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ORIGINAL RESEARCH PAPER

Disruption of non-anchored cell wall protein NCW-1 promotes cellulase production by increasing cellobiose uptake in *Neurospora crassa*

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Abstract
Objectives To elucidate the mechanism of cellulase signal transduction in filamentous fungi including the components of the cellulase induction pathway.
Results *Neurospora crassa ncw-1* encodes a non-anchored cell wall protein. The absence of *ncw-1* increased cellulase gene expression and this is not due to relieving carbon catabolite repression mediated by the *cre-1* pathway. A mutant lacking genes encoding both three major β-glucosidase enzymes and NCW-1 (Δ3βGΔ*ncw-1*) was constructed. Transcriptome analysis of the quadruple mutant demonstrated enhanced expression of cellodextrin transporters after *ncw-1* deletion, indicating that *ncw-1* affects cellulase expression and production by inhibiting the uptake of the cellodextrin.
Conclusions NCW-1 is a novel component that plays a critical role in the cellulase induction signaling pathway.

Keywords Cellobiose uptake · Cellodextrin transporter · Cellulase induction · Cell wall protein · *Neurospora crassa*

Introduction

Filamentous fungi secrete a wide variety of lignocellulolytic enzymes that convert complex biomass into fermentable sugars. This capacity has been exploited by industry to produce biofuels and biochemicals (Sun and Cheng 2002). However, the high cost of lignocellulolytic enzyme production is a major bottleneck in the utilization of lignocellulosic biomass. A premise of the current work is that a better understanding of cellulase induction pathway in filamentous fungi could provide novel strategies to increase cellulase yield.

The filamentous fungus, *Neurospora crassa*, a native degrader of lignocellulose, has been investigated as a novel model for cellulase induction and synthesis. Using this system, β-glucosidases have been shown to play critical roles in cellulase induction. Deletion of three major β-glucosidases led to rapid and efficient induction of cellulase on cellobiose (Znameroski et al. 2012). In addition, two cellodextrin

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transporters, CDT-1 and CDT-2, contribute to both cellobiose uptake and cellulase inducer sensing (Znameroski et al. 2014). Although β-glucosidase and the cellodextrin transporter have been shown to be key components of the cellulase induction pathway (Zhang et al. 2013; Zhou et al. 2012), little is known about how this pathway is regulated and what other components might participate in this pathway.

The fungal cell wall is the interphase with the surrounding environment and plays crucial roles in signal sensing and transduction. *N. crassa* NCU05137 encodes a non-anchored cell wall protein (NCW-1). Deletion of this gene (or its homolog PDE01641) led to enhanced cellulase production in *N. crassa* (Tian et al. 2009) and *Penicillium decumbens* (Zhang et al. 2012). However, the mechanism by which deletion of *ncw-1* enhanced cellulase production is unclear. To decipher this mechanism, we further investigated the critical roles of NCW-1 during cellulase induction. Here, we demonstrate that the improvement of cellulase production in the Δ*ncw-1* is not a consequence of relief from carbon catabolite repression, and provide evidence that NCW-1 is indeed involved in cellulase induction by influencing cellodextrin transporter expression.

Materials and methods

Strains and culture conditions

Neurospora crassa wild-type (WT) strain and all single-gene deletion strains were obtained from the Fungal Genetics Stock Center. *N. crassa* strains were grown on Vogel's minimal media (MM) (Tian et al. 2009). For liquid cultures, 10^7 conidia were inoculated into 100 ml Vogel's salts with 2% (w/v) Avicel or 2% (w/v) sucrose in a 250 ml Erlenmeyer flask and cultivated at 25 °C in constant light and shaking (200 rpm).

Complementation of Δ*ncw-1* and subcellular localization of NCW-1-GFP in *N. crassa*

WT genomic DNA was extracted as described previously (Lin et al. 2011). To complement Δ*ncw-1*, the ORF of *ncw-1* was cloned by PCR with primers ORF-F and ORF-R, and then the fragment was inserted into the *Xba*I and *Pac*I sites of pMF272 to form pMF272-Pc-ncw-1-gfp. The promoter of *ncw-1* was cloned with primers PF and PR, and inserted into the *Not*I and *Xba*I sites of pMF272-Pc-ncw-1-gfp to result in pMF272-Pn-ncw-1-gfp. Plasmid DNA (10 μg) was transformed into a (*his-3*; Δ*ncw-1*) strain as described (Wang et al. 2015). The resulting complemented strains were termed Pc-*ncw-1* and Pn-*ncw-1*. Observations of NCW-1 localization were performed with a fluorescence microscope.

To compare the differences in cell surface structure between the WT and Δ*ncw-1*, fungal conidia were incubated in MM at 25 °C for 40 h. The harvested mycelia were fixed in 0.1 M phosphate buffer (pH 7.2) containing 2.5% (w/v) glutaraldehyde. The samples were rinsed in thrice-distilled PBS, and dehydrated with a series of ethanol solutions (30–100%, v/v). After which the samples were dried by critical point drying (Leica CPD300) and coated with platinum (Hitachi E-1045). Observations were conducted using a scanning electron microscope (Hitachi SU8010) operating at 3 kV.

Enzyme activity and cellobiose consumption assay

Total extracellular proteins were determined with the Bradford assay (Bio-Rad Protein Assay kit). The endoglucanase and endoxylanse activities were assayed using the azo-CMC and azo-xylan kits (Megazyme), respectively, according to manufacturer's instructions. *p*-Nitrophenyl-D-cellobioside (pNPC) and *p*-nitrophenyl-*β*-D-glucopyranoside (pNPG) were used as the substrates for the measurement of exoglucanase and β-glucosidase activities as described previously (Wang et al. 2015). The amount of cellobiose remaining in the supernatant was determined by HPLC with Aminex HPX-87H column (Bio-Rad) (Znameroski et al. 2012). All assays were performed in triplicate.

Biomass assay and rheological measurement

Mycelia grown on 2% (w/v) Avicel for 7 days were harvested, dried, and weighed. The relative proportion of mycelia to residual Avicel was calculated by solubilizing fungal biomass in boiling acetic nitric reagent as described (Li et al. 2014). The residual mass was washed thoroughly, dried, and reweighed.

Rheological properties of fermentation broth were determined by using a Brookfield digital HBDV-III Ultra rheometer according to the manufacturer's instructions. Viscosity was measured as the torque

exerted by the mycelial sample on the rheometer impeller at 150 rpm. Torque was recorded after the reading had stabilized (45–60 s). All assays were performed in triplicate.

RNA sequencing and data analysis

For gene expression analysis, mycelia were pre-cultured for 20 h in MM and transferred into Vogel's salts containing 1% (w/v) cellobiose for an additional 4 h. Total RNA from frozen samples was extracted and treated as described previously (Li et al. 2014). RNA-seq was performed on the Illumina HiSeq 2000 platform of Beijing Genomics Institute (BGI, Shenzhen, China). Filtered clean reads were mapped against predicted transcripts from the *N. crassa* OR74R genome (version 12) using Tophat (version 2.0.8b). The alignment results were stored in SAM format files for subsequent analysis. The counts of reads that uniquely mapped to only one gene were calculated for each gene by Htseq-count (http://www-huber.embl.de/users/anders/HTSeq) using SAM files and genome annotation as input. Abundance for each transcript was calculated using the reads per kilobase per million (RPKM). Genes with altered expression were performed by using R package NOISeq (version 2.6.0) (Q value $\geq$ 0.95 used as threshold). Profiling data are listed in Supplementary Table 1 and the sequence data produced in this study can be accessed (GEO: GSE73838).

Quantitative real time-PCR

Quantitative PCR was performed using a CFX96 real-time PCR detection system (Bio-Rad) with reagents from TOYOBO (One-step qPCR Kit). All assays were performed in triplicate with *actin* (NCU04173) as the endogenous standard, according to the manufacturer's instruction. The primers used in this study are listed in Supplementary Table 2.

Results and discussion

Deletion of *ncw-1* increases biomass accumulation and broth viscosity

During 7 days growth on 2% (w/v) Avicel medium the broth of the *ncw-1* mutant became more viscous than that of WT strain. The viscosity of Δ*ncw-1* was significantly increased by twofold compared to that of WT strain (Fig. 1a). It is well known that both mycelial morphology and biomass concentration have significant influences on the rheological properties of a fermentation broth. Scanning electron microscopy demonstrated that Δ*ncw-1* exhibited much smoother surface than the WT (Supplementary Fig. 1). Nevertheless, the *ncw-1* mutant still showed a clump type of morphology in submerged culture, which was indistinguishable from that of WT. So we speculated that the *ncw-1* mutant might grow faster on Avicel. Figure 1b shows that the biomass of the Δ*ncw-1* mutant was 47% higher than that of WT strain. Thus, the change in rheological properties is probably due in part to increased growth.

Additionally, the complementation analysis indicated that complementing Δ*ncw-1* with a genomic clone of *ncw-1* produced a strain indistinguishable from the wild type in all the phenotypic assays performed in this study (data not shown).

NCW-1 mainly localizes to the cell wall and hyphal tip

In order to obtain more detailed information about subcellular localization, we constructed a *C*-terminal GFP-tagged NCW-1 in the Δ*ncw-1* strain under the control of the *ccg-1* promoter (Pc-*ncw*-1 strain). Fluorescence microscopy indicated that the NCW-1 was localized to the surface of spores and hyphae. Intriguingly, NCW-1-GFP also accumulated at the hyphal tip area (Fig. 1c), where protein secretion occurs (Wosten et al. 1991). Since the cell wall at the hyphal tip is mostly composed of primary wall material with a comparatively porous structure, we hypothesized that NCW-1 could rapidly pass through this region and diffuse into the medium, which could also explain why NCW-1 comprises as much as 1.5% of the supernatant protein by weight (Phillips et al. 2011).

The enhanced cellulase production in the Δ*ncw-1* mutant is not due to decreased carbon catabolite repression

The zinc finger transcription factor *cre-1* is a key regulator in carbon catabolite repression. Deletion of *cre-1* or its homolog could lead to enhanced cellulase

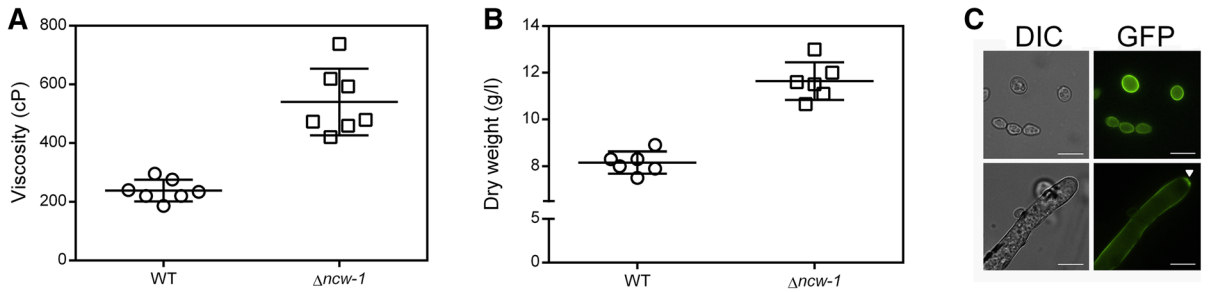

Fig. 1 Phenotype of Δ*ncw-1* grown on Avicel. Conidia from Δ*ncw-1* and wild-type (WT) strains were separately inoculated into Avicel medium and batch cultured for 7 days. The culture viscosity (**a**) and biomass accumulation (**b**) were measured. Values represent the means of at least three replicates, *error bars* show standard deviation. **c** Microscopic observation of NCW-1 subcellular localization in *N. crassa*. *Scale bar* is 10 μm

production (Sun and Glass 2011). Since the disruption of *ncw-1* enhances expression of cellulase genes in *N. crassa*, it is imperative to know whether NCW-1 exerts its function on cellulase expression via CRE1. The results indicated that the Δ*ncw-1*Δ*cre-1* strain exhibited stronger protein production capability than either the Δ*cre-1* strain or the Δ*ncw-1* strain (Fig. 2). The endoglucanase activity, β-glucosidase activity and secreted protein in the double deletion strain were 39, 44 and 77% higher than those of Δ*ncw-1*. Surprisingly, the xylanase activity of Δ*ncw-1*Δ*cre-1* increased to about 1.5-fold that of the reference strain Δ*ncw-1*. These results confirm that deleting both *ncw-1* and *cre-1* had a significant synergistic effect on lignocellulase production. On the other hand, a real-time PCR analysis indicated that the disruption of *ncw-1* dramatically increased production of the *cre1* transcript on Avicel medium (Supplementary Fig. 2). In conclusion, these data indicate that enhanced cellulase gene expression in the Δ*ncw-1* is not the result of relief from catabolite repression mediated by *cre-1*.

Deletion of NCW-1 improves cellobiose uptake in *N. crassa*

Based on previous findings, a *N. crassa* mutant carrying deletions of genes encoding three major β-glucosidases (Δ3βG) is an excellent system for dissecting the cellulase induction pathway (Znameroski et al. 2012). To test the effect of *ncw-1* on cellulase gene expression under cellobiose condition, a Δ3βGΔ*ncw-1* quadruple mutant was constructed by crossing. Cellobiose consumption and cellulase production assays were performed in Δ3βGΔ*ncw-1* and Δ3βG strains. The consumption assay indicated that the Δ3βGΔ*ncw-1* strain had dramatically increased cellobiose consumption compared with its parental strain. Cellobiose was almost depleted after 48 h of Δ3βGΔ*ncw-1* cultivation, while there was still 6 g cellobiose/l in the culture of Δ3βG (Fig. 3a). Surprisingly, the Δ3βGΔ*ncw-1* strain displayed a faster cellulase induction and a significant enhancement of cellulase production when transferred to cellobiose. The protein production of Δ3βGΔ*ncw-1* was up to 141 μg/ml after 24 h induction, which was significantly higher than that of Δ3βG strain (below 10 μg/ml) (Fig. 3b).

To observe the role of *ncw-1* in cellulase expression, transcriptomic analysis of Δ3βGΔ*ncw-1* strain on cellobiose was performed using Δ3βG as the control. Consistent with the cellulase production phenotype on 1% (w/v) cellobiose, the cellulase genes (NCU07340, NCU09680 and NCU00762), hemicellulase genes (NCU02855, NCU05955 and NCU07326), lytic polysaccharide monooxygenase genes (NCU08760, NCU02916, NCU02240 and NCU01050) and the cellobiose dehydrogenase gene (NCU00206) have significantly higher expression levels in Δ3βGΔ*ncw-1* (Fig. 4a). Compared with Δ3βG, the expression level of *cdt1* and *cdt2* in Δ3βGΔ*ncw-1* were up-regulated 19- and 15-fold, respectively (Fig. 4b), further showing that cellobiose uptake is dramatically accelerated in Δ3βGΔ*ncw-1* consistent with more efficient transport of cellobiose into the cell. However, a detailed mechanistic description of how the *ncw-1* influences *cdt* gene expression requires further study.

Cellulase- and hemicellulase-related transcriptional factors *clr1*, *clr2* and *xlr1* were also up-

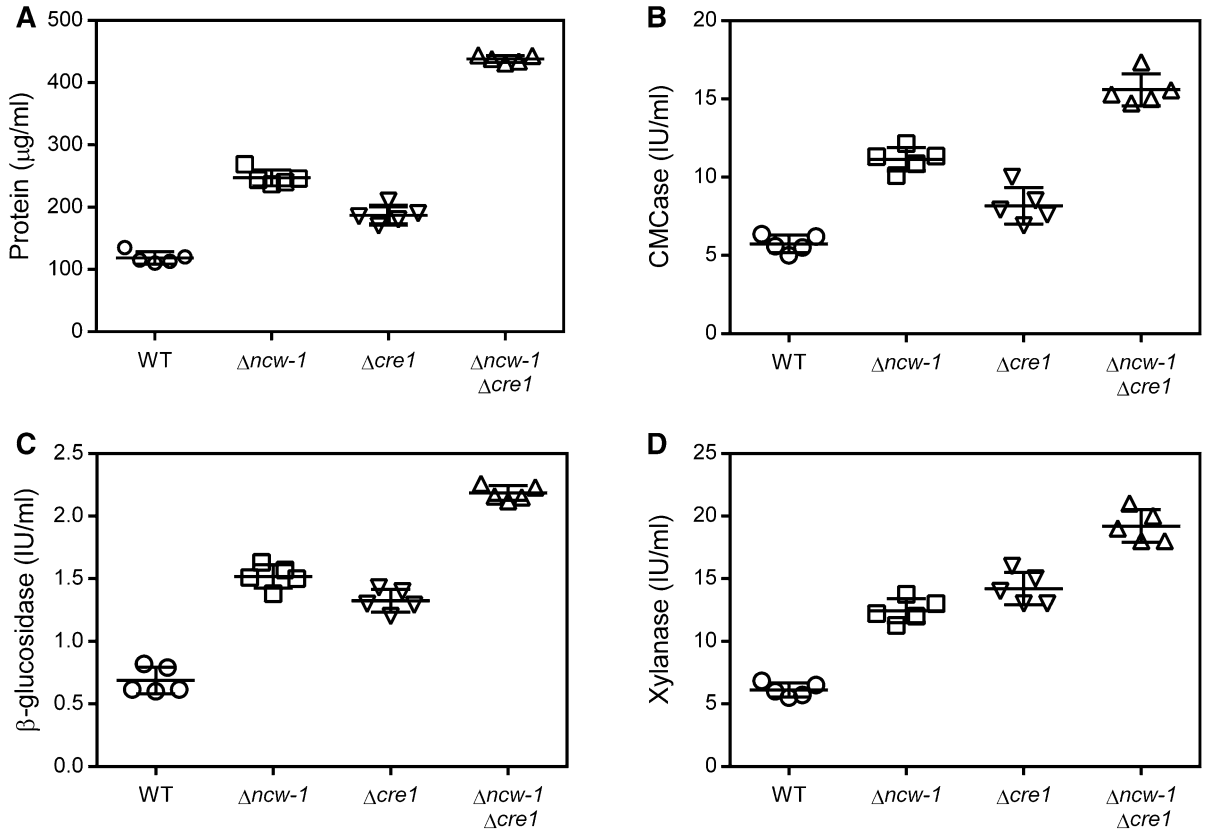

Fig. 2 Phenotype of Δ*cre-1*Δ*ncw-1* double mutant when grown on Avicel medium. Conidia from Δ*cre-1*Δ*ncw-1*, Δ*cre-1*, Δ*ncw-1* and wild-type (WT) strains were separately inoculated into Avicel medium and batch cultured for 7 days. The total extracellular protein concentration (**a**), endoglucanase activity (**b**), β-glucosidase activity (**c**) and xylanase activity (**d**) were then measured. Values represent the means of at least three replicates, *error bars* show standard deviation

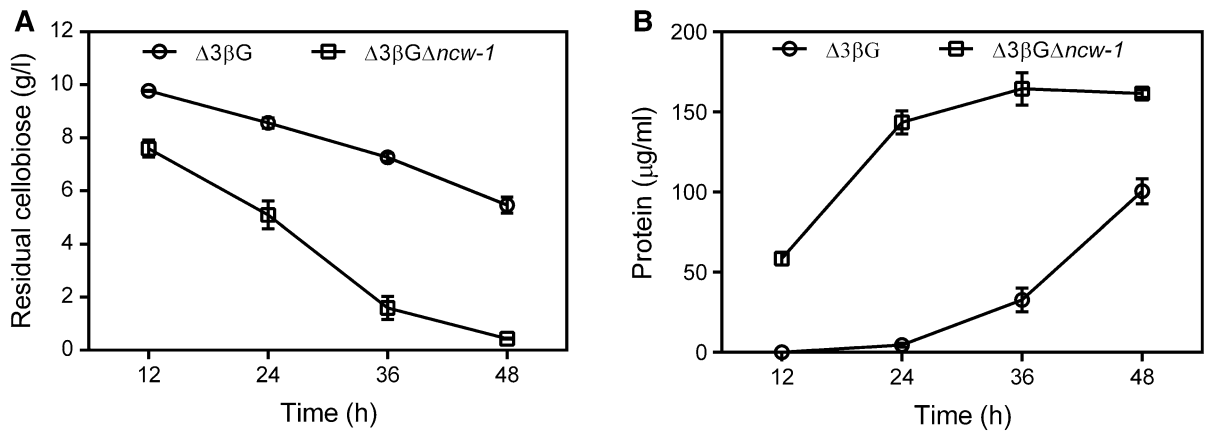

Fig. 3 Cellobiose consumption (**a**) and protein production (**b**) by the Δ3βGΔ*ncw-1* strain on 1% (w/v) cellobiose medium. All strains were grown for 20 h on 2% (w/v) sucrose, followed by a transfer to 1% (w/v) cellobiose for 48 h. Values represent the means of at least three replicates, *error bars* show standard deviation

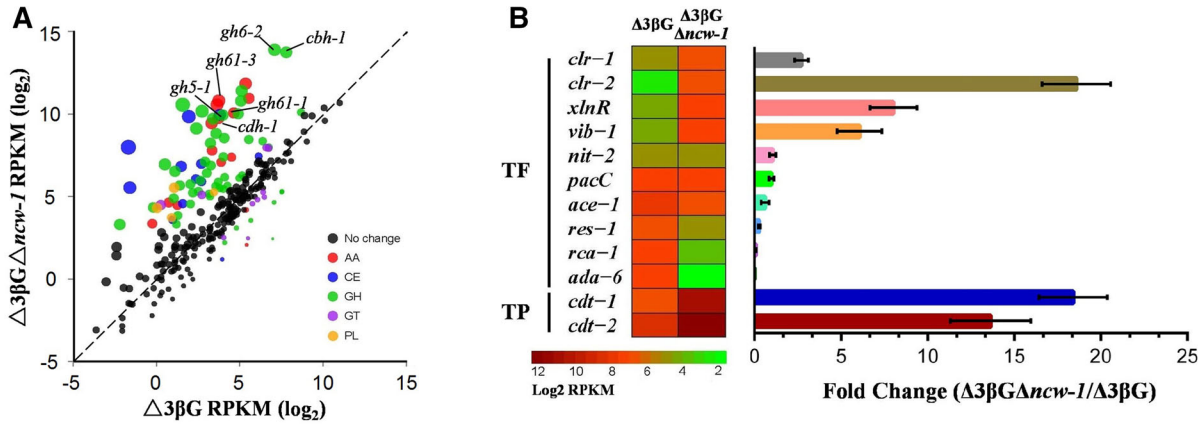

Fig. 4 Transcriptome analysis of the Δ3βGΔ*ncw-1* strain on cellobiose. **a** The transcriptional pattern of CAZy genes responding to 1% (w/v) cellobiose in Δ3βGΔ*ncw-1* and Δ3βG strains. The *size of dots* represents the fold change between Δ3βGΔ*ncw-1* and Δ3βG. Lytic polysaccharide monooxygenases (AA, *red points*), Carbohydrate esterases (CE, *blue points*), Glycosyltransferases (GT, *purple points*), Glycoside hydrolases (GH, *green points*), Polysaccharide lyases (PL, *orange points*). *Black points* represent genes with no differences in the expression. *cbh-1* (NCU07340), *gh6-2* (NCU09680), *gh61-3* (NCU02916), *gh5-1* (NCU00762), *gh61-1* (NCU02240) and *cdh-1* (NCU00206). **b** Expression patterns of the key transcriptional factors and cellodextrin transporters involved in cellulase induction in Δ3βGΔ*ncw-1* and Δ3βG strains. *TF* transcription factor, *TP* transporter protein. All strains were grown for 20 h on 2% (w/v) sucrose, followed by a transfer to 1% (w/v) cellobiose for 4 h. Values represent the means of triplicates; *error bars* show standard deviation

regulated twofold, 17-fold and sevenfold in Δ3βGΔ*ncw-1* on 1% (w/v) cellobiose, respectively (Fig. 4b). Likewise, the transcription factor *vib-1,* which is essential for cellulase production and is involved in repressing both glucose signaling and CCR (Xiong et al. 2014), was also up-regulated sixfold. Meanwhile, several cellulase repressor genes were significantly down-regulated. The *N. crassa rca-1* (NCU01312) was down-regulated 14-fold, deletion of this gene significantly improves lignocellulase production when grown on plant biomass (Wang et al. 2015). In addition, the transcription factor *res-1*, which is required for endoplasmic reticulum stress response during cellulase synthesis (Fan et al. 2015), was down-regulated 3.8-fold. The most significant change in expression occurred with *ada-6*, which was down-regulated 51-fold in the hypercellulase-producing strain Δ3βGΔ*ncw-1*. This result accords with our unpublished data that disruption of *ada-6* increases cellulase production by threefold in *N. crassa*. Taken together, the cellulase expression machinery was dramatically stimulated in Δ3βGΔ*ncw-1*. Overall, our findings suggest that NCW-1 affects cellulase induction principally by increasing uptake of cellobiose, a principal inducer of cellulase production in *N. crassa* and other cellulolytic filamentous fungi.

In conclusion, *N. crassa ncw-1* is involved in the cellulase induction pathway. The improved cellulase production produced by deleting *ncw-1* is not a consequence of relief from CCR of *cre1* pathway, which suggests a novel strategy for improving cellulase production by co-disruption of *ncw-1* and *cre-1*. Furthermore, NCW-1 could suppress cellulase induction via influencing cellulase inducer uptake. Thus, the characterization of *ncw-1* in this study provides new insights into the cellulase induction pathway in filamentous fungi and may facilitate industrial strain improvement for cellulase production.

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Supporting information Supplementary Table 1—Primers used in this study.

Supplementary Table 2—Relative expression level of all genes detected in Δ3βG and Δ3βGΔ*ncw-1* on 1 % (w/v) cellobiose.

Supplementary Fig. 1—Hyphal morphologies of Δ*ncw-1* and wild type strains.

Supplementary Fig. 2—The expression levels of cellulase genes and transcriptional factor *cre-1* in Δ*ncw-1* strain monitored by quantitative RT-PCR after grown on Avicel for 2 days.

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