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 $CGGN_8CCG$ 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 $Zn(II)_2Cys_6$ 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 $Zn(II)_2Cys_6$ 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

93 homolog STE12 (Nolting and Poggeler, 2006a, Nolting and Poggeler, 2006b).

94 Considering that ClrB is responsible for cellulose-induced expression in *A. nidulans*,
95 it is reasonable to assume that McmA and ClrB work together in the regulation of
96 cellulase gene expression, possibly via interaction. However, the observation that
97 mutation of *mcmA* apparently does not affect mannanolytic activity, even though the
98 target genes of CLR-2/ClrB/ManR include various β-mannanase and β-mannosidase
99 genes (Coradetti *et al.*, 2012; Ogawa *et al.*, 2012; Coradetti *et al.*, 2013; Ogawa *et al.*,
100 2013; Yamakawa *et al.*, 2013), argues against this hypothesis.

101 In this study, we investigated participation of McmA and ClrB in the regulation of
102 major cellulase genes in *A. nidulans* by means of RT-qPCR, electrophoretic mobility
103 shift assay (EMSA), and mutational analysis of the ClrB/McmA binding site *in vivo*,
104 with special reference to cooperative binding of the two factors to the *eglA* and *eglB*
105 promoters. In addition, we identified McmA-independent ClrB-binding sites in the
106 *mndB* (β-mannosidase) promoter and investigated their functional significance *in vivo*.
107 To our knowledge, this is the first report showing involvement of McmA-dependent and
108 -independent mechanisms in the regulation of the cellulase and hemicellulase genes that
109 are under control of ClrB. Our data aid in understanding the complex regulatory system
110 governing cellulase and hemicellulase production.
111

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 *bglI* (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*$_{I70A}$ 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*$_{I70A}$ mutation on *cbhA* and *bglI* 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

149 expression of *eglA* (Endo *et al.*, 2008). McmA binds to the sequences
150 5'-CCTTTTTAGG-3' (-148 to -139) within the CeRE and 5'-TCCGTTTTGG-3' (-164 to
151 -155) adjacent to the CeRE (Fig. 2A) (Yamakawa *et al.*, 2013). Strong dependence on
152 the CeRE of induced expression of *eglA* suggested that ClrB is recruited to the
153 CeRE-containing region. Furthermore, ClrB recruitment might depend on McmA
154 because it is a member of the MADS box transcription factors that generally function
155 together with cofactors (Messenguy and Dubois, 2003).
156 FLAG-tagged DNA-binding domain of ClrB (FLAG-$ClrB_{118}$) and His_6-tagged
157 McmA (His-McmA) were produced in *E. coli* to examine their binding properties to the
158 *eglA* promoter by EMSA. FLAG-$ClrB_{118}$ (aa 1 to 118) contained the $Zn(II)_2Cys_6$
159 DNA-binding motif (aa 37 to 65) and the predicted coiled-coil motif (aa 75 to 94).
160 SDS-PAGE of purified FLAG-$ClrB_{118}$ yielded a single protein band, while the
161 His-McmA preparation contained another protein with low molecular mass (Fig. S2),
162 consistent with our previous study (Yamakawa *et al.*, 2013).
163 The DNA-binding ability of FLAG-$ClrB_{118}$ was examined in the presence and
164 absence of His-McmA to explore whether both proteins show cooperative binding to the
165 *eglA* probe, which was a CeRE-containing region of the *eglA* promoter spanning -251 to
166 -142 (Fig. 2A). As shown in Fig. 2B, FLAG-$ClrB_{118}$ produced faint shifted bands with

167 increasing intensity along with increasing amounts of protein (Fig. 2B, lanes 2 to 4).
168 His-McmA yielded two shifted bands due to the presence of two binding sites, as
169 described previously (Fig. 2B, lane 5) (Yamakawa *et al.*, 2013). When FLAG-ClrB$_{118}$
170 was added in the presence of a fixed amount of McmA, the McmA-DNA complexes
171 observed in lane 5 disappeared, even at the lowest amount, while two new bands
172 appeared at higher positions (Fig. 2B, lanes 6 to 8). The major shifted band at the top
173 had a much stronger intensity than the shifted bands formed by FLAG-ClrB$_{118}$ or
174 His-McmA alone. These results suggested that FLAG-ClrB$_{118}$ and His-McmA
175 cooperatively form a stable protein–DNA complex, probably through a protein–protein
176 interaction. However, the physical interaction between ClrB and McmA *in vitro* was not
177 detected by either pull-down assay using Ni-NTA agarose beads or immunoprecipitation
178 assay using anti-FLAG agarose beads (data not shown). Taken together, these results
179 indicate that weak interactions between ClrB and McmA as well as ClrB and DNA
180 likely contribute to the formation of a stable ClrB/McmA/DNA complex.
181 A supershift assay was performed to confirm the existence of FLAG-ClrB$_{118}$ and
182 His-McmA in the protein–DNA complex formed in the presence of both transcription
183 factors (Fig. 2C). Addition of His·Tag Monoclonal Antibody and Monoclonal
184 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$_{118}$ 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$_6$GG) with two-base spacing, suggesting that the CCG triplet might be required for interaction between FLAG-ClrB$_{118}$ 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$_{118}$ was observed with probe 31 carrying the CeRE, but not the upstream McmA-binding site (Fig. 3A, B). In contrast, FLAG-ClrB$_{118}$ 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$_3$TCN$_6$GG sequence similar to the CeRE was present just upstream of the CeRE, and His-McmA bound to TCN$_6$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$_{118}$ 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$_{143}$ (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$_{118}$ recruitment was more obvious (Figs. S2 and 3C). These results indicate that the N-terminal 143-aa region supports recruitment of FLAG-ClrB$_{118}$ 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$_{118}$ was not observed. Faulty ClrB recruitment is the predicted primary cause for the decreased *eglA* expression levels observed in the McmA$_{I70A}$ mutant in Fig. 1.

Cooperative binding of McmA and ClrB to the eglB promoter

Cooperative DNA binding of His-McmA and FLAG-ClrB$_{118}$ 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-$ClrB_{118}$, 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-$ClrB_{118}$ 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-$ClrB_{118}$ are within B3 and B4. In addition, these results confirmed that the binding of His-McmA to the DNA is not sufficient for FLAG-$ClrB_{118}$ 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′-$CCGN_2TCN_6GG$-3′ instead of 5′-$CCGN_2CCN_6GG$-3′, which was located in the overlapping region of probes B3 and B4. In addition, probe B4 contained a palindromic sequence, 5'-$CCGN_2CCN_6GGN_2CGG$-3' (-116 to -97), comprised of two overlapping copies of the CeRE sequence in opposite directions.

239 To identify the functional *cis-element* required for *eglB* induction, the intact B3 and B4
240 fragments and their mutated derivatives were examined to determine whether they are
241 functional in induction using the promoter probe vector pBAT. pBAT carries the *A.*
242 *oryzae* taka-amylase A structural gene *taaG2* but lacks the promoter region including
243 the AmyR-binding site required for induction (Tani *et al.*, 2001b; Tani *et al.*, 2001a).
244 The intact B3 and B4 fragments and their mutated derivatives, were inserted into the
245 *Xba* I/*Spe* I site of pBAT located upstream of the *taaG2* structural gene and the resulting
246 plasmids were integrated into the *argB* locus in *A. nidulans* (Fig. 4C and S3). Culture
247 supernatants of the strains carrying the pBAT derivatives were subjected to α-amylase
248 assay following 40 h of cultivation with or without carboxymethyl cellulose (CMC)
249 (Fig. 4D). α-Amylase production was induced by CMC when the intact B3 and B4 as
250 well as B4 down mut, which lacked the palindromic CeRE, were used. In contrast,
251 CMC-induction was abolished by mutation of the CeRE-like sequence in B3 mut, B4 up
252 del, B4 up mut, and B4 double mut (Fig. 4D). These results indicate that the functional
253 site in the *eglB* promoter is the CeRE-like sequence present in B3 and B4.
254 To confirm that McmA is required for ClrB recruitment to the identified functional
255 *cis*-element at -264 to -278, EMSA was performed using short DNA fragments spanning
256 -287 to -244 (44 bp) of the *eglB* promoter containing either the intact CeRE-like

257 sequence or mutated sequences (Table S2 and Fig. 5A). No protein-DNA complex was
258 formed when only FLAG-ClrB$_{118}$ was mixed with either the wild type (wt) or mutant
259 probes (Fig. 5B, lanes 3, 7, 11, and 15), while addition of His-McmA$_{143}$ enabled the
260 recruitment of FLAG-ClrB$_{118}$ to the wt probe (Fig. 5B, lane 4). FLAG-ClrB$_{118}$
261 recruitment was not detected with mutant probes CGGmt and CGGCTGmt carrying a
262 CGG to ATT substitution (Fig. 5B, lanes 8 and 16). As described below, ClrB could
263 bind to the CGGN$_8$CCG sequences in the *mndB* promoter without the assistance of
264 McmA (Fig. 6). A similar motif, CGGN$_8$CTG, was present in the wt *eglB* probe, with
265 the CGG overlapping the CeRE like sequence. Mutation of the CTG did not affect
266 FLAG-ClrB$_{118}$ recruitment (Fig. 5B, lane 12).
267 In order to confirm that the recruitment of FLAG-ClrB$_{118}$ observed *in vitro*
268 correlates with *in vivo* induction by CMC, the short DNA fragments used in the EMSA
269 in Fig. 5 were examined for *in vivo* function using the promoter probe vector pBAT.
270 α-Amylase production was induced approximately 3-fold by CMC when the wt and
271 CTGmt fragments were used, while no induction was observed with the CGGmt and
272 CGGCTGmt fragments (Fig. 5C), indicating that the CCGN$_2$TCN$_6$GG motif at -264 to
273 -278 is the functional site of action responsible for *eglB* induction.
274

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-$ClrB_{118}$ by itself bound to some of the restriction fragments and that the 5'-$CGGN_8CCG$-3' sequence was intact in these fragments (data not shown). The 5'-$CGGN_8CCG$-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'-$CGGN_8CCG$-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

293 possible binding site, carrying the wild-type sequence, the CGG to ATT substitution at
294 the 5' end, the CCG to AAT substitution at the 3' end, or substitutions at both ends. As
295 shown in Fig. 6, EMSA using the wild-type promoter sequence probes (Bs1-WT,
296 Bs2-WT, and Bs3-WT) resulted in the formation of a single DNA–protein complex,
297 with the weakest binding observed for Bs2-WT. Lack of either one of the CGG/CCG
298 triplets led to complete loss of complex formation. Thus, FLAG-ClrB$_{118}$ binds to the
299 5'-CGGN$_8$CCG-3' sequences without assistance of McmA, implying that they are
300 responsible for McmA-independent induction. The weak binding of FLAG-ClrB$_{118}$ to
301 the Bs2-WT probe is probably caused by differences in the internal N$_8$ and/or the
302 outside flanking sequences. In fact, 22 out of 29 bases of the Bs1- and Bs3-containing
303 regions were identical (CNAGAGCTNGACGGAGNNNANCCCGTNCC), although in
304 opposite direction, while only 12 bases were conserved in Bs2 among the 22 bases
305 shared in Bs1 and Bs3.
306 The promoter probe vector pBAT was used to verify that the three CGGN$_8$CCG
307 sequences on the *mndB* promoter function in induction *in vivo*. The three ClrB-binding
308 fragments identified by EMSA were inserted into the *Bam*H I site of pBAT and the
309 resulting plasmids were integrated into the *argB* locus of *A. nidulans* (Fig. S3).
310 Following 40 h of cultivation, culture supernatants were subjected to α-amylase

311 assay (Fig. 6E). α-Amylase production was induced approximately 4-fold by CMC in
312 the Bs1+ and Bs1- strains that carried the Bs1 site in opposite directions, indicating that
313 the Bs1 site is functional *in vivo*. The Bs3 site also appeared functional, although to a
314 lower extent than Bs1, as indicated by the 1.6- and 2.1-fold increase in Bs3+ and Bs3-,
315 respectively. This induction was completely lost in the *clrBΔ* strain. As expected, the
316 contribution of the Bs2 site appeared to be insignificant, because of the weak binding of
317 FLAG-ClrB$_{118}$ to this site. However, this site might be weakly functional *in vivo*, based
318 on the statistically significant induction ratio of approximately 1.5 in Bs2-.
319
320 *Binding of the ClrB monomer to CeRE with McmA assistance*
321 The functional forms of Zn(II)$_2$Cys$_6$ type transcription factors often consist of a
322 homodimer or a heterodimer with another same-type transcription factor (MacPherson
323 *et al.*, 2006). Considering that the functional CeREs of the *eglA* and *eglB* promoters
324 contained a single CGG/CCG triplet while the functional ClrB binding sites in *mndB*
325 promoter were comprised of an inverted triplet repeat, ClrB might bind to CeRE as a
326 monomer with the assistance of McmA and to CGGN$_8$CCG as a homodimer. In order to
327 determine whether the ClrB monomer is recruited to CeRE, His-ClrB$_{183}$ (aa 1 to 183),
328 which was larger in size than FLAG-ClrB$_{118}$, was expressed in *E. coli*, purified, and

used in EMSA.

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 $CGGN_8CCG$ sequence without the assistance of McmA (Fig. 6). Inverted and direct repeats of CCG/CGG triplets are

typical binding sites of $Zn(II)_2Cys_6$ 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 $Zn(II)_2Cys_6$ transcription factors as homodimers to palindromic recognition triplets.

Cellobiose-induced expression of *manB* and *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 *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 β-mannan-induced expression of the mannanase genes (Ogawa *et al*., 2012; Ogawa *et al*., 2013). However, in *A. nidulans*, the *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 $CGGN_8CCG$ 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 $CGGN_8CCG$ 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 $CGGN_8CCG$. 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 $CGGN_8CCG$ and CeRE sequences partially overlap in *eglB*, *cbhD*, and AN10124 forming

396 $CGGN_8CCGN_2CCN_6GG$, suggesting that ClrB recruitment to these sites might be
397 enhanced by McmA. Expression of *cbhA* was weakly affected by the *mcmA* mutation
398 although it does not have a complete CeRE. However, it has a CeRE-like sequence
399 $CCGN_2TCN_6GG$, which was identical to the functional CeRE-like sequence in the *eglB*
400 promoter. It should be noted that McmA does bind to TCN_6GG, as reported previously
401 (Yamakawa *et al.*, 2013), and as shown in Fig. 5. The *bglI* and *xlnC* promoters do not
402 possess CeRE or CeRE-like sequences; however, their expression was affected by the
403 *mcmA* mutation. Analysis of the promoter regions revealed the presence of a
404 $CCGN_2CTN_6AG$ motif overlapping the $CGGN_8CCG$ at -440 to -427 in *bglI* and the
405 $CCGN_2CTN_6GG$ at -265 to -251 in *xlnC*. Although experimental evidence is lacking,
406 these sequences might function as weak, cooperative binding sites for McmA and ClrB.
407 The *xlnC* promoter possesses a $CGGN_8CCG$ motif at -214 to -201; however, its
408 expression, albeit at a low level, was strictly dependent on McmA under our
409 experimental conditions. This might be due to very weak binding to the site (as is the
410 case for the Bs2 site of *mndB*), considering that it has low similarity to the Bs1 and Bs3
411 sites (Fig. 6). Furthermore, it should be noted that binding of a transcription factor *in*
412 *vitro* to a certain promoter region is supportive, but not conclusive that gene expression
413 is regulated by the binding. A typical example of this is the palindromic CeRE on the B4

414 fragment. We detected strong McmA-assisted recruitment of ClrB to this site (data not
415 shown); however, the site was not functional *in vivo* (Fig. 4). Consequently, regulation
416 of the ClrB target genes can be classified into three types: ClrB and McmA absolutely
417 required (AN3860), McmA not required (*mndB*), and McmA partially required, as
418 observed for the other genes examined in this study.

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

428 In *S. cerevisiae*, it has been reported that activity of Mcm1p must be regulated
429 because a new isoform was induced by salt stress (Kuo *et al.*, 1997). Mammalian serum
430 response factor MADS protein is regulated by protein kinase C-α via phosphorylation at
431 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).

467 *RT-qPCR analysis*

468 Strains ABP, MCMI70A, and ΔClrB were pregrown at 37°C for 24 h in standard
469 minimal medium containing the appropriate supplements. Polypeptone NF at a
470 concentration of 1% was used instead of glucose as the carbon source. The mycelia
471 were collected and rinsed with minimal medium without a carbon source. Then, the
472 mycelia (0.5 g wet weight) were transferred to 40 ml of fresh minimal medium
473 containing 0.1% cellobiose as the inducer. The mycelia were harvested 1, 3, and 6 h
474 after transfer, frozen in liquid nitrogen, and ground to fine powder with an SK mill
475 SK-100 (Tokken Inc., Kashiwa, Japan). RNA extraction and RT-qPCR were carried out
476 as previously described (Yamakawa *et al.*, 2013) using THUNDERBIRD® SYBR®
477 qPCR mix (Toyobo Co. Ltd., Osaka, Japan) with the primers listed in Table S2.
478
479 *Expression and purification of recombinant ClrB and McmA proteins*
480 FLAG- and His_6-tagged tagged ClrB derivatives (FLAG-$ClrB_{118}$ and His_6-$ClrB_{183}$) and
481 His_6-tagged McmA derivatives (His-McmA, His-$McmA_{I70A}$, and His-$McmA_{143}$) were
482 produced in *E. coli*. The numbers after the transcription factors represent the C-termini
483 of the recombinant proteins; for example, FLAG-$ClrB_{118}$ includes amino acid residues 1
484 to 118. I70A designates a substitution of I70 to A. Purification of the His-tagged McmA

485 derivatives, His-McmA and His-McmA$_{I70A}$, produced in *E. coli* was conducted as
486 previously reported (Yamakawa *et al.*, 2013).
487 Recombinant plasmids for expression of FLAG-ClrB$_{118}$, His-ClrB$_{183}$, and
488 His-McmA$_{143}$ were constructed by conventional molecular cloning methods with *E. coli*
489 XL1-Blue and JM109 as the hosts. The FLAG-ClrB$_{118}$ expression plasmid carried the
490 PCR-amplified and *Hin*d III/*Eco*R I-digested DNA fragment encoding the 118
491 N-terminal amino acid residues containing the Zn(II)$_2$Cys$_6$ DNA-binding motif
492 inserted between the corresponding restriction enzyme sites of pT7-FLAGTM-1
493 (Sigma-Aldrich Co., St. Louis, MO, USA). The His-ClrB$_{183}$ expression plasmid was
494 generated by introducing the PCR-amplified and *Nde* I/*Xho* I-digested DNA fragment
495 encoding the 183 N-terminal amino acid residues between the corresponding
496 restriction enzyme sites of pET-15b (Merck Millipore, Darmstadt, Germany). The
497 McmA$_{143}$ expression plasmid was generated by introducing the PCR-amplified and *Nde*
498 I/*Xho* I-digested DNA fragment encoding the 143 N-terminal amino acid residues
499 between the corresponding restriction enzyme sites of pET-33b(+) (Merck Millipore,
500 Darmstadt, Germany). PCR amplification was carried out with primer sets ClrB-1-f and
501 ClrB-1-r-354 for FLAG-ClrB$_{118}$, HM(Nde1)sense and HM(Xho1)anti-sense for
502 His-ClrB$_{183}$, and McmA1-f and McmA143-r for His-McmA$_{143}$ (Table S1) with *A.*

503 *nidulans* cDNA, synthesized using the SuperScript^{TM} First-Strand Synthesis System for
504 RT-PCR (Thermo Fisher Scientific Inc., Waltham, MA, USA) as the template.
505 The plasmids were introduced into *E. coli* BL21 (DE3) for expression of
506 recombinant proteins. Cultivation of the strains, expression of the recombinant proteins,
507 and cell extract preparation were performed as previously described (Yamakawa *et al.*,
508 2013), except that the cells were resuspended in TBS buffer (50 mM Tris-HCl [pH 7.4]
509 and 150 mM NaCl) prior to sonication. When required, kanamycin or ampicillin was
510 added to the growth media at a concentration of 50 μg ml^{-1}.
511 His-McmA, His-McmA$_{143}$, His-McmA$_{I70A}$, and His-ClrB$_{183}$ were purified from the
512 cell extracts using Ni-NTA agarose (Qiagen, Hilden, Germany), as previously described
513 (Yamakawa *et al.*, 2013). FLAG-ClrB$_{118}$ was purified using ANTI-FLAG® M2 Affinity
514 Gel (Sigma-Aldrich Co., St. Louis, MO, USA) following the manufacturers' instructions.
515 FLAG-ClrB$_{118}$ bound to the gel was eluted with 100 μg ml^{-1} FLAG peptide
516 (Sigma-Aldrich) and dialyzed overnight at 4°C against TBS containing 10% glycerol
517 followed by dialysis against TBS containing 50% glycerol for another 3 h. Purified
518 proteins were stored at -20°C.
519 Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad,
520 Hercules, CA, USA) with bovine IgG as the standard.

521

522 *EMSA*

523 Biotin-labeled DNA probes were generated in different ways depending on the probe.
524 The *eglA* probe covered a 110-bp region (-251 to -142 referring to the translational start
525 site as +1) of the *eglA* promoter including the CeRE region. This probe was obtained by
526 PCR using the *eglA* promoter primers (eglA promoter110s and biotin-eglA-promoter-r;
527 Table S1) with chromosomal DNA of *A. nidulans* as the template. The latter primer had
528 a biotin moiety at the 5' end. For analysis of the *eglB* promoter, four DNA fragments
529 spanning -991 to -703, -758 to -453, -503 to -251, and -287 to -4 (referring to the
530 translational start site as +1) were PCR-amplified with the primer sets for *eglB* promoter
531 analysis (Table S1). The PCR products were digested with *Bam*H I designed within the
532 sense-strand primers and the resulting 5'-overhangs were filled in by Klenow fragment
533 using a dNTP mixture containing biotin-14-dCTP (Thermo Fisher Scientific) instead of
534 dCTP. The probes for analysis of the *eglB* and *mndB* promoters were prepared by
535 annealing two complementary oligonucleotides that were designed to produce
536 5'-overhangs after annealing (Table S1). Annealing reactions were performed as
537 follows: equimolar amounts of two oligonucleotides were mixed in annealing buffer (10
538 mM Tris·HCl, 1 mM EDTA, and 50 mM NaCl [pH 8]) and boiled for 5 min. Then, the

539 mixture was slowly cooled down to room temperature and the 5'-overhangs were
540 biotinylated as described above.
541 EMSA was performed using the LightShift® Chemiluminescent EMSA Kit (Thermo
542 Fisher Scientific). All binding reactions were carried out in the presence of 100 ng μl^{-1}
543 of Poly(dI-dC) (Thermo Fisher Scientific), 1 nM of biotin-labeled DNA probe, 1 mM
544 $ZnCl_2$, and various concentrations of the recombinant proteins. DNA–protein
545 complexes were separated by electrophoresis on a 5% or 6% polyacrylamide gel and
546 transferred to a Hybond-N+ membrane (GE Healthcare Japan, Tokyo, Japan). The
547 membrane was processed for detection of biotinylated probes per the manufacturer's
548 instructions. Chemiluminescent signals were visualized using a LAS 3000 system
549 (Fujifilm Co., Tokyo, Japan).
550 Supershift assays to detect His-McmA and FLAG-$ClrB_{118}$ in DNA–protein
551 complexes were performed using the His·Tag Monoclonal Antibody (EMD Millipore,
552 Billerica, MA, USA) and the Monoclonal ANTI-FLAG® M2 antibody (Sigma-Aldrich).
553 The Strep·Tag® II Monoclonal Antibody (Merck Millipore) was used as a negative
554 control. In this assay, each antibody was added to the binding reaction mixture prior to
555 addition of the probe. The reaction mixture was incubated at room temperature for 20
556 min. The following steps were the same as for EMSA, as described above.

Reporter assay to identify the cis element required for induction of eglB and *mndB*

For identification of the *in vivo* functional *cis*-element in the *eglB* 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 *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 *taaG2* without a functional promoter, so that insertion of a DNA fragment with a functional *cis* element at the multiple cloning site leads to *taaG2* expression under control of the inserted element (Tani *et al.*, 2001a).

Short DNA fragments containing the *in vivo* functional CeRE-like sequence of the *eglB* promoter or each of the three ClrB binding sites in the *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 *Bam*H I site in pBAT (Fig. S3). pBAT derivatives carrying the *eglB* and *mndB* promoter fragments were digested at the *Eco*R V site within the *A. nidulans argB* gene and introduced into *A. nidulans* ABPU1 and ΔClrB. Transformants with single plasmid integration at the *argB*

575 locus were selected by Southern blot analysis (Fig. S3C).

576 The transformants were examined for α-amylase production in response to CMC as

577 previously described (Endo *et al.*, 2008). The amount of enzyme that liberated 1 μmol

578 of reducing sugar equivalent to glucose per min was defined as one unit of activity.

579

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585

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734

735

736 **Figure legends**

737

738 Fig. 1. Dependence of the expression of cellulolytic and hemi-cellulolytic enzyme genes
739 on ClrB and McmA. The expression levels, relative to the β-actin gene (*actA*), of
740 endoglucanase (*eglA* and *eglB*), putative cellobiohydrolase (*cbhA* and *cbhD*), putative
741 lytic polysaccharide monooxygenase (AN3860), putative β-glucosidase (AN10124 and
742 *bglI*), β-mannosidase (*mndB*), β-mannanase (*manB*), and xylanase (*xlnC*) genes are
743 shown. The x-axes represent the time (h) after induction by cellobiose. Data are from
744 three biological replicates. Letters above the bars indicate a significant difference ($p <$
745 0.05, one-way ANOVA).

746

747 Fig. 2. Binding of ClrB and McmA to the *eglA* promoter. (A) Schematic representation
748 of the DNA probe used for EMSA. Two McmA-binding sites are boxed, one of which is
749 located in the CeRE (cellulose-responsive element). Possible binding sites for CreA are
750 indicated by dark gray boxes. The numbers indicate nucleotide positions relative to the
751 translational start site designated as +1. (B) Binding analysis of ClrB to the probe in the
752 absence (lanes 1 to 4) and presence (lanes 5 to 8) of McmA. The amounts (μg per 20 μl
753 of reaction mixture) of FLAG-ClrB$_{118}$ and His-McmA are indicated above the lanes (-,

754 no addition). DNA–protein complexes formed with FLAG-ClrB$_{118}$ or His-McmA are
755 indicated by thin arrows. The thick arrows indicate complexes containing both
756 FLAG-ClrB$_{118}$ and His-McmA. (C) Supershift assay to confirm the inclusion of
757 FLAG-ClrB$_{118}$ and His-McmA in the DNA–protein complex. FLAG-ClrB$_{118}$ and
758 His-McmA (1.0 μg and 0.4 μg, respectively) were incubated with the probe in the
759 presence of either the Anti-His tag (lane 2), Anti-Flag tag (lane 3), or Anti-Strep tag
760 (lane 4) antibodies as indicated. The Anti-Strep tag antibody was used as a negative
761 control.
762
763 Fig. 3. Identification of the sequence required for recruitment of FLAG-ClrB$_{118}$ and the
764 ability of McmA derivatives His-McmA$_{143}$ and His-McmA$_{I70A}$ to form a DNA–protein
765 complex with FLAG-ClrB$_{118}$. (A) DNA sequence of probes 31 and 32. The CeRE is
766 boxed and the McmA-binding sites are underlined. The CCG triplet within the CeRE is
767 shown in bold and italics. This triplet was replaced by AAT in the 31-CCG probe. (B, C)
768 Effect of mutation of the CCG triplet on FLAG-ClrB$_{118}$ recruitment. The C-terminal
769 truncated McmA derivative His-McmA$_{143}$ was used in (C). (D) Effect of the I70A
770 mutation in McmA on ClrB recruitment. The amounts of protein used were 1.5 μg, 0.8
771 μg, and 0.8 μg for FLAG-ClrB$_{118}$, His-McmA, His-McmA$_{143}$, and His-McmA$_{I70A}$,

772 respectively, in a 20-μl reaction mixture.

773

774 Fig. 4. Binding of ClrB and McmA to the *eglB* promoter and *in vivo* function of the
775 binding sites. (A) Schematic drawing of DNA probes (B1–B4) derived from the *eglB*
776 promoter used in EMSA analysis. Two overlapping CeRE sequences sharing the single
777 McmA-binding site are indicated by arrows. The numbers indicate nucleotide positions
778 relative to the translation start site. (B) Binding of FLAG-$ClrB_{118}$ and His-McmA to the
779 probes. One microgram of each protein was used in a 20-μl reaction mixture. (C)
780 Schematic drawing of the DNA fragments inserted into the pBAT reporter plasmid.
781 Checked patterns indicate the positions of CeRE and CeRE-like sequences, which were
782 the targets of the mutational studies. Asterisks indicate that the corresponding CeRE or
783 CeRE-like sequence was mutated. The base changes introduced by mutation are
784 indicated by the bold letters. (D) *In vivo* function of the CeRE and CeRE-like sequences
785 on the *eglB* promoter. The DNA fragments shown in (C) were evaluated for their ability
786 to confer CMC-induced production of α-amylase using the reporter gene *taaG2*
787 (taka-amylase A) in pBAT (Fig. S3). Statistical significance was analyzed using a
788 two-tailed (equal variances) *t*-test; *, $p < 0.01$.

789

790 Fig. 5. Mutational analysis of the combinatorial binding site of ClrB and McmA on the
791 *eglB* promoter. (A) Wild-type and mutated probes used for EMSA. The CeRE-like
792 sequences located at -278 to -264 are underlined. Triplets in bold and italics indicate the
793 mutations. Mutant probes carried a CGG to ATT (CGGmt) or CTG to AAT (CTGmt)
794 substitution. (B) Recruitment of FLAG-ClrB$_{118}$ to the probes by His-McmA$_{143}$; 1.0 μg
795 of FLAG-ClrB$_{118}$ and His-McmA$_{143}$ were used. (C) *In vivo* functionality of the
796 CeRE-like sequence. The DNA fragments used in (B) were inserted into pBAT and
797 introduced into *A. nidulans*. α-Amylase activity was measured following cultivation in
798 the presence and absence of CMC. Statistical significance was analyzed using a
799 two-tailed (equal variances) *t*-test; *, $p < 0.01$.
800
801 Fig. 6. Binding of ClrB to the CGGN$_8$CCG sites in the *mndB* promoter and their *in vivo*
802 activity. (A) Sequences and locations of the three CGGN$_8$CCG sites. The CGG/CCG
803 triplets are shown in bold. The locations are indicated by the positions of the 5' and 3'
804 ends referring to the translational start site as +1. Bs: binding site. (B)–(D) Effects of
805 mutations in the CGG and CCG triplets on binding of FLAG-ClrB$_{118}$ (1.2 μg). Mutant
806 probes carried a CGG to ATT (Bs1-CGG, Bs2-CGG, and Bs3-CGG) or CCG to AAT
807 (Bs1-CCG, Bs2-CCG, and Bs3-CCG) substitution. Bs1-, Bs2-, and Bs3-dbl probes had

808 mutations in both CGG and CCG. (E) Activity of the ClrB-binding sites on the *mndB*
809 promoter as a *cis* element for induction. DNA fragments carrying the three ClrB-binding
810 sites (Bs1–Bs3) in the *mndB* promoter were inserted upstream of the reporter gene
811 *taaG2* (taka-amylase A) in pBAT and introduced into *A. nidulans* (Fig. S3). α-Amylase
812 activity produced by the strains carrying pBAT and its derivatives was determined
813 following cultivation in the presence and absence of CMC. +, forward insertion; -,
814 reverse insertion. Statistical significance was analyzed using a two-tailed (equal
815 variances) *t*-test; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.
816
817 Fig. 7. McmA-assisted recruitment of the ClrB monomer to the CeRE-like sequence on
818 the *eglB* promoter. (A) Binding of a ClrB dimer to the CGG/CCG inverted repeat on the
819 *mndB* promoter. The FLAG-$ClrB_{118}$ homodimer is indicated by a gray triangle, the
820 His-$ClrB_{183}$ homodimer by a white triangle, and the FLAG-$ClrB_{118}$ and His-$ClrB_{183}$
821 heterodimer by a black triangle. (B) McmA-assisted binding of the ClrB monomer to
822 the CeRE and CeRE-like sequences in the *eglA* and *eglB* promoters. The gray triangles
823 indicate complexes formed by His-$McmA_{143}$ and DNA, the black triangles indicate a
824 FLAG-$ClrB_{118}$ and His-$McmA_{143}$ hetero complex, and the gray triangles indicate a

825 His-ClrB$_{183}$ and His-McmA$_{143}$ hetero complex. The amounts of protein used were 1.0

826 μg, 1.5 μg, and 1.0 μg for FLAG-ClrB$_{118}$, His-ClrB$_{183}$, and His-McmA$_{143}$, respectively

827

Table 1. Numbers and locations of $CGGN_8CCG$ and CeRE in the promoters of genes encoding cellulolytic and hemi-cellulolytic enzymes.

Gene	$CGGN_8CCG$	CeRE	CeRE-like
eglA	1 (-144 to -131)	1 (-187 to -173)	0
eglB	1 (-127 to -114)[#]	2[##] (-116 to -102, -97 to -111)	2 (-818 to -804, -264 to -278)
cbhA	2 (-392 to -379, -185 to -172)	0	1 (-496 to -510)
cbhD	1 (-753 to -740)[#]	1 (-742 to -728)	1 (-847 to -861)
AN3860	0	1 (-850 to -864)	1 (-408 to -422)
AN10124	2 (-392 to -379[#], -226 to -213)	1 (-390 to -404)	0
bglI	2 (-498 to -485, -440 to -427)	0	0
mndB	3 (-560 to -547, -314 to -301, -222 to -209)	0	0
manB	0	1 (-264 to -250)	0
xlnC	1 (-214 to -201)	0	0

Numbers of $CGGN_8CCG$, $CCGN_2CCN_6GG$ (CeRE), and $CCGN_2TCN_6GG$ or $CCGN_2CCN_6GA$ (CeRE-like) are shown with their locations in parentheses. [#] $CGGN_8CCG$ and CeRE overlap ($CGGN_8CCGN_2CCN_6GG$), [##] Two CeREs overlap in the opposite direction.

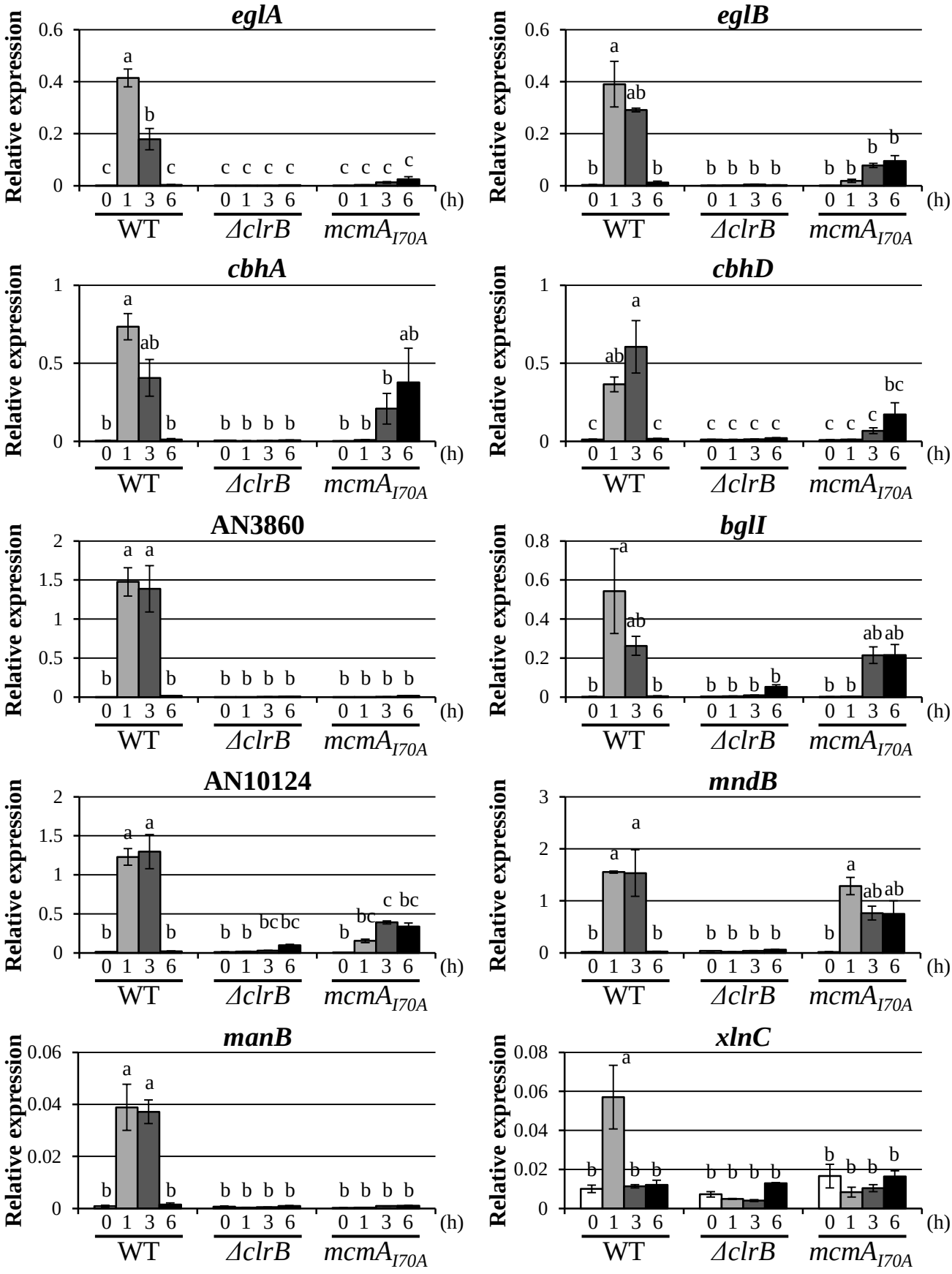

eglA
eglB
cbhA
cbhD
AN3860
bglI
AN10124
mndB
manB
xlnC
Relative expression
0 1 3 6 (h)
WT
ΔclrB
mcmA I70A

Fig. 1 Li et al.

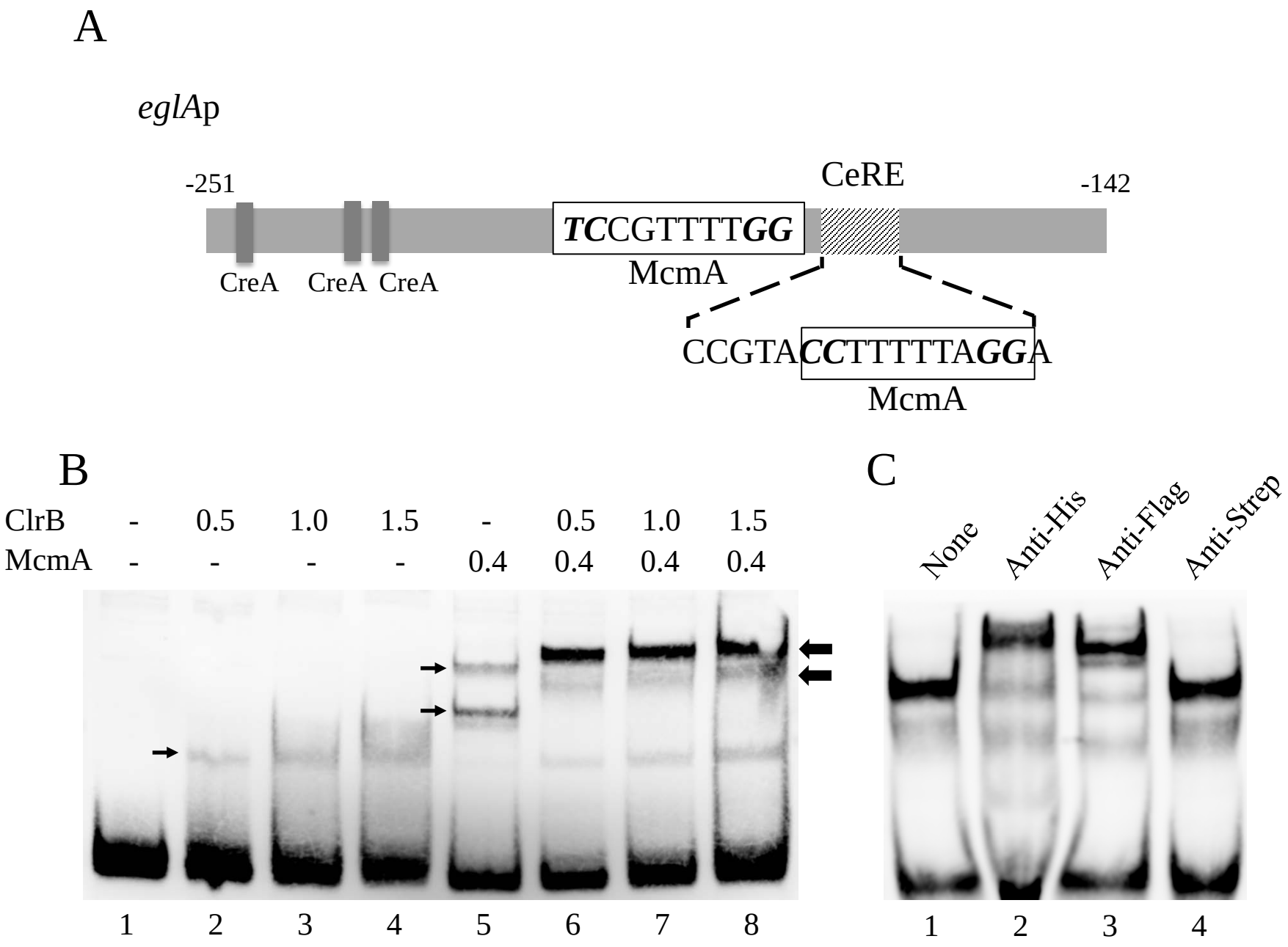

A
eglAp
-251
CeRE
-142
TCCGTTTTGG
CreA
CreA
CreA
McmA
CCGTACCTTTTTAGGA
McmA
B
ClrB - 0.5 1.0 1.5 - 0.5 1.0 1.5
McmA - - - - 0.4 0.4 0.4 0.4
1 2 3 4 5 6 7 8
C
None
Anti-His
Anti-Flag
Anti-Strep
1 2 3 4

Fig. 2 Li et al.

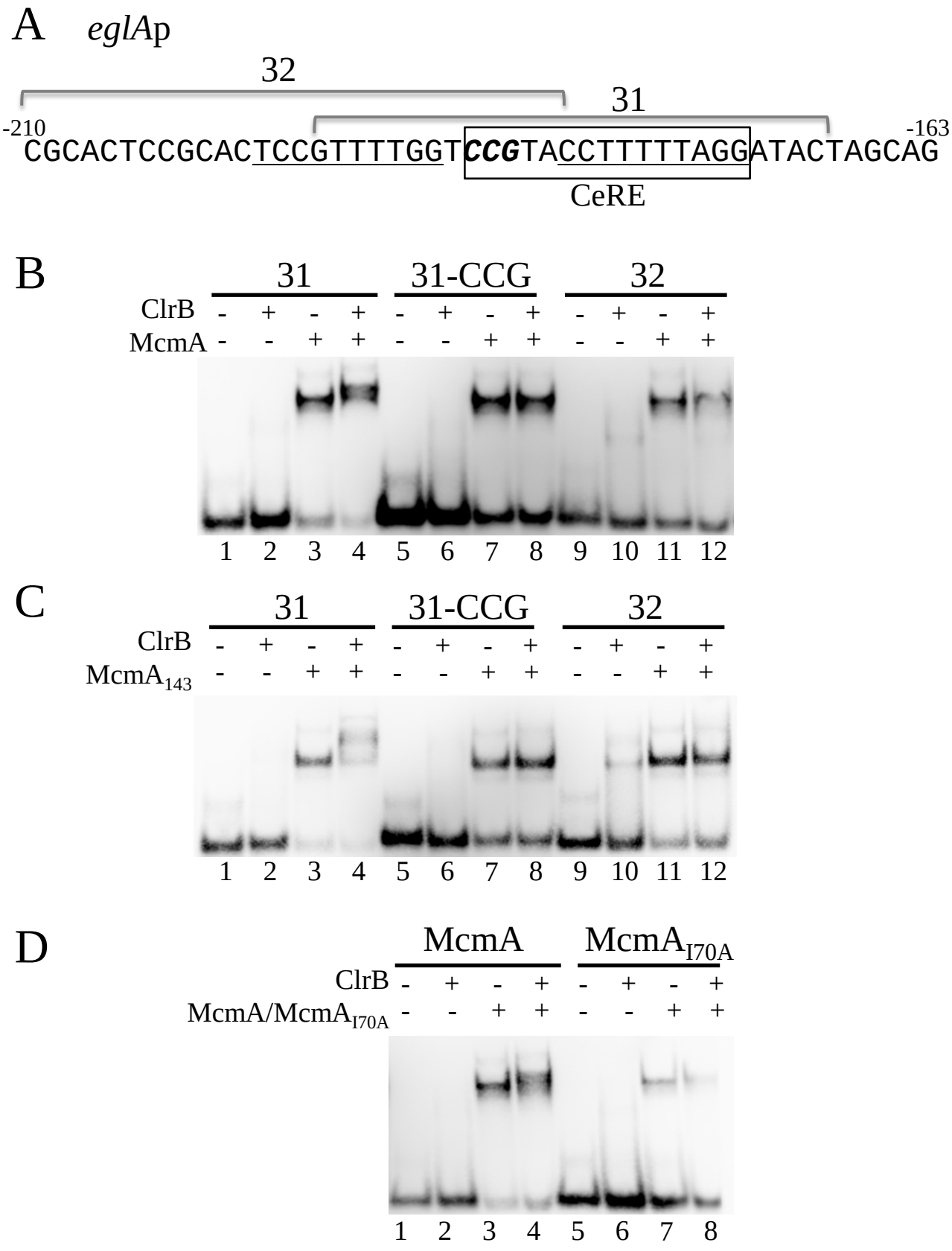
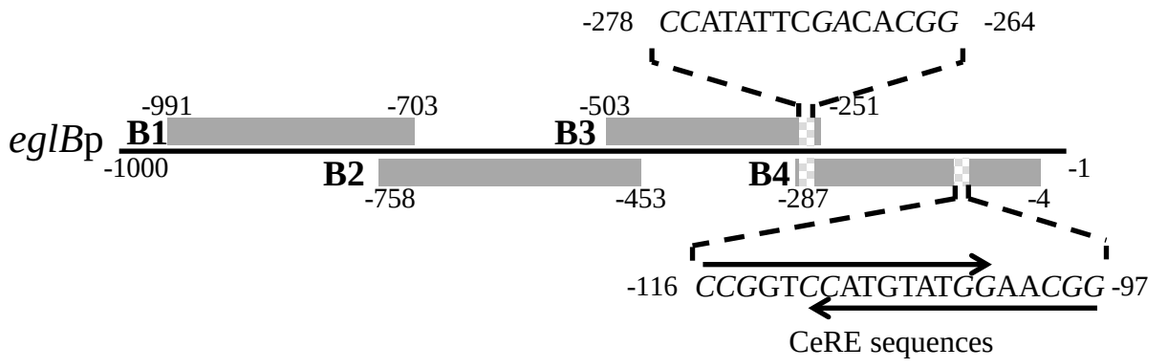
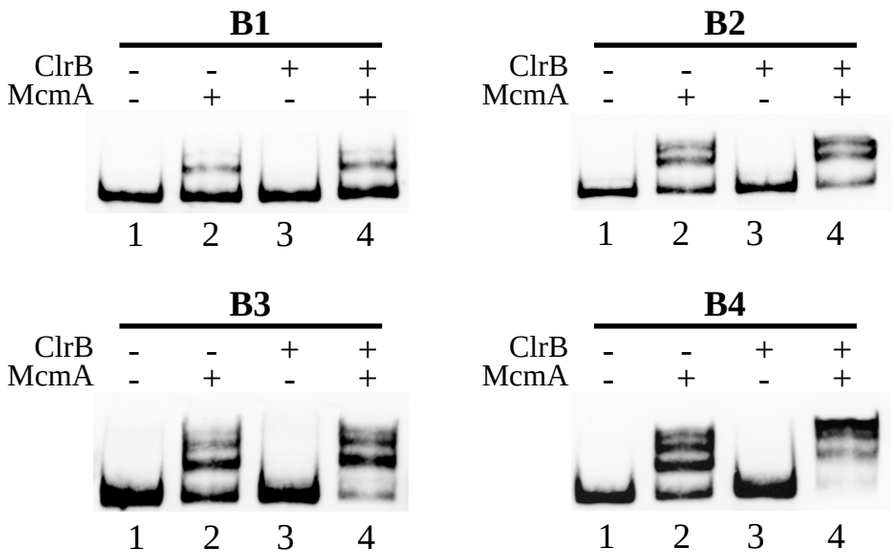

A
eglAp
32
31
-210
-163
CGCACTCCGCACTCCGTTTTGGTCCGTACCTTTTTAGGATACTAGCAG
CeRE
B
31
31-CCG
32
ClrB
McmA
1 2 3 4 5 6 7 8 9 10 11 12
C
31
31-CCG
32
ClrB
McmA143
1 2 3 4 5 6 7 8 9 10 11 12
D
McmA
McmAI70A
ClrB
McmA/McmAI70A
1 2 3 4 5 6 7 8

Fig. 3 Li et al.

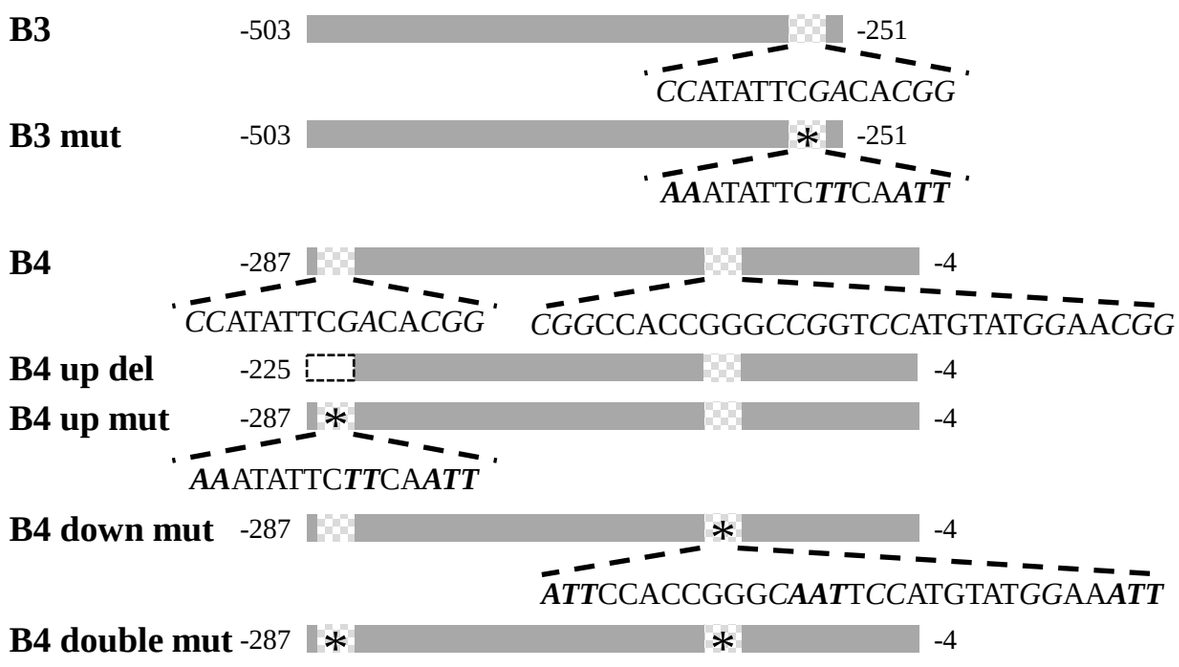
A


-278 CCATATTCGACACGG -264
-991
-703
-503
-251
eglBp B1
B3
-1000
B2
B4
-1
-758
-453
-287
-4
-116 CCGGTCCATGTATGGAACGG -97
CeRE sequences

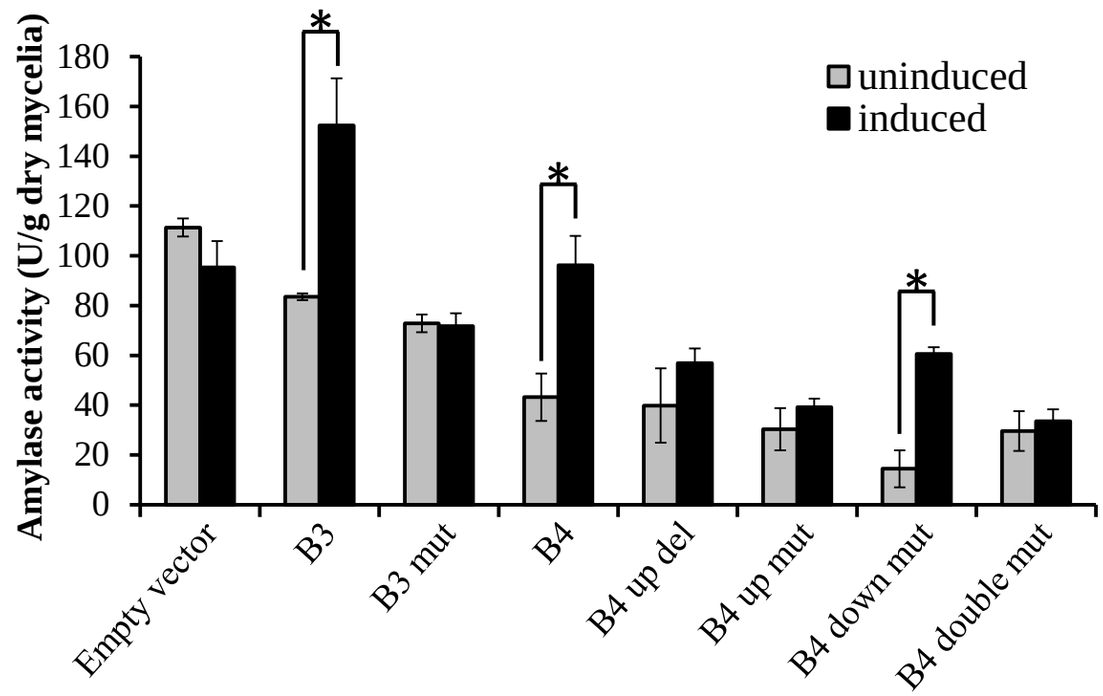
B


B1
B2
B3
B4
ClrB
McmA
1 2 3 4

C


B3
-503
-251
CCATATTCGACACGG
B3 mut
-503
*
-251
AAATATTCTTCAATT
B4
-287
-4
CCATATTCGACACGG
CGGCCACCGGGCCGGTCCATGTATGGAACGG
B4 up del
-225
-4
B4 up mut
-287
*
-4
AAATATTCTTCAATT
B4 down mut
-287
*
-4
ATTCCACCGGGCAATTCCATGTATGGAAATT
B4 double mut
-287
*
*
-4

D

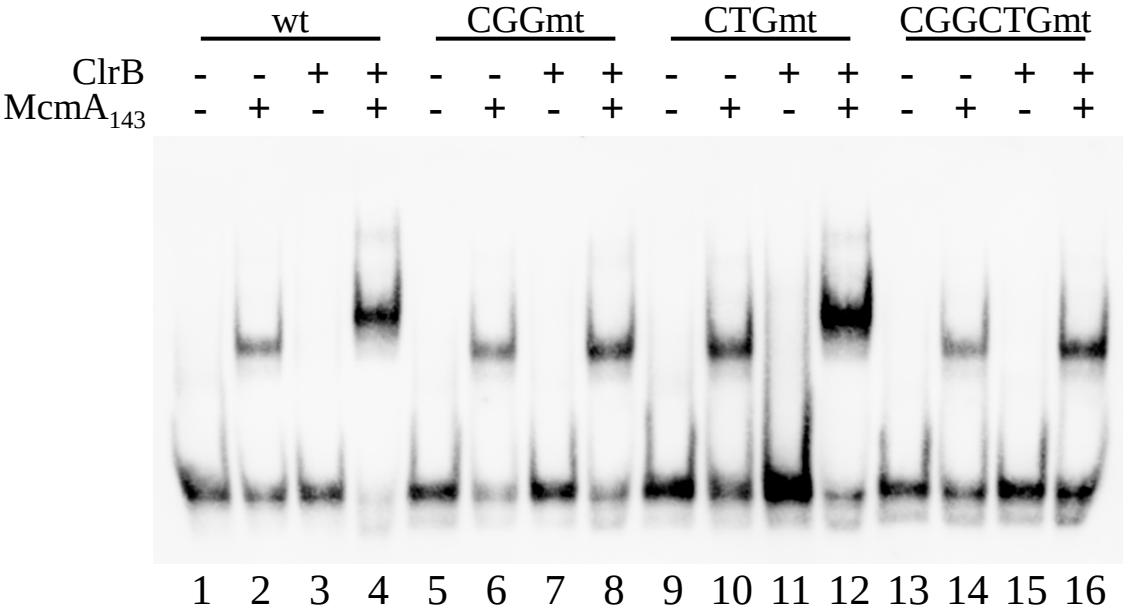

Amylase activity (U/g dry mycelia)
0
20
40
60
80
100
120
140
160
180
*
*
*
uninduced
induced
Empty vector
B3
B3 mut
B4
B4 up del
B4 up mut
B4 down mut
B4 double mut

A

*eglB*p-B3 CeRE-like

```
            -287                                                -244
wt          GTGGCACTTCCATATTCGACACGGAGAGGAAACTGAGAATTATC
CGGmt       GTGGCACTTCCATATTCGACAATTAGAGGAAACTGAGAATTATC
CTGmt       GTGGCACTTCCATATTCGACACGGAGAGGAAAAATAGAATTATC
CGGCTGmt    GTGGCACTTCCATATTCGACAATTAGAGGAAAAATAGAATTATC
```

B



C

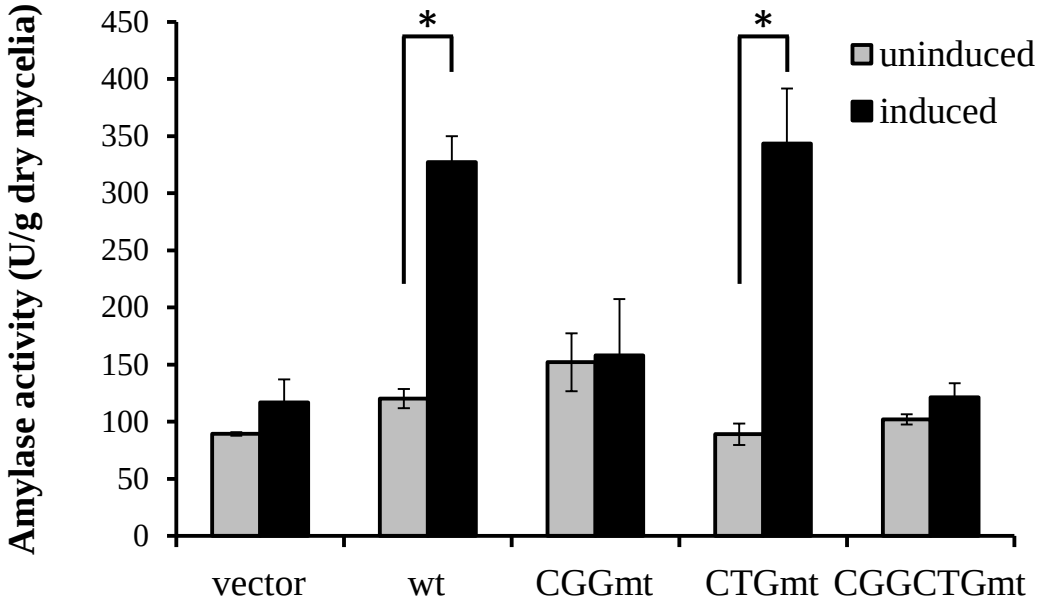


Fig. 5 Li et al.

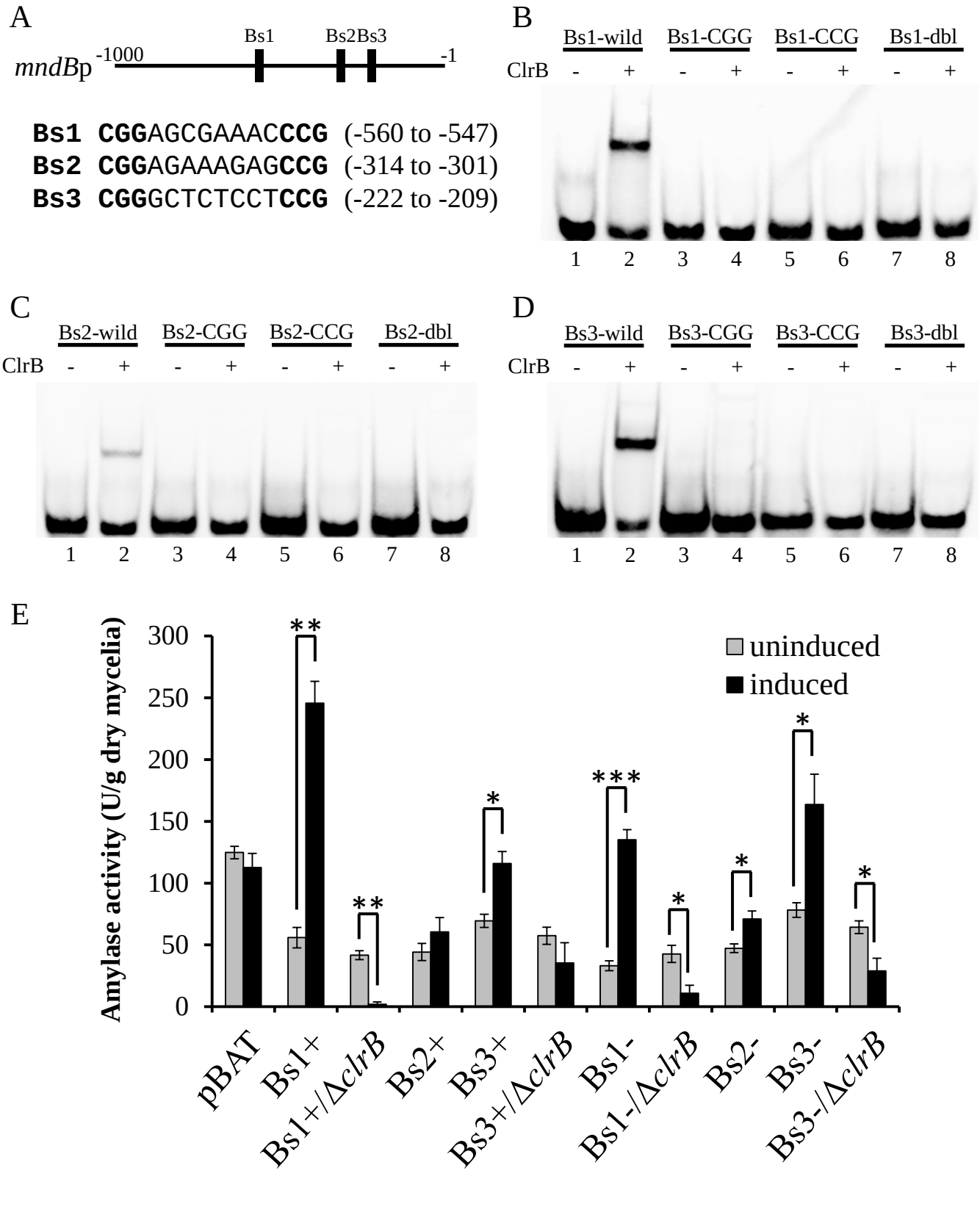

A
mndBp
-1000
Bs1
Bs2Bs3
-1
Bs1 CGGAGCGAAACCCG (-560 to -547)
Bs2 CGGAGAAAGAGCCG (-314 to -301)
Bs3 CGGGCTCTCCTCCG (-222 to -209)
B
Bs1-wild
Bs1-CGG
Bs1-CCG
Bs1-dbl
ClrB - + - + - + - +
1 2 3 4 5 6 7 8
C
Bs2-wild
Bs2-CGG
Bs2-CCG
Bs2-dbl
ClrB - + - + - + - +
1 2 3 4 5 6 7 8
D
Bs3-wild
Bs3-CGG
Bs3-CCG
Bs3-dbl
ClrB - + - + - + - +
1 2 3 4 5 6 7 8
E
Amylase activity (U/g dry mycelia)
0
50
100
150
200
250
300
uninduced
induced
**
**
*
***
*
*
*
*
pBAT
Bs1+
Bs1+/ΔclrB
Bs2+
Bs3+
Bs3+/ΔclrB
Bs1-
Bs1-/ΔclrB
Bs2-
Bs3-
Bs3-/ΔclrB

Fig. 6 Li et al.

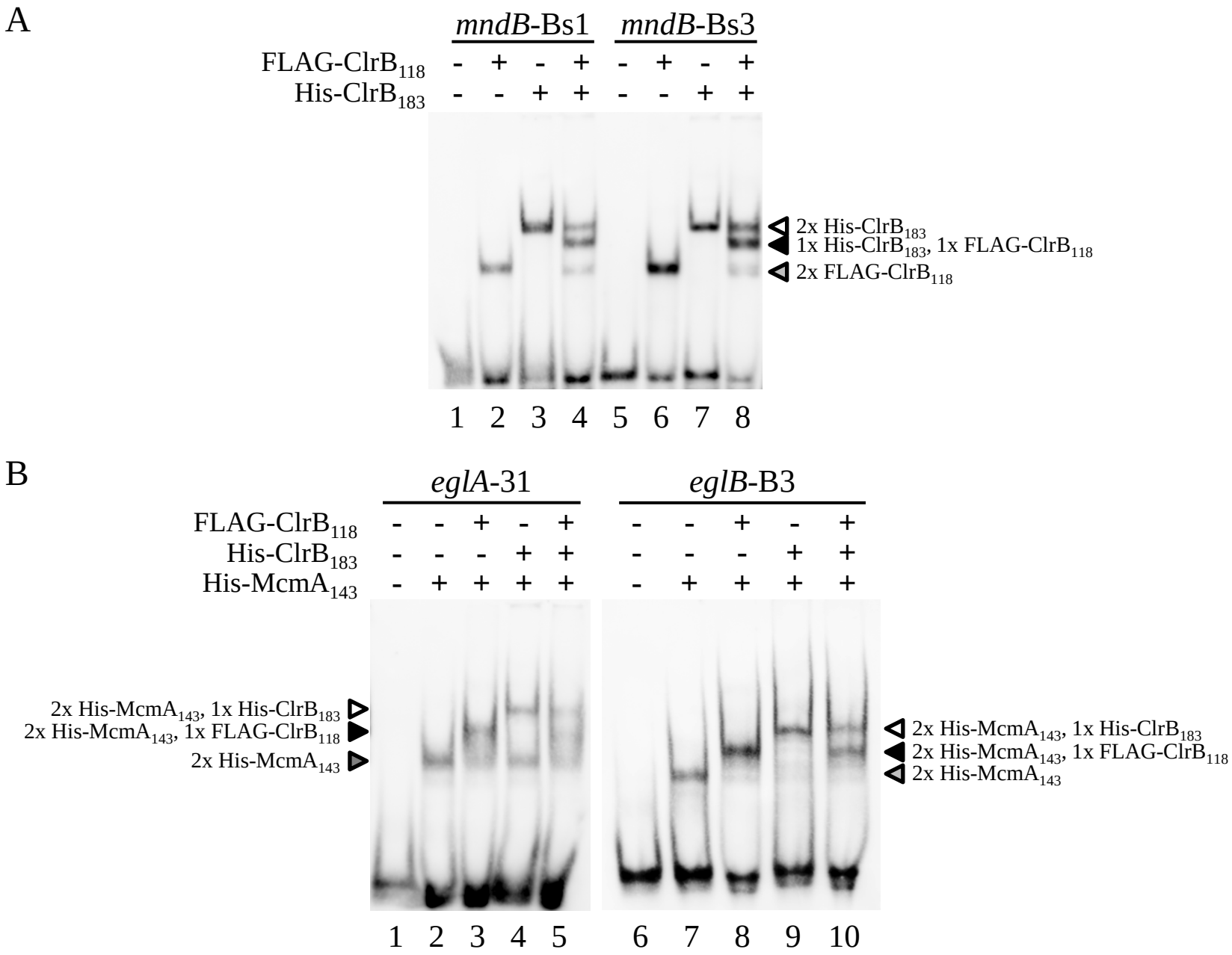

A
mndB-Bs1
mndB-Bs3
FLAG-ClrB118 - + - + - + - +
His-ClrB183 - - + + - - + +
2x His-ClrB183
1x His-ClrB183, 1x FLAG-ClrB118
2x FLAG-ClrB118
1 2 3 4 5 6 7 8
B
eglA-31
eglB-B3
FLAG-ClrB118 - - + - + - - + - +
His-ClrB183 - - - + + - - - + +
His-McmA143 - + + + + - + + + +
2x His-McmA143, 1x His-ClrB183
2x His-McmA143, 1x FLAG-ClrB118
2x His-McmA143
2x His-McmA143, 1x His-ClrB183
2x His-McmA143, 1x FLAG-ClrB118
2x His-McmA143
1 2 3 4 5 6 7 8 9 10

Fig. 7 Li et al.