McmA-dependent and -independent regulatory systems governing expression of
ClrB-regulated cellulase and hemicellulase genes in *Aspergillus nidulans*

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Summary

Fungal cellulolytic and hemicellulolytic enzymes are promising tools for industrial hydrolysis of cellulosic biomass; however, the regulatory network underlying their production is not well understood. The recent discovery of the transcriptional activators ClrB and McmA in *Aspergillus nidulans* implied a novel regulatory mechanism driven by their interaction, experimental evidence for which was obtained from transcriptional and DNA-binding analyses in this study. We found that ClrB was essential for induced expression of all the genes examined in this study, while McmA dependency of their expression was gene-dependent. DNA-binding studies revealed McmA assisted in the recruitment of ClrB to the cellulose-responsive element (CeRE) in the promoters of *eglA* and *eglB*, expression of which was significantly reduced in the *mcmA* mutant. The CCG triplet within the CeRE served as the recognition sequence for the ClrB monomer. In contrast, ClrB did not require McmA for binding as a homodimer to the CGGN<sub>8</sub>CCG sequences in the promoter of *mndB*, expression of which was affected less in the *mcmA* mutant than in all other examined genes. Thus, there are two types of ClrB-mediated regulation: McmA-assisted and McmA-independent. This novel McmA–ClrB synergistic system provides new insights into the complex regulatory network involved in cellulase and hemicellulase production.
Introduction

Cellulase gene expression in filamentous fungi is generally induced by cellulose and xylan. The physiological inducers are mono- and disaccharides derived from polysaccharides such as cellobiose, sophorose, and gentiobiose, as well as D-xylose, and they vary depending on the species (Sternberg and Mandels, 1979; Kurasawa et al., 1992; Chikamatsu et al., 1999). *Aspergillus niger* XlnR is the first identified transcription factor that regulates both xylanolytic and cellulolytic systems in response to D-xylose (van Peij et al., 1998a; van Peij et al., 1998b; Gielkens et al., 1999). Orthologs of *A. niger* XlnR also regulate the genes encoding xylanolytic and cellulolytic enzymes in other filamentous fungi such as *A. oryzae* and *Hypocrea jecorina* (Marui et al., 2002b; Marui et al., 2002a; Stricker et al., 2006; Noguchi et al., 2009), but XlnR-independent induction of cellulase genes is also observed (Marui et al., 2002a; Brunner et al., 2007; Endo et al., 2008).

XlnR-independent, cellulose-inductive expression of cellulases is regulated by another Zn(II)$_2$Cys$_6$ transcription factor, CLR-2, in *Neurospora crassa*, and by its ortholog ClrB in *A. nidulans* (Coradetti et al., 2012). RNA sequencing analysis indicated that these factors also regulate genes encoding hemicellulolytic enzymes including mannanases. ManR, which was initially identified as an essential transcriptional activator for the β-mannan utilization system in *A. oryzae*, is an ortholog
of CLR-2/ClrB and is responsible for cellulose-inductive expression of the cellulase
genes (Ogawa et al., 2012; Ogawa et al., 2013). Although the three factors are orthologs,
CLR-2 and ClrB have different mechanisms of regulation. Induction of CLR-2 target
genes requires another Zn(II)$_2$Cys$_6$ transcription factor, CLR-1, which is responsible for
cellulose-triggered expression of $clr$-$2$; however, the CLR-1 ortholog, ClrA, is not
required for cellulase induction in $A. nidulans$ (Coradetti et al., 2012). In addition, while
artificially forced expression of $clr$-$2$ leads to inducer-independent expression of the
target genes, ClrB requires an inducer, even under similar conditions (Coradetti et al.,
2013).

The regulation of cellulase genes involves more factors, including ClbR in $A.$
aculeatus (Tani et al., 2012; Kunitake et al., 2013; Tani et al., 2014), the Hap complex
in $A. nidulans$ and $H. jecorina$ (Tanaka et al., 2000; Zeilinger et al., 2001), and the pH
response transcription factor PacC orthologs in $Trichoderma reesei$ and $A. nidulans$ (He
et al., 2014; Kunitake et al., 2016). CreA in $A. nidulans$ and its ortholog Cre1 in $T.$
reesei mediate carbon catabolite repression of cellulase genes (Ilmen et al., 1996;
Lockington et al., 2002). These factors are not essential for cellulose-induced
expression of the cellulase genes, but rather modulate the expression level of the genes
in response to intracellular or environmental conditions.
We previously reported that McmA regulates extracellular enzyme production, including cellulases and asexual/sexual development in *A. nidulans*, and that it binds to the CeRE of the *eglA* promoter (Yamakawa et al., 2013; Li et al., 2016). McmA is a member of the SRF-type MADS box protein family conserved among eukaryotic organisms (Shore and Sharrocks, 1995). The proteins in this family generally regulate gene expression through interaction with other cofactors; so that a single MADS box protein can participate in a variety of cellular functions by changing its partner. Mcm1p in *Saccharomyces cerevisiae* is one of the best-studied SRF-type MADS box proteins. It interacts with transcription factors including α1, α2, Ste12p, Yox1p, Yhp1p, Fkh2p, Arg80p, and Arg81p, and regulates genes involved in mating, cell cycle, and arginine metabolism, depending on the partner transcription factor (Messenguy and Dubois, 2003). However, little information is available on cooperative regulation by Mcm1p orthologs and cofactors in filamentous fungi except that orthologs in several plant pathogens regulate sexual development, microconidia production, virulence, and/or secondary metabolism (Nolting and Poggeler, 2006a; Nolting and Poggeler, 2006b; Mehrabi et al., 2008; Zhou et al., 2011; Ortiz and Shim, 2013). Among these studies, experimental evidence on the cofactors was provided only in *Sordaria macrospora*, in which MCM1 interacts with SmtA-1 that carries an α1 domain and with the Ste12p
homolog STE12 (Nolting and Poggeler, 2006a, Nolting and Poggeler, 2006b).

Considering that ClrB is responsible for cellulose-induced expression in A. nidulans, it is reasonable to assume that McmA and ClrB work together in the regulation of cellulase gene expression, possibly via interaction. However, the observation that mutation of mcmA apparently does not affect mannanolytic activity, even though the target genes of CLR-2/ClrB/ManR include various β-mannanase and β-mannosidase genes (Coradetti et al., 2012; Ogawa et al., 2012; Coradetti et al., 2013; Ogawa et al., 2013; Yamakawa et al., 2013), argues against this hypothesis.

In this study, we investigated participation of McmA and ClrB in the regulation of major cellulase genes in A. nidulans by means of RT-qPCR, electrophoretic mobility shift assay (EMSA), and mutational analysis of the ClrB/McmA binding site in vivo, with special reference to cooperative binding of the two factors to the eglA and eglB promoters. In addition, we identified McmA-independent ClrB-binding sites in the mndB (β-mannosidase) promoter and investigated their functional significance in vivo. To our knowledge, this is the first report showing involvement of McmA-dependent and -independent mechanisms in the regulation of the cellulase and hemicellulase genes that are under control of ClrB. Our data aid in understanding the complex regulatory system governing cellulase and hemicellulase production.
Results

Involvement of McmA in regulation of ClrB-regulated cellulase genes in A. nidulans

Our previous study revealed that McmA is involved in the regulation of the A. nidulans cellulase genes eglA (AN1285), eglB (AN3418), and cbhA (AN5176) (Yamakawa et al., 2013), which are also under control of ClrB (Coradetti et al., 2012; Coradetti et al., 2013). The ClrB target genes include a wide range of genes encoding cellulolytic and hemicellulolytic enzymes as well as transporters and uncharacterized proteins. These findings led to the simple question whether McmA is necessary for inducible expression of all ClrB target genes.

To answer this question, eight cellulase genes and three hemicellulase genes were selected for RT-qPCR analysis, namely the endoglucanase genes eglA and eglB, putative cellobiohydrolase genes cbhA and cbhD (AN1273), a putative lytic polysaccharide monooxygenase gene (AN3860), putative β-glucosidase genes AN10124 and bgII (AN2227), the β-mannosidase gene mndB (AN3368), the mannanase gene manB (AN3297), and the xylanase gene xlnC (AN1818). All of these genes are expressed in a ClrB-dependent manner in response to cellulose (Coradetti et al., 2013).

While cellobiose-induced expression of all genes was observed in the reference strain ABP, the induction was abolished in the clrB disruptant (Fig. 1), which is consistent
with the results of a previous study using cellulose as the inducer (Coradetti et al., 2013).

On the other hand, the effect of the mcmA<sub>I70A</sub> mutation on expression differed depending on the gene. Cellobiose-induced expression of AN3860 was abolished in the mcmA mutant as well as in the clrB disruptant, while that of eglA, eglB, cbhD, and AN10124 was detectable, although significantly decreased and delayed. The effect of the mcmA<sub>I70A</sub> mutation on cbhA and bglII was very weak in terms of the expression level, but expression was obviously delayed. Cellobiose induction of the hemicellulase genes, manB and xlnC, was abolished in both the clrB disruptant and mcmA mutant, although the expression levels of these two genes were extremely low compared to that of the cellulase genes. The expression profile of mndB in the mcmA mutant differed completely from that of the other genes; expression was rapidly induced at 1 h to a level comparable to that in the reference strain. Taken together, the expression profiles of the genes in the mcmA mutant suggested that McmA-dependent and -independent mechanisms are involved in cellobiose induction.

Cooperative binding of McmA and ClrB to the eglA promoter

The cis element CeRE (5'-CCGTACCTTTTTAGG-3'), which is located at -187 to -173 of the eglA promoter relative to the translational start site (+1), is essential for induced
expression of *eglA* (Endo *et al.*, 2008). McmA binds to the sequences 5′-CCTTTTTAGG-3′ (-148 to -139) within the CeRE and 5′-TCCGTTTTGG-3′ (-164 to -155) adjacent to the CeRE (Fig. 2A) (Yamakawa *et al.*, 2013). Strong dependence on the CeRE of induced expression of *eglA* suggested that ClrB is recruited to the CeRE-containing region. Furthermore, ClrB recruitment might depend on McmA because it is a member of the MADS box transcription factors that generally function together with cofactors (Messenguy and Dubois, 2003).

FLAG-tagged DNA-binding domain of ClrB (FLAG-ClrB\textsubscript{118}) and His\textsubscript{6}-tagged McmA (His-McmA) were produced in *E. coli* to examine their binding properties to the *eglA* promoter by EMSA. FLAG-ClrB\textsubscript{118} (aa 1 to 118) contained the Zn(II)\textsubscript{2}Cys\textsubscript{6} DNA-binding motif (aa 37 to 65) and the predicted coiled-coil motif (aa 75 to 94).

SDS-PAGE of purified FLAG-ClrB\textsubscript{118} yielded a single protein band, while the His-McmA preparation contained another protein with low molecular mass (Fig. S2), consistent with our previous study (Yamakawa *et al.*, 2013).

The DNA-binding ability of FLAG-ClrB\textsubscript{118} was examined in the presence and absence of His-McmA to explore whether both proteins show cooperative binding to the *eglA* probe, which was a CeRE-containing region of the *eglA* promoter spanning -251 to -142 (Fig. 2A). As shown in Fig. 2B, FLAG-ClrB\textsubscript{118} produced faint shifted bands with
increasing intensity along with increasing amounts of protein (Fig. 2B, lanes 2 to 4). His-McmA yielded two shifted bands due to the presence of two binding sites, as described previously (Fig. 2B, lane 5) (Yamakawa et al., 2013). When FLAG-ClrB$_{118}$ was added in the presence of a fixed amount of McmA, the McmA-DNA complexes observed in lane 5 disappeared, even at the lowest amount, while two new bands appeared at higher positions (Fig. 2B, lanes 6 to 8). The major shifted band at the top had a much stronger intensity than the shifted bands formed by FLAG-ClrB$_{118}$ or His-McmA alone. These results suggested that FLAG-ClrB$_{118}$ and His-McmA cooperatively form a stable protein–DNA complex, probably through a protein–protein interaction. However, the physical interaction between ClrB and McmA in vitro was not detected by either pull-down assay using Ni-NTA agarose beads or immunoprecipitation assay using anti-FLAG agarose beads (data not shown). Taken together, these results indicate that weak interactions between ClrB and McmA as well as ClrB and DNA likely contribute to the formation of a stable ClrB/McmA/DNA complex.

A supershift assay was performed to confirm the existence of FLAG-ClrB$_{118}$ and His-McmA in the protein–DNA complex formed in the presence of both transcription factors (Fig. 2C). Addition of His-Tag Monoclonal Antibody and Monoclonal ANTI-FLAG® M2 led to the formation of supershift bands and the disappearance of the
protein–DNA complex (Fig. 2C, lanes 2 and 3 from the left). Addition of Strep·Tag® II Monoclonal Antibody did not affect the mobility of the protein–DNA complex (Fig. 2C, lane 4). These results confirm the formation of a protein–DNA complex containing both FLAG-ClrB\textsubscript{118} and His-McmA.

Requirement of the CCG triplet for McmA-assisted ClrB recruitment to CeRE

The CeRE is composed of a CCG triplet and an McmA-binding site (CCN\textsubscript{6}GG) with two-base spacing, suggesting that the CCG triplet might be required for interaction between FLAG-ClrB\textsubscript{118} and CeRE. To obtain experimental evidence for the involvement of the CCG triplet, EMSA was performed with a mutant probe lacking the CCG triplet. His-McmA-assisted recruitment of FLAG-ClrB\textsubscript{118} was observed with probe 31 carrying the CeRE, but not the upstream McmA-binding site (Fig. 3A, B). In contrast, FLAG-ClrB\textsubscript{118} was not recruited to the 31-CCG probe in which CCG was replaced with AAT (Fig. 3A, B), confirming that the CCG triplet is required for McmA-assisted recruitment of ClrB. As shown in Fig. 3A, the CCGN\textsubscript{3}TCN\textsubscript{6}GG sequence similar to the CeRE was present just upstream of the CeRE, and His-McmA bound to TCN\textsubscript{6}GG within the sequence, as previously reported (Yamakawa et al., 2013). However, this sequence did not function in McmA-assisted ClrB recruitment,
suggesting that a two-base spacing between the CCG triplet and the McmA-binding site is crucial (Fig. 3B, probe 32). FLAG-ClrB\textsubscript{118} could bind probe 32 with very low affinity, but not probe 31, in the absence of His-McmA. The presence of three CCG triplets in the probe might be the cause of this weak binding, which was also observed with the longer probe, as shown in Fig. 2. Additionally, the C-terminally truncated McmA derivative His-McmA\textsubscript{143} (aa 1 to 143), which carries the conserved MADS box domain (aa 53 to 134), was subjected to EMSA. The results were basically identical to those with full-length McmA, except that the shift of the DNA-protein complex caused by FLAG-ClrB\textsubscript{118} recruitment was more obvious (Figs. S2 and 3C). These results indicate that the N-terminal 143-aa region supports recruitment of FLAG-ClrB\textsubscript{118} to the probe.

Fig. 3D shows the impact of the I70A mutation in McmA on ClrB recruitment. Reduced intensity of the DNA–McmA complex was clearly visible; however, more importantly, recruitment of FLAG-ClrB\textsubscript{118} was not observed. Faulty ClrB recruitment is the predicted primary cause for the decreased egl\textit{A} expression levels observed in the McmA\textsubscript{I70A} mutant in Fig. 1.

Cooperative binding of McmA and ClrB to the egl\textit{B} promoter

Cooperative DNA binding of His-McmA and FLAG-ClrB\textsubscript{118} was further examined with
eglB promoter fragments as the probes. Probes B1 to B4 covered regions -991 to -703, -758 to -453, -503 to -252, and -287 to -4, respectively (Fig. 4A). No protein–DNA complex was formed upon addition of FLAG-ClrB118, indicating that it has very weak affinity for the eglB promoter sequence (Fig. 4B). In contrast, His-McmA could bind to all the probes by forming multiple protein–DNA complexes of different mobility, indicating the presence of two binding sites in probes B1, two or three in B2, three in B3, and three or four in B4.

His-McmA-assisted recruitment of FLAG-ClrB118 to the B3 and B4 probes, but not to the B1 and B2 probes (Fig. 4B), was detected based on a significant decrease in the unbound probes, suggesting that the sites for cooperative binding of His-McmA and FLAG-ClrB118 are within B3 and B4. In addition, these results confirmed that the binding of His-McmA to the DNA is not sufficient for FLAG-ClrB118 recruitment, as described above for the eglA promoter (Fig. 3). Probe B3 contained a CeRE-like sequence (-264 to -278) in the opposite direction to the eglA CeRE, 5'-CCGN2TCN6GG-3' instead of 5'-CCGN2CCN6GG-3', which was located in the overlapping region of probes B3 and B4. In addition, probe B4 contained a palindromic sequence, 5'-CCGN2CCN6GGN2GG-3' (-116 to -97), comprised of two overlapping copies of the CeRE sequence in opposite directions.
To identify the functional cis-element required for eglB induction, the intact B3 and B4 fragments and their mutated derivatives were examined to determine whether they are functional in induction using the promoter probe vector pBAT. pBAT carries the A. oryzae taka-amylase A structural gene taaG2 but lacks the promoter region including the AmyR-binding site required for induction (Tani et al., 2001b; Tani et al., 2001a).

The intact B3 and B4 fragments and their mutated derivatives, were inserted into the Xba I/Spe I site of pBAT located upstream of the taaG2 structural gene and the resulting plasmids were integrated into the argB locus in A. nidulans (Fig. 4C and S3). Culture supernatants of the strains carrying the pBAT derivatives were subjected to α-amylase assay following 40 h of cultivation with or without carboxymethyl cellulose (CMC) (Fig. 4D). α-Amylase production was induced by CMC when the intact B3 and B4 as well as B4 down mut, which lacked the palindromic CeRE, were used. In contrast, CMC-induction was abolished by mutation of the CeRE-like sequence in B3 mut, B4 up del, B4 up mut, and B4 double mut (Fig. 4D). These results indicate that the functional site in the eglB promoter is the CeRE-like sequence present in B3 and B4.

To confirm that McmA is required for ClrB recruitment to the identified functional cis-element at -264 to -278, EMSA was performed using short DNA fragments spanning -287 to -244 (44 bp) of the eglB promoter containing either the intact CeRE-like
sequence or mutated sequences (Table S2 and Fig. 5A). No protein-DNA complex was formed when only FLAG-ClrB_{118} was mixed with either the wild type (wt) or mutant probes (Fig. 5B, lanes 3, 7, 11, and 15), while addition of His-McmA_{143} enabled the recruitment of FLAG-ClrB_{118} to the wt probe (Fig. 5B, lane 4). FLAG-ClrB_{118} recruitment was not detected with mutant probes CGGmt and CGGCTGmt carrying a CGG to ATT substitution (Fig. 5B, lanes 8 and 16). As described below, ClrB could bind to the CGGN$_8$CCG sequences in the mndB promoter without the assistance of McmA (Fig. 6). A similar motif, CGGN$_8$CTG, was present in the wt eglB probe, with the CGG overlapping the CeRE like sequence. Mutation of the CTG did not affect FLAG-ClrB$_{118}$ recruitment (Fig. 5B, lane 12).

In order to confirm that the recruitment of FLAG-ClrB$_{118}$ observed in vitro correlates with in vivo induction by CMC, the short DNA fragments used in the EMSA in Fig. 5 were examined for in vivo function using the promoter probe vector pBAT. α-Amylase production was induced approximately 3-fold by CMC when the wt and CTGmt fragments were used, while no induction was observed with the CGGmt and CGGCTGmt fragments (Fig. 5C), indicating that the CCGN$_2$TCN$_6$GG motif at -264 to -278 is the functional site of action responsible for eglB induction.
McmA-independent binding of ClrB to the mndB promoter

The mndB gene (AN3368) is adjacent to clrB (AN3369) in the opposite direction and is under control of ClrB (Coradetti et al., 2013). While expression of the cellulase genes examined in this study was delayed and decreased in the mcmA mutant, mndB expression was not significantly affected by the mutation at 1 h and 3 h (Fig. 1). At 6 h, mndB mRNA was still detected in the mutant, while it disappeared in the reference strain. The prolonged expression in the mutant is likely caused by delayed and decreased expression of the genes encoding β-glucosidase. Furthermore, the mndB promoter possesses no CeRE-like sequences. These observations suggested that McmA is not necessary for induced expression of mndB, and thus, ClrB may bind to the mndB promoter without the assistance of McmA.

Pilot EMSAs using probes generated by restriction enzyme digestion of the mndB promoter region revealed that FLAG-ClrB118 by itself bound to some of the restriction fragments and that the 5'-CGGN₈CCG-3' sequence was intact in these fragments (data not shown). The 5'-CGGN₈CCG-3' sequences were located at -560 to -547 (Bs1), -314 to -301 (Bs2), and -222 to -209 (Bs3) (Fig. 6A). To confirm that the 5'-CGGN₈CCG-3' sequence is the target of ClrB, EMSA was performed using a set of probes generated by annealing synthetic oligonucleotides (Table S1). Four probes were designed for each
possible binding site, carrying the wild-type sequence, the CGG to ATT substitution at the 5’ end, the CCG to AAT substitution at the 3’ end, or substitutions at both ends. As shown in Fig. 6, EMSA using the wild-type promoter sequence probes (Bs1-WT, Bs2-WT, and Bs3-WT) resulted in the formation of a single DNA–protein complex, with the weakest binding observed for Bs2-WT. Lack of either one of the CGG/CCG triplets led to complete loss of complex formation. Thus, FLAG-ClrB118 binds to the 5’-CGGN₈CCG-3’ sequences without assistance of McmA, implying that they are responsible for McmA-independent induction. The weak binding of FLAG-ClrB₁₁₈ to the Bs2-WT probe is probably caused by differences in the internal N₈ and/or the outside flanking sequences. In fact, 22 out of 29 bases of the Bs1- and Bs3-containing regions were identical (CNAGAGCTNGAGGGAGNNNANCCCGTNCC), although in opposite direction, while only 12 bases were conserved in Bs2 among the 22 bases shared in Bs1 and Bs3.

The promoter probe vector pBAT was used to verify that the three CGGN₈CCG sequences on the mndB promoter function in induction in vivo. The three ClrB-binding fragments identified by EMSA were inserted into the BamHI site of pBAT and the resulting plasmids were integrated into the argB locus of A. nidulans (Fig. S3). Following 40 h of cultivation, culture supernatants were subjected to α-amylase
assay (Fig. 6E). α-Amylase production was induced approximately 4-fold by CMC in the Bs1+ and Bs1- strains that carried the Bs1 site in opposite directions, indicating that the Bs1 site is functional in vivo. The Bs3 site also appeared functional, although to a lower extent than Bs1, as indicated by the 1.6- and 2.1-fold increase in Bs3+ and Bs3-, respectively. This induction was completely lost in the clrBA strain. As expected, the contribution of the Bs2 site appeared to be insignificant, because of the weak binding of FLAG-ClrB118 to this site. However, this site might be weakly functional in vivo, based on the statistically significant induction ratio of approximately 1.5 in Bs2-.

Binding of the ClrB monomer to CeRE with McmA assistance

The functional forms of Zn(II)$_2$Cys$_6$ type transcription factors often consist of a homodimer or a heterodimer with another same-type transcription factor (MacPherson et al., 2006). Considering that the functional CeREs of the eglA and eglB promoters contained a single CGG/CCG triplet while the functional ClrB binding sites in mndB promoter were comprised of an inverted triplet repeat, ClrB might bind to CeRE as a monomer with the assistance of McmA and to CGGN$_8$CCG as a homodimer. In order to determine whether the ClrB monomer is recruited to CeRE, His-ClrB$_{183}$ (aa 1 to 183), which was larger in size than FLAG-ClrB$_{118}$, was expressed in E. coli, purified, and
When the *mndB* Bs1 or Bs3 probes were used, FLAG-ClrB$_{118}$ demonstrated a single shift band of greater mobility than when His-ClrB$_{183}$ was used, as expected based on the size of the ClrB derivatives (Fig. 7A, lanes 2, 3, 6, and 7). In contrast, a different shift band was detected when a mixture of FLAG-ClrB$_{118}$ and His-ClrB$_{183}$ was used (Fig. 7A, lane 4 and 8), the mobility of which was intermediate between those formed with FLAG-ClrB$_{118}$ or His-ClrB$_{183}$. These results indicate that ClrB binds to the CGGN$_8$CCG sequence as a homodimer.

Similar analysis was performed in the presence of McmA using the *eglA*-31 and *eglB*-B3 probes; however, no intermediate shift band was observed (Fig. 7B). This indicates that the ClrB monomer was recruited to the CCG triplet in the CeRE with the assistance of McmA (Fig. 7B, lane 5 and 10).
Discussion

Previously, we reported the involvement of McmA in the regulation of cellulase genes in *A. nidulans* and suggested that ManR/ClrB was the most probable cofactor of McmA (Yamakawa *et al.*, 2013). This suggestion was based on the fact that the CeRE, to which McmA binds, is the sole *cis* element essential for induced expression of *eglA* (Endo *et al.*, 2008). If the regulation is achieved by direct binding to the cellulase promoters, ClrB should bind to the CeRE. In this study, we provide experimental evidence of McmA-assisted ClrB recruitment to the CeRE and CeRE-like sequence in the *eglA* and *eglB* promoters. In addition to the presence of McmA, the CCG triplet in the CeRE or the CeRE-like sequence was required for ClrB recruitment. This implies that interactions between McmA and ClrB as well as ClrB and DNA are necessary for stable DNA–protein complex formation. In addition, structural changes to the DNA caused by McmA binding might assist ClrB recruitment, as SRF-MADS proteins are known to cause severe DNA bending (West *et al.*, 1997).

In contrast to *eglA* and *eglB*, the *mndB* gene does not possess either a CeRE or a CeRE-like sequence in its promoter region and it was expressed in an McmA-independent manner (Fig. 1). ClrB bound to the CGGN$_3$CCG sequence without the assistance of McmA (Fig. 6). Inverted and direct repeats of CCG/CGG triplets are
typical binding sites of Zn(II)$_2$Cys$_6$ transcription factors (Todd and Andrianopoulos, 1997). Therefore, there are two types of ClrB binding; McmA-assisted binding to a single CCG/CGG triplet in the CeRE or CeRE-like sequence and McmA-independent binding to a CCG/CGG inverted repeat. Notably, McmA-assisted binding of a ClrB monomer via a single CCG/CGG triplet contrasts with the general mode of binding for Zn(II)$_2$Cys$_6$ transcription factors as homodimers to palindromic recognition triplets.

Cellobiose-induced expression of $\text{manB}$ and $\text{xlnC}$ was strongly dependent on ClrB and McmA although their expression levels were significantly lower than those of the cellulase genes (Fig. 1). Additional hemicellulase genes show low level ClrB- and McmA-dependent expression based on previously reported genome-wide transcriptomic analyses (Coradetti et al., 2013; Li et al., 2016). Nevertheless, mutation of $\text{mcmA}$ does not affect the production of mannanolytic and xylanolytic enzymes when cells are grown on glucomannan and xylan (Yamakawa et al., 2013). It is possible that high-level xylan-induced expression of the xylanase genes is mainly regulated by XlnR without the assistance of McmA.

ManR, the ClrB ortholog in $A.\ oryzae$, is essential for cellulose- and $\beta$-mannan-induced expression of the mannanase genes (Ogawa et al., 2012; Ogawa et al., 2013). However, in $A.\ nidulans$, the $\text{man134A}$ (AN2710) gene, which encodes a
novel and major endo-mannanase, is strongly induced by galactomannan but only very weakly by CMC (Shimizu et al. 2015), suggesting that the regulatory systems for induction of the mannanase genes are different in these species. Our preliminary studies revealed that β-mannan-induced expression of the mannanase genes in *A. nidulans* is mainly regulated by the ClrB paralog, ManS, which is not present in *A. oryzae* (unpublished results). Further studies are required to elucidate the precise mechanisms involved in mannanase induction in *Aspergillus*.

Table 1 shows the numbers and locations of the CeRE, CeRE-like, and CGGN₈CCG sequences in the promoter regions of the genes examined in Fig. 1. Gene expression upon exposure to cellobiose was significantly correlated with the presence of CeRE, CeRE-like, and/or CGGN₈CCG sequences, although this does not conclusively prove that the sequences are functional *in vivo*. As mentioned above, *mndB*, expression of which was least affected by the *mcmA* mutation, lacks any CeRE or CeRE-like sequences in its promoter region. In contrast, AN3860 expression absolutely required McmA and its promoter does not possess CGGN₈CCG. Expression of *eglA, eglB, cbhD,* and AN10124, which possess both *cis* elements, was induced by cellobiose, albeit to a significantly lower level in the *mcmA* mutant than in the wild type. The CGGN₈CCG and CeRE sequences partially overlap in *eglB, cbhD,* and AN10124 forming
CGGN$_8$CCGN$_2$CCN$_6$GG, suggesting that ClrB recruitment to these sites might be enhanced by McmA. Expression of $cbhA$ was weakly affected by the $mcmA$ mutation although it does not have a complete CeRE. However, it has a CeRE-like sequence $CCGN_2$TCN$_6$GG, which was identical to the functional CeRE-like sequence in the $eglB$ promoter. It should be noted that McmA does bind to TCN$_6$GG, as reported previously (Yamakawa et al., 2013), and as shown in Fig. 5. The $bgll$ and $xlnC$ promoters do not possess CeRE or CeRE-like sequences; however, their expression was affected by the $mcmA$ mutation. Analysis of the promoter regions revealed the presence of a $CCGN_2$CTN$_6$AG motif overlapping the CGGN$_8$CCG at -440 to -427 in $bgll$ and the $CCGN_2$CTN$_6$GG at -265 to -251 in $xlnC$. Although experimental evidence is lacking, these sequences might function as weak, cooperative binding sites for McmA and ClrB. The $xlnC$ promoter possesses a CGGN$_8$CCG motif at -214 to -201; however, its expression, albeit at a low level, was strictly dependent on McmA under our experimental conditions. This might be due to very weak binding to the site (as is the case for the Bs2 site of $mndB$), considering that it has low similarity to the Bs1 and Bs3 sites (Fig. 6). Furthermore, it should be noted that binding of a transcription factor in vitro to a certain promoter region is supportive, but not conclusive that gene expression is regulated by the binding. A typical example of this is the palindromic CeRE on the B4
fragment. We detected strong McmA-assisted recruitment of ClrB to this site (data not shown); however, the site was not functional in vivo (Fig. 4). Consequently, regulation of the ClrB target genes can be classified into three types: ClrB and McmA absolutely required (AN3860), McmA not required (mndB), and McmA partially required, as observed for the other genes examined in this study.

In T. reesei, the egl2 and cbh2 genes encoding a glycoside hydrolase family 5 (GH5) endoglucanase and a GH6 cellobiohydrolase, respectively, play an important role in efficient formation of the inducer from cellulose, while egl1 (GH7) and cbh1 (GH7) are not involved in inducer formation (Seiboth et al., 1997). The egl2 and cbh2 homologs of A. nidulans (eglA and cbhD, respectively) were affected more severely by the mcmA mutation than eglB and cbhA, the homologs of egl1 and cbh1. Therefore, McmA might play a more important role in the production of cellulolytic enzymes with cellulose as the carbon source than in that with cellobiose, because reduction in eglB and cbhA expression should cause delayed production of inducing molecules such as cellobiose.

In S. cerevisiae, it has been reported that activity of Mcm1p must be regulated because a new isoform was induced by salt stress (Kuo et al., 1997). Mammalian serum response factor MADS protein is regulated by protein kinase C-α via phosphorylation at Ser162 (Iyer et al., 2006). The corresponding Ser73 in McmA and its surrounding
amino acid sequence are highly conserved, suggesting that McmA activity might be
modulated by protein kinase C. Currently, information on McmA is very limited, but
there might be an isoform of McmA that specifically acts in McmA-dependent
regulation by ClrB.

In conclusion, we show that cellulase gene regulation by ClrB involves two classes
of regulatory systems according to McmA-dependency in *A. nidulans*. Given the
widespread presence of McmA and ClrB orthologs among filamentous fungi including
the industrially important *A. niger, A. oryzae*, and *T. reesei*, this combinatorial
regulatory system may be conserved in a wide range of filamentous fungi. However, our
study did not uncover the physiological implications of McmA dependency in the
regulation of genes encoding cellulolytic and hemicellulolytic enzymes. Further studies
will provide a more comprehensive understanding of the complex regulatory system of
cellulase and hemicellulose degradation, leading to innovative applications in
biotechnology industries.
Experimental procedures

A. nidulans strains

The *mcmA* mutant (MCMI70A) and the *clrB* disruptant (ΔClrB) of *A. nidulans* were used in RT-qPCR analysis to clarify the involvement of McmA and ClrB in the expression of various cellulase and hemicellulase genes. Strain MCMI70A (*biA1 pyrG89; wA3; ΔmcmA::mcmAI70A::pyr4 argB2::argB::eglAp-taaG2; pyroA4*) expresses the McmA protein with the I70A substitution (Yamakawa *et al.*, 2013). The *clrB* disruptant strain ΔClrB (*pyrG89 biA1; wA3; argB2; pyroA4; ΔclrB::pyrG*) was constructed by replacing the *clrB*-coding region of *A. nidulans* ABPU1 (*pyrG89 biA1; wA3; argB2; pyroA4*) with *A. nidulans* *pyrG* (Fig. S1). Strain ABP (*biA1; wA3; argB2; pyroA4*), which was generated by replacing the *pyrG89* allele of *A. nidulans* ABPU1 with the wild-type *pyrG* gene, was used as a reference strain. Oligonucleotide primers used for strain construction are listed in Table S1. *A. nidulans* ABPU1 was used as the host in the reporter assay for *eglB* and *mndB* promoter activity as described below.

Transformation of *A. nidulans* was carried out by protoplast transformation (Ballance and Turner, 1985) with slight modifications, as previously described (Makita *et al.*, 2009). *A. nidulans* strains were grown at 37°C in standard minimal medium with appropriate supplements unless otherwise noted (Rowlands and Turner, 1973).
RT-qPCR analysis

Strains ABP, MCMI70A, and ΔClrB were pregrown at 37°C for 24 h in standard minimal medium containing the appropriate supplements. Polypeptone NF at a concentration of 1% was used instead of glucose as the carbon source. The mycelia were collected and rinsed with minimal medium without a carbon source. Then, the mycelia (0.5 g wet weight) were transferred to 40 ml of fresh minimal medium containing 0.1% cellobiose as the inducer. The mycelia were harvested 1, 3, and 6 h after transfer, frozen in liquid nitrogen, and ground to fine powder with an SK mill SK-100 (Tokken Inc., Kashiwa, Japan). RNA extraction and RT-qPCR were carried out as previously described (Yamakawa et al., 2013) using THUNDERBIRD® SYBR® qPCR mix (Toyobo Co. Ltd., Osaka, Japan) with the primers listed in Table S2.

Expression and purification of recombinant ClrB and McmA proteins

FLAG- and His6-tagged tagged ClrB derivatives (FLAG-ClrB118 and His6-ClrB183) and His6-tagged McmA derivatives (His-McmA, His-McmA170A, and His-McmA143) were produced in E. coli. The numbers after the transcription factors represent the C-termini of the recombinant proteins; for example, FLAG-ClrB118 includes amino acid residues 1 to 118. I70A designates a substitution of I70 to A. Purification of the His-tagged McmA
derivatives, His-McmA and His-McmA\textsubscript{170A}, produced in \textit{E. coli} was conducted as previously reported (Yamakawa et al., 2013).

Recombinant plasmids for expression of FLAG-ClrB\textsubscript{118}, His-ClrB\textsubscript{183}, and His-McmA\textsubscript{143} were constructed by conventional molecular cloning methods with \textit{E. coli} XL1-Blue and JM109 as the hosts. The FLAG-ClrB\textsubscript{118} expression plasmid carried the PCR-amplified and \textit{Hind} III/\textit{Eco} R I-digested DNA fragment encoding the 118 N-terminal amino acid residues containing the Zn(II)\textsubscript{2}Cys\textsubscript{6} DNA-binding motif inserted between the corresponding restriction enzyme sites of pT7-FLAGTM-1 (Sigma-Aldrich Co., St. Louis, MO, USA). The His-ClrB\textsubscript{183} expression plasmid was generated by introducing the PCR-amplified and \textit{Nde} I/\textit{Xho} I-digested DNA fragment encoding the 183 N-terminal amino acid residues between the corresponding restriction enzyme sites of pET-15b (Merck Millipore, Darmstadt, Germany). The McmA\textsubscript{143} expression plasmid was generated by introducing the PCR-amplified and \textit{Nde} I/\textit{Xho} I-digested DNA fragment encoding the 143 N-terminal amino acid residues between the corresponding restriction enzyme sites of pET-33b(+) (Merck Millipore, Darmstadt, Germany). PCR amplification was carried out with primer sets ClrB-1-f and ClrB-1-r-354 for FLAG-ClrB\textsubscript{118}, HM(Nde1)sense and HM(Xho1)anti-sense for His-ClrB\textsubscript{183}, and McmA1-f and McmA143-r for His-McmA\textsubscript{143} (Table S1) with \textit{A.}
*nidulans* cDNA, synthesized using the SuperScript™ First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific Inc., Waltham, MA, USA) as the template.

The plasmids were introduced into *E. coli* BL21 (DE3) for expression of recombinant proteins. Cultivation of the strains, expression of the recombinant proteins, and cell extract preparation were performed as previously described (Yamakawa *et al.*, 2013), except that the cells were resuspended in TBS buffer (50 mM Tris-HCl [pH 7.4] and 150 mM NaCl) prior to sonication. When required, kanamycin or ampicillin was added to the growth media at a concentration of 50 μg ml⁻¹.

His-McmA, His-McmA₁₄₃, His-McmA₁₇₀₈, and His-ClrB₁₈₃ were purified from the cell extracts using Ni-NTA agarose (Qiagen, Hilden, Germany), as previously described (Yamakawa *et al.*, 2013). FLAG-ClrB₁₁₈ was purified using ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich Co., St. Louis, MO, USA) following the manufacturers’ instructions. FLAG-ClrB₁₁₈ bound to the gel was eluted with 100 μg ml⁻¹ FLAG peptide (Sigma-Aldrich) and dialyzed overnight at 4°C against TBS containing 10% glycerol followed by dialysis against TBS containing 50% glycerol for another 3 h. Purified proteins were stored at -20°C.

Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) with bovine IgG as the standard.
Biotin-labeled DNA probes were generated in different ways depending on the probe.

The *eglA* probe covered a 110-bp region (-251 to -142 referring to the translational start site as +1) of the *eglA* promoter including the CeRE region. This probe was obtained by PCR using the *eglA* promoter primers (*eglA* promoter110s and biotin-eglA-promoter-r; Table S1) with chromosomal DNA of *A. nidulans* as the template. The latter primer had a biotin moiety at the 5' end. For analysis of the *eglB* promoter, four DNA fragments spanning -991 to -703, -758 to -453, -503 to -251, and -287 to -4 (referring to the translational start site as +1) were PCR-amplified with the primer sets for *eglB* promoter analysis (Table S1). The PCR products were digested with *Bam*H I designed within the sense-strand primers and the resulting 5'-overhangs were filled in by Klenow fragment using a dNTP mixture containing biotin-14-dCTP (Thermo Fisher Scientific) instead of dCTP. The probes for analysis of the *eglB* and *mndB* promoters were prepared by annealing two complementary oligonucleotides that were designed to produce 5'-overhangs after annealing (Table S1). Annealing reactions were performed as follows: equimolar amounts of two oligonucleotides were mixed in annealing buffer (10 mM Tris·HCl, 1 mM EDTA, and 50 mM NaCl [pH 8]) and boiled for 5 min. Then, the
mixture was slowly cooled down to room temperature and the 5'-overhangs were biotinylated as described above.

EMSA was performed using the LightShift® Chemiluminescent EMSA Kit (Thermo Fisher Scientific). All binding reactions were carried out in the presence of 100 ng μl⁻¹ of Poly(dI-dC) (Thermo Fisher Scientific), 1 nM of biotin-labeled DNA probe, 1 mM ZnCl₂, and various concentrations of the recombinant proteins. DNA–protein complexes were separated by electrophoresis on a 5% or 6% polyacrylamide gel and transferred to a Hybond-N+ membrane (GE Healthcare Japan, Tokyo, Japan). The membrane was processed for detection of biotinylated probes per the manufacturer’s instructions. Chemiluminescent signals were visualized using a LAS 3000 system (Fujifilm Co., Tokyo, Japan).

Supershift assays to detect His-McmA and FLAG-ClrB₁₁₈ in DNA–protein complexes were performed using the His·Tag Monoclonal Antibody (EMD Millipore, Billerica, MA, USA) and the Monoclonal ANTI-FLAG® M2 antibody (Sigma-Aldrich). The Strep·Tag® II Monoclonal Antibody (Merck Millipore) was used as a negative control. In this assay, each antibody was added to the binding reaction mixture prior to addition of the probe. The reaction mixture was incubated at room temperature for 20 min. The following steps were the same as for EMSA, as described above.
Reporter assay to identify the cis element required for induction of egI\textit{B} and \textit{mndB}

For identification of the \textit{in vivo} functional cis-element in the egI\textit{B} promoter, the B3 and B4 fragments and derivatives containing mutations in the candidate cis-elements (Fig. 4) were generated by PCR amplification. The combination of primers used for construction of the fragments is shown in Fig. S3A. The DNA fragments were inserted into the \textit{Xba I}/Spe I site of the promoter probe vector pBAT using the GeneArt® Seamless Cloning and Assembly Enzyme Mix (Thermo Fisher Scientific). This vector carries the Taka-amylase A structural gene \textit{taaG2} without a functional promoter, so that insertion of a DNA fragment with a functional cis element at the multiple cloning site leads to \textit{taaG2} expression under control of the inserted element (Tani \textit{et al.}, 2001a).

Short DNA fragments containing the \textit{in vivo} functional CeRE-like sequence of the \textit{eglB} promoter or each of the three ClrB binding sites in the \textit{mndB} promoter were produced by annealing sets of complementary oligonucleotides listed in Table S1. The oligonucleotide sets were designed to produce 5'-overhangs (GATC) at both termini after annealing, which enabled insertion of the fragments at the \textit{BamH I} site in pBAT (Fig. S3). pBAT derivatives carrying the \textit{eglB} and \textit{mndB} promoter fragments were digested at the \textit{EcoR V} site within the \textit{A. nidulans argB} gene and introduced into \textit{A. nidulans} ABPU1 andΔClrB. Transformants with single plasmid integration at the \textit{argB}
locus were selected by Southern blot analysis (Fig. S3C).

The transformants were examined for α-amylase production in response to CMC as previously described (Endo et al., 2008). The amount of enzyme that liberated 1 μmol of reducing sugar equivalent to glucose per min was defined as one unit of activity.

Acknowledgements

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(xylanase regulator 1) regulates both the hydrolytic enzyme system and 
analyses of the AmyR binding site of the Aspergillus nidulans agdA 
promoter; requirement of the CGG direct repeat for induction and high affinity 
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family: the Zn(II)$_2$Cys$_6$ binuclear cluster DNA binding motif. Fungal Genet Biol 
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jecorina HAP 2/3/5 protein complex binds to the inverted CCAAT-box 
(ATTGG) within the cbh2 (cellulbiohydrolase II-gene) activating element. Mol
Figure legends

Fig. 1. Dependence of the expression of cellulolytic and hemi-cellulolytic enzyme genes on ClrB and McmA. The expression levels, relative to the β-actin gene (actA), of endoglucanase (eglA and eglB), putative cellbiohydrolase (cbhA and cbhD), putative lytic polysaccharide monooxygenase (AN3860), putative β-glucosidase (AN10124 and bgII), β-mannosidase (mndB), β-mannanase (manB), and xylanase (xlnC) genes are shown. The x-axes represent the time (h) after induction by cellobiose. Data are from three biological replicates. Letters above the bars indicate a significant difference (p < 0.05, one-way ANOVA).

Fig. 2. Binding of ClrB and McmA to the eglA promoter. (A) Schematic representation of the DNA probe used for EMSA. Two McmA-binding sites are boxed, one of which is located in the CeRE (cellulose-responsive element). Possible binding sites for CreA are indicated by dark gray boxes. The numbers indicate nucleotide positions relative to the translational start site designated as +1. (B) Binding analysis of ClrB to the probe in the absence (lanes 1 to 4) and presence (lanes 5 to 8) of McmA. The amounts (μg per 20 μl of reaction mixture) of FLAG-ClrB118 and His-McmA are indicated above the lanes (−, +).
no addition). DNA–protein complexes formed with FLAG-ClrB_{118} or His-McmA are indicated by thin arrows. The thick arrows indicate complexes containing both FLAG-ClrB_{118} and His-McmA. (C) Supershift assay to confirm the inclusion of FLAG-ClrB_{118} and His-McmA in the DNA–protein complex. FLAG-ClrB_{118} and His-McmA (1.0 μg and 0.4 μg, respectively) were incubated with the probe in the presence of either the Anti-His tag (lane 2), Anti-Flag tag (lane 3), or Anti-Strep tag (lane 4) antibodies as indicated. The Anti-Strep tag antibody was used as a negative control.

Fig. 3. Identification of the sequence required for recruitment of FLAG-ClrB_{118} and the ability of McmA derivatives His-McmA_{143} and His-McmA_{I70A} to form a DNA–protein complex with FLAG-ClrB_{118}. (A) DNA sequence of probes 31 and 32. The CeRE is boxed and the McmA-binding sites are underlined. The CCG triplet within the CeRE is shown in bold and italics. This triplet was replaced by AAT in the 31-CCG probe. (B, C) Effect of mutation of the CCG triplet on FLAG-ClrB_{118} recruitment. The C-terminal truncated McmA derivative His-McmA_{143} was used in (C). (D) Effect of the I70A mutation in McmA on ClrB recruitment. The amounts of protein used were 1.5 μg, 0.8 μg, and 0.8 μg for FLAG-ClrB_{118}, His-McmA, His-McmA_{143}, and His-McmA_{I70A}, respectively.
respectively, in a 20-µl reaction mixture.

Fig. 4. Binding of ClrB and McmA to the eglB promoter and *in vivo* function of the binding sites. (A) Schematic drawing of DNA probes (B1–B4) derived from the eglB promoter used in EMSA analysis. Two overlapping CeRE sequences sharing the single McmA-binding site are indicated by arrows. The numbers indicate nucleotide positions relative to the translation start site. (B) Binding of FLAG-ClrB_{118} and His-McmA to the probes. One microgram of each protein was used in a 20-µl reaction mixture. (C) Schematic drawing of the DNA fragments inserted into the pBAT reporter plasmid. Checked patterns indicate the positions of CeRE and CeRE-like sequences, which were the targets of the mutational studies. Asterisks indicate that the corresponding CeRE or CeRE-like sequence was mutated. The base changes introduced by mutation are indicated by the bold letters. (D) *In vivo* function of the CeRE and CeRE-like sequences on the eglB promoter. The DNA fragments shown in (C) were evaluated for their ability to confer CMC-induced production of α-amylase using the reporter gene *taaG2* (taka-amylase A) in pBAT (Fig. S3). Statistical significance was analyzed using a two-tailed (equal variances) *t*-test; *, *p* < 0.01.
Fig. 5. Mutational analysis of the combinatorial binding site of ClrB and McmA on the eglB promoter. (A) Wild-type and mutated probes used for EMSA. The CeRE-like sequences located at -278 to -264 are underlined. Triplets in bold and italics indicate the mutations. Mutant probes carried a CGG to ATT (CGGmt) or CTG to AAT (CTGmt) substitution. (B) Recruitment of FLAG-ClrB_{118} to the probes by His-McmA_{143}; 1.0 μg of FLAG-ClrB_{118} and His-McmA_{143} were used. (C) In vivo functionality of the CeRE-like sequence. The DNA fragments used in (B) were inserted into pBAT and introduced into A. nidulans. α-Amylase activity was measured following cultivation in the presence and absence of CMC. Statistical significance was analyzed using a two-tailed (equal variances) t-test; *, p < 0.01.

Fig. 6. Binding of ClrB to the CGGN$_8$CCG sites in the mndB promoter and their in vivo activity. (A) Sequences and locations of the three CGGN$_8$CCG sites. The CGG/CCG triplets are shown in bold. The locations are indicated by the positions of the 5' and 3' ends referring to the translational start site as +1. Bs: binding site. (B)–(D) Effects of mutations in the CGG and CCG triplets on binding of FLAG-ClrB$_{118}$ (1.2 μg). Mutant probes carried a CGG to ATT (Bs1-CGG, Bs2-CCG, and Bs3-CCG) or CCG to AAT (Bs1-CCG, Bs2-CCG, and Bs3-CCG) substitution. Bs1-, Bs2-, and Bs3-dbl probes had
mutations in both CGG and CCG. (E) Activity of the ClrB-binding sites on the *mndB*
promoter as a *cis* element for induction. DNA fragments carrying the three ClrB-binding
sites (Bs1–Bs3) in the *mndB* promoter were inserted upstream of the reporter gene
*taaG2* (taka-amylase A) in pBAT and introduced into *A. nidulans* (Fig. S3). α-Amylase
activity produced by the strains carrying pBAT and its derivatives was determined
following cultivation in the presence and absence of CMC. +, forward insertion; -, reverse insertion. Statistical significance was analyzed using a two-tailed (equal
variances) *t*-test; *, *p* < 0.05, **, *p* < 0.01, ***, *p* < 0.001.

Fig. 7. McmA-assisted recruitment of the ClrB monomer to the CeRE-like sequence on
the *eglB* promoter. (A) Binding of a ClrB dimer to the CGG/CCG inverted repeat on the
*mndB* promoter. The FLAG-ClrB<sub>118</sub> homodimer is indicated by a gray triangle, the
His-ClrB<sub>183</sub> homodimer by a white triangle, and the FLAG-ClrB<sub>118</sub> and His-ClrB<sub>183</sub>
heterodimer by a black triangle. (B) McmA-assisted binding of the ClrB monomer to
the CeRE and CeRE-like sequences in the *eglA* and *eglB* promoters. The gray triangles
indicate complexes formed by His-McmA<sub>143</sub> and DNA, the black triangles indicate a
FLAG-ClrB<sub>118</sub> and His-McmA<sub>143</sub> hetero complex, and the gray triangles indicate a
His-ClrB\textsubscript{183} and His-McmA\textsubscript{143} hetero complex. The amounts of protein used were 1.0 μg, 1.5 μg, and 1.0 μg for FLAG-ClrB\textsubscript{118}, His-ClrB\textsubscript{183}, and His-McmA\textsubscript{143}, respectively.
Table 1. Numbers and locations of CGGN$_8$CCG and CeRE in the promoters of genes encoding cellulolytic and hemi-cellulolytic enzymes.

<table>
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<th>CeRE-like</th>
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<td>eglA</td>
<td>1 (-144 to -131)</td>
<td>1 (-187 to -173)</td>
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<tr>
<td>eglB</td>
<td>1 (-127 to -114)$^#$</td>
<td>2## (-116 to -102, -97 to -111)</td>
<td>2 (-818 to -804, -264 to -278)</td>
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<tr>
<td>xlnC</td>
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Numbers of CGGN$_8$CCG, CCGN$_2$CCN$_6$GG (CeRE), and CCGN$_2$TCN$_6$GG or CCGN$_2$CCN$_6$GA (CeRE-like) are shown with their locations in parentheses. $^#$ CGGN$_8$CCG and CeRE overlap (CGGN$_8$CCGN$_2$CCN$_6$GG), ## Two CeREs overlap in the opposite direction.
Fig. 1 Li et al.
A

eglAp

-251

CreA CreA CreA

TCCGT TTTTGG

McmA

CeRE

-142

CCGT ACCCTTTTAAAGGA

McmA

B

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C

Fig. 2 Li et al.
### A

**eglAp**

-210

CGCACTCCGCACTCCGTTTTGGT

**CeRE**

-163

\[\text{31} \quad \text{32}\]

### B

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<td><strong>31-CCG</strong></td>
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<td><strong>32</strong></td>
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### C

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<tr>
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<td>-</td>
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<tr>
<td><strong>31-CCG</strong></td>
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<td>+</td>
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<tr>
<td><strong>32</strong></td>
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### D

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<td>McmA/McmA_{170A}</td>
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<td>ClrB</td>
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<tr>
<td>McmA/McmA_{170A}</td>
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</tr>
</tbody>
</table>

Fig. 3 Li et al.
Figure A: Schematic representation of the eglBp promoter region showing the locations of CeRE sequences.

- **B1**
  - -991
  - -703
  - -503
  - -251

- **B2**
  - -758
  - -453
  - -251

- **B3**
  - -116
  - -97

- **B4**
  - -264
  - -278

**CeRE sequences**: CCGTCCATGTATGGACGG

Figure B: Gel shift assay showing the binding of ClrB and McmA to different promoters.

- **B1**
  - ClrB McmA: - + + - - - + +

- **B2**
  - ClrB McmA: - + + - - - + +

- **B3**
  - ClrB McmA: - + + - - - + +

- **B4**
  - ClrB McmA: - + + - - - + +

Figure C: DNA sequence variations in the promoters.

- **B3**
  - -503
  - -251

- **B3 mut**
  - -503
  - -251

- **B4**
  - -287
  - -4

- **B4 up del**
  - -225
  - -4

- **B4 up mut**
  - -287
  - -4

- **B4 down mut**
  - -287
  - -4

- **B4 double mut**
  - -287
  - -4

**CeRE sequences (B3 mut)**: AAATATTCTTAATT

**CeRE sequences (B4 up mut)**: AAATATTCTTAATT

**CeRE sequences (B4 down mut)**: AAATATTCTTAATT

**CeRE sequences (B4 double mut)**: AAATATTCTTAATT

Figure D: Bar graph showing amylase activity (U/g dry mycelia) in different treatments.

- **Empty vector**
  - uninduced: 80 ± 10
  - induced: 80 ± 10

- **B3**
  - uninduced: 80 ± 10
  - induced: 160 ± 20

- **B3 mut**
  - uninduced: 80 ± 10
  - induced: 160 ± 20

- **B4**
  - uninduced: 80 ± 10
  - induced: 160 ± 20

- **B4 up del**
  - uninduced: 80 ± 10
  - induced: 160 ± 20

- **B4 up mut**
  - uninduced: 80 ± 10
  - induced: 160 ± 20

- **B4 down mut**
  - uninduced: 80 ± 10
  - induced: 160 ± 20

- **B4 double mut**
  - uninduced: 80 ± 10
  - induced: 160 ± 20

* indicates significant difference.
A

\[ \text{eglBp-B3 CeRE-like} \]

\[ -287 \text{ to } -244 \]

wt: \( \text{GGCACTTCCATATTCGACA CCGAGAGGAAA CTGAGAATTATC} \)

CGGmt: \( \text{GGCACTTCCATATTCGACA AATTAGAGGAAA CTGAGAATTATC} \)

CTGmt: \( \text{GGCACTTCCATATTCGACA CGGAGAGGAAA AATTAGAATTATC} \)

CGGCTGmt: \( \text{GGCACTTCCATATTCGACA AATTAGAGGAAA AATTAGAATTATC} \)

B

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<tr>
<th></th>
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<th>CGGmt</th>
<th>CTGmt</th>
<th>CGGCTGmt</th>
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<td>+</td>
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C

![Graph showing Amylase activity (U/g dry mycelia)]

- **uninduced**
- **induced**

Fig. 5 Li et al.
A

\( mndBp^{-1000} \)  

Bs1  `CGGAGCGAAACCG` (-560 to -547)  
Bs2  `CGGAGAAAGAGCCG` (-314 to -301)  
Bs3  `CGGGCTCTCCCTCGG` (-222 to -209)

B

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<th>Bs1-CGG</th>
<th>Bs1-CGG</th>
<th>Bs1-dbl</th>
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C

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<th>Bs2-dbl</th>
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D

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E

**Fig. 6 Li et al.**

Amylase activity (U/g dry mycelia)

- Uninduced
- Induced
**A**

<table>
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<tr>
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<th>mndB-Bs1</th>
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<tr>
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**B**

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<td>- - + - +</td>
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<tr>
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<tr>
<td>His-McmA$_{143}$</td>
<td>- + + + +</td>
<td>- + + + +</td>
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2x His-McmA$_{143}$, 1x His-ClrB$_{183}$
2x His-McmA$_{143}$, 1x FLAG-ClrB$_{118}$
2x His-McmA$_{143}$

Fig. 7 Li et al.