *T. reesei* [2]. In this study, we showed that *T. koningii* ACEI not only acted as a repressor of the main cellulase and xylanase genes but also repressed the *xyr1* gene transcription. Aro *et al.* [2] speculated that the transcription regulating function of the truncated form of ACEI differed from that of the full-length ACEI, and ACEI functioned in a context-dependent manner with potential dual–repressor-activator function, depending on the interactions with anther factor(s) that influences its function [2].

In conclusion, the function of ACEI was verified in *T. koningii* YC01 through RNAi, and silencing of ACEI

resulted in elevated production of cellulase and xylanase under inducing condition. When the expression of ACEI was partially silenced, the mRNA levels of the main cellulase and xylanase genes increased in accordance with the enzyme activities. This work demonstrates that RNAi may act as an effective tool for improving the properties of filamentous fungi.

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REFERENCES

1. Akel, E., B. Metz, B. Seiboth, and C. P. Kubicek. 2009. Molecular regulation of arabinan and L-arabinose metabolism in *Hypocrea jecorina* (*Trichoderma reesei*). *Eukaryot. Cell* **8:** 1837–1844.
2. Aro, N., M. Ilmén, A. Saloheimo, and M. Penttilä. 2003. ACEI of *Trichoderma reesei* is a repressor of cellulase and xylanase expression. *Appl. Environ. Microbiol.* **69:** 56–65.
3. Aro, N., T. Pakula, and M. Penttilä. 2005. Transcriptional regulation of plant cell wall degradation by filamentous fungi. *FEMS Microbiol. Rev.* **29:** 719–739.
4. Aro, N., A. Saloheimo, M. Ilmén, and M. Penttilä. 2001. ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. *J. Biol. Chem.* **276:** 24309–24314.
5. Bailey, M. J., P. Biely, and K. Poutanen. 1992. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* **23:** 257–270.
6. Belinky, P. A., A. Matityahu, Y. Hadar, and C. G. Dosoretz. 2008. Gene silencing by RNA interference in the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **74:** 5359–5365.
7. Deshpande, M. V., K. E. Eriksson, and L. G. Pettersson. 1984. An assay for selective determination of exo-1, 4,-beta-glucanases in a mixture of cellulolytic enzymes. *Anal. Biochem.* **138:** 481–487.
8. Fire, A., S. Xu, M. Montgomery, S. Kostas, S. Driver, and C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391:** 806–811.
9. Fitzgerald, A., J. A. L. van Kan, and K. M. Plummer. 2004. Simultaneous silencing of multiple genes in the apple scab fungus, *Venturia inaequalis*, by expression of RNA with chimeric inverted repeats. *Fungal Genet. Biol.* **41:** 963–971.
10. Ghose, T. 1987. Measurement of cellulase activities. *Pure Appl. Chem.* **59:** 257–268.
11. Ilmén, M., A. Saloheimo, M. L. Onnela, and M. E. Penttilä. 1997. Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. *Appl. Environ. Microbiol.* **63:** 1298–1306.
12. Jiang, X., A. Geng, N. He, and Q. Li. 2011. New isolate of *Trichoderma viride* strain for enhanced cellulolytic enzyme complex production. *J. Biosci. Bioeng.* **111:** 121–127.
13. Kadotani, N., H. Nakayashiki, Y. Tosa, and S. Mayama. 2003. RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*. *Mol. Plant Microbe Interact.* **16:** 769–776.
14. Khalaj, V., H. Eslami, M. Azizi, N. Rovira-Graells, and M. Bromley. 2007. Efficient downregulation of *alb1* gene using an AMA1-based episomal expression of RNAi construct in *Aspergillus fumigatus*. *FEMS Microbiol. Lett.* **270:** 250–254.
15. Liu, H., T. R. Cottrell, L. M. Pierini, W. E. Goldman, and T. L. Doering. 2002. RNA interference in the pathogenic fungus *Cryptococcus neoformans*. *Genetics* **160:** 463–470.
16. Luo, K. Q. and D. C. Chang. 2004. The gene-silencing efficiency of siRNA is strongly dependent on the local structure of mRNA at the targeted region. *Biochem. Biophys. Res. Commun.* **318:** 303–310.
17. Mach-Aigner, A. R., M. E. Pucher, M. G. Steiger, G. E. Bauer, S. J. Preis, and R. L. Mach. 2008. Transcriptional regulation of *xyr1*, encoding the main regulator of the xylanolytic and cellulolytic enzyme system in *Hypocrea jecorina*. *Appl. Environ. Microbiol.* **74:** 6554–6562.
18. Mach, R. and S. Zeilinger. 2003. Regulation of gene expression in industrial fungi: *Trichoderma*. *Appl. Microbiol. Biotechnol.* **60:** 515–522.
19. Mandels, M. and J. Weber. 1969. The production of cellulases. *Adv. Chem.* **95:** 391–414.
20. Mouyna, I., C. Henry, T. L. Doering, and J. P. Latge. 2004. Gene silencing with RNA interference in the human pathogenic fungus *Aspergillus fumigatus*. *FEMS Microbiol. Lett.* **237:** 317–324.
21. Nakari-Setälä, T., M. Paloheimo, J. Kallio, J. Vehmaanperä, M. Penttilä, and M. Saloheimo. 2009. Genetic modification of carbon catabolite repression in *Trichoderma reesei* for improved protein production. *Appl. Environ. Microbiol.* **75:** 4853–4860.
22. Nakayashiki, H. and Q. B. Nguyen. 2008. RNA interference: Roles in fungal biology. *Curr. Opin. Microbiol.* **11:** 494–502.
23. Namekawa, S. H., K. Iwabata, H. Sugawara, F. N. Hamada, A. Koshiyama, H. Chiku, *et al*. 2005. Knockdown of LIM15/DMC1 in the mushroom *Coprinus cinereus* by double-stranded RNA-mediated gene silencing. *Microbiology* **151:** 3669–3678.
24. Nguyen, Q. B., N. Kadotani, S. Kasahara, Y. Tosa, S. Mayama, and H. Nakayashiki. 2008. Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, *Magnaporthe oryzae*, using a high-throughput RNA-silencing system. *Mol. Microbiol.* **68:** 1348–1365.
25. Nidetzky, B., W. Steiner, M. Hayn, and M. Claeyssens. 1994. Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: A new model for synergistic interaction. *Biochem. J.* **298 (Pt 3):** 705–710.
26. Penttilä, M., H. Nevalainen, M. Ratto, E. Salminen, and J. Knowles. 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* **61:** 155–164.

27. Qin, L.-N., F.-R. Cai, X.-R. Dong, Z.-B. Huang, Y. Tao, J.-Z. Huang, and Z.-Y. Dong. 2012. Improved production of heterologous lipase in *Trichoderma reesei* by RNAi mediated gene silencing of an endogenic highly expressed gene. *Bioresour. Technol.* **109:** 116–122.
28. Rauscher, R., E. Würleitner, C. Wacenovsky, N. Aro, A. R. Stricker, S. Zeilinger, *et al*. 2006. Transcriptional regulation of *xyn1*, encoding xylanase I, in *Hypocrea jecorina*. *Eukaryot. Cell* **5:** 447–456.
29. Saloheimo, A., N. Aro, M. Ilmén, and M. Penttilä. 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:** 5817–5825.
30. Schmoll, M. and C. P. Kubicek. 2003. Regulation of *Trichoderma* cellulase formation: Lessons in molecular biology from an industrial fungus. A review. *Acta Microbiol. Immunol. Hung.* **50:** 125–145.
31. Seiboth, B., L. Hartl, M. Pail, E. Fekete, L. Karaffa, and C. P. Kubicek. 2004. The galactokinase of *Hypocrea jecorina* is essential for cellulase induction by lactose but dispensable for growth on D-galactose. *Mol. Microbiol.* **51:** 1015–1025.
32. Steiger, M. G., R. L. Mach, and A. R. Mach-Aigner. 2010. An accurate normalization strategy for RT-qPCR in *Hypocrea jecorina* (*Trichoderma reesei*). *J. Biotechnol.* **145:** 30–37.
33. Strauss, J., R. L. Mach, S. Zeilinger, G. Hartler, G. Stöffler, M. Wolschek, and C. P. Kubicek. 1995. Cre1, the carbon catabolite repressor protein from *Trichoderma reesei*. *FEBS Lett.* **376:** 103–107.
34. Stricker, A. R., K. Grosstessner-Hain, E. Würleitner, and R. L. Mach. 2006. Xyr1 (xylanase regulator 1) regulates both the hydrolytic enzyme system and D-xylose metabolism in *Hypocrea jecorina*. *Eukaryot. Cell* **5:** 2128–2137.
35. Stricker, A. R., R. L. Mach, and L. H. de Graaff. 2008. Regulation of transcription of cellulases- and hemicellulases-encoding genes in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*). *Appl. Microbiol. Biotechnol.* **78:** 211–220.
36. Takeno, S., E. Sakuradani, A. Tomi, M. Inohara-Ochiai, H. Kawashima, T. Ashikari, and S. Shimizu. 2005. Improvement of the fatty acid composition of an oil-producing filamentous fungus, *Mortierella alpina* 1S-4, through RNA interference with delta12-desaturase gene expression. *Appl. Environ. Microbiol.* **71:** 5124–5128.
37. Whisson, S. C., A. O. Avrova, P. Van West, and J. T. Jones. 2005. A method for double-stranded RNA-mediated transient gene silencing in *Phytophthora infestans*. *Mol. Plant Pathol.* **6:** 153–163.
38. Zamore, P. D. and Y. Tomari. 2005. Perspective: Machines for RNAi. *Gene Dev.* **19:** 517–529.