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ctf1 *Trichoderma reesei*

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Strains from *Trichoderma reesei* have been used for cellulase production with a long history. It has been well known that cellulase biosynthesis by the fungal species is controlled through regulators, and elucidation of their regulation network is of great importance for engineering *T. reesei* with robust cellulase production. However, progress in this regard is still very limited. In this study, *T. reesei* RUT-C30 was transformed with an artificial zinc finger protein (AZFP) library, and the mutant *T. reesei* M2 with improved cellulase production was screened. Compared to its parent strain, the filter paper activity and endo-β-glucanase activity in cellulases produced by *T. reesei* M2 increased 67.2% and 35.3%, respectively. Analysis by quantitative reverse transcription polymerase chain reaction indicated significant downregulation of the putative gene *ctf1* in *T. reesei* M2, and its deletion mutants were thus developed for further studies. An increase of 36.9% in cellulase production was observed in the deletion mutants, but when *ctf1* was constitutively overexpressed in *T. reesei* RUT-C30 under the control of the strong *pdc1* promoter, cellulase production was substantially compromised. Comparative transcriptomic analysis revealed that the deletion of *ctf1* upregulated transcription of gene encoding the regulator VIB1, but downregulated transcription of gene encoding another regulator RCE1, which consequently upregulated genes encoding the transcription factors XYR1 and ACE3 for the activation of genes encoding cellulolytic enzymes. As a result, *ctf1* was characterized as a gene encoding a repressor for cellulase production in *T. reesei* RUT-C30, which is significant for further elucidating molecular mechanism underlying cellulase biosynthesis by the fungal species for rational design to develop robust strains for cellulase production. And in the meantime, AZFP transformation was validated to be an effective strategy for identifying functions of putative genes in the genome of *T. reesei*.

artificial zinc finger proteins, cellulase production, *ctf1*, transcription regulator, *Trichoderma reesei*

Lignocellulosic biomass is sustainable and environmentally friendly for biorefinery to produce fuels and chemicals, alleviating dependence on fossil resources such as crude oil, and in the meantime, mitigating greenhouse gas emissions, since, theoretically, CO_2 released during the consumption of bio-based products can be fixed by plants through

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2.1 |

Escherichia coli DH5α (TransGen, Beijing, China) was used for plasmid propagation, which was cultured in a shaker at 37°C and 200 rpm using lysogeny broth (LB) medium. The transformation of *T. reesei* was performed by *Agrobacterium tumefaciens* mediated transformation (ATMT) (Zhong, Wang, Wang, & Jiang, 2007). *A. tumefaciens* AGL-1 transformants with the pCB303-ZFP library for fungal transformation were grown at 28°C and 200 rpm using LB medium supplemented with kanamycin. The induction medium containing 200 μmol/L acetosyringone (Sangon Biotech, China) was prepared as previously reported (Michielse, Hooykaas, van den Hondel, & Ram, 2008). *T. reesei* RUT-C30 (ATCC 56765) was used as the parent strain, which was engineered with the AZFP library for screening mutants with improved cellulase production. Details on the library development and assessment of its quality, capacity, and transformation efficiency are available in our previous work (Zhang et al., 2016).

All *T. reesei* mutants were cultured first on solid medium containing 3% malt extract and 2% agar at 28°C for 5–7 days to produce conidia, which were harvested, and then inoculated with 10^5/ml into a 250-ml Erlenmeyer flask containing 50 ml minimal medium supplemented with 0.1% peptone and 2% glucose to grow mycelia at 28°C and 200 rpm for 36 hr. The minimal medium composed of (g/L): $(NH_4)_2SO_4$, 5; KH_2PO_4, 15; $MgSO_4$, 0.6; $CaCl_2$, 0.8; $FeSO_4{\cdot}7H_2O$, 0.0005; $MnSO_4{\cdot}H_2O$, 0.0016; $ZnSO_4{\cdot}7H_2O$, 0.0014; $CoCl_2$, 0.0002 (Liu et al., 2016).

2.2 | A

Mycelial culture of 5 ml was inoculated into a 250-ml Erlenmeyer flask containing 50 ml minimal medium supplemented with 2% (w/v) microcrystalline cellulose for cellulase production at 28°C and 200 rpm, which was characterized by the activities of filter paper (FPase), endo-glucanase (CMCase), exo-glucanase (*p*NPCase) and β-glucosidase (*p*NPGase) following the protocol developed previously (Gao et al., 2017; Wood & Bhat, 1988). In addition, extracellular proteins were assayed using the BCA Kit (Sangon Biotech) after mycelia were removed by centrifugation.

When cellulose is used as substrate for cellulase production by *T. reesei*, mycelial biomass cannot be measured directly. To address such a challenge, mycelial growth was characterized indirectly by measuring intracellular proteins that were extracted by 1 M NaOH according to the protocol developed previously (Aro, Ilmen, Saloheimo, & Penttila, 2003).

2.3 | A *Azfp-* 2 *T. reesei* 2

Thermal asymmetrical interlaced polymerase chain reaction (TAIL-PCR) was used to clone the genomic DNA fragments flanking with *Azfp*-M2 insert from *T. reesei* M2 following the protocol previously described (Liu & Chen, 2007). The specific right-border primers (RB-1, RB-2, and RB-3),

le/F21(0.4)92TJ/F51Tf17.43326m060Tc(T)Tj/F21Tf.51210TD(.)Tj/F11Tf.42680TD-.0331T

transcription analysis were amplified from the genomic DNA of *T. reesei* RUT-C30 with the primers listed in Table S1. Then, the fragments were ligated into the *NcoI*/*XbaI* sites of the plasmid pCB304 under the control of the constitutive *pdc1* promoter (Li et al., 2012; Zhang et al., 2016) to develop vectors pCZF-10530, pCZF-123146, and pCZF-131902 (Figure S1). The recombinant plasmids were transformed into *T. reesei* RUT-C30 by ATMT to explore their impact on cellulase production. Since these genes were integrated into the genome of *T. reesei* RUT-C30 randomly for overexpression, three transformants were selected on the minimal medium plate supplemented with 2% glucose and 300 μg/L hygromycin B for genetic manipulation with each gene, which were verified by PCR, and qRT-PCR analysis was further employed to quantify the transcription of *ctf1* in the transformants (Figure S2).

2.7 | *ctf1*

Mutants for *ctf1* deletion (*T. reesei* Δ*ctf1*) and its *ctf1* complementation were developed through homologous recombination, which were verified by PCR (Figure S3). Two mutants for *ctf1* deletion (*T. reesei* Δ*ctf1*-T1 and T2) and *ctf1* complementation to the deletion mutants (*T. reesei ctf1*-rec-T1 and T2) were developed, respectively, to study the role of *ctf1* on cellulase biosynthesis in *T. reesei*. Such an experimental design could assess potential impact of those genetic manipulations on functions of other genes in the genome of *T. reesei* RUT-C30. Primers used for the construction of *ctf1* deletion and complementation cassettes and verification of the transformants were given in Table S1.

First, the hygromycin B phosphotransferase gene expression cassette ptrpC-*hph*-ttrpC was amplified from the vector pSilent-1 (Nakayashiki et al., 2005). Upstream and downstream fragments of *ctf1* were obtained through PCR amplification from the genome of *T. reesei* RUT-C30 using the KOD Polymerase (Toyobo, Japan). The amplicons were then mixed at a molar ratio of 1:3:1 for 5′-flanking region: ptrpC-*hph*-ttrpC: 3′-flanking region, which were linked together through another round of PCR reaction. The PCR product was used as the template for the third round of PCR to construct the *ctf1* deletion cassette with the nested primers Δ*ctf1*-F/Δ*ctf1*-R. The *ctf1* deletion cassette was transformed into the protoplast of *T. reesei* RUT-C30 using the method described elsewhere (Li, Du, Zhong, & Wang, 2010). Transformants were cultured and screened on the minimal medium plate supplemented with 2% glucose and 300 μg/L hygromycin B, and the deletion of *ctf1* was confirmed by PCR (Figure S3A).

For the complementation of *ctf1* in *T. reesei* Δ*ctf1*, a 5181 bp DNA fragment containing the upstream, *ctf1* and a 774 bp TtrpC terminator DNA fragment were amplified from the genome of *T. reesei* RUT-C30 using the primer pairs *ctf1*-upstream-F/R and TtrpC-F/R, respectively. Then, the two DNA fragments were inserted into the *ctf1* expression cassette by the overlap extension PCR. Similarly, the 1606 bp *pdc1* promoter, the 1633 bp downstream region and the 610 bp selecting marker gene *bar* encoding phosphinothricin acetyltransferase were amplified from the genome of *T. reesei* RUT-C30 and the pBar vector (Zhang et al., 2016), respectively. Furthermore, the three fragments were inserted into the *bar* expression cassette through the overlap extension PCR. Finally, the *ctf1* expression cassette, the *bar* expression cassette and the pUG6 fragment amplified from the pUG6 vector were linked together by the RecET direct cloning technology to form the pUG6-*ctf1*-bar vector (Wang et al., 2016), which was transformed into the protoplast of *T. reesei* Δ*ctf1*, and transformants were screened on the minimal medium plate supplemented with 2% glucose and 300 μg/L glufosinate ammonium. The *ctf1* complementation mutants were verified by PCR using the primer pairs *ctf1*-up-verif-F/bar-verif-R and bar-verif-F/*ctf1*-down-verif-R (Figure S3B).

Mutants with *ctf1* deletion and complementation were inoculated with 2 μl spore suspension containing 10^6 spores/ml into the minimal medium plates supplemented with 2% (w/v) carbon source including cellulose, lactose, glycerol and glucose, respectively, which were incubated at 28°C for 3–5 days to assess cellulase production through comparing the size of transparent zones developed by the enzymatic degradation of cellulose. Followed the preliminary assessment, submerged culture was performed in flask at 28°C and 180 rpm using the minimal medium supplemented with 2% (w/v) cellulose to further quantify cellulase production.

2.8 | A- *ctf1*

cDNA libraries prepared from messenger RNA (mRNA) of *T. reesei* Δ*ctf1* were organized based on the protocol developed by Illumina Inc. (San Diego, CA), which were sequenced by the HiSeq 2000 platform at BGI (Shenzhen, China). Sequenced reads were mapped against transcripts predicted for the genome of *T. reesei* RUT-C30 from JGI Genome Portal (https://genome.jgi.doe.gov/TrireRUTC30_1/TrireRUTC30_1.home.html) (Kim, Landmead, & Salzberg, 2015). The transcription of differentially expressed genes (DEGs) was normalized through the number of reads/fragments per kb of the exon region per million mapped reads (FPKM). Furthermore, Log_2R (FPKM ratio of the samples) was used to characterize the transcription of genes, and DEGs were screened with the threshold $-1 \geq Log_2R \geq 1$. The false discovery rate was used to determine the *p*-value of the multiple test (Benjamini, Drai, Elmer, Kafkafi, & Golani, 2001), and $p \leq .01$ was used to evaluate the statistic significance.

2.9 | A- 1

To express the DNA binding domain of CTF1 in *E. coli*, the DNA fragment encoding the CTF1 binding domain with amino acids 1–120 was amplified from the first-strand cDNA of *T. reesei* RUT-C30, which was inserted into the pGEX4T-1 vector by the In-Fusion HD Cloning

Kit (Takara) to obtain the pGEX4T-1-CTF1$_{1\text{-}120}$ plasmid. Subsequently, the plasmid was introduced into *E. coli* BL21 for protein expression. Purification and verification of the GST-fused protein CTF1$_{1\text{-}120}$ were performed according to the methods described by Cao et al. (2017). The binding of the DNA-binding domain of CTF1 with targeted genes was verified through the electrophoretic mobility shift assay (EMSA) (Ruscher et al., 2000).

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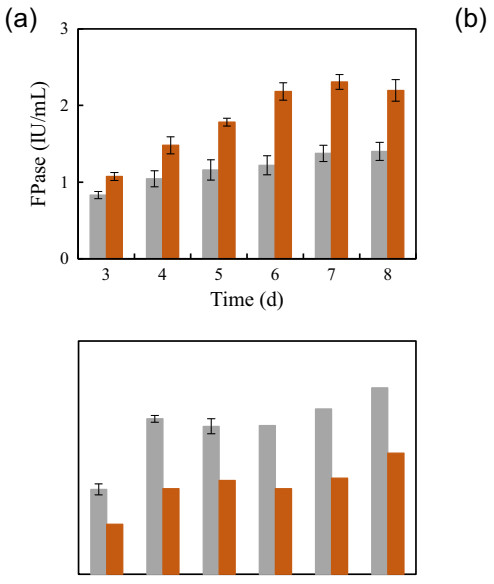
3.1 | A

Approximately 600 transformants engineered with AZFPs were obtained (Zhang et al., 2016), and three of them were screened by naked eye from culture grown on cellulose plates based on the size of transparent zones developed for preliminary assessment on their cellulase production (Figure S4), through which *T. reesei* M2 was selected for further evaluation by submerged culture in the minimal medium supplemented with 2% cellulose.

As can be seen in Figure 1, the activities of FPase, CMCase, and *p*NPCase in cellulases produced by *T. reesei* M2 increased 67.2%, 35.3%, and 9.7%, respectively, compared to that detected in cellulases produced by *T. reesei* RUC-C30 when both were cultured for 8 days, although 50% lower activity in *p*NPGase was detected in cellulases produced by the mutant. On the other hand, no significant difference was observed for their production of extracellular proteins.

The expression of AZFP in *T. reesei* M2 (AZFP-M2) was confirmed by Western blot (Figure 2a), suggesting that the enhanced production of cellulases by *T. reesei* M2 might be caused by AZFP-M2, since potentially it could bind onto the nucleotide sequences of target genes to regulate their functions. To explore the potential targets, the gene encoding AZFP-M2 was amplified from the genome of *T. reesei* M2, and sequenced for alignment with amino acid residues. As can be seen in Figure 2b, AZFP-M2 is composed of four ZFP domains followed by the Gal4 effector domain, and four amino acid residues at sites −1, 2, 3, and 6 with each ZFP domain could bind to a specific DNA sequence, which was deduced to be 5′-GTTGYAHGAGGG-3′, in which Y and H represent C or T and A, C or T, respectively (Park et al., 2003). We further aligned the genome of *T. reesei* RUT-C30 with the binding sites of AZFP-M2, and a total of 21 binding sites were detected (Table S3). Genes with those binding sites include TrireRUTC30: 131902 encoding formaldehyde transketolase, TrireRUTC30: 87447 encoding triacylglycerol lipase,



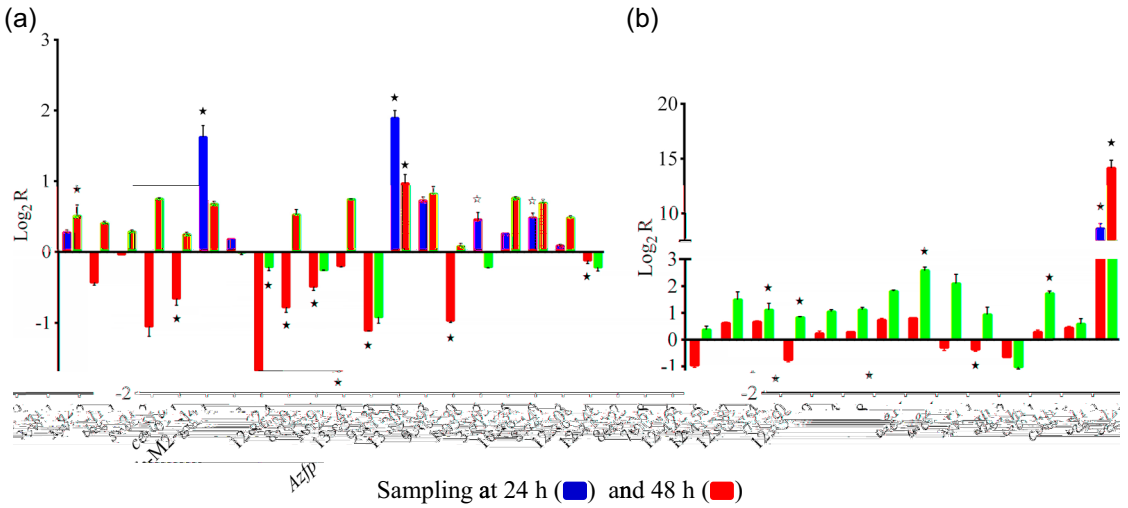
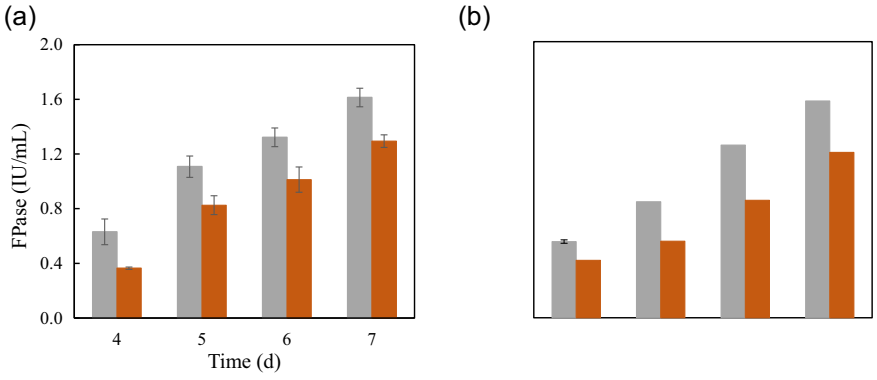


FIGURE 3 Transcriptional analysis by quantitative reverse transcription polymerase chain reaction for genes potentially targeted by AZFP-M2 (a) and major genes encoding cellulolytic enzymes in *T. reesei* M2 (b). Error bars show the standard deviations, and the asterisk (*) indicates the significance of $p < .05$. Mycelia were cultured at 28°C and 180 rpm in flasks using minimal medium supplemented with 2% cellulose, and sampled at 24 hr and 48 hr, respectively. The expression of genes was normalized to that for the housekeeping gene *tef1*. AZFP, artificial zinc finger protein [Color figure can be viewed at wileyonlinelibrary.com]

proteins as well, leading to an average decrease of 30% and 25% in the FPase activity and the amount of extracellular proteins, respectively, compared to that detected during the culture of *T. reesei* RUT-C30 at 6 days. On the other hand, no significant impact on cellulase production was observed when TrireRUTC30:123146 or TrireRUTC30:131902 was overexpressed in *T. reesei* RUT-C30 (Figure S6). Therefore, it is very likely that *ctf1* could act as a repressor for cellulase biosynthesis by *T. reesei*, and the effect of its deletion on cellulase production was further explored to validate such a speculation.

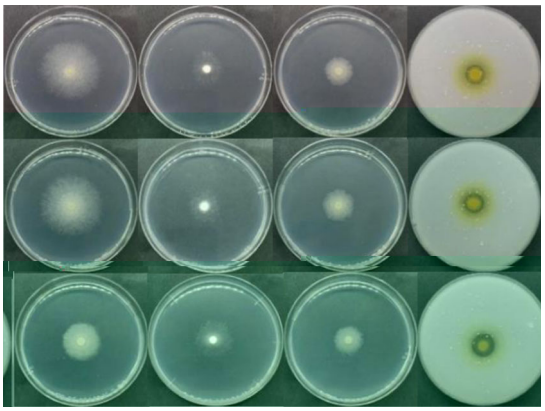
3.3 | *ctf1*

Two *ctf1* deletion mutants were selected. As shown in Figure 5a, *T. reesei* Δ*ctf1* (T1) developed a much larger transparent zone on the cellulose plate, which is highlighted by the dark background, but such an effect was compromised when *ctf1* was complemented to the deletion mutant, indicating that the deletion of *ctf1* might improve cellulase production to hydrolyze cellulose more effectively. On the other hand, dense and compact mycelia were observed on the plate supplemented with glucose, indicating that the deletion of *ctf1* affected the mycelial morphology of *T. reesei*, which could benefit process design for its submerged culture to production cellulases more efficiently, since such a morphology would decrease the viscosity of the fermentation broth for more efficient mixing and oxygen mass transfer. No significant difference was observed for mycelial growth and cellulase production on the plates supplemented with lactose and glycerol, since lactose is a less effective inducer compared to cellulose, and glycerol is a neutral carbon source without inducing effect for cellulase production.



The activities of cellulolytic enzymes were further evaluated for *T. reesei* Δ*ctf1* mutants. When grown on the minimal medium supplemented with 2% cellulose for 5 days, *T. reesei* Δ*ctf1* mutant (T1) displayed 36.9%, 12.1%, 23.7%, and 26.3% higher activities of FPase, CMCase, *p*NPCase, and *p*NPGase than those detected in cellulases produced by *T. reesei* RUT-C30 (Figure 5b–e). On the other hand, the production of extracellular proteins was also enhanced, although the increase was not significant as that observed for the cellulolytic enzymes (Figure 5f). The growth of the mutants was further characterized through analyzing total intracellular proteins. Although more intracellular proteins were detected at the early stage due to biosynthesis and intracellular accumulation of cellulases, their content was relatively constant at the late stage with the

Glucose

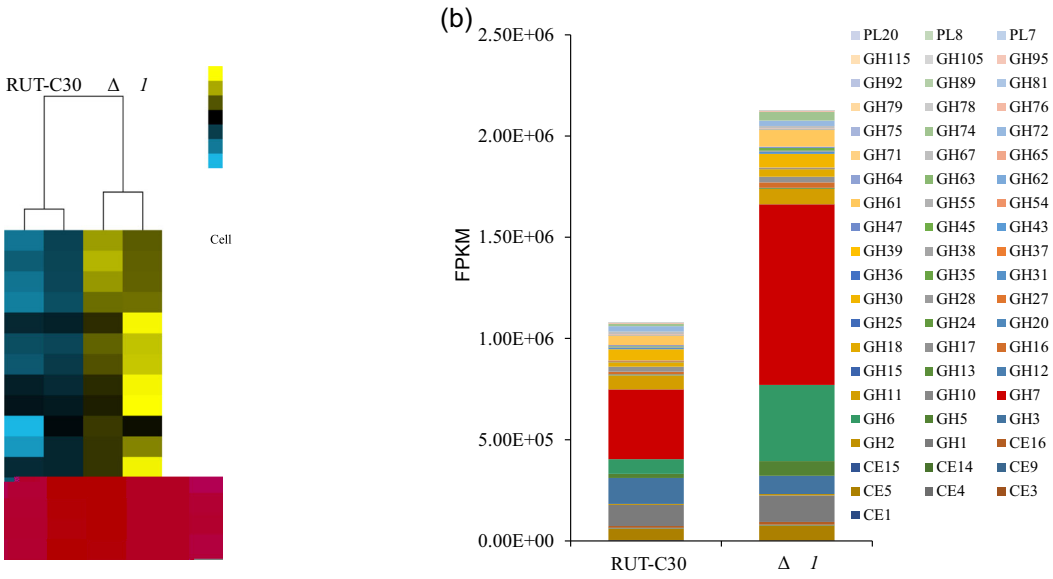


excretion of those cellulolytic enzymes, and no significant difference was observed between the mutants and *T. reesei* RUT-C30 (Figure 5e). These experimental results indicate that *ctf1* might negatively regulate cellulase biosynthesis in *T. reesei*. Similar experimental results were also observed for another *ctf1* deletion and complementation mutants (*T. reesei* Δ*ctf1*-T2 and *ctf1-rec*-T2), which are shown in Figure S7.

Hydrolysis of corn stover was performed using raw cellulases produced by *T. reesei* Δ*ctf1*-T1 to quantitatively evaluate the impact of *ctf1* deletion on the production of other components associated with cellulose hydrolysis, and experimental results indicated that cellulases produced by *T. reesei* Δ*ctf1* hydrolyzed alkali-pretreated corn stover more effectively with more glucose released (Figure S8).

3.4 | [illegible] 1 [illegible]

To determine molecular mechanism underlying the improvement on cellulase production observed in *T. reesei* Δ*ctf1* mutants, we assessed the genome-wide expression through RNA-seq for *T. reesei* Δ*ctf1* and RUT-C30 grown vigorously at 24 hr, and screened DEGs based on the threshold: $-1 \geq \mathrm{Log}_2\mathrm{R} \geq 1$. In total, 646 genes were differentially expressed, in which 377 were down-regulated, and 269 were upregulated. These DEGs were subjected to gene ontology enrichment analysis, which were enriched mainly into pathways for the metabolism of carbohydrates, amino acids, and lipids as well as transport and catabolism (Figure S9). Moreover, 28 genes encoding transcription factors were identified and highlighted in Table 1, including TrireRUTC30:125610 encoding the transcription factor VIB1 (Zhang et al., 2018), TrireRUTC30:98455, and TrireRUTC30:6520 encoding transcriptional activator ACE III and RCE 1 for the expression of cellulolytic en-



significant induction of CBH I and CBH II as well as EG I and EG II with the GH6 and GH7 families.

3.5 | 1 *T. reesei*

For mutants with *ctf1* deleted, the expression of *vib1* and *ace3* was significantly upregulated, but the expression of *rce1* was downregulated. Therefore, we speculated direct interactions between *ctf1* and these transcription factors, and in vitro EMSA was performed to validate such a speculation. Bands were observed in the EMSA analysis when $CTF1_{1\text{-}120}$ was mixed with the cy5-labeled DNA probes corresponding to the promoter region of *vib1*, *rce1*, and *ace3*, and the binding strength increased as the concentration of those proteins was increased from 0.1 to 0.6 μM (Figure 7). Therefore, we confirmed that CTF1 mediates cellulase production by *T. reesei* RUT-C30 indirectly through binding with the promoter region of *vib1*, *rce1*, and *ace3*.

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Engineering transcription factors for global perturbation has been developed to alter phenotypes for robust production of different products (Alper, Moxley, Nevoigt, Fink, & Stephanopoulos, 2006; Kwon et al., 2006; Santos & Stephanopoulos, 2008). In this study, *T. reesei* M2 with improved cellulase production was developed by the AZFP transformation, and CTF1 was discovered to repress cellulase production by *T. reesei*, which was verified through the impact on cellulase production by engineering *T. reesei* RUT-C30 with the deletion and overexpression of *ctf1*, respectively. The RNA-seq analysis demonstrated that CTF1 might exert negative regulation on cellulase production through modulating the expression of transcription factors regulating the expression of genes related to cellulase production by *T. reesei*.

The deletion of *ctf1* from *T. reesei* RUT-C30 led to a significant increase in the transcription of *vib1*, *ace3*, and *cre1*, but a considerably decrease in the transcription of *rce1*. As a regulator of CCR, CRE1 regulates the expression of transcription factors such as XYR1 to control the expression of genes encoding cellulolytic enzymes in *T. reesei* (Portnoy et al., 2011). *T. reesei* RUT-C30 was developed through mutagenesis, and its *cre1* was partially truncated, but the remaining *cre1* in *T. reesei* RUT-C30 can be transcribed into mRNA to synthesize a truncated CRE1, which consequently exerts positive regulation on the expression of genes encoding cellulolytic enzymes (Mello-de-Sousa et al., 2014). Recently, the transcription factor VIB1 was identified in *Neurospora crassa*, which not only functions to repress CRE1-mediated CCR and glucose sensing and metabolism, but also indirectly regulate the expression of hydrolytic enzyme genes (Xiong, Sun, & Glass, 2014). It was found that *vib1* deletion had a negative effect on cellulase production by *T. reesei* (Ivanova et al., 2017), and our recent study indicated that cellulase production could be improved by the overexpreesion of *vib1* (Zhang et al., 2018). Therefore, CTF1 might exert impact on CRE1 indirectly through the mediation of VIB1, since no evidence is available so far for their direct interactions.

The open reading frame of *ctf1* encodes a protein that is composed of 842 amino acid residues. The online software Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) from the Pfam database predicted two major functional domains in CTF1, which include a Zn (II)2Cys6 binuclear cluster for DNA binding at site R61-K100 with a confidence of 2.2×10^{-13} and a specific transcription factor domain at site R340-D434 with a confidence of 1.5×10^{-32}. Our in vitro binding experiment showed that CTF1 DNA binding domains did not bind to those genes encoding major cellulolytic enzymes such as *cbh1*, *cbh2*, *egl1*, and *egl2* (data not shown), but they specifically bound to *vib1*, *ace3*, and *rce1*, indicating that CTF1 directly regulates the expression of genes encoding transcription factors, and consequently mediates cellulase biosynthesis by *T. reesei* indirectly. These

could play different roles in regulating the expression of genes associated with cellulase production in *T. reesei*.

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The mutant *T. reesei* M2 was developed from *T. reesei* RUT-C30 by AZFP engineering. Analysis for the putative target genes of the AZFP identified the function of the putative gene *ctf1*, which encodes a novel transcription factor, and its deletion led to improved cellulase production by *T. reesei*. Comparative transcriptomic analysis suggested that *ctf1* might function through its interactions with multiple regulators. Our results from this study highlight the significance of such a strategy for developing robust strains for more efficient cellulase production as well as for elucidating functions of unknown genes in the genome of *T. reesei* for strain development through rational design.

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The authors declare that there are no conflict of interests.

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Additional supporting information may be found online in the Supporting Information section.

: Meng Q-S, Zhang F, Liu C-G, Zhao X-Q, Bai F-W. Identification of a novel repressor encoded by the putative gene *ctf1* for cellulase biosynthesis in *Trichoderma reesei* through artificial zinc finger engineering. *Biotechnology and Bioengineering*. 2020;1–14. https://doi.org/10.1002/bit.27321