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Regulation of genes encoding cellulolytic enzymes by Pal-PacC signaling in *Aspergillus nidulans*

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Abstract Cellulosic biomass represents a valuable potential substitute for fossil-based fuels. As such, there is a strong need to develop efficient biotechnological processes for the enzymatic hydrolysis of cellulosic biomass via the optimization of cellulase production by fungi. Ambient pH is an important factor affecting the industrial production of cellulase. In the present study, we demonstrate that several *Aspergillus nidulans* genes encoding cellulolytic enzymes are regulated by Pal-PacC-mediated pH signaling, as evidenced by the decreased cellulase productivity of the *palC* mutant and *pacC* deletants of *A. nidulans*. The deletion of *pacC* was observed to result in delayed induction and decreased expression of the cellulase genes based on time course expression analysis. The genome-wide identification of PacC-regulated genes under cellobiose-induced conditions demonstrated that genes expressed in a PacC-dependent manner included 82 % of ClrB (a transcriptional activator of the cellulase genes)-regulated genes, including orthologs of various transporter and β-glucosidase genes considered to be involved in cellobiose uptake or production of stronger inducer molecules. Together with the significant overlap between ClrB- and PacC-regulated genes, the results suggest that PacC-mediated regulation of the cellulase genes involves not only direct regulation by binding to their promoter regions but also indirect regulation via modulation of the expression of genes involved in ClrB-dependent transcriptional activation. Our findings are expected to contribute to the development of more efficient industrial cellulase production methods.

Keywords *Aspergillus nidulans* · PalC · PacC · Cellulolytic enzymes · ClrB

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Introduction

Cellulosic biomass is a prospective substitute for fossil fuels. Extensive global efforts are invested in the establishment of technologies for the conversion of cellulosic biomass into biofuels and biomaterials. These include the development of technologies for the efficient enzymatic hydrolysis of cellulosic biomass, as this process enables higher yields of fermentable monosaccharides compared with acid hydrolysis. The main challenge associated with enzymatic hydrolysis is the requirement of high amounts of cellulase and the need for expensive pretreatment due to the crystalline nature of cellulose. Filamentous fungi, such as *Trichoderma reesei* and *Aspergillus* strains, are known to be excellent cellulase producers. These fungi are utilized for industrial cellulase production; however, the levels of cellulase productivity obtained via the use of these fungi remain insufficient for efficient enzymatic hydrolysis of cellulosic biomass. A clearer understanding of the mechanisms involved in the regulation of cellulase production by fungi is crucial for the improvement of industrial production processes to obtain higher yields.

In cellulase-producing fungi, the induction of cellulolytic enzyme-encoding genes involves inducer-triggered activation of transcriptional activators. Cellobiose, which is released

from cellulose by cellobiohydrolase, generally acts as an inducing molecule, although true inducers may include transglycosylation products produced from cellobiose by β-glucosidases. For example, sophorose and gentiobiose are highly efficient inducers in *T. reesei* and *Penicillium purpurogenum*, respectively (Sternberg and Mandels 1980; Kurasawa et al. 1992). *Aspergillus niger* XlnR, which was initially identified as a xylanase regulator (van Peij et al. 1998b), regulates the genes encoding cellulolytic enzymes in filamentous fungi. XlnR and its orthologues regulate D-xylose-triggered induction of genes encoding not only xylanolytic but also cellulolytic enzymes in *Aspergillus* as well as in *Hypocrea jecorina* (*T. reesei*) (van Peij et al. 1998a; Gielkens et al. 1999a, b; Marui et al. 2002b; Stricker et al. 2006; Noguchi et al. 2009). XlnR is also involved in cellobiose-triggered induction of the genes encoding cellulolytic and xylanolytic enzymes in *Aspergillus oryzae* (Marui et al. 2002a).

Recent studies have identified several novel transcriptional activators involved in the regulation of cellulolytic enzyme production, namely, CLR-1 and CLR-2 in *Neurospora crassa* and their orthologues ClrA and ClrB in *A. nidulans* (Coradetti et al. 2012), ManR in *A. oryzae* (Ogawa et al. 2012), ClbR in *Aspergillus aculeatus* (Kunitake et al. 2013), and McmA in *A. nidulans* (Yamakawa et al. 2013). ManR was identified as a mannanase regulator and found to be orthologous to CLR-2/ClrB. Furthermore, RNA sequencing and DNA microarray analysis showed that CLR-2/ClrB/ManR regulates genes encoding cellulolytic and mannanolytic enzymes (Coradetti et al. 2013; Ogawa et al. 2013). Production of cellulolytic enzymes may be regulated by direct and/or indirect interaction between these activators. However, the precise mechanisms of induction may differ according to the species, as described for CLR-2 and ClrB, e.g., the constitutive expression of CLR-2 leads to inducer-independent expression of the genes encoding cellulolytic enzymes in *N. crassa*, whereas ClrB-dependent expression requires the inducing signal in *A. nidulans* even if it is constitutively expressed (Coradetti et al. 2013).

Cellulase production is not only affected by the available carbon source but also by ambient pH, as in *Aspergillus fumigatus* and *N. crassa* (Eberhart et al. 1977; Stewart and Parry 1981). In *T. reesei*, both cellulase production and inducer uptake are affected by ambient pH (Sternberg and Mandels 1979). The pH signaling pathway, which is well studied in *A. nidulans*, involves the *pal* gene products (PalA, B, C, F, H, and I) and the C_2H_2 transcription factor PacC (Peñalva et al. 2014). The Pal proteins sense alkaline pH in the environment and transmit a signal accordingly, which leads to the activation of PacC by PalB-catalyzed proteolysis. Activated PacC regulates the expression of several genes in order to adapt to the alkaline environment. In *A. nidulans*, PacC upregulates the transcription of a xylanase gene *xlnA* under

alkaline pH but downregulates another xylanase gene *xlnB* and an α-L-arabinofuranosidase gene *abfB* (MacCabe et al. 1998; Gielkens et al. 1999a, b). Under acidic pH, the transcription of *xlnB* and *abfB* is de-repressed from PacC-mediated repression.

Ambient pH is one of the key factors affecting the industrial production of biomass-degrading enzymes, and it is clear that mechanisms underlying the regulation of pH must be understood in detail for further enhancement of the productivity of these industrial processes. In this study, we demonstrate that a number of genes encoding cellulolytic enzymes are under the control of the Pal-PacC-mediated pH signaling pathway and suggest that multiple genes may be regulated indirectly by PacC, possibly via modulation of ClrB activity.

Materials and methods

Construction of *A. nidulans* strains

A. nidulans strains used in this study are listed in Table 1. ABPU1, a progeny of a cross between FGSC A89 and FGSC A773 (Motoyama et al. 1997), was used as the parent strain for construction of the *xlnR* and *pacC* deletion strains. FGSC A517 was obtained from the Fungal Genetics Stock Center. ABP was constructed by replacing the *pyrG89* alleles of ABPU1 with *pyrG* by protoplast transformation.

The *palC*-complementing strain RepalC1 was constructed by integrating the plasmid pRpalC into the *pyroA4* locus of A517 (Fig. S1a in the Supplementary Material). pRpalC carries the PCR-amplified wild-type *palC* fragment between the *SpeI* and *NotI* sites of pPyroA (Yamakawa et al. 2013). The *xlnR* deletion strain DXlnR1 and the *pacC* deletion strain DPacC1 were constructed by replacing the respective genes with the *pyrG* gene via homologous recombination. The deletion cassettes were composed of the upstream and downstream regions of the target genes with *pyrG* inserted between them (Figs. S1b and c in the Supplementary Material).

All the primers used in the above are listed in Table S1 in the Supplementary Material. The chromosomal DNA of ABPU1 was used as the template. PCR-amplified regions were sequenced to confirm the absence of non-designed mutations prior to transformation into *A. nidulans*. Transformation of *A. nidulans* was performed by protoplast transformation as previously described (Makita et al. 2009). Transformants were subjected to Southern blotting analysis, and those harboring the engineered genes at the target loci as designed were used in this study.

A. nidulans strains were grown at 37 °C in standard minimal medium (Rowlands and Turner 1973) with appropriate supplements unless otherwise noted. *Escherichia coli* strains DH5α (TaKaRa Bio Inc., Shiga, Japan) and XL-1 Blue

Table 1 *A. nidulans* strains used in this study

Strain	Genotype	Reference
ABPU1	*biA1 pyrG89; wA3; argB2; pyroA4*	(Motoyama et al. 1997)
ABP	*biA1; wA3; argB2; pyroA4*	This study
FGSC A517	*riboA1 yA2 adE20; methG1 suC11adE20*	(Käfer 1977)
	palC4 pabaB22 pyroA4	
RepalC1	*riboA1 yA2 adE20; methG suC11adE20*	This study
	palC4 pabaB22 pyroA4::palC::pyroA	
DXlnR1	*biA1 pyrG89; wA3; argB2; pyroA4 ΔxlnR::pyrG*	This study
DPacC1	*biA1 pyrG89; wA3; argB2; pyroA4; ΔpacC::pyrG*	This study

(Agilent Technologies Inc., Santa Clara, CA, USA) were used for DNA manipulations.

Plate assay for extracellular cellulase and xylanase production

A. nidulans strains were point-inoculated on minimal agar plates containing 0.1 % Triton X-100 (Sigma-Aldrich Co. LLC., St. Louis, MO, USA), in which carboxymethyl cellulose sodium salt (CMC, 1 %) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) plus Polypepton P1 (1 %) (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) was used instead of glucose as carbon sources for the cellulase assay, and oat spelt xylan (1 %) was used for the xylanase assay. Enzyme activity was visualized by staining with 0.1 % Congo-red (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) followed by de-staining with 0.7 M NaCl.

Transcriptional analysis of genes encoding cellulolytic enzymes

A. nidulans strains were grown in standard minimal medium (pH 4.2) containing 2 % Polypepton P1 instead of glucose as a carbon source for 20 h at 37 °C. The mycelia were collected by filtration using sterile 75-mesh nylon filter (NBC Meshtec, Tokyo, Japan) and washed with minimal medium without carbon sources. The mycelia (0.5 g, wet weight) were transferred into 40 ml of fresh minimal media of pH 8.2 containing 0.1 % cellobiose as the inducer and cultivated for 1, 3, or 6 h. The media were buffered at pH 8.2 with 25 mM Tris-HCl. The mycelia were frozen in liquid nitrogen and ground to fine powder. Total RNA extraction, cDNA synthesis, and qRT-PCR were carried out as previously described (Yamakawa et al. 2013). The amount of each mRNA was determined using the standard curve method. *actA* (ANIA_06542) mRNA was used as the internal reference to evaluate the mRNA levels for each gene. All experiments were repeated three times to ensure reproducibility of the results. Primers used are listed in Table S2 in the Supplementary Material.

RNA-sequencing analysis

mRNA libraries and cDNA libraries were prepared using the Illumina TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA) according to the standard protocols. Briefly, each total RNA sample (1 µg) was enriched for mRNA using oligo (dT)-tagged beads. Samples of RNA were fragmented into smaller pieces and used to synthesize cDNA. cDNA library construction involved end repair, A-tailing, adapter ligation, and amplification. The mean length for each cDNA library was approximately 250 to 300 bp. Sequencing was performed in a pair-end 2×25 base mode on a Miseq system (Illumina, San Diego, CA, USA), running six samples per run (multiplexing).

The sequences were analyzed using a CLC genomics workbench (CLC Bio, Aarhus, Denmark) and subsequently demultiplexed and trimmed. Read quality was checked, and only reads with quality values higher than Q30 were used for mapping. The *A. nidulans* genome data of PRJEA40559 obtained from NCBI (http://www.ncbi.nlm.nih.gov/bioproject/PRJEA40559) was used as the template for mapping. From the mapping data, Reads Per Kilobase of exon per Million mapped reads (RPKM) values were calculated using the CLC genomics workbench program. Independently, raw mapped read counts were normalized using iDEGES/edgeR in the R package TCC (Sun et al. 2013). Genes with a false discovery rate (FDR) of below 0.05 were considered differentially expressed genes (DEGs).

The RNA sequencing data was deposited to the DDBJ BioProject database with BioProject ID of PRJDB3191.

Preparation of PacC recombinant protein

A. nidulans cDNA mixture was synthesized using Super Script First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific Inc., Waltham, MA, USA). A DNA fragment encoding PacCc (5–254 aa) was amplified by PCR using cDNA as the template and pacC-F1_*Hin*dIII and pacC-R1_*Bam*HI primer set (Table S3 in the Supplementary Material). The amplified DNA fragment was digested with

*Hin*dIII and *Bam*HI and cloned into pT7-FLAG™-1 (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) to produce pT7-FLAG-PacC1. *E. coli* KRX strain (Promega Corporation, Madison, WI, USA) was used for plasmid construction and recombinant protein expression. *E.coli* KRX harboring pT7-FLAG-PacC1 was grown in LB broth containing 50 mg/l ampicillin to $OD_{600} = 0.6$. Expression of FLAG-tagged PacCc (FLAG-PacCc) was induced by addition of 0.1 % rhamnose and 1 mM isopropyl β-D-thiogalactopyranoside for 8 h at 25 °C. The cells were collected by centrifugation at $5000 \times g$ for 5 min at 4 °C and re-suspended in buffer consisting 10 mM NaH_2PO_4 (pH 7.0), 150 mM NaCl, and 10 % glycerol and lysed by sonication. Cell extract was obtained by centrifugation of the cell lysate at $14,000 \times g$ for 10 min at 4 °C. FLAG- PacCc was purified by column chromatography using ANTI-FLAG M2 Antibody Affinity Gel (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) as described in the manual. The purified fraction was dialyzed two times against buffer consisting of 20 mM HEPES-NaOH (pH 7.0), 150 mM NaCl, and 10 % glycerol, and then dialyzed against the same buffer, except with an increased glycerol concentration of 50 %. Protein concentration was measured by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Electrophoretic mobility shift assay

DNA fragments corresponding to the promoter regions of cellulase and transcription factor-encoding genes were constructed by amplification with primer sets listed in Table S4 in the Supplementary Material and digested with *Bam*HI or *Bgl*II. DNA fragments were end-labeled by DNA polymerase reaction using Klenow fragment and dNTP mixture containing biotin-14-dCTP (Thermo Fisher Scientific Inc., Waltham, MA, USA), to fill in the flanking ends.

The binding reaction mixture consisted of 10 mM HEPES-KOH (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 20 μM $ZnCl_2$, 1 mM dithiothreitol, 10 % glycerol, 1 μg of poly (dI-dC), 20 fmol of biotin-labeled DNA probe, and 25–100 ng FLAG::$PacC_{5-245}$. The binding mixtures were incubated for 20 min at room temperature and loaded onto 4 % polyacrylamide gel in 0.5 × TBE. After electrophoresis, DNA in the gel was blotted on to Hybond N+ (GE Healthcare UK Ltd., Buckinghamshire, England). Signal detection was performed using LightShift® Chemiluminescent EMSA Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), according to the manufacturer's protocol, and LAS-3000 mini (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Competition experiments were performed using 100 ng of FLAG-PacCc with increasing amounts of unlabeled competitor DNA (20-fold to 250-fold molar excess). The DNA fragment containing ipnA2, one of the PacC binding sites of the isopenicillin N synthase gene promoter (Espeso and Peñalva

1996), was used as the specific competitor (ipnA2wt). The PacC binding consensus GCCAAG in ipnA2wt was replaced to TGATCC in the non-specific competitor (ipnA2mt). The competitor DNA fragments were obtained by annealing the oligonucleotides ipnA2-F and ipnA2-R for ipnA2-wt and ipnA2mt2-F and ipnA2mt2-R for ipnA2-mt. DNA fragments corresponding to the −1006 to −626 region of the cellobiohydrolase A gene (*cbhA*) with or without mutation in the putative PacC binding sequences were also used as unlabeled competitors. Two putative PacC binding sites within the region were mutated by overlapping PCR. The primers PcbhA-F1_emsa, PcbhA1-1mt-R, PcbhA1-1mt-F, and PcbhA-R1_emsa were used for mutagenizing the upstream putative binding site. For mutation of the downstream putative binding site, PcbhA-F1_emsa, PcbhA1-2mt-R, PcbhA1-2mt-F, and PcbhA-R1_emsa were used. Oligonucleotides used for construction of the competitor DNA fragments are listed in Table S4 in the Supplementary Material.

Results

The Pal-PacC signaling pathway in xylanase and cellulase regulation in *A. nidulans*

A. nidulans FGSC A517 was found to exert a small degree of cellulase and xylanase activity on CMC (carboxymethyl cellulose) and xylan (Fig. 1b, c). As the transcription factor XlnR and its orthologues have been shown to be involved in cellulase and xylanase expression in *A. niger*, *A. oryzae*, and *T. reesei* (van Peij et al. 1998a; van Peij et al. 1998b; Marui et al. 2002a, 2002b; Stricker et al. 2006), we suspected that an uncharacterized mutation in the *xlnR* gene may have been present in the A517 strain. However, *xlnR* deletion affected xylanase production but not cellulase production in *A. nidulans* (Fig. 1e, f). In order to roughly localize the mutant allele that caused the defect in cellulase production, A517 was crossed with the *xlnR* deletion strain DXlnR1. Among the 192 progenies with reduced cellulase production, 187 displayed extremely reduced growth at pH 8.2, indicating that the mutant allele in A517 that had led to the reduced cellulase production was *palC4* or closely linked to *palC4*. The *palC+* gene was introduced into the *pyroA* locus of A517 to examine whether *palC4* was the cause of the reduced cellulase production. The *palC+* gene complemented not only the growth defect at pH 8.2 but also the defects in cellulase and xylanase production (Fig. 1b, c), confirming that *palC4* is the cause of the reduction in enzyme production.

Since PalC is a component of the pH signaling pathway that activates the wide-domain transcription factor PacC in response to alkaline pH (Galindo et al. 2007, Galindo et al. 2012; Peñalva et al. 2014), PacC should be involved in the *palC*-mediated regulation of the cellulolytic and xylanolytic enzyme production

Fig. 1 Growth and extracellular enzyme production of the wild-type (*ABPU1* or *ABP*), A517, palC-complementing (*RepalC1*), ΔxlnR (*DXlnR1*), and ΔpacC (*DPacC1*) strains. **a**, **d**, **g** Growth on minimal medium containing glucose as the carbon source at pH 6.5. **h** Growth on minimal medium at pH 8.2. **b**, **e**, **i** Plate assay for endoglucanase activity. **c**, **f**, **j** Plate assay for xylanase activity
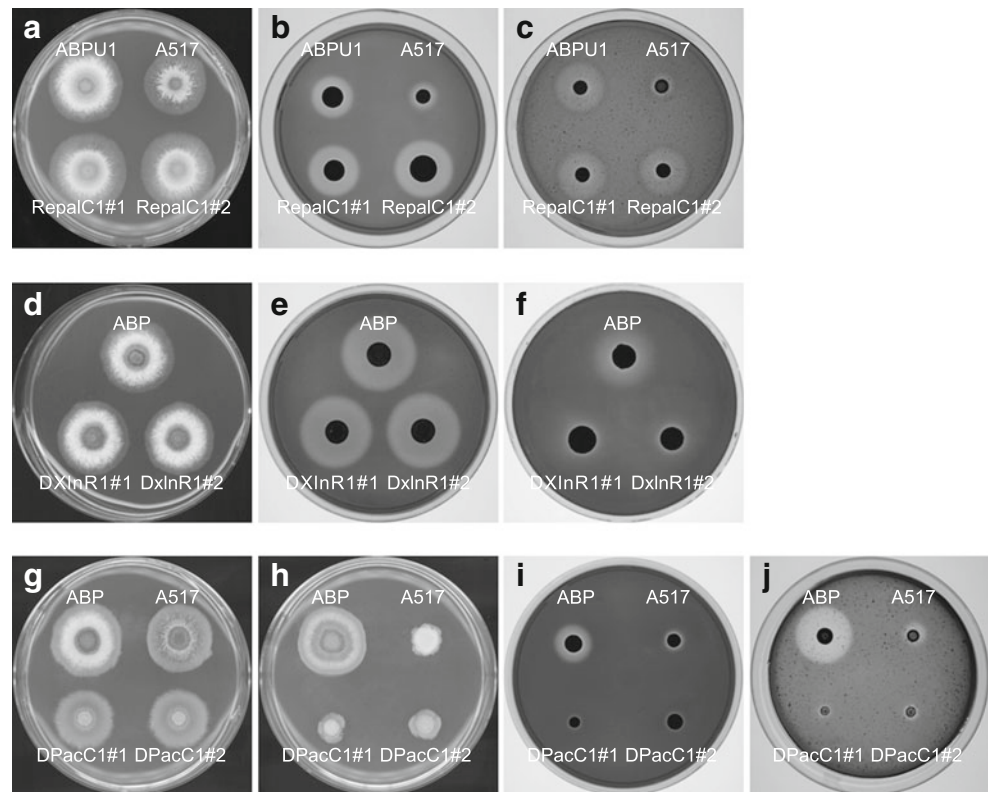

in *A. nidulans*. To confirm this, the *pacC* gene was deleted by replacing it with *pyrG* (Fig. S1c in the Supplementary Material). As expected, a significant decrease in cellulase and xylanase production on CMC and xylan plates (pH 6.5) was observed in the Δ*pacC* strains (DPacC1). Moreover, their reduced growth was evident when compared with the *pacC*[+] strain (ABP) (Fig. 1g, h, i, j). Further analyses were performed at the transcriptional level after short exposure to cellobiose with the aim of eliminating possible effects caused by the poor growth of the Δ*pacC* strain, in order to focus on the relationship between cellulase production and PacC.

Genome-wide transcription analysis to elucidate the impact of *pacC* deletion under cellulase-inducing conditions

RNA sequencing analysis was performed to identify DEGs between the ABP (*pacC*[+]) and DPacC1 (Δ*pacC*) strains under cellobiose-induced conditions for 1 h at pH 8.2. Genes that showed decreased expression following *pacC* deletion were identified based on statistical analysis using the R package TCC (Sun et al. 2013). Since incorporation of genes with low expression may result in incorrect interpretation, we focused on the 6957 genes with RPKM values of over 10 in the *pacC*[+] strain. When the false discovery rate of below 0.05 was applied, 738 out of the 6957 genes were assigned as DEGs with decreased expression in the Δ*pacC* strain (Table S5 in

the Supplementary Material). This indicates that the 738 genes were directly or indirectly upregulated by PacC.

Total expression of CAZy genes encoding the major classes of plant cell wall-degrading enzymes is shown in Fig. 2.
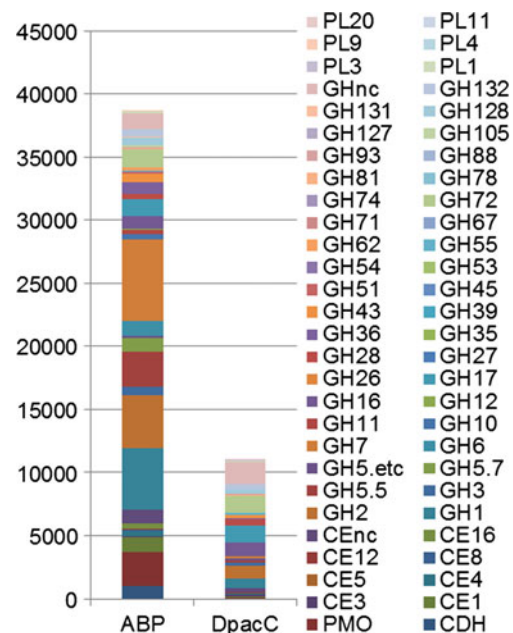
Fig. 2 Effect of pacC deletion on the total expression of the CAZy genes related to plant cell wall degradation. Expression level of each family represents a sum of RPKM values of the genes in the family

The sum of RPKM in the $\Delta pacC$ strain decreased to approximately one third of that in the $pacC^+$ strain. A decrease of greater than 10-fold was observed for AA8 (cellobiose dehydrogenases), AA9 (lytic polysaccharide monooxygenases), GH5 (β-endoglucanases and β-endomannanases), GH6 (cellobiohydrolases), GH7 (β-endoglucanases and cellobiohydrolases), GH10 (β-endoxylanases), GH11 (β-endoxylanases), GH26 (β-endomannanases), GH36 (α-galactosidases), GH62 (α-arabinofuranosidases), GH131 (β-glucanases), CE1 (acetyl xylan esterases and feruloyl esterases), CE12 (pectin acetylesterases and acetyl xylan esterases), and CE16 (acetyl esterases). This indicates that the expression of the genes encoding cellulolytic and hemicellulolytic enzymes genes was more significantly affected by the $pacC$ deletion. The 72 CAZy genes with RPKM values of over 10 and the false discovery rate of below 0.05 are listed in Table 2.

The transcription factor ClrB/ManR regulates the expression of genes encoding cellulolytic and hemicellulolytic enzymes in *Aspergillus*. Deletion of *clrB* has been shown to cause a decrease in the expression of 141 genes, which was classified as the *clrB* regulon (Coradetti et al. 2013). As three genes, AN12193, AN12293, and AN11947, were not found in the genome database used in this study, the expression levels of the remaining 138 genes were compared with those of the *clrB* regulon. Among the 138 genes examined, 101 genes were sufficiently expressed in the previous study under induction by Avicel (with RPKM of over 4) (Coradetti et al. 2013). In our experimental conditions, 67 genes out of the 101 genes were sufficiently expressed with RPKM of over 10, and 55 out of the 67 genes (82 %) exhibited decreased expression in DPacC1 ($\Delta pacC$) (Table 3). Possible reasons for the lack of decrease in the expression of the remaining 12 genes following *pacC* deletion are discussed in the Discussion section. With regards to the CAZy genes, 39 genes were regulated by ClrB, and 31 out of the 39 were regulated by PacC (Table 3). No genes encoding cellulolytic enzymes were included in the remaining 8 genes (*agdB* (α-glucosidase), *chiA* (endochitinase), *pgxB* (exo-polygalacturonase), *plyA* (pectate lyase), *plyF* (pectate lyase), *pmeB* (pectin methyl esterase), ANIA_00452 (xyloglucan-specific endoglucanase), and ANIA_02804 (β-galactosidase)). The expression of *plyF* was increased whereas that of the others was not significantly affected by *pacC* deletion.

Effect of *pacC* deletion on time course induction of the genes encoding cellulolytic enzymes

Nine genes encoding cellulolytic enzymes were chosen for time course expression analysis, with reference to the RNA sequencing analysis by Coradetti et al. (2013): endoglucanase genes *eglA* (ANIA_01285) and *eglB* (ANIA_03418), the cellobiohydrolase genes *cbhA* (ANIA_05176), *cbhB*

(ANIA_00494), *cbhC* (ANIA_05282), and *cbhD* (ANIA_01273), the lytic polysaccharide monooxygenase gene ANIA_03860, and the β-glucosidase genes ANIA_10124 and *bglI* (ANIA_02227). RNA was extracted at 0, 1, 3, and 6 h following cellobiose induction at pH 8.2, and the mRNA levels of the nine genes were quantified by RT-qPCR. The expression of these genes was clearly induced at pH 8.2 in ABP ($pacC^+$) by addition of cellobiose, except for *cbhB* (Fig. 3). As the expression level of *cbhB* was extremely low compared with the others, we focused on the remaining eight genes.

The *pacC* deletion caused a delay in the induction of gene expression, as is evident from the fact that the most significant decrease in expression was observed at 1 h (Fig. 3). In ABP ($pacC^+$), expression levels increased 47-fold to 650-fold, depending on the genes, at 1 h after cellobiose induction, whereas at 3 h, the increase was 0.51-fold to 3.0-fold that of the expression levels at 1 h. In contrast, the initial increase at 1 h was 1.17-fold to 118-fold in DPacC1 ($\Delta pacC$), which was followed by a 5.7-fold to 58-fold increase by 3 h. The *pacC* deletion also caused a decrease in the peak expression levels. The most significant decrease of 29-fold was observed for *cbhA*, followed by *cbhC* (26-fold), *eglB* (7.5-fold), *cbhD* (5.2-fold), AN3860 (2.6-fold), AN10124 (2.2-fold), and *eglA* (1.9-fold). The exception was *bglI*, whose expression was not significantly decreased by the deletion.

Binding of PacC to promoter regions of cellulase genes in vitro

Examination of the sequences of the 1000-bp-upstream regions of the genes (in Fig. 3) revealed that all the genes, with a single exception, possessed the PacC binding consensus 5′-GCCARG-3′ or 5′-GCCGAG-3′ (Table S6 in the Supplementary Material) (Espeso et al. 1997; Espeso and Arst 2000). The exception was *cbhD*, which did not have the PacC binding consensus in the intergenic region (1251 bp) between *cbhD* and its upstream gene ANIA_11936, suggesting that regulation by PacC may occur in an indirect manner.

PacC binding to the promoter regions was examined by EMSA using FLAG-PacCc produced in *E. coli*. As shown in Fig. 4a, FLAG-PacCc bound to at least one of the probes generated from each promoter; however, the binding affinities were significantly different. FLAG-PacCc displayed the highest affinity to *cbhA* probe 1 and ANIA_10124 probe 2, followed by *cbhC* probe 1 and *bglI* probe 2. On the other hand, FLAG-PacCc exhibited very weak binding to the *eglB* and *cbhD* promoters, although *pacC* deletion strongly affected the expression of these genes, as shown in Fig. 3. Such weak binding may be sufficient for PacC to activate expression in vivo. However, we suspect that the indirect regulation by

Table 2 CAZy genes regulated by PacC

gene_id (ANIA)	$pacC^+$ RPKM	$\Delta pacC$ RPKM	Gene	Description[a]	CAZy
10124	4881	637		β-Glucosidase	GH1
03418	4017	137	eglB	Endo-β-1,4-glucanase	GH7
03368	4002	886	mndB	β-Mannosidase	GH2
01285	2778	211	eglA	Endo-β-1,4-glucanase	GH5
05176	2381	7	cbhA	Cellobiohydrolase	GH7
03860	2232	42		Lytic polysaccharide monooxygenase	AA9
01273	1109	17	cbhD	Cellobiohydrolase	GH6
08782	1046	99		Esterase	CEnc
07230	986	3		Cellobiose dehydrogenase	AA3
05267	855	11	faeC	Ferulic acid esterase	CE1
09035	765	55	aglG	α-Galactosidase	GH36
06427	688	2	manC	Endo-β-1,4-mannanase	GH5
05320	579	17		GDSL esterase/lipase	CEnc
01477	531	38		β-1,4-Xylosidase	GH43
06422	430	19		GDSL esterase/lipase	CE16
02227	414	10	bglI	β-1,4-Glucosidase	GH3
03613	383	1	xlnA	Endo-β-1,4-xylanase	GH11
03297	378	11	manB	Endo-β-1,4-mannanase	GH5
06093	367	43	axeA	Acetylxylan esterase	CE1
00558	331	151	gelB	β-1,3-Glucanosyltransferase	GH72
09380	312	12		Chitin deacetylase	CE4
01818	311	14	xlnC	Endo-β-1,4-xylanase	GH10
10419	306	3		Endo-β-1,4-glucanase	AA9
02953	258	130		Endo-α-1,4-polygalactosaminidase	GH114
08175	217	68			AA11
07908	211	2	axhA	α-L-Arabinofuranosidase	GH62
00726	197	47	sunB	SUN domain protein	GH132
08761	171	56	pgxA	Exo-polygalacturonase	GH28
07505	156	25	agdD	α-1,4-Xylosidase	GH31
06470	140	37		N,O-diacetylmuramidase	GH25
01015	133	53		Glycogen phosphorylase	GT35
00393	119	65	dfgC	Endo-mannanase	GH76
09383	116	26	urhC	Unsaturated rhamnogalacturonan hydrolase	GH105
02528	115	11		Rhamnogalacturonan acetylesterase	CE12
09069	113	42		Polysaccharide synthase	GT2
07401	110	2	xlnE	Endo-β-1,4-xylanase	GH10
01277	104	37	abfC	α-Arabinofuranosidase	GH51
01455	101	42		Oligosaccharyl transferase subunit	GT66
06428	89	2		Lytic polysaccharide monooxygenase	AA9
01742	85	30	mndA	β-1,4-Mannosidase	GH2
06819	83	31			GH16
08138	83	15	aglC	α-1,4-Galactosidase	GH36
03903	83	19	bglH	β-1,4-Glucosidase	GH3
02828	82	3	bglL	β-1,4-Glucosidase	GH3
07598	77	1			GH131
03733	76	40	mns1A	Class I α-mannosidase 1A	GH47
05282	74	1	cbhC	Cellobiohydrolyase	GH6
07828	63	1	urhB		GH105

Table 2 (continued)

gene_id (ANIA)	$pacC^+$ RPKM	$\Delta pacC$ RPKM	Gene	Description[a]	CAZy
				Unsaturated rhamnogalacturonan hydrolase	
00741	55	4	pelA	Pectate lyase	PL1
07533	49	27	exgD	Exo-β-1,3-glucanase	GH5
08068	44	4		Endo-β-1,4-glucanase	GH5
06620	41	10		Endo-β-1,3(4)-glucanase	GH16
02388	40	3		Lytic polysaccharide monooxygenase	AA9
02060	39	11	abxA	Exo-arabinanase	GH93
02664	38	10		β-1,4-Xylosidase	GH43
04843	37	16	agdG	α-1,6-Glucosidase	GH13
08007	36	4	abnC	Endo-α-1,5-arabinanase	GH43
02955	35	5		Glycosyl transferase	GT4
05663	32	14			GT31
03196	32	2	urhA	Unsaturated rhamnogalacturonan hydrolase	GH105
04738	30	14		Polysaccharide export protein	GT69
02632	30	1	axhB	α-L-Arabinofuranosidase	GH62
05727	29	8	galA	Endo-β-1,4-galactanase	GH53
00031	26	7			GH16
07413	23	1	manE	Endo-β-1,4-mannanase	GH26
05309	21	1		Cutinase	CE5
02217	21	9	bxlC	β-1,4-Xylosidase	GH3
04864	18	9	alg6	Glucosyltransferase	GT57
00494	17	8	cbhB	Cellobiohydrolyase	GH7
11143	14	4	glaA	Glucoamylase	GH15
01542	11	2	xgcA	Xyloglucan cellobiohydrolase	GH74
02690	10	4		Endo-β-1,3(4)-glucanase	GH16

[a] Descriptions are based on AspGD (http://www.aspergillusgenome.org/) and CAZy (http://www.cazy.org/Welcome-to-the-Carbohydrate-Active.html) web sites

PacC via direct regulation of unidentified gene(s) that affect cellulase gene expression is also possible.

To assess whether the observed FLAG-PacCc/DNA complexes are formed by specific binding of FLAG-PacCc, competition assay was performed using the double-strand oligonucleotide (ipnA2wt) containing the PacC binding site (ipnA2) of the *ipnA* gene (Espeso and Peñalva 1996) as a specific competitor. As a non-specific competitor, the ipnA2mt oligonucleotide, which carried a mutated PacC binding site, was used. Figure 4b shows the result with the cbhA-1 fragment as a probe. Addition of ipnA2wt at a 20-fold molar excess caused a significant decrease in the formation of the FLAG-PacCc/cbhA-1 complex, while ipnA2mt addition did not affect the complex formation even at a 50-fold excess. These indicate that the binding of FLAG-PacCc to cbhA-1 is specific. The cbhA-1 fragment possesses two putative PacC binding sites (GCCAGG and GCCAAG), while only a single FLAG-PacCc/DNA complex was detected (Fig. 4a). The

competition assay using the mutated cbhA-1 fragments as competitors revealed that FLAG-PacCc preferably bound to the downstream site (GCCAAG), since cbhA1m1 was as effective as cbh1wt in reduction of the complex formation while cbhA2mt failed to function as a specific competitor (Fig. 4b).

The results of the competition assay with the other promoter fragments as the probes and ipnA2 as the specific competitor are shown in Fig. S2 in the Supplementary Material. The results indicated that all the FLAG-PacCc/DNA complex formation observed in Fig. 4a was caused by specific binding of FLAG-PacCc.

Effect of *pacC* deletion on the expression of transcription factor-encoding genes

The genome-wide transcription analysis at 1 h after the cellobiose induction revealed that most of the ClrB-regulated genes were also regulated by PacC (Table 3). In addition, the time

Table 3 Effect of *pacC* deletion on expression of genes in the ClrB regulon[a]

Gene_ID (ANIA)	*pacC*+ RPKM	Δ*pacC* RPKM	Fold change[b]	Gene	Description	PacC
02814	5802	185	0.032		MFS lactose permease	D[c]
10124	4881	637	0.130		β-Glucosidase	D
03418	4017	137	0.034	*eglB*	Endo-β-1,4-glucanase	D
03368	4002	886	0.220	*mndB*	β-Mannosidase	D
01285	2778	211	0.076	*eglA*	Endo-β-1,4-glucanase	D
05176	2381	7	0.003	*cbhA*	Cellobiohydrolyase	D
03860	2232	42	0.019		Lytic polysaccharide monooxygenase	D
01273	1109	17	0.015	*cbhD*	Cellobiohydrolase	D
02660	1051	106	0.100			D
07230	986	3	0.003		Cellobiose dehydrogenase	D
09035	765	55	0.072	*aglG*	α-Galactosidase	D
06427	688	2	0.003	*manC*	Endo-β-1,4-mannanase	D
00610	671	213	0.316			D
05320	579	17	0.029		GDSL esterase/lipase	D
07353	421	61	0.143		Galactose 1-dehydrogenase	D
02227	414	10	0.023	*bglI*	β-1,4-Glucosidase	D
03297	378	11	0.029	*manB*	Endo-β-1,4-mannanase	D
10419	306	3	0.009		Endo-β-1,4-glucanase	D
08599	302	101	0.334			D
03298	221	44	0.197		Oxidoreductase	D
00233	172	26	0.152		MFS sugar transporter	D
08761	171	56	0.326	*pgxA*	Exo-polygalacturonase	D
08891	167	171	1.017	*pgxB*	Exo-polygalacturonase	I[c]
01109	166	175	1.035		MFS sugar transporter	I
07505	156	25	0.157	*agdD*	α-1,4-Xylosidase	D
01577	120	2	0.016		MFS lactose permease	D
02528	115	11	0.093		Rhamnogalacturonan acetylesterase	D
08404	113	0	0.004			D
07401	110	2	0.020	*xlnE*	Endo-β-1,4-xylanase	D
06035	103	65	0.623			I
02112	100	18	0.183			D
00761	95	11	0.116			D
06428	89	2	0.026		Lytic polysaccharide monooxygenase	D
11077	88	3	0.036		Sugar O-acetyltransferase	D
03903	83	19	0.224	*bglH*	β-1,4-Glucosidase	D
07598	77	1	0.013			D
05282	74	1	0.013	*cbhC*	Cellobiohydrolyase	D
07828	63	1	0.008	*urhB*	Unsaturated rhamnogalacturonan hydrolase	D
08514	61	2	0.028	*tdiB*	Asterriquinone prenyltransferase	D
08953	58	36	0.617	*agdB*	α-1,4-Glucosidase	I
00741	55	4	0.068	*pelA*	Pectate lyase	D
03609	46	4	0.091		Cytochrome P450	D
10447	38	4	0.111			D
03321	31	3	0.097		Aspartyl protease	D
02632	30	1	0.023	*axhB*	α-L-Arabinofuranosidase	D
05727	29	8	0.270	*galA*	Endo-β-1,4-galactanase	D
10430	25	1	0.029		Polyketide synthase	D

Table 3 (continued)

Gene_ID (ANIA)	$pacC^+$ RPKM	$\Delta pacC$ RPKM	Fold change[b]	Gene	Description	PacC
07067	24	12	0.477			I
04860	24	22	0.887	pmeB	Pectin methylesterase	I
11153	24	2	0.101			D
07413	23	1	0.024	manE	Endo-β-1,4-mannanase	D
00452	23	19	0.806		Xyloglucan-specific endoglucanase	I
02804	22	20	0.889		β-Galactosidase	I
08516	22	0	0.015	tdiD	L-Tryptophan aminotransferase	D
05309	21	1	0.042		Cutinase	D
02217	21	9	0.444	bxlC	β-1,4-Xylosidase	D
07646	18	20	1.088	plyA	Pectate lyase	I
00494	17	8	0.455	cbhB	Cellobiohydrolase	D
07046	17	22	1.289	lipA	Lipase	I
08513	17	0	0.020	tdiA	Nonribosomal peptide synthetase (NRPS)	D
09361	16	8	0.457			D
03211	15	2	0.156		NPP1 domain protein	D
08241	13	13	0.983	chiA	Chitinase	I
02652	12	1	0.114			D
03877	12	3	0.244			D
01542	11	2	0.156	xgcA	Xyloglucan cellobiohydrolase	D
06106	10	41	3.952	plyF	Pectate lyase	I

[a] Genes strictly regulated by ClrB (Coradetti et al. 2013). Among these, sufficiently expressed genes (as defined in the Results section) are shown in the table

[b] Fold change refers to the ratio DpacC1 ($\Delta pacC$)/ABP ($pacC^+$) of the gene expression levels, calculated from the m values of iDEGES/edgeR analysis

[c] D and I indicate PacC-dependent and PacC-independent genes, respectively

course analysis shown in Fig. 2 indicated that the delay in the induction in the $\Delta pacC$ strain was a common feature in all the genes examined. These observations suggest that PacC exerts a regulative role in cellulase and hemicellulase gene expression via regulation of the ClrB function at the transcriptional and/or post-transcriptional levels at the initial stages of induction of gene expression. Other transcription factors that may cause such indirect regulation are ClrA, McmA, and CreA, which are reported to be involved in the regulation of the genes encoding cellulolytic enzymes in *A. nidulans* (Lockington et al. 2002; Coradetti et al. 2012; Yamakawa et al. 2013).

Time course expression of the transcription factor-encoding genes was examined by RT-qPCR. Expression of *creA* and *mcmA* increased 2.0-fold and 2.7-fold at 1 h on *pacC* deletion, whereas that of *clrA* decreased by 36 %. Although the difference was not statistically significant, the expression of *clrA* and *clrB* also decreased by 42 and 33 % at 0 h. (Fig. 5). In addition, all the genes possessed PacC binding consensus sequences in the 1000-bp upstream region (Table S6 in the Supplementary Material), and FLAG-PacCc exhibited specific binding to their promoter regions (Fig. 6

and Fig. S2 in the Supplementary Material). It is possible that the decrease in expression of the positive regulator genes *clrA* and *clrB* and the increase in the expression of the negative regulator *creA* in concert caused the delay in the induction in the $\Delta pacC$ strain. However, direct regulation of the transcription factor genes by PacC is unlikely considering that the expression levels were comparable to that of the $pacC^+$ strain at 3 h. As McmA, a positive regulator, is suggested to cooperatively regulate the cellulase genes in concert with ClrB, the increase in *mcmA* expression would not be expected to significantly affect the induction of gene expression due to the simultaneous decrease in the levels of ClrB.

Discussion

The transcription factor PacC regulates the expression of extracellular enzyme-encoding genes. For example, the alkaline phosphatase and alkaline protease genes *palD* and *prtA* are positively regulated by PacC whereas the acid phosphatase gene *pacA* is negatively regulated by this transcription factor (Peñalva et al. 2014). In many cases, PacC activates
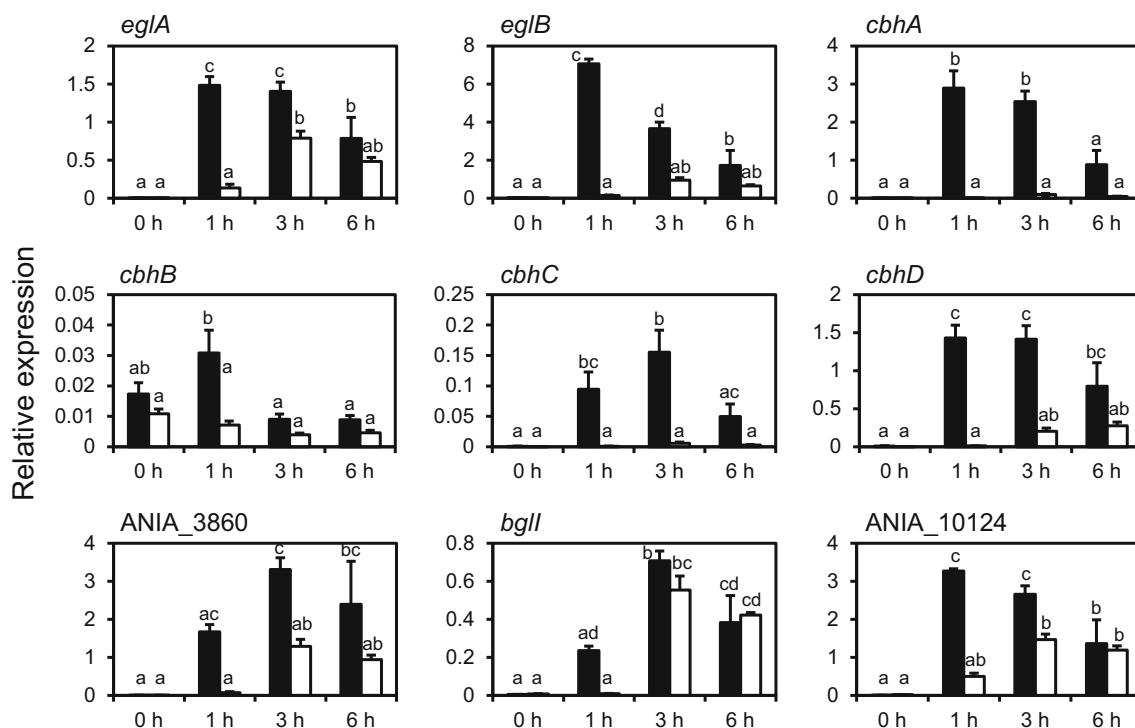
Fig. 3 Effect of pacC deletion on the time course expression of cellulase genes. Expression levels of the genes encoding cellulolytic enzymes in the pacC+ (ABP, *black bar*) and ΔpacC (DPacC1, *white bar*) strains are shown relative to that of the actA gene. Expression of the genes was induced by cellobiose for 0, 1, 3, and 6 h at pH 8.2. *Error bars* represent standard error. Letters above the bar indicate significant differences ($p < 0.05$, one-way ANOVA)

production of extracellular enzymes that function optimally under neutral to alkaline conditions; however, it represses the production of acidophilic enzymes under the same conditions. We examined the involvement of PacC in the regulation of genes encoding cellulolytic enzymes in *A. nidulans* and found that most genes under the control of ClrB (Coradetti et al. 2013) were positively regulated by PacC. Recently effect of deletion of *pac1*, the ortholog of *pacC*, on expression of cellulase and hemicellulase genes in *T. reesei* was studied by two different groups with somewhat contradictory results. That is, while the deletion leads to enhanced transcription of the major cellulase genes such as *cbh1* and *egl1* based on the report by He et al. (2014), expression of these genes is not affected by the deletion in the study by Häkkinen et al. (2015). Therefore, the role of Pac1 in cellulase regulation in *T. reesei* is currently not very clear.
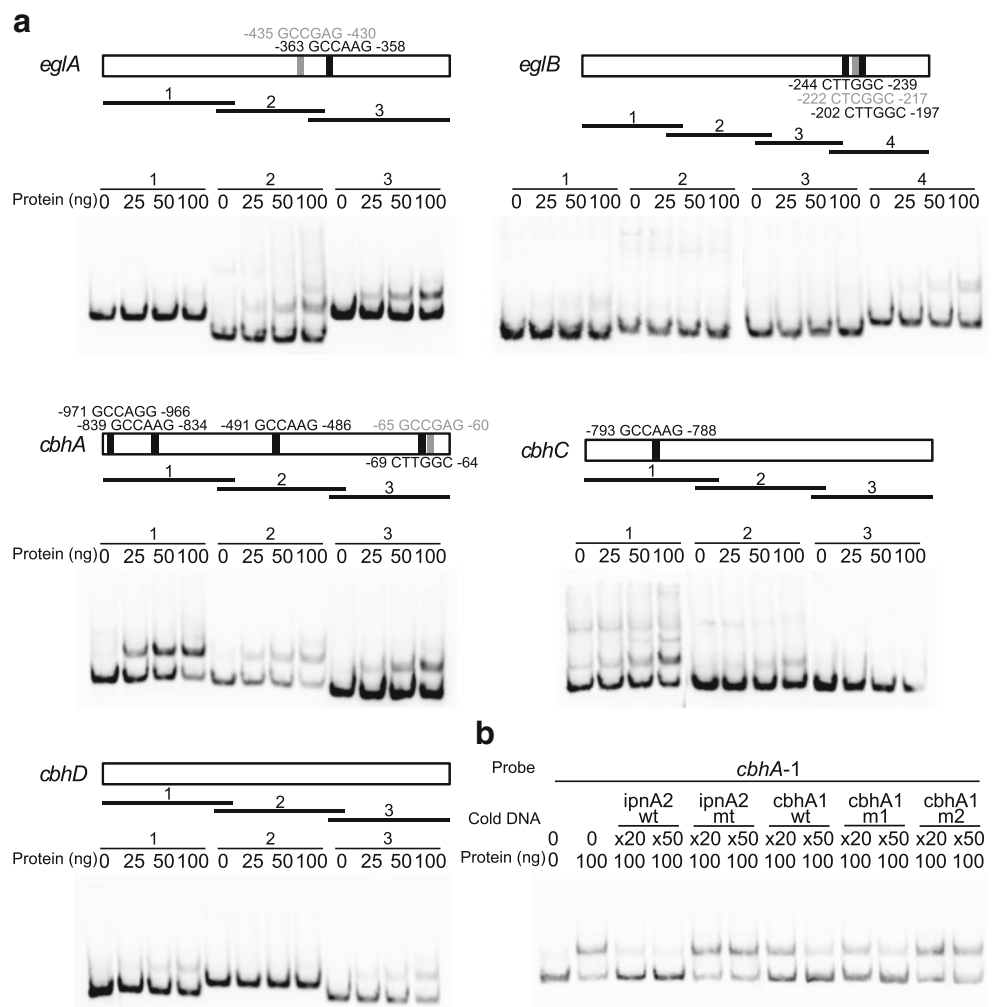
Genes encoding cellulolytic enzymes may be regulated by PacC in an indirect as well as direct manner. For example, the expression of 7 genes encoding cellulolytic enzymes is upregulated at alkaline conditions in *Humicola grisea*. The *egl3* gene is one of these upregulated genes; however, its promoter does not possess PacC binding sites and PacC does not bind to its promoter in vitro (Mello-de-Sousa et al. 2011). Similarly, in *A. nidulans*, *cbhD* was clearly regulated by PacC; however, a PacC binding site was not found to be present in its promoter region. FLAG-PacCc was able to specifically bind the *cbhD*

promoter, but the binding affinity was extremely weak. In addition, the presence of a high affinity specific binding site may not directly imply that the site is functional in vivo, considering that expression of *clrB* that possesses a high affinity binding site was not affected by the pacC deletion. Using the Regulatory Sequence Analysis Tools (RSAT) (Thomas-Chollier et al. 2008), it was shown that the PacC consensus sequence GCCARG is distributed among the upstream regions of over 60 % of genes in the *A. nidulans* genome, implying the existence of a number of non-functional consensus sequences.

Indirect regulation by PacC may be caused by differences in perceived availability of a low molecular weight inducer such as cellobiose by the cell. When cellulose or CMC is used as the inducer, the direct regulation of endoglucanase- and/or cellobiohydrolase-encoding genes by PacC may result in slower production of cellobiose, which in turn leads to a delay and decrease in the expression of all cellobiose-inducible genes. However, the delay and decrease in expression of the genes encoding cellulolytic enzymes observed following cellobiose induction in this study suggests that proteins other than endoglucanases and cellobiohydrolases are also involved in indirect regulation by PacC.

The significant overlap between the ClrB-regulated and PacC-regulated genes suggests that ClrB activity may be affected by PacC-regulated gene(s). Cellobiose triggers the
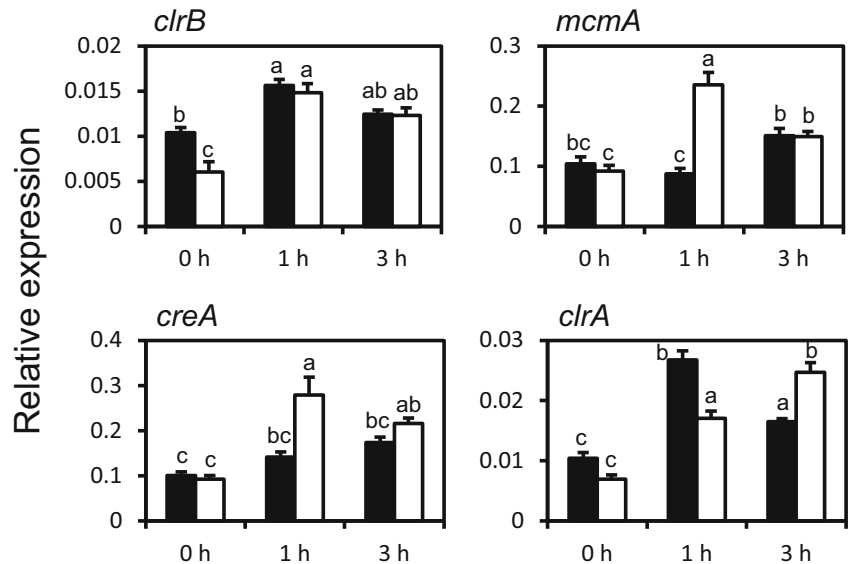
Fig. 4 In vitro DNA binding of PacC to cellulase gene promoters. **a** EMSA was performed using FLAG-PacCc with the DNA fragments derived from the promoter regions as the probe. **b** Competition assay with cbhA-1 as the probe using 100 ng of FLAG-PacCc. Unlabeled ipnA2wt and ipnA2mt were used as specific and non-specific competitors, respectively. The competitor cbhA1wt was the unlabeled cbhA-1 fragment, while cbhA1m1 and cbhA1m2 carried a mutation in the upstream and downstream PacC binding motifs, respectively. The competitors were added at 20-fold and 50-fold molar excess relative to the labeled-probe cbhA-1

ClrB-dependent expression of genes encoding cellulolytic enzymes. Therefore, genes involved in the hydrolysis, conversion, or uptake of cellobiose represent possible candidates responsible for such indirect regulation by PacC. A single β-glucosidase gene ANIA_09183 (*bglR*), the product of which was found to be an extracellular enzyme based on PSORT II

Fig. 5 Time course transcriptional analysis of transcription factor genes involved in regulation of cellulase genes. RT-qPCR was performed using the same cDNA samples as in Fig. 3. *Black* and *white bars* represent expression levels of the genes relative to actA in pacC+ (ABP) and ΔpacC (DPacC1). *Error bars* represent standard error. *Letters above the bar* indicate significant differences ($p < 0.05$, one-way ANOVA)

Fig. 6 In vitro DNA binding of PacC to the promoters of transcription factor genes involved in the regulation of cellulase genes
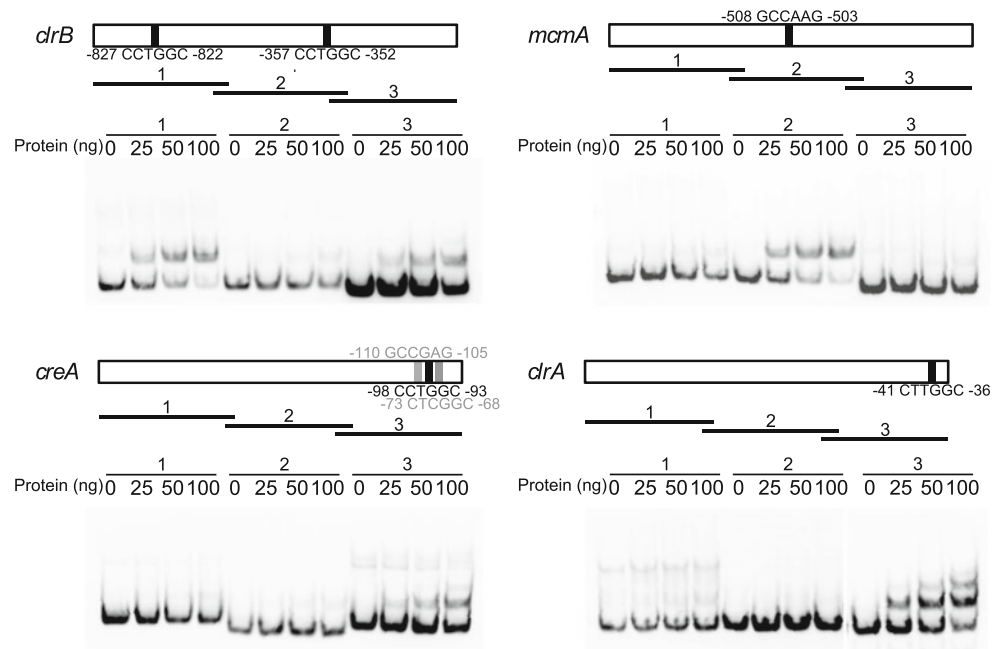
prediction (http://psort.hgc.jp/form2.html), exhibited an 80-fold higher expression on *pacC* deletion. However, as cellobiose disappeared more rapidly in ABP (*pacC*[+]) than in DPacC1 (Δ*pacC*) (data not shown), cellobiose hydrolysis appeared not to be accelerated by *pacC* deletion. It is reported in other fungi that transglycosylation products derived from cellobiose, such as sophorose and gentiobiose, exhibited stronger inducing activity than cellobiose (Kurasawa et al. 1992; Mach et al. 1995). If this is also the case in *A. nidulans*, decreased expression of the β-glucosidase genes may cause decreased expression of the genes encoding cellulolytic enzymes. Expression of four β-glucosidase genes (ANIA_02828 (*bglL*), ANIA_03903 (*bglH*), ANIA_02227 (*bglI*), and ANIA_10124) was decreased as a result of *pacC* deletion (Table 3). In particular, the decrease in *bglL* and *bglI* expression was significant (30-fold and 40-fold). BglL is distinct from other β-glucosidases in that it is not a member of the ClrB regulon and its product is predicted to be extracellular, whereas the others are included in the ClrB regulon and their products are predicted to be cytoplasmic. It is worth noting that *bglL* and ANIA_10124 encode orthologues of *T. reesei* Cel3A and Cel1A, respectively, which produce sophorose from cellobiose (Mach et al. 1995; Saloheimo et al. 2002).

With regards to cellobiose uptake, four transporter genes (ANIA_02814, ANIA_08347, and ANIA_06831) orthologous to the transporter genes involved in the regulation of the genes encoding cellulolytic enzymes in other fungi exhibited decreased expression in the Δ*pacC* strain (Table S5 in the Supplementary Material) by 32-fold, 9.2-fold, and 58-fold, respectively. ANIA_02814 and ANIA_08347 were orthologous to *N. crassa cdt-1* and *cdt-2* (Galazka et al. 2010), and ANIA_06831 to *T. reesei crt1* (Zhang et al.

2013). *N. crassa* CDT-1 and CDT-2 function not only as cellodextrin transporters but also as receptors for cellobiose (Znameroski et al. 2014). *T. reesei* Crt1 is essential for cellulase induction; however, its transporter activity for cellobiose and sophorose is not detectable (Zhang et al. 2013).

It is reasonable to surmise that the decrease in expression of the β-glucosidase genes and/or the transporter genes affects the activity of ClrB. An understanding of the inducing effects of various β-glucobioses as well as the transglycosylation activity of the β-glucosidases and identification of the transporter gene(s) involved in the inducer uptake and/or perception would be essential to clarify the involvement of these genes in indirect regulation by PacC.

The RNA sequencing analysis of the *pacC*[+] and Δ*pacC* strains under cellobiose-inducing conditions revealed that 82 % of the ClrB-regulated genes were also under control of PacC. The remaining genes were *agdB* (α-glucosidase), *chiA* (endochitinase), *lipA* (lipase), *pgxB* (exo-polygalacturonase), *plyA* (pectate lyase), *plyF* (pectate lyase), *pmeB* (pectin methyl esterase), ANIA_00452 (xyloglucan-specific endoglucanase), ANIA_02804 (β-galactosidase), ANIA_01109 (transporter), ANIA_07067 (transporter), and ANIA_06035. These genes exhibited relatively low expression levels with RPKM values below 25 (except for *agdB*, *pgxB*, ANIA_01109, and ANIA_06035) under the experimental conditions in the present study. The fold decreases in the expression levels of these genes as a result of *clrB* deletion were relatively small, ranging from 2.9 to 6.8, with only one exception (ANIA_02804) as reported in a previous study (Coradetti et al. 2013). Low expression levels as well as weak dependency on ClrB may have hampered the detection of PacC dependency. It should be noted that the *A. oryzae* orthologues of the 12 genes also

exhibit low dependency on ManR, the ClrB orthologue; fold decreases in their expression following *manR* deletion are within the range of 0.3 to 3 (Ogawa et al. 2013).

The *agdB* gene encodes isomaltose-forming α-glucosidase (Kato et al. 2002b), and its expression is under the control of the transcription factor AmyR (Nakamura et al. 2006). AmyR-mediated induction is triggered not only by isomaltose and maltose but also by glucose, which acts as a repressive carbon source; glucose triggers the nuclear localization of AmyR, leading to the expression of Taka-amylase A gene (*taaG2*) at low glucose concentrations, whereas high concentrations of glucose repress *taaG2* expression in a CreA-dependent manner (Kato et al. 2002a; Makita et al. 2009; Murakoshi et al. 2012). Therefore, low levels of glucose liberated from cellulose and cellobiose may induce *agdB* transcription independently of ClrB.

There were significant differences between the expression levels of multiple genes in the *clrB* regulon in this study and previous studies by Coradetti et al. (2012, 2013), which may be attributable to the differences in growth conditions. Besides, *cbhB* induction was not evident in this study, and a number of genes exhibited differential expression as compared with the previous study. The expression of the CAZy genes ANIA_10124 (β-glucosidase), *mndB* (β-mannosidase), *aglG* (α-galactosidase), *manB* (β-mannanase), and *manC* (β-mannanase) was 10-fold higher in our experimental conditions, whereas *axhB* (α-L-arabinofuranosidase), ANIA_05309 (CE5), *plyF* (pectate lyase), ANIA_00567 (AA3), *pelB* (pectin lyase), *manF* (β-mannanase), and *chiC* (chitinase) exhibited a greater than 10-fold decrease in their expression levels. The reasons for the differences between the expression levels in response to cellobiose induction under alkaline conditions and Avicel induction under neutral conditions are currently unclear.

In conclusion, we show that most of the genes regulated by ClrB are also under the control of PacC and suggest that both direct and indirect regulation by PacC may be involved in modulating the expression of these genes. However, our study did not identify the genes conclusively involved in this indirect regulation. Further studies focusing on the identification of the cellobiose transporter (or transceptor) involved in cellulase induction should provide a deeper understanding of the indirect regulation by PacC.

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