



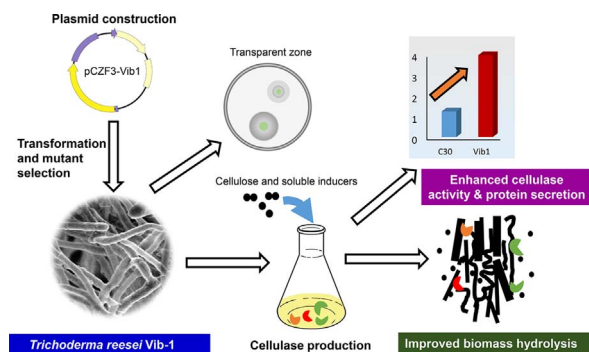
# Improvement of cellulase production in *Trichoderma reesei* Rut-C30 by overexpression of a novel regulatory gene *Trvib-1*



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## GRAPHICAL ABSTRACT



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## ABSTRACT

*Trichoderma reesei* is a widely used cellulase producer, and development of robust strains for improved cellulase production is of great interest. In this study, the gene *Trvib-1* encoding a putative transcription factor was overexpressed in *T. reesei* Rut-C30, and effects on cellulase production by the manipulation as well as corn stover degradation by the crude enzyme were investigated. Cellulase production and protein secretion were significantly improved in the culture of the recombinant *T. reesei* Vib-1, which were 200% and 219%, respectively, higher than that produced by the parent strain. Cellulase induction was enhanced in the presence of pure cellulose as well as various soluble inducers. Glucose released from the pretreated corn stover hydrolyzed by the crude enzyme in the recombinant strain was improved 40%. These results indicate that the overexpression of *Trvib-1* is a feasible strategy for producing cellulase to enhance bioconversion efficiency of lignocellulosic biomass.

## 1. Introduction

Lignocellulosic biomass is abundant natural resource for bioconversion applications, which is mainly composed of cellulose, hemicellulose, and lignin. Biofuels and bio-based chemicals produced from lignocellulosic biomass could alleviate the requirement for fossil fuels, which are also of environmental and social concerns (Kawaguchi

et al., 2016). Biological degradation of lignocellulose into fermentable sugars by cellulase enzymes is a promising and environmental-friendly approach (Gupta et al., 2016). However, so far the major challenge for lignocellulosic biorefinery is the high price of cellulase, and development of efficient strains for cellulase production is of great interest for lignocellulosic biorefinery (Taha et al., 2016; Zhao et al., 2016).

*Trichoderma reesei* Rut-C30 is an efficient producer of extracellular

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proteins, and is widely employed in cellulase production (Peterson and Nevalainen, 2012). The genome of *T. reesei* contains at least 200 genes encoding glycoside hydrolases (Häkkinen et al., 2012), which are involved in decomposing lignocellulose to release fermentable sugars. At least three types of cellulases are produced by *T. reesei*, namely, cellobiohydrolases (CBHs), endoglucanases (EGs) and  $\beta$ -glucosidases (BGLs), which hydrolyze cellulose synergistically to release glucose (Gupta et al., 2016). *T. reesei* also produces xylanase to digest hemicellulose into xylose. In addition, accessory proteins cooperate with cellulases to assist degradation of lignocellulosic biomass and also received increasing concerns (Gao et al., 2011).

Production of cellulase in *T. reesei* requires induction by cellulose or oligosaccharides. The oligosaccharide inducers include sophorose, lactose or cellobiose, as well as low cost inducer-mixture of glucose and  $\beta$ -disaccharides (MGD) prepared by transglycosylation reaction (Ilmen et al., 1997; Li et al., 2016c). The expression of cellulase genes in filamentous fungi generally depends on the regulators functioning as the active or repress transcription factors. So far, several transcription factors involved in the regulation of cellulase expression in *T. reesei* have been identified (Portnoy et al., 2011), including Xyr1, Ace2, Ace3 and Cre1. Among these regulators, Xyr1 and Cre1 are considered as the crucial activator and repressor, respectively, for cellulase production. Recently, new transcription factors such as Crz1, Rce1, have been identified for regulating cellulase production (Cao et al., 2017; Chen et al., 2016; Zhang et al., 2017). In our recent work, the artificial zinc finger protein (AZFP) library was employed to screen cellulase hyperproducer, and the identified AZFP mutant *T. reesei*-U3 showed enhanced cellulase production (Zhang et al., 2016). Further analysis of the putative target genes of the AZFP-U3 in the mutant revealed that *Trvib-1* showed increased transcription levels in *T. reesei*-U3, which promotes us to further investigate this novel transcriptional regulator.

VIB-1 is a homolog protein of *Saccharomyces cerevisiae* NDT80 functioning as a transcriptional activator of genes involved in meiosis (Montano et al., 2002). VIB-1 was mainly studied in the filamentous fungus *Neurospora crassa*, which is a major regulator of responses to nitrogen and carbon starvation. Mutations in *vib-1* fully relieved growth inhibition conferred by *het-c* vegetative incompatibility, which significantly reduced hyphal compartmentation and death rates in *N. crassa* (Xiang and Glass, 2002). VIB-1 is also involved in the production of extracellular proteases upon carbon and nitrogen starvation in *N. crassa* (Dementhon et al., 2006). Recently, it was found that VIB-1 regulates cellulase production in *N. crassa* through modulating the expression of other regulators such as CLR2 and COL26, and the function of VIB-1 is related to glucose signaling and carbon catabolite repression (CCR). It was also proved in the same study that the VIB-1 homolog TrVIB-1 in *T. reesei* was able to complement the deletion of *vib-1* in *N. crassa* (Xiong et al., 2014). However, so far no result on the function of *Trvib-1* in *T. reesei* has been published, and it will be interesting to explore whether overexpression of *Trvib-1* in *T. reesei* can be used to develop cellulase hyperproducer.

In this study, *Trvib-1* was overexpressed in *T. reesei* Rut-C30, and induction of cellulase production was investigated in the resultant recombinant. The crude enzyme produced by the mutant was further tested for biomass hydrolysis. Our results demonstrated that elevated expression of *Trvib-1* is an efficient approach to improve cellulase production in *T. reesei*, and we also revealed novel aspects of regulation by the VIB-1 family regulatory protein in filamentous fungi.

## 2. Materials and methods

### 2.1. Strains, plasmids, and growth conditions

*Escherichia coli* DH5 $\alpha$  was used for DNA manipulations and plasmid propagation. *Agrobacterium tumefaciens* AGL-1 was used for the *Agrobacterium tumefaciens*-mediated genetic transformation (ATMT) of *T. reesei*. The AGL-1 transformants with the target plasmid were

selected on the Luria-Bertani (LB) plates containing 50  $\mu$ g/mL kanamycin and 50  $\mu$ g/mL rifampicin (Sigma-Aldrich, USA).

*T. reesei* Rut-C30 was obtained from the ARS Culture Collection (NRRL, USA). Conidial suspensions were prepared by cultivating the strain on malt extract agar plate (Sangon Biotech, China) (20 g/L each of malt extract and agar) for 7 days. After being collected, the conidia were suspended, then gauze-filtered, and stored in sterilized water containing 20% glycerol at  $-80^{\circ}\text{C}$ .

### 2.2. Over-expression of gene *vib-1* in *T. reesei* Rut-C30

The PCR products from pCAMBIA1302 and pCB303 amplified using the primers (CAMBIA-F/CAMBIA-R, pCB303-F/pCB303-R) were ligated by Seamless Cloning Master Mix (Sangon Biotech, China), and the ligated product was named as pCZF1. After the pCZF1 was digested by *Bam*HI, the fragment (named as pCZF2) containing pCAMBIA backbone and hygromycin expression cassette was recovered. Meanwhile, the *pdcl* promoter (Ppdc) and the *cox 4* terminator (Tcox4) were amplified from the genome of *T. reesei* Rut-C30 with primer pairs of Ppdc-F/Ppdc-R and Tcox4-F/Tcox4-R, respectively, which were then fused to yield Tcox4-Ppdc by overlap extension PCR. Subsequently, the pCZF2 and Tcox4-Ppdc was combined to produce pCZF3 by Seamless Cloning Master Mix, in which the target fragment was inserted between the *Nco*I and *Xba*I sites for expression driven by the *pdcl* promoter.

Total RNA of *T. reesei* Rut-C30 was extracted and reversely transcribed into the cDNA according to the procedure of PrimeScript RT Reagen Kit (Takara, Japan), and the cDNA prepared was used as a template to amplify *Trvib-1*. The nucleotide sequence of *Trvib-1* was obtained by PCR with the primers (*vib1*-F/*vib1*-R). For over-expression of *Trvib-1* under the control of the constitutive promoter *pdcl*, the PCR product of *Trvib-1* amplified from the cDNA of *T. reesei* Rut-C30 was ligated into the vector pCZF3 digested by *Nco*I and *Xba*I using Seamless Cloning Master Mix, then the pCZF3-Vib1 identified with the primer pair *ver3*-F/*ver3*-R was transformed into *A. tumefaciens* AGL-1. Subsequently, the *A. tumefaciens* AGL-1 strain harboring pCZF3-Vib1 were co-cultured with *T. reesei* Rut-C30 for transforming the *Trvib-1* sequence into the parent strain *T. reesei* Rut-C30 according to a previously described protocol (Zhang et al., 2016). The putative transformants were picked up from the PDA medium with hygromycin (200  $\mu$ g/mL) and cephalixin (300  $\mu$ g/mL), and transferred to the malt extract agar plate followed by sub-culturing for five successive rounds. The conidia from putative transformants were grown in the PDA medium with 50  $\mu$ g/mL hygromycin B to verify the mitotic stability of transformants containing the *Vib-1* cassette. Furthermore, the *Vib-1* cassette were rescued from the stable transformants by PCR with the primer pair *ver3*-F/*ver3*-R, and the PCR products were further confirmed by sequencing. The primers used for construction and verification of pCZF3-Vib1 are listed in Table S1.

To determine the copy number of *Trvib-1* in the transformants, genomic DNA of the parent strain *T. reesei* Rut-C30 and the transformants was isolated and used as the template for quantitative PCR (qPCR). The qPCR method was essentially the same as that described by Solomon (Solomon et al., 2008). The qPCR analysis was performed by a Bio-Rad real-time PCR system with SYBR Premix Ex Taq kit (Takara, Japan) according to the manufacturer's instructions. The *Trvib-1* and *tefla* (translation elongation factor 1-alpha) genes were used to represent the single copy gene, respectively, by blasting *Trvib-1* and *tefla* sequences against *T. reesei* Rut-C30 genome sequence using the *T. reesei* Rut-C30 genome database ([http://genome.jgi.doe.gov/TrireRUTC30\\_1/TrireRUTC30\\_1.home.html](http://genome.jgi.doe.gov/TrireRUTC30_1/TrireRUTC30_1.home.html)). The integrated site of the pCZF3-Vib1 was determined by the thermal asymmetric interlaced PCR (TAIL-PCR) according to the procedure described elsewhere (Liu et al., 1995). The primers used for qPCR and TAIL-PCR analysis are listed in Table S1 and S2.

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