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A novel transcriptional regulator, ClbR, controls the cellobiose- and cellulose-responsive induction of cellulase and xylanase genes regulated by two distinct signaling pathways in *Aspergillus aculeatus*

Emi Kunitake · Shuji Tani · Jun-ichi Sumitani · Takashi Kawaguchi

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Abstract The cellobiose- and cellulose-responsive induction of the FIII-avicelase (*cbhI*), FII-carboxymethyl cellulase (*cmc2*), and FIa-xylanase (*xynIa*) genes is not regulated by XlnR in *Aspergillus aculeatus*, which suggests that this fungus possesses an unknown cellulase gene-activating pathway. To identify the regulatory factors involved in this pathway, we constructed a random insertional mutagenesis library using *Agrobacterium tumefaciens*-mediated transformation of *A. aculeatus* NCP2, which harbors a transcriptional fusion between the *cbhI* promoter (P_{CBHI}) and the orotidine 5′-phosphate decarboxylase gene (*pyrG*). Of the ~6,000 transformants screened, one 5-FOA-resistant transformant, S4-22, grew poorly on cellulose-containing media and exhibited reduced cellobiose-induced expression of *cbhI*. Southern blot analysis and nucleotide sequencing of the flanking regions of the T-DNA inserted in S4-22 indicated that the T-DNA was inserted within the coding region of a previously unreported $Zn(II)_2Cys_6$-transcription factor, which we designated the cellobiose response regulator (ClbR). The disruption of the *clbR* gene resulted in a significant reduction in the expression of *cbhI* and *cmc2* in response to cellobiose and cellulose. Interestingly, the cellulose-responsive induction of FI-carboxymethyl cellulase (*cmc1*) and FIb-xylanase (*xynIb*) genes that are under the control of XlnR, was also reduced in the *clbR*-deficient mutant, but there was no effect on the induction of these genes in response to D-xylose or L-arabinose. These data demonstrate that ClbR participates in both XlnR-dependent and XlnR-independent cellobiose- and cellulose-responsive induction signaling pathways in *A. aculeatus*.

Keywords XlnR-independent signaling pathway · XlnR-dependent signaling pathway · Cellulase induction · Xylanase induction · Cellobiose response regulator ClbR · *Aspergillus aculeatus*

Introduction

Aspergillus aculeatus no. F-50 [NBRC 108796], which was isolated from soil, produces a number of cellulose- and hemicellulose-degrading enzymes that effectively hydrolyze pulp in combination with *Hypocrea jecorina* (Murao et al. 1979). One reason of the synergism could be due to high activity of *A. aculeatus* enzymes on hydrolyzing cellooligosaccharides into monosaccharides (Sakamoto et al. 1985). However, the enzymes produced by *A. aculeatus* are not in sufficient quantities for industrial use. Comprehensive analyses of their induction mechanisms are expected to provide critical knowledge that will enhance the production of these useful enzymes and thus aid in their development for industrial applications.

The $Zn(II)_2Cys_6$ binuclear cluster DNA-binding motif has been found in several fungal specific transcription factors in ascomycetes (Todd and Andrianopoulos 1997), especially in factors that regulate the expression of glycoside hydrolase genes. These transcription factors include AmyR for amylase genes in *Aspergillus oryzae* and *Aspergillus nidulans* (Gomi et al. 2000; Tani et al. 2001), XlnR/XYR1 for cellulase, hemicellulase, and accessory enzyme genes that are involved in xylan degradation in *Aspergillus niger*

E. Kunitake · S. Tani (✉) · J.-i. Sumitani · T. Kawaguchi
Graduate School of Life and Environmental Sciences, Osaka Prefecture University,
1-1 Gakuen-cho, Naka-ku,
Sakai, Osaka 599-8531, Japan
e-mail: shuji@biochem.osakafu-u.ac.jp

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and *H. jecorina* (van Peij et al. 1998a, b; Stricker et al. 2006), Ace2 for cellulase genes in *H. jecorina* (Aro et al. 2001), AraR for the L-arabinose reductase gene in *A. niger and A. nidulans* (Battaglia et al. 2011a, b), BglR for the β-glucosidase gene in *H. jecorina* (Nitta et al. 2012), and CLR-1 and CLR-2 for cellulase genes in *Neurospora crassa* (Coradetti et al. 2012). To date, although many transcription factors have been identified, when we started this study, two transcription factors that regulate cellulase and hemicellulase genes in response to the presence of cellulose, XlnR/XYR1 and Ace2, had been identified; however, *Aspergillus* does not possess an *ace2* ortholog. XYR1 in *H. jecorina* regulates all states of gene expression (basal, derepressed, and induced) for the major cellulose- and hemicellulose-degrading enzyme-encoding genes in response to pure oligosaccharides, such as sophorose, β-cellobiono-1,5-lactone, D-xylose, xylobiose, galactose, and lactose (Aro et al. 2005; Karaffa et al. 2006; Kubicek and Penttilä 1998; Morikawa et al. 1995; Stricker et al. 2006, 2007). However, some cellulase genes are not regulated by XYR1/XlnR in *Fusarium graminearum* (Brunner et al. 2007), in *N. crassa* (Sun et al. 2012), and in *A. nidulans* (Endo et al. 2008). The functions of these transcription factors are not completely conserved, although the amino acid sequences are highly similar in all orthologs analyzed. Therefore, we first disrupted the *xlnR* gene to determine whether XlnR governs the regulation of cellulase and hemicellulase genes in *A. aculeatus* (Tani et al. 2012). In our previous report on the role of XlnR in *A. aculeatus*, the *xlnR*-deficient mutant formed significantly small colonies on xylan and xylose media and less conidia on xylan and cellulose media. These data suggested that XlnR regulates both the expression of cellulose- and hemicellulose-degrading enzymes in *A. aculeatus*. Transcription analyses of their genes showed that cellulose, D-xylose, and L-arabinose induced the expression of the FI-carboxymethyl cellulase (*cmc1*) and FIb-xylanase (*xynIb*) genes, which are under the control of XlnR, while cellulose and cellobiose induced the FIII-avicelase (*cbhI*), FII-carboxymethyl cellulase (*cmc2*), and FIa-xylanase (*xynIa*) genes, which are regulated by an unknown XlnR-independent signaling pathway.

In this study, to identify novel transcription factors involved in the XlnR-independent signaling pathway, we established a screening system to positively isolate cellulase induction-deficient mutants from a random insertional mutagenesis library using a P_{CBHI}-*pyrG* reporter fusion. We identified ClbR, a new activator with a $Zn(II)_2Cys_6$ binuclear cluster DNA-binding motif. We further demonstrated that ClbR controls the cellobiose- and cellulose-responsive induction of cellulase and xylanase genes regulated by both XlnR-dependent and XlnR-independent signaling pathways in *A. aculeatus*.

Materials and methods

Strains and media

All *A. aculeatus* strains used in this study are derived from wild-type *A. aculeatus* no. F-50. Unless otherwise stated, all strains were propagated at 30 °C in appropriately supplemented minimal medium (MM) (Adachi et al. 2009).

The *A. aculeatus* wild-type strain was spontaneously mutated and selected with chlorate and 5-fluoroorotic acid (5-FOA) to identify an *A. aculeatus* nitrate assimilation-deficient and uridine auxotrophic mutant NP1 strain (*niaD1*; *pyrG1*) (Cove 1976a, b; Goosen et al. 1987). *A. aculeatus* NCP2 (*niaD1*::*niaD*::P_{CBHI}-*pyrG*; *pyrG1*) was produced from the NP1 strain and then used as the host for insertional mutagenesis. The *A. aculeatus ku80* mutant, MR12 (*pyrG1*; Δ*ku80*), was used as the host for disruption and complementation of the *clbR* gene. *Agrobacterium tumefaciens* C58C1 harboring pBIG2RHPH2 was used for *A. tumefaciens*-mediated transformation (AMT) (Tsuji et al. 2003). *Escherichia coli* DH5αF′ was used for plasmid construction.

Gene expression analysis by quantitative RT-PCR

To obtain RNA for gene expression analysis, *A. aculeatus* strains were grown in MM containing 1 % (*w*/*v*) glucose with or without the appropriate auxotrophy supplements at 30 °C with shaking for 24–30 h. The mycelia were harvested by filtration through Miracloth (Calbiochem, La Jolla, CA) and washed with MM that did not contain a carbon source. Then, the mycelia were grown in MM containing 0.1 % (*w*/*v*) cellobiose together with 50 μg/ml 1-deoxynojirimycin (DNJ) for the indicated times, 1 % (*w*/*v*) D-glucose for 3 h, 0.1 % (*w*/*v*) polypeptone (Nihon Pharmaceutical, Tokyo, Japan) for 3 h, 1 % (*w*/*v*) D-xylose for 3 h, 1 % (*w*/*v*) L-arabinose for 3 h, or 0.5 % (*w*/*v*) alkali-swollen cellulose (ASC) for the indicated times. The harvested mycelia were immediately frozen in liquid nitrogen and ground to a fine powder using a Micro Smash homogenizing system (Tomy, Tokyo, Japan). Total RNA was isolated from powdered mycelia using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (1 μg) was treated with DNase I and was then primed with oligo-dT using the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa, Kyoto, Japan). Quantitative real-time PCR (qPCR) was performed in a Thermal Cycler Dice Real-Time System (TaKaRa). For the amplification reactions, a SYBR Green I assay using SYBR Premix Ex Taq (TaKaRa) was performed in a reaction volume of 20 μl. The primers used for qPCR are listed in Table 1. The following thermal settings were used: a 30-s initial denaturation step at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. The specificity of the PCR amplifications was confirmed by melting curve analyses. The concentration of each gene product was determined

Table 1 Primers used in this study

Name	Sequence (5′ to 3′)
For the construction of the reporter system	
AacbhNot	ATAAGAATGCGGCCGCACTCCGAGCGCCACATAGAA
AacbhNhe	CTAGCTAGCGGTTGCCAACTGATCGAGG
pyrGNhe	CTAGCTAGCATGTCTTCGAAGTCCCACCTC
pyrGSpeR	GGACTAGTCTGGTAATACTATGCTGGCTGC
niaDpF	CAGCGGCTCACAAGGAAT
cbhIp-niaDtR	GGCGCTCGGAGTTGGGACAAGAAGGGGTGGAA
pyrGt-niaDtF	GCATAGTATTACCAGATCGAGGCAACCCAGC
niaDtRnot	TATGCGGCCGCACGCCTGATCGAAGAAGC
niaDt-cbhIpF	CACCCCTTCTTGTCCCAACTCCGAGCGCCA
niaDt-pyrGtR	TGGGTTGCCTCGATCTGGTAATACTATGCTGGCTGC
AaniaDF2	AGYGAYAACTGGTACCAYATYWAYGACAA
AaniaDR2	CGGAACCANGGRTTRTTCATCAT
For the disruption of *clbR*	
22aA-F_Xba	AATCTAGAGACTCCAGTCAACCTCTCGC
22aAptrA-R2	GATCCCGTAATCCGAAGAATAGCGATGCAGG
22a-ptrA-F2	CGCTATTCTTCGGATTACGGGATCCCATTGG
22a-ptrA-R	GCGTAGTAGCATGGGGTGACGATGAGCC
22aDptrA-F	GTCACCCCATGCTACTACGCGAGATTCACG
22aD-xho-R	TCACTCGAGTACTCATGACCACCATCGTC
22aA-F2	TCCATCATGGTTCATTCCG
22aD-R2	TCATCTTTGTTGTGACATGCTG
For the complementation of the *clbR* null strain	
co22aN-R_Not	TTAGCGGCCGCAGAATGGAAGACACACGCC
co22aC-F_Pac	GGCTTAATTAACTCGAATCCCTGGTGAGC
co22aC-R	GTGTGCGTACCGTATGGG
pyrGF + NotI	TAGCGGCCGCAATGCTCTCTATC
pyrGR + PacI	CCTTAATTAACCGTTACACATTTCCACTCA
FAr-F1_EcoRI	AAGAATTCATGCCTACGTATCCCATTCG
FAr-R2_HindIII	CCCAAGCTTTTACTTTCGGCATTGTTCATCG
For cDNA cloning	
FAr-R1_HindIII	CCCAAGCTTTCAAGCGTTCCCCGTACTC
For quantitative RT-PCR	
QcbhF	ACCATGTGCACAGGCGATAC
QcbhR	TGCTGTTGTCGACGGTCTTT
Qcmc2F	CCCTGCTGCAGAGCTATGTG
Qcmc2R	GGCAGTGGAGGTGGAGGTAG
QxynIBF	GAACTTCGTCGGTGGAAAGG
xynIBR	CCAGGGTTGTACTCCCCGTA
Qcmc1F	TGGCTCGCAGAAGACCTACA
Qcmc1R	ACCAGTTCGAGACGGAGAGC
QcgaF-F	TCTGGCGGAGCATATTGAAC
QcgaF-R	GCTGCTCATCCATGTGTGC
QgpdF	CTCCTCCACTGGTGCTGCT
QgpdR	GACTTCTCGAGACGGCAGG

using the standard curve method. The glyceraldehyde-3-phosphate dehydrogenase A gene (*gpdA*) was used as a reference gene. The reliability of the reference gene under the test conditions was evaluated according to NormFinder (Andersen et al. 2004). More than three biological replicates were performed for each experiment, and each was tested in triplicate.

Construction of the reporter system

Vectors were constructed using standard cloning methods, and critical regions were sequenced prior to use. The construction of transcriptional fusions between the *cbhI* promoter (P_{CBHI}) and the orotidine 5′-phosphate decarboxylase gene (*pyrG*), P_{CBHI}-*pyrG* is described below. The *cbhI* promoter, containing *Not*I and *Nhe*I recognition sites, was amplified by PCR using the primers AacbhNot and AacbhNhe. The *A. nidulans pyrG* gene, containing the *Nhe*I and *Spe*I recognition sites, was amplified with the primers pyrGNhe and pyrGSpe. The amplified segments were digested with *Not*I and *Nhe*I or *Nhe*I and *Spe*I and ligated into the *Not*I-*Spe*I sites in the pBluescript II KS(+) plasmid (pBSKS (+)) to yield pNCP. P_{CBHI}-*pyrG* was PCR-amplified with the primers niaDt-cbhIpF and niaDt-pyrGtR using pNCP as the template. Fragments of the nitrate reductase gene (*niaD*) containing the 5′- and 3′-regions required for its expression were amplified by PCR with the primers niaDpF and chbI-niaDtR using *A. aculeatus* genomic DNA as a template. The 3′ region of the *niaD* gene that is required for homologous recombination was amplified with the primers pyrGt-niaDtF and niaDtRnot. The three amplified segments were then fused by PCR with the primers niaDpF and niaDtRnot. A single copy of the obtained fragments was introduced into the *niaD* locus in the *A. aculeatus* NP1 strain to yield *A. aculeatus* NCP2. The primers used to amplify these fragments are shown in Table 1.

Screening strategy for novel regulator(s)

T-DNA tagging using AMT was performed following the method optimized for AMT in the *pyrG* mutant, as described previously (Kunitake et al. 2011), with minor revisions. The conidia (2×10^7) of *A. aculeatus* NCP2 were grown in 100 ml of liquid induction media (IM) supplemented with 0.01 % (*w*/*v*) uridine for 24 h. The germinated conidia were then mixed with 1×10^9 *A. tumefaciens* bacteria (OD_{660}=0.4) in 100 ml of liquid IM containing 400 μM acetosyringone, 200 μg/ml rifampicin, and 0.01 % (*w*/*v*) uridine. The culture was then co-cultivated for 48 h with shaking at 120 rpm. The mycelia were harvested by filtration and transferred onto selection media supplemented with 0.2 % (*w*/*v*) uridine and 100 μg/ml hygromycin B. The transformants were transferred to MM containing 0.01 % (*w*/*v*) uridine, 1.5 mM 5-FOA, and 1 % (*w*/*v*) wheat bran as the sole carbon source. The 5-FOA-resistant strains were grown on MM containing 0.2 % (*w*/*v*) uridine and 1 % (*w*/*v*) glucose, avicel, ASC, or birchwood xylan to evaluate their carbon utilization properties.

clbR disruption and complementation

The *A. aculeatus clbR*-deficient mutant (*pyrG1*; Δ*ku80*; Δ*clbR*::*ptrA*) was created by replacing the *clbR* gene with the pyrithiamine resistance gene (*ptrA*). To construct the *clbR* disruption cassette, the 5′ and 3′ regions of the *clbR* gene that are required for homologous recombination were amplified from *A. aculeatus* genomic DNA using the primer pairs 22aA-F_Xba and 22aAptrA-R2 for the 5′ region and 22aDptrA-F and 22aD-Xho-R for the 3′ region. The *ptrA* fragment was amplified using pPTRI (TaKaRa) as the template and the primers 22a-ptrA-F2 and 22a-ptrA-R. The three fragments were joined by fusion PCR using the primers 22aA-F2 and 22aD-R2, and the fused product was subcloned into the *Eco*RV site of pBSKS(+), which yielded pDclbR(ptrA)-2. The *clbR* disruption cassette was amplified with the primers 22aA-F2 and 22aD-R2 using pDclbR (ptrA)-2 as the template. The fragments were introduced into the MR12 strain using the protoplast-PEG method (Adachi et al. 2009). The disruption of endogenous *clbR* was confirmed by Southern blot analysis using DNA probes that were amplified with the primers 22aA-F2 and 22aAptrA-R2 or 22aDptrA-F and 22aD-R2 from pDclbR(ptrA)-2 as a template. The *clbR*-complemented strain (*pyrG1*; Δ*ku80*; Δ*clbR*::*clbR*::*pyrG*) was produced by inserting *clbR* into its endogenous locus using *pyrG* as a marker. To construct the *clbR* cassette that was used for complementation, the *clbR* ORF, including enough of its flanking region to express *clbR* (5′clb), was amplified from genomic DNA with the primers 22aA-F_Xba and co22aN-R_Not. Similarly, the 3′ region of *clbR* (3′clb) was amplified with the primers co22aC-F_PacI and co22aC-R. *A. nidulans pyrG* (AnpyrG) was amplified using pPL6 (Punt et al. 1991) as a template with the primers pyrGF+NotI and pyrGR+PacI. The 3′clb and AnpyrG fragments were digested with *Pac*I. These *Pac*I-digested fragments, which had one blunt end, were simultaneously ligated into the *Eco*RV site in pBSKS(+)ΔKN, disrupting the *Kpn*I and *Not*I sites and thus enabling blue-white selection. The constructed plasmid harboring AnpyrG-3′clb was digested with *Not*I and *Xba*I and then ligated to the 5′clb fragment that had been digested with the same enzymes. The resulting plasmid harbored the *clbR* complementation cassette 5′clb-AnpyrG-3′clb and was named pCFAP. The full-length complementation cassette was amplified from pCFAP using the primers 22aA-F_XbaI and co22aC-R and was introduced into the Δ*clbR* strain using the protoplast-PEG method. Insertion of the complementation cassette into the *clbR* locus was confirmed by Southern blot analysis using a DNA probe amplified by the primers FAr-F1_EcoRI and FAr-R2_HindIII and using pCFAP as a template. To construct the plasmid, the *clbR* cDNA was PCR-amplified using the primers FAr-F1_EcoRI and FAr-R1_HindIII. The amplified product was digested with *Eco*RI and *Hin*dIII and then subcloned into the *Eco*RI-*Hin*dIII site of pBSKS(+).

Other methods

Genomic DNA isolation and Southern blot analyses were performed as described previously (Adachi et al. 2009).

Dig-labeled DNA probes were amplified by PCR with the primer pairs listed in Table 1. Inverse PCR was performed using genomic DNA digested with *Eco*RI or *Xba*I-*Spe*I to amplify the right or left flanking regions of the T-DNA, respectively, as described previously (Kunitake et al. 2011). The genomic sequence of the *clbR* gene was obtained from our in-house draft genome database of *A. aculeatus*. The *clbR* cDNA sequence was determined by analyzing two independently constructed plasmids (pClbRcDNA). The Paircoil2 algorithm was used to predict coiled-coil domains (McDonnell et al. 2006), with a cutoff score of 0.03.

Results

Positive screening to identify novel regulator(s) involved in the XlnR-independent signaling pathway

The *cbhI* promoter from *A. aculeatus* was used as bait to hunt for novel regulators involved in the cellobiose- and cellulose-induced control of the XlnR-independent signaling pathway. To enable the positive screening of mutants deficient in the regulation of cellulase production, we constructed an *A. aculeatus* reporter strain, NCP2, in which one copy of a P_{CBHI}-*pyrG* fusion was inserted into the *niaD* locus. Expression analysis confirmed that *cbhI* and *cmc2* were induced by cellobiose and cellulose in the same manner in both NCP2 and NP1 (data not shown). Furthermore, the reporter P_{CBHI}-*pyrG* demonstrated the inducibility of P_{CBHI} in NCP2; i.e., the NCP2 strain grew on MM containing glucose with 5-FOA (repressed condition) but not on MM containing wheat bran and 5-FOA (induced condition). We expected that mutants with defects in the regulation of *cbhI* induction would be able to grow on wheat bran media containing 5-FOA. Therefore, we used NCP2 as a host for insertional mutagenesis. We first transformed germinated NCP2 conidia with *A. tumefaciens* harboring the binary vector pBIG2RHPH2 and selected transformants with hygromycin B to form mononuclear conidia. Conidia from the transformants were transferred onto MM containing wheat bran with 5-FOA. 5-FOA-resistant transformants were purified twice on plates using the mono-spore isolation technique to obtain homokaryons. Their growth was examined on plates containing 1 % (*w*/*v*) glucose, avicel, ASC, or birchwood xylan as the sole carbon source. We screened a library consisting of estimated 6,000 transformants and identified four 5-FOA-resistant strains that exhibited reduced growth on avicel and ASC media and normal growth on the other carbon sources tested. Because cellobiose together with DNJ induced *cbhI* most efficiently of all the tested disaccharides composed of β-glucosidic bonds (Tani et al. 2012), *cbhI* transcription under the cellobiose-inducing condition were quantified in the four mutants by qPCR. DNJ, an inhibitor for β-glucosidase, was added for effective gene induction to prevent carbon catabolite repression by glucose. One mutant, S4-22, reduced *cbhI* expression in response to cellobiose by approximately 80 % compared to the levels observed in NCP2 (Fig. 1). These data strongly suggested that the T-DNA insertion in S4-22 disrupted a gene required to induce *cbhI* in response to cellobiose.

Recovery of the T-DNA flanking sequences

We first investigated the T-DNA integration pattern in the S4-22 genome by Southern blot analysis because multiple copies of T-DNA fragments with vector backbones can be integrated into multiple loci in the recipient genome (Kunitake et al. 2011). Treatment with restriction enzymes with no recognition sites in pBIG2RHPH2 (*Sal*I, *Spe*I, *Xho*I, *Bam*HI, and *Hin*dIII) yielded single hybridized bands that were greater than 10 kb (Fig. 2a). Single digestion by *Eco*RI or *Pst*I (unique sites in the vector) and double digestion by *Xba*I and *Spe*I (unique to the vector and absent from the vector, respectively) yielded a 9.6-kb vector band and an additional band most likely derived from the transformant chromosome (Fig. 2a). These results suggested that two copies of the T-DNA and the vector backbone were inserted into a single locus in S4-22 (Fig. 2b). When the vector backbone is inserted along with the T-DNA, inverse PCR can be used to rescue the sequence flanking the T-DNA (Kunitake et al. 2011). To rescue the left border of the flanking region of the T-DNA, inverse PCR was performed using the genomic DNA of S4-22. Two segments were amplified: a 9.0-kb segment containing only a vector sequence, and a 1.5-kb segment consisting of a 354-bp vector

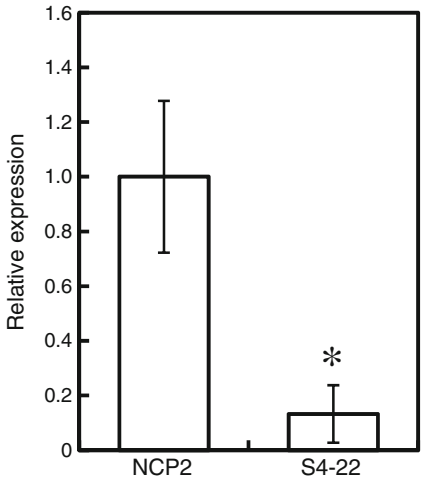

Fig. 1 qPCR analysis of *cbhI* expression induced by 0.1 % cellobiose together with 50 μg/ml DNJ in S4-22 compared to the parental strain NCP2. The level of *gpdA* was used as an endogenous control in both samples. The levels of *cbhI* expression in NCP2 were assigned a value of 1. The relative transcription levels of *cbhI* shown are the means of three independent experiments, and the *error bars* indicate the standard deviations. *$p<0.05$

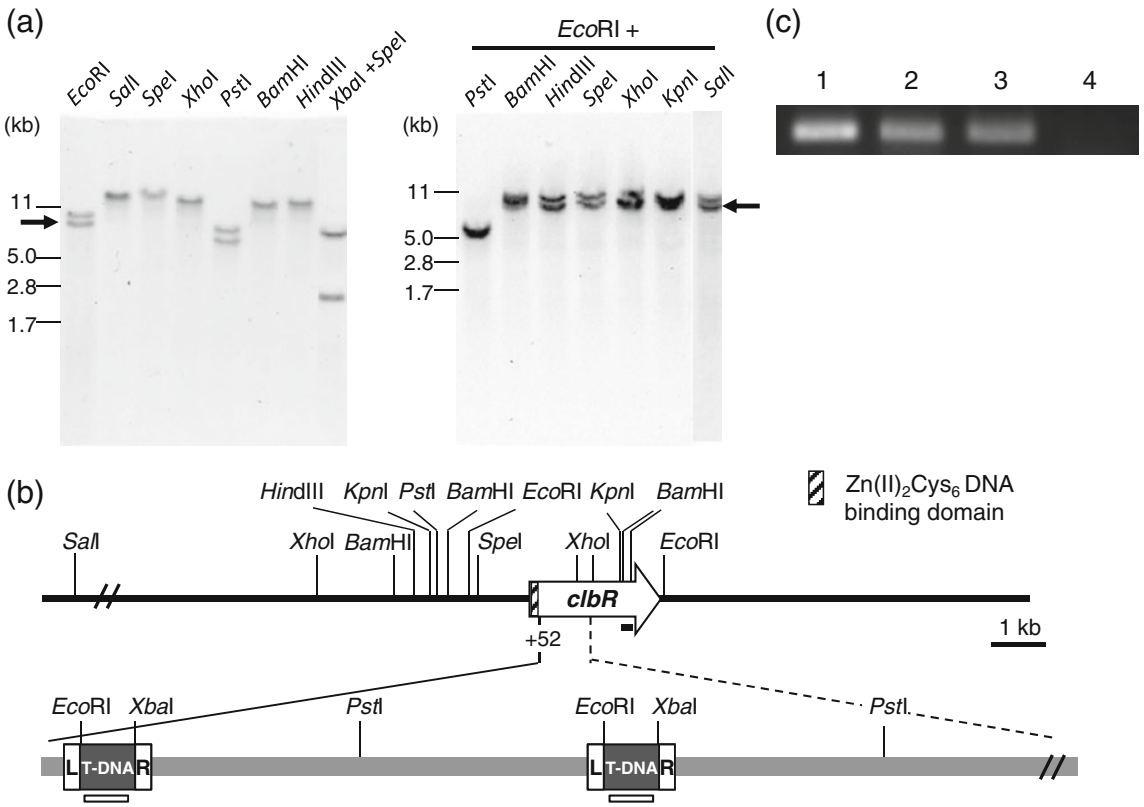

Fig. 2 The T-DNA integration pattern in S4-22 genomic DNA. **a** Southern blotting analysis was performed using S4-22 genomic DNA digested with the indicated restriction enzymes. Arrows indicate the signal from the vector backbone. **b** Restriction enzyme map of the *clbR* locus and the deduced T-DNA integration pattern. The DNA probe used for Southern blot analysis is indicated as an open bar under the T-DNA region. *L* left border; *R* right border. **c** PCR analysis to identify the region deleted by the T-DNA insertion. The *solid bar* under the *clbR* gene in (**b**) indicates the region amplified by PCR. The following DNA samples were used as templates: pClbRcDNA (*lane 1*), NCP2 genomic DNA (*lane 2*), S4-22 genomic DNA (*lane 3*), and Δ*clbR* genomic DNA (*lane 4*)

sequence and 1.1 kb of S4-22 genomic DNA. The genomic DNA sequence revealed that the T-DNA was inserted 52 nt after the translational start site and just behind the $Zn(II)_2Cys_6$ binuclear cluster domain of a putative transcription factor (Fig. 2b). Trial to rescue the right border-flanking region of the T-DNA resulted in the amplification of the vector sequence only. Because T-DNA insertion is often accompanied by the deletion of the recipient genomic region in *A. aculeatus* (Kunitake et al. 2011), Southern blot analysis was performed to identify the region deleted by the T-DNA insertion. Double digestions with *Eco*RI and *Hin*dIII, *Spe*I, or *Sal*I (no sites near the right side of the ORF) yielded identical band patterns: a 9.6-kb band derived from the intact pBIG2RHPH2 and a larger fragment. Double digestions with *Eco*RI and *Bam*HI, *Xho*I, or *Kpn*I (sites within the ORF) yielded a broad doublet. By cross-referencing these results to the restriction enzyme map around the T-DNA insertion site, we predicted that two copies of the T-DNA with the vector backbone had inserted into the genome, as shown in Fig. 2b, suggesting that the 3′-region of the putative transcription factor gene harboring the *Xho*I, *Kpn*I, and *Bam*HI sites remained in the genome. Because PCR successfully amplified segments from 1,753 to 1,859 nt of the identified gene (Fig. 2c lane 3), we concluded that the T-DNA insertion disrupted only the gene encoding the putative transcription factor. These data strongly suggest that the putative transcription factor is involved in *cbhI* induction. Therefore, this gene was designated *ce*l*lo*b*i*ose response *r*egulator (ClbR) (DDBJ Acc. no. AB689701).

Sequencing of the *clbR* gene

The sequence of the genomic and complementary DNAs indicated that the *clbR* gene is 2,407 bp long and is interrupted by four introns. The deduced ClbR protein contains 727 amino acids with a calculated molecular mass of 80.8 kDa. The ClbR protein contains a $Zn(II)_2Cys_6$ binuclear cluster domain (amino acids 14–51, PF00172) and a fungal specific transcription factor domain (amino acids 303–480, PF04082) (Fig. 3a and b). An RRRVWW motif, located at position 408–413, is a variant of the RRRLWW motif that was first identified in the UaY regulator in *A. nidulans* (Suarez et al. 1995). The Paircoil2 algorithm predicted that the ClbR protein contains two parallel coiled-coil regions, at positions 109–147 and 623–661.

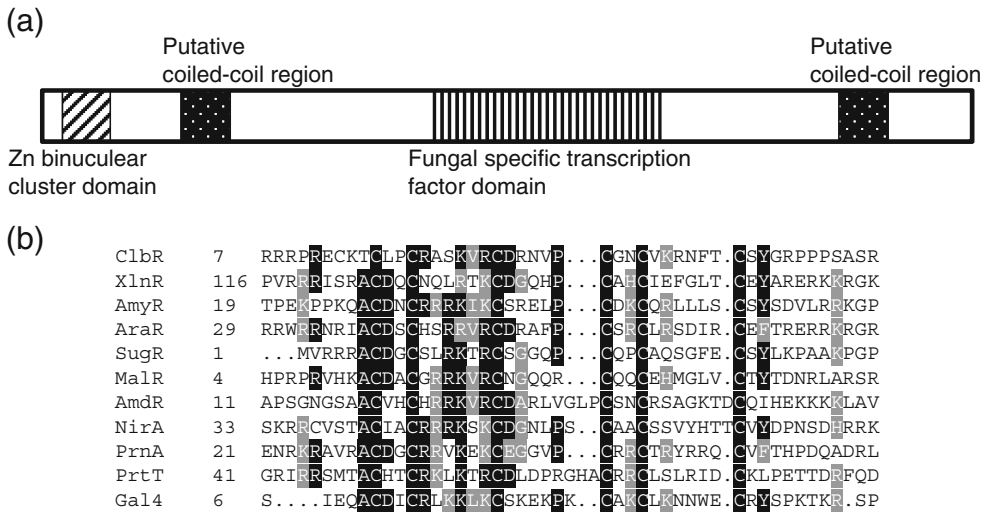

Fig. 3 Conserved domains in ClbR. **a** Diagram of the putative domain organization of ClbR. **b** Comparison of the $Zn(II)_2Cys_6$ DNA-binding domains of transcriptional activators in *Aspergillus* species and *Saccharomyces cerevisiae* (XlnR (O42804, *A. niger*); AmyR, (BAA25754, *A. oryzae*); AraR (CAK38947, *A. niger*); SugR (AAS66030, *Aspergillus parasiticus*); MalR (BAE64052, *A. oryzae*); AmdR (P15699, *A. nidulans*); NirA (P28348, *A. nidulans*); PrnA (CAA11374, *A. nidulans*); PrtT (CAK44694, *A. niger*); Gal4 (P04386, *S. cerevisiae*)). Identical amino acids and similar amino acids are *highlighted by black and gray backgrounds*, respectively

ClbR regulates the cellulose induction of genes regulated by both XlnR-dependent and -independent signaling pathways

We disrupted *clbR*, as described in the "Materials and methods" section, and confirmed the disruption by Southern blot analysis, as shown in Fig. 4. To assess the functional role of ClbR, transcripts of *cbhI* and *cmc2* induced under the cellobiose-inducing condition were quantified at 3, 6, and 9 h postinduction in the control strain (MR12) and *clbR*-deficient mutant (Δ*clbR*). The expression levels of *cbhI* and *cmc2* were assessed during the early phase of induction because their expression tends to be reduced during 9 h postinduction in MR12 strains, most likely due to the depletion of the inducer. In the Δ*clbR* strain, the induction of the *cbhI* and *cmc2* genes in response to cellobiose was reduced to 20–40 % of that in the host strain at 3 and 6 h postinduction, similar to the reduction observed in strain S4-22. Both *cbhI* and *cmc2* expression were restored to normal levels by gene complementation (Fig. 5). In contrast, the *clbR* disruption had no effect on the expression of *cmc1* and *xynIb* induced by D-xylose and L-arabinose (Fig. 6). These results verified that ClbR is involved in the cellobiose-responsive induction of the *cbhI* and *cmc2* genes under the

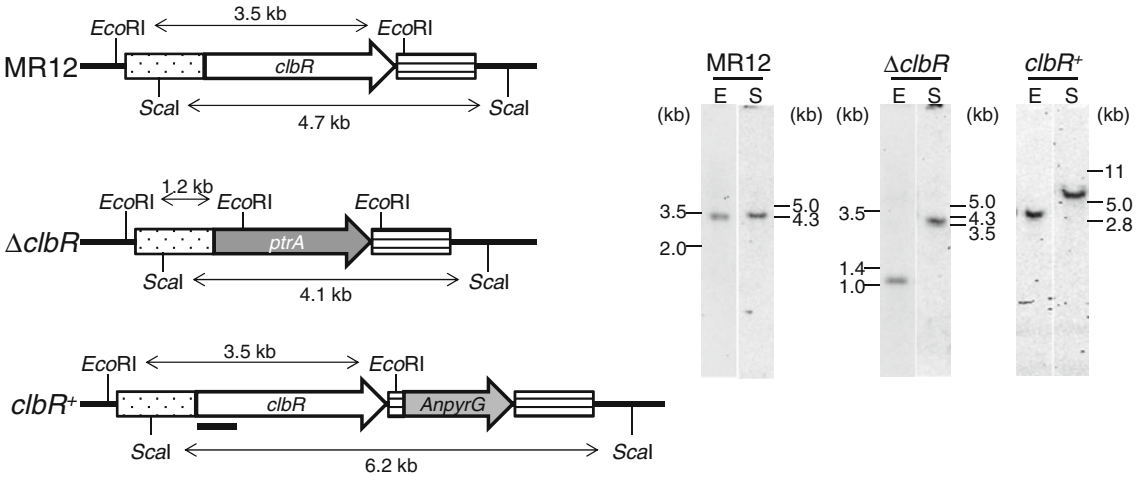

Fig. 4 Construction of the *clbR*-deficient mutant (Δ*clbR*) and the complemented strain (*clbR*$^+$). The illustrations depict restriction enzyme maps of the *clbR* loci in the MR12, Δ*clbR*, and *clbR*$^+$ strains. In the MR12 and Δ*clbR* strains, the DNA regions highlighted with a *dotted and boarded boxes* were used as DNA probes for Southern blot analysis to detect DNA fragments digested with *Eco*RI and *Sca*I, respectively. The DNA probe used for Southern blot analysis of *clbR*$^+$ is indicated with a *solid bar*. The *Eco*RI and *Sca*I digestions are denoted as "E" and "S" in the Southern blot results (*right panel*)

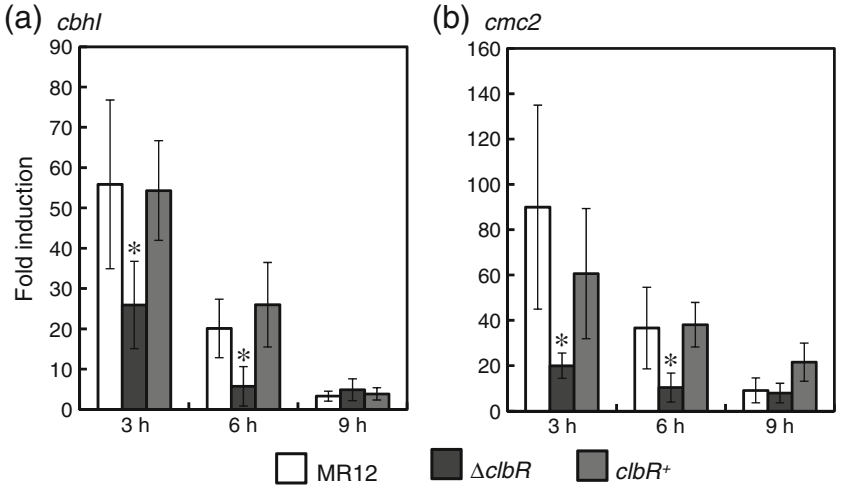

Fig. 5 qPCR analysis of *cbhI* (**a**) and *cmc2* (**b**) expression in the presence of 0.1 % cellobiose together with 50 μg/ml DNJ in Δ*clbR* (*filled bars*) and *clbR*$^{+}$ (*gray bars*) compared to those in the MR12 parental strain (*open bars*). Expression levels of *gpdA* were used as an endogenous control in all samples. Fold induction corresponds to the ratio of the mean expression levels of *cbhI* and *cmc2* under cellobiose-inducing condition divided by those under uninduced condition (0.1 % polypeptone), respectively. The relative transcription levels are the means of at least three independent experiments, and the *error bars* indicate the standard deviations. *$p<0.05$

control of an XlnR-independent signaling pathway. Similar levels of *clbR* transcripts were detected under *cbhI*-induced (cellobiose), -uninduced (D-xylose), and -repressed conditions (D-glucose) (Fig. 7). Thus, the function of ClbR is presumably controlled at the post-transcriptional or translational level.

ASC induces genes that are regulated by the XlnR-independent signaling pathway (*cbhI*, *cmc2*, and *xynIa*) and by the XlnR-dependent signaling pathway (*cmc1* and *xynIb*). Therefore, we assessed the ASC-responsive induction of these genes in the MR12 and Δ*clbR* strains at 4, 8, 12, and 24 h postinduction. The growth defect of the Δ*clbR* strain in ASC media was diminished by adding 0.1 % polypeptone as a neutral carbon source. The total amounts of intracellular protein in the MR12 and Δ*clbR* strains cultured for the indicated periods confirmed that both strains grew equally well under the tested condition (data not shown). All genes in MR12 were significantly induced by 8 h, reached their maximum levels by 12 h, and decayed by 24 h (Fig. 8). In contrast, in the Δ*clbR* strain, the expression of all tested genes significantly reduced at the early phase of induction and attained nearly the peak level observed in the control strain at 24 h postinduction, except for *xynIb*, which expressed at low level at any tested timepoints. These reductions were restored in a strain that was complemented

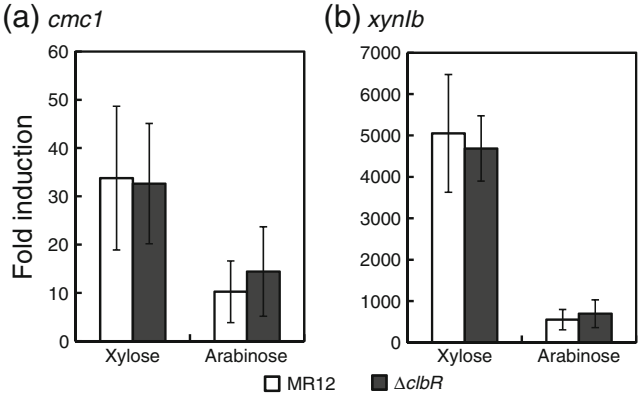

Fig. 6 The *cmc1* (**a**) and *xynIb* (**b**) expression levels in MR12 (*open bars*) and Δ*clbR* (*filled bars*) after a 3-h induction with 1 % D-xylose or 1 % L-arabinose. *gpdA* was used as an endogenous control in all samples. Fold induction corresponds to the ratio of the mean expression levels of *cmc1* and *xynIb* under xylose- or arabinose-inducing condition divided by those under uninduced condition (0.1 % polypeptone), respectively. The data shown are the means of at least three independent experiments, and the *error bars* indicate standard deviations

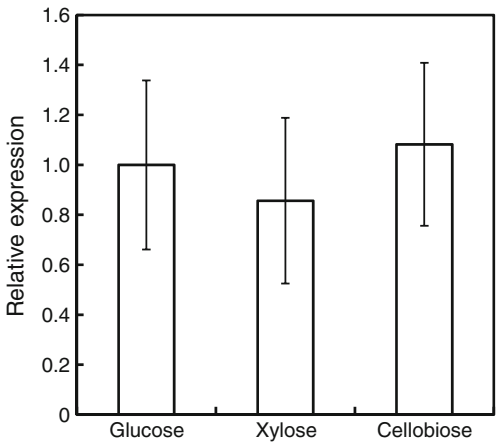

Fig. 7 qPCR analysis of *clbR* in MR12 grown for 3 h in MM containing 1 % D-glucose, 1 % D-xylose, or 0.1 % cellobiose and 50 μg/ml DNJ. *gpdA* was used as an endogenous control in all samples. The level of *clbR* under the glucose condition was assigned a value of 1. The results shown are the means of three independent experiments, and the *error bars* indicate the standard deviations

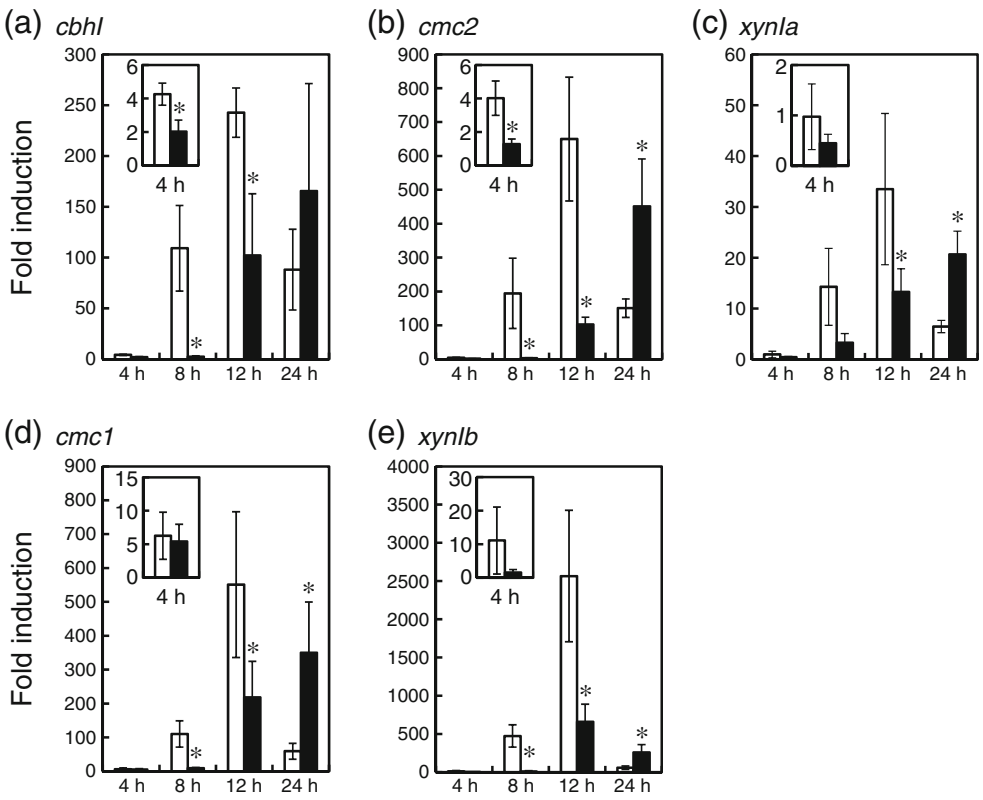

Fig. 8 Cellulase and xylanase gene expression in response to 0.5 % ASC in MR12 (*open bars*) and Δ*clbR* (*filled bars*). *gpdA* was used as an endogenous control in all samples. Fold induction corresponds to the ratio of the mean expression levels of each gene under ASC-inducing condition divided by those under uninduced condition (0.1 % polypeptone), respectively. The data shown are the means of at least three independent experiments, and *error bars* indicate standard deviations. *$p<0.05$. **a** *cbhI*; **b** *cmc2*; **c** *xynIa*; **d** *cmc1*; **e** *xynIb*

with *clbR* (data not shown). These data demonstrate that ClbR controls the early phase induction in response to the presence of cellobiose and cellulose through both XlnR-dependent and XlnR-independent signaling pathways in *A. aculeatus*.

Discussion

In *A. aculeatus*, the expression of cellulase and xylanase genes, which is induced by cellulose, is mediated through both XlnR-dependent and -independent signaling pathways. In this study, using gene-tagging mutagenesis in the *A. aculeatus* NCP2 strain, which harbors a P_{CBHI}-*pyrG* reporter fusion, we identified a novel gene, *clbR*, as a putative regulator involved in cellobiose and cellulose induction. ClbR belongs to the $Zn(II)_2Cys_6$ binuclear cluster protein family, which is known to contain transcription factors that are specific to fungi. The disruption of *clbR* decreased especially the early phase of the cellobiose- and cellulose-responsive induction of the *cbhI*, *cmc2*, and *xynIa* genes and the cellulose-responsive induction of the *cmc1* and *xynIb* genes. These data indicate that ClbR participates in gene induction in response to cellulose through both XlnR-independent and -dependent signaling pathways. However, ClbR is not involved in the XlnR-dependent D-xylose- or L-arabinose-responsive induction of *cmc1* and *xynIb*. Our data demonstrate that ClbR plays a major role in transcriptional activation in response to cellobiose- and cellulose-specific induction. However, all tested genes remained inducible in the Δ*clbR* strain, also suggesting that a distinct cellulose signaling pathway independent of ClbR exists in *A. aculeatus*. Coradetti et al. identified transcription factors, CLR-1 and CLR-2, were required for cellulose utilization and the induction of cellulase genes in *N. crassa* (Coradetti et al. 2012). They also proved that ClrB, CLR-2 homolog in *A. nidulans*, plays an essential role in cellulase gene induction, in contrast ClrA, CLR-1 homolog in *A. nidulans*, has less important role on cellulase gene induction. These factors might control the gene expression in response to cellulosic compounds cooperatively or independently, although further analysis is required.

The Δ*clbR* strain was identified by screening 6,000 transformants. In a previous study, two albino mutants were discovered in 11,000 transformants (Kunitake et al. 2011). The albino mutant has a dominant-negative phenotype; thus, it was identified relatively easily by insertional mutagenesis. However, the Δ*clbR* strain has a moderate phenotype; the disruption reduced the inducibility of the *cbhI* promoter by 80 %. This reporter system enabled us to identify strains with a dominant-negative mutation, but it would be difficult to identify strains with an ambiguous deficient phenotype using this system. The successful identification of ClbR has encouraged us to use this positive screening system in the future, which may lead to the identification of additional factors that only partially participate in the induction of *cbhI*. The *A. aculeatus* genome size is estimated as 35 Mb

and the average ORF size is 1.5 kb; thus, >54,000 transformants would be required to achieve >90 % saturation of the *A. aculeatus* genome with T-DNA insertions (Krysan et al. 1999). We are currently performing screening experiments and functional analyses of other candidate genes.

A comparison of amino acid sequences using the FASTA algorithm revealed that ClbR orthologs are found only in the genomes of *Aspergillus*, *Penicillium*, and *Talaromyces* species, which belong to the *Eurotiales* (Fig. 9). Although ClbR orthologs are found in all genomic databases containing *Aspergillus* species, the orthologous open reading frames are only inferred in many species. These inferred sequences were not used for phylogenetic analysis. *A. aculeatus* possesses a ClbR paralog, ClbR2 (DDBJ Acc. no. AB689702), which is 42 % identical to ClbR at the amino acid sequence level. Orthologs of ClbR2 are found in a wide array of fungi, including *Onygenales* in *Eurotiomycetes* and *Pleosporales* and *Capnodiales* in *Dothideomycetes*, but do not appear to be present in other orders. This observation suggests that ClbR2 was duplicated in *Eurotiales* after the divergence of this order. Although we have not analyzed the function of ClbR2 in *A. aculeatus*, our observation that the *clbR* disruption resulted in the reduction of *cbhI*, *cmc1*, *cmc2*, *xynIa*, and *xynIb* expression suggests that the function of ClbR2 does not completely overlap with that of ClbR.

In many cases, $Zn(II)_2Cys_6$ binuclear cluster DNA-binding proteins bind to CGG or CCG triplets (Noël and Turcotte 1998). The cellulose response element (CeRE) in the *A. aculeatus cbhI* promoter contains several CCG triplets (Tani et al. 2012). Therefore, we examined whether ClbR binds to CeRE in vitro. MalE recombinant proteins fused to a ClbR N-terminal fragment (MalE-$ClbR_{1\text{-}250}$) or ClbR whole protein (MalE-ClbRw) were expressed in *E. coli* Rosetta-gami B (DE3) pLacI, partially purified, and then subjected to an electrophoretic mobility shift assay. Our preliminary data showed that neither recombinant

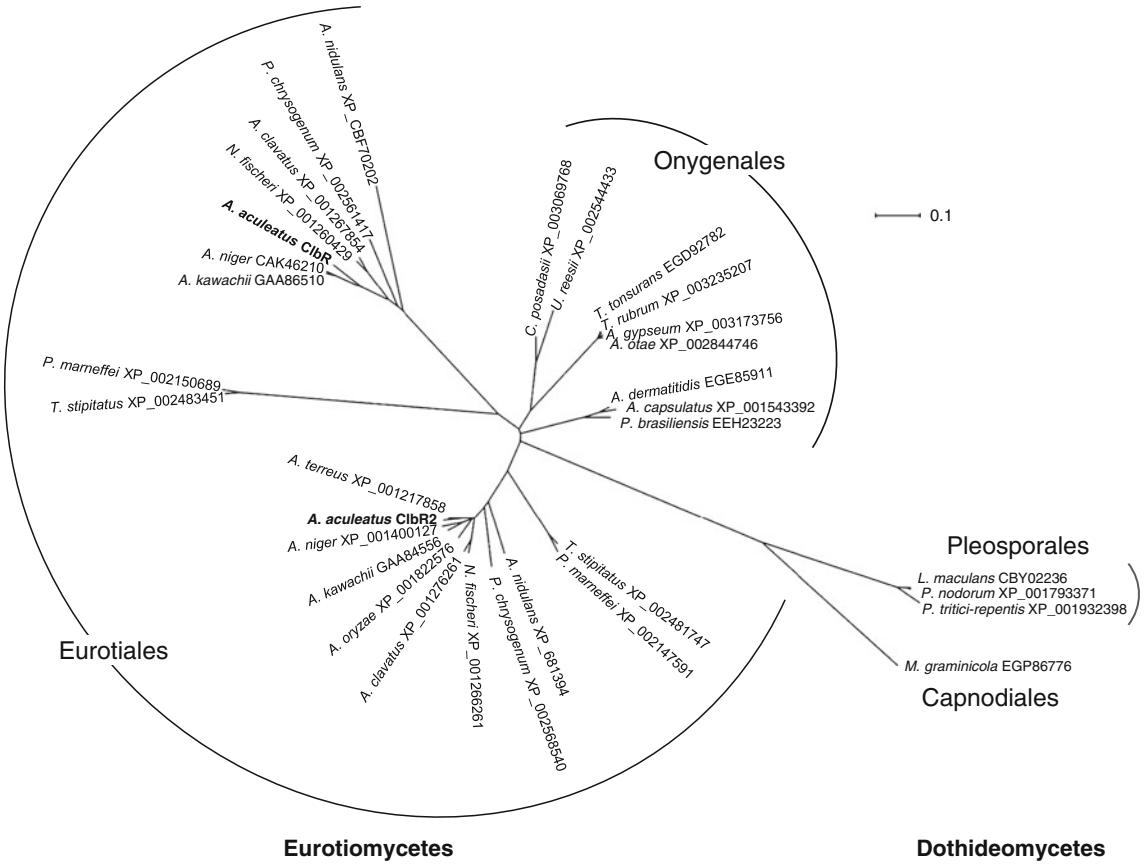

Fig. 9 The phylogenetic relationship of ClbR orthologs. A phylogenetic tree of sequences orthologous to ClbR and ClbR2 (cutoff e-value > E-50) was built using ClustalW and Dendroscope. The sequences were identified by BLAST analysis. *Eurotiales*: *A. clavatus*, *Aspergillus clavatus*; *A. kawachii*, *Aspergillus kawachii*; *A. terreus*, *Aspergillus terreus*; *N. fischeri*, *Neosartorya fischeri*; *P. chrysogenum*, *Penicillium chrysogenum*; *P. marneffei*, *Penicillium marneffei*; *T. stipitatus*, *Talaromyces stipitatus*; *Onygenales*: *A. capsulatus*, *Ajellomyces capsulatus*; *A. dermatitidis*, *Ajellomyces dermatitidis*; *A. gypseum*, *Arthroderma gypseum*; *A. otae*, *Arthroderma otae*; *C. posadasii*, *Coccidioides posadasii*; *P. brasiliensis*, *Paracoccidioides brasiliensis*; *T. rubrum*, *Trichophyton rubrum*; *T. tonsurans*, *Trichophyton tonsurans*; *U. reesii*, *Uncinocarpus reesii*; *Pleosporales*: *L. maculans*, *Leptosphaeria maculans*; *P. nodorum*, *Phaeosphaeria nodorum*; *P. tritici-repentis*, *Pyrenophora tritici-repentis*; *Capnodiales*: *M. graminicola*, *Mycosphaerella graminicola*

MalE-ClbR$_{1-250}$ nor MalE-ClbRw specifically bound to CeRE in the *cbhI* promoter. Furthermore, these proteins did not specifically bind to the 1-kb *xynIb* promoter region. These results suggest that ClbR alone does not bind to the CeRE sequence in vitro. However, further analyses of the binding properties of ClbR should be performed. For example, the Zn(II)$_2$Cys$_6$ binuclear cluster protein involved in oleate induction, Oaf1p, forms a heterodimer with Pip2p, and this heterodimerization is necessary for recognition site binding and subsequent gene induction (Karpichev and Small 1998). Our results demonstrated that ClbR is involved in cellulose-responsive induction regulated by XlnR. Because the coiled-coil domain contributes to protein–protein interactions, e.g., to form homodimers or heterodimers, ClbR might interact with another transcription factor, such as XlnR, and then bind to the promoter region and activate transcription of the genes. It is also possible that ClbR interacts with ClrA or ClrB to transduce signals from cellulosic compounds in *A. aculeatus*. A logical next step will be to investigate the mode of action of ClbR in response to cellobiose and cellulose.

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