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Regulation of cellulolytic genes by McmA, the SRF-MADS box protein in *Aspergillus nidulans*

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ABSTRACT

Cellobiose triggers the production of two endoglucanases, EglA and EglB, in *Aspergillus nidulans*. The cellulose responsive element (CeRE) *cis*-element that is essential for induction has been identified on the *eglA* promoter, but transcription factors that bind to CeRE have not yet been identified.

CeRE contained a consensus sequence CC(A/T)$_6$GG for binding of the SRF-type MADS box proteins. Introduction of a missense mutation into *mcmA*, encoding for the sole SRF-MADS protein in *A. nidulans*, caused a significant reduction in cellulase induction. Real-time RT-PCR analysis revealed that inductive expression of not only *eglA* but also *eglB* and *cbhA* by cellobiose were under control of McmA.

The McmA protein expressed in *Escherichia coli* specifically bound to two regions of the *eglA* promoter: CeRE and its upstream proximal region. These results, together with our previous study on the *eglA* promoter structure, imply that McmA regulates *eglA* expression by binding directly to its promoter. This is the first evidence for participation of an SRF-MADS protein in cellulase regulation.

1. Introduction

Renewable carbon sources have currently attracted attention as a result of depletion of fossil fuels with the passage of time. Because the most abundant renewable carbon source in nature is cellulose, the bioconversion of cellulose to biofuels or other useful compounds is highly sought after. Hydrolysis of cellulose is catalyzed by the coordinated action of three types of enzymes, endo-β-1,4-glucanase, cellobiohydrolase, and β-glucosidase, leading to the liberation of D-glucose. Filamentous fungi such as the *Trichoderma* and *Aspergillus* species are a good source of cellulases and have been used for years in industrial production. However, despite a long history of research, our knowledge is still insufficient to draw a clear picture of regulatory mechanisms, especially in terms of induction of cellulolytic genes.

Cellulase production in filamentous fungi is generally induced by small molecules derived from cellulose such as sophorose, gentiobiose, and cellobiose depending on the organism [1–4]. D-xylose also serves as an inducer of cellulases in *Aspergillus niger* and *Aspergillus oryzae*, and the induction is mediated by the transcription factor XlnR [5–8], which was originally identified as an activator of xylanolytic genes in *A. niger* [9]. Cellobiose induction of cellulases in *A. oryzae* is dependent on XlnR [7], and sophorose induction in *Trichoderma reesei* (*Hypocrea jecorina*) is dependent on the XlnR ortholog Xyr1 [10]. Based on these findings, it has been widely believed that XlnR orthologs are the main regulators of not only xylanolytic but also cellulolytic enzymes in filamentous fungi. However, XlnR is not the sole transcriptional activator responsible for the induction of cellulolytic genes. First, *celC* of *A. oryzae* is induced by cellobiose even in the XlnR disruptant [7], indicating the presence of XlnR-independent cellobiose induction. Second, D-xylose, but not cellobiose, triggers reversible phosphorylation of XlnR in *A. oryzae* [11]. This suggests involvement of a co-acting factor in case of cellobiose induction. Third, *eglA* of *A. nidulans* is not induced by D-xylose, the inducer of XlnR-mediated transcription [1]. Extensive promoter analysis of *eglA* has revealed a novel *cis*-element responsible for cellobiose induction (cellulose responsive element; CeRE), the sequence of which does not contain the XlnR binding consensus [12]. These observations indicate the presence of other transcription factors responsible for cellobiose induction in *Aspergillus*.

MADS box proteins are transcription factors that are widely distributed among eukaryotes and are involved in the regulation of a vast range of cellular functions through interaction with their cofactors [13]. They are classified into two subfamilies: the SRF-type subfamily and MEF2-type subfamily, based on amino acid sequence of the conserved MADS box. In *Saccharomyces cerevisiae*, two SRF-type proteins, Mcm1p and Arg80p, are present and they

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regulate mating type specific genes (Mcm1p), cell cycle (Mcm1p), and arginine metabolism (Mcm1p and Arg80p) [14–17].

In the course of studies on the *eglA* regulation, we found that CeRE contains the binding consensus for the SRF-MADS protein CC(A/T)$_6$GG [12,18]. *A. nidulans* has only a single SRF-MADS protein called McmA. In this study, we investigated the involvement of McmA in the regulation of cellulolytic genes.

2. Materials and methods

2.1. Aspergillus nidulans strains

A. nidulans D6B (*biA1 pyrG89*; *wA3*; *argB2::argB::eglA*p-*taaG2*; *pyroA4*) [12] was used as the parent strain for construction of the *mcmA*$_{I70A}$ mutant (MCMI70A) (*biA1 pyrG89*; *wA3*; Δ*mcmA::mcmA*$_{I70A}$*::pyr4 argB2::argB::eglA*p-*taaG2*; *pyroA4*). For construction of the MCMI70A strain, wild-type *mcmA* (AN8676) gene of D6B was replaced with the *mcmA*$_{I70A}$ allele that encodes the McmA protein with an I70 to A substitution, by transforming *A. nidulans* D6B with the *Kpn* I fragment derived from pMCMAI70A (Fig. 1B). Substitution of *mcmA* to *mcmA*$_{I70A}$ was confirmed by Southern hybridization, followed by DNA sequencing of the mutated *mcmA* locus. MCMI70AC (*biA1 pyrG89*; *wA3*; Δ*mcmA:: mcmA*$_{I70A}$*::pyr4 argB2::argB::eglA*p-*taaG2*; *pyroA4::pyroA::mcmA*$^{+}$) was generated by introducing wild-type *mcmA* into the *pyroA* locus of MCMI70A. This was achieved by transformation of MCMI70A with the plasmid carrying the PCR-amplified *mcmA* fragment (−742 to +1902) between the *Kpn* I and *Eco*R I sites of pPyroA, which carried *A. nidulans pyroA* inserted between the *Pst* I and *Bam*H I sites of pBluescript KSII(+). The PCR primers used for construction of the strains in this study are listed in Supplementary Table S1.

Transformation of *A. nidulans* was carried out by protoplast transformation [19] except that the protoplasts were generated using a mixture of Yatalase (Takara Co.) and Lysing enzyme (Sigma) at final concentrations of 3 mg/ml and 0.3 mg/ml [20]. *A. nidulans* strains were grown at 37 °C in standard minimal medium with appropriate supplements unless otherwise noted [21].

2.2. Plate assay for extracellular enzyme production

Plate assays for cellulase, xylanase, and mannanase were performed on standard minimal agar plates containing 0.01% TritonX-100. Carboxymethyl cellulose (CMC, 0.5%), birch wood xylan (1.0%), and konjac glucomannan (0.5%) were used as the sole carbon source for cellulase, xylanase, and mannanase assay, respectively. Activity was visualized by staining with 0.1% Congo red followed by de-staining with 0.7 M NaCl. In case of the xylanase assay, the plate was treated with 5% acetic acid for better visualization.

2.3. α-amylase reporter assay of eglA promoter activity

D6B and MCMI70A carries *A. oryzae* α-amylase gene (*taaG2*) fused to the *eglA* promoter, so that *eglA* promoter activity can be measured as α-amylase activity [12]. The strains were grown in standard minimal medium containing 1% polypeptone P1 as the carbon source. The mycelia were collected, washed, and transferred to fresh medium containing 1% polypeptone P1, and then cultivated at 37 °C for 12 h with or without addition of 0.1% cellobiose. Culture supernatant was subjected to the α-amylase assay as described previously [22].

2.4. Real time PCR analysis of cellulase gene expression

Culture conditions for the *A. nidulans* strains were essentially identical to those used for the reporter assay as described above, except that 0.5 g of the mycelia was transferred to 40-ml medium containing 0.1% cellobiose as the inducer. The mycelia were harvested at 1, 3, and 6 h after inoculation in the cellobiose medium, frozen in liquid nitrogen, and ground with an SK-mill (SK-100, Tokken, Japan). Total RNA was isolated using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol followed by treatment with DNA-free™ DNase (Ambion). Quality of the isolated RNA was checked by agarose gel electrophoresis.cDNA was synthesized using the PrimeScript® RT reagent kit (Perfect Real Time, Takara Bio Inc., Japan) with Oligo(dT) as the primer. Real

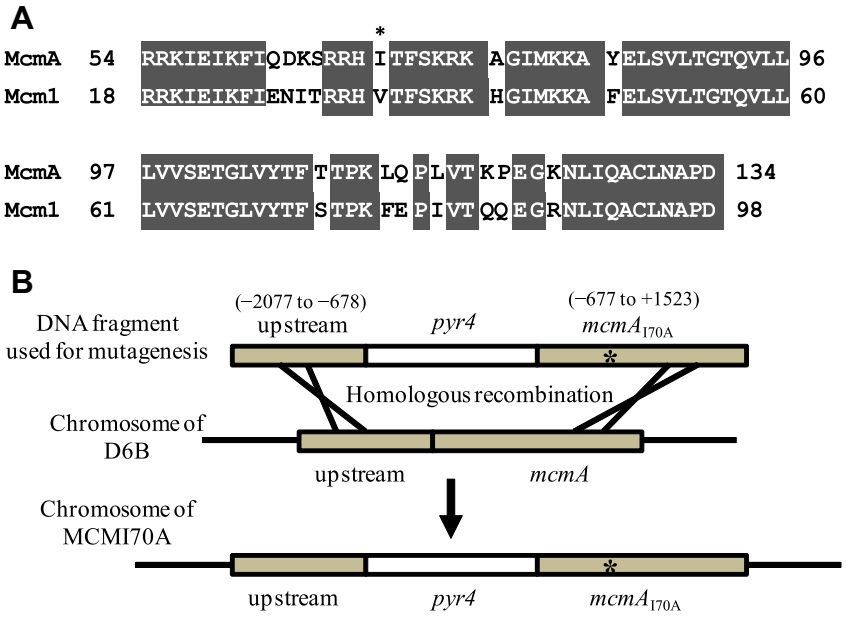

Fig. 1. Construction of the *mcmA* mutant strain MCMI70A. (A) Alignment of MADS domain of *A. nidulans* McmA and *S. cerevisiae* Mcm1p. Identical amino acid residues are shaded. The residue I70, which was replaced to alanine in the *mcmA* mutant, is shown by an asterisk. (B) Schematic representation of the procedure to construct the *mcmA*$_{I70A}$ mutant (MCMI70A). *N. crassa pyr4* was used as a marker.

time PCR was carried out with a LightCycler® 1.5 ST300 (Roche Applied Science Inc.) using SYBR® Premix Ex Taq™ (Takara Bio Inc., Japan). The whole experiment, from culturing to Real time PCR, was repeated three times to confirm reproducibility of the results. The actin mRNA (encoded by AN6542) was used as the internal reference to evaluate mRNA abundance of each gene. Primers used for the analysis are listed in Supplementary Table S1.

2.5. Expression of His_6-tagged McmA and $McmA_{I70A}$ proteins in E. coli

A. nidulans cDNA mixture was synthesized using the Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen). Using the cDNA mixture as the template, *mcmA* cDNA was amplified with the primer set, Mcm1FNde and Mcm224R (Supplementary Table S1). The plasmid pEMCMA that carried the His-tagged *mcmA* cDNA was generated by introduction of the *Nde* I and *Xho* I digested *mcmA* cDNA between the *Nde* I and *Xho* I sites of pET33b(+). pEMCMI70A carrying the His-tagged $mcmA_{I70A}$ cDNA was obtained using the QuikChange® Multi Site-Directed Mutagenesis Kit. The primers used for mutagenesis were HismcmAI70AF and HismcmAI70AR (Supplementary Table S1). These plasmids were constructed using *E. coli* JM109 as the host, followed by the introduction of the plasmids into *E. coli* BL21(DE3) for recombinant protein expression.

Expression of the tagged proteins was induced by addition of IPTG. The cells were collected by centrifugation 3 h after induction, re-suspended in a buffer consisting of 20 mM Tris–HCl, 300 mM NaCl (pH 6.0), and lyzed by repetitive freeze-thawing followed by sonication. Cell extracts were obtained by centrifugation of the cell lysate at 10,000*g* for 30 min at 4 °C. Then the cell extracts were applied to a column of Ni-NTA Agarose (Qiagen). After washing with 20 mM Tris–HCl (pH 8.0) followed by 20 mM Tris–HCl (pH 8.0) containing 100 mM imidazole, the His-tagged proteins were eluted with 20 mM Tris–HCl (pH8.0) containing 200 mM imidazole.

2.6. Electrophoretic mobility shift assay (EMSA)

Probe DNA fragments were constructed from pairs of complementary synthetic oligonucleotides listed in Supplementary Table S1. The oligonucleotides were 45 bases long and annealing of the complementary oligonucleotides left a 5-base flanking single strand at each end. After annealing the complementary oligonucleotides, the resultant double-stranded DNA fragments were end-labeled by DNA polymerase reaction using Klenow fragment and dNTP mixture containing [α-^{32}P] dCTP, to fill in the flanking ends. Unincorporated [α-^{32}P] dCTP was removed using a Probe Quant G-50 Micro Column (GE Healthcare).

The reaction mixture (20 μl) for McmA–DNA binding consisted of 60 mM KCl, 40 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 50 ng/μl poly(dI–dC), the labeled probe DNA fragments (3000 cpm), and 0.05–0.2 μg/μl McmA. After incubation for 5 min at 25 °C, the samples were subjected to 4% polyacrylamide gel electrophoresis using a buffer consisting of 40 mM Tris-acetate (pH 8.0) and 1 mM EDTA. Signals were detected using the BAS 2500 II system (Fujifilm).

3. Results

3.1. Effect of the mcmA mutation on endoglucanase production

In our previous study, we identified the *cis*-element CeRE essential for regulation of the endoglucanase A gene (*eglA*; AN1285) in *A. nidulans* [12]. The CeRE sequence consists of 5′-CCGTACCTTTTAGGA and similar sequences are distributed among other cellulase promoters in *A. nidulans* and *A. oryzae*. CeRE contained the consensus sequence 5′-CC(A/T)$_6$GG, for the binding of SRF-type MADS box proteins such as Mcm1p in *S. cerevisiae* [18].

A. nidulans has only one SRF-type MADS (AN8676). The cDNA sequence is in the GenBank database with the accession number of AY957455. The *mcmA* gene encoded a protein of 224 amino acid residues, and its MADS box region (residues 54–134) shared 64 identical residues out of 81 with that of Mcm1p (residues 18–98) (Fig. 1A).

While deletion of *MCM1* is lethal in *S. cerevisiae*, a mutant having a substitution of V34 to alanine has been reported to be viable, albeit with significant defects in the regulation of a number of genes [23]. The residue corresponded to I70 of McmA (Fig. 1A). We constructed the *mcmA* mutant strain MCMI70A by homologous recombination in which the wild-type *mcmA* gene of the D6B strain was replaced with the $mcmA_{I70A}$ allele, which encoded the McmA protein with I70A substitution (Fig. 1B). The I70A mutation led to the formation of small colonies with dense mycelia (Fig. 2A). Endoglucanase, endomannanase, and endoxylanase production by the mutant was examined by plate assay. The mutant produced extremely reduced endoglucanase activity on the CMC agar plate (Fig. 2B), while endomannanase (Fig. 2C), and endoxylanse (Fig. 2D) production was not significantly affected. The defects in colony morphology and cellulase production were recovered by introduction of the wild-type *mcmA* into MCMI70A (Fig. 2, MCMI70AC).

Because the I70A mutant produced smaller colonies than the parental strain D6E, the growth defect might be the cause of the reduced endoglucanase production on the CMC agar plate. To clarify whether the mutation affected expression of the *eglA* gene, reporter assay of the *eglA* promoter activity was performed. *A. nidulans* strains used here carry the *eglA* promoter::*taaG2* fusion gene integrated at the *argB* locus, so that α-amylase is produced in the presence of cellulase inducers such as cellobiose [12]. After 12 h of induction by cellobiose, α-amylase activity in the culture supernatant was measured and normalized by the weight of dry

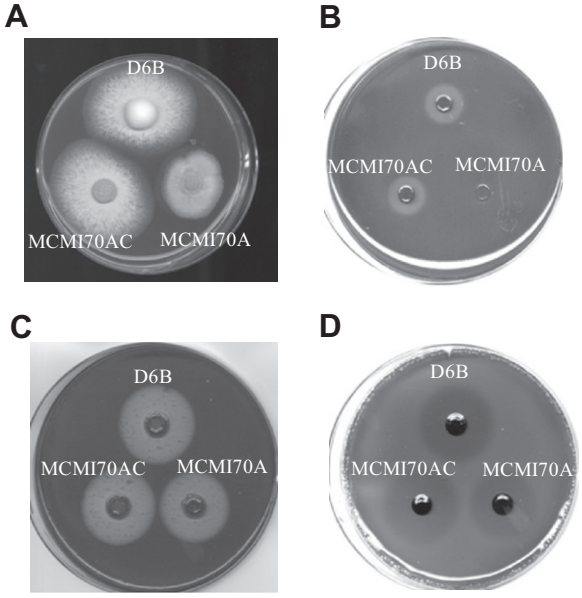

Fig. 2. Growth and extracellular enzyme production of D6B (*mcmA*$^+$), MCMI70A ($mcmA_{I70A}$), and MCMI70AC ($mcmA_{I70A}$/ *mcmA*$^+$). (A) Growth on glucose minimal medium. B–D, extracellular enzyme production at 2 days of growth. (B) Endoglucanase on CMC. (C) Endomannanase production on konjac glucomannan. (D) Endoxylanase production on xylan. Halos around the colonies, which indicate production of the enzymes, were visualized by Congo red staining.

mycelia. The average activity of two independent experiments with D6B was 425 U/g dry mycelia, while that with MCMI70A was 77 U/g dry mycelia, indicating that the I70A mutation affected transcription from the *eglA* promoter. It should be noted that the I70A mutant produced approximately 20% less mycelia by dry weight than the parental strain D6B, implying that growth defect of the mutant is not very serious under our experimental conditions.

3.2. Effect of mcmA mutation on the inductive expression of cellulolytic genes

To clarify whether McmA was involved in the regulation of not only *eglA* but also other endoglucanase and cellobiohydrolase genes at the transcriptional level, the effect of the $mcmA_{I70A}$ mutation on the expression of *eglA*, *eglB*, *cbhA* (AN5176), and *cbhB* (AN0494) was examined by quantitative RT-PCR. The *cbhA* and *cbhB* genes encode cellobiohydrolase [24]. Time courses of expression of the genes in the D6B strain, after addition of 0.1% cellobiose as the inducer, are shown in Fig. 3A. The expression profiles of *eglA*, *eglB*, and *cbhA* were very similar; their expression levels increased after 1 h of cellobiose addition, peaked at 3 h, and decreased at 6 h. Expression of *cbhB* was very low throughout and probably not induced by cellobiose. Based on these observations, we examined the expression levels of *eglA*, *eglB*, *cbhA*, and *cbhB* in MCMI70A and MCMI70AC after 1, 3, and 6 h of cellobiose induction (Fig. 3B and C). In MCMI70A, the expression levels of *eglA*, *eglB*, and *cbhA* increased to constant levels after 3 h of the cellobiose addition. However, the highest expression levels of *eglA*, *eglB*, and *cbhA* were approximately 30-, 10-, and 5-times lower in MCMI70A than those in D6B. Re-introduction of the wild-type *mcmA* gene partially compensated for the defects as observed in the case of MCMI70AC. This might suggest that the $McmA_{I70A}$ protein perturbed the function of McmA by competing to bind to DNA as the recombinant $McmA_{I70A}$ protein possessed DNA binding activity comparable to the wild type as described below.

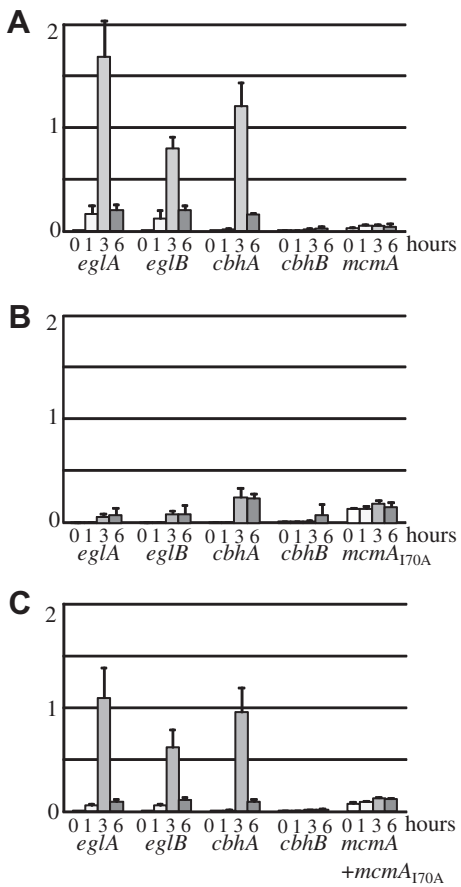

Fig. 3. Effect of the *mcmA* mutation on abundance of cellulase mRNAs. Expression of the cellulase genes was induced by addition of cellobiose, and the *eglA*, *eglB*, *cbhA*, *cbhB*, and *mcmA* mRNA levels were quantified by real time RT-PCR using the actin mRNA as the internal reference. Error bars represent standard deviation. Time courses of mRNA abundance of the genes in D6B ($mcmA^+$), MCMI70A ($mcmA_{I70A}$), and MCMI70AC ($mcmA_{I70A}$/ $mcmA^+$) after induction with cellobiose, are shown in the graphs A, B, and C.

3.3. Binding of McmA to CeRE and its proximal region

If McmA directly and positively regulates *eglA* expression, it should bind to CeRE because it is the sole *cis*-element essential to inductive expression of the gene. To support this hypothesis, His_6-tagged McmA (His-McmA) and $McmA_{I70A}$ (His-$McmA_{I70A}$) were expressed in *E. coli* and purified using Ni–NTA agarose. His-McmA and His-$McmA_{I70A}$ proteins were obtained with an estimated molecular mass of 37 kDa based on SDS–PAGE, which was approximately 30% larger than the predicted molecular mass of 27 kDa (Fig. 4B).

The ability of the recombinant proteins to bind to CeRE was assessed by electrophoretic mobility shift assay (EMSA). Nucleotide sequences of the wild type and mutant probes are shown in Fig. 4A. They corresponded to the −175 to −126 region of the *eglA* promoter except for US (negative control probe), which covered the region from −273 to −224. Mutant probes M1–M3 were used to demonstrate specific binding of His-McmA to the wild-type probe.

When the wild-type fragment (WT) containing CeRE (underlined in Fig. 4A) was used, His-McmA produced an intensive shifted band (Fig. 4C, thick arrow). In addition, a minor shifted band was also observed (Fig. 4C, narrow arrow), suggesting that McmA binds to two sites on this probe. It was inferred that the binding sites were 5′-CCTTTTTAGG in CeRE and 5′-TCCGTTTTGG just upstream of CeRE, based on the consensus binding sequence $CC(A/T)_6GG$ for SRF-MAD proteins. His-$McmA_{I70A}$ also bound to the wild-type probe, indicating that the I70A substitution did not significantly affect DNA binding. This was expected because it had already been reported that the V34A substitution in Mcm1p, which corresponds to I70A in McmA (Fig. 1A), only slightly affects DNA binding [23].

In order to demonstrate that McmA binds to 5′-CCTTTTTAGG and 5′-TCCGTTTTGG, the mutant probes M1, M2, and M3 were employed in the EMSA assay. When either of the possible binding sites was mutated, the slower-migrating shifted band disappeared, and when both binding sites were mutated the faster-migrating shifted band was barely detectable (Fig. 4D). A faint shifted band was also visible with US as the probe. These observations implied that McmA preferred 5′-CCTTTTTAGG and 5′-TCCGTTTTGG as the binding sites but the binding specificity was not strictly maintained. As MADS proteins generally regulate gene expression via interaction with specific cofactors, McmA might also require a cofactor to exhibit high specificity to the binding sequences on the *eglA* promoter.

4. Discussion

The model fungus *A. nidulans* produces two major endoglucanases, EglA and EglB, upon induction by CMC or cellobiose [1]. In the previous study [12], a precise analysis of the *eglA* promoter was performed leading to the identification of a novel *cis*-element, CeRE, required for inductive expression. Based on the finding that CeRE contained consensus binding sequences for SRF-type MADS

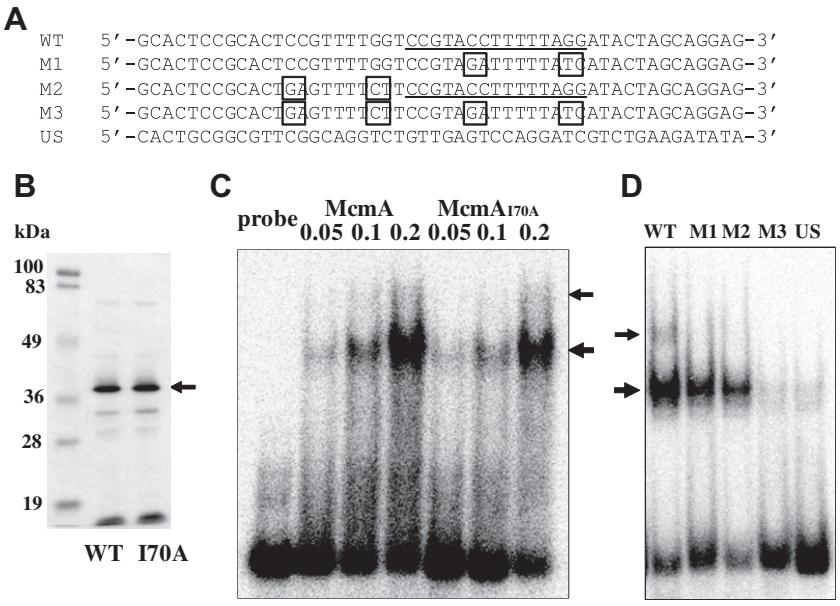

Fig. 4. *In vitro* DNA binding of McmA to the *eglA* promoter. (A) DNA probes used for electrophoretic mobility shift assay (EMSA). WT is the −175 to −126 region of the *eglA* promoter with the CeRE sequence underlined. M1–M3 have mutations as indicated by the boxes. US (negative control probe) corresponds to the region covering −273 to −224. (B) SDS–PAGE of His-tagged McmA and $McmA_{I70A}$ (shown by the arrow) partially purified by Ni-NTA agarose. (C) EMSA with WT as a probe. The protein concentrations of the His-tagged McmA and $McmA_{I70A}$ preparations (μg/μl) in the DNA–protein binding mixture are shown. Major and minor DNA–protein complexes are shown by the thick and narrow arrows. (D) EMSA with the mutant probes M1–M3. The concentration of the His-tagged McmA preparation was 0.2 μg/μl.

box proteins, we investigated the involvement of McmA, the sole SRF-type MADS box protein in *A. nidulans*, in the regulation of *eglA* and other cellulolytic genes in this study, and provided evidence to show that McmA regulates some cellulolytic genes.

It should be noted that expression of *eglA*, *eglB*, and *cbhA* was still induced at low levels even in MCMI70A, and their mRNA levels did not decrease at 6 h (Fig. 4B). This suggests that the McmAI70A protein is partially active. Alternatively, the induction might be mediated by unknown McmA-independent regulatory machinery.

In general, MADS box proteins regulate gene expression through interaction with various cofactors. For example, *S. cerevisiae* Mcm1p interacts with various proteins such as α1, α2, Ste12p, Yox1p, Yhp1p, Fkh2p, Arg80p, and Arg81p, and regulates different sets of genes depending on the interacting protein [13]. This indicates the presence of an unidentified factor that functions in the regulation of cellulolytic genes through an interaction with McmA.

XlnR regulates xylanolytic and cellulolytic genes in response to xylose in *A. niger* and *A. oryzae* [5–8,25]. Xylose-triggered phosphorylation of *A. oryzae* XlnR is a possible molecular mechanism underlying xylose induction [11]. However, although cellobiose induces XlnR-dependent gene expression in *A. oryzae*, it does not trigger XlnR phosphorylation [7,11]. We originally hypothesized that the XlnR-mediated cellobiose induction involved an interaction between XlnR and McmA; however, this is not the case at least in *A. nidulans eglA* because mutation of the sole XlnR binding site on its promoter does not affect the cellobiose induction of EglA (endo et al., 2008).

ManR, a recently identified regulator of mannanolytic genes in *A. oryzae* [26], also regulates cellulolytic genes (submitted for publication). ManR ortholog in *A. nidulans* was identical to the recently identified ClrB, a homolog of *Neurospora crassa* CLR-2, which is essential for cellulase gene expression [27]. These findings imply that *A. nidulans* ClrB may also regulate both mannanolytic and cellulolytic genes. Therefore, we here use the name ManR/ClrB. Considering that ManR/ClrB regulates cellulase expression and that the CeRE containing region of the *eglA* promoter is the sole region responsible for inductive expression, it is reasonable to assume that ManR/ClrB is recruited to the CeRE containing region and interacts with McmA. Alternatively, McmA might regulate *manR*/*clrB*. We are currently trying to obtain evidence to affirm interaction between McmA and ManR/ClrB.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.031.

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