Deletion of the middle region of the transcription factor ClrB in *Penicillium oxalicum* enables cellulase production in the presence of glucose

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**ABSTRACT**

Enzymes that degrade lignocellulose to simple sugars are of great interest in research and for biotechnology because of their role in converting plant biomass to fuels and chemicals. The synthesis of cellulolytic enzymes in filamentous fungi is tightly regulated at the transcriptional level, with the transcriptional activator ClrB/CLR-2 playing a critical role in many species. In *Penicillium oxalicum*, clrB overexpression could not relieve the dependence of cellulase expression on cellulose as an inducer, suggesting that clrB is controlled post-transcriptionally. In this study, using a reporter gene system in yeast, we identified the C-terminal region of ClrB/CLR-2 as a transcriptional activation domain. Expression of *clrB*ID, encoding a ClrB derivative in which the DNA-binding and transcriptional activation domains are fused together to remove the middle region, led to cellulase production in the absence of cellulose in *P. oxalicum*. Strikingly, the *clrB*ID-expressing strain produced cellulase on carbon sources that normally repress cellulase expression, including glucose and glycerol. Results from deletion of the carbon catabolite repressor gene creA in the *clrB*ID-expressing strain suggested that the effect of *clrB*ID is independent of CreA's repressive function. A similar modification of *clrB* in *Aspergillus niger* resulted in the production of a mannanase in glucose medium. Taken together, these results indicate that ClrB suppression under non-inducing conditions involves its middle region, suggesting a potential strategy to engineer fungal strains for improved cellulase production on commonly used carbon sources.

Enzymes degrading lignocellulose to simple sugars are of great interest in research due to their critical role in converting plant cell wall biomass to fuels and chemicals (1,2). In industry, cellulases are
mainly produced as secreted proteins by filamentous fungi such as *Trichoderma reesei* (3) and *Penicillium* species (4,5). Therefore, understanding the biological processes related to cellulase production in these fungi is important for engineering strains for higher production capacities (6,7).

The synthesis of cellulases in filamentous fungi is mainly regulated at the transcriptional level. In general, the transcription of a set of cellulase genes is repressed in the presence of easy-to-use carbon sources (e.g. glucose) and coordinately induced by cellulosic materials (8). Several transcription factors (e.g. XlnR/Xyr1/XLR-1, CLR-1, CLR-2/ClrB, Ace2, CreA/Cre1/CRE-1, Ace1 and VIB-1) have been identified to participate in this regulation, of which many are conserved in different fungal species (see reviews (9,10)). The Zn$_2$Cys$_6$-type transcription factor CLR-2/ClrB is essential for cellulase induction in several ascomycete fungi, including *Neurospora crassa*, *Aspergillus nidulans* (11), and *Penicillium oxalicum* (12). CLR-2 directly binds to the promoter of 164 genes in *N. crassa* cultivated on cellulose, which include around 30 lignocellulolytic enzyme genes (13). Overexpression of *clrB/clr*-2 led to significantly enhanced cellulase production on cellulose (12,14), and even constitutive cellulase production under non-inducing conditions in *N. crassa* (14). On the other hand, the global carbon-catabolite repressor CreA/Cre1/CRE-1 represses cellulase expression in the presence of preferred carbon sources in many fungal species (15,16). In addition to direct inhibition of cellulase gene transcription, CreA/Cre1/CRE-1 also represses the expression of cellulase activator genes including *clrB/clr*-2 (12,17).

Many species belonging to *Penicillium* and *Aspergillus* genera, which are closely related in phylogeny (18), are efficient producers of cellulytic enzymes. Some cellulases produced by *Penicillium* and *Aspergillus* species showed excellent catalytic efficiencies (19,20) or inhibitor tolerances (21,22), which are valuable in the bioconversion of lignocellulosic materials. So far, several *Penicillium* species such as *P. oxalicum* (23) and *Penicillium funiculosum* (24) have been used for the production of commercial cellulase preparations. While many mechanisms for the regulation of cellulase expression are conserved, some *Penicillium* and *Aspergillus* species have evolved unique characteristics in cellulase production. For example, lactose as an effective cellulase inducer in *T. reesei* does not induce cellulase expression in *Penicillium echinulatum* (25) and *P. oxalicum* (26). Also, different from the case in *N. crassa*, overexpression of *clrB* could not relieve the dependence of cellulase expression on inducer in *P. oxalicum* and *A. nidulans*, although their expression on cellulose was improved (12,14,27). Therefore, current cellulase production by *Penicillium/Aspergillus* species relies on insoluble cellulosic materials as the inducer, which is not favorable for submerged fermentation due to operation difficulty, enzyme adsorption and toxicity problems (28). Expression of a gene encoding a chimeric transcriptional activator in *P. oxalicum* led to cellulase production under non-inducing conditions, but cellulase expression in the resulted strains was still repressed by glucose (27).

In this study, we explored the possibility of enabling *P. oxalicum* to produce cellulase on glucose by mutating the sequence of ClrB. An internal deletion mutant of ClrB was constructed based on the identification of its transcriptional activation domain, and a strain expressing this mutant was able to produce cellulase on glucose and glycerol. The global transcriptional change in the mutant strain relative to reference strain was studied and compared to that in a creA-deletion mutant. These results deepen our understanding of
cellulase gene expression in filamentous fungi, and provide a novel strategy for engineering cellulase-producing strains.

**Results**

**The C-terminal region in ClrB/CLR2 is a transcriptional activation domain**

Overexpression of *clrB* gene in *P. oxalicum* using a constitutive *gpdA* promoter significantly improved cellulase production on cellulose, while cellulase activity was still hard to detect in the absence of cellulose (Fig. 1). We hypothesized that the transcriptional activation ability of ClrB was inhibited at the protein level under non-inducing conditions. If that is the case, inactivation or removal of the regulatory region responsible for this inhibition is expected to cause constitutive transcriptional activation by ClrB.

For many Zn<sup>2+</sup>Cys<sub>6</sub>-type transcription factors, the C-terminal region is responsible for transcriptional activation, and the middle region containing a “middle homology region” is involved in regulating their activities (29). Direct fusion of the transcriptional activation domain to DNA-binding domain by removing the middle regulatory region could render several regulators constitutively active (30,31). If a similar mechanism exists in ClrB, fusion of its N-terminal DNA-binding domain (12,32) (Fig. S1) and transcriptional activation domain might lead to cellulose-independent cellulase expression. However, the location of transcriptional activation domain in ClrB remains unknown. Therefore, we started with the identification of transcriptional activation domain in ClrB using a yeast reporter system.

The full-length sequence of *P. oxalicum* ClrB (780 amino acids) and its truncated versions were fused to the C-terminal of the DNA-binding domain of *Saccharomyces cerevisiae* transcription factor Gal4 (Fig. 2A). *S. cerevisiae* cells expressing functional chimeric transcriptional activators could activate the expression of four reporter genes containing Gal4-binding sites on their promoters. Fusion of the DNA binding domain-removed ClrB (amino acids 123-780), but not the full-length ClrB, to Gal4 binding domain successfully activated the expression of reporter genes. Subsequently, the 123-780 region of ClrB was divided into different fragments for the construction of chimeric transcription factors. The C-terminal 685-780 fragment was found to enable the expression of reporter genes when fused to Gal4 binding domain, suggesting this region contains the transcriptional activation domain. Further dividing this region to two smaller fragments (685-727 and 728-780) revealed that each fragment had a transcriptional activation ability. None of the fragments from the middle region of ClrB (123-684) resulted in the expression of reporter genes, suggesting the C-terminal region is likely the only transcriptional activation domain in ClrB.

The C-terminal region is less conserved than the rest of the protein among ClrB homologs (see Fig. S2 for details). Actually, the transcriptional activation domain of *P. oxalicum* ClrB shows only random sequence similarity with the C-terminal region of *N. crassa* CLR-2 (Fig. S2). To test if the transcriptional activation function of C-terminal region is conserved in ClrB homologs, the 684-812 fragment of CLR-2, as well as its two sub-fragments, were fused to Gal4 binding domain. All three fusion proteins could activate the expression of reporter genes (Fig. 2B), suggesting the conserved functional domain architecture of ClrB/CLR-2 homologs.

**Expression of an internal deletion mutant of clrB eliminated the dependence of cellulase production on cellulose**

The N-terminal DNA-binding domain and C-terminal transcriptional activation domain of ClrB
Mutation of ClrB causes de-repressed cellulase expression

were fused together and tested for its function under cellulase non-inducing condition. Compared with full-length ClrB, the predicted fusion protein ClrB<sup>ID</sup> (named for Internal Deletion) lacks the amino acids from positions 173 to 684 (Fig. 3A). To avoid the interference of native ClrB, the ClrB<sup>ID</sup>-encoding gene <i>clrB</i><sup>ID</sup> driven by <i>gpdA</i> promoter was introduced into a <i>clrB</i>-deletion mutant, resulting strain gClrB<sup>ID</sup>. The gClrB<sup>ID</sup> strain was able to produce cellulase in the medium without the addition of carbon source (Fig. 3B and Fig. S3A). As a control, cellulase production was barely detected in reference strain M12. Consistently, proteins with molecular masses of major cellulases (33) were detected in the culture supernatant of gClrB<sup>ID</sup> but not in M12 (Fig. 3C), and the transcript abundances of major cellulase genes (<i>cel7A-2</i>, <i>cel7B</i> and <i>cel5B</i>) were elevated by more than 150-fold in gClrB<sup>ID</sup> relative to M12 (Fig. 3D). Moreover, the cellulase activity of gClrB<sup>ID</sup> in no carbon medium was comparable to that of M12 in cellulose medium (Fig. 1), suggesting that the dependence of cellulase synthesis on cellulose was relieved in gClrB<sup>ID</sup>. The gClrB<sup>ID</sup> strain produced cellulase on glucose and glycerol. While cellulase expression in strain gClrB<sup>ID</sup> does not require cellulose as the inducer, the process might still be repressed by preferred carbon sources such as glucose and glycerol. Thus, the cellulase activity of gClrB<sup>ID</sup> in the medium with glucose as a sole carbon source was further examined. Within the first 12 hours after shifting the mycelia to glucose medium, residual glucose with a concentration higher than 10 g/L could be detected in the culture (Fig. 4A). During this time, the reference strain M12 and <i>clrB</i>-deletion mutant Δ<i>clrB</i> showed no cellulase activity (Fig. 4B and 4C; Fig. S3B). However, gClrB<sup>ID</sup> produced significant amounts of cellulase in the presence of glucose, which was more than 10-times higher than that of M12 in cellulose medium (Fig. 1). Strikingly, the transcript abundances of major cellulase genes on glucose increased by more than 800-fold in gClrB<sup>ID</sup> relative to M12 (Fig. 4D). The cellulase-producing phenotype was also observed in the medium with glycerol as carbon source (Fig. 4F). Taken together, the expression of <i>clrB</i><sup>ID</sup> could activate cellulase expression under classical cellulase-repressing conditions.

To verify the existence of full-length and mutated ClrB proteins in the cells, <i>clrB</i> and <i>clrB</i><sup>ID</sup> genes with enhanced green fluorescent protein (EGFP)-encoding sequence fused to the downstream were expressed in the <i>clrB</i>-deletion mutant Δ<i>clrB</i>. The obtained strains were named gClrB-EGFP and gClrB<sup>ID</sup>-EGFP, respectively. The expression of <i>clrB-egfp</i> restored cellulase production on cellulose, and <i>clrB</i><sup>ID</sup>-egfp expression led to cellulase production on glucose (Fig. S4), indicating that the tag did not affect the activity of ClrB and ClrB<sup>ID</sup>. The results of fluorescence microscopy and Western blot revealed the presence of full-length and internal-mutated ClrB when the strains were grown in glucose medium (Fig. 5). Interestingly, fluorescence signal was observed throughout mycelium in gClrB-EGFP strain but only in nuclei in gClrB<sup>ID</sup>-EGFP. Thus, it is likely that the internal deletion affected the activity of ClrB at levels beyond protein abundance.

**The gClrB<sup>ID</sup> strain produced cellulase on glucose and glycerol**

While cellulase expression in strain gClrB<sup>ID</sup> does not require cellulose as the inducer, the process might still be repressed by preferred carbon sources such as glucose and glycerol. Thus, the cellulase activity of gClrB<sup>ID</sup> in the medium with glucose as a sole carbon source was further examined. Within the first 12 hours after shifting the mycelia to glucose medium, residual glucose with a concentration higher than 10 g/L could be detected in the culture (Fig. 4A). During this time, the reference strain M12 and <i>clrB</i>-deletion mutant Δ<i>clrB</i> showed no cellulase activity (Fig. 4B and 4C; Fig. S3B). However, gClrB<sup>ID</sup> produced significant amounts of cellulase in the presence of glucose,
changes (> 50-fold or 100-fold) was higher than that of down-regulated genes (Fig. 6B), which was consistent with the transcriptional activating function of ClrB. As expected, Gene Ontology (GO) terms related to the degradation of cellulose, pectin and xylan were enriched for the significantly up-regulated genes (Table S1). Actually, the 658 up-regulated genes included 14 of the 18 cellulases encoded by the P. oxalicum genome (34). In addition, 19 of the 51 hemicellulases were also significantly up-regulated in gClrB ID (Table S2). GO terms for transmembrane transport (including amino acid transport) and oxidation-reduction process were enriched for the down-regulated genes (Table S1). The reason and physiological effect of the down-regulation of these genes remain unclear.

In our previous work, the regulon of ClrB in P. oxalicum has been studied by comparing the transcriptomes of wild type and a clrB-deletion mutant on cellulose (12). By including this previous data, a hierarchical clustering analysis of cellulase genes was performed, which classified the genes into three groups (Fig. 6C). Group II (the biggest group) genes were significantly up-regulated by clrB ID expression on glucose, and were induced by cellulose in a clrB-dependent manner. This group included genes encoding the most abundant cellulases detected in the secretome of P. oxalicum (e.g. Cel7A-2/PDE_07945, Cel6A/PDE_07124, Cel5B/PDE_09226 and Cel7B/PDE_07929 (33)). Group I genes were also significantly up-regulated by clrB ID expression on glucose, but were not efficiently induced by cellulose at the time of sampling. Group III genes were cellulose-induced, but their expression was either not enhanced or less enhanced by the expression of clrB ID. Interestingly, all the four lytic polysaccharide monooxygenases, which degrade cellulose using an oxidative mechanism (35), were clustered into group III, implying the requirement of regulators other than ClrB for full induction of their expression. In summary, clrB ID expression triggered the transcription of a major portion of cellulase genes on glucose.

clrB ID expression had an additive effect with creA deletion on carbon catabolite de-repression of cellulase genes

Gene deletion or mutation of carbon catabolite repressor CreA/Cre1/CRE-1 is reported to result in de-repressed cellulase expression in several fungal species (36-38). To investigate whether de-repressed cellulase production by strain gClrB ID is related to the alleviation of CreA-mediated carbon catabolite repression, cellulase expression in gClrB ID was compared with those in creA-deletion mutants. The creA gene was deleted in reference strain M12 and gClrB ID respectively, resulting strains ∆creA and gClrB IDΔcreA. When glucose was used as a sole carbon source, no cellulase production was detected for strain ∆creA before glucose depletion (Fig. 4B and C). The transcript abundances of major cellulase genes and clrB were significantly elevated in ∆creA relative to M12, but the times of increase were lower than those in gClrB ID by more than an order of magnitude (Fig. 4D and E). Therefore, although the deletion of creA results in carbon catabolite de-repression of cellulase genes, this relief was not sufficient for high-level cellulase production on glucose. In addition, strain gClrB IDΔcreA showed further increased cellulase production compared to gClrB ID before glucose depletion (Fig. 4B and 4C), suggesting that CreA still repressed cellulase expression in strain gClrB ID.

A typical phenotype of carbon catabolite repression-resistant cellulase-expressing strains is the growth on cellulose medium supplemented with 2-deoxyglucose (2-DOG), a non-metabolizable analog of glucose (17,39). Wild-type strains cannot grow on such medium because 2-DOG represses the expression of cellulase genes and therefore the
Mutation of ClrB causes de-repressed cellulase expression

For mutants in which catabolite repression was impaired, e.g. creA-deletion mutant, cellulase production was not repressed by 2-DOG and therefore the strain could utilize cellulose (17,40). As expected, gClrBIΔcreA and the double mutant gClrBIΔcreA, but not M12, could grow and produce hydrolysis halos on the medium containing cellulose and 0.01 g/L 2-DOG (Fig. 7). Increasing the concentration of 2-DOG to 0.03 g/L showed that gClrBIΔcreA had a higher resistance to this inhibitor than ΔcreA. This confirmed that clrBI expression had a greater effect on cellulase de-repression than creA deletion (Fig. 4D). In the presence of 0.05 g/L 2-DOG, only gClrBIΔcreA, but not the two single mutants, could grow on cellulose (Fig. 7). That is to say, clrBI expression and creA deletion have an additive effect on releasing the 2-DOG repression of cellulase expression, which is consistent with the highest cellulase production level of strain gClrBIΔcreA in liquid glucose medium (Fig. 4B and 4C).

Moreover, clrBI expression and creA deletion affected the expression of overlapping but distinct sets of genes. CreA/Cre1 was reported to repress a broad range of alternative carbon source-utilizing genes in filamentous fungi (15,41). In N. crassa, deletion of cre-1 resulted in dramatic up-regulation of genes encoding amylases and a high affinity glucose transporter NCU04963 on sucrose (16). Therefore, the transcript abundances of the homologues of these genes in P. oxalicum, including amy13A/PDE_01201 (encoding α-amylase), amy15A/PDE_09417 (glucoamylase) (42), hgtB/PDE_01388 (high affinity glucose transporter), as well as amyrase transcriptional activator gene amyR/PDE_03964 (12), were determined. All the four genes were up-regulated in ΔcreA strain relative to M12 on glucose (Fig. 8), suggesting they are also targets of CreA-mediated catabolite repression in P. oxalicum. Interestingly, the expression of these four genes (especially amyR and hgtB) was lower in gClrBI than that in M12, which was consistent with the RNA-seq result (Table S3). Taken together, comparative analysis of the effects caused by clrBI expression and creA deletion suggested that the de-repressed cellulase expression in gClrBI is not due to a relief from CreA-mediated carbon catabolite repression.

clrBI expression moderately enhanced cellulase production on cellulose

In cellulose medium as an inducing condition, ClrB is expected to be turned to a functional transcriptional activator. In addition, the expression of clrB is induced by cellulose, followed by a reduction at latter stage of cultivation (43). Whether the expression of clrBI could further improve cellulase production under this condition was studied. Compared to M12, gClrBI showed 1.6- to 8.3-fold increases in cellulase production at different time points (Fig. 9A and 9B; Fig. S3C). Transcript abundance determination also showed significant up-regulation of major cellulase genes in gClrBI (Fig. 9C). It should be noted that the cellulase activity of gClrBI around 0.02 pNPCase units per mL at 24 h) was similar to that of the gClrB strain which overexpresses the intact clrB using the same promoter (Fig. 1). Therefore, the internal sequence deletion of clrB seemed not to play an important role in improving cellulase production on cellulose.

Deletion of creA has been previously demonstrated to remarkably improve cellulase production on cellulose by P. oxalicum (12). The cellulase activity of ΔcreA was much higher than that of gClrBI on cellulose, particularly at the later stage of fermentation (Fig. 9A and 9B). Even at the early stage (4 hours after shifting mycelia to cellulose medium), cellulase gene expression in ΔcreA was more than two-fold higher than that in gClrBI (Fig. 9C). The expression of clrB was slightly higher in ΔcreA relative to reference strain
Mutation of ClrB causes de-repressed cellulase expression

M12 (Fig. 9D), suggesting that the enhancement of cellulase expression by creA deletion was not mediated by the up-regulation of clrB. Similar with that in M12 background, deletion of creA in gClrB™ resulted in a large increase in cellulase production (Fig. 9A and 9B). These results showed that CreA strongly inhibits cellulase expression on cellulose regardless of the regulatory strength of ClrB.

The changes in gene transcription caused by clrB™ expression and creA deletion on cellulose were further studied by RNA-seq. A total of 452 and 485 genes were significantly up-regulated and down-regulated in gClrB™ strain relative to M12, respectively. Thus, clrB™ expression affected the transcription of fewer genes on cellulose than that on glucose (Fig. 6B). Comparison of the significantly up-regulated genes in gClrB™ relative to M12 between glucose medium and cellulose medium revealed 138 genes up-regulated under both conditions (Fig. 10A). These genes included 13 cellulase genes and 12 hemicellulase genes, and were significantly enriched for GO terms involved in lignocellulose degradation (Table S1). The expression of 13 hemicellulase genes were up-regulated in gClrB™ on cellulose but not on glucose (Fig. 10A), suggesting that their transcription might require the cooperation of ClrB and other regulators which were active on cellulose. No specific GO term was enriched for the 520 genes up-regulated in gClrB™ uniquely on glucose, while heme-binding proteins and oxidoreductases were enriched for the 314 genes up-regulated uniquely on cellulose.

Deletion of creA resulted in 825 and 834 genes significantly up-regulated and down-regulated on cellulose, respectively. The up-regulated gene set included 162 genes that were also up-regulated in gClrB™, of which 13 encode cellulases and 22 encode hemicellulases (Fig. 10B). In addition, 12 hemicellulase genes (e.g. xyl3A/PDE_00049 encoding a β-xylosidase) were uniquely up-regulated in ΔcreA (Table S2), suggesting a broader regulatory function of CreA than ClrB. GO terms related to ribosome biogenesis and conidiation were enriched for the 663 genes up-regulated in ΔcreA but not gClrB™ (Table S1), indicating affected growth and development in the ΔcreA strain. When all the gene expression data were separated on a principal component (PC) analysis plot, the 15 samples (involving three strains and two conditions run in triplicates) were clustered into three big groups by the first and second PCs (Fig. 10C). The gClrB™ strain was mildly separated from M12 to the same direction along the PC2 axis on glucose and cellulose, suggesting an overlapped effect of clrB™ expression between the two conditions. On cellulose, the ΔcreA strain was clearly separated from M12 and gClrB™ on both axes, again supporting the strong regulatory role of CreA under this condition.

Expression of clrB™ analogue in Aspergillus niger led to de-repressed lignocellulolytic enzyme production

To examine whether the effect of clrB™ expression on cellulase production is conserved in the family Aspergillaceae, a similar manipulation of clrB as that in gClrB™ was performed in A. niger, which is widely used for enzyme production in industry (44). The amino acid sequence of putative ClrB in A. niger (An12g01870, named AnClrB) has an identity of 58% with P. oxalicum ClrB. According to the result of sequence alignment (Fig. S5), the sequences encoding N-terminal (amino acids 1 to 158) and C-terminal (679 to 777) regions of AnClrB were fused together and overexpressed in strain N593. Two transformants, gAnClrB™-1 and gAnClrB™-2, were obtained and compared with the parent strain. As a control, a mutant overexpressing the intact AnClrB-encoding gene using the same promoter was constructed and named gAnClrB.

In the medium with glucose as a sole carbon
source, all the recombinant strains showed cellulase production before glucose depletion, which was not observed for the parent strain (Fig. 11A and 11B). SDS-PAGE followed by mass spectrometry analysis of the culture supernatants revealed the production of endoglucanase EglA (An14g02760) by the three recombinant strains but not the parental strain (Fig. 11C, Table S4). Thus, overexpression of AnClrB-encoding gene is sufficient to trigger some cellulase expression on glucose in A. niger. In contrast, a protein band identified as β-mannanase Man5A (An05g01320) was uniquely detected in the culture supernatants of gAnClrBID-1 and gAnClrBID-2 but not gAnClrB (Fig. 11C, Table S4), suggesting that internal deletion of clrB resulted in de-repressed expression of man5A.

Discussion

The crucial and conserved function of ClrB/CLR-2 in the transcriptional activation of cellulase genes have been well documented in several filamentous fungi (11-13,45). However, the understanding about how this protein functions as a transcriptional regulator is still limited. Previously, the N-terminal regions of ClrB in P. oxalicum (amino acids 1-163) and A. nidulans (1-118) were shown to bind to cellulase gene promoters in vitro (12,32), and CLR-2 in N. crassa was found to act as a homodimer (13). These characteristics are typical for Zn2Cys6-type transcription factors (29,46). Another feature of many members in this family is the transcriptional activation function of the C-terminal region (46). In contrast to the DNA-binding domain, transcriptional activation domains do not have a well-defined structure and are hard to predict by computational methods (47). In this study, the C-terminal 96 amino acids in P. oxalicum ClrB and 129 amino acids in N. crassa CLR-2 were identified to constitute transcriptional activation domains, both of which seemed to contain smaller functional units (Fig. 2). The transcriptional activation domains from the two species share only random similarity, indicating the rapid evolution of this region. In addition, none of the two domains are rich in acidic amino acids, although this was observed for many other transcriptional activation domains. Whether the activation domains in ClrB/CLR-2 work in known manners to activate transcription (e.g. recruitment of RNA polymerase, Mediator and/or nucleosome modifiers) could be studied in the future.

In N. crassa, overexpression of full-length CLR-2 was sufficient to cause cellulase production in the medium without carbon source, but this phenomenon could not be observed in A. nidulans with the same manipulation (14). A similar result with that in A. nidulans was obtained in P. oxalicum (Fig. 1) (27). Therefore, the transcriptional activation ability of ClrB in A. nidulans and P. oxalicum is likely to be post-transcriptionally inhibited in the absence of cellulose signal. Here, the cellulose-independent cellulase producing phenotype of strain gClrBID suggested that the middle region accounting for 65.6% of the length of ClrB is dispensable for its activity (Fig. 3). Instead, the region is responsible for the regulation of ClrB in response to upstream signal. In the absence of cellulose, the middle region itself, or some other inhibitory molecules interacting with this region (like the case of Gal80 in S. cerevisiae (48)), might participate in suppressing the activity of ClrB. The suppression seems not to act through mediating its degradation, as EGFP-tagged ClrB could be detected in the cell in glucose medium, despite the inactive cellulase expression under this condition (Fig. 5 and Fig. S4B).

The results of clrB manipulation in A. niger have some differences from those in P. oxalicum (Fig. 11). Overexpression of full-length clrB in A. niger resulted in the production of cellobiohydrolase and endoglucanase EglA on glucose, which was not observed in P. oxalicum.
Mutation of ClrB causes de-repressed cellulase expression

However, de-repressed production of mannanase Man5A in A. niger was only detected in the strain expressing internal-deleted AnclrB, which was similar with the case of cellulase in P. oxalicum. The different responses of cellulase and mannanase to ClrB mutation in A. niger might be due to distinct regulatory modes of ClrB on different target genes, which has been reported in A. nidulans regarding the regulation of cellulolytic and mannanolytic genes by ClrB (32). Together, the results in A. niger reveal that the mechanism for regulating lignocellulolytic enzyme production by ClrB is partially conserved across fungal species.

Several reports have suggested that inactive cellulase expression under repressing conditions are mainly due to insufficient transcriptional activation but not CreA/CRE1-mediated carbon catabolite-repression. As mentioned above, overexpression of clr-2 in N. crassa led to cellulase production on repressing carbon source (sucrose) at a level similar to that on cellulose (14). In T. reesei, overexpression of the cellulase transcriptional activator gene xyr1 by a copper responsive promoter also resulted in full relief of cellulase production from repression on glucose (49). In this study, the similar result was observed after mutating the sequence of clrB (Fig. 4 and Fig. 6). Of note, the cre-1/cre1/creA gene was not disrupted in the above three cases. In contrast, single deletion of creA in P. oxalicum was not sufficient for cellulase production on glucose despite the up-regulation of cellulase gene expression (Fig. 4B-D). This consequence of creA deletion appears similar, although somewhat variable in severity among different fungal species. In T. reesei, deletion of cre1 led to detectable cellulase production on glucose, but the level was much lower than that under inducing condition (36). In N. crassa, deletion of cre-1 even did not increase the expression of cellulase genes on sucrose (16). Taken together, the primary reason for cellulase gene repression on preferred carbon source is the insufficient transcriptional activation due to low abundance and/or low activity of transcriptional activator, while CreA/Cre1/CRE-1 plays an additional role in repression.

Internal deletion of clrB resulted in only moderately enhanced cellulase production on cellulose relative to reference strain M12 (Fig. 9A-C). This is not surprising since under inducing conditions, the activity of native ClrB is already at high levels. In contrast, deletion of creA had a markedly enhancing effect on cellulase expression on cellulose (Fig. 9A-C). The similar results were previously reported in T. reesei (36) and N. crassa (16), where cre1/cre-1 deletion significantly increased cellulase expression under inducing conditions. It is worth being noted that creA deletion did not affect cellulase expression in the medium without carbon source (Fig. 3B-D). Therefore, CreA/Cre1/CRE-1 seems to repress cellulase expression as long as the cell senses the signal from glucose (either exogenously added or gradually released from cellulose) or other preferred carbon sources. This mechanism might prevent excessive synthesis of cellulases during the growth on cellulose.

In conclusion, this study describes a novel clrB gain-of-function mutant whose expression led to cellulase production under repressing conditions in P. oxalicum. The result provides new insights into the understanding of the control of cellulase gene expression in filamentous fungi. The detailed mechanism including the sensing of cellulose signal and its transduction to ClrB is to be clarified in the future. From the view of industrial application, soluble simple sugars like glucose and sucrose are more suitable for large-scale cellulase production in bioreactors (28). Combining the expression of clrB and engineering of other targets is expected to yield industrial Penicillium strains with high-level cellulase production ability on glucose.
1. Similarly, the fused to a sequence encoding (GGGS) together. To express EGFP-tagged proteins, the protoplast transformation (55) as indicated in Table cassettes were transformed to parental strains via cassette. The gene deletion or overexpression Agar plates containing Vogel’s salt creA, the upstream sequence of from Adrian Tsang, Concordia University, Canada). and transformed to expressing cassette fused to downstream of the target gene, and expressing cassette fused to select marker gene (54) was used as the selection marker. To delete creA, the upstream sequence of creA, selection marker gene hph, and the downstream sequence of creA were fused together to obtain the gene deletion cassette. To express clrBID, the A. nidulans gpdA promoter, the fragment of clrB gene encoding the N-terminal 172 amino acids, the region encoding C-terminal 96 amino acids followed by terminator, and selection marker gene bar (53) were fused together. To express EGFP-tagged proteins, the sequence encoding (GGGS) linker and EGFP was fused downstream of the target gene, and hph gene (54) was used as the selection marker. To delete creA, the upstream sequence of creA, selection marker gene hph, and the downstream sequence of creA were fused together to obtain the gene deletion cassette. The gene deletion or overexpression cassettes were transformed to parental strains via protoplast transformation (55) as indicated in Table 1. Similarly, the AnclrB-overexpressing cassette fused to a ptrA marker gene (56) and AnclrBID-expressing cassette fused to hph were constructed and transformed to A. niger strain N593 (a kind gift from Adrian Tsang, Concordia University, Canada). Agar plates containing Vogel’s salt (57), 2% (w/v) glucose and 1 M sorbitol supplemented with 2.5 mg/mL glufosinate ammonium (for bar marker) or 0.5 μg/mL pyrithiamine (for ptrA marker) or 350 μg/mL hygromycin B (for hph marker) were used for transformant screening. The transformants were purified by plate streaking and the obtained strains were identified via PCR and DNA sequencing. All the primers used for gene manipulation cassette construction are listed in Table S5.

Identification of transcriptional activation domain in ClrB/CLR-2—The clrB/tri-2 gene fragments of different lengths were amplified from the cDNA of P. oxalicum or genomic DNA of N. crassa, and fused to vector pGBK7T (Takara Bio, Japan) using the ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China). To create sequence homology between the PCR product and vector, 16 bp-long sequences matching the ends of pGBK7T linearized with EcoRI I and BamH I were added to the 5' ends of primers. All the primers used for vector construction are listed in Table S6. The resulted recombinant plasmids extracted from Escherichia coli cells were confirmed by Sanger sequencing, and then separately transformed to S. cerevisiae strain Y2HGold (MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-UAS-Gal1TATA-His3, GAL2-UAS-Gal2TATA-Ade2, URA3::MEL1-UAS-Mel1TATA-AUR1-C, MEL1; Takara Bio USA, Inc.). Transformants were screened on SD/-Trp medium. The recombinant yeast strains were then inoculated to agar plates of different compositions to test the expression of reporter genes. The test plate contained (per liter) 6.7 g yeast nitrogen base (Solarbio, Beijing, China), 20 g glucose, 1.3 g Yeast Synthetic Drop-out Medium Supplements without histidine, leucine, tryptophan and uracil (Sigma-Aldrich, St. Louis, United States), 63 mg leucine, 0.5 mg aureobasidin A and 40 mg X-a-gal. The control plate was different from the test plate by replacing aureobasidin A and X-a-gal with 20 mg/L histidine. Transcriptional activation of reporter genes AUR1-C, HIS3, ADE2 and MEL1 resulted in resistance to aureobasidin A, growth on histidine deficient medium, formation of white colony and hydrolysis of X-a-gal (blue color), respectively. S. cerevisiae cells expressing the empty pGBK7T vector was
Mutation of ClrB causes de-repressed cellulase expression

Cultivation of P. oxalicum and A. niger strains—The fungal strains were cultivation on wheat bran extract slants for 4 days to collect conidia. For cultivation in liquid media, the conidia were inoculated to and pre-cultivated in Vogel’s salts supplemented with 2% (w/v) glucose with a final concentration of 10⁶ conidia per mL. 0.1% (w/v) peptone was added to the medium for pre-cultivation of A. niger strains. The 300 mL-flasks containing 50 mL cultures were incubated on a rotary shaker at 200 rpm at 30°C. After 22 hours of pre-cultivation, the mycelia were collected on filter paper by vacuum filtration. Mycelia were resuspended in Vogel’s salts supplemented with indicated carbon sources with a concentration of 6.0 g (for glucose and cellulose media) or 10.0 g (for the medium without carbon source) wet cell weight per liter. The cultivation was continued for 4 to 96 hours for the analyses of gene transcription, cellulase activity and extracellular proteins. For cultivation on agar plates, 1.5 μL of conidial suspension at a concentration of 10⁷/mL were spotted to Vogel’s medium supplemented with 1.5% (w/v) agar powder and 2-DOG of different concentrations as indicated in the text. Photographs of plates were taken after 48 hours of cultivation at 30°C. Uracil at a concentration of 2 g/l was added to the medium for all cultivations.

Cellulase assays, SDS-PAGE and protein identification—The liquid cultures were taken from shake flasks and centrifuged at 4°C for 10 min. The supernatants were used for the determination of cellulase activities and SDS-PAGE analyses. For the measurement of cellobiohydrolase (a major type of cellulases) activity, 50 μL of 1 mg/mL p-nitrophenyl-D-cellobioside (pNPC, Sigma-Aldrich, USA) in 0.2 M acetate buffer (pH 4.8) supplemented with 10 mg/mL D-glucono-1,5-δ-lactone was mixed with 100 μL diluted culture supernatant and incubated at 50°C for 30 min. Then, the reaction was stopped by adding 150 μL 10% Na₂CO₃, and the absorbance of the reaction system at 420 nm was determined. Filter paper activity was measured as previously described (55). One unit of enzyme activity was defined as the amount of enzyme required to release one μmol product (p-nitrophenyl or glucose equivalent) from the substrate per minute under the assayed conditions. Polyacrylamide gel at a concentration of 12.5% (w/v) was used for protein separation, and Coomassie brilliant blue R250 (Sangon, Shanghai, China) or silver (58) (for A. niger samples) was used for gel staining. Protein bands of interest were cut from SDS-PAGE gels and analyzed on a MALDI-TOF/TOF 5800 mass spectrometer (AB SCIEX, USA) by Shanghai Applied Protein Technology Co. Ltd. The MS data were processed using Data Explorer 4.5 (AB SCIEX, USA). Then, the extracted peak lists were searched using the Mascot search engine (version 2.2, Matrix Science) against the Uniprot database (December 7th, 2018) restricted to the taxonomy A. niger (59,116 sequences). Search parameters included use of trypsin to generate peptides, a maximum of one missed cleavage permitted, carbamidomethyl (C) as fixed modification, oxidation (M) as variable modification, 100 ppm peptide mass tolerance, 0.4 Da fragment mass tolerance, and p<0.05 as threshold score for accepting individual spectra.

Quantitative reverse transcription PCR (qRT-PCR)—The mycelia were collected on filter paper by vacuum filtration, and then ground to powder in liquid nitrogen. Total RNA extraction and cDNA synthesis were performed using the RNAiso Plus reagent (Takara Bio, Japan) and PrimeScript RT reagent Kit with gDNA eraser (Takara Bio, Japan) according to the manufacturer’s instructions. The 20 μL qRT-PCR reaction mixture was prepared using SYBR Premix Ex Taq (Perfect Real Time, Takara Bio, Japan), and the amplification was carried out on LightCycler 480 system with
Mutation of ClrB causes de-repressed cellulase expression

Software version 4.0 (Roche, Mannheim, Germany). The PCR program included 95°C for 2 min for initial denaturation, 40 cycles of 95°C for 10 s followed by 61°C for 30 s, and a dissociation stage in which the temperature increased from 65°C to 95°C with a gradient of 0.1°C/s. Fluorescence signal was gathered at the end of each extension step at 80°C. The copy number of transcripts was calculated by comparing the Cp value to standard curve of each gene, respectively. The transcript level of actin gene (Gene ID: PDE_01092) was used as an internal reference for data normalization. The primers used for qRT-PCR are shown in Table S7.

**Microscopy analysis**—The conidia were inoculated to Vogel’s salts supplemented with 2% (w/v) glucose and cultivated for nine hours. The mycelia were stained with Hoechst 33342 (Sigma-Aldrich, USA) with a final concentration of 10 μg/mL for 20 min, washed and resuspended in 2% (w/v) glucose medium. Images were acquired with a laser scanning confocal microscope (LSM 880, Zeiss, Germany).

**Western blotting**—The mycelia pre-cultivated in Vogel’s salts supplemented with 2% (w/v) glucose for 22 hours were transferred to the same medium, further cultivated for 4 hours, and ground to powder in liquid nitrogen. Total protein was extracted from the mycelia by mixing with the extraction buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, pH 7.5) on ice for 30 min, and then centrifugation at 12,000 rpm at 4°C for 10 min to collect the supernatant. Equal amounts of total protein were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 5% (w/v) skim milk, the membrane was incubated with GFP Tag Antibody (rabbit polyclonal, Proteintech Group, USA; 1:1,000 dilution) and then with HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) (Proteintech Group, USA; 1:5,000 dilution). Target bands were visualized using an enhanced chemiluminescent reagent kit (Wuhan Sanying, China).

**RNA-seq**—High-throughput sequencing of RNA samples were performed by Whbioacme Co. Ltd, Wuhan, China. mRNA libraries for sequencing were prepared using KAPA mRNA Capture Kit and KAPA Stranded RNA-Seq Kit (Roche, Germany) according to the manufacturer's instructions. Paired-end sequencing was performed on Illumina Hiseq X Ten with a read length of 150 bp. All clean reads obtained after raw data processing were mapped to the reference genome of 114–2 (34) using hisat2 2.0.0-beta (59) with default parameters for paired-end reads. Raw counts of mapped reads on gene-level were quantified using featureCounts v1.5.0-p1 (60). FPKM (fragments per kilobase per million mapped fragments) was used to represent the gene expression values. Deseq2 (61) was used to compare the gene expression levels between samples and perform statistical analysis. Genes of significantly differential expressions were identified with combined thresholds (FDR < 0.001 and fold change > 2). Genesis 1.8.1 (62) was used for hierarchical clustering analysis of genes after adjusting FPKM values by log2 transformation and within-gene normalization. Blast2GO (63) was used for GO enrichment analysis of gene sets with a threshold of FDR < 0.05. PC analysis was performed using the precomp function in R 3.4.4 (https://www.R-project.org) and the result was visualized using ggbiplot (https://www.rdocumentation.org/packages/ggbiplot/versions/0.55).

**Protein sequence analysis**—Conserved protein domains in ClrB were predicted using the InterProScan tool (http://www.ebi.ac.uk/interpro/) (64). Transcriptional activation domain was predicted using the 9aaTAD tool (http://www.med.muni.cz/9aaTAD/) (65) by choosing the Moderately stringent Pattern.
Secondary structure was predicted using the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/) (66). For sequence comparison, ClrB in P. oxalicum and its orthologs in A. niger, A. nidulans (GenBank acc. No. XP_660973.1), Aspergillus oryzae (BAM66380.1) and N. crassa (XP_962712.2) were aligned using ClustalX 2.1 (67).
Mutation of ClrB causes de-repressed cellulase expression

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Data Availability: The RNA-seq data have been deposited in the Gene Expression Omnibus database under the accession number GSE120416. The mass spectrometry data have been deposited in the iProX database (http://www.iprox.org) under the Project ID IPX0001796000.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.
Mutation of ClrB causes de-repressed cellulase expression

References
1. Sanderson, K. (2011) Lignocellulose: A chewy problem. Nature 474, S12-14
2. Harris, P. V., Xu, F., Kree, N. E., Kang, C., and Fukuyama, S. (2014) New enzyme insights drive advances in commercial ethanol production. Curr. Opin. Chem. Biol. 19, 162-170
3. Bischof, R. H., Ramoni, J., and Seiboth, B. (2016) Cellulases and beyond: the first 70 years of the enzyme producer Trichoderma reesei. Microb. Cell Fact. 15, 106
4. Gusakov, A. V. (2011) Alternatives to Trichoderma reesei in biofuel production. Trends Biotechnol. 29, 419-425
5. Gusakov, A. V., and Sinitsyn, A. P. (2012) Cellulases from Penicillium species for producing fuels from biomass. Biofuels 3, 463-477
6. Liu, G., Qin, Y., Li, Z., and Qu, Y. (2013) Development of highly efficient, low-cost lignocellulolytic enzyme systems in the post-genomic era. Biotechnol. Adv. 31, 962-975
7. Druzhinina, I. S., and Kubicek, C. P. (2017) Genetic engineering of Trichoderma reesei cellulases and their production. Microb. Biotechnol. 10, 1485-1499
8. Schmoll, M., and Kubicek, C. P. (2003) Regulation of Trichoderma cellulase formation: lessons in molecular biology from an industrial fungus. A review. Acta Microbiol. Immunol. Hung. 50, 125-145
9. Benocci, T., Aguilar-Pontes, M. V., Zhou, M., Seiboth, B., and de Vries, R. P. (2017) Regulators of plant biomass degradation in ascomycetous fungi. Biotechnol. Biofuels 10, 152
10. Huberman, L. B., Liu, J., Qin, L., and Glass, N. L. (2016) Regulation of the lignocellulolytic response in filamentous fungi. Fungal Biol. Rev. 30, 101-111
11. Coradetti, S. T., Craig, J. P., Xiong, Y., Shock, T., Tian, C., and Glass, N. L. (2012) Conserved and essential transcription factors for cellulase gene expression in ascomycete fungi. Proc. Natl. Acad. Sci. U. S. A. 109, 7397-7402
12. Li, Z., Yao, G., Wu, R., Gao, L., Kan, Q., Liu, M., Yang, P., Liu, G., Qin, Y., Song, X., Zhong, Y., Fang, X., and Qu, Y. (2015) Synergistic and dose-controlled regulation of cellulase gene expression in Penicillium oxalicum. PLoS Genet. 11, e1005509
13. Craig, J. P., Coradetti, S. T., Starr, T. L., and Glass, N. L. (2015) Direct target network of the Neurospora crassa plant cell wall deconstruction regulators CLR-1, CLR-2, and XLR-1. mBio 6, e01452-01415
14. Coradetti, S. T., Xiong, Y., and Glass, N. L. (2013) Analysis of a conserved cellulase transcriptional regulator reveals inducer-independent production of cellulolytic enzymes in Neurospora crassa. Microbiologyopen 2, 595-609
15. Antonieto, A. C., dos Santos Castro, L., Silva-Rocha, R., Persinoti, G. F., and Silva, R. N. (2014) Defining the genome-wide role of CRE1 during carbon catabolite repression in Trichoderma reesei using RNA-Seq analysis. Fungal Genet. Biol. 73, 93-103
16. Sun, J., and Glass, N. L. (2011) Identification of the CRE-1 cellulolytic regulon in Neurospora crassa. PLoS One 6, e25654
17. Xiong, Y., Sun, J., and Glass, N. L. (2014) VIB1, a link between glucose signaling and carbon catabolite repression, is essential for plant cell wall degradation by Neurospora crassa. PLoS Genet. 10, e1004500
Mutation of ClrB causes de-repressed cellulase expression

18. Houbraken, J., de Vries, R. P., and Samson, R. A. (2014) Modern taxonomy of biotechnologically important Aspergillus and Penicillium species. Adv. Appl. Microbiol. 86, 199-249
19. Taylor, L. E., 2nd, Knott, B. C., Baker, J. O., Alahuhta, P. M., Hobdey, S. E., Linger, J. G., Lunin, V. V., Amore, A., Subramanian, V., Podkaminer, K., Xu, Q., VanderWall, T. A., Schuster, L. A., Chaudhari, Y. B., Adney, W. S., Crowley, M. F., Himmel, M. E., Decker, S. R., and Beckham, G. T. (2018) Engineering enhanced cellobiohydrolase activity. Nature Commun. 9, 1186
20. Morozova, V. V., Gusakov, A. V., Andrianov, R. M., Pravilnikov, A. G., Osipov, D. O., and Sinitsyn, A. P. (2010) Cellulases of Penicillium verruculosum. Biotechnol. J. 5, 871-880
21. Rajasree, K. P., Mathew, G. M., Pandey, A., and Sukumaran, R. K. (2013) Highly glucose tolerant β-glucosidase from Aspergillus unguis: NII 08123 for enhanced hydrolysis of biomass. J. Ind. Microbiol. Biotechnol. 40, 967-975
22. Li, Z., Pei, X., Zhang, Z., Wei, Y., Song, Y., Chen, L., Liu, S., and Zhang, S. H. (2018) The unique GH5 cellulase member in the extreme halotolerant fungus Aspergillus glaucus CCHA is an endoglucanase with multiple tolerance to salt, alkali and heat: prospects for straw degradation applications. Extremophiles 22, 675-685
23. Liu, G., Qin, Y., Li, Z., and Qu, Y. (2013) Improving lignocellulolytic enzyme production with Penicillium: from strain screening to systems biology. Biofuels 4, 523-534
24. Guais, O., Borderies, G., Pichereaux, C., Maestracci, M., Neugnot, V., Rossignol, M., and Francois, J. M. (2008) Proteomics analysis of "Rovabiot Excel", a secreted protein cocktail from the filamentous fungus Penicillium funiculosum grown under industrial process fermentation. J. Ind. Microbiol. Biotechnol. 35, 1659-1668
25. Sehnem, N. T., de Bittencourt, L. R., Camassola, M., and Dillon, A. J. P. (2006) Cellulase production by Penicillium echinulatum on lactose. Appl. Microbiol. Biotechnol. 72, 163-167
26. Wei, X., Zheng, K., Chen, M., Liu, G., Li, J., Lei, Y., Qin, Y., and Qu, Y. (2011) Transcription analysis of lignocellulolytic enzymes of Penicillium decumbens 114-2 and its catabolite-repression-resistant mutant. C. R. Biol. 334, 806-811
27. Gao, L., Xia, C., Xu, J., Li, Z., Yu, L., Liu, G., Song, X., and Qu, Y. (2017) Constitutive expression of chimeric transcription factors enables cellulase synthesis under non-inducing conditions in Penicillium oxalicum. Biotechnol. J. 12, 1700119
28. Ellilä, S., Fonseca, L., Uchima, C., Cota, J., Goldman, G. H., Saloheimo, M., Sacon, V., and Siikahimo, M. (2017) Development of a low-cost cellulase production process using Trichoderma reesei for Brazilian biorefineries. Biotechnol. Biofuels 10, 30
29. MacPherson, S., Larochelle, M., and Turcotte, B. (2006) A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiol. Mol. Biol. Rev. 70, 583-604
30. Baumgartner, U., Hamilton, B., Piskacek, M., Ruis, H., and Rottensteiner, H. (1999) Functional analysis of the Zn2Cys6 transcription factors Oaf1p and Pip2p. Different roles in fatty acid induction of β-oxidation in Saccharomyces cerevisiae. J. Biol. Chem. 274, 22208-22216
31. Friden, P., Reynolds, C., and Schimmel, P. (1989) A large internal deletion converts yeast LEU3 to a constitutive transcriptional activator. Mol. Cell. Biol. 9, 4056-4060
32. Li, N., Kunitake, E., Aoyama, M., Ogawa, M., Kanamaru, K., Kimura, M., Koyama, Y., and Kobayashi, T. (2016) McmA-dependent and -independent regulatory systems governing expression
Mutation of ClrB causes de-repressed cellulase expression of ClrB-regulated cellulase and hemicellulase genes in *Aspergillus nidulans*. *Mol. Microbiol.* **102**, 810-826

33. Liu, G., Zhang, L., Qin, Y., Zou, G., Li, Z., Yan, X., Wei, X., Chen, M., Chen, L., Zheng, K., Zhang, J., Ma, L., Li, J., Liu, R., Xu, H., Bao, X., Fang, X., Wang, L., Zhong, Y., Liu, W., Zheng, H., Wang, S., Wang, C., Xun, L., Zhao, G. P., Wang, T., Zhou, Z., and Qu, Y. (2013) Long-term strain improvements accumulate mutations in regulatory elements responsible for hyper-production of cellulolytic enzymes. *Sci Rep* **3**, 1569

34. Liu, G., Zhang, L., Wei, X., Zou, G., Qin, Y., Ma, L., Li, J., Zheng, H., Wang, S., Wang, C., Xun, L., Zhao, G. P., Zhou, Z., and Qu, Y. (2013) Genomic and secretomic analyses reveal unique features of the lignocellulolytic enzyme system of *Penicillium decumbens*. *PLoS One* **8**, e55185

35. Hemsworth, G. R., Johnston, E. M., Davies, G. J., and Walton, P. H. (2015) Lytic polysaccharide monooxygenases in biomass conversion. *Trends Biotechnol.* **33**, 747-761

36. Nakari-Setala, T., Palohimo, M., Kallio, J., Vehmaanpera, J., Penttila, M., and Saloheimo, M. (2009) Genetic modification of carbon catabolite repression in *Trichoderma reesei* for improved protein production. *Appl. Environ. Microbiol.* **75**, 4853-4860

37. Huberman, L. B., Coradetti, S. T., and Glass, N. L. (2017) Network of nutrient-sensing pathways and a conserved kinase cascade integrate osmolarity and carbon sensing in *Neurospora crassa*. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E8665-E8674

38. Fujii, T., Inoue, H., and Ishikawa, K. (2013) Enhancing cellulase and hemicellulase production by genetic modification of the carbon catabolite repressor gene, creA, in *Acremonium cellulolyticus*. *AMB Express* **3**, 73

39. Anwar, M. N., Suto, M., and Tomita, F. (1996) Isolation of mutants of *Penicillium purpurogenum* resistant to catabolite repression. *Appl. Microbiol. Biotechnol.* **45**, 684-687

40. Le Crom, S., Schackwitz, W., Pennacchio, L., Magnuson, J. K., Culley, D. E., Collett, J. R., Martin, J., Druzhinina, I. S., Mathis, H., Monot, F., Seiboth, B., Cherry, B., Rey, M., Berka, R., Kubicek, C. P., Baker, S. E., and Margeot, A. (2009) Tracking the roots of cellulase hyperproduction by the fungus *Trichoderma reesei* using massively parallel DNA sequencing. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 16151-16156

41. Ruijter, G. J., and Visser, J. (1997) Carbon repression in *Aspergilli*. *FEMS Microbiol. Lett.* **151**, 103-114

42. Hu, Y., Xue, H., Liu, G., Song, X., and Qu, Y. (2015) Efficient production and evaluation of lignocellulolytic enzymes using a constitutive protein expression system in *Penicillium oxalicum*. *J. Ind. Microbiol. Biotechnol.* **42**, 877-887

43. Lei, Y., Liu, G., Li, Z., Gao, L., Qin, Y., and Qu, Y. (2014) Functional characterization of protein kinase CK2 regulatory subunits regulating *Penicillium oxalicum* asexual development and hydrolytic enzyme production. *Fungal Genet. Biol.* **66**, 44-53

44. Andersen, M. R., Salazar, M. P., Schaap, P. J., van de Vondervoort, P. J., Culley, D., Thykaer, J., Frisvad, J. C., Nielsen, K. F., Albang, R., Albermann, K., Berka, R. M., Braus, G. H., Braus-Stromeyer, S. A., Corrochano, L. M., Dai, Z., van Dijck, P. W., Hofmann, G., Lasure, L. L., Magnuson, J. K., Menke, H., Meijer, M., Meijer, S. L., Nielsen, J. B., Nielsen, M. L., van Ooyen, A. J., Pel, H. J., Poulsen, L., Samson, R. A., Stam, H., Tsang, A., van den Brink, J. M., Atkins, A.,
Mutation of ClrB causes de-repressed cellulase expression

Aerts, A., Shapiro, H., Pangilinan, J., Salamov, A., Lou, Y., Lindquist, E., Lucas, S., Grimwood, J., Grigoriev, I. V., Kubicek, C. P., Martinez, D., van Peij, N. N., Roubos, J. A., Nielsen, J., and Baker, S. E. (2011) Comparative genomics of citric-acid-producing Aspergillus niger ATCC 1015 versus enzyme-producing CBS 513.88. Genome Res. 21, 885-897

45. Ogawa, M., Kobayashi, T., and Koyama, Y. (2013) ManR, a transcriptional regulator of the β-mannan utilization system, controls the cellulose utilization system in Aspergillus oryzae. Biosci. Biotechnol. Biochem. 77, 426-429

46. Schjerling, P., and Holmberg, S. (1996) Comparative amino acid sequence analysis of the C6 zinc cluster family of transcriptional regulators. Nucleic Acids Res. 24, 4599-4607

47. Tjian, R., and Maniatis, T. (1994) Transcriptional activation: a complex puzzle with few easy pieces. Cell 77, 5-8

48. Lohr, D., Venkov, P., and Zlatanova, J. (1995) Transcriptional regulation in the yeast GAL gene family: a complex genetic network. FASEB J. 9, 777-787

49. Lv, X., Zheng, F., Li, C., Zhang, W., Chen, G., and Liu, W. (2015) Characterization of a copper responsive promoter and its mediated overexpression of the xylanase regulator 1 results in an induction-independent production of cellulases in Trichoderma reesei. Biotechnol. Biofuels 8, 67

50. Qin, Y., Zheng, K., Liu, G., Chen, M., and Qu, Y. (2013) Improved cellulolytic efficacy in Penicilium decumbens via heterologous expression of Hypocrea jecorina endoglucanase II. Arch. Biol. Sci. 65, 305-314

51. Oakley, B. R., Rinehart, J. E., Mitchell, B. L., Oakley, C. E., Carmona, C., Gray, G. L., and May, G. S. (1987) Cloning, mapping and molecular analysis of the pyrG (orotidine-5’-phosphate decarboxylase) gene of Aspergillus nidulans. Gene 61, 385-399

52. Yu, J. H., Hamari, Z., Han, K. H., Seo, J. A., Reyes-Dominguez, Y., and Scagazzocchio, C. (2004) Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet. Biol. 41, 973-981

53. Fan, Y., Zhang, S., Krueer, N., and Keyhani, N. O. (2011) High-throughput insertion mutagenesis and functional screening in the entomopathogenic fungus Beauveria bassiana. J. Invertebr. Pathol. 106, 274-279

54. Cullen, D., Leong, S. A., Wilson, L. J., and Henner, D. J. (1987) Transformation of Aspergillus nidulans with the hygromycin-resistance gene, hph. Gene 57, 21-26

55. Gao, L., Li, Z., Xia, C., Qu, Y., Liu, M., Yang, P., Yu, L., and Song, X. (2017) Combining manipulation of transcription factors and overexpression of the target genes to enhance lignocellulolytic enzyme production in Penicillium oxalicum. Biotechnol. Biofuels 10, 100

56. Kubodera, T., Yamashita, N., and Nishimura, A. (2002) Transformation of Aspergillus sp. and Trichoderma reesei using the pyrithiamine resistance gene (ptrA) of Aspergillus oryzae. Biosci. Biotechnol. Biochem. 66, 404-406

57. Vogel, H. J. A. (1956) A convenient growth medium for Neurospora (Medium V). Microb. Genet. Bull. 13, 42-43

58. Chevallet, M., Luche, S., and Rabilloud, T. (2006) Silver staining of proteins in polyacrylamide gels. Nat. Protoc. 1, 1852-1858

59. Kim, D., Langmead, B., and Salzberg, S. L. (2015) HISAT: a fast spliced aligner with low memory
Mutation of ClrB causes de-repressed cellulase expression.

60. Liao, Y., Smyth, G. K., and Shi, W. (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923-930

61. Love, M. I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550

62. Sturn, A., Quackenbush, J., and Trajanoski, Z. (2002) Genesis: cluster analysis of microarray data. Bioinformatics 18, 207-208

63. Gotz, S., Garcia-Gomez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., Robles, M., Talon, M., Dopazo, J., and Conesa, A. (2008) High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res. 36, 3420-3435

64. Jones, P., Binns, D., Chang, H. Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A. F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S. Y., Lopez, R., and Hunter, S. (2014) InterProScan 5: genome-scale protein function classification. Bioinformatics 30, 1236-1240

65. Piskacek, S., Gregor, M., Nemethova, M., Grabner, M., Kovarik, P., and Piskacek, M. (2007) Nine-amino-acid transactivation domain: establishment and prediction utilities. Genomics 89, 756-768

66. Buchan, D. W., Minneci, F., Nugent, T. C., Bryson, K., and Jones, D. T. (2013) Scalable web services for the PSIPRED Protein Analysis Workbench. Nucleic Acids Res. 41, W349-357

67. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947-2948

68. Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M., and Henrissat, B. (2014) The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 42, D490-495
Table 1 *P. oxalicum* strains used in this study.

| Strain       | Genotype or characteristics                        | Parent strain | Reference |
|--------------|-----------------------------------------------------|---------------|-----------|
| M12          | pyrG                                                | 114-2         | (50)      |
| gClrB        | pyrG, AngpdA(p)-clrB::AnpyrG                        | M12           | This study|
| ΔclrB        | pyrG, ΔclrB::AnpyrG                                 | M12           | This study|
| gClrB<sup>ID</sup> | pyrG, ΔclrB::AnpyrG, AngpdA(p)-clrB<sup>ID</sup>::bar | ΔclrB         | This study|
| gClrB<sup>ID</sup>-EGFP | pyrG, ΔclrB::AnpyrG, AngpdA(p)-clrB<sup>ID</sup>-egfp::hph | ΔclrB         | This study|
| ΔcreA        | pyrG, ΔcreA::hph                                    | M12           | This study|
| gClrB<sup>ID</sup>ΔcreA | pyrG, ΔclrB::AnpyrG, AngpdA(p)-clrB<sup>ID</sup>::bar, ΔcreA::hph | gClrB<sup>ID</sup> | This study|
Mutation of ClrB causes de-repressed cellulase expression

Figure 1. Cellulase production of clrB-overexpressing strain gClrB and reference strain M12. The cellobiohydrolase (measured as \( p \)-nitrophenyl cellobiosidase, \( p \)NPCase) activities of culture supernatants in 2% (w/v) cellulose medium, the medium without carbon source, or 2% (w/v) glucose medium are shown. For glucose medium, around 8 g/L of glucose was detected in the culture supernatants after 12 h. Data represent mean±SD from triplicate cultivations.
Mutation of ClrB causes de-repressed cellulase expression

Figure 2. Identification of transcriptional activation domains in ClrB and CLR-2. (A) Transcriptional activation function of different regions in *P. oxalicum* ClrB. (B) Transcriptional activation function of the C-terminal region in *N. crassa* CLR-2. Yeast cells expressing the indicated fusion proteins were cultivated on “test” (SD/-Trp/-His/+aureobasidin A/+X-α-gal) and “control” (SD/-Trp) agar plates. For each construct, two independent yeast transformants were inoculated to agar plates. Adenine hemisulfate was added to the control plate in panel B, resulting in the white color colony of control strain containing empty vector.
Mutation of ClrB causes de-repressed cellulase expression

Figure 3. Expression of clrB internal deletion mutant clrB<sup>ID</sup> results in cellulose-independent cellulase production. (A) Diagram representing the domain architectures of ClrB and ClrB<sup>ID</sup>. MHR, “middle homology region” domain. (B) pNPCase activities of culture supernatants of strains in the medium without carbon source. (C) SDS-PAGE analysis of culture supernatants 48 h after shifting mycelia to the medium without carbon source. Coomassie Brilliant Blue was used for gel staining. (D) Expression levels of cellulase genes determined by qRT-PCR. Transcript abundances (relative to strain M12) four hours after shifting mycelia to the medium without carbon source are shown. In B and D, data represent mean±SD from triplicate cultivations.
Mutation of ClrB causes de-repressed cellulase expression

Figure 4. Expression of clrB<sup>ID</sup> results in cellulase production on glucose and glycerol. (A) Concentrations of residual glucose in culture supernatants of strains in 2% (w/v) glucose medium. (B) pNPCase activities of culture supernatants in glucose medium. (C) SDS-PAGE analysis of culture supernatants 12 h after shifting mycelia to glucose medium. Coomassie Brilliant Blue was used for gel staining. (D) Expression levels of cellulase genes determined by qRT-PCR. Transcript abundances (relative to strain M12) four hours after shifting mycelia to glucose medium are shown. (E) Expression levels of cellulase regulator genes (relative to strain M12) determined by qRT-PCR. (F) pNPCase activities of culture supernatants in 2% (w/v) glycerol medium. In A, B and D-F, data represent mean±SD from triplicate cultivations. The legends in panels B and E are the same with those in A and D, respectively.
Mutation of ClrB causes de-repressed cellulase expression

Figure 5. Detection of full-length and internal-deleted ClrB proteins in glucose medium. ClrB and ClrB<sup>ID</sup> were tagged by EGFP at C-termini. (A) Fluorescence microscopy analysis of ClrB-EGFP and ClrB<sup>ID</sup>-EGFP. The parent strain ΔclrB was used as a control. The nuclei were stained with Hoechst 33342. Scale bar = 20 μm. (B) Western blotting analysis of ClrB-EGFP and ClrB<sup>ID</sup>-EGFP. As a loading control, cell extracts were subjected to SDS-PAGE and Coomassie Brilliant Blue (CBB) staining.
Mutation of ClrB causes de-repressed cellulase expression

Figure 6. Comparative transcriptome analysis of gClrB<sup>ID</sup> strain and reference strain M12 on 2% (w/v) glucose. (A) Scatterplot of FPKM values for all genes in gClrB<sup>ID</sup> and M12. Significantly up-regulated and down-regulated genes (FDR < 0.001 and fold-change > 2) are shown in red and blue colors, respectively. (B) The number of genes of significantly differential expression with indicated fold changes in comparison of gClrB<sup>ID</sup> versus M12. (C) Hierarchical clustering analysis of cellulase genes according to their transcriptional profiles. Blue, white and red colors represent maximum negative expression, zero expression and maximum positive expression after data normalization, respectively. The suffix of gene ID (locus tag prefix: PDE), family number in CAZy database (68), and enzyme activity (CBH, cellobiohydrolase; EG, endo-β-1,4-glucanase) are shown for each gene. Proteins detected in the secretome of <i>P. oxalicum</i> in a previous study (33) are shown in bold. The ΔclrB strain in panel C was constructed by deleting clrB gene in wild-type (WT) strain 114-2 (12).
Mutation of ClrB causes de-repressed cellulase expression

**Figure 7.** The resistance of *clrB* and *creA* mutants to 2-deoxyglucose. Growth and cellulose hydrolysis by strains on agar plates containing 1% (w/v) ball-milled cellulose and increasing concentrations of 2-deoxyglucose are shown. The plates were incubated at 30°C for 48 hours.
Figure 8. The effect of \( clrB^{ID} \) expression on the transcription of CreA target genes. Transcript abundances of genes (relative to reference strain M12) four hours after shifting mycelia to 2% (w/v) glucose medium were determined by qRT-PCR. Data represent mean±SD from triplicate cultivations.
Figure 9. Contributions of clrB internal deletion and creA deletion to enhanced cellulase production on 2% (w/v) cellulose. (A) pNPCase activities of culture supernatants of strains in cellulose medium. (B) SDS-PAGE analysis of culture supernatants 96 h after shifting mycelia to cellulose medium. Coomassie Brilliant Blue was used for gel staining. (C) Expression levels of cellulase genes determined by qRT-PCR. Transcript abundances (relative to M12) four hours after shifting mycelia to cellulose medium are shown. (D) Expression levels of cellulase regulator genes (relative to M12) determined by qRT-PCR. In A, C and D, data represent mean±SD from triplicate cultivations. The legend in panel D is the same with that in C.
Figure 10. Comparison of the effects of clrB\textsuperscript{id} expression and creA deletion on the transcriptome. (A) Overlaps between genes up-regulated in gClrB\textsuperscript{id} strain on glucose and on cellulose. (B) Overlaps between genes up-regulated in gClrB\textsuperscript{id} strain and in ΔcreA on cellulose. (C) PC analysis plot of transcriptome data. The two most significant variances among samples are shown. Each dot represents one biological triplicate.
**Figure 11.** The effect of *AnclrB* manipulation on extracellular enzyme production in *A. niger*. The parent strain N593, full-length *AnclrB*-overexpressing strain gAnClrB, and two independent transformants expressing internal-deleted *AnclrB* were analyzed in 2% (w/v) glucose medium. (A) Concentrations of residual glucose in culture supernatants of strains. (B) *p*NPCase activities of culture supernatants. (C) SDS-PAGE analysis of culture supernatants 12 h after shifting mycelia to glucose medium. Silver nitrate was used for gel staining. The two protein bands identified by mass spectrometry are indicated. In A and B, data represent mean±SD from triplicate cultivations. The legend in panel B is the same with that in A.
Deletion of the middle region of the transcription factor ClrB in *Penicillium oxalicum* enables cellulase production in the presence of glucose

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