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ORIGINAL ARTICLE

CreA-independent carbon catabolite repression of cellulase genes by trimeric G-protein and protein kinase A in *Aspergillus nidulans*

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Abstract

Cellulase production in filamentous fungi is repressed by various carbon sources. In our preliminary survey in *Aspergillus nidulans*, degree of de-repression differed depending on carbon sources in a mutant of *creA*, encoding the transcriptional repressor for carbon catabolite repression (CCR). To further understand mechanisms of CCR of cellulase production, we compared the effects of *creA* deletion with deletion of protein kinase A (*pkaA*) and G (*ganB*) genes, which constitute a nutrient sensing and signaling pathway. In plate culture with carboxymethyl cellulose and D-glucose, deletion of *pkaA* and *ganB*, but not *creA*, led to significant de-repression of cellulase production. In submerged culture with cellobiose and D-glucose or 2-deoxyglucose, both *creA* or *pkaA* single deletion led to partial de-repression of cellulase genes with the highest level by their double deletion, while *ganB* deletion caused de-repression comparable to that of the *creA*/*pkaA* double deletion. With ball-milled cellulose and D-glucose, partial de-repression was detected by deletion of *creA* but not of *pkaA* or *ganB*. The *creA*/*pkaA* or *creA*/*ganB* double deletion led to earlier expression than the *creA* deletion. Furthermore, the effect of each deletion with D-xylose or L-arabinose as the repressing carbon source was significantly different from that with D-glucose, D-fructose, and D-mannose. Consequently, this study revealed that PkaA and GanB participate in CreA-independent CCR and that contribution of CreA, PkaA, and GanB in CCR differs depending on the inducers, repressing carbon sources, and culture conditions (plate or submerged). Further study of CreA-independent mechanisms is needed to fully understand CCR in filamentous fungi.

Keywords *Aspergillus nidulans* · Carbon catabolite repression · Cellulase · PkaA · GanB

Introduction

Microorganisms possess a carbon catabolite repression (CCR) system, which ensures preferential utilization of easily metabolizable carbon sources such as D-glucose by preventing the utilization of difficult to metabolize carbon sources. As a result, the CCR system determines the hierarchy of carbon substrate utilization in filamentous fungi, which are known to utilize a huge variety of carbon substrates. A number of genes involved in the carbon catabolism, including industrially important carbohydrate-degrading-enzymes such as cellulase, amylase, and xylanase are regulated by CCR. The transcriptional repressor that mediates CCR in *Aspergillus nidulans* is CreA (Dowzer and Kelly 1989), with orthologues such as CRE-1 in *Neurospora crassa* (de la Serna et al. 1999) and CRE1 in *Trichoderma reesei* (Strauss et al. 1995). CreA has a C_2H_2-type DNA-binding motif and directly binds to

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5′-SYGGRG-3′ on the promoters of target genes to prevent transcription (Cubero and Scazzocchio 1994). CreA activity is regulated according to nuclear/cytoplasmic trafficking and stability. That is, CreA is excluded from the nucleus under de-repressing conditions, and is degraded in the cytoplasm (Brown et al. 2013; de Assis et al. 2018; Tanaka et al. 2018). The protein kinases SnfA and SchA are involved in the exclusion of CreA from the nucleus (Brown et al. 2013), while the cAMP-dependent protein kinase (PKA) encoded by *pkaA* counteracts SnfA function (de Assis et al. 2015).

CreA directly and indirectly regulates gene expression. It represses the expression of the *xlnR* gene encoding the major transcriptional activator of the xylanolytic enzyme genes in *A. niger*, such that the genes regulated by XlnR, including the xylanase genes *xlnA* and *xlnB*, are downregulated (Orejas et al. 2001; Tamayo et al. 2008). In the case of proline utilization, CreA directly represses *prnB* expression, which encodes the proline permease. This causes inducer exclusion, such that the expression of *prnC* and *prnD* genes remains at a low level, even in the presence of proline (Cubero et al. 2000).

The CreA-dependent system described above is not the sole system governing CCR. The transcription of *A. nidulans* α-L-rhamnosidase genes *rhaA* and *rhaE* is repressed and the uptake of the inducer molecule L-rhamnose is inhibited by D-glucose even in the *creA*^{d}*30* mutant (Tamayo-Ramos et al. 2012). Uptake of phenylacetate has also been reported to be inhibited by D-glucose in a CreA-independent manner (Fernández-Canón and Luengo 1997). A recent report found that an Arrestin-like protein CreD and a ubiquitin ligase HulA, identified via the characterization of the suppressor mutants of the *creB* and *creC* mutations (Boase and Kelly 2004), regulate D-glucose-induced endocytosis of the maltose transporter MalP, thus affecting CCR of α-amylase genes in *A. oryzae* (Hiramoto et al. 2015). CreB encodes a putative deubiquitinase and CreC encodes a WD40 protein, which interact with each other (Boase et al. 2003; Lockington and Kelly 2002). Although CreA was initially suggested to be a possible target of CreB, a recent study denied such possibility based on the result that CreA was not ubiquitinated (Alam el al. 2017). In fact, mutation or deletion of *creB* does not affect CreA stability in *A. nidulans* or *A. oryzae* (Ries et al. 2016; Tanaka et al. 2018), while a mutation in CreC has been found to lead to the destabilization of CreA (Ries et al. 2016). The *creA* and *creB* double deletion mutant has been found to lead to a higher production of α-amylase, xylanase, and β-glucosidase activity than single *creA* or *creB* deletion mutants in *A. oryzae* (Ichinose et al. 2014, 2018). These reports point towards the presence of a CreB-dependent but CreA-independent CCR. Alternatively, CreB might target a CreA-interacting protein that is required for function of CreA (Alam and Kelly 2017).

Although many of the abovementioned studies used D-glucose as a representative repressing substrate, many other carbon substrates also show repressing activity at varying strengths. Moreover, the level of repression exerted by certain repressing substrates can differ from gene to gene. Currently, no concrete regulatory mechanisms are known to determine the hierarchy of carbon source utilization, indicating that our understanding of CCR is still not satisfactory. In this study, we compared the effects on CCR of cellulase genes caused by the deletion of *creA* and *pkaA*, as well as of *ganA, ganB*, and *fadA*, which encode Gα, a subunit of the trimeric G protein, in the presence of various carbon substrates. Our results demonstrated that CreA, PkaA, and GanB play distinct roles in CCR depending on the repressing carbon sources as well as the growth conditions.

Materials and methods

Strains and media

The *A. nidulans* strains used in this study are listed in Table S1. ABPU1 was the parent strain of all the deletion mutants. ABU was generated by introduction of the wild-type *pyroA* gene into ABPU1. The *creA* and *pkaA* single deletion strains (Δ*creA* and Δ*pkaA*, respectively) were constructed by replacing the genes with *A. nidulans pyroA* (Fig. S1A and B). The *creA* and *pkaA* double deletion strain Δ*creA*/Δ*pkaA* was constructed using the marker recycling strategy (Akada et al. 2006); first, the *pkaA* gene in the Δ*creA* strain was deleted using a deletion cassette specially designed for marker recycling, with *A. oryzae pyrG* as a selectable marker, then the *pyrG* gene was eliminated by selecting 5-fluoroorotic acid (FOA)-resistant strains (Fig. S2A). The *ganA, ganB*, and *fadA* single deletion strains (Δ*ganA*, Δ*ganB*, and Δ*fadA*, respectively) were also constructed from ABPU1 using the marker recycling strategy (Fig. S2B, C, D). The Δ*creA*/Δ*ganB* double deletion strain was constructed from the Δ*ganB* strain by the marker recycling strategy using *A. oryzae pyrG* as a selectable marker (Fig. S2E).

Plasmids carrying the deletion cassettes were constructed by joining PCR-generated DNA fragments via the conventional method using restriction enzymes and DNA ligase for the *pkaA* single deletion and via the seamless cloning technique for the rest using the GeneArt™ Seamless Cloning and Assembly kit (Thermo fisher Scientific, Waltham, MA, USA) or the In-Fusion® HD Cloning Kit (Takara, Shiga, Japan). The primers used for the construction of the plasmids are listed in Table S2. Plasmid construction was performed using *Escherichia coli* DH5α and XL1-Blue strains. Plasmids for complementation of the deletions were also constructed via the seamless cloning technique.

Transformation of *A. nidulans* was carried out by protoplast transformation (balance and Turner 1985), with slight modifications, as previously described (Makita et al. 2009). Deletion of the target genes was first examined by PCR (data not shown), and then confirmed by Southern blot analysis (Figs. S1 and S2). The presence of the re-introduced genes in the complementary strains was confirmed by PCR. *A. nidulans* strains were grown at 37 °C in the standard minimal medium (MM) with the appropriate supplements to fulfill auxotrophy, unless otherwise stated (Rowlands and Turner 1973).

Plate assay of amylase and cellulase activity

An aliquot of suspension containing 10^4 conidia of each *A. nidulans* strain was spotted onto agar plates of the standard minimal medium containing 0.1% TritonX-100 with 1% starch or 0.5% carboxymethylcellulose (CMC). In addition, each repressing monosaccharide was added at a final concentration of 1%. After incubation at 37 °C for 3 days, amylase activity was visualized using an iodine test. Cellulase activity was visualized by staining with 0.1% Congo red followed by de-staining with 0.7 M NaCl.

Transcriptional analysis

A. nidulans strains were grown in MM containing 1% Bacto™ Peptone (Becton Dickinson, Franklin Lakes, NJ, USA) and 0.1% Bacto™ Yeast Extract (Becton Dickinson) instead of D-glucose for 22 h at 37 °C. The mycelia were collected and washed with MM without carbon sources. The mycelia were transferred into fresh MM containing 3 mM cellobiose and 50 µM 1-deoxynojirimycin (DNJ) with or without repressing carbon substrates at 2.5 mM for 2-deoxyglucose (2DG) and 30 mM for hexoses and pentoses, then cultivated at 37 °C for 1.5 h. DNJ was added to prevent the hydrolysis of cellobiose. When ball-milled cellulose (BMC) was used, the mycelia were transferred into fresh MM containing 0.5% BMC with or without D-glucose at 1%, and cultivated for 6 and 12 h. The BMC used in this study was prepared from KC flock W-300G (Nippon Paper Industries, Tokyo, Japan) by wet ball milling. The average degree of polymerization was 190, which was calculated by dividing the total sugars by the reducing sugars. Total and reducing sugar concentrations were determined by the phenol–sulfuric acid method and Somogyi–Nelson method, respectively (Dubois et al. 1951; Somogyi 1952). The mycelia were harvested, frozen in liquid nitrogen, and ground into a fine powder using an SK mill SK-100 (Tokken, Chiba, Japan). RNA extraction was carried out as previously described (Kunitake et al. 2016). Genomic DNA removal and cDNA synthesis were carried out using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Quantitative PCR was performed as previously described (Kunitake et al. 2016) using the THUNDERBIRD SYBR qPCR Mix (Toyobo) with the StepOnePlus™ Real-time PCR system (Thermo Fisher Scientific). The primers used for qPCR are listed in Table S3.

Zymography of cellulase activity

After cultivation with BMC as the inducer, 15 µl of culture supernatant was subjected to 12% native-PAGE containing 0.1% CMC. The gels were then rinsed with 50 mM sodium phosphate buffer (pH 6.5) three times and incubated in the same buffer for 3 h at 37 °C. The protein bands containing cellulase activity were visualized by staining with 0.1% Congo-Red for 30 min, followed by de-staining with 1 M NaCl.

Results

PkaA and Gα-mediated but CreA-independent CCR

Screening of the protein kinase deletion library of *A. nidulans*, generated by De Souza et al. (De Souza et al. 2013a), by plate assay for cellulase production revealed that the deletion of *pkaA*, encoding protein kinase A, led to a significant de-repression of cellulase production with carboxymethylcellulose (CMC) plus D-glucose as the carbon sources. PkaA is partially involved in CCR by counteracting SnfA that promotes exclusion of CreA from the nucleus (de Assis et al. 2015). However, the *creA204* mutation, which possesses an amino acid substitution in the DNA-binding domain of CreA (Shroff et al. 1996), did not cause such de-repression of cellulase production in our preliminary studies (data not shown). This suggested that PkaA is involved in CreA-independent CCR. To obtain further evidence of a PkaA-dependent and CreA-independent CCR, amylase and cellulase production in the *creA, pkaA*, and *creA*/*pkaA* knockouts, derived from the same parent strain ABPU1, was compared by the plate assay with D-glucose as the repressing carbon source. In addition, the knockouts of the genes *ganA, ganB*, and *fadA*, encoding the trimeric G-protein α subunit (Gα), were examined considering that protein kinase A is generally under the control of Gα in eukaryotes. The Δ*creA* and Δ*pkaA* strains grew poorly on starch, D-glucose, CMC, starch plus D-glucose, and CMC plus D-glucose, where the double knockout Δ*creA*/Δ*pkaA* exhibited the slowest growth (Fig. 1a).

Despite the growth defect, the Δ*creA*, Δ*creA*/Δ*pkaA*, and Δ*ganB*/Δ*creA* strains were found to have extremely higher amylase activity, based on the sizes of the clear zones (halos) around their colonies, than the other strains on starch and starch plus D-glucose, while such an elevated production was

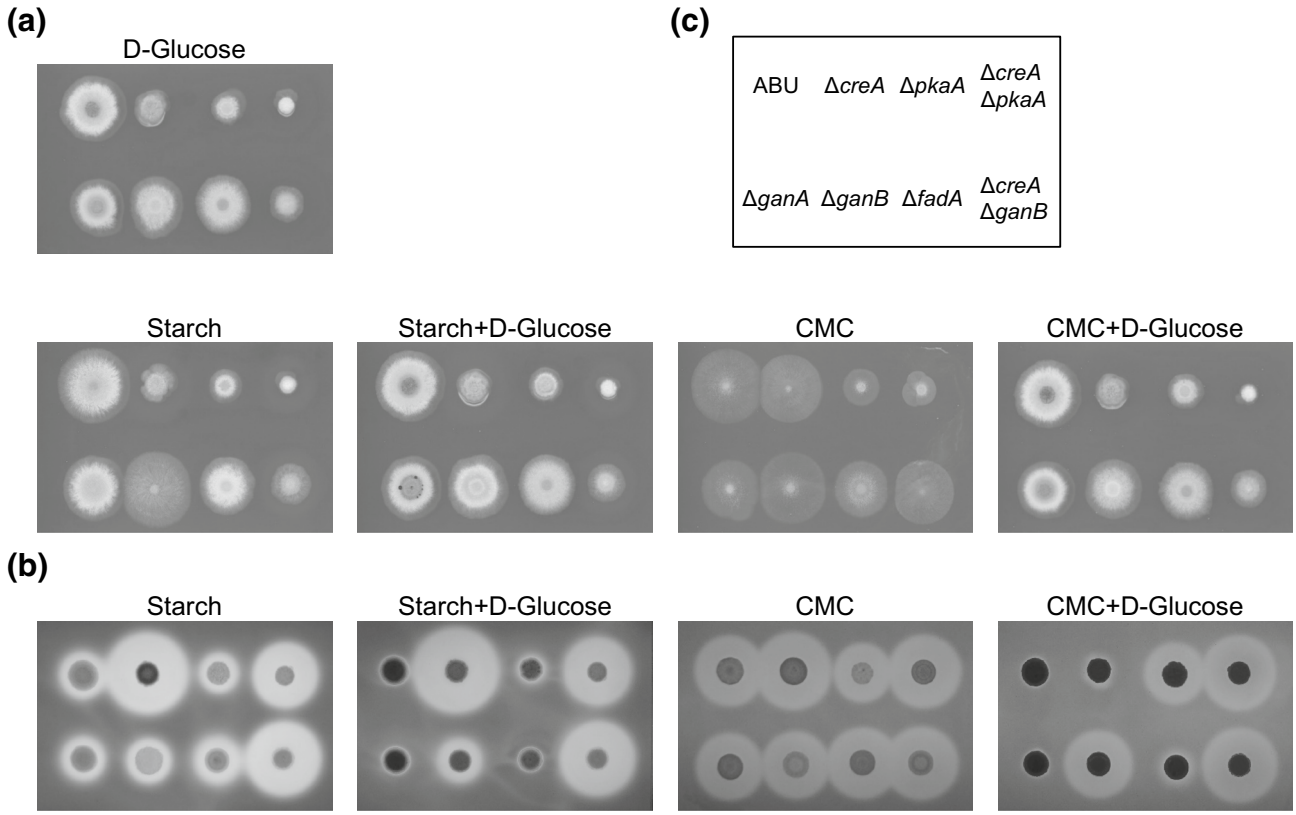

Fig. 1 Growth (**a**) and amylase and cellulase production (**b**) of the deletion strains on agar plates containing starch or CMC with D-glucose. Positions of strains are shown in (**c**)

not observed in the Δ*pkaA* strain (Fig. 1b). Consequently, PkaA does not participate in the CCR of amylase, or if it does, only plays a minor role. In contrast, the effects of the *creA, pkaA*, and *ganB* deletions on cellulase production were completely different from those on amylase production. High cellulase production in the presence of D-glucose was observed in the Δ*pkaA*, Δ*ganB*, Δ*creA*/Δ*pkaA*, and Δ*creA*/Δ*ganB* strains, but not in the Δ*creA* strain (Fig. 1b). The Δ*creA*/Δ*pkaA* and Δ*creA*/Δ*ganB* strains produced slightly higher cellulase activity than the Δ*pkaA* and Δ*ganB* strains, despite that the double deletants grew more poorly than the single deletants. Defects in CCR of amylase or cellulase production were recovered by reintroduction of the *creA, pkaA*, and *ganB* genes into the deletants (Fig. S3). As a single exception, trial to reintroduce *creA* into the Δ*creA*/Δ*ganB* strain failed because protoplasts were not formed.

Consequently, CCR of amylase production is CreA dependent, PkaA independent, and partially GanB dependent while CCR of cellulase production is PkaA and GanB dependent, but surprisingly almost CreA independent. De-repression of the cellulase activity by the *ganB* deletion suggests that the extracellular D-glucose signal sensed by G-protein-coupled receptors (GPCRs) is mainly transmitted to GanB, but not to GanA or FadA.

Effects of deletion of *creA, pkaA*, and Gα genes on CCR of cellulase genes in submerged culture

Although cellulase production in the presence of D-glucose was not de-repressed in the Δ*creA* strain, the Δ*creA*/Δ*pkaA* and Δ*creA*/Δ*ganB* strains produced slightly higher cellulase activity than the Δ*pkaA* and Δ*ganB* strains (Fig. 1b). This suggests that CreA plays a minor role in CCR of cellulase production, but we could not conclude this because plate assay is not quantitative enough. To obtain quantitative data, we investigated expression of cellulase genes by RT-qPCR in submerged culture with cellobiose as an inducer with 2-deoxyglucose (2DG) as a repressing carbon source.

In the pilot experiments, we determined experimental conditions for cellobiose induction and 2DG repression. When induced with 3 mM cellobiose for 1.5 h, the expression of *eglA* and *cbhA*, encoding an endoglucanase and a cellobiohydrolase, was found to be several fold higher in the Δ*creA* and Δ*pkaA* strains compared to that of the reference strain ABU (*creA*$^{+}$ *pkaA*$^{+}$), even without D-glucose addition (Fig. S4). The low-level expression in ABU was recovered by the addition of 50 µM 1-deoxynojirimycin (DNJ), an inhibitor of β-glucosidase, indicating that D-glucose released from cellobiose represses cellulase gene expression (Fig.

S4). Therefore, DNJ was added in all subsequent transcriptional analyses using cellobiose as the inducer. In addition, a non-metabolizable D-glucose analog, 2DG, was used as a repressing carbon substrate. When cellobiose, DNJ, and various concentrations of 2DG were added, repression levels of *eglA* and *cbhA* were not significantly different between 0.5 mM and 2.5 mM 2DG in the ABU strain, as well as in the Δ*creA* and Δ*pkaA* strains (Fig. S5). Based on these results, further experiments were performed using 3 mM cellobiose, 50 µM DNJ, and 2.5 mM 2DG.

The addition of 2DG caused a remarkable decrease in the expression levels of *eglA* and *cbhA* not only in ABU but also in the Δ*creA*, Δ*pkaA*, Δ*creA*/Δ*pkaA*, and Δ*ganB* strains to different degrees (Fig. 2a, b). The differences in their expression levels without 2DG were not negligible; the expression of *cbhA* in the Δ*creA*/Δ*pkaA* strain was 3.3-fold higher than in the reference strain (Fig. 2b). Considering such a difference in the absence of 2DG, the expression of *eglA* and *cbhA* in the presence of 2DG relative to that in its absence in each strain was compared to evaluate the degree of de-repression caused by each deletion (Fig. 2c, d). The expression of *eglA* and *cbhA* in ABU in the presence of 2DG was only 0.6% and 0.7%, respectively, compared to that without 2DG. The relative expression of the genes in the presence of 2DG increased to 11% and 8% in the Δ*creA* strain, 17% and 20% in the Δ*pkaA* strain, and 20% and 19% in the Δ*creA*/Δ*pkaA* strain. As for the Gα genes, the expression of *eglA* and *cbhA* in the presence of 2DG was 1% and 2% in both the Δ*ganA* and Δ*fadA* strains, 25% and 34% in the Δ*ganB* strain, and 11% and 14% in the Δ*creA*/Δ*ganB* strain. Additionally, the increase in the de-repression levels in the Δ*creA*/Δ*pkaA* strain compared to the Δ*creA* strain ($p = 0.0698$ for *eglA*, and 0.0009 for *cbhA* by the Welch's *t* test) was consistent with the observation in the plate assay (Fig. 1b). De-repression level of the genes was the highest in the Δ*ganB* strain, and unexpectedly, the level decreased in the Δ*creA*/Δ*ganB* strain. As to

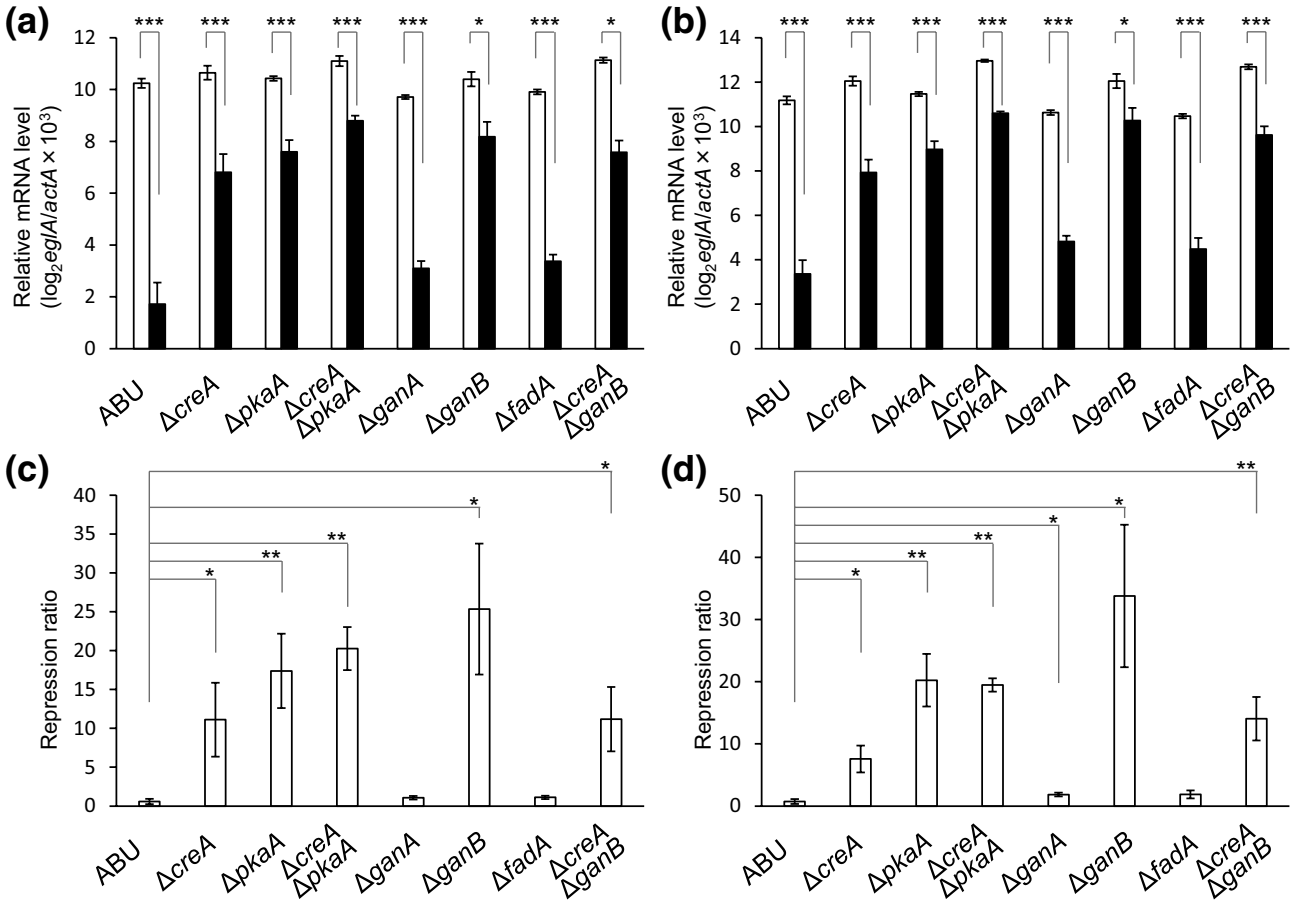

Fig. 2 Expression of *eglA* (**a, c**) and *cbhA* (**b, d**), evaluated by RT-qPCR, under 3 mM cellobiose-induced conditions with or without 2.5 mM 2-DG. **a, b** Relative expression levels of the genes were normalized with *actA* and shown by a logarithmic scale. White bar, cellobiose; black bar, cellobiose with 2DG. **c, d** Expression ratio in the presence of 2DG compared to in its absence. Error bars indicate the standard errors of more than three biological independent experiments. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Welch's *t* test)

creA, pkaA, and *ganB*, the deletions did not significantly affect expression of the undeleted genes (Fig. S6).

Although it was not obvious whether CreA is involved in CCR of cellulase production in the plate assay (Fig. 1b), transcriptional analysis clearly revealed its involvement. However, considering that deletion of *pkaA* or *ganB* caused stronger de-repression than that of *creA*, our results here again confirmed the presence of CreA-independent CCR of the cellulase genes.

Effects of the *creA, pkaA*, and *ganB* deletions on CCR under ball-milled cellulose-induced conditions

In the above transcriptional analyses, the inducer of the expression of the cellulase genes used was cellobiose, which is produced by endoglucanases and cellobiohydrolases from cellulose. When only cellulose is present, the enzymes produced at a basal level and/or induced by starvation are thought to produce cellobiose to boost cellulase expression. Thus, the effects of *creA* and/or *pkaA* deletion on cellulose induction may differ from cellobiose induction. To assess this issue, the effects of the deletions on the production of endoglucanases (EglA and EglB) and the expression of *eglA* and *cbhA* were examined by zymography and transcriptional analysis using 0.5% ball-milled cellulose (BMC) as the inducer and 1% D-glucose as the repressing carbon substrate. BMC was prepared from crystalline cellulose by grinding with ceramic ball, resulting in segmentation of the polymers. Its average degree of polymerization was 190. As shown in Fig. 3a, cellulase production in the absence of D-glucose did not significantly differ among the ABU, Δ*creA*, Δ*pkaA*, and Δ*ganB* strains, while that in the Δ*creA*/Δ*pkaA* strain was clearly elevated at 6 h of induction. The lower activity band represents EglA and the upper two bands represent EglB in differently glycosylated forms (Chikamatsu et al. 1999; Endo et al. 2008). In the presence of D-glucose, endoglucase activity was not detected in the ABU, Δ*pkaA*, and Δ*ganB* strains, but was detected in the Δ*creA* strain at 12 h. In the Δ*creA*/Δ*pkaA* and Δ*creA*/Δ*ganB* strains, the activity was detected at 6 h and further increased at 12 h.

The expression of *eglA* and *cbhA* exhibited very similar results; their expression was significantly repressed by D-glucose in the ABU, Δ*pkaA*, and Δ*ganB* strains, while it was detected at 12 h in the Δ*creA* strain and at 6 h with an increased production at 12 h in the Δ*creA*/Δ*pkaA* and Δ*creA*/Δ*ganB* strains (Fig. 3b). Although de-repression from

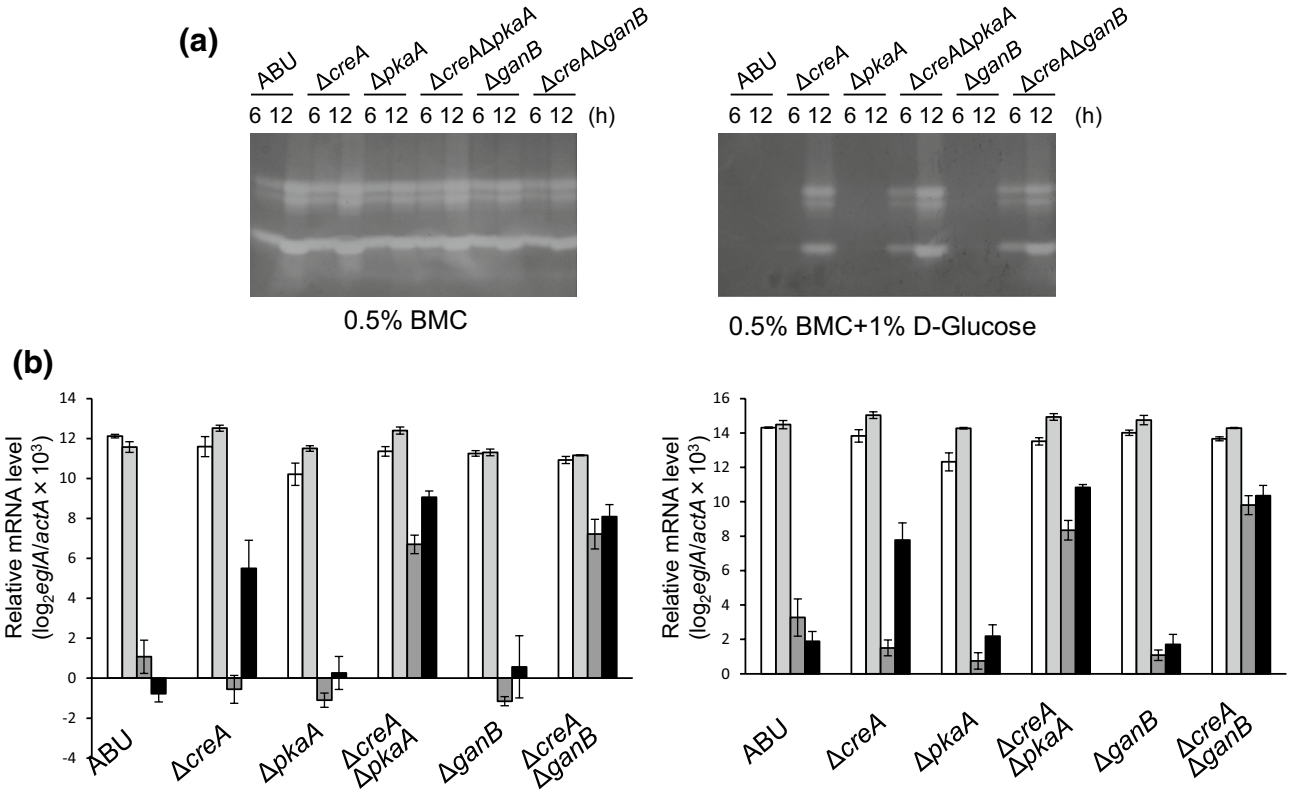

Fig. 3 Cellulase production and *eglA* and *cbhA* expression under 0.5% BMC-induced conditions with or without 1% D-glucose. Each strain was cultivated in MM plus BMC with or without D-glucose for 6 h and 12 h. Culture supernatant (15 µl) of each deletion strain was subjected to zymography (**a**), and total RNA extracted from the mycelia was subjected to RT-qPCR analysis (**b**). White bar, BMC for 6 h; light gray bar, BMC for 12 h, dark gray bar, BMC + D-glucose for 6 h; black bar, BMC + D-glucose for 12 h

CCR was not observed in the Δ*pkaA* and Δ*ganB* strains, the CreA-independent participation of PkaA and GanB in CCR is evident considering the earlier expression of *eglA* and *cbhA*, as well as the earlier production of endoglucanases in the Δ*creA*/Δ*pkaA* and Δ*creA*/Δ*ganB* strains compared to the Δ*creA* strain. It should be noted that the effects of the *creA, pkaA*, and *ganB* deletion on the cellulase expression in the submerged culture were the opposite of those in the plate assay (Figs. 1b, 3a).

Effects of the *creA, pkaA*, and *ganB* deletions on CCR with various carbon sources

Not only D-glucose but also other carbon sources repress the expression of cellulase genes. Among the monosaccharides examined in this study, D-fructose, D-mannose, and D-xylose were found to cause a strong repression of cellulase production in the reference strain ABU, and D-galactose and L-arabinose were found to be weakly repressing carbon sources (Fig. 4b). The effects of the deletion of the *creA, pkaA, creA*/*pkaA*, and Gα genes on the repression of cellulase production and cellulase gene expression were examined, since these genes may be involved in CCR via different mechanisms depending on the carbon sources used. Growth of the deletion mutants on various carbon sources was similar to that on D-glucose, as shown in Fig. 1a. A single exception was the Δ*pkaA* and Δ*ganB* strains in which little growth was observed on both D-galactose and CMC plus D-galactose. Interestingly, the growth defect on D-galactose was recovered to a level comparable to that of other carbon sources in the Δ*creA*/Δ*pkaA* strain. Furthermore, the growth defect of the Δ*creA*/Δ*ganB* strain was hardly observed on both D-galactose and CMC plus D-galactose. These results suggest that the cAMP signaling pathway plays an essential role in the D-galactose catabolism.

The cellulase production of the deletion mutants on the plates containing various monosaccharides revealed a striking difference from the data with D-glucose shown in Fig. 1b, where de-repression was observed on all the monosaccharides by the *creA* deletion. Additive de-repression by double deletion of *creA* and *pkaA* as well as *creA* and *ganB* was evident on D-fructose and D-mannose, while cellulase

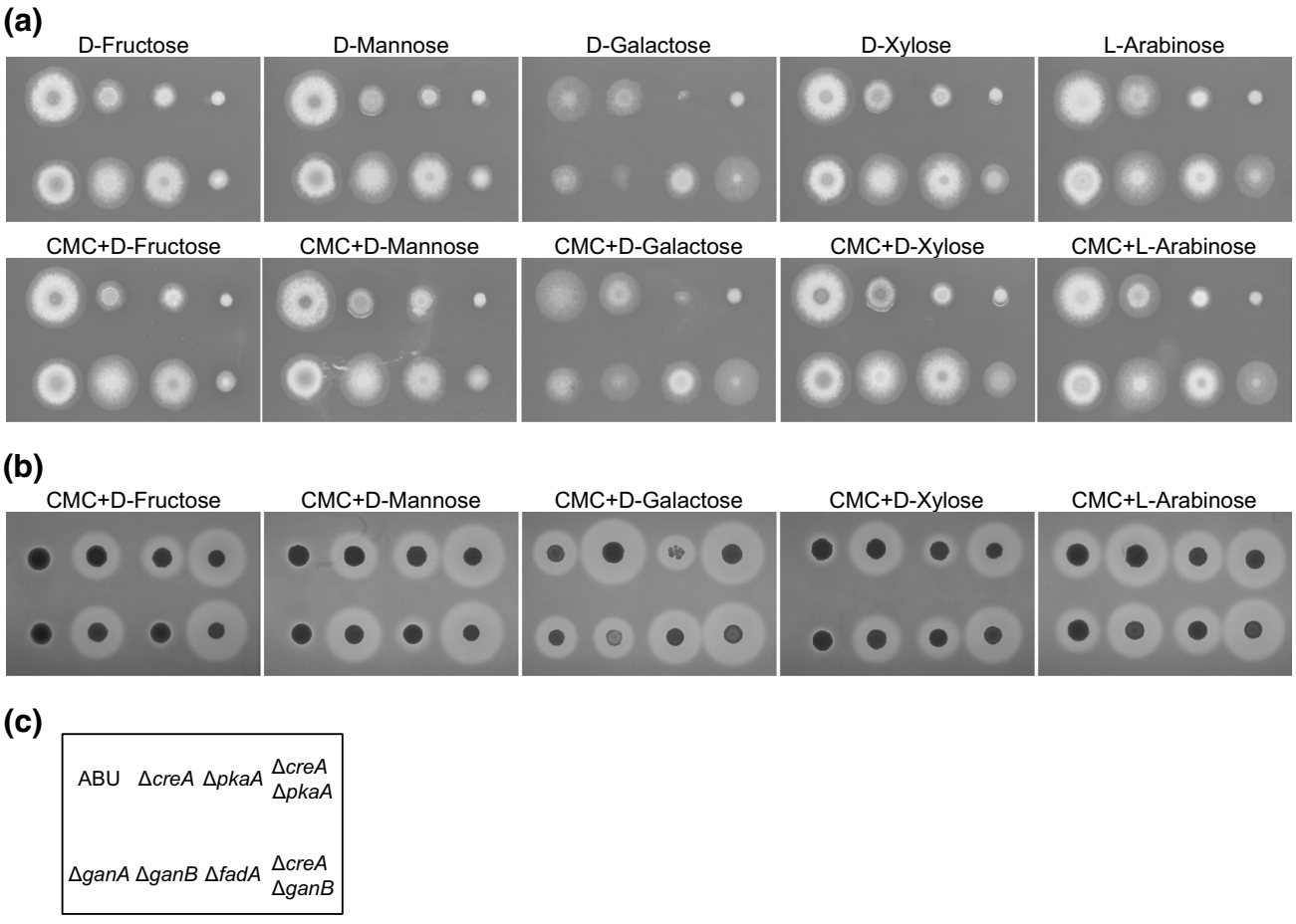

Fig. 4 Growth on (**a**) and repression of cellulase production by (**b**) various monosaccharides. Strains were cultured on MM agar plates containing indicated sugars as carbon sources. Positions of strains are shown in **c**

production in Δ*creA*, Δ*creA*/Δ*pkaA*, and Δ*creA*/Δ*ganB* was comparable to each other on D-xylose and L-arabinose (Fig. 4b). These observations imply that CCR caused by pentoses might be different from that by hexoses, and that contribution of PkaA is very minor or negligible on pentoses. The *pkaA* deletion led to a weakly de-repressed production of cellulase on all the monosaccharides, while the *ganB* deletion led to stronger de-repression than the *pkaA* deletion. In addition, the *fadA* deletion led to a weak de-repression on D-xylose. Due to the extremely impaired growth of the Δ*pkaA* and Δ*ganB* strains, de-repression on D-galactose could not be evaluated.

The effects of various repressing monosaccharides on the expression of *eglA* and *cbhA* were examined in the submerged culture with cellobiose as the inducer (Fig. 5a, b). D-glucose, D-fructose, and D-mannose appeared to be strong repressing carbon sources in the reference strain ABU, and the Δ*creA* strain was found to exhibit a higher expression of the cellulase genes than the Δ*pkaA* strain in the presence of hexoses. Compared to the hexoses, the pentoses D-xylose and L-arabinose were weaker repressing carbon sources in ABU. The deletion of *creA* led to the highest levels of expression, while the effects of the *pkaA* deletion were very minor on D-xylose, with no changes observed on L-arabinose.

The expression levels in the presence of the monosaccharides relative to those in their absence were compared (Fig. 5c, d), which revealed significant differences between CCR by the hexoses and by the pentoses. In the case of CCR by the hexoses, the effects of the *creA* and *pkaA* deletions were additive, leading to higher expression levels of *eglA* and *cbhA* in the Δ*creA*/Δ*pkaA* strain than in the Δ*creA* and Δ*pkaA* strains, although this additive action was not very

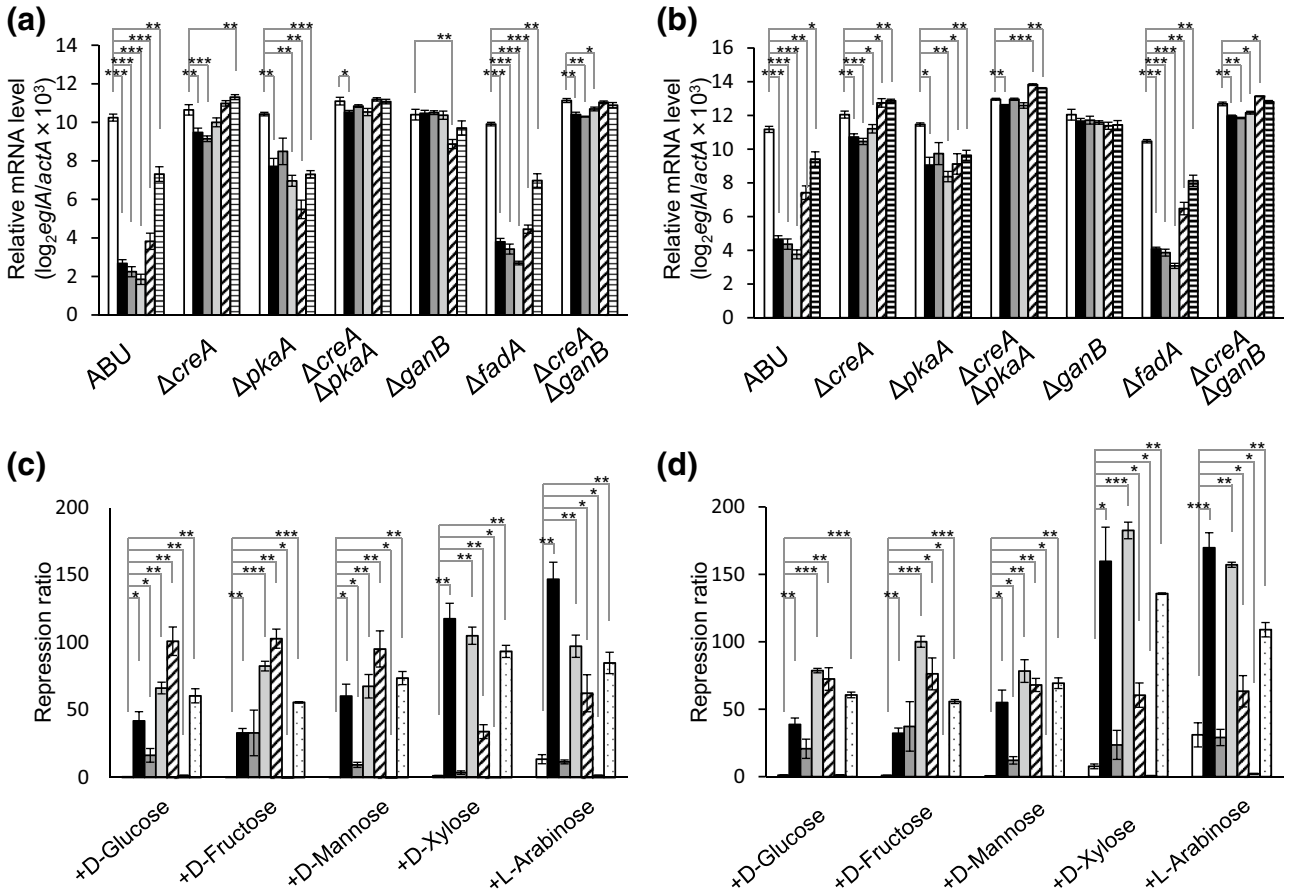

Fig. 5 Transcriptional analysis of *eglA* (**a, c**) and *cbhA* (**b, d**) on 3 mM cellobiose-induced conditions in the presence of various repressing monosaccharides (30 mM). The data without the repressing carbon sources are the same as those used in Fig. 2. **a, b** Relative expression levels normalized with *actA*. White bar, cellobiose; black bar, cellobiose + D-glucose; dark gray bar, cellobiose + D-fructose; light gray bar, cellobiose + D-mannose; shaded bar, cellobiose + D-xylose; horizontal striped bar, cellobiose + L-arabinose. **c, d** Expression ratio in the presence of a repressing monosaccharide compared to in its absence. White bar, ABU; black bar, Δ*creA*; dark gray bar, Δ*pkaA*; light gray bar, Δ*creA*Δ*pkaA*; shaded bar, Δ*ganB*; horizontal striped bar, Δ*fadA*; dotted bar, Δ*creA*Δ*ganB*. Error bars indicate the standard errors of three biological independent experiments. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Welch's *t* test)

evident in *eglA* expression upon D-mannose addition, which was the only exception. The Δ*ganB* and Δ*creA*/Δ*ganB* strains also exhibited the expression of the genes to a greater extent than the Δ*creA* and Δ*pkaA* strains. On the other hand, the deletion of *creA* led to an extremely high expression of the genes in the presence of the pentoses, which exceeded the expression level in the absence of the repressing carbon sources. The *ganB* deletion was found to cause de-repression: however, the degree was much lower than in the *creA* deletion. Consequently, CCR by the hexoses appeared to be mediated by the additive actions of CreA and PkaA, while that by the pentoses was mainly CreA-mediated. Furthermore, a higher de-repression was found in the Δ*ganB* strain than the Δ*pkaA* strain on the pentoses, implying that GanB has a role in addition to PkaA regulation in cellulase gene expression.

Discussion

The expression of genes related to the utilization of carbon in filamentous fungi is induced in the presence of an inducer, while this expression is repressed by CCR if easily metabolizable carbon substrates co-exist with the inducer. The repressing activity differs depending on the carbon substrates, among which D-glucose and D-xylose are known to be the strongest. This suggests that CCR plays an important role in determining the order of carbon sources to be utilized in the conditions where various carbon substrates are present, as is often the case in natural environments. CCR is regulated by CreA-dependent as well as CreA-independent mechanisms in *Aspergillus*, as described in the introduction. In this study, we provided evidence of the involvement of PkaA and GanB in CreA-independent CCR by observing the effects of deletions of genes encoding the proteins on the production and gene expression of cellulases.

PkaA partially regulates nuclear-cytoplasmic shuttling of CreA by counteracting SnfA, which directly phosphorylates CreA in *A. nidulans* (Brown et al. 2013; de Assis et al. 2018). If the role of PkaA in CCR is limited to this function, de-repression caused by the *pkaA* deletion should be weaker than that by the *creA* deletion. However, the production of cellulase on the plate culture was significantly de-repressed from D-glucose repression as a result of *pkaA* and *ganB* deletions, but not by *creA* deletion (Fig. 1), indicating that PkaA and GanB participate CreA-independently in CCR in the case of the cellulase production. GanB is the Gα subunit of trimeric G protein, which generally responds to extracellular signals sensed by GPCRs and regulates adenylate cyclase, which produces cAMP. In contrast, the CreA-dependent system is thought to respond to intracellular D-glucose as the CreA orthologue Mig1 in *S. cerevisiae* is regulated by hexokinase 2, which is reported to be an intracellular D-glucose sensor (Vega et al. 2016). CCR of cellulase genes in *A. nidulans* may be regulated by a double lock system caused by both extracellular and intracellular D-glucose. It should be noted that the effects of the *creA, pkaA*, and *ganB* deletions were the opposite in the production of amylase. This suggests that GanB/PkaA-mediated CCR specifically functions for the regulation of cellulase genes.

The expression of *eglA* and *cbhA* in the submerged culture also confirmed the differential contribution of CreA and PkaA in CCR, considering that the highest level of de-repression of *eglA* and *cbhA* expression as well as cellulase production was observed in the Δ*creA*/Δ*pkaA* strain compared to the single deletion strains (Figs. 2, 3, 5). However, while de-repression as a result of the *creA* deletion was not observed in the plate culture (Fig. 1), it was detected in the submerged culture with cellobiose plus 2DG (Fig. 2), with BMC plus D-glucose (Fig. 3), and with cellobiose plus D-glucose (Fig. 5). These findings suggest that the degree of contribution of CreA and PkaA changes depending on the cultural conditions. De-repression due to *pkaA* and *ganB* deletions was detected with cellobiose plus D-glucose (Fig. 5) but not with BMC plus D-glucose (Fig. 2). De-repression of *eglA* and *cbhA* in the Δ*ganB* strain was the most marked. While full and 72% de-repression of *eglA* and *cbhA*, respectively, occurred with cellobiose plus D-glucose (Fig. 5), the expression was found to be completely repressed with BMC plus D-glucose (Fig. 3). Although we were unable to identify the possible causes of this difference in the literature, the *creA* but not *ganB* deletion might have led to an increased basal-level production of cellulases required for the production of the physiological inducer cellobiose from BMC.

The Δ*ganB* strain exhibited approximately 6.1-fold and 3.4-fold higher de-repression of *eglA* and *cbhA* ($p = 0.003$ and 0.005, respectively, by Welch's t test) compared to the Δ*pkaA* strain in the cellobiose plus D-glucose culture (Fig. 5). The higher level of de-repression implies that the role of GanB in CCR is not limited to the regulation of PkaA activity. The *ganB* deletion may affect the phospholipase C-dependent signaling pathway, which is also known to be under the regulation of trimeric G protein in eukaryotes, although the involvement of this phospholipase in cellulase regulation has not been elucidated in filamentous fungi. Another possible pathway may be GanB to FbxA via cAMP. FbxA is an F-box protein, which are known to be involved in ubiquitination, and has cAMP-binding motifs. This protein is required for the full expression of xylanase genes in *A. nidulans* and has been previously suggested to be involved in CCR (Colabardini et al. 2012).

Catabolite repression is caused not only by D-glucose but also by various other carbon substrates. As shown in Fig. 5, CreA, PkaA, and GanB are clearly involved in the CCR caused by hexoses. On the other hand, full de-repression

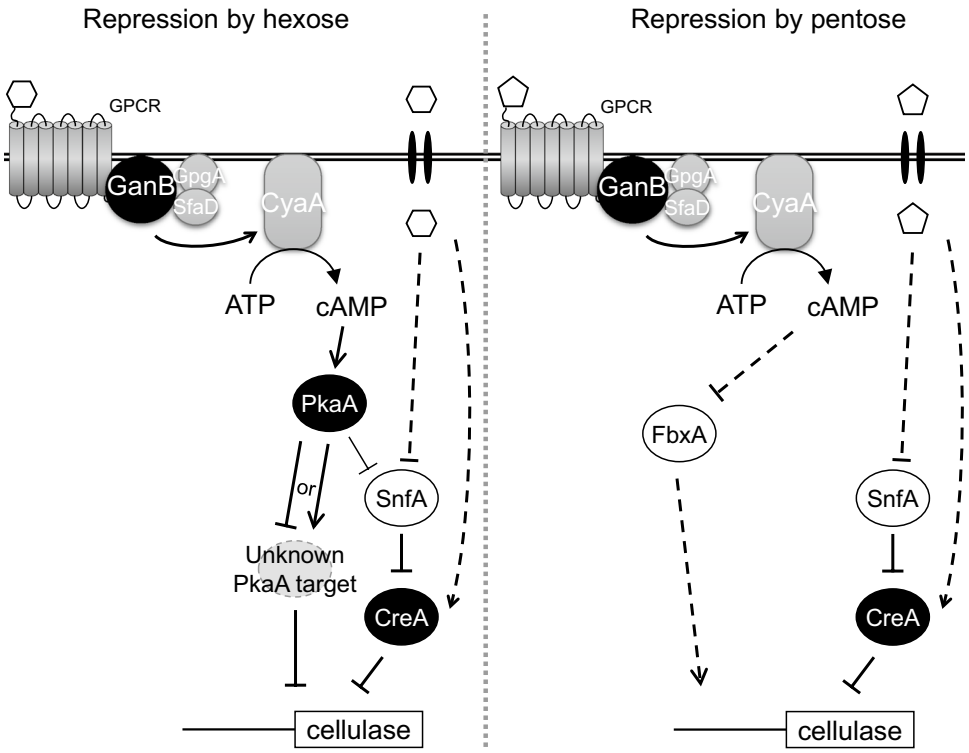

Fig. 6 Model for CCR of cellulase genes mediated by CreA, PkaA, and GanB. In the presence of a hexose such as D-glucose (left), sugar recognition by GPCR causes activation of cAMP synthesis via GanB. PkaA activated by cAMP indirectly represses cellulase gene expression by regulating an unknown factor and SnfA. In the presence of a pentose such as D-xylose (right), CreA mainly functions in CCR, but GanB-dependent, PkaA-independent system is also involved. Possible pathway for the GanB-dependent system may include FbxA, a protein with a cAMP-binding motif. The dashed lines represent hypothetical pathways

was observed in the Δ*creA* strain by pentoses. The contribution by PkaA was either very weak or nonexistent. The significant effect caused by the *creA* deletion may be at least partially due to the XlnR-dependent activation of cellulase gene expression, since XlnR participates in cellulase gene expression in *Aspergillus* species (Gielkens et al. 1999; Marui et al. 2002; Noguchi et al. 2009; van Peij et al. 1998), and is activated not only by D-xylose, but also by commercially available L-arabinose (de Souza et al. 2013b; Ishikawa et al. 2018; Noguchi et al. 2011). Interestingly, GanB was found to be involved in CCR caused by all the monosaccharides examined (Fig. 5). However, the contribution of PkaA was very small on pentoses. This is consistent with previous findings that conidial germination was impaired on various carbon sources in a *ganB* mutant (Lafon et al. 2005), implying that hexoses and pentoses were sensed by the GPCRs which exist upstream of GanB. There are 16 genes encoding GPCRs in *A. nidulans*. However, monosaccharide-sensing by GPCRs are not well characterized, except for some reports which state that the GprH of *A. nidulans* is a possible D-glucose sensor (Brown et al. 2015; Lafon et al. 2005). Further studies on the ligand–receptor relationship in GPCRs may increase our understanding of CCR. Possible signaling pathways in CCR, based on this study and literatures, are illustrated in Fig. 6.

In conclusion, this study revealed that CreA, PkaA, and GanB have distinct functions in CCR. This finding provides an insight into the mechanisms determining the hierarchy of carbon source utilization in filamentous fungi, where the identification of the factors upstream and downstream of GanB and PkaA is crucial to improve our understanding of the tactics employed by *A. nidulans* for its survival in nature. From a biotechnological point of view, the double knockout of *creA* and *pkaA* exhibited the best performance in cellulase gene expression; however, the resulting significantly impaired growth of the strain would be a limitation for industrial application. Further research on CCR would be needed to construct a CCR-free strain with better growth for industrial use.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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