Revisiting overexpression of a heterologous β-glucosidase in *Trichoderma reesei*: fusion expression of the *Neosartorya fischeri Bgl3A* to *cbh1* enhances the overall as well as individual cellulase activities

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**Abstract**

**Background**: The filamentous fungus *Trichoderma reesei* has the capacity to secrete large amounts of cellulase and is widely used in a variety of industries. However, the *T. reesei* cellulase is weak in β-glucosidase activity, which results in accumulation of cellobiose inhibiting the endo- and exo-cellulases. By expressing an exogenous β-glucosidase gene, the recombinant *T. reesei* cellulase is expected to degrade cellulose into glucose more efficiently.

**Results**: The thermophilic β-glucosidase *NfBgl3A* from *Neosartorya fischeri* is chosen for overexpression in *T. reesei* due to its robust activity. *In vitro*, the *Pichia pastoris*-expressed *NfBgl3A* aided the *T. reesei* cellulase in releasing much more glucose with significantly lower amounts of cellobiose from crystalline cellulose. The *NfBgl3A* gene was hence fused to the *cbh1* structural gene and assembled between the strong *cbh1* promoter and *cbh1* terminator to obtain pRS-*NfBgl3A* by using the DNA assembler method. pRS-*NfBgl3A* was transformed into the *T. reesei* uridine auxotroph strain TU-6. Six positive transformants showed β-glucosidase activities of 2.3–69.7 U/mL (up to 175-fold higher than that of wild-type). The largely different β-glucosidase activities in the transformants may be ascribed to the gene copy numbers of *NfBgl3A* or its integration loci. The *T. reesei*-expressed *NfBgl3A* showed highly similar biochemical properties to that expressed in *P. pastoris*. As expected, overexpression of *NfBgl3A* enhanced the overall cellulase activity of *T. reesei*. The CBHI activity in all transformants increased, possibly due to the extra copies of *cbh1* gene introduced, while the endoglucanase activity in three transformants also largely increased, which was not observed in any other studies overexpressing a β-glucosidase. *NfBgl3A* had significant transglycosylation activity, generating sophorose, a potent cellulase inducer, and other oligosaccharides from glucose and cellobiose.

**Conclusions**: We report herein the successful overexpression of a thermophilic *N. fischeri* β-glucosidase in *T. reesei*. In the same time, the fusion of *NfBgl3A* to the *cbh1* gene introduced extra copies of the cellobiohydrolase 1 gene. As a result, we observed improved β-glucosidase and cellobiohydrolase activity as well as the overall cellulase activity. In addition, the endoglucanase activity also increased in some of the transformants. Our results may shed light on design of more robust *T. reesei* cellulases.

**Keywords**: *Trichoderma reesei*, Cellulase, β-Glucosidase, DNA assembler, *Neosartorya fischeri*
Background

Trichoderma reesei is one of the most important industrial filamentous fungi for its robust secretion of cellulases and is widely used in textile, paper, animal feed, and biofuel industries [1]. Trichoderma reesei produces a complete set of extracellular cellulase system including two exo-glucanases (cellobiohydrolases), five endoglucanases, and one β-glucosidase [2], which synergistically act to deconstruct cellulose into simple sugars [3]. The predominant components of the T. reesei secretome are cellobiohydrolase I (CBHI) and II (CBHII), which together account for 80–85% of the overall cellulases [4] and processively degrade crystalline cellulose from the reducing (CBHI) or non-reducing (CBHII) end, generating cellobiose as the main product [5]. Accumulated cellobiose inhibits the activity of endo- and exo-glucanases [6, 7], which can nonetheless be relieved through cellobiose hydrolysis by a β-glucosidase. Therefore, β-glucosidase plays an important role in cellulose degradation [8, 9]. Although T. reesei has a complete cellulase system, its β-glucosidase activity is weak and is thought to be a critical bottleneck of the T. reesei cellulase efficiency [10].

As an approach to circumvent the intrinsic insufficiency of β-glucosidase activity, expressions of either a heterologous or an endogeneous β-glucosidase have been tried, in which the supplemented β-glucosidases are frequently observed to improve the degrading efficiency of T. reesei cellulases [11–13]. With the increased cellulase activity, saccharification of pretreated cornstalk and barley straw is enhanced [13]. Despite a general cellulase activity-enhancing effect, it is noted from these studies that the β-glucosidases of varying origins have largely differing effects on the cellulase performance. This suggests that, by exploring the rich microbial diversity for novel β-glucosidases with potent capability, the T. reesei cellulase activity can be significantly improved.

We have previously reported the cloning and biochemical characterization of a new β-glucosidase NfBgl3A from a thermophilic filamentous fungus Neurosartorya fischeri [14]. This enzyme is robust, thermostable, and resistant to many chemicals and ions, making it an ideal candidate for supplementing T. reesei with the needed β-glucosidase activity. In this study, the Pichia pastoris-expressed NfBgl3A was first added in vitro to the T. reesei cellulase and evaluated for its ability to improve the cellulase performance. Then, the NfBgl3A β-glucosidase gene was overexpressed in T. reesei by fusing with the cbh1 gene and the overall and individual cellulase activities of the transformants were systematically analyzed.

Results

NfBgl3A enhanced T. reesei cellulase in hydrolyzing crystalline cellulose in vitro

The P. pastoris-expressed NfBgl3A is a robust thermophilic β-glucosidase (optimal temperature: 80 °C) with a specific activity of 2189 ± 1.7 U/mg against p-nitrophenol-β-d-glucopyranoside (pNPG). At the temperature optimum for T. reesei cellulases (50 °C), it still retains significant activity as high as above 50% [14]. The T. reesei TU-6 cellulase (i.e. the fermentation broth of TU-6 on day 6 post Avicel induction, 51.4 μg/mL) alone released mainly glucose (3747.6 ± 57.3 μM) and large amounts of cellobiose (158.4 ± 12.9 μM) with much lower amounts of cellobiose oligosaccharides with degrees of polymerization from three to six (Fig. 1). Increasingly adding the P. pastoris-expressed NfBgl3A into the TU-6 cellulase (20, 25, 33, and 50% percent, w/w) enhanced release of glucose from 4441.2 ± 46.7 (20% NfBgl3A) to 5334.4 ± 42.5 μM (50% NfBgl3A), whereas the amounts of cellobiose dramatically decreased to between 13.2 ± 2.1 (20% NfBgl3A) and 39.4 ± 5.7 (50% NfBgl3A) μM (Fig. 1). The amounts of cellobiotaose, cellobiose, and cellobiose were nearly unchanged for the reactions supplemented with NfBgl3A, whereas cellobiose slightly increased from 2.6 ± 0.9 (no NfBgl3A) to between 3.7 ± 0.9 (20% NfBgl3A) to 19.2 ± 0.7 μM (50% NfBgl3A) (Fig. 1). The results clearly demonstrated that NfBgl3A could enhance the T. reesei cellulase activity by decreasing cellobiose accumulation which in turn relieved the inhibition of cellulase.

Construction of the NfBgl3A expressing plasmid by DNA assembler

Since in vitro supplementing with NfBgl3A largely enhanced the T. reesei cellulase activity, it was expected that extracellular expression and extracellular secretion of NfBgl3A in T. reesei would improve the cellulase efficiency. A recombinant plasmid for overexpressing NfBgl3A in T. reesei was thus constructed using the fast DNA assembler method, which was initially employed to assemble metabolic pathways in Saccharomyces cerevisiae. The DNA assembler method utilizes the yeast highly efficient in vivo homology recombination system [15] and has been applied to construct high throughput gene knockout cassettes in T. reesei [16] but has seldom been used in constructing expressing cassettes in T. reesei. We used this method to rapidly construct the recombinant plasmid, namely pRS-NfBgl3A, expressing the new thermophilic β-glucosidase NfBgl3A using the multicopy plasmid pRS424 as the backbone (Fig. 2a). The expression cassette includes the cbh1 promoter (1700 bp), the cbh1 structural gene (1542 bp), the NfBgl3A gene (2315 bp), and the cbh1 terminator.
The cbh1 promoter, cbh1 structural gene, Nbgl3A gene, and the cbh1 terminator were all PCR amplified from corresponding T. reesei or N. fischeri genomic DNAs and co-transformed with the Xhol-linearized pRS424 into S. cerevisiae AH109. Long primers with >30 bp overhangs were designed (Table 1), which ensured efficient homologous recombination of the adjacent DNA fragments and correct assembly of the genes into an integrated expressing cassette. A Kex2 endoproteinase recognition site (RDKR, corresponding to the nucleotide sequence CGCGACAAGCGC) [17, 18] was designed between CBH1 and Nbgl3A, which allows automatic cleavage of Nbgl3A from the fusion protein CBH1–Nbgl3A during secretion. The integrity of the recombinant plasmid pRS-Nbgl3A was verified by both restriction digest by NotI (Fig. 2b) and DNA sequencing (data not shown).

The Nbgl3A transformants have largely different β-glucosidase activity

pRS-Nbgl3A was co-transformed with pSKpyr4, which provides uridine prototrophy, into T. reesei TU-6. After regeneration of the protoplasts, nine transformants (T1 to T9) were randomly picked out. The integration of Nbgl3A expression cassette in the genome of TU-6 was verified by PCR (Fig. 3a). Among these transformants, six strains, that were T1, T3, T4, T7, T8, and T9, showed the brightest band corresponding to the expected size (445 bp) and were consequently selected for further analyses and comparison with their parent strain TU-6.

TU-6 had low β-glucosidase activity over the whole induction phase (0.26–0.58 U/mL) as reported previously.

(1500 bp). The cbh1 gene was fused N-terminal to Nbgl3A gene, facilitating the latter’s expression and secretion, as well as introducing extra copies of this main cellobiohydro-lase gene. Note that the introns in the cbh1 and Nbgl3A structural genes were not removed because we believe that T. reesei should have the ability to correctly recognize and splice the mRNA precursors of the two fusion genes both originating from filamentous fungi.
(Fig. 3b). T4 had the highest β-glucosidase activity over the fermentation period (day 2 to 7 post cellulose induction), while T1, T3, and T7 showed similar β-glucosidase activity to each other but much higher than T8 and T9 (Fig. 3b). The β-glucosidase activity of T4, T1, T3, and T7 remarkably increased from day 2 to 6 post induction, whereas those of T8, T9, and TU-6 did not increase significantly (Fig. 3b). The highest β-glucosidase activity (69.7 ± 0.4 U/mL) for T4 was at day 6 post Avicel induction, which was approximately 175-fold of TU-6 (Fig. 3b). The maximum β-glucosidase activities of T1 (51.9 ± 0.2 U/mL), T7 (52.9 ± 1.4 U/mL), and T3 (42.5 ± 1.3 U/mL) were 130-, 132- and 106-fold of TU-6, respectively.

Table 1  Primers used in this study

| Primer  | Sequence (5′–3′)                                      | Usage                        |
|---------|------------------------------------------------------|------------------------------|
| Trcbh1pF | TGAGCGCGCGTAATCGACTCCTGACTATAGGCGAATTGATATCTAGAGTTGTGAAGTCGG | Cloning of cbh1 promoter     |
| Trcbh1Pr | GCACAAATACGACTCCCGGCGCTGGCCGATGCCGATCTCGGCGTGTTGACTATT   | Cloning of cbh1 promoter     |
| TrCBH1F  | CCAGCCGCGTAGCGCTGACAGGACGAGCCGACGTTCTGCAGGGCTGGACATTACGAC | Cloning of cbh1 structural gene |
| TrCBH1R  | CCACCTACGCTCCGGGCGAGCCGAGGGCTGGGCGAGGACGAGAC          | Cloning of cbh1 structural gene |
| Fbgl3AF  | TTACTACTCTCAGTGCCTGCGAGCGCGACAAGCGCTACGGTTCTGGTGGCAGCAACTGGGATC | Cloning of NfBgl3A gene      |
| Fbgl3AR  | TACGGGCTACTCCGAAGAATCTACCGGTGCGAGCTTGGGTACCCACACCCACCAACA CTTCGGTGGAGGTGCGAGTACG | Cloning of NfBgl3A gene      |

Fig. 3 Transformation and expression of NfBgl3A in T. reesei. a Determination of NfBgl3A integration in the genome of T. reesei transformants by PCR using the primers Yz-Trchb1F/Yz-NfBgl3AR. The expected size is 445 bp, corresponding to a fragment spanning the joint region of cbh1 structural gene and NfBgl3A. Lane M DNA molecular mass marker; lane T1–T9, the transformants of T1–T9. b The β-glucosidase activities in the fermentation broth of TU-6 and six transformants.

Correlation of the NfBgl3A copy numbers with the β-glucosidase activity of the transformants

The largely differing β-glucosidase activity in the NfBgl3A transformants suggested that NfBgl3A might be expressed with varying degrees. Indeed, the SDS-PAGE analysis of the culture supernatants indicated that more NfBgl3A was secreted by transformants T1, T3, and T4 (Fig. 4a). Since the copy number is one of the critical factors affecting heterologous gene expression [19], we determined the copy number of NfBgl3A in the transformants.

The qPCR analysis of the transformants indicated that the copy numbers of the NfBgl3A gene were different
in these strains. T4 and T7 had four copies of the NfB-gl3A gene, while T3 had three copies (Fig. 4b). The high copy numbers of these strains were in accordance with high level β-glucosidase expression in T. reesei. Although T1 had only one copy of NfBgl3A gene, its β-glucosidase activity was similar to that of T7 and even higher than T3, whereas T8 and T9 had only one gene copy of NfBgl3A but much lower β-glucosidase activity. This suggested that the integration locus other than the gene copy numbers might also affect the expression of the β-glucosidase.

The β-glucosidase NfBgl3A expressed in T. reesei has similar biochemical property to that expressed in P. pastoris

The fermentation broth of T4 on day 6 post induction with the highest β-glucosidase activity was used as crude NfBgl3A (trNfBgl3A). The crude enzyme showed similar biochemical properties to that expressed in P. pastoris [14]. trNfBgl3A had an optimum pH of 5.0 and temperature of 80 °C (Fig. 5a, c), which were the same as that expressed in P. pastoris. trNfBgl3A had approximately 80 % activity at pH 4.5–5.5. Additionally, trNfBgl3A was stable over a broad range of pH, retaining >65 % of the activity after incubation at pH 3.0–9.0 for 1 h (Fig. 5b). trNfBgl3A was thermostable at 70 °C for 1 h (Fig. 5d).

Improved overall and individual cellulase activities in the pRS-NfBgl3A transformed T. reesei

Since the highest β-glucosidase activity was detected on day 6 post Avicel induction (Fig. 3b), the fermentation broth of TU-6 and its NfBgl3A transformants at this time was subjected to protein concentration determination and cellulase assays using a variety of substrates. The protein concentration of the fermentation broth of TU-6 was 51.4 μg/mL. For T1, T3, T4, T7, T8, and T9, the values were 84.0, 109.2, 217.3, 78.8, 55.4, and 63.8 μg/mL, respectively. As mentioned before, T4 had the highest β-glucosidase activity, followed by T1, T3, and T7 (Figs. 3b, 6a). T8 and T9 had lower activities, which were nevertheless still higher than that of TU-6. Most of the pRS-NfBgl3A transformed T. reesei had improved overall cellulase activity against Avicel (Fig. 6b) and filter paper (Fig. 6c). Higher cellulase activity was commonly (T4, T1, T3, and T7) but not always (compare T7 and T8) associated with better β-glucosidase activity (Fig. 6b, c). While the elevated protein concentration in the transformants seemed to generally have a positive effect on the overall cellulase activity, a clearly proportional relationship could not be identified. Nevertheless, the higher the β-glucosidase activity was, the higher the protein concentration in the fermentation broth of the transformants (T1, T3, T4, and T7).

The CBHI activity increased in all transformants, with T1 (2.9 ± 0.1 U/mL), T3 (2.6 ± 0.1 U/mL), and T4 (2.6 ± 0.2 U/mL) being the highest, followed by T7 (1.7 ± 0.1 U/mL), T9 (1.3 ± 0.1 U/mL) and T8 (1.1 ± 0.1 U/mL) (Fig. 6d). TU-6 had a much lower CBHI activity of 0.4 ± 0.0 U/mL. This can be explained by extra copies of the cbh1 gene introduced in the transformants. It appeared that the endo-glucanase activity (CMCase activity, Fig. 6e) was affected in some transformants. While TU-6 had an endo-glucanase activity of 2.1 ± 0.2 U/mL, T4, T3, and T1 had much higher activities of 8.1 ± 0.1, 6.7 ± 0.2, and 4.8 ± 0.2 U/mL, respectively. T9 had a slightly increased activity of 2.9 ± 0.1 U/mL. Conversely, the endo-glucanase activities decreased for T7 (1.3 ± 0.0 U/mL) and T8 (0.8 ± 0.0 U/mL).

In accordance with the in vitro NfBgl3A-supplementation experiment, extracellular expression of NfBgl3A enhanced release of glucose by all transformants from
5873.2 ± 62.1 (T9) to 8666.9 ± 67.0 μM (T4) compared with 3747.6 ± 57.3 μM by TU-6, as analyzed by HPAEC-PAD (Fig. 7). Note that the concentrations of released glucose by all transformants, particularly by T1 and T4, were significantly higher than those released by TU-6 supplemented with different amounts of *P. pastoris*-expressed *Nf*Bgl3A. Higher amounts of released glucose are basically positively related to stronger β-glucosidase activities (T4 and T1). For all transformants, the concentrations of cellobiotetraose, cellopentaose, and cellohexaose were similar to those of TU-6. No increase of cellotriose was observed for any of the transformants, which may be ascribed to the low ratio of *Nf*Bgl3A against the total protein: the densitometry analysis by Image-Pro plus 6.0 software (Media Cybernetics Company, America) revealed that *Nf*Bgl3A expressed in T4 did not exceed 20 % of the total protein (data not shown). This complied with the in vivo supplementation experiments, in which only supplementation with >20 % *Nf*Bgl3A increased release of cellotriose (Fig. 1). Cellobiose in all transformants decreased to 19.7 ± 2.4 to 35.5 ± 9.1 μM, which were much lower than that released by TU-6 (158.4 ± 12.9 μM) (Fig. 7).

*Nf*Bgl3A displays significant transglycosylation ability in vitro
The *P. pastoris*-expressed *Nf*Bgl3A was tested for transglycosylation activity using glucose (150 mM) and cellobiose (250 mM) as the substrates, respectively. In a time-course analysis, *Nf*Bgl3A produced mainly cellobiose from glucose at the end of the reaction (300 min); however, significant amounts of sophorose and cellotriose also began to accumulate after 180 min (Fig. 8a). When cellobiose was used as the substrate, sophorose and cellotriose were also observed (Fig. 8b).
Discussion
It is well known that the T. reesei cellulase is weak in β-glucosidase activity, which may be ascribed to the relatively low expression level [20] as well as to the low specific activity (0.58 ± 0.032 U/mL) [9] of the extracellular Bgl1 (GenBank accession number: AAA18473.1). Inefficient β-glucosidase activity results into accumulation of cellobiose, which inhibits cellobiohydrolase and endoglucanase activities. Relieving such an inhibition by cellobiose requires more potent β-glucosidase in T. reesei cellulase. This can be accomplished by supplementing with commercially available β-glucosidases such as that from Aspergillus niger [21], or alternatively, by overexpressing either an endogenous or a heterologous β-glucosidase gene in T. reesei. Successful overexpressions include the T. reesei BGLI [4, 6] and BGLII [5], the P. decumbens Bgl1 [7], and the Periconia sp. β-glucosidase [8]. NfBgl3A is a thermophilic β-glucosidase from N.
ref. [13]. The transformants of NfBgl3A transformants showed a higher β-glucosidase activity, some of the transformants in our study. The high specific activity of T. reesei 34.31 ± 3.13 U/mL, which is nevertheless still lower than the parent strain TU-6 displayed low level of β-glucosidase activity, some of the NfBgl3A transformants (T4, T1, T7, and T3) had very high β-glucosidase activity. The varied β-glucosidase activities in different transformants may arise from the copy numbers of NfBgl3A integrated into the genome or are from the genomic loci of NfBgl3A integration, which has been commonly observed for filamentous fungi [14]. The β-glucosidase activities in NfBgl3A-overexpressing transformants were much higher than those of transformants expressing the T. reesei-BGL1 [11], P. decumbens-BGL1 [12], and Periconia sp.-BGL1 [13]. The transformants of P. decumbens-Bgl3A had a β-glucosidase activity of as high as 34.31 ± 3.13 U/mL, which is nevertheless still lower than the NfBgl3A transformants T4. Since the β-glucosidases appear to be similarly well expressed in T. reesei in these studies, the high specific activity of NfBgl3A is regarded to account for the higher β-glucosidase activity of the transformants in current study.

As expected, the overall cellulase activity was improved in most transformants to different extents. The maximal folds of activity improvement were 2.5-fold for T4 on Avicel and 2.4-fold for T1 on filter paper. Note that increased CBHI activity was also observed for all NfBgl3A-overexpressing transformants. This was not observed in previous studies, in which the cellobiohydrolases were only moderately changed or unchanged [11–13]. This is not unexpected since the NfBgl3A gene was fused to cbh1 gene in our study, which introduced additional copies of cbh1 in the transformants and is in contrast with the previous studies, in which the β-glucosidase genes were not fused to cbh1. Therefore, the extra copies of cbh1 gene should at least partially account for the increased cellobiohydrolase activity. The endoglucanase activities of the transformants, as reflected by the activities against CMC, were either largely up-regulated (for T1, T3, and T4), slightly up-regulated (for T9) or down-regulated (T7 and T8). This is also in contrast to previous β-glucosidase overexpression experiments, in which the endoglucanase activity of the transformants were down-regulated or kept unchanged [11–13]. The folds of overall cellulase activity on crystalline cellulose (Avicel and filter paper) are neither simple addition nor multiplication of individual activities. This could be explained by the complexity of the canonical cellulase system model, which has cellobiohydrolase, endoglucanase, and β-glucosidase [22]. In brief, the overall cellulase activity is a representation of the synergy of these different but complementary types of enzymes, which is related to both the enzyme components’ concentrations, ratios, and the reaction conditions such as substrate characters and concentrations.

The activities of endoglucanases in some transformants were largely altered, which were not observed in former β-glucosidase overexpression studies, either. Integration of NfBgl3A might have disrupted expression of a certain gene(s), which in turn affected expression of cellulases. In addition, a β-glucosidase may have transglycosylation activity that allows the enzyme to generate either cellobiose or other oligosaccharides with altered glycosidic linkages (such as sorghorose) [23] acting as inducers of cellulase. Indeed, we found that NfBgl3A have significant transglycosylation activities on both glucose and cellobiose, generating sorghorose as one of the products (Fig. 7a, b). It is well-known that sorghorose acts as a strong inducer of cellulase expression in T. reesei. We found addition of minor amounts of sorghorose (36.5 μM) in an Avicel-inducing TU-6 culture significantly enhanced the endo-glucanase activity from 2.8 ± 0.2 to 4.0 ± 0.4 U/mL at day 5 post Avicel induction (data not shown). Therefore, it is possible that sorghorose (or other oligosaccharides) generated by NfBgl3A might be involved in altering the expression profile of T. reesei. One may think that the growth condition of T. reesei (28–30 °C, and comparably lower concentrations of glucose and cellobiose in the

\[ \text{Fig. 7} \quad \text{Hydrolysis of Avicel by TU-6 and its transformants as analyzed by HPAEC-PAD. The fermentation broth of strain TU-6 and its transformants harboring nfbgl3A on day 6 post the induction was used to hydrolyze 20 mg/mL of Avicel. The reactions were carried out at 50 °C for 24 h. The released glucose and cellobiose/cellooligosaccharides were analyzed using HPAEC-PAD. C2-C6 represent cellobiose to cellohexa-} \]
is not optimal for NfBgl3A’s transglycosylation activity; however, note that NfBgl3A is a highly active enzyme and retains significant activity at 30 °C (Fig. 5c). In addition, sophorose is a potent inducer of cellulase; therefore, even minor amounts of sophorose might result in more effective induction of cellulase in presence of Avicel cellulose in those transformants. In spite of these pieces of evidence, nevertheless, more experiments are required in the future to better understand the mechanisms.

Fig. 8 NfBgl3A displayed transglycosylation activity. NfBgl3A was incubated with 150 mM of glucose (a) or 250 mM of cellobiose (b) at pH 5.0 and 70 °C. Samples were taken out periodically and analyzed by HPAEC-PAD. G1 glucose; C2–C6 cellobiose to cellohexaose.
underlying the findings. Our study revisited overexpression of a heterologous β-glucosidase gene in *T. reesei* by fusing it to the major cellulohydrolase gene *cbh1* and discovered that the overall as well as the individual cellulase activities in *T. reesei* were enhanced.

### Conclusions

The weak β-glucosidase activity is a bottleneck of the *T. reesei* cellulase in biomass deconstruction. In this study, the NfBgl3A gene coding for a highly active and thermo-tolerant β-glucosidase (NfBgl3A) from *N. fischeri* was successfully overexpressed in *T. reesei* TU-6 by fusing C-terminal to the major cellulohydrolase gene *cbh1*. Compared with in vitro addition of NfBgl3A to the *T. reesei* cellulase, overexpressing NfBgl3A in *T. reesei* with this special design not only enhanced the glucose yield more significantly, but also improved the cellulase-degrading performance of the major cellulase components, including the celllobiohydrolase and endoglucanase. NfBgl3A had transglycosylation activity and generated sophorose, a potent inducer of *T. reesei* cellulase, as a product from both glucose and cellobiose. This was hypothesized to be one of the reasons accounting for increased endoglucanase activity in some of the transformants.

### Methods

#### Microbial strains and culture conditions

The *Escherichia coli* Trans I-T1 (Transgen, Beijing, China) strain was used throughout this study for plasmid propagation. The *S. cerevisiae* AH109 (Clontech, San Francisco, CA) auxotrophic for adenine, histidine, leucine, methionine, tryptophan and uracil was used as the host for DNA assembler [15]. The *E. coli* was cultured in Luria-Bertani (LB) medium with appropriate concentrations of ampicillin at 37 °C when needed. The *S. cerevisiae* was cultivated in yeast peptone dextrose medium with adenine (YPDA) at 30 °C. The thermophilic filamentous fungus *Neosartorya fischeri* strain P1 (CGMCC 3.15369) [24] is stored in our lab. For extraction of its genomic DNA, *N. fischeri* P1 was grown in potato dextrose medium at 45 °C. The *T. reesei* strain was used throughout this study for plasmid propagation. The *E. coli* AH109 (Clontech, San Francisco, CA) auxotrophic for adenine, histidine, leucine, methionine, tryptophan and uracil was used as the backbone vector for yeast and *E. coli*, was used as the backbone plasmid. The plasmid was linearized by restriction digestion with Xhol (TaKaRa, Dalian, China). The *cbh1* promoter, structural gene, and the terminator were amplified from the genomic DNA of *T. reesei* TU-6 (primers listed in Table 1). The NfBgl3A gene was amplified from the genomic DNA of *N. fischeri* P1.

Supplementing with NfBgl3A in *T. reesei* cellulase

NfBgl3A was recombinantly produced in *P. pastoris* and purified according to the method described in [14]. Increasing amounts of NfBgl3A (0, 20, 25, 33, and 50 %, w/w) were added in vitro to the TU-6 cellulase on day 6 post Avicel induction and incubated with 20 mg/mL of Avicel in a total volume of 1.0 mL. The reactions were carried out at 50 °C for 24 h. After the reaction, the enzymes were removed from the reaction system by using a Nanoprep centrifugal 3 K device (Pall, New York, NY) and the released glucose and celloligosaccharides were analyzed using HPAEC-PAD (high performance anion exchange chromatography with pulsed amperometric detection).

For HPAEC-PAD analysis, 500 μL of appropriately diluted hydrolysates were applied to a Thermo Scientific Dionex ICS-5000 (Dionex Corporation, Sunnyvale, CA) high-performance liquid chromatography (HPLC) instrument equipped with a CarboPac PA100 guard column (4 by 50 mm) and an analytical column (4 by 250 mm). The flow rate was 1 mL/min and the analysis was carried out at 22 °C. The samples were resolved in a mobile phase gradient from 0 to 100 mM NaOH using glucose and celloligosaccharides (cellobiose to cellobextrinase) as standards.

### Construction of the NfBgl3A-expressing plasmid by DNA assembler

The DNA assembler method [15], which utilizes the highly efficient in vivo homologous recombination machinery of the baker's yeast, was chosen to rapidly construct the NfBgl3A-expressing plasmid. The pRS424 plasmid (New England Biolabs, Beverly, MA), a shuttle vector for yeast and *E. coli*, was used as the backbone plasmid. The plasmid was linearized by restriction digestion with Xhol (TaKaRa, Dalian, China). The *cbh1* promoter, structural gene, and the terminator were amplified from the genomic DNA of *T. reesei* TU-6 (primers listed in Table 1). The NfBgl3A gene was amplified from the genomic DNA of *N. fischeri* P1.

The Xhol-linearized pRS424 plasmid and the four DNA fragments, including *cbh1* promoter, *cbh1* structural gene, *cbh1* terminator, and the NfBgl3A gene, were mixed and transformed into AH109 by a LiAc-mediated chemical transformation method using a Yeastmaker Yeast Transformation System 2 kit (Clontech, San Francisco, CA). Briefly, 5 μL each of the linearized pRS424 and the four DNA fragments were mixed with equal volumes of Herring Sperm DNA (Promega, Madison, WI) and then added into 100 μL of *S. cerevisiae* competent cells, followed by adding 500 μL of PEG/LiAc. The mixture was incubated at 30 °C for 30 min. Thereafter, the mixture was added with 20 μL of DMSO, incubated at 42 °C for...
15 min, and then centrifuged. The supernatant was discarded and the cells were re-suspended with 1 mL of YPDA medium and recovered by incubating at 30 °C for 90 min. Aliquots of 50 μL were spread on synthetic drop-out nutrient medium without tryptophan (SD-Trp) plates for selection of positive transformants. The colonies were initially screened for assembly of the DNA fragments by PCR with the gene specific primers of Yz-Trchb1F/Yz-NfBgl3AR (Table 1) spanning the joint regions of cbh1 promoter and the NfBgl3A gene. The positive colonies were picked out, inoculated into the SD-Trp liquid medium, and cultured at 30 °C with rigorous shaking. The yeast plasmid was extracted using a Yeast Plasmid Miniprep kit (TIANGEN, Beijing, China) and chemically transformed into the Escherichia coli Trans I-T1 competent cells for propagation. Since a NptI restriction site was added to both the cbh1 promoter and terminator (Fig. 2a), the recombinant plasmids were verified for assembly of the five DNA fragments by NptI restriction digestion followed by agarose gel electrophoresis. The recombinant pRS424 plasmid with the correct assembly of the cbh1 promoter-structural gene, NfBgl3A gene, and the cbh1 terminator was named pRS-NfBgl3A.

Transformation of T. reesei
A pBluescript SK(+) plasmid, which was constructed by inserting the pyr4 expressing cassette from T. reesei into the pBluescript SK++ [28], was used for supplementation of the uridine auxotrophy [29]. The T. reesei TU-6 was cotransformed with the NptI-linearized pRS-NfBgl3A and ClaI/EcoRI-digested pSKpyr4 plasmids using the PEG-mediated protoplast transformation method essentially the same as that described previously [27]. Briefly, T. reesei Tu-6 was cultured in 100 mL of minimal medium supplemented with 2 % glucose at 28 °C for 16 h. The mycelia were collected by filtration through 8-layers of gauze. Then 10 mg/mL of lysing enzyme (Sigma-Aldrich, St. Louis, MO) and 1 mg/mL of Cellulase ONOZUKA R-10 were simultaneously added to the mycelia and the mixture was incubated at 30 °C with gentle shaking until large amounts of protoplasts were released. Five μg each of pSKpyr4 and pRS-NfBgl3A were used to co-transform the TU-6 protoplasts and the plates were incubated at 28 °C for 5–7 days until transformants could be clearly visualized. The successful transformation of the NfBgl3A-expressing cassette into TU-6 was determined by PCR using the primers Yz-Trchb1F/Yz-NfBgl3AR (Table 1).

Induction of NfBgl3A expression in T. reesei
Six NfBgl3A positive transformants (namely T1, T3, T4, T7, T8, and T9) were used for flask fermentation. Spores (10⁷) were inoculated into 50 mL of liquid minimal medium supplemented with 2 % glucose and cultured at 28 °C with agitation at 200 rpm for 48 h. The mycelia were filtered by passing the culture through a 200-mesh sifter and washed twice with the minimal medium without any carbon source. Two grams of the mycelia were added to 100 mL of the minimal medium supplemented with 2 % of Avicel. The culture was continued at 28 °C to induce production of the cellulases. From day 2 to 7 post inoculation, 2 mL of the culture were periodically collected for assay of the cellulase activities.

Assay of β-glucosidase and endo/exo-glucanase activities
For assay of the β-glucosidase activity, pNPG was used as the substrate. The reaction consisted of 400 μL of 1.25 mM pNPG dissolved in McIlvaine buffer (200 mM Na₂HPO₄, 100 mM citric acid, pH 5.0) and 100 μL of appropriately diluted enzymes. The mixture was incubated at 50 °C for 10 min and terminated by adding 1.5 mL of 1 M Na₂CO₃. After cooling down to room temperature, the optical density at 405 nm (OD₄₀₅) was measured. One unit of β-glucosidase activity was defined as the amount of enzyme that released 1 μmol of p-nitrophenol in one minute.

The cellulase activity can be presented as the overall cellulase activity or divided into endo-glucanase, exo-glucanase (or cellobiohydrolase), and β-glucosidase activity. For the overall activity, filter paper and Avicel were used as the substrate in the assays. For assay of the endo-glucanase activity, sodium carboxymethyl cellulose (CMC-Na) was used as the substrate. The reactions contained 100 μL of appropriately diluted enzymes and 900 μL of 1 % (w/v) substrate (for Avicel and CMC-Na) or one strip of Whatman No.1 filter paper (6 x 1 cm) in the McIlvaine buffer (pH 5.0) and were incubated at 50 °C for 1 h. Then 1.5 mL of the DNS reagent was added to the mixture and boiled for 5 min to terminate the reaction [30]. The OD₅₄₀ of the reactions was measured. One unit of the overall cellulase or endo-glucanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per min under the assay conditions.

To determine the activity of CBHI, the dominant cellulases produced by T. reesei, 4-methylumbeliferoyl-β-D-celllobioside (MUC) was used as the substrate [31]. Neither CBHII nor EGII react with the MUC substrate [32]. The standard reaction system contained 200 μL of 2 mM MUC with 25 μL of 1 M glucose, with or without 50 mM cellobiose, and 25 μL of appropriately diluted enzymes in the McIlvaine buffer (pH 5.0). The reaction was incubated at 50 °C for 20 min, followed by addition of 250 μL of 1 M Na₂CO₃. In the reactions with glucose only, high concentration of glucose inhibited the action of β-glucosidase on MUC. Therefore, MU was released by CBHI and endoglucanase I (EGI). In the reactions with glucose and cellobiose, cellobiose inhibited the
activity of CBHI. Therefore, the activity of CBHI could be calculated based on the difference of MUC released in the two reactions. One unit of CBHI activity was defined as the amount of enzyme that released 1 μmol of MU per min under the assay conditions.

Determining copy numbers by qPCR
To determine the copy numbers of the integrated NfBgl3A gene in the transformants, the fungal genomic DNAs were isolated using a Fungal DNA extraction kit (TianGen, Beijing, China) and were used as the template for quantitative PCR (qPCR). The qPCR method was essentially the same as that described by Solomon [33]. The Tef1α (translation elongation factor 1-alpha) gene was used to represent a single copy gene [34]. The qPCR was performed with the SYBR Green Real-time PCR Master Mix (TOYO-OBO, Osaka, Japan) in a CFX96 Real Time PCR Detection System (Bio-Rad, Hercules, CA), using 96-well white PCR plate sealed with ABsolute qPCR seals (Thermo, Waltham, MA). The primers used for the genes were listed in Table 1. A melt curve analysis was performed at the end of each run from 55 to 95 °C with a ramp speed of 0.5 °C to ensure specific sequences amplification of all primers and only one melting temperature on the melting curve. To determine the efficiencies between 90–110 % of all reactions, the genomic DNA samples were diluted serially to construct standard curves and a temperature gradient was used in RT-PCR. The 25 μL reactions each contained 1 μL of diluted DNA as the template, 12.5 μL of 2× SYBR Green Real time PCR Master Mix, 1 μL each of the primers (0.4 M), and 9.5 μL of H₂O. Equal volume of water instead of DNA was used as a negative control. The qPCR was performed as follows: 94 °C for 30 s, 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The data were analyzed using Bio-Rad IQ5 2.1 Standard Edition Optical System Software. The products of qPCR were also analyzed by electrophoresis on 2.0 % agarose gels.

Hydrolysis of crystalline cellulose Avicel by TU-6 and its transformants
The fermentation broth of TU-6 and the transformants on day 6 post induction was used to hydrolyze Avicel. The reaction mixture contained 20 mg/mL of Avicel and appropriately diluted broth. The total volume of all these reaction was 1.0 mL. The reactions were carried out at 50 °C for 24 h. After the reaction, the enzymes were removed from the reaction system by using a Nanosep centrifugal 3K device before HPAEC-PAD analysis.

Biochemical characterization of the β-glucosidase NfBgl3A expressed in T. reesei
To biochemically characterize the recombinant β-glucosidase NfBgl3A expressed in T. reesei, the crude enzyme (i.e. the fermentation broth) of T4 on Day 6 post Aivcel induction was used for analyses. Using pNPG as the substrate, the pH optimum of the T. reesei-expressed NfBgl3A (trNfBgl3A) was determined in a series of buffers including glycine–HCl (pH 1.0–3.0), McIlvaine buffer (pH 3.0–8.0), Tris–HCl (pH 7.0–9.0), and glycine–NaOH (pH 9.0–12.0) all with a concentration of 100 mM. To determine the optimal temperature of trNfBgl3A, the reaction was performed at temperatures ranging from 30 to 90 °C at pH 5.0. For pH stability analysis, trNfBgl3A was pre-incubated in a wide range of pH from 1.0 to 12.0 without substrate at 37 °C for 1 h. Then the residual activities were measured under optimal conditions (pH 5.0 and 80 °C). The thermostability of trNfBgl3A was determined by measuring the residual β-glucosidase activity after pre-incubating the crude enzyme at 70, 75 or 80 °C for different periods of time.

Transglycosylation of NfBgl3A
To determine if NfBgl3A has transglycosylation capacity, the purified NfBgl3A recombinantly produced in P. pastoris was incubated with 150 mM of glucose or 250 mM of cellulose at pH 5.0 and 70 °C for a serial of time (0, 60, 180, and 300 min). The total volume of all these reaction was 1.0 mL, which included 900 μL of glucose or cellulose and 100 μL of appropriately diluted NfBgl3A. After the reaction, the enzyme was removed from the reaction system by a Nanosep centrifugal 3K device before HPAEC-PAD analysis. Glucose, sorbose, and cellobiose (cellobiose to cellohexaose) were used as standards.

Abbreviations
GH: glycoside hydrolase; DNS: 3,5-dinitrosalicylic acid; HPAEC-PAD: high-performance anion exchange chromatography with pulsed amperometric detector.

Authors’ contributions
XS and BY conceived and designed the experiments. XX, XQ, and YW performed the experiments. XX, RM, and HL analyzed the data. XX and XS wrote the manuscript. RM, HL, and BY reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.
Availability of data and materials

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References

1. Cherry JR, Fidantsef AL. Directed evolution of industrial enzymes: an update. Curr Opin Biotech. 2003;14:438–43.
2. Martinez D, Berka RM, Henrissat B, Saloheimo M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, et al. Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). Nat Biotechnol. 2008;26:553–60.
3. Sipos B, Beniko Z, Dienes D, Reczey K, Viikari L, Siika-aho M. Characterisation of specific activities and hydrolytic properties of cell-wall-degrading enzymes produced by Trichoderma reesei Rut. C30 on different carbon sources. Appl Biochem Biotechnol. 2010;161:347–64.
4. Miettinen-Cinonnen A, Palohmeio M, Lanto R, Suominen P. Enhanced production of cellulohydrolyses in Trichoderma reesei and evaluation of the new preparations in biofinishing of cotton. J Biotechnol. 2005;116:305–17.
5. Kovuila A, Reikanninen T, Ruohonen L, Valkeajarvi A, Claeyssens M, Teleman O, Kleywegt GJ, Rouvinen J, Jones TA, Teeri TT. The active site of Trichoderma reesei cellobiohydrolase II: the role of tyrosine 169. Protein Eng. 1996;9:691–9.
6. Gruno M, Valjamae P, Pettersson G, Johansson G. Inhibition of the Trichoderma reesei cellulases by cellobiose is strongly dependent on the nature of the substrate. Biotechnol Bioeng. 2004;86:503–11.
7. Penttila M, Nevalainen H, Ratto M, Salminen E, Knowles J. A versatile transformation system for the cellulolytic filamentous fungus Hypocrea jecorina. Seiboth B. Gene targeting in a nonhomologous end joining deficient Trichoderma reesei Rut-C30 with high specific activity, broad substrate specificity and significant hydrolysis ability of soymilk. Bioreasour Technol. 2014;153:361–4.
8. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev. 2002;66:673–9.
9. Shaof Z, Zhao H. DNA assemblers, an in vivo genetic method for rapid construction of biochemical pathways. Nucleic Acids Res. 2009;37:e16.
10. Schuster A, Bruno KS, Collett JR, Baker SE, Seiboth B, Kubicek CP, Schmolz M. A versatile toolkit for high throughput functional genomics with Trichoderma reesei. Biotechnol Biofuels. 2012;5:1.
11. Palohmeio M, Mantyla A, Kallio J, Suominen P. High-yield production of a bacterial xylanase in the filamentous fungus Trichoderma reesei requires a carrier polypeptide with an intact domain structure. Appl Environ Microb. 2003;69:7073–82.
12. Li XL, Skory CD, Ximenes EA, Jordan DB, Diem BS, Hughes SR, Cotta MA. Expression of an AT-rich xylanase gene from the anaerobic fungus Omnobium sp strain PC-2 in and secretion of the heterologous enzyme by Hypocrea jecorina. Appl Microbiol Biot. 2007;74:1264–75.
13. Graesse S, Haas H, Friedlin E, Kurnsteiner H, Stoffler G, Redi B. Regulated system for heterologous gene expression in Penicilium chrysogenum. Appl Environ Microb. 1997;63:753–6.
14. Yang X, Ma R, Shi P, Huang H, Bai Y, Wang Y, Yang P, Fan Y, Yao B. Molecular characterization of a highly-active thermostable β-glucosidase from Neosartorya fischeri P1 and its application in the hydrolysis of soybean isoflavone glycosides. PLoS One. 2014;9:e106785.
15. Shao Z, Zhao H. DNA assemblers, an in vivo genetic method for rapid construction of biochemical pathways. Nucleic Acids Res. 2009;37:e16.
16. Schuster A, Bruno KS, Collett JR, Baker SE, Seiboth B, Kubicek CP, Schmolz M. A versatile toolkit for high throughput functional genomics with Trichoderma reesei. Biotechnol Biofuels. 2012;5:1.
17. Palohmeio M, Mantyla A, Kallio J, Suominen P. High-yield production of a bacterial xylanase in the filamentous fungus Trichoderma reesei requires a carrier polypeptide with an intact domain structure. Appl Environ Microb. 2003;69:7073–82.
18. Li XL, Skory CD, Ximenes EA, Jordan DB, Diem BS, Hughes SR, Cotta MA. Expression of an AT-rich xylanase gene from the anaerobic fungus Omnobium sp strain PC-2 in and secretion of the heterologous enzyme by Hypocrea jecorina. Appl Microbiol Biot. 2007;74:1264–75.
19. Graesse S, Haas H, Friedlin E, Kurnsteiner H, Stoffler G, Redi B. Regulated system for heterologous gene expression in Penicilium chrysogenum. Appl Environ Microb. 1997;63:753–6.
20. Foreman PK, Brown D, Dankmeyer L, Dean R, Diener S, Dunn-Coleman NS, Goedegebuur F, Houfek TD, England GJ, Kelley AS, et al. Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus Trichoderma reesei. J Biol Chem. 2003;278:31988–97.
21. Chauve M, Mathis H, Orc C, Casanove D, Monot F, Lopes Ferreira N. Comparative kinetic analysis of two fungal β-glucosidases. Biotechnol Biofuels. 2010;3:33.
22. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev. 2002;66:673–9.
23. Uchiyama T, Miyazaki K, Yaoi K. Characterization of a novel β-glucosidase from a compost microbial metagenome with strong transglycosylation activity. J Biol Chem. 2013;288:18325–34.
24. Wang H, Shi P, Luo H, Huang H, Yang P, Yao B. A thermostable α-galactosidase from Neosartorya fischeri P1 with high specific activity, broad substrate specificity and significant hydrolysis ability of soymilk. Bioreasour Technol. 2014;153:361–4.
25. Gruber F, Visser J, Kubicek CP, de Graaff LH. The development of a heterologous transformation system for the cellulolytic fungus Trichoderma reesei based on a pyrG-negative mutant strain. Curr Genet. 1990;18:71–6.
26. Guangtao Z, Hartl L, Schuster A, Polak S, Schmolz M, Wang T, Seidl V, Seiboth B. Gene targeting in a nonhomologous end joining deficient Hypocrea jecorina. J Biotechnol. 2009;139:146–51.
27. Penttila M, Nevalainen H, Ratto M, Salminen E, Knowles J. A versatile transformation system for the cellulolytic filamentous fungus Trichoderma reesei. Gene. 1987;61:155–64.
28. Smith JL, Bayliss FT, Ward M. Sequence of the cloned pyr4 gene of Trichoderma reesei and its use as a homologous selectable marker for transformation. Curr Genet. 1991;19:27–33.
29. Qin LN, Cai FR, Dong XR, Huang ZB, Tao Y, Huang JZ, Dong ZY. Improved production of heterologous lipase in Trichoderma reesei by RNAi mediated gene silencing of an endogenically highly expressed gene. Bioreasour Technol. 2012;109:116–22.
30. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31:426–8.
31. van Tilbeurgh H, Claeyssens M. Detection and differentiation of cellulase components using low molecular mass fluorescent substrates. FEBS Lett. 1985;187:283–8.
32. Reinkainen T. The cellulose-binding domain of cellobiohydrolase I from Trichoderma reesei: Espoo: VTT Publications. 1994.
33. Solomon P, Ipcho S, Hane J, Tan K-C, Oliver R. A quantitative PCR approach to determine gene copy number. Fungal Genet Rep. 2008;55:5–8.
34. Nakani T, Atlatori E, Penttila M. Isolation of Trichoderma reesei genes highly expressed on glucose-containing media: characterization of the tef1 gene encoding translation elongation factor 1 alpha. Gene. 1993;136:313–8.